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OXIDATIVE STRESS AND IMMUNITY IN ATTENTION DEFICIT HYPERACTIVITY DISORDER

OXIDATIEVE STRESS EN IMMUNITEIT BIJ AANDACHTSTEKORT HYPERACTIVITEITSSTOORNIS

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LIST OF ABBREVIATIONS

8-OHdG	8-hydroxy-2’-deoxyguanosine
8-oxoG	8-hydroxyguanine, 8-oxo-7,8-dihydroguanine
AA	Arachidonic acid
AAS	Atomic absorption spectroscopy
ACh	Acetylcholine
ADD	Attention deficit disorder
ADDH	Attention deficit disorder with hyperactivity
ADHD	Attention deficit hyperactivity disorder
ADHD-RS	ADHD Rating Scale
ADME	Absorption, distribution, metabolism and excretion
ADRA2A	Alpha-2A-adrenergic receptor
AE	Adverse event
AFC	Artificial food colours
ALA	α-linolenic acid
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMF	Amphetamine
AML	General Medical Laboratory
ANOVA	Analysis of variance
ANSM	French Health Products Safety Agency
AOA	Antioxidant activity
AOPP	Advanced oxidation protein products
APC	Antigen presenting cell
ASD	Autism spectrum disorder
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BC	Buffy coat
BD	Becton, Dickinson and Company
BDNF	Brain-derived neurotrophic factor
BHT	Butylhydroxytoluene
BSE	Bovine spongiform encephalopathy
CAT	Catalase
CBA	Cytometric bead array
CBCL	Child behaviour checklist
Cc	Weight capsule content
CCI	Controlled cortical impact
CD	Conduct disorder
CI	Confidence interval

CNS	Central nervous system
CNV	Copy number variant
ConA	Concanavalin A
CoQ10	Co-enzyme Q10
COX	Cyclooxygenase
Cp	Concentration procyanidins
CRF	Clinical research file
Crn	Creatinine
CRP	C-reactive protein
CRS	Conners' rating scale
CSF	Cerebrospinal fluid
CTD	Common technical document
Da	Dalton
DAD	Diode array detector
DAT	Dopamine active transporter
DC	Dendritic cell
dfs	Dilution factor reference solution
dfT	Dilution factor test solution
DHA	Docosahexaenoic acid
DIBD	Development international birth date
DISC	Diagnostic interview schedule for children
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DRD	Dopamine receptor D
DSM	Diagnostic and statistical manual of mental disorders
DSUR	Development safety update report
EC	Ethical committee
ECD	Electrochemical detection
EDTA	Ethylenediaminetetraacetic acid
EFA	Essential fatty acid
EGCG	Epigallocatechin-gallate
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ENS	Enteric nervous system
EPA	Eicosapentaenoic acid
EPD	Enzyme-potentiated desensitisation
Es	Extinction reference solution
ET	Extinction test solution
EtOH	Ethanol
EU	European Union
FAMHP	Federal Agency for Medicines and Health Products
FBS	Foetal bovine serum
FFQ	Food frequency questionnaire

fMRI	Functional MRI
GABA	γ-aminobutyric acid
GAD	Glutamic acid decarboxylase
GCP	Good clinical practice
GGT	Gamma-glutamyl transferase
GI	Gastrointestinal
GIDM	Gastrointestinal dialysis model
GLA	γ-linolenic acid
GLUT	Glucose transporter
GMP	Good manufacturing practice
GPx	Glutathione peroxidase
GR	Glutathione reductase
GRAS	Generally recognised as safe
GSH	Glutathione
GSSG	Glutathione disulphide
GST	Glutathione S-transferase
GWAS	Genome-wide association study
H_2O_2	Hydrogen peroxide
H_3PO_4	Phosphoric acid
HD	Hyperkinetic disorder
HEK	Human embryonic kidney
HEPES	Hydroxyethyl piperazine ethane sulfonic acid
HLA	Human leukocyte antigen
HNMT	Histamine N-methyl transferase
$HO^{\bullet}$	Hydroxyl radical
HPLC	High-pressure liquid chromatography
HRP	Horseradish peroxidase
IB	Investigator’s Brochure
ICAM	Intercellular adhesion molecule
ICD	International classification of diseases
ICH	International Council for Harmonisation
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMP	Investigational medicinal product
IMPD	Investigational medicinal product dossier
iNOS	Inducible nitric oxide synthase
I.p.	Intraperitoneally
IP	Investigational product
IQ	Intelligence quotient
IQR	Interquartile range
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KH_2PO_4	Potassium phosphate monobasic

LAF	Laminar airflow
LC	Liquid chromatography
LOD	Loss on drying
LOX	Lipoxygenase
LPS	Lipopolysaccharide
M	Microfold
M1	δ-(3,4-dihydroxy-phenyl)-γ-valerolactone
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MeOH	Methanol
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MMP	Matrix metalloprotease
MPH	Methylphenidate
MRI	Magnetic resonance imaging
MS	Mass spectrophotometry
ms	Mass reference
mT	Mass test product
Na_2HPO_4	Disodium hydrogen phosphate
NaH_2PO_4	Monosodium dihydrogen ortho-phosphate
NET1	Norepinephrine transporter gene 1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NMR	Nuclear magnetic resonance
nNOS	Neuronal NOS
NO	Nitric oxide
NO_2^-	Nitrite
NOAEL	No-observed-adverse-effects level
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
$O_2^{\bullet -}$	Superoxide anion radical
OCD	Obsessive–compulsive disorder
ODD	Oppositional defiant disorder
ODS	Octadecyl silane
ORAC	Oxygen radical absorbance capacity
OSA	Octane sulphonic acid
OSI	Oxidative stress index
OUT	Operational taxonomic units
P/S	Penicillin/Streptomycin
PAH	Polycyclic aromatic hydrocarbon
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCB	Polychlorinated biphenyl

PCQ	Physical Complaints Questionnaire
PCR	Polymerase chain reaction
PE	Phycoerythrin
PES	Polyethersulfone
PET	Positron emission tomography
PMA	Phorbol-12-myristate-13 acetate
PUFA	Polyunsaturated fatty acid
PWM	Pokeweed mitogen
QP	Qualified person
qPCR	Quantitative polymerase chain reaction
R	Correlation coefficient
RBC	Red blood cell
RED	Restricted elimination diet
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROH	Alcohol
ROOH	Peroxide
ROS	Reactive oxygen species
RP	Reversed phase
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RR	Relative risk
rRNA	Ribosomal RNA
RS	Reference standard
RT	Room temperature
RT-qPCR	Real-time quantitative polymerase chain reaction
SAE	Serious adverse event
SAM	S-adenosyl-l-methionine
SD	Standard deviation
SEQ	Social-Emotional Questionnaire
SmPC	Summary of medicinal product characteristics
SNAP-25	Synaptosomal-associated protein of 25 kDa
SOD	Superoxide dismutase
SOP	Standard operating procedure
SPSS	Statistical Package for the Social Sciences
STAT	Signal transducer and activator of transcription
SUSAR	Suspected unexpected serious adverse reaction
TAS	Total antioxidant status
TBA	Thiobarbituric acid
TGF	Transforming growth factor
T_H	T helper
THP-1	Human acute monocytic leukaemia cell line
TLR	Toll-like receptor
TMB	3,3’,5,5’tetramethylbenzidine

TMP	1,1,3,3-tetramethyoxypropane
TNF	Tumour necrosis factor
TOF	Time Of Flight
TOS	Total oxidant status
T_{Reg}	Regulatory T cell
TSE	Transmissible spongiform encephalopathy
USP	US Pharmacopeia
UV	Ultraviolet
UZ Ghent	Ghent university hospital
UZA	Antwerp university hospital
VCAM	Vascular cell adhesion molecule
XO	Xanthine oxidase
ZNA	Hospital network Antwerp

CHAPTER 1

GENERAL INTRODUCTION

Verlaet AAJ, Maasakkers CM, Hermans N, Savelkoul HFJ. Rationale for Dietary Antioxidant Treatment of ADHD. Nutrients (2018), 10(4).

Verlaet AAJ, Noriega DB, Hermans N, Savelkoul HFJ. Nutrition, immunological mechanisms and dietary immunomodulation in ADHD. Eur Child Adolesc Psychiatry (2014), 23(7).

Everyone is a genius. But if you judge a fish on its ability to climb a tree, it will live its whole life believing that it is stupid. – Albert Einstein

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PART 1

ATTENTION DEFICIT HYPERACTIVITY DISORDER

Attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterised by developmentally inappropriate levels of impulsive behaviour, hyperactivity and/or inattention [1, 2]. Though ADHD is a common disorder in childhood, adolescence and adulthood, this thesis focusses on childhood ADHD. Numerous associations, based upon literature, between potential aetiopathological factors and ADHD are mentioned throughout this thesis. However, it should be mentioned that such associations do not address causality and that not all associations are necessarily biologically relevant.

1.1 Diagnosis

1.1.1 History

The first detailed description of ADHD symptoms appeared in a *Lancet* publication in 1902, with symptoms mentioned being "aggression, defiance, emotionality, limited sustained attention, and deficient rule-governed behaviour" [3]. Up to the 1960s these symptoms were thought to be caused by an underdeveloped brain or by brain damage and they were indicated as "minimal brain dysfunction" and "minimal brain damage" [4]. Later, a more descriptive formulation of the symptoms without referring to a cause was used, like "attention deficit disorder with hyperactivity" (ADDH) in the Diagnostic and Statistical Manual of Mental Disorders (DSM), 3th edition (1980). It has however been questioned whether a clinical diagnosis can be based solely on behavioural criteria [5, 6].

1.1.2 Criteria

The DSM is used widely for ADHD diagnosis. The DSM-5 (2013) defines ADHD as a neurodevelopmental disorder with a specific, persistent and pervasive behavioural pattern, consisting of disruptive or developmentally inappropriate levels of impulsive behaviour, hyperactivity and/or inattention. ADHD symptoms can be divided into two major domains, inattention and hyperactivity-impulsivity, with at least six out of nine symptoms (Table 1.1) in one domain being required for diagnosis (five for patients aged 17 or older) [1, 2]. Symptoms should interfere with functioning or development and should result

in significant psychological, social, educational, and/or occupational impairment [1, 7]. The symptoms must have an onset before the age of twelve and persist for at least six months, with several symptoms in multiple settings, like in school and at home. In addition, ADHD symptoms should not occur exclusively during the course of schizophrenia or another psychotic disorder and should not be better explained by any other disorder with similar manifestations (e.g. personality disorder, mood disorder, anxiety disorder, or learning disability) [1, 2].

Similar symptoms of inattention, hyperactivity and impulsivity are described as Hyperkinetic Disorder by the International Classification of Diseases (ICD) [8, 9]. This thesis is however restricted to DSM terminology.

Table 1.1. Characteristic symptoms of inattention and hyperactivity-impulsivity [1, 2].

Inattention	Hyperactivity-impulsivity
1. Often does not follow through on instructions and fails to finish schoolwork, chores, or duties in the workplace (e.g. loses focus, side-tracked).	1. Often runs about or climbs in situations where it is not appropriate (adolescents or adults may be limited to feeling restless).
2. Often has trouble holding attention on tasks or play activities.	2. Often leaves seat in situations when remaining seated is expected.
3. Often does not seem to listen when spoken to directly.	3. Often fidgets with or taps hands or feet, or squirms in seat.
4. Often fails to give close attention to details or makes careless mistakes in schoolwork, at work, or with other activities.	4. Is often unable to play or take part in leisure activities quietly.
5. Often has trouble organising tasks and activities.	5. Is often "on the go" acting as if "driven by a motor".
6. Often avoids, dislikes, or is reluctant to do tasks that require mental effort over a long period of time (such as schoolwork or homework).	6. Often interrupts or intrudes on others (e.g. butts into conversations or games).
7. Often loses things necessary for tasks and activities (e.g. school materials, pencils, books, tools, wallets, keys, paperwork, eyeglasses, mobile telephones).	7. Often blurts out an answer before a question has been completed.
8. Is often easily distracted.	8. Often has trouble waiting his/her turn.
9. Is often forgetful in daily activities.	9. Often talks excessively.

Based on the number and kind of symptoms present, three types of ADHD occur:

1. Predominantly inattentive type (“attention deficit disorder”, ADD): at least six symptoms of inattention, but not hyperactivity-impulsivity, were present the past six months;
2. Predominantly hyperactive-impulsive type: at least six symptoms of hyperactivity-impulsivity, but not inattention, were present for the past six months;

3. Combined type: at least six symptoms of both inattention and hyperactivity-impulsivity were present for the past six months.

Each of these subtypes is associated with different sex ratios (e.g. patients with inattentive subtype being predominantly female), comorbidities (e.g. combined subtype being associated with more co-occurring psychiatric disorders) and forms of functional impairment (e.g. academic impairment mainly being related to inattentive problems) [1, 4].

1.1.3 Tools

The Child Behaviour Checklist (CBCL, Figure 1.1A) is one of the validated tools for the broad inventarisation of behavioural and emotional problems in childhood, including ADHD and various comorbidities [10, 11]. The Conners' Rating Scale (CRS, Figure 1.1B) can also be used to distinguish between children with and without ADHD or related behaviour and is often used in pharmacological research [12-14]. The ADHD rating scale (ADHD-RS, Figure 1.1C) is used as well, but might not perform equally across different populations [15]. Another tool is the Diagnostic Interview Schedule for Children (DISC, not shown), a structured interview to diagnose more than 30 psychiatric disorders in children and adolescents, such as ADHD and oppositional defiant disorder (ODD) [16]. In Belgium and The Netherlands (as well as later in this thesis), also the Social-Emotional Questionnaire (SEQ, see Chapter 2, 3.3.2) is used [17].

It is unclear which diagnostic tool, or combination of tools, should be preferred. In addition, though rating scales are very helpful in the diagnosis of ADHD, as well as for documenting a possible response to treatment, a valid diagnosis requires more than a simple checklist, as ADHD is not just a sum of symptoms [18, 19]. The diagnosis should be made by a trained health care professional, based on a parent and child interview, school information, psychological tests and physical examination [20, 21]. The confusion of ADHD with for instance mood disorders or learning disabilities illustrates the need for unequivocal diagnostic tools. Unfortunately, biological diagnostic (e.g. serum) markers for ADHD do not yet exist [22].

1.2 Prevalence

ADHD is the most common childhood-onset neurocognitive behavioural disorder and one of the most prevalent chronic paediatric conditions [23]. Different interpretations of DSM guidelines and different sources of information however result in different estimates of ADHD prevalence, ranging between 2

A

Please print **CHILD BEHAVIOR CHECKLIST FOR AGES 6-18** For office use only ID #

CHILD'S FULL NAME First Middle Last

CHILD'S GENDER ☐ Boy ☐ Girl | CHILD'S AGE | CHILD'S ETHNIC GROUP OR RACE

TODAY'S DATE Mo. ___ Day ___ Year ___ | CHILD'S BIRTHDATE Mo. ___ Day ___ Year ___

GRADE IN SCHOOL ______

NOT ATTENDING SCHOOL ☐

Please fill out this form to reflect your view of the child's behavior even if other people might not agree. Feel free to print additional comments beside each item and in the space provided on page 2. **Be sure to answer all items.**

PARENTS' USUAL TYPE OF WORK, even if not working now. (Please be specific — for example, auto mechanic, high school teacher, homemaker, laborer, lathe operator, shoe salesman, army sergeant.)

PARENT 1 (or FATHER) TYPE OF WORK ______

PARENT 2 (or MOTHER) TYPE OF WORK ______

THIS FORM FILLED OUT BY: (print your full name)

Your gender: ☐ Male ☐ Female

Your relation to the child: ☐ Biological Parent ☐ Step Parent ☐ Grandparent ☐ Adoptive Parent ☐ Foster Parent ☐ Other (specify)

I. Please list the sports your child most likes to take part in. For example: swimming, baseball, skating, skate boarding, bike riding, fishing, etc.

Compared to others of the same age, about how much time does he/she spend in each?

Compared to others of the same age, how well does he/she do each one?

	Less Than Average	Average	More Than Average	Don't Know	Below Average	Average	Above Average	Don't Know
☐ None								
a. ______	☐	☐	☐	☐	☐	☐	☐	☐
b. ______	☐	☐	☐	☐	☐	☐	☐	☐
c. ______	☐	☐	☐	☐	☐	☐	☐	☐

B

TEACHER'S RATING SCALE

* * *

Child's Name______________ **Date of Rating:**_______**AM PM Teacher's Name:**_______________

Please rate the child twice daily - once at the end of the morning and again at the end of the school day - for 5 consecutive days (not necessarily in the same week). Please base your ratings on current observations, not past impressions, etc. After the initial ratings, the form may be used again to monitor progress on a weekly or monthly basis. Thank you for your cooperation.

	Not at all	Just a little	Pretty much	Very much
Classroom Behavior				
Constantly fidgeting	0	1	2	3
Hums and makes other odd noises	0	1	2	3
Demands must be met immediately - easily frustrated	0	1	2	3
Coordination poor	0	1	2	3
Restless or overactive	0	1	2	3
Excitable, impulsive	0	1	2	3
Inattentive, easily distracted	0	1	2	3

C

ADHD Rating Scale-IV: Home Version

Child's Name:______________________ **Sex:** ☐ **M** ☐ **F** **Age:**_______ **Grade:**________

Completed by: ☐ Mother ☐ Father ☐ Guardian ☐ Grandparent

Circle the number that *best describes* your child's home behavior over the past 6 months.	Never or Rarely	Sometimes	Often	Very Often
1. Fails to give close attention to details or makes careless mistakes in schoolwork.	0	1	2	3
2. Fidgets with hands or feet or squirms in seat.	0	1	2	3
3. Has difficulty sustaining attention in tasks or play activities.	0	1	2	3
4. Leaves seat in classroom or in other situations in which remaining seated is expected.	0	1	2	3
5. Does not seem to listen when spoken to directly.	0	1	2	3

Figure 1.1. Example of (A) the Child Behaviour Checklist (CBCL), (B) Conners' Teacher Rating Scale (CTRS) and (C) ADHD rating scale (ADHD-RS).

and 18% of all children [24, 25]. According to DSM-4, the worldwide prevalence of ADHD in children and adolescents was estimated 5.9-7.1% in 2012 [26]. In Belgium, only ADHD prevalence in adulthood has been investigated (4.1%) [27]. The majority (50-75%) of patients is diagnosed with the combined subtype. The inattentive (20%-30%) and hyperactive-impulsive subtype (less than 15%) are less common. Symptoms and related impairment persist into adolescence and adulthood in 30-80% of the affected children [4, 28-30]. In addition, adult ADHD is characterised more often by inattention than by hyperactivity-impulsivity [4].

Rather than depending on culture, prevalence estimates vary greatly depending on the methodology used (e.g. diagnostic criteria related to the number and type of symptoms required, rating scales versus clinical interviews, etc.) [4, 9, 28]. For instance, prevalence estimates based on teacher reports alone are 5.47% higher than when based on the recommended procedure and those without requirement of impairment 2.32% higher than when impairment is required. In addition, prevalence rates based on DSM-4 are higher than those based on ICD-10 due to different requirements regarding symptoms in specific dimensions [9, 31, 32]. Methodology probably also accounts for the apparent increase of the overall ADHD prevalence [25, 28, 33]. For example, extension of the age-of-onset criterion from 7 (DSM-4) to 12 years (DSM-5) increased in the prevalence rate from 7.38% to 10.84% in children of 12 to 15 years old. Youth with a later age of onset was more likely to be from lower income and ethnic minority families, but did not differ regarding ADHD severity and patterns of comorbidity [34]. Results from a prospective birth cohort however suggest that the prevalence estimate is not affected by the extended age-of-onset criterion [35].

Finally, also sample characteristics affect prevalence estimates. ADHD is for example more frequently diagnosed in younger samples [4, 36]. In addition, the prevalence in boys is two to four times higher than in girls, though underdiagnosis due to the predominantly inattentive ADHD subtype (symptoms of which are less intruding) and less common disturbing comorbidities in girls could partly explain this observation [4, 37-39].

1.3 Comorbid conditions

Up to 65% of ADHD patients has one or more comorbid conditions [22]. In US paediatric ADHD patients, one comorbid condition was reported in 33%, two in 16% and three or more in 18% [40]. ODD (29% of ADHD patients) and conduct disorder (CD; 36%) are the most frequently reported comorbid psychiatric conditions in ADHD [41], but patients are also at higher risk for depression, anxiety, tics, bipolar disorder, learning disorders like dyslexia and dyscalculia, motor disorders, substance abuse and aggressive and criminal behaviour [24, 28, 40, 42-45]. On top of this, ADHD is associated with cognitive shortfalls, leading to memory difficulties and functional impairment [24]. Also autism frequently co-occurs with ADHD [46]. In addition, other physical symptoms like headache, bellyache, asthma, eczema, sleep disorders and enuresis are frequent complaints among patients [47-51].

1.4 Impact

Despite equivocal results, several studies found a reduction in quality of life due to ADHD [52]. This is not surprising, as the disorder affects several aspects in patients' everyday life [28]. Indeed, disruptive behaviour, distractibility and poor self-discipline and self-regulation can have a high impact on relationships, school or work performance and self-esteem [24]. In adolescence and adulthood, ADHD is associated with academic failure, unemployment and antisocial and criminal behaviour [53-55]. Furthermore, patients more frequently visit a general practitioner or specialist and are more often referred to special education [56-58]. This substantially increases demands for social and healthcare services, but also compromises professional productivity of parents [58-60]. Direct medical costs of ADHD patients and their mothers are 11 and 5 times higher, resp., than those of children without behavioural problems [59]. In addition, comorbid psychiatric disorders result in a more severe psychopathology and a higher risk of long-term impairment [40, 54, 61]. ADHD thus poses a considerable personal, social and financial burden to patients, their environment and society as a whole [28, 62].

1.5 Aetiology

ADHD is a complex and multifactorial disorder, influenced by genetic, biochemical, psychological and environmental factors. Dopaminergic and noradrenergic dysfunction are involved in ADHD [28], but also immunity and oxidative stress appear to play a role (see Chapter 1, part 3) [63-65]. Moreover, also nutrition plays a role in hyperactivity and attention disorders, at least in specific subgroups (see Chapter 1, Part 2) [66, 67]. However, no straightforward indication can yet be given about its exact pathophysiology, because factors involved are interchangeable and no individual factor is either necessary or sufficient to trigger ADHD [22, 28, 68-70]. Additive and interactive effects between risk factors increase the vulnerability to the disorder [28]. It is also unclear whether distinct ADHD subtypes and ADHD with and without certain comorbidities have a distinct aetiology [22], but heterogeneity might be a central factor in the clinical variability of the disorder (e.g. predominantly inattentive vs. predominantly hyperactive-impulsive type) [71].

1.5.1 Neurobiology

Catecholamines (e.g. epinephrine, norepinephrine and dopamine; monoamines derived from the amino acid tyrosine, Figure 1.2) are active in specific brain areas like the prefrontal cerebral cortex, caudate nucleus and cerebellum and therefore involved in the regulation of cognitive processes like

attention, impulse control, arousal and behaviour [72, 73]. Though the neurobiology underlying ADHD is not entirely clear, various associations have been observed between ADHD and structural or functional alterations in the brain, especially in these areas or catecholaminergic pathways [74].

Figure 1.2. Structure of catecholamines.

Structural alterations include a disturbed white matter microstructure, reduced grey matter density and reduced brain volume [74, 75]. Moreover, various brain areas, including the hippocampus, nucleus caudatus and amygdala, are smaller in patients than in controls [76]. These subcortical structures are involved in motor control, attention, reward and cognition [28, 74]. As these differences are more pronounced in children than in adults, this demonstrates delayed brain maturation in ADHD [76]. In addition, also a reduced blood flow to these areas was observed [24, 28, 77, 78].

Regarding functional alterations, imaging studies associated an underactivated frontoparietal network and overactivated regions in the visual, dorsal attention and default mode networks with ADHD [79, 80]. In addition, animal studies suggest that destruction of dopamine pathways in the prefrontal cortex results in hyperactivity [81]. Moreover, urinary epinephrine and norepinephrine levels were about 5 and 1.5 times higher in patients than in controls, with norepinephrine concentration correlating with hyperactivity and inattention [82, 83]. Moreover, ADHD medication affects catecholamine uptake or release (see Chapter 1, 1.6.1) [28, 73].

1.5.2 Genetic factors

Genetic factors play a dominant role in ADHD aetiology, with a heritability of up to 80%, based on adoption, twin and familial association studies [84]. Still, only a limited number of genes with small effect sizes have been repeatedly identified to be associated with ADHD, including genes involved in dopamine, norepinephrine and serotonin transmission and metabolism [64, 71, 84].

Genetic variations were for example found for the dopamine active transporter (*DAT*) gene. Activated neurons release dopamine and take it up again from the synaptic cleft by DAT, thereby stopping signal transduction. On average, the activity of this transporter is 14% higher in patients than in controls. As a result, time for dopamine to exert an effect in the synaptic cleft is significantly shorter in patients than in controls [69, 85]. In addition, dopamine receptors are involved, which are located on the

dendrite of the next neuron and signal within this neuron when dopamine is present in the synaptic cleft, thereby activating the potential. ADHD patients are characterised by a lower expression of the dopamine receptor D_4 (*DRD4*) gene in brain areas involved in attention. Consequently the signal is transduced less to the next neuron [69]. Other dopamine receptor genes related to ADHD are *DRD1*, *DRD2* and *DRD5* [84, 86]. Also the norepinephrine transporter gene 1 (*NET1*), responsible for norepinephrine reuptake, and the α-2A-adrenergic receptor (*ADRA2A*), regulating (nor)epinephrine release and function, as well as serotonin transporter and receptor genes *5-HTT/SLC6A4* and *HTR1B*, the dopamine β-hydroxylase gene and the synaptosomal-associated protein of 25 kDa (SNAP-25) gene, appear to play a role in ADHD [68, 69, 71, 87]. However, none of these genes has large effects [22, 28]. Specific ADHD susceptibility genes are thus still missing. In addition, the complexity of genetics in ADHD is exemplified by increased rates of copy number variants (CNVs, i.e. chromosomal duplications and deletions), not only in 16% of patients, but also in 7% of controls [68, 88]. It is also unclear whether distinct ADHD phenotypes have distinct genotypes [22]. Moreover, various genetic variants involved in ADHD also underly other neurodevelopmental and psychiatric disorders like schizophrenia and depression, as well as health risk behaviours such as smoking [89, 90].

1.5.3 Environmental factors

The contribution of environmental factors to ADHD is estimated to be 20-30% [22, 28].

Various perinatal environmental factors predispose to the development of metabolic, mental and behavioural disorders later in life [91-93]. Specifically, delivery complications, prematurity, hypoxia and prenatal alcohol and tobacco smoke are risk factors for ADHD (see also Chapter 1, 3.1.5 and 3.2.6) [28]. For example, ADHD patients were 3 times more likely to have had a low birth weight and 2.1 and 2.5 times more likely to have been exposed to cigarettes and alcohol, resp., *in utero* than controls, even after adjustment for potential confounding factors, like familial psychopathology [94, 95]. In primates, even moderate alcohol consumption during pregnancy was found to affect both attention and neuromotor functioning [96]. Moreover, various studies conclude that also postnatal secondhand cigarette smoke exposure increases attention deficit and hyperactive behaviour [97]. Also medical conditions of the mother (e.g. obesity, multiple sclerosis and asthma), season of birth (e.g. odds ratio of 4.5 for ADHD when born in September) and maternal inflammation are associated with ADHD [39, 91, 98]. Factors that could mediate the effect between maternal diseases and ADHD are for instance genetics, maternal medication intake and more contact with health care services [39]. Indeed, use of specific medications during pregnancy, like antiasthmatic drugs, acetaminophen (paracetamol), antipsychotics and selective serotonin reuptake inhibitors, is associated with an increased risk of birth

defects, hyperkinetic disorder and altered neurobehavioral development in offspring. Acetaminophen, used by more than half of all mothers while pregnant, may for instance act as a hormone disruptor, interfering with thyroid function, which is important for brain development [99-101]. Its use during pregnancy increased the risk for ADHD-like behaviour at the age of 7 (relative risk (RR): 1.13), with stronger associations with increasing frequency and not confounded by e.g. infection during pregnancy [99].

In addition, various studies point at an association between pre- and postnatal air pollution (e.g. traffic-related) and ADHD. Of particular concern are the polycyclic aromatic hydrocarbons (PAHs), but also lead and arsenic are present in air pollution [102, 103]. In fact, up to 30% higher blood lead levels were found in ADHD patients compared to controls [104, 105], associated with hyperactivity and impulsivity symptoms [105], though contradicted by another study [106]. Hair lead levels have been associated with physician-diagnosed ADHD and teacher ratings on inattention [107]. Lead is a neurotoxicant, affecting neurotransmitter pathways and thereby dysregulating for example the dopaminergic system and causing neurodevelopmental effects [102, 106, 108-110]. Other typical neurotoxicants are organophosphates or organochlorine compounds (e.g. polychlorinated biphenyls, PCBs), which are widely used as pesticides. Even children with rather typical levels of pesticide exposure (e.g. by eating pesticide-treated fruits and vegetables) have a higher risk of ADHD [111].

Moreover, an adverse psychosocial environment is a risk factor for ADHD symptom development. Examples are low social class, paternal criminality, maternal mental disorder, foster placement and family dysfunction [28].

The RR for ADHD, attention deficiency and hyperactivity decreased significantly with breastfeeding for 12-20 weeks (28 weeks for hyperactivity) in a prospective cohort study (e.g. RR for ADHD of 0.57 as compared to breastfeeding for less than two weeks) [112]. Indeed, ADHD children were breastfed significantly less often compared to healthy controls, even after correcting for variables like maternal education. Breastfeeding could protect against ADHD due to enhanced stimulation of cognitive development. However, mothers could also be less willing or able to breastfeed a restless child [113].

Other environmental factors that have been mentioned with respect to ADHD (see Chapter 1, Part 2) are lesions to the prefrontal cortex, food additives (e.g. specific artificial food colours and benzoate preservative), a western dietary pattern (i.e., higher intake of saturated fat, refined sugars and sodium, and lower intake of omega-3 fatty acids, fibre and folate) and elevated or reduced levels of specific micronutrients (e.g. magnesium or zinc, see Chapter 1, 2.1) [22, 24, 114, 115].

1.6 Treatment

Treatment for ADHD aims at reducing symptoms in order to improve patient functioning [116]. The combination of behavioural and pharmacological therapy appears the most beneficial form of treatment, also in case of comorbid disorders [28, 117]. Though pharmacological treatment is generally only advised in case of severe symptoms and impairment that warrant direct medication and when psychological and behavioural interventions are insufficient [20], currently, the main treatment for ADHD is pharmacological, using stimulant drugs such as methylphenidate (MPH, e.g. Ritalin®, Equasym®, Medikinet®) and dexamphetamine (e.g. Dexedrine) or non-stimulant drugs like atomoxetine (e.g. Strattera®) [28, 116]. MPH is the most frequently used medication for ADHD, with an increase in daily doses for patients of 6-12 years from 1.5 million to 2.2 million between 2006 and 2016 in Belgium [118].

1.6.1 Mechanism of action

Psychostimulants like MPH are indirectly working sympathomimetics, binding competitively with the presynaptic dopamine transporter and to a lesser extent with the norepinephrine transporter, especially in the prefrontal cortex and striatum (Figure 1.3A). This leads to decreased presynaptic reuptake of dopamine and norepinephrine, increased concentrations of these catecholamines in the synaptic cleft, and thereby increased neurotransmission. In addition, MPH prevents excessive accumulation of dopamine in the cytoplasm of cells. It thereby reduces dopamine neurotoxicity and thus has neuroprotective effects. MPH decreases ADHD symptoms in up to 80% of patients [119, 120].

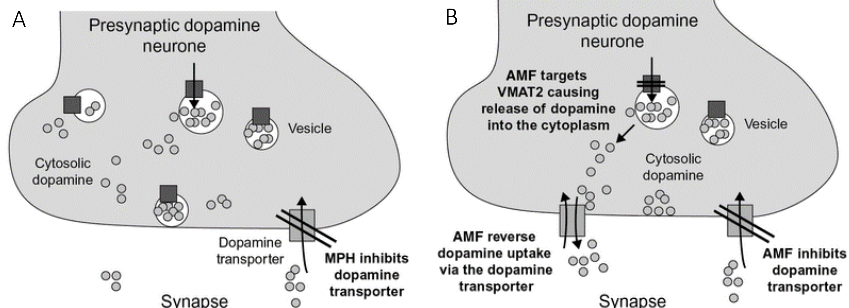

Figure 1.3. Mechanism of action of (A) methylphenidate (MPH) and (B) amphetamine (AMF) [121].

Like MPH, dexamphetamine binds competitively with the presynaptic transporter, but additionally stimulates catecholamine release (Figure 1.3B) and inhibits monoamine oxidase (MAO), the enzyme involved in norepinephrine and dopamine metabolism. It is therefore prescribed when MPH is insufficiently effective [121-123]. Atomoxetine selectively inhibits reuptake of norepinephrine in the

prefrontal cortex, but is generally less effective in reducing ADHD symptoms [123]. Due to their effects on dopamine release, opioids including codeine, an ingredient in e.g. specific cough syrups, could be effective in ADHD as well [124]. Nevertheless, their putative therapeutic effects in ADHD have been insufficiently studied.

1.6.2 Drawbacks

Although the benefits of MPH until now outweigh the risks, a meta-analysis of randomised clinical trials confirmed non-serious adverse effects like insomnia and decreased appetite in 25% of patients treated with MPH [125, 126]. Headache, stomach ache and irritability are other common side effects, in addition to indications of stunted growth [116, 119, 126]. Psychotic symptoms and mood disorders are reported as side effects in 0.25% [127, 128]. Moreover, MPH may induce transient T helper (T_H) cell suppression and hypergammaglobulinaemia (immunoglobulin (Ig) E and IgG), and could thereby disturb immune maturation and aggravate atopic diseases [129, 130]. In addition, as MPH increases heart rate and blood pressure, caution is advised regarding its use, especially in those with a family history or other known risk factors for cardiovascular disease [131, 132]. Nevertheless, a meta-analysis concluded that there is no evidence of MPH being associated with an increase in serious adverse events (SAEs) [125].

Moreover, neurotransmitters enable synaptic communication, but are also involved in central nervous system (CNS) development, including morphogenesis and the proliferation, migration and differentiation of neurons. Therefore, any interference with neurotransmitter systems potentially produces acute and/or long-lasting alterations in CNS structure and function [133].

In addition, publication bias in reported efficacy of MPH possibly exists, while evidence of long-term efficacy is lacking [126]. Possibly, part of the efficacy of the medication is a result of the extra attention that has been given to the child or a placebo response. Furthermore, non-adherence to therapy is high and parents are disinclined to use MPH [116, 134, 135], stating that “they are unwilling to accept the premise that medication is the only viable treatment option for their child” [136]. Finally, until now, full symptom reduction in ADHD patients is yet to be achieved and the burden of ADHD-associated consequences, like effects on academic performance, has not been diminished. Other therapeutic options are therefore warranted [116, 134, 137].

PART 2

NUTRITION AND BEHAVIOUR

Not only medication, but also for instance various foods contain psychoactive substances. One example is caffeine, a well-known stimulant found in e.g. coffee and tea. Mixed effects of caffeine on ADHD symptoms have been found. As MPH and amphetamines are generally more effective, caffeine is not basically recommended for ADHD [138-140]. Other examples are alcohol, affecting the prefrontal cortex, and nicotine, acting as an indirect dopamine agonist and thereby improving attention and arousal [141, 142]. However, as this thesis focusses on childhood ADHD, alcohol and nicotine will not be discussed further.

The occurrence of adverse physical reactions to foods (e.g. eczema or gastrointestinal (GI) disturbances) in combination with the high comorbidity of aberrant behaviour and physical complaints, stimulated speculations that foods might not only affect organs like the skin and GI tract. Food might also have an impact on the brain, resulting in behavioural effects, especially in (genetically) vulnerable subjects. Based on these speculations, avoidance of offending foods could lead to a decrease of symptoms [64, 143-145]. Next to adverse reactions to foods and food additives, altered levels of specific micronutrients and essential fatty acids (EFAs) have also been described in children with ADHD as compared to controls or as a risk factor for paediatric ADHD development and are therefore implicated in behaviour [24, 144]. Various mechanisms could underlie behavioural responses to food, like immunological mechanisms as well as direct effects on neurotransmitter systems [13, 64, 146].

Nutritional approaches could thus be important in controlling ADHD symptoms. However, this theory remains controversial and clinicians do not attach great importance to environmental factors, such as exposure to foods, in the diagnosis of ADHD [64, 145]. Nevertheless, research has been conducted regarding alternative therapies for ADHD, including specific diets and food supplements (e.g. iron and zinc).

2.1 Micronutrients

The micronutrients discussed below are those hypothesised most frequently to be involved in ADHD, though these associations do not appear driven solely by dietary micronutrient inadequacy [147]. Studies on specific micronutrients are scarce, so that overall insufficient evidence exists regarding deficiencies or beneficial effects of supplementation, except for reduced ferritin, magnesium, vitamin D and zinc levels [24, 145]. In addition, the variety of (micro)nutrients that appear to be involved in ADHD illustrates that administration of multi-ingredient formulas might be more beneficial [148-150].

2.1.1 Calcium

Despite relatively small differences (2% on average), mean serum calcium levels were significantly lower in paediatric ADHD patients than in healthy controls in two studies [151, 152]. Two other studies however found no significant difference [153, 154]. Calcium not only plays a role in proper bone formation and muscle contraction, but also in neurotransmitter release from neurons [155].

2.1.2 Copper

Plasma copper concentrations tended to be higher in ADHD patients than in controls, but this difference was not statistically significant [106]. Copper is involved in catecholamine metabolism via dopamine β-hydroxylase and MAO, with higher copper concentrations leading to lower dopamine levels in rats [106, 156].

2.1.3 Iron

Serum ferritin levels were found to be significantly lower in paediatric ADHD patients than in control subjects, with differences between 20 and 50% and abnormal levels (< 30 ng/ml) in e.g. 84% of children with ADHD and 18% of controls [83, 152, 157-160]. Moreover, serum ferritin was found to inversely correlate with ADHD symptom scores [161] and one study also found lower transferrin levels in ADHD [162]. However, other studies including a large population-based study found no association between peripheral ferritin concentrations and ADHD symptoms [163, 164]. In addition, though iron supplementation improved attention and behaviour significantly in non-anaemic paediatric ADHD patients with low serum ferritin levels [165], results of studies on iron supplementation in ADHD are overall equivocal [108, 166-168]. Iron is involved in the structure and function of the CNS. It acts for example as cofactor for tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis and has neuroprotective effects against lead exposure [24]. Moreover, iron deficiency was suggested to decrease the effectiveness of psychostimulant treatment [166].

2.1.4 Magnesium

Magnesium serum levels were found to be deficient in for example 33.6% of ADHD patients compared to 15.8% of controls, and hair levels in 77.6% compared to 20.7% [152, 159, 169, 170]. One study reported higher serum magnesium levels in patients as compared to controls [171]. Saliva and hair levels of magnesium are reported to be lower in patients than in controls [169, 172]. Magnesium is involved in e.g. synaptic signal transduction and blood flow [159, 172, 173].

2.1.5 Phosphorus

Mean serum phosphorus levels were significantly lower (on average 5%) in paediatric ADHD patients than in healthy controls in two studies [151, 152], while two other studies found no significant difference [153, 154]. Phosphorus is involved in bone formation, while phosphates (compounds containing PO_4^{3-}) are a component of DNA, ATP and phospholipids [155].

2.1.6 Vitamin A

Plasma levels of retinol and its precursor β-carotene were not significantly different between paediatric ADHD patients and controls [174]. Vitamin A is essential for embryonic development, including the heart and CNS. Inadequate vitamin A status during pregnancy was found to be involved in some paediatric congenital abnormalities [175].

2.1.7 Vitamin B12

One study found lower serum vitamin B12 levels in child and adolescent ADHD patients as compared to controls (371 ± 156 pg/ml vs. 429 ± 174 pg/ml, resp.). Significance was only assessed (and confirmed) for the difference between ADHD patients, autism patients and controls [176]. Also called cobalamin, this water-soluble vitamin has a key role in the synthesis of myelin and is involved as a cofactor in DNA synthesis and fatty acid metabolism [177, 178].

2.1.8 Vitamin D

Inverse associations between umbilical cord [179] and maternally circulating vitamin D levels during pregnancy [180, 181] and ADHD symptoms in their children were found, suggesting a protective effect of prenatal vitamin D. Other studies however found no association between maternal vitamin D status and ADHD in offspring, though this might be due to their low statistical power [182, 183]. Serum vitamin D levels were significantly lower (about 30%) in paediatric ADHD patients compared to controls [151-154, 159, 176, 184-188], with a deficiency in e.g. 73.9 % of patients and 51.4% of controls [184]. Lower

sunlight exposure possibly causes this difference, as the prevalence of ADHD is lower in areas with a higher solar intensity [184, 189]. However, other studies reported no significant association between ADHD and vitamin D level [190, 191], though after adjustment for confounders, vitamin D levels correlated significantly with attention deficit [191]. In addition, vitamin D supplementation in ADHD patients with vitamin D deficiency caused improvements in inattention, hyperactivity and impulsivity [188] and also lower serum vitamin D receptor levels have been observed [154]. A recent systematic review supports the relation between vitamin D deficiency and ADHD, but overall effect sizes are small [192]. Next to its effects on bone and muscle strength, this lipid soluble vitamin has a beneficial effect on intestinal absorption of minerals, such as calcium and phosphorus, though conflicting results have been published on both. In addition, it is a neurosteroid involved in neuronal differentiation and catecholamine regulation, while it regulates Ca^{2+} and redox signalling pathways which play key roles in development [193-195].

2.1.9 Vitamin E

Significantly higher (± 30%) plasma α- and γ-tocopherol levels were found in paediatric ADHD [174]. Vitamin E is essential for the development and maintenance of the human nervous system [196].

2.1.10 Zinc

ADHD patients displayed on average 10-40% lower zinc levels in serum, plasma, erythrocytes, hair, urine and nails, possibly related to symptom severity [69, 83, 106, 159, 197-200]. One publication mentions higher hair zinc levels in ADHD [201]. Zinc levels below the normal range were found in up to 30% of patients compared to 0-3% of controls [202, 203]. In animals, an association has been found between zinc deficiency and aggressive behaviour, anxiety and low levels of attention [204]. Zinc supplementation as monotherapy can be beneficial, though especially in case of deficiency, while in combination with stimulant medication, zinc supplementation reduces the optimal medication dose [145, 167, 205, 206]. Zinc is essential for CNS structure and function, including neuronal development, blood-brain barrier (BBB) integrity and dopamine transport (i.e. the dopamine transporter is regulated by Zn^{2+}) [24, 106, 109]. Overall, zinc deficiency is associated with neurologic dysfunction [204].

2.2 Omega-3 fatty acids

Most research investigating the effect of nutrition on ADHD is undoubtly related to omega-3 (ω-3) fatty acids, polyunsaturated fatty acids (PUFAs) with a double bond at the third carbon-atom counted from

the methyl end, which could be linked to ADHD because of their importance in brain development and function, including neurotransmitter and cerebral vascular function [24, 207, 208]. Docosahexaenoic acid (DHA; 22:6 ω-3, Figure 1.4) is abundant in the brain, comprising up to 40% of fatty acids [209]. DHA is involved as a structural component for neuronal membranes and in nerve cell myelination [109, 210, 211]. DHA precursor eicosapentaenoic acid (EPA; 20:5 ω-3) represents less than 1% of the fatty acids in the brain [209]. EPA acts as a second messenger in neurotransmitter systems and is also a precursor for eicosanoids. These substances are involved in the regulation of blood flow, ion channels, synaptic transmission, apoptosis and many other biological processes [210].

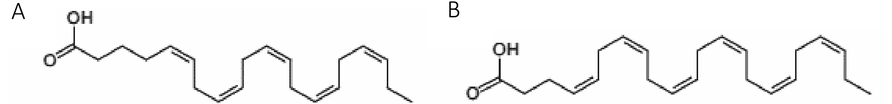

Figure 1.4. Structure of (A) eicosapentaenoic acid (EPA) and (B) docosahexaenoic acid (DHA).

ω-3 fatty acids are considered essential fatty acids. Though EPA and DHA can be metabolised from α-linolenic acid (ALA; 18:3 ω-3), this is a limited and inefficient process. Dietary sources are thus crucial [209]. Indeed, preclinical studies show that removing ω-3 fatty acids from the diet results in abnormal dopaminergic regulation and cognitive deficits, including impairments in attention and learning [209, 212].

Significantly lower plasma and membrane concentrations of PUFAs and especially of ω-3 fatty acids have been found in ADHD, suggesting that supplementation can reduce ADHD symptoms [174, 207-209, 213]. For instance, a 30% lower concentration of ω-3 fatty acids was found in erythrocyte membrane phospholipids in patients versus controls [214]. Still, another study found that EFA status was unchanged in ADHD [174]. Nevertheless, despite consumption of similar amounts of ω-3 and ω-6 PUFAs, a higher ratio of ω-6/ω-3 PUFAs (arachidonic acid (AA)/EPA) has been observed in patients compared to controls (e.g. pooled mean difference for the ω-6/ω-3 ratio between children and youth with ADHD and controls of 1.97) [207, 215, 216]. Therefore, altered PUFA metabolism was suggested in ADHD. Indeed, an association between the fatty acid desaturase 2 (*FADS2*) gene and ADHD has been described, indicating an impairment in fatty acid metabolism [145, 217]. In addition, low ω-3 PUFA levels were associated with decreased plasma levels of magnesium and zinc, which are cofactors in the desaturase enzymes for the conversion of ALA to EPA and DHA [174].

Despite numerous studies, evidence on a potential beneficial effect of ω-3 supplementation on ADHD is limited. Multiple reviews and meta-analyses report small, beneficial effects of ω-3 PUFAs (sometimes in combination with ω-6 PUFAs) on inattention, hyperactivity, cognition and/or ADHD overall [167, 213,

218-221], but others do not support a therapeutic effect [145, 168, 208, 219, 222, 223]. A meta-regression analysis including 18 studies with over 1500 participants found effects on inattention, but not on hyperactivity or impulsivity [209, 224]. In addition, especially the duration and composition of supplementation appear of influence on the behavioural effect, with EPA doses of at least 500 mg/day or the combination of ω-3 with ω-6 PUFAs (γ-linolenic acid (GLA; 18:3 ω-6) and EPA, possibly with antioxidants like vitamin C or E) generating the most promising results [24, 28, 213, 224-226]. Trials with only DHA or an intervention period of less than three months are less effective [167]. Possibly, ω-3 PUFAs are more effective in case of learning problems than in case of ADHD [227]. Future research should address weaknesses in this area, including small sample sizes, variability of selection criteria, supplementation type and dosage, potentially active placebos and short follow-up periods [208, 224]. Finally, evidence of publication bias limits the generalisability of these findings [224].

2.3 Food additives

Besides dietary supplementation, eliminating certain foods or food components has potentially beneficial outcomes in ADHD. Though results have been inconsistent and studies have various limitations (e.g. imperfect blinding and non-standardised outcome measures), a meta-analysis of double-blind placebo-controlled trials supports that artificial food colours (AFC) promote hyperactivity in hyperactive children [228], though the extent of the exacerbation varies between patients [229-231]. A population-based study on AFC and sodium benzoate (a preservative) including 277 3-year-olds indeed found a substantial effect on hyperactivity, but independent of pre-existing hyperactivity or atopy. It was not determined whether the AFC, preservative, or both engendered the effect [232]. Nevertheless, there is enough evidence to support that a subgroup of children with ADHD (about 10%) can improve on a diet free of the preservative sodium benzoate (E211) and the AFC tartrazine (E102), quinoline yellow (E104), sunset yellow (E110), carmoisine (E122), ponceau 4R (E124) and allura red (E129) (Figure 1.5) [230, 233]. In addition, the European Parliament decided (ordinance 1333/2008) that if a food product contains one or more of the mentioned AFC, labelling must include the statement that it 'may have an adverse effect on activity and attention in children' [144, 234, 235].

Both dose-response effects and effects on a general population were observed [232, 236]. Histamine is involved as an intermediate, as the central histamine H3 receptor is involved in hyperactivity and dopamine release in the frontal cortex, as polymorphisms in the histamine N-methyl transferase (*HNMT*) gene, impairing histamine clearance, were found to affect the behavioural responses to food additives, and as a food additive challenge causes non-IgE-dependent histamine release from

circulating basophils [229]. In addition, food colourings can alter concentrations of micronutrients, for example causing zinc deficiency in extreme cases, which has been linked to ADHD (see Chapter 1, 2.1.10) [72, 109]. However, it thus appears that food additives may cause behavioural effects in a general population of children, rather than specifically in ADHD patients [232, 236].

Figure 1.5. Chemical structure of (A) sodium benzoate (E211), (B) tartrazine (E102), (C) quinoline yellow (E104), (D) sunset yellow (E110), (E) carmoisine (E122), (F) ponceau 4R (E124) and (G) allura red (E129).

2.4 Food allergy and intolerance

A large variety of foods and food components can provoke or exacerbate behavioural responses, though not every child responds to the same products in a similar manner [143, 144, 231, 237-239]. A diet excluding not all provoking foods might have limited or no effect. This is possibly the reason why specifically adapted diets (e.g. Feingold or gluten-free diet) generally lack statistically significant results [237, 240, 241].

The few foods diet, also known as oligoantigenic or restricted elimination diet (RED), aims to eliminate all provoking foods from the diet. This diet is supported by the theory that hyperactivity can be (caused by) a food allergy, at least in some children [48, 242]. A few double-blind studies evaluated the effect of such dietary restrictions, eliminating a wide range of foods including for instance cow's milk, chocolate and wheat [237]. Though characterised by small participant groups, they show a significant benefit in a subgroup of ADHD patients [143, 144, 231, 237-239]. Depending on the extensiveness and duration of the elimination and population characteristics, 24-82% of subjects achieved significant improvements upon a RED diet [237, 238], with a large effect size of 0.80 found in a meta-analysis of five double-blind placebo-controlled RED studies [47, 48, 237-239, 242].

Though diagnosing food sensitivity is complex, a 2 to 3 week individually constructed RED seems justified in selected patients who are willing to make the effort [143, 145]. In 2001, RED was included

in a basic treatment plan for ADHD in the UK, at least for cases with an indication of dietary involvement [243]. Still, diet is generally not considered part of ADHD therapy and RED remains a controversial dietary intervention [18, 24, 28, 36, 134, 143-145].

PART 3

OXIDATIVE STRESS AND IMMUNITY IN ADHD

3.1 Oxidative stress

Reactive oxygen species (ROS) are oxygen-derived radicals and non-radicals that are oxidising agents and/or easily converted into radicals [244]. Similarly, reactive nitrogen species (RNS) are derived from nitrogen. Different mechanisms generate ROS and RNS *in vivo*. Mitochondria are the most important source of ROS, generated during energy production, but also enzymatic mechanisms, exposure to ultraviolet (UV)-radiation, pollutants, cigarette smoke and the immune system contribute substantially. Low levels of ROS are involved in regulating gene expression and signalling pathways, among other functions, and are therefore required for normal cellular function. However, as radicals are highly reactive molecules which can damage carbohydrates, nucleic acids, lipids and proteins and thereby alter their functions, an excess of ROS can damage cells [245-248].

Multiple mechanisms exist to reduce ROS accumulation. First, intracellular ROS formation is prevented by creating a high oxygen gradient between intracellular and extracellular compartments. In addition, free radicals can be detoxified by non-enzymatic and enzymatic antioxidants [249]. Other mechanisms exist to repair, in a direct and indirect way, oxidative damage to cellular structures. During direct repair, specific enzymes reduce oxidised products, such as proteins. During indirect repair, the damaged part is recognised and eliminated [250].

The shift in the balance between oxidants and antioxidant mechanisms in favour of oxidants is termed “oxidative stress”. High levels of oxidative stress can generate damage to e.g. lipids, proteins and DNA, thereby for example altering signal transduction and gene expression, inhibiting protein function and promoting cell death [251, 252]. More specifically, oxidative stress can impair neuronal proliferation and mediate apoptosis, and therefore lead to progressive neuronal damage and deterioration of normal cerebral functions [63, 72, 253, 254]. Biomarkers of oxidative damage are more reflective of the actual oxidative stress situation compared to antioxidant levels, since even relative high antioxidant levels can still be too low to balance high oxidant levels. In addition, due to their reactivity and thus short lifetime, measurement of ROS themselves is not feasible *in vivo* [108].

3.1.1 Indications from other psychiatric disorders

Numerous studies have indicated increased oxidative stress in various psychiatric disorders, like autism [255], obsessive-compulsive disorder (OCD) [256], depression [257], bipolar disorder and schizophrenia [258], and point at the potential use of antioxidants as therapeutic agent. For example, the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx), and malondialdehyde (MDA), a biomarker of lipid peroxidation, seem implicated in the pathogenesis of schizophrenia. SOD is a class of enzymes neutralising the unstable $O_2^{-\bullet}$ to the more stable H_2O_2, releasing O_2. GPx, a selenium-dependent enzyme, catalyses the reduction of H_2O_2 and peroxides (ROOH) resp. to water and alcohols (ROH), while converting GSH into GSSG [259]. In addition, patients with OCD had significantly higher MDA levels and lower antioxidant enzyme activities [249], while antidepressant treatment in panic disorder decreased xanthine oxidase (XO) levels, a ROS generating enzyme and important source of free radicals, and increased SOD activity [249, 260]. Plasma levels of reduced glutathione (GSH), the major intracellular antioxidant, were significantly lower in autism patients than in controls, as were cysteine and cysteinylglycine, metabolic precursors of GSH, suggesting insufficient GSH synthesis. In addition, levels of glutathione disulphide (GSSG, oxidised glutathione) were increased [261, 262]. Like ADHD, autism is a complex neurodevelopmental disorder with a strong genetic component in which a defective dopamine neurotransmission plays a role. ADHD and autism co-occur with a high frequency [46, 263, 264].

3.1.2 Oxidants and oxidative damage in ADHD

Various studies point at elevated levels of oxidative damage in ADHD (Table 1.2). Methodological differences might underlie contradictive results [85, 174, 264].

For instance, increased lipid peroxidation in ADHD is evidenced by raised urinary acrolein-lysine levels as well as by exhaled ethane levels, a marker of PUFA oxidation [265, 266]. Results on MDA are inconsistent, with higher plasma levels [264] as well as lower plasma and serum levels in paediatric ADHD patients as compared to controls [85, 174]. MDA levels did not correlate with ADHD subtype [264]. Total deoxyribonucleic acid (DNA) damage in ADHD as compared to controls was increased as determined by the concentration of 8-oxo-7,8-dihydroguanine (8-oxoG, the oxidised product of a free guanine base) in lymphocytes, [267-269], but reduced as determined by the concentration of 8-hydroxy-2'-deoxyguanosine (8-OHdG, the oxidised product of deoxyguanosine) in serum [85]. No significant differences are reported regarding protein oxidation end-products as measured by levels of exhaled butane [266].

Table 1.2. Levels of oxidative stress markers in ADHD patients as compared to controls.

Marker	Sample	Compared to control	Paper	Number of participants	Mean age of participants (years)
8-OHdG	Serum	↓	[85]	A = 30, C = 30	A = 8.7, C = 9.1
8-oxoG	Lymphocytes	↑	[267]	A = 61, C = 56	11.5
Acrolein-lysine	Urine	↑	[265]	A = 10, C = 73	A = 11, C = 10
AOPP	Plasma	=	[85]	A = 30, C = 30	A = 8.7, C = 9.1
Butane	Exhaled air	=	[266]	A = 10, C = 12	A = 10.3, C = 10.8
Ethane	Exhaled air	↑	[266]	A = 10, C = 12	A = 10.3, C = 10.8
MDA	Plasma	↑	[264]	A = 35, C = 35	A = 10.0, C = 10.2
	Serum	↓	[85]	A = 30, C = 30	A = 8.7, C = 9.1
	Plasma	↓	[174]	A = 37, C = 35	A = 9.0, C = 5.5
NO	Plasma	↑	[264]	A = 35, C = 35	A = 10.0, C = 10.2
	Plasma	=	[270]	A = 45, C = 45	A = 9.2, C = 9.2
	Serum	↓	[271]	A = 30, C = 51	9
NOS	Serum	↑	[63]	A = 35, C = 35	A = 10.0, C = 10.2
OSI	Plasma	↑	[70]	A = 56, C = 62	A = 9.3, C = 9.7
	Plasma	↑	[253]	A = 48, C = 24	A = 9.9, C = 10.0
	Serum	↑	[272]	A = 76, C = 78	A = 11.2, C = 12.2
TOS	Plasma	↑	[70]	A = 56, C = 62	A = 9.3, C = 9.7
	Plasma	↑	[253]	A = 48, C = 24	A = 9.9, C = 10.0
	Serum	↑	[272]	A = 76, C = 78	A = 11.2, C = 12.2
XO	Serum	↑	[63]	A = 35, C = 35	A = 10.0, C = 10.2

↑: increased as compared to controls; ↓: decreased as compared to controls; =: no difference as compared to controls; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; 8-oxoG: 8-oxo-7,8-dihydroguanine; A: ADHD; AOPP: advanced oxidation protein products; C: controls; MDA: malondialdehyde; NO: nitric oxide; NOS: nitric oxide synthase; OSI: oxidative stress index; TOS: total oxidant status; XO: xanthine oxidase.

Higher serum XO activity was observed in paediatric ADHD patients, though not related to ADHD subtype [63]. In addition, NO levels and nitric oxide synthase (NOS) activity were higher in ADHD patients than in controls, but while NOS activity was positively correlated with teacher hyperactivity ratings, ADHD subtype did not correlate with NO level or NOS activity [63, 264]. NOS inhibitor treatment improved rat hyperactivity and inattention [273]. However, also reduced NO levels as well as no difference have been reported [270, 271]. NO is a vasodilator and is also implicated in various physiological functions such as norepinephrine and dopamine release, memory and learning [274-276]. However, as a ROS, it can damage a wide range of biological molecules [63, 271].

Finally, higher total oxidant status (TOS, representing the cumulative effect of all oxidants present in the investigated sample) and oxidative stress index (OSI) were found in ADHD [70, 253, 272].

3.1.3 Antioxidants in ADHD

Plasma and saliva activity levels of the antioxidant enzymes glutathione-S-transferase (GST), GPx, SOD and catalase (CAT) were significantly lower in children and adolescents with ADHD as compared to controls (Table 1.3), indicating lower antioxidant activity [63, 158, 264, 277, 278]. Still, though GPx activity levels were higher in patients with no family history of ADHD [264], antioxidant enzyme activity overall did not correlate with ADHD subtype [63, 264]. Other studies however reported higher GST activity in ADHD [279] and no significant differences in SOD and CAT activity [264, 279]. Significant associations were observed between specific GST polymorphisms and ADHD subtypes, but not with overall ADHD incidence [280]. GST conjugates GSH with xenobiotic substrates or toxic endogenous compounds (e.g. reactive peroxidised lipids) [158]. CAT is a haem containing intracellular enzyme present in all aerobic cells, catalysing the breakdown of H_2O_2 to water and oxygen [259]. Evidence concerning thiol levels is conflicting, as lower, equal and higher levels in the plasma and serum of ADHD patients have been reported as compared to controls [70, 85], and higher in saliva [172]. Moreover, urinary epinephrine and norepinephrine concentrations were not only found to correlate positively with the degree of hyperactivity of ADHD children, but also with plasma GSSG levels [82]. Other studies found no differences regarding selenium, caeruloplasmin, retinol and β-carotene levels between patients and controls [106, 172, 174], or even higher plasma α- and γ-tocopherol levels in ADHD patients [174]. The hydroxyl group on the chromanol ring of tocopherols is responsible for their effective antioxidant action [281]. In a lipophilic environment and especially in cell membranes, vitamin E works as a chain breaking antioxidant, preventing the propagation of free radical damage by scavenging peroxyl radicals, forming a lipid hydroperoxide and tocopheroxyl radical. It is the principal membrane antioxidant in mammalian cells, guaranteeing cell membrane integrity [281-283].

Finally, though several studies report a lower total antioxidant status (TAS, representing the cumulative effect of all antioxidants present in the investigated sample) in paediatric ADHD as compared to reference values [267] and as compared to controls [253, 272, 278], also higher TAS and no difference have been reported [70, 279]. Moreover, one study reported statistically significant decreased salivary antioxidant activity (AOA) only in the combined and the hyperactive-impulsive ADHD subtypes as compared to normal subjects [278].

It should be mentioned that higher antioxidant levels might be attributed to a reactive increase to protect against more oxidative stress [70, 85, 158, 264]. Again, methodological differences might underlie contradictive results, e.g. analytical methods, participant selection criteria or analysed matrix.

Table 1.3. Levels of antioxidants or activity of antioxidant enzymes in ADHD patients as compared to controls.

Marker	Sample	Compared to control	Paper	Number of participants	Mean age of participants (years)
AOA	Saliva	=	[278]	A = 47, C = 35	A = 8.7, C = 9.0
β-carotene	Plasma	=	[174]	A = 37, C = 35	A = 9.0, C = 8.5
CAT	Plasma	=	[279]	A = 40, C = 35	A = 10.3, C = 9.9
	Plasma	=	[264]	A = 35, C = 35	A = 10.0, C = 10.2
	Plasma	↓	[158]	A = 30, C = 30	A = 7.4, C = 7.5
	Saliva	↓	[278]	A = 32, C = 35	A = 9.0, C = 8.7
Caeruloplasmin	Saliva	=	[172]	A = 20, C = 20	A = 9, C = 9
GPx	Plasma	↓	[264]	A = 35, C = 35	A = 10.0, C = 10.2
	Plasma	↓	[158]	A = 30, C = 30	A = 7.4, C = 7.5
GST	Plasma	↑	[279]	A = 40, C = 35	A = 10.3, C = 9.9
	Serum	↓	[63]	A = 35, C = 35	A = 10.0, C = 10.2
	Plasma	↓	[158]	A = 30, C = 30	A = 7.4, C = 7.5
Retinol	Plasma	=	[174]	A = 37, C = 35	A = 9.0, C = 8.5
Selenium	Plasma	=	[106]	A = 58, C = 50	A = 9.4, C = 8.9
SOD	Plasma	=	[264]	A = 35, C = 35	A = 10.0, C = 10.2
	Plasma	↓	[158]	A = 30, C = 30	A = 7.4, C = 7.5
	Serum	↓	[277]	A = 22, C = 20	A = 10.2, C = 12.8
TAS	Plasma	↑	[279]	A = 40, C = 35	A = 10.3, C = 9.9
	Plasma	↓	[267]	A = 61, C = 62	11.5
	Plasma	=	[70]	A = 56, C = 62	A = 9.3, C = 9.7
	Saliva	↓	[278]	A = 32, C = 35	A = 9.0, C = 8.7
	Serum	↓	[272]	A = 76, C = 78	A = 11.2, C = 12.2
Thiols	Saliva	↑	[172]	A = 20, C = 20	A = 9, C = 9
	Serum	↑	[284]	A = 90, C = 65	A = 10.3, C = 10.9
	Plasma	↓	[70]	A = 56, C = 62	A = 9.3, C = 9.7
	Serum	=	[85]	A = 30, C = 30	A = 8.7, C = 9.1
Tocopherol	Plasma	↑	[174]	A = 37, C = 35	A = 9.0, C = 8.5

↑: increased as compared to controls; ↓: decreased as compared to controls; =: no difference as compared to controls; A: ADHD; AOA: antioxidant activity; C: controls; CAT: catalase; GPx: glutathione peroxidase; GST: glutathione-S-transferase; SOD: superoxide dismutase; TAS: total antioxidant status.

3.1.4 ADHD therapy

MPH seems to protect against *in vitro* oxidative DNA and lipid damage and hypoxia-induced mitochondrial damage [285, 286]. However, acute MPH treatment of young rats decreased SOD activity in the cerebral prefrontal cortex and increased it in the cerebral cortex, while CAT activity decreased in hippocampus. With chronic treatment however, SOD activity increased in the hippocampus and cerebral cortex and decreased in the striatum, while GPx activity was not affected [287]. In juvenile rats, chronic MPH treatment decreased non-enzymatic plasma antioxidant level and

increased plasma SOD and CAT activities [288], but did not affect superoxide level in submitochondrial particles [289], while a dose-dependent increase in protein carbonyls was observed in specific brain regions [290]. In addition, chronic MPH administration resulted in overall increased activity of mitochondrial enzymes in the brain of young rats, more adenosine triphosphate (ATP) production and thus oxygen consumption, and accordingly, can lead to higher ROS production [291]. Despite being neuroprotective by preventing excessive dopamine accumulation, MPH thus potentially even increases oxidative stress [70, 120, 292]. The effect of MPH on oxidative stress markers however, based on rat studies, depends on the subject's age (e.g. juvenile vs. adult rats), as well as the dose (1 vs. 10 mg/kg MPH) and duration (acute vs. chronic exposure) of treatment [287, 289-291]. For instance, in case of acute exposure, only the MPH highest dose (10 mg/kg) increased hippocampal lipid peroxidation [290].

It is unclear whether results from rats can be extrapolated to humans. In fact, blood TAS levels from child and adolescent ADHD patients were significantly higher after a 12-week MPH treatment than pre-treatment, accompanied by a significantly lower post-treatment OSI value [70].

3.1.5 Environmental factors, nutrition and micronutrient status

Several environmental, nutritional and micronutrient factors that have been related to ADHD possibly involve oxidative stress in their mechanism of action increasing ADHD risk [104, 109, 158, 293]. Prenatal tobacco smoke exposure and western dietary habits are for example associated with oxidative stress [63, 294]. In addition, lead interferes with the recycling of GSSG to GSH by GSH reductase (GR), thereby depleting cellular GSH [295].

Other examples include lower ferritin, magnesium and zinc levels in ADHD [69, 83, 106, 157, 158, 169, 172, 197, 202, 203]. Ferritin, an intracellular protein, stores iron and regulates its release, thereby limiting the amount of free iron that can produce oxidative radicals [108, 296]. As lower ferritin levels imply less iron regulation, it possibly indicates more oxidative stress rather than iron deficiency [108, 157]. Magnesium is required in enzyme systems protecting against oxidative damage, such as 8-oxoG endonuclease [172, 173]. Zinc is a cofactor in antioxidant defence systems, like CuZn-SOD [295, 297].

Besides, also EPA and DHA have specific antioxidant activities [298, 299]. For example, DHA supplementation significantly increased CAT, GSH and GPx levels in the cerebrum of rats [298]. Moreover, EPA or DHA supplementation significantly decreased urinary F2-isoprostanes, a measure of lipid peroxidation, in type 2 diabetic subjects [299]. Supplementation with 635 mg EPA and 195 mg DHA per day for eight weeks, as compared to olive oil, significantly increased SOD and GR (but not CAT)

activity in paediatric ADHD patients [300]. Moreover, also polyphenols (see Chapter 1, 4.1) are well-known for their antioxidant capacity [301].

Also a link between oxidative stress and food additives has been found [72, 109]. For example, artificial food colours and flavours reduced blood and liver concentrations of GSH, GST and SOD in rats. Moreover, also by altering concentrations of micronutrients, including essential cofactors for antioxidant enzymes, food additives could influence the development or expression of ADHD through effects on oxidative stress [72].

3.1.6 Oxidative imbalance?

Results regarding oxidative damage and antioxidant status in ADHD are equivocal. Studies overall had their limitations, like a limited number of biomarkers analysed and a small sample size. Nevertheless, it is interesting that not only more oxidative damage was observed in various studies, but also reduced antioxidant levels or antioxidant enzyme activity, pointing at a coping defect, i.e. the inability to increase antioxidant defence in case of increased oxidative stress [108, 302]. A recent meta-analysis, though preliminary due to the small number of studies, concluded that ADHD patients are not characterised by deficient antioxidant production, but by insufficient response to oxidative stress, leading to oxidative damage [108]. Association with specific ADHD symptoms seems to depend on the biomarker analysed [63, 197, 204, 264, 302-304]. Interestingly, subcortical volumetric reductions in ADHD can be partly explained by increased vulnerability to oxidative stress [75].

3.2 Immunity

The immune system is a network of cells, tissues and organs, defending the body against bacteria, viruses, parasites and toxins, and repairing damaged tissue. Moreover, it plays a role in the overall homeostasis of the body [305].

The immune system in vertebrates is subdivided into the innate and adaptive immune system [306, 307]. Innate immunity is the first line of immune defence, providing immediate but less specific responses based on general molecular recognition mechanisms to detect microbes, but also includes epithelial barriers, antimicrobial substances, complement proteins and cytokines. Innate immunity can activate the adaptive response [306, 307]. Cells providing the innate immune response are mainly neutrophils, monocytes, macrophages, dendritic cells (DCs) and natural killer (NK) cells. Adaptive immunity gives highly specific responses to epitopes of both microbial and non-microbial substances

and develops a memory, so that faster and stronger responses to later challenges can be mounted [308, 309]. The main components of adaptive immunity are lymphocytes, effector cells and their secreted products. Adaptive immunity comprises both cell-mediated and humoral immunity (Figure 1.6), which function to eliminate different types of microbes [310].

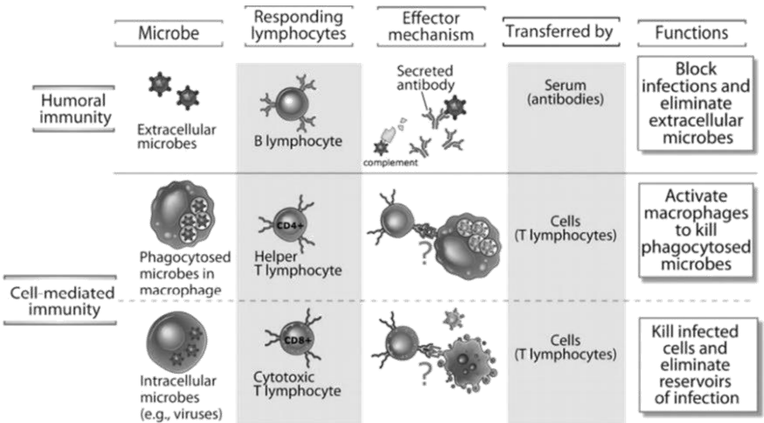

Figure 1.6. Types of adaptive immunity [309].

Moreover, intestinal microbiota provide protection against pathogens, strengthen the intestinal barrier and guide induction, maturation and function of the immune response [311-313]. Gut microbiota not only affect local but also systemic immune responses, well beyond mucosal tissues and in organs distal from the intestine [311, 314, 315].

Since the 1980s, potential immune involvement in ADHD has been suspected due to the increased prevalence of allergic diseases in ADHD patients. It has been hypothesised that allergic reactions cause an imbalance in CNS cholinergic and adrenergic activity and thereby lead to ADHD symptoms in a subgroup of children [316, 317]. Also in recent years, there has been an interest in the potential role of atopy and (allergic) immunopathology in ADHD, with indications of chronic immune-mediated neurologic inflammation, probably requiring a predisposing genetic background [318-321].

3.2.1 Indications from other psychiatric disorders

A disrupted immune balance and inflammation have been indicated in various psychiatric disorders, like autism [322, 323], schizophrenia [324], bipolar disorder [325] and depression [326]. For example in autism, a pro-inflammatory cytokine profile of the cerebrospinal fluid (CSF) was observed, indicating innate neuroimmune reactions [322]. In addition, specific auto-Igs are linked to several neuropsychiatric disorders, while patients with autoimmune diseases have a higher prevalence of psychiatric symptoms [327, 328]. Moreover, knowledge on the effect of microbiota on brain and

behaviour is growing [311, 329, 330]. Germ-free mice for instance display alterations in central neurochemistry and behaviour, while probiotics alleviated autism-like symptoms (Figure 1.7) [331].

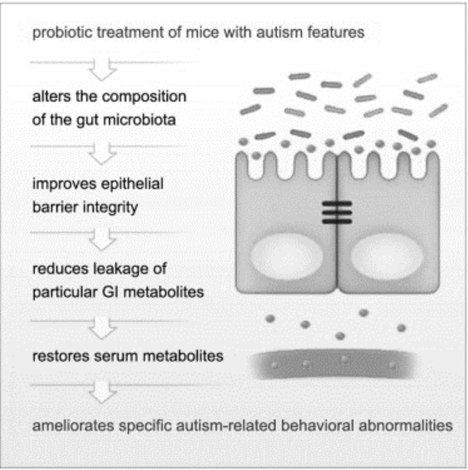

Figure 1.7. Mechanism of probiotic effect on autism in mice [332].

3.2.2 Genetics

Like asthma and other (atopic) diseases, ADHD is highly hereditary [28, 64]. Not only polymorphisms in specific genes related to dopamine and norepinephrine regulation are involved, but also related to histamine (Figure 1.8) [64]. For example, polymorphisms in the *HNMT* gene, impairing histamine clearance, were found to affect behavioural responses to food additives (see Chapter 1, 2.3) [229].

Figure 1.8. Structure of histamine.

Moreover, mice with a reduced expression of SNAP-25 presented ADHD-like symptoms and associated striatal dopamine deficiency [87]. SNAP-25, a presynaptic plasma membrane protein highly expressed in the nervous system, is important in the regulation of neurotransmitter release and is suggested to be involved in histamine release from mast cells [71, 333]. In addition, signal transducer and activator of transcription 6 (STAT6)-deficient mice exhibited increased locomotor activity and decreased expression of striatal dopamine transporter. STAT6 is a transcription factor stimulated by certain cytokines like interleukin (IL)-4 and is important for immune regulation and brain development [309, 334]. Moreover, major histocompatibility complex (MHC)-linked genes appear to play a role in ADHD, based on significant correlations between ADHD and specific genes which are involved in autoimmune diseases like arthritis and type I diabetes [335, 336]. Also, an association between polymorphisms of

the IL-1 receptor antagonist gene and ADHD was found. IL-1 in the brain is involved in the differentiation of dopamine neurons [337]. Finally, based on gene expression profiles, subcortical volumetric reductions in ADHD can be partly explained by increased vulnerability of these regions to apoptosis and autophagy [75].

3.2.3 Perinatal influences

Medical conditions of the mother (e.g. obesity, asthma and specific autoimmune diseases like multiple sclerosis; Figure 1.9), season of birth (possibly related to maternal respiratory infections in early pregnancy) and maternal inflammation caused by for example an allergic disorder are associated with ADHD in the offspring [39, 91, 98]. In addition, prenatal exposure to viral infections leading to altered dopaminergic development (e.g. measles, rubella or influenza) has also been implicated as a risk factor for neurodevelopmental disorders [39]. This implies that specific maternal immune components might be involved in ADHD aetiology [39, 91]. Moreover, supplementation with probiotics (e.g. *Lactobacillus rhamnosus GG*) during the first months of life may reduce the risk of ADHD development later in childhood [338].

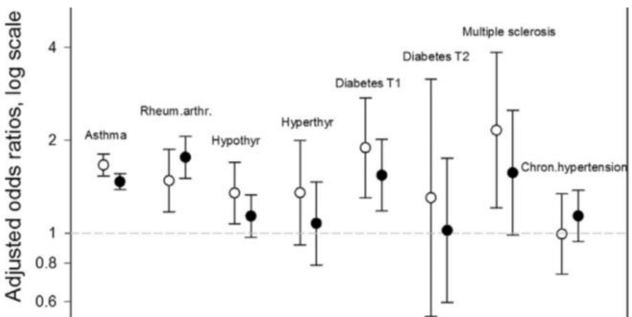

Figure 1.9. Adjusted odds ratios (and 95% confidence intervals) between maternal diseases and ADHD in female (white circles) and male (black circles) offspring [39].

3.2.4 Comorbidities

ADHD has a high comorbidity with both T_H1- and T_H2-mediated disorders and thus with inflammation [32, 339-341]. However, while acute inflammation marks an activated immune defence against infections, chronic (and often low-grade) inflammation appears typical for ADHD. As compared to controls, ADHD patients for example have a higher incidence of stomach aches and ear infections (T_H1-mediated) [341], as well as of asthma and atopic eczema (T_H2-mediated) [32, 340, 342, 343]. Still, a possible relation between ADHD and streptococcal infection or otitis media require further confirmation [339, 344-346].

The association between atopic diseases (eczema, asthma and rhinitis) and ADHD has been subject of controversy, though a number of studies reported significant associations [64, 347, 348]. For example, a population-based study observed a higher prevalence of allergic diseases (asthma, rhinitis, atopic dermatitis and urticaria) in ADHD, independent of environmental or lifestyle factors [317, 349, 350]. Other studies reported that paediatric patients with an allergic disorder had a significantly increased risk of ADHD or mental health problems [32, 342, 350-353]. For example, in patients with atopic dermatitis as compared to controls, a significantly higher prevalence of ADHD-related symptoms was found, such as attention problems and impulsivity [32, 354-356]. The association between ADHD and asthma is less clear: though various studies found a positive association, the majority did not correct for comorbidity with eczema [52, 357-359]. Nevertheless, a common pathophysiological mechanism was suggested to underlie asthma and ADHD [64]. The association between rhinitis and ADHD is equivocal [348, 357], though pollen caused neurobehavioral regression in ADHD patients [360]. In addition, atopic subjects responded more often with ADHD-like symptoms to foods and artificial food dyes and/or preservatives, pointing towards allergic hypersensitivity [242]. Immunological recognition of foods provoking ADHD-like symptoms, rather than a direct biochemical or pharmacological pathway, was also suggested based on the diversity of agents causing a similar response in different children [145, 361]. Still, several studies found no association between ADHD and allergy [49, 362, 363]. In addition, the "chicken or egg" debate on the relationship between psychological problems and allergic diseases is ongoing [364-366]. It might however be no coincidence that ADHD often remits with age [28], as atopic diseases frequently remit spontaneously after several years [367]. Nevertheless, though these hypersensitivities appear to involve components of the immune system, they were independent of a family history of atopy and lifestyle factors such as breastfeeding and early day care exposure [52, 64, 341, 351, 353, 357].

Autoimmunity could be related to ADHD as well. For example, despite an overall low comorbidity between autoimmune diseases and ADHD, a higher prevalence of ankylosing spondylitis, ulcerative colitis and thyroid disease was found in ADHD [317]. Results on antiganglia antibodies in ADHD are equivocal [19, 52, 143, 344, 357, 368, 369].

3.2.5 Immune analyses

ADHD patients are reported to have a CSF cytokine profile intermediate to that of patients with OCD (characterised by a skewing to T_H1-mediated cytokines) and schizophrenics (skewing to T_H2-mediated cytokines) [370]. In addition, serum levels of interferon (IFN)-γ and IL-13 were significantly higher in ADHD patients than in controls. Levels of IL-2, IL-6, IL-10 and IL-16 were non-significantly increased,

while IL-1β showed a non-significant decrease and tumour necrosis factor (TNF)-α levels were clearly similar in both groups. The slightly altered levels of all cytokines were normalised by medication. The TNF-α/IFN-γ ratio was lower in ADHD than in control children and increased by ADHD medication, although overall no dysfunction in pro- and anti-inflammatory cytokine balance was found [320]. Moreover, elevated levels of circulating inflammatory markers like IL-6 in premature babies are associated with inattention at the age of two [371].

Plasma levels of the C4b complement protein, important in the defence against viral and bacterial infections, were decreased in ADHD patients [372]. As compared to controls, patients also had increased activity of adenosine deaminase, an enzyme involved in T cell maturation and function and thus a marker of cellular immunity [63]. Furthermore, antibodies against glutamic acid decarboxylase (GAD65, important for inhibitory neurotransmission) have been detected in the serum of ADHD patients, as well as reactions of serum antibodies with DAT and with cells in the cerebellum and basal ganglia, suggesting direct effects on brain function, despite discussion [344, 373, 374]. One study found an association between ADHD and positive skin prick test results to common aeroallergens [363], but this was countered by others [19, 375]. In addition, IgE nor IgG levels were related to behavioural improvement by dietary restriction [143]. IgE is involved in immunity against parasites as well as allergy via mast cell degranulation, causing immediate hypersensitivity. IgG is the most common antibody isotype with multiple subclasses and different effector functions: IgG_1 and IgG_3 opsonise and thereby promote phagocytosis, IgG_2 activates the complement system and stimulates NK cells and functions of IgG_4 are related to those of IgE [309].

Converging evidence suggests that the gut microbiome is altered in ADHD, with a decreased microbial diversity and altered composition [376]. For instance, in ADHD patients versus controls, increases in the genus *Bifidobacterium* were found, associated with an increased metabolic function of cyclohexadienyl dehydratase. This enzyme is involved phenylalanine synthesis, an essential amino acid and dopamine precursor [377-379]. Other studies found a decrease in *Faecalibacterium* in treatment-naïve children with ADHD as compared to controls [380] and elevated levels of *Bacteroidaceae* [376]. *Faecalibacterium* is known for its anti-inflammatory effects and low levels have also been associated with a Western-type diet and atopic disorders [380], while *Bacteroidetes* generally support immunity and neurochemistry by generating essential vitamins and cofactors [376]. Finally, supplementation with *Lactobacillus rhamnosus GG* during the first months of life may reduce the risk of ADHD development later in childhood [338]. It was suggested that microbial changes in ADHD are unrelated to caesarean delivery or antibiotic use [381]. Commensal microbiota play a crucial role in the

development of intestinal immunity, e.g. via the induction of regulatory T cells (T_{Regs}) [65, 382], but also in CNS development and function [380, 383]. Moreover, gut microbiota are involved in susceptibility to allergies [384, 385].

3.2.6 Environmental factors, nutrition and micronutrient status

Several environmental, nutritional and micronutrient factors that have been related to ADHD, could influence the development or expression of this disorder through effects on immune regulation [104, 109]. Smoking (which is also related to asthma development) and western dietary habits are for example associated with inflammation [63, 340, 386]. Moreover, food colourings are associated with immune reactivity. Especially tartrazine has been reported to be a trigger for asthma and urticarial attacks [72, 387]. Furthermore, (maternal) obesity causes low-grade chronic inflammation, due to cytokine production (e.g. IL-6 and TNF-α) by adipocytes [388].

Other examples include lower vitamin D and zinc levels in ADHD [69, 83, 106, 153, 184, 185, 197, 202, 203], since both are involved in immune responsivity, e.g. in T cell activation [194, 389]. In addition, vitamin D status affects microbial development and function [313]. Besides, ω-3 fatty acids exert anti-inflammatory effects by inducing apoptosis in T_H1 cells, promoting T_H2 effector cell differentiation and enhancing T_{Reg} development [390, 391]. A high intake of ω-3 PUFAs has been shown to decrease the production of pro-inflammatory mediators and facilitate anti-inflammatory processes, leading to neuroprotective effects [209, 392-395]. For example, supplementation with 3.1–8.4 g EPA+DHA/day decreases ROS production by stimulated human neutrophils by 30–55%, while 1.5 g EPA+DHA/d lowered intercellular adhesion molecule (ICAM)-1 expression on stimulated human blood monocytes [392]. Fish oil supplementation providing 2g EPA+DHA/d in healthy humans decreased the production of TNF, IL-1, and IL-6. Supplementation with 635 mg EPA and 195 mg DHA per day for eight weeks, as compared to olive oil, significantly decreased C-reactive protein (CRP) and IL-6 levels in paediatric ADHD patients [300]. ω-3 PUFAs exert these effects via direct (e.g. replacing AA and inhibiting its metabolism, and by giving rise to resolvins, a family of anti-inflammatory mediators) and indirect actions (e.g. downregulating the activity of nuclear factor-κB (NF-κB) and thereby altering the expression of inflammatory genes) [392, 396]. For instance, EPA is a precursor of eicosanoids, which have anti-inflammatory, antithrombotic and vasodilation properties, but which can also modulate neurotransmitter uptake, synaptic transmission and apoptosis [24, 210].

In addition, breastfeeding stimulates infant immune development and protects for instance against future development of allergic diseases [397-399].

3.2.7 Immune imbalance?

Three mechanisms were hypothesised to explain the association between atopic eczema and ADHD:

1. eczema influences ADHD development by elevated cytokine levels or by stress, raising glucocorticoid levels, as both can interfere with brain development and neurotransmitter systems;
2. ADHD increases stress levels, thereby increasing vulnerability to develop eczema via neuroimmunological mechanisms;
3. a mutual risk factor connects both diseases, like genetics or prenatal stress [319].

Other researchers rejected the hypothesis of ADHD being caused by allergic disorders, and hypothesised ADHD being a (non-)allergic hypersensitivity disorder itself, i.e. that (non-)allergic hypersensitivity to environmental factors triggers ADHD-type symptoms in some children, sharing mechanisms with immunological reactions (e.g. activated mast cells and basophilic granulocytes releasing cytokines and histamine by exposure to triggering factors) [64]. This hypothesis is substantiated by ADHD symptoms arising after eating certain foods and the therapeutic effect of certain elimination diets. Indeed, food hypersensitivity, including true allergy, and ADHD may share common aetiologic pathways [48, 64, 231, 237]. A shared genetic aetiology may thus underly both atopy and ADHD [64].

3.2.7.1 Humoral mechanisms

Atopic subjects responded more often with ADHD-like symptoms to foods [242], which points towards allergic hypersensitivity. However, since atopic diseases, in particular eczema, can be mediated by various mechanisms, the association with ADHD provides no information on the mechanism affecting ADHD risk [64, 400]. Indeed, if ADHD symptoms are triggered by food allergies in a subgroup of patients, these reactions do not seem to follow the typical IgE implication, and also IgG measurements are overall not proven to be useful [19, 143, 363, 375, 401]. For example, no association was found between the severity of IgE-mediated atopy and ADHD scores, based on history of anaphylaxis and skin-prick tests [19, 229]. Behavioural reactions to food components may occur independently of atopy, as behavioural hypersensitivity reactions occur in both allergic and non-allergic children [64, 232, 237, 333]. The connection between ADHD and allergies therefore seems based on a non-IgE-mediated mechanism, perhaps by a non-IgE-dependent histamine release from mast cells and basophilic granulocytes. Still, allergy is associated with reduced numbers of T_{Regs}, which inhibit IgE production by IL-10 [309, 402]. In addition, further clarification on a role for autoimmunity in ADHD pathogenesis is required due to discussion on elevated antiganglia antibodies in ADHD [19, 52, 143, 344, 357, 368, 369].

3.2.7.2 Cellular mechanisms

The pathophysiology of atopic disease involves, next to hypersecretion of IgE, also increased eosinophilic and basophilic activity, increased IL-4- and IL-5-mediated T_H2 expression, increased levels of pro-inflammatory cytokines, like IL-6 and TNF-α, and reduced T_{Reg}-mediated IL-10 secretion [403-405]. In addition, dopamine receptors, causally implicated in ADHD, are abundant on human T cells, activate STAT6 and trigger selective secretion of immune-regulatory cytokines like IL-10 [64, 406, 407]. Moreover, a significant increase in tolerance towards provoking foods following enzyme-potentiated desensitisation (EPD) was observed as compared to placebo. EPD reduces cellular responsiveness to antigens, including food additives, instead of giving rise to some form of immunisation [408]. Cell-mediated hypersensitivity (with NO production by macrophages), rather than humoral mechanisms, thus seems involved in behavioural reactions to food and to underlie the association between atopy and ADHD [317].

Cellular immune mechanisms in ADHD are also supported by reduced cortisol production in children with ADHD [409, 410], though contradicted by another study [411]. The stress hormone cortisol suppresses T_H1 responses and stimulates T_H2 activity, most likely due to enhanced T_{Reg} immunomodulation [318, 412, 413]. Reduced cortisol levels in ADHD could implicate a defective T_{Reg} immune modulation, elevated T_H1 and depressed T_H2 cytokine levels [413].

Finally, though the underlying mechanism of the association between autoimmunity and ADHD is not well understood, next to shared genetics, again pro-inflammatory cytokines could be involved, as higher levels of TNF-α, IL-6 and IL-8 are for example related to ulcerative colitis [317, 336].

3.3 Interconnection of oxidative and immune imbalance

Both unresolved oxidative stress and immune dysbalance may thus contribute to the clinical pathology of ADHD, both by injuring neuronal cells or altering neuronal development, e.g. via altered gene expression, direct DNA damage and promotion of cell death. In addition, inflammation and oxidative stress are strongly interconnected in a self-perpetuating cycle (Figure 1.10) [63]. Oxidative stress for instance stimulates pro-inflammatory pathways involving NF-κB activation, cytokine production and lymphocyte activation and proliferation, while inflammation can cause oxidative stress, as activated immune cells produce ROS, for example because of their role in phagocyte killing [414]. Cytokines can for instance stimulate the expression of iNOS and thereby increase $NO^{\bullet}$ production [415]. In fact, the two main sources of ROS within the CNS are mitochondria and activated microglia, resident innate

immune cells in the brain [72]. Also, various immune functions (e.g. T cell proliferation and macrophage function) are sensitive to GSH depletion [416, 417], though supplementation with cysteine may only be useful in case of abnormally low GSH levels [416]. Moreover, naïve T cells can be polarised by oxidative stress toward the T_H2 phenotype, potentially by upregulation of STAT6 and NF-κB activation, while increased antioxidant levels blunt the T_H2 response [418].

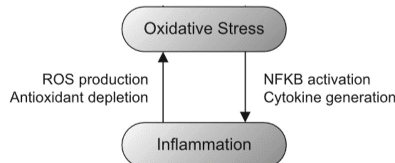

Figure 1.10. Interconnection between oxidative stress and inflammation [414].
NF-κB: nuclear factor κB; ROS: reactive oxygen species.

PART 4

POLYPHENOLS

Polyphenols are natural products widely distributed in plants and composed of several phenol groups (benzene rings with a hydroxyl group attached), subdivided into different groups (Figure 1.11) [419].

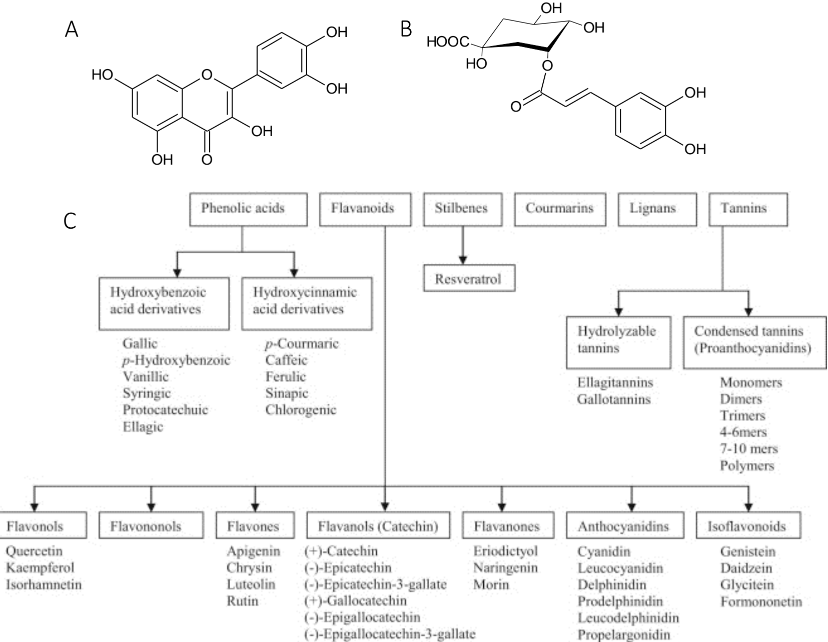

Figure 1.11. (A) Quercetin, (B) chlorogenic acid and (C) polyphenol classification [420].

Several plant foods are especially rich in polyphenols, like cacao, tea, apples, berries and wine [421, 422]. However, questions remain on their bioavailability, metabolism, distribution and excretion, and thereby on their activity *in vivo* [423]. For example, procyanidins are highly stable in the gastric environment, so that most ingested procyanidins reach the small intestine intact [424]. Generally, 5-10% of consumed polyphenols is absorbed in the small intestine, though this percentage depends highly upon the type and structure of the polyphenol. Absorption is restricted to those with low polymerisation degree. Of the compounds absorbed in the small intestine, 5-10% reach the plasma unchanged while the majority enters the blood stream as conjugates [425, 426]. Colonic microbiota play an important role in the metabolism of polyphenols that are not absorbed in the small intestine (e.g. *Bifidobacterium* and *Lactobacillus;* hydrolysis of glycosides, glucuronides, sulphates, amides and

esters, as well as aromatic ring fission, reduction, decarboxylation, demethylation, etc.), with degradation to phenolic acids, which can be absorbed. Interindividual differences in microbial composition can therefore affect polyphenol bioavailability and activity [426-428]. Components can also be metabolised by phase I (oxidation, reduction and hydrolysis) and phase II (methylation, glucuronidation, sulphation) reactions by enterocytes and hepatocytes [426]. During and after absorption, polyphenols are thus extensively modified (Figure 1.12), but also metabolites can contribute to their *in vivo* activity. Moreover, polyphenols and their metabolites affect host microbial composition, e.g. by inhibition of *Clostridium* or stimulation of *Bifidobacterium, Lactobacillus* and *Eubacterium* species [426, 428, 429].

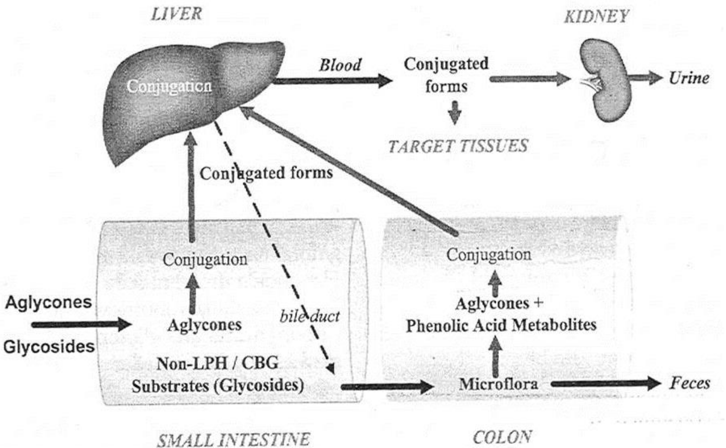

Figure 1.12. Global scheme of the metabolism of polyphenols. Adapted from [427].

4.1 Antioxidant effects

The most well-known characteristic of polyphenols is their antioxidant capacity [301]. Their physicochemical properties, i.e. aromatic rings with one or more hydroxyl groups, render them highly reducing, making polyphenols important free radical scavengers. The resulting resonance-stabilised phenoxyl radical is less reactive due to charge delocalisation over the aromatic moiety (Figure 1.13) [430]. In addition, polyphenols can exert their effects through other antioxidant mechanisms as well: low doses of phytochemicals like polyphenols can activate signalling pathways leading to increased expression of cytoprotective genes. One example is induction of phase II xenobiotic metabolising enzymes like GST, e.g., via the transcription factor nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) [431]. This further increases antioxidant capacity and provides more long-lived protective effects

as opposed to direct antioxidant effects [432, 433]. In addition, polyphenols may already exert antioxidant action in the GI tract, e.g. by scavenging ROS originating from the diet and gut phagocyte activation [434, 435].

Figure 1.13. Polyphenol-radical stabilisation through charge delocalisation (resonance stabilisation).

Though many polyphenols show antioxidant activity *in vitro* [436-441], a direct effect on antioxidant defence might be small *in vivo* due to their low absorption rate in the GI tract [425] as well as due to the higher rate constants of free radical reactions with the target molecules than those with polyphenols [442]. In addition, many metabolites have altered antioxidant capacities as compared to their parent compound [443].

In vitro tests also indicate dose-dependent prooxidative effects of polyphenols [444, 445], with low concentrations (e.g. 50 µM epigallocatechin-gallate (EGCG)) inducing mild oxidative stress and thereby stimulating GSH synthesis, while higher doses (e.g. 400 µM EGCG) only increased oxidative stress [446]. Mild exposure to prooxidants thus activates intracellular antioxidant systems, while high exposure causes cell death. Polyphenols may thus exert beneficial effects by acting as low dose prooxidants. Though evidence for systemic prooxidative effects of polyphenols in humans is not yet available [443, 444, 447], such results have been published for other natural antioxidants. A well-known example is an increased risk of lung and stomach cancer with a daily intake of 20-30 mg β-carotene, especially in smokers [448].

Clinical trials with specific polyphenols (140-500 mg/day) or more complex mixtures of polyphenols, including plant extracts, foods and beverages with interesting *in vitro* antioxidant profiles, are generally small-scale (< 50 participants) and short-term (< 1 month), and without dietary exclusion criteria [449, 450]. Their results are often inconsistent and difficult to compare, due to a huge difference in supplemented doses and analysed biomarkers. For example, evidence of flavonoid antioxidant effects in humans is limited and variable [427, 434]. In addition, dietary confounding factors can influence the results, while characterisation of the supplemented product is often incomplete or lacking; standardisation on the main bioactive compounds should be a prerequisite. Moreover, it is hard to establish dose-effect relationships and significant health improving effects in healthy persons, on whom many studies were conducted. Nevertheless, many examples of antioxidant effects are available.

For instance, intake of more than 500 mg/day of green tea extract or drinking more than 4 cups/day of green tea, rich in flavan-3-ols (6-5390 mg/l), significantly decreased *in vivo* oxidative damage biomarkers like MDA and 8-OHdG [427, 451-455]. In addition, 10 g cocoa powder (rich in flavanols like catechin, epicatechin and procyanidins B2 and C1) twice daily for 6 weeks significantly reduced serum MDA levels in type 2 diabetic patients [456].

4.2 Immune modulating effects

Polyphenols have anti-allergic and anti-inflammatory effects by influencing both activation and differentiation of multiple immune cell types, including T_{Reg} cells, T_H1 cells, NK cells, DCs and macrophages, and by modulating the T_H1/T_H2 balance [86, 457, 458]. For example, *in vitro* and *ex vivo* studies have demonstrated that one of the effects of polyphenols is the inhibition of key regulators of the inflammatory response, including cyclooxygenase-2 (COX-2), TNF-α, IL-1β and IL-6 [459-462]. *In vitro* experiments using Jurkat T cells treated with EGCG found increased Foxp3 and IL-10 gene expression, required for T_{Reg} functioning [463]. *In vitro* assays using naïve T cells from C57BL/6 mice under T_H17 differentiation conditions and Baicalin treatment showed a decreased expression of IL-17 [464]. Other *in vitro* research indicates that oligomeric apple epicatechins inhibit the release of histamine from mast cells, luteolin and apigenin inhibit T_H2-type cytokine production by basophils and quercetin suppresses pro-inflammatory cytokine production in the presence of lipopolysaccharide (LPS) in THP-1 monocytes and macrophages [465-467]. These findings are merely examples of the great deal of available results and indicate that, independent of treatment or the cellular model used, the modulation of cytokine production appears a common factor in the immunomodulatory effects of polyphenols. In addition, in *in vivo* research, vegetable, fruit and tea consumption is inversely associated with blood inflammation markers, like CRP, IL-6 and adhesion factors, and coupled to the enhancement of anti-inflammatory cytokines like IL-10 [468, 469].

Treatment of U266 cells with green tea extract showed a dose- and time-dependent decrease of IgE production [470], demonstrating that polyphenols are also relevant in the modulation of B cell function [471]. Moreover, a quercetin-enriched diet attenuated respiratory symptoms in sensitised guinea pigs [472], while intake of oligomeric apple epicatechins (10 mg/kg per day) during 8 weeks reduced symptoms of human atopic dermatitis [473].

Polyphenols might exert their immune modulatory effects through antioxidant actions [474-477] and prebiotic-like effects on colonic microbiota composition or activity, by altering microbial membrane

permeability, formation of polyphenol-metal ion complexes, or inhibition of DNA and RNA synthesis [426, 429]. Though robust data from human intervention trials are lacking, polyphenol supplementation might mainly be useful in case of diseases associated with inflammation and oxidative stress [466].

4.3 Implications for ADHD therapy

Increasing understanding regarding disadvantages of stimulant medication in children stimulates the investigation of alternative therapeutics [478]. Advances in ADHD treatment are required, but are impeded as its exact aetiology remains unclear [80]. Nevertheless, the association of oxidative and immune dysregulation with ADHD provides potential for nutritional supplements in ADHD therapy. Though their exact contribution to ADHD aetiology is unclear, their correction (e.g. modulation of immune system activity) using nutritional approaches might have a beneficial effect on ADHD prognosis. Oxidative and immune dysregulation should therefore be considered in ADHD diagnosis and therapeutic plans [63, 249].

Due to their antioxidant capacities as well as immunoregulatory effects, dietary polyphenols appear appropriate in ADHD therapy. Various polyphenolic extracts with antioxidant and immune modulating properties have been investigated in the treatment of ADHD, though most studies were relatively small or conducted over a short time span [479-484]. In addition, as most research on their antioxidant and immune effects has been performed *in vitro*, questions remain on their activity *in vivo* [423]. Examples of extracts investigated in ADHD are Pycnogenol®, *Ginkgo biloba* extract, *Hypericum perforatum* extract and *Passiflora incarnata* extract.

4.3.1 Pycnogenol®

In a randomised double-blind placebo-controlled trial, 61 boys and girls (6-14 years) with ADHD were treated with 1 mg/kg body weight/day Pycnogenol® (n = 44) or placebo (n = 17) for 1 month. Pycnogenol® significantly improved teacher hyperactivity and inattention ratings, though parent ratings did not reach statistical significance. After a one-month washout period, scores returned to baseline. Various mechanisms may play a role, as Pycnogenol® has been shown to have immune modulating and antioxidant properties [485, 486], while one of its metabolites could be able to cross the BBB [73, 487]. Below, Pycnogenol® is discussed extensively (see Chapter 1, 4.4).

4.3.2 *Ginkgo biloba*

Ginkgo biloba extract is made from the leaves of the Ginkgo tree and widely used to improve cognition. The main pharmacological effects of this extract in the therapy of cognitive decline are ascribed to terpene trilactones and flavonoids. In a double-blind randomised study including fifty ADHD patients, 80–120 mg/day *Ginkgo biloba* extract (composition not specified) for six weeks was much less effective than MPH treatment. Still, Ginkgo caused fewer adverse events (AEs) than MPH [488]. A three to five-week supplementation with a higher dose (max. 240 mg EGb 761® *Ginkgo biloba* extract) in an open study improved ADHD symptoms as well as brain electrical activity. EGb 761® is standardised to contain 22-27% ginkgo flavonoid glycosides (primarily quercetin, kaempferol and isorhamnetin), 5-7% terpene lactones (2.8-3.4% ginkgolides A, B and C; 2.6-3.2% bilobalide) and less than 5 ppm ginkgolic acids, which have toxic potential [489]. Next to immune and antioxidant effects, *Ginkgo biloba* extract has been found to improve cerebrovascular blood flow and to affect several central neurotransmitter systems [479, 480, 488, 490, 491].

4.3.3 *Hypericum perforatum*

Hypericum perforatum extract (St. John's wort) has been shown to be beneficial in mild depression and contains various active compounds, including hypericin and hyperforin (presumed to be the main antidepressant constituents) and flavonoids like kaempferol, rutin and quercetin [492]. This extract (standardised to 0.3% hypericin, 900 mg/day for 8 weeks) did not significantly improve ADHD symptoms as compared to placebo in a randomised double-blind trial with 54 patients [493, 494].

4.3.4 *Passiflora incarnata*

Passiflora incarnata extract is indicated for restlessness, anxiety and sleep problems. Flavonoids including luteolin, quercetin, vitexin and rutin have been reported to be the major constituents of this extract. *Passiflora incarnata* extract (0.4 mg/kg/day twice daily; the total flavonoid content being 4% (w/w)) and MPH (1 mg/kg/day twice daily) performed equally well regarding parent and teacher ratings in a randomised controlled trial with 34 ADHD patients, both demonstrating a significant clinical benefit over 8 weeks [495].

4.3.5 *Crocus sativus L.*

Crocus sativus L., also known as saffron, is known for its antidepressant and memory enhancing effects, potentially via dopamine and norepinephrine reuptake inhibition. In addition, it reduces blood pressure and has anti-inflammatory, radical scavenging and neuroprotective capabilities. Its active constituents

include, among others, crocin, safranal and various flavonoids like rutin and quercetin [496, 497]. In a very recent 6-week randomised double-blind study in 54 ADHD patients (6-17 years old), *Crocus sativus* extract showed the same efficacy as MPH (both 20 mg/day if < 30 kg; 30 mg/day if > 30 kg) based on parent and teacher behaviour ratings. Also the frequency of adverse effects was similar in both treatment groups [496].

4.4 Pycnogenol®

Pycnogenol® (US Pharmacopeia Maritime Pine Extract, Horphag Research, Geneva, Switzerland) is a patented herbal extract from the outer bark of French maritime pine (*Pinus pinaster Ait. subsp. atlantica*). The fresh bark is powdered and extracted with ethanol and water. After purification of the raw extract, the aqueous solution of the extracted constituents is spray-dried [498]. Pycnogenol® received GMP (good manufacturing practice) certification from the French Health Products Safety Agency (ANSM).

4.4.1 Composition and toxicology

Pycnogenol® is standardised to contain 70 ± 5% (w/w) procyanidins, flavonoid oligomers and polymers, consisting of 2 to 12 units of catechin and epicatechin (C6-C3-C6 basic structure; Figure 1.14). Catechin, epicatechin and taxifolin represent the flavonoids in Pycnogenol®, of which catechin is the most common. Common dimers are procyanidin B1 (epicatechin-(4β→8)-catechin) and B3 (catechin-(4α→8)-catechin). Less common dimers are B6 (catechin-(4α→6)-catechin) and B7 (epicatechin-(4α→6)-epicatechin). Trimer C2 (catechin-(4α→8)-catechin-(4α→8)-catechin) has also been identified. Phenolic acids present in Pycnogenol® are derivatives of benzoic acid (p-hydroxybenzoic acid, protocatechuic acid, gallic acid, vanillic acid) or cinnamic acid (caffeic acid, ferulic acid, p-cumaric acid), found in free form and as glucosides [499, 500]. Inorganic substances include calcium, potassium and iron as well as traces of manganese, zinc and copper [501].

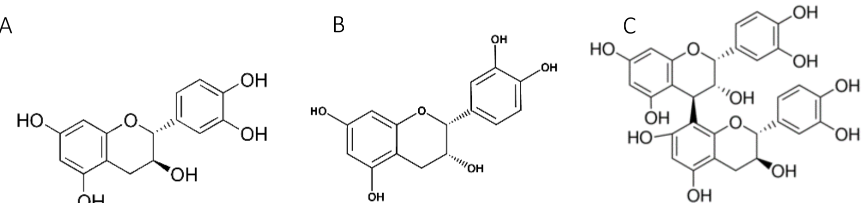

Figure 1.14. Structure of (A) catechin, (B) epicatechin and (C) procyanidin dimer B1.

Pycnogenol® has been affirmed generally recognised as safe (GRAS), with a very low toxicity, a no-observed-adverse-effects level (NOAEL) of 100 mg/kg/day, absence of mutagenic, genotoxic and teratogenic effects, no perinatal toxicity and no negative effects on fertility. The safety of Pycnogenol® is based on data obtained from over 140 clinical studies including more than 12,000 participants. There are no known contraindications for Pycnogenol® or interactions with concomitant therapies [73, 501-517]. In addition, AEs occur infrequently (1.8%), are unrelated to the dose or duration of use and are primarily mild. The most frequently reported AEs are GI discomfort, dizziness and headache [517].

4.4.2 Pharmacokinetics

Studies indicate high bioavailability of individual components in Pycnogenol®. For example, 46% of catechin metabolites (glucuronides and sulphates) were recovered in urine [518]. In addition, Pycnogenol® intake is associated with increased urinary excretion of ferulic acid [519].

After a single oral 300 mg dose, 15 constituents and metabolites were detected in the plasma of human adults between 30 min and 14 h post-dosing, each with a different T_{max}, 5 of which could be identified (Table 1.4, Figure 1.15). For example, catechin was detected between 30 min and 14 h after Pycnogenol® intake, indicating absorption in the small intestine of catechin genuinely present in the extract as well as additional generation by metabolic breakdown of higher procyanidin oligomers. The early T_{max} of caffeic and ferulic acid implies absorption in the small intestine of these monomers as well. On the contrary, M1 (δ-(3,4-dihydroxy-phenyl)-γ-valerolactone) was only detectable after 6 h, with a peak concentration around 10 h, indicating metabolism and colonic absorption [520, 521]. Indeed, catechin (also released by cleavage of procyanidins) is metabolised by gut microbiota to M1 (Figure 1.16). The ten unknown compounds detected could not be identified as known free polyphenol monomers in Pycnogenol®, but might be conjugated monomers, oligomer metabolites or procyanidin dimers [520]. For instance, though polymerisation greatly impairs intestinal absorption, procyanidin B1 has been detected in human plasma before [419, 522, 523].

Table 1.4. C_{max} and T_{max} of known constituents/metabolites of Pycnogenol® detected in plasma [520].

Constituent	C_{max} (ng/ml)	T_{max} (h)
Caffeic acid	17	4
Catechin	107	4
Ferulic acid	15	1
Taxifolin	33	8
M1	4	10

(+) Catechin Taxifolin Caffeic acid Ferulic acid

M1

Figure 1.15. Chemical structure of the known Pycnogenol® compounds detected in human plasma [520].

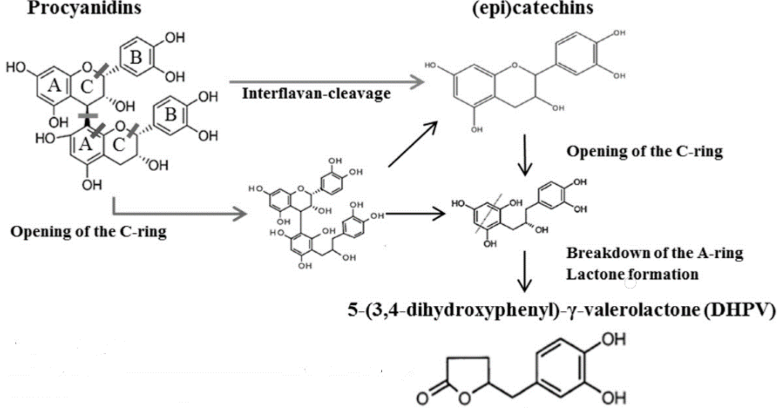

Figure 1.16. Microbial metabolism of procyanidins [524].

As for polyphenols in general, procyanidins in Pycnogenol® undergo phase II metabolism, as many compounds were found as conjugates of sulphate and/or glucuronic acid [500, 501, 520, 525, 526]. Still, considerable differences between individuals and compounds have been observed regarding Pycnogenol® polyphenol pharmacokinetics, with the degree of conjugation with sulphate and glucuronic acid in serum ranging from 54.29% ± 26.77% for catechin to 98.34% ± 4.40% for M1. Again, results of *in vitro* studies thus need to be interpreted with caution. In addition, after 200 mg/day Pycnogenol® supplementation for three weeks, the highest polyphenol concentrations were not detected in serum: catechin and taxifolin primarily resided within blood cells, while M1, ferulic acid and caffeic acid were mainly present in synovial fluid [525]. Next to M1, the catechin metabolite M2 (δ-(3-methoxy-4-hydroxy-phenyl)-γ-valerolactone) has been detected in human urine samples after oral Pycnogenol® intake [526].

In vitro human and murine cell culture studies revealed that M1 enters cells (macrophages, monocytes, erythrocytes and endothelial cells) via facilitated transport, probably mediated by the glucose transporter-1 (GLUT-1). This facilitated uptake causes M1 accumulation to levels that could be

bioactive, with further intracellular metabolism (e.g. conjugation with GSH) [487, 527]. Via the same transporter, M1 could be able to cross the BBB [73, 487]. Most other polyphenols lack this ability and are therefore not effective in reducing oxidative stress in the brain [528, 529].

4.4.3 Biological actions and clinical efficacy

Compounds in Pycnogenol® or metabolites thereof have various biological activities, including antioxidant, anti-inflammatory and antihypertensive actions, e.g. by binding proteins and thereby altering their structure and activity, by increasing erythrocyte membrane fluidity and by altering gene expression [487, 498, 518, 530-532]. Regarding its antioxidant effects, Pycnogenol® displays stronger biological activity as a mixture than when separated into its individual components, indicating that the components have additive effects or interact synergistically [533].

4.4.3.1 Antioxidant effects

In vitro studies have shown that Pycnogenol® improves clearance of the hydroxyl radical ($HO^{\bullet}$) and superoxide anion radical ($O_2^{\bullet -}$) [534, 535], chelates transition metals, quenches $NO^{\bullet}$ and reduces iNOS messenger ribonucleic acid (mRNA) expression as well as iNOS and XO activity [518, 536]. Moreover, it stimulates the activity of SOD, GPx and CAT [458, 534], reduces DNA damage and H_2O_2-induced chromosome breakage [537, 538] and protects endogenous α-tocopherol, vitamin C and GSH [458, 518, 534, 539]. Moreover, though catechin scavenges $O_2^{\bullet -}$ with effectiveness similar to ascorbic acid, M1 was found to be significantly more active than catechin or ascorbic acid [540]. In addition, M1 concentration-dependently inhibits nitrite (NO_2^-) production and iNOS expression [541].

In vivo studies demonstrated Pycnogenol® (10 mg/kg intraperitoneally (i.p.)) to reduce liver and pancreas protein carbonyl levels, increase GSH levels and GR, GST, CAT, SOD and GPx activity [542-544] and to reduce serum NO in diabetic rats [543]. In other animal studies, Pycnogenol® (20-100 mg/kg i.p. or 200 mg/kg per day orally) decreased MDA concentrations, DNA damage and iNOS activity and increased GSH levels, for example in kidney and skin tissues [518, 545-547].

In an *in vivo* human study in 25 healthy adults, Pycnogenol® (150 mg/d for six weeks) significantly increased plasma phenol level. Plasma antioxidant capacity was significantly increased by ± 40%, as determined by oxygen radical absorbance capacity (ORAC), and returned to baseline after a washout period of four weeks [548]. In addition, in human randomised controlled trials, Pycnogenol® supplementation as compared to placebo prevented oxidative damage to lipids (i.e. 150 mg daily for three months in elderly significantly decreased F2-isoprostane levels by 30% [503]) and DNA (i.e. 1 mg/kg daily for one month in 6-12 year old ADHD patients significantly reduced lymphocyte 8-oxoG as

determined by the comet assay by on average 35% [267], while it significantly increased GSH levels by 27% and decreased GSSG levels by 22% [486]). Mechanisms of action could be "a direct scavenging activity, chelating ability, stimulation of DNA repair system, or their combinations" [267]. However, Pycnogenol® (150 mg/day for three months) had no effect on oxidative DNA damage in elderly [549]. Additional age-related studies are needed to further elucidate these findings.

4.4.3.2 Immune modulation

In vitro, Pycnogenol® dose-dependently decreased histamine, TNF-α and IL-6 release by rat peritoneal mast cells. Therefore, Pycnogenol® might have potential to improve mast cell-mediated immediate-type allergy [471]. Pycnogenol® supplementation (150 mg/day for five days) in healthy adults inhibited COX-2 and 5-lipoxygenase (5-LOX) gene expression and reduced leukotriene production in leukocytes after *ex vivo* stimulation [460]. Other *in vitro* and *ex vivo* studies found that it blocks NF-κβ activation and translocation [471, 518, 550-552], limits induction of vascular cell adhesion protein (VCAM)-1 and ICAM-1 [553], reduces oxidative burst intensity [538] and inhibits upregulation of COX-1 and -2 and matrix metalloprotease (MMP) 1, 2, and 9, with M1 being more potent than catechin [459, 554, 555]. Moreover, Pycnogenol® was found to inhibit pro-inflammatory cytokine expression (e.g. IL-1, IL-2 and IL-8) [550, 554, 556] and acute inflammatory cell infiltration [557].

In animal models, Pycnogenol® (50 mg/kg intravenous injection or 30-100 mg/kg orally) decreased the inflammatory cell count, mucus secretion and IL-4, IL-5, IL-13 and IgE levels in asthmatic rats [536, 558], while plasma TNF-α level decreased in septic and diabetic rats (10-100 mg/kg i.p.) [542, 547]. In addition, Pycnogenol® stimulated wound healing in diabetic rats (30 mg dermally) [559] and restored immune function and prolonged survival of mice with diet-induced immune dysfunction (50-100 mg/kg orally) [485].

In the *in vivo* human situation, Pycnogenol® has potential for improving inflammation and allergy symptoms as well. For instance, 100 mg/day for six months significantly reduced inhalation corticosteroid use, symptom frequency and specialist consultations in adult asthma patients, while decreasing the specific IgE titre by 15.2% [560]. In addition, 100 mg/day for three months significantly reduced plasma CRP levels by 71% as compared to placebo in osteoarthritis patients [561].

4.4.3.3 Vascular health

In vitro studies found that, by stimulating endothelial NOS (eNOS) and thus NO release, Pycnogenol® dose-dependently augments endothelium-dependent vasorelaxation [518, 562], which was confirmed by *in vivo* animal studies using 10 mg/kg daily [518, 563].

Moreover, a human randomised controlled trial with healthy volunteers found that 2 weeks of daily oral administration of Pycnogenol® (180 mg/day) as compared to placebo significantly augmented forearm blood flow response to acetylcholine (ACh), an endothelium-dependent vasodilator, by on average 40%. Administration of a NOS inhibitor abolished this effect, again suggesting that Pycnogenol® augments vasodilation by stimulating eNOS [564]. In patients with coronary artery disease, a randomised controlled crossover study found that a daily dose of 200 mg Pycnogenol® for 8 weeks improved endothelial function as measured by flow-mediated dilatation, without effects on blood pressure [506]. *In vivo* human and animal studies also found that, e.g. by enhancing the capillary diameter, Pycnogenol® improves microcirculation [518]. For instance, in a randomised controlled trial, 150 mg/day for four weeks improved fingernail microcirculation by 53.8% in patients with coronary artery disease [565]. Finally, Pycnogenol® (100 mg/day for eight weeks) significantly decreased oedema by 40%, a symptom of chronic venous insufficiency [566].

4.4.3.4 Neurologic effects

In vitro, Pycnogenol® protects nerve cells against β-amyloid-induced apoptosis, possibly by decreasing free radical generation [567], suggesting it may reduce the risks of neurodegenerative diseases such as Alzheimer's disease.

Animal studies also found positive effects of Pycnogenol® on mental health, e.g. via neuroprotective effects. For example, Pycnogenol® (dose unknown) increased nerve growth factor content in rat hippocampus and cortex, areas important for learning and memory, and improved spatial memory impairment [568]. In addition, it attenuated cognitive performance decline (e.g. water maze task) in oxidative stress-related neurodegeneration, while increasing choline acetyltransferase activity in the hippocampus and GSH level and decreasing protein carbonyl levels (10 mg/kg i.p. once daily for 3 weeks) [569]. Following traumatic brain injury (controlled cortical impact, CCI) in rats, Pycnogenol® (100 mg/kg i.p. or a single intravenous injection of 1, 5 or 10 mg/kg up to 4 hours after injury) had neuroprotective effects. Pycnogenol® for instance significantly reduced the loss of key pre- and postsynaptic proteins, attenuated brain oxidative damage (protein carbonyls and lipid peroxidation) and pro-inflammatory cytokine levels and protected synaptic function [570-572]. However, Pycnogenol® did not improve cognitive ability following CCI [573].

In humans, Pycnogenol® supplementation was found to enhance mental performance, for instance supported by improved cognitive functions in healthy professionals (150mg/day for 12 weeks) as compared to a control group [574]. In elderly subjects (mean age 67.8 years) without chronic disease, 150 mg/day Pycnogenol® for 3 months significantly increased quality of working memory as compared

to placebo, while decreasing plasma F2-isoprostane concentration [503]. In addition, after 44 healthy adults (age range 55-70) with high oxidative stress levels received Pycnogenol® for 12 months (100 mg/day, open study), less cognitive decline and oxidative stress as well as better daily and cognitive functioning was noticed as opposed to subjects with comparable oxidative stress levels [530].

4.4.3.5 ADHD

A case report describes improved ADHD symptoms in a 10-year-old boy with coadministration of Pycnogenol® and dextroamphetamine [575]. A double-blind placebo-controlled crossover study in adults (n = 24) however failed to demonstrate a beneficial effect of three weeks Pycnogenol® supplementation (1 mg/lb body weight) over placebo. Nevertheless, the relevance of this result is questionable, as also no difference between MPH and placebo was found [576].

In a randomised double-blind placebo-controlled trial, 61 boys and girls (6-14 years) with ADHD were treated with 1 mg/kg/day Pycnogenol® (n = 44) or placebo (n = 17) for one month. Patients were not supplemented with any other drug, nor with vitamins C or E during the study. Trebacticka *et al.* reported that active treatment significantly improved teacher hyperactivity and inattention ratings on the Child Attention Problem rating scale compared to placebo, but only inattention ratings (marginally) on the Conners' Teacher Rating Scale. A trend for reduced hyperactivity was found based on parent ratings (Conners' Parent Rating Scale). Psychologist assessment of visual-motoric coordination and concentration revealed significant improvements. No SAEs were reported and there were no changes in standard blood chemistry parameters, suggesting good tolerance [73].

Exact mechanisms by which Pycnogenol® improves brain function and reduces ADHD symptoms are not entirely clear yet. Various mechanisms may play a role. For example, this could be related to increased production of NO, which is involved in the regulation of norepinephrine and dopamine release and uptake [73], while NO also improves cerebral blood circulation [562]. However, though Pycnogenol® was found to stimulate eNOS [564], it is unknown whether it also affects neuronal NOS (nNOS) [73]. Moreover, Pycnogenol® significantly decreased urinary dopamine levels in ADHD patients as compared to baseline. Levels of epinephrine and norepinephrine, which were significantly elevated as compared to healthy controls, only showed a trend toward reduced levels, with a stronger decreasing effect for higher original concentrations [82]. In addition, Pycnogenol® reduced elevated lymphocyte 8-oxoG and plasma GSSG levels, and increased GSH levels and the GSH/GSSG ratio (possibly by increasing the activity of GR) in ADHD patients as compared to placebo [73, 267, 486, 544]. TAS increased non-significantly [267]. Plasma copper and iron levels decreased significantly. No effect on zinc, selenium, ferritin and transferrin was found [304]. After a one-month washout, behavioural scores

as well as 8-oxoG levels and GSH/GSSG ratio returned to baseline [73, 267, 486]. Reduction of 8-oxoG levels and increment of TAS correlated with an improvement in inattention scores, while GSH/GSSG ratio correlated negatively with urinary dopamine levels [82, 267, 486]. In addition, urinary catecholamine concentrations were found to correlate with the degree of hyperactivity as well as with plasma GSSG levels before treatment [82]. Pycnogenol® could thus normalise behaviour by affecting catecholamine metabolism and reducing oxidative stress.

Nevertheless, the relatively small sample size and short supplementation period limit generalisation of Trebaticka's findings in ADHD patients [73]. Other limitations are the very small placebo group, no active control and standardisation of Pycnogenol® being limited to total procyanidin content without determination of major polyphenolic constituents [73, 267, 486]. Moreover, it is possible that antioxidant-rich extracts such as Pycnogenol® cause more obvious improvements in a subset of ADHD patients, e.g. those with more severe oxidative stress [576]. Further research is thus needed to investigate its efficacy, mechanism of action and possibly also its value as compared to MPH treatment.

PART 5

AIM OF THIS THESIS

MPH, the first-choice medication for ADHD, is linked to concerns about adverse effects and possible publication bias in reported efficacy. ADHD aetiology involves dopaminergic dysfunction, but ADHD has also been associated with increased oxidative stress. Moreover, an immune dysbalance appears part of the aetiology or symptom exacerbation of ADHD, at least in a subgroup of patients. Cellular rather than humoral immune mechanisms seem to be involved, with a disturbed immune regulation being more likely than a single (sub)cellular defect. In addition, unresolved oxidative stress and immune dysbalance are interconnected in a self-perpetuating cycle. However, results are not completely consistent and research on these topics is limited and overall had its limitations, e.g. regarding biomarkers analysed or sample size. Specific immune markers other than antibodies, such as subsets of DCs and T_H cells and their cytokines, have not been systematically studied in ADHD, despite evidence on associations in e.g. autism [323, 577]. In addition, the exact contribution of oxidative stress and immune dysbalance to ADHD and their causal correlation are still unclear, as well as whether such influences are limited to a subgroup or affect the majority of children with ADHD. Moreover, immune and oxidative effects of both standard therapy and nutritional supplementation in ADHD are neglected topics in research.

Since oxidative stress and immune dysbalance appear aetiopathological factors in various (psychiatric) conditions, they might not be used as a specific diagnostic requirement for any exclusive disorder. Nevertheless, if immune pathways and/or oxidative stress contribute to ADHD development and/or manifestation, even if only in specific subgroups, ADHD diagnosis and especially treatment should be reconsidered to improve patient care. In fact, these imbalances provide potential for nutritional approaches as efficacious and safe methods for ADHD prevention and management, resulting in fewer side effects and possibly also improving comorbid complaints. One example is Pycnogenol® (*Pinus pinaster* bark extract), an herbal polyphenol-rich extract with potent antioxidant and anti-inflammatory properties. Pycnogenol® is considered to have therapeutic benefits in ADHD and increased antioxidant levels, reduced oxidative damage and improved neurochemical status in ADHD patients [73, 82, 486]. Still, specific diets or nutrient supplementation in ADHD therapy remains controversial.

Further research is thus required to explain the role of immunity and oxidative stress in the aetiology of this complex neurobiological disorder and also the possible interconnection between oxidative and immune imbalances deserves attention. In addition, more research on nutritional supplements and dietary factors in ADHD therapy is implicated, using acceptable sample sizes as well as substantiated treatment options and durations. Systematic comparison of such interventions to MPH treatment and placebo is highly useful, as a novel therapy based on improvement of immune and/or oxidative biomarkers might have potential in ADHD therapy, possibly even more than conventional therapy, which has various drawbacks.

Research should be performed in a multidisciplinary setting, using proper biomarkers and double-blind placebo-controlled designs, where applicable. For example, to obtain a good evaluation of *in vivo* oxidative stress status, an investigation of several sensitive, reliable and accurate biological parameters covering the different aspects of *in vivo* oxidative damage and antioxidative defence is required. Moreover, assessing correlations between behaviour and comorbid complaints [13], generally neglected by previous research, is crucial to increase understanding of ADHD aetiology and mechanisms by which nutritional therapy can lead to improvements [73, 578]. Properly selected patient groups with a reliable ADHD diagnosis, as well as age- and sex-matched controls, where appropriate, should be included. Moreover, immune analyses in ADHD should focus on cell-mediated mechanisms rather than IgE-mediated allergies. Using both biological markers and standardised questionnaires, responses to specific treatments should be monitored.

The first aim of this thesis is to get a better understanding of oxidative stress and immune dysbalance in ADHD. **Chapter 2** therefore describes a case-control comparison between untreated paediatric ADHD patients and controls (Figure 1.17). A broad diversity of oxidative stress and immune analyses in biological samples have been taken into account. Analyses include determination of erythrocyte reduced glutathione (GSH) levels, plasma lipid soluble antioxidant (retinol, retinylpalmitate, α-tocopherol, γ-tocopherol, β-carotene and co-enzyme Q10), malondialdehyde (MDA), immunoglobulin (IgE, IgG and IgM) and cytokine (interleukin (IL)-1β, IL-5, IL-6, IL-8, IL-10, tumour necrosis factor (TNF) and interferon (IFN)-γ) levels and urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels. Questionnaires on non-biological factors, which could possibly explain (part of) the association between oxidative stress and/or immune dysbalance and ADHD, include the Social-Emotional Questionnaire (SEQ), the Physical Complaints Questionnaire (PCQ) and a Food Frequency Questionnaire (FFQ). The main research question is whether ADHD patients present more oxidative stress and inflammation, unrelated to dietary habits.

General hypotheses
Chapter 1

ADHD aetiology involves oxidative stress and immune dysbalance

Nutritional approaches with antioxidant and/or immune effects have potential in ADHD therapy

Verlaet *et al*. Eur Child Adolesc Psychiatry (2014), 23(7)
Verlaet *et al*. Nutrients (2018), 10(4)

***In vivo* case-control comparison**
Chapter 2

ADHD patients – controls

- Oxidative stress and immune analyses
- Questionnaires on non-biological factors

Verlaet *et al*. Eur Child Adolesc Psychiatry (2018)

Characterisation of Pycnogenol®: validation of analytical methods, quality control
Chapter 3

- Spectrophotometric method: total procyanidins
- Fingerprint chromatography: catechin, taxifolin, caffeic acid, ferulic acid

Pycnogenol®, catechin *in vitro*
Chapter 4

- HEK cells: TLR binding
- THP-1 cells: cytokine secretion
- Pycnogenol®, catechin: as purchased & metabolised in gastrointestinal simulation model

Verlaet *et al*. Nutrients (2019), 11(2)

***In vivo* randomised controlled trial**
Chapter 5

- Design and protocol
- Registration requirements
- Ethical approval
- Safety monitoring

Verlaet *et al*. Trials (2017), 18(1)

Figure 1.17. Schematic overview of this PhD thesis.

During this PhD project, a review was published supporting dietary antioxidant treatment of ADHD [579]. The herbal extract Pycnogenol® was for instance shown to have various antioxidant and immune modulating properties. Standardisation, characterisation and quality control of nutritional supplements are however essential for credible research and to be able to compare results of various studies. **Chapter 3** therefore describes the optimisation and validation of United States Pharmacopeial

(USP) analytical methods for Pycnogenol® (i.e. a fingerprint chromatographic method and a spectrophotometric method for total procyanidin content) as well as quality control of Pycnogenol®. The research question is whether Pycnogenol® complies with USP requirements.

Newest research indicates that Pycnogenol® exerts its immunomodulatory effects at least in part through the Toll-like receptor (TLR)4-NF-κB pathway [580]. However, the mechanism by which Pycnogenol® exerts its effects via TLR4 and whether other TLRs and signalling mechanisms are involved remains to be elucidated. **Chapter 4** describes *in vitro* research with the HEK cell line transfected with pNIFTY plasmids and TLR1/2, TLR2/6, TLR4 and TLR5 to investigate TLR binding, as well as with the THP-1 cell line to investigate the effect on cytokine secretion (IL-1β, IL-6, IL-8, IL-10, and TNF-α). Pycnogenol® extract was not only used as purchased, but also metabolised by an *in vitro* gastrointestinal dialysis model with colon phase. Research questions are whether Pycnogenol® might exert its immune modulating properties through other cell membrane TLRs than TLR4, whether Pycnogenol® stimulates anti-inflammatory cytokine secretion and whether digestion in the presence of microbiota affects its immune modulating effects. The same analyses were performed for catechin, the main monomer in Pycnogenol®.

Finally, knowledge on therapeutic options for ADHD behaviour must be improved. Though some results are promising, further controlled studies are warranted to investigate effects of Pycnogenol® on ADHD and comorbid symptoms. After all, patients are interested in effects on behaviour as well as side effects of a treatment, compared to standard therapy. **Chapter 5** describes the setup of a randomised double-blind placebo and active product controlled 10-week clinical trial with three parallel treatment arms in paediatric ADHD patients, including the design and protocol, registration and ethical approval requirements and safety monitoring. The ultimate goal of this trial is to investigate the efficacy, mechanism of action and value of Pycnogenol® in ADHD therapy as compared to MPH treatment and placebo, including effects on immunity, antioxidant levels, oxidative damage and comorbid psychiatric and physical complaints, and to evaluate the tolerability of Pycnogenol® compared to MPH. The main research question is whether Pycnogenol® is more effective than placebo and not less effective than MPH in improving behaviour. Other research questions are whether, as compared to placebo and MPH, Pycnogenol® increases antioxidant levels, reduces oxidative damage, improves immune and neurochemical status and reduces comorbid physical and psychiatric complaints. Final results of this trial, which is currently ongoing, will only be available after publication of this PhD thesis.

REFERENCES

1. APA. American Psychiatric Association: DSM-5 Attention Deficit/Hyperactivity Disorder Fact Sheet. 2013; Available from: http://www.dsm5.org/Documents/ADHD%20Fact%20Sheet.pdf [Accessed 8 February 2018].
2. APA, American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders, Fourth Edn, Text Revision. 2000, Washington, DC.
3. Still, G.F., Some abnormal psychical conditions in children: the Goulstonian lectures. Lancet, 1902. 1: p. 1008-1012.
4. Spencer, T.J., J. Biederman, and E. Mick, Attention-deficit/hyperactivity disorder: diagnosis, lifespan, comorbidities, and neurobiology. J Pediatr Psychol, 2007. 32: p. 631-642.
5. APA, American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders, Third Edn. 1980, Washington, DC.
6. Rubinstein, R.A. and R.T. Brown, An evaluation of the validity of the diagnostic category of attention deficit disorder. Am J Orthopsychiatry, 1984. 54: p. 398-414.
7. NICE, Attention Deficit Hyperactivity Disorder: diagnosis and management of ADHD in children, young people and adults. NICE Clinical Guideline 72. 2008, National Institute for Health and Clinical Excellence: London, UK.
8. WHO. World Health Organisation: International Classification of Diseases (ICD). 26.11.2013]; Available from: http://www.who.int/classifications/icd/en [Accessed 8 March 2018].
9. Polanczyk, G., et al., The Worldwide Prevalence of ADHD: A Systematic Review and Metaregression Analysis. Am J Psychiatry, 2007. 164(6): p. 942-948.
10. Biederman, J., et al., Convergence of the Child Behavior Checklist with structured interview-based psychiatric diagnoses of ADHD children with and without comorbidity. J Child Psychol Psychiat, 1993. 34: p. 1241 - 1251.
11. Biederman, J., et al., The CBCL as a screen for psychiatric comorbidity in paediatric patients with ADHD. Arch Dis Child, 2005. 90: p. 1010-1015.
12. Furman, L., What is Attention-Deficit Hyperactivity Disorder (ADHD)? J Child Neurol, 2005. 20(12): p. 994-1002.
13. Pelsser, L.M.J. and J.K. Buitelaar, Favourable effect of a standard elimination diet on the behaviour of young children with attention deficit-hyperactivity disorder (ADHD), a pilot study. Ned Tijdschr Geneesk, 2002. 46(52): p. 2543-2547.
14. Conners, C.K., et al., The revised Conners' Parent Rating Scale (CPRS-R): factor structure, reliability, and criterion validity. J Abnorm Child Psychol, 1998. 26(4): p. 257-268.
15. Reid, R., et al., Assessing Culturally Different Students for Attention Deficit Hyperactivity Disorder Using Behavior Rating Scales. J Abnorm Child Psychol, 1998. 26(3): p. 187-198.
16. Schaffer, D.F.R.C., et al., NIMH Diagnostic Interview Schedule for Children Version IV (NIMH DISC-IV): Description, Differences From Previous Versions, and Reliability of Some Common Diagnoses. J Am Acad Child Adolesc Psychiatry, 2000. 39(1): p. 28-38.
17. TestWeb. Sociaal-Emotionele Vragenlijst (SEV). 2013 Available from: http://testweb.bsl.nl/tests/sev/ [Accessed 5 July 2016].
18. Thapar, A. and G. Lewis, Should we be rethinking how we assess and manage ADHD? J Am Acad Child Adolesc Psychiatry, 2009. 48: p. 1051-1052.
19. Gaitens, T., B.J. Kaplan, and B. Freigang, Absence of an Association between IgE-mediated Atopic Responsiveness and ADHD Symptomatology. J Child Psychol Psychiatry, 1998. 39(3): p. 427-431.
20. Taylor, E., et al., European clinical guidelines for hyperkinetic disorder -- first upgrade. Eur Child Adolesc Psychiatry, 2004. 13 (Suppl): p. 17-30.
21. Barkley, R., Attention-Deficit/Hyperactivity Disorder: A Handbook for Diagnosis and Treament. 2 ed. 1998, New York: Guilford Press.
22. Wallis, D., H.F. Russell, and M. Muenke, Review: Genetics of Attention Deficit/Hyperactivity Disorder. J Pediatr Psychol, 2008. 33(10): p. 1085-1099.
23. Bope, E. and D. Kellerman, Attention-Deficit/Hyperactivity Disorder, in Conn's Current Therapy. 2013, Saunders Elsevier: Philadelphia. p. 1050-1053.
24. Sinn, N., Nutritional and dietary influences on attention deficit hyperactivity disorder. Nutr Rev, 2008. 66(10): p. 558-568.
25. Polanczyk, G.V., et al., ADHD prevalence estimates across three decades: an updated systematic review and meta--regression analysis. Int J Epidemiol, 2014. 43(2): p. 434-442.
26. Willcutt, E.G., The prevalence of DSM-IV attention-deficit/hyperactivity disorder: a meta-analytic review. Neurotherapeutics, 2012. 9(3): p. 490-499.

27. De Ridder, T., et al., The prevalence of ADHD in the Belgian general adult population: an epidemiological explanatory study. Tijschr Psychiatrie, 2008. 80: p. 499-508.
28. Biederman, J. and S.V. Faraone, Attention-deficit hyperactivity disorder. Lancet, 2005. 366: p. 237-248.
29. Staller, J. and S.-. Faraone, Attention-deficit hyperactivity disorder in girls: epidemiology and management. CNS Drugs, 2006. 20: p. 107-123.
30. Kessler, R.C., et al., The Prevalence and Correlates of Adult ADHD in the United States: Results From the National Comorbidity Survey Replication. Am J Psychiatry, 2006. 163: p. 716-723.
31. Lee, S.I., et al., Predictive validity of DSM-IV and ICD-10 criteria for ADHD and hyperkinetic disorder. J Child Psychol Psychiat, 2008. 49(1): p. 70-78.
32. Schmitt, J., et al., Atopic Eczema and Attention-Deficit Hyperactivity Disorder in a Population-Based Sample of Children and Adolescents. JAMA, 2009. 301(7): p. 724-726.
33. Schubert, I., I. Köster, and G. Lehmkuhl, The changing prevalence of attention-deficit/hyperactivity disorder and methylphenidate prescriptions: a study of data from a random sample of insurees of the AOK Health Insurance Company in the German State of Hesse, 2000-2007. Dtsch Arztebl Int, 2010. 107: p. 615-621.
34. Vande Voort, J.L., et al., Impact of the DSM-5 Attention-Deficit/Hyperactivity Disorder Age-of-Onset Criterion in the US Adolescent Population J Am Acad Child Adolesc Psychiatry, 2017. 53(7): p. 736-744.
35. Polanczyk, G.V. and S. Kollins, Implications of extending the ADHD age-of-onset criterion to age 12: Results from a prospectively studied birth cohort. J Am Acad Child Adolesc Psychiatry, 2010. 49(3): p. 210-216.
36. Brown, R.T., et al., Prevalence and assessment of attention-deficit/hyperactivity disorder in primary care settings. Pediatrics, 2001. 107: p. E43.
37. Merikangas, K.R., et al., Prevalence and treatment of mental disorders among US children in the 2001-2004 NHANES. Pediatrics, 2010. 125: p. 75-81.
38. Sauver, J.L.S., et al., Early Life Risk Factors for Attention-Deficit/Hyperactivity Disorder/ A Population-Based Cohort Study. Mayo Clin Proc, 2004. 79(9): p. 1124-1131.
39. Instanes, J.T., et al., Attention-Deficit/Hyperactivity Disorder in Offspring of Mothers With Inflammatory and Immune System Diseases. Biol Psychiatry, 2017. 81(5): p. 452-459.
40. Larson, K., et al., Patterns of comorbidity, functioning, and service use for US children with ADHD, 2007. Pediatrics, 2011. 127: p. 462-470.
41. Elia, J., P. Ambrosini, and W. Berrettini, ADHD characteristics: I. Concurrent co-morbidity patterns in children & adolescents. Child Adolesc Psychiatry Ment Health, 2008. 2: p. 15.
42. Danckaerts, M., et al., The quality of life of children with attention deficit/hyperactivity disorder: a systematic review. Eur Child Adolesc Psychiatry, 2010. 19: p. 83-105.
43. Taurines, R., et al., Developmental comorbidity in attention-deficit/hyperactivity disorder. Atten Defic Hyperact Disord, 2010. 2(4): p. 267-289.
44. Fliers, E.A., et al., Genome-wide association study of motor coordination problems in ADHD identifies genes for brain and muscle function. World J Biol Psychiatry, 2012. 13(3): p. 211-222.
45. Mangeot, S.D., et al., Sensory modulation dysfunction in children with attention-deficit-hyperactivity disorder. Development Med Child Neurol, 2001. 43(6): p. 399-406.
46. Rommelse, N., et al., Shared heritability of attention-deficit/hyperactivity disorder and autism spectrum disorder. Eur Child Adolesc Psychiatry, 2010. 19: p. 281-295.
47. Kaplan, B.J., et al., Dietary replacement in preschool-aged hyperactive boys. Pediatrics, 1989. 83: p. 7-17.
48. Carter, C.M., et al., Effects of a few food diet in attention deficit disorder. Arch Dis Child, 1993. 69: p. 564-568.
49. Hammerness, P., et al., Reexamining the familial association between asthma and ADHD in girls. J Atten Disord, 2005. 8: p. 133-143.
50. Niederhofer, H. and K. Pittschieler, A preliminary investigation of ADHD symptoms in persons with celiac disease. J Atten Disord, 2006. 10: p. 200-204.
51. Kumperscak, H.G., et al., Prevalence of Celiac Disease Is Not Increased in ADHD Sample. J Atten Disord, 2016.
52. Yuksel, H., A. Sogut, and O. Yilmaz, Attention Deficit and Hyperactivity Symptoms in Children with Asthma. J Asthma, 2008. 45(7): p. 545-547.
53. Biederman, J., et al., The longitudinal course of comorbid oppositional defiant disorder in girls with ADHD: findings from a controlled 5-year prospective longitudinal follow-up study. J Dev Behav Pediatr, 2008. 29(6): p. 501-507.
54. Harpin, V.A., The effect of ADHD on the life of an individual, their family, and community from preschool to adult life. Arch Dis Child, 2005. 90: p. 12-17.
55. Kleinman, N.L., et al., Incremental employee health benefit costs, absence days, and turnover among employees with ADHD and among employees with children with ADHD. J Occup Environ Med, 2009. 51: p. 1247-1255.
56. Marks, D.J., et al., Profiles of service utilization and the resultant economic impact in preschoolers with attention deficit/hyperactivity disorder. J Pediatr Psychol, 2009. 34: p. 681-689.

57. Leibson, C.L., et al., Use and costs of medical care for children and adolescents with and without attention-deficit/hyperactivity disorder. JAMA, 2001. 285: p. 60-66.
58. De Ridder, A. and D. De Graeve, Healthcare use, social burden and costs of children with and without ADHD in Flanders, Belgium. Clin Drug Investig, 2006. 26: p. 75-90.
59. Hakkaart-van Roijen, L., et al., Societal costs and quality of life of children suffering from attention deficient hyperactivity disorder (ADHD). Eur Child Adolesc Psychiatry, 2007. 16: p. 316-326.
60. Wehmeier, P.M., A. Schacht, and R.A. Barkley, Social and emotional impairment in children and adolescents with ADHD and the impact on quality of life. J Adolesc Health, 2010. 46: p. 209-217.
61. Daviss, W.B., A Review of Co-Morbid Depression in Pediatric ADHD: Etiologies, Phenomenology, and Treatment. J Child Adol Psychopharmacol, 2008. 18(6): p. 565-571.
62. Pelham, W.E. and E.M.R. Foster, J.A., The Economic Impact of Attention-Deficit/Hyperactivity Disorder in Children and Adolescents. J Pediatr Psychol, 2007. 32(6): p. 711-727.
63. Ceylan, M., et al., Changes in oxidative stress and cellular immunity serum markers in attention-deficit/hyperactivity disorder. Psychiatry Clin Neurosci, 2012. 66: p. 220-226.
64. Pelsser, L.M.J., J.K. Buitelaar, and H.F.J. Savelkoul, ADHD as a (non) allergic hypersensitivity disorder: a hypothesis. Pediatr Allergy Immunol, 2009. 20(2): p. 107-112.
65. Verlaet, A.A.J., et al., Nutrition, immunological mechanisms and dietary immunomodulation in ADHD. Eur Child Adolesc Psychiatry, 2014. 23(7): p. 519-529.
66. Schonwald, A., ADHD and Food Additives Revisited. AAP Grand Rounds, 2008. 19: p. 17.
67. de Theije, C.G., et al., Food allergy and food-based therapies in neurodevelopmental disorders. Pediatr Allergy Immunol, 2014. 25(3): p. 218-226.
68. Poelmans, G., et al., Integrated Genome-Wide Association Study Findings: Identification of a Neurodevelopmental Network for Attention Deficit Hyperactivity Disorder. Am J Psychiatr, 2011. 168: p. 365–377.
69. Faraone, S.V., C. Bonvicini, and C. Scassellati, Biomarkers in the diagnosis of ADHD - promising directions. Curr Psych Reports, 2014. 16(497): p. 1-20.
70. Guney, E., et al., Attention Deficit Hyperactivity Disorder and oxidative stress: A short term follow up study. Psychiatry Res, 2015. 229: p. 310-317.
71. Corradini, I., et al., SNAP-25 in Neuropsychiatric Disorders. Ann NY Acad Sci, 2009. 1152: p. 93-99.
72. Lopresti, A.L., Oxidative and nitrosative stress in ADHD: possible causes and the potential of antioxidant-targeted therapies. ADHD, 2015. 7: p. 237-247.
73. Trebatická, J., et al., Treatment of ADHD with French maritime pine bark extract, Pycnogenol. Eur Child Adolesc Psychiatry, 2006. 15: p. 329-335.
74. ADHD-Institute. Neurobiology - ADHD is associated with structural, functional and neurotransmitter alterations in the brain.; Available from: http://adhd-institute.com/burden-of-adhd/aetiology/neurobiology/ [Accessed 13 September 2017].
75. Hess, J.L., et al., Why is there selective subcortical vulnerability in ADHD? Clues from postmortem brain gene expression data. Mol Psychiatry, 2018. 23(8): p. 1787-1793.
76. Hoogman, M., et al., Subcortical brain volume differences in participants with attention deficit hyperactivity disorder in children and adults: a cross-sectional mega-analysis. Lancet Psych, 2017. 4(310-319).
77. Biederman, J. and S.V. Faraone, Attention-deficit hyperactivity disorder. Lancet, 2005. 366(9481): p. 237-48.
78. Biederman, J., Attention-deficit/hyperactivity disorder: a selective overview. Biol Psychiatry, 2005. 57(11): p. 1215-20.
79. Cortese, S., et al., Toward systems neuroscience of ADHD: a meta-analysis of 55 fMRI studies. Am J Psychiatry, 2012. 169: p. 1038-1055.
80. Biederman, J., Attention-Deficit/Hyperactivity Disorder: A Selective Overview. Biological Psychiatry, 2005. 57(11): p. 1215-1220.
81. Konrad, K., S. Gauggel, and J. Schurek, Catecholamine functioning in children with traumatic brain injuries and children with attention-deficit/hyperactivity disorder. Cogn Brain Res, 2003. 16: p. 425-433.
82. Dvořáková, M., et al., Urinary catecholamines in children with attention deficit hyperactivity disorder (ADHD): Modulation by a polyphenolic extract from pine bark (Pycnogenol). Nutr Neurosci, 2007. 10: p. 151-157.
83. Scasselati, C., et al., Biomarkers and Attention-Deficit/Hyperactivity Disorder: A Systematic Review and Meta-analyses. J Am Acad Child Adolesc Psychiatry, 2012. 51(10): p. 1003-1019.
84. Faraone, S., et al., Molecular genetics of attention-deficit/hyperactivity disorder. Biol Psychiatry, 2005. 57: p. 1313-1323.
85. Oztop, D., et al., Oxidative stress in children with attention deficit hyperactivity disorder. Clin Biochem, 2012. 45: p. 745-748.
86. Trebatická, J. and Z. Ďuračková, Psychiatric Disorders and Polyphenols: Can They Be Helpful in Therapy? Ox Med Cell Longevity, 2015. 248529: p. 1-16.

87. Russell, V.A., Overview of animal models of attention deficit hyperactivity disorder (ADHD). Curr Protoc Neurosci, 2011. 9: p. 9.35.
88. Williams, N.M., et al., Rare chromosomal deletions and duplications in attention-deicit hyperactivity disorder: a genome-wide analysis. Lancet 2010. 376: p. 1401-1408.
89. Thapar, A., et al., Psychiatric gene discoveries shape evidence on ADHD's biology. Mol Psychiatry, 2016. 21(9): p. 1202-1207.
90. Demontis, D., et al., Discovery Of The First Genome-Wide Significant Risk Loci For ADHD. Nat Genet, 2019. 51(1): p. 63-75.
91. Pineda, D.A., et al., Environmental influences that affect attention deficit/hyperactivity disorder - Study of a genetic isolate. Eur Child Adolesc Psychiatry, 2007. 16(5): p. 337-346.
92. Roza, S.J., et al., What Is Spared by Fetal Brain-Sparing? Fetal Circulatory Redistribution and Behavioral Problems in the General Population. Am J Epidemiol, 2008. 168: p. 1145-1152.
93. Hüppi, P.S., Neuroimaging of brain development - Discovering the origins of neuropsychiatric disorders? Ped Res, 2008. 64: p. 325.
94. Mick, E., et al., Case-control study of attention-deficit hyperactivity disorder and maternal smoking, alcohol use, and drug use during pregnancy. J Am Acad Child Adolesc Psychiatry, 2002. 41(4): p. 378-385.
95. Mick, E., et al., Impact of low birth weight on attention-deficit hyperactivity disorder. J Dev Behav Pediatr, 2002. 23(1): p. 16-22.
96. Schneider, M.L., E.C. Roughton, and G.R. Lubach, Moderate Alcohol Consumption and Psychological Stress during Pregnancy Induce Attention and Neuromotor Impairments in Primate Infants. Child Development, 1997. 68(5): p. 747-759.
97. Pagani, L.S., Environmental tobacco smoke exposure and brain development: The case of attention deficit/hyperactivity disorder. Neurosci Biobehav Rev, 2014. 44: p. 195-205.
98. Mick, E., J. Biederman, and S.V. Faraone, Is season of birth a risk factor for attention-deficit hyperactivity disorder? J Am Acad Child Adolesc Psychiatry, 1996. 35: p. 1470-1476.
99. Liew, Z., et al., Acetaminophen use during pregnancy, behavioral problems, and hyperkinetic disorders. JAMA Pediatr, 2014. 168: p. 313-320.
100. Blais, L., et al., Asthma exacerbations during the first trimester of pregnancy and congenital malformations: Revisiting the association in a large representative cohort. Thorax, 2015. 70: p. 647-652.
101. Bauer, A.Z., et al., Prenatal paracetamol exposure and child neurodevelopment: A review. Horm Behav, 2018. 101: p. 125-147.
102. Myhre, O., et al., Early life exposure to air pollution particulate matter (PM) as risk factor for attention deficit/hyperactivity disorder (ADHD): Need for novel strategies for mechanisms and causalities. Toxicol Appl Parmacol, 2018. 354: p. 196-214.
103. Guxens, M., et al., Air pollution during pregnancy and childhood cognitive and psychomotor development: six European birth cohorts. Epidemiol, 2014. 25(5): p. 636-647.
104. Braun, J.M., et al., Exposures to Environmental Toxicants and Attention Deficit Hyperactivity Disorder in U.S. Children. Environ Health Perspect, 2006. 114(12): p. 1904-1909.
105. Nigg, J.T., et al., Low blood lead levels associated with clinically diagnosed attention-deficit/hyperactivity disorder and mediated by weak cognitive control. Biol Psychiatry, 2008. 63: p. 325-331.
106. Viktorinova, A., et al., Changed plasma levels of zinc and copper to zinc ratio and their possible associations with parent- and teacher-rated symptoms in children with attention-deficit hyperactivity disorder. Biol Trace Element Res, 2016. 169: p. 1-7.
107. Tuthill, R.W., Hair lead levels related to children's classroom attention-deficit behaviour. Arch Env Health, 1996. 51(3): p. 214-220.
108. Joseph, N., et al., Oxidative Stress and ADHD: A Meta-Analysis. J Atten Disord, 2015. 19(11): p. 915-924.
109. Dufault, R., et al., Mercury exposure, nutritional deficiencies and metabolic disruptions may affect learning in children. Behav Brain Functions, 2009. 5: p. 44-59.
110. Davidson, P.W., G.J. Myers, and B. Weiss, Mercury Exposure and Child Development Outcomes. Pediatrics, 2004. 113(Suppl 3): p. 1023-1029.
111. Kuehn, B.M., Increased Risk of ADHD Associated With Early Exposure to Pesticides, PCBs. JAMA, 2010. 304(1): p. 27-28.
112. Julvez, J., et al., Attention behaviour and hyperactivity at age 4 and duration of breast-feeding. Acta Pediatr, 2007. 96: p. 842-847.
113. Mimouni-Bloch, A., et al., Breastfeeding may protect from developing attention-deficit/hyperactivity disorder. Breastfeeding Med, 2013. 8(4): p. 363-367.
114. Howard, A.L., et al., ADHD is associated with a "Western" dietary pattern in adolescents. J Atten Disord, 2011. 15(5): p. 403-411.
115. Yan, S., et al., Dietary patterns are associated with attention-deficit/hyperactivity disorder (ADHD) symptoms among preschoolers in mainland China. Eur J Clin Nutr, 2018. 72(11): p. 1517-1523.

116. Antshel, K.M., et al., Advances in understanding and treating ADHD. BMC Med, 2011. 9: p. 72.
117. Zwi, M., et al., Parent training interventions for Attention Deficit Hyperactivity Disorder (ADHD) in children aged 8 to 15. Cochrane Library, 2011(12).
118. RIZIV, Farmanet databank: vergoede specialiteiten methylfenidaat (ATC N06BA04) afgeleverd via Belgische openbare apotheken, 2006-2016. 2018: Brussel.
119. Ougrin, D., S. Chatterton, and R. Banarsee, Attention deficit hyperactivity disorder (ADHD): review for primary care clinicians. London J Prim Care, 2010. 3: p. 45-51.
120. Volz, T.J., Neuropharmacological mechanisms underlying the neuroprotective effects of methylphenidate. Curr Neuropharmacol, 2008. 6: p. 379-385.
121. Hodgkins, P., et al., Amfetamine and methylphenidate medications for attention-deficit/hyperactivity disorder: complementary treatment options. ECAP, 2012. 21: p. 477-492.
122. Pliszka, S.R., The neuropsychopharmacology of attention-deficit/hyperactivity disorder. Biol Psychiatry, 2005. 57: p. 1385-1390.
123. Findling, R.L., Evolution of the treatment of attention-deficit/hyperactivity disorder in children: a review. Clin Therap, 2008. 30: p. 942-957.
124. Blum, K.G., M.S., et al., Neurogenetics of acute and chronic opiate/opioid abstinence: treating symptoms and the cause. Front Bioscience, 2017. 22: p. 1247-1288.
125. Storebø, O.J., et al., Methylphenidate for attention-deficit/hyperactivity disorder in children and adolescents: Cochrane systematic review with meta-analyses and trial sequential analyses of randomised clinical trials. BMJ, 2015. 351.
126. Schachter, H.M., et al., How efficacious and safe is shortacting methylphenidate for the treatment of attention-deficit disorder in children and adolescents? A meta-analysis. CMAJ, 2011. 165(11): p. 1475-1488.
127. Shibib, S. and N. Chalhoub, Stimulant induced psychosis. Child Adolesc Ment Health, 2009. 14: p. 20-23.
128. NICE, Attention deficit hyperactivity disorder: Diagnosis and management of ADHD in children, young people and adults. National Clinical Practice Guideline. 2009, The British Psychological Society and The Royal College of Psychiatrist: Leicester, London.
129. Auci, D.L., S. Fikrig, and J. Rodriquez, Methylphenidate and the immune system. J Am Acad Child Adolesc Psychiatry, 1997. 36(8): p. 1015-1016.
130. Schmidt, A.J., et al., Impact of drugs approved for treating ADHD on the cell survival and energy metabolism: an in-vitro study in human neuronal and immune cells. J Psychopharmacol, 2010. 24(12): p. 1829-1833.
131. Martinez-Raga, J., et al., Risk of serious cardiovascular problems with medications for attention-deficit hyperactivity disorder. CNS Drugs, 2013. 27: p. 15-30.
132. Silva, R.R., J.W. Skimming, and R. Muniz, Cardiovascular safety of stimulant medications for pediatric attention-deficit hyperactivity disorder. Clin Pediatr (Phila), 2010. 49: p. 840-851.
133. Heyer, D.B. and R.M. Meredith, Environmental toxicology: Sensitive periods of development and neurodevelopmental disorders. NeuroToxicol, 2017. 58: p. 23-41.
134. Steer, C.R., Managing attention deficit/hyperactivity disorder: unmet needs and future directions. Arch Dis Child, 2005. 90(Suppl I): p. i19-i25.
135. Charach, A. and R. Fernandez, Enhancing ADHD medication adherence: challenges and opportunities. Curr Psychiatr Rep, 2013. 15: p. 371-379.
136. Taylor, M., T. O'Donoghue, and S. Houghton, To medicate or not to medicate? The decision-making process of Western Australian parents following their child's diagnosis with an attention deficit hyperactivity disorder. Int J Disabil Developm Educ, 2006. 53(1): p. 111-128.
137. Kortekaas-Rijlaarsdam, A.F., et al., Does methylphenidate improve academic performance? A systematic review and meta-analysis. Eur Child Adolesc Psychiatry, 2019. 28(2): p. 155-164.
138. Nehlig, A., Is Caffeine a Cognitive Enhancer? J Alzheimer's Disease, 2010. 20: p. S85-S94.
139. Roth Leon, M., Effects of caffeine in cognitive, psychomotor, and affective performance of children with Attention-Deficit/Hyperactivity Disorder. J Atten Disord, 2000. 4(1): p. 27-47.
140. Lara, D.R., Caffeine, mental health, and psychiatric disorders. J Alzheimer's Disease, 2010. 20: p. S239-S248.
141. Conners, C.K., et al., Nicotine and attention in adults Attention Deficit Hyperactivity Disorder (ADHD). Psychopharmacol Bull, 1996. 32(1): p. 67-73.
142. McClernon, F.J. and S.H. Kollins, ADHD and Smoking From Genes to Brain to Behavior. Ann NY Acad Sci, 2008. 1141: p. 131-147.
143. Pelsser, L.M.J., et al., Effects of a restricted elimination diet on the behaviour of children with attention-deficit hyperactivity disorder (INCA study): a randomised controlled trial. Lancet, 2011. 377: p. 494-503.
144. Stevens, L., et al., Dietary Sensitivities and ADHD Symptoms: Thirty-five Years of Research. Clin Pediatr, 2011. 50(4): p. 279-293.
145. Millichap, J.G. and M.M. Yee, The diet factor in attention-deficit/hyperactivity disorder. Pediatrics, 2012. 129: p. 330-337.

146. Lau, K., et al., Synergistic Interactions between Commonly Used Food Additives in a Developmental Neurotoxicity Test. Toxicol Sci, 2006. 90(1): p. 178-187.
147. Holton, K.F., et al., Evaluation of dietary intake in children and college students with and without attention-deficit/hyperactivity disorder. Nutr Neurosci, 2018. [Epub ahead of print].
148. Kaplan, B.J., et al., Vitamins, Minerals, and Mood. Psychol Bull, 2007. 133(5): p. 747-760.
149. Rucklidge, J.J., et al., Moderators of treatment response in adults with ADHD treated with a vitamin-mineral supplement. Progr Neuro-Psychopharmacol Biol Psych, 2014. 50: p. 163-171.
150. Gordon, H.A., et al., Clinically Significant Symptom Reduction in Children with Attention-Deficit/Hyperactivity Disorder Treated with Micronutrients: An Open-Label Reversal Design Study. J Child Adolesc Psychopharmacol, 2015. 25(10): p. 783-798.
151. Avcil, S., et al., Vitamin D Deficiency and a Blunted Parathyroid Hormone Response in Children with Attention-Deficit/Hyperactivity Disorder. Clin Lab, 2017. 63(3): p. 435-443.
152. Bener, A., et al., Higher prevalence of iron deficiency as strong predictor of attention deficit hyperactivity disorder in children. Ann Med Health Sci Res, 2014. 4(Suppl 3): p. S291-297.
153. Goksugur, S.B., et al., Vitamin D Status in Children with Attention Deficit Hyperactivity Disorder. Pediatr Int, 2014 56(4): p. 515-519.
154. Sahin, N., et al., Vitamin D and vitamin D receptor levels in children with attention-deficit/hyperactivity disorder. Neuropsychiatr Dis Treat, 2018. 14: p. 581-585.
155. Ross, A.C., et al., Modern nutrition in health and disease. 10 ed, ed. M.E. Shils. 2006, Baltimore: Lippincott Williams & Wilkins.
156. Yu, W., et al., Copper (Cu2+) induces degeneration of dopaminergic neurons in the nigrostriatal system of rats. Neurosci Bull, 2008. 24(2): p. 73-78.
157. Konofal, E., et al., Iron deficiency in children with attention-deficit/hyperactivity disorder. Arch Pediatr Adolesc Med, 2004. 158: p. 1113-1115.
158. El Adham, E.K., A.I. Hassan, and A.A. El Aziz El- Mahdy, Nutritional and Metabolic Disturbances in Attention Deficit Hyperactivity Disease. Res J Med & Med Sci, 2011. 6(1): p. 10-16.
159. Villagomez, A. and U. Ramtekkar, Iron, Magnesium, Vitamin D, and Zinc Deficiencies in Children Presenting with Symptoms of Attention-Deficit/Hyperactivity Disorder. Children (Basel), 2014. 1(3): p. 261-279.
160. Wang, Y., et al., Iron status in attention-deficit/hyperactivity disorder: a systematic review and meta-analysis. PLoS One, 2017. 12(e0169145).
161. Calarge, C., et al., Serum ferritin and amphetamine response in youth with attention-deficit/hyperactivity disorder. J Child Adolesc Psychopharmacol, 2010. 20(6): p. 495-502.
162. Kwon, H.J., et al., Transferrin in korean children with attention deficit hyperactivity disorder. Psychiatry Investig, 2011. 8(4): p. 366-371.
163. Romanos, M., et al., No cross-sectional and longitudinal association of ferritin and symptoms of attention-deficit-/hyperactivity disorder in a large population-based sample of children: results from the GINIplus and LISAplus studies. Atten Defic Hyperact Disord, 2013. 5(3): p. 313-320.
164. Menegassi, M., et al., Food intake and serum levels of iron in children and adolescents with attention-deficit/hyperactivity disorder. Rev Bras Psiquiatr, 2010. 32(2): p. 132-138.
165. Konofal, E., et al., Effects of iron supplementation on attention deficit hyperactivity disorder in children. Pediatr Neurol, 2008. 38: p. 20-26.
166. Cortese, S., et al., Iron and attention deficit/hyperactivity disorder: What is the empirical evidence so far? A systematic review of the literature. Expert Rev Neurother, 2012. 12(10): p. 1227-1240.
167. Hurt, E.A., L.E. Arnold, and N. Lofthouse, Dietary and nutritional treatments for attention-deficit/hyperactivity disorder: Current research support and recommendations for practitioners. Curr Psychiatry Rep, 2011. 13: p. 323-332.
168. Skokauskas, N., et al., Complementary medicine for children and young people who have attention deficit hyperactivity disorder. Curr Opin Psychiatry, 2011. 24: p. 291-300.
169. Kozielec, T. and B. Starobrat-Hermelin, Assessment of magnesium levels in children with attention deficit hyperactivity disorder (ADHD). Magn Res, 1997. 10(2): p. 143-148.
170. Mahmoud, M.M., et al., Zinc, ferritin, magnesium and copper in a group of Egyptian children with attention deficit hyperactivity disorder. Ital J Pëdiatr, 2011. 37(60).
171. Irmisch, G., et al., Modified magnesium and lipoproteins in children with attention deficit hyperactivity disorder (ADHD). World J Biol Psychiatr, 2011. 12: p. S63-S65.
172. Archana, E., et al., Altered Biochemical parameters in saliva of pediatric attention deficit hyperactivity disorder. Neurochem Res, 2012. 37: p. 330-334.
173. Croteau, D.L. and V.A. Bohr, Repair of oxidative damage to nuclear and mitochondrial DNA in mammalian cells. J Biol Chem, 1997. 272(41): p. 25409-25412.
174. Spahis, S., et al., Lipid profile, fatty acid composition and pro- and anti-oxidant status in pediatric patients with attention-deficit/hyperactivity disorder. Prostaglandins Leukot Essent Fatty Acids, 2008. 79: p. 47-53.

175. Zile, M.H., Function of Vitamin A in Vertebrate Embryonic Development. J Nutr, 2001. 131(3): p. 705-708.
176. Garipardic, M., et al., Association of Attention Deficit Hyperactivity Disorder and Autism Spectrum Disorders with Mean Platelet Volume and Vitamin D. Med Sci Monit, 2017. 23: p. 1378-1384.
177. Miller, A., et al., Vitamin B12, demyelination, remyelination and repair in multiple sclerosis. J Neurol Sci, 2005. 233(1-2): p. 93-97.
178. Yamada, K., Cobalt: Its Role in Health and Disease., in Interrelations between Essential Metal Ions and Human Diseases. Metal Ions in Life Sciences., A. Sigel, H. Sigel, and R.K. Sigel, Editors. 2013, Springer.
179. Mossin, M.H., et al., Inverse associations between cord vitamin D and attention deficit hyperactivity disorder symptoms: A child cohort study. Aust N Z J Psychiatry, 2017. 51(7): p. 703-710.
180. Morales, E., et al., Vitamin D in Pregnancy and Attention Deficit Hyperactivity Disorder-like Symptoms in Childhood. Epidemiol, 2015. 26(4): p. 458-465.
181. Daraki, V., et al., High maternal vitamin D levels in early pregnancy may protect against behavioral difficulties at preschool age: the Rhea mother-child cohort, Crete, Greece. Eur Child Adolesc Psychiatry, 2018. 27(1): p. 79-88.
182. Strøm, M., et al., Vitamin D measured in maternal serum and offspring neurodevelopmental outcomes: a prospective study with long-term follow-up. Ann Nutr Metab, 2014. 64(3-4): p. 254-261.
183. Gustafsson, P., et al., Vitamin D Status at Birth and Future Risk of Attention Deficit/Hyperactivity Disorder (ADHD). PLoS One, 2015. 10(10): p. e0140164.
184. Sharif, M.R., et al., The relationship between serum vitamin D level and attention deficit hyperactivity disorder. Iran J Child Neurol, 2015. 9(4): p. 48-53.
185. Kamal, M., A. Bener, and M.S. Ehlayel, Is high prevalence of vitamin D deficiency a correlate for attention deficit hyperactivity disorder? Atten Defic Hyperact Disord, 2014. 6(2): p. 73-78.
186. Bener, A. and M. Kamal, Predict attention deficit hyperactivity disorder? Evidence-based medicine. Glob J Health Sci, 2013. 6(2): p. 47-57.
187. Bala, K.A., et al., Hormone disorder and vitamin deficiency in attention deficit hyperactivity disorder (ADHD) and autism spectrum disorders (ASDs). J Pediatr Endocrinol Metab, 2016. 29(9): p. 1077-1082.
188. Elshorbagy, H.H., et al., Impact of Vitamin D Supplementation on Attention-Deficit Hyperactivity Disorder in Children. Ann Pharmacother, 2018. 52(7): p. 623-631.
189. Arns, M., et al., Geographic variation in the prevalence of attention-deficit/hyperactivity disorder: the sunny perspective. Biol Psychiatry, 2013. 74: p. 585-590.
190. Tolppanen, A.M., et al., The association of 25-hydroxyvitamin D3 and D2 with behavioural problems in childhood. PLoS One, 2012. 7(7): p. e40097.
191. Reinehr, T., et al., 25-Hydroxvitamin D concentrations are not lower in children with bronchial asthma, atopic dermatitis, obesity, or attention-deficient/hyperactivity disorder than in healthy children. Nutr Res, 2018. 52: p. 39-47.
192. Khoshbakht, Y., R. Bidaki, and A. Salehi-Abargouei, Vitamin D Status and Attention Deficit Hyperactivity Disorder: A Systematic Review and Meta-Analysis of Observational Studies. Adv Nutr, 2018. 9(1): p. 9-20.
193. Eyles, D.W., T.H. Burne, and J.J. McGrath, Vitamin D, effects on brain development, adult brain function and the links between low levels of vitamin D and neuropsychiatric disease. Front Neuroendocrinol., 2013. 34(1): p. 47-64.
194. Holick, M.F., High prevalence of vitamin D inadequacy and implications for health. Mayo Clinic Proc, 2006. 81(3): p. 353-373.
195. Berridge, M.J., Vitamin D deficiency: infertility and neurodevelopmental diseases (attention deficit hyperactivity disorder, autism, and schizophrenia). Am J Physiol Cell Physiol, 2018. 314(2): p. C135-C151.
196. Traber, M.G. and H. Sies, Vitamin E in humans: demand and delivery. Annu Rev Nutr, 1996. 16: p. 321-347.
197. Arnold, L.E. and R.A. DiSilvestro, Zinc in attention-deficit/hyperactivity disorder. J Child Adolesc Psychopharmacol, 2005. 15: p. 619-627.
198. Bekaroğlu, M., et al., Relationships between serum free fatty acids and zinc, and attention deficit hyperactivity disorder: a research note. J Child Psychol Psychiatry, 1996. 37(2): p. 225-227.
199. Yorbik, O., et al., Potential effects of zinc on information processing in boys with attention deficit hyperactivity disorder. Prog Neuropsychopharmacol Biol Psychiatry, 2008. 32(3): p. 662-667.
200. Zhou, F., et al., Dietary, nutrient patterns and blood essential elements in Chinese children with ADHD. NUtrients, 2016. 8(6): p. E352.
201. Tippairote, T., et al., Hair Zinc and Severity of Symptoms Are Increased in Children with Attention Deficit and Hyperactivity Disorder: a Hair Multi-Element Profile Study. Biol Trace Elem Res, 2017. 179(2): p. 185-194.
202. Toren, P., et al., Zinc deficiency in Attention-Deficit Hyperactivity Disorder. Biol Psychiatry, 1996. 40: p. 1308-1310.
203. Kiddie, J., et al., Nutritional Status of Children with Attention Deficit Hyperactivity Disorder: A Pilot Study. Int J Pediatr, 2010: p. 1-7.

204. DiGirolamo, A.M. and M. Ramirez-Zea, Role of zinc in maternal and child mental health. Am J Clin Nutr, 2009. 89(suppl): p. 940S-645S.
205. Arnold, L.E., et al., Zinc for attention-deicit/hyperactivity disorder: placebo-controlled double-blind pilot trial alone and combined with amphetamine. J Child Adolesc Psychopharmacol, 2011. 21: p. 1-19.
206. Ghanizadeh, A. and M. Berk, Zinc for treating of children and adolescents with attention-deficit yperactivity disorder: a systematic review of randomized controlled clinical trials. Eur J Clin Nutr, 2013. 67(122-124).
207. Ramakrishnan, U., B. Imhoff-Kunsch, and A.M. DiGirolamo, Role of docosahexaenoic acid in maternal and child mental health. Am J Clin Nutr, 2009. 89(suppl): p. 958S-962.
208. Gillies, D., et al., Polyunsaturated fatty acids (PUFA) for attention deficit hyperactivity disorder (ADHD) in children and adolescents. Cochrane Database Systematic Rev, 2012. 7.
209. Bos, D.J., et al., Effects of omega-3 polyunsaturated fatty acids on human brain morphology and function: What is the evidence? Eur Neuropsychopharmacol, 2016. 26(3): p. 546-561.
210. Richardson, A.J., The importance of omega-3 fatty acids for behaviour, cognition and mood. Scand J Nutr, 2003. 47(2): p. 92-98.
211. Wu, A., Z. Ying, and F. Gomez-Pinilla, Dietary omega-3 fatty acids normalize BDNF levels, reduce oxidative damage, and counteract learning disability after traumatic brain injury in rats. J Neurotrauma, 2004. 21(10): p. 1457-1467.
212. Chalon, S., et al., Polyunsaturated fatty acids and cerebral function: focus on monoaminergic neurotransmission. Lipids, 2001. 36(9): p. 937-944.
213. Hawkey, E. and J.T. Nigg, Omega-3 fatty acid and ADHD: Blood level analysis and meta-analytic extension of supplementation trials. Clin Psychol Rev, 2014. 34: p. 496-505.
214. Chen, J.R., et al., Dietary patterns and blood fatty acid composition in children with attention-deficit hyperactivity disorder in Taiwan. J Nutr Biochem, 2004. 15(8): p. 467-472.
215. LaChance, L., et al., Omega-6 to Omega-3 Fatty Acid Ratio in Patients with ADHD: A Meta-Analysis. J Can Acad Child Adolesc Psychiatry, 2016. 25(2): p. 87-96.
216. Colter, A.L., C. Cutler, and K.A. Meckling, Fatty acid status and behavioural symptoms of Attention Deficit Hyperactivity Disorder in adolescents: A case-control study. Nutr J, 2008. 7: p. 8-18.
217. Brookes, K.J., et al., Association of fatty acid desaturase genes with attention-deficit/hyperactivity disorder. Biol Psychiatry, 2006. 60(10): p. 1053-1061.
218. Bos, D.J., et al., Reduced symptoms of inattention after dietary omega-3 fatty acid supplementation in boys with and without attention deficit/hyperactivity disorder. Neuropshychopharmacology, 2015. 40: p. 2298-2306.
219. Ortega, R.M., E. Rodriguez-Rodriguez, and A.M. Lopez-Sobaler, Effects of omega-3 fatty acids supplementation in behaviour and non-neurodegenerative neuropsychiatric disorders. Br J Nutr, 2012. 107: p. S261-S270.
220. Grassmann, V., R.F. Santos-Galduroz, and S.C.F. Galduroz, Effects of low doses of polyunsaturated fatty acids on the attention deficit/hyperactivity disorder of children: a systematic review. Curr Neuropharmacol, 2013. 11: p. 186-196.
221. Schuchardt, J.P., et al., Significance of long-chain polyunsaturated fatty acids (PUFAs) for the development and behaviour of children. Eur J Pediatr, 2010. 169: p. 149-164.
222. Matsudaira, T., et al., Biochemical and psychological effects of omega-3/6 supplements in male adolescents with Attention-Deficit/Hyperactivity Disorder: a randomized, placebo-controlled, clinical trial. J Child Adol Psychopharmacol, 2015. 25(10): p. 775-782.
223. Transler, C., et al., The impact of polyunsaturated fatty acids in reducing child attention deficit and hyperactivity disorders. J Atten Disord, 2010. 14(3): p. 232-246.
224. Puri, B.K. and J.G. Martins, Which polyunsaturated fatty acids are active in children with attention-deficit hyperactivity disorder receiving PUFA supplementation? A fatty acid validated meta-regression analysis of randomized controlled trials. Prostagland Leukotr Essent Fatty Acids, 2014. 90: p. 179-189.
225. Königs, A. and A.J. Kiliaan, Critical appraisal of omega-3 fatty acids in attention-deficit/hyperactivity disorder treatment. Neuropsychiatr Dis Treat, 2016. 12: p. 1869-1882.
226. Joshi, K., et al., Supplementation with flax oil and vitamin C improves the outcome of Attention Deficit Hyperactivity Disorder (ADHD). Prostagland Leukot Essent Fatty Acids, 2006. 74(1): p. 17-21.
227. Richardson, A.J. and P. Montgomery, The Oxford-Durham study: a randomized, controlled trial of dietary supplementation with fatty acids in children with developmental coordination disorder. Pediatrics, 2005. 115: p. 1360-1366.
228. Schab, D.W. and N.H.T. Trinh, Do Artificial Food Colors Promote Hyperactivity in Children with Hyperactive Syndromes? A Meta-Analysis of Double-Blind Placebo-Controlled Trials. Develop and Behavior Pediatr, 2004. 25(6): p. 423-434.
229. Stevenson, J., et al., The role of histamine degradation gene polymorphisms in moderating the effects of food additives on children’s ADHD symptoms. Am J Psychiatr, 2010. 167(9): p. 1108-1115.

230. Arnold, L.E., N. Lofthouse, and E. Hurt, Artificial Food Colors and Attention-Deficit/Hyperactivity Symptoms: Conclusions to Dye for. Neurotherapeutics, 2012. 9(3): p. 599-609.
231. Stevens, L.J., et al., Mechanisms of behavioral, atopic, and other reactions to artificial food colors in children. Nutr Rev, 2013. 71: p. 268-281.
232. Bateman, B., et al., The effects of a double blind, placebo controlled, sample of preschool children challenge on hyperactivity in a general population artificial food colourings and benzoate preservative. J Arch Dis Child, 2004. 89(6): p. 506-511.
233. Nigg, J.T., et al., Meta-analysis of Attention-Deficit/Hyperactivity Disorder or Attention-Deficit/Hyperactivity Disorder Symptoms, Restriction Diet, and Synthetic Food Color Additives. J Am Acad Child Adolesc Psychiatry, 2012. 51: p. 86-97.
234. Gov.uk. Food labelling and packaging. Food and drink warnings.; Available from: https://www.gov.uk/food-labelling-and-packaging/food-and-drink-warnings [Accessed 15 May 2018].
235. Parlement, E., Verordening (EG) Nr. 1333/2008 van het Europees Parlement en de Raad van 16 december 2008 inzake levensmiddelenadditieven., E. Unie, Editor. 2008.
236. McCann, D., et al., Food additives and hyperactive behaviour in 3-year-old and 8/9-year-old children in the community: a randomised, double-blinded, placebo-controlled trial. Lancet, 2007. 370(9598): p. 1560-1567.
237. Egger, J., et al., Controlled trial of oligo-antigenic treatment in the hyperkinetic syndrome. Lancet, 1985. 1: p. 540-545.
238. Schmidt, M.H., et al., Does oligoantigenic diet influence hyperactive/conduct-disordered children - a controlled trial. Eur Child Adolesc Psychiatry, 1997. 6(2): p. 88-95.
239. Breakey, J., The role of diet and behaviour in childhood. J Paediatr Child Health, 1997. 33: p. 190-194.
240. Rowe, K.S. and K.J. Rowe, Synthetic food coloring and behavior: A dose response effect in a double-blind, placebo-controlled, repeated-measures study. J Pediatr Psychol, 1994. 125(5pt1): p. 694-698.
241. Swain, A., et al., Salycilates, Oligoantigenic Diets, and Behaviour. Lancet, 1985. 2(8445): p. 41-42.
242. Boris, M. and F. Mandel, Foods and additives are common causes of the attention deficit hyperactive disorder in children. Ann Allergy, 1994. 73: p. 462-467.
243. Hill, P. and E. Taylor, An auditable protocol for treating attention deicit/hyperactivity disorder. Arch Dis Child, 2001. 84: p. 404-409.
244. Halliwell, B., Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. Free Rad Res, 1996. 25: p. 57-74.
245. Firuzi, O., et al., Antioxidant Therapy: Current Status and Future Prospects. Curr Med Chem, 2011. 18(25): p. 3871-3888.
246. Emiliani, F.E., T.W. Sedlak, and A. Sawa, Oxidative stress and schizophrenia: recent breakthroughs from an old story. Curr Opin Psychiatry, 2014. 27(3): p. 185-190.
247. Halliwell, B., Antioxidant defence mechanisms: From the beginning to the end (of the beginning). Free Rad Res, 1999. 31: p. 261-272.
248. Willcox, J.K., S.L. Ash, and G.L. Catignani, Antioxidants and prevention of chronic disease. Crit Rev Food Sci, 2004. 44: p. 275-295.
249. Ng, F., et al., Oxidative stress in psychiatric disorders: evidence base and therapeutic implications. Int J Neuropsychopharmacol., 2008. 11: p. 851-876.
250. Davies, K.J.A., Oxidative stress, antioxidant defences, and damage removal, repair, and replacement systems. IUBMB Life, 2000. 50: p. 279-289.
251. Birben, E., et al., Oxidative stress and antioxidant defense. World Allergy Organ J, 2012. 5: p. 9-19.
252. Dröge, W., Free Radicals in the Physiological Control of Cell Function. Physiol Rev, 2002. 82(1): p. 47-95.
253. Kul, M., et al., Evaluation of oxidative metabolism in child and adolescent patients with attention deficit hyperactivity disorder. Psychiatry Investig, 2015. 12(3): p. 361-366.
254. Delion, S., et al., Chronic dietary a-linolenic acid deficiency alters dopaminergic and serotoninergic neurotransmission in rats. J Nutr, 1994. 124: p. 2466-2476.
255. Chauhan, A. and V. Chauhan, Oxidative stress in autism. Pathophysiol, 2006. 13: p. 171-181.
256. Rabe-Jabłońska, J. and A. Dietrich-Muszalska, Relationship between oxidative stress and obsessive-compulsive disorder., in Studies on psychiatric disorders., A. Dietrich-Muszalska, V. Chauhan, and S. Grignon, Editors. 2015, Springer New York: New York, NY. p. 131-141.
257. Palta, P., et al., Depression and oxidative stress: results from a meta-analysis of observational studies. Psychosom Med, 2014. 76: p. 12-19.
258. Salim, S., Oxidative Stress and Psychological Disorders. Curr Neuropharmacol, 2014. 12: p. 140-147.
259. Aruoma, O.I., Free radicals, oxidative stress, and antioxidants in human health and disease. JAOCS, 1998. 75: p. 199-212.
260. Herken, H., et al., Nitric oxide, adenosine deaminase, xanthine oxidase and superoxide dismutase in patients with panic disorder: alterations by antidepressant treatment. Hum Psychopharmacol, 2006. 21(1): p. 53-59.

261. James, S.J., et al., Metabolic biomarkers of increased oxidative stress and impaired methylation capacity in children with autism. Am J Clin Nutr, 2004. 80(6): p. 1611-1617.
262. Bradstreet, J.J., et al., Biomarker-Guided Interventions of Clinically Relevant Conditions Associated with Autism Spectrum Disorders and Attention Deficit Hyperactivity Disorder. Altern Med Rev, 2010. 15(1): p. 15-32.
263. Rehnström, K., et al., No association between common variants in glyoxalase 1 and autism spectrum disorders. . Am J Med Genet B Neuropsychiatr Genet, 2008. 147B(1): p. 124-127.
264. Ceylan, M., et al., Oxidative imbalance in child and adolescent patients with attention-deficit/hyperactivity disorder. Prog Neuropsychopharmacol Biol Psychiatry, 2010. 34(8): p. 1491-1494.
265. Kawatani, M., H. Tsukahara, and M. Mayumi, Evaluation of oxidative stress status in children with pervasive developmental disorder and attention deficit hyperactivity disorder using urinary-specific biomarkers. Redox Rep, 2011. 16(1): p. 45-46.
266. Ross, B.M., et al., Increased levels of ethane, a non-invasive marker of n-3 fatty acid oxidation, in breath of children with attention deficit hyperactivity disorder. Nutr Neurosci, 2003. 6(5): p. 277-281.
267. Chovanová, Z., et al., Effect of polyphenolic extract, Pycnogenol, on the level of 8-oxoguanine in children suffering from attention deficit/hyperactivity disorder. Free Radical Res, 2006. 40: p. 1003-1010.
268. Chiou, C.-C., et al., Urinary 8-hydroxydeoxyguanosine and its analogs as DNA marker of oxidative stress: development of an ELISA and measurement in both bladder and prostate cancers. Clin Chim, 2003. 334: p. 87-94.
269. Wu, L.L., et al., Urinary 8-OHdG: a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. Clin Chim Acta, 2004. 339: p. 1-9.
270. Özgür, B.G., et al., The probable role of adrenomedullin and nitric oxide in childhood attention deficit hyperactivity disorder. Nord J Psychiatry, 2017. 71(7): p. 521-524.
271. Varol Tas, F., et al., Nitric oxide levels in disruptive behavioral disorder. Neuropsychobiol, 2006. 53: p. 176-180.
272. Sezen, H., et al., Increased oxidative stress in children with attention deficit hyperactivity disorder. Redox Rep., 2016. 21(6): p. 248-253.
273. Apside, R., et al., Non-selective attention and nitric oxide in putative animal models of attention-deficit hyperactivity disorder. Behav Brain Res, 1998. 95: p. 123-133.
274. Akyol, O., et al., Nitric oxide as a physiopathological factor in neuropsychiatric disorders. In Vivo, 2004. 18: p. 377-390.
275. Van Hove, C.E., et al., Vasodilator efficacy of nitric oxide depends on mechanisms of intracellular calcium mobilization in mouse aortic smooth muscle cells. Br J Pharmacol, 2009. 158(3): p. 920-930.
276. Moncada, S., R.M.J. Palmer, and E.A. Higgs, Nitric Oxide: Physiology, Pathophysiology, and Pharmacology. Pharmacol Rev, 1991. 43(2): p. 109-142.
277. Russo, A.J., Decreased Serum Cu/Zn SOD Associated with High Copper in Children with Attention Deficit Hyperactivity Disorder (ADHD). J Central Nervous System Dis, 2010. 2: p. 9-14.
278. Ruchi, K., et al., Antioxidant activity in children with ADHD - A comparison in untreated and treated subjects with normal children. Int Med J Malaysia, 2011. 10(1): p. 31-35.
279. Çelik, K., et al., Plasma catalase, glutathione-s-transferase and total antioxidant activity levels of children with attention deficit and hyperactivity disorder. Adv Biosci Biotechnol, 2013. 4: p. 183-187.
280. Lee, J.Y., et al., Association of glutathione S-transferases M1, T1 and P1 gene polymorphisms with attention deficit and hyperactivity disorder in Korean children. General Physiol Biophys, 2016. 586(2): p. 228-233.
281. Naguib, Y., et al., Antioxidant activities of natural vitamin E formulations. J Nutr Sci Vitaminol, 2003. 49: p. 217-220.
282. Ruperez, F.J., et al., Evolution of oxidative stress parameters and response to oral vitamins E and C in streptozotocin-induced diabetic rats. J Pharm Pharmacol, 2008. 60(7): p. 971-878.
283. Mason, J.B., Vitamins, trace minerals, and other micronutrients., in Goldman's Cecil Medicine, Goldman, Editor. 2011, Saunders Elsevier.
284. Avcil, S., et al., Dynamic thiol/disulfide homeostasis in children with attention deficit hyperactivity disorder and its relation with disease subtypes. Compr Psychiatry., 2017. 73: p. 53-60.
285. Schmidt, A.J., et al., Impact of psychostimulants and atomoxetine on the expression of 8-hydroxyguanine glycosylase 1 in human cells. J Neural Transm, 2010. 117(6): p. 793-797.
286. Zhu, M., et al., Methylphenidate ameliorates hypoxia-induced mitochondrial damage in human neuroblastoma SH-SY5Y cells through inhibition of oxidative stress. Life Sci, 2018. 197: p. 40-45.
287. Gomes, K.M., et al., Antioxidant Enzymes Following Acute or Chronic Methylphenidate Treatment in Young Rats. Neurochem Res, 2008. 33: p. 1024-1027.
288. Schmitz, F., et al., Chronic methylphenidate administration alters antioxidant defenses and butyrylcholinesterase activity in blood of juvenile rats. Mol Cell Biochem, 2012. 361(1-2): p. 281-288.
289. Gomes, K.M., et al., Superoxide production after acute and chronic treatment with methylphenidate in young and adult rats. Neurosci Lett, 2009. 465: p. 95-98.

290. Martins, M.R., et al., Methylphenidate treatment induces oxidative stress in young rat brain. Brain Res, 2006. 1078(1): p. 189-197.
291. Fagundes, A.O., et al., Chronic administration of methylphenidate activates mitochondrial respiratory chain in brain of young rats. Int J Develop Neurosci, 2007. 25: p. 47-51.
292. Motaghinejad, M., M. Motevalian, and B. Shabab, Effects of chronic treatment with methylphenidate on oxidative stress and inflammation in hippocampus of adult rats. Neurosci Lett, 2016. 619(21): p. 106-113.
293. Morales, E., et al., Association of early-life exposure to household gas appliances and indoor nitrogen dioxide with cognition and attention behavior in preschoolers. Am J Epidemiol, 2009. 169(11): p. 1327-1336.
294. Heinonen, I., et al., The effects of equal caloric high fat and western diet on metabolic syndrome, oxidative stress and vascular endothelial function in mice. Acta Physiol, 2014. 211(3): p. 515-527.
295. Jomova, K. and M. Valko, Advances in metal-induced oxidative stress and human disease. Toxicol, 2011. 283: p. 65-87.
296. Hariri, M. and L. Azadbakht, Magnesium, iron and zinc supplementation for the treatment of attention deficit hyperactivity disorder: a systematic review on the recent lieterature. Int J Prev Med, 2015. 6(83).
297. Powell, S.R., The Antioxidant Properties of Zinc. J Nutr, 2000. 130(5): p. 1447S-1454S.
298. Hossain, M.S., et al., Antioxidative effects of docosahexaenoic acid in the cerebrum versus cerebellum and brainstem of aged hypercholesterolemic rats. J Neurochem, 1999. 72(3): p. 1133-1138.
299. Cunnane, S.C., et al., Fish, docosahexaenoic acid and Alzheimer’s disease. Prog Lipid Res, 2009. 48(5): p. 239-256.
300. Hariri, M., et al., Effect of n-3 supplementation on hyperactivity, oxidative stress and inflammatory mediators in children with attention-deficit-hyperactivity disorder. Malays J Nutr, 2012. 18(3): p. 329-335.
301. Scalbert, A., I.T. Johnson, and M. Saltmarsh, Polyphenols: antioxidants and beyond. Am J Clin Nutr, 2005. 81: p. 215s-217s.
302. Selek, S., et al., Oxidative imbalance in adult attention deficit/hyperactivity disorder. Biol Psychol, 2008. 79: p. 256-259.
303. Bulut, M., et al., Malondialdehyde levels in adult attention-deficit hyperactivity disorder. J Psychiatry Neurosci, 2007. 32: p. 435-438.
304. Viktorinova, A., et al., Natural polyphenols modify trace element status and improve clinical symptoms of attention-deficit hyperactivity disorder. Biomed Pharmacother, 2009. p. 1-6.
305. Matzinger, P., The danger model: A renewed sense of self. Science, 2002. 296: p. 301-305.
306. Lin, E., J.E. Freedman, and L.M. Beaulieu, Innate immunity and toll-like receptor antagonsists: A potential role in the treatment of cardiovascular diseases. Cardiovasc Ther, 2009. 27(117-123).
307. Si-Tahar, M., L. Touqui, and M. Chignard, Innate immunity and inflammation - Two facets of the same anti-infectious reaction. Clin Exp Immunol, 2009. 156(194-198).
308. Kumar, H., T. kawai, and S. Akira, Pathogen recognition in the innate immune response. Biochem J, 2009. 420: p. 1-16.
309. Abbas, A.K., A.H. Lichtman, and S. Pillai, Cellular and Molecular Immunology. 2015, Philadelphia: Elsevier Saunders.
310. Kimura, S., et al., Evaluation of urinary 8-hydroxydeoxyguanine in healthy Japanese people. Basic Clin Pharmacol Toxicol, 2006. 98(496-502).
311. Belkaid, Y. and T. Hand, Role of the Microbiota in Immunity and Inflammation. Cell, 2014. 157: p. 121-141.
312. Mangiola, F., et al., Gut microbiota in autism and mood disorders. World J Gastroenterol, 2016. 22(1): p. 361-368.
313. Sandgren, A.M. and R.J.M. Brummer, ADHD-originating in the gut? The emergence of a new explanatory model. Med Hypotheses, 2018. 120: p. 135-145.
314. Wu, H.-J. and E. Wu, The role of gut microbiota in immune homeostasis and autoimmunity. Gut Microbes, 2012. 3(1): p. 4-14.
315. Hooper, L.V., D.R. Littman, and A.J. Macpherson, Interactions between the microbiota and the immune system. Science, 2012. 336(1268-1273).
316. Marshall, P., Attention Deficit Disorder and Allergy: A Neurochemical Model of the Relation Between the Illnesses. Psychol Bull, 1989. 106(3): p. 434-446.
317. Chen, M., et al., Comorbidity of allergic and autoimmune diseases among patients with ADHD: A nationwide population-based study. J Atten Disord, 2013. 21(3): p. 219-227.
318. Martino, M., et al., Immunomodulation mechanism of antidepressants: interactions between serotonin/norepinephrine balance and Th1/Th2 balance. Curr Neuropharmacol, 2012. 10: p. 97-123.
319. Buske-Kirschbaum, A., et al., Psychoendocrine and psychoneuroimmunological mechanisms in the comorbidity of atopic eczema and attention deficit/hyperactivity disorder. Psychoneuroendocrinology, 2013. 38: p. 12-23.
320. Oades, R., et al., Attention-deficit hyperactivity disorder (ADHD) and glial integrity: S100B, cytokines and kynurenine metabolism - effects of medication. Behav Brain Functions, 2010. 6(29): p. 2-14.

321. Zhou, R.Y., et al., Attention deficit hyperactivity disorder may be a highly inflammation and immune-associated disease. Mol Med Rep, 2017. 16(4): p. 5071-5077.
322. Vargas, D.L., et al., Neuroglial activation and neuroinflammation in the brain of patients with autism. Ann Neurol, 2005. 57: p. 67-81.
323. Sasayama, D., et al., Negative Correlation between Serum Cytokine Levels and Cognitive Abilities in Children with Autism Spectrum Disorder. J. Intell. 2017, 5, 19; , 2017. 5(19): p. 19-26.
324. Muller, N. and M.J. Schwarz, Immune System and Schizophrenia. Curr Immunol Rev, 2010. 6: p. 213-220.
325. Rege, S. and S.J. Hodgkinson, Immune dysregulation and autoimmunity in bipolar disorder: Synthesis of the evidence and its clinical application. Aust N Z J Psychiatry, 2013. 47: p. 1136-1151.
326. Dantzer, R., et al., From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci, 2008. 9: p. 46-56.
327. Hoffmann, C., et al., Autoantibodies in Neuropsychiatric Disorders. Antibodies 2016. 5: p. 9.
328. Kayser, M.S. and J. Dalmau, The emerging link between autoimmune disorders and neuropsychiatric disease. J Neuropsychiatry Clin Neurosci, 2011. 23: p. 90-97.
329. Hughes, D.A., Dietary antioxidants and human immune function. Nutrition Bulletin, 2000. 25: p. 35-41.
330. Dinan, T., et al., Collective unconscious: How gut microbes shape human behavior. J Psych Res, 2015. 63: p. 1-9.
331. Carding, S., et al., Dysbiosis of the gut microbiota in disease. Microb Ecol Health Dis, 2015. 26: p. 26191-16200.
332. Hsiao, E.Y., et al., Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell, 2013. 155(7): p. 1451-1463.
333. Chen, H., et al., Characterization of Gene Expression in Resting and Activated Mast Cells. J Exp Med, 1998. 188(9): p. 1657-1668.
334. Tsai, S.J., Signal transducer and activator of transcription 6 (STAT6) and attention-deficit hyperactivity disorder: A speculative hypothesis. Med Hypotheses, 2006. 67(6): p. 1341-1343.
335. Ogdie, M.N., et al., A Genome wide Scan for Attention-Deficit/Hyperactivity Disorder in an Extended Sample: Suggestive Linkage on 17p11. Am J Hum Genet, 2003. 72: p. 1268-1279.
336. Aureli, A., et al., Investigation on the possible relationship existing between the HLA-DR gene and attention deficit hyperactivity disorder and/or mental retardation. Int J Immunopathol Pharma, 2008. 21: p. 985-991.
337. Segman, R.H., et al., Preferential transmission of interleukin-1 receptor antagonist alleles in attention deficit hyperactivity disorder. Mol Psychiatry, 2002. 7: p. 72-74.
338. Pärtty, A., et al., A possible link between early probiotic intervention and the risk of neuropsychiatric disorders later in childhood: a randomized trial. Pediatr Res, 2015. 77(6): p. 823-828.
339. Millichap, J.G., Etiologic Classification of Attention-Deficit/Hyperactivity Disorder. Pediatrics, 2008. 121: p. e358 -e365.
340. Fasmer, O.B., et al., Adult attention deficit hyperactivity disorder is associated with asthma. BMC Psychiatr, 2011. 11: p. 128-134.
341. Stevens, L.J., et al., Essential fatty acid metabolism in boys with attention-deficit hyperactivity disorder. Am J Clin Nutr, 1995. 62(4): p. 761-768.
342. Chen, M.H., et al., Is atopy in early childhood a risk factor for ADHD and ASD? A longitudinal study. J Psychosom Res, 2014. 77: p. 316-321.
343. Scahill, L. and A. deGraft-Johnson, Food Allergies, Asthma, and Attention Deficit Hyperactivity Disorder. JCAPN, 1997. 10: p. 36-40.
344. Peterson, B.S., et al., Preliminary findings of antistreptococcal antibody titers and basal ganglia volumes in tic, obsessive-compulsive, and attention-deficit/hyperactivity disorders. Arch Gen Psychiatry, 2000. 57: p. 364-372.
345. Murphy, T., R. Kurlan, and J. Leckman, The immunobiology of Tourette's disorder, pediatric autoimmune neuropsychiatric disorders associated with Streptococcus, and related disorders: a way forward. J Child Adolesc Psychopharmacol, 2010. 20(4): p. 317-331.
346. Adesman, A.R., et al., Otitis media in children with learning disabilities and in children with attention deficit disorder with hyperactivity. Pediatrics, 1990. 85(3 Pt 2): p. 442-446.
347. Cochrane, S., et al., Factors influencing the incidence and prevalence of food allergy. Allergy, 2009. 64: p. 1246-1255.
348. Brawley, A., et al., Allergic rhinitis in children with attention-deficit/hyperactivity disorder. Ann Allergy Asthma Immunol, 2004. 92(6): p. 663-667.
349. Chen, M., et al., Attention deficit hyperactivity disorder, tic disorder, and allergy: Is there a link? A nationwide population-based study. J Child Psychol Psychiatry, 2013. 54(5): p. 545-551.
350. Tsai, J.D., et al., Association between atopic diseases and attention-deficit/hyperactivity disorder in childhood: a population-based case-control study. Ann Epidemiol, 2013. 23(4): p. 185-188.
351. Shyu, C.-S., et al., Prevalence of attention-deficit/hyperactivity disorder in patients with pediatric allergic disorders: A nationwide, population-based study. J Microbiol, 2012. 45: p. 237-242.

352. Romanos, M., et al., Association of attention-deficit/hyperactivity disorder and atopic eczema modified by sleep disturbance in a large population-based sample. J Epidemiol Community Health, 2010. 64: p. 269-273.
353. Schmitt, J., et al., Infant-onset eczema in relation to mental health problems at age 10 years: results from a prospective birth control study (German Infant Nutrition Intervention plus). J Allergy Clin Immunol, 2010. 125: p. 404-410.
354. Roth, N., et al., Coincidence of Attention Deficit Disorder and Atopic Disorders in Children: Empirical Findings and Hypothetical Background. J Abnorm Child Psychol, 1991. 19(1): p. 1-13.
355. Catal, F., et al., Psychiatric disorders and symptoms severity in preschool children with atopic eczema. Allergol Immunopathol, 2016. 44: p. 120-124.
356. Genuneit, J., et al., Infant atopic eczema and subsequent attention-deficit/hyperactivity disorder - A prospective birth cohort study. Pediatr Allergy Immunol, 2014. 25(1): p. 51-56.
357. Schmitt, J., A. Buske-Kirschbaum, and V. Roessner, Is atopic disease a risk factor for attention-deficit/hyperactivity disorder? A systematic review. Allergy, 2010. 65(12): p. 1506-1524.
358. Cuffe, S.P., C.G. Moore, and R. McKeown, ADHD and health services utilization in the national health interview survey. J Atten Disord, 2009. 12: p. 330-340.
359. Cutuli, J.J., et al., Asthma and behavior in homeless 4 to 7 year olds. Pediatrics, 2010. 125: p. 145-151.
360. Boris, M. and A. Goldblatt, Pollen Exposure as a Cause for the Deterioration of Neurobehavioral Function in Children with Autism and Attention Deficit Hyperactive Disorder: Nasal Pollen Challenge. J Nutr Env Med, 2004. 14(1): p. 47-54.
361. Egger, J., A. Stolla, and L.M. McEwen, Hyposensitisation for food-induced hyperkinetic syndrome. Lancet, 1993. 341(8842): p. 115.
362. Biederman, J., et al., Associations between childhood asthma and ADHD: issues of psychiatric comorbidity and familiality. J Am Acad Child Adolesc Psychiatry, 1994. 33: p. 842-848.
363. McGee, R., W.R. Stanton, and M.R. Sears, Allergic Disorders and Attention Deficit Disorder in Children. J Abnorm Child Psychol, 1993. 21(1): p. 79-88.
364. Chida, Y., M. Hamer, and A. Steptoe, A bidirectional relationship between psychosocial factors and atopic disorders: a systematic review and meta-analysis. Psychosom Med, 2008. 70: p. 102-116.
365. Chen, M.H., et al., Higher risk of mood disorders among adolescents with ADHD and asthma: A nationwide prospective study. J Affect Disord, 2013. 156: p. 232-235.
366. Chang, H.Y., et al., Allergic diseases in preschoolers are associated with psychological and behavioural problems. Allergy Asthma Immunol Res, 2013. 5: p. 315-321.
367. Wahn, U. World Allergy Organization: Disease summaries - The allergic march - Introduction. 2007; Available from: http://www.worldallergy.org/professional/allergic_diseases_center/allergic_march/ [Accessed 20 September 2017].
368. Sanchez-Carpintero, R., et al., A preliminary study of the frequency of anti-basal ganglia antibodies and streptococcal infection in attention deficit/hyperactivity disorder. J Neurol, 2009. 256: p. 1103-1108.
369. Toto, M., et al., Antibasal Ganglia Antibodies and Antistreptolysin O in Noncomorbid ADHD. J Atten Disord, 2012. 19(11): p. 965-970.
370. Mittleman, B.B., et al., Cerebrospinal Fluid Cytokines in Pediatric Neuropsychiatric Disease. J Immunol, 1997. 159(6): p. 2994-2999.
371. O'Shea, T.M., et al., Elevated blood levels of inflammation-related proteins are associated with an attention problem at age 24 mo in extremely preterm infants. Pediatr Res, 2014. 75(6): p. 781-787.
372. Warren, R.P., et al., Is Decreased Blood Plasma Concentration of the Complement C4B Protein Associated with Attention-Deficit Hyperactivity Disorder. J Am Acad Child Adolesc Psychiatry, 1995. 34(8): p. 1009-1014.
373. Rout, U., N. Mungan, and D. Dhossche, Presence of GAD65 autoantibodies in the serum of children with autism or ADHD. Eur Child Adolesc Psychiatry, 2012. 21: p. 141-147.
374. Donfrancesco, R., et al., Anti-Yo Antibodies in Children With ADHD: First Results About Serum Cytokines. J Atten Disord, 2016. [Epub ahead of print].
375. Blank, R. and H. Remschmidt, Hyperkinetic Syndrome: The Role of Allergy among Psychological and Neurological Factors. Eur Child Adol Psychiatry, 1994. 3(4): p. 220-228.
376. Prehn-Kristensen, A., et al., Reduced microbiome alpha diversity in young patients with ADHD. PLoS One, 2018. 13(7): p. 1-19.
377. Mülek, M., et al., Profiling a gut microbiota-generated catechin metabolite's fate in human blood cells using a metabolomic approach. J Pharm Biomed Anal, 2015. 114: p. 71-81.
378. Wang, Y. and L.H. Kasper, The role of microbiome in central nervous system disorders. Brain Behav Immun, 2014. 38: p. 1-12.
379. Aarts, E., et al., Gut microbiome in ADHD and its relation to neural reward anticipation. PLoS One, 2017. 12(9): p. e0183509.
380. Jiang, H.Y., et al., Gut microbiota profiles in treatment-naïve children with attention deficit hyperactivity disorder. Behav Brain Res, 2018. 347(408-413).

381. Axelsson, P.B., et al., Investigating the effects of cesarean delivery and antibiotic use in early childhood on risk of later attention deficit hyperactivity disorder. J Child Psychol Psychiatry, 2019. 60(2): p. 151-159.
382. Tillisch, K., The effects of gut microbiota on CNS function in humans. Gut Microbes, 2014. 5(3): p. 404-410.
383. Principi, N. and S. Esposito, Gut microbiota and central nervous system development. J Infect, 2016. 73(6): p. 536-546.
384. Petra, A.I., et al., Gut-Microbiota-Brain Axis and Its Effect on Neuropsychiatric Disorders With Suspected Immune Dysregulation. Clin therap, 2015. 37(5): p. 984-995.
385. Abrahamsson, T.R., R.Y. Wu, and M.C. Jenmalm, Gut microbiota and allergy: the importance of the pregnancy period. Pediatr Res, 2015. 77(1-2): p. 214-219.
386. Lopez-Garcia, E., et al., Major dietary patterns are related to plasma concentrations of markers of inflammation and endothelial dysfunction. Am J Clin Nutr, 2004. 80(4): p. 1029-1035.
387. Vojdani, A. and C. Vojdani, Immune reactivity to food coloring. Altern Ther Health Med, 2015. 21(Suppl 1): p. 52-62.
388. Castro, A.M., L.E. Macedo-de la Concha, and C.A. Pantoja-Meléndez, Low-grade inflammation and its relation to obesity and chronic degenerative diseases. Revista Médica del Hospital General de México, 2017. 80(2): p. 101-105.
389. Shankar, A.H. and A.S. Prasad, Zinc and immune function: the biological basis of altered resistance to infection. Am J Clin Nutr, 1998. 68(suppl): p. 447S-463S.
390. Kim, S.J., et al., Omega-3 and omega-6 fatty acids suppress ER- and oxidative stress in cultured neurons and neuronal progenitor cells from mice lacking PPT1. Neurosci Lett, 2010. 479(3): p. 292-296.
391. Han, S.C., et al., Fermented fish oil suppresses T helper 1/2 cell response in a mouse model of atopic dermatitis via generation of CD4+CD25+Foxp3+ T cells. BMC Immunol, 2012. 13: p. 44.
392. Calder, P.C., n-3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr, 2006. 83(Suppl): p. 1505S-1519S.
393. Barber, M.D., et al., The effect of an oral nutritional supplement enriched with fish oil on weight-loss in patients with pancreatic cancer. Br J Cancer, 1999. 81: p. 80-86.
394. Gibney, M.J. and B. Hunter, The effects of short- and long-term supplementation with fish oil on the incorporation of n-3 polyunsaturated fatty acids into cells of the immune system in healthy volunteers. Eur J Clin Nutr, 1993. 47: p. 255-259.
395. Gogos, C.A., et al., Dietary omega-3 polyunsaturated fatty acids plus vitamin E restore immunodeficiency and prolong survival for severely ill patients with generalized malignancy: A randomized control trial. Cancer, 1998. 82: p. 395-402.
396. Calder, P.C., Polyunsaturated fatty acids and inflammation. Biochem Soc Trans, 2005. 33(Pt 2): p. 423-427.
397. Hanson, L.A., M. Korotkova, and E. Telemo, Breast-feeding, infant formulas, and the immune system. Ann Allergy Asthma Immunol, 2003. 90(6 Suppl): p. 59-63.
398. Andersson, Y., et al., Formula Feeding Skews Immune Cell Composition toward Adaptive Immunity Compared to Breastfeeding. J Immunol, 2009. 183(7): p. 4322-4328.
399. Stuebe, A., The Risks of Not Breastfeeding for Mothers and Infants. Rev Obstet Gynecol, 2009. 2(4): p. 222-231.
400. Romanos, M., et al., Itches and scratches - is there a link between eczema, ADHD, sleep disruption and food hypersensitivity? Allergy, 2011. 66: p. 1407-1409.
401. Bekdas, M., et al., Subclinical immune reactions to viral infections may correlate with child and adolescent diagnosis of attention-deficit/hyperactivity disorder: a preliminary study from Turkey. Afr Health Sci, 2014. 14(2): p. 439-445.
402. Nauta, A.J., et al., Mechanisms of allergy and asthma. Eur J Pharmacol, 2008. 585(1-3): p. 354-360.
403. Stone, K.D., C. Prussin, and D.D. Metcalfe, IgE, mast cells, basophils, and eosinophils. J Allergy Clin Immunol, 2010. 125: p. S73-S80.
404. van Beek, A.A., et al., Recent developments in basophil research: do basophils initiate and perpetuate type 2 T-helper cell responses? Int Arch Allergy Immunol, 2012. 160: p. 7-17.
405. Raison, C., L. Capuron, and A. Miller, Cytokines sing the blues: inflammation and the pathogenesis of depression. Trends Immunol, 2006. 27: p. 24-31.
406. Besser, M.J., Y. Ganor, and M. Levite, Dopamine by itself activates either D2, D3 or D1/D5 dopaminergic receptors in normal human T-cells and triggers the selective secretion of either IL-10, TNFa or both. J Neuroimmun, 2005. 169: p. 161-171.
407. Yukawa, K., et al., Down-regulation of dopamine transporter and abnormal behaviour in STAT6-deficient mice. Int J Mol Med, 2005. 15: p. 819-825.
408. Egger, J., A. Stolla, and L.M. McEwen, Controlled trial of hyposensitisation in children with food-induced hyperkinetic syndrome. Lancet, 1992. 339: p. 1150-1153.
409. Ma, L., et al., The function of hypothalamus-pituitary-adrenal axis in children with ADHD. Brain Res, 2011. 1368: p. 159-162.

410. Isaksson, J., K.W. Nilsson, and F. Lindblad, Early psychosocial adversity and cortisol levels in children with attention-deficit/hyperactivity disorder. ECAP, 2013. 22(7): p. 425-432.
411. Işık, U., et al., Serum levels of cortisol, dehydroepiandrosterone, and oxytocin in children with attention-deficit/hyperactivity disorder combined presentation with and without comorbid conduct disorder. Psych Res, 2018. 261: p. 212-219.
412. Feder, A., et al., Psychobiological mechanisms of resilience to stress., in Handbook of adult resilience., J.W. Reich, A.J. Zautra, and J.S. Hall, Editors. 2010, The Guilford Press: New York. p. 35-54.
413. Xiang, L. and G.D.J. Marshall, Immunomodulatory effect of in vitro stress hormones on Foxp3, Th1/Th2 cytokine and costimulatory molecule mRNA expression in human peripheral blood mononuclear cells. Neuroimmunomod, 2011. 18: p. 1-10.
414. Vaziri, N.D., Causal link between oxidative stress, inflammation, and hypertension. IJKD, 2008. 2: p. 1-10.
415. Halliwell, B. and J.M.C. Gutteridge, Free radicals in biology and medicine., C. Press, Editor. 1999, Oxford University Press: Oxford.
416. Dröge, W. and R. Breitkreutz, Glutathione and immune function. Proc Nutr Soc, 2000. 59: p. 595-600.
417. Furukawa, T., S.N. Meydani, and J.B. Blumberg, Reversal of age-associated decline in immune responsiveness by dietary glutathione supplementation in mice. Mech Ageing Dev, 1987. 38(2): p. 107-117.
418. King, M.R., et al., Oxidative stress promotes polarisation of human T cell differentiation toward a T helper 2 phenotype. J Immunol, 2006. 176: p. 2765-2772.
419. Manach, C. and J.L. Donovan, Pharmacokinetics and metabolism of dietary flavonoids in humans. Free Rad Res, 2004. 38: p. 771-785.
420. Hardman, E.W., Diet components can suppress inflammation and reduce cancer risk. Nutr Res Pract, 2014. 8(3): p. 233-240.
421. Kähkönen, M.P., et al., Antioxidant activity of plant extracts containing phenolic compounds. J Agric Food Chem, 1999. 47: p. 3954-3962.
422. Scalbert, A. and W. G., Dietary intake and bioavailability of polyphenols. J Nutr, 2000. 13: p. 2073S-2085S.
423. Fernandez-Panchon, M.S., et al., Antioxidant activity of phenolic compounds: From in vitro results to in vivo evidence. Crit Rev Food Sci Nutr, 2008. 48(7): p. 649-671.
424. Rios, L.Y., et al., Cocoa procyanidins are stable during gastric transit in humans. Am J Clin Nutr, 2002. 76: p. 1106-1110.
425. Clifford, M.N., Diet-derived Phenols in plasma and tissues and their implications for health. Planta Medica, 2004. 70(12): p. 1103-1114.
426. Cardona, F., et al., Benefits of polyphenols on gut microbiota and implications in human health. J Nutr Biochem, 2013. 24(8): p. 1415-1422.
427. Crozier, A., I.B. Jaganath, and M.N. Clifford, Dietary phenolics: chemistry, bioavailability and effects on health. Nat Prod Rep, 2009. 26(8): p. 1001-1043.
428. Marín, L., et al., Bioavailability of dietary polyphenols and gut microbiota metabolism: antimicrobial properties. Biomed Res Int, 2015. 905215.
429. Duda-Chodak, A., et al., Interaction of dietary compounds, especially polyphenols, with the intestinal microbiota: a review. Eur J Nutr, 2015. 54(3): p. 325-341.
430. Hermans, N., Development of analytical methods and in vivo oxidative stress models for the evaluation of nutritional antioxidants, PhD thesis in Pharmaceutical Sciences. 2005, University of Antwerp: Antwerp.
431. Ma, Q., Role of Nrf2 in Oxidative Stress and Toxicity. Annu Rev Pharmacol Toxicol, 2013. 53: p. 401-426.
432. Tumer, T.B., et al., Direct and Indirect Antioxidant Activity of Polyphenol- and Isothiocyanate-Enriched Fractions from Moringa oleifera. J Agric Food Chem, 2015. 63(5): p. 1505-1513.
433. Lee, M.T., et al., Antioxidant capacity of phytochemicals and their potential effects on oxidative status in animals — A review. Asian-Australas J Anim Sci, 2017. 30(3): p. 299-308.
434. Halliwell, B., J. Rafter, and A. Jenner, Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? Am J Clin Nutr, 2005. 81(1): p. 268s-276s.
435. Pietta, P.G., Flavonoids as antioxidants. J Nat Prod, 2000. 63(7): p. 1035-1042.
436. Cos, P., Evaluation of hydroxycinnamic acids and flavonoids as antioxidants and pro-oxidants., PhD thesis in Pharmaceutical Sciences. 2001, University of Antwerp: Antwerp.
437. Cos, P., et al., Cytotoxicity and lipid peroxidation-inhibiting activity of flavonoids. Planta Medica, 2001. 67(6): p. 515-519.
438. Cos, P., et al., Comparative study of eight well-known polyphenolic antioxidants. J Pharm Pharmacol, 2003. 55(9): p. 1291-1297.
439. Cos, P., et al., In vitro antioxidant profile of phenolic acid derivatives. Free Rad Res, 2002. 36(6): p. 711-716.
440. Shahat, A.A., et al., Antiviral and antioxidant activity of flavonoids and proanthocyanidins from Crataegus sinaica. Planta Medica, 2002. 68(6): p. 539-541.
441. De Bruyne, T., et al., Biological evaluation of proanthocyanidin dimers and related polyphenols. J Nat Prod, 1999. 62(7): p. 954-958.

442. Taubert, D., et al., Reaction rate constants of superoxide scavenging by plant antioxidants. Free Rad Bio Med, 2003. 35(12): p. 1599-1607.
443. Halliwell, B., Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies? Arch Biochem Biophys, 2008. 476(2): p. 107-112.
444. Babich, H., et al., Research strategies in the study of the pro-oxidant nature of polyphenol nutraceuticals. J Toxicol, 2011. 2011.
445. Fukumoto, L.R. and G. Mazza, Assessing antioxidant and prooxidant activities of phenolic compounds. J Agric Food Chem, 2000. 48(8): p. 3597-3604.
446. Raza, H. and A. John, Green tea polyphenol epigallocatechin-3-gallate differentially modulates oxidative stress in PC12 cell compartments. Toxicol Appl Pharmacol, 2005. 207(3): p. 212-20.
447. Holst, B. and G. Williamson, Nutrients and phytochemicals: from bioavailability to bioefficacy beyond antioxidants. Curr Opinion Biotechnol, 2008. 19(2): p. 73-82.
448. Druesne-Pecollo, N., et al., Beta-carotene supplementation and cancer risk: a systematic review and metaanalysis of randomized controlled trials. Int J Cancer, 2010. 127(1): p. 172-184.
449. Pellegrini, N., et al., Total Antioxidant Capacity of Plant Foods, Beverages and Oils Consumed in Italy Assessed by Three Different In Vitro Assays. J Nutr, 2003. 133: p. 2812-2819.
450. Pellegrini, N., et al., Total antioxidant capacity of spices, dried fruits, nuts, pulses, cereals and sweets consumed in Italy assessed by three different in vitro assays. Mol Nutr Food Res, 2006. 50: p. 1030-1038.
451. Hakim, I.A., et al., Effect of increased tea consumption on oxidative DNA damage among smokers: a randomized controlled study. J Nutr, 2003. 133: p. 3303S-3309S.
452. Basu, A., et al., Green tea supplementation affects body weight, lipids, and lipid peroxidation in obese subjects with metabolic syndrome. J Am Coll Nutr, 2010. 29(1): p. 31-40.
453. Luo, H., et al., Phase IIa chemoprevention trial of green tea polyphenols in high-risk individuals of liver cancer: modulation of urinary excretion of green tea polyphenols and 8-hydroxydeoxyguanosine. Carcinogenesis, 2006. 27(2): p. 262-268.
454. Freese, R., et al., Green tea extract decreases plasma malondialdehyde concentration but does not affect other indicators of oxidative stress, nitric oxide production, or hemostatic factors during a high-linoleic acid diet in healthy females. Eur J Nutr, 1999. 38(3): p. 149-157.
455. Neveu, V., et al., Phenol-Explorer: an online comprehensive database on polyphenol contents in foods. Database 2010.
456. Parsaeyan, N., et al., Beneficial effects of cocoa on lipid peroxidation and inflammatory markers in type 2 diabetic patients and investigation of probable interactions of cocoa active ingredients with prostaglandin synthase-2 (PTGS-2/COX-2) using virtual analysis. J Diabetes Metabol Dis, 2014. 13: p. 30-38.
457. Pae, M. and D. Wu, Immunomodulating effects of epigallocatechin-3-gallate from green tea: mechanisms and applications. Food Funct, 2013. 4(9): p. 1287-1303.
458. Bayeta, E. and B.H.S. Lau, Pycnogenol inhibits generation of inflammatory mediators in macrophages. Nutr Res, 2000. 20(2): p. 249-259.
459. Schafer, A., et al., Inhibition of COX-1 and COX-2 activity by plasma of human volunteers after ingestion of French maritime pine bark extract (Pycnogenol). Biomed Pharmacother, 2006. 60(1): p. 5-9.
460. Canali, R., et al., The anti-inflammatory pharmacology of Pycnogenol in humans involves COX-2 and 5-LOXmRNA expression in leukocytes. Int Immunopharmacol, 2009. 9(10): p. 1145-1149.
461. Yahfoufi, N., et al., The Immunomodulatory and Anti-Inflammatory Role of Polyphenols. Nutrients, 2018. 10: p. 1618-1640.
462. Lagha, A.B. and D. Grenier, Tea polyphenols inhibit the activation of NF-κB and the secretion of cytokines and matrix metalloproteinases by macrophages stimulated with Fusobacterium nucleatum. Sci Rep, 2016. 6.
463. Wong, C.P., et al., Induction of regulatory T cells by green tea polyphenol EGCG. Immunol Lett, 2011. 139(1-2): p. 7-13.
464. Yang, J., et al., Identification of Baicalin as an immunoregulatory compound by controlling T(H)17 cell differentiation. PLoS One, 2011. 6(2): p. e17164.
465. Kanda, T., et al., Inhibitory effects of apple polyphenol on induced histamine release from RBL-2H3 cells and rat mast cells. Biosci Biotechnol Biochem, 1998. 62(7): p. 1284-1289.
466. Gonzalez-Gallego, J., et al., Fruit polyphenols, immunity and inflammation. Br J Nutr, 2010. 104: p. S15-S27.
467. Cho, S.Y., et al., Quercetin suppresses proinflammatory cytokines production through MAP kinases and NF-κB pathway in lipopolysaccharide-stimulated macrophage. Mol Cell Biochem, 2003. 243: p. 153-160.
468. Maki, T., et al., The relationship of coffee and green tea consumption with high-sensitivity C-reactive protein in Japanese men and women. Clin Chem Lab Med, 2010. 48(6): p. 849-854.
469. Hosseini, B., et al., Effects of fruit and vegetable consumption on inflammatory biomarkers and immune cell populations: a systematic literature review and meta-analysis. Am J Clin Nutr, 2018. 108: p. 136-155.
470. Hassanain, E., et al., Green tea (Camelia sinensis) suppresses B cell production of IgE without inducing apoptosis. Ann Clin Lab Sci, 2010. 40(2): p. 135-143.

471. Choi, Y.H. and G.H. Yan, Pycnogenol inhibits immunoglobulin E-mediated allergic response in mast cells. Phytother Res, 2009. 23(12): p. 1691-1695.
472. Dorsch, W., et al., Antiasthmatic effects of Galphimia glauca, gallic acid, and related compounds prevent allergen- and platelet-activating factor-induced bronchial obstruction as well as bronchial hyperreactivity in guinea pigs. Int Arch Allergy Immunol, 1992. 97(1): p. 1-7.
473. Kojima, T., et al., Anti-allergic effect of apple polyphenol on patients with atopic dermatitis: A pilot study. Allergol Internat, 2000. 49: p. 69-73.
474. Al-Hanbali, M., et al., Epicatechin suppresses IL-6, IL-8 and enhances IL-10 production with NF-κB nuclear translocation in whole blood stimulated system. Neuroendocrinol Letters 2009. 30: p. 131-138.
475. Mitjans, M., et al., Novel epicatechin derivatives with antioxidant activity modulate interleukin-1β release in lipopolysaccharide-stimulated human blood. Bioorgan Medicin Chem Letters, 2004. 14: p. 5031-5034.
476. Jang, I.-S., et al., Effects of Vitamin C or E on the Pro-inflammatory Cytokines, Heat Shock Protein 70 and Antioxidant Status in Broiler Chicks under Summer Conditions. AJAS, 2014. 27(5): p. 749-756.
477. Chae, H.S., et al., The Effect of Antioxidants on the Production of Pro-Inflammatory Cytokines and Orthodontic Tooth Movement. Mol Cells, 2011. 32(2): p. 189-196.
478. Sarris, J., et al., Complementary medicines (herbal and nutritional products) in the treatment of Attention Deficit Hyperactivity Disorder (ADHD): A systematic review of the evidence. Compl Ther Med, 2011. 19: p. 216-227.
479. Droy-Lefaix, M.T., Effect of the antioxidant action of Ginkgo biloba extract (EGb 761) on aging and oxidative stress. Age, 1997. 20(3): p. 141-149.
480. Puebla-Pérez, A.M., X. Lozoya, and M.M. Villaseñor-García, Effect of Ginkgo biloba extract, EGb 761, on the cellular immune response in a hypothalamic-pituitary-adrenal axis activation model in the rat. Int Immunopharmacol, 2003. 3(1): p. 75-80.
481. Aghili, T., et al., Effects of Hypericum perforatum extract on IgG titer, leukocytes subset and spleen index in rats. Avicenna J Phytomed, 2014. 4(6): p. 413-419.
482. Orčić, D.Z., et al., Antioxidant activity relationship of phenolic compounds in Hypericum perforatum L. Chem Cent J, 2011. 5: p. 34.
483. Ingale, S.P. and S.B. Kasture, Antioxidant and antiparkinsonian activity of Passiflora incarnata leaves. Orient Pharm Exp Med, 2014. 14(3): p. 231-236.
484. Beaumont, D.M., et al., The effects of chrysin, a Passiflora incarnata extract, on natural killer cell activity in male Sprague-Dawley rats undergoing abdominal surgery. AANA J, 2008. 76(2): p. 113-117.
485. Lee, J., et al., Pycnogenol attenuates the symptoms of immune dysfunction through restoring a cellular antioxidant status in low micronutrient-induced immune deficient mice. Nutr Res Pract, 2014. 8(5): p. 533-538.
486. Dvořáková, M., et al., The effect of polyphenolic extract from pine bark, Pycnogenol, on the level of glutathione in children suffering from attention deficit hyperactivity disorder (ADHD). Redox Report, 2006. 11: p. 163-172.
487. Kurlbaum, M., M. Mülek, and P. Högger, Facilitated uptake of a bioactive metabolite of maritime pine bark extract (pycnogenol) into human erythrocytes. PLoS ONE, 2013. 8(4).
488. Salehi, B., et al., Ginkgo biloba for attention-deficit/hyperactivity disorder in children and adolescents: a double blind, randomized controlled trial. Progr Neuro-Psychopharmacol Biol Psych, 2010. 34(1): p. 76-80.
489. Uebel-von Sandersleben, H., et al., Ginkgo biloba extract EGb 761 in children with ADHD. Z Kinder Jugendpsychiatr Psychother, 2014. 42(5): p. 337-347.
490. Naik, S.R. and V.S. Panda, Antioxidant and hepatoprotective effects of Ginkgo biloba phytosomes in carbon tetrachloride-induced liver injury in rodents. Liver Int, 2007: p. 393-399.
491. Bridi, R., et al., The antioxidant activity of standardized extract of Ginkgo biloba (EGb 761) in rats. Phytother Res, 2001. 15: p. 449-451.
492. Klemow, K.M., et al., Chapter 11: Medical Attributes of St. John's Wort (Hypericum perforatum). in Herbal Medicine: Biomolecular and Clinical Aspects., I.F.F. Benzie and S. Wachtel-Galor, Editors. 2011, Taylor & Francis: Boca Raton.
493. Weber, W., et al., Hypericum perforatum (St John's Wort) for attention-deficit/hyperactivity disorder in children and adolescents: a randomized controlled trial. JAMA, 2008. 229(22): p. 2633-2641.
494. Maksyutina, N.P. and T.A. Koget, Polyphenols of the herb Hypericum perforatum and the preparation novoimanin. Chem Nat Comp, 1971. 7(3): p. 338-341.
495. Akhondzadeh, S., M.R. Mohammadi, and F. Momeni, Passiflora incarnata in the treatment of attention-deficit hyperactivity disorder in children and adolescents. Therapy, 2005. 2(4): p. 609-614.
496. Baziar, S., et al., Crocus sativus L. Versus Methylphenidate in Treatment of Children with Attention-Deficit/Hyperactivity Disorder: A Randomized, Double-Blind Pilot Study. J Child Adol Psychopharmacol, 2019. 29(3): p. 205-212.
497. Srivastava, R., et al., Crocus sativus L.: A comprehensive review. Pharmacogn Rev, 2010. 4(8): p. 200-208.
498. Rohdewald, P.J., Pycnogenol, French Maritime Pine Bark Extract. Encyclop Diet Suppl, 2005: p. 545-553.

499. Rohdewald, P., Pycnogenol. Flavonoids in Health and Disease, ed. C.A. Rice-Evans and L. Packer. 1998, New York, NY: Marcel Dekker, Inc.
500. Kurlbaum, M. and P. Hogger, Plasma protein binding of polyphenols from maritime pine bark extract (USP). J Pharm Biomed Anal, 2011. 54(1): p. 127-132.
501. Rohdewald, P., A review of the French maritime pine bark extract (Pycnogenol), a herbal medication with a diverse clinical pharmacology. Int J Clin Pharmacol Ther, 2002. 40(4): p. 158-168.
502. Putter, M., et al., Inhibition of smoking-induced platelet aggregation by aspirin and pycnogenol. Thromb Res, 1999. 95(4): p. 155-161.
503. Ryan, J., et al., An examination of the effects of the antioxidant Pycnogenol on cognitive performance, serum lipid profile, endocrinological and oxidative stress biomarkers in an elderly population. J Psychopharmacol, 2008. 22(5): p. 553-562.
504. Hosseini, S., et al., A randomized, double-blind, placebo-controlled, perspective, 16 week crossover study to determine the role of Pycnogenol in modifying blood pressure in mildly hypertensive patients. Nutr Res, 2001. 21: p. 1251-1260.
505. Yang, H.M., et al., A randomised, double-blind, placebo-controlled trial on the effect of Pycnogenol on the climacteric syndrome in peri-menopausal women. Acta Obstet Gynecol Scand, 2007. 86(8): p. 978-985.
506. Enseleit, F., et al., Effects of Pycnogenol on endothelial function in patients with stable coronary artery disease: a double-blind, randomized, placebo-controlled, cross-over study. Eur Heart J, 2012. 33(13): p. 1589-1597.
507. Kohama, T. and M. Negami, Effect of low-dose French maritime pine bark extract on climacteric syndrome in 170 perimenopausal women: a randomized, double-blind, placebo-controlled trial. J Reprod Med, 2013. 58(1): p. 39-46.
508. Belcaro, G., et al., Pycnogenol(R) Supplementation Improves Health Risk Factors in Subjects with Metabolic Syndrome. Phytother Res, 2013. 27(10): p. 1572-1578.
509. Rohdewald, P., A review of the French maritime pine bark extract (Pycnogenol), a herbal medication with a diverse clinical pharmacology. Int J Clin Pharmacol Ther, 2002. 40(4): p. 158-68.
510. Hosseini, S., et al., A randomized, double-blind, placebo-controlled, perspective, 16 week crossover study to determine the role of Pycnogenol in modifying blood pressure in mildly hypertensive patients. Nutr Res, 2001b. 21: p. 1251-1260.
511. Liao, M.-F., et al., A randomized, double-blind, placebo-controlled trial on the effect of Pycnogenol® on the climacteric syndrome in peri-menopausal women. Acta Obstetricia et Gynecologica Scandinavica, 2007. 86: p. 978-985.
512. Putter, M., et al., Inhibition of smoking-induced platelet aggregation by aspirin and pycnogenol. Thromb Res, 1999. 95(4): p. 155-61.
513. Enseleit, F., et al., Effects of Pycnogenol on endothelial function in patients with stable coronary artery disease: a double-blind, randomized, placebo-controlled, cross-over study. Eur Heart J, 2012. 33(13): p. 1589-97.
514. Kohama, T. and M. Negami, Effect of low-dose French maritime pine bark extract on climacteric syndrome in 170 perimenopausal women: a randomized, double-blind, placebo-controlled trial. J Reprod Med, 2013. 58(1-2): p. 39-46.
515. Belcaro, G., et al., Pycnogenol(R) Supplementation Improves Health Risk Factors in Subjects with Metabolic Syndrome. Phytother Res, 2013. 27(10): p. 1572-1578.
516. Pella, D., Slovak Study. 2005, Horphag Research: Geneva, Switzerland.
517. ABC, American Botanical Council - PYCNOGENOL® Consumer/Patient Information Sheet. Available from: www.herbalgram.com [Accessed 28 November 2016].
518. Packer, L., G. Rimbach, and F. Virgili, Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (Pinus maritima) bark, Pycnogenol. Free Rad Biol Med, 1999. 27(5/6): p. 704-724.
519. Virgili, F., et al., Ferulic acid excretion as a marker of consumption of a French maritime pine (Pinus maritima) bark extract. Free Radic Biol Med, 2000. 28(8): p. 1249-56.
520. Grimm, T., et al., Single and multiple dose pharmacokinetics of maritime pine bark extract (Pycnogenol) after oral administration to healthy volunteers. BMC Clin Pharmacol, 2006. 6(4): p. 1-12.
521. Williamson, G. and M.N. Clifford, Colonic metabolites of berry polyphenols: the missing link to biological activity? Br J Nutr, 2010. 104(Suppl 3): p. S48-S66.
522. Sano, A., et al., Procyanidin B1 is detected in human serum after intake of proanthocyanidin-rich grape seed extract. Biosci Biotechnol Biochem, 2003. 67(5): p. 1140-1143.
523. Manach, C., et al., Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am J Clin Nutr, 2005. 81(1): p. 230S-242S.
524. Lee, C.C., et al., 5-(3′,4′-Dihydroxyphenyl-γ-valerolactone), a Major Microbial Metabolite of Proanthocyanidin, Attenuates THP-1 Monocyte-Endothelial Adhesion. Int J Mol Sci, 2017. 18(7).
525. Mülek, M., et al., Distribution of Constituents and Metabolites of Maritime Pine Bark Extract (Pycnogenol®) into Serum, Blood Cells, and Synovial Fluid of Patients with Severe Osteoarthritis: A Randomized Controlled Trial. Nutrients, 2017. 9(5).

526. Duweler, K.G. and P. Rohdewald, Urinary metabolites of French maritime pine bark extract in humans. Pharmazie, 2000. 55(5): p. 364-368.
527. Uhlenhut, K. and P. Högger, Facilitated cellular uptake and suppression of inducible nitric oxide synthase by a metabolite of maritime pine bark extract (Pycnogenol). Free Radic Biol Med, 2012. 53: p. 305-313.
528. Iuga, C., J.R. Alvarez-Idaboy, and A. Vivier-Bunge, ROS Initiated Oxidation of Dopamine under Oxidative Stress Conditions in Aqueous and Lipid Environments. J Phys Chem B, 2011.
529. Gilgun-Sherki, Y., E. Melamed, and D. Offen, Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. Neuropharmacol, 2001. 40: p. 959-975.
530. Belcaro, G., et al., The COFU3 Study. Improvement in cognitive function, attention, mental performance with Pycnogenol® in healthy subjects (55-70) with high oxidative stress. J Neurosurg Sci, 2015. 59(4): p. 437-446.
531. Baldwin, A., et al., Blood-brain barrier breach following cortical contusion in the rat. J Neurosurg. 85(3): p. 476-481.
532. Sivoňová, M., et al., The effect of Pycnogenol on the erythrocyte membrane fluidity. General Physiol Biophys, 2004. 23(1): p. 39-51.
533. Yoshida, A., et al., Direct assessment by electron spin resonance spectroscopy of the antioxidant effects of French maritime pine bark extract in the maxillofacial region of hairless mice. J Clin Biochem Nutr, 2011. 49: p. 79-86.
534. Wei, Z.H., Q.L. Peng, and B.H.S. Lau, Pycnogenol enhances endothelial cell antioxidant defenses. Redox Rep, 1997. 3(4): p. 219-224.
535. Noda, Y., et al., Hydroxyl and superoxide anion radical scavenging activities of natural source antioxidants using the computerized JES-FR30 ESR spectrometer system. Biochem Mol Biol Int, 1997. 42(1): p. 35-44.
536. Shin, I.S., et al., Inhibitory effects of Pycnogenol® (French maritime pine bark extract) on airway inflammation in ovalbumin-induced allergic asthma. Food Chem Toxicol, 2013. 62: p. 681-686.
537. Taner, G., et al., Assessment of the cytotoxic, genotoxic, and antigenotoxic potential of Pycnogenol® in in vitro mammalian cells. Food Chem Toxicol, 2013. 61: p. 203-208.
538. Nelson, A.B., et al., Pycnogenol inhibits macrophage oxidative burst, lipoprotein oxidation, and hydroxyl radical-induced DNA damage. Drug Dev Ind Pharm, 1998. 24(2): p. 139-144.
539. Cossins, E., R. Lee, and L. Packer, ESR studies of vitamin C regeneration, order of reactivity of natural source phytochemical preparations. Biochem Mol Biol Int, 1998. 45(3): p. 583-597.
540. Grimm, T., A. Schafer, and P. Hogger, Antioxidant activity and inhibition of matrix metalloproteinases by metabolites of maritime pine bark extract (pycnogenol). Free Radic Biol Med, 2004. 36(6): p. 811-22.
541. Uhlenhut, K. and P. Hogger, Facilitated cellular uptake and suppression of inducible nitric oxide synthase by a metabolite of maritime pine bark extract (Pycnogenol). Free Radic Biol Med, 2012. 53(2): p. 305-13.
542. Parveen, K., et al., Protective effects of Pycnogenol on hyperglycemia-induced oxidative damage in the liver of type 2 diabetic rats. Chem Biol Interact, 2010. 186(2): p. 219-227.
543. Parveen, K., et al., Modulatory effects of Pycnogenol in a rat model of insulin-dependent diabetes mellitus: biochemical, histological, and immunohistochemical evidences. Protoplasma, 2013. 250(1): p. 347-360.
544. Berryman, A.M., et al., Influence of treatment of diabetic rats with combinations of pycnogenol, beta-carotene, and alpha-lipoic acid on parameters of oxidative stress. J Biochem Mol Toxicol, 2004. 18(6): p. 345-352.
545. Bayomy, N.A., et al., Effect of pycnogenol and spirulina on vancomycin-induced renal cortical oxidative stress, apoptosis, and autophagy in adult male albino rat. Can J Physiol Pharmacol, 2016. 94(8): p. 838-848.
546. Lee, I.C., et al., Protective effects of pine bark extract on hexavalent chromium-induced dermatotoxicity in rats. Phytother Res, 2012. 26(10): p. 1534-1540.
547. Taner, G., et al., Modulating effects of Pycnogenol® on oxidative stress and DNA damage induced by sepsis in rats. Phytother Res, 2014. 28(11): p. 1692-1700.
548. Devaraj, S., et al., Supplementation with a pine bark extract rich in polyphenols increases plasma antioxidant capacity and alters the plasma lipoprotein profile. Lipids, 2002. 37(10): p. 931-934.
549. Dvorakova, M., et al., How does pycnogenol(R) influence oxidative damage to DNA and its repair ability in elderly people? Prague Med Rep, 2010. 111(4): p. 263-71.
550. Cho, K.J., et al., Effect of bioflavonoids extracted from the bark of Pinus maritima on proinflammatory cytokine interleukin-1 production in lipopolysaccharide-stimulated RAW 264.7. Toxicol Appl Pharmacol, 2000. 168(1): p. 64-71.
551. Grimm, T., et al., Inhibition of NF-κB activation and MMP-9 secretion by plasma of human volunteers after ingestion of maritime pine bark extract (Pycnogenol). J Inflam, 2006. 3(1): p. 1-6.
552. Saliou, C., et al., Solar ultraviolet-induced erythema in human skin and nuclear factor-kappa-B-dependent gene expression in keratinocytes are modulated by a French maritime pine bark extract. Free Radic Biol Med, 201. 30(2): p. 154-160.
553. Peng, Q., Z. Wei, and B.H. Lau, Pycnogenol inhibits tumor necrosis factor-alpha-induced nuclear factor kappa B activation and adhesion molecule expression in human vascular endothelial cells. Cell. Mol. Life Sci., 2000. 57: p. 834-841.

554. Peng, Y.J., et al., Pycnogenol attenuates the inflammatory and nitrosative stress on joint inflammation induced by urate crystals. Free Rad Bio Med, 2012. 52(4): p. 765-774.
555. Grimm, T., A. Schäfer, and H. P., Antioxidant activity and inhibition of matrix metalloproteinases by metabolites of maritime pine bark extract (Pycnogenol). Free Radic Biol Med, 2004. 36(6): p. 811-822.
556. Cho, K.J., et al., Inhibition mechanisms of bioflavonoids extracted from the bark of Pinus maritima on the expression of proinflammatory cytokines. Ann N Y Acad Sci, 2001. 928: p. 141-146.
557. Peng, Y.J., et al., Pycnogenol attenuates the inflammatory and nitrosative stress on joint inflammation induced by urate crystals. Free Radic Biol Med, 2012. 52(4): p. 765-74.
558. Liu, Z., et al., Pycnogenol Ameliorates Asthmatic Airway Inflammation and Inhibits the Function of Goblet Cells. DNA Cell Biol, 2016 35(11): p. 730-739.
559. Dogan, E., et al., The Effect of Pycnogenol on Wound Healing in Diabetic Rats. Ostomy Wound Manage, 2017. 63(4).
560. Belcaro, G., et al., Pycnogenol improvements in asthma management. Panminerva Med, 2011. 53(3): p. 57-64.
561. Belcaro, G., et al., Variations in C-reactive protein, plasma free radicals and fibrinogen values in patients with osteoarthritis treated with Pycnogenol. Redox Rep, 2008. 13(6): p. 271-276.
562. Fitzpatrick, D.F., B. Bing, and P. Rohdewald, Endothelium-dependent vascular effects of Pycnogenol. J Cardiovasc Pharmacol, 1998. 32: p. 509-515.
563. Rezzani, R., et al., Effects of melatonin and Pycnogenol on small artery structure and function in spontaneously hypertensive rats. Hypertension, 2010. 55(6): p. 1373-1380.
564. Nishioka, K., et al., Pycnogenol, French maritime pine bark extract, augments endothelium-dependent vasodilation in humans. Hypertens Res., 2007. 30(9): p. 775-780.
565. Wang, S., T. Duanjun, and Z. Yusheng, The effect of Pycnogenol® on the microcirculation, platelet function and ischaemic myocardium in patients with coronary artery diseases. Eur Bull Drug Res, 1999. 7: p. 19-25.
566. Belcaro, G., A Clinical Comparison of Pycnogenol, Antistax, and Stocking in Chronic Venous Insufficiency. Int J Angiol, 2015. 24(4): p. 268-274.
567. Peng, Q.L., A.R. Buz'Zard, and B.H. Lau, Pycnogenol protects neurons from amyloid-beta peptide-induced apoptosis. Brain Res Mol Brain Res, 2002. 104(1): p. 55-65.
568. Hasegawa, N. and M. Mochizuki, Improved effect of Pycnogenol on impaired spatial memory function in partial androgen deficiency rat model. Phytother Res, 2009. 23(6): p. 840-843.
569. Ishrat, T., et al., Effects of Pycnogenol and vitamin E on cognitive deficits and oxidative damage induced by intracerebroventricular streptozotocin in rats. Behav Pharmacol, 2009. 20(7): p. 567-575.
570. Scheff, S.W., M.A. Ansari, and K.N. Roberts, Neuroprotective effect of Pycnogenol following traumatic brain injury. Exp Neurol, 2013. 239(1): p. 183-191.
571. Norris, C.M., et al., Pycnogenol protects CA3-CA1 synaptic function in a rat model of traumatic brain injury. Exp Neurol, 2016. 276: p. 5-12.
572. Ansari, M.A., K.N. Roberts, and S.W. Scheff, Dose- and Time-Dependent Neuroprotective Effects of Pycnogenol® following Traumatic Brain Injury. J Neurotrauma, 2013. 30(17): p. 1542-1549.
573. Scheff, S.W. and K.N. Roberts, Cognitive assessment of Pycnogenol therapy following traumatic brain injury. Neurosci Lett, 2016. 634: p. 126-131.
574. Belcaro, G., et al., Pycnogenol improves cognitive function, attention, mental performance and specific professional skills in healthy professionals age 35-55. J Neurosurg Sci, 2014. 58(4): p. 239-248.
575. Heimann, S.W., Pycnogenol for ADHD? J Am Acad Child Adolesc Psychiatry, 1999. 38: p. 357-358.
576. Tenenbaum, S., et al., An experimental comparison of Pycnogenol and methylphenidate in adults with attention-deficit/hyperactivity disorder (ADHD). J Attention Disorders, 2002. 6: p. 49-60.
577. Furlano, R., et al., Colonic CD8 and gamma delta T-cell infiltration with epithelial damage in children with autism. J Pediatr, 2001. 138(3): p. 366-372.
578. Pelsser, L.M.J., et al., Effects of food on physical and sleep complaints in children with ADHD: a randomised controlled pilot study. Eur J Pediatr, 2010. 169(9): p. 1129-1138.
579. Verlaet, A.A.J., et al., Rationale for Dietary Antioxidant Treatment of ADHD. Nutrients, 2018. 10(4): p. 405-424.
580. Fan, B., et al., Pycnogenol attenuates the release of proinflammatory cytokines and expression of perilipin 2 in lipopolysaccharide-stimulated microglia in part via inhibition of NF-κB and AP-1 activation. PLoS One, 2015. 10(9).

CHAPTER 2

OXIDATIVE STRESS AND IMMUNITY IN ADHD: A CASE-CONTROL COMPARISON

Verlaet AAJ, Breynaert A, Ceulemans B, De Bruyne T, Fransen E, Pieters L, Savelkoul HFJ, Hermans N. Oxidative stress and immune aberrancies in attention-deficit/hyperactivity disorder (ADHD): a case-control comparison. Eur Child Adolesc Psychiatry (2018).

I'd rather attempt to do something great and fail, than to attempt nothing and succeed. – Robert H. Schuller

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PART 1

ABSTRACT

Objective: The objective of this study is to compare oxidative stress and immune biomarkers between ADHD patients and controls without ADHD.

Methods: A case-control comparison between 57 paediatric (6-12 years) untreated ADHD patients from the Antwerp University Hospital and 69 controls without ADHD from random schools in Flanders, Belgium, was conducted. Erythrocyte glutathione (GSH), plasma lipid soluble antioxidants (retinol, α-tocopherol, γ-tocopherol, retinyl palmitate, β-carotene and co-enzyme Q10) were determined by HPLC with electrochemical detection, plasma malondialdehyde (MDA) by HPLC with fluorescence detection, plasma cytokines (interleukin (IL)-1β, IL-5, IL-6, IL-8, IL-10, tumour necrosis factor (TNF), and interferon (INF)-γ) and immunoglobulins (IgE, IgG, and IgM) by flow cytometry and urinary 8-hydroxy-2'deoxyguanosine (8-OHdG) levels by ELISA. Dietary habits were determined by a food frequency questionnaire.

Results: Plasma MDA levels were on average 0.031 µM higher in patients than in controls ($p < 0.05$) and a trend for higher urinary 8-OHdG was observed. Erythrocyte GSH and plasma retinyl palmitate levels as well as IgG and IgE levels were higher in patients than in controls as well (an increment of on average 93.707 µg/ml, 0.006 µg/ml, 301.555 µg/ml and 125.004 µg/ml, resp., $p < 0.05$). Finally, a trend for lower plasma IL-5 levels was observed. After Bonferroni correction for multiple testing, the difference in GSH levels remained statistically significant (nominally significant for retinyl palmitate), while significance was lost for MDA, IgG and IgE levels. Dietary habits do not appear to cause the observed differences.

Conclusion: These results point at the potential involvement of slight oxidative stress and immune disturbances in ADHD.

PART 2

INTRODUCTION

ADHD is a multifactorial disorder, influenced by genetic, environmental, biochemical and psychological factors, but no straightforward indication can yet be given about its exact pathophysiology. Dopaminergic and noradrenergic dysfunction are involved [1], but ADHD is also associated with immune and oxidative imbalances, as reviewed recently [2]. Various studies for instance demonstrate increased levels of oxidative damage markers and decreased activity of antioxidant enzymes in ADHD [3-7]. In addition, ADHD has a high comorbidity with both T_H1- and T_H2-mediated disorders [8-13]. Oxidative and immune mechanisms may contribute to ADHD via neuronal damage and abnormal neurotransmitter regulation, but decisive evidence on their exact contributions to this disorder is yet to be published [13-15]. Research is limited (e.g. regarding sample size and biomarker analyses), results are often inconsistent and immune markers other than antibodies, like cellular activation and cytokine levels, have not been systematically studied in ADHD [4-7, 16-19]. Moreover, more insight in underlying factors and mechanisms could eventually lead to safer treatment options. A thorough investigation of oxidative and immune aberrancies and their interconnection in ADHD is thus needed.

In the present study, a broad diversity of biomarkers of *in vivo* antioxidant status (erythrocyte GSH and plasma retinol, α-tocopherol, γ-tocopherol, retinyl palmitate, β-carotene and co-enzyme Q10 (coQ10)), oxidative damage (plasma MDA and urinary 8-OHdG) and immune status (plasma cytokines (IL-1β, IL-5, IL-6, IL-8, IL-10, TNF and INF-γ) and immunoglobulins (IgE, IgG and IgM)) were compared between untreated paediatric ADHD patients and controls without ADHD. It is hypothesised that ADHD patients have more oxidative stress and a disturbed immune balance. In addition, also non-biological factors like comorbid conditions and dietary habits were compared between children with and without ADHD with the use of multiple questionnaires. Results can increase insight in ADHD aetiology and can be used for inception of new therapies.

PART 3

MATERIALS AND METHODS

This study was approved by the Antwerp University Hospital (UZA) Ethics Committee (approved study number: 13/18/209; Belgian registration number: B300201317799; Appendix 2.1, see bookmark for QR code) and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

3.1 Participants

3.1.1 Criteria

Subjects were children aged 6-12 years. They were untreated ADHD patients (cases) from the paediatric outpatient department of Child Neurology of UZA and controls without ADHD from 10 random schools in Flanders. In- and exclusion criteria are listed in Table 2.1. Patients were diagnosed with ADHD according to DSM-4 or DSM-5 criteria by experienced staff [20, 21]. Participants were recruited between June 2013 and September 2017 via a letter in schools and UZA and a poster in UZA waiting rooms. All participants and their legally accepted representatives agreed with and signed the written informed consent (Appendix 3.1, see bookmark for QR code). Upon inclusion, participants received a randomly assigned participation code. Personal data were stored separately to maintain confidentiality.

3.1.2 Sample size requirements

Sample size calculation was based on standard deviation (SD) values in previous studies [7, 13]. It was determined that 63 children per group were required to demonstrate group differences of 0.5 SD regarding the proposed biomarkers (power 0.80, significance level 0.05, 2-sided, independent groups).

Table 2.1. In- and exclusion criteria for participation.

Criteria	ADHD	Control
Age 6-12 years	✓	✓
Diagnosis ADHD/ADD according to DSM criteria	✓	×
Diagnosis autism-spectrum disorder	×	×
Chronic systemic disorder (diabetes, epilepsy, schizophrenia, autoimmunity, renal disorder, …)	×	×
Severe mental conditions, IQ < 70, pervasive developmental disorder	×	×
Use of medication or vitamin/mineral/omega-3 supplement for more than 1 week during the 3 months preceding inclusion	×	×
Responsible caregiver to provide information about the patient's functional status	✓	✓

✓ inclusion; × exclusion; DSM: Diagnostic and Statistical Manual of Mental Disorders; IQ: intelligence quotient.

3.2 Sample collection and processing

3.2.1 Blood

Blood (12 ml) was collected by means of peripheral venous sampling in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes (Becton, Dickinson and Company (BD), USA) and placed on ice immediately. After centrifugation (2000g, 4°C, 10 min), plasma was frozen immediately in -70°C and stored until analysis. Erythrocytes were 1:1 diluted with phosphate buffer (50 mM Na_2HPO_4/NaH_2PO_4 (disodium hydrogen phosphate, ≥99,0%, Sigma Aldrich, USA; monosodiumdihydrogenorthophosphate, ≥99,0%, Fluka, Switzerland), pH 7,4) with 2 mM EDTA (> 99,5%, Fluka, Switzerland) and frozen in -70°C until analysis.

3.2.2 Urine

Urine was collected in sterile urine containers, placed on ice immediately and stored at -70°C until analysis.

3.3 Questionnaires and basic information

Participants' birth date, gender, weight and height were recorded at the time of sample collection. In addition, parents were asked to fill out several questionnaires. Researchers involved in digitalising questionnaire answers, calculating scores and statistics were blinded for participant groups.

3.3.1 Pregnancy Questionnaire

This questionnaire was used to assess the course of the pregnancy, premature birth, breastfeeding and chronic illness by means of twelve questions (Appendix 4.4.1, see bookmark for QR code).

3.3.2 Social-Emotional Questionnaire (SEQ)

The SEQ, containing 72 questions, was used to assess child behaviour as perceived by parents (Appendix 4.5.1, see bookmark for QR code). This questionnaire focusses on four clusters of social-emotional problems, including ADHD (18 questions) and frequently occurring psychiatric comorbidities of ADHD, i.e. social behaviour problems (ODD and CD; 26 questions), anxiety (including general and social anxiety; 18 questions) and autism (10 questions), with items covering the most important core symptoms of these clusters according to the DSM-4. The SEQ can be used for screening, diagnosis and treatment evaluation. Items were rated on a five-point scale, concerning the past six months: never (0), less than once a month (1), every month (2), every week (3) or (almost) every day (4). Scores per condition and subcondition were classified into five levels of severity, based on norm categories: normal, normal-high, subclinical, clinical and clinical-high. The latter two levels, clinical and clinical-high, can lead to a diagnosis of the specific disorder. The reliability and validity of the SEQ are good [22].

3.3.3 Physical Complaints Questionnaire (PCQ)

The 39 questions of the PCQ (Appendix 4.3.1, see bookmark for QR code) focus on various physical and sleep complaints, some of them frequently co-occurring with ADHD, in eight domains: pain (e.g. headache), unusual thirst or perspiration, eczema, asthma or rhinitis, skin problems, tiredness, GI problems and sleep problems [23, 24]. Questions were rated on the same scale as the SEQ, concerning the past six months. Questions were added regarding the occurrence of allergies, colds and ear infections. Allergy and atopy were determined based on their clinical manifestation and a physician’s diagnosis of allergy in the subject and at least one first grade relative, resp. [23]. The PCQ has unknown validity and reliability, but was already used in research on the effect of nutrition on ADHD [24].

3.3.4 Food Frequency Questionnaire (FFQ)

The dietary habits of participants were assessed by an FFQ concerning 7 food groups: fruits, vegetables, cereal and potato products, dairy, meat and fish, drinks and miscellaneous. This FFQ was based upon an FFQ assessing infant dietary habits in an unpublished pilot study [25]. The original FFQ consisted of three questions on meal frequency (breakfast, lunch and dinner) in addition to 60 questions on food

product frequency (concerning the intake of seven food groups, including portion sizes), which were rated on a six-point scale (never or less than once a month, once to three times a month, once a week, twice to four times a week, five to six times a week or every day). Fifteen additional questions assessed more specific information, such as bread type. Comparison of this FFQ to a three-day food diary, the gold standard to investigate dietary habits, revealed acceptable validity, but potential overestimation of vegetable and potato product intake. For the present FFQ, 35 questions were extracted from the original questionnaire as well as additional questions on e.g. type of bread, without portion size questions. The rating scale was adapted to a five-point scale: never or less than once a month (0), once to three times a month (1), once a week (2), twice to five times a week (3) or (almost) every day (4) (Appendix 4.2.1, see bookmark for QR code).

3.4 Biological analyses

Technicians blinded for the participant groups performed biological analyses for patients and controls concurrently.

3.4.1 Glutathione analysis

GSH is the most important intracellular antioxidant. Therefore, erythrocyte GSH content was analysed *in duplo* after sample haemolysis and purification as described by Magielse *et al.* by a validated high-pressure liquid chromatography (HPLC) method with electrochemical detection (ECD) [26, 27], in which detection is based on both retention time and electrochemical properties of components [28].

3.4.1.1 Materials

Na_2HPO_4 (≥ 99.0%), 1-octanesulphonic acid (OSA) sodium salt (~ 98%) and GSH (≥ 99.0%) were purchased from Sigma-Aldrich, USA. NaH_2PO_4 (≥ 99.0%) and EDTA (> 99.5%) were purchased from Fluka, Switzerland. Methanol (MeOH, HPLC quality) was purchased from Fisher Scientific, UK, and phosphoric acid (H_3PO_4, 85%) from Acros Organics, USA. Water (H_2O) was purified by Milli-Q from Merck Millipore, USA.

3.4.1.2 Sample and standard preparation

Thawed RBC samples were centrifuged (5000g, 4°C, 10 min) to separate intracellular content from cell debris. The supernatant was filtered over a 3 kDa filter with polyethersulfone (PES) membrane (VWR, USA) by centrifugation (14000g, 4°C, 20 min) to remove proteins [29]. Filtrates were diluted (1:10) with mobile phase A (see below) and acidified with 5% (v:v) H_3PO_4 [85%].

Standard solutions for the calibration curve were made in phosphate buffer (50 mM Na_2HPO_4/NaH_2PO_4; pH 7.4; + EDTA 2 mM), standard 1 containing 2.5 mM GSH, diluting 1:1 up to standard 5 (Table 2.2). Standards were diluted 1:10 with mobile phase A and 5% H_3PO_4 [85%] was added. A blank sample was prepared with phosphate buffer.

Table 2.2. Concentrations of GSH standards.

GSH (mM)	
STD 1	2.5
STD 2	1.25
STD 3	0.625
STD 4	0.313
STD 5	0.156

GSH: reduced glutathione; STD: standard. Standard ranges used for different samples can slightly differ.

3.4.1.3 HPLC analysis

Samples, standards and blanks were analysed by a HPLC system from Agilent, USA (type 1260 quaternary pump, 1260 auto-injector and 1290 temperature controller), linked to an ESA-5600A CoulArray 8-channel electrochemical detector (Thermo Fisher Scientific, USA). Mobile phase A (NaH_2PO_4 25 mM and OSA 1.4 mM in 3% MeOH, pH 2.7) and B (NaH_2PO_4 25 mM and OSA 1.4 mM in 50% MeOH, pH 2.7) were used at a flow of 0.7 ml/min. The elution profile was set at a 25 min linear gradient from 0 to 50% B, a 5 min linear gradient from 50 to 100% B and a 5 min isocratic elution at 100% B before returning to initial conditions. Samples were cooled at 4°C. Three electrochemical cells were set at 600, 850 and 900 mV. A C18 reversed phase (RP) column (250 x 4.6 mm, 5 µm) from Merck, USA was used. Resulting chromatograms (Figure 2.1) were analysed with CoulArrayWin software. The retention time of GSH was approximately 8 min.

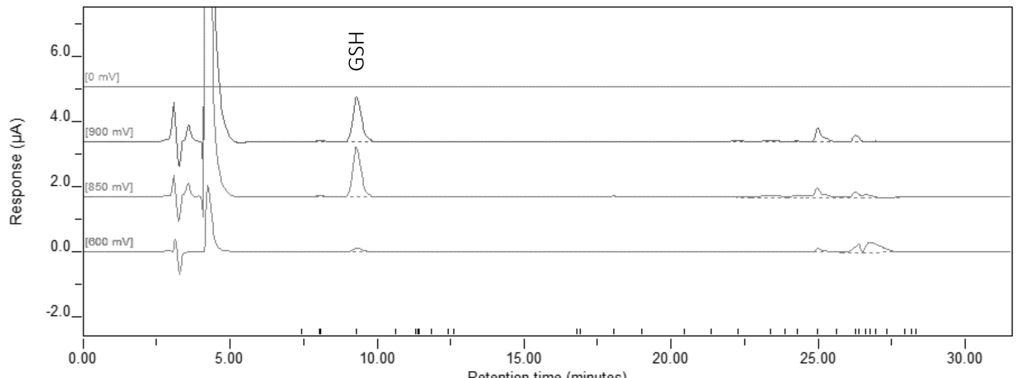

Figure 2.1. Resulting chromatogram from the analysis of GSH in erythrocytes.
GSH: reduced glutathione.

3.4.2 Lipid soluble antioxidant analysis

The lipid soluble antioxidants α- and γ-tocopherol, β-carotene, retinol, retinyl palmitate and coQ10 are non-enzymatic defence mechanisms against oxidants [30-33]. Therefore, plasma levels of these antioxidants were analysed *in duplo* after extraction as described by Hermans *et al.* by a validated HPLC-ECD method [28].

3.4.2.1 Materials

Retinol (≥ 95%), γ-tocopherol (≥ 96%), α-tocopherol (≥ 96%), retinyl palmitate (≥ 93%), β-carotene (≥ 93%) and coQ10 (≥ 98%) were purchased from Sigma-Aldrich, USA. Ammonium acetate (CH_3COONH_4, ≥98%) was purchased from Merck, USA. MeOH (HPLC quality), ethanol (EtOH, absolute), hexane (HPLC quality) and acetic acid (99.97%) were purchased from Thermo Scientific, USA. 1-propanol (HPLC quality) was purchased from Lab-Scan Analytical Sciences, Ireland. Water (H_2O) was purified by Milli-Q from Merck Millipore, USA.

3.4.2.2 Sample and standard preparation

Thawed plasma samples (100 µl) were diluted with an equal volume of EtOH and lipid soluble antioxidants were extracted with 500 µl hexane by vortexing 5 min. After centrifugation (4000g, 4°C, 10 min), supernatant was isolated. Extraction was repeated twice for the remaining sample. After evaporation of the combined extracts by vacuum centrifugation (Savant, Thermo Scientific, USA), the residue was dissolved in 100 µl EtOH.

Standard solutions for the calibration curve were made in EtOH, with standard 1 containing 1.80 µg/ml retinol, 2.40 µg/ml γ-tocopherol, 42.40 µg/ml α-tocopherol, 0.08 µg/ml retinyl palmitate, 1.84 µg/ml β-carotene and 3.10 µg/ml coQ10, diluting 1:1 up to standard 5 (Table 2.3). EtOH was used as blank. 100 µl H_2O was added to 100 µl of each standard and blank, which were then extracted with hexane, evaporated and dissolved as samples.

Table 2.3. Concentrations of lipid soluble antioxidant standards.

Concentration (µg/ml)	Retinol	γ-tocopherol	α-tocopherol	Retinyl palmitate	β-carotene	CoQ10
STD 1	1.80	2.40	42.40	0.08	1.84	3.10
STD 2	0.90	1.20	21.20	0.04	0.92	1.55
STD 3	0.45	0.60	10.60	0.02	0.46	0.775
STD 4	0.225	0.30	5.30	0.01	0.23	0.388
STD 5	0.113	0.15	2.65	0.005	0.115	0.194

CoQ10: co-enzyme Q10; STD: standard. Standard ranges used for different samples can slightly differ.

3.4.2.3 HPLC analysis

Dissolved residues were analysed by an HPLC system from Agilent, USA (type 1260 quaternary pump, 1260 auto-injector and 1290 temperature controller), linked to an ESA-5600A CoulArray 8-channel electrochemical detector (Thermo Fisher Scientific, USA). Mobile phase A (MeOH/H_2O/ammonium acetate 1 M (pH 4 with acetic acid); 90:8:2 v:v:v) and B (MeOH/1-propanol/ammonium acetate 1 M (pH 4 with acetic acid); 78:20:2 v:v:v) were used at a flow of 0.6 ml/min. The elution profile was set at a 21 min linear gradient from 0 to 80% B, a 10 min linear gradient from 80 to 100% B and a 14 min isocratic elution at 100% B before returning to initial conditions. Samples were cooled at 4°C. The electrochemical cells were set at 200, 480, 600, 700, 800, -1000, 240 and 500 mV. A Hypersil octadecyl silane (ODS, C18) RP analytical column (150 x 3 mm, 3 µm) from Thermo Scientific, USA, was used, heated to 37°C. Resulting chromatograms (Figure 2.2) were analysed with CoulArrayWin software. Retention times were approximately 3.9 min for retinol, 13.5 min for γ-tocopherol, 14.9 min for α-tocopherol, 27.3 min for retinyl palmitate, 30.6 min for β-carotene and 38.9 min for coQ10.

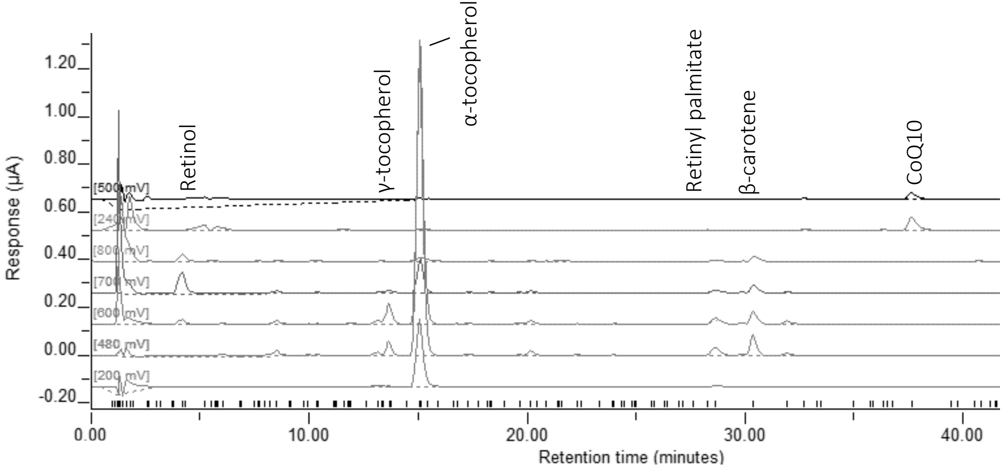

Figure 2.2. Resulting chromatogram from the analysis of retinol, γ-tocopherol, α-tocopherol, retinyl palmitate, β-carotene and coQ10 in plasma.
CoQ10: co-enzyme Q10.

3.4.3 MDA analysis

Oxidative damage to fatty acids leads to the generation of lipid hydroperoxides, which are unstable and therefore subjected to fragmentation into different products, including reactive aldehydes like MDA [34]. Plasma MDA is therefore a marker of *in vivo* oxidative stress and lipid peroxidation. It was measured *in duplo* as described by Hermans *et al.* by a validated HPLC method with fluorescence detection, which is more specific than spectrophotometric analysis [28, 35, 36].

3.4.3.1 Principle

At high temperature and acidity, thiobarbituric acid (TBA) and MDA react with each other, generating the pink fluorescent MDA-$(TBA)_2$ complex (Figure 2.3). Addition of butylhydroxytoluene (BHT) during sample preparation and heating samples for 40 min at 95°C limits artefact lipid peroxidation [36].

MDA TBA MDA-$(TBA)_2$ complex

Figure 2.3. Reaction of MDA and TBA at high temperature and acidity, resulting in MDA-$(TBA)_2$.
MDA: malondialdehyde; TBA: thiobarbituric acid.

3.4.3.2 Materials

BHT (≥ 99.0%), 1,1,3,3-tetramethyoxypropane (TMP, 99%) and TBA (≥ 98%) were purchased from Sigma-Aldrich, USA. Potassium phosphate monobasic (KH_2PO_4, analytical quality) and sodium hydroxide (NaOH, analytical quality) were purchased from Merck, USA. MeOH (HPLC quality), EtOH (absolute) and H_3PO_4 [85%] were purchased from Thermo Fisher Scientific, USA. Water (H_2O) was purified by Milli-Q from Merck Millipore, USA.

3.4.3.3 Sample and standard preparation

Before each use, glass reaction tubes were prewashed with a mixture of TBA 0.046 M, H_3PO_4 1.22 M and H_2O in a 1:3:2 (v:v:v) ratio (99.9°C, 1 h), followed by rinsing, one hour of heating (99.9°C) filled with ultra-pure water and an extensive rinse, to reduce blank peaks by contamination.

Thawed plasma samples (50 µl) were transferred into prewashed glass tubes and 25 µl BHT 1% (w:v) in EtOH, 250 µl H_3PO_4 1.22 M, 425µl H_2O and 250 µl TBA 0.046 M were consecutively added. The mixture was heated for 40 min at 95°C and afterwards cooled on ice. Protein precipitation was performed by addition of 40 µl NaOH 1 M and 360 µl MeOH to 200 µl of the reaction mixture. After centrifugation (9500g, 4°C, 5 min), the supernatant was isolated to be analysed.

TMP (Figure 2.4) was used as standard since MDA is unstable. Under hot and acidic conditions, TMP is hydrolysed completely to MDA. Standard solutions for the calibration curve were prepared in H_2O, with standard 1 containing 2.4 µM TMP and diluting 1:1 with H_2O up to standard 6 (Table 2.4). H_2O was used as blank. Standards and blanks followed the same reaction procedure as samples.

CH_3O OCH_3
H_3CO OCH_3

Figure 2.4. Chemical structure of 1,1,3,3-tetramethyoxypropane (TMP).

Table 2.4. Concentrations of TMP standards.

TMP (µM)	
STD 1	2.4
STD 2	1.2
STD 3	0.6
STD 4	0.3
STD 5	0.15

TMP: 1,1,3,3-tetramethyoxypropane; STD: standard. Standard ranges used for different samples can slightly differ.

3.4.3.4 HPLC analysis

Samples, standards and blanks were analysed by an HPLC system from Agilent, USA (type 1260 quaternary pump, 1260 auto-injector and 1290 temperature controller), linked to a FP-1520 fluorescence detector (Jasco, The Netherlands). Mobile phase A (KH_2PO_4 25 mM, pH 4) and B (MeOH) were used at a flow of 0.8 ml/min. The elution profile was set at an 8 min isocratic flow at 45% B, a 2 min linear gradient from 45 to 90% B and a 10 min isocratic elution at 90% B before returning to initial conditions. Samples were cooled at 4°C. The excitation and emission wavelengths were set at 532 and 553 nm, resp. A C18 RP column (250 x 4.6 mm, 5 µm) from Merck was used. Resulting chromatograms (Figure 2.5) were analysed with Agilent OpenLAB ChemStation. The retention time of the MDA-$(TBA)_2$ complex was approximately 6 min.

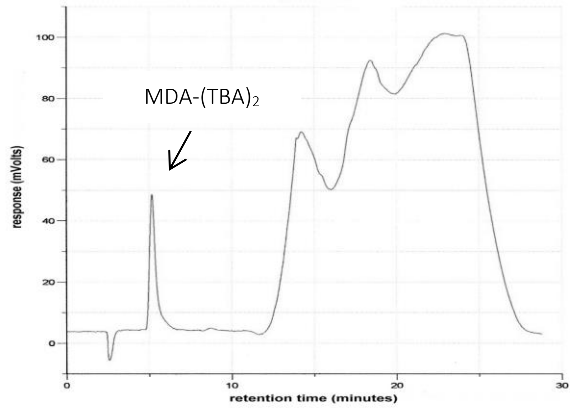

Figure 2.5. Resulting chromatogram of the MDA-$(TBA)_2$ complex in plasma.
MDA: malondialdehyde; TBA: thiobarbituric acid.

3.4.4 8-OhdG analysis

Especially the highly reactive HO• interacts with nuclear and mitochondrial DNA, with guanine of all nucleobases being most prone to free radical reactions [37]. Reaction of HO• with guanine adds a hydroxyl group at position 8 of the guanine molecule. This finally leads to generation of 8-hydroxy-2'-deoxy-guanosine (8-OHdG), one of the predominant metabolites of free radical-induced DNA damage, though also produced from nucleotides in cellular pools by nucleotide excision repair [36-38]. When DNA is repaired, 8-OHdG is excreted in urine without further metabolism [37, 39]. Therefore, urinary 8-OHdG (corrected for urinary creatinine concentration) was analysed *in duplo* as a marker of *in vivo* oxidative stress by an enzyme-linked immunosorbent assay (ELISA) kit [40]. The advantage of urinary 8-OHdG analysis is avoidance of DNA digestion during sample preparation and thereby absence of artefact 8-OHdG production. The reliability of this assay however depends on the selectivity of the antibodies for 8-OHdG, as interferences are inherent to the urine matrix [36]. The assay used therefore applies the anti-8-OHdG N45.1 clone, which has the highest possible specificity [40].

3.4.4.1 Principle

A competitive ELISA was used, in which sample 8-OHdG competes with plate-bound 8-OHdG for binding added murine monoclonal antibody. After washing and addition of both secondary antibody linked with horse-radish peroxidase (HRP) to bind the primary antibody and substrate (3,3',5,5'-tetramethylbenzidine, TMB), colour development is inversely proportional to 8-OHdG concentration in the sample.

Creatinine reacts with picric acid under alkaline conditions, but this reaction also occurs non-specifically with other components in biological samples. However, the specific colour produced by creatinine degrades rapidly under acidic conditions, so that the difference in colour intensity is a direct estimate of creatinine concentrations.

3.4.4.2 Materials

The urinary 8-OHdG ELISA kit (Northwest Life Science Specialties, USA) included 8-OHdG standards, murine-anti-8-OHdG monoclonal primary antibody, anti-murine-HRP, secondary antibody, phosphate-buffered saline (PBS) antibody buffer, TMB, H_2O_2/citrate/PBS diluting buffer, PBS wash buffer and 1 M H_3PO_4 stop solution. PBS was purchased from Lonza, Switzerland. Water (H_2O) was purified by Milli-Q from Merck Millipore, USA.

The Creatinine Assay Kit (Oxford Biomedical Research, USA) included creatinine standards, picrate reagent, alkali solution and acid reagent.

3.4.4.3 Sample and standard preparation and analysis

Thawed urine samples were centrifuged (2000g, RT, 12 min). 50 µl sample, standard or blank (PBS) was transferred to the 8-OHdG-coated plate and an equal amount of murine anti-8-OHdG monoclonal antibody was added. The plate was incubated (37°C, 1 h), emptied and washed with PBS. 100 µl antimurine antibody conjugated with HRP was added and the plate was incubated (37°C, 1 h), emptied and washed again. Afterwards, 100 µl TMB was added, the plate was incubated (RT, 15 min) and 100 µl stop solution was added, after which absorbance was measured at 450 nm using a microplate reader (BioTek Synergy Mx, USA). The logarithmic calibration curve was made with standard solution 1 being 200 ng/ml 8-OHdG, diluted till standard solution 6 (Table 2.5).

Table 2.5. Concentrations of 8-OHdG standards.

8-OHdG (ng/ml)	
STD 1	200
STD 2	80
STD 3	20
STD 4	8
STD 5	2
STD 6	0.5

8-OHdG: 8-hydroxy-2'-deoxyguanosine; STD: standard. Standard ranges used for different samples can slightly differ.

For creatinine analysis, thawed urine samples were centrifuged (2000g, RT, 12 min) and 25 µl standards, blank (H_2O), or 1:30 diluted samples were transferred to the plate. 180 µl alkali solution diluted 1:5 with picrate reagent was added, followed by incubation (RT, 10 min) and measurement of absorbance at 490 nm using a microplate reader (BioTek Synergy Mx, USA). Then, 15 µl acid reagent was added, after which the plate was incubated (RT, 5 min) and absorbance was measured again at 490 nm. A calibration curve was made with standard solution 1 being 10.0 mg/dl, 2 being 3.0 mg/dl and 3 being 1.0 mg/dl creatinine.

3.4.5 Cytokine analysis

3.4.5.1 Materials

The Cytometric Bead Array (CBA) Human Soluble Protein Master Buffer Kit (BD, USA) included capture beads, phycoerythrin (PE) detection reagent and standards for human IL-1β, IL-5, IL-6, IL-8, IL-10, TNF and IFN-γ, capture bead diluent for plasma, detection reagent diluent, assay diluent, wash buffer and cytometer setup beads.

3.4.5.2 Sample and standard preparation and analysis

Capture beads for the concerned cytokines were mixed with wash buffer, vortexed and centrifuged (200g, RT, 5 min). Supernatant was discarded and capture beads were resuspended in capture bead diluent for plasma. After 15 min incubation at RT, 10 µl of the concerned capture bead mixture was added to 10 µl sample, incubating 1 h at RT. After addition of 10 µl PE detection reagent diluted in detection reagent diluent and overnight incubation (4°C), 200 µl wash buffer was added. Supernatant was discarded after centrifugation (200g, RT, 5 min) and the bead pellet was resuspended in 150 µl wash buffer.

Standard solutions for the calibration curve were made in assay diluent, with standard 1 containing 2500 pg/ml IL-1β, IL-5, IL-6, IL-8, IL-10, TNF and IFN-γ, diluting 1:1 up to standard 9 (Table 2.6). Assay diluent was used as blank. Standards and blanks followed the same procedure as samples.

The flow cytometer (BD FACSCanto™ II with BD FACS DIVA™ Software) was setup with cytometer setup beads. Number of events to be counted was set at 300 per cytokine.

Table 2.6. Concentrations of IL-1β, IL-5, IL-6, IL-8, IL-10, TNF and IFN-γ standards.

Cytokine (pg/ml)	
STD 1	2500
STD 2	1250
STD 3	625
STD 4	312.5
STD 5	156.25
STD 6	78.13
STD 7	39.06
STD 8	19.53
STD9	9.77

STD: standard. Standard ranges used for different samples can slightly differ.

3.4.6 Antibody analysis

3.4.6.1 Materials

The CBA Human Immunoglobulin Master Buffer Kit and CBA Human Soluble Protein Master Buffer Kit (BD, USA) both included assay diluent, capture bead diluent, detection reagent diluent, wash buffer and instrument setup beads. The CBA Human Ig Flex Set (BD, USA) included standards, capture beads and PE detection reagent for human IgM and total IgG. The CBA Human IgE Flex Set (BD, USA) included the standard, detection reagent and capture beads for human IgE.

3.4.6.2 Sample and standard preparation and analysis

Thawed plasma samples were diluted with assay buffer (1:1000 for IgM, 1:200,000 for total IgG and 1:4 for IgE). IgE capture beads were diluted with wash buffer, vortexed and centrifuged (200g, RT, 5 min). Supernatant was discarded and beads resuspended in capture bead diluent. Other capture beads were mixed and diluted with capture bead diluent. After 15 min incubation at RT, 10 µl of capture bead mixture was added to 10 µl diluted sample, incubating 1 h at RT. 200 µl wash buffer was added and supernatant was discarded after centrifugation (200g, RT, 5 min). After addition of 10 µl PE detection reagent diluted in detection reagent diluent and overnight incubation (4°C), 200 µl wash buffer was added. Again, supernatant was discarded after centrifugation (200g, RT, 5 min) and the bead pellet was resuspended in 60 µl wash buffer.

Standard solutions were prepared by reconstitution of Ig standard in 1000 µl assay diluent followed by 15 min equilibration at RT. For total IgG, 50 µl reconstituted standard was further diluted with 1.55 ml assay diluent. Standard 1 contained 910.00 ng/ml IgM and 430.00 ng/ml total IgG, diluting 1:1 up to standard 9 (Table 2.7). IgE standard was reconstituted with assay diluent and diluted 1:1 till standard 9, standard 1 containing 500.00 ng/ml IgE (Table 2.7). Assay diluent was used as blank.

Table 2.7. Concentrations of Ig standards.

Concentration (ng/ml)	**IgM**	**Total IgG**	**IgE**
STD 1	910.00	430.00	500.00
STD 2	455.00	215.00	250.00
STD 3	227.50	107.5	125.00
STD 4	113.75	53.75	62.50
STD 5	56.88	13.44	31.25
STD 6	28.44	6.72	15.63
STD 7	14.22	3.36	7.81
STD 8	7.11	1.68	3.91
STD 9	3.55	0.84	1.95

Ig: immunoglobulin; STD: standard. Standard ranges used for different samples can slightly differ.

The flow cytometer (BD FACSCanto™ II with BD FACS DIVA™ Software) was setup with cytometer setup beads. Number of events to be counted was set at 300 per antibody.

3.5 Statistics

SPSS (Statistical Package for the Social Sciences, SPSS Inc., Chicago, USA) version 23.0.0.0 was used for statistical analyses. Data were checked for outliers and normality (Shapiro-Wilk Test and QQ-plot). For

normally distributed continuous variables, group means were expressed as mean ± SD. Continuous variables with a non-normal distribution were described by median ± interquartile range (IQR). Ordinal variables were presented by percentages, e.g. stacked bar charts representing percentages within each category. Differences between patients and controls were tested for significance by Independent Samples t-Test (in case of normal distribution), Mann-Whitney U test (in case of non-normal distribution) or multiple regression analysis for continuous variables and Cochran-Armitage trend test or Chi-Square test for ordinal variables. Correlations between variables were expressed as Pearson's or Spearman's correlation coefficient. Bonferroni correction for multiple testing was performed. For all regression models, residuals versus the predicted values were plotted to check the assumptions of linearity and homoskedasticity. Using QQ-plots, the normality of the residuals was checked. A p-value < 0.05 was considered significant.

PART 4

RESULTS

In total, 126 participants were included in this comparison: 57 ADHD patients and 69 controls. No significant differences were found between patients and controls regarding the demographic variables age, height and weight (Independent Samples t-Tests, Table 2.8) and also gender ratio was not significantly different (Chi-Square test). Participants were mainly Caucasian (84% in the patient group, 86% in the control group; data not shown).

Table 2.8. Demographic variables per group.

	ADHD	Control	Test value	P-value (2-sided)
Age (years; mean ± SD)	8.98 ± 1.75	8.37 ± 1.69	T(121)=-1.959	0.052
Height (m; mean ± SD)	1.36 ± 0.11	1.36 ± 0.12	T(114)=-0.105	0.916
Weight (kg; mean ± SD)	32.54 ± 9.48	31.15 ± 7.64	T(114)=-0.878	0.382
Gender (n; male/female)	41/16	45/24	χ(1)=0.649	0.420

SD: standard deviation.

4.1 Questionnaires

Five pregnancy questionnaires were never received, as well as one SEQ, four PCQs and four FFQs.

4.1.1 Pregnancy questionnaire

No statistically significant differences were found between ADHD patients and controls based on the pregnancy questionnaire (Table 2.9). Early birth was defined as birth at least two weeks before due date. The median breastfeeding duration was 8 weeks (IQR = 24) for patients and 13 weeks (IQR = 23) for controls (U=1401.5, p = 0.442). Chronic illnesses reported include celiac disease, asthma and reflux.

Table 2.9. Percentage of positive responses on specific questions of the pregnancy questionnaire.

	ADHD	Control	Test value	P-value (2-sided)
Complications pregnancy	11.1	10.4	$\chi(1)=0.014$	0.907
Medication pregnancy	15.1	10.6	$\chi(1)=0.538$	0.463
Smoking pregnancy	16.7	11.9	$\chi(1)=0.553$	0.457
Alcohol pregnancy	11.1	16.9	$\chi(1)=0.814$	0.367
Substance abuse pregnancy	0.0	0.0	-	-
Complications delivery	22.6	17.5	$\chi(1)=0.486$	0.489
Early birth	21.2	30.3	$\chi(1)=1.257$	0.262
Cry-baby	18.0	17.5	$\chi(1)=0.006$	0.940
Breastfeeding	72.2	82.1	$\chi(1)=1.679$	0.195
Breastfeeding ≥ 4 weeks	63.0	71.1	$\chi(1)=0.921$	0.337
Breastfeeding ≥ 12 weeks	50.0	59.1	$\chi(1)=2.021$	0.364
Chronic illness	5.6	6.0	$\chi(1)=0.009$	0.923

4.1.2 Social-emotional questionnaire

The median scores on all SEQ conditions and subconditions (Table 2.10) were significantly higher in the ADHD group than in controls ($p < 0.05$), except for social anxiety (Mann-Whitney U tests). Similar results were obtained regarding the percentage of diagnoses based on SEQ scores after score classification into levels of severity (Table 2.11): the percentage of participants diagnosed with a certain disorder according to SEQ guidelines was higher in the ADHD group ($p < 0.05$), except for social anxiety (Chi-Square tests). Based on SEQ scores, patients in this study are mostly classified as having combined type ADHD (Table 2.12). Finally, all SEQ scores correlate significantly positively with each other, except for "Social anxiety" and "Conduct Disorder – Aggression" (Table 2.13).

Table 2.10. Median scores and IQR per condition and subcondition of the SEQ.

	ADHD		Control			P-value
	Median	IQR	Median	IQR	Test value	(2-sided)
ADHD total	40.0	22.8	12.0	11.5	U=329.5	*< 0.001**
Inattention	15.0	8.0	4.0	4.0	U=199.5	*< 0.001**
Hyperactivity	16.0	12.5	5.0	6.0	U=664.0	*< 0.001**
Impulsivity	9.0	8.5	4.0	4.8	U=649.0	*< 0.001**
Autism	6.0	6.0	2.0	2.3	U=827.5	*< 0.001**
Social problem behaviour total	17.0	19.0	10.0	11.0	U=963.0	*< 0.001**
Oppositional Defiant Disorder	7.0	12.5	4.0	4.8	U=1168.5	*< 0.001**
Conduct Disorder – Aggression	1.0	3.8	0.0	2.0	U=1393.5	0.004*
Conduct Disorder – Antisocial	9.0	8.0	4.0	6.0	U=928.0	*< 0.001**
Anxiety total	16.5	16.0	7.0	10.0	U=982.5	*< 0.001**
General anxiety	6.0	6.0	3.0	4.0	U=902.5	*< 0.001**
Social anxiety	3.0	6.5	3.0	3.9	U=1621.0	0.114
Depression	5.0	5.0	2.0	4.0	U=1017.5	*< 0.001**

*Statistically significant if p < 0.05; italic p-values: statistically significant after Bonferroni correction. IQR: interquartile range; SEQ: social-emotional questionnaire.

Table 2.11. Percentages of diagnoses based on SEQ scores.

	ADHD	Control	Test value	P-value (2-sided)
ADHD total	59.6	1.5	χ(1)=59.334	*< 0.001**
Inattention	71.9	1.5	χ(1)=83.605	*< 0.001**
Hyperactivity	49.1	5.9	χ(1)=52.023	*< 0.001**
Impulsivity	26.3	1.5	χ(1)=34.804	*< 0.001**
Autism	24.6	1.5	χ(1)=30.622	*< 0.001**
Social problem behaviour total	29.8	5.9	χ(1)=22.696	*< 0.001**
Oppositional Defiant Disorder	29.8	4.4	χ(1)=19.522	*< 0.001**
Conduct Disorder – Aggression	17.5	4.4	χ(1)=10.651	0.017*
Conduct Disorder – Antisocial	36.8	7.4	χ(1)=22.245	*< 0.001**
Anxiety total	28.1	4.4	χ(1)=20.554	*< 0.001**
General anxiety	33.3	4.4	χ(1)=26.386	*< 0.001**
Social anxiety	12.3	7.4	χ(1)=12.350	0.352
Depression	31.6	4.4	χ(1)=15.954	*< 0.001**

*Statistically significant if p < 0.05; italic p-values: statistically significant after Bonferroni correction; SEQ: social-emotional questionnaire.

Table 2.12. Percentage of participants per ADHD subtype, based on SEQ scores.

	ADHD	Control
ADHD – predominantly inattentive type	26.3	0.0
ADHD – predominantly hyperactive-impulsive type	3.5	4.4
ADHD – combined type	45.6	1.5

Table 2.13. Correlations (Spearman's rho) between SEQ scores.

	ADHD total	ADHD inattention	ADHD hyperactivity	ADHD impulsivity	**Autism**	**Social problem behaviour**	Oppositional Defiant disorder	Conduct Disorder – Aggression	Conduct Disorder – Antisocial	**Anxiety total**	General anxiety	Social anxiety	Depression
ADHD total	-	_0.926*_	_0.893*_	_0.901*_	_0.754*_	_0.726*_	_0.608*_	_0.421*_	_0.752*_	_0.626*_	_0.674*_	0.255°	_0.585*_
ADHD inattention	_0.926*_	-	_0.745*_	_0.775*_	_0.680*_	_0.651*_	_0.526*_	_0.379*_	_0.667*_	_0.566*_	_0.631*_	0.221#	_0.518*_
ADHD hyperactivity	_0.893*_	_0.745*_	-	_0.771*_	_0.644*_	_0.563*_	_0.462*_	_0.312*_	_0.630*_	_0.497*_	_0.563*_	0.184#	_0.443*_
ADHD impulsivity	_0.901*_	_0.775*_	_0.771*_	-	_0.744*_	_0.786*_	_0.677*_	_0.481*_	_0.792*_	_0.661*_	_0.687*_	0.302°	_0.608*_
Autism	_0.754*_	_0.680*_	_0.644*_	_0.744*_	-	_0.660*_	_0.538*_	_0.401*_	_0.672*_	_0.675*_	_0.674*_	_0.454*_	_0.554*_
Social problem behaviour total	_0.726*_	_0.651*_	_0.563*_	_0.786*_	_0.660*_	-	_0.882*_	_0.683*_	_0.904*_	_0.623*_	_0.653*_	0.249°	_0.620*_
Oppositional Defiant Disorder	_0.608*_	_0.526*_	_0.462*_	_0.677*_	_0.538*_	_0.882*_	-	_0.521*_	_0.687*_	_0.650*_	_0.587*_	_0.329*_	_0.668*_
Conduct Disorder - Aggression	_0.421*_	_0.379*_	_0.312*_	_0.481*_	_0.401*_	_0.683*_	_0.521*_	-	_0.564*_	0.280°	_0.403*_	0.014	0.295°
Conduct Disorder - Antisocial	_0.752*_	_0.667*_	_0.630*_	_0.792*_	_0.672*_	_0.904*_	_0.687*_	_0.564*_	-	_0.574*_	_0.636*_	0.206#	_0.556*_
Anxiety total	_0.626*_	_0.566*_	_0.497*_	_0.661*_	_0.675*_	_0.623*_	_0.650*_	0.280°	_0.574*_	-	_0.851*_	_0.727*_	_0.884*_
General anxiety	_0.674*_	_0.631*_	_0.563*_	_0.687*_	_0.674*_	_0.653*_	_0.587*_	_0.403*_	_0.636*_	_0.851*_	-	_0.390*_	_0.698*_
Social anxiety	0.255°	0.221#	0.184#	0.302°	_0.454*_	0.249°	_0.329*_	0.014	0.206#	_0.727*_	_0.390*_	-	_0.493*_
Depression	_0.585*_	_0.518*_	_0.443*_	_0.608*_	_0.554*_	_0.620*_	_0.668*_	0.295°	_0.556*_	_0.884*_	_0.698*_	_0.493*_	-

*Statistically significant if $p < 0.001$; ° Statistically significant if $p < 0.01$; # Statistically significant if $p < 0.05$; italic Spearman's rho: statistically significant after Bonferroni correction; SEQ: social-emotional questionnaire.

4.1.3 Physical complaints questionnaire

As compared to controls, parents of ADHD patients reported significantly more stomach ache, tiredness, circles under eyes, sensitivity for sounds, sloppy handwriting, wanting to touch, compulsive behaviour, tics and sleep complaints (difficulties falling asleep and waking up, as well as waking up at night) and less eye complaints ($p < 0.05$, Cochran-Armitage trend tests, Table 2.14, Figure 2.6). Trends were observed for more headache, throat, nose and ear problems and high muscle tension ($p > 0.05$). After clustering several physical complaints (e.g. total pain: headache, stomach ache and growing pains), significantly more total pain and sleep complaints were reported for patients than for controls, as well as a trend for more GI complaints (Cochran-Armitage trend tests, Table 2.15).

A trend was noticed for more physician's diagnosed allergies as reported by parents in patients than in controls (23.1% vs. 11.8%, $\chi(1)=2.715$, $p = 0.099$). Allergens mentioned were grasses, pollen, animal dander and medication. Suspicion of undiagnosed allergies by parents, the use of antibiotics and the prevalence of ear infections were not significantly different between both groups ($p > 0.05$, Chi-Square tests, data not shown).

4.1.4 Food frequency questionnaire

Dietary habits of patients and controls appeared very similar, except for consumption of sweet milk drinks and fish, which were less in patients ($p < 0.05$, Cochran-Armitage trend tests, Table 2.16 and Figure 2.7). In addition, trends were observed for a lower consumption of fruit juice, raw vegetables, vegetable puree, soy and fried foods, and a higher consumption of light soda by patients. No significant difference was observed in percentages of participants meeting recommendations for daily fruit (55.6% in ADHD vs. 48.5% in controls) and vegetable intake (63.0% vs 70.6%). Also, no significant differences were found regarding the type of milk, yoghurt, bread, pasta or chocolate generally consumed.

Table 2.14. P-values for distributions of physical complaints per group.

	Test value	P-value (2-sided)
Headache	$\chi(1)=2.811$	0.094
Stomach ache	$\chi(1)=10.551$	_0.001*_
Nausea, vomiting	$\chi(1)=0.310$	0.578
Thirst	$\chi(1)=0.242$	0.623
Sweating	$\chi(1)=0.125$	0.725
Feeling warm	$\chi(1)=1.798$	0.180
Sensitive skin	$\chi(1)=0.359$	0.549
Eczema	$\chi(1)=0.004$	0.952
Throat, nose and ear problems	$\chi(1)=3.442$	0.064
Colds	$\chi(1)=1.237$	0.226
Asthma	$\chi(1)=0.999$	0.317
Diarrhoea	$\chi(1)=1.164$	0.281
Constipation	$\chi(1)=0.009$	0.925
Flatulence	$\chi(1)=0.072$	0.789
Bedwetting	$\chi(1)=0.010$	0.920
Growing pains	$\chi(1)=0.120$	0.729
Tiredness	$\chi(1)=4.000$	0.045*
Nose bleedings	$\chi(1)=0.015$	0.903
Red spots on the face	$\chi(1)=0.575$	0.448
Red border around the mouth	$\chi(1)=0.751$	0.386
Red ears	$\chi(1)=0.488$	0.485
Circles under eyes	$\chi(1)=4.444$	0.035*
Sensitivity for sounds	$\chi(1)=6.864$	0.009*
High pain threshold	$\chi(1)=0.284$	0.594
High muscle tension	$\chi(1)=2.805$	0.094
Sloppy handwriting	$\chi(1)=24.018$	_< 0.001*_
Not wanting to be touched	$\chi(1)=2.033$	0.154
Wanting to touch	$\chi(1)=11.274$	_0.001*_
Obsessive behaviour	$\chi(1)=2.180$	0.140
Compulsive behaviour	$\chi(1)=4.417$	0.036*
Tics	$\chi(1)=8.753$	0.003*
Problems falling asleep	$\chi(1)=14.093$	_< 0.001*_
Waking up at night	$\chi(1)=12.126$	_< 0.001*_
Difficulties awaking	$\chi(1)=12.211$	_< 0.001*_
Stomach problems	$\chi(1)=0.945$	0.331
Nasal complaints	$\chi(1)=0.259$	0.611
Wheezing	$\chi(1)=0.816$	0.366
Eye complaints	$\chi(1)=4.396$	0.036*
Skin problems	$\chi(1)=0.815$	0.367

*Statistically significant if $p < 0.05$; italic p-values: statistically significant after Bonferroni correction.

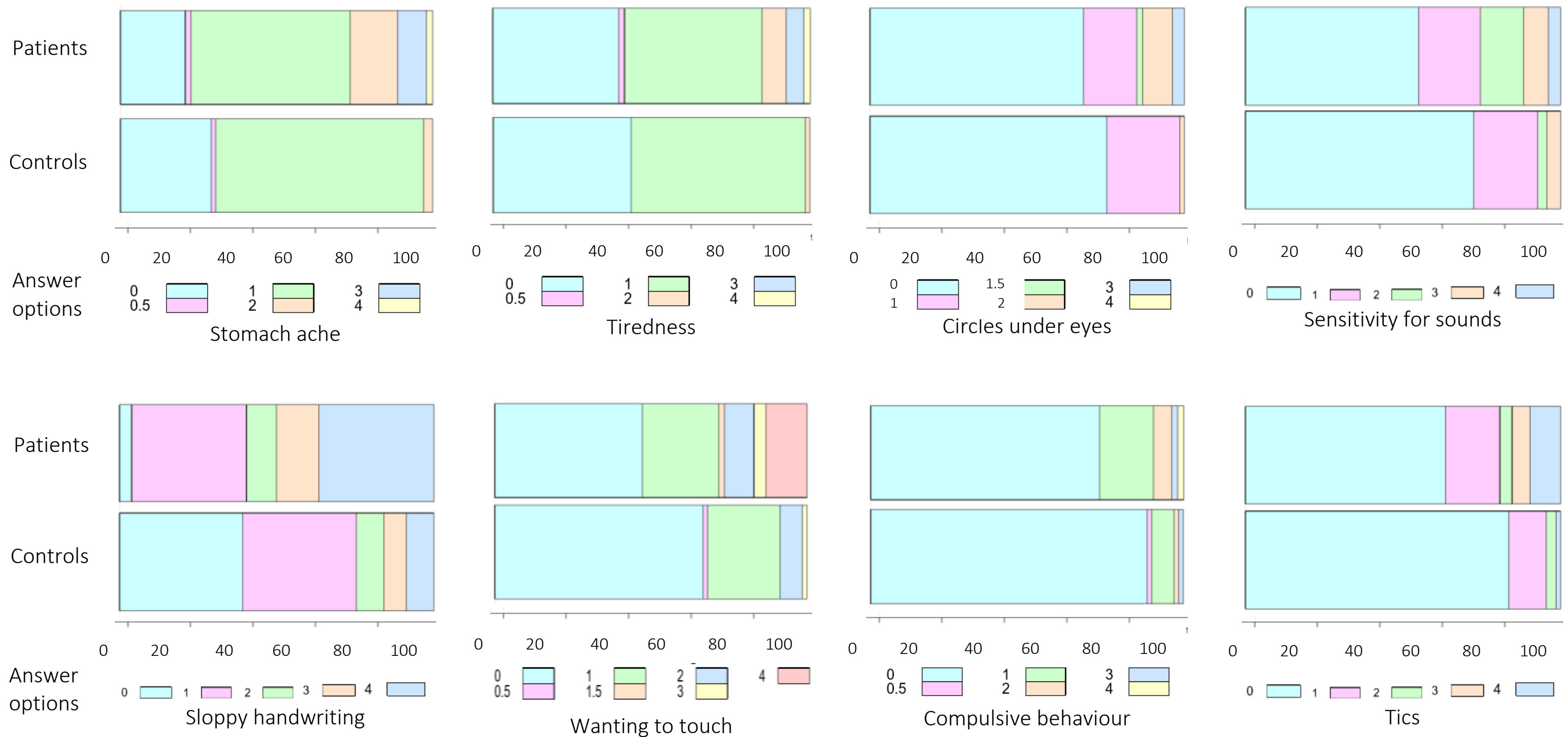

Figure 2.6 (part 1). Distributions of physical complaints per group (% of answers per question) for complaints with significant results in Table 2.14. Answer options: never (0), less than once a month (1), every month (2), every week (3) or (almost) every day (4).

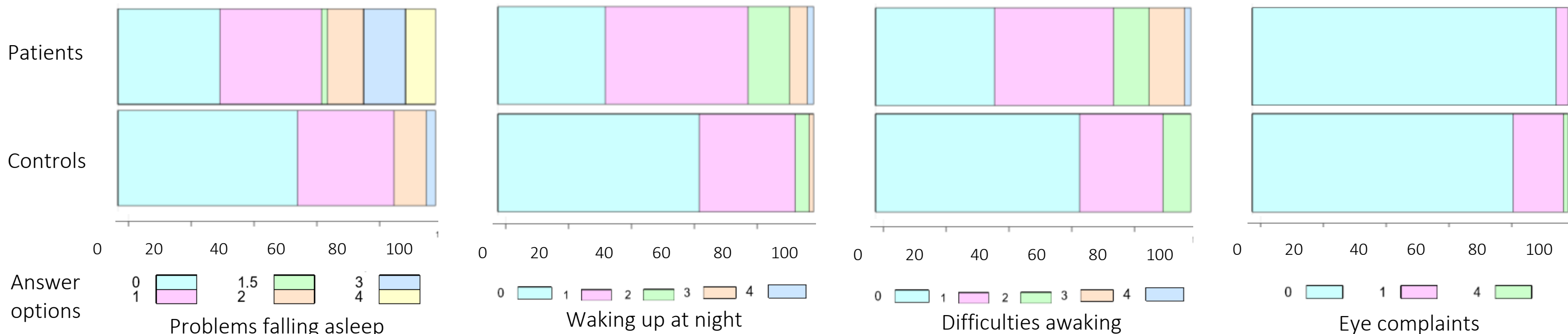

Figure 2.6 (part 2). Distributions of physical complaints per group (% of answers per question) for those complaints with significant results in Table 2.14. Answer options: never (0), less than once a month (1), every month (2), every week (3) or (almost) every day (4).

Table 2.15. Clustered physical complaints scores per group.

	Test value	P-value (2-sided)
Total pain	χ(1)=3.964	0.046*
Total gastrointestinal problems	χ(1)=2.895	0.089
Total skin complaints	χ(1)=2.167	0.141
Total sleep complaints	χ(1)=15.414	*< 0.001**

*Statistically significant if $p < 0.05$; italic p-values: statistically significant after Bonferroni correction.

Table 2.16. P-values for distributions of dietary habit scores per group.

	Test value	P-value (2-sided)
Fresh fruit	$\chi(1)=3.081$	0.079
Fruit juice	$\chi(1)=1.535$	0.215
Canned fruit	$\chi(1)=0.556$	0.456
Dried fruit	$\chi(1)=0.883$	0.347
Raw vegetables	$\chi(1)=2.968$	0.085
Cooked vegetables	$\chi(1)=2.134$	0.144
Vegetable puree	$\chi(1)=2.895$	0.089
Vegetable drinks	$\chi(1)=2.146$	0.143
Soup	$\chi(1)=0.474$	0.491
Bread	$\chi(1)=0.065$	0.799
Cereals	$\chi(1)=1.088$	0.297
Pasta	$\chi(1)=0.098$	0.754
Rice	$\chi(1)=2.339$	0.126
Potatoes	$\chi(1)=0.226$	0.635
Milk	$\chi(1)=0.014$	0.904
Sweet milk drinks	$\chi(1)=3.954$	0.047*
Yoghurt	$\chi(1)=0.358$	0.549
Soy	$\chi(1)=3.140$	0.076
Milk drinks	$\chi(1)=2.247$	0.134
Butter	$\chi(1)=0.589$	0.443
Margarine	$\chi(1)=1.329$	0.249
Cheese	$\chi(1)=0.712$	0.399
Eggs	$\chi(1)=1.661$	0.198
Meat	$\chi(1)=0.144$	0.704
Fish	$\chi(1)=6.180$	0.013*
Water	$\chi(1)=0.032$	0.857
Tea	$\chi(1)=1.341$	0.247
Light soda	$\chi(1)=3.575$	0.059
Soda	$\chi(1)=0.136$	0.713
Nuts and seeds	$\chi(1)=0.015$	0.903
Chocolate	$\chi(1)=0.772$	0.380
Sweet snacks	$\chi(1)=0.003$	0.958
Snacks	$\chi(1)=0.373$	0.541
Vegetarian products	$\chi(1)=1.463$	0.226
Fried foods	$\chi(1)=3.439$	0.064
Type of milk	$\chi(1)=0.144$	0.704
Type of yoghurt	$\chi(1)=0.927$	0.336
Type of bread	$\chi(1)=0.139$	0.709
Type of pasta	$\chi(1)=1.095$	0.295
Type of chocolate	$\chi(1)=0.130$	0.718
Daily fruit intake	$\chi(1)=0.595$	0.440
Daily vegetable intake	$\chi(1)=0.794$	0.373

*Statistically significant if $p < 0.05$.

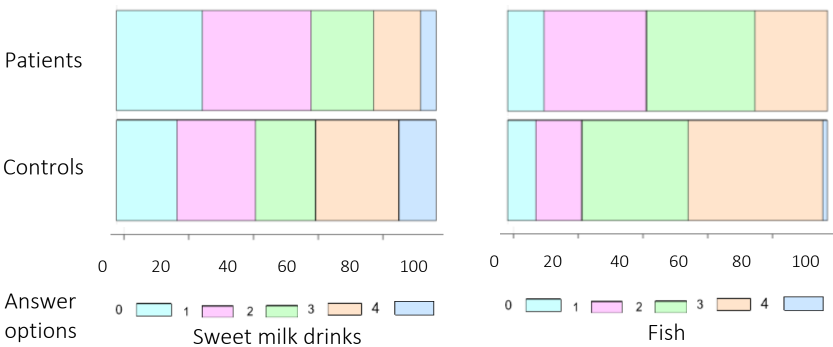

Figure 2.7. Distributions of dietary habits per group (% of answers per question) for food items with significant results in Table 2.16. Answer options: never or less than once a month (0), once to three times a month (1), once a week (2), twice to five times a week (3) or (almost) every day (4).

4.2 Biological analyses

Mean levels of each analysed oxidative damage and antioxidant biomarker are presented per group in Table 2.17, those of each analysed immune biomarker in Table 2.18. A comparison of these levels between groups must account for different processing times as potential confounder (Table 2.19), as processing time could influence biomarker levels. Therefore, a linear regression model was fitted with the biomarker as outcome variable, disease status as predicting variable and processing time as covariate. For example, for GSH, a significant effect of both processing time (data not shown) and disease status was found. The adjusted differences between both groups for oxidative damage, antioxidant and immune biomarkers can also be found in Tables 2.17 and 2.18. For example, assuming an equal processing time, erythrocyte GSH level was on average 93.7 µg/ml higher in patients than in controls ($p < 0.05$). Also plasma retinyl palmitate, MDA, IgG and IgE levels were significantly higher in patients than in controls when accounting for processing time ($p < 0.05$, linear regression). Trends for higher urinary 8-OHdG and lower plasma IL-5 levels were observed as well. IL-10/TNF ratio was not significantly different between patients and controls ($p > 0.05$, linear regression, data not shown).

To test whether antioxidant levels depended on the amount of oxidative damage, and whether this dependence differed between groups, again a linear regression model was fitted, with the antioxidant marker as outcome variable, disease status as predicting variable and oxidative damage biomarker as covariate (Tables 2.20 and 2.21). As the interactions between group and the oxidative damage biomarkers were not significant, the effect of MDA and 8-OHdG on antioxidant levels did not differ significantly between cases and controls. After removal of the interaction term, antioxidant levels were

not predicted by the amount of oxidative damage, except for β-carotene by MDA levels as well as α-tocopherol and GSH by 8-OHdG levels ($p < 0.05$, linear regression).

In addition, as for patients versus controls, a comparison was made between participants with and without a physician's diagnosed allergy as reported by parents. When accounting for processing time, none of the antioxidant, oxidative damage or immune biomarkers was significantly different between participants with and without allergies ($p > 0.05$, linear regression, data not shown).

A positive correlation was found between plasma MDA level accounted for processing time and SEQ impulsivity score ($p < 0.05$; Table 2.22). In addition, multiple correlations were found between the various oxidative damage, antioxidant and immune biomarkers (Table 2.23). For example, IL-1β correlated positively with all other measured cytokines, while α-tocopherol correlated positively with 8-OHdG, γ-tocopherol, coQ10, retinol and IL-1β ($p < 0.05$). IgE and IgG did not correlate with any of the other analysed biomarkers.

Table 2.17. Mean levels ± SD for oxidative damage and antioxidant biomarkers in patients and controls and adjusted mean difference, accounting for processing time, by linear regression.

	ADHD		Control		Adjusted			P-value
	Mean	SD	Mean	SD	difference	95% CI	Test value	(2-sided)
8-OHdG (ng/mg Crn)	13.749	5.168	12.229	4.259	1.851	-0.290 – 3.992	F(1,116)=2.932	0.089
MDA (µM)	0.374	0.078	0.348	0.056	0.031	0.004 – 0.058	F(1,118)=5.030	0.027*
α-tocopherol (µg/ml)	10.302	2.199	9.708	1.709	0.319	-0.470 – 1.109	F(1,117)=0.641	0.425
β-carotene (µg/ml)	0.590	0.229	0.657	0.267	-0.020	-0.118 – 0.079	F(1,118)=0.153	0.696
γ-tocopherol (µg/ml)	0.462	0.170	0.420	0.147	0.041	-0.024 – 0.106	F(1,117)=1.586	0.210
CoQ10 (µg/ml)	0.578	0.143	0.534	0.134	0.043	-0.013 – 0.099	F(1,119)=2.337	0.129
GSH (µg/ml)	805.662	129.761	670.742	112.641	93.707	45.684 – 141.729	F(1,111)=14.951	*< 0.001**
Retinol (µg/ml)	0.310	0.043	0.316	0.050	-0.003	-0.022 – 0.016	F(1,116)=0.084	0.772
Retinyl palmitate (µg/ml)	0.019	0.013	0.011	0.008	0.006	0.002 – 0.011	F(1,108)=7.780	0.006*

*Statistically significant if p < 0.05; italic p-values: statistically significant after Bonferroni correction. CI: confidence interval; 8-OHdG: 8-hydroxy-2'-deoxy-guanosine; coQ10: co-enzyme Q10; Crn: creatinine; GSH: reduced glutathione; MDA: malondialdehyde; SD: standard deviation.

Table 2.18. Mean levels ± SD for immune biomarkers in patients and controls and adjusted mean difference, accounting for processing time, by linear regression.

	ADHD		Control		Adjusted			P-value
	Mean	SD	Mean	SD	difference	95% CI	Test value	(2-sided)
IFN-γ (pg/ml)	5.802	0.228	5.815	0.291	-0.031	-0.139 – 0.078	F(1,116)=0.316	0.575
IL-1β (pg/ml)	5.463	0.253	5.480	0.286	-0.036	-0.149 – 0.076	F(1,114)=0.406	0.525
IL-5 (pg/ml)	5.442	0.312	5.603	0.410	-0.130	-0.284 – 0.023	F(1,112)=2.819	0.096
IL-6 (pg/ml)	6.669	0.368	6.669	0.481	-0.057	-0.236 – 0.122	F(1,110)=0.403	0.527
IL-8 (pg/ml)	7.321	1.131	7.291	1.193	0.009	-0.464 – 0.481	F(1,115)=0.001	0.972
IL-10 (pg/ml)	5.549	0.528	5.602	0.710	-0.061	-0.309 – 0.187	F(1,116)=0.237	0.627
TNF (pg/ml)	6.308	0.553	6.418	0.492	-0.102	-0.304 – 0.101	F(1,120)=0.993	0.321
IgE (µg/ml)	250.358	270.350	132.348	158.592	125.004	25.298 – 224.709	F(1,97)=0.779	0.015*
IgG (µg/ml)	2198.126	985.728	2100.781	116.924	301.555	143.382 – 746.491	F(1,111)=0.182	0.016*
IgM (µg/ml)	328.257	190.206	294.117	163.720	17.293	-54.126 – 88.712	F(1,114)=0.230	0.632

*Statistically significant if p < 0.05. CI: confidence interval; IFN: interferon; Ig: immunoglobulin; IL: interleukin; SD: standard deviation; TNF: tumour necrosis factor.

Table 2.19. Mean ± SD sample processing time (hours) for each sample type.

	ADHD		Control	
	Mean	SD	Mean	SD
Plasma	4.05	3.08	6.82	2.49
Erythrocytes	4.20	2.76	6.80	2.46
Urine	3.93	3.34	6.37	2.93

SD: standard deviation.

Table 2.20. Interaction as well as main effect of MDA on antioxidant levels.

	Interaction	Main effect MDA		
	P-value (2-sided)	β	SE	P-value (2-sided)
α-tocopherol (µg/ml)	0.819	-1.380	2.726	0.614
β-carotene (µg/ml)	0.548	0.731	0.337	0.032*
γ-tocopherol (µg/ml)	0.248	0.103	0.219	0.614
CoQ10 (µg/ml)	0.357	0.282	0.184	0.129
GSH (µg/ml)	0.194	22.853	181.038	0.900
Retinol (µg/ml)	0.589	0.060	0.066	0.365
Retinyl palmitate (µg/ml)	0.614	0.011	0.015	0.475

*Statistically significant if p < 0.05. β: effect size; coQ10: co-enzyme Q10; GSH: reduced glutathione; MDA: malondialdehyde; SE: standard error.

Table 2.21. Interaction as well as main effect of 8-OHdG on antioxidant levels.

	Interaction	Main effect 8-OHdG		
	P-value (2-sided)	β	SE	P-value (2-sided)
α-tocopherol (µg/ml)	0.906	0.073	0.033	0.026*
β-carotene (µg/ml)	0.069	-0.004	0.004	0.315
γ-tocopherol (µg/ml)	0.361	0.001	0.003	0.748
CoQ10 (µg/ml)	0.824	-0.004	0.002	0.090
GSH (µg/ml)	0.698	5.218	1.999	0.010*
Retinol (µg/ml)	0.738	0.001	0.001	0.251
Retinyl palmitate (µg/ml)	0.895	< 0.001	< 0.001	0.145

*Statistically significant if p < 0.05. β: effect size; 8-OHdG: 8-hydroxy-2'-deoxy-guanosine; coQ10: co-enzyme Q10; GSH: reduced glutathione; SE: standard error.

Table 2.22. Correlations (Pearson's r) between oxidative damage, antioxidant and immune biomarkers and ADHD SEQ scores.

	ADHD total	Inattention	Hyperactivity	Impulsivity
8-OHdG	0.011	0.001	0.034	0.019
MDA	0.111	0.069	0.079	0.208*
α-tocopherol	-0.114	-0.044	-0.154	-0.178
β-carotene	-0.008	-0.021	0.009	-0.033
γ-tocopherol	-0.043	-0.021	-0.036	-0.077
CoQ10	0.051	0.048	0.019	0.047
GSH	-0.037	-0.013	-0.044	-0.060
Retinol	0.004	0.042	0.040	-0.042
Retinyl palmitate	0.032	0.011	0.020	0.049
IFN-γ	-0.037	-0.057	-0.001	-0.072
IL-1β	0.000	-0.065	0.053	0.019
IL-5	-0.144	-0.157	-0.127	-0.132
IL-6	-0.063	-0.065	-0.057	-0.069
IL-8	-0.078	-0.111	-0.059	-0.054
IL-10	-0.121	-0.104	-0.096	-0.140
TNF	-0.043	-0.068	-0.003	-0.050
IgE	-0.007	-0.015	-0.011	-0.013
IgG	-0.109	-0.098	-0.101	-0.100
IgM	-0.100	-0.027	-0.091	-0.141

*Statistically significant if $p < 0.05$. 8-OHdG: 8-hydroxy-2'-deoxy-guanosine; coQ10: co-enzyme Q10; GSH: reduced glutathione; IFN: interferon; Ig: immunoglobulin; IL: interleukin; MDA: malondialdehyde; TNF: tumour necrosis factor.

Table 2.23. Correlations (Pearson's r) between oxidative stress and immune biomarkers.

	8-OHdG	MDA	α-tocopherol	β-carotene	γ-tocopherol	CoQ10	GSH	Retinol	Retinyl palmitate	IFN-γ	IL-1β	IL-5	IL-6	IL-8	IL-10	TNF	IgE	IgG	IgM
8-OHdG	-	0.023	0.193#	-0.091	0.029	-0.159	0.189	0.132	0.151	0.026	-0.005	-0.041	0.262°	0.028	0.145	0.108	-0.091	0.114	-0.036
MDA	0.023	-	-0.032	0.190#	0.048	0.144	0.043	0.076	0.085	-0.057	-0.053	-0.168	0.054	-0.050	-0.021	-0.159	0.035	0.070	0.067
α-tocopherol	0.193#	-0.032	-	0.025	0.221#	_0.414*_	0.063	0.250°	-0.017	0.003	0.223#	0.072	0.170	0.140	0.175	0.003	-0.101	0.096	-0.002
β-carotene	-0.091	0.190#	0.025	-	-0.077	0.138	0.134	0.111	0.157	-0.040	0.099	0.021	-0.001	-0.016	-0.061	0.153	0.031	0.119	0.189#
γ-tocopherol	0.029	0.048	0.221#	-0.077	-	0.236#	0.036	0.202#	0.271°	0.136	0.079	0.042	0.147	0.005	0.052	0.014	0.082	-0.117	0.089
CoQ10	-0.159	0.144	_0.414*_	0.138	0.236#	-	0.083	0.183#	0.106	-0.145	0.135	0.119	-0.106	-0.154	-0.076	-0.141	0.098	-0.268	0.056
GSH	0.189	0.043	0.063	0.134	0.036	0.083	-	0.002	0.066	0.050	0.040	-0.127	0.252#	-0.088	-0.019	0.076	-0.032	0.201	0.048
Retinol	0.132	0.076	0.250°	0.111	0.202#	0.183#	0.002	-	0.013	0.121	0.079	-0.108	-0.009	0.063	0.136	-0.046	-0.042	-0.148	0.103
RP	0.151	0.085	-0.017	0.157	0.271°	0.106	0.066	0.013	-	0.122	-0.024	0.032	0.177	0.001	-0.022	0.017	0.097	-0.074	0.076
IFN-γ	0.026	-0.057	0.003	-0.040	0.136	-0.145	0.050	0.121	0.122	-	0.268°	0.128	0.230#	0.266°	0.297°	0.211#	0.014	0.289	-0.088
IL-1β	-0.050	-0.053	0.223#	0.099	0.079	0.135	0.040	0.079	-0.024	0.268°	-	_0.357*_	_0.352*_	0.305°	_0.388*_	0.224#	-0.094	-0.005	0.068
IL-5	-0.041	-0.168	0.072	0.021	0.042	0.119	-0.127	-0.108	0.032	0.128	_0.357*_	-	0.209#	0.199#	0.295°	0.227#	0.125	-0.226	-0.135
IL-6	0.262°	0.054	0.170	-0.001	0.147	-0.106	0.252#	-0.009	0.177	0.230#	_0.352*_	0.209#	-	0.321°	_0.366*_	0.156	0.031	0.301	0.108
IL-8	0.028	-0.050	0.140	-0.016	0.005	-0.154	-0.088	0.063	0.001	0.266°	0.305°	0.199#	0.321°	-	_0.333*_	0.233#	-0.091	0.123	-0.034
IL-10	0.145	-0.021	0.175	-0.061	0.052	-0.076	-0.019	0.136	-0.022	0.297°	_0.388*_	0.295°	_0.366*_	_0.333*_	-	0.023	-0.001	-0.014	0.034
TNF	0.108	-0.159	0.003	0.153	0.014	-0.141	0.076	-0.046	0.017	0.211#	0.224#	0.227#	0.156	0.233#	0.023	-	-0.045	0.241	0.008
IgE	-0.091	0.035	-0.101	0.031	0.082	0.098	-0.032	-0.042	0.097	0.014	-0.094	0.125	0.031	-0.091	-0.001	-0.045	-	-0.006	-0.037
IgG	0.114	0.070	0.096	0.119	-0.117	-0.268	0.201	-0.148	-0.074	0.289	-0.005	-0.226	0.301	0.123	-0.014	0.241	-0.006	-	-0.172
IgM	-0.036	0.067	-0.002	0.189#	0.089	0.056	0.048	0.103	0.076	-0.088	0.068	-0.135	0.108	-0.034	0.034	0.008	-0.037	-0.172	-

*Statistically significant if $p < 0.001$; ° Statistically significant if $p < 0.01$; # Statistically significant if $p < 0.05$; italic Pearson's r: statistically significant after Bonferroni correction; coQ10: co-enzyme Q10; GSH: GSH: reduced glutathione; IFN: interferon; Ig: immunoglobulin; IL: interleukin; IQR: interquartile range; MDA: malondialdehyde; RP: retinyl palmitate; TNF: tumour necrosis factor.

PART 5

DISCUSSION

To the best of our knowledge, this is the first comparison of erythrocyte GSH and plasma coQ10, retinyl palmitate, IL-5, IL-8 and IgM between ADHD patients and controls. Significantly increased plasma MDA levels were found in ADHD patients as compared to controls, as well as a trend for higher urinary 8-OHdG levels. In addition, significantly increased erythrocyte GSH as well as plasma retinyl palmitate and total IgE and IgG levels were found in patients as compared to controls, along with a trend for more diagnosed allergies and a trend for lower plasma IL-5 levels. After Bonferroni correction for multiple testing, the result for GSH remained statistically significant, while only a borderline significant difference was found for retinyl palmitate. Significance was lost for MDA, IgG and IgE levels. Nevertheless, these data are consistent with a potential role of slight oxidative stress that affects the immune system in children with ADHD as compared to controls. As a result, paediatric ADHD patients might develop more IgE- and non-IgE-mediated allergies [10].

5.1 Questionnaires

No significant differences were found regarding demographic variables between patients and controls. In addition, no difference regarding breastfeeding was found, in contrast to literature [41, 42]. Possibly, the power of the present study was too low.

Though the SEQ grading system is different for boys and girls (i.e. higher cut-off scores for boys), the comparison between the ADHD and control group is valid due to an equal gender distribution in both groups. The expected higher scores on various SEQ (sub)conditions in the ADHD group, even after Bonferroni correction to take into account false-positive significant differences due to multiple testing (in this case: 13 conditions and subconditions, so significant if $p < 0.05/13$), is a confirmation of comorbidity in ADHD, and multiple positive correlations between various SEQ scores imply strong interrelations between these comorbid conditions. It is possible that parents of ADHD patients are more focussed on the behaviour of their child, thereby rating its behaviour differently than parents of controls. However, high comorbidity rates could also be due to selection of the patient group via a

university hospital. Previous research already found high comorbidity rates in ADHD [43, 44]. Still, as CD for example was linked to lower antioxidant levels before, these comorbidities could influence the results of this study [5].

The two highest SEQ severity levels, clinical and clinical-high, can lead to a diagnosis of the specific disorder, though based solely on the SEQ, without careful examination by a health care professional. This explains why only 59.6% of the children in the ADHD group were classified as diagnosed with ADHD based on parental SEQ scores (total ADHD score; 75.4% based on subscores). In practice, all children in the patient group were diagnosed with ADHD by a trained physician. In addition, while 24.6% of the patients were classified as autistic based on SEQ scores, none had an autism diagnosis. As in literature, the combined and inattentive ADHD subtypes were found to be the most prevalent subtypes [45].

After Bonferroni correction, still significantly more stomach ache, sloppy handwriting, wanting to touch and sleep complaints (difficulties falling asleep and awaking, as well as waking up at night) were reported for the ADHD group. Impaired handwriting, defined as illegible and/or written with an inappropriate speed, was already reported in ADHD, possibly explained by inattention and impaired fine motor skills [46]. Moreover, physical symptoms like stomach ache, asthma, eczema and sleep disorders are frequent complaints among ADHD patients [47-51]. The effects of certain elimination diets on behaviour might be even attributable to improved sleep patterns [52]. In the present study however, complaints related to asthma and eczema were not reported more often in the ADHD group. In addition, physical complaints could also be related to comorbidities of ADHD, like anxiety [53].

Dietary habits of patients and controls appeared very similar, as the only significant differences found concern less consumption of sweet milk drinks and fish by patients ($p < 0.05$). Again, Bonferroni correction for multiple testing should be taken into account (35 questioned items, so significant if $p < 0.05/35$). Therefore, these differences can be neglected. Though dietary habits can influence oxidative and inflammatory status [54, 55], they thus probably do not explain differences observed regarding the biomarkers analysed. In addition, similar dietary habits reflect a similar socioeconomic status of both groups, despite different recruitment sources [56]. Still, slightly lower fish consumption (43.4% patients consumed fish less than once a week as compared to 23.2% controls) could cause lower EPA and DHA levels, which are involved in neuronal membrane structure, anti-inflammatory eicosanoid formation and dopamine function [57, 58]. In fact, a Western dietary pattern, characterised by low fish consumption, among other factors, has been related to ADHD diagnosis [59]. Moreover, multiple reviews and meta-analyses report beneficial effects of PUFAs on inattention, hyperactivity, cognition and ADHD overall [60-65].

5.2 Oxidative damage

Significantly higher adjusted plasma MDA levels in ADHD patients were observed, while MDA correlated weakly with the SEQ impulsivity score. Though after Bonferroni correction for multiple testing, significance was lost for the adjusted difference and correlation, these results still indicate potential involvement of oxidative damage in ADHD. Another study (without Bonferroni correction) also reported more plasma MDA in paediatric ADHD, but no correlation with ADHD subtype was found [4]. Other studies, however, found lower plasma or serum MDA levels [16, 19] or no significant difference regarding lipid peroxidation products, though measured by thiobarbituric acid reactive substances (TBARS), which is a less specific method compared to the HPLC method used in the present study [66]. Adult MDA levels did correlate with total hyperactivity-impulsivity score, suggesting an association between adult ADHD subtype and lipid peroxidation [14]. Increased lipid peroxidation in ADHD is also evidenced by raised urinary acrolein-lysine levels as well as by exhaled ethane levels [6, 7]. Elevated lipid peroxidation is potentially related to lower omega-3 fatty acid levels in ADHD [16, 62, 67-69].

A trend for higher adjusted urinary 8-OHdG levels was observed in ADHD. Literature on oxidative DNA damage in ADHD is contradictory, as increased 8-oxoG levels in lymphocytes were found [17], as well as reduced 8-OHdG levels in serum [19].

5.3 Antioxidants

When correcting for processing time, ADHD patients had significantly higher erythrocyte GSH levels than controls, even after Bonferroni correction, though GSH levels did not correlate significantly with any of the SEQ ADHD scores. In a randomised double-blind placebo-controlled trial, administration of a procyanidin rich extract (Pycnogenol®, 1 mg/kg body weight/day) significantly increased whole blood GSH levels in paediatric ADHD patients from 0.103 ± 0.019 mM to 0.130 ± 0.008 mM, but no GSH levels were determined for healthy controls [70]. The striking difference in concentration level between this and the present study (2-3 mM) is probably caused by methodological differences, especially sample type (whole blood vs. erythrocytes). Higher antioxidant levels do not necessarily imply less oxidative damage, as these could be a compensation mechanism for increased oxidative stress [4]. In fact, as higher MDA levels were found in the ADHD group, this compensation mechanism appears insufficient to restore the redox balance. In addition, levels of GST and GPx, both necessary for GSH activities, were found to be lower in ADHD [4, 5, 13], though one study found higher GST levels [71]. These reduced

levels, leading to low GSH use by these enzymes and thus lack of an efficient antioxidant defence, could also explain higher GSH levels and elevated oxidative damage. In autism however, plasma levels of GSH and its metabolic precursors, cysteine and cysteinylglycine, were significantly reduced, suggesting insufficient GSH synthesis [72].

Vitamin A, including retinol and retinyl palmitate, is required for various biological events, including cell differentiation and survival. However, excessive vitamin A intake can have negative effects, including redox impairment, mitochondrial dysfunction and neurotoxicity. For instance, *in vitro* as well as *in vivo*, vitamin A possesses antioxidant abilities [73-76]. However, depending on the dose and other factors like nutritional status and pathology, it may also exert prooxidative effects, leading to cell death and inflammation [74, 77]. This prooxidant inversion also accounts for other well-known antioxidants, including β-carotene, a vitamin A precursor [73]. In the present study however, physiological levels have been measured, which can be assumed to exert antioxidant rather than prooxidant effects. As retinyl palmitate is the storage form of vitamin A and as low levels were found in plasma, it is questionable whether the borderline significant difference that was observed after Bonferroni correction is biologically relevant. No difference was found for adjusted plasma retinol levels as well as for its precursor β-carotene, as confirmed by Spahis *et al.* [16]. No difference regarding retinol is not surprising as retinol concentrations in plasma are strictly regulated due to toxicity at high levels [78]. Based on these results, it is doubtful that vitamin A is involved in ADHD, irrespective of pro- or antioxidant effects.

Spahis *et al.* found significantly higher plasma α- and γ-tocopherol levels in ADHD patients [16], but another study, like the present study when accounting for processing time, found no difference regarding serum tocopherol levels [79].

5.4 Redox imbalance

Research generally points towards more oxidative stress in ADHD. Although non-enzymatic antioxidant levels generally do not appear reduced in ADHD, despite conflicting results, levels or activities of antioxidant enzymes were found to be lower in various studies [4, 5, 13, 17, 71, 80-84]. Therefore, oxidative damage biomarkers appear more reflective of the actual oxidative stress situation compared to antioxidant levels, since even relatively high antioxidant levels can still be too low to balance high oxidant levels [85]. Joseph *et al.* (2015) hypothesised an insufficient reaction to oxidative stress in ADHD, and thus more oxidative damage, rather than a deficient antioxidant production [85]. This is

applicable to the results of this case-control study as well, as also increased levels of certain antioxidants were found, despite slightly more oxidative damage, especially to lipids.

5.5 Immune biomarkers

In patients as compared to controls and when accounting for processing time, no significantly different plasma cytokine levels were found, despite a trend for lower IL-5 levels (without Bonferroni correction). In addition, cytokine levels adjusted for processing time did not correlate significantly with any of the SEQ ADHD scores. No significant difference was found regarding IL-10/TNF ratio either, which is indicative of anti-/pro-inflammatory status. In autism, IL-6 and IFN-γ levels correlated negatively with full-scale IQ, verbal comprehension index and working memory index [86], but these cognitive abilities were not assessed in the present study. Significantly higher adjusted plasma IgG and IgE levels in ADHD were observed, indicating an immune imbalance, but significance was lost after Bonferroni correction. In addition, no correlation with any of the SEQ ADHD scores was found. Still, the high variability in IgE levels is striking. The observed higher IgG levels in ADHD support involvement of inflammation [87]. To our knowledge, antibody levels were not analysed this extensively in ADHD before.

It should be noted that the use of medication was an exclusion criterion of this study, so that patients with intense allergy symptoms were likely to be excluded. It is therefore no surprise that, despite slightly higher IgE levels and even indications for lower IL-5 levels in ADHD, no significantly aberrant systemic cytokine levels were found, which would indicate active inflammation. In addition, cytokine concentrations in mucosal secretions are predominantly the result of local cytokine production, causing a potential lack of correlation between low plasma IL-5 levels and, presumably, high mucosal concentrations reflecting local T_H2 expansion [88]. In addition, as the analysed cytokine levels in both groups were very low, it is questionable whether any observed difference would be biologically relevant.

Due to the use of various methodologies in literature (e.g. analytical methods and sample matrices), it is hard to compare the results of different studies. For example, despite the increased prevalence of atopies in ADHD patients in literature [10, 89], their association appears based on a non-IgE-mediated mechanism [24, 90-92]. A role for IgG in ADHD pathophysiology was countered before as well [24]. Moreover, indications of more T_H1 cytokines have been found in previous research, e.g. higher detection rates for IFN-γ and TNF-β in CSF [93]. Another study however reported no significant differences regarding serum TNF-α, IL-1β, IL-6 and IL-10 levels, but did find higher levels of IFN-γ in

ADHD patients than in controls [94]. In adults, no significantly altered plasma IL-6 and TNF-α concentrations were found [95].

Further research with consistent methodology is thus required to draw a final conclusion. Nevertheless, cytokines could be important in ADHD as they can pass the BBB, affect synaptic plasticity and neurogenesis and even cause T cell-mediated neuroinflammation and neurodegeneration, which is consistent with reduced cortical volume and folding in ADHD, and can lead to behavioural effects [94, 96, 97].

5.6 Interconnection between redox and immune status

Oxidative and inflammatory processes are closely related [13]. A redox imbalance is responsible for changes in the nervous and immune system [13, 82, 98-103]. Moreover, immune cells are an important source of ROS, RNS and pro-inflammatory compounds like cytokines, which stimulate NF-κB activation, leading to production of more oxidants and inflammatory compounds, and thereby establishing a vicious circle [13, 103]. Still, an increase in locally produced cytokines could be too low to be detected in plasma. In addition, though elevated oxidative stress can cause higher basal (without antigen stimulation) levels of inflammatory cytokines [104], oxidative stress in ADHD might be too limited for clear effects on plasma cytokine levels. Still, antioxidants could exert beneficial effects through an improvement of oxidative stress and immune cell functions, and might therefore have potential in ADHD therapy [2].

ADHD-associated increased oxidative stress might, in case of a chronic state, lead to immune dysfunction resulting in elevated T_H2 induction and thereby increased IgE levels. Although IgE is found in every individual, and levels increase with age until reaching a stable level at adulthood, elevated concentrations of total IgE reflect a predisposition to develop IgE-mediated allergic diseases, despite not being related directly to allergic status [105, 106]. Oxidative stress might thus facilitate the development of allergic conditions in ADHD patients [2, 100]. Indeed, the observed higher IgE levels were supported by a trend for more diagnosed allergies in patients than in controls, as well as by the association between ADHD and atopic disorders in literature [32, 332, 334, 335].

The shifted immune balance due to oxidative stress thus results in a modified humoral immune response. Though IgE levels are more strongly T cell-regulated and have a much shorter half-life than IgG, IgG levels will also be changed under chronic conditions [107].

5.7 Correlations

It is striking that various immune, oxidative damage and antioxidant biomarkers (e.g. GSH) differed significantly between patients and controls (though significance was often lost after Bonferroni correction), while no correlation was found with SEQ scores. This could be related to several high SEQ scores in control subjects and low SEQ scores in ADHD subjects and underscores the discrepancy between physician's diagnosed ADHD and parent rated behaviour.

Considering the strength of the correlations between SEQ scores and biomarkers, especially impulsivity appeared related to oxidative lipid damage. This could imply that the ADHD spectrum not only varies in terms of behavioural manifestations, but also regarding underlying mechanisms and thus potentially also appropriate treatment. For instance, in case of impulsivity, a stronger insufficiency might exist against oxidative stress, causing more oxidative damage compared to predominantly inattentive patients.

Multiple correlations were found between the various oxidative damage, antioxidant and immune biomarkers. Only a small part of the variability of one parameter can however be explained by the variability of the other, as the strength of the correlations was generally weak (except the correlation between coQ10 and α-tocopherol, which was moderate). Indeed, linear regression showed that antioxidant levels were not predicted by the amount of oxidative damage. When accounting for groups, MDA did have a significant effect on β-carotene level and 8-OHdG on GSH and α-tocopherol. However, significance was lost every time after Bonferroni correction. Nevertheless, to illustrate the complexity of their interrelations, the significant correlations between the analysed oxidative damage and antioxidant biomarkers and immune biomarkers are shown in Figures 2.8 and 2.9, demonstrating for example that various parts of the antioxidant defence system are closely intertwined. It is for instance known that coQ10 regenerates α-tocopherol from its radical form in lipoproteins and membranes [32, 33]. The positive correlations, though weak, between 8-OHdG and α-tocopherol as well as between MDA and β-carotene support higher antioxidant levels as a compensation mechanism for increased oxidative stress in ADHD. However, these correlations also imply that if a biomarker is significantly different between patients and controls (e.g. retinyl palmitate), this difference might be due to its correlation with another biomarker (e.g. γ-tocopherol). The same accounts for immune biomarkers. Still, due to multiple correlations being analysed, Bonferroni correction should be applied, leaving only the correlations with bold arrows (marginally) significant.

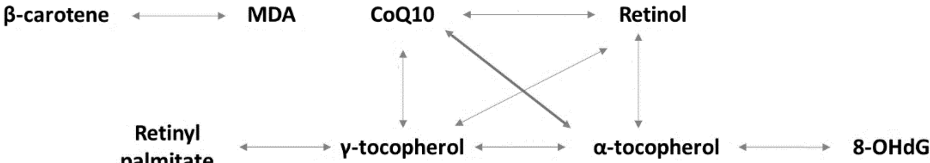

Figure 2.8. Statistically significant correlations between oxidative damage and antioxidant biomarkers.
Bold arrows indicate significant correlations after Bonferroni correction for multiple testing. CoQ10: co-enzyme Q10.

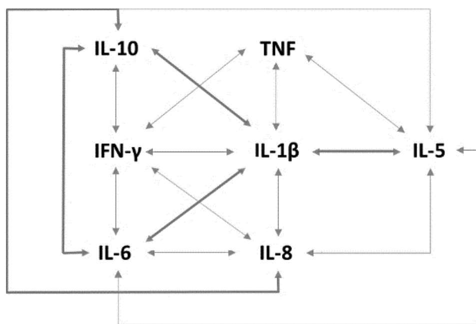

Figure 2.9. Statistically significant correlations between immune biomarkers.
Bold arrows indicate significant correlations after Bonferroni correction for multiple testing. IFN: interferon; IL: interleukin; TNF: tumour necrosis factor.

5.8 Strengths and limitations

This case-control comparison has some limitations. For example, the nature of this study does not address the investigation of causality. It is therefore unknown whether increased oxidative stress or immune dysbalance are causative factors in the pathophysiology of ADHD or a consequence of the disorder [13]. For instance, increased oxidative stress in ADHD could be the result of the restlessness related with ADHD [83]. Still, case-control studies are a good start, though not providing solid proof, to unravel involved mechanisms of action.

Furthermore, though all included patients in this study were diagnosed by a child neurologist as having ADHD according to DSM criteria (DSM-4 or DSM-5), several patients would not be classified as such based solely on parental SEQ scores. In addition, several controls had a positive SEQ ADHD score, without official diagnosis or parental complaints. This underscores the subjectivity of questionnaires and might explain the lack of correlations between biomarkers and SEQ (sub)scores.

Another limitation is that the FFQ questioned consumption frequency but not portion size, which makes it impossible to draw conclusions on actual intake. In addition, the probability of recall bias and

overestimation of vegetable and potato product intake should be taken into account [25], but both are expected to be similar in patients and controls.

An important strength of this study is the use of regression analysis correcting for differences in processing time due to practical issues (e.g. sample transport to the analytical facility from the neighbouring UZA was faster than from schools). Systematically recording processing time and correcting for this potential confounder was found to be crucial to obtain valid results and should thus be implemented in future research. In addition, Bonferroni correction for multiple testing is essential, but often not performed in literature.

Finally, a broad diversity of oxidative stress and immune biomarkers was taken into account, with the comparison of erythrocyte GSH and plasma coQ10, retinyl palmitate, IL-5, IL-8 and IgM between ADHD patients and controls not being published before.

PART 6

CONCLUSION

An evaluation of redox and immune status in ADHD was performed. Significantly higher plasma MDA levels were found in patients as compared to controls, as well as a trend for higher urinary 8-OHdG levels. Erythrocyte GSH and plasma retinyl palmitate, IgG and IgE levels were significantly higher in patients than in controls. Finally, a trend for lower plasma IL-5 levels was observed. After Bonferroni correction for multiple testing, the result for GSH remained statistically significant, while only a borderline significant difference was found for retinyl palmitate. Significance was lost for MDA, IgG and IgE levels. Still, though only slightly elevated, an indication of more oxidative damage was thus found in ADHD. Antioxidant levels however did not differ or were even higher in patients than in the control group. In addition, slightly higher IgE levels were supported by a trend for more diagnosed allergies in patients than in controls and might point at an immune dysbalance in ADHD. However, due to the nature of this study, it is unknown whether redox and immune dysbalance play a causative role in ADHD. Dietary habits do not appear to explain the observed biomarker differences. However, more psychiatric comorbidities were found in ADHD patients, which could possibly influence the results of this study. Further confirmation of these results is required, as well as further investigation of potential differences between ADHD subtypes. Finally, systematically correcting for processing time was found to be crucial to obtain valid results.

REFERENCES

1. Biederman, J. and S.V. Faraone, Attention-deficit hyperactivity disorder. Lancet, 2005. 366: p. 237-248.
2. Verlaet, A.A.J., et al., Rationale for Dietary Antioxidant Treatment of ADHD. Nutrients, 2018. 10(4): p. 405-424.
3. Ng, F., et al., Oxidative stress in psychiatric disorders: evidence base and therapeutic implications. Int J Neuropsychopharmacol., 2008. 11: p. 851-876.
4. Ceylan, M., et al., Oxidative imbalance in child and adolescent patients with attention-deficit/hyperactivity disorder. Prog Neuropsychopharmacol Biol Psychiatry, 2010. 34(8): p. 1491-1494.
5. El Adham, E.K., A.I. Hassan, and A.A. El Aziz El- Mahdy, Nutritional and Metabolic Disturbances in Attention Deficit Hyperactivity Disease. Res J Med & Med Sci, 2011. 6(1): p. 10-16.
6. Ross, B.M., et al., Increased levels of ethane, a non-invasive marker of n-3 fatty acid oxidation, in breath of children with attention deficit hyperactivity disorder. Nutr Neurosci, 2003. 6(5): p. 277-281.
7. Kawatani, M., H. Tsukahara, and M. Mayumi, Evaluation of oxidative stress status in children with pervasive developmental disorder and attention deficit hyperactivity disorder using urinary-specific biomarkers. Redox Rep, 2011. 16(1): p. 45-46.
8. Güngör, S., et al., The Frequency of Celiac Disease In Attention-Deficit Hyperactivity Disorder. J Pediatr Gastroenterol Nutr, 2013. 56(2): p. 211-4.
9. Minter, K., et al., Early Childhood Otitis Media in Relation to Children's Attention-Related Behavior in the First Six Years of Life. Pediatrics, 2001. 107: p. 1037-1042.
10. Pelsser, L.M.J., J.K. Buitelaar, and H.F.J. Savelkoul, ADHD as a (non) allergic hypersensitivity disorder: a hypothesis. Pediatr Allergy Immunol, 2009. 20(2): p. 107-112.
11. Schmitt, J., A. Buske-Kirschbaum, and V. Roessner, Is atopic disease a risk factor for attention-deficit/hyperactivity disorder? A systematic review. Allergy, 2010. 65(12): p. 1506-1524.
12. Tsai, S.J., Signal transducer and activator of transcription 6 (STAT6) and attention-deficit hyperactivity disorder: A speculative hypothesis. Med Hypotheses, 2006. 67(6): p. 1341-1343.
13. Ceylan, M., et al., Changes in oxidative stress and cellular immunity serum markers in attention-deficit/hyperactivity disorder. Psychiatry Clin Neurosci, 2012. 66: p. 220-226.
14. Bulut, M., et al., Malondialdehyde levels in adult attention-deficit hyperactivity disorder. J Psychiatry Neurosci, 2007. 32: p. 435-438.
15. Kerschensteiner, M., E. Meinl, and R. Hohlfeld, Neuro-immune crosstalk in CNS diseases. Neuroscience, 2009. 158: p. 1122–1132.
16. Spahis, S., et al., Lipid profile, fatty acid composition and pro- and anti-oxidant status in pediatric patients with attention-deficit/hyperactivity disorder. Prostaglandins Leukot Essent Fatty Acids, 2008. 79: p. 47-53.
17. Chovanová, Z., et al., Effect of polyphenolic extract, Pycnogenol, on the level of 8-oxoguanine in children suffering from attention deficit/hyperactivity disorder. Free Radical Res, 2006. 40: p. 1003-1010.
18. Thapar, A. and G. Lewis, Should we be rethinking how we assess and manage ADHD? J Am Acad Child Adolesc Psychiatry, 2009. 48: p. 1051-1052.
19. Oztop, D., et al., Oxidative stress in children with attention deficit hyperactivity disorder. Clin Biochem, 2012. 45: p. 745-748.
20. APA, American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders, Fourth Edn, Text Revision. 2000, Washington, DC.
21. APA. American Psychiatric Association: DSM-5 Attention Deficit/Hyperactivity Disorder Fact Sheet. 2013; Available from: http://www.dsm5.org/Documents/ADHD%20Fact%20Sheet.pdf [Accessed 5 July 2016].
22. TestWeb. Sociaal-Emotionele Vragenlijst (SEV). 2013 Available from: http://testweb.bsl.nl/tests/sev/ [Accessed 5 July 2016].
23. Pelsser, L.M.J. and J.K. Buitelaar, Favourable effect of a standard elimination diet on the behaviour of young children with attention deficit-hyperactivity disorder (ADHD), a pilot study. Ned Tijdschr Geneesk, 2002. 46(52): p. 2543-2547.
24. Pelsser, L.M.J., et al., Effects of a restricted elimination diet on the behaviour of children with attention-deficit hyperactivity disorder (INCA study): a randomised controlled trial. Lancet, 2011. 377: p. 494-503.
25. Jeunen, E., Onderzoek naar voedingsproblemen bij preterm geboren kleuters., K.H. Kempen, MSc thesis. 2011: Geel.
26. Magielse, J., Investigation of the biological activity of plant food supplements in animal models: evaluation of the antioxidant efficacy of Cynara scolymus and the hepatoprotective activity of Desmodium adscendens., PhD thesis in Farmaceutical Sciences. 2013, University of Antwerp: Antwerp.

27. Magielse, J., et al., Investigation of the in vivo antioxidative activity of Cynara scolymus (artichoke) leaf in the streptozotocin-induced diabetic rat. Mol Nutr Food Res, 2014. 58: p. 211-215.
28. Hermans, N., et al., Method development and validation for monitoring in vivo oxidative stress: Evaluation of lipid peroxidation and fat-soluble vitamin status by HPLC in rat plasma. J Chromatogr B, 2005. 822: p. 33-39.
29. Lee, R. and P. Britz-McKibbin, Differential Rates of Glutathione Oxidation for Assessment of Cellular Redox Status and Antioxidant Capacity by Capillary Electrophoresis-Mass Spectrometry: An Elusive Biomarker of Oxidative Stress. Anal Chem, 2009. 81: p. 7047-7056.
30. Conaway, H.H., P. Henning, and U.H. Lerner, Vitamin a metabolism, action, and role in skeletal homeostasis. Endocrine Rev, 2013. 34: p. 766-797.
31. Naguib, Y., et al., Antioxidant activities of natural vitamin E formulations. J Nutr Sci Vitaminol, 2003. 49: p. 217-220.
32. Littarru, G.P. and L. Tiano, Bioenergetic and antioxidant properties of coenzyme Q10: recent developments. Mol Biotechnol, 2007. 37: p. 31-37.
33. Molyneux, S.L., et al., Coenzyme Q10: is there a clinical role and a case for measurement? Clin Biochem Rev, 2008. 29: p. 71-82.
34. Grotto, D., et al., Importance of the lipid peroxidation biomarkers and methodological aspects for malondialdehyde quantification. Quimica Nova, 2009. 32(1): p. 169-174.
35. Bulut, M., et al., Lipid peroxidation markers in adult attention deficit hyperactivity disorder: new findings for oxidative stress. Psychiatry Res, 2013. 209: p. 638-642.
36. Hermans, N., et al., Challenges and pitfalls in antioxidant research. Curr Med Chem, 2007. 14: p. 417-430.
37. Wu, L.L., et al., Urinary 8-OHdG: a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. Clin Chim Acta, 2004. 339: p. 1-9.
38. Yano, T., et al., Significance of the urinary 8-OHdG level as an oxidative stress marker in lung cancer patients. Lung Cancer, 2009. 63(1): p. 111-114.
39. Chiou, C.-C., et al., Urinary 8-hydroxydeoxyguanosine and its analogs as DNA marker of oxidative stress: development of an ELISA and measurement in both bladder and prostate cancers. Clin Chim, 2003. 334: p. 87-94.
40. Aruoma, O.I., Free radicals, oxidative stress, and antioxidants in human health and disease. JAOCS, 1998. 75: p. 199-212.
41. Mimouni-Bloch, A., et al., Breastfeeding may protect from developing attention-deficit/hyperactivity disorder. Breastfeeding Med, 2013. 8(4): p. 363-367.
42. Julvez, J., et al., Attention behaviour and hyperactivity at age 4 and duration of breast-feeding. Acta Pediatr, 2007. 96: p. 842-847.
43. Biederman, J., Attention-deficit/hyperactivity disorder: a life-span perspective. J Clin Psychiatry, 1998. 59: p. 4-16.
44. Jensen, P.S., D. Martin, and D.P. Cantwell, Comorbidity in ADHD: Implications for research, practice, and DSM-V. J Am Acad Child Adolesc Psychiatry, 1997. 36(8): p. 1065-1079.
45. Rowland, A.S., et al., The shifting subtypes of ADHD: Classification depends on how symptom reports are combined. TJ Abnorm Child Psychol, 2008. 36: p. 731-743.
46. Racine, M.B., et al., Handwriting performance in children with attention deficit hyperactivity disorder (ADHD). J Child Neurol, 2008. 23(4): p. 399-406.
47. Kaplan, B.J., et al., Dietary replacement in preschool-aged hyperactive boys. Pediatrics, 1989. 83: p. 7-17.
48. Carter, C.M., et al., Effects of a few food diet in attention deficit disorder. Arch Dis Child, 1993. 69: p. 564-568.
49. Hammerness, P., et al., Reexamining the familial association between asthma and ADHD in girls. J Atten Disord, 2005. 8: p. 133-143.
50. Niederhofer, H. and K. Pittschieler, A preliminary investigation of ADHD symptoms in persons with celiac disease. J Atten Disord, 2006. 10: p. 200-204.
51. Kumperscak, H.G., et al., Prevalence of Celiac Disease Is Not Increased in ADHD Sample. J Atten Disord, 2016.
52. Romanos, M., et al., Itches and scratches - is there a link between eczema, ADHD, sleep disruption and food hypersensitivity? Allergy, 2011. 66: p. 1407-1409.
53. Ghanizadeh, A., Sensory processing problems in children with ADHD, a systematic review. Psych Invest, 2013. 8: p. 89-94.
54. Dandona, P., et al., Macronutrient intake induces oxidative and inflammatory stress: potential relevance to atherosclerosis and insulin resistance. Exp Mol Med, 2010. 42(4): p. 245-253.
55. Folchetti, L.D., et al., Association of fruits and vegetables consumption and related-vitamins with inflammatory and oxidative stress markers in prediabetic individuals. Diabetol Metab Syndr, 2014. 6.
56. Fismen, A.S., et al., Trends in food habits and their relation to socioeconomic status among Nordic adolescents 2001/2002-2009/2010. PLoS One, 2016. 11(2).
57. Richardson, A.J., The importance of omega-3 fatty acids for behaviour, cognition and mood. Scand J Nutr, 2003. 47(2): p. 92-98.

58. Sinn, N., Nutritional and dietary influences on attention deficit hyperactivity disorder. Nutr Rev, 2008. 66(10): p. 558-568.
59. Howard, A.L., et al., ADHD is associated with a "Western" dietary pattern in adolescents. J Atten Disord, 2011. 15(5): p. 403-411.
60. Hurt, E.A., L.E. Arnold, and N. Lofthouse, Dietary and nutritional treatments for attention-deficit/hyperactivity disorder: Current research support and recommendations for practitioners. Curr Psychiatry Rep, 2011. 13: p. 323-332.
61. Stevenson, J., et al., Research review: The role of diet in the treatment of attention-deficit/hyperactivity disorder - an appraisal of the evidence on efficacy and recommendations on the design of future studies. J Child Psychol Psychiatry, 2014. 55(5): p. 416-427.
62. Hawkey, E. and J.T. Nigg, Omega-3 fatty acid and ADHD: Blood level analysis and meta-analytic extension of supplementation trials. Clin Psychol Rev, 2014. 34: p. 496-505.
63. Ortega, R.M., E. Rodriguez-Rodriguez, and A.M. Lopez-Sobaler, Effects of omega-3 fatty acids supplementation in behaviour and non-neurodegenerative neuropsychiatric disorders. Br J Nutr, 2012. 107: p. S261-S270.
64. Grassmann, V., R.F. Santos-Galduroz, and S.C.F. Galduroz, Effects of low doses of polyunsaturated fatty acids on the attention deficit/hyperactivity disorder of children: a systematic review. Curr Neuropharmacol, 2013. 11: p. 186-196.
65. Schuchardt, J.P., et al., Significance of long-chain polyunsaturated fatty acids (PUFAs) for the development and behaviour of children. Eur J Pediatr, 2010. 169: p. 149-164.
66. Joshi, K., et al., Supplementation with flax oil and vitamin C improves the outcome of Attention Deficit Hyperactivity Disorder (ADHD). Prostagland Leukot Essent Fatty Acids, 2006. 74(1): p. 17-21.
67. Gillies, D., et al., Polyunsaturated fatty acids (PUFA) for attention deficit hyperactivity disorder (ADHD) in children and adolescents. Cochrane Database Systematic Rev, 2012. 7.
68. Bos, D.J., et al., Effects of omega-3 polyunsaturated fatty acids on human brain morphology and function: What is the evidence? Eur Neuropsychopharmacol, 2016. 26(3): p. 546-561.
69. Ramakrishnan, U., B. Imhoff-Kunsch, and A.M. DiGirolamo, Role of docosahexaenoic acid in maternal and child mental health. Am J Clin Nutr, 2009. 89(suppl): p. 958S-962.
70. Dvořáková, M., et al., The effect of polyphenolic extract from pine bark, Pycnogenol, on the level of glutathione in children suffering from attention deficit hyperactivity disorder (ADHD). Redox Report, 2006. 11: p. 163-172.
71. Çelik, K., et al., Plasma catalase, glutathione-s-transferase and total antioxidant activity levels of children with attention deficit and hyperactivity disorder. Adv Biosci Biotechnol, 2013. 4: p. 183-187.
72. James, S.J., et al., Metabolic biomarkers of increased oxidative stress and impaired methylation capacity in children with autism. Am J Clin Nutr, 2004. 80(6): p. 1611-1617.
73. Pravkin, S.K., E.N. Yakusheva, and D.G. Uzbekova, In vivo analysis of antioxidant and prooxidant properties of retinol acetate. Bull Exp Biol Med, 2013. 156(2): p. 220-223.
74. De Oliveira, M.R., The neurotoxic effects of vitamin A and retinoids. Ann Brazl Acad Sci, 2015. 87: p. 1361-1373.
75. Ciaccio, M., et al., Vitamin A Inhibits Doxorubicin-Induced Membrane Lipid Peroxidation in Rat Tissues in Vivo. Arch Biochem Biophys, 1993. 302(1): p. 103-108.
76. Schwarz, K.B., et al., Possible antioxidant effect of vitamin A supplementation in premature infants. J Pediatr Gastroenterol Nutr, 1997. 24(4): p. 408-414.
77. Petiz, L.L., et al., Vitamin A Oral Supplementation Induces Oxidative Stress and Suppresses IL-10 and HSP70 in Skeletal Muscle of Trained Rats. Nutrients, 2017. 9(4): p. 353.
78. Olson, J.A., Vitamin A and carotenoids as antioxidants in a physiological context. J Nutr Sci Vitamol, 1993. 39: p. S57-S65.
79. Landaas, E.T., et al., Vitamin levels in adults with ADHD. BJPsych Open, 2016. 2: p. 337-384.
80. Ruchi, K., et al., Antioxidant activity in children with ADHD - A comparison in untreated and treated subjects with normal children. Int Med J Malaysia, 2011. 10(1): p. 31-35.
81. Russo, A.J., Decreased Serum Cu/Zn SOD Associated with High Copper in Children with Attention Deficit Hyperactivity Disorder (ADHD). J Central Nervous System Dis, 2010. 2: p. 9-14.
82. Kul, M., et al., Evaluation of oxidative metabolism in child and adolescent patients with attention deficit hyperactivity disorder. Psychiatry Investig, 2015. 12(3): p. 361-366.
83. Sezen, H., et al., Increased oxidative stress in children with attention deficit hyperactivity disorder. Redox Rep., 2016. 21(6): p. 248-253.
84. Guney, E., et al., Attention Deficit Hyperactivity Disorder and oxidative stress: A short term follow up study. Psychiatry Res, 2015. 229: p. 310-317.
85. Joseph, N., et al., Oxidative Stress and ADHD: A Meta-Analysis. J Atten Disord, 2015. 19(11): p. 915-924.
86. Sasayama, D., et al., Negative Correlation between Serum Cytokine Levels and Cognitive Abilities in Children with Autism Spectrum Disorder. J. Intell. 2017, 5, 19; , 2017. 5(19): p. 19-26.
87. Abbas, A.K., A.H. Lichtman, and S. Pillai, Cellular and Molecular Immunology. 2015, Philadelphia: Elsevier Saunders.

88. Dugernier, T.L., et al., Compartmentalization of the Inflammatory Response during Acute Pancreatitis Correlation with Local and Systemic Complications. Am J Respir Crit Care Med, 2003. 168: p. 148-157.
89. Chen, M., et al., Attention deficit hyperactivity disorder, tic disorder, and allergy: Is there a link? A nationwide population-based study. J Child Psychol Psychiatry, 2013. 54(5): p. 545-551.
90. Gaitens, T., B.J. Kaplan, and B. Freigang, Absence of an Association between IgE-mediated Atopic Responsiveness and ADHD Symptomatology. J Child Psychol Psychiatry, 1998. 39(3): p. 427-431.
91. McGee, R., W.R. Stanton, and M.R. Sears, Allergic Disorders and Attention Deficit Disorder in Children. J Abnorm Child Psychol, 1993. 21(1): p. 79-88.
92. Blank, R. and H. Remschmidt, Hyperkinetic Syndrome: The Role of Allergy among Psychological and Neurological Factors. Eur Child Adol Psychiatry, 1994. 3(4): p. 220-228.
93. Mittleman, B.B., et al., Cerebrospinal Fluid Cytokines in Pediatric Neuropsychiatric Disease. J Immunol, 1997. 159(6): p. 2994-2999.
94. Oades, R., et al., Attention-deficit hyperactivity disorder (ADHD) and glial integrity: S100B, cytokines and kynurenine metabolism - effects of medication. Behav Brain Functions, 2010. 6(29): p. 2-14.
95. Corominas-Roso, M., et al., IL-6 and TNF-α in unmedicated adults with ADHD: Relationship to cortisol awakening response. Psychoneuroendocrinol, 2017. 79: p. 67-73.
96. Buske-Kirschbaum, A., et al., Psychoendocrine and psychoneuroimmunological mechanisms in the comorbidity of atopic eczema and attention deficit/hyperactivity disorder. Psychoneuroendocrinology, 2013. 38: p. 12-23.
97. Martino, M., et al., Immunomodulation mechanism of antidepressants: interactions between serotonin/norepinephrine balance and Th1/Th2 balance. Curr Neuropharmacol, 2012. 10: p. 97-123.
98. Lopresti, A.L., Oxidative and nitrosative stress in ADHD: possible causes and the potential of antioxidant-targeted therapies. ADHD, 2015. 7: p. 237-247.
99. Delion, S., et al., Chronic dietary a-linolenic acid deficiency alters dopaminergic and serotoninergic neurotransmission in rats. J Nutr, 1994. 124: p. 2466-2476.
100. King, M.R., et al., Oxidative stress promotes polarisation of human T cell differentiation toward a T helper 2 phenotype. J Immunol, 2006. 176: p. 2765-2772.
101. Dröge, W. and R. Breitkreutz, Glutathione and immune function. Proc Nutr Soc, 2000. 59: p. 595-600.
102. Furukawa, T., S.N. Meydani, and J.B. Blumberg, Reversal of age-associated decline in immune responsiveness by dietary glutathione supplementation in mice. Mech Ageing Dev, 1987. 38(2): p. 107-117.
103. Vaziri, N.D., Causal link between oxidative stress, inflammation, and hypertension. IJKD, 2008. 2: p. 1-10.
104. Mallone, R., et al., Isolation and preservation of peripheral blood mononuclear cells for analysis of islet antigen-reactive T cell responses: position statement of the T-Cell Workshop Committee of the Immunology of Diabetes Society. Clin Exp Immunol, 2010. 163: p. 33-49.
105. Martins, T., et al., New childhood and adult reference intervals for total IgE. J Allergy Clin Immunol, 2014. 133: p. 589-591.
106. Bernstein, I.L., et al., Allergy diagnostic testing: An updated practice parameter. Ann Allergy Asthma Immunol, 2008. 100(3 Suppl 3): p. S1-S148.
107. Singer, R.E., et al., Association of Systemic Oxidative Stress with suppressed Serum IgG to Commensal Oral Biofilm and Modulation by Periodontal Infection. Antioxid Redox Signal, 2009. 11: p. 2973-2983.

CHAPTER 3

CHARACTERISATION OF PYCNOGENOL®: VALIDATION OF ANALYTICAL METHODS AND QUALITY CONTROL

Don't waste your time on jealousy. Sometimes you're ahead, sometimes you're behind. The race is long, and in the end, it's only with yourself. – Baz Luhrmann

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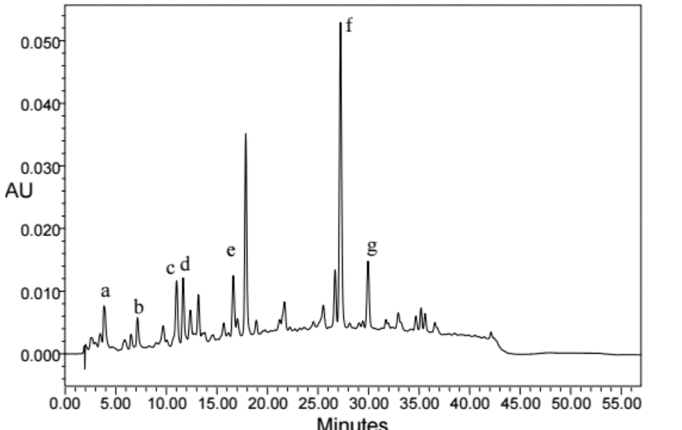
PART 1

INTRODUCTION

As ADHD is associated with immune imbalances and increased oxidative stress, this offers potential for nutritional supplementation as therapy [1-8]. One example is Pycnogenol®, a commercially available extract from *Pinus pinaster* bark with antioxidant and immune modulating activities [9-12]. This extract was used in an *in vitro* study to investigate membrane receptor binding and effects on cytokine secretion (Chapter 4) and in a randomised controlled trial on the effect of Pycnogenol® on ADHD (Chapter 5). Standardisation, characterisation and quality control of herbal nutritional supplements are however essential for credible research and to be able to compare results of various studies.

Pycnogenol® is standardised to contain 70 ± 5% procyanidins. The United States Pharmacopeia (USP 38) includes a spectrophotometric method for the determination of total procyanidin content, and a fingerprint chromatographic method (Figure 3.1) for Pycnogenol®, a.k.a. Maritime Pine Extract [13]. These methods were optimised (fingerprint chromatography only) and validated with commercially available reference compounds, and Pycnogenol® quality control was performed, to be used for *in vitro* research (Chapter 4) as well as for release and stability testing on study capsules (Chapter 5). In addition, specific phenolic constituents (e.g. catechin) were determined to obtain more details on the composition of the studied extract.



a - Gallic acid
b - Protocatechuic acid
c - Procyanidin B1
d - Catechin
e - Caffeic acid
f - Taxifolin
g - Ferulic acid

Figure 3.1. United States Pharmacopeia (USP) Maritime Pine Extract Reference Standard chromatogram.

PART 2

MATERIALS AND METHODS

2.1 Total procyanidin content

2.1.1 Materials

Butanol (99%, extra pure) was purchased from Acros Organics, USA, ferric ammonium sulphate 12H_2O from VWR, USA, hydrochloric acid (37%) from Fisher Chemical, UK, and USP Maritime Pine extract reference standard (RS; 0.673 mg procyanidins/mg extract, loss on drying (LOD): 4.82%) from the USP, USA. Pycnogenol® dry extract was offered by Horphag Research, Switzerland. Capsules containing 20 mg Pycnogenol® extract mixed with filler (175 mg microcrystalline cellulose) and lubricant (2 mg magnesium stearate) to be used in the clinical trial (Chapter 5) were produced by Qualiphar NV, Belgium.

2.1.2 Sample and standard preparation

USP Maritime Pine extract RS was dissolved in methanol to a final concentration of 0.15 mg/ml as reference stock solution. 1.0 ml of this stock solution was transferred to a 10 ml dark vial, to which 6.0 ml reagent solution A (a 95:5 mixture of butanol and hydrochloric acid) and 0.25 ml reagent solution B (a mixture of ferric ammonium sulphate, water and hydrochloric acid in a 1:50:8.75 (w:v:v) ratio) were added. The vial was sealed, mixed and heated to 99°C in a water bath for 40 min and then cooled to RT on ice. This solution was diluted with reagent solution A to 10 ml volume and mixed. This reference solution was made *in duplo*.

The test stock solution for Pycnogenol® extract was prepared by dissolving 125.0 mg Pycnogenol® powder in 100 ml MeOH, placing it for 20 min in an ultrasonic bath, centrifugation (2500 rpm, RT, 10 min) and diluting the upper layer 1:20 with MeOH. The test stock solution for capsules containing Pycnogenol® was prepared by dissolving 125.0 mg capsule content in 20 ml MeOH, placing it for 20 min in an ultrasonic bath, centrifugation (2500 rpm, RT, 10 min) and diluting the upper layer 1:10 with MeOH. Preparation of both test solutions (as well as blank solution, using only MeOH) then follows the same procedure as the reference solution (1.0 ml transferred to a 10 ml dark vial, etc.).

2.1.3 Spectrophotometric determination

The absorbances of the test and reference solutions were determined by means of an UV-Vis spectrophotometer (Perkin Elmer, Lamda 35) at 551 nm using the blank solution as compensation liquid, following these calculations:

- Response (R) = ms x Cp x (100-LOD) / Es x dfs x 100
- Mg procyanidins/capsule = ET x R x dfT x Cc / mT

Whereby: ms = mass reference Pycnogenol® extract (mg)
Cp = procyanidin concentration in reference extract (mg/mg)
Es = extinction reference solution
dfs = dilution factor reference solution
ET = extinction test solution
dfT = dilution factor test solution
mT = mass test product (mg)
Cc = weight capsule content

2.2 HPLC fingerprint

2.2.1 Materials

MeOH (HPLC grade) was purchased from Fisher Chemical, UK, H_3PO_4 (85%) from Acros Organics, USA. The reference standards caffeic acid (99.2%), taxifolin (95.4%) and ferulic acid (99.8%) were purchased from Sigma Aldrich, USA, while catechin (100%) was purchased from Roth, Germany. Pycnogenol® dry extract was offered by Horphag Research, Switzerland.

2.2.2 Sample and standard preparation

Reference stock solutions were prepared by dissolving reference standards separately in MeOH to a final concentration of 1.5 mg/ml for catechin and taxifolin and 0.5 mg/ml for caffeic acid and ferulic acid. These stock solutions were placed in an ultrasonic bath for 15 min, after which three concentrations of two reference measuring solutions (catechin + taxifolin, caffeic acid + ferulic acid) were prepared by diluting stock solutions in MeOH 20%. Resulting concentrations can be found in Table 3.1.

Table 3.1. Concentrations of the four reference standards in the final reference solutions.

	Catechin	Taxifolin	Caffeic acid	Ferulic acid
S1 (mg/ml)	0.03	0.03	0.02	0.02
S2 (mg/ml)	0.06	0.06	0.012	0.012
S3 (mg/ml)	0.075	0.075	0.006	0.006

The USP method indicates to dissolve Pycnogenol® in 100% MeOH, which however resulted in poor chromatographic peaks. Therefore, this was optimised. To prepare the test solution, 2 ml MeOH was added to 200.0 mg sample. After 15 min ultrasonic treatment, MilliQ water was added to 10 ml volume and the solution was centrifuged (2500 rpm, RT, 10 min). Pycnogenol® was thus dissolved in 20% MeOH instead of 100% MeOH, which improved chromatographic peaks.

2.2.3 HPLC analysis

Samples were analysed by an HPLC system and diode array detector (DAD) from Agilent, USA (type 1260 quaternary pump and auto-injector). Mobile phase A (0.1% H_3PO_4 in water) and B (MeOH) were used at a flow of 1 ml/min. The elution profile was set at a 40 min linear gradient from 8 to 34% B, a 5 min linear gradient from 34 to 98% B and a 5 min isocratic elution at 98% B before returning to initial conditions. The detector was set at 280 nm. A C8 Lichrospher RP column (250 x 4.6 mm x 5 µm) at 40°C was used. Resulting chromatograms were manually integrated. The retention times of catechin, caffeic acid, taxifolin and ferulic acid were approximately 14.3, 20.7, 31.2 and 34.0 min, resp. The amount of the different components was calculated by means of a calibration curve.

2.2.4 Response function of the calibration model

At least 5 reference solutions of the component to be determined were prepared and injected twice. The following parameters were determined:

- Correlation coefficient r of the calibration curve (y = ax + b) must be larger than 0.999
- Slope (a): t-test on the slope: t-value > t (table) with $\alpha = 0.05$, df = n-2
- Intercept (b): t-test on the intercept, 95% confidence interval of the intercept
- Graphical inspection of the residuals

2.3 Statistics

Excel 2016 was used for statistical analyses. Differences in analysed quantities between days were tested for significance by analysis of variance (ANOVA). Differences in variations between days were

tested for significance by the Cochrans test. RSD_{max} was determined based on Horwitz. For assessing linearity (see Chapter 3, 2.2.4), the least square line and the correlation coefficient were calculated. Calibration curves were tested on slope ($a \neq 0$) and intercept ($b = 0$) by the Student's t-test and regression analysis, resp. A lack-of-fit (LOF) test was performed using a F-test and residuals were graphically examined to evaluate the lack of fit of the linear model.

PART 3

RESULTS

3.1 Validation

3.1.1 Total procyanidin content

3.1.1.1 Precision

Repeatability was tested by analysing six capsules on the same day (Table 3.2). For intermediate precision, six analyses were performed on three different days, each day with freshly prepared standard solutions. Based on the 18 measurements, the average procyanidin content per 20 mg capsule was 14.93 mg (CI: 14.78 mg – 15.08 mg), with a standard deviation of 0.30 mg and RSD% of 1.98%. The calculated $RSD\%_{within\ day}$ was 1.87%, $RSD\%_{between\ days}$ 2.02%. It can be concluded that, based on ANOVA ($F_{calculated} = 2.06 < F_{critical} = 3.682$), the results obtained on the 3 different days are not significantly different. Based on the Cochrans test ($C_{calculated} = 0.351 < C_{tabel} = 0.707$), the variation of the method can be considered equal for the analysis on the 3 different days.

Table 3.2. Repeatability and intermediate precision for total procyanidin analysis.

	Day 1	Day 2	Day 3
Test 1 (mg)	15.17	14.78	14.66
Test 2 (mg)	15.10	14.84	14.95
Test 3 (mg)	14.70	15.06	14.49
Test 4 (mg)	15.31	14.46	14.72
Test 5 (mg)	14.93	14.65	15.24
Test 6 (mg)	15.51	15.26	14.94
Average (mg)	15.12	14.84	14.83
SD (mg)	0.28	0.28	0.27
RSD%	1.78	1.91	1.79

RSD: relative standard deviation; SD: standard deviation.

3.1.1.2 Linearity-repeatability on different concentration levels

Since the determination of total procyanidin content is an unchanged USP method, validation of linearity by means of the reference standard is not required. Linearity-repeatability on different concentration levels was therefore tested by analysing repeatability on 50% and 150% of the amount

of sample, to demonstrate the method's validity in case the sample would contain a slightly lower or higher concentration.

Based on the 30 measurements (Tables 3.2 and 3.3), the average procyanidin content per 20 mg capsule to be used in the clinical trial (see Chapter 5) was 14.82 mg (CI: 14.65 mg – 14.98 mg), with a standard deviation of 0.44 mg and RSD% of 2.96%. The calculated $RSD\%_{within\ level}$ was 2.33%, $RSD\%_{between\ levels}$ 3.07%. Because the $RSD\%_{between\ levels}$ is in the order of the $RSD\%_{between\ days}$, the method can be considered precise. Based on the Cochrans test ($C_{calculated} = 0.505 < C_{tabel} = 0.506$), the variation of the method can be considered equal for the analysis on the 3 different levels.

Table 3.3. Linearity-repeatability for total procyanidin content.

	50%	**150%**
Test 1 (mg)	15.59	14.11
Test 2 (mg)	14.51	14.67
Test 3 (mg)	14.31	14.33
Test 4 (mg)	14.89	14.39
Test 5 (mg)	15.15	13.95
Test 6 (mg)	15.64	14.17
Average (mg)	15.02	14.27
SD (mg)	0.55	0.25
RSD%	3.65	1.78

RSD: relative standard deviation; SD: standard deviation.

3.1.1.3 Accuracy

The accuracy was tested on three concentration levels in triplicate by spiking the sample with Pycnogenol® extract. First, the procyanidin concentration in the extract was determined using USP Maritime Pine extract RS (see Chapter 3, 3.2.1). Based on the analysis of three concentration levels on three different days (Table 3.4), the average recovery was 100.9% (CI: 97.6% – 104.2%) with a standard deviation of 4.3%. The method can thus be considered accurate.

Table 3.4. Recovery (%).

Concentration	Day 1	Day 2	Day 3
100%	105.47	104.82	102.41
125%	100.92	104.15	103.69
150%	95.82	97.17	93.56

3.1.2 HPLC fingerprint

3.1.2.1 Response function of the calibration model

Since the HPLC fingerprint method was optimised, validation of linearity by means of the reference standard was required.

Catechin

The linearity for catechin was investigated by injecting six references of different concentrations (11.23, 18.72, 28.08, 37.44, 46.8 and 93.6 µg/ml) in duplicate. The assay showed good linearity up to at least 93.6 µg/ml catechin. The calibration curve was y = 12.76x - 8.12 (r = 0.9999). Graphical inspection of the residuals revealed random scatter with the highest deviation/area of 1.4% (< 5%). T-test on the intercept showed a statistical difference from zero (t = 2.65 > 2.306, CI: -14.95 – -1.30). A calibration curve of at least 3 points therefore must be made for the determination of catechin in the sample. T-test on the slope showed a statistical difference from zero (t = 198.6 > 2.306).

Taxifolin

The linearity for taxifolin was investigated by injecting six references of different concentrations (14.54, 24.23, 36.35, 48.46, 60.58 and 121.16 µg/ml) in duplicate. The assay showed good linearity up to at least 121.16 µg/ml taxifolin. The calibration curve was y = 46.98x – 33.38 (r = 0.9995). Graphical inspection of the residuals revealed random scatter with the highest deviation/area of 4.4% (< 5%). T-test on the intercept showed no statistical difference from zero (t = 1.18 < 2.306, CI: -96.49 – 29.74). T-test on the slope showed a statistical difference from zero (t = 102.3 > 2.306).

Caffeic acid

The linearity for caffeic acid was investigated by injecting six references of different concentrations (2.57, 4.29, 6.43, 8.57, 10.72 and 21.43 µg/ml) in duplicate. The assay showed good linearity up to at least 21.43 µg/ml caffeic acid. The calibration curve was y = 60.64x – 11.34 (r = 0.9999). Graphical inspection of the residuals revealed random scatter with the highest deviation/area of 1.3% (< 5%). T-test on the intercept showed a statistical difference from zero (t = 4.45 > 2.306, CI: -17.02 – -5.66). A calibration curve of at least 3 points therefore must be made for the determination of caffeic acid in the sample. T-test on the slope showed a statistical difference from zero (t = 259.31 > 2.306).

Ferulic acid

The linearity for ferulic acid was investigated by injecting six references of different concentrations (2.31, 3.85, 5.77, 7.70, 9.60 and 19.24 µg/ml) in duplicate. The assay showed good linearity up to at

least 19.24 µg/ml ferulic acid. The calibration curve was y = 55.70x – 11.51 (r = 0.9999). Graphical inspection of the residuals revealed random scatter with the highest deviation/area of 0.95% (< 5%). T-test on the intercept showed a statistical difference from zero (t = 8.62 > 2.306, CI: -14.48 – -8.53). A calibration curve of at least 3 points therefore must be made for the determination of ferulic acid in the sample. T-test on the slope showed a statistical difference from zero (t = 408.85 > 2.306).

3.1.2.2 Precision

Repeatability was tested by analysing six capsules on the same day. For intermediate precision, six analyses were performed on three different days, each day with freshly prepared standard solutions.

Catechin

Based on the 18 measurements (Table 3.5), the average catechin content per capsule was 0.2607 mg (CI: 0.2571 mg – 0.2644 mg), with a standard deviation of 0.0073 mg and RSD% of 2.79%. The calculated $RSD\%_{within\ day}$ was 1.20%, $RSD\%_{between\ days}$ 3.23%. It can be concluded that, based on ANOVA ($F_{calculated}$ = 38.65 > $F_{critical}$ = 3.682), the results obtained on the 3 different days are significantly different. Because the $RSD\%_{between\ days}$ is smaller than the $RSD\%_{MAX}$ (3.3%), the method is acceptable. Based on the Cochrans test ($C_{calculated}$ = 0.610 < C_{tabel} = 0.707), the variation of the method can be considered equal for the analysis on the 3 different days.

Table 3.5. Repeatability and intermediate precision for catechin analysis.

	Day 1	**Day 2**	**Day 3**
Test 1 (mg)	0.2684	0.2634	0.2535
Test 2 (mg)	0.2705	0.2577	0.2581
Test 3 (mg)	0.2693	0.2552	0.2512
Test 4 (mg)	0.2706	0.2573	0.2568
Test 5 (mg)	0.2691	0.2550	0.2501
Test 6 (mg)	0.2710	0.2551	0.2610
Average (mg)	**0.2698**	**0.2573**	**0.2551**
SD (mg)	**0.0010**	**0.0032**	**0.0042**
RSD%	**0.37**	**1.25**	**1.66**

RSD: relative standard deviation; SD: standard deviation.

Taxifolin

Based on the 18 measurements (Table 3.6), the average taxifolin content per capsule was 0.2782 mg (CI: 0.2762 mg – 0.2802 mg), with a standard deviation of 0.0040 mg and RSD% of 1.44%. The calculated $RSD\%_{within\ day}$ was 0.82%, $RSD\%_{between\ days}$ 1.62%. It can be concluded that, based on ANOVA ($F_{calculated}$ = 18.33 > $F_{critical}$ = 3.682), the results obtained on the 3 different days are significantly different. Because the $RSD\%_{between\ days}$ is smaller the $RSD\%_{MAX}$ (3.2%), the method is acceptable. Based on the Cochrans

test ($C_{calculated}$ = 0.684 < C_{tabel} = 0.707), the variation of the method can be considered equal for the analysis on the 3 different days.

Table 3.6. Repeatability and intermediate precision for taxifolin analysis.

	Day 1	Day 2	Day 3
Test 1 (mg)	0.2809	0.2751	0.2748
Test 2 (mg)	0.2825	0.2762	0.2797
Test 3 (mg)	0.2811	0.2744	0.2762
Test 4 (mg)	0.2838	0.2768	0.2797
Test 5 (mg)	0.2825	0.2724	0.2727
Test 6 (mg)	0.2847	0.2734	0.2810
Average (mg)	**0.2826**	**0.2747**	**0.2773**
SD (mg)	**0.0015**	**0.0017**	**0.0033**
RSD%	**0.53**	**0.61**	**1.18**

RSD: relative standard deviation; SD: standard deviation.

Caffeic acid

Based on the 18 measurements (Table 3.7), the average caffeic acid content per capsule was 0.0576 mg (CI: 0.0571 mg – 0.0582 mg), with a standard deviation of 0.0011 mg and RSD% of 1.94%. The calculated $RSD\%_{within\ day}$ was 1.24%, $RSD\%_{between\ days}$ 2.17%. It can be concluded that, based on ANOVA ($F_{calculated}$ = 13.40 > $F_{critical}$ = 3.682), the results obtained on the 3 different days are significantly different. Because the $RSD\%_{between\ days}$ is smaller the $RSD\%_{MAX}$ (4.1%), the method is acceptable. Based on the Cochrans test ($C_{calculated}$ = 0.598 < C_{tabel} = 0.707), the variation of the method can be considered equal for the analysis on the 3 different days.

Table 3.7. Repeatability and intermediate precision for caffeic acid analysis.

	Day 1	Day 2	Day 3
Test 1 (mg)	0.05744	0.05706	0.05955
Test 2 (mg)	0.05771	0.05755	0.05924
Test 3 (mg)	0.05760	0.05649	0.05841
Test 4 (mg)	0.05818	0.05671	0.05974
Test 5 (mg)	0.05729	0.05592	0.05735
Test 6 (mg)	0.05708	0.05574	0.05795
Average (mg)	**0.05755**	**0.05658**	**0.05871**
SD (mg)	**0.00038**	**0.00068**	**0.00096**
RSD%	**0.66**	**1.21**	**1.63**

RSD: relative standard deviation; SD: standard deviation.

Ferulic acid

Based on the 18 measurements (Table 3.8), the average ferulic acid content per capsule was 0.05574 mg (CI: 0.05537 mg – 0.05612 mg), with a standard deviation of 0.00076 mg and RSD% of 1.37%. The

calculated $RSD\%_{within\ day}$ was 1.41%, $RSD\%_{between\ days}$ 1.41%. It can be concluded that, based on ANOVA ($F_{calculated} = 0.0499 < F_{critical} = 3.682$), the results obtained on the 3 different days are not significantly different. Based on the Cochrans test ($C_{calculated} = 0.631 < C_{tabel} = 0.707$), the variation of the method can be considered equal for the analysis on the 3 different days.

Table 3.8. Repeatability and intermediate precision for ferulic acid analysis.

	Day 1	Day 2	Day 3
Test 1 (mg)	0.05586	0.05587	0.05472
Test 2 (mg)	0.05578	0.05623	0.05647
Test 3 (mg)	0.05569	0.05399	0.05429
Test 4 (mg)	0.05629	0.05579	0.05679
Test 5 (mg)	0.05602	0.05551	0.05537
Test 6 (mg)	0.05624	0.05578	0.05670
Average (mg)	**0.05598**	**0.05553**	**0.05572**
SD (mg)	**0.00025**	**0.00079**	**0.0011**
RSD%	**0.44**	**1.42**	**1.93**

RSD: relative standard deviation; SD: standard deviation.

3.1.2.3 Linearity-repeatability on different concentration levels

Linearity-repeatability on different concentration levels was tested by analysing repeatability on 50% and 150% of the amount of sample, to demonstrate the method's validity in case the sample would contain a slightly lower or higher concentration.

Catechin

Based on the 30 measurements (Tables 3.5 and 3.9), the average catechin content per 20 mg capsule was 0.2576 mg (CI: 0.2549 mg – 0.2603 mg), with a standard deviation of 0.0073 mg and RSD% of 2.82%. The calculated $RSD\%_{within\ level}$ was 1.24%, $RSD\%_{between\ levels}$ 3.05%. It can be concluded that, based on ANOVA ($F_{calculated} = 31.48 > F_{critical} = 2.759$), the results obtained on the 3 different levels are significantly different. Because the $RSD\%_{between\ levels}$ is in the order of the $RSD\%_{between\ days}$, the method can be considered precise. Based on the Cochrans test ($C_{calculated} = 0.353 < C_{tabel} = 0.506$), the variation of the method can be considered equal for the analysis on the 3 different levels.

Table 3.9. Linearity-repeatability for catechin.

	50%	150%
Test 1 (mg)	0.2527	0.2500
Test 2 (mg)	0.2455	0.2593
Test 3 (mg)	0.2542	0.2542
Test 4 (mg)	0.2518	0.2546
Test 5 (mg)	0.2515	0.2562
Test 6 (mg)	0.2470	0.2566
Average (mg)	**0.2505**	**0.2552**
SD (mg)	**0.0034**	**0.0031**
RSD%	**1.37**	**1.22**

RSD: relative standard deviation; SD: standard deviation.

Taxifolin

Based on the 30 measurements (Tables 3.6 and 3.10), the average taxifolin content per 20 mg capsule was 0.2754 mg (CI: 0.2734 mg – 0.2773 mg), with a standard deviation of 0.0052 mg and RSD% of 1.90%. The calculated $RSD\%_{within\ level}$ was 0.99%, $RSD\%_{between\ levels}$ 2.04%. It can be concluded that, based on ANOVA ($F_{calculated} = 20.83 > F_{critical} = 2.759$), the results obtained on the 3 different days are significantly different. Because the $RSD\%_{between\ levels}$ is in the order of the $RSD\%_{between\ days}$, the method can be considered precise. Based on the Cochrans test ($C_{calculated} = 0.334 < C_{tabel} = 0.506$), the variation of the method can be considered equal for the analysis on the 3 different levels.

Table 3.10. Linearity-repeatability for taxifolin.

	50%	150%
Test 1 (mg)	0.2718	0.2667
Test 2 (mg)	0.2665	0.2771
Test 3 (mg)	0.2720	0.2729
Test 4 (mg)	0.2675	0.2734
Test 5 (mg)	0.2708	0.2747
Test 6 (mg)	0.2651	0.2745
Average (mg)	**0.2690**	**0.2732**
SD (mg)	**0.0030**	**0.0035**
RSD%	**1.10**	**1.29**

RSD: relative standard deviation; SD: standard deviation.

Caffeic acid

Based on the 30 measurements (Tables 3.7 and 3.11), the average caffeic acid content per 20 mg capsule was 0.0571 mg (CI: 0.0566 mg – 0.0575 mg), with a standard deviation of 0.0013 mg and RSD% of 2.20%. The calculated $RSD\%_{within\ level}$ was 1.41 %, $RSD\%_{between\ levels}$ 2.33%. It can be concluded that, based on ANOVA ($F_{calculated} = 11.41 > F_{critical} = 2.759$), the results obtained on the 3 different days are

significantly different. Because the $RSD\%_{between\ levels}$ is in the order of the $RSD\%_{between\ days}$, the method can be considered precise. Based on the Cochrans test ($C_{calculated} = 0.343 < C_{tabel} = 0.506$), the variation of the method can be considered equal for the analysis on the 3 different levels.

Table 3.11. Linearity-repeatability for caffeic acid.

	50%	150%
Test 1 (mg)	0.05758	0.05433
Test 2 (mg)	0.05540	0.05646
Test 3 (mg)	0.05693	0.05603
Test 4 (mg)	0.05716	0.05568
Test 5 (mg)	0.05759	0.05633
Test 6 (mg)	0.05527	0.05601
Average (mg)	**0.0567**	**0.05581**
SD (mg)	**0.0011**	**0.00077**
RSD%	**1.86**	**1.39**

RSD: relative standard deviation; SD: standard deviation.

Ferulic acid

Based on the 30 measurements (Tables 3.8 and 3.12), the average ferulic acid content per 20 mg capsule was 0.0555 mg (CI: 0.0550 mg – 0.0560 mg), with a standard deviation of 0.0013 mg and RSD% of 2.27%. The calculated $RSD\%_{within\ level}$ was 1.85 %, $RSD\%_{between\ levels}$ 2.34%. It can be concluded that, based on ANOVA ($F_{calculated} = 4.65 > F_{critical} = 2.759$), the results obtained on the 3 different days are significantly different. Because the $RSD\%_{between\ levels}$ is in the order of the $RSD\%_{between\ days}$, the method can be considered precise. Based on the Cochrans test ($C_{calculated} = 0.331 < C_{tabel} = 0.506$), the variation of the method can be considered equal for the analysis on the 3 different levels.

Table 3.12. Linearity-repeatability for ferulic acid.

	50%	150%
Test 1 (mg)	0.05636	0.0544
Test 2 (mg)	0.05324	0.0573
Test 3 (mg)	0.05387	0.0563
Test 4 (mg)	0.05332	0.0578
Test 5 (mg)	0.05414	0.0549
Test 6 (mg)	0.05269	0.0567
Average (mg)	**0.0539**	**0.0562**
SD (mg)	**0.0013**	**0.0013**
RSD%	**2.40**	**2.35**

RSD: relative standard deviation; SD: standard deviation.

3.1.2.4 Accuracy

The accuracy was tested on three concentration levels in triplicate by spiking the sample with Pycnogenol® extract. First, the concentration of the four monomers in the extract was determined (see Chapter 3, 3.2.2).

Catechin

Based on analysis of three concentration levels in triplicate (Table 3.13), the average recovery for catechin was 99.97% (CI: 98.88% – 101.06%) with a standard deviation of 1.41%. The method can thus be considered accurate.

Table 3.13. Recovery catechin (%).

Concentration	Day 1	Day 2	Day 3
100%	98.87	101.37	99.85
125%	102.31	97.44	100.08
150%	99.27	100.59	99.95

Taxifolin

Based on analysis of three concentration levels in triplicate (Table 3.14), the average recovery for taxifolin was 99.39% (CI: 98.27% – 100.50%) with a standard deviation of 1.44%. The method can thus be considered accurate.

Table 3.14. Recovery taxifolin (%).

Concentration	Day 1	Day 2	Day 3
100%	98.11	100.10	99.05
125%	101.01	96.82	99.40
150%	98.32	100.79	100.88

Caffeic acid

Based on analysis of three concentration levels in triplicate (Table 3.15), the average recovery for caffeic acid was 99.42% (CI: 98.27% – 100.58%) with a standard deviation of 1.51%. The method can thus be considered accurate.

Table 3.15. Recovery caffeic acid (%).

Concentration	Day 1	Day 2	Day 3
100%	99.28	101.01	99.07
125%	100.88	97.86	101.67
150%	97.43	99.55	98.06

Ferulic acid

Based on analysis of three concentration levels in triplicate (Table 3.16), the average recovery for ferulic acid was 102.55% (CI: 100.72% – 104.38%) with a standard deviation of 2.38%. The recovery for ferulic acid is statistically different from 100% but within the acceptance levels (97% -103%). The method can thus be considered accurate.

Table 3.16. Recovery ferulic acid (%).

Concentration	Day 1	Day 2	Day 3
100%	102.09	105.78	101.53
125%	104.98	103.86	102.82
150%	98.98	103.76	99.12

3.2 Characterisation of the Pycnogenol® test extract

3.2.1 Total procyanidin content

Based on analysis *in triplo* on three different days (Table 3.17), the average procyanidin content in the Pycnogenol® extract was determined to be 78.3% ± 3.0%.

Table 3.17. Procyanidin content (%) in Pycnogenol® extract.

	Day 1	Day 2	Day 3
Test 1 (%)	75.45	79.73	76.16
Test 2 (%)	77.28	82.70	78.01
Test 3 (%)	72.90	80.79	81.69
Average (%)	75.21	81.07	78.62

3.2.2 HPLC fingerprint

The resulting chromatograms for the reference standards for catechin + taxifolin and caffeic acid + ferulic acid, determined at 280 nm, can be found in Figure 3.2. The fingerprint chromatogram for the 20 mg Pycnogenol® capsules, determined at 280 nm, can be found in Figure 3.3.

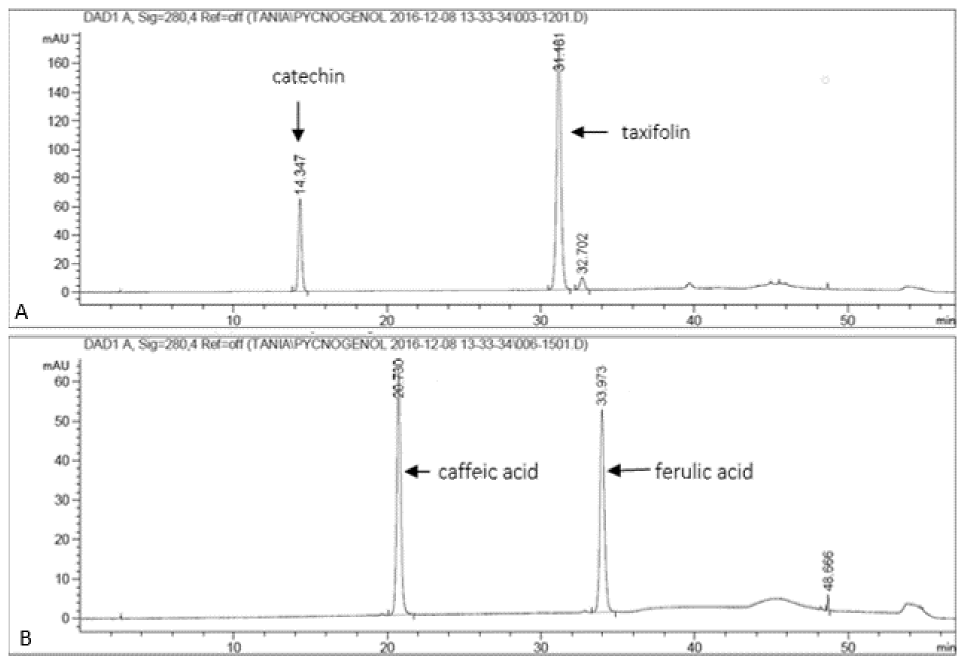


Figure 3.2. Chromatograms for the reference standards for (A) catechin + taxifolin and (B) caffeic acid + ferulic acid.

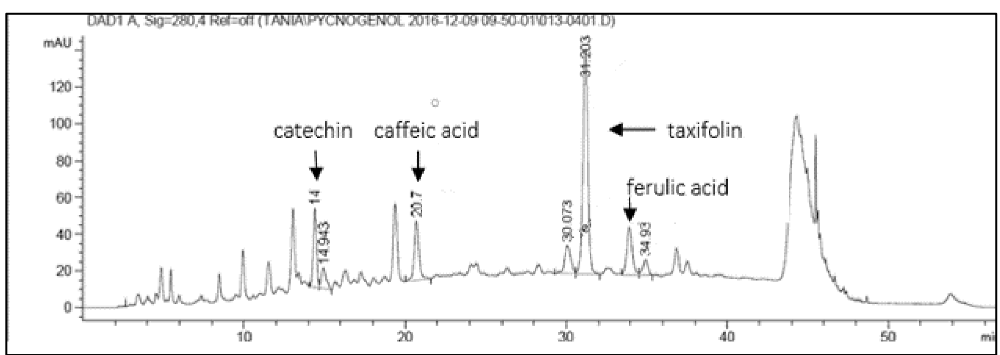


Figure 3.3. HPLC fingerprint chromatogram for Pycnogenol® capsules.

Based on analysis *in triplo* on three different days, the average content of catechin, taxifolin, caffeic acid and ferulic acid in the Pycnogenol® extract was determined (Table 3.18).

Table 3.18. Monomer content (%) in Pycnogenol® extract.

	Average	SD
Catechin (%)	1.276	0.031
Taxifolin (%)	1.375	0.012
Caffeic acid (%)	0.287	0.005
Ferulic acid (%)	0.274	0.003

SD: standard deviation.

PART 4

DISCUSSION AND CONCLUSION

Both methods for quality control of Pycnogenol® extract and capsules (total procyanidin level and HPLC analysis of catechin, taxifolin, caffeic acid and ferulic acid) were validated and were shown to have a good repeatability and intermediate precision ($RSD\%_{within\ day}$ of < 2% and $RSD\%_{between\ days}$ of < 4%), linearity (determined for the HPLC method only as this method was optimised), linearity-repeatability on different concentration levels (between 50% and 150% of the sample) and accuracy.

Pycnogenol® was found to comply with USP requirements for Maritime Pine Extract, as the average procyanidin content in the Pycnogenol® extract was determined to be 78.3% ± 3.0%, including 1.276% ± 0.031% catechin, 1.375% ± 0.012% taxifolin, 0.287% ± 0.005% caffeic acid and 0.274% ± 0.003% ferulic acid. Analysis of the trial's 20 mg Pycnogenol® capsules (see Chapter 5) revealed that one capsule on average contains 14.82 ± 0.44 mg procyanidins, including 0.2576 ± 0.0073 mg catechin, 0.2754 ± 0.0052 mg taxifolin, 0.0571 ± 0.0013 mg caffeic acid and 0.0555 ± 0.0013 mg ferulic acid.

REFERENCES

1. Ceylan, M., et al., Changes in oxidative stress and cellular immunity serum markers in attention-deficit/hyperactivity disorder. Psychiatry Clin Neurosci, 2012. 66: p. 220-226.
2. Ng, F., et al., Oxidative stress in psychiatric disorders: evidence base and therapeutic implications. Int J Neuropsychopharmacol., 2008. 11: p. 851-876.
3. Droy-Lefaix, M.T., Effect of the antioxidant action of Ginkgo biloba extract (EGb 761) on aging and oxidative stress. Age, 1997. 20(3): p. 141-149.
4. Puebla-Pérez, A.M., X. Lozoya, and M.M. Villaseñor-García, Effect of Ginkgo biloba extract, EGb 761, on the cellular immune response in a hypothalamic-pituitary-adrenal axis activation model in the rat. Int Immunopharmacol, 2003. 3(1): p. 75-80.
5. Aghili, T., et al., Effects of Hypericum perforatum extract on IgG titer, leukocytes subset and spleen index in rats. Avicenna J Phytomed, 2014. 4(6): p. 413-419.
6. Orčić, D.Z., et al., Antioxidant activity relationship of phenolic compounds in Hypericum perforatum L. Chem Cent J, 2011. 5: p. 34.
7. Ingale, S.P. and S.B. Kasture, Antioxidant and antiparkinsonian activity of Passiflora incarnata leaves. Orient Pharm Exp Med, 2014. 14(3): p. 231-236.
8. Beaumont, D.M., et al., The effects of chrysin, a Passiflora incarnata extract, on natural killer cell activity in male Sprague-Dawley rats undergoing abdominal surgery. AANA J, 2008. 76(2): p. 113-117.
9. D'Andrea, G., Pycnogenol: A blend of procyanidins with multifaceted therapeutic applications? Fitoterapia, 2010. 81: p. 724-736.
10. Dvořáková, M., et al., The effect of polyphenolic extract from pine bark, Pycnogenol, on the level of glutathione in children suffering from attention deficit hyperactivity disorder (ADHD). Redox Report, 2006. 11: p. 163-172.
11. Canali, R., et al., The anti-inflammatory pharmacology of Pycnogenol in humans involves COX-2 and 5-LOXmRNA expression in leukocytes. Int Immunopharmacol, 2009. 9(10): p. 1145-1149.
12. Wilson, D., et al., A randomized, double-blind, placebo-controlled exploratory studyto evaluate the potential of pycnogenol for improving allergic rhinitis. Phytother Res, 2010. 24(8): p. 1115-1119.
13. Convention, U.S.P., Maritime Pine Extract, in United States Pharmacopeia 38. 2014: Rockville, MD, USA. p. 6144.

CHAPTER 4

TOLL-LIKE RECEPTOR-DEPENDENT IMMUNOMODULATORY ACTIVITY OF PYCNOGENOL®

Verlaet A, van der Bolt N, Meijer B, Breynaert A, Naessens T, Konstanti P, Smidt H, Hermans N, Savelkoul HFJ, Teodorowicz G. Toll-Like Receptor-dependent immunomodulatory activity of Pycnogenol®. Nutrients (2019), 11(2).

The secret of getting ahead is getting started. – Mark Twain

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PART 1

ABSTRACT

Background: Pycnogenol®, an extract of French maritime pine bark, is widely used as a dietary supplement. It has been shown to exert anti-inflammatory actions by inhibiting the Toll-like receptor 4 (TLR4) pathway.

Aim: The aim of this study was to investigate whether Pycnogenol® might exert its immune modulating properties through other cell membrane TLRs (TLR1/2, TLR5 and TLR2/6) than TLR4. Moreover, the effect of gastrointestinal digestion on the immune modulating effects of Pycnogenol® was investigated.

Results: It was shown that non-metabolised Pycnogenol® dose-dependently acts as agonist of TLR1/2 and TLR2/6 and as partial agonist of TLR5. Pycnogenol® on its own does not agonise or antagonise TLR4. However, after formation of complexes with lipopolysaccharides (LPS), it is a potent activator of TLR4 signalling. Gastrointestinal metabolism of Pycnogenol® revealed immunosuppressive potential of the retentate fraction against TLR1/2 and TLR2/6 when compared to the control fraction containing microbiota and enzymes only. The dialysed fraction containing Pycnogenol® metabolites revealed the capacity to induce anti-inflammatory IL-10 secretion. The analysis of microbiota revealed that Pycnogenol® affects microbial composition.

Conclusion: This study showed that gastrointestinal digestion of Pycnogenol® reveals its biological activity as a potential inhibitor of TLRs signalling. The results suggest that metabolised Pycnogenol® acts as partial agonist of TLR1/2 and TLR2/6 in the presence of the microbiota-derived TLR agonists (retentate fraction) and that it possesses anti-inflammatory potential reflected by induction of IL-10 from THP-1 macrophages (dialysate fraction).

PART 2

INTRODUCTION

2.1 *In vitro* models

Activation of pattern recognition receptors (PRRs) on innate immune cells, e.g. by recognition of pathogen/damage associated molecular patterns (PAMPs/DAMPs), leads to activation of signalling pathways inside the cell, with the involved transcription factors depending on the receptor, resulting in the transcription of specific genes [1]. An example of a class of PRRs are Toll-like receptors (TLRs; receptors for e.g. LPS (TLR4), peptidoglycan (TLR2) and flagellin (TLR5)), which can use NF-κB as transcription factor to stimulate cell proliferation, differentiation, migration and survival. NF-κB is also involved in the transcriptional regulation of various pro-inflammatory cytokines (e.g. IL-1β, IL-6, IL-8 and TNF-α) and iNOS [2, 3].

2.1.1 THP-1 cell line

The human acute monocytic leukaemia cell line, THP-1, was established in 1980 from a patient with acute monocytic leukaemia. Regarding its morphological and differentiation properties, this cell line resembles monocytes and macrophages. THP-1 cells are useful for food- and non-food-derived compound screening purposes to establish their effects on monocyte and macrophage regulation and function, like apoptosis and pro-inflammatory responses [4-10], both in the resting and inflammatory state. Monocytes typically react faster to external stimuli, while macrophages react stronger in terms of mRNA expression, cytokine production, phagocytic activity, etc. [7]. For example, simultaneous exposure of THP-1 cells to LPS and other test compounds is a promising *in vitro* tool to investigate modulating effects of these compounds on the induction and severity of inflammation [7-10].

2.1.2 HEK cell line

The human embryonic kidney (HEK) 293 cell line (or shortly: HEK cells) was established in 1973 from an adrenal cell in a human embryonic kidney cell culture [11]. As opposed to initial assumptions that HEK cells were generated from a fibroblastic, endothelial or epithelial cell, they have various properties of immature neurons [12]. HEK cells are known for their easy and reliable growth and transfection potential and used as a host for induced and stable gene expression, for example by the biotechnology

industry to produce therapeutic proteins [13]. Using the HEK cell line transfected with specific TLRs and expressing specific reporter plasmids, a compound can thus be tested for its ability to bind this TLR.

2.2 Immune modulating effects of Pycnogenol®

Pycnogenol® is widely used as a dietary supplement as it was shown to exert multiple putative biological and pharmacological effects in various health conditions. Next to its well documented antioxidant and free radical scavenging properties [14-19], Pycnogenol® reduces the release of pro-inflammatory cytokines from macrophages [16, 20-25]. In addition, Pycnogenol® reduces the expression of cell adhesion molecules [26], MMP-9 secretion [22, 27, 28], COX and LOX expression [22, 27-31] and levels of NO and iNOS expression (see Chapter 1, 4.4.3) [16, 17, 32, 33]. Newest research points out that Pycnogenol® exerts its immunomodulatory effects at least in part through the TLR4-NF-κB pathway [22, 25, 34-36]. Nevertheless, the mechanism by which Pycnogenol® exerts its inhibitory effects via TLR4 and whether other TLRs and signalling mechanisms play part remains to be elucidated.

The aim of the present study was to elucidate the effect of Pycnogenol® and its primary monomer catechin on membrane TLR activity and pro-inflammatory cytokine secretion. In addition, the effect of *in vitro* GI metabolism on the immunomodulatory effects of Pycnogenol® was investigated.

PART 3

MATERIALS AND METHODS

3.1 Sample preparation

3.1.1 Non-metabolised samples

Pycnogenol® was obtained from Horphag Research Ltd, Switzerland, and (+)-catechin hydrate (> 98%) was purchased from Sigma, USA. These preparations were tested to have undetectably low contamination with endotoxin according to the manufacturers. 50 mg/ml of the spray-dried Pycnogenol® and catechin stocks were freshly dissolved in PBS (Lonza, Belgium), dimethyl sulfoxide (DMSO) or ethanol (96%; Thermo Scientific, USA), vortexed for 1 min and centrifuged for 1 min at 1000g. The supernatant was sterilised by filtration using 0.22 µm Millex®-GV filters (Millipore, USA). Further dilutions of the samples were made in different types of culture medium.

3.1.2 PYC-LPS complexes

Pycnogenol®-LPS complexes were made using a stock solution containing 400 µg/ml Pycnogenol® which was mixed with 50 pg/ml LPS from Escherichia coli 055:B5 (Sigma Aldrich, The Netherlands). This stock solution was further diluted in medium containing 50 pg/ml LPS and incubated for 1 h at RT to allow complex formation. Recombinant Factor C Endotoxin Detection Assay (Hyglos GmbH, Germany) was used to compare the LPS levels of the Pycnogenol®-LPS solution and only LPS at the same concentration.

3.1.3 Gastrointestinal metabolism

Pycnogenol® extract was metabolised by a GI dialysis model with colon phase (GIDM-colon) to mimic the *in vivo* situation after oral ingestion. The experimental setup was based on an *in vitro* continuous flow dialysis model, as described and validated by Breynaert *et al.* [37].

In summary, physiological conditions of the gastric phase were simulated for 1 h at pH 2 by addition of 6 M HCl and pepsin solution (37°C, shaking conditions). The intestinal phase was simulated using Amicon stirred cells (Figure 4.1) with a semi-permeable dialysis membrane (1000 Da cut-off) in an anaerobic glove-box (5% CO_2, 5% H_2 and 90% N_2) at 37°C. Dialysis mimics one-way GI absorption by

passive diffusion from lumen to mucosa. The small intestine was mimicked for 2.5 h at 37°C, pH 7.5, while using pancreatic enzymes and bile solution. More specifically, a dialysis tubing (14 kDa cut-off) containing 1 M $NaHCO_3$ required for the gastric digest to reach pH 7.5 was added to the sample in the dialysis cell. After 30 min, 15 ml of pancreatin-bile mixture was added with an end concentration of 0.052% (w:v) pancreatin and 0.1% (w:v) bile. Afterwards, the colonic phase with retentate samples from the small intestine was simulated at pH 5.8 by addition of 1 M HCl and by addition of microbial faecal culture obtained from pooled faeces from healthy donors (n = 3; non-smoking, non-vegetarian adults (25-58 years old) with normal defaecation, without history of GI disease and without antibiotics use during the 3 months before faeces collection).

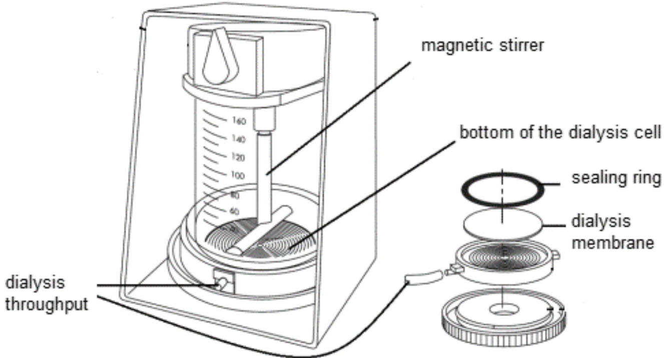

Figure 4.1. A dialysis cell.

Both retentate and dialysate samples were taken at specific time points and freeze-dried prior to analysis. GIDM digestion was performed *in duplo* for Pycnogenol® (starting dose of 0.024 g up to a high dose of 0.2 g), in addition to a blank without Pycnogenol® (containing enzymes and faecal suspension).

3.2 HPLC analysis

As the high concentration of Pycnogenol® (50 mg/ml) did not dissolve completely in PBS, HPLC analysis for the identification of catechin, caffeic acid, taxifolin and ferulic acid as well as detection of the overall percentage of procyanidins by spectrophotometry were performed according to the (slightly adapted) USP methods for Maritime Pine Extract (samples were dissolved in 20% MeOH instead of 100% MeOH to improve chromatographic peaks; see Chapter 3).

3.3 Endotoxin Detection Assay

Recombinant Factor C Endotoxin Detection Assay was used for quantitative determination of LPS contamination in the Pycnogenol® extract as well as in catechin. This is an enzymatic assay using recombinant Factor C in combination with a fluorogenic substrate. Factor C is the LPS receptor of the blood-clotting cascade in horseshoe crabs. The detection range of the assay is 0.005-50 EU/ml. The assay was performed according to the manufacturer's instructions. Results were obtained using SoftMax Pro software (Molecular Devices, USA) and calculated standard curves. Samples were spiked with 5 EU/ml LPS to validate if sample components interfered with the assay and heated to exclude false positive results by protease contamination of the samples. Spiking recovery between 50% and 200% excludes interference of sample components with the assay. To calculate LPS units from EU to pg/ml, 1 EU is approximately equivalent to 100 pg LPS.

3.4 Cell cultures

HEK293 cells stably transfected with TLR1/2, TLR2/6, TLR4, TLR5 as well as empty pNIFTY plasmids were obtained from CAYLA-InvivoGen (France). TLR activation upon ligand stimulation was determined by assessing NF-κB activation. The latter was achieved by stably transfecting the HEK293 cells with pNIFTY, a family of NF-κB inducible reporter plasmids expressing the luciferase gene. The selection antibiotics Zeocin™ and Blasticidin (Invitrogen, New Zealand) were used to generate stable clones. HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; ThermoFisher, USA), supplemented with 1% Penicillin/Streptomycin (P/S), 10% Foetal Bovine Serum (FBS) and 2 mM L-Glutamine from Gibco (Invitrogen, New Zealand) and incubated at 37°C in 5% CO_2 in a humidified incubator.

THP-1 cells (American Type Culture Collection, USA) were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium with L-Glutamine and 25 mM hydroxyethyl piperazineethanesulfonic acid (Hepes; Lonza, Belgium), supplemented with 10% FBS and 1% P/S. Cells were subcultured twice a week and medium was changed before every experiment. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 and were used below passage number 25. THP-1 cells were seeded at $1*10^5$ cells/well in Costar®3596 flat bottom 96-well plates (Corning Inc, USA). Macrophage phenotype was induced by stimulating THP-1 cells with 10 ng/ml PMA (Sigma Aldrich, The Netherlands) for another 48 h before stimulation. Differentiated cells were washed twice in culture medium and incubated for another 48 h before stimulation.

3.5 Cytotoxicity assay

Cytotoxicity of Pycnogenol® and catechin solutions was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation assay (Promega, USA). HEK293 cells and THP-1 macrophages were plated at a density of $6*10^4$ and $1*10^5$ cells/well, resp. in flat bottom 96-well plates (Corning Inc., USA) and incubated with Pycnogenol® and catechin extracts at different concentrations for 18 h. Afterwards, cells were incubated with 15 µl CellTiter96®Aqueous One Solution for 1 hour at 37°C in a humidified atmosphere with 5% CO_2. Absorbance was recorded at 485 nm using the FilterMax F5 Multi-Mode Microplate Reader (Molecular Devices, USA). Viability of cells stimulated with sample conditions was normalised in relation to the negative control (cells cultured in pure medium). All experiments were performed *in triplo*.

3.6 Quantification of cytokines in THP-1 supernatants

THP-1 cells were stimulated with different concentrations of Pycnogenol® (400, 100, 25, 6.25 µg/ml), catechin (250, 50, 5, 0.5 µg/ml) or metabolised samples taken at specific time points (4 h, 10 h, 27 h and 36 h) diluted in culture medium. These doses were obtained from pilot experiments in which more doses were tested. The experiment was performed with and without costimulation with PAM3 at a concentration of 0.1 µg/ml. After 18 h of incubation, human cytokine concentrations (IL-1β, IL-6, IL-8, IL-10 and TNF-α) in the culture supernatants were determined using Cytometric Bead Array (CBA) kit (Human Inflammatory Cytokine Kit, BD Bioscience, USA) according to the manufacturer's instructions. The samples were analysed by flow cytometry (BD FACS Canto II, BD Bioscience). The results were normalised to cytokine levels of unstimulated macrophages cultured in the medium.

3.7 Expression of surface markers on THP-1 macrophages

THP-1 macrophages were incubated for 18 h with dialysates collected after 4 h and 10 h of metabolism with and without triacylated lipopeptide (PAM3, InvivoGen, USA) as costimulation and as described before [38]. After incubation, cells were harvested and the expression of receptors (cluster of differentiation (CD)83, TLR1, TLR5 and TLR6) was analysed using FACS Canto II (BD Pharmingen, USA). The antibodies anti-human CD83 (BioLegend, USA), TLR1, TLR5 and TLR6 (BD Pharmingen, USA) were used.

3.8 Activation or inhibition of TLRs

HEK293 cells were seeded (6*10^4 cells/well) in Costar®3610 clear flat-bottom 96 well plates (Corning Inc., USA) and cultured 24 h. Cells were stimulated with different concentrations of Pycnogenol® (400, 100, 25, 6.25 µg/ml) and catechin (250, 50, 5, 0.5 µg/ml) or metabolised samples taken at specific time points (4 h, 10 h, 27 h and 36 h) diluted in culture medium. Their known ligands were used as positive controls: triacylated lipopeptide (PAM3, InvivoGen, USA), PAM2 (PAM2, InvivoGen, USA), LPS from Escherichia coli 055:B5 (Sigma Aldrich, The Netherlands) and flagellin (InvivoGen, USA) for TLR1/2, TLR2/6, TLR4 and TLR5, resp. After 18 h incubation, the luciferase activity in HEK293 cells was measured and related to the cells grown in medium as control. Bright-Glo Luciferase Assay System (Promega, USA) was added to medium (1:1) and vortexed for 5 min at 250 rpm before luminescence was read from top with 750 ms integration time using a microplate reader (Molecular Devices, USA).

3.9 Microbial analysis

Bacterial DNA was isolated from the retentate samples, which were collected from the colonic phase at time 4 h and 10 h. The samples were added to a bead-beating tube with 300 µL Stool Transport and Recovery (STAR) buffer (Roche Diagnostics GmbH, Germany), 0.25g of sterilised zirconia beads (0.1 mm) and 3 glass beads (2.5 mm; Sigma, USA). Samples were homogenised by three lots of bead-beating (60 s × 5.5 ms). Homogenised samples were incubated at 95°C for 15 min and centrifuged (14,000g, 4°C, 5 min). Supernatants were collected and transferred to sterile tubes. Pellets were re-processed, this time adding 200 µL of STAR buffer, and supernatants from both steps were pooled. DNA was purified with a customised kit (XAS 1220; Promega) using 250 µl of the final supernatant pool. DNA was eluted in 50 µl DNAse- and RNAse-free water, and its concentration was measured using the DS-11 FX+ Spectrophotometer/Fluorometer (DeNovix Inc., USA) [39].

For the amplification of the V5-V6 region of the bacterial 16S ribosomal RNA (rRNA) gene, polymerase chain reaction (PCR) was carried out *in duplo* with conditions set as described previously [40]. Briefly, the master mix contained 1 µl of a unique barcoded primer, 784F-n and 1064R-n (10 µM each per reaction), 1 µl deoxyribonucleotide triphosphate (dNTP) mixture, 0.5 µl Phusion Green Hot Start II High-Fidelity DNA Polymerase (2 U/µl; Thermo Scientific, The Netherlands), 10 µl 5× Phusion Green HF Buffer (Thermo Scientific, The Netherlands), 36.5 µl DNAse- and RNAse-free water (Promega, USA) and 1 µL template DNA (20 ng/µl). The amplification program included 30s of initial denaturation at 98°C, followed by 25 cycles of denaturation at 98 °C for 10s, annealing at 42°C for 10s, elongation at 72°C

for 10s and extension at 72°C for 7 min. The PCR products were visualised in 1% agarose gel (~280 bp) and purified with CleanPCR kit (CleanNA, The Netherlands). The concentrations of the purified amplicons were measured with Qubit dsDNA BR Assay Kit (Invitrogen, USA) and 200 ng from each sample was used to create the final amplicon library. Samples were sequenced (paired-end, read length: 2 x 150 base pairs) with an Illumina HiSeq.2500 platform (GATC Biotech, Germany).

Real-time PCR (RT-PCR) amplification and detection were performed on a CFX384™ RT-PCR detection system (Bio-Rad, USA). Broad range primers were used, targeting the bacterial 16S rRNA gene [41]. The reaction mixture was composed of 5 µl iQ™ SYBR® Green Supermix, 0.2 µl forward and reverse primers (10 nmol), 1.6 µl nuclease-free water and 3 µl DNA template (1 ng/µl). The program for amplification was initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 20 s, annealing at 52°C for 30 s and elongation at 72°C for 30 s. Standard curves contained 10^1–10^9 16S rRNA copies/µl and were included *in triplo*.

3.10 Statistics

3.10.1 Cell cultures

All data were expressed as mean ± SD. Statistical analysis on cell cultures were performed by GraphPad Prism software by one-way ANOVA with Tukey post-hoc comparison. P-values < 0.05 were considered statistically significant. Significant differences were indicated by asterisks: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.10.2 qPCR analysis

qPCR results were normally distributed. Statistical analysis on qPCR data and cell cultures were performed by GraphPad Prism software by one-way ANOVA with Tukey post-hoc comparison. P-values < 0.05 were considered statistically significant.

Sequencing data filtering and taxonomy assignment were performed using the NG-Tax pipeline using the default settings [40]. Taxonomy was assigned to the identified Operational Taxonomic Units (OTU) using the Silva_111_SSU database [42]. The total number of obtained reads was 266972, while 33912 was the minimum number of reads obtained for a sample. The microbiota data were analysed at the genus level and transformed into the relative abundances prior to downstream analysis. In total, 32 bacterial genera were identified from the four samples.

3.10.3 Data availability

The 16S rRNA gene sequencing dataset is publicly available at the European Nucleotide Archive (ENA) database with access code PRJEB29793.

PART 4

RESULTS

4.1 LPS detection and cytotoxicity

Pycnogenol® was dissolved (50 mg/ml) in three different solvents: ethanol (96%), DMSO and PBS. The cytotoxicity of these solutions was determined using HEK293 cells and THP-1 macrophages. DMSO, even at low concentrations, was found to significantly decrease the viability of both cell lines and therefore Pycnogenol® in DMSO was not used for further experiments. PBS and ethanol solutions of Pycnogenol® were tested on HEK cells expressing TLR4, TLR1/2, TLR2/6 and TLR5. It was found that the ethanol solution impaired valid modulation of TLR1/2 and TLR5 (data not shown). Therefore, PBS was chosen as the most optimal solvent. Moreover, the use of an aqueous solvent like PBS mimics the *in vivo* situation after oral administration of PYC.

The putative endotoxin (LPS) contamination of the Pycnogenol® preparation was below the detection limit of 0.05 EU/ml, even at a high concentration of Pycnogenol® (1 mg/ml) (Figure 4.2A), showing that samples were essentially not contaminated with LPS. Cytotoxicity of all tested preparations (LPS, Pycnogenol®, Pycnogenol®-LPS and metabolised samples) at concentrations corresponding with further experiments was examined for both the HEK and THP-1 cell line. None of the above-mentioned preparations were found to decrease the viability of these two cell lines (Figure 4.2B,C).

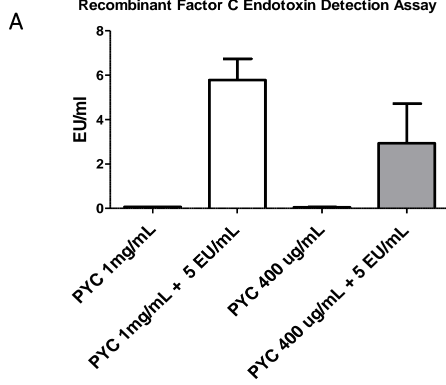

Figure 4.2 (part 1). Pycnogenol® contains non-detectable amounts of LPS and does not influence the viability of HEK293 and THP-1 cells. (A) LPS concentration in Pycnogenol® without and after spiking with 5 EU/ml LPS (n = 4 as technical replicates). Data are expressed as mean ± SD.
LPS: lipopolysaccharide; PYC: Pycnogenol®.

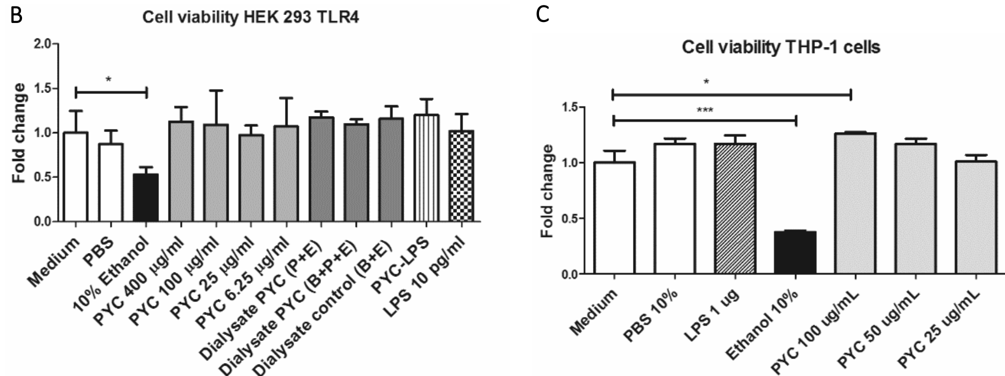

Figure 4.2 (part 2). Pycnogenol® contains non-detectable amounts of LPS and does not influence the viability of HEK293 and THP-1 cells. (B) Viability of HEK293 cells. (C) Viability of THP-1 macrophages. Results of (B) and (C) are expressed as fold change of absorbance (490 nm) compared to unstimulated cells (medium control = 1; n = 4 as technical replicates). Ethanol was used as positive control. Data are expressed as mean ± SD. Significant differences are indicated by asterisks: * $p < 0.05$; *** $p < 0.001$.
B: bacteria; E: enzymes; LPS: lipopolysaccharide; PBS: phosphate-buffered saline; PYC/P: Pycnogenol®; TLR: Toll-like receptor.

4.2 Non-metabolised samples

4.2.1 Pycnogenol® composition

Spectrophotometric analysis revealed that the Pycnogenol® extract dissolved in PBS contained 60.2% procyanidins as opposed to 78.3 ± 3% in the original extract dissolved in MeOH (see Chapter 3, 3.2.1), confirming a solubility of about 80% for procyanidins. HPLC analysis revealed that the solubility of catechin, caffeic acid, taxifolin and ferulic acid in Pycnogenol® 50 mg/ml solution was not complete, though still acceptable (80.4 – 92.2%, % of analysis of the MeOH solution, Table 4.1), while the fingerprint chromatogram was similar to the reference (Figure 4.3). It was therefore assumed that, despite a lower concentration, the overall composition of the dissolved extract was not drastically changed by its incomplete solubility.

Table 4.1. Solubility of catechin, caffeic acid, taxifolin and ferulic acid at 50 mg/ml Pycnogenol® in PBS as compared to solubility in MeOH determined by HPLC.

Component	Solubility (%)
Catechin	88.8
Caffeic acid	92.2
Taxifolin	80.4
Ferulic acid	82.7

HPLC: high-performance liquid chromatography; MeOH: methanol; PBS: phosphate-buffered saline.

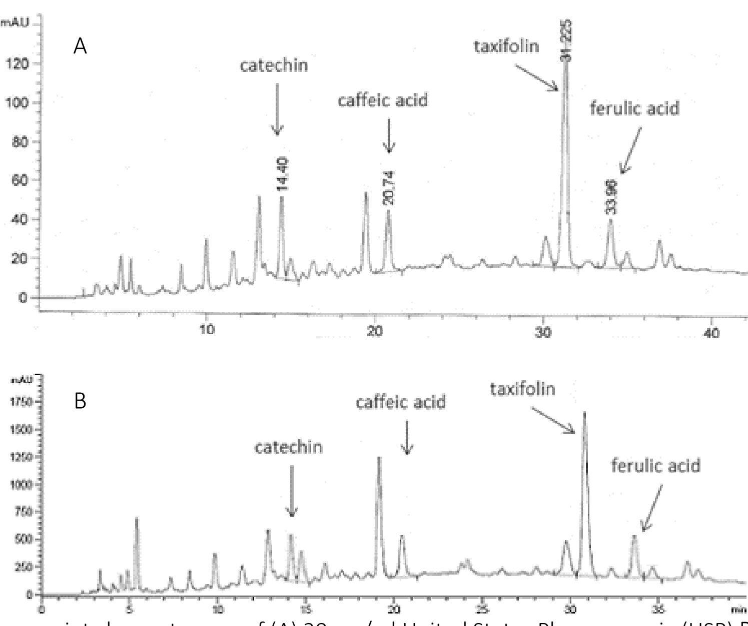

Figure 4.3. Fingerprint chromatogram of (A) 20 mg/ml United States Pharmacopeia (USP) Pycnogenol® reference in 20% MeOH and (B) 50 mg/ml Pycnogenol® in PBS.
MeOH: methanol; PBS: phosphate-buffered saline.

4.2.2 Effects on TLRs

The effect of Pycnogenol® and catechin on TLR activation or inhibition was measured using stably transfected HEK293 cells expressing TLR2, TLR1/2, TLR2/6, TLR4 and TLR5. Responses were determined by quantifying luminescence upon selective ligand binding, induced by the pNIFTY reporter gene via the NF-κB pathway (Figure 4.4). The stimulation of cells with Pycnogenol® showed dose-dependent increase of luminescence of the cells expressing the heterodimeric receptor TLR2/6. The highest concentration of Pycnogenol® (400 µg/ml) induced 3.2-fold activation for TLR2/6 when compared to the negative control being culture medium (Figure 4.4B). The highest concentration of Pycnogenol® induced activation of TLR1/2 and TLR5, which was 4.9- and 3.2-fold higher, resp., than for the medium control, but no dose dependency was observed (Figure 4.4A,C). Little activation of TLR2 (Figure 4.4D) and TLR4 (Figure 4.4E) was observed (around 1.4 times higher than that observed for medium control) upon incubation of HEK293 cells with 400 µg/ml Pycnogenol®. Catechin showed no agonistic effects on any of the membrane TLRs investigated, suggesting that catechin is not the constituent of Pycnogenol® responsible for the observed activation of TLR1/2 and TLR2/6 (Figure 4.4A-D). Nevertheless, catechin dose-dependently reduced the activity of TLR5 (0.6- and 0.7-fold) and TLR1/2 (0.3- and 0.4-fold) below the activation levels of the negative control being medium.

To investigate whether Pycnogenol® and catechin function as antagonists or partial agonists of studied TLRs, costimulation of each receptor with Pycnogenol®/catechin and its known ligand as positive control was performed. Pycnogenol® dose-dependently inhibited the activation of TLR5 when costimulated with flagellin, reducing the level of activation to 0.29- and 0.63-fold in the studied concentrations of 400 and 100 µg/ml, resp. (Figure 4.4F). The affinity of TLR5 for flagellin is therefore apparently much higher than for Pycnogenol®. Catechin reduced the activation of TLR5 only at the highest concentration (250 µg/ml). Costimulation of the other receptors did not affect the level of receptor activation (data not shown). Therefore, these data suggest that Pycnogenol® acts as agonist of TLR1/2 and TLR2/6 and as partial agonist of TLR5.

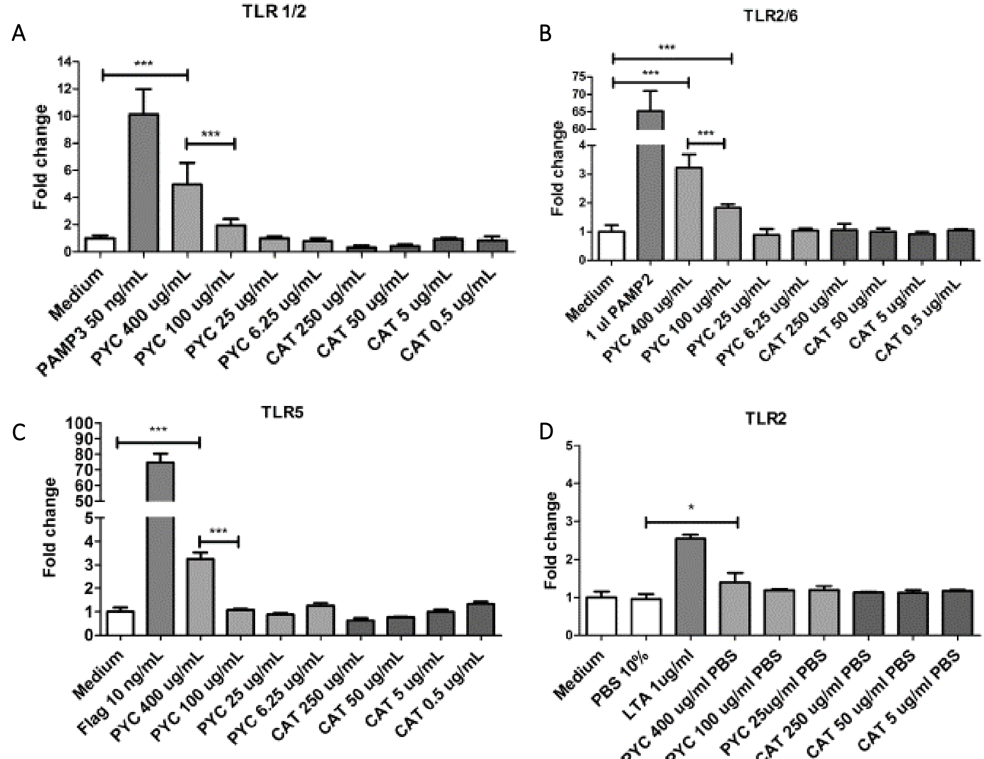

Figure 4.4 (part 1). Pycnogenol® acts as agonist of TLR1/2 and TLR2/6 and as partial agonist of TLR5. HEK293 cells expressing (A) TLR1/2, (B) TLR2/6, (C) TLR5 and (D) TLR2 were incubated 24 h with different concentrations of Pycnogenol® and catechin or their known ligands as positive controls (PAM3, PAM2, flagellin and LTA, resp.). All corresponding positive ligands induced TLR activation, confirming functionality of the assay. Results are expressed as fold change of fluorescence intensity relative to unstimulated cells (medium control = 1; n = 4 as technical replicates). All data are expressed as mean ± SD. Significant differences are indicated by asterisks: * $p < 0.05$; *** $p < 0.001$.

CAT: catechin; Flag: flagellin; LPS: lipopolysaccharide; LTA: lipoteichoic acid; PBS: phosphate-buffered saline; PYC: Pycnogenol®; TLR: Toll-like receptor.

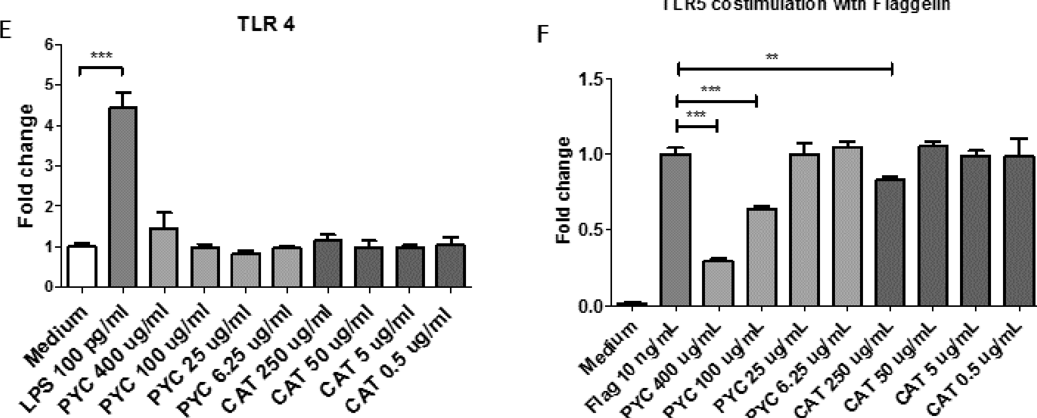

Figure 4.4 (part 2). Pycnogenol® acts as agonist of TLR1/2 and TLR2/6 and as partial agonist of TLR5. (E) HEK293 cells expressing TLR4 were incubated 24 h with different concentrations of Pycnogenol® and catechin or LPS as positive control. LPS induced TLR4 activation, confirming functionality of the assay. (F) HEK293 cells expressing TLR5 were incubated 24 h with different concentrations of Pycnogenol® and catechin in combination flagellin as positive control. Results are expressed as fold change of fluorescence intensity relative to unstimulated cells (medium control = 1; n = 4 as technical replicates). All data are expressed as mean ± SD. Significant differences are indicated by asterisks: ** $p < 0.01$; *** $p < 0.001$.
CAT: catechin; Flag: flagellin; LPS: lipopolysaccharide; PYC: Pycnogenol®; TLR: Toll-like receptor.

4.2.3 Effect on cytokine production

Pycnogenol® (but not catechin) was shown, in a dose-dependent manner, to induce pro-inflammatory cytokine (IL-8, TNF-α and IL1-β) secretion from THP-1 macrophages, confirming an agonistic effect on TLR signalling (Figure 4.5).

4.2.4 Costimulation of Pycnogenol® and LPS

Interestingly, a clear stimulating dose-response curve was observed for Pycnogenol® on TLR4 activity when TLR4$^+$ HEK cells were stimulated with mixture of Pycnogenol® and LPS (10 pg/ml). 400, 100 and 25 µg/ml Pycnogenol® in combination with LPS induced a 4.9-fold, 3.5- and 1.7-fold increase in TLR4 activation, resp., when compared to pure LPS (10 pg/ml) (Figure 4.6A). As described above, 400 µg/ml Pycnogenol® on its own significantly induced TLR4 activation, albeit at a low level (Figure 4.4E). Also, pre-incubation of cells with similar doses of Pycnogenol® followed by stimulation with LPS did not influence the level of TLR4 activation when compared to the LPS control (Figure 4.6B). The observation of increased activation of TLR4 by costimulation with Pycnogenol®-LPS mixture may be explained by the formation of complexes between Pycnogenol® and LPS, beneficially affecting the activation of TLR4. This has been confirmed by a decreased concentration of LPS measured in the Pycnogenol®-LPS preparation after 30 min of pre-incubation (Figure 4.6C).

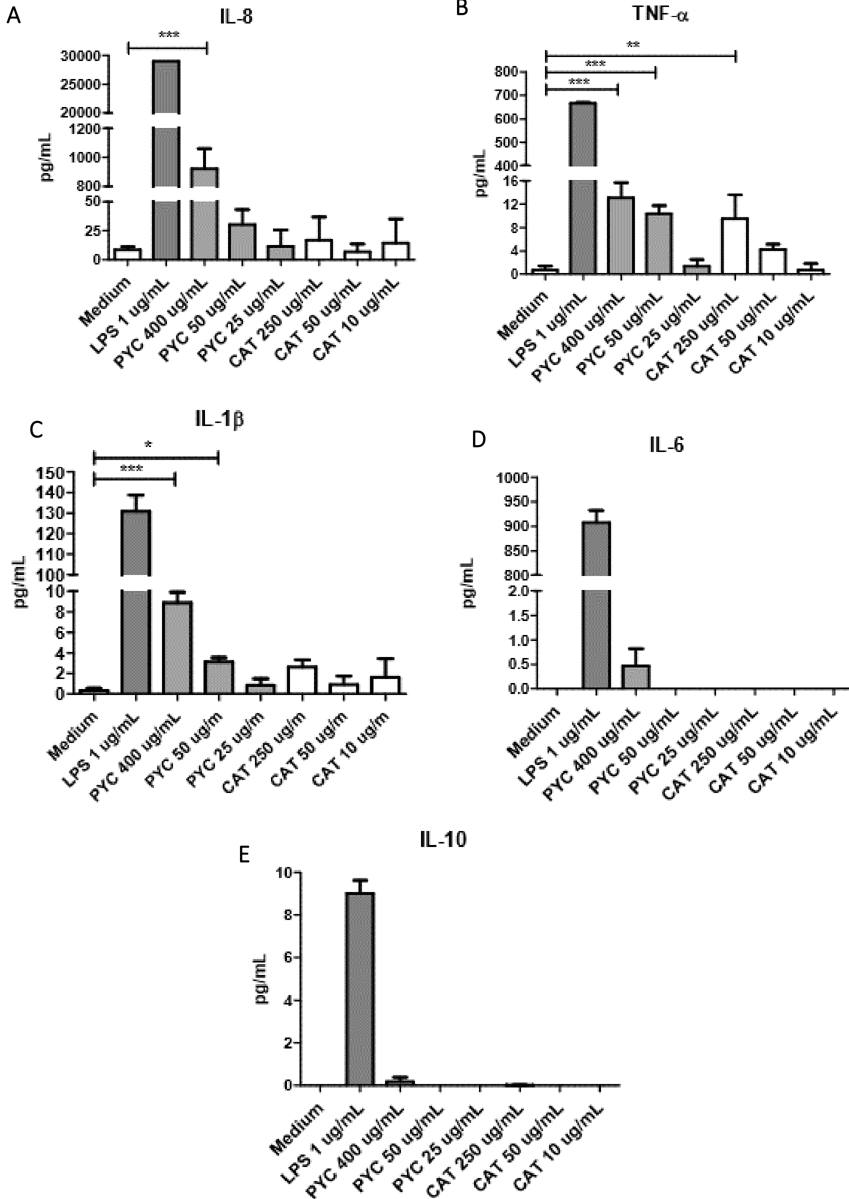

Figure 4.5. Effect of non-metabolised Pycnogenol® on pro-inflammatory cytokine secretion from THP-1 macrophages. THP-1 macrophages were incubated 24 h with various concentrations of Pycnogenol® or catechin, and the concentration of (A) IL-8, (B) TNF-α, (C) IL-1β, (D) IL-6 and (E) IL-10 in supernatant was determined by flow cytometry. All data (n = 4 technical replicates) are expressed as mean ± SD. Significant differences are indicated by asterisks: * $p < 0.05$; ** $p < 0.01$; *** $P < 0.001$.
CAT: catechin; IL: interleukin; LPS: lipopolysaccharide; PYC: Pycnogenol®; TLR: Toll-like receptor; TNF: tumour necrosis factor.

Costimulation of TLR4+ HEK cells with catechin and LPS did not show any boosting effect on TLR4 activation. When costimulated with LPS, catechin at a concentration of 250 µg/ml showed inhibiting activity against TLR4 activation which was 30% lower than that of the LPS control (Figure 4.6D).

The enhanced activation of TLR4 in HEK293 cells by costimulation of cells with Pycnogenol® and LPS was confirmed by the boosted cytokine secretion by THP-1 macrophages stimulated with Pycnogenol®-LPS complexes (Figure 4.6E-G). Costimulation with LPS and different doses of Pycnogenol® resulted in

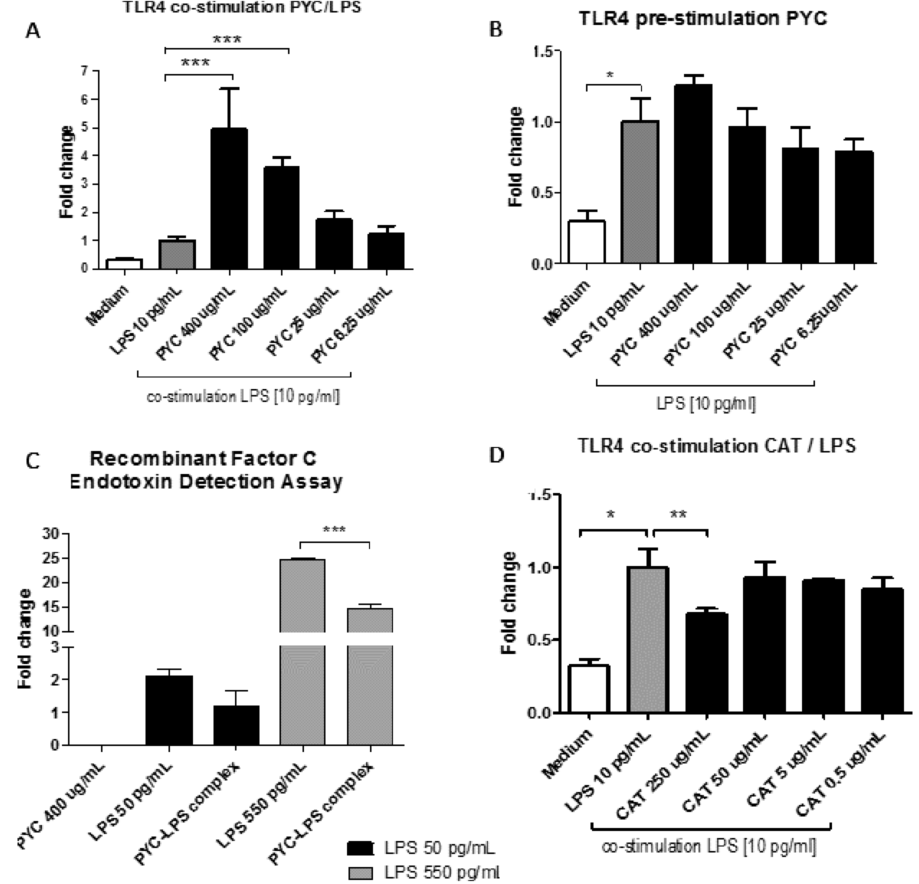

Figure 4.6 (part 1). Pycnogenol® and LPS form complexes, which boost activation of TLR4. (A) Pycnogenol® pre-incubated with LPS (10 ng/ml) for 30 min before addition to TLR4+ HEK293 cells followed by incubation for 24 h. (B) TLR4+ HEK293 cells pre-incubated with Pycnogenol® followed by washing and incubation with LPS (10 ng/ml) for 24 h. (C) LPS concentration in Pycnogenol® (400 µg/ml), LPS standard and after 30 min incubation of Pycnogenol® with LPS standard (50 and 550 pg/ml) expressed in LPS units EU/ml. (D) Catechin pre-incubated with LPS (10 ng/ml) for 30 min before addition to TLR4+ HEK293 cells for 24 h. Results of (A), (B) and (D) are expressed as fold change of fluorescence intensity relative to cells stimulated with 10 ng/ml LPS. All data (n = 4 technical replicates from two independent experiments) are expressed as mean ± SD. Significant differences are indicated by asterisks: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. CAT: catechin; LPS: lipopolysaccharide; PYC: Pycnogenol®; TLR: Toll-like receptor.

higher levels of IL-1β, IL-8 and TNF-α when compared to stimulation only with LPS at the same concentration. When costimulated with LPS, 400 and 100 µg/ml Pycnogenol® caused a 26- and 3-fold increase in IL-1β, a 7- and 2.5-fold increase in IL-8, and a 75- and 21-fold increase in TNF-α concentration, resp. No effect of costimulation of catechin with LPS on cytokine levels was observed (Figure 4.6H).

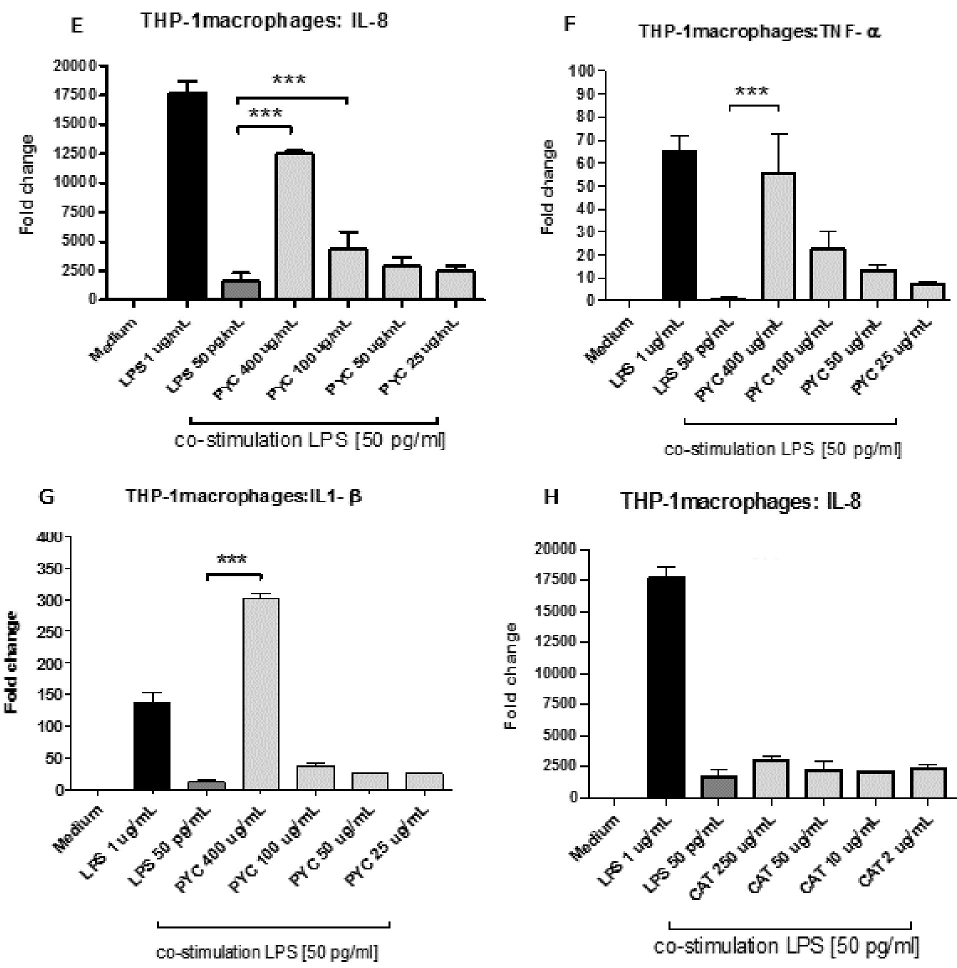

Figure 4.6 (part 2). Pycnogenol® and LPS form complexes, which boost activation of TLR4. Concentrations of (E) IL-8, (F) TNF-α and (G) IL-1β in supernatants of THP-1 macrophages after incubation with LPS (1 µg/ml and 50 pg/ml) and with Pycnogenol®-LPS complexes formed during 30 min pre-incubation of LPS (50 pg/ml) with different concentrations of Pycnogenol®. (H) Concentration of IL-8 in supernatants of THP-1 macrophages after incubation with LPS (1 µg/ml and 50 pg/ml) and with both catechin and LPS after 30 min pre-incubation of LPS (50 pg/ml) with different concentrations of catechin. Results are expressed relative to cells stimulated with 50 µg/ml LPS. All data (n = 4 technical replicates from two independent experiments) are expressed as mean ± SD. Significant differences are indicated by asterisks: *** $p < 0.001$. CAT: catechin; IL: interleukin; LPS: lipopolysaccharide; PYC: Pycnogenol®; TNF: tumour necrosis factor.

4.3 Metabolised samples

4.3.1 Effects on TLRs

Pycnogenol® was subjected to GI metabolism in the presence of human faecal suspension. Retentate and dialysate fractions from different time points (4 h, 10 h, 27 h and 36 h) were tested for their potential to activate TLRs using both HEK293 cells and cultured THP-1 macrophages.

When comparing the control retentate samples containing enzymes and bacteria to the test retentate samples containing Pycnogenol®, enzymes and bacteria, test samples collected at time points 10 h, 27 h and 36 h showed significant suppressive effects upon stimulation of HEK293 cells expressing TLR1/2 and TLR2/6 (Figure 4.7A,C). The control samples stimulated TLR1/2 on average four times more than 50 ng/ml PAM3 positive control for time points 10 h, 27 h and 36 h. This level of stimulation was reduced by the presence of Pycnogenol® metabolites to a 1.4 times higher stimulation than the PAM3 positive control. The same significant reduction of receptor activation was observed upon the stimulation of HEK293 cells expressing TLR2/6. No clear effect of the retentate containing Pycnogenol® metabolites on the stimulation of HEK293 cells expressing TLR4 and TLR5 was observed (Figure 4.7E,G).

When comparing the control dialysate fractions containing enzymes and bacteria with the fractions enriched with Pycnogenol®, significantly higher stimulation was observed by the fraction containing Pycnogenol® metabolites collected at time point 4 h for HEK293 cells expressing TLR1/2, TLR2/6 and TLR4 (Figure 4.7B,D,F). These differences declined with time of metabolism, showing even an inverted tendency for TLR2/6, where the activation level of the receptor was significantly reduced by fractions enriched with Pycnogenol® when compared to control at time point 36 h. A similar but non-significant tendency was observed for TLR1/2 and TLR4. No effect of the dialysates containing Pycnogenol® metabolites on the stimulation of HEK293 cells expressing TLR5 was observed (Figure 4.7H).

4.3.2 Effects on cytokine production

The dialysed fractions collected at time points 4 h and 10 h were incubated with THP-1 macrophages to study their immunogenicity by measuring the cytokine profile (Figure 4.8). The levels of TNF-α, IL-1β, IL-6 and IL-10 in the supernatants were evaluated after 18 h incubation with or without PAM3 as costimulant. Neither the control dialysate fraction containing enzymes and bacteria nor the dialysate fraction containing also Pycnogenol® metabolites induced the secretion of any of the pro-inflammatory cytokines (TNF-α, IL-1β, IL-6; Figure 4.8A,E,G) while the dialysate fraction containing Pycnogenol®

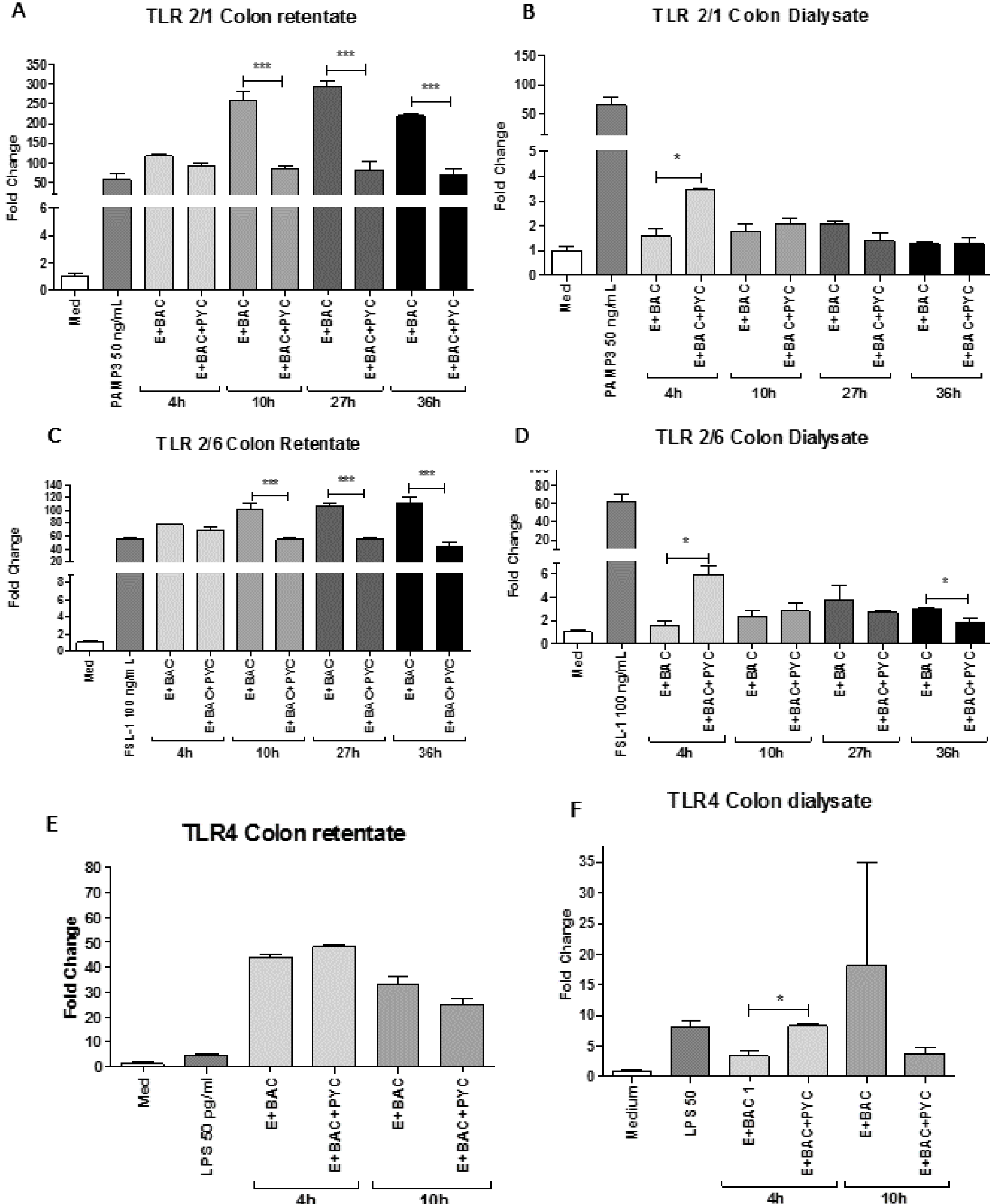

Figure 4.7 (part 1). The effect of GI metabolism on the interaction of Pycnogenol® with TLRs. During GI metabolism, retentate and dialysate samples were taken at time points 4 h, 10 h, 27 h and 36 h and used for stimulation of HEK293 cells expressing (A,B) TLR1/2, (C,D) TLR2/6 and (E,F) TLR4, which were also stimulated with their known ligands as positive control (PAM3, PAM2 (FSL-1) and LPS, resp.). After 24 h incubation, luciferase activity was measured and expressed as fold change of fluorescence intensity relative to cells grown in medium. Data are expressed as mean ± SD of n = 4 technical replicates from two independent experiments. Significant differences are indicated by asterisks: * $p < 0.05$; *** $p < 0.001$.
BAC: intestinal bacteria; E: enzymes; Med: medium; PYC: Pycnogenol®; TLR: Toll-like receptor.

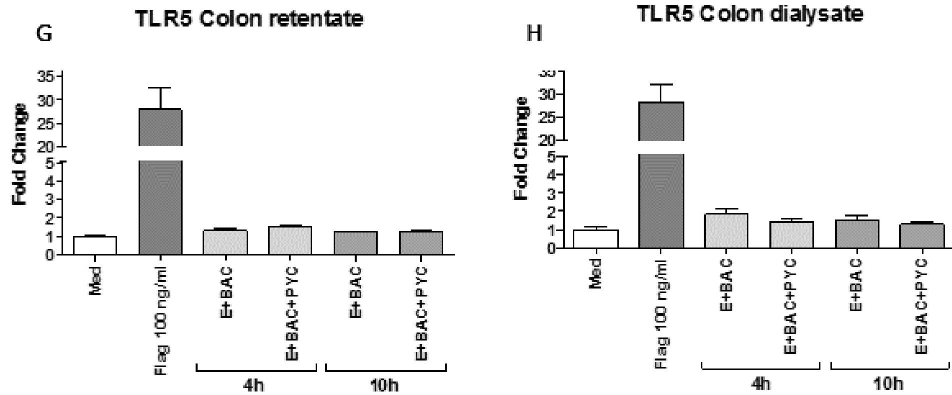

Figure 4.7 (part 2). The effect of GI metabolism on the interaction of Pycnogenol® with TLRs. During GI metabolism, retentate and dialysate samples were taken at time points 4 h, 10 h, 27 h and 36 h and used for stimulation of HEK293 cells expressing (G,H) TLR5, which were also stimulated with their known ligand as positive control (flagellin). After 24 h incubation, luciferase activity was measured and expressed as fold change of fluorescence intensity relative to cells grown in medium. Data are expressed as mean ± SD of n = 4 technical replicates from two independent experiments.

BAC: intestinal bacteria; E: enzymes; Med: medium; PYC: Pycnogenol®; TLR: Toll-like receptor.

metabolites significantly induced the production of IL-10 compared to cells incubated with control dialysate fractions (Figure 4.8C). The significant induction of IL-10 was also observed in the presence of PAM3 for both the control dialysate containing enzymes and bacteria as well as the dialysate sample containing Pycnogenol® metabolites. However, the samples containing Pycnogenol® showed significantly lower capacity to induce IL-10 when compared to the control. Nevertheless, both samples were shown to significantly shift the balance of cytokines produced by PAM3-stimulated macrophages towards regulatory IL-10 secretion (Figure 4.8D).

4.3.3 Effects on the expression of CD83, TLR1,TLR5 and TLR6

THP-1 macrophages were incubated for 18 h with dialysates of metabolised Pycnogenol® and the level of expression of the receptors CD83, TLR1, TLR5 and TLR6 was evaluated (Figure 4.9). A significantly enhanced TLR1 and TLR5 expression was observed upon incubation of THP-1 macrophages with the dialysate fraction containing Pycnogenol® metabolites compared to the control dialysate fraction containing only enzymes and bacteria (Figure 4.9B,C). This effect was visible only for time point 4 h of GI metabolism and was abolished after 10 h. The expression of the costimulatory molecule CD83 on the surface of THP-1 macrophages was not affected by the dialysate fraction containing Pycnogenol® metabolites or by the control fraction.

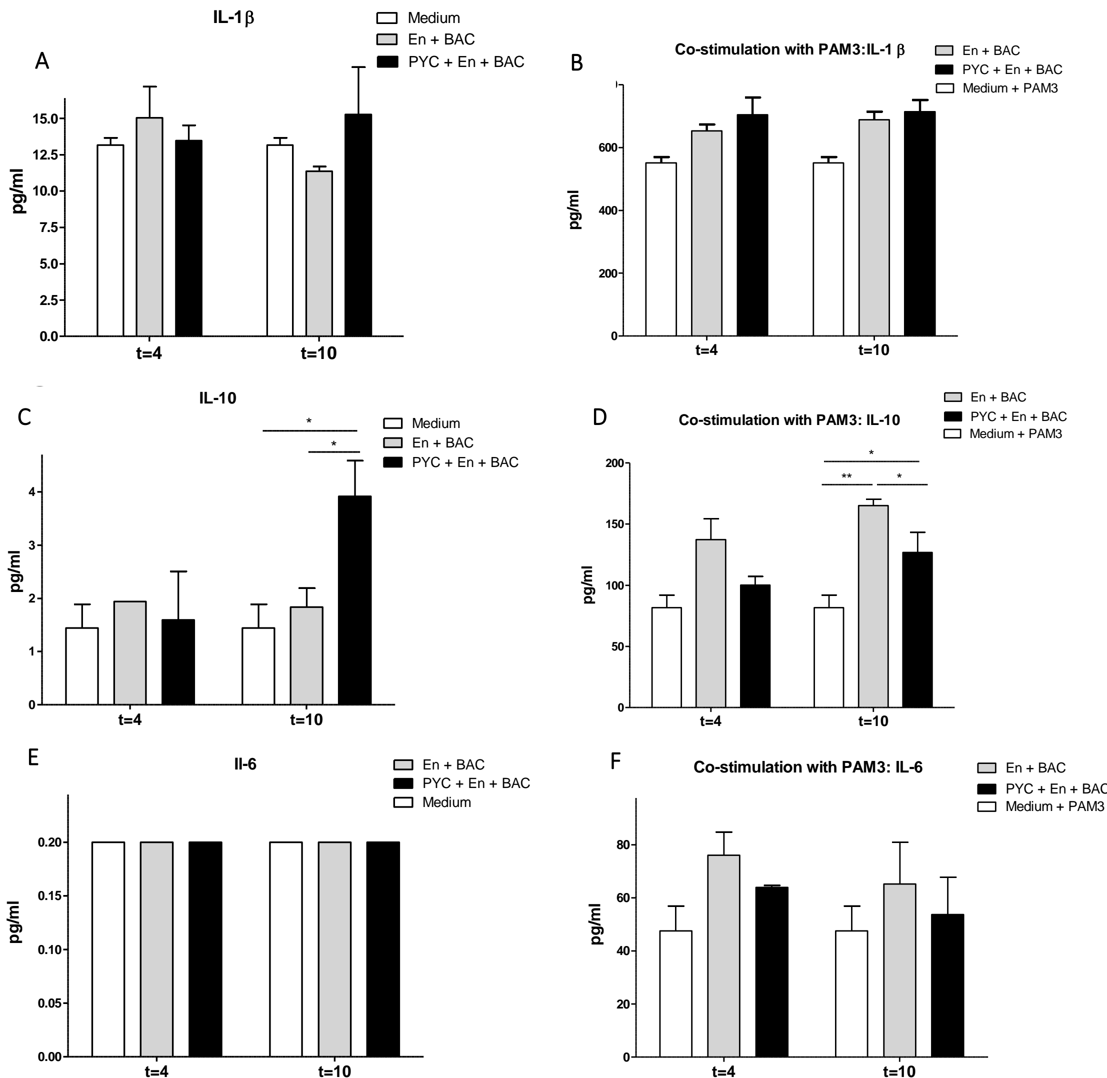

Figure 4.8 (part 1). Effect of metabolised Pycnogenol® on cytokine secretion. THP-1 macrophages were incubated with dialysates collected after 4 h and 10 h of metabolism with (A,C,E) and without (B,D,F) costimulation with the known ligand PAM3 (0.1 µg/ml). After incubation, the concentration of (A,B) IL-1β, (C,D) IL-10 and (E,F) IL-6 in the supernatants was determined. All data are expressed as mean ± SD of n = 4 independent replicates. Significant differences are indicated by asterisks: * $p < 0.05$; ** $p < 0.01$.
BAC: intestinal bacteria; E: enzymes; IL: interleukin; PYC: Pycnogenol®.

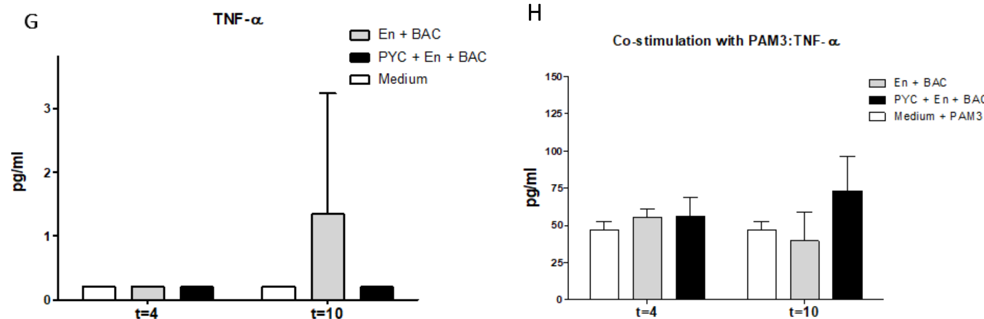

Figure 4.8 (part 2). Effect of metabolised Pycnogenol® on cytokine secretion. THP-1 macrophages were incubated with dialysates collected after 4 h and 10 h of metabolism with (G) and without (H) costimulation with the known ligand PAM3 (0.1 µg/ml). After incubation, the concentration of TNF-α in the supernatants was determined. All data are expressed as mean ± SD of n = 4 independent replicates.
BAC: intestinal bacteria; E: enzymes; PYC: Pycnogenol®; TNF: tumour necrosis factor.

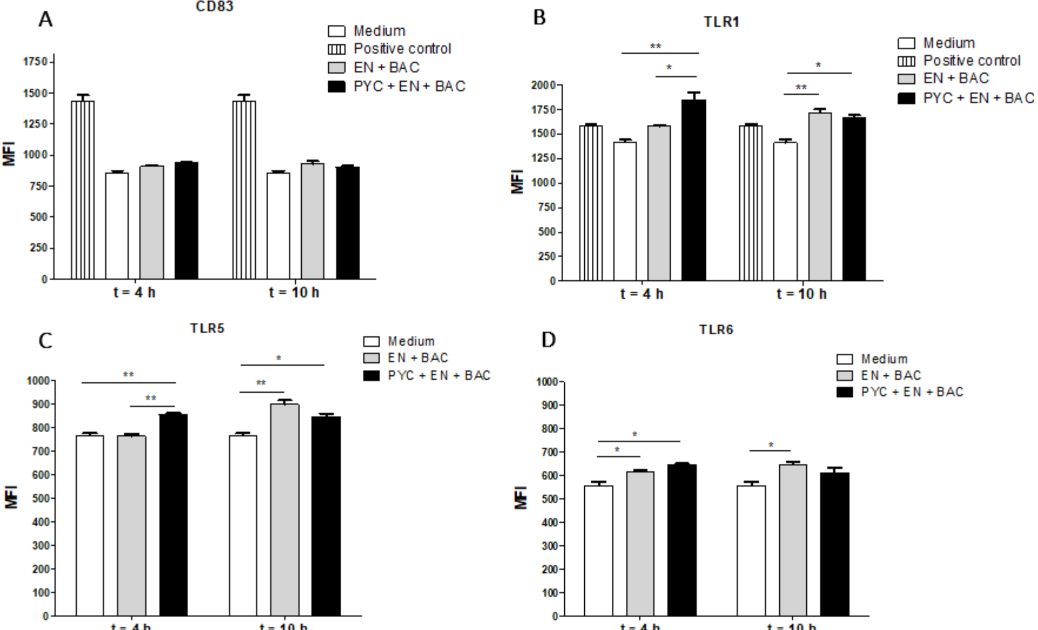

Figure 4.9. Effect of metabolised Pycnogenol® (dialysates) on the expression of (A) CD83, (B) TLR1, (C) TLR5 and (D) TLR6 on THP-1 macrophages. THP-1 macrophages were incubated for 18 h with dialysates collected after 4 h and 10 h of metabolism or PAM3 as positive control. After incubation, cells were harvested and the expression of CD83, TLR1, TLR5 and TLR6 was determined and expressed as differences in mean fluorescence intensity (MFI). All data are expressed as mean ± SD of n = 4 independent replicates. Significant differences are indicated by asterisks: * $p < 0.05$; ** $p < 0.01$.
BAC: intestinal bacteria; CD: cluster of differentiation; E: enzymes; PYC: Pycnogenol®; TLR: Toll-like receptor.

4.3.4 Effects on the microbial profile

To assess the effect of Pycnogenol® on the bacterial composition of retentate samples, Illumina HiSeq sequencing of PCR-amplified 16S rRNA gene amplicons was used (Figure 4.10). In the 4 h sample, the addition of Pycnogenol® resulted for instance in an increase for the genera *Collinsella* (d = +8% points), *Streptococcus* (+4%) and *Bifidobacterium* (+3%) and a decrease for *Eubacterium hallii* (-11%), *Anaerostipes* (-5%) and *Lachnospiraceae_UCG-004* (-5%). For the samples from time point 10 h, the Pycnogenol® sample for instance presented increased abundances for the genera *Enterococcus* (+5) and *Streptococcus* (+4%), while *Blautia* (-9%), *Dorea* (-4%) and *Veillonella* (-4%) were decreased (Figure 4.10C). No differences could be observed for *Collinsella*. Consistent changes at both time points were observed for *Streptococcus*, *Sutterella*, *Bacteroides* and *Bifidobacterium,* which increased in the Pycnogenol® sample, while relative abundances of *Dorea*, *Lachnospiraceae_UCG-004*, *Anaerostipes* and *Eubacterium hallii* were decreased.

Furthermore, alpha diversity was calculated using the Shannon index, which accounts for both evenness and richness of a community, and Faith Phylogenetic diversity, which yielded results that were comparable between the different samples (Figure 4.10B). This suggests that the diversity of the community is stable, despite the changes in the bacterial composition. To determine whether the absolute number of bacteria was different due to Pycnogenol®, RT-qPCR was performed. No differences in the absolute concentration of the bacteria between samples were observed (Figure 4.10A).

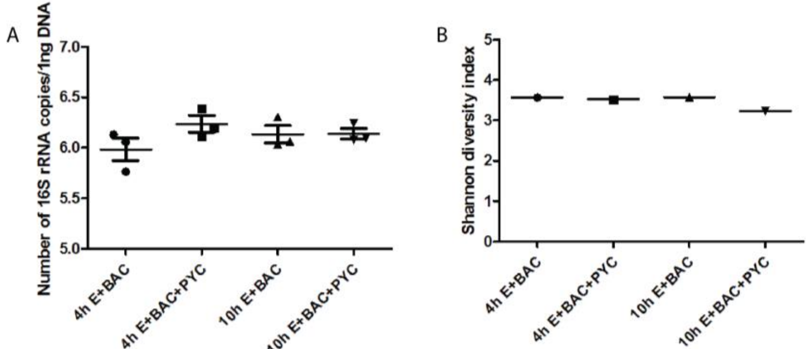

Figure 4.10 (part 1). Effect of Pycnogenol® metabolites on microbial composition. (A) Absolute abundance of bacteria present in the four samples, determined by qPCR, with total counts log 10 transformed. (B) Alpha diversity. In A, mean and SD are shown of n = 3 independent determinations.
BAC: intestinal bacteria (human faecal suspension); E: enzymes; PYC: Pycnogenol®.

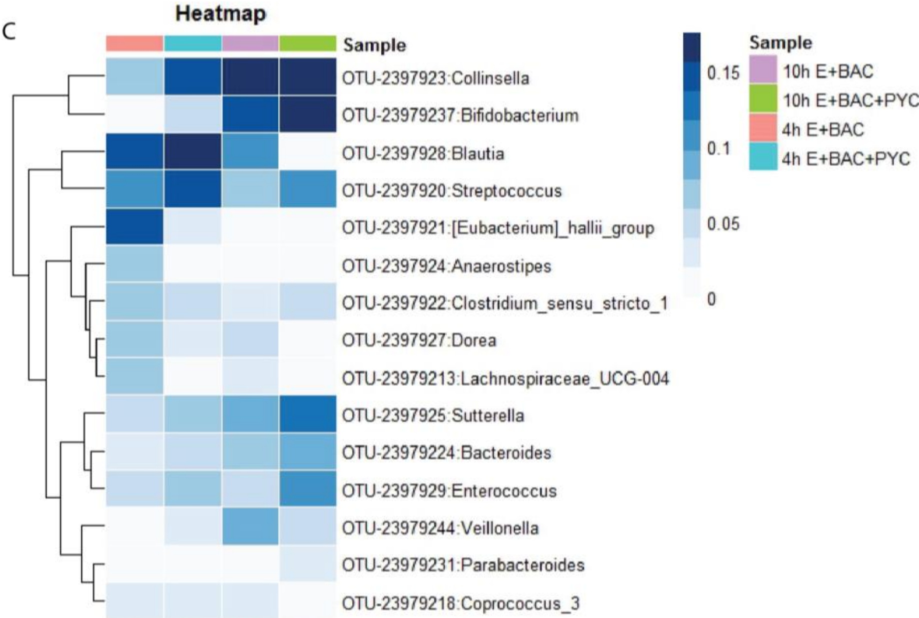

Figure 4.10 (part 2). Effect of Pycnogenol® metabolites on microbial composition. (C) Heatmap representing the abundances of the fifteen most abundantly present bacterial genera.
BAC: intestinal bacteria; E: enzymes; OTU: Operational Taxonomic Units; PYC: Pycnogenol®.

4.3.5 Effects on Pycnogenol® composition

As expected, fingerprint chromatographic analysis of Pycnogenol® GIDM dialysates (Figure 4.11) revealed a different phenolic composition of the metabolised sample as compared to Pycnogenol® reference and control sample without Pycnogenol®. This is possibly due to microbial metabolism, though also medium interference should be taken into account. The exact composition of dialysates could not be revealed by HPLC analysis, due to the presence of various unknown compounds in Pycnogenol® [43].

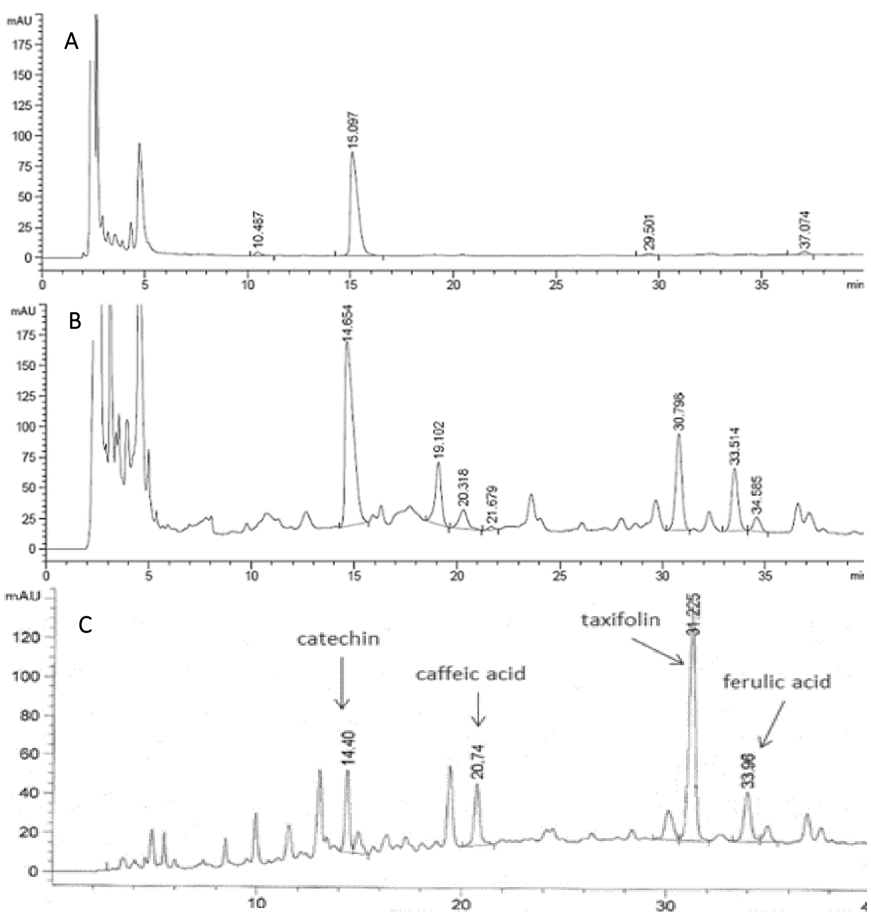

Figure 4.11. Fingerprint chromatograms of (A) 4 h GIDM blank dialysate sample containing only enzymes and bacteria, (B) 4 h GIDM dialysate sample containing Pycnogenol®, enzymes and bacteria, (C) 20 mg/ml USP Pycnogenol® reference in 20% MeOH.

GIDM: gastrointestinal dialysis model; USP: United States Pharmacopeia.

PART 5

DISCUSSION

TLRs on macrophages are involved in inflammatory responses through recognition of different structures of DAMPs and PAMPs. Till now, 13 TLRs (TLR1-TLR13) have been described to be involved in various phases of the inflammatory response including release of pro-inflammatory cytokines/ chemokines and antimicrobial peptides [44-47]. These signals attract immune cells like macrophages, NK cells and mast cells, which as a consequence may release ROS and RNS [48]. ROS production not only directly causes cell injury and initiates associated degenerative processes, but can also act as a signal for other processes, such as pro-inflammatory pathways involving NF-κB activation. The antioxidant properties of Pycnogenol® have been elucidated in a variety of *in vitro* and *in vivo* studies [14, 15, 17, 33, 49-54]. Additional to its radical scavenging activity, inhibition of NF-κB-dependent gene expression as well as inhibition of production and activity of various pro-inflammatory mediators and adhesion molecules was observed after incubation of different cell types with Pycnogenol® [22, 26, 28, 30, 35, 55]. Despite many studies showing that Pycnogenol® attenuates inflammation via suppressing oxidative stress, the mechanism of action remains largely unknown. The study of Luo *et al.* [34] showed that Pycnogenol® attenuated LPS-induced lipid droplet formation through the TLR4 and NF-κB pathway. Additionally, other studies suggested the involvement of Pycnogenol® in suppression of the TLR4-related pro-inflammatory cascade [35, 55].

In the present study with TLR4-transfected HEK293 cells, no inhibitory (antagonistic) action of Pycnogenol® against LPS-induced TLR4 activation was observed, which is in contradiction with data presented in literature. The differences between our results and the outcomes of others [34, 56], who showed that Pycnogenol® reduces TLR4 signalling, are most likely caused by mechanistic differences in the cell lines used. Liu and colleagues for instance used mouse macrophages, while in the present study, HEK293 cells transfected with human TLR4 were used [56]. There are a few possible mechanisms of blocking of the TLR signalling pathway, including (a) extracellular conformational blocking of the binding of selective ligands to the receptor, resulting in impaired signal transduction, and (b) intracellular small molecule inhibitors that block intracellular TLR signal transduction [57]. It has been reported that Pycnogenol® blocks the TLR-JNK (c-Jun N-terminal kinase) pathway, but the exact mechanisms of action as well as the compounds responsible have not been elucidated. Therefore, it is

possible that some of the small size Pycnogenol® compounds are able to cross cell membranes and act on specific intracellular adapter proteins or compartments along the TLR signalling pathways, which has been described in the literature as the mechanism of action of certain drugs. Such a mechanism includes the inhibition of mitogen-activated protein kinase (MAPK) signalling and phospholipase A2, antiproliferative effects and reduction of MMP-9 activity or binding to the intracellular TIR domain of TLR4, which all lead to diminished LPS-induced TLR4 signalling and inflammation [57, 58]. Taking these possibilities into account, HEK cells may not express the appropriate transporter(s) for certain Pycnogenol® compounds, which could explain the differences in the observed results.

It was found that Pycnogenol® and catechin do not function as agonist or antagonist for TLR4 on their own in the HEK293 model. However, in the presence of costimulators like LPS, Pycnogenol® acts as an agonist. It was shown that Pycnogenol® and LPS form complexes that show enhanced binding and/or activation of TLR4. It is likely that LPS and Pycnogenol® form complexes through hydrophobic interactions, as both have large hydrophobic portions [59]. Previous studies confirm the potential of Pycnogenol® to interact with proteins (xanthine oxidase) through hydrophobic bonding [60]. Although it is not known which compound(s) in Pycnogenol® extract form complexes with LPS, our data indicate that it is not its main monomer catechin.

In the present study, the involvement of TLRs other than TLR4 (TLR1/2, TLR2/6 and TLR5) in the immunomodulatory effects of Pycnogenol® was elucidated. Moreover, for the first time, the bioactivity of non-metabolised Pycnogenol® was compared with samples after GI metabolism.

It was shown that, without costimulation with known agonists, non-metabolised Pycnogenol® induced activation of TLR1/2, TLR2/6 and TLR5, though only in concentrations higher than 25 µg/ml. Costimulation of HEK293 cells expressing TLR1/2 and TLR2/6 with agonists of these receptors (PAM3 and PAM2, resp.) in the presence of Pycnogenol® did not result in any reduction of receptor activation, suggesting no antagonistic action of Pycnogenol® against these receptors. Pycnogenol® thus acts as agonist of TLR1/2 and TLR2/6. However, Pycnogenol® dose-dependently inhibited the activation of TLR5 when HEK293 cells were costimulated with flagellin. This finding suggests that Pycnogenol® may be a partial agonist of TLR5. In the presence of Pycnogenol®, the full agonist of TLR5 (flagellin) is unable to bind to the receptor and activate it fully. Without the presence of the full agonist, Pycnogenol® acts as a ligand that binds to TLR5 receptor binding site to induce some conformational change without leading to full activation of the receptor (Fig 4.3C). This phenomenon was already described for underacylated and underphosphorylated derivatives of lipid A that have partial TLR4 agonist properties, resulting in weak agonistic properties but also antagonistic properties in the presence of

full agonist [61, 62]. The agonistic effect of non-metabolised Pycnogenol® against TLR1/2 and TLR2/6 may for example be responsible for the immunomodulatory effects when administrated orally. Therefore, in the presented work, the question relevant for the *in vivo* situation, what happens to the immunomodulatory properties of Pycnogenol® after microbial metabolism, was investigated.

The investigation of immunomodulatory properties of metabolised Pycnogenol® concerned the analysis of retentate and dialysate fractions. The retentate samples containing Pycnogenol® metabolised in the presence of enzymes and bacteria showed a significantly lower level of activation of TLR1/2 and TLR2/6 compared to control samples with enzymes and bacteria only (Figure 4.7A,C). These data suggest that GI metabolites of Pycnogenol® (retentate fraction) show antagonistic or partial agonistic effects on TLR1/2 and TLR2/6. TLR1/2 and TLR2/6 signalling was impaired by Pycnogenol® metabolites. Therefore, in the presence of Pycnogenol® metabolites, the full agonist (bacteria and their fragments present in the retentate) was unable to bind to the receptor, thereby not leading to full activation as was observed in the control samples without Pycnogenol®. This effect was not caused by the differences in bacterial numbers in the samples (Figure 4.10A), but might be partially explained by differences in microbial composition caused by Pycnogenol® (Figure 4.10C).

The dialysate fraction of time point 4 h containing Pycnogenol® metabolites was able to activate TLR1/2, TLR2/6 and TLR4, but this effect was not observed for any further time points (Figure 4.7B,D,F). The activation was small (less than 5-fold), albeit significant, as compared to the more than 40-fold increased activation with retentate fractions. When THP-1 macrophages were exposed to dialysed Pycnogenol® metabolites, an enhanced expression of TLR1 and TLR5 was observed for time point 4 h compared to cells exposed to the control samples with enzymes and bacteria only. These findings suggest that metabolites of Pycnogenol® can activate TLR-dependent signal transduction pathways. However, this activation is limited, as reflected by the almost complete absence of pro-inflammatory cytokine levels. Still, enhanced IL-10 secretion was observed by THP-1 macrophages incubated with metabolised Pycnogenol® (dialysate fraction). IL-10 is often secreted as a feedback inhibitor during inflammation initiated by LPS or tissue damage [63], though in our study, levels of pro-inflammatory cytokines were not altered (Figure 4.8). An increased level of IL-10 secreted by THP-1 macrophages may be explained by the activation of TLRs, as it was already shown for intestinal epithelial cells [64]. Nevertheless, additional studies are needed to confirm this hypothesis. An increased release of IL-10 by THP-1 macrophages upon stimulation with the dialysate fraction containing Pycnogenol® metabolites confirms the anti-inflammatory properties of Pycnogenol® reported by other studies [16,

17, 20, 21, 24, 36, 65-69] and suggests that Pycnogenol® may help in balancing intestinal homeostasis, especially during chronic inflammatory conditions.

Because Pycnogenol® is a complex mixture of polyphenols, it is difficult to speculate about the specific compounds or metabolites which may exert the described effects. One of the known metabolites of catechin, M1 (δ-(3,4-dihydroxy-phenyl)-γ-valerolactone), was already shown to be taken up by macrophages via facilitated transport, where it accumulates and undergoes further intracellular metabolism to levels that could be bioactive [32, 43, 70]. Our results show that catechin acts as antagonist of TLR4 and TLR5, but only at the highest concentration (250 µg/ml). In addition, Pycnogenol® displayed stronger biological activity as a mixture than its primary monomer. Previous research has already indicated that its components act synergistically [71].

Another explanation for the observed inhibition of TLR signalling by Pycnogenol® may be the influence of Pycnogenol® and its metabolites on gut microbiota composition. Although this result needs to be interpreted with caution because a strong effect of digestion time on microbial composition was also observed, the sample containing Pycnogenol® showed differences in microbial composition when compared to the control sample for both analysed time points. It was observed that Pycnogenol® modulates the microbial composition for instance by increasing the presence of *Bifidobacterium*, *Streptococcus*, *Sutterella* and *Bacteroides*. Therefore, the different diversity of PAMPs may also influence the levels of activation of the different TLRs by the metabolised samples. There are currently no data on the specific effect of Pycnogenol® on microbial composition, but it has been shown that dietary polyphenols affect gut microbiota [72-75] as well as the expression of TLRs on immune cells [73]. Here, we showed that metabolised Pycnogenol® samples (retentates) are able to modify microbial composition and to inhibit TLR signalling (TLR1/2 and TLR2/6), although downstream signalling mechanisms of these activities need to be further investigated.

PART 6

CONCLUSION

In conclusion, this study showed that non-metabolised Pycnogenol® acts as agonist of TLR1/2 and TLR2/6 and as partial agonist of TLR5. Pycnogenol® on its own does not agonise or antagonise TLR4. However, after formation of complexes with LPS, it is a potent activator of TLR4 signalling.

GI digestion of Pycnogenol® reveals the biological activity of its metabolites as potential inhibitors of TLR1/2 and TLR2/6 signalling (retentate fraction) and inducers of IL-10 secretion from THP-1 macrophages (dialysate fraction). Moreover, Pycnogenol® was shown to influence the intestinal microbial composition, though a more detailed study, including larger numbers of faeces donors, is required to get more insight into the microbial composition promoted by the presence of Pycnogenol® and its metabolites.

Based on the presented data, microbially metabolised Pycnogenol® can show local gut (retentate) but also systemic (dialysate) immunomodulatory effects via (1) inhibition of TLR signalling by acting as an antagonist or a partial agonist; (2) alteration of microbial composition by directly or indirectly (via metabolites) affecting specific microbial growth; and (3) affecting signal transduction, by interfering with transcription factors involved in inflammatory and immune response activation (upregulation of surface markers on immune cells and increased expression of IL-10). The molecular mechanisms underlying these immunomodulatory activities are not completely characterised and need to be elucidated in further studies. The present data represent a pioneer study on the potential role of Pycnogenol® in promoting specific health conditions due to its immunomodulatory activity, next to its well-documented antioxidant and free radical scavenging properties.

REFERENCES

1. Abbas, A.K., A.H. Lichtman, and S. Pillai, Cellular and Molecular Immunology. 2015, Philadelphia: Elsevier Saunders.
2. Guha, M. and N. Mackman, LPS induction of gene expression in human monocytes. Cell Signall, 2001. 13: p. 85-94.
3. Kikuchi, T., et al., Clarithromycin suppresses lipopolysaccharide-induced interleukin-8 production by human monocytes through AP-1 and NF-kappa B transcription factors. J Antimicrob Chemother, 2002. 49(5): p. 745-755.
4. Charlab, R., et al., Effect of unique Mycobacterium leprae phenolic glycolipid-I (PGL-I) on tumour necrosis factor production by human mononuclear cells. Leprosy Rev, 2001. 72: p. 63-69.
5. Schroecksnadel, S., M. Jenny, and D. Fuchs, Myelomonocytic THP-1 cells for in vitro testing of immunomodulatory properties of nanoparticles. J Biomed Nanotechnol, 2011. 7: p. 209-210.
6. Shin, H.Y., et al., Regulation of cytokine production by treating with Chinese tonic herbs in human peripheral blood mononuclear and human acute monocytic leukemia cells. J Food Drug Anal, 2010. 18: p. 414-424.
7. Chanput, W., et al., Transcription profiles of LPS-stimulated THP-1 monocytes and macrophages: a tool to study inflammation modulating effects of food-derived compounds. Food Funct, 2010. 1(3): p. 254 - 261.
8. Yang, C.W., et al., Curcumin induces the apoptosis of human monocytic leukemia THP-1 cells via the activation of JNK/ERK Pathways. BMC Complement Altern Med, 2012. 22.
9. Chanput, W., et al., β-Glucans are involved in immune-modulation of THP-1 macrophages. Mol Nutr Food Res, 2012. 5(822-833).
10. Håversen, L., et al., Induction of proinflammatory cytokines by longchain saturated fatty acids in human macrophages. Atherosclerosis, 2009. 202: p. 382-393.
11. Graham, F.L., et al., Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol, 1977. 36(1): p. 59-74.
12. Shaw, G., et al., Preferential transformation of human neuronal cells by human adenoviruses and the origin of HEK 293 cells. FASEB J, 2002. 16(8): p. 869-871.
13. Swiech, K., V. Picanco-Castro, and D.T. Covas, Human cells: new platform for recombinant therapeutic protein production. Protein Expr Purif, 2012. 84: p. 147-153.
14. Wei, Z.H., Q.L. Peng, and B.H.S. Lau, Pycnogenol enhances endothelial cell antioxidant defenses. Redox Rep, 1997. 3(4): p. 219-224.
15. Noda, Y., et al., Hydroxyl and superoxide anion radical scavenging activities of natural source antioxidants using the computerized JES-FR30 ESR spectrometer system. Biochem Mol Biol Int, 1997. 42(1): p. 35-44.
16. Shin, I.S., et al., Inhibitory effects of Pycnogenol® (French maritime pine bark extract) on airway inflammation in ovalbumin-induced allergic asthma. Food Chem Toxicol, 2013. 62: p. 681-686.
17. Packer, L., G. Rimbach, and F. Virgili, Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (Pinus maritima) bark, Pycnogenol. Free Rad Biol Med, 1999. 27(5/6): p. 704-724.
18. Devaraj, S., et al., Supplementation with a pine bark extract rich in polyphenols increases plasma antioxidant capacity and alters the plasma lipoprotein profile. Lipids, 2002. 37(10): p. 931-934.
19. Ryan, J., et al., An examination of the effects of the antioxidant Pycnogenol on cognitive performance, serum lipid profile, endocrinological and oxidative stress biomarkers in an elderly population. J Psychopharmacol, 2008. 22(5): p. 553-562.
20. Choi, Y.H. and G.H. Yan, Pycnogenol inhibits immunoglobulin E-mediated allergic response in mast cells. Phytother Res, 2009. 23(12): p. 1691-1695.
21. Cho, K.J., et al., Effect of bioflavonoids extracted from the bark of Pinus maritima on proinflammatory cytokine interleukin-1 production in lipopolysaccharide-stimulated RAW 264.7. Toxicol Appl Pharmacol, 2000. 168(1): p. 64-71.
22. Peng, Y.J., et al., Pycnogenol attenuates the inflammatory and nitrosative stress on joint inflammation induced by urate crystals. Free Rad Bio Med, 2012. 52(4): p. 765-774.
23. Cho, K.J., et al., Inhibition mechanisms of bioflavonoids extracted from the bark of Pinus maritima on the expression of proinflammatory cytokines. Ann N Y Acad Sci, 2001. 928: p. 141-146.
24. Liu, Z., et al., Pycnogenol Ameliorates Asthmatic Airway Inflammation and Inhibits the Function of Goblet Cells. DNA Cell Biol, 2016 35(11): p. 730-739.
25. Fan, B., et al., Pycnogenol attenuates the release of proinflammatory cytokines and expression of perilipin 2 in lipopolysaccharide-stimulated microglia in part via inhibition of NF-κB and AP-1 activation. PLoS One, 2015. 10(9).

26. Peng, Q., Z. Wei, and B.H. Lau, Pycnogenol inhibits tumor necrosis factor-alpha-induced nuclear factor kappa B activation and adhesion molecule expression in human vascular endothelial cells. Cell. Mol. Life Sci., 2000. 57: p. 834-841.
27. Schafer, A., et al., Inhibition of COX-1 and COX-2 activity by plasma of human volunteers after ingestion of French maritime pine bark extract (Pycnogenol). Biomed Pharmacother, 2006. 60(1): p. 5-9.
28. Grimm, T., A. Schäfer, and H. P., Antioxidant activity and inhibition of matrix metalloproteinases by metabolites of maritime pine bark extract (Pycnogenol). Free Radic Biol Med, 2004. 36(6): p. 811-822.
29. Canali, R., et al., The anti-inflammatory pharmacology of Pycnogenol in humans involves COX-2 and 5-LOXmRNA expression in leukocytes. Int Immunopharmacol, 2009. 9(10): p. 1145-1149.
30. Kim, Y.J., Y.A. Kim, and T. Yokozawa, Pycnogenol modulates apoptosis by suppressing oxidative stress and inflammation in high glucose-treated renal tubular cells. Food Chem Toxicol, 2011. 49(9): p. 2196-2201.
31. Wilson, D., et al., A randomized, double-blind, placebo-controlled exploratory studyto evaluate the potential of pycnogenol for improving allergic rhinitis. Phytother Res, 2010. 24(8): p. 1115-1119.
32. Uhlenhut, K. and P. Hogger, Facilitated cellular uptake and suppression of inducible nitric oxide synthase by a metabolite of maritime pine bark extract (Pycnogenol). Free Radic Biol Med, 2012. 53(2): p. 305-13.
33. Parveen, K., et al., Modulatory effects of Pycnogenol in a rat model of insulin-dependent diabetes mellitus: biochemical, histological, and immunohistochemical evidences. Protoplasma, 2013. 250(1): p. 347-360.
34. Luo, H., et al., Pycnogenol attenuates atherosclerosis by regulating lipid metabolism through the TLR4-NF-κB pathway. Exp Mol Med, 2015. 47.
35. Gu, J.Q., et al., Pycnogenol, an extract from French maritime pine, suppresses Toll-like receptor 4-mediated expression of adipose differentiation-related protein in macrophages. Am J Physiol Endocrinol Metab, 2008. 295: p. E1390-E1400.
36. Grimm, T., et al., Inhibition of NF-κB activation and MMP-9 secretion by plasma of human volunteers after ingestion of maritime pine bark extract (Pycnogenol). J Inflam, 2006. 3(1): p. 1-6.
37. Breynaert, A., et al., Development and Validation of an in vitro Experimental GastroIntestinal Dialysis Model with Colon Phase to Study the Availability and Colonic Metabolisation of Polyphenolic Compounds. Planta Medica, 2015. 81(12-13): p. 1075-1083.
38. van Splunter, M., et al., Bovine Lactoferrin Enhances TLR7-Mediated Responses in Plasmacytoid Dendritic Cells in Elderly Women: Results From a Nutritional Intervention Study With Bovine Lactoferrin, GOS and Vitamin D. Front Immunol, 2018. 9: p. 1-12.
39. Zoetendal, E.G., et al., Isolation of DNA from bacterial samples of the human gastrointestinal tract. Nat Protoc, 2006. 1(2): p. 870-873.
40. Ramiro-Garcia, J., et al., NG-Tax, a highly accurate and validated pipeline for analysis of 16S rRNA amplicons from complex biomes. Version 1. F1000Research, 2016. 5: p. 1-44.
41. Nadkarni, M.A., et al., Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiol, 2002. 148: p. 257-266.
42. Quast, C., et al., The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res, 2013. 41: p. D590-D596.
43. Grimm, T., et al., Single and multiple dose pharmacokinetics of maritime pine bark extract (Pycnogenol) after oral administration to healthy volunteers. BMC Clin Pharmacol, 2006. 6(4): p. 1-12.
44. Jiménez-Dalmaroni, M.J., M.E. Gerswhin, and I.E. Adamopoulos, The critical role of toll-like receptors--From microbial recognition to autoimmunity: A comprehensive review. Autoimmun Rev, 2016. 15(1): p. 1-8.
45. Aalaei-andabili, S.H. and N. Rezaei, Toll like receptor (TLR)-induced differential expression of microRNAs (MiRs) promotes proper immune response against infections: a systematic review. J Infect, 2013. 67(4): p. 251-264.
46. Lin, E., J.E. Freedman, and L.M. Beaulieu, Innate immunity and toll-like receptor antagonsists: A potential role in the treatment of cardiovascular diseases. Cardiovasc Ther, 2009. 27(117-123).
47. Hug, H., M.H. Mohajeri, and G. La Fata Toll-Like Receptors: Regulators of the Immune Response in the Human Gut. Nutrients, 2018. 10.
48. Lucas, K. and M. Maes, Role of the Toll Like receptor (TLR) radical cycle in chronic inflammation: possible treatments targeting the TLR4 pathway. Mol Neurobiol, 2013. 48(1): p. 190-204.
49. Bayeta, E. and B.H.S. Lau, Pycnogenol inhibits generation of inflammatory mediators in macrophages. Nutr Res, 2000. 20(2): p. 249-259.
50. Parveen, K., et al., Protective effects of Pycnogenol on hyperglycemia-induced oxidative damage in the liver of type 2 diabetic rats. Chem Biol Interact, 2010. 186(2): p. 219-227.
51. Berryman, A.M., et al., Influence of treatment of diabetic rats with combinations of pycnogenol, beta-carotene, and alpha-lipoic acid on parameters of oxidative stress. J Biochem Mol Toxicol, 2004. 18(6): p. 345-352.
52. Bayomy, N.A., et al., Effect of pycnogenol and spirulina on vancomycin-induced renal cortical oxidative stress, apoptosis, and autophagy in adult male albino rat. Can J Physiol Pharmacol, 2016. 94(8): p. 838-848.
53. Lee, I.C., et al., Protective effects of pine bark extract on hexavalent chromium-induced dermatotoxicity in rats. Phytother Res, 2012. 26(10): p. 1534-1540.

54. Taner, G., et al., Modulating effects of Pycnogenol® on oxidative stress and DNA damage induced by sepsis in rats. Phytother Res, 2014. 28(11): p. 1692-1700.
55. Gao, B., et al., Pycnogenol Protects Against Rotenone-Induced Neurotoxicity in PC12 Cells Through Regulating NF-κB-iNOS Signaling Pathway. DNA and Cell Biology, 2015. 34(10): p. 643-649.
56. Liu, R., et al., Pycnogenol Reduces Toll-Like Receptor 4 Signaling Pathway-Mediated Atherosclerosis Formation in Apolipoprotein E-Deficient Mice. J Cardiovasc Pharmacol, 2016. 68(4): p. 292-303.
57. Gao, W., et al., Inhibition of Toll-Like Receptor Signaling as a Promising Therapy for Inflammatory Diseases: A Journey from Molecular to Nano Therapeutics. Front Physiol, 2017. 8: p. 1-20.
58. Kuznik, A., et al., Mechanism of endosomal TLR inhibition by antimalarial drugs and imidazoquinolines. J Immunol, 2011. 186(6): p. 4794-4804.
59. Na, Y.J., et al., Lactoferrin works as a new LPS-binding protein in inflammatory activation of macrophages. Int Immunopharmacol, 2004. 4(9): p. 1187-1199.
60. Moini, H., Q. Guo, and L. Packer, Enzyme inhibition and protein-binding action of the procyanidin-rich french maritime pine bark extract, pycnogenol: Effect on xanthine oxidase. J Agric Food Chem, 2000. 48: p. 5630-5639.
61. Piazza, M., et al., A synthetic lipid A mimetic modulates human TLR4 activity. ChemMedChem, 2012. 7(2): p. 213-217.
62. Bryant, C.E., et al., The molecular basis of the host response to lipopolysaccharide. Nat Rev Microbiol, 2010. 8(1): p. 8-14.
63. Moore, K.W., et al., Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol, 2001. 19: p. 683-765.
64. Latorre, E., et al., Toll-like receptors 2 and 4 modulate intestinal IL-10 differently in ileum and colon. United European Gastroenterol J., 2018. 6(3): p. 446-453.
65. Saliou, C., et al., Solar ultraviolet-induced erythema in human skin and nuclear factor-kappa-B-dependent gene expression in keratinocytes are modulated by a French maritime pine bark extract. Free Radic Biol Med, 201. 30(2): p. 154-160.
66. Nelson, A.B., et al., Pycnogenol inhibits macrophage oxidative burst, lipoprotein oxidation, and hydroxyl radical-induced DNA damage. Drug Dev Ind Pharm, 1998. 24(2): p. 139-144.
67. Peng, Y.J., et al., Pycnogenol attenuates the inflammatory and nitrosative stress on joint inflammation induced by urate crystals. Free Radic Biol Med, 2012. 52(4): p. 765-74.
68. Belcaro, G., et al., Pycnogenol improvements in asthma management. Panminerva Med, 2011. 53(3): p. 57-64.
69. Belcaro, G., et al., Variations in C-reactive protein, plasma free radicals and fibrinogen values in patients with osteoarthritis treated with Pycnogenol. Redox Rep, 2008. 13(6): p. 271-276.
70. Kurlbaum, M., M. Mülek, and P. Högger, Facilitated uptake of a bioactive metabolite of maritime pine bark extract (pycnogenol) into human erythrocytes. PLoS ONE, 2013. 8(4).
71. Yoshida, A., et al., Direct assessment by electron spin resonance spectroscopy of the antioxidant effects of French maritime pine bark extract in the maxillofacial region of hairless mice. J Clin Biochem Nutr, 2011. 49: p. 79-86.
72. Serra, D., L.M. Almeida, and T.C.P. Dinis, Dietary polyphenols: A novel strategy to modulate microbiota-gut-brain axis. Trends Fod Sci Technol, 2018. 78: p. 224-233.
73. Pérez-Cano, F.J., et al., Flavonoids Affect Host-Microbiota Crosstalk through TLR Modulation. Antiox, 2014. 3(4): p. 649-670.
74. Ahmed Nasef, N., S. Mehta, and L.R. Ferguson, Dietary interactions with the bacterial sensing machinery in the intestine: the plant polyphenol case. Front Genet, 2014. 5: p. 64.
75. Shimizu, M., Multifunctions of dietary polyphenols in the regulation of intestinal inflammation. J Food Drug Anal, 2017. 25(1): p. 93-99.

CHAPTER 5

EFFECT OF A POLYPHENOL-RICH PLANT EXTRACT ON ADHD: TRIAL SETUP AND PROCEDURES

Verlaet AAJ, Ceulemans B, Verhelst H, Van West D, De Bruyne T, Pieters L, Savelkoul HFJ, Hermans N. Effect of Pycnogenol® on attention-deficit hyperactivity disorder (ADHD): study protocol for a randomised controlled trial. Trials (2017), 18(1).

Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time. – Thomas A. Edison

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PART 1

INTRODUCTION

MPH, the first-choice medication for ADHD, is linked to concerns about adverse effects and possible publication bias in reported efficacy [1, 2]. However, ADHD is associated with immune imbalances and increased oxidative stress, which offer potential for nutritional supplementation as therapy [3-10]. Due to its antioxidant, anti-inflammatory and immune modulating activities, a commercially available extract from *Pinus pinaster* bark with a high content of phenolic compounds, Pycnogenol®, was selected for this study [11-14]. One small randomised trial suggests therapeutic benefit from Pycnogenol® in ADHD [15]. However, this trial had several limitations (e.g. small sample size, very small placebo group and short supplementation period) and the mechanism of action involved remains unclear [12, 15-17].

Therefore, a trial was designed to address the potential of Pycnogenol® in ADHD by investigating its efficacy, mechanism of action and value as compared to MPH treatment. This clinical trial will be conducted in accordance with the guidelines for Good Clinical Practice (GCP) and applicable legislation. GCP is an international quality standard for the design, conduct, recording, report and audit of trials with human subjects, in order to assure the rights, safety and well-being of these subjects and that the trial data are accurate and traceable [18]. These principles cover, amongst other aspects, ethics committee procedures, obtaining of informed consent from trial subjects, adherence to the trial protocol, administrative documentation, documentation regarding the IMP, data collection, trial subjects' medical records (source documents), documentation and reporting of AEs, preparation for inspections and audits and archiving trial documentation.

This chapter therefore describes the setup of a randomised double-blind placebo and active product controlled 10-week clinical trial with three parallel treatment arms in paediatric ADHD patients, including the protocol, registration requirements, ethical approval and safety monitoring. The ultimate goal of this trial is to investigate the efficacy, mechanism of action and value of Pycnogenol® in ADHD therapy as compared to MPH treatment and placebo, including effects on immunity, antioxidant levels, oxidative damage and comorbid psychiatric and physical complaints, and to evaluate the tolerability of Pycnogenol® compared to MPH. Final results of this trial, which is currently ongoing, will only be available after publication of this PhD thesis. The main research question is whether Pycnogenol® is

more effective than placebo and not less effective than MPH in improving behaviour. Other research questions are whether, as compared to placebo and MPH, Pycnogenol® increases antioxidant levels, reduces oxidative damage, improves immune and neurochemical status and reduces comorbid physical and psychiatric complaints.

PART 2

PROTOCOL

The protocol describes how the clinical trial will be conducted, including the design, methodology, statistics and organisation, and ensures participant safety and integrity of collected data. According to GCP requirements, a protocol should include [18-20]:

- Title page with protocol number and date;
- Name and address of the sponsor and investigator(s);
- Background information;
- Objectives;
- Study design and duration;
- Selection procedures, in- and exclusion criteria;
- Treatment of participants, treatment allocation, dosage, accountability;
- Primary and secondary endpoints;
- Assessment of efficacy, safety and AEs;
- Dropout, withdrawal, discontinuation and unblinding conditions, definition of end of trial;
- Statistics, including methods, power calculation and missing data;
- Quality control and assurance;
- Ethics;
- Data handling and storage;
- Publication policy;
- Timetable;
- References.

In addition, the protocol should be signed by the sponsor (trial initiator) and principal investigator. The protocol presented in this thesis is a summary of the official trial protocol, which contains more detailed information on subject safety, AEs, publication policy, etc.

2.1 Synopsis

Sponsor:	University of Antwerp Universiteitsplein 1 2610 Wilrijk, Belgium	Represented by: Prof. dr. N. Hermans
Principal Investigator:	B. Ceulemans, M.D., PhD Paediatric Neurology, University Hospital Antwerp Wilrijkstraat 10, 2650 Edegem, Belgium	
Coordinating investigator:	N. Hermans, PhD Department of Pharmaceutical Sciences, University of Antwerp Universiteitsplein 1, 2610 Wilrijk, Belgium	
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Title of the clinical trial:	Effect of a polyphenol-rich plant extract on Attention Deficit Hyperactivity Disorder (ADHD): a randomised double-blind placebo and active product controlled multicentre trial.	
Phase:	Phase III	

Type of trial, trial design, methodology:	Multicentre randomised double-blind placebo- controlled parallel-group design with three treatment arms (Pycnogenol®/Medikinet® Retard/placebo)
Number of subjects:	48 per treatment group (total 144)
Principal inclusion criteria:	• 6-12 years old (both inclusive) • Diagnosis of ADHD/ADD • Responsible caregiver able to provide information about the patient's functional status • Sufficient level of Dutch • Written informed consent from the legally accepted representative
Principal exclusion criteria:	• Diagnosis of Autism Spectrum Disorder (ASD) • Situational hyperactivity, pervasive developmental disorder, schizophrenia, personality disorder, IQ < 70, CD, dyskinesias, tics or Tourette's syndrome, personal/family history of psychotic disorder, bipolar illness, depression or suicide attempt • Any chronic medical disorder or acute inflammatory disease, glaucoma, heart disease or rhythm disorder, high blood pressure, or peripheral vascular disease • Contraindications for the use of MPH • Pregnancy • Use of any of these medications during the past 3 months: clonidine, guanethidine, blood thinners, antidepressants, medication with decongestant, blood pressure medicine, seizure medicine or diet pills • Use of MAO inhibitor in the past 2 weeks • Use of vitamin/mineral/herbal/omega-3 supplements or any medication > 1 week during the past 3 months
Primary trial objective:	To assess the efficacy of Pycnogenol® for improving ADHD behaviour as compared to placebo and Medikinet® Retard.

Study end points:

Primary end point:

- Summed ADHD score of the ADHD Rating Scale (ADHD-RS) rated by teachers

Secondary end points:

- Summed ADHD score of the ADHD-RS rated by parents
- Summed ADHD score of the SEQ rated by parents and teachers
- Percentage of responders

Other variables:

- Social behaviour problems and anxiety subscales of the SEQ rated by parents and teachers
- Physical and sleep complaints measured by the PCQ rated by parents
- Erythrocyte GSH level
- Lipid soluble antioxidant status
- Antioxidant enzyme activity
- Urinary 8-OHdG level
- Plasma MDA level
- Plasma cytokine levels
- Plasma antibody levels
- White blood cell count, measurement of their functional response
- Intestinal microbial composition
- Gene expression quantification by RT-qPCR
- Serum neuropeptide Y (NPY) level
- Serum zinc level
- Urinary catecholamine level
- Long-term follow up on treatment choice, behaviour and comorbid physical/psychiatric complaints
- Acceptability: dropouts, adherence, adverse events

Criteria for evaluation:	Efficacy: • Summed ADHD score of the ADHD-RS and SEQ, percentage of responders Tolerability: • Adverse effects • Physical and sleep complaints measured by the PCQ
Name investigational medicinal product (IMP), dosage, administration:	Pycnogenol®, oral intake 20 mg/day if body weight < 30 kg, 40 mg/day if body weight ≥ 30 kg. First two weeks: all participants 20 mg/day
IMP or therapy used as comparator, dosage, administration:	Placebo, oral intake Medikinet® Retard, oral intake First week: 10 mg/day methylphenidate hydrochloride Second week: 20 mg/day methylphenidate hydrochloride Afterwards: 20 mg/day methylphenidate hydrochloride if < 30 kg; 30 mg/day methylphenidate hydrochloride if ≥ 30 kg
Duration of treatment:	10 weeks
GCP conformance:	This trial will be conducted in accordance with the valid versions of the protocol and the internationally recognised Good Clinical Practice Guidelines (ICH-GCP).

2.2 Abstract

Background: Methylphenidate (MPH), the first-choice medication for ADHD, is associated with serious adverse effects like arrhythmia. Evidence on the association of ADHD with immune and oxidant-antioxidant imbalances offers potential for antioxidant and/or immunomodulatory nutritional supplements as ADHD therapy. One small randomised trial in ADHD suggests, despite various limitations, therapeutic benefit from Pycnogenol®, an herbal, polyphenol-rich extract.

Methods: This phase III trial is a 10-week randomised double-blind placebo and active treatment controlled multicentre trial with three parallel treatment arms to compare the effect of Pycnogenol®,

MPH and placebo on the behaviour of 144 paediatric ADHD and ADD patients. Evaluations of behaviour (measured by the ADHD-Rating Scale (ADHD-RS, primary endpoint) and the Social-Emotional Questionnaire (SEQ)), immunity (plasma cytokine and antibody levels, white blood cell counts and faecal microbial composition), oxidative stress (erythrocyte glutathione, plasma lipid soluble antioxidants and malondialdehyde and urinary 8-hydroxy-2'-deoxyguanosine levels, as well as antioxidant enzyme activity and gene expression), serum zinc and neuropeptide Y level, urinary catecholamines and physical complaints (Physical Complaints Questionnaire) will be performed in week 10 as compared to baseline. Acceptability evaluations will be based on adherence, dropouts and reports of adverse events. Dietary habits will be taken into account.

Discussion: This trial takes into account comorbid behavioural and physical symptoms as well as a broad range of innovative immune and oxidative stress biomarkers expected to provide fundamental knowledge on ADHD aetiology and therapy. Research on microbiota in the context of an intervention trial in ADHD is novel. Moreover, the active control arm is rather unseen in research on nutritional supplements, but of great importance, as patients and parents are often concerned about side effects of MPH.

Trial registration: Clinical Trial Registry number NCT02700685 (registered 18th of January 2016), EudraCT 2016-000215-32 (registered 4th of October 2016).

2.3 Background (as presented in the study protocol)

ADHD is a common neurodevelopmental behavioural disorder with childhood onset and core symptoms of hyperactivity, impulsivity and inattention [21]. ADHD has a worldwide prevalence of 5.9-7.1% and is associated with other psychiatric disorders, such as ODD, autism and anxiety [22, 23].

MPH, the first-choice medication for ADHD, is a CNS stimulant. It increases attentiveness and reduces hyperactivity and impulsivity by inhibition of dopamine reuptake in the prefrontal cortex and striatum, without triggering its release. MPH is prescribed for chronic use to a large proportion of ADHD patients, but is linked to possible publication bias in reported efficacy [2, 24, 25]. In addition, parents are often disinclined to use MPH due to negative publicity and its frequent side effects and, subsequently, non-adherence to therapy is high [2, 24-26]. A recent review reports adverse effects like insomnia and decreased appetite in about 25% of patients using MPH [27]. Other therapeutic options are therefore warranted, at least for a subgroup of patients [24-27].

ADHD is a complex and multifactorial disorder, influenced by genetics and environment. Its exact pathophysiology remains however unclear. Brain and peripheral catecholamines are dysregulated in ADHD (dopaminergic dysfunction is for instance involved), but also associations with immune and oxidant-antioxidant imbalances exist [3, 28]. Various studies demonstrated for example increased levels of plasma MDA and exhalant ethane (oxidative stress markers) and decreased activity of antioxidant enzymes such as GPx and CAT [29-32]. Also, catecholamine metabolism is considered a source of free radical formation [33]. In addition, ADHD has been hypothesised to be a hypersensitivity disorder, with a disrupted immune regulation contributing to its aetiology [28]. I.e., ADHD has a high comorbidity with both T_H1- and T_H2-mediated disorders and several genes that have been linked to ADHD have immune functions [3, 28, 34-37]. Ceylan *et al.* observed increased levels of adenosine deaminase, a marker of cellular immunity, and decreased levels of the antioxidant enzyme GST. These results indicate involvement of oxidative changes and cellular immunity in ADHD [3].

Still, specific immune biomarkers other than antibodies have not been systematically studied in ADHD, despite growing evidence on associations in autism [38, 39]. In addition, immune and oxidative effects of both standard therapy and nutritional supplementation in ADHD is a neglected topic in research. Yet, immune and oxidative imbalances linked with ADHD offer potential for appropriate supplementation in ADHD therapy [40].

Due to its antioxidant and immune modulating properties, a commercially available standardised extract from French maritime pine (*Pinus pinaster*) bark with a high content of polyphenolic compounds (including phenolic acids and procyanidins), Pycnogenol®, was selected for this study [11, 14, 15]. One small randomised trial suggests its therapeutic benefit in ADHD. Still, this trial had some limitations (e.g. short supplementation period) and the mechanisms of action involved remain unclear [15]. In addition, the efficacy and value of Pycnogenol® in ADHD as compared to MPH treatment remain to be confirmed.

The present trial protocol and amendments were and will be prepared in accordance with the Declaration of Helsinki (version of October 2013).

2.4 Methods

2.4.1 Objective

To evaluate the effect of Pycnogenol® on ADHD behaviour and comorbid physical and psychiatric symptoms, as well as on immunity, oxidative damage, antioxidant status and neurochemical parameters, in addition to its acceptability as compared to placebo and MPH treatment.

2.4.2 Hypotheses

- In ADHD therapy, Pycnogenol® is more effective than placebo and not less effective than MPH;
- As compared to placebo and MPH, Pycnogenol® increases antioxidant levels, reduces oxidative damage, improves immune and neurochemical status and reduces comorbid physical and psychiatric complaints;
- The tolerability of Pycnogenol® is higher than that of MPH.

2.4.3 Design

This is a phase III randomised double-blind placebo and active product controlled, multicentre clinical trial with three parallel treatment arms, to compare effects on ADHD behaviour between Pycnogenol®, MPH (Medikinet® Retard) and placebo, using the ADHD-RS as a primary outcome measure. Secondary outcomes are comorbid physical and psychiatric complaints (including side effects), oxidative stress, immunity, neurochemical parameters and acceptability of the interventions. Following screening and baseline assessments, 144 patients (6-12 years) will receive one of the three treatments for 10 weeks (Figure 5.1). Evaluations will be performed in week 5 and 10 as compared to baseline. Dietary habits will be taken into account. Moreover, long-term follow up is planned (6 months) to assess eventual treatment choice and concurrent behaviour and comorbid physical/psychiatric complaints.

Two study visits with similar evaluations and sample collections will be conducted: at baseline and after 10 weeks. Evaluations scheduled in week 10 could be performed in week 11 as well, to increase trial feasibility. Enough capsules for 11 weeks treatment will be provided. To analyse biomarkers of interest, 16 ml venous blood will be collected at the start and the end of the intervention, as well as urine. Faecal samples will be collected from participant subgroups (n = 60). Next to baseline and final evaluations, an extra evaluation of behaviour and physical symptoms will be conducted in week 5 by means of questionnaires. Filling out questionnaires takes approximately 30 min. Two reminders will be sent in case questionnaires are not received within one week after the required date. After every blood and urine collection and in case questionnaires are completed, participants receive two movie tickets.

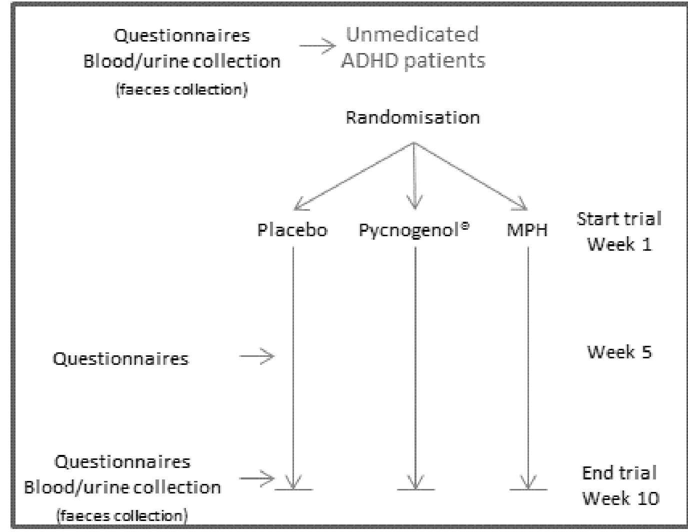

Figure 5.1. Design of the trial.
MPH: methylphenidate.

2.4.4 Inclusion and randomisation

Recruitment started in September 2017. The trial population (eligibility criteria in Table 5.1) will consist of ADHD patients, both diagnosed *de novo* and formerly treated, recruited at the University Hospitals of Antwerp (UZA) and Ghent (UZ Ghent) and the Hospital Network Antwerp (ZNA). With an expected inclusion rate of 30-50 patients per year (10-20 participants in UZA, 15-20 in UZ Ghent and 5-10 in ZNA), about 3 years will be required for subject recruitment. Though, as compared to inclusion rate, a ten-fold higher diagnosis rate of ADHD and ADD is expected in these centres, in- and exclusion criteria of the proposed trial (e.g. regarding autism or the recent intake of supplements or medication) are expected to exclude at least half of all newly diagnosed patients, while a consent rate of 30% is expected, taking into account potential reluctance regarding the use of medication or supplements, as well as "risk" for placebo treatment [41, 42]. In addition, patients from random primary schools in Flanders will be invited for this trial by letters and diagnosed in one of the trial centres before inclusion. In case of slow recruitment, also "ZitStil" (information centre on ADHD/ADD), revalidation centres, independent child psychiatrists/paediatricians and other hospitals can be involved. Assessments of patients will take place at the outpatient departments of Child Neurology of UZA and UZ Ghent and the University Child and Adolescent Psychiatry department of Hospital Network Antwerp. Diagnosis of all subjects will be confirmed by a child neurologist or psychiatrist according to DSM-5 guidelines.

The allocation sequence will be generated by the participating pharmacies. Participants will be randomised, stratified by trial centre, to one of the three treatment arms (placebo, Pycnogenol® or

Medikinet® Retard) by randomisation software (randomization.com, original generator, different starting number across trial sites and taking into account body weights below and above 30 kg). The number of participants per trial site is not limited. Participants will choose a sealed envelope within their weight category, containing their randomisation number. Trial participants, physicians, care providers, outcome assessors and data analysts will be blinded to avoid bias. The involved physicians and hospital pharmacies will assure confidentiality by retaining the randomisation code at all times in a sealed envelope, only to be used in case of emergency or SAEs. Patients will receive contact information from the three trial centres, so that questions could be answered by a trial investigator other than their attending physician.

Table 5.1. Inclusion and exclusion criteria for patient selection.

Inclusion criteria	Exclusion criteria
1. Age 6-12 years (both inclusive)	1. Diagnosis of autism spectrum disorder
2. ADHD diagnosis a. by the investigating physician b. based upon the ADHD-RS	2. Pervasive developmental disorder, personality disorder, IQ < 70, CD, tics, schizophrenia, dyskinesia, personal or family history of psychotic disorder, bipolar illness, depression or suicide attempt
3. Responsible caregiver to provide information about the patient's functional status	3. Chronic medical disorder or acute inflammatory disease, glaucoma, heart disease, high blood pressure, or peripheral vascular disease.
4. Patient and responsible caregiver have sufficient knowledge of Dutch	4. Use of MAO inhibitor 14 days before inclusion. Use of clonidine, guanethidine, seizure medication, antidepressants, blood thinners, blood pressure or diet medication 3 months before inclusion.
5. Written informed consent by the patient's legally accepted representative	5. Use of vitamin/mineral/herbal/omega-3 supplements or any medication for longer than 1 week 3 months before inclusion
	6. Other contraindications for MPH or Pycnogenol®, as defined in the Summary of Product Characteristics and Investigator's Brochure, resp.

ADHD-RS: ADHD Rating Scale; CD: conduct disorder; IQ: intelligence quotient; MAO: monoamine oxidase; MPH: methylphenidate.

Withdrawal, the presence of SAEs, development of a severe and unstable disease, failure to provide follow up information or to come to the follow up appointment and intake of concomitant medication/supplements during more than one week will lead to drop-out. Drop-outs will not be replaced.

2.4.5 Treatment

Patients will receive all capsules required for the complete study at inclusion, at a dose based on their body weight (1 or 2 oral capsules at breakfast):

- MPH (Medikinet® Retard, methylphenidate hydrochloride modified release, Medice GmbH): Patients with a body weight < 30 kg will receive 20 mg/day, those with a body weight ≥ 30 kg 30 mg/day. Treatment during the first week always contains 10 mg, increasing 10 mg per week to limit side effects.
- Pycnogenol®: Patients with a body weight < 30 kg will receive 20 mg/day, those with a body weight ≥ 30 kg 40 mg/day, aiming at a daily dose of 1 mg/kg and taking into account formulation issues [15]. Treatment during the first two weeks always contains 20 mg.
- Placebo: Placebo contains excipients only.

Pycnogenol® and placebo will be produced in capsules identical to Medikinet® Retard. Capsules to be used are hard gelatine capsules (Capsugel NV, Belgium) and excipients are microcrystalline cellulose and magnesium stearate, which are also used for marketed Pycnogenol® capsules. All treatments will be provided in identical jars, labelled with the subject's trial number and week of intake. Encapsulation, primary packaging and labelling will be performed by Qualiphar NV under full GMP regulations. Compliance will be determined based on accountability of investigational products (IPs) and self-reported adherence.

In case of AEs, the investigator, principal caregiver and participant can decide to discontinue the trial medication. No dose adjustment will be performed. With a standardised questionnaire, AEs will be documented at week 5 and 10, taking into account the patient's medical records as well. Also spontaneously reported AEs will be recorded. Abnormal test results are not considered AEs, unless inducing clinical symptoms or requiring therapeutic intervention. In case of a SAE, the trial code will be broken and treatment discontinued.

Premature termination of the trial will be considered if the risk-benefit balance for trial subjects changes markedly, it is no longer ethical to continue treatment with the investigational medicinal product (IMP), an unacceptable high number of SAEs occurs (i.e. if 10% of the participants experience a potentially related SAE), the sponsor (trial initiator, not provider of financial support) or principal investigator considers that the trial must be discontinued for safety reasons, it is no longer practicable to complete the trial, or a high number of drop-outs (> 40 %) is noticed. The sponsor decides on whether to discontinue the trial in consultation with the principal investigator and/or statistician.

2.4.6 Outcomes

2.4.6.1 Primary outcome

The primary objective is to assess the efficacy of Pycnogenol® for improving ADHD behaviour as rated by teachers compared to placebo and Medikinet® Retard. The primary outcome is therefore the summed ADHD score of the ADHD-RS as rated by teachers (Table 5.2 and Appendix 4.1, see bookmark for QR code). Power calculation is based on this scale. Teachers will fill out this questionnaire before the start of the intervention and after 5 and 10 weeks.

2.4.6.2 Secondary outcomes

Secondary outcomes related to ADHD behaviour are:

- Summed ADHD score of the ADHD-RS, rated by parents, as a reflection of the child's ADHD/ADD behaviour at home;
- Summed ADHD score of the SEQ (Appendix 4.5.2, see bookmark for QR code), rated by parents and teachers.
- Scores on ADHD subscales of the ADHD-RS and SEQ, rated by parents and teachers (hyperactivity, impulsivity and inattention);
- Percentage of responders, rated by parents and teachers, defined as participants with a reduction of at least 20% of their baseline summed ADHD-RS score [43].

Other secondary outcomes are:

Psychiatric complaints

- Social behaviour problems subscale of the SEQ, rated by parents and teachers, to evaluate to what extent symptoms of ODD and CD are displayed [44];
- Anxiety subscale of the SEQ, rated by parents and teachers, to evaluate symptoms of general anxiety, social anxiety and anxiety-depression [44].

Physical complaints

- Physical and sleep complaints, including various potential side effects, measured by the PCQ (Appendix 4.3.2, see bookmark for QR code) [45].

Neurochemistry

- Urinary catecholamines (dopamine, norepinephrine and epinephrine) and their metabolites, determined by HPLC with electrochemical (detection [33];
- Serum neuropeptide Y (NPY), analysed by ELISA [57].

Antioxidant levels

- Erythrocyte GSH level, the most important intracellular antioxidant, analysed by HPLC with electrochemical detection [46] (see Chapter 2, 3.4.1);
- Lipid soluble antioxidants: plasma vitamin E (α- and γ-tocopherol), vitamin A (β-carotene, retinol, retinyl palmitate) and coQ10, analysed by HPLC with electrochemical detection [47-50] (see Chapter 2, 3.4.2);
- Antioxidant enzyme activity (CAT, SOD and GPx), analysed by ELISA [29, 51];
- Gene expression, quantified by RT-qPCR, focusing on networks counteracting oxidative stress (GPx, CAT, SOD, XO) and stress-related proteins (Clusterin) [52, 53];
- Serum zinc level, analysed by atomic absorption spectroscopy (AAS) [54].

Oxidative damage

- Plasma MDA level, a marker of lipid peroxidation, analysed by HPLC with fluorescence detection [55] (see Chapter 2, 3.4.3);
- Urinary 8-OHdG level, a marker of oxidative DNA damage (though also considered a general marker of *in vivo* oxidative damage, as it is also produced from nucleotides in cellular pools), corrected for urinary creatinine concentration, analysed by ELISA [56] (see Chapter 2, 3.4.4).

Immunity

- Plasma cytokines for monocytes (IL-1β, IL-6, IL-8, IL-10, IL-12, TNF-α) and T cells (IL-4, IL-5, IL-6, IL-10, IFN-γ) as well as antibody levels (IgA_{1-2}, IgG_{1-4}, IgE), analysed by flow cytometry, as markers of immune activation state and skewing [40, 58, 59] (see Chapter 2, 3.4.5 and 3.4.6);
- Identification of peripheral blood mononuclear cells (PBMCs; like CD4, CD8 and B cells) and neutrophils and measurement of their functional responses (e.g. cytokine release) after stimulation, as a marker of immune activation state, skewing and responsivity by flow cytometry [40, 58, 59];
- Intestinal microbial composition, assessed using extreme throughput multiplexed sequencing of 16S rRNA gene pools PCR-amplified from faecal samples [60, 61].

Table 5.2. Investigations and data acquisition during the trial.

Evaluations/Interventions	Screening	Baseline Week 0	Week 5	Week 10
Inclusion and exclusion criteria	X	X		
Current use of medication/supplements	X	X	X	X
Informed consents	X	X		
Randomisation		X		
Treatment				
Treatment distribution		X		
Medication count		X		X
ADHD-RS		X	X	X
SEQ		X		X
PCQ		X	X	X
FFQ		X		X
Sample collection (blood, urine, faeces)		X		X
GSH analysis		X		X
Lipid soluble antioxidants analysis		X		X
Antioxidant enzyme activity		X		X
Genetics analysis		X		X
MDA analysis		X		X
8-OHdG analysis		X		X
Cytokine analysis		X		X
Antibody analysis		X		X
PBMC count and reactivity analysis		X		X
Microbial composition analysis		X		X
Catecholamine analysis		X		X
NPY analysis		X		X
Zinc analysis		X		X

8-OHdG: 8-hydroxy-2'-deoxyguanosine; ADHD-RS: ADHD Rating Scale; FFQ: food frequency questionnaire; GSH: reduced glutathione; MDA: malondialdehyde; NPY: neuropeptide Y; PBMC: peripheral blood mononuclear cell; PCQ: Physical Complaints Questionnaire; SEQ: Social-Emotional Questionnaire.

The safety of both MPH and Pycnogenol® has been investigated and demonstrated before and is therefore beyond the scope of this trial. A final objective is however to investigate the acceptability of Pycnogenol® compared to Medikinet® Retard and placebo, based on the prevalence of side effects, treatment adherence (defined as > 90% ingestion as scheduled) and proportion of drop-outs. Though seemingly trivial, adherence and dropouts are important real-life indicators of the effectiveness of a treatment, based on both the achievement of positive effects and the absence of adverse effects, and therefore highly valuable.

Dietary habits of participants, such as consumption of vegetables, chocolate, fruit, etc., possibly influence the results of the trial and will therefore be assessed by an FFQ at the start and end of intervention [62, 63], to assess potential dietary adaptations during the study as well as baseline differences between treatment groups. The highest educational achievement of both parents will be

determined as a proxy for socioeconomic status, while also questions regarding the course of pregnancy will be asked (Appendix 4.4.2, see bookmark for QR code).

Long-term follow up to assess eventual treatment choice and concurrent behaviour and comorbid physical/psychiatric complaints will be based on parental questionnaires 6 months after study termination (ADHD-RS, SEQ, PCQ and few additional questions). Though open label, this provides additional information on the efficacy of experimental and standard treatment, long-term Pycnogenol® treatment, or potential symptom relapse after ceasing Pycnogenol® intake [15, 64].

Analyses of oxidative stress and antioxidants, as well as NPY, catecholamines and questionnaires, will be performed at the Laboratory of Nutrition and Functional Food Science of the University of Antwerp. Immune profiling and genetics will be performed at the Cell Biology and Immunology Department and the Microbiology department of Wageningen University. Plasma zinc status (and possibly catecholamine status) will be determined by the General Medical Laboratory (AML) in Antwerp.

2.4.7 Statistics

The minimum number of patients required, based upon the effect size of 1.38 found by Trebaticka *et al.* (teacher evaluation), is only 13 patients per group to reach statistical power of 80% and a significance level of 0.016 (0.05 with Bonferroni correction), not taking into account dropouts [15]. However, based on results of other previous studies on nutrition and supplementation in ADHD, a more modest improvement of 0.75 SD on behaviour rating scales is rather expected [65]. For the estimation of the required sample size, following assumptions were therefore made:

- Patients improve 0.75 SD on the ADHD-RS summed ADHD score as rated by teachers if using Pycnogenol® for 10 weeks [15, 65], which corresponds to a 20% improvement with active treatment as compared to placebo;
- Power of 80%, drop-out of 20%;
- Two-sided testing at a significance level of 0.05 with Bonferroni post-hoc testing correction.

Based on these considerations, 48 patients per treatment will be necessary (n = 144 in total).

Data will be checked for outliers. Missing data will not be accounted for. The three groups will be compared regarding baseline characteristics to check for differences at the start. A two-way ANOVA will be performed to investigate a potential interaction between treatment and weight. In case of a significant interaction, the two weight categories will be studied separately. In case of a non-significant interaction, both weight categories will be studied together. Change in ADHD-RS score as rated by

teachers (primary outcome measure) will be compared between the three treatments by means of a one-way ANOVA (categories: treatment, time; $\alpha = 0.05$) with post-hoc testing. Changes regarding secondary target variables will be compared between the three treatment groups by one-way ANOVA with post-hoc analysis with multiple testing correction, Kruskal-Wallis or Fisher's exact test, depending on normality and the type of variable. Separate analyses for subgroups (e.g. based on gender, severity or subtype of ADHD, dietary habits, etc.) will also be performed. Effect of sample processing time will be tested and, if significant, taken into account by multiple regression. Non-inferiority of Pycnogenol® as compared to Medikinet® Retard will be demonstrated when the difference in effect on ADHD-RS score (after 10 weeks vs. at baseline) is no more than 5 points [66]. This wide margin might be justified due to frequent side effects of MPH. Non-inferiority will only be accepted if supported by both intention-to-treat and per protocol analyses [67, 68].

2.4.8 Trial management and research team

The University of Antwerp (Laboratory of Nutrition and Functional Food Science) is the sponsor of this trial, with Nina Hermans being the coordinating investigator. As principle investigator, Berten Ceulemans, as well as investigators Dirk Van West and Helene Verhelst, will be primarily responsible for patient inclusion. Nina Hermans and Annelies Verlaet are responsible for the analysis of oxidative stress and neurologic biomarkers and questionnaire results, as well as for data management. Huub Savelkoul is responsible for the analysis of immune biomarkers and genetics. No data monitoring committee will be set up. Berten Ceulemans, Helene Verhelst, Dirk Van West, Nina Hermans and Annelies Verlaet will discuss potential issues regarding e.g. the progress of the trial, including safety data, accurate reporting and compliance with GCP and the protocol. Practical and financial agreements were signed between the sponsor, investigators and any other parties involved with the trial.

2.5 Discussion

2.5.1 Design

This randomised controlled trial addresses the potential of an herbal extract in ADHD by investigating its efficacy, mechanism of action and value as compared to standard treatment and placebo. Results can be partly compared to a previously conducted study [15, 64, 69]. A double-blind design was chosen to avoid bias, due to the subjectivity of questionnaire responses. In addition, both parents and teachers will rate the behaviour of participants. Behavioural assessment by teachers is preferred as primary

objective due to the higher sensitivity of teachers' ratings [70, 71]. A 10-week treatment is considered long enough to see clear effects of both Pycnogenol® and Medikinet® Retard, though still minimising patient burden and thus maximising compliance [15]. The parallel design was also chosen to reduce patient burden and as it is unclear how long the wash out period for Pycnogenol® should be in case of a crossover design. Compliance control by additional blood sampling as well as objective behaviour assessment (e.g. movement trackers) rather than questionnaires would increase participants' burden. In addition, such compliance control is expensive and difficult for Pycnogenol®, as diet might limit the relevance of plasma polyphenol analysis.

2.5.2 Inclusion

Though the symptoms of adult ADHD are similar to the restlessness, distractibility and impulsivity of childhood ADHD, the expression of symptoms changes as the individual matures. Moreover, as ADHD is generally diagnosed in childhood, persisting into adulthood in about 60% of individuals with childhood onset, children rather than adults were chosen for this trial [72].

2.5.3 Treatment

Patients and especially their parents are often worried about side effects of MPH, the standard medication for ADHD. It is therefore important not only to investigate the behavioural effect of Pycnogenol® compared to placebo, but also compared to MPH, as well as to take into account side effects and effects on comorbid complaints. This active control is rather unseen in research on nutritional supplements. In one previous trial in adult ADHD, the effect of Pycnogenol® was compared to MPH and placebo. However, neither MPH nor Pycnogenol® outperformed placebo, possibly due to the short treatment period of 3 weeks [17].

Despite being the first-choice medication for ADHD, MPH is associated with various side effects (including SAEs), some of them frequently occurring, including irritability, insomnia, loss of appetite and headache (see Chapter 1, 1.3) [27]. The overall frequency of side effects due to Pycnogenol® is very low (1.8%) and unrelated to dose or duration of use (see Chapter 1, 4.4.1). GI discomfort, the most frequently occurring adverse effect, may be avoided by taking Pycnogenol® with or after meals. In children with ADHD, 2 of 41 Pycnogenol® supplemented participants experienced side effects (rise of slowness and moderate gastric discomfort), while various biochemical parameters (bilirubin, glucose, GGT, ALP, AST, ALT, uric acid and fatty acid profile) remained normal after Pycnogenol® supplementation (1 mg/kg for 4 weeks) [15]. Pycnogenol® did not cause any significant changes in blood pressure or heart rate in 4 clinical studies (total n = 185). There have been no reports of SAEs

since its introduction into the European market around 1970 [61]. Therefore, the use of Pycnogenol® in children is considered to be safe. A small discomfort for the patient will be the puncture to get blood samples.

The Pycnogenol® dosage is based upon the previous clinical trial, using 1 mg/kg body weight [15]. In the present trial, due to practical reasons, 0.67-1.33 mg/kg body weight will be applied.

Though the Belgian law and the Declaration of Helsinki (2013) discourage the use of a placebo treatment in case an active treatment is available, ethical problems regarding the placebo group are not anticipated as MPH treatment often causes side effects. In addition, a 10-week waiting time between ADHD diagnosis and the start of treatment (the period in which the clinical trial will be performed as much as possible) is not exceptional in routine care (oral communication with prof. dr. Ceulemans, UZA). Moreover, since placebo effects could be large due to e.g. the subjectivity of questionnaire responses, despite blinding of patients and their parents and teachers, it is uttermost important to include a placebo arm. Furthermore, various placebo-controlled trials, also in paediatric ADHD patients, have been performed and published recently, like a six-week treatment with *Ginkgo biloba* extract as compared to placebo [73] or an eight-week treatment with ALA as compared to placebo [74]. Though ADHD is a serious condition with a vast impact on the patient and its environment, it is not life-threatening. A 10-week placebo treatment will not have a negative impact on the patient or academic achievements in further life. Finally, participation is voluntary and all participants and their principal caregiver will be informed about the potential risks and benefits, including placebo treatment. After 10 weeks, routine care for ADHD is started.

2.5.4 Outcomes

The ADHD-RS is validated and internationally accepted and consists of 9 inattention and 9 impulsivity and hyperactivity questions based on the DSM, each marked out on a four-point rating scale [11]. Use of the ADHD-RS allows comparison of results to those of previously performed trials [65]. In addition to analysing teacher and parent ratings separately, their comparison will provide information on differences between treatments. For instance, the extended release formulation of MPH is effective for about 8 h, while the effect of Pycnogenol® on behaviour is not expected to wear off suddenly.

In addition to the ADHD-RS, the SEQ (see Chapter 2, 3.3.2) is used in this trial. Though this increases the number of questions on behaviour significantly (72 questions), the SEQ also assesses frequently occurring psychiatric comorbidities of ADHD [44].

The PCQ (see Chapter 2, 3.3.3) includes questions on various potential AEs [45, 65]. In addition, parents will be asked whether the participant experienced any illness during the trial, which illness it was, whether any medication was taken, and the type, dose and duration of medication intake.

The FFQ (Appendix 4.2.2, see bookmark for QR code) consists of 50 questions on different food groups to be rated on a nine-point scale by parents at the start and end of the intervention, to assess baseline dietary habits and potential adaptations during the study, as well as to relate potential differential effects of Pycnogenol® to dietary polyphenol intake [62, 63]. Insight in global dietary habits (e.g. whether the participant consumes fresh fruit daily) is therefore aimed for. In addition, the frequency of eating breakfast, lunch and dinner is asked for, as well as further information on the type of milk, rice, chocolate, etc.

Zinc is essential for CNS structure and function, including regulation of the dopamine transporter [75-77]. In addition, it is a cofactor in antioxidant defence systems, like CuZn-SOD [78, 79]. Reduced serum, plasma, urine and hair zinc levels have been found in ADHD patients compared to controls [80]. Serum zinc is therefore a relevant marker in this study.

NPY participates in thermoregulation, feeding regulation and circadian rhythms, while it frequently colocalises with catecholamines. In addition, elevated plasma NPY levels in ADHD patients indicate that NPY could be a potential biomarker of ADHD [81, 82].

2.5.5 Originality

Most research on nutritional supplements or medication in ADHD predominantly assesses effects on ADHD behaviour. This trial however takes into account comorbid behavioural and physical symptoms, such as ODD, anxiety and side effects, as well as a broad range of innovative immune, oxidative and neurochemical biomarkers, which have not been systematically studied in ADHD. Research on microbiota in the context of an intervention trial in ADHD patients is novel, too.

In addition, as opposed to most studies, this trial includes both placebo and active control. Inception of therapeutic intervention with large treatment groups and comparison to MPH treatment is highly useful, as an alternative approach to ADHD based on underlying mechanisms might have more potential than the current therapy, which has serious limitations.

Assessment of improvement in behaviour and comorbid complaints, and their correlations [45], as well as determining effects on immune and oxidative status, is crucial to increase understanding of ADHD pathophysiology, potentially effective therapies and mechanisms by which polyphenol-rich extracts

might lead to improvements. Previous research overall failed to perform these assessments [15, 83]. Finally, also intervention acceptability will be taken into account. Results of this project will therefore increase insight in ADHD aetiology and (dietary) treatment options, which is highly desired by medical staff, parents and patients.

PART 3

PREPARATION OF TRIAL-RELATED FILES

This clinical trial involves various files and questionnaires (clinical research files, CRFs) to be signed or filled out by the participant, his/her primary caregiver and teacher as well as by involved investigators, but also information folders, manuals and standard operating procedures (SOPs). In addition, an Investigator's Brochure (IB) for Pycnogenol®, Summary of Product Characteristics (SmPC) for Medikinet® Retard and Investigation Medicinal Product Dossier (IMPD) for both Pycnogenol® and placebo were required. Here, an overview is given of all files that were prepared. All documents are identical in the different trial sites, except for e.g. the contact information of the local investigator.

3.1 Recruitment advertising

A poster (Figure 5.2) for hospital waiting rooms was developed, as well as a flyer (Figure 5.3) with more specific information for potentially interested ADHD patients and their parents.

Figure 5.2. Poster for waiting rooms.

Ben je tussen 6 en 12 jaar?

Heb je ADHD of ADD?

ONDERZOEK
ADHD/ADD en voeding

Kunnen voedingssupplementen helpen bij ADHD/ADD?

Wat is het doel van het onderzoek?

Het doel is om **patiënten met ADHD zo goed mogelijk te helpen**. De resultaten van dit onderzoek zijn **cruciaal** om meer wetenschappelijk inzicht te verkrijgen in ADHD, en om de rol van bepaalde plantaardige voedingssupplementen in de behandeling ervan te evalueren.

Het effect van een **plantaardig voedingssupplement** (Pycnogenol®) op ADHD en bijhorende psychische en fysieke symptomen wordt onderzocht, in vergelijking met methylfenidaat (standaardbehandeling bij ADHD) en placebo. Ook immuniteit, oxidatieve stress en voedingsgewoonten bij ADHD worden onderzocht. Het immuunsysteem is het verdedigingssysteem van het lichaam. Bij oxidatieve stress is er meer dan een normale hoeveelheid schadelijke stoffen, vrije radicalen genaamd, aanwezig in het lichaam. Deze stoffen beschadigen delen van de cel.

Waarom dit onderzoek?

Resultaten van verschillende studies wijzen op een andere immuunstatus en meer oxidatieve stress bij ADHD patiënten, in vergelijking met personen zonder ADHD. Omdat het plantaardig voedingssupplement in deze studie zowel het immuunsysteem kan verbeteren als oxidatieve stress kan verminderen, lijkt dit **supplement ideaal bij ADHD**. Bovendien blijkt uit een voorgaande, kleine studie al dat het voedingssupplement ook de **symptomen van ADHD kan verbeteren**. Verder onderzoek is echter noodzakelijk om een volledig beeld te krijgen van deze effecten, zodat de **behandeling van ADHD aangepast en verbeterd** kan worden.

Waarom deelnemen?

Om waardevol en betrouwbaar wetenschappelijk onderzoek te kunnen doen, is het zeer belangrijk dat er voldoende patiënten meedoen. Hiervoor zijn wij op zoek naar **kinderen met ADHD of ADD** die willen meewerken aan het onderzoek. Het betreft hier zuiver wetenschappelijk onderzoek, zonder commerciële doeleinden.

Deelname van uw kind aan dit onderzoek is volledig **vrijwillig**. U kunt uw kind later op ieder moment uit het onderzoek terugtrekken.

Wat houdt deelname in?

Bij deelname krijgt uw kind **willekeurig één van de drie behandelingen gedurende 10 weken:**

een voedingssupplement, medicatie of placebo.

Voedingssupplement: Pycnogenol®, schorsextract van de Franse maritieme pijnboom. Een gecontroleerde klinische studie bij kinderen suggereert een positief effect van het gebruik van Pycnogenol® bij ADHD.

Medicatie: Methylfenidaat, de standaardbehandeling bij ADHD.

Placebo: Heeft geen werkzame eigenschappen.

Aan de start van het onderzoek en na 10 weken wordt een **urine- en bloedstaal** afgenomen. Mogelijk vragen we ook een stoelgangstaal. Op dezelfde tijdstippen en na 5 weken vragen wij u enkele **vragenlijsten** in te vullen.

De reguliere behandeling van ADHD kan na deze 10 weken gewoon gestart worden.

Figure 5.3. Part of the information flyer.

3.2 Informed consent forms

Trial subjects cannot be enrolled unless they and their legal representatives have voluntarily consented to take part in the trial after having been informed verbally and in writing in comprehensible language of all aspects of the trial relevant to his/her decision by a trial investigator (informed consent). These aspects include the nature, scope and possible consequences of the trial as well as reasons to conduct a placebo-controlled trial. Informed consent should be documented by means of a written, dated and signed informed consent form. In case the teacher of the trial subject agrees to participate in the trial and to fill out various questionnaires, he/she should also sign the informed consent form.

The originally signed consent form should be archived in the investigator site file. Trial subjects receive copies of the written information sheet, confirmation of insurance and the informed consent form. An example of the informed consent forms can be found in Figure 5.4 and Appendix 3.2 (see bookmark for QR code).

An informed consent form should contain the following information [18, 84]:

- Title of the trial;
- Contact information of the principal and local investigator, sponsor and emergency help;
- Explanations that the trial involves research and those aspects that are experimental;
- Rationale and objectives, type and design of the study, duration, anticipated number of participants, dose and expected mechanism of action of the investigated product;
- Implications of participation, e.g. financially and compared to standard care, procedures to be followed;
- The subject's responsibilities;
- Voluntary participation: withdrawal can occur at any time without justification and without jeopardising the further course of treatment or quality of further care;
- (Lack of guarantee on) potential advantages, random treatment assignment, risk of placebo treatment;
- Other potential treatment options and their potential benefits and risks;
- Reasonably foreseeable risks or inconveniences, possible AEs including those unknown;
- Confidentiality according to GCP and national law;
- Coding of study data ensuring anonymity to third parties;
- Access to the medical record and the information recorded within the framework of the clinical trial by authorised representatives of the sponsor (e.g. monitors or auditors) or the competent supervisory or federal authorities;
- Rights of the participants, including inspection of the own personal information;
- Notification of the participant’s general practitioner for safety reasons;
- Potential treatment after the trial, including the option for continuation of study medication (if this option is available);
- Costs and financing, including the anticipated payment and expenses (if any) for participation;
- Trial insurance;
- Approval by the central and local ethics committee;
- Consent form, stating that all questions about the trial are answered to the satisfaction of the participant (or legal representative), to be signed and dated by both the participant (or legal representative) and the investigator.

A

ADHD-UAntwerpen

Formulier voor geïnformeerde toestemming **Deelnemersn°: _____**

Titel: **Effect van een polyfenolrijk plantenextract op Aandachtstekort-Hyperactiviteitsstoornis (ADHD): een gerandomiseerde, dubbelblinde, placebo en actief product gecontroleerde multicentrische klinische studie.**
01/01/2018 – 31/12/2021, FWO referentienummer: G038218N, Peoplesoft ID: 36193.

Protocolnr.: 2017/11

Organisator: Universiteit van Antwerpen

Onderzoeker: Prof. Dr. Berten Ceulemans

Je wordt gevraagd om mee te doen aan dit onderzoek omdat jij ADHD hebt. Een onderzoek is een manier om meer te weten te komen over hoe ADHD ontstaat en wat we eraan kunnen doen. Met deze informatie kun je beslissen of je wel of niet aan dit onderzoek mee wilt doen.

We willen meer weten over hoe ADHD ontstaat en of we het met voeding kunnen behandelen. Zo kunnen we kinderen met ADHD beter verzorgen.

Er doen ongeveer 144 kinderen aan dit onderzoek mee.

Wat zal er tijdens dit onderzoek met mij gebeuren?

Als je aan het onderzoek mee wilt doen, krijg je één van de drie mogelijke behandelingen. Je kunt een pilletje met een soort vitamine krijgen, een pilletje met een medicijn, of een pilletje zonder iets in. Deze behandeling moet je dan 10 weken volgen. Ook wordt je gewogen en wordt je lengte gemeten aan het begin en het einde van deze 10 weken. Dan neemt de dokter of verpleegkundige ook wat bloed van je af, met een naald. Je moet ook een plasje en een beetje stoelgang inleveren.

B

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Antwerpen

ADHD-UAntwerpen

Formulier voor geïnformeerde toestemming **Deelnemersn°: _____**

Titel: **Effect van een polyfenolrijk plantenextract op Aandachtstekort-Hyperactiviteitsstoornis (ADHD): een gerandomiseerde, dubbelblinde, placebo en actief product gecontroleerde multicentrische klinische studie.**
01/01/2018 – 31/12/2021, FWO referentienummer: G038218N, Peoplesoft ID: 36193.

Protocolnr.: 2017/11

Organisator: Universiteit van Antwerpen

Onderzoeker: Prof. Dr. Berten Ceulemans

Inleiding

Uw kind wordt gevraagd om deel te nemen aan een klinisch-wetenschappelijk onderzoek naar het effect van een plantaardig voedingssupplement (Pycnogenol®, een schorsextract van de Franse pijnboom, *Pinus pinaster*) op de symptomen van ADHD (attention-deficit/hyperactivity disorder of aandachtstekort–hyperactiviteitstoornis) in vergelijking met de standaardbehandeling voor ADHD (methylfenidaat) en placebo.

Dit onderzoek wordt uitgevoerd omdat resultaten van verschillende studies wijzen op een andere immuunstatus en meer oxidatieve stress bij ADHD patiënten, in vergelijking met gezonde personen. Het immuunsysteem is het verdedigingssysteem van het lichaam met als doel indringers zoals bacteriën en virussen te bestrijden. Oxidatieve stress is een toestand in het lichaam waarbij meer dan een normale hoeveelheid schadelijke stoffen, vrije radicalen genaamd, aanwezig zijn. Deze stoffen beschadigen delen van de cel.

Omdat Pycnogenol® zowel het immuunsysteem kan verbeteren als oxidatieve stress kan

Figure 5.4. Example of the information and consent forms for (A) patients and (B) their legal representative (first page).

3.3 Documents for the primary caregiver and teacher

Various files, forms and questionnaires in this trial are specifically for the primary caregiver and/or teacher of the patient to acquire information, as a reminder or to inform them on specific details of the trial. Examples are a brochure for the caregiver with information on capsule intake, questionnaire completion, potential side effects and contact information (Figure 5.5), a letter informing the subject's teacher on the objective of the trial and asking for participation (not shown), a document with instructions for faeces collection (not shown) and various questionnaires, i.e. on the course of pregnancy and the educational level of both parents, as well as the ADHD-RS, SEQ, PCQ and FFQ (Figures 5.6, 5.7 and 5.8).

UZA Kennis / Ervaring / Zorg

UZ

ZNA

Ben je tussen 6 en 12 jaar?
Heb je ADHD of ADD?

ONDERZOEK
ADHD/ADD en voeding

Zijn voedingssupplementen zinvol bij ADHD/ADD?

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Deelnemersnummer: ______

Eerste bloedafname: ___/___/_____ om ___u___

Invullen vragenlijsten week 5: ___/___/_____

Vervolgafspraak: ___/___/_____ om ___u___

De reguliere behandeling van ADHD kan, indien gewenst, na deze 10 weken gewoon gestart worden. Indien na deze 10 weken blijkt dat uw kind in de methylfenidaat- of placebogroep terechtkwam, kan u gratis Pycnogenol® supplementen krijgen voor 10 weken behandeling (op eigen initiatief en verantwoordelijkheid).

Inname capsules

Deelname aan dit onderzoek vereist het **strikt volgen van de opgelegde behandeling** (Pycnogenol®, methylfenidaat of placebo) gedurende 10 weken. Uw kind wordt verwacht **elke dag 1 capsule** in te nemen, vanaf de dag na de bloedafname **tot en met de dag van de tweede bloedafname** (ook indien deze bloedafname niet exact na 10 weken ingepland werd):

- Week 1: Bij het ontbijt: inname van 1 capsule uit de "week 1" container
- Week 2-einde: Bij het ontbijt: inname van 1 capsule uit de "week 2-10" container

Wij vragen u de capsules niet te openen, en alle resterende capsules mee te nemen bij de vervolgafspraak voor de 2e bloedafname. Het is heel normaal dat er capsules overblijven aan het eind van het onderzoek. Tijdens deelname aan het onderzoek, vragen we ook geen andere voedingssupplementen in te nemen. Wanneer uw kind een capsule is vergeten innemen, mogen er op een later tijdstip niet twee capsules samen ingenomen worden. De vergeten capsule laat u gewoon in de pot. Bewaar de capsules in de gesloten pot, op kamertemperatuur en buiten het bereik van kinderen of huisdieren.

Figure 5.5. Information brochure for the primary caregiver (two example pages).

A

UZA
Kennis / Ervaring / Zorg

ADHD-UAntwerpen

Algemene vragenlijst: start   Deelnemersn°: _____

Het is van belang dat u alle vragen zo goed mogelijk beantwoordt, ook als u niet helemaal zeker bent of als u de vraag raar vindt.

Deelnemersnummer: ____________________   Datum: __________________

Vragenlijst ingevuld door: Vader / Moeder / Grootouder / Andere, nl.: ___________

1. Waren er problemen tijdens de zwangerschap?   JA / NEE

   Welke? ____

2. Heeft de moeder van de deelnemer tijdens de zwangerschap medicijnen gebruikt?   JA / NEE

   Welke en waarom? ____

3. Heeft de moeder van de deelnemer tijdens de zwangerschap gerookt?   JA / NEE

4. Heeft de moeder van de deelnemer tijdens de zwangerschap alcohol gebruikt?   JA / NEE

5. Heeft de moeder van de deelnemer tijdens de zwangerschap verdovende middelen gebruikt?   JA / NEE

6. Waren er complicaties bij de bevalling?   JA / NEE

   Welke? ____

B

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ADHD-UAntwerpen

ADHD ratingschaal-IV: Thuisversie (1): start   Deelnemersn°: _____

Deelnemersnummer: ________________   Datum: ________________

Vragenlijst ingevuld door: Moeder / Vader / Grootouder / Andere, nl.: ___________

Kruis aan welk van de antwoordmogelijkheden het beste het gedrag van uw kind omschrijft in de huiselijke situatie van de **afgelopen 6 maanden.**

	Het kind:	**Nooit of zelden**	**Soms**	**Vaak**	**Heel vaak**
1	Slaagt er niet in om veel aandacht te besteden aan details of maakt slordige fouten in schoolwerk				
2	Friemelt met handen of voeten of draait op zijn/haar stoel				
3	Heeft moeite de aandacht bij taken of spelletjes te houden				
4	Staat op in de klas of in andere situaties waarin wordt verwacht dat het kind blijft zitten				
5	Lijkt niet te luisteren als er tegen hem/haar wordt gepraat				
6	Rent rond of klimt veel in situaties waarin dit ongepast is				
7	Volgt instructies niet op en slaagt er niet in om werk af te maken				
8	Heeft moeite rustig te spelen of mee te doen aan vrijetijdsactiviteiten				
9	Heeft moeite met het organiseren van taken en activiteiten				
10	Is altijd gehaast of doet alsof hij/zij is aangedreven door een motor				
11	Vermijdt taken (bijv. schoolwerk, huiswerk) die langdurige mentale inspanning vereisen				
12	Praat overdreven veel				

Figure 5.6. Example of (A) the general questionnaire and (B) ADHD-RS (first pages).

A

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Sociaal-Emotionele Vragenlijst – Ouders (1): start Deelnemersn°: ____

Waarom deze vragenlijst?
Elk kind heeft op zijn tijd wel eens (gedrags)problemen. Wij willen graag weten welke vaak en welke minder vaak voorkomen. Daarom noemt deze lijst een groot aantal (72) mogelijke gedragsproblemen van kinderen. Wij vragen u aan te kruisen welke problemen het kind weleens heeft.

Hoe vult u de vragenlijst in?
U leest de bewering eerst goed door. Vervolgens geeft u aan in welke mate het genoemde gedrag of probleem bij het kind voorkomt. Baseer u hierbij op **de laatste 6 maanden**. U kunt de volgende antwoorden geven:

Nooit: Gedrag komt niet of nauwelijks voor.
Af en toe: Gedrag komt incidenteel voor.
Geregeld: Gedrag komt maandelijks voor.
Vaak: Gedrag komt wekelijks voor.
Heel vaak: Gedrag komt dagelijks voor.

Per bewering kruist u één antwoord aan. Wilt u alstublieft geen beweringen overslaan?

N.B. Sommige gedragingen komen alleen voor bij oudere kinderen, zoals 's nachts over straat zwerven. Indien een vraag duidelijk niet van toepassing is vanwege de *leeftijd of omgeving (bv. school)*, dan kunt u "Nooit" aankruisen. Dat geldt ook als u het antwoord op een vraag beslist niet weet.

Deelnemersnummer: ________________ Datum: ________________

Vragenlijst ingevuld door: Vader / Moeder / Leerkracht / Andere, nl.: ____________

	Het kind:	**Nooit**	**Af en toe**	**Geregeld**	**Vaak**	**Heel vaak**
1	Wordt makkelijk afgeleid					
2	Praat aan één stuk door					
3	Heeft moeite om rustig te spelen					
4	Is vergeetachtig					
5	Begint gauw vechtpartijtjes of is snel in vechtpartijtjes betrokken					

B

Fysieke klachten: Thuisversie (1): start Deelnemersn°: _____

Wilt u alstublieft bij iedere vraag aanduiden of uw kind het symptoom "Nooit", "Af en toe", "Geregeld", "Vaak" of "Heel vaak" heeft? Wilt u alstublieft uw antwoorden baseren op de laatste zes maanden?

Nooit: Symptoom komt niet of nauwelijks voor.
Af en toe: Symptoom komt incidenteel voor.
Geregeld: Symptoom komt maandelijks voor.
Vaak: Symptoom komt wekelijks voor.
Heel vaak: Symptoom komt dagelijks voor.

	Klacht:	**Nooit**	**Af en toe**	**Geregeld**	**Vaak**	**Heel vaak**
1	Hoofdpijn					
2	Buikpijn					
3	Misselijkheid, overgeven					
4	Ongebruikelijke dorst					
5	Ongebruikelijk veel zweten					
6	Ongebruikelijk warm hebben					
7	Gevoelige huid					
8	Eczeem					
9	Neus/keel/oorklachten					
10	Verkoudheid					
11	Astma					
12	Diarree					
13	Obstipatie					
14	Winderigheid					

Figure 5.7. Example of (A) SEQ and (B) PCQ (first pages).

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Voedselvragenlijst: start

Deelnemersn°: _____

Deelnemersnummer: ____________________ Datum: ________________

Vragenlijst ingevuld door: Vader / Moeder / Grootouder / Andere, nl.: ___________

Gelieve onderstaande vooraf aandachtig te lezen. Hoe deze vragenlijst in te vullen:

Duid in de tabellen op de volgende pagina's aan hoe vaak uw kind de vermelde producten eet.
Denk hierbij aan uw kinds gemiddelde over een volledig jaar.

Ter verduidelijking wordt een voorbeeld uitgewerkt:

Voorbeeld 1: Uw kind eet nooit andijvie.

Voorbeeld 2: Uw kind eet in het seizoen (mei-juni) ongeveer 2 maal per week asperges.
Als u dit over een gans jaar bekijkt, komt dit neer op een gemiddelde van 8 tot 10 maal per jaar.
U kruist het vakje "Minder dan 1 dag per maand aan".

Voorbeeld3: Uw kind eet gemiddeld om de twee dagen een appel.
U kruist het vakje "2-4 dagen per week" aan.

Duid slechts één keuze aan voor elke vraag.

Voedingsproduct:	Nooit	Minder dan 1 dag per maand	1-3 dagen per maand	1 dag per week	2-4 dagen per week	5-6 dagen per week	1 maal per dag	2-3 maal per dag	Meer dan 3 maal per dag
Water (leidingwater, flessenwater, …)									
Koffie, thee									

Figure 5.8. Example of the FFQ (first page).

3.4 Documents for the investigator

Various forms were developed to be filled out by the involved investigators. Examples are the screening checklist (Figure 5.9A), which contains all in- and exclusion criteria to be confirmed before inclusion of a patient, and the prescription form (Figure 5.9B), for participants to receive trial medication from the hospital pharmacy. Other examples include forms for participants' personal information (e.g. full name, address), basic information (gender, birth date, date and time of blood and urine collection, etc.), vital signs (blood pressure, heart rate, weight and length), spontaneously reported AEs and deviation from the protocol, among others.

A

Screening deelname ADHD/ADD
Pycno 2015-14

Datum:
Arts:

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Initialen mogelijke deelnemer		Geboortedatum:		Tijd sinds diagnose:	
Opmerkingen					
Criteria*	○6-12 jaar oud ○ADHD of ADD diagnose (of positieve ADHD-RS) ○Geen autisme of chronische ziekte (diabetes, epilepsie, autoimmuun-ziekte, maag-darm/nier/cardiovasculaire aandoening, etc.) of acute ontsteking ○Geen glaucoom, hartaandoening, hartritmestoornis, hoge bloeddruk, of perifere vasculaire aandoening (bv. syndroom van Raynaud). ○Geen ernstige mentale aandoening of retardatie (IQ < 70) ○Geen situationele hyperactiviteit, pervasieve ontwikkelingsstoornis, schizofrenie, andere psychotische aandoeningen (stemmings-, angst- of persoonlijkheids-stoornis), persoonlijkheidsverandering door medische aandoening, onvoldoende stimulerende omgeving, antisociale gedragsstoornis, chorea of dyskinesie. ○Geen tics of syndroom van Tourette, persoonlijke of familiale geschiedenis van psychoses, bipolaire stoornis, depressie of zelfmoordpoging ○Geen zwangerschap of plannen voor zwangerschap ○Ten minste 3 maanden voorafgaand deelname geen medicatie of vitaminen/mineralen/visolie (omega-3 vetzuur) supplementen gedurende langer dan 1 week, geen gebruik van clonidine, guanethidine, bloedverdunners, antidepressiva, mediatie tegen verkoudheid of allergie met decongestivum, medicatie tegen hoge of lage bloeddruk, beroertemedicatie, of dieetpillen ○Geen gebruik van MAO inhibitor gedurende de laatste 14 dagen ○Geen contraindicatie voor MPH of Pycnogenol of deelname aan andere studie ○Verantwoordelijke volwassene kan informatie geven over functionele status ○Voldoende kennis Nederlands ○Geen afhankelijkheid van de onderzoeker of verblijf in instelling				

*Duid aan welke criteria wel gehaald werden (aanduiden), en welke niet (open laten).
Enkel deelname indien voldaan aan alle criteria.

B

Universiteit Antwerpen

Klinische Studie PYCNO 2015-14

VOORSCHRIFT

Hoofdonderzoeker:

Onderzoeker:

Patiënt naam:	
Geboortedatum:	
Deelnemersnummer:	
Gewicht (< of ≥ 30 kg)	

Behandeling gedurende 10 weken.
Orale inname.
1 capsule per dag bij het ontbijt.
≥ 30 kg: 2 capsules per dag bij het ontbijt vanaf week 3.

Handtekening voorschrijver: ______________________

Naam voorschrijver: ______________________

Datum: ______________________

Afgeleverd: ________(datum)________(paraf, initialen)

Figure 5.9. Example of (A) the screening checklist and (B) prescription form for trial medication.

3.5 Standard operating procedures and related forms

All investigators were given instructions on compliance with the protocol and GCP in general. In addition, the rules and regulations of the trial (based on official regulations, laws and trial specific standards) were written down in various manuals, SOPs and related files. For instance, SOPs and manuals were created regarding inclusion of participants, obtaining of informed consents, CRF completion and filing, data entry and security, sample collection and pick-up, treatment storage, randomisation and disposal, AEs, breaches and deviations, internal audits, etc. Every SOP should be read and signed by the involved trial personnel. These SOPs and manuals serve to implement and maintain quality assurance and control, by ensuring that the whole trial team and the different trial sites are operating in a correct and formalised way, thereby increasing comparability, reducing the risk of errors and guaranteeing participant safety. SOPs thus ensure that the trial is conducted and data are generated, recorded and reported in compliance with the protocol, GCP and regulatory requirements.

Additionally, a signature list should be signed by every involved investigator, lab technician, etc., to be able to trace signatures on every document. Other examples are a training log to be signed by everyone who was trained for a specific activity in the trial and a list with contact information of all personnel involved. Finally, also a drug dispensation and accountability form was developed for the involved hospital pharmacies, to keep record of the "products' deliveries to the trial site, the inventory at the site, the use by each subject and the return to the sponsor or alternative disposition of unused product(s). These records include dates, quantities, batch/serial numbers, expiration dates (if applicable) and the unique code numbers assigned to the IPs and trial subjects" [18].

3.6 Investigator's Brochure

When planning a clinical trial, sufficient safety and efficacy data from non-clinical and/or clinical studies should be available "to support human exposure by the route, at the dosages, for the duration and in the trial population to be studied". The IB (Figure 5.10) compiles all preclinical and clinical data about an IP relevant to the use of this product in human subjects and summarises potential risks and benefits, if any. This document is intended to provide the investigator with information underlying specific features of the protocol, such as dosage and safety monitoring procedures. This information must be presented in an objective and concise manner, so that the investigator can make his/her own risk-benefit assessment. The IB should include [18]:

- Title page: IB number and release date, sponsor, chemical or generic and trade name of the IP;
- Summary: main physical, chemical, pharmacokinetic, pharmaceutical, pharmacological, toxicological and clinical information;
- Introduction: active ingredients, pharmacological class and rationale for research;
- Physical, chemical and pharmaceutical properties, formulation (incl. excipients) and storage;
- Non-clinical studies: pharmacology (incl. therapeutic activity and safety), toxicology and pharmacokinetic studies with their methodology (species tested, number of animals, dosing and route of administration), results (nature, severity, time of onset, reversibility and duration of effects) and relevance to humans;
- Effects in humans: pharmacokinetics, pharmacodynamics, dose-response, safety and efficacy studies, incl. population subgroups and interactions, with a summary of every completed clinical trial and implications of results, as well as marketing experience;
- Reference safety information: guidance for the investigator regarding possible risks and adverse reactions and regarding specific precautions possibly required in the clinical trial.

If the IP is marketed as a medicinal product and studied for its intended indication, an IB might not be necessary and a basic information brochure or package leaflet can be sufficient. In case a marketed product is studied for a different indication, a specific IB should still be prepared.

The IB should be reviewed at least once a year and should be updated with any new and important information [18].

may play a role in UV-induced erythema. Pycnogenol added to keratinocyte cell culture inhibited UV-induced NF-κB-dependent gene expression in a concentration-dependent manner [68]. However, NF-κB-DNA-binding activity was not prevented, suggesting that Pycnogenol affects the transactivation capacity of NF-κB. Inhibition of NF-κB-dependent gene expression by Pycnogenol may contribute to its mechanism of protecting human skin against solar UV-simulated light-induced erythema [68].

Pycnogenol may also be beneficial for inflammatory skin disorders. Human keratinocytes were treated with Pycnogenol or control in cell culture. Pycnogenol downregulated calgranulin A and B genes, which are upregulated in patients with psoriasis [69]. Also, patients with psoriasis, atopic dermatitis, and lupus erythematosus have upregulation of ICAM-1 expression in keratinocytes. A cell culture experiment revealed that Pycnogenol inhibited of IFN-γ-induced expression of ICAM-1 and inhibited adherence of T-cells to keratinocytes [70].

Pycnogenol suppresses melanin biosynthesis via its antioxidative properties. It suppressed superoxide, nitric oxide, peroxynitrite and hydroxyl radical in melanoma cell culture [71].

Animal studies - Pycnogenol dose-dependently and significantly reduced the incidence and severity of skin irritation and histopathological lesions in a rat model of hexavalent chromium-induced dermotoxicity. Also, it reduced MDA concentration, and increased GST and catalase activity [72].

Wound-healing was examined in 2 rat experiments. Pycnogenol (at concentrations of 1, 2, and 5%) significantly shortened the time of wound healing by 1.6 days, 2.8 days and 3.3 days. Pycnogenol gel also dose-dependently reduced the scar diameter [73]. Pycnogenol 2% decreased MDA and increased SOD in the wound [74].

3.1.9 Allergy

In vitro studies - Pycnogenol dose-dependently reduced histamine release from rat peritoneal mast cells and decreased anti-dinitrophenyl (DNP) IgE-induced calcium uptake into rat peritoneal mast cells (which is required to perpetuate the allergic reaction) [75].

Animal studies - Immune system dysfunction in mice, induced by a diet containing 7.5% of recommended nutrients, resulted in an abnormal pattern of cytokine secretion, enhanced hepatic lipid peroxidation, low lymphocyte proliferation and shorter survival time. Oral administration of Pycnogenol restored immune function and prolonged survival [76].

In a rat model of allergy, oral Pycnogenol significantly inhibited DNP IgE-mediated passive cutaneous anaphylaxis [75]. Along with in vitro data [75], this demonstrates potential for Pycnogenol in mast-cell immediate-type allergies.

3.1.10 Liver function

Animal studies - Intraperitoneal injection of Pycnogenol in rats could dose-dependently prevented liver tissue damage and lipid peroxidation when given 2 weeks before an ip injection of carbon tetrachloride [77]. Hepatoprotective effects of Pycnogenol were also observed in a rats model of non-alcoholic steatohepatitis, as oral Pycnogenol decreased liver triglycerides and macrovesicular steatosis [78].

3.2 Pharmacokinetics

3.2.1 Transdermal bioavailability

Transdermal bioavailability was evaluated by applying 5% w/v Pycnogenol in polyethylene glycol topically to human cadaver skin (n = 10). Six substances (gallic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillin, catechin, and an unidentified substance) were absorbed through the skin. The absorption was rapid (30 minutes). Taxifolin was also detected, but only in perfusates from 3 of the 10 donors. The authors concluded that Pycnogenol can be used for topical application [81].

3.2.2 Biodistribution

Plasma protein binding can alter PK and pharmacodynamic properties. Hence, the plasma protein binding of Pycnogenol's constituents/metabolites were assessed in vitro. The greatest protein binding was for catechin = taxifolin > procyanidin B1 > ferulic acid > caffeic acid > p-cumaric acid > vanillic acid > p-hydroxy benzoic acid > M1 > gallic acid > M2 > protocatechuic acid [82]. The clinical significance of this needs to be elucidated. Others have shown that the protein bound fraction of polyphenols is protected from degradation; in turn, the protein is protected against peroxidation [83].

M1 is not a constituent of Pycnogenol but rather is generated from (procyanidins cleaved to) catechin units by gut microflora after ingestion (ring fission to valerolactones) [84]. An in vitro human cell culture study revealed that M1 is transported into cells (macrophages, monocytes, and endothelial cells) via facilitated transport, most probably by the GLUT-1 transporter, where it accumulates and undergoes further intracellular metabolism, to levels that could be bioactive [16]. This explains why M1, which is only present in low concentrations in the plasma after Pycnogenol ingestion, could produce a biological effect. The M1 forms glutathione conjugates in human blood cells besides other conjugates [85, 86].

3.3 Toxicology

The toxicity of Pycnogenol is regarded as very low. The acute toxicity is low after oral administration in mice, rats, and guinea pigs. The representative LD_{50} values are 2.3, 4.2, and 2.0 g/kg, respectively [87]. In chronic toxicity tests, oral application of up to 2000 mg/kg/day in rats did not produce clinically meaningful changes in blood status, body weight, or food consumption. Also, the rats exhibited normal behavior [87]. The no-adverse-effects level (NOAEL) has been established at 100 mg/kg/day [4]. The level of mutagenicity and genotoxicity of Pycnogenol was tested with the Ames test, the micronucleus assay in mouse bone marrow cells in vivo, and with the chromosome aberration assay in human lymphocytes in vitro. Some evidence of slight mutagenicity was suspected in one *in vitro* micronucleus study, but this was countered by other results of the same study, as well as by various other *in vitro* studies, as well as by *in vivo* animal and human research. In fact, other results suggest that Pycnogenol is non-mutagenic and non-genotoxic, and even protects genetic material against oxidative damage [87].

Figure 5.10. Example pages of the Investigator's Brochure (IB).

3.7 Investigational Medicinal Product Dossier

An IMP is defined as "a pharmaceutical form of an active substance or placebo being tested or used as a reference in a clinical trial, including products already with a marketing authorisation but used or assembled (formulated or packaged) in a way different from the authorised form, or when used for an unauthorised indication, or when used to gain further information about the authorised form" [20]. An IMP should be manufactured, handled and stored according to GMP guidelines. For instance, its packaging should prevent contamination and deterioration during transport and storage. In addition, it should be coded and labelled clearly according to regulatory requirements, while protecting the blinding, if applicable.

The IMPD (Figure 5.11) is required for clinical trials in the European Union (EU) and provides information on the quality, production and control of any IMP as well as data from non-clinical studies and from its clinical use. The IMPD focusses on patient safety, including impurities, storage conditions, microbial contamination and toxic degradation products. The IMPD should be prepared in the Common Technical Document (CTD) format and should include [85, 86]:

- Introduction: proprietary and non-proprietary name of the drug substance, company, dosage form(s), route of administration, proposed indication(s);
- Information on the drug substance: chemical name, structure, physicochemical properties, manufacturer, manufacturing process, source material(s), analytical procedures, stability, etc.;
- Information on the drug product (IMP): composition, pharmaceutical development, manufacturer, excipient quality, analytical procedures, packaging, labelling, stability, etc.;
- Non-clinical pharmacology, pharmacokinetics and toxicology;
- Clinical pharmacology and pharmacokinetics;
- Overall risk and benefit assessment;
- Appendices, e.g. material safety data sheet, certificate of analysis, TSE/BSE certificate, qualified person (QP) certificate, manufacturing authorisation (in this case: authorisation for Qualiphar NV to handle an IMP to be used in a human clinical trial, including labelling and storage), GMP certificate of the active substance (in this case: certificate that the manufacturing site of Pycnogenol® extract complies with all GMP requirements), etc.

Cross-reference to the IB for the preclinical and clinical parts of the IMPD is allowed. The IMPD should be updated with any new information [87].

Labelling specifications (Figure 5.12) on the IMP should be included in the IMPD as well. Labelling should be done in all national languages of the countries the trial will be performed in and should include [85]:

- The main contact(s) for information on the product, clinical trial and emergency unblinding;
- Pharmaceutical dosage form, route of administration and quantity of dosage units;
- Batch and/or code number to identify the contents and packaging operation;
- Trial reference code for identification of the trial, site, investigator and sponsor;
- Trial subject identification number/treatment number;
- Directions for use;
- “For clinical trial use only”;
- Storage conditions;
- Expiry or retest date.

Used part of the plant: When trees are cut for timber production, the bard is stripped and immediately transferred to the extraction plant. The quality of the raw material (bark) is controlled to French standard regulations (ANSM), as is the production of Pycnogenol powder. Because the bark is obtained from a monoculture grown over 30-50 years, the quality of the raw material used for the production of Pycnogenol is consistent and not subjected to seasonal and annual variations.

Processing of the bark: The bark is cleaned after crushing into small pieces by sieving, removing soil, branches and needles. The bark is controlled for conformity with the USP 38 monograph "Pine Bark" and crushed again to produce a powder suitable for extraction.

Extraction and spray-drying: The bark is subjected to a series of extraction steps involving water and ethanol (95% V/V ethanol) as eluents in a patented, fully automated extraction process. After purification of the raw extract, the aqueous solution of the extracted constituents is spray-dried, resulting in a fine brownish powder.

The extraction process is summarized in the flow chart in Figure 1.

2.1.S.2.3 Control of Materials

Reagents, solvents and other materials used in the preparation process for Pycnogenol are listed in Table 1.

Table 1: Reagents, solvents and other materials

Material	Grade	Specific test item	Possible impurity
Outer bark of Pinus pinaster	USP	USP	Branches, needles, soil (USP)
Water	Potable water*	See 2.5.3	See 2.5.3
Ethanol	96%	PCC 006 & PCC 038	See 2.5.4

*As tap water is used, French state ensures its analysis, but 3 additional points in the production facility are also tested by the manufacturer.

2.1.S.2.4 Controls of Critical Steps and Intermediates

See red arrows in extraction process flow chart (Figure 1).

2.1.S.2.5 Process Validation and/or Evaluation

The apparatus and production methods used are validated in GMP procedures.

2.1.S.2.6 Manufacturing Process Development

The manufacturing process of Pycnogenol powder for the proposed study does not differ from that used for the production of batches used for marketing or in non-clinical studies.

2.1.S.3.1 Elucidation of Structure and Other Characteristics

Pycnogenol is standardized to contain 70 ± 5% procyanidins. Pycnogenol, Pinus pinaster Ait. subsp. Atlantica outer bark extract, meets the detailed specifications for Maritime Pine Extract of the US Pharmacopeia (USP 38 Dietary Supplements). A spectrophotometric method is available in the USP for determination of Pycnogenol® procyanidin content, by the Bates-Smith Assay (USP 38). Thin-Layer Chromatography (TLC) and HPLC fingerprint (both USP 38) confirm the identity of Pycnogenol with the USP Maritime Pine Extract reference standard.

HPLC, LC-MS and NMR methods revealed that Pycnogenol contains mainly procyanidins, phenolic acids, catechin and taxifolin. The procyanidins are biopolymers consisting of units of catechin and epicatechin, with chain lengths ranging from 2 to 12 monomeric units. The more common dimers, B1 and B3, have been identified as consisting of epicatechin-catechin and catechin-catechin units linked with a C4-C8 bond. Less common are dimers B6 and B7, which are catechin-catechin and epicatechin-epicatechin units linked with a C4-C6 bond. A trimer C2, consisting of catechin-epicatechin-catechin has also been identified. Catechin, epicatechin, and taxifolin represent the "monomeric" procyanidins, of which catechin is the most common. The phenolic acids in Pycnogenol are derivatives of benzoic acid (p-hydroxybenzoic acid, protocatechic acid, gallic acid, vanillic acid) or cinnamic acid (caffeic acid, ferulic acid, p-cumaric acid). The phenolic acids are found in free form and as glucosides or glucose esters.

Free glucose is present in small amounts, and other sugars including arabinose, rhamnose, and xylose are detected following glycolysis. Inorganic substances include calcium, potassium, and iron with traces of manganese, zinc and copper.

Pharmacological studies employing *in vitro*, animal, and/or human models have found that Pycnogenol has potent antioxidant activity; anti-inflammatory actions; alters neurotransmitter levels in children with ADHD; improves measures of cognition; reduces neuroinflammation; etc.

2.1.S.3.2 Impurities

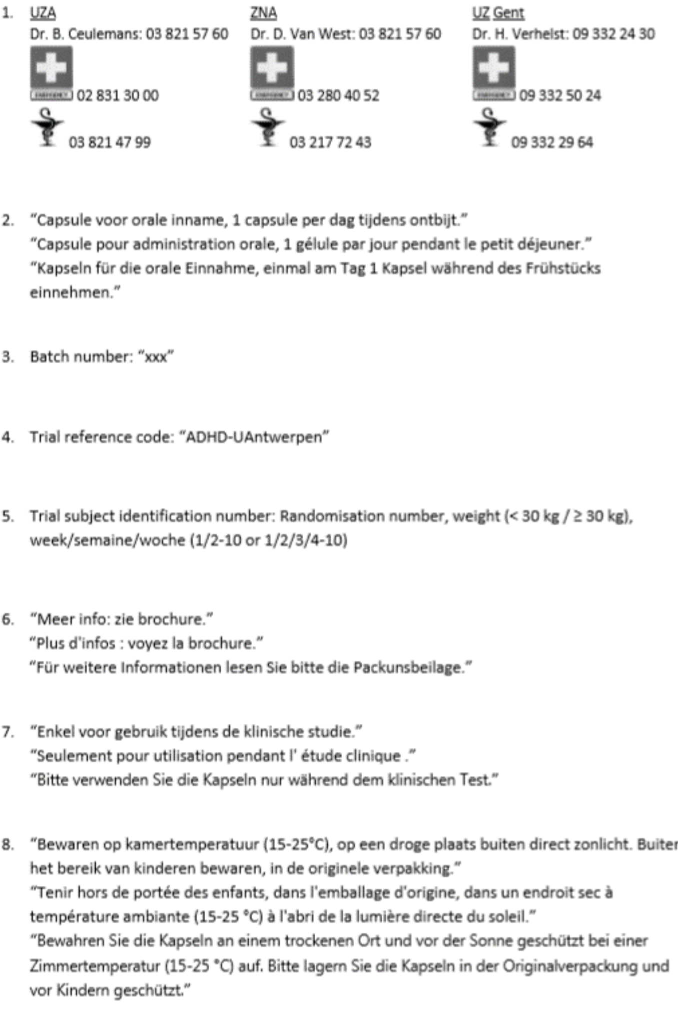
Residual solvents: Water (not more than 8%), ethanol (not more than 100 ppm).

Microbial contamination: Limits (total aerobic microbial count, total combined molds and yeasts count, enterobacterial count, Salmonella, E. coli, P. aeruginosa and S. aureus, and C. albicans) are USP 38 conform.

Organic impurities: Total ash is not more than 0.7%, water insoluble residues not more than 10%. Pesticide residue: test of USP 38 passed.

Traces of iron, selenium, zinc, heavy metals, arsenic, cadmium, chromium, copper, lead, mercury and nickel are conform with USP 38.

Figure 5.11. Example pages of the Investigational Medicinal Product Dossier (IMPD).



1. UZA
   Dr. B. Ceulemans: 03 821 57 60
   02 831 30 00
   03 821 47 99

   ZNA
   Dr. D. Van West: 03 821 57 60
   03 280 40 52
   03 217 72 43

   UZ Gent
   Dr. H. Verhelst: 09 332 24 30
   09 332 50 24
   09 332 29 64

2. "Capsule voor orale inname, 1 capsule per dag tijdens ontbijt."
   "Capsule pour administration orale, 1 gélule par jour pendant le petit déjeuner."
   "Kapseln für die orale Einnahme, einmal am Tag 1 Kapsel während des Frühstücks einnehmen."

3. Batch number: "xxx"

4. Trial reference code: "ADHD-UAntwerpen"

5. Trial subject identification number: Randomisation number, weight (< 30 kg / ≥ 30 kg), week/semaine/woche (1/2-10 or 1/2/3/4-10)

6. "Meer info: zie brochure."
   "Plus d'infos : voyez la brochure."
   "Für weitere Informationen lesen Sie bitte die Packunsbeilage."

7. "Enkel voor gebruik tijdens de klinische studie."
   "Seulement pour utilisation pendant l' étude clinique ."
   "Bitte verwenden Sie die Kapseln nur während dem klinischen Test."

8. "Bewaren op kamertemperatuur (15-25°C), op een droge plaats buiten direct zonlicht. Buiten het bereik van kinderen bewaren, in de originele verpakking."
   "Tenir hors de portée des enfants, dans l'emballage d'origine, dans un endroit sec à température ambiante (15-25 °C) à l'abri de la lumière directe du soleil."
   "Bewahren Sie die Kapseln an einem trockenen Ort und vor der Sonne geschützt bei einer Zimmertemperatur (15-25 °C) auf. Bitte lagern Sie die Kapseln in der Originalverpackung und vor Kindern geschützt."

9. "Exp. Yyyy/mm"

Figure 5.12. Treatment label content.

3.8 Summary of Product Characteristics

The SmPC of a marketed product can replace the IMPD and IB if the IMP has a marketing authorisation in the EU and if the IMP will be used for the same indication, in the same form and with a dosing regimen that can be accepted based on the SmPC. The SmPC is a legal document approved during the marketing authorisation of a medicinal product. It contains all required information for a healthcare professional on how to use the product, including qualitative and quantitative information on benefits and risks, effects in subpopulations and pharmaceutical information. Package leaflets are based on the

SmPC. Like the IB and IMPD, the SmPC is updated as new data emerge. Example pages from the SmPC of Medikinet® Retard can be found in Figure 5.13.

The SmPC includes [88]:

- Name of the medicinal product;
- Qualitative and quantitative composition;
- Pharmaceutical form;
- Clinical particulars: therapeutic indications, dosing, method of administration, contraindications, precautions, interactions, pregnancy and lactation, driving ability, side effects, overdose, etc.;
- Pharmacological properties: pharmacodynamics, pharmacokinetics and preclinical safety;
- Pharmaceutical particulars: excipients, incompatibilities, shelf life, storage, container and disposal.

1. Name of the medicinal product

Medikinet XL 10 mg modified-release capsules, hard

Medikinet XL 20 mg modified-release capsules, hard

2. Qualitative and quantitative composition

Medikinet XL 10 mg modified-release capsules, hard: Each modified-release capsule, hard contains 10 mg methylphenidate hydrochloride, equivalent to 8.65 mg methylphenidate. Excipient with known effect: 127.14 mg – 145.42 mg sucrose/modified release capsule, hard.

Medikinet XL 20 mg modified-release capsules, hard: Each modified-release capsule, hard contains 20 mg methylphenidate hydrochloride, equivalent to 17.30 mg methylphenidate. Excipient with known effect: 114.65 mg – 131.13 mg sucrose/modified-release capsule, hard.

For the full list of excipients, see section 6.1.

3. Pharmaceutical form

Modified-release capsule, hard.

Medikinet XL 10 mg modified-release capsules, hard: White opaque capsule body/mauve opaque capsule cap (15.9 mm) containing white and blue pellets.

Medikinet XL 20 mg modified-release capsules, hard: Mauve opaque capsule body/mauve opaque capsule cap (15.9 mm) containing white and blue pellets.

4. Clinical particulars

4.1 Therapeutic indications

Attention-Deficit/Hyperactivity Disorder (ADHD): Methylphenidate is indicated as part of a comprehensive treatment programme for attention-deficit/ hyperactivity disorder (ADHD) in children

Appropriate educational placement is essential, and psychosocial intervention is generally necessary. Where remedial measures alone prove insufficient, the decision to prescribe a stimulant must be based on rigorous assessment of the severity of the child's symptoms. The use of methylphenidate should always be used in this way according to the licensed indication and according to prescribing / diagnostic guidelines.

4.2 Posology and method of administration

Posology

Treatment must be initiated under the supervision of a specialist in childhood and/or adolescent behavioural disorders.

Pre-treatment screening: Prior to prescribing, it is necessary to conduct a baseline evaluation of a patient's cardiovascular status including blood pressure and heart rate. A comprehensive history should document concomitant medications, past and present co-morbid medical and psychiatric disorders or symptoms, family history of sudden cardiac/unexplained death and accurate recording of pre-treatment height and weight on a growth chart (see sections 4.3 and 4.4)

Ongoing monitoring: Growth, psychiatric and cardiovascular status should be continuously monitored (see also section 4.4).

- Blood pressure and pulse should be recorded on a centile chart at each adjustment of dose and then at least every 6 months;
- Height, weight and appetite should be recorded at least 6 monthly with maintenance of a growth chart;
- Development of de novo or worsening of pre-existing psychiatric disorders should be monitored at every adjustment of dose and then least every 6 months and at every visit.

Patients should be monitored for the risk of diversion, misuse and abuse of methylphenidate.

Dose titration: Careful dose titration is necessary at the start of treatment with methylphenidate. This is normally achieved using an immediate-release formulation taken in divided doses. The recommended starting daily dose is 5 mg once daily or twice daily (e.g. at breakfast and lunch), increasing if necessary by weekly increments of 5-10 mg in the daily dose according to tolerability and degree of efficacy observed. Medikinet XL 10 mg once daily may be used in place of immediate-release methylphenidate hydrochloride 5 mg twice daily from the beginning of treatment where the treating physician considers that twice daily dosing is appropriate from the outset and twice daily treatment administration is impracticable.

Figure 5.13. Example pages of the Summary of Product Characteristics (SmPC).

PART 4

INTERNATIONAL REGISTRATION

For transparency, every clinical trial should be registered in an international database before the inclusion of participants, while also updates should be provided and primary results submitted. This information is thus publicly available and thereby fulfils various purposes. For instance, it provides objective information to referring clinicians and potential participants, it reduces publication bias and it facilitates systematic reviews. In addition, registration is required for ethical approval and publication. This trial has been registered at Clinicaltrials.gov (NCT02700685) and EudraCT (European Medicines Agency, 2016-000215-32). To further increase visibility and transparency, the trial protocol was published (Verlaet, A.A.J., *et al.* Effect of Pycnogenol® on attention-deficit hyperactivity disorder (ADHD): study protocol for a randomised controlled trial. Trials (2017), 18(1): p. 145-153) [89].

4.1 Clinicaltrials.gov

The information in Appendix 1.1, 1.2, 1.3 and 1.4 (see bookmark for QR code) was registered at clinicaltrials.gov (NCT02700685).

4.2 EudraCT

Analogously to the registration at clinicaltrials.gov, information on this clinical trial has been registered at EudraCT, the European Union Clinical Trials Register (n° 2016-000215-32).

PART 5

ETHICAL APPROVAL

Before the start of participant inclusion, a clinical trial requires ethical approval for every site it will be performed in. No deviations from the protocol should be initiated without prior ethical approval, except when only involving administrative or logistic changes (e.g. change of telephone number) or if necessary to eliminate immediate hazards to participants. In addition, the ethics committee should review each ongoing trial at least once a year, while new information that changes the risk-benefit balance for subjects and all adverse drug reactions that are both serious and unexpected should be reported immediately. Ethical approval for the present trial has been obtained in UZA (leading ethical committee, EC 15/35/365), ZNA (EC approval 4656) and UZ Ghent (EC 2016/0969). Here, an overview is given of all information and documents that were given ethical approval, as well as the initial approval letters.

5.1 Cover letter

In this letter (example in Figure 5.14), the title of the trial, rationale and short description should be presented. In case of an amendment, also reasons for the amendment can be given, as well as responses to questions of the ethics committee. Finally, all files to be approved should be listed and the letter should be signed by one of the investigators.

5.2 Site-specific information

Next to the protocol, the site-specific information document (example in Figure 5.15) gives a concise overview of the trial, including EudraCT number, title, sponsor, investigators, financial support, trial site(s), phase and kind of research (e.g. diagnostic or therapeutic), summary, rationale, interventions, in- and exclusion criteria, expected advantages and risks, etc. As this document is site-specific, it differs slightly between e.g. UZA and UZ Ghent.

Commissie voor Medische Ethiek UZ Gent 15 april 2016
De Pintepark II - 2de verdieping
De Pintelaan 185, 9000 Gent

Onderzoek naar Pinus pinaster schorsextract (Pycnogenol®) als therapeutisch alternatief voor methylfenidaat bij kinderen met ADHD: een parallele trial.

FWO MAND 2013 - 11U8314N (Verlaet Annelies) - PeopleSoft ID: 31579

Geachte Voorzitter van het Comité voor Medische Ethiek,
Geachte Leden,

ADHD wordt steeds vaker geassocieerd met immuunverstoringen en verhogingen in oxidatieve stress. Bovendien heeft methylfenidaat, de meest gebruikte behandeling bij ADHD, verschillende bijwerkingen en staan ouders vaak afkerig tegen het gebruik ervan. De eerste fase van dit groter project (UZA studie 13/18/209: FWO mandaat 2013 – 11U8314N – Annelies Verlaet) richtte zich op de bepaling van immuunverstoringen en oxidatieve stress bij ADHD. Omdat immuunverstoringen en verhogingen in oxidatieve stress mogelijkheden bieden voor voedingssupplementen als behandeling voor ADHD, wordt in deze tweede fase het effect van een polyfenolrijk extract van Pinus pinaster schors (Pycnogenol®, met zowel antioxidatieve als immuuneffecten) op gedrag, co-morbide symptomen en immuun en oxidatieve status van ADHD patiënten vergeleken met methylfenidaat en placebo in een parallelle trial. Resultaten kunnen gebruikt worden voor het ontwikkelen van een nieuwe therapie, daar behandeling met methylfenidaat niet optimaal is.

Doordat een open label behandeling selectiebias en een minder sterke vergelijking oplevert (bv. naar aanvaardbaarheid, verbeteren van symptomen en bijwerkingen), is namelijk besloten om te randomiseren naar drie verschillende behandelingen (methylfenidaat, Pycnogenol of placebo) die dubbel blind toegepast zullen worden. Kinderen die al medicatie nemen zullen uitgesloten worden van de studie.

Figure 5.14. Cover letter example (UZ Ghent, first page).

ETHISCH COMITE
Universitair Ziekenhuis
De Pintelaan 185
9000 Gent
ethisch.comite@UGent.be
tel. +32 9 332 33 36 - +32 9 332 68 54 - +32 9 332 26 88
fax +32 9 332 49 62

Document A (interventioneel academisch onderzoek)

VERZOEK TOT ADVIES VAN HET ETHISCH COMITE BETREFFENDE EEN ONDERZOEKSPROJECT BIJ DE MENS

EUDRACT NUMMER (INDIEN INTERVENTIONEEL GENEESMIDDELENONDERZOEK): **2016-000215-32**

1. TITEL VAN HET ONDERZOEK

EFFECT OF PYCNOGENOL® ON ATTENTION-DEFICIT HYPERACTIVITY DISORDER (ADHD): A RANDOMIZED, DOUBLE BLIND, PLACEBO AND ACTIVE PRODUCT CONTROLLED MULTICENTER TRIAL

2. **GEGEVENS VAN DE ONDERZOEKER(S) [de eerste onderzoeker moet een persoon zijn die vast verbonden is aan de dienst (geen ASO) of universiteit] :**

- NAAM: HELENE VERHELST
- FUNCTIE: ADJUNCT-KLINIEKHOOFD, KINDERNEUROLOOG
- UZ DIENST : KINDERGENEESKUNDE
  OF FACULTEIT/VAKGROEP: /
- TELEFOONNUMMER: 09 332 24 30
- FAX: /
- E-MAIL: HELENE.VERHELST@UGENT.BE
- NAAM UZ DIENSTHOOFD: PROF DR S VAN DAELE
  OF NAAM VAKGROEPVOORZITTER

Figure 5.15. Example of the site-specific information form (UZ Ghent, first page).

5.3 Protocol and summary of the protocol

Submission of the official protocol is required for the ethics committee to get an overview on how the trial will be conducted (see Chapter 5, Part 2). The EudraCT clinical trial application form (version "request for opinion of the ethics committee") should be submitted as well (see Chapter 5, 4.2).

5.4 Documents aimed at the participant

The ethics committee should check whether all information (i.e. information- and consent forms, recruitment advertisements, brochures, letters and questionnaires) presented to participants, caregivers, etc. is objective, complete, clearly stated using easy to understand language and not deceptive. Forms concerning personal and basic information of participants should be submitted as well, to check confidentiality requirements (see Chapter 5, 3.1, 3.2 and 3.3).

5.5 List of all ethics committees and local investigators

A list of all involved ethics committees (leading and local), as well as the curriculum vitae of every involved investigator, should be submitted for approval, as every individual involved in a trial should be qualified by education, training and experience to perform his/her respective task(s).

5.6 Insurance certificate

Based on the Belgian law of 7 May 2004 regarding human clinical trials, the trial sponsor is, even if faultless, liable for all damage caused to the participant or his/her beneficiaries, whether directly or indirectly related to the experiment. This liability is covered by a specific trial insurance, provided by the trial sponsor. The University of Antwerp offers an insurance contract for clinical trials on humans (Vanbreda/Amlin Europe NV policy number 199.535.692, Figure 5.16).

Attest aansprakelijkheidsverzekering

Ondergetekende bevestigt hiermee dat een verzekering objectieve aansprakelijkheid als opdrachtgever van een experiment op de menselijke persoon (wet 7 mei 2004) op naam van **Universiteit Antwerpen** werd onderschreven via de bemiddeling van Vanbreda Risk & Benefits NV, Plantin en Moretuslei 297, 2140 Antwerpen en op basis van de volgende gegevens:

1. **Verzekeraar**
   Amlin Europe NV

2. **Contractnummer**
   199.535.692

3. **Verzekerde periode**
   1/01/2016 – 31/12/2016

4. **Voorwerp van de dekking**
   Clinical trials waarvoor Universiteit Antwerpen opdrachtgever is en die in overeenstemming zijn met de Wet Experimenten op de menselijke persoon dd. 7/5/2004.

   Dekking wordt automatisch en zonder voorafgaandelijke meldingsplicht verleend voor experimenten (uitsluitend uitgevoerd in België) welke onder toepassing van risicoklasse 1 tot en met 5 vallen. Studies welke onder toepassing vallen van risicoklasse 6 en 7 blijven evenwel uitgesloten (volgens klasse-indeling in bijlage aan deze polis).

5. **Verzekerde waarborgen**
   per experiment : 2.500.000 EUR
   per verzekeringsjaar : 5.000.000 EUR

Antwerpen, 6 oktober 2015

Jan Van Hecke
Deputy Director

Figure 5.16. University of Antwerp insurance certificate for clinical trials.

5.7 Investigational product data

For evaluation of the safety and risk-benefit balance, all available information regarding the IPs should be submitted to the ethics committee. In case of the present trial, this included the IB of Pycnogenol® (see Chapter 5, 3.6), IMPD of Pycnogenol® and placebo (see Chapter 5, 3.7) and SmPC of Medikinet® Retard (see Chapter 5, 3.8).

5.8 Approval by the ethics committee

In case the ethics committee has no further questions and approves all submitted documents and thereby the proposed trial procedures, a favourable opinion for the clinical trial will be given (Figure 5.17 and Appendix 2.2, see bookmark for QR code).

A

UZA / Wilrijkstraat 10 / 2650 Edegem
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Tel +32 3 821 30 00 / Fax +32 3 829 05 20
www.uza.be / BE0874.619.603

ETHISCH COMITE

Prof. dr. B. CEULEMANS

Neurologie

VOORZITTER
Prof. dr. Patrick Cras

SECRETARIAAT
tel: 03 821 35 44

Onderzoek naar Pinus pinaster schorsextract (Pycnogenol®) als alternatieve of complementaire behandeling voor methylfenidaat bij kinderen met ADHD: een parallele trial. (FWO MAND 2013 - 11U8314N, Annelies Verlaet)
nieuwe titel: Onderzoek naar Pinus pinaster schorsextract (Pycnogenol®) als therapeutisch alternatief voor methylfenidaat bij kinderen met ADHD: een parallele trial. (FWO MAND 2013 - 11U8314N, Annelies Verlaet)

Belgisch Registratienummer: B300201526445

datum	ons kenmerk	contactpersoon
23/11/2015	15/35/365	Annelies Van Looy / Kim Vernimmen ethisch.comite@uza.be

DEFINITIEF GUNSTIG ADVIES

Geachte Collega,

Het Ethisch Comité van het Universitair Ziekenhuis Antwerpen en de Universiteit Antwerpen werd aangesteld als Centraal Ethisch Comité voor het uitbrengen van het enkel advies voor bovenvermelde studie wat betreft de punten 3, 4, 6 en 7 van §4, art 11 van de wet van 7 mei 2004.
De antwoorden op de brief dd. 12/10/2015 werden besproken op de vergadering dd. 23/11/2015. Het Ethisch Comité is van oordeel dat er voldoende rekening gehouden werd met de gemaakte opmerkingen.

De volgende bijlagen werden volgens de ICH- GCP richtlijnen door het Ethisch Comité goedgekeurd:

- Bijsluiter Pycnogenol®
- CV Onderzoeker Lokaal Centrum Prof. Van West (ZNA)

B

ETHICS COMMITTEE JUDGEMENT

On December 09, 2015, the Commissie voor Medische Ethiek ZNA,
Institutional Review Board – ZNA/OCMW Antwerpen, Lindendreef 1, 2020 Antwerpen,
(Erkenning nr. 009; OG 031), has examined the following protocol:

Protocol title:	Onderzoek naar pinus pinaster schorsextract (Pycnogenol®) als alternatieve of complementaire behandeling voor methylfenidaat bij kinderen met ADHD: een parallele trial. (Effect of Pycnogenol® on attention-deficit hyperactivity disorder (ADHD)) - FWO MAND 2013 – 11U8314N **Nieuwe titel**: Onderzoek naar pinus pinaster schorsextract (Pycnogenol®) als therapeutisch alternatief voor methylfenidaat bij kinderen met ADHD: een parallele trial. (FWO MAND 2013 – 11U8314N)
Protocol number:	2015/01 Version: 1
Eudract number:	B300201526445
Protocol date:	July 15, 2015
Pharm. company:	UA
Submitted by:	prof. dr. D. Van West, Kinder- en Jeugdpsychiatrie UKJA (prof. dr. B. Ceulemans, mevr. A. Verlaet UA)

☐ initial ☐ continuing ■ protocol amendments ☐ end of study

Following documents have been submitted as required by European Directive 2001/20/EC and as stated in the Belgian law regarding experiments in humans dd. 07.05.2004:

■ protocol, updated version 2 dd. 30.10.15
☐ investigators brochure
■ informed consent document, child 6-12 years, Dutch version dd. 30.10.15
informed consent document, parents – PYC vs placebo vs MPH, Dutch version dd. 30.10.15
informed consent document, teacher PYC vs placebo vs MPH, Dutch version dd. 30.10.15
☐ insurance declaration
☐ amendment
■ letter of notification from EC UZA reg. full approval, dd. 23.11.15
■ letter of notification in answer to remarks dd. 30.10.15
■ adviesaanvraag EC UZA, updated version 2
■ synopsis of the protocol, Dutch version 2 dd. 30.10.15
■ letter to volunteer, Dutch version 2 dd. 30.10.15
■ leaflet, Pycnogenol®, Dutch version 2 dd. 30.10.15
■ questionnaire, algemeen, Dutch version 2 dd. 30.10.15
■ questionnaire, overige klachtenlijst, laatste 6 maanden, Dutch version 2 dd. 30.10.15
■ questionnaire, overige klachtenlijst, laatste 5 weken, Dutch version 2 dd. 30.10.15
■ informatieformulier ADHD/ADD, Dutch version 2, dd. 30.10.15

Figure 5.17 (Part 1). Initial approval letters of the ethics committees of (A) UZA and (B) ZNA (first pages).

C

COMMISSIE VOOR MEDISCHE ETHIEK

Voorzitter:
Prof. Dr. D. Matthys

Secretaris:
Prof. Dr. J. Decruyenaere

Afz: Commissie voor Medische Ethiek

Pediatrie Neurologie
Kliniekgebouw 5
Dr. Helene VERHELST
ALHIER

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UW KENMERK	**ONS KENMERK**	**DATUM**	**KOPIE**
	2016/0969	10-jan-17	Zie "CC"

BETREFT

Advies Amendement voor studie met als titel:
Effect of Pycnogenol on attention-deficit hyperactivity disorder (ADHD): A randomized, double blind, placebo and active product controlled multicenter trial.

Belgisch Registratienummer: B670201629713 (AV)
Fase (Phase): 3

Geachte collega,

Wij ontvingen uw aanvraag op 2/12/2016 voor een amendement betreffende bovenvermelde studie. Het betreft:

- Begeleidende brief Amendement dd.10/11/2016
- Request for authorisation of a clinical trial dd.10/11/2016
- Notification of a substantial amendment dd.10/11/2016
- Amendement op ICF
  - ICF versie 6 Gent september 2016
- Amendement op Protocol
  - Protocol Amendement 1 september 2016

Figure 5.17 (Part 2). Initial approval letter of the ethics committee of (C) UZ Ghent (first page).

PART 6

APPROVAL BY THE COMPETENT FEDERAL AUTHORITY

Before the start of a clinical trial, all necessary documentation should be submitted to and approved by the competent federal authority. The main product under research in this clinical trial is Pycnogenol®. Though a nutritional supplement, it is considered a medicinal product in this trial, as it is compared to Medikinet® Retard, which is a registered medicinal product. In this case, the Federal Agency for Medicines and Health Products (FAMHP) is therefore the competent federal authority. The following documents were submitted to the FAMHP:

- Cover letter;
- Trial protocol (see Chapter 5, Part 2);
- EudraCT clinical trial application form (version “request for authorisation to the competent authority”; see Chapter 5, 4.2);
- IB for Pycnogenol® (see Chapter 5, 3.6);
- IMPD for Pycnogenol® and placebo (see Chapter 5, 3.7);
- SmPC for Medikinet® Retard (see Chapter 5, 3.8);
- Label content (see Chapter 5, 3.7);
- Approval of the leading ethics committee (see Chapter 5, 5.8);
- Manufacturing authorisation (see Chapter 5, 3.7);
- GMP certificate of the active substance (see Chapter 5, 3.7);
- Insurance certificate (see Chapter 5, 5.6).

In the cover letter (example in Figure 5.18), the title of the trial, EudraCT number, protocol number, trial particulars and referral to the “reference safety information” (e.g. in the IB) should be presented. In addition, special populations and Belgian manufacturing sites and their activities should be mentioned, as well as whether the trial is the first application of a new active substance, whether the IMP is a narcotic or psychotropic substance, whether the trial is part of a Paediatric Investigation Plan and whether this application is a resubmission. Finally, NIMPs (non-IMPs), exploratory studies, deviant labelling, radiopharmaceuticals and answers to minor objections to previous applications with the same IMP should be mentioned when applicable [85].

FAGG – Federaal Agentschap voor Geneesmiddelen en Gezondheidsproducten
Eurostation II
Victor Hortaplein 40/40 31 March 2016
1060 BRUSSEL

EUDRACT number: 2016-000215-32
Sponsor protocol number: Pycno 2015-14
FWO MAND 2013 - 11U8314N (Verlaet Annelies) - PeopleSoft ID: 31579
UZA Ethical committee study 15/35/365

Effect of Pycnogenol® on Attention-Deficit Hyperactivity Disorder (ADHD): A randomized, double blind, placebo and active product controlled multicenter clinical trial

Dear Ms/Mr,

Attention-deficit hyperactivity disorder (ADHD) has been associated with disturbed immunity and elevated oxidative stress. In addition, methylphenidate is the most frequently used medication for ADHD, despite adverse effects, possible publication bias in reported efficacy, no evidence of long-term efficacy and parents being disinclined to use it. Because immune and oxidative imbalances offer potential for nutritional supplementation as efficacious and safe ADHD therapy, this project investigates the effect of a polyphenol rich extract of Pinus Pinaster bark (Pycnogenol®) on behaviour, co-morbid symptoms and immune and oxidative stress status in ADHD patients (6-12 years) as compared to methylphenidate (psychotropic medication) and placebo in a parallel trial.

Pycnogenol® has a high content of polyphenolic compounds, including phenolic acids, catechins and procyanidins. Next to its interesting antioxidant effects, Pycnogenol® has anti-inflammatory and immunomodulatory activities. Few observational studies and one small randomised trial (Trebaticka et al., 2006) suggest therapeutic benefit from Pycnogenol® in ADHD. However, this trial had several limitations (e.g. very small placebo group and short supplementation period) and the mechanisms of action involved remain unclear. Further research is thus needed to investigate its efficacy, mechanism of action and value as compared to MPH treatment.

Figure 5.18. Cover letter example (first page).

PART 7

ANNUAL SAFETY REPORT

Periodic analyses of safety information are crucial for adequate risk assessment. Every suspected unexpected serious adverse reaction (SUSAR) occurring in a clinical trial should be reported to the competent authority. In addition, the sponsor of a clinical trial in Belgium should report Belgian SUSARs in the concerned trial to the ethics committee. Moreover, once a year during the full duration of the trial (i.e. from authorisation by the competent authority until the last visit of the last patient or the otherwise defined end of trial), the sponsor should supply a safety report to both the competent authority and responsible ethics committee(s). This report should include all safety-related data for the IMP under investigation, regardless of country, pharmaceutical form, dosage, indication or patient population. It should be based upon AEs in the own trial and other ongoing or recently finished clinical trials as compared to the reference safety information, as well as data from observational studies, non-clinical studies, postmarketing studies, related safety reports, manufacturing changes, recent literature, etc., and actions proposed or being taken to address safety concerns. Based on the conclusion of this report, the reference safety information might require an update and thus resubmission [90, 91].

The start of the annual period for the report is based upon the Development International Birth Date (DIBD), the date of the first authorisation to conduct a clinical trial with the IMP in any country worldwide. Each report should be supplied within 60 days after the data lock point (the anniversary of the DIBD) [90, 92, 93].

The annual safety report must be submitted in the format of the Development Safety Update Report (DSUR) in combination with a cover letter including the EudraCT number of the concerning clinical trial.

The DSUR (example pages in Figure 5.19) should include [90, 92, 93]:

- Title page: DSUR number, IMP, reporting period and sponsor;
- Executive summary: IMP specifications (e.g. therapeutic class, mode of action), marketing approval, estimated subject exposure, overall safety assessment, risks, actions taken, etc.;
- Table of contents;
- Introduction: reporting period, IMP specifications, indication(s) and population(s) being studied, scope of clinical trials covered by the report, etc.;
- Worldwide marketing approval status: date of first approval, indication(s) and location(s);
- Actions taken in the reporting period for safety reasons: recall, protocol modifications;
- Changes to reference safety information: contraindications, interactions;
- Inventory of clinical trials ongoing and completed by the sponsor during the reporting period: phase, status, design, population, dosage, etc.;
- Estimated cumulative exposure: number of subjects exposed and demographic characteristics in clinical trial(s) and the marketed setting;
- Data in line listings and summary tabulations: reference information, SAEs (subject age and sex, dosage, description serious adverse reaction, etc.);
- Significant findings from clinical trials by the sponsor during the reporting period;
- Safety findings from non-interventional studies by the sponsor, e.g. observational studies;
- Other clinical trial/study safety information by the sponsor, e.g. meta-analyses;
- Safety findings from marketing experience, incl. off-label use, special populations, etc.;
- Non-clinical data by the sponsor, e.g. carcinogenicity or immunotoxicity studies;
- Literature: safety findings from non-clinical and clinical studies in literature;
- Other DSURs by the sponsor if more than one DSUR on the IMP, e.g. on different indications;
- Lack of efficacy, e.g. relative to established therapies;
- Region-specific information based on national and regional requirements;
- Late-breaking information: safety findings after the data lock point, e.g. case reports;
- Overall safety assessment: interpretation of all non-clinical, clinical and epidemiologic information compared to previous knowledge as well as risk-benefit considerations;
- Summary of important risks, e.g. those that might lead to labelled contraindications;
- Conclusions: changes to the previous knowledge on IMP efficacy and safety, actions taken;
- Appendices, e.g. IB or line listings of serious adverse reactions.

2. WORLDWIDE MARKETING APPROVAL STATUS

Medikinet XL is produced by Medice Arzneimittel Pütter GmbH & Co. KG, Kuhloweg 37, 58638 Iserlohn, Germany (Marketing authorisation numbers: PL 11243/005, PL 11243/006; 11/12/2013). Medikinet XL 10 mg and 20 mg modified-release capsules, for oral use, contain 10 mg and 20 mg methylphenidate hydrochloride, equivalent to 8.65 and 17.30 mg methylphenidate, resp. (pharmacotherapeutic group: psychoanaleptics, psychostimulants, agents used for ADHD and nootropics; centrally acting sympathomimetics; ATC Code: N06BA04). The main therapeutic indication is ADHD.

Careful dose titration is necessary at the start of treatment with methylphenidate. This is normally achieved using an immediate-release formulation taken in divided doses. The recommended starting daily dose is 5 mg once daily or twice daily (e.g. at breakfast and lunch), increasing if necessary by weekly increments of 5-10 mg in the daily dose according to tolerability and degree of efficacy observed. Medikinet XL 10 mg once daily may be used in place of immediate-release methylphenidate hydrochloride 5 mg twice daily from the beginning of treatment where the treating physician considers that twice daily dosing is appropriate from the outset and twice daily treatment administration is impracticable. The regimen that achieves satisfactory symptom control with the lowest total daily dose should be employed.

3. ACTIONS TAKEN IN THE REPORTING PERIOD FOR SAFETY REASONS

No significant actions related to safety have been taken by the sponsor, regulators, Data and Safety Monitoring Boards or independent ethics committees that could have an impact on the conduct of a specific trial or the whole clinical development programme. Participant inclusion for the concerning clinical trial was started in September 2017.

4. CHANGES TO REFERENCE SAFETY INFORMATION

No significant safety-related changes were made to reference safety information (SmPC) within the reporting period. Participant inclusion for the concerning clinical trial was started in September 2017.

5. INVENTORY OF CLINICAL TRIALS ONGOING AND COMPLETED DURING THE REPORTING PERIOD

This report concerns a single study: Pycno 2015-14 ("Effect of Pycnogenol® on Attention-Deficit Hyperactivity Disorder (ADHD). A randomized, double blind, placebo and active product controlled multicentre trial"). In addition to the concerning study, no other studies using Pycnogenol or methylphenidate as IMP were being performed during the reporting period by the University of Antwerp.

The concerning phase III clinical trial, ongoing, is a 10 week randomised, double blind, placebo and active product controlled multicentre clinical trial in Belgium with three parallel treatment arms (Pycnogenol®, placebo and Medikinet Retard). The aim is to investigate the effect of Pycnogenol on behaviour, co-morbid physical and psychiatric symptoms, as well as on immunity, oxidative stress and antioxidant status (to investigate mechanism of action), in 144 ADHD patients, as compared to placebo and methylphenidate treatment. Evaluations of behaviour, immunity, oxidative stress and co-morbid complains (blood/urine/faeces collection, ADHD-Rating Scale, Physical Complaints Questionnaire, Social-Emotional Questionnaire) will be performed at baseline and after 10 weeks. Certain questionnaires will additionally be filled out after 5 weeks. Atopy and dietary habits will be taken into account. In addition, the acceptability of Pycnogenol® treatment as compared to methylphenidate will be determined based on side effects, drop outs and adherence.

Patients consulting Prof. Dr. Ceulemans (UZA), Prof. Dr. Van West (ZNA) or Dr. Verhelst (UZ Ghent) and complying with in- and exclusion criteria (6-12 years old; ADHD or ADD but no ASD; no chronic medical condition, medication use or contra-indication for the use of MPH; no mental retardation; no use of nutritional supplements during the past 3 months) will be proposed to participate in this trial. In addition, information letters will invite children with ADHD via schools and patient organisations.

Patients randomized to Pycnogenol treatment will receive 20 mg Pycnogenol® (1 oral capsule at breakfast) per day in case of a body weight of < 30 kg, 40 mg Pycnogenol® (2 oral capsules at breakfast) in case of a body weight of ≥ 30 kg. During the first two weeks, all patients will receive 20 mg Pycnogenol® per day (due to practical reasons, for blinding). Patients randomized to methylphenidate treatment (Medikinet® Retard) will also receive a dose based on their body weight (1 or 2 oral capsules at breakfast). Capsules during the first week always contain 10 mg methylphenidate hydrochloride (modified release), during the second week 20 mg methylphenidate hydrochloride (modified release). In case of a body weight < 30 kg, patients receive 20 mg methylphenidate hydrochloride (modified release) per day afterwards. In case of a body weight of ≥ 30 kg, patients receive 30 mg methylphenidate hydrochloride (modified release; 2 capsules: 10 and 20 mg) per day afterwards. Patients randomized to placebo will receive 1 or 2 oral placebo capsules at breakfast.

Oxidative stress and neurological biomarker analyses will be performed at the University of Antwerp, immune analyses at Wageningen University.

Participant inclusion was started in September 2017; the last visit of the last patients is planned in August 2020.

6. ESTIMATED CUMULATIVE EXPOSURE

6.1 CUMULATIVE SUBJECT EXPOSURE IN THE TRIAL

Participant inclusion was started in September 2017. Participants are randomised, stratified by trial centre, to one of the three treatments (placebo, Pycnogenol® or Medikinet® Retard). Randomisation to one of the three treatment groups is done separately at every trial site (blocks of variable size

Figure 5.19. Example pages of the annual safety report.

PART 8

DISCUSSION

The presented trial is conducted in accordance with GCP guidelines and applicable legislation, in order to assure the rights, safety and well-being of its subjects and that the trial data are accurate and traceable [18]. Though appropriate quality control and protection of participants are incontrovertibly required, the administrative workload for the setup as well as during the ongoing clinical trial should not be underestimated. In addition, financial resources might limit many university research groups to set up a clinical trial without monetary help from authorities or industry. However, research in which industry is involved, even if solely financial, is often seen as biased.

In the design of the present intervention study, besides placebo and Pycnogenol® treatment, also MPH treatment was included, which extended the application considerably. In case of comparison to placebo only, Pycnogenol® would be regarded as a nutritional supplement, so that less strict regulations apply from trial registration to treatment encapsulation. However, though a nutritional supplement, Pycnogenol® is considered a medicinal product in the current trial, as it is compared to MPH, which is a registered and approved medicinal product. Though increasing the relevance of the study, the application process for trial approval was consequently extended considerably, while encapsulation for example required full implementation of GMP principles, thereby increasing costs as well.

Various trial-related files can be found in the digital Appendix of this PhD thesis (see bookmark for QR code).

REFERENCES

1. Storebø, O.J., et al., Methylphenidate for attention-deficit/hyperactivity disorder in children and adolescents: Cochrane systematic review with meta-analyses and trial sequential analyses of randomised clinical trials. BMJ, 2015. 351.
2. Schachter, H.M., et al., How efficacious and safe is shortacting methylphenidate for the treatment of attention-deficit disorder in children and adolescents? A meta-analysis. CMAJ, 2011. 165(11): p. 1475-1488.
3. Ceylan, M., et al., Changes in oxidative stress and cellular immunity serum markers in attention-deficit/hyperactivity disorder. Psychiatry Clin Neurosci, 2012. 66: p. 220-226.
4. Ng, F., et al., Oxidative stress in psychiatric disorders: evidence base and therapeutic implications. Int J Neuropsychopharmacol., 2008. 11: p. 851-876.
5. Droy-Lefaix, M.T., Effect of the antioxidant action of Ginkgo biloba extract (EGb 761) on aging and oxidative stress. Age, 1997. 20(3): p. 141-149.
6. Puebla-Pérez, A.M., X. Lozoya, and M.M. Villaseñor-García, Effect of Ginkgo biloba extract, EGb 761, on the cellular immune response in a hypothalamic-pituitary-adrenal axis activation model in the rat. Int Immunopharmacol, 2003. 3(1): p. 75-80.
7. Aghili, T., et al., Effects of Hypericum perforatum extract on IgG titer, leukocytes subset and spleen index in rats. Avicenna J Phytomed, 2014. 4(6): p. 413-419.
8. Orčić, D.Z., et al., Antioxidant activity relationship of phenolic compounds in Hypericum perforatum L. Chem Cent J, 2011. 5: p. 34.
9. Ingale, S.P. and S.B. Kasture, Antioxidant and antiparkinsonian activity of Passiflora incarnata leaves. Orient Pharm Exp Med, 2014. 14(3): p. 231-236.
10. Beaumont, D.M., et al., The effects of chrysin, a Passiflora incarnata extract, on natural killer cell activity in male Sprague-Dawley rats undergoing abdominal surgery. AANA J, 2008. 76(2): p. 113-117.
11. D'Andrea, G., Pycnogenol: A blend of procyanidins with multifaceted therapeutic applications? Fitoterapia, 2010. 81: p. 724-736.
12. Dvořáková, M., et al., The effect of polyphenolic extract from pine bark, Pycnogenol, on the level of glutathione in children suffering from attention deficit hyperactivity disorder (ADHD). Redox Report, 2006. 11: p. 163-172.
13. Canali, R., et al., The anti-inflammatory pharmacology of Pycnogenol in humans involves COX-2 and 5-LOXmRNA expression in leukocytes. Int Immunopharmacol, 2009. 9(10): p. 1145-1149.
14. Wilson, D., et al., A randomized, double-blind, placebo-controlled exploratory studyto evaluate the potential of pycnogenol for improving allergic rhinitis. Phytother Res, 2010. 24(8): p. 1115-1119.
15. Trebatická, J., et al., Treatment of ADHD with French maritime pine bark extract, Pycnogenol. Eur Child Adolesc Psychiatry, 2006. 15: p. 329-335.
16. Chovanová, Z., et al., Effect of polyphenolic extract, Pycnogenol, on the level of 8-oxoguanine in children suffering from attention deficit/hyperactivity disorder. Free Radical Res, 2006. 40: p. 1003-1010.
17. Tenenbaum, S., et al., An experimental comparison of Pycnogenol and methylphenidate in adults with attention-deficit/hyperactivity disorder (ADHD). J Attention Disorders, 2002. 6: p. 49-60.
18. Agency, E.M., Guideline for good clinical practice E6(R2). 2015.
19. California, U.o. Clinical Trial Protocol Development.; Available from: https://hub.ucsf.edu/protocol-development [Accessed 15 June 2018].
20. Commission, E., Detailed guidance on the request to the competent authorities for authorisation of a clinical trial on a medicinal product for human use, the notification of substantial amendments and the declaration of the end of the trial (CT-1) (2010/C 82/01). Off J Eur Union, 2010. 82.
21. Pelham, W.E. and E.M.R. Foster, J.A., The Economic Impact of Attention-Deficit/Hyperactivity Disorder in Children and Adolescents. J Pediatr Psychol, 2007. 32(6): p. 711-727.
22. Polanczyk, G.V., et al., ADHD prevalence estimates across three decades: an updated systematic review and meta--regression analysis. Int J Epidemiol, 2014. 43(2): p. 434-442.
23. Biederman, J. and S.V. Faraone, Attention-deficit hyperactivity disorder. Lancet, 2005. 366: p. 237-248.
24. Antshel, K.M., et al., Advances in understanding and treating ADHD. BMC Med, 2011. 9: p. 72.
25. Claes, S., et al., Het toenemend gebruik van psychofarmaca. Visietekst werkgroep Metaforum Leuven. 2010, Katholieke Universiteit Leuven.
26. Steer, C.R., Managing attention deficit/hyperactivity disorder: unmet needs and future directions. Arch Dis Child, 2005. 90(Suppl I): p. i19-i25.

27. Storebø, O.J., et al., Methylphenidate for attention-deficit/hyperactivity disorder in children and adolescents: Cochrane systematic review with meta-analyses and trial sequential analyses of randomised clinical trials. BMJ, 2015. 351: p. h5203.
28. Pelsser, L.M.J., J.K. Buitelaar, and H.F.J. Savelkoul, ADHD as a (non) allergic hypersensitivity disorder: a hypothesis. Pediatr Allergy Immunol, 2009. 20(2): p. 107-112.
29. Ceylan, M., et al., Oxidative imbalance in child and adolescent patients with attention-deficit/hyperactivity disorder. Prog Neuropsychopharmacol Biol Psychiatry, 2010. 34(8): p. 1491-1494.
30. El Adham, E.K., A.I. Hassan, and A.A. El Aziz El- Mahdy, Nutritional and Metabolic Disturbances in Attention Deficit Hyperactivity Disease. Res J Med & Med Sci, 2011. 6(1): p. 10-16.
31. Ross, B.M., et al., Increased levels of ethane, a non-invasive marker of n-3 fatty acid oxidation, in breath of children with attention deficit hyperactivity disorder. Nutr Neurosci, 2003. 6(5): p. 277-281.
32. Kawatani, M., H. Tsukahara, and M. Mayumi, Evaluation of oxidative stress status in children with pervasive developmental disorder and attention deficit hyperactivity disorder using urinary-specific biomarkers. Redox Rep, 2011. 16(1): p. 45-46.
33. Dvořáková, M., et al., Urinary catecholamines in children with attention deficit hyperactivity disorder (ADHD): Modulation by a polyphenolic extract from pine bark (Pycnogenol). Nutr Neurosci, 2007. 10: p. 151-157.
34. Güngör, S., et al., The Frequency of Celiac Disease In Attention-Deficit Hyperactivity Disorder. J Pediatr Gastroenterol Nutr, 2013. 56(2): p. 211-4.
35. Minter, K., et al., Early Childhood Otitis Media in Relation to Children's Attention-Related Behavior in the First Six Years of Life. Pediatrics, 2001. 107: p. 1037-1042.
36. Schmitt, J., A. Buske-Kirschbaum, and V. Roessner, Is atopic disease a risk factor for attention-deficit/hyperactivity disorder? A systematic review. Allergy, 2010. 65(12): p. 1506-1524.
37. Tsai, S.J., Signal transducer and activator of transcription 6 (STAT6) and attention-deficit hyperactivity disorder: A speculative hypothesis. Med Hypotheses, 2006. 67(6): p. 1341-1343.
38. Furlano, R., et al., Colonic CD8 and gamma delta T-cell infiltration with epithelial damage in children with autism. J Pediatr, 2001. 138(3): p. 366-372.
39. Noriega, D.B. and H.F.J. Savelkoul, Immune dysregulation in autism spectrum disorder. Eur J Ped, 2014. 173(1): p. 33-43.
40. Verlaet, A.A.J., et al., Nutrition, immunological mechanisms and dietary immunomodulation in ADHD. Eur Child Adolesc Psychiatry, 2014. 23(7): p. 519-529.
41. Jennings, C.G., et al., Does offering an incentive payment improve recruitment to clinical trials and increase the proportion of socially deprived and elderly participants? Trials, 2015. 16: p. 80.
42. Kerkhoff, L.A., et al., Trends in Consent for Clinical Trials in Cardiovascular Disease. J Am Heart Assoc, 2016. 5(6): p. e003582.
43. Buitelaar, J.K., S.A. Montgomery, and B.J. van Zwieten-Boot, Attention deficit hyperactivity disorder: guidelines for investigating efficacy of pharmacological intervention. European Neuropsychopharmacology, 2003. 13: p. 297-304.
44. TestWeb. Sociaal-Emotionele Vragenlijst (SEV). 2013 Available from: http://testweb.bsl.nl/tests/sev/ [Accessed 5 July 2016].
45. Pelsser, L.M.J. and J.K. Buitelaar, Favourable effect of a standard elimination diet on the behaviour of young children with attention deficit-hyperactivity disorder (ADHD), a pilot study. Ned Tijdschr Geneesk, 2002. 46(52): p. 2543-2547.
46. Pastore, A., et al., Analysis of glutathione: implication in redox and detoxification. Clin Chim Acta, 2003. 333(1): p. 19-39.
47. Conaway, H.H., P. Henning, and U.H. Lerner, Vitamin a metabolism, action, and role in skeletal homeostasis. Endocrine Rev, 2013. 34: p. 766-797.
48. Naguib, Y., et al., Antioxidant activities of natural vitamin E formulations. J Nutr Sci Vitaminol, 2003. 49: p. 217-220.
49. Littarru, G.P. and L. Tiano, Bioenergetic and antioxidant properties of coenzyme Q10: recent developments. Mol Biotechnol, 2007. 37: p. 31-37.
50. Molyneux, S.L., et al., Coenzyme Q10: is there a clinical role and a case for measurement? Clin Biochem Rev, 2008. 29: p. 71-82.
51. Sezen, H., et al., Increased oxidative stress in children with attention deficit hyperactivity disorder. Redox Rep., 2016. 21(6): p. 248-253.
52. Franceschi, C., et al., Genes involved in immune response/inflammation, IGF1/insulin pathway and response to oxidative stress play a major role in the genetics of human longevity: the lesson of centenarians. Mech Ageing Dev, 2005. 126(2): p. 351-361.
53. Rahman, I., S.R. Yang, and S.K. Biswas, Current concepts of redox signaling in the lungs. Antioxid Redox Signal, 2006. 8: p. 681-689.

54. Whitehouse, R.C., et al., Zinc in plasma, neutrophils, lymphocytes, and erythrocytes as determined by flameless atomic absorption spectrophotometry. Clin Chem., 1982. 28(3): p. 475-480.
55. Dalle-Donne, I., et al., Biomarkers of oxidative damage in human disease. Clin Chem, 2006. 52: p. 601-623.
56. Aruoma, O.I., Free radicals, oxidative stress, and antioxidants in human health and disease. JAOCS, 1998. 75: p. 199-212.
57. Özcan, Ö., et al., Plasma Leptin, Adiponectin, Neuropeptide Y Levels in Drug Naive Children With ADHD. J Atten Disord, 2018. 22(9): p. 896-900.
58. Furukawa, T., S.N. Meydani, and J.B. Blumberg, Reversal of age-associated decline in immune responsiveness by dietary glutathione supplementation in mice. Mech Ageing Dev, 1987. 38(2): p. 107-117.
59. Grimm, T., et al., Inhibition of NF-κB activation and MMP-9 secretion by plasma of human volunteers after ingestion of maritime pine bark extract (Pycnogenol). J Inflam, 2006. 3(1): p. 1-6.
60. Booijink, C.C., et al., High temporal and inter-individual variation detected in the human ileal microbiota. Environ Microbiol., 2010. 12(12): p. 3213-3227.
61. González, T.J.B., et al., Study of the Aminoglycoside Subsistence Phenotype of Bacteria Residing in the Gut of Humans and Zoo Animals. Front Microbiol., 2015. 6.
62. de Vriese, S., et al., The Belgian food consumption survey: aims, design and methods. Archives of Public Health, 2005. 63: p. 1-16.
63. De Keyzer, W., et al., Relative validity of a short qualitative food frequency questionnaire for use in food consumption surveys. Eur J Public Health, 2012. 23(5): p. 737-742.
64. Heimann, S.W., Pycnogenol for ADHD? J Am Acad Child Adolesc Psychiatry, 1999. 38: p. 357-358.
65. Pelsser, L.M.J., et al., Effects of a restricted elimination diet on the behaviour of children with attention-deficit hyperactivity disorder (INCA study): a randomised controlled trial. Lancet, 2011. 377: p. 494-503.
66. Berek, M., et al., Improved functionality, health related quality of life and decreased burden of disease in patients with ADHD treated with OROS® MPH: is treatment response different between children and adolescents? Child Adolesc Psych Mental Health, 2011. 5(26).
67. Christensen, E., Methodology of superiority vs. equivalence trials and non-inferiority trials. J Hepatol, 2007. 46: p. 947-954.
68. Piaggio, G., et al., Reporting of noninferiority and equivalnce randomized trials: an extension of the CONSORT statement. JAMA, 2006. 295: p. 1152-1160.
69. Greenblatt, J., Nutritional supplements in ADHD. J Am Acad Child Adolesc Psychiatry, 1999. 38: p. 1209-1210.
70. Power, T.J., et al., The Predictive Validity of Parent and Teacher Reports of ADHD Symptoms. J Psychopathol Behav Assessment, 1998. 20(1): p. 57-81.
71. Tripp, G., E.A. Schaughency, and B. Clarke, Parent and Teacher Rating Scales in the Evaluation of Attention-deficit Hyperactivity Disorder: Contribution to Diagnosis and Differential Diagnosis in Clinically Referred Children. J Developm Behav Ped, 2006. 27(3): p. 209-218.
72. Adler, L.A., Clinical Presentations of Adult Patients With ADHD. J Clin Psychiatry, 2004. 65(S3): p. 8-11.
73. Shakibaei, F., et al., Ginkgo biloba in the treatment of attention-deficit/hyperactivity disorder in children and adolescents. A randomized, placebo-controlled, trial. Complement Ther Clin Pract., 2015. 21(2): p. 61-67.
74. Dubnov-Raz, G., et al., The effect of alpha-linolenic acid supplementation on ADHD symptoms in children: a randomized controlled double-blind study. Front Hum Neurosci, 2014. 7(8): p. 780.
75. Sinn, N., Nutritional and dietary influences on attention deficit hyperactivity disorder. Nutr Rev, 2008. 66(10): p. 558-568.
76. Viktorinova, A., et al., Changed plasma levels of zinc and copper to zinc ratio and their possible associations with parent- and teacher-rated symptoms in children with attention-deficit hyperactivity disorder. Biol Trace Element Res, 2016. 169: p. 1-7.
77. Dufault, R., et al., Mercury exposure, nutritional deficiencies and metabolic disruptions may affect learning in children. Behav Brain Functions, 2009. 5: p. 44-59.
78. Powell, S.R., The Antioxidant Properties of Zinc. J Nutr, 2000. 130(5): p. 1447S-1454S.
79. Jomova, K. and M. Valko, Advances in metal-induced oxidative stress and human disease. Toxicol, 2011. 283: p. 65-87.
80. Scasselati, C., et al., Biomarkers and Attention-Deficit/Hyperactivity Disorder: A Systematic Review and Meta-analyses. J Am Acad Child Adolesc Psychiatry, 2012. 51(10): p. 1003-1019.
81. Oades, R.D., R. Daniels, and W. Rascher, Plasma neuropeptide-Y levels, monoamine metabolism, electrolyte excretion and drinking behavior in children with attention-deficit hyperactivity disorder. Psychiatry Res., 1998. 80: p. 177-186.
82. Lesch, K.P., et al., Genome-wide copy number variation analysis in attention-deficit/hyperactivity disorder: association with neuropeptide Y gene dosage in an extended pedigree. Mol Psychiatry, 2011. 16: p. 491-503.
83. Pelsser, L.M.J., et al., Effects of food on physical and sleep complaints in children with ADHD: a randomised controlled pilot study. Eur J Pediatr, 2010. 169(9): p. 1129-1138.

84. Leuven, U. Informed consent formulier (ICF) opstellen voor EC Onderzoek. 2018 18 June 2018]; Available from: https://www.uzleuven.be/nl/ethische-commissie/onderzoek/icf-richtlijnen [Accessed 17 April 2018].
85. FAMHP, Omzendbrief 575, FAHMP, Editor. 2010: Brussel.
86. Agency, E.M., ICH Topic M 4 Q Common Technical Document for the Registration of Pharmaceuticals for Human Use - Quality. 2003.
87. Union, E. About Investigational Medicinal Product Dossiers. 2018; Available from: http://www.imp-dossier.eu/ [Accessed 17 April 2018].
88. Commission, E., Notice to applicants: A guideline on Summary of Prduct Characteristics. 2009 [Accessed 27 April 2017].
89. Verlaet, A.A.J., et al., Effect of Pycnogenol® on attention-deficit hyperactivity disorder (ADHD): study protocol for a randomised controlled trial. Trials, 2017. 18(1): p. 145-153.
90. ICH and FDA, International Conference on Harmonisation - Guidance for Industry: E2F Development Safety Update Report. 2011.
91. Commission, E., Communication from the Commission — Detailed guidance on the collection, verification and presentation of adverse event/reaction reports arising from clinical trials on medicinal products for human use (‘CT-3’) O.J.E. Union, Editor. 2011.
92. FAMHP, Omzendbrief 593. 2012: Brussel.
93. FAMHP, Omzendbrief 586. 2011: Brussel.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

Success is not the key to happiness. Happiness is the key to success. If you love what you are doing, you will be successful. – Albert Schweitzer

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PART 1

INTRODUCTION

ADHD is an aetiologically complex and multifactorial disorder, influenced by genetic, biochemical, psychological and environmental factors. Every risk factor has a small effect on the vulnerability to the disorder and none of these factors appears either necessary or sufficient to trigger ADHD by itself. Its exact pathophysiology is still unclear and might differ between patients [1-6]. In children as compared to adults, ADHD has a higher prevalence and is characterised more often by hyperactivity-impulsivity [1, 7-9]. Medication like MPH is prescribed to a rapidly growing number of children, despite serious concerns about adverse effects and possible publication bias in reported efficacy as well as without evidence of long-term efficacy [10-12].

The principal aim of this thesis is to get a better understanding of oxidative stress and immune dysbalance in ADHD as well as therapeutic options. This thesis shows that nutritional factors, oxidative stress and immune dysbalance play a potential role in ADHD aetiology. Nutritional approaches like polyphenol-rich foods or extracts could therefore have a positive impact on ADHD behaviour and possibly also related complaints due to their antioxidant and immune modulating activity. The ongoing randomised double-blind placebo and active product controlled clinical trial will provide further understanding of the pathogenesis and treatment options of ADHD.

The specific results of each part of this thesis have been discussed separately before. This general discussion will focus on the overall findings and, in combination with literature, on their consequences on the knowledge on ADHD. This discussion elaborates on the overall methodology and results of this thesis, the potential mechanisms by which redox and immune imbalance can be involved in ADHD aetiology, potential mechanisms and efficacy of polyphenol-based approaches in ADHD therapy, implications and additional research options. Moreover, as various answers lead to even more questions, perspectives for future research are discussed.

PART 2

REDOX AND IMMUNE IMBALANCE IN ADHD

2.1 Results and implications

The results of the comparison between patients and controls (Chapter 2) are consistent with a role of low-level oxidative stress that could affect the immune system in children with ADHD as compared to controls without ADHD. As a result, paediatric ADHD patients might develop more IgE and non-IgE-mediated allergies [13]. Indeed, though no significantly different plasma cytokine levels were found, which is possibly due to medication use as an exclusion criterion or the absence of overt systemic inflammation, slightly higher IgG levels in ADHD point to the existence of a low-grade inflammation [14]. Though results on specific biomarkers are often inconsistent between studies, the results of this case-control comparison generally support conclusions of preceding research. These conclusions comprise that “changes in oxidative metabolism and cellular immunity may have a role in the aetiopathogenesis of ADHD” [15], that “patients with ADHD have normal levels of antioxidant production, but that their response to oxidative stress is insufficient, leading to oxidative damage” [16] and that patients have a higher incidence of allergies, including asthma and atopic eczema [13, 17-21].

Increased lipid peroxidation in ADHD was characterised by elevated MDA levels in patients as compared to controls (though significance was lost after Bonferroni correction, which takes into account false-positive significant differences due to multiple testing). It is striking that levels of vitamin E, the most important inhibitor of lipid peroxidation in membranes and lipoproteins, were not reduced in ADHD patients. In other neuropsychiatric disorders, like depression and schizophrenia, lower vitamin E levels have been observed, unrelated to dietary vitamin E intake [22, 23], while results in ADHD are equivocal [24, 25]. However, again, comparable or even higher antioxidant levels do not necessarily imply less oxidative damage [26-28]. In addition, a broad range of antioxidants is present *in vivo*, making vitamin E regeneration another possible explanation.

It is questionable whether the borderline higher retinyl palmitate levels observed in patients are biologically relevant. This is because of the relatively large error range due to the low plasma levels measured in the case-control study, as retinyl palmitate is the main vitamin A storage form in the liver [29-31]. In addition, no significant difference was observed for retinol in the present study, though this

result might be due to plasma retinol generally being strictly regulated due to toxicity at high levels [32]. Retinol reduces radicals directly, thereby acting as a chain breaking antioxidant [33]. Another form of vitamin A however, retinoic acid, is involved in gene expression regulation via activation of nuclear receptors [34]. Transcriptional regulation of dopamine receptors and hippocampal neurogenesis by retinoic acid are potential mechanisms of action by which retinoic acid could be involved in ADHD [35]. Nevertheless, retinoic acid was not analysed in ADHD before.

In addition to inconsistent results, large variations in biomarker levels have been found between and within studies in literature. For example, for serum and plasma MDA, levels between 0.001 and 4 µM have been found in paediatric ADHD patients [24, 26, 36], while the present study found levels around 0.35 µM. These differences do not appear to be caused by sample type, but rather due to differences regarding the analytical methods used. Nevertheless, also the direction and magnitude of the difference between patients and controls differs in literature and as compared to the present comparison. For instance, up to 3 SD higher MDA levels have been found in patients as compared to controls [26], but also 1-2 SD lower levels [24, 36]. In the present comparison, MDA levels uncorrected for processing time were only 0.5 SD higher in patients than in controls.

In children, total IgE levels are highly variable (as reflected by large SDs) and depend on age, with levels generally increasing between birth and adulthood [37]. Paediatric ADHD patients were found to have increased total IgE plasma levels (367 ± 483 kU/L) compared to healthy aged-matched controls (262 ± 420 kU/L) [38], as was observed in the case-control study (Chapter 2). Earlier studies using cut-off levels of 100 kU/L to indicate increased IgE levels however found no differences between ADHD and control children [39]. During the development of allergic inflammation, increased T_H2 activity is reflected by increased levels of IL-4, IL-5 and IL-13, which are widely associated with young age and which lead to increased total IgE levels. In addition, IL-5 production was found to be strongly linked to total serum IgE levels in the general population [40]. The results obtained in the case-control comparison appear inconsistent with this suggestion. However, though no significantly different plasma cytokine levels were observed, an increase in locally produced cytokines could be too low to be detected in plasma [41]. In addition, significantly deviant systemic cytokine levels would indicate active inflammation [42], while chronic low-grade inflammation appears typical for ADHD.

Though dietary habits and perinatal events can influence oxidative and inflammatory status [43-47], differences observed regarding the biomarkers analysed cannot be explained by differences in dietary habits, the course of pregnancy, alcohol use or smoking during pregnancy, premature birth or breastfeeding. Previous research however reported associations between specific dietary patterns and

ADHD symptoms [48-52] and an apparent protective effect of breastfeeding on the occurrence of ADHD [53, 54], while delivery complications, prematurity, hypoxia and prenatal alcohol and tobacco smoke are known risk factors for ADHD [1]. Possibly, the power of the present case-control study was too low to resolve these possibilities.

2.2 Study population

The patient group was selected through a tertiary referral academic hospital. No demographic differences were found as compared to controls, which were recruited from random schools. In addition, the absence of major dietary differences is an indication of a similar socioeconomic status of both groups, which makes their comparison valid [55]. Nevertheless, when extrapolating results to the general paediatric population, in- and exclusion criteria of this study should be taken into account. For instance, despite an overall high comorbidity between autism and ADHD and despite some patients having a high autism score as rated by parents, autism diagnosis was an exclusion criterion in this study. It is thus unknown whether results can be extrapolated to ADHD patients with comorbid autism. In addition, this comparison was performed in a paediatric population, thus impeding extrapolation to adult ADHD.

There were difficulties with patient recruitment, causing delay, as patients often use supplements before their official diagnosis and due to a lack of positive reinforcement to participate in the case-control study (no direct benefit for the patient). In addition, it should be noted that about 15% of the ADHD patients were included various months after their official diagnosis. As the use of medication was an exclusion criterion of this study, their urge for medication was apparently not very high. This possibly points at relatively mild ADHD symptoms in this subgroup, potentially causing some selection bias.

The relatively high comorbidity with physical complaints may not reflect clinical practice but could be explained by the use of a specific questionnaire. For instance, stomach ache is not routinely asked for when ADHD is concerned and is probably not spontaneously reported by parents in a psychiatric or psychological setting.

PART 3

IN VITRO EFFECTS OF PYCNOGENOL®

3.1 *In vitro* models

Due to financial and/or ethical constraints regarding specific animal and human *in vivo* studies, which additionally are characterised by inter- and intraindividual variation, and as interindividual variation is characteristic for *ex vivo* systems, *in vitro* research is sometimes more informative to resolve basic mechanisms of action. Though *in vitro* systems have limited predictive power for *in vivo* situations (i.e. artificial cultivation), acceptable correlations between *in vitro* and *in vivo* readings have been found for immune analyses [56, 57]. In addition, *in vitro* models are useful for the screening of promising compounds, can give more insight in specific mechanistic pathways and ensure reproducibility of results as circumstances can be standardised. Nevertheless, *in vivo* research remains essential, considering pharmacokinetic phenomena [58].

Though studying effects of immune modulating compounds using PBMC-derived monocytes and macrophages might be a more realistic model than THP-1 cells, this is difficult to apply due to variation between blood samples and the limited survival of these cells in culture, as they require inflammatory mediators like IL-1β to prevent apoptosis [59-61]. THP-1 cells, immortalised, can grow and divide indefinitely under proper conditions [59, 60]. In addition, they are relatively safe to use due to the absence of infectious viruses or toxic products and their homogenous genetic background minimises the degree of cell phenotype variability and thus increases reproducibility of results [62, 63]. Still, THP-1 cells do not resemble monocytes or macrophages from subjects with diseases like chronic inflammation [64]. Nevertheless, more similarities are found between THP-1 cells and PBMCs in the macrophage than monocyte state [65-69]. Cell sensitivity regarding cytotoxicity is similar between THP-1 cells and PBMCs [70].

3.2 Methodology and results

The concentrations of Pycnogenol® and catechin used in Chapter 4 are physiologically very high [71], which is however not exceptional for *in vitro* research [72]. For the analyses, it was assumed that all

constituents were fully dissolved. However, high concentrations of Pycnogenol® and catechin did not dissolve completely in PBS. HPLC analysis revealed that the solubility of catechin, caffeic acid, taxifolin and ferulic acid was not complete, though sufficient to realistically expect activity, while the fingerprint chromatogram was similar to the reference. Nevertheless, Pycnogenol® is a complex mixture of polyphenols, with various compounds yet to be identified [71]. It is therefore possible that specific biologically active compounds in Pycnogenol® are not dissolved and therefore not incubated *in vitro*.

Previous research has already shown that Pycnogenol® displays stronger biological activity as a mixture than when separated into its individual components, indicating that its components act synergistically and/or additively [73]. This potentially explains differences in observed effects between Pycnogenol® and its main monomer catechin. After all, monomeric catechin constitutes less than 2% of the extract (see Chapter 3) [74].

Results of the unmetabolised extract should be interpreted with caution because absorption, distribution, metabolism (e.g. by hepatic enzymes and intestinal microbiota) and excretion (ADME) kinetics were not taken into account. As opposed to for example catechin and taxifolin [75], components in Pycnogenol® with a high polymerisation degree and thus molecular weight are not absorbed as such in the GI tract and are therefore not able to exert biological effects on cells other than those in the lumen or those lining the GI tract. In addition, two known metabolites (M1 and M2) are formed by microbiota from catechin units, potentially after depolymerisation, and procyanidins in Pycnogenol® undergo phase II metabolism [75-79].

To account for absorption and microbial metabolism, analyses were also performed with dialysates and retentates from the GIDM. However, an important limitation of the GIDM is the absence of active transport of digestion products.

PART 4

POTENTIAL MECHANISM OF ACTION

The main contributing factor to ADHD is thought to be abnormal neurotransmitter regulation, particularly dopamine. Decisive evidence on the exact contributions of redox and immune imbalance in ADHD is yet to be reported. Arguably, both are not even directly related to ADHD, but solely an artefact of related factors, like genetic or environmental factors or comorbid disorders [80]. In addition, results from case-control research provide information on associations but do not address the investigation of causality. Nevertheless, as oxidative stress and immune activity influence brain environment [80, 81], they can be considered important targets in ADHD [15, 82, 83].

4.1 Redox imbalance

Multiple effects of oxidative stress can be related to ADHD aetiology (Figure 6.1) [15].

The brain is especially sensitive to oxidative stress because of its high oxygen consumption (high metabolic rate), neuron membranes contain a high proportion of easily peroxidisable PUFAs and neurons have a low GSH content [16, 80, 84, 85]. Oxidative stress can impair neuronal proliferation and mediate apoptosis and can therefore lead to progressive neuronal damage and deterioration of normal cerebral functions.

Lipid peroxidation is a free radical-mediated oxidative deterioration to which PUFAs are especially vulnerable due to their unconjugated double bonds [86]. By damaging fatty acid side chains, lipid peroxidation affects both membranes and lipoproteins. Increased oxidative stress can therefore lead, by peroxidation of membrane lipids and membrane-associated proteins (e.g. neurotransmitter receptors), to structural neuronal damage with an altered membrane fatty acid composition, decreased membrane fluidity, inactivation of membrane bound enzymes, increased membrane leakiness and altered neurotransmitter receptor and transporter functioning. Oxidative stress can thereby lead to abnormal neurotransmitter regulation [15, 80, 87-89]. Lipid peroxidation, as evidenced by elevated MDA levels in patients as compared to controls (Chapter 2), could therefore affect

neurotransmission in ADHD. Still, results on plasma or serum MDA levels in paediatric ADHD patients are not consistent in literature [24, 26, 36].

Furthermore, oxidative stress may interfere with dopamine synthesis, neuronal cell migration and neuronal plasticity. This is possible via genetic mutations or by limiting brain-derived neurotrophic factors (BDNFs) required for neurogenesis and neuronal plasticity [15, 87, 90, 91].

In addition, dopamine is highly susceptible to auto-oxidation when antioxidant defence is weak [15, 92]. Dopamine oxidation leads to products such as dopamine quinone, which can cause mitochondrial dysfunction and inflammation by activation of microglia (monocyte-derived macrophage-like brain cells) and alteration of gene expression [16, 93-95]. Such dopamine cytotoxicity can thus lead to brain damage [15, 96].

Also, the role of MAO in neuropsychiatric disorders may be mediated by oxidative stress. Reduced platelet MAO B activity has been observed in ADHD patients as compared to controls. This possibly leads to excessive accumulation of dopamine in the neuronal cytoplasm and thus to dopamine neurotoxicity [97]. In contrast, deprenyl (a MAO B inhibitor) was found to reduce ADHD symptoms, pointing at elevated MAO activity. Accelerated dopamine breakdown by MAO leads not only to dopamine deficiency, but also to by-products like hydrogen peroxide (H_2O_2), ammonia and aldehydes. These by-products increase oxidative stress and have neurotoxic potential [98]. Both elevated and reduced MAO activity could thus lead to neurotoxic effects.

Oxidative stress and dopamine dysfunction thus appear interrelated in ADHD. The causal relationship however remains unclear: it is unknown whether increased oxidative stress causes dopamine dysfunction and ADHD symptoms or the other way around. For instance, ADHD patients have an increased risk for obesity, which is correlated with oxidative stress. Higher oxidative stress levels could thus rather be a consequence of obesity than a cause of ADHD, or both [99, 100], though the weight and height of patients and controls did not differ significantly (Chapter 2). In addition, catecholamine metabolism may be a source of free radical formation. Abnormal catecholamine levels (e.g. by altered MAO activity) could therefore be responsible for a disturbed antioxidant status and elevations in oxidative stress markers, next to behavioural effects [101-104]. A causative role of oxidative stress in ADHD is however supported by indications of genetic factors influencing oxidative metabolism. Oxidative stress was for example higher in patients who had a family member with ADHD, indicating a higher genetic load as compared to patients without a family history of ADHD [26].

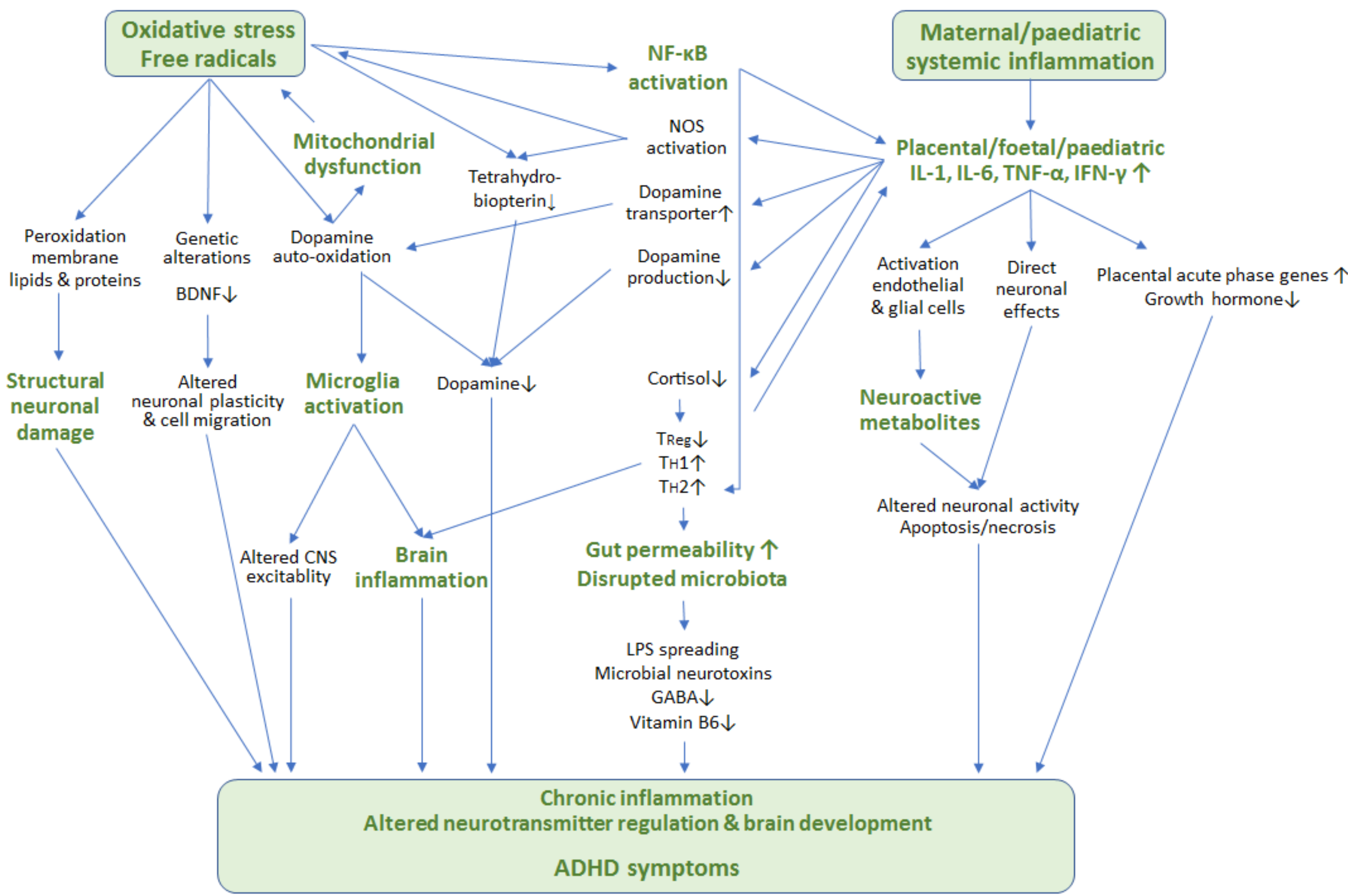

Figure 6.1. Schematic overview of potential mechanisms of action involved in redox and immune imbalance underlying ADHD. This scheme is not exhaustive. BDNF: brain-derived neurotropic factor; CNS: central nervous system; GABA: γ-aminobutyric acid; IFN: interferon; IL: interleukin; LPS: lipopolysaccharide; NF-κB: nuclear factor κB; NOS: nitric oxide synthase; T_H: helper T cell; TNF: tumour necrosis factor; T_{Reg}: regulatory T cell.

Finally, a redox imbalance might not only be responsible for changes in the nervous system, but also in the immune system, as oxidative and inflammatory processes are closely related [15, 80, 87, 88, 105-108].

4.2 Immune imbalance

During brain development, cytokines are crucially involved in neuronal proliferation, differentiation, migration, homing and positioning, and synaptogenesis [109]. However, chronic inflammation can also result in neuronal alterations, including alteration in energy balance, neurotransmitter production and cytoskeletal structure, thereby triggering neurodegeneration [110].

In the case-control study, no significantly different plasma cytokine levels were found between patients and controls (Chapter 2). Still, indications of more T_H1-derived cytokines have for example been found in previous research [111]. Significantly deviant systemic cytokine levels would, however, indicate active inflammation, but was not the case. An increase in locally produced cytokines could be too low to be detected in plasma. Nevertheless, circulating cytokines like IFN-γ, IL-1, IL-6 and TNF-α can pass the BBB. However, an exclusively direct effect of cytokines on neurons is rather unlikely due to the scarcity of specific receptors. Still, cytokines can exert their effects by activation of glial and endothelial cells. These cells subsequently produce neuroactive metabolites modulating neuronal activity and can have neurotoxic potential by priming cells for apoptosis or necrosis [112-118]. In addition, from animal studies it was suggested that peripheral inflammation leads to a microglia-dependent increase in CNS excitability mediated by TNF-α [119]. Moreover, cytokines stimulate NOS, which uses tetrahydrobiopterin as a cofactor, and stimulate the production of ROS, to which tetrahydrobiopterin is sensitive. Cytokines thereby reduce the availability of this cofactor for hydroxylase enzymes producing monoamine neurotransmitters. Finally, cytokines increase dopamine and norepinephrine transporter expression [117, 118].

Maternal immune activation during pregnancy as a consequence of infection may induce the presence of pro-inflammatory cytokines and acute phase proteins, the influx of inflammation associated cells and bouts of fever. Elevated cytokines, due to prenatal immune activation, could also affect foetal and paediatric neuronal development and activity [120, 121]. For example, elevated levels of foetal and/or placental pro-inflammatory cytokines like IL-6 and TNF-α in response to maternal immune activation can cause a CNS inflammatory response in the foetus and promote oxidative stress, mitochondrial damage, apoptosis and neurotoxicity [98]. Dependent on timing, the increase in pro-inflammatory

cytokine levels can alter foetal brain development and cause long-term brain abnormalities, including pathological changes in brain morphology akin to those observed in autism. Pro-inflammatory cytokines thereby possibly increase the risk for ADHD later in life [109, 120-127]. In fact, early life presence of inflammatory cytokines has a major role in the pathophysiology of brain white matter damage, as for example IL-6 can interfere with prefrontal cortex maturation [114, 125, 128]. This is consistent with findings of grey matter heterotopia and reduced cortical volume and folding in ADHD, which are considered to be involved in the development of behavioural effects [114, 129, 130]. Maternal immune activation could thus predispose, via altered foetal brain development, to dopamine-associated neuropsychiatric disorders [120, 121]. There is indeed evidence suggesting neuroanatomical abnormalities in the main dopaminergic and glutamatergic systems after prenatal infection, which could contribute to the development of ADHD [131].

Additionally, maternal immune activation can affect neurodevelopment indirectly. An increase in placental IL-6 leads to a reduction of growth hormone and insulin-like growth factor. In addition, this increase activates the Janus kinase (JAK)-signal transducer and activator of transcription 3 (STAT3) pathway, resulting in the expression of acute phase genes. These placental changes in gene expression mediated by maternal IL-6 can have downstream effects on foetal (neuro)development [132].

Moreover, stress (including maternal psychological stress) is associated with cortisol. This stress hormone suppresses IL-12 and IL-18 production by antigen presenting cells (APCs), thus inhibiting T_H1 responses, while increasing IL-4-mediated T_H2 activity [112, 133, 134]. High cortisol concentrations shift the immune response from pro-inflammatory T_H1 predominance to a more balanced cytokine profile, most likely due to enhanced regulatory T cell (T_{Reg}) immunomodulation and accompanied by IL-10 production [112, 133, 135]. Chronic elevation of cortisol levels can, because of negative feedback, however result in increased (neuro)inflammation and impaired cognitive functions [112, 114, 129, 133, 135, 136]. A disturbed T_H1/T_H2 balance has been observed in other neuropsychiatric disorder as well, including depression and schizophrenia [137, 138].

In children with ADHD, cortisol production however seems reduced [129, 139], though contradicted by another study [140]. Therefore, it can be hypothesised that patients have a defective T_{Reg} immunomodulation, elevated T_H1 and depressed T_H2 cytokine levels [135]. The resulting defective downregulation of inflammation may thus contribute to an enhanced pro-inflammatory cytokine synthesis and reduction of anti-inflammatory IL-10, and thereby to brain white matter damage [112]. Both chronic high and low cortisol levels and the resulting neuro-inflammation could therefore

promote emotional, cognitive and behavioural impairments [112, 115, 129, 135]. Cortisol homeostasis in a relatively narrow range therefore appears important for normal brain development and function.

In addition, a defective T_{Reg} regulation due to reduced cortisol levels could even result in T_H2-driven inflammation. This makes the patient "allergy-prone" (thus more likely to have asthma, allergic rhinitis, atopic dermatitis and allergic conjunctivitis) by inducing activated B cells, mast cells and eosinophils [112]. A theory is therefore that dietary factors and inhalants can contribute to ADHD via T_H2-like hypersensitivity reactions [13, 20]. Indeed, the observed higher IgE levels were supported by a trend for more diagnosed allergies in patients than in controls (Chapter 2), though significance for higher IgE levels was lost after Bonferroni correction (to correct for significant differences by chance due to multiple testing). Though no significantly different plasma cytokine levels were observed, an increase in locally produced cytokines could be too low to be detected in plasma.

In fact, a large variety of foods and food components can provoke or exacerbate behavioural responses [141-146]. As allergy or hypersensitivity reactions induce inflammation [147-151], consumption of offending foods can provoke increased gut permeability, GI inflammation and elevated inflammatory cytokine levels [113, 114]. The overproduction of cytokines can thereby lead to chronic T cell-mediated neuroinflammation, triggering neurodegeneration and cognitive dysfunction [13, 112, 114, 152, 153]. In ADHD, this immune dysbalance is corroborated by more frequent physical comorbidities [154-156]. This is supported by more physical complaints in patients than in controls (Chapter 2), elevations of pro-inflammatory cytokines [114] (not observed in the case-control comparison in Chapter 2, though local cytokine production might not be reflected in systemic cytokine levels [41]), increased adenosine deaminase activity [15] and possibly increased eosinophilic activity [18, 114]. Extensive modulation of the diet (e.g. by RED) could be effective in ADHD by having major consequences on immune functioning. Examples include counteracting effects of gut epithelium damage as well as restoring the T_H1/T_H2 balance and immune regulation by T_{Reg}. Dietary modulation can thereby reduce chronic immune dysregulation and neuroinflammation [114, 157-160]. Similar effects can be related to solar intensity [161]. Exposure to UVB through sunlight enables the production of vitamin D_3 in the body, which has immune modulating effects by inducing T_{Reg} differentiation and by suppressing isotype switching to IgE in B cells [162].

Similarly, the mechanism underlying the association of atopic eczema and ADHD could be related to an increased secretion of IgE, increased eosinophilic activity and predominantly T_H2 type cytokine secretion. This leads to an overproduction of inflammatory cytokines that pass the BBB and activate neuro-immune mechanisms involved in neuronal circuits that relate to behavioural and emotional

modulation. Next to elevated cytokine levels, increased stress levels due to atopic eczema and thereby raised glucocorticoids can interfere with the development of the brain and neurotransmitter systems and increase the risk of developing ADHD [114].

Moreover, chronically elevated oxidative stress can lead to elevated T_H2 induction and thereby increased IgE levels. More specifically, potentially via upregulation of STAT6 and NF-κB activation, naïve T cells can be polarised to T_H2 by oxidative stress. T_H2 cytokines stimulate B cell isotype switching to IgE. Depletion of GSH for instance inhibits T cell proliferative response, while elevated ROS and MDA levels have been shown to promote T_H2 differentiation [163]. On the contrary, high antioxidant levels blunt the T_H2 response and thereby reduce IL-4 production. High concentrations of total IgE reflect a predisposition to develop IgE-mediated allergic diseases, despite not being related directly to allergic status [164, 165]. Oxidative stress might thus facilitate the development of allergic conditions in ADHD patients [105, 166]. Indeed, the observed higher IgE levels in the case-control comparison (though significance was lost after Bonferroni correction; Chapter 2) were supported by a trend for more diagnosed allergies in patients than in controls, as well as by the association between ADHD and atopic disorders in literature. Moreover, immune cells are an important source of not only pro-inflammatory compounds like inflammatory cytokines, but also of ROS and RNS, thereby possibly establishing a vicious circle [15, 108].

Finally, GI inflammation is associated with a disrupted intestinal microbial composition [167]. Significantly altered commensal microbiota have been observed in various disorders, like diabetes and autism [168, 169]. Such a dysbiosis can be a cause rather than a consequence [153, 170-172]. Commensal microbiota play a crucial role in the development of intestinal immunity, e.g. via the induction of T_{Regs} [168, 172, 215], but also in CNS development and function [173, 174]. The bidirectional relationship between gut microbiota and the brain, called the microbiota-gut-brain axis, appears responsible for the association between ADHD and the observed altered gut microbiome, by affecting neuroimmune mechanisms. For instance, via a dysregulated enteric nervous system (ENS), intestinal neurotransmitters, metabolites of absorbed food components and cytokines, the CNS can be affected [175-177]. It was speculated that microbial dysbiosis causes colonisation by bacteria producing neurotoxins (Figure 6.2) [170]. In addition, microbial dysbiosis and subsequent alteration of intestinal permeability cause production and spreading of LPS, cytokines and chemokines [178]. This further enhances pro-inflammatory cytokine production, altering neuropeptide synthesis [170]. Moreover, commensal bacteria produce γ-aminobutyric acid (GABA) and a significant amount of vitamin B6. GABA is the main CNS inhibitory neurotransmitter. It influences both immune and neural

receptors within the ENS and CNS and is implicated in the pathogenesis of anxiety and depression. Moreover, reduced cerebral GABA concentrations have been observed in ADHD [153, 179]. Metabolism of for instance norepinephrine and dopamine depends on the coenzyme pyridoxal phosphate, the active form of vitamin B6. In paediatric ADHD patients, reduced activity of pyridoxal phosphate-dependent enzymes was found [176]. Finally, in ADHD patients versus controls, increases in *Bifidobacterium* have been observed, related to an increased metabolic function of cyclohexadienyl dehydratase. This enzyme is involved in phenylalanine synthesis, an essential amino acid and dopamine precursor [180-182]. It is however still unknown whether a generic modification of gut microbiota is involved in various neurologic disorders or whether a specific alteration plays a particular role for a certain condition [173].

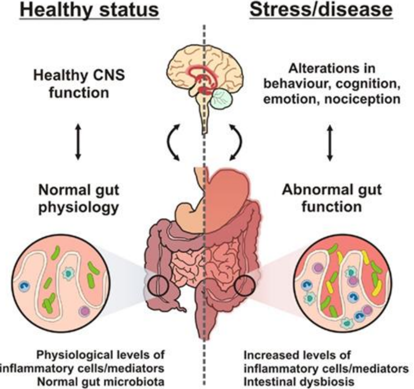

Figure 6.2. Brain-gut-microbe communication in health and disease [183].
CNS: central nervous system.

4.3 Polyphenol-rich plant extracts

Various polyphenols have antioxidant and immune modulating properties [184-188], but they can also exert other biological effects. Specific polyphenols or metabolites have been shown to cross the BBB and to influence neuronal survival and regeneration [189, 190]. Supplementation with these natural food constituents could therefore potentially constitute a promising approach for ADHD, though interpretation of their mechanism of action can be complex.

In paediatric ADHD, Pycnogenol® (1 mg/kg body weight/day during one month) was already shown to improve hyperactivity and inattention ratings (see Chapter 1, 4.3.1) [101]. Exact mechanisms by which Pycnogenol® can improve brain function and behaviour and possibly comorbid complaints are unclear.

Various mechanisms may play a role, including antioxidant and anti-inflammatory effects [77]. In addition, it is possible that polyphenol-rich extracts such as Pycnogenol® cause more obvious improvements in a subset of ADHD patients, e.g. those with more severe oxidative stress [191].

Pycnogenol® was shown to significantly decrease urinary dopamine levels in ADHD patients as compared to baseline. Levels of epinephrine and norepinephrine, which were significantly elevated as compared to healthy controls, only showed a trend toward reduced levels with Pycnogenol® treatment, with a stronger decreasing effect for higher original concentrations [102]. Normalisation of norepinephrine levels was also found in another polyphenol supplementation study [4, 192]. This effect could be related to stimulation of eNOS, which is involved in the regulation of norepinephrine and dopamine release and uptake [101]. Additionally, NO improves cerebral blood circulation [193]. Moreover, M1 (δ-(3,4-dihydroxy-phenyl)-γ-valerolactone, a catechin metabolite) has been reported to suppress iNOS, thereby possibly reducing inflammation [194].

Pycnogenol® could also normalise catecholamine metabolism by reducing brain oxidative stress. In fact, Pycnogenol® reduced lymphocyte 8-oxoG and plasma GSSG levels and increased GSH levels and the GSH/GSSG ratio (possibly by increasing GR activity) in ADHD patients [101, 103, 104, 195]. After a one-month washout, behavioural scores as well as 8-oxoG levels and GSH/GSSG ratio returned to baseline [101, 103, 104]. Reduction of 8-oxoG levels correlated with an improvement in inattention, while GSH/GSSG ratio correlated negatively with urinary dopamine levels [102-104]. In addition, urinary catecholamine levels correlated with the degree of hyperactivity as well as with plasma GSSG levels before treatment [102]. It could therefore be suggested that Pycnogenol® can have a beneficial effect on ADHD symptoms because of its direct scavenging and chelating ability, stimulation of DNA repair and/or combinations thereof [104].

Catecholamine metabolism is a source of ROS ($O_2^{\bullet-}$ and H_2O_2). Normalisation of catecholamine levels could therefore be responsible for an improved antioxidant status and reductions in oxidative stress markers, next to behavioural improvements [101-104].

Moreover, Pycnogenol® can be beneficial in ADHD because of its immune modulating effects [199, 200]. Though its immune effects in ADHD have not been analysed before, Pycnogenol® has potential for improving inflammation and allergy symptoms. For example, 100 mg/day for six months significantly reduced inhalation corticosteroid use and specific IgE titre in adult asthma patients [199]. In mice, Pycnogenol® (50-100 mg/kg orally) increased T and B cell mitogen response and partly restored the T_H1/T_H2 imbalance (low T_H1 and high T_H2 cytokine secretion) after diet-induced immune

dysfunction. Meanwhile, it reduced hepatic lipid peroxidation and increased α-tocopherol levels [201]. Though only a slight immune imbalance and limited elevation of lipid peroxidation levels were observed in ADHD (Chapter 2), Pycnogenol® is therefore expected to restore the T_H1/T_H2 balance, possibly by its antioxidant activity and by influencing activation and differentiation of T_{Regs} [188, 190, 202, 203]. In addition, *in vitro* GI digestion of Pycnogenol® in the presence of enzymes and microbiota revealed its' biological activity as a potential inhibitor of macrophage TLR signalling. More specifically, Pycnogenol® was shown to potentially exert local gut as well as systemic immunomodulatory effects by inhibition of TLR signalling, as partial agonist of TLR1/2 and TLR2/6, by influencing gut microbial composition and the expression of surface markers on immune cells and by induction of IL-10 secretion (Chapter 4).

Also, previous research indicates that polyphenols and their metabolites can affect host microbial composition, e.g. by stimulation of *Bifidobacterium* and *Lactobacillus* species [212-214]. Intestinal microbiota are important for the induction, maturation and function of the immune response, even well beyond mucosal tissues and in organs distal from the intestine [168, 170, 172, 215]. In addition, knowledge on the effect of microbiota on brain and behaviour is growing [215-217]. For instance, probiotics alleviated autism-like symptoms in mice [153].

M1, one of the active metabolites of Pycnogenol®, is able to the cross the BBB and accumulates in cells like monocytes and macrophages to levels that could be bioactive [101, 194, 204, 205]. Pycnogenol® might therefore exert its effects locally in the brain. For instance, Pycnogenol® had a neuroprotective effect by attenuating brain oxidative stress and pro-inflammatory cytokine levels following traumatic brain injury in rats [206]. In oxidative stress-related neurodegeneration in rats, it attenuated cognitive performance decline [207]. Pycnogenol® was also shown to enhance human mental performance, while reducing oxidative stress [208-210]. Moreover, Pycnogenol® can exert neuroprotective effects by improving nerve growth factor content in the hippocampus and cortex, areas important for learning and memory [211].

PART 5

IMPLICATIONS

Like early genetic and environmental factors, multiple immune and oxidative influences of small effect can act together “to create a spectrum of neurobiological risk by altering brain structure and function that mediate the emergence of ADHD” [15]. Various reports indeed support redox and immune mechanisms to contribute to ADHD development and/or exacerbation, at least in a subgroup of patients. A disturbed immune regulation is more likely than a single (sub)cellular defect and oxidative damage markers appear more reflective of the actual oxidative stress situation compared to antioxidant levels [218]. This chronic low-grade pro-inflammatory immune dysregulation and redox imbalance probably require a predisposing genetic background. By affecting neuronal structure, they can impair dopamine synthesis and neurotransmission. If immune pathways and/or oxidative stress contribute to ADHD development and/or manifestation, even if low-grade and only in specific subgroups, this could be taken into account in ADHD diagnosis and treatment to improve patient care. In this case, ADHD symptoms could also be addressed with a nutritional approach before prescribing medications or in combination with medication, which might appear more acceptable to parents as well. In fact, based on both *in vitro* and *in vivo* research, polyphenol-rich extracts such as Pycnogenol®, characterised by antioxidant and anti-inflammatory properties, appear to have potential in addressing ADHD and comorbid symptoms. However, the exact contribution of the immune system and oxidative stress to ADHD should still be revealed and the effect of Pycnogenol® remains to be confirmed by *in vivo* intervention research. In addition, oxidative stress and immune imbalance represent a common aetiopathological factor in various disorders. They can therefore not be used as a specific diagnostic requirement for any exclusive condition.

PART 6

INTERVENTION TRIAL

Since Pycnogenol® is compared to both MPH and placebo, it is considered a medicinal product in the current trial. This extended the application process for trial approval considerably, while encapsulation for example required full implementation of GMP principles.

6.1 Participant recruitment

Similar recruitment problems appeared in the intervention trial as in the case-control comparison, such as patients often using supplements before their official diagnosis. One of the reasons parents of ADHD patients decided not to participate in the ongoing clinical trial was reluctance to use medication. In addition, a 10-week placebo treatment did not seem very appealing to patients experiencing a heavy burden of ADHD. Moreover, again several patients were included months after their official diagnosis. These factors might cause some selection bias. Nevertheless, such selection bias could occur in any placebo-controlled trial or study with medication use as an exclusion criterion or with a placebo or active treatment as control. Moreover, adequate assessment of ADHD symptom severity allows comparison between studies.

As in the case-control comparison, a heterogeneous sample of ADHD patients is expected in this trial. Therefore, power should be sufficient to perform subgroup analyses (e.g. based on gender, severity or subtype of ADHD or dietary habits).

6.2 Treatment

One of the advantages of polyphenol supplementation in ADHD could be a more continuous effect as compared to MPH. The effect of MPH can be noticeable within 30 min, which is not expected for Pycnogenol®. However, behavioural effects of MPH wear off after a specific amount of time, dependent on the formula given (e.g. elimination half-life of Medikinet® Retard 20 mg is 3.2 h) [219, 220]. Medication therefore does not reduce behavioural problems in the early morning or in the evening. Though it might take several weeks for a clear behavioural effect of Pycnogenol® to be noticed, no

behavioural worsening at night is expected due to its less abrupt cessation of effect as compared to MPH. Various components present in Pycnogenol® have half-lives up to 6 h with a presence of up to 12 h after a single intake [71]. In addition, many ADHD patients quit medication intake despite initial positive behavioural effects, often due to adverse side effects [221]. Polyphenol supplementation is, however, even hypothesised to reduce physical and sleep complaints [222]. Moreover, while dietary adaptations like a RED ask for an enormous commitment of both parents and child, a nutritional supplement like Pycnogenol® is, like medication, easy to apply.

In routine clinical practice, MPH doses are titrated individually to maximise behavioural effects and to minimise unwanted side effects. In case this approach is included in a randomised double-blind controlled trial, it would be optimal to also provide an individually titrated dose of the comparison treatment(s). This would without doubt provide a more realistic comparison between treatments and possibly leads to higher effect sizes as compared to placebo. In the ongoing randomised trial, financial and logistic possibilities were too limited to provide such personalised treatments. However, this approach should be considered in future research.

6.3 Outcomes

Stimulant treatment does not improve comorbid physical complaints and could even cause some complaints [220]. In addition, ADHD patients with physical problems even tend to respond less to medication [223]. Pycnogenol® is however expected to reduce ADHD symptoms as well as comorbid physical and psychiatric complaints. Previous dietary approaches, e.g. a RED, were shown to not only improve ADHD-related behaviour, but also ODD and physical and sleep complaints in the majority of participants. Patients with a positive behavioural effect due to a RED initially reported even more physical complaints than those with less behavioural effects of the diet [224-226]. Assessment of comorbid complaints thus appears important and will further increase insight in ADHD.

Nevertheless, as observed in the case-control comparison, subjectivity of questionnaire responses should be taken into account and objective behaviour assessment could be considered in further research. In addition, again an FFQ questioning consumption frequency but not portion size was chosen, which makes it impossible to draw conclusions on actual intake. However, as parents are asked a large number of questions in the trial already, even more questions might cause reluctance and thereby unanswered questions or unreliable questionnaire results. Since the purpose of the FFQ is only

to get an overall idea of e.g. fruit and vegetable intake, questions on portion size were therefore considered redundant.

6.4 Statistics

The importance of regression analysis correcting for e.g. differences in processing time was shown in the case-control comparison. Therefore, processing time will again be systematically recorded and corrected for, which should become common practice in biomarker research. The same accounts for Bonferroni correction for multiple testing.

PART 7

FUTURE PERSPECTIVES

Development of interventions that successfully target functional deficits is vital to achieve positive long-term outcomes in children with ADHD. In fact, a therapy specifically aimed at improvement of the immune system and/or oxidative stress regulation might have potential in ADHD treatment, possibly even more than conventional therapy by stimulant medication. However, though oxidative and immune mechanisms may contribute to ADHD via neuronal damage and abnormal neurotransmitter regulation, decisive evidence on their exact contributions is lacking [15, 82, 83]. To date, it is unclear to what extent and in which patients such mechanisms occur. Moreover, clinicians pay little, if any, attention to oxidative and immune markers in the diagnosis or therapy of ADHD [13]. The hypothesis of ADHD being an oxidative and immune disorder as well as underlying mechanisms thus need to be thoroughly tested. In addition, though modulation of immune system activity and redox status might have potential in ADHD treatment and might be more acceptable to parents than prescription drugs, nutritional supplementation in ADHD remains controversial, but at the same time warrants further research [157, 227, 228].

7.1 Redox and immune imbalance

7.1.1 Biomarkers

Though various similar studies have been performed, the originality of the present case-control comparison is in assessing various biomarkers that have not been systematically studied in ADHD, like IgM and specific cytokines, and a broad diversity of oxidative stress markers. Nevertheless, various other potential research options are possible. Indeed, despite multiple correlations between various biomarkers, only small parts of the variability of one parameter could be explained by the variability of the other. Additionally, linear regression showed that antioxidant levels were not predicted directly by the amount of oxidative damage. This could imply that other pathways and thus biomarkers might be involved and could be more informative.

Future research should focus on a broad range of both oxidative damage and antioxidant markers, including antioxidant enzymes, as well as immune markers focussing on both IgE- and T cell-mediated mechanisms (e.g. different subsets of T_H cells and DCs and their cytokine products) [229, 230]. Such a more comprehensive characterisation of oxidative stress and immune status in ADHD is needed. Studies in these domains are overall limited regarding biomarkers investigated and highly various regarding populations and methodologies. Moreover, the ADHD spectrum might not only vary in terms of behavioural manifestations, but also regarding underlying mechanisms and thus potentially appropriate treatment. It should therefore be determined whether specific underlying factors apply to the majority of children with ADHD or only to a specifically identified subgroup [231]. For instance: are elevated levels of a specific oxidative damage biomarker typical for patients with pronounced impulsivity? Using biomarker levels, patient subgroups might be defined further and even more precisely based on ADHD causative and triggering factors in order to predict their responses to specific treatments [149].

7.1.1.1 Oxidative stress status

It would have been interesting to have more information on antioxidant enzyme capacity, like GPx, CAT and SOD activity. These analyses could increase insight in the mechanism underlying both elevated antioxidant and oxidative damage levels. For instance, lower levels of GST and GPx could lead to low GSH expenditure and thus lack of an efficient antioxidant defence and could thereby explain higher GSH levels and elevated oxidative damage [15, 26, 96]. However, as analyses of antioxidant enzyme activities were not prioritised in the beginning and since their stability in biological samples is restricted, these were not taken into account.

Since it is difficult and labour intensive to measure each antioxidant in a biological sample separately, TAS was analysed by various research groups. TAS determines the capacity to inhibit ROS-induced oxidative damage [5, 104, 232-234]. As antioxidants have additive and synergistic effects, TAS analysis can be informative [5]. However, methodological differences hamper comparison between studies. Moreover, this analysis shows a global picture, while depletion of one antioxidant could be masked by another. The analysis of a broad range of antioxidants in a biological sample thus gives a more complete overview of the antioxidant status. In addition, TAS is a measure for the total reducing capacity of the biological sample, but not all reductants are antioxidants and vice versa (i.e., while reductants chemically scavenge ROS, the effect of antioxidants in the biological context depends on the redox potential and reaction rate, and thus concentration achieved, while specific antioxidants can also induce protective enzymes) [235-237]. Finally, elevated TAS is not necessarily positive, as it may be an

adaptation to increased oxidative stress. Like specific antioxidants, TAS does not reflect oxidative damage *in vivo* [218, 238]. When monitoring oxidative stress *in vivo*, several biological parameters covering different aspects of both oxidative damage and antioxidant defence should be analysed by well-validated assays in order to draw reliable conclusions. This is because a change in one parameter might disturb the oxidant-antioxidant balance, thereby causing an augmentation or reduction of other parameters.

When free radicals react with PUFAs via their carbon-carbon double bond, lipid hydroperoxides are formed. These are unstable and therefore subjected to fragmentation into different products, such as MDA and 4-hydroxynonenal (4-HNE) [239]. F2-isoprostanes are a marker of oxidative lipid damage as well, formed when essential fatty acids, especially AA, are oxidised [238]. Both 4-HNE and F2-isoprostanes are therefore other oxidative damage biomarkers, which could be measured in ADHD, both by means of ELISA.

Oxidative damage to proteins includes primary damage to enzymes, receptors and transport proteins, as well as secondary damage to other biomolecules (e.g. increased mutation frequency due to damaged DNA-repair enzymes) [55,56]. An example of a marker of oxidative damage to proteins is 3-nitrotyrosine (3-NT), formed by a reaction of RNS, such as peroxynitrite ($ONOO^-$), with tyrosine (Figure 6.3) [54]. Due to altered protein conformation, solubility and degradation, tyrosine nitration can have various functional and biological consequences. Examples are enzyme inactivation (e.g. in case of GR) or induction of an immunological response [240, 241]. 3-NT analysis is therefore desirable, though not very straightforward. Different methods for 3-NT analysis in plasma and plasma protein samples have been described, but aspects like protein hydrolysis or derivatisation can cause artefact 3-NT formation. Due to low plasma 3-NT concentrations (around 1 nM), LC-MS/MS is the most sensitive and specific method without sample derivatisation [242]. To eliminate problems regarding incomplete proteolysis, the ratio 3-NT/tyrosine should be determined. Still, as the concentration of tyrosine is about 10^6 times higher than that of 3-NT, it is difficult to analyse both in one run.

OH + N•=O → → O, N–O• + HO• → → OH, N⁺(=O)–O⁻ (R substituents)

Figure 6.3. Proposed reaction mechanism of tyrosine and NO• leading to 3-nitrotyrosine (3-NT) formation, after formation of an iminoxyl radical [243].

A potent antioxidant which might be interesting as well, is vitamin C, or ascorbic acid, the most prevalent water-soluble antioxidant in human plasma. Ascorbic acid for instance scavenges ROS and regenerates α-tocopherol, β-carotene and GSH [244-247]. *Ex vivo,* ascorbic acid is however very sensitive to oxidation, though addition of metaphosphoric acid to the sample might improve its stability. Levels of ascorbic acid can be determined by HPLC with electrochemical detection [248].

7.1.1.2 Immune status

IL-4, IL-5 and IL-13 are found adjacent on chromosome 5 in humans and are regulated in a coordinated though independent fashion. Despite being markers for T_H2 cell activity and sharing common regulatory pathways and receptors, these cytokines perform distinct functions [249, 250]. For instance, basophils and eosinophils exert their function rather through IL-4 than IL-13. Broadly, IL-4 mainly stimulates naïve T cell differentiation towards the T_H2 subtype and IgE production by B cells, and together with IL-13, it recruits leukocytes and stimulates peristalsis in the GI tract. IL-13 also increases mucus secretion. IL-5 stimulates B cells, mast cells and granulocytes [14, 112, 251]. Therefore, it would have been interesting to determine plasma levels of IL-4 and IL-13 as well. However, plasma levels of these cytokines are generally low and difficult to interpret. In addition, though elevated oxidative stress can cause higher basal levels of inflammatory cytokines [252], oxidative stress in ADHD might be too limited for effects on plasma cytokine levels. Therefore, mucosal cytokine levels (e.g. in saliva or faeces), reflecting local cytokine production, could provide additional information on immune activation in ADHD [253].

MHC involvement is likely to influence the development of an adaptive immune response in ADHD [254, 255]. Antigen presentation by APCs, T_H cell proliferation and T_{Reg} and memory B cell activation should be therefore explored. With respect to T_{Regs}, the importance of breaking tolerance in ADHD, as in allergy, is interesting but not yet studied. This could also provide a clue on the mechanism(s) underlying spontaneous remission of both atopic diseases and ADHD. Additionally, it should be determined whether an allergic or a non-allergic hypersensitivity mechanism is involved in children with food-induced behavioural symptoms.

Unless in case of e.g. active inflammation, analysis of systemic immune markers, like in plasma, might not always be informative, as these are not sensitive enough to small and local changes. *Ex vivo* stimulation of isolated PBMCs with e.g. LPS, pokeweed mitogen (PWM) or concanavalin A (conA) and analysis of the proliferative and cytokine production response, to investigate monocyte and T and B cell reactivity, could therefore be more informative on immune reactivity than systemic cytokine or antibody levels. In addition, further technology development allowing measurement of mucosal

cytokine levels (e.g. in saliva or faeces) could provide additional information on immune activation in ADHD [253].

Further research on the genetic background and interconnection of oxidative stress and immune dysregulation in ADHD is implicated as well. In this respect, qPCR analyses focussing on PBMC cytokine (e.g. IL-1β, TNF-α, IL-10), intracellular signalling (incl. NF-κB) and (anti)oxidant pathways (e.g. GPx, SOD) can be related to plasma cytokine and antibody levels, identification of PBMC subsets and their functional responses (e.g. cytokine release) and markers of oxidative damage and antioxidant status of both patients and controls. This approach can indicate genetic variations and biological processes related to immune and (anti)oxidative mechanisms and their interrelations. It can thus provide crucial information on ADHD pathophysiology at the genetic and molecular level. It might thereby lead to the identification of new markers or insights and potentially effective therapies [256].

To further investigate the interconnection between redox and immune imbalance in ADHD, PBMC GSH levels are interesting to determine, as various immune functions are sensitive to GSH depletion (e.g. NK and B cell reactivity) [106, 107]. Also interesting would be analyses on PBMCs from ADHD patients and controls before (to assess intrinsic differences like numbers of various T cell subsets, monocytes and NK and B cells) and after (to assess differences in antioxidant metabolism and effects on the proliferation, maturation and activation) *ex vivo* treatment with Pycnogenol®.

7.1.2 Design

Since a case-control design does not address the investigation of causality, a cohort design should be considered, despite the large number of children to be included. The advantage of a case-control design is that it’s easier to include sufficient numbers of cases. In addition, by using multivariate analysis, data on how variables influence each other can be extracted, generating relevant research questions on causality, which can be studied further.

ADHD is a continuum, with individual patients being highly variable, i.e. inattention vs. hyperactivity or low vs. high level of behavioural impairment. Subgroup analyses and correlations to specific behaviours are therefore advisable but require enough statistical power. In addition, division into subgroups can be bothersome as sometimes a discrepancy appeared between individual questionnaire answers and (the absence of) physician-based diagnosis (Chapter 2). Still, it is plausible that subgroups vary regarding underlying mechanisms and thus potentially also regarding appropriate treatment.

A striking discrepancy between physician's diagnosed ADHD and parent rated behaviour was found in the case-control comparison (Chapter 2), with several high SEQ scores in control subjects and low scores in patients. This underscores the importance of properly selected patient groups with a reliable ADHD diagnosis to be included in further research, as well as clear information on comorbidities. Efforts should be made to include acceptable sample sizes based on power calculation and to use suitable methods and durations of behaviour monitoring, like classroom observations and internationally accepted questionnaires.

For comparison with literature, the SEQ could be replaced by a more frequently used international questionnaire. One example is the CRS, which also assesses ADHD and related behaviour like ODD and CD and is often used in pharmacological research [224, 257, 258]. However, the validity and reliability of the Dutch translation of this questionnaire are as yet insufficient [259].

7.2 Pycnogenol® in ADHD

Questions remain regarding the mechanism of action of polyphenol-rich extracts like Pycnogenol® affecting behaviour. More knowledge is for instance required on potentially bioactive metabolites. As *in vivo* research is however characterised by inter- and intraindividual variation, an *in vitro* gastrointestinal simulation model like the GIDM is a feasible option for further in-depth investigation [260]. The lack of active transport in the GIDM could be partly resolved by implementing Caco2 cells.

In culture, the human epithelial colorectal adenocarcinoma cell line Caco2 differentiates into monolayers with various functions of the small intestinal villus epithelium [261]. This cell line for instance exhibits tight junctions and a brush border on the apical surface and expresses typical hydrolases and nutrient transporters [262]. Caco2 cells grown on permeable filters are therefore the golden standard and most popular model for *in vitro* prediction of *in vivo* small intestinal permeability and absorption of orally administered drugs or food components [261-263]. Since Caco2 cells express several cytochrome P450 isoforms and phase II enzymes, like sulfotransferases and GST, this cell line can also be used to study presystemic metabolism [262]. Still, Caco2 cells do not express every intestinal metabolising enzyme to an appreciable extent and, unlike the small intestine and the colon, does not secrete mucus and contains no microfold (M) cells. In addition, this cell line may alter its performance over time, making good quality control essential [261]. Finally, it has to be kept in mind that *in vitro* assays are useful for the elucidation of mechanisms of action, while *in vivo* research remains essential considering pharmacokinetic phenomena [58].

Another option would be to incubate THP-1 or HEK cells with plasma of human volunteers treated with Pycnogenol® as well as controls. However, interindividual variation is characteristic for *ex vivo* systems [56]. Indeed, prominent variability of polyphenol pharmacokinetics has been observed [75].

Immune responses of *in vitro* models are commonly determined by measuring cytokine levels in culture medium after challenging cells. Though relevant in terms of biological functions, the relatively long exposure time usually required (generally hours, as compared to minutes for gene expression and days for cell differentiation) to obtain cytokine levels above threshold can lead to the initiation of other signalling pathways. In addition, cytokine secretion is only partly related to the expression of genes and their upstream transcription factors, due to transcription and translation regulation: levels of transcripts and proteins correlate significantly for only half of all genes, depending on their cellular location and biological function [61, 264, 265]. For example, the level of available cytokine in the supernatant is lowered by binding to its receptor [266]. Gene expression of relevant cytokines, enzymes and transcription factors is therefore also interesting to investigate, e.g. by RT-qPCR, making it possible to differentiate between primary stimulus-cell and secondary cell-cell signals [61]. The major advantage of this technology is the ability to quantitatively compare gene expression over a 10^7 fold with a good sensitivity, specificity and reproducibility [267].

To further investigate the interconnection between redox and immune effects of Pycnogenol®, both immune (e.g. cytokine levels and gene expression) and redox effects (e.g. GSH, MDA and 8-OHdG levels) could be determined after stimulation of THP-1 macrophages with Pycnogenol® [185, 268].

Further research should also focus on which components pass the BBB, by positron emission tomography (PET) scans [269], as well as on the brain activity of children before and after polyphenol supplementation, by functional magnetic resonance imaging (fMRI) scans [270].

Though beyond the scope of the ongoing project, investigating a potential synergistic effect of MPH and Pycnogenol® is interesting as well [271].

7.3 Other research options

7.3.1 Microbiota

The gut may play an important role in ADHD, as many patients report GI problems. Microbiota, gut and immune function are closely intertwined and a disturbed immune function appears involved in at least a subgroup of ADHD patients. Moreover, polyphenols and their metabolites can affect host microbial composition [212-214]. Further research is therefore required to determine whether there is a causal connection between microbiota, gut and ADHD.

Probiotics are live microorganisms that, upon ingestion in specific and sufficient numbers, confer non-specific health benefits to the host. In addition to directly affecting gut microbial composition, they reconstitute the GI barrier by increasing mucin production by goblet cells and strengthening tight junctions [170]. Moreover, they modulate the immune response by inhibiting production of pro-inflammatory cytokines like TNF-α by DCs and by increasing expression of anti-inflammatory mediators like IL-10, thereby promoting T_{Reg} production [153]. To date, one study found a significantly reduced risk for ADHD development after *Lactobacillus rhamnosus GG* supplementation during the first six months of life [272].

7.3.2 Study design

Research on alternative treatment options to investigate their efficacy, mechanism of action and value should be performed in a double-blind placebo-controlled manner, especially in case of questionnaires. Attention should be paid to adequate sample sizes and treatment periods. A double-blind placebo-controlled design is essential to illustrate that potential effects are not attributable to expectations or hopes of parents or patients. Standardisation and characterisation of treatments is essential for drawing valid conclusions and for comparability between studies. Treatment adherence should preferably be assessed by electronic monitoring and time-recording devices, which are superior to medication counts or self-reported adherence [273].

PART 8

GENERAL CONCLUSION

ADHD is a multifactorial disorder, influenced by genetic, biochemical, psychological and environmental factors. MPH is prescribed to a rapidly growing number of patients despite serious concerns about adverse effects and possible publication bias in reported efficacy, without evidence of long-term efficacy. Moreover, this disorder has been associated with immune and oxidant-antioxidant imbalances, though research has been inconsistent and studies mostly have severe limitations.

A comparison of redox and immune status between ADHD patients and controls without ADHD showed significantly higher plasma MDA levels in patients, as well as a trend for higher urinary 8-OHdG levels. Erythrocyte GSH and plasma retinyl palmitate, IgG and IgE levels were significantly higher in patients than in controls. Finally, a trend for lower plasma IL-5 levels was observed. After Bonferroni correction (correcting for significance “by chance” due to multiple testing) however, the result for GSH remained statistically significant (borderline significant for retinyl palmitate), while significance was lost for MDA, IgG and IgE levels. Though thus only slightly elevated, an indication of more oxidative damage was found in ADHD. Antioxidant levels did not differ or were even higher in patients than in controls. This could indicate a compensation mechanism for increased oxidative stress, though apparently insufficient to restore the redox balance. In addition, consistently, albeit not significant, higher IgE levels were supported by a trend for more allergies in patients than in controls and might point at an immune dysbalance in ADHD. Though specific biomarker levels only slightly differed between patients and controls, these results are consistent with a role of oxidative stress that affects the immune system in children with ADHD as compared to controls. However, due to the nature of this study, it is unknown whether redox and immune dysbalance play a causative role in ADHD, though both may contribute to ADHD via neuronal damage and abnormal neurotransmitter regulation. Dietary habits do not appear to explain the observed biomarker differences. However, more psychiatric comorbidities were found in ADHD patients, which possibly influences the results of this study. The exact pathophysiology of ADHD thus remains unclear and might even differ between patients. Further confirmation of these results is required, as well as further investigation of potential differences between ADHD subtypes. Finally, systematically correcting for processing time appears crucial to obtain valid results.

Despite various unresolved questions, nutritional approaches like polyphenol-rich foods or extracts could have a positive impact on ADHD behaviour and possibly also on related complaints, at least in a subgroup of patients, due to their antioxidant and immune modulating activity. In fact, after *in vitro* GI metabolism in the presence of enzymes and microbiota, Pycnogenol® was shown to potentially exert local gut as well as systemic immune modulating effects via inhibition of TLR signalling, by acting as partial agonist of TLR1/2 and TLR2/6, via alteration of gut microbial composition, via upregulation of surface markers on immune cells and via induction of IL-10 secretion.

Therefore, a randomised double-blind placebo and active product controlled clinical trial was set up to evaluate effects of an herbal polyphenol-rich extract (Pycnogenol®), MPH and placebo on ADHD over a 10-week period. Secondary outcomes are effects on comorbid physical and psychiatric complaints and levels of immune, antioxidant, oxidative damage and neurologic biomarkers. Possibly influencing dietary habits are questioned. As the trial is still ongoing, conclusions cannot be drawn yet. However, it is hypothesised that Pycnogenol® is more effective than placebo and not less effective than MPH and that, as compared to placebo and MPH, Pycnogenol® increases antioxidant levels, reduces oxidative damage, improves immune and neurochemical status and reduces comorbid physical and psychiatric complaints.

Results of this thesis (especially when supplemented with the results of the ongoing clinical trial) thus increase insight in ADHD aetiology and treatment options, which is highly desired by medical staff, parents and patients. These findings pioneer further biochemical studies on ADHD. The broad clinical context of this research is of interest to the general public and results are suitable to be communicated to a non-expert as well as expert audience.

REFERENCES

1. Biederman, J. and S.V. Faraone, Attention-deficit hyperactivity disorder. Lancet, 2005. 366: p. 237-248.
2. Wallis, D., H.F. Russell, and M. Muenke, Review: Genetics of Attention Deficit/Hyperactivity Disorder. J Pediatr Psychol, 2008. 33(10): p. 1085-1099.
3. Poelmans, G., et al., Integrated Genome-Wide Association Study Findings: Identification of a Neurodevelopmental Network for Attention Deficit Hyperactivity Disorder. Am J Psychiatr, 2011. 168: p. 365–377.
4. Faraone, S.V., C. Bonvicini, and C. Scassellati, Biomarkers in the diagnosis of ADHD - promising directions. Curr Psych Reports, 2014. 16(497): p. 1-20.
5. Guney, E., et al., Attention Deficit Hyperactivity Disorder and oxidative stress: A short term follow up study. Psychiatry Res, 2015. 229: p. 310-317.
6. Corradini, I., et al., SNAP-25 in Neuropsychiatric Disorders. Ann NY Acad Sci, 2009. 1152: p. 93-99.
7. Spencer, T.J., J. Biederman, and E. Mick, Attention-deficit/hyperactivity disorder: diagnosis, lifespan, comorbidities, and neurobiology. J Pediatr Psychol, 2007. 32: p. 631-642.
8. Staller, J. and S.-. Faraone, Attention-deficit hyperactivity disorder in girls: epidemiology and management. CNS Drugs, 2006. 20: p. 107-123.
9. Kessler, R.C., et al., The Prevalence and Correlates of Adult ADHD in the United States: Results From the National Comorbidity Survey Replication. Am J Psychiatry, 2006. 163: p. 716-723.
10. RIZIV, Farmanet databank: vergoede specialiteiten methylfenidaat (ATC N06BA04) afgeleverd via Belgische openbare apotheken, 2006-2016. 2018: Brussel.
11. Storebø, O.J., et al., Methylphenidate for attention-deficit/hyperactivity disorder in children and adolescents: Cochrane systematic review with meta-analyses and trial sequential analyses of randomised clinical trials. BMJ, 2015. 351.
12. Schachter, H.M., et al., How efficacious and safe is shortacting methylphenidate for the treatment of attention-deficit disorder in children and adolescents? A meta-analysis. CMAJ, 2011. 165(11): p. 1475-1488.
13. Pelsser, L.M.J., J.K. Buitelaar, and H.F.J. Savelkoul, ADHD as a (non) allergic hypersensitivity disorder: a hypothesis. Pediatr Allergy Immunol, 2009. 20(2): p. 107-112.
14. Abbas, A.K., A.H. Lichtman, and S. Pillai, Cellular and Molecular Immunology. 2015, Philadelphia: Elsevier Saunders.
15. Ceylan, M., et al., Changes in oxidative stress and cellular immunity serum markers in attention-deficit/hyperactivity disorder. Psychiatry Clin Neurosci, 2012. 66: p. 220-226.
16. Joseph, N., et al., Oxidative Stress and ADHD: A Meta-Analysis. J Atten Disord, 2015. 19(11): p. 915-924.
17. Schmitt, J., et al., Atopic Eczema and Attention-Deficit Hyperactivity Disorder in a Population-Based Sample of Children and Adolescents. JAMA, 2009. 301(7): p. 724-726.
18. Fasmer, O.B., et al., Adult attention deficit hyperactivity disorder is associated with asthma. BMC Psychiatr, 2011. 11: p. 128-134.
19. Chen, M.H., et al., Is atopy in early childhood a risk factor for ADHD and ASD? A longitudinal study. J Psychosom Res, 2014. 77: p. 316-321.
20. Scahill, L. and A. deGraft-Johnson, Food Allergies, Asthma, and Attention Deficit Hyperactivity Disorder. JCAPN, 1997. 10: p. 36-40.
21. Chen, M., et al., Attention deficit hyperactivity disorder, tic disorder, and allergy: Is there a link? A nationwide population-based study. J Child Psychol Psychiatry, 2013. 54(5): p. 545-551.
22. Yao, J.K., R.D. Reddy, and D.P. Van Kammen, Oxidative damage and schizophrenia. CNS Drugs, 2001. 15: p. 287-310.
23. Owen, A.J., et al., Low plasma vitamin E levels in major depression: diet or disease? Eur J Clin Nutr, 2004. 59: p. 304-306.
24. Spahis, S., et al., Lipid profile, fatty acid composition and pro- and anti-oxidant status in pediatric patients with attention-deficit/hyperactivity disorder. Prostaglandins Leukot Essent Fatty Acids, 2008. 79: p. 47-53.
25. Landaas, E.T., et al., Vitamin levels in adults with ADHD. BJPsych Open, 2016. 2: p. 337-384.
26. Ceylan, M., et al., Oxidative imbalance in child and adolescent patients with attention-deficit/hyperactivity disorder. Prog Neuropsychopharmacol Biol Psychiatry, 2010. 34(8): p. 1491-1494.
27. Ernster, L. and G. Dallner, Biochemical, physiological and medical aspects of ubiquinone function. Biochim Biophys Acta, 1995. 1271: p. 195-204.
28. Hermans, N., et al., Study of potential systemic oxidative stress animal models for the evaluation of antioxidant activity: status of lipid peroxidation and fat-soluble antioxidants. Pharm Pharmacol, 2007. 59(1): p. 131-136.

29. Palace, V.P., et al., Antioxidant potentials of vitamin A and carotenoids and their relevance to heart disease. Free Radical Bio Med, 1998. 26: p. 746-761.
30. O'Byrne, S.M. and W.S. Blaner, Retinol and retinyl esters: biochemistry and physiology: Thematic Review Series: Fat-Soluble Vitamins: Vitamin A. . J Lipid Res, 2013. 54: p. 1731-1743.
31. Thurnham, D.I. and C.A. Northrop-Clewes, Optimal nutrition: vitamin A and the carotenoids. Proc Nutr Soc, 1999. 58: p. 449-457.
32. Olson, J.A., Vitamin A and carotenoids as antioxidants in a physiological context. J Nutr Sci Vitamol, 1993. 39: p. S57-S65.
33. Sies, H. and W. Stahl, Vitamins E and C, b-carotene, and other carotenoids as antioxidants. Am J Clin Nutr, 1995. 62: p. 1315s-1321s.
34. Lidén, M. and U. Eriksson, Understanding Retinol Metabolism: Structure and Function of Retinol Dehydrogenases. J Biol Chem, 2006. 281: p. 13001-13004.
35. Ludot, M., S. Mouchabac, and F. Ferreri, Inter-relationships between isotretinoin treatment and psychiatric disorders: Depression, bipolar disorder, anxiety, psychosis and suicide risks. World J Psychiatr, 2015. 5(2): p. 222-227.
36. Oztop, D., et al., Oxidative stress in children with attention deficit hyperactivity disorder. Clin Biochem, 2012. 45: p. 745-748.
37. De Amici, M. and G. Ciprandi, The Age Impact on Serum Total and Allergen-Specific IgE. Allergy Asthma Immunol Res, 2013. 5(3): p. 170-174.
38. Wang, L., et al., Attention deficit–hyperactivity disorder is associated with allergic symptoms and low levels of hemoglobin and serotonin. Sci Rep, 2018. 8(1): p. 1-7.
39. Akyol, S.T., et al., Association between allergic diseases and attention deficit hyperactivity disorder in childhood. Asthma Allergy Imunol, 2015. 13: p. 65-70.
40. Crestani, E., et al., Association of IL-5 cytokine production and in vivo IgE levels in infants and parents. J Allergy Clin Immunol, 2007. 120: p. 820-826.
41. Dugernier, T.L., et al., Compartmentalization of the Inflammatory Response during Acute Pancreatitis Correlation with Local and Systemic Complications. Am J Respir Crit Care Med, 2003. 168: p. 148-157.
42. Joseph, J., et al., Serum interleukin-5 levels are elevated in mild and moderate persistent asthma irrespective of regular inhaled glucocorticoid therapy. BMC Pulm Med, 2004. 4: p. 1-6.
43. Dandona, P., et al., Macronutrient intake induces oxidative and inflammatory stress: potential relevance to atherosclerosis and insulin resistance. Exp Mol Med, 2010. 42(4): p. 245-253.
44. Folchetti, L.D., et al., Association of fruits and vegetables consumption and related-vitamins with inflammatory and oxidative stress markers in prediabetic individuals. Diabetol Metab Syndr, 2014. 6.
45. Andersson, Y., et al., Formula Feeding Skews Immune Cell Composition toward Adaptive Immunity Compared to Breastfeeding. J Immunol, 2009. 183(7): p. 4322-4328.
46. Hanson, L.A., M. Korotkova, and E. Telemo, Breast-feeding, infant formulas, and the immune system. Ann Allergy Asthma Immunol, 2003. 90(6 Suppl): p. 59-63.
47. Wilinska, M., et al., Oxidative stress and total antioxidant status in term newborns and their mothers. Ann Agric Environ Med, 2015. 22(4): p. 736-740.
48. Yan, S., et al., Dietary patterns are associated with attention-deficit/hyperactivity disorder (ADHD) symptoms among preschoolers in mainland China. Eur J Clin Nutr, 2018. 72(11): p. 1517-1523.
49. Zhou, F., et al., Dietary, nutrient patterns and blood essential elements in Chinese children with ADHD. NUtrients, 2016. 8(6): p. E352.
50. Kim, Y. and H. Chang, Correlation between attention deficit hyperactivity disorder and sugar consumption, quality of diet, and dietary behavior in school children. Nutr Res Pract, 2011. 5(3): p. 236-245.
51. Howard, A.L., et al., ADHD is associated with a “Western” dietary pattern in adolescents. J Atten Disord, 2011. 15(5): p. 403-411.
52. Holton, K.F., et al., Evaluation of dietary intake in children and college students with and without attention-deficit/hyperactivity disorder. Nutr Neurosci, 2018. [Epub ahead of print].
53. Mimouni-Bloch, A., et al., Breastfeeding may protect from developing attention-deficit/hyperactivity disorder. Breastfeeding Med, 2013. 8(4): p. 363-367.
54. Julvez, J., et al., Attention behaviour and hyperactivity at age 4 and duration of breast-feeding. Acta Pediatr, 2007. 96: p. 842-847.
55. Fismen, A.S., et al., Trends in food habits and their relation to socioeconomic status among Nordic adolescents 2001/2002-2009/2010. PLoS One, 2016. 11(2).
56. de Roock, S., et al., Gut derived lactic acid bacteria induce strain specific CD4+ T cell responses in human PBMC. Clin Nutr, 2011. 30(6): p. 845-851.
57. Foligne, B., et al., Correlation between in vitro and in vivo immunomodulatory properties of lactic acid bacteria. World J Gastroentero, 2007. 13(2): p. 236-243.

58. Holst, B. and G. Williamson, Nutrients and phytochemicals: from bioavailability to bioefficacy beyond antioxidants. Curr Opinion Biotechnol, 2008. 19(2): p. 73-82.
59. Mangan, D.F. and S.M. Wahl, Differential regulation of human monocyte programmed cell death (apoptosis) by chemotactic factors and pro-inflammatory cytokines. J Immunol, 1991. 147: p. 3408-3412.
60. Mangan, D.F., G.R. Welch, and S.M. Wahl, Lipopolysaccharide, tumor necrosis factor-α, and IL-1β prevent programmed cell death (apoptosis) in human peripheral blood monocytes. J Immunol, 1991. 146: p. 1541-1546.
61. Chanput, W., et al., Transcription profiles of LPS-stimulated THP-1 monocytes and macrophages: a tool to study inflammation modulating effects of food-derived compounds. Food Funct, 2010. 1(3): p. 254 - 261.
62. Cousins, R.J., et al., A global view of the selectivity of zinc deprivation and excess on genes expressed in human THP-1 mononuclear cells. Proc Nat Acad Sci USA, 2003. 100: p. 6952-6957.
63. Rogers, P.D., et al., Pneumolysin-dependent and -independent gene expression identified by cDNA microarray analysis of THP-1 human mononuclear cells stimulated by Streptococcus pneumoniae. Infection Immunity, 2003. 71: p. 2087-2094.
64. Li, L.M., et al., Role of microRNA-214-targeting phosphatase and tensin homolog in advanced glycation end product-induced apoptosis delay in monocytes. J Immunol, 2011. 186(2552-2560).
65. Kunnari, A.M., et al., The expression of human resistin in different leucocyte lineages is modulated by LPS and TNFα. Regul Pept, 2009. 157: p. 57-63.
66. Mancl, M.E., et al., Two discrete promoters regulate the alternatively spliced human interferon regulatory factor-5 isoforms: Multiple isoforms with distinct cell type-specific expression, localization, regulation, and function. J Biol Chem, 2005. 280: p. 21078-21090.
67. Hijiya, N., et al., Possible involvement of toll-like receptor 4 in endothelial cell activation of larger vessels in response to lipopolysaccharide. Pathobiol, 2002. 70: p. 18-25.
68. Daigneault, M., et al., The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. PloS one, 2010. 5.
69. Tsuchiya, S., Y. Kobayashi, and Y. Goto, Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. Cancer Res, 1982. 42: p. 1530-1536.
70. Han, X.Q., et al., Isolation, structure characterization, and immunomodulating activity of a hyperbranched polysaccharide from the fruiting bodies of Ganoderma sinense. J Agricult Food Chem, 2012. 60: p. 4276-4281.
71. Grimm, T., et al., Single and multiple dose pharmacokinetics of maritime pine bark extract (Pycnogenol) after oral administration to healthy volunteers. BMC Clin Pharmacol, 2006. 6(4): p. 1-12.
72. Liu, R., et al., Pycnogenol Reduces Toll-Like Receptor 4 Signaling Pathway-Mediated Atherosclerosis Formation in Apolipoprotein E-Deficient Mice. J Cardiovasc Pharmacol, 2016. 68(4): p. 292-303.
73. Yoshida, A., et al., Direct assessment by electron spin resonance spectroscopy of the antioxidant effects of French maritime pine bark extract in the maxillofacial region of hairless mice. J Clin Biochem Nutr, 2011. 49: p. 79-86.
74. Yesil-Celiktas, O., et al., Determination of polyphenolic constituents and biological activities of bark extracts from different Pinus species. J Sci Food Agric, 2009. 89: p. 1339-1345.
75. Mülek, M., et al., Distribution of Constituents and Metabolites of Maritime Pine Bark Extract (Pycnogenol®) into Serum, Blood Cells, and Synovial Fluid of Patients with Severe Osteoarthritis: A Randomized Controlled Trial. Nutrients, 2017. 9(5).
76. Duweler, K.G. and P. Rohdewald, Urinary metabolites of French maritime pine bark extract in humans. Pharmazie, 2000. 55(5): p. 364-8.
77. Rohdewald, P., A review of the French maritime pine bark extract (Pycnogenol), a herbal medication with a diverse clinical pharmacology. Int J Clin Pharmacol Ther, 2002. 40(4): p. 158-168.
78. Kurlbaum, M. and P. Hogger, Plasma protein binding of polyphenols from maritime pine bark extract (USP). J Pharm Biomed Anal, 2011. 54(1): p. 127-32.
79. Grimm, T., et al., Single and multiple dose pharmacokinetics of maritime pine bark extract (pycnogenol) after oral administration to healthy volunteers. BMC Clin Pharmacol, 2006. 6(4): p. 1-12.
80. Lopresti, A.L., Oxidative and nitrosative stress in ADHD: possible causes and the potential of antioxidant-targeted therapies. ADHD, 2015. 7: p. 237-247.
81. Sarlus, H., et al., Allergy influences the inflammatory status of the brain and enhances tau-phosphorylation. J Cell Mol Med, 2012. 16(10): p. 2401-2412.
82. Bulut, M., et al., Malondialdehyde levels in adult attention-deficit hyperactivity disorder. J Psychiatry Neurosci, 2007. 32: p. 435-438.
83. Kerschensteiner, M., E. Meinl, and R. Hohlfeld, Neuro-immune crosstalk in CNS diseases. Neuroscience, 2009. 158: p. 1122–1132.
84. Fagundes, A.O., et al., Chronic administration of methylphenidate activates mitochondrial respiratory chain in brain of young rats. Int J Develop Neurosci, 2007. 25: p. 47-51.

85. Ng, F., et al., Oxidative stress in psychiatric disorders: evidence base and therapeutic implications. Int J Neuropsychopharmacol., 2008. 11: p. 851-876.
86. Farmamozaïek., I. De peroxidatie van lipiden, celmembraandestructie en atherosclerose.; Available from: http://www.farmamozaiek.be/farmamozaiek/?q=node/3835 [Accessed 16 December 2015].
87. Kul, M., et al., Evaluation of oxidative metabolism in child and adolescent patients with attention deficit hyperactivity disorder. Psychiatry Investig, 2015. 12(3): p. 361-366.
88. Delion, S., et al., Chronic dietary a-linolenic acid deficiency alters dopaminergic and serotoninergic neurotransmission in rats. J Nutr, 1994. 124: p. 2466-2476.
89. Halliwell, B. and J.M.C. Gutteridge, Free radicals in biology and medicine., C. Press, Editor. 1999, Oxford University Press: Oxford.
90. Wu, A., Z. Ying, and F. Gomez-Pinilla, Dietary curcumin counteracts the outcome of traumatic brain injury on oxidative stress, synaptic plasticity, and cognition. Exp Neurol, 2006. 197: p. 309-317.
91. Dufault, R., et al., Mercury exposure, nutritional deficiencies and metabolic disruptions may affect learning in children. Behav Brain Functions, 2009. 5: p. 44-59.
92. Iuga, C., J.R. Alvarez-Idaboy, and A. Vivier-Bunge, ROS Initiated Oxidation of Dopamine under Oxidative Stress Conditions in Aqueous and Lipid Environments. J Phys Chem B, 2011.
93. Selek, S., et al., Oxidative imbalance in adult attention deficit/hyperactivity disorder. Biol Psychol, 2008. 79: p. 256-259.
94. Miyazaki, I. and M. Asanuma, Dopaminergic neuro-specific oxidative stress caused by dopamine itself. Acta Medica Okayama, 2008. 62(4): p. 141-150.
95. Kuhn, D.M., D.M. Francescutti-Verbeem, and D.M. Thomas, Dopamine quinones activate microglia and induce a neurotoxic gene expression profile. Relationship to metamphetamine-induced nerve ending damage. Ann N Y Acad Sci, 2006. 1074: p. 31-41.
96. El Adham, E.K., A.I. Hassan, and A.A. El Aziz El- Mahdy, Nutritional and Metabolic Disturbances in Attention Deficit Hyperactivity Disease. Res J Med & Med Sci, 2011. 6(1): p. 10-16.
97. Nedic, G., et al., Platelet monoamine oxidase activity in children with attention-deficit/hyperactivity disorder. Psychiatry Res, 2010. 175(3): p. 252-255.
98. Bortolato, M., K. Chen, and J.C. Shih, Monoamine oxidase inactivation: from pathophysiology to therapeutics. Adv Drug Deliv Rev, 2008. 60(13-14): p. 1527-1533.
99. Albayrak, Ö., et al., Psychiatric GWAS Consortium: ADHD Subgroup. Common obesity risk alleles in childhood attention-deficit/hyperactivity disorder. Am J Med Genet B Neuropsychiatr Genet, 2013. 162B: p. 295-305.
100. Furukawa, S., et al., Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest, 2004. 114(12): p. 1752-1761.
101. Trebatická, J., et al., Treatment of ADHD with French maritime pine bark extract, Pycnogenol. Eur Child Adolesc Psychiatry, 2006. 15: p. 329-335.
102. Dvořáková, M., et al., Urinary catecholamines in children with attention deficit hyperactivity disorder (ADHD): Modulation by a polyphenolic extract from pine bark (Pycnogenol). Nutr Neurosci, 2007. 10: p. 151-157.
103. Dvořáková, M., et al., The effect of polyphenolic extract from pine bark, Pycnogenol, on the level of glutathione in children suffering from attention deficit hyperactivity disorder (ADHD). Redox Report, 2006. 11: p. 163-172.
104. Chovanová, Z., et al., Effect of polyphenolic extract, Pycnogenol, on the level of 8-oxoguanine in children suffering from attention deficit/hyperactivity disorder. Free Radical Res, 2006. 40: p. 1003-1010.
105. King, M.R., et al., Oxidative stress promotes polarisation of human T cell differentiation toward a T helper 2 phenotype. J Immunol, 2006. 176: p. 2765-2772.
106. Dröge, W. and R. Breitkreutz, Glutathione and immune function. Proc Nutr Soc, 2000. 59: p. 595-600.
107. Furukawa, T., S.N. Meydani, and J.B. Blumberg, Reversal of age-associated decline in immune responsiveness by dietary glutathione supplementation in mice. Mech Ageing Dev, 1987. 38(2): p. 107-117.
108. Vaziri, N.D., Causal link between oxidative stress, inflammation, and hypertension. IJKD, 2008. 2: p. 1-10.
109. Goines, P.E. and P. Ashwood, Cytokine dysregulation in autism spectrum disorders (ASD): possible role of the environment. Neurotoxicol Teratol, 2013. 36: p. 67-81.
110. Esatbeyoglu, T., et al., Curcumin - From molecule to biological function. Angew Chem Int Ed, 2012. 51: p. 5308-5332.
111. Mittleman, B.B., et al., Cerebrospinal Fluid Cytokines in Pediatric Neuropsychiatric Disease. J Immunol, 1997. 159(6): p. 2994-2999.
112. Martino, M., et al., Immunomodulation mechanism of antidepressants: interactions between serotonin/norepinephrine balance and Th1/Th2 balance. Curr Neuropharmacol, 2012. 10: p. 97-123.
113. Marshall, P., Attention Deficit Disorder and Allergy: A Neurochemical Model of the Relation Between the Illnesses. Psychol Bull, 1989. 106(3): p. 434-446.
114. Buske-Kirschbaum, A., et al., Psychoendocrine and psychoneuroimmunological mechanisms in the comorbidity of atopic eczema and attention deficit/hyperactivity disorder. Psychoneuroendocrinology, 2013. 38: p. 12-23.

115. Sonar, S.A. and G. Lal, Differentiation and Transmigration of CD4 T Cells in Neuroinflammation and Autoimmunity. Front Immunol, 2017. 8(1695): p. 1-9.
116. Song, C., Z. Merali, and H. Anisman, Variations of nucleus accumbens dopamine and serotonin following systemic interleukin-1, interleukin-2 or interleukin-6 treatment. Neuroscience, 1999. 88(3): p. 823-826.
117. Miller, A.H., et al., Cytokine Targets in the Brain: Impact on Neurotransmitters and Neurocircuits. Depression Anxiety, 2013. 30: p. 297-306.
118. Guyon, A., et al., How cytokines can influence the brain: A role for chemokines? J Neuroimmunol, 2008. 198: p. 45-55.
119. Riazi, K., et al., Microglial activation and TNFalpha production mediate altered CNS excitability following peripheral inflammation. Proc Natl Acad Sci U S A, 2008. 105(44): p. 17151-17156.
120. Smith, S.E., et al., Maternal immune activation alters fetal brain development through interleukin-6. J Neurosci, 2007. 27: p. 10695-10702.
121. Meyer, U., J. Feldon, and B.K. Yee, A review of the fetal brain cytokine imbalance hypothesis of schizophrenia. Schizophr Bull, 2009. 35: p. 959-972.
122. Pineda, D.A., et al., Environmental influences that affect attention deficit/hyperactivity disorder - Study of a genetic isolate. Eur Child Adolesc Psychiatry, 2007. 16(5): p. 337-346.
123. Instanes, J.T., et al., Attention-Deficit/Hyperactivity Disorder in Offspring of Mothers With Inflammatory and Immune System Diseases. Biol Psychiatry, 2017. 81(5): p. 452-459.
124. Entringer, S., C. Buss, and P.D. Wadhwa, Prenatal stress and developmental programming of human health and disease risk: concepts and integration of empirical findings. Curr Opin Endocrinol Diabetes Obes, 2010. 17: p. 507-516.
125. LeMay, L.G., A.J. Vander, and M.J. Kluger, The effects of psychological stress on plasma interleukin-6 activity in rats. Physiol Behav, 1990. 47(5): p. 957-961.
126. Amiri, A. and A. Turner-Henson, The Roles of Formaldehyde Exposure and Oxidative Stress in Fetal Growth in the Second Trimester. J Obstet Gynecol Neonatal Nurs, 2017. 46(1): p. 51-62.
127. Fatemi, S.H., et al., Gene expression is altered in hippocampus following prenatal viral infection in mouse at E16. Eur Neuropsychopharmacol, 2009. 19(9): p. 648-653.
128. Corominas-Roso, M., et al., IL-6 and TNF-α in unmedicated adults with ADHD: Relationship to cortisol awakening response. Psychoneuroendocrinol, 2017. 79: p. 67-73.
129. Ma, L., et al., The function of hypothalamus-pituitary-adrenal axis in children with ADHD. Brain Res, 2011. 1368: p. 159-162.
130. Nopoulos, P., et al., Developmental Brain Anomalies in Children With Attention-Deficit Hyperactivity Disorder. J Child Neurol, 2000. 15: p. 102-108.
131. Baharnoori, M., W.G. Brake, and L.K. Srivastava, Prenatal immune challenge induces developmental changes in the morphology of pyramidal neurons of the prefrontal cortex and hippocampus I rats. Schizophr Res, 2009. 107(1): p. 99-109.
132. Hsiao, E.Y. and P.H. Patterson, Activation of the maternal immune system induces endocrine changes in the placenta via IL-6. Brain Behav Immun, 2011. 25(4): p. 604-615.
133. Feder, A., et al., Psychobiological mechanisms of resilience to stress., in Handbook of adult resilience., J.W. Reich, A.J. Zautra, and J.S. Hall, Editors. 2010, The Guilford Press: New York. p. 35-54.
134. Schlotz, W. and D.I. Phillips, Fetal origins of mental health: evidence and mechanisms. Brain Behav Immun, 2009. 23(7): p. 905-916.
135. Xiang, L. and G.D.J. Marshall, Immunomodulatory effect of in vitro stress hormones on Foxp3, Th1/Th2 cytokine and costimulatory molecule mRNA expression in human peripheral blood mononuclear cells. Neuroimmunomod, 2011. 18: p. 1-10.
136. Raison, C., L. Capuron, and A. Miller, Cytokines sing the blues: inflammation and the pathogenesis of depression. Trends Immunol, 2006. 27: p. 24-31.
137. Gabbay, V., et al., Immune system dysregulation in adolescent major depressive disorder. J Affect Disord, 2009. 115: p. 177-182.
138. Kim, Y.-K., et al., Th1, Th2 and Th3 cytokine alteration in schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry, 2004. 28: p. 1129-1134.
139. Isaksson, J., K.W. Nilsson, and F. Lindblad, Early psychosocial adversity and cortisol levels in children with attention-deficit/hyperactivity disorder. ECAP, 2013. 22(7): p. 425-432.
140. Işık, U., et al., Serum levels of cortisol, dehydroepiandrosterone, and oxytocin in children with attention-deficit/hyperactivity disorder combined presentation with and without comorbid conduct disorder. Psych Res, 2018. 261: p. 212-219.
141. Boris, M. and F. Mandel, Foods and additives are common causes of the attention deficit hyperactive disorder in children. Ann Allergy, 1994. 73: p. 462-467.
142. Carter, C.M., et al., Effects of a few food diet in attention deficit disorder. Arch Dis Child, 1993. 69: p. 564-568.

143. Egger, J., et al., Controlled trial of oligo-antigenic treatment in the hyperkinetic syndrome. Lancet, 1985. 1: p. 540-545.
144. Kaplan, B.J., et al., Dietary replacement in preschool-aged hyperactive boys. Pediatrics, 1989. 83: p. 7-17.
145. Schmidt, M.H., et al., Does oligoantigenic diet influence hyperactive/conduct-disordered children - a controlled trial. Eur Child Adolesc Psychiatry, 1997. 6(2): p. 88-95.
146. Breakey, J., The role of diet and behaviour in childhood. J Paediatr Child Health, 1997. 33: p. 190-194.
147. Bengmark, S., Gut microbiota, immune development and function. Pharmacol Res, 2013. 69: p. 87-113.
148. Jyonouchi, H., Non-IgE mediated food allergy. Inflamm Allergy Drug Targets, 2008. 7: p. 173-180.
149. Bradstreet, J.J., et al., Biomarker-Guided Interventions of Clinically Relevant Conditions Associated with Autism Spectrum Disorders and Attention Deficit Hyperactivity Disorder. Altern Med Rev, 2010. 15(1): p. 15-32.
150. Dupont, C., et al., Food-induced alterations of intestinal permeability in children with cow's milk-sensitive enteropathy and atopic dermatitis. JPGN, 1989. 8: p. 459-465.
151. Lupp, C., M.L. Robertson, and M.E. Wickham, Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host & Microbe, 2007. 2: p. 119-129.
152. Egger, J., A. Stolla, and L.M. McEwen, Controlled trial of hyposensitisation in children with food-induced hyperkinetic syndrome. Lancet, 1992. 339: p. 1150-1153.
153. Carding, S., et al., Dysbiosis of the gut microbiota in disease. Microb Ecol Health Dis, 2015. 26: p. 26191-16200.
154. Stevens, L.J., et al., Essential fatty acid metabolism in boys with attention-deficit hyperactivity disorder. Am J Clin Nutr, 1995. 62(4): p. 761-768.
155. Ryan, C.W., Association Between Colonic Symptoms and Inattention/Hyperactivity in British Children. Child Adolesc Mental Health, 2008. 13(4): p. 188-192.
156. McKeown, C., et al., Association of constipation and fecal incontinence with attention-deficit/hyperactivity disorder. Pediatrics, 2013. 132(5): p. e1210-e1215.
157. Stevens, L., et al., Dietary Sensitivities and ADHD Symptoms: Thirty-five Years of Research. Clin Pediatr, 2011. 50(4): p. 279-293.
158. De Angelis, M., et al., The food-gut human axis: the effects of diet on gut microbiota and metabolome. Curr Med Chem, 2017. [Epub ahead of print].
159. Guzman, J.R., V.S. Conlin, and C. Jobin, Diet, Microbiome, and the Intestinal Epithelium: An Essential Triumvirate? Biomed Res Int, 2013. 2013: p. 425146.
160. Sudo, N., et al., Dietary nucleic acids promote a shift in Th1/Th2 balance toward Th1-dominant immunity. Clin Exp Allergy, 2000. 30(7): p. 979-987.
161. Arns, M., et al., Geographic variation in the prevalence of attention-deficit/hyperactivity disorder: the sunny perspective. Biol Psychiatry, 2013. 74: p. 585-590.
162. Milovanovic, M., et al., Vitamin D receptor binds to the ε germline gene promoter and exhibits transrepressive activity. J Allergy Clin Immunol, 2010. 126: p. 1016-1023.
163. Bennett, S.J. and H.R. Griffiths, Chapter 2 Regulation of T-Cell Functions by Oxidative Stress., in Studies on Arthritis and Joint Disorders, Oxidative Stress in Applied Basic Research and Clinical Practice, M.J. Alcaraz and E. al, Editors. 2013, Springer Science+Business Media.
164. Martins, T., et al., New childhood and adult reference intervals for total IgE. J Allergy Clin Immunol, 2014. 133: p. 589-591.
165. Bernstein, I.L., et al., Allergy diagnostic testing: An updated practice parameter. Ann Allergy Asthma Immunol, 2008. 100(3 Suppl 3): p. S1-S148.
166. Verlaet, A.A.J., et al., Rationale for Dietary Antioxidant Treatment of ADHD. Nutrients, 2018. 10(4): p. 405-424.
167. Buttó, L.F. and D. Haller, Dysbiosis in intestinal inflammation: Cause or consequence. Int J Med Microbiol, 2016. 306(5): p. 302-309.
168. Wu, H.-J. and E. Wu, The role of gut microbiota in immune homeostasis and autoimmunity. Gut Microbes, 2012. 3(1): p. 4-14.
169. De Angelis, M., et al., Autism spectrum disorders and intestinal microbiota. Gut Microbes, 2015. 6(3): p. 207-213.
170. Mangiola, F., et al., Gut microbiota in autism and mood disorders. World J Gastroenterol, 2016. 22(1): p. 361-368.
171. Musso, G., R. Gambino, and M. Cassader, Obesity, Diabetes, and Gut Microbiota. The hygiene hypothesis expanded? Diabetes Care, 2010. 33(10): p. 2277-2284.
172. Hooper, L.V., D.R. Littman, and A.J. Macpherson, Interactions between the microbiota and the immune system. Science, 2012. 336(1268-1273).
173. Principi, N. and S. Esposito, Gut microbiota and central nervous system development. J Infect, 2016. 73(6): p. 536-546.
174. Jiang, H.Y., et al., Gut microbiota profiles in treatment-naïve children with attention deficit hyperactivity disorder. Behav Brain Res, 2018. 347(408-413).

175. Petra, A.I., et al., Gut-Microbiota-Brain Axis and Its Effect on Neuropsychiatric Disorders With Suspected Immune Dysregulation. Clin therap, 2015. 37(5): p. 984-995.
176. Sandgren, A.M. and R.J.M. Brummer, ADHD-originating in the gut? The emergence of a new explanatory model. Med Hypotheses, 2018. 120: p. 135-145.
177. Kim, B.J., et al., Environmental changes, microbiota, and allergic diseases. Allergy Asthma Immunol Res, 2014. 6(5): p. 389-400.
178. Banks, W.A. and M.A. Erickson, The blood-brain barrier and immune function and dysfunction. Neurobiol Dis, 2010. 37(1): p. 26-32.
179. Edden, R.A.E., et al., Reduced GABA concentration in attention-deficit/hyperactivity disorder. Arch Gen Psychiatry, 2012. 69(7): p. 750-753.
180. Mülek, M., et al., Profiling a gut microbiota-generated catechin metabolite's fate in human blood cells using a metabolomic approach. J Pharm Biomed Anal, 2015.
181. Wang, Y. and L.H. Kasper, The role of microbiome in central nervous system disorders. Brain Behav Immun, 2014. 38: p. 1-12.
182. Aarts, E., et al., Gut microbiome in ADHD and its relation to neural reward anticipation. PLoS One, 2017. 12(9): p. e0183509.
183. Grenham, S., et al., Brain-gut-microbe communication in health and disease. Front Physiol, 2011. 2: p. 1-15.
184. Brewer, M.S., Natural Antioxidants: Sources, Compounds, Mechanisms of Action, and Potential Applications. Comp Rev Food Sci Food Safety, 2011. 10(4): p. 221-247.
185. Packer, L., G. Rimbach, and F. Virgili, Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (Pinus maritima) bark, Pycnogenol. Free Rad Biol Med, 1999. 27(5/6): p. 704-724.
186. Kim, J., et al., Cocoa phytochemicals: Recent advances in molecular mechanisms on health. Crit Rev Food Sci Nutr, 2014. 54: p. 1458-1472.
187. Manach, C. and J.L. Donovan, Pharmacokinetics and metabolism of dietary flavonoids in humans. Free Rad Res, 2004. 38: p. 771-785.
188. Pae, M. and D. Wu, Immunomodulating effects of epigallocatechin-3-gallate from green tea: mechanisms and applications. Food Funct, 2013. 4(9): p. 1287-1303.
189. Pogačnik, L., et al., Potential for brain accessibility and analysis of stability of selected flavonoids in relation to neuroprotection in vitro. Brain Res, 2015. 1651: p. 17-26.
190. Trebatická, J. and Z. Ďuračková, Psychiatric Disorders and Polyphenols: Can They Be Helpful in Therapy? Ox Med Cell Longevity, 2015. 2015.
191. Tenenbaum, S., et al., An experimental comparison of Pycnogenol and methylphenidate in adults with attention-deficit/hyperactivity disorder (ADHD). J Attention Disorders, 2002. 6: p. 49-60.
192. Scasselati, C., et al., Biomarkers and Attention-Deficit/Hyperactivity Disorder: A Systematic Review and Meta-analyses. J Am Acad Child Adolesc Psychiatry, 2012. 51(10): p. 1003-1019.
193. Fitzpatrick, D.F., B. Bing, and P. Rohdewald, Endothelium-dependent vascular effects of Pycnogenol. J Cardiovasc Pharmacol, 1998. 32: p. 509-515.
194. Uhlenhut, K. and P. Högger, Facilitated cellular uptake and suppression of inducible nitric oxide synthase by a metabolite of maritime pine bark extract (Pycnogenol). Free Radic Biol Med, 2012. 53: p. 305-313.
195. Berryman, A.M., et al., Influence of treatment of diabetic rats with combinations of pycnogenol, beta-carotene, and alpha-lipoic acid on parameters of oxidative stress. J Biochem Mol Toxicol, 2004. 18(6): p. 345-352.
196. Parveen, K., et al., Protective effects of Pycnogenol on hyperglycemia-induced oxidative damage in the liver of type 2 diabetic rats. Chem Biol Interact, 2010. 186(2): p. 219-227.
197. Parveen, K., et al., Modulatory effects of Pycnogenol in a rat model of insulin-dependent diabetes mellitus: biochemical, histological, and immunohistochemical evidences. Protoplasma, 2013. 250(1): p. 347-360.
198. Viktorinova, A., et al., Natural polyphenols modify trace element status and improve clinical symptoms of attention-deficit hyperactivity disorder. Biomed Pharmacother, 2009. p. 1-6.
199. Belcaro, G., et al., Pycnogenol improvements in asthma management. Panminerva Med, 2011. 53(3): p. 57-64.
200. Belcaro, G., et al., Variations in C-reactive protein, plasma free radicals and fibrinogen values in patients with osteoarthritis treated with Pycnogenol. Redox Rep, 2008. 13(6): p. 271-276.
201. Lee, J., et al., Pycnogenol attenuates the symptoms of immune dysfunction through restoring a cellular antioxidant status in low micronutrient-induced immune deficient mice. Nutr Res Pract, 2014. 8(5): p. 533-538.
202. Bayeta, E. and B.H.S. Lau, Pycnogenol inhibits generation of inflammatory mediators in macrophages. Nutr Res, 2000. 20(2): p. 249-259.
203. Wong, C.P., et al., Induction of regulatory T cells by green tea polyphenol EGCG. Immunol Lett, 2011. 139(1-2): p. 7-13.
204. Kurlbaum, M., M. Mülek, and P. Högger, Facilitated uptake of a bioactive metabolite of maritime pine bark extract (pycnogenol) into human erythrocytes. PLoS ONE, 2013. 8(4).
205. Grimm, T., A. Schafer, and P. Hogger, Antioxidant activity and inhibition of matrix metalloproteinases by metabolites of maritime pine bark extract (pycnogenol). Free Radic Biol Med, 2004. 36(6): p. 811-22.

206. Scheff, S.W., M.A. Ansari, and K.N. Roberts, Neuroprotective effect of Pycnogenol following traumatic brain injury. Exp Neurol, 2013. 239(1): p. 183-191.
207. Ishrat, T., et al., Effects of Pycnogenol and vitamin E on cognitive deficits and oxidative damage induced by intracerebroventricular streptozotocin in rats. Behav Pharmacol, 2009. 20(7): p. 567-575.
208. Belcaro, G., et al., Pycnogenol improves cognitive function, attention, mental performance and specific professional skills in healthy professionals age 35-55. J Neurosurg Sci, 2014. 58(4): p. 239-248.
209. Ryan, J., et al., An examination of the effects of the antioxidant Pycnogenol on cognitive performance, serum lipid profile, endocrinological and oxidative stress biomarkers in an elderly population. J Psychopharmacol, 2008. 22(5): p. 553-562.
210. Belcaro, G., et al., The COFU3 Study. Improvement in cognitive function, attention, mental performance with Pycnogenol® in healthy subjects (55-70) with high oxidative stress. J Neurosurg Sci, 2015. 59(4): p. 437-446.
211. Hasegawa, N. and M. Mochizuki, Improved effect of Pycnogenol on impaired spatial memory function in partial androgen deficiency rat model. Phytother Res, 2009. 23(6): p. 840-843.
212. Cardona, F., et al., Benefits of polyphenols on gut microbiota and implications in human health. J Nutr Biochem, 2013. 24(8): p. 1415-1422.
213. Duda-Chodak, A., et al., Interaction of dietary compounds, especially polyphenols, with the intestinal microbiota: a review. Eur J Nutr, 2015. 54(3): p. 325-341.
214. Marín, L., et al., Bioavailability of dietary polyphenols and gut microbiota metabolism: antimicrobial properties. Biomed Res Int, 2015. 905215.
215. Belkaid, Y. and T. Hand, Role of the Microbiota in Immunity and Inflammation. Cell, 2014. 157: p. 121-141.
216. Hughes, D.A., Dietary antioxidants and human immune function. Nutrition Bulletin, 2000. 25: p. 35-41.
217. Dinan, T., et al., Collective unconscious: How gut microbes shape human behavior. J Psych Res, 2015. 63: p. 1-9.
218. Forman, H.J., Glutathione - from antioxidant to post-translational modifier. Arch Biochem Biophys, 2016. 595: p. 64-67.
219. Cox, D.J., et al., Rebound effects with long-acting amphetamine or methylphenidate stimulant medication preparations among adolescent male drivers with attention-deficit/hyperactivity disorder. J Child Adolesc Psychopharmacol, 2008. 18(1): p. 1-10.
220. Steer, C.R., Managing attention deficit/hyperactivity disorder: unmet needs and future directions. Arch Dis Child, 2005. 90(Suppl I): p. i19-i25.
221. Charach, A., A. Ickowicz, and R. Schachar, Stimulant treatment over five years: adherence, effectiveness, and adverse effects. J Am Acad Child Adolesc Psychiatry, 2004. 43: p. 559-567.
222. Yang, H.M., et al., A randomised, double-blind, placebo-controlled trial on the effect of Pycnogenol on the climacteric syndrome in peri-menopausal women. Acta Obstet Gynecol Scand, 2007. 86(8): p. 978-985.
223. Barkley, R.A., Taking charge of ADHD. 1995, New York: Guilford.
224. Pelsser, L.M.J. and J.K. Buitelaar, Favourable effect of a standard elimination diet on the behaviour of young children with attention deficit-hyperactivity disorder (ADHD), a pilot study. Ned Tijdschr Geneesk, 2002. 46(52): p. 2543-2547.
225. Pelsser, L.M.J., et al., Effects of food on physical and sleep complaints in children with ADHD: a randomised controlled pilot study. Eur J Pediatr, 2010. 169(9): p. 1129-1138.
226. Pelsser, L.M.J., et al., Effects of a restricted elimination diet on the behaviour of children with attention-deficit hyperactivity disorder (INCA study): a randomised controlled trial. Lancet, 2011. 377: p. 494-503.
227. Millichap, J.G. and M.M. Yee, The diet factor in attention-deficit/hyperactivity disorder. Pediatrics, 2012. 129: p. 330-337.
228. Sinn, N., Nutritional and dietary influences on attention deficit hyperactivity disorder. Nutr Rev, 2008. 66(10): p. 558-568.
229. Furlano, R., et al., Colonic CD8 and gamma delta T-cell infiltration with epithelial damage in children with autism. J Pediatr, 2001. 138(3): p. 366-372.
230. Sasayama, D., et al., Negative Correlation between Serum Cytokine Levels and Cognitive Abilities in Children with Autism Spectrum Disorder. J. Intell. 2017, 5, 19; , 2017. 5(19): p. 19-26.
231. Pelsser, L.M.J., J.K. Buitelaar, and H.F.J. Savelkoul, ADHD as a (non) allergic hypersensitivity disorder: a hypothesis. Pediatr Allergy Immunol, 2008. 20(2): p. 107-112.
232. Çelik, K., et al., Plasma catalase, glutathione-s-transferase and total antioxidant activity levels of children with attention deficit and hyperactivity disorder. Adv Biosci Biotechnol, 2013. 4: p. 183-187.
233. Ruchi, K., et al., Antioxidant activity in children with ADHD - A comparison in untreated and treated subjects with normal children. Int Med J Malaysia, 2011. 10(1): p. 31-35.
234. Sezen, H., et al., Increased oxidative stress in children with attention deficit hyperactivity disorder. Redox Rep., 2016. 21(6): p. 248-253.
235. Cheng, Z. and Y. Li, Reducing power: the measure of antioxidant activities of reductant compounds? Redox Rep, 2004. 9(4): p. 213-217.

236. Forman, H.J. and F. Ursini, What is an Antioxidant: Reductant, Nucleophile, Electrophile, Scavenger or Hormetic? Searching for Consensus between Chemistry and Biology. J Wine Res, 2011. 22(2): p. 139-141.
237. Ma, Q., Role of Nrf2 in Oxidative Stress and Toxicity. Annu Rev Pharmacol Toxicol, 2013. 53: p. 401-426.
238. Hermans, N., et al., Challenges and pitfalls in antioxidant research. Curr Med Chem, 2007. 14: p. 417-430.
239. Grotto, D., et al., Importance of the lipid peroxidation biomarkers and methodological aspects for malondialdehyde quantification. Quimica Nova, 2009. 32(1): p. 169-174.
240. Ryberg, H. and K. Caidahl, Chromatographic and mass spectrometric methods for quantitative determination of 3-nitrotyrosine in biological samples and their application to human samples. J Chromatogr B, 2007. 851: p. 160-171.
241. Souza, J.M., G. Peluffo, and R. Radi, Protein tyrosine nitration - Functional alteration or just a biomarker? Free Rad Bio Med, 2008. 45: p. 357-366.
242. Delatour, T., et al., Simultaneous determination of 3-nitrotyrosine and tyrosine in plasma proteins of rats and assessment of artifactual tyrosine nitration. J Chromatogr B, 2002. 779(189-199).
243. Papavasileiou, K.D., et al., A DFT study of the nitric oxide and tyrosyl radical interaction: A proposed radical mechanism. Chemphyschem, 2007. 8: p. 2595-2602.
244. Wintergerst, E.S., S. Maggini, and D.H. Hornig, Immune-Enhancing Role of Vitamin C and Zinc and Effect on Clinical Conditions. Ann Nutr Metab, 2006. 50: p. 85-94.
245. Padayatty, S.J. and M. Levin, New insights into the physiology and pharmacology of vitamin C. CMAJ, 2001. 164(3): p. 353-355.
246. Hamilton, I.M.J., et al., Interactions between vitamins C and E in human subjects. Br J Nutr, 2000. 84: p. 261-267.
247. Carr, A. and B. Frei, Does vitamin C act as a pro-oxidant under physiological conditions? Faseb, 1999. 13: p. 1007-1024.
248. Li, X. and F. A.A., Fast HPLC-ECD analysis of ascorbic acid, dehydroascorbic acid and uric acid. Anal Techn Biomed life Sci, 2009. 877(10): p. 853-856.
249. Kelly, B.L. and R.M. Locksley, Coordinate regulation of the IL-4, IL-13, and IL-5 cytokine cluster in Th2 clones revealed by allelic expression patterns. J Immunol, 2000. 165(6): p. 2982-2986.
250. Bao, K. and R.L. Reinhardt, The differential expression of IL-4 and IL-13 and its impact on type-2 Immunity. Cytokine, 2015. 75(1): p. 25-37.
251. Classen, A., J. Lloberas, and A. Celada, Macrophage activation: classical versus alternative. Methods Mol Biol, 2009. 531: p. 29-43.
252. Mallone, R., et al., Isolation and preservation of peripheral blood mononuclear cells for analysis of islet antigen-reactive T cell responses: position statement of the T-Cell Workshop Committee of the Immunology of Diabetes Society. Clin Exp Immunol, 2010. 163: p. 33-49.
253. Soto-Méndez, M.J., et al., Associations among Inflammatory Biomarkers in the Circulating, Plasmatic, Salivary and Intraluminal Anatomical Compartments in Apparently Healthy Preschool Children from the Western Highlands of Guatemala. PLoS One, 2015. 10(6): p. e0129158.
254. Ogdie, M.N., et al., A Genome wide Scan for Attention-Deficit/Hyperactivity Disorder in an Extended Sample: Suggestive Linkage on 17p11. Am J Hum Genet, 2003. 72: p. 1268-1279.
255. Aureli, A., et al., Investigation on the possible relationship existing between the HLA-DR gene and attention deficit hyperactivity disorder and/or mental retardation. Int J Immunopathol Pharma, 2008. 21: p. 985-991.
256. Baars, E.W., Evidence-based curative health promotion: a systems biology-orientated treatment of seasonal allergic rhinitis with Citrus/Cydonia comp., PhD thesis at Wageningen University. 2011: Wageningen.
257. Furman, L., What is Attention-Deficit Hyperactivity Disorder (ADHD)? J Child Neurol, 2005. 20(12): p. 994-1002.
258. Conners, C.K., et al., The revised Conners' Parent Rating Scale (CPRS-R): factor structure, reliability, and criterion validity. J Abnorm Child Psychol, 1998. 26(4): p. 257-268.
259. Prodia, Diagnostische fiche CRS-R/CRS-3. 2014.
260. Breynaert, A., et al., Development and Validation of an in vitro Experimental GastroIntestinal Dialysis Model with Colon Phase to Study the Availability and Colonic Metabolisation of Polyphenolic Compounds. Planta Medica, 2015. 81(12-13): p. 1075-1083.
261. Hubatsch, I., E.G.E. Ragnarsson, and P. Artursson, Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. Nature Protocols, 2007. 2(9): p. 2111-2119.
262. Meunier, V., et al., The human intestinal epithelial cell line Caco-2; pharmacological and pharmacokinetic applications. Cell Biol Toxicol, 1995. 11(3): p. 187-194.
263. Yee, S., In Vitro Permeability Across Caco-2 Cells (Colonic) Can Predict In Vivo (Small Intestinal) Absorption in Man-Fact or Myth. Pharm Res, 1997. 14(6): p. 763-766.
264. Ghazalpour, A., et al., Comparative analysis of proteome and transcriptome variation in mouse. PLoS Genetics, 2011. 7(6): p. 1-17.
265. Anderson, L. and J. Seilhamer, A comparison of selected mRNA and protein abundances in human liver. Electrophoresis, 1997. 18: p. 533-537.

266. Sprague, A.H. and R.A. Khalil, . Biochemical, Inflammatory cytokines in vascular dysfunction and vascular disease. Biochem Pharmacol, 2009. 78: p. 539-552.
267. Skrzypski, M., Quantitative reverse transcriptase real-time polymerase chain reaction (qRT-PCR) in translational oncology: Lung cancer perspective. Lung Cancer, 2008. 59: p. 147-154.
268. Bito, T., et al., Pine bark extract Pycnogenol downregulates IFN-g induced adhesion of T-cells to human keratinocytes by inhibiting inducibla ICAM-1 expression. Free Rad Biol Med, 2000. 28(2): p. 219-227.
269. Sloan, C.D.K., et al., Analytical and Biological Methods for Probing the Blood-Brain Barrier. Annu Rev Anal Chem, 2012. 5: p. 505-531.
270. Cortese, S., et al., Toward systems neuroscience of ADHD: a meta-analysis of 55 fMRI studies. Am J Psychiatry, 2012. 169: p. 1038-1055.
271. Kaplan, B.J., et al., Vitamins, Minerals, and Mood. Psychol Bull, 2007. 133(5): p. 747-760.
272. Pärtty, A., et al., A possible link between early probiotic intervention and the risk of neuropsychiatric disorders later in childhood: a randomized trial. Pediatr Res, 2015. 77(6): p. 823-828.
273. Farmer, K.C., Methods for measuring and monitoring medication regimen adherence in clinical trials and clinical practice. Clin therap, 1999. 21: p. 1074-1090.

SUMMARY

Everything is hard before it is easy. – Johann Wolfgang von Goethe

Attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterised by developmentally inappropriate levels of impulsivity, hyperactivity and/or inattention. **Chapter 1** starts with an overview of ADHD problemacy. ADHD is a complex and multifactorial disorder, influenced by genetic, biochemical, psychological and environmental factors, but its aetiology is not completely understood. Various studies report increased levels of oxidative damage markers, decreased activity of antioxidant enzymes and an altered gut microbiome in ADHD. Moreover, ADHD has a high comorbidity with T cell-mediated disorders (affecting different subsets including T_H1, T_H2 and T_{Reg}) and several genes linked to ADHD have immune functions. However, results are not completely consistent and research on these topics overall had its limitations, e.g. regarding biomarkers analysed. Nevertheless, both unresolved oxidative stress and immune dysbalance may contribute to the clinical pathology of ADHD, for instance by injuring neuronal cells. Moreover, methylphenidate (MPH), the first-choice medication for ADHD, is linked to adverse effects and possible publication bias in reported efficacy. Due to their antioxidant capacities as well as immunoregulatory effects, dietary polyphenols therefore appear appropriate in ADHD therapy. One example is Pycnogenol®, a patented herbal extract from the outer bark of French maritime pine (*Pinus pinaster Ait. subsp. atlantica*), standardised to contain 70 ± 5% (w/w) procyanidins. Pycnogenol® supplementation significantly improved teacher hyperactivity and inattention ratings in paediatric ADHD, but this study had various limitations.

The first objective of this thesis was to get a better understanding of oxidative stress and immune dysbalance in ADHD. In **Chapter 2**, 57 untreated paediatric ADHD patients and 69 controls were therefore compared regarding a broad diversity of oxidative stress and immune biomarkers. After Bonferroni correction for multiple testing, only significantly higher reduced glutathione (GSH) levels in erythrocytes were found in ADHD patients (and nominally significant for plasma retinyl palmitate). No differences were observed for the plasma lipid soluble antioxidants retinol, α-tocopherol, γ-tocopherol, β-carotene and co-enzyme Q10, nor for the oxidative damage markers urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) and plasma malondialdehyde (MDA), or for the plasma cytokines interleukin (IL)-1β, IL-6, IL-8, IL-10, tumour necrosis factor (TNF) and interferon (IFN)-γ or the antibodies IgE, IgG or IgM as markers of immunity. Strikingly, a trend for higher IgE levels was supported by a trend for more diagnosed allergies in patients. Dietary habits did not appear to cause the observed differences. As antioxidant levels did not differ or were even higher in patients than in controls, this could be a compensation mechanism for increased oxidative stress. These results thus point at the potential involvement of slight oxidative stress and immune disturbance in ADHD. However, due to the nature of this study, it is unknown whether redox and immune dysbalance play a causative role.

Standardisation, characterisation and quality control of nutritional supplements is essential for research with complex herbal supplements to be credible and to be able to compare results of various studies. Chapter 3 therefore describes the optimisation and validation of United States Pharmacopeial (USP) analytical methods for Pycnogenol® (i.e., a spectrophotometric method for total procyanidin content and a fingerprint chromatographic method) as well as quality control of Pycnogenol®. For fingerprint HPLC, Pycnogenol® was dissolved in 20% methanol (instead of 100% as described in the USP), optimising resulting chromographic peaks. Both methods were shown to have an acceptable repeatability and intermediate precision, linearity and accuracy. Analysis of the available Pycnogenol® extract revealed that it contained 65-75% procyanidins, as required, and 1.3% catechin, 1.4% taxifolin, 0.3% caffeic acid and 0.3% ferulic acid.

Research indicates that Pycnogenol® exerts its immune modulating effects at least in part through the Toll-like receptor (TLR)4-NF-κB pathway. However, the exact mechanism by which Pycnogenol® exerts its effects via TLR4 and whether other TLRs are involved remained to be elucidated. In Chapter 4, the effect of Pycnogenol® and its primary monomer catechin on membrane TLR activity and pro-inflammatory cytokine secretion was examined using Human Embryonic Kidney (HEK) cells and the human monocytic leukaemia cell line THP-1. It was shown that non-metabolised Pycnogenol® acts as agonist of TLR1/2 and TLR2/6 and as partial agonist of TLR5, stimulating activation of NF-κB and pro-inflammatory cytokine secretion. Moreover, formation of lipopolysaccharide (LPS)-Pycnogenol® complexes was shown to be required to stimulate TLR4 to the NF-κB-dependent release of pro-inflammatory cytokines. *In vitro* metabolism of Pycnogenol® by the gastrointestinal dialysis model with colon phase (GIDM-colon) however caused immuno-suppressive potential against TLR1/2 and TLR2/6 by the retentate fraction and potential to induce secretion of anti-inflammatory IL-10 by the dialysate fraction. For catechin, no clear effects could be observed on TLR activation and pro-inflammatory cytokine production. This study stresses the importance of the effect of metabolism on the biological activity of Pycnogenol®.

Though some previously reported results are promising, further controlled studies to investigate effects of Pycnogenol® on ADHD are required. In addition, though Pycnogenol® has been shown to have immune modulating and antioxidant properties, while at least one of its metabolites was demonstrated to be able to cross the blood-brain barrier, exact mechanisms by which it improves brain function and reduces ADHD symptoms remain to be elucidated. Chapter 5 therefore describes the setup of a randomised double-blind placebo and active product controlled 10-week clinical trial with three parallel treatment arms in paediatric ADHD patients, including the design and protocol,

preparation of all trial-related files, registration and ethical approval requirements and safety monitoring. The ultimate goal of this trial is to investigate the efficacy, mechanism of action and value of Pycnogenol® in ADHD therapy as compared to MPH treatment and placebo, including effects on immunity, antioxidant levels, oxidative damage and comorbid psychiatric and physical complaints, and to evaluate the tolerability of Pycnogenol® compared to MPH. Final results of this trial, which is currently ongoing, will only be available after publication of this PhD thesis.

Finally, in **Chapter 6**, the methodology and results of the previous chapters are discussed in relation to the existing literature. This discussion elaborates on potential mechanisms by which redox and immune imbalance can be involved in ADHD aetiology, potential mechanisms and efficacy of polyphenols in ADHD therapy and perspectives for future research. Overall, this thesis and the present literature show that nutritional factors, oxidative stress and immune dysbalance play a potential role in ADHD aetiology. Decisive evidence on the exact contributions of redox and immune imbalance in ADHD is yet to be reported, though multiple effects of oxidative stress and low-grade inflammation can be related to ADHD symptoms. Nutritional approaches like polyphenol-rich foods or extracts can therefore have a positive impact on ADHD-related behaviour and possibly also comorbid complaints due to their antioxidant and immune modulating activity.

This thesis overall increases insight in the aetiology of ADHD. The ongoing randomised double-blind placebo and active product controlled clinical trial will provide further understanding of the pathogenesis and treatment options of ADHD.

SAMENVATTING

It always seems impossible until it's done. – Nelson Mandela

Aandachtstekort hyperactiviteitsstoornis (ADHD) is een neurologische ontwikkelingsstoornis gekenmerkt door een verhoogd niveau van impulsiviteit, hyperactiviteit en/of aandachtstekort. **Hoofdstuk 1** begint met een overzicht van de ADHD-problematiek. ADHD is een complexe en multifactoriële aandoening, beïnvloed door genetische, biochemische, psychologische en omgevingsfactoren, maar de onderliggende etiologie is niet helemaal duidelijk. Verschillende studies rapporteren een verhoogd gehalte aan oxidatieve schade merkers, een verlaagde activiteit van antioxidatieve enzymen en veranderde intestinale microbiota bij ADHD. Bovendien heeft ADHD een hoge comorbiditeit met T cel-gemedieerde ziekten (gekoppeld aan T_H1, T_H2 en T_{Reg} cellen) en hebben verschillende genen die gelinkt worden aan ADHD specifieke immuunfuncties. Resultaten zijn echter niet helemaal consistent en onderzoek rond deze thema's heeft algemeen tekortkomingen, bv. betreffende de geanalyseerde biomerkers. Toch kunnen zowel oxidatieve stress als een verstoorde immuunbalans bijdragen aan de klinische pathologie van ADHD, bijvoorbeeld door schade te veroorzaken aan neuronale cellen. Bovendien wordt methylfenidaat (MPH), de meest gebruikte medicatie bij ADHD, gelinkt aan bijwerkingen en publicatiebias qua gerapporteerde efficaciteit. Door hun antioxidatieve en immuunregulerende eigenschappen lijken polyfenolen geschikt voor de behandeling van ADHD. Een voorbeeld is Pycnogenol®, een gepatenteerd extract van de schors van Franse maritieme pijnboom (*Pinus pinaster Ait. subsp. atlantica*), gestandaardiseerd om 70 ± 5% (w/w) procyanidinen te bevatten. Pycnogenol® verbeterde significant hyperactiviteit en aandachtstekort beoordeeld door leerkrachten in pediatrische ADHD-patiënten, maar deze studie had verschillende beperkingen.

Het eerste doel van deze thesis was om een beter inzicht te krijgen in de rol van oxidatieve stress en immuunverstoring bij ADHD. In **Hoofdstuk 2** werden daarom 57 onbehandelde ADHD-patiënten en 69 controles vergeleken m.b.t. een brede waaier aan biomerkers van oxidatieve stress en immuniteit. Na Bonferroni correctie voor meervoudige testing werd enkel een significant hogere concentratie gereduceerd glutathion (GSH) in rode bloedcellen gevonden bij ADHD-patiënten (en nominaal significant voor plasma retinylpalmitaat). Er werden geen verschillen gevonden voor de vetoplosbare antioxidanten retinol, α-tocoferol, γ-tocoferol, β-caroteen en co-enzym Q10 in plasma, noch voor de merkers van oxidatieve schade 8-hydroxy-2'-deoxyguanosine (8-OHdG) in urine en malondialdehyde (MDA) in plasma, of voor de cytokines interleukine (IL)-1β, IL-5, IL-6, IL-8, IL-10, tumor necrose factor (TNF) en interferon (IFN)-γ of de antilichamen IgE, IgG en IgM in plasma als merkers van immuniteit. Opvallend was een trend voor hogere IgE waarden in combinatie met een trend voor meer gediagnosticeerde allergieën bij patiënten. Voedingsgewoonten leken niet aan de basis te liggen van de gevonden verschillen. Antioxidant waarden waren dus niet verschillend of zelfs hoger bij patiënten

dan bij controles. Dit kan wijzen op een compensatiemechanisme bij verhoogde oxidatieve stress. Deze resultaten wijzen daarom op de mogelijke betrokkenheid van geringe oxidatieve stress en immuunverstoringen bij ADHD. Door de aard van deze studie is het echter onduidelijk of een verstoorde redox- en immuunbalans een oorzakelijke rol spelen.

Standaardisatie, karakterisatie en kwaliteitscontrole van voedingssupplementen is essentieel om betrouwbaar onderzoek te voeren met complexe supplementen en om resultaten van verschillende studies te kunnen vergelijken. **Hoofdstuk 3** beschrijft de optimalisatie en validatie van de analytische methoden voor Pycnogenol® uit de Amerikaanse farmacopee (een spectrofotometrische methode voor de bepaling van totaal procyanidinegehalte en een *fingerprint* chromatografische methode) alsook de kwaliteitscontrole van Pycnogenol®. Voor de *fingerprint* HPLC-methode werd Pycnogenol® opgelost in 20% methanol (i.p.v. 100% zoals beschreven in de farmacopee) om de chromatografische pieken te optimaliseren. Beide methoden hadden een aanvaardbare herhaalbaarheid en intermediaire precisie, lineariteit en accuraatheid. Analyse van het beschikbare Pycnogenol® extract toonde aan dat het 65-75% procyanidinen bevat, zoals vereist, en 1.3% catechine, 1.4% taxifoline, 0.3% koffiezuur en 0.3% ferulazuur.

Onderzoek geeft aan dat Pycnogenol® zijn immuunmodulerende effecten ten minste deels uitoefent via het Toll-like receptor (TLR)4-NF-κB mechanisme. Het exacte mechanisme via TLR4, alsook of andere TLRs betrokken zijn, was nog niet opgehelderd. In **Hoofdstuk 4** werd het effect van Pycnogenol® en zijn belangrijkste monomeer, catechine, op membraan TLR-activiteit en pro-inflammatoire cytokine secretie onderzocht m.b.v. de Humane Embryonale Nier (HEK) en de humane monocyten leukemie (THP-1) cellijnen. Er werd aangetoond dat niet-gemetaboliseerd Pycnogenol® extract optreedt als agonist voor TLR1/2 en TLR2/6 en als partieel agonist voor TLR5, en dat het daarbij de activatie van NF-κB en secretie van pro-inflammatoire cytokines stimuleert. Bovendien werd aangetoond dat lipopolysaccharide (LPS)-Pycnogenol® complexvorming noodzakelijk is voor stimulatie van TLR4 voor NF-κB-afhankelijke secretie van pro-inflammatoire cytokinen. *In vitro* metabolisatie van Pycnogenol® door het gastro-intestinaal dialysemodel met colonfase (GIDM-colon) zorgde echter voor immuun-suppressieve effecten van de retentaatfractie t.o.v. TLR1/2 en TLR2/6 en voor stimulerende effecten van de dialysaatfractie op secretie van het anti-inflammatoire cytokine IL-10. Voor catechine werden geen duidelijke effecten gevonden op TLR-activatie of productie van pro-inflammatoire cytokinen. Deze studie benadrukt het belang van het effect van metabolisatie op de biologische activiteit van Pycnogenol®.

Hoewel bepaalde eerder gerapporteerde resultaten veelbelovend zijn, is verder gecontroleerd onderzoek naar effecten van Pycnogenol® op ADHD nodig. Hoewel immuunmodulerende en antioxidatieve eigenschappen van Pycnogenol® aangetoond zijn en ten minste één van de metabolieten de bloed-hersen barrière kan passeren, dienen exacte mechanismen waardoor Pycnogenol® de hersenfunctie kan verbeteren en ADHD-symptomen kan verminderen nog te worden opgehelderd. **Hoofdstuk 5** beschrijft daarom de opzet van een gerandomiseerde dubbelblinde, placebo en actief product gecontroleerde studie gedurende 10 weken met drie parallelle behandelingsarmen bij pediatrische ADHD-patiënten, inclusief het ontwerp en protocol, voorbereiding van alle studiegerelateerde documenten, vereisten voor registratie en ethische goedkeuring en opvolging van veiligheid. Het uiteindelijke doel van deze studie is om de efficaciteit, het werkingsmechanisme en de waarde van Pycnogenol® in de behandeling van ADHD te onderzoeken in vergelijking met MPH en placebo, inclusief effecten op immuniteit, antioxidantwaarden, oxidatieve schade en comorbide psychiatrische en fysieke klachten, en om de verdraagbaarheid van Pycnogenol® in vergelijking met MPH te evalueren. Finale resultaten van deze studie, die momenteel loopt, zullen pas beschikbaar zijn na publicatie van deze doctoraatsthesis.

Tenslotte worden in **Hoofdstuk 6** de methodologie en resultaten van de voorgaande hoofdstukken bediscussieerd in relatie tot de bestaande literatuur. Deze discussie weidt uit over mogelijke mechanismen waardoor redox- en immuunverstoringen kunnen betrokken zijn bij de etiologie van ADHD, mogelijke mechanismen en efficaciteit van therapie gebaseerd op polyfenolen in de behandeling van ADHD en perspectieven voor toekomstig onderzoek. Globaal tonen deze thesis en de huidige literatuur aan dat nutritionele factoren, oxidatieve stress en immuunverstoring een mogelijke rol spelen in de etiologie van ADHD. Doorslaggevend bewijs rond de exacte bijdrage van een redox- en immuunverstoring bij ADHD moet nog geleverd worden, hoewel verscheidene effecten van oxidatieve stress en laaggradige ontsteking kunnen gerelateerd worden aan ADHD-symptomen. Nutritionele benaderingen zoals polyfenolrijke voedingsmiddelen of extracten kunnen daarom een positief effect hebben op ADHD-gerelateerd gedrag en mogelijk ook op comorbide klachten door hun antioxidatieve en immuunmodulerende activiteit.

Deze thesis verhoogt de kennis rond de etiologie van ADHD. De lopende gerandomiseerde dubbel-blinde, placebo en actief product gecontroleerde klinische studie zal verder bijdragen aan het inzicht in de pathogenese en behandelingsopties van ADHD.

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'Cause if I fall, you'll fall. And if I rise, we'll rise together. Don't worry about me. – Frances

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X

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SCIENTIFIC CURRICULUM VITAE

If you never try, you'll never know. – Coldplay

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Education

2013-2019 PhD in Pharmaceutical Sciences *Antwerp University (BE)*
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2011-2012 Preparation of application for PhD scholarship

2009-2011 MSc Nutrition and Health *Wageningen University (NL)*
Specialisation: Nutrition in Health and Disease
Thesis: Evaluation of the expansion of Phadiatop® Infant.
Thesis: Menopause and diet.
Internship: Physical activity in adults with controlled and uncontrolled asthma. (Porto University)

2006-2009 BSc Nutrition and Health *Wageningen University (NL)*
Awarded ‘cum laude’; minor Nutritional Aspects of Immunology
Thesis: Immunological mechanisms and dietary immunomodulation in ADHD.

2000-2006 Secondary education: Latin – Mathematics *OLVP Bornem (BE)*

Publications in peer-reviewed journals

Verlaet A, van der Bolt N, Meijer B, Breynaert A, Naessens T, Konstanti P, Smidt H, Hermans N, Savelkoul HFJ, Teodorowicz G. Toll-Like Receptor-dependent immunomodulatory activity of Pycnogenol®. Nutrients (2019), 11(2).

Verlaet AAJ, Breynaert A, Ceulemans B, De Bruyne T, Fransen E, Pieters L, Savelkoul HFJ, Hermans N. Oxidative stress and immune aberrancies in attention-deficit/hyperactivity disorder (ADHD): a case-control comparison. Accepted for publication in Eur Child Adolesc Psychiatry (2018).

Verlaet AAJ, Maasakkers CM, Hermans N, Savelkoul HFJ. Rationale for Dietary Antioxidant Treatment of ADHD. Nutrients (2018), 10(4).

Hermans N, Van der Auwera A, Breynaert A, Verlaet A, De Bruyne T, Van Gaal L, Pieters L, Verhoeven V. A red yeast rice-olive extract supplement reduces biomarkers of oxidative stress, OxLDL and Lp-PLA2, in subjects with metabolic syndrome: a randomised, double-blind, placebo-controlled trial. Trials (2017), 18(1).

Verlaet AAJ, Ceulemans B, Verhelst H, Van West D, De Bruyne T, Pieters L, Savelkoul HFJ, Hermans N. Effect of Pycnogenol® on attention-deficit hyperactivity disorder (ADHD): study protocol for a randomised controlled trial. Trials (2017), 18(1).

De Bie J, Langbeen A, Verlaet AAJ, Florizoone F, Immig I, Hermans N, Fransen E, Bols PEJ, Leroy JLMR. The effect of a negative energy balance status on β-carotene availability in serum and follicular fluid of nonlactating dairy cows. Journal of Dairy Science (2016), 99(7).

Van Hoeck V, Rizos D, Gutierrez-Adan A, Pintelon I, Jorssen E, Dufort I, Sirard MA, Verlaet A, Hermans N, Bols PEJ, Leroy JLMR. Interaction between differential gene expression profile and phenotype in bovine blastocysts originating from oocytes exposed to elevated non-esterified fatty acid concentrations. Reproduction, Fertility and Development (2015), 27(2).

Verlaet AAJ, Noriega DB, Hermans N, Savelkoul HFJ. Nutrition, immunological mechanisms and dietary immunomodulation in ADHD. Eur Child Adolesc Psychiatry (2014), 23(7).

Magielse J, Verlaet A, Breynaert A, Manuel Y Keenoy B, Apers S, Pieters L, Hermans N. Investigation of the *in vivo* antioxidative activity of Cynara scolymus (artichoke) leaf in the streptozotocin-induced diabetic rat. Mol Nutr Food Res (2014), 58(1).

Verlaet A, Moreira A, Sá-Sousa A, Barros R, Santos R, Moreira P, Fonseca J. Physical activity in adults with controlled and uncontrolled asthma as compared to healthy adults: a cross-sectional study. Clinical and Translational Allergy (2013), 3(1).

Verlaet A, Jansen A, Plaisier A, van Huystee B, Vissers YM, Savelkoul HFJ en Ruinemans-Koerts J. Diagnostische waarde van de IgE-screeningstest (Phadiatop Infant) bij kinderen tot 4 jaar. Nederlands Tijschrift voor Allergie & Astma (2012), 12(4).

Contributions at scientific conferences and symposia

2019 Oral presentation "Oxidatieve stress en immuniteit bij aandachtstekort hyperactiviteitsstoornis (ADHD): potentieel voor een polyfenolrijk extract?"
Nederlandse Vereniging voor Geneeskruidenonderzoek (NVGO) symposium *(Garderen, NL)*

Oral presentation "Oxidative stress and immunity in ADHD: a case-control comparison"
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2012 Poster presentation "Physical activity in adults with controlled and uncontrolled asthma as compared to healthy adults: a cross-sectional study"
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