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Exploring the therapeutic potential of French Maritime Pine Bark Extract in Attention-Deficit Hyperactivity Disorder and Arterial Stiffness

Onderzoek naar het therapeutische potentieel van de Franse Maritieme Pijnboomschors bij Aandachtstekortstoornis met Hyperactiviteit en Arteriële Stijfheid

Proefschrift voorgelegd tot het behalen van de graad van Doctor in de Farmaceutische Wetenschappen aan de Universiteit Antwerpen, te verdedigen door

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“I never lose. Either I win or learn”

Nelson Mandela

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List of abbreviations

8-OHdG	8-hydroxy-2'-deoxyguanosine
8-oxoG	8-hydroxyguanine, 8-oxo-7,8-dihydroguanine
5-HT	Serotonin receptor
AA	Arachidonic Acid
AAS	Atomic Absorption Spectroscopy
ACE	Angiotensin-Converting Enzyme
ACh	Acetylcholine
ADD	Attention Deficit Disorder
ADHD	Attention Deficit Hyperactivity Disorder
ADHD-RS	ADHD Rating Scale
ADME	Absorption, Distribution, Metabolism and Excretion
ADRA	Alpha-2A-Adregeneric Receptor
AE	Adverse Event
AGE	Advanced Glycation End product
AML	General Medical Laboratory
ANOVA	Analysis of Variance
ANSM	French Health Products Safety Agency
APA	American Psychological Association
AS	Arterial Stiffness
ASV	Amplicon Sequence Variant
BBB	Blood-Brain Barrier

BC	Buffy Coat
BHT	Butylhydroxytoluene
BL	Blank
BLAST	Basic Local Alignment Search Tool
BMI	Body Mass Index
BP	Blood Pressure
CAT	Catalase
CBG	Cytosolic β-glucosidase
CD	Conduct Disorder
CDT	Cyclohexadienyl Dehydratase
CFU	Colony Forming Unit
CI	Confidence Interval
CNS	Central Nervous System
CNV	Copy Number Variant
CoQ10	Co-enzyme Q10
COX	Cyclooxygenase
CRP	C-reactive protein
CVD	Cardiovascular Disease
Da	Dalton
DAD	Diode Array Detector
DAT	Dopamine Active Transporter
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco PBS

DRD	Dopamine Receptor D
DSM	Diagnostic and Statistical Manual of Mental Disorders
EC	Ethical Committee
ECD	Electrochemical Detection
ECGM	Endothelial Cell Growth Medium
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
ENA	European Nucleotide Archive
eNOS	Endothelial Nitric Oxide Synthase
ET-1	Endothelin-1
F/B	Firmicutes/Bacteroidetes
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
FFQ	Food Frequency Questionnaire
FMD	Flow-mediated dilation
GABA	Gamma Aminobutyric Acid
GCP	Good Clinical Practice
GDPR	General Data Protection Regulation
GI	Gastrointestinal
GIDM-Colon	Gastrointestinal Dialysis Model with Colon phase
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
HAoEC	Human Aortic Endothelial Cell
HAoSMC	Human Aortic Smooth Muscle Cell
HI	Hyperactivity
HPLC	High-Pressure Liquid Chromatography
HRF	Heterocyclic ring fission
HPA	Hypothalamus-Pituitary-Adrenal
HRP	Horseradish Peroxidase
HTR	5-hydroxytryptamine
I	Impulsivity
IA	Inattention
I.p.	Intraperitoneally
ICAM	Intracellular Adhesion Molecule
IFN	Interferon
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IQR	Interquartile range
ISAPP	International Scientific Association for Probiotics and Prebiotics
LAF	Laminar airflow
LC	Liquid Chromatography

LMM	Linear Mixed Model
LOD	Loss On Drying
LOX	Lipoxygenase
LPH	Lactase Phloridizin Hydrolase
LPS	Lipopolysaccharide
M1	δ-(3,4-dihydroxy-phenyl)-γ-valerolactone
M2	δ-(3- methoxy-4-hydroxy-phenyl)-γ-valerolactone
MAO	Monoamine Oxidase
MDA	Malondialdehyde
MGBA	Microbiota-Gut-Brain-Axis
MMP	Matrix Metalloprotease
MPH	Methylphenidate hydrochloride
MRI	Magnetic Resonance Imaging
MS	Mass Spectrophotometry
MTT	3-(4, 5-dimethylthiozol-2-yl)-2, 5-diphenyltetrazolium bromide
NC	Negative Control
NCBI	National Center for Biotechnology Information
NCD	Non-Communicable Disease
NET	Noradrenaline Transporter
NF-κB	Nucelar factor kappa-light-chain-enhancer of activated B cells
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOAEL	No-Observed-Adverse-Effect-Level
NOS	Nitric Oxide Synthase

NPY	Neuropeptide Y
Nrf2	Nuclear factor erythroid 2–related factor 2
NSAID	Non-Steroidal Anti-Inflammatory Drug
ODD	Obsessive-Compulsive Disorder
ODS	Octadecyl silane
OSA	Octane Sulphonic Acid
OSI	Oxidative Stress Index
P/S	Penicillin/Streptomycin
PAC	Proanthocyanidin
PBMC	Peripheral Blood Mononuclear Cell
PBE	French Maritime Pine Bark Extract
PCoA	Principal Coordinate Analysis
PCQ	Physical Complaints Questionnaire
PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography
PI3K	Phosphoinositide-3-kinase
PON	Paraoxonase
PPI	Proton pump inhibitor
PUFA	Polyunsaturated fatty acid
PYC	Pycnogenol®
p38-MAPK	p38 mitogen-activated protein kinase
QC	Quality Control
QM	Quinone methide reaction
qPCR	Quantitative PCR

R	Correlation coefficient
RBC	Red blood cell
RDA	Retro-Diels-Alder reaction
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen species
RP	Reversed phase
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT	Retention time
RT-qPCR	Real-time qPCR
SAE	Serious AE
SCFA	Short-Chain-Fatty-Acid
SD	Standard Deviation
SEQ	Social Emotional Questionnaire
SOD	Superoxide Dismutase
SMCGM	Smooth Muscle Cell Growth Medium
SNAP-25	Synaptosomal-associated protein of 25 kDA
SNP	Single Nucleotide Polymorphism
SPSS	Statistical Package for the Social Sciences
T_H	T-Helper
TLR	Toll-like Receptor
TNF-α	Tumor Necrosis Factor alpha
TOF	Time Of Flight

TOS	Total Oxidant Status
TPH	Tryptophan Hydroxylase
TPS	Total Protein Stain
USP	US Pharmacopeia
UV	Ultraviolet
UZ Ghent	Ghent University Hospital
UZA	Antwerp University Hospital
VCAM	Vascular Cell Adhesion Molecule
VMAT	Vesicular Monoamine Transporter
WHO	World Health Organisation
XO	Xanthine Oxidase
ZNA	Hospital Network Antwerp

Chapter 1 **General introduction and outline of this thesis**

1.1 Non-communicable diseases

Non-communicable diseases (NCDs) are responsible for 80% of the disease burden in the European Union (EU) countries and are considered to be a major public health concern in both developing and developed regions worldwide (Fraga et al., 2019). In Belgium, NCDs contribute to nearly 90% of all deaths over the decade to 2019 and 40% of this number could be attributed to modifiable risk factors (WHO, 2022). According to the World Health Organisation (WHO), NCDs are disorders not directly transmissible from one person to another, and are usually of long duration as well as slow in progression (WHO, 2023). In fact, NCDs are recognised as "the dominant public health challenge of the 21st century" and a "public health emergency in slow motion" (Jagannathan et al., 2019). The top four NCDs causing the highest number of deaths are cardiovascular diseases (CVDs; 17.9 million deaths annually), followed by cancer (9 million), respiratory diseases (3.9 million) and diabetes (1.6 million). Furthermore, the term NCDs has been extended to cover an array of health problems such as dermatological, gastrointestinal and neurodevelopmental disorders (Budreviciute et al., 2020). Moreover, the human and financial costs of NCDs are high and expected to rise, especially considering an ageing population. Along with the well-known risk factors such as overweight, alcohol consumption and an unhealthy diet, recent evidence points out the fundamental role of inflammation and oxidative stress in the pathophysiology of NCDs (Nattagh-Eshtivani et al., 2022). Therapeutic agents that could help to alleviate NCDs burden are therefore highly warranted. Hence, most of the NCDs are preventable and lifestyle modifications and interventions to reduce the risk of NCDs are pivotal in the primary intervention (Budreviciute et al., 2020).

Research work by Newmann and Cragg (Newman & Cragg, 2020) highlights the utility of natural products in the development of drugs for human use and depicts the significance of natural product-based drugs in the treatment of human diseases (Newman, 2022). Furthermore, emerging scientific evidence supports the use of dietary polyphenols as an effective solution to mitigate NCDs concerns (Koch 2019). In addition to the nutrients found in fruits and vegetables such as minerals and vitamins, there are a number of plant-derived components, polyphenols, that confer health benefits (Fraga et al., 2019). In fact, the majority of fruits and vegetables contain these polyphenols as secondary metabolic byproducts. Plants often synthesise polyphenols to protect themselves against different types of internal (e.g. free radicals) or environmental (ultraviolet (UV)

rays, fungi, insects or animals) stressors. Additionally, polyphenols play a pivotal role in the organoleptic characteristics of plants, especially in food and cosmetic formulations. Though, for a long period of time polyphenols were deemed not essential in human nutrition, nowadays, these natural compounds are probably the most extensively described in scientific literature (Koch, 2019).

1.2 Polyphenols

Polyphenols are naturally occurring compounds found abundantly in roots, leaves, fruit pulp and peel across the plant kingdom and in several plant foods like tea, apples, berries and wine (Durazzo et al., 2019; Lv et al., 2021). More than 8000 polyphenolic structures have been identified and isolated to date, making it one of the most widespread and varied classes of secondary metabolites (Durazzo et al., 2019). Polyphenols can be classified according their plant source (e.g. apple polyphenols, grape polyphenols,..) but can also be grouped based on their chemical structure, characterised by the presence of an aromatic ring substituted with at least one hydroxyl group. The most common variations in the chemical skeleton include the degree of oxidation, hydroxylation, methylation and glycosylation (Rambaran, 2020). The main classes of polyphenols include phenolic acids, flavonoids and non-flavonoids with subclasses of the latter including stilbenoids, lignans, condensed and hydrolysable tannins (Lv et al., 2021; Rambaran 2020; Singla et al., 2019; Tijjani et al., 2020) (Figure 1.1). Flavonoids constitute the main subclass and can further be divided into six subclasses: flavonols, flavones, flavanols, flavanones, isoflavones and anthocyanidins (Bié et al., 2023). Polymers comprised of different classes of polyphenols are frequently found in plants as well (e.g. flavono-lignans), making this classification a simplified representation of the real situation.

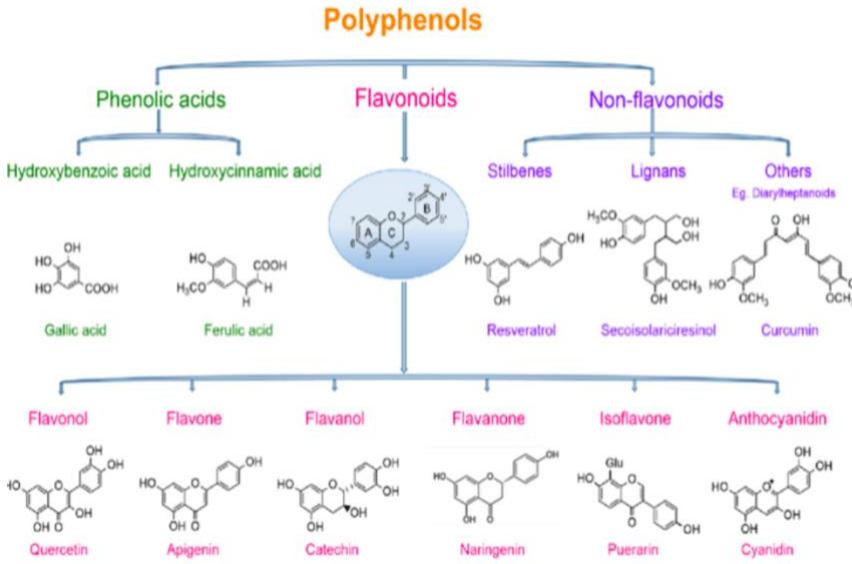

Figure 1.1: Chemical classification of polyphenols. The polyphenols can be divided into phenolic acids, flavonoids and non-flavonoids, all with their own subdivisions. Adapted from Rambaran, 2020.

Over 4000 flavonoids have been isolated until now, mainly flavonols, flavanones, isoflavones, flavones, procyanidins and anthocyanins (Lv et al., 2021). These bioactive compounds are widely spread in many plant-based foods and beverages and can be considered as basic and important components of a balanced human diet (Koirala et al., 2016; Panche et al., 2016). Flavonoids all share the same structural C6-C3-C6 backbone, formed by two aromatic rings linked by a three-carbon chain forming a closed pyran ring (heterocyclic ring containing an oxygen atom for most flavonoids) (Rana et al., 2022). The large variety of flavonoid structures arises from the numerous combinations of multiple hydroxyl groups, methyl groups, glycosides and acetylated group substituents on the basic C6-C3-C6 backbone (Bié et al., 2023). All these flavonoids can undergo further modifications including methylation, glycosylation and polymerisation (Koirala et al., 2016). Alterations in hydroxylation pattern and oxidation state are based on the presence or absence of a double bond between C2 and C3 and the formation of carbonyl group by C4. Flavonoids subdivision is based on differences in the form of moiety they contain on the pyran ring (Panche et al., 2016). Flavones, flavonols and flavanones represent the largest subgroup among all polyphenols and constitute the majority of flavonoid compounds (Bié et al., 2023). A specific kind of oligomerisation of flavan-3-ols through C4-C6 or C4-C8 linkage leads to the condensed tannins or procyanidins. These

oligomers and polymers can be made up of 2-200 catechin and/or epicatechin subunits (Rue et al., 2018). Anthocyanidins and anthocyanins (glycosylated form of anthocyanidins) on the other hand, differ from other flavonoids by the presence of two double bonds in their heterocyclic rings. Variation in the number of hydroxylated groups and the nature and number of bonded sugar units result in a variety of anthocyanins (Singla et al., 2019). Non-flavonoids can be further classified into three subgroups: stilbenes, diarylheptanoids and lignans with resveratrol, curcumin and podophyllotoxin as common examples. Phenolic acids, characterised by a carboxyl group linked to a benzene ring, can be divided into two main subgroups: benzoic and cinnamic acids derivatives. Examples of hydroxybenzoic derivatives are gallic, p-hydroxybenzoic, vanillic and syringic acids, whereas caffeic, ferulic, sinapic, and p-coumaric acids belong to hydroxycinnamic acids (Durazzo et al., 2019; Singla et al., 2019).

Generally, over the last decades dietary polyphenols have been suggested to display a plethora of biological activities including antioxidant, anti-inflammatory, anti-microbial, anti-proliferative, pro-apoptotic activity and hormonal regulation capacity (del Bo et al., 2019; Leri et al., 2020). Moreover, they have become an emerging field of interest due to their favourable effects on human health and in the prevention of chronic diseases. It has become clear that long-term intake can be beneficial on the incidence of several cancers and other chronic diseases, like CVDs, type II diabetes, and neurodegenerative diseases (del Bo et al., 2019). This wide spectrum of biological activities of dietary polyphenols is already extensively described in numerous scientific reports, including both *in vitro* and *in vivo* studies (Leri et al., 2020; Ruskovska et al., 2020).

Despite many promising results obtained in *in vitro* or animal experiments regarding their favourable effects, there is still not enough convincing evidence from human studies, especially in larger populations. Moreover, little is still known about their behaviour *in vivo*, their kinetics, intestinal absorption and their biotransformation. It is important to note that the aforementioned health benefits are all dependent on the bioavailability (according to the commonly accepted definition bioavailability refers to the proportion of the nutrient that is digested, absorbed and available for use at the site of action (Bié et al., 2023)) and bioactivity of these compounds since their beneficial effects can be hindered by for instance a reduced bioavailability, reduced intestinal absorption and rapid biotransformation by gut microbiota (De Bruyne et al., 2019; Goszcz et al., 2017; Leri et al., 2020). Nevertheless, smaller biotransformation products (e.g. phenolic acids) can be absorbed and also exert potential health effects. The

extensive biotransformation processes of polyphenols have been highlighted in several studies before and will be discussed in detail later (see Chapter 2).

1.3 French Maritime Pine Bark Extract: a polyphenol-rich source

French Maritime Pine Bark Extract (PBE; PYC; Pycnogenol®, Horphag Research Ltd., Geneva, Switzerland) is a patented polyphenol-rich herbal extract originating from the outer bark of the maritime pine *Pinus pinaster*, which grows along the coast of southwest France (Figure 1.2). 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 (Rohdewald, 2005). The result is a very fine, red/brown coloured and water-soluble powder. PBE is available in various dosage forms, but it is mostly orally administered as a capsule or tablet.

Figure 1.2: *Pinus pinaster* or maritime pine. Picture from Euforgen (Pinus Pinaster – EUFORGEN European Forest Genetic Resources Program, 2022).

PBE received good manufacturing practice (GMP) certification from the French Health Products Safety Agency (ANSM) (Verlaet et al., 2019) and has been accepted as "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 effects on fertility. The safety profile of PBE was based on data acquired from over 140 clinical studies with more than 12,000 participants (Verlaet et al., 2019). No known contraindications for PBE or interactions with existing therapies are reported (Rohdewald, 2002; Verlaet et al., 2019). Besides, adverse events (AEs) occur rarely (1.8%), are unrelated to the dose or duration of use and are primarily mild (Verlaet et al., 2019). The only frequently reported AEs were dizziness, headache and gastrointestinal (GI) problems, though all relatively mild in nature and easy to overcome: nausea for example can be prevented when PBE is ingested with some food (Oliff et al., 2019).

It is a proprietary commercially available, standardised extract containing 70 ± 5% procyanidins comprising of catechin and epicatechin subunits with varying chain lengths between 2-12 subunits (D'Andrea, 2010). Common dimers are procyanidin B1 (epicatechin-(4β→8)-catechin) and B3 (catechin-(4α→8)-catechin). Since it is a complex polyphenol mixture, also various, other polyphenolic compounds such as caffeic and ferulic acid and their glycosides as well as the flavonoids (+)-taxifolin, (+)-catechin and (-)-epicatechin are present (Figure 1.3). Inorganic substances include calcium, potassium and iron as well as traces of manganese, copper and zinc (Ferreira-Santos et al., 2020).

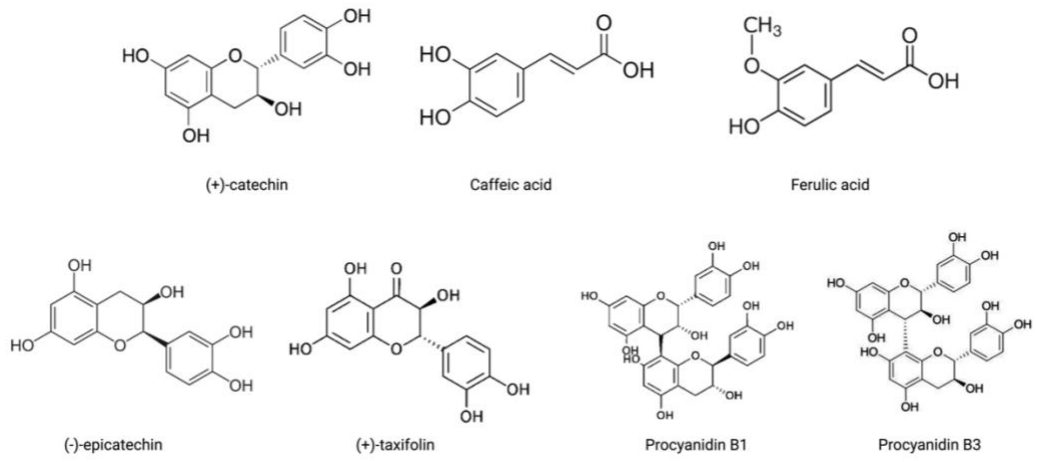

Figure 1.3: Structures of compounds present in PBE. PBE: French Maritime Pine Bark Extract. Created in BioRender.

The overall distribution of these constituents in the extract was determined by means of High Pressure Liquid Chromatography with Diode Array Detection analysis (HPLC-DAD)

by D'Andrea et al. (D'Andrea, 2010) and is presented in Table 1.1. From the HPLC analysis it can be observed that the fraction consisting of dimeric procyanidins is the major component in PBE, next to a significant amount of the catechin monomer as well as trimeric and tetrameric procyanidins. Pic X refers to a still unidentified constituent.

Table 1.1: Overall distribution of constituents in French Maritime Pine Bark Extract (PBE). Adapted from D'Andrea, 2010.

Constituent	Percentage content (%)
Dimeric procyanidins	40.9
(+)-catechin	18.9
Pic X	12.8
Gallic acid	3.2
Taxifolin	2.1
Caffeic acid	1.9
Ferulic acid	0.5
(-)-epicatechin	0.2
Coumaric acid	0.2
Others (e.g. trimeric/ tetrameric procyanidins)	19.0

Pic X is an unknown constituent.

1.3.1 Pharmacokinetics and biotransformation of PBE

Dietary polyphenols have thus been studied in relation to multiple NCDs (Jelena et al., 2018). The beneficial effects of polyphenols are mainly due to their biotransformation products since polyphenols as such have a low bioavailability (Leri et al., 2020). According to Stromsnes et al. (Stromsnes et al., 2021), the plasma concentration is rarely found to be above 1 µM after consumption of 10 to 100 mg of a single phenolic compound. Nevertheless, the total polyphenolic concentration in plasma can be augmented depending on the presence of biotransformation products formed. A study from Grimm et al. (Grimm et al., 2006) analysed the pharmacokinetics of PBE native constituents and biotransformation products after oral administration to human subjects (single dose of 300 mg or 200 mg each day for five days). Fifteen constituents

and biotransformation products could be detected in plasma of human adults between 30 min and 14 h post-dosing, each with a different T_{max} (time to reach maximal plasma concentration), of which only five constituents or biotransformation products were identified: (+)-catechin, caffeic and ferulic acid, taxifolin and δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (M1) (Table 1.2).

Additionally, plasma time profiles and steady state appearance of 10 so far unknown compounds were described (Grimm et al., 2006). Monomeric compounds were absorbed quickly after ingestion and could be quantified in the plasma of all volunteers in concentrations ranging between 1 - 100 ng/mL. For instance, (+)-catechin was detected between 30 min and 14 h after oral ingestion, indicating absorption in the small intestine of (+)-catechin present as such in the extract as well as additional generation by metabolic breakdown of higher procyanidin oligomers. According to the early T_{max}, caffeic and ferulic acid are also absorbed in the small intestine. Although polymerisation highly compromises intestinal absorption, procyanidin B1 (10.6 ± 2.5 nM; measured with LC-MS) has been found in plasma two hours after ingestion of a grape seed extract composed of 0.9% procyanidin B1 as measured by HPLC analysis (Sano et al., 2003). In addition, several other studies have demonstrated that procyanidins can be absorbed and subsequently appear in plasma. Even though most of these *in vivo* studies suggested that only procyanidin dimers can be absorbed, trimers have also been detected in urine and plasma of rats (Appeldoorn, 2009). According to Mendoza-Wilson et al. (Mendoza-Wilson et al., 2016) absorption of epicatechin (0.17%), dimers (0.13%), trimers (0.1%), tetramers (0.05%) and pentamers (0.04%) have been reported on the IEC-18 cell line, which is a useful model of intestinal epithelium for the study of permeability and paracellular transport.

Pronounced biotransformation processes occur, resulting in the formation of new biotransformation products like M1 which could only be detected after six hours, reaching a peak concentration around 10 h after ingestion, suggesting biotransformation reactions by the gut microbiota, and colonic absorption (D'Andrea, 2010; Verlaet et al., 2019; Williamson & Clifford, 2010). Moreover, (+)-catechin itself can undergo biotransformation reactions by gut microbiota, resulting in M1 as illustrated in Figure 1.4. Next to M1, another (+)-catechin metabolite M2 (δ-(3-methoxy-4-hydroxy-phenyl)-γ-valerolactone) has been detected in human urine samples after oral PBE intake (Grimm et al., 2006). In general, M1 and M2, the primary ring-fission biotransformation products

of catechin, are taken up by the host and appear in plasma and urine (Hodgson et al., 2014; Pede et al., 2023).

Ten other unknown constituents and/or biotransformation products were thus observed but are yet to be characterised and might be conjugated monomers, oligomer metabolites or procyanidin dimers (Grimm et al., 2006).

Table 1.2: Calculated pharmacokinetic parameters (mean ± SD) of constituents or biotransformation products after intake of a single dose (300 mg) PBE. Adapted from Grimm et al., 2006.

Constituent	C_{max} (ng/mL)	T_{max} (h)
Caffeic acid	16.67 ± 13.29	3.70 ± 2.40
Catechin	107.22 ± 55.49	3.20 ± 1.70
Ferulic acid	14.78 ± 5.89	1.20 ± 1.10
Taxifolin	33.34 ± 12.54	8.20 ± 2.50
M1	4.11 ± 2.08	10.00 ± 1.90

C_{max}: maximal plasma concentration; M1: δ-(3,4-dihydroxy-phenyl)-γ-valerolactone; T_{max}: time until maximal plasma concentration; PBE: French Maritime Pine Bark Extract.

Moreover, after a 200 mg/day supplementation for three weeks, the highest polyphenol concentrations were not detected in serum (Verlaet et al., 2019). The *in vivo* distribution of the individual polyphenols was previously investigated and revealed that catechin and taxifolin primarily resided in blood cells while M1, caffeic and ferulic acid were mainly present in synovial fluid found in the cavities of synovial joints (Mülek et al., 2017). M1 has also been found to accumulate in erythrocytes, presumably by facilitated uptake by the glucose transporter-1 (GLUT-1) and could therefore be able to cross the blood-brain-barrier (BBB), making this biotransformation product effective at reducing oxidative stress in the brain (Kurlbaum et al., 2013).

As for polyphenols in general, the procyanidin fraction in PBE also undergoes pronounced biotransformation. Besides formation of new biotransformation products like the microbially formed M1 from catechin (Figure 1.4), also phase II biotransformation reactions occur since many compounds were found as conjugates of sulphates and/or glucuronides (Düweler & Rohdewald, 2000; Grimm et al., 2006;

Kurlbaum & Högger, 2011; Rohdewald, 2002). However, considerable differences remain regarding PBE polyphenol pharmacokinetics, with the degree of conjugation with sulphate and glucuronic acid ranging from 54.29% ± 26.77% for catechin (meaning that 54.29% of the total (+)-catechin fraction is conjugated) and to 98.34% ± 4.40% for M1 (98.34% of the total M1 fraction is conjugated) (Verlaet et al., 2019). Results of *in vitro* studies thus need to be interpreted with caution.

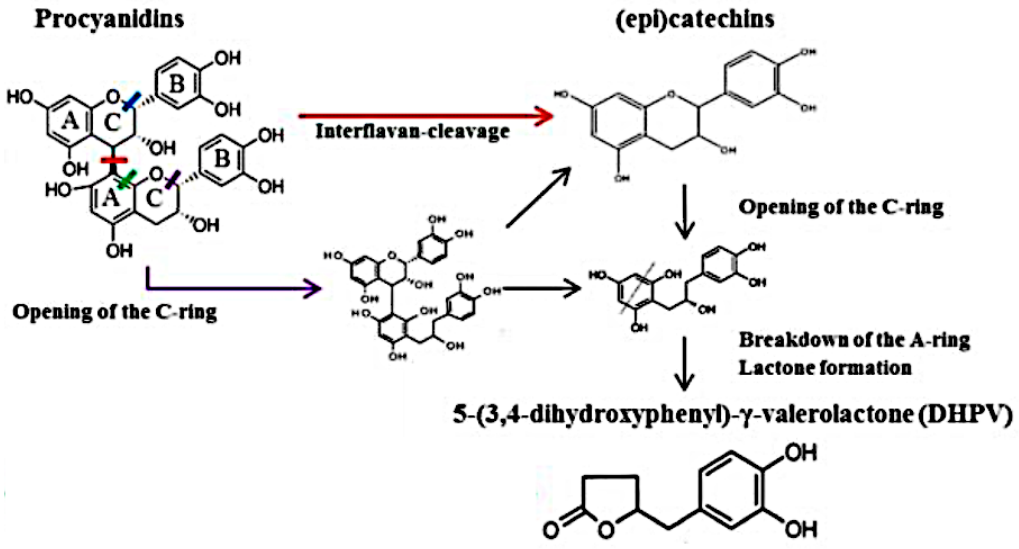

Figure 1.4: Microbial transformation of catechin. Adapted from Lee et al., 2017.

1.3.2 Biological effects of PBE

Polyphenols are subject of increasing scientific interest due to their plethora of health-promoting properties. Evidence suggests that polyphenols can modulate or inhibit a large number of cell signalling pathways in the human body (Rasouli et al., 2017). Polyphenol activities are not only the result of direct effects of the parent compounds but often depend on the bioactivity of their biotransformation products (di Lorenzo et al., 2021). To date, full insight on the structure-activity relationships between the polyphenolic compounds and biotransformation products and health benefits in several pathologies are not fully understood (Bié et al., 2023). The most relevant mode-of-actions of PBE are depicted in Figure 1.5 and will be discussed below.

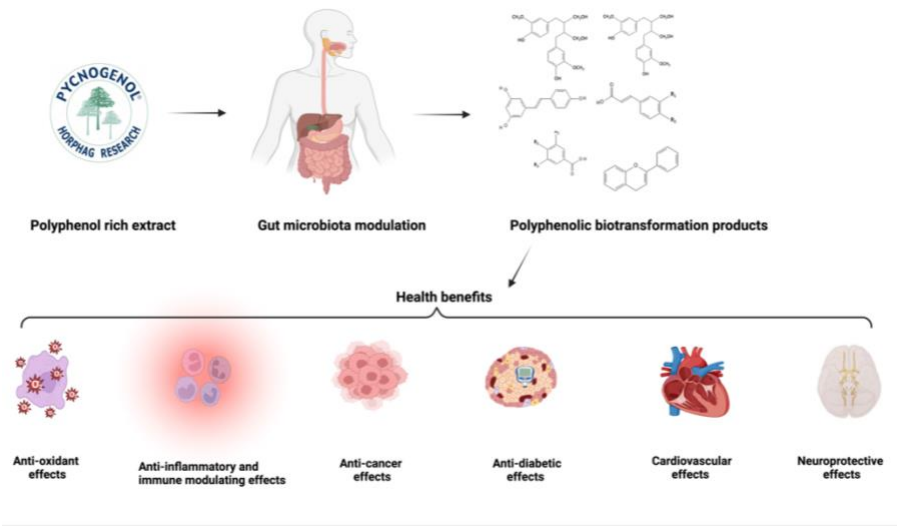

Figure 1.5: Health benefits attributed to French Maritime Pine Bark Extract, also known as Pycnogenol®. Created in BioRender.

1.3.2.1 Antioxidant effects

One of the most well-known properties of dietary polyphenols is their antioxidant capacity and several mechanisms of action can be distinguished which can be attributed to their physicochemical properties (Lv et al., 2021; Scalbert et al., 2005). Firstly, the phenolic hydroxyl groups of polyphenolic constituents render them highly reducing, making them important free radical scavengers of $O_2^{\bullet -}$ (superoxide anion radical), $OH^{\bullet}$ (hydroxyl radical), $ROO^{\bullet}$ (peroxyl radical) and other active radicals. The resulting phenoxyl radical is subsequently stabilised through charge delocalisation over the aromatic moiety which makes the radical less reactive (Figure 1.6) (Magielse et al., 2014).

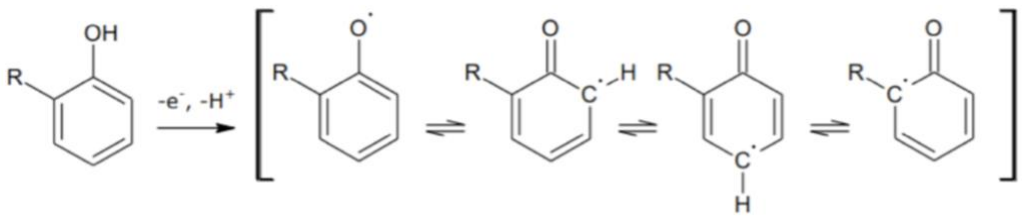

Figure 1.6: Polyphenolic compounds have the potency to scavenge free radicals with their aromatic rings, thereby forming phenoxyl radicals stabilised by charge delocalisation (resonance stabilisation). Figure adapted from Magielse et al., 2014.

Besides acting as free radical scavengers, polyphenols also exert their antioxidative effect through other mechanisms such as reducing the production of radicals by inhibiting the enzymes required to produce free radicals. Furthermore, polyphenols can also chelate metal ions that induce free radical production thereby reducing the generation of free radicals. In addition, polyphenols can already act in the GI tract by scavenging reactive oxygen species (ROS) originating from the diet and gut phagocyte activation (Halliwell et al., 2005; Pietta, 2000). Also, inhibition of oxidation reactions by increasing the activity of antioxidant enzymes or the expression of antioxidant proteins and exerting synergistic antioxidant effects with other substances are possible mechanisms through which polyphenols exert their antioxidative effects. For example, low doses of polyphenols can activate signalling pathways such as the Nfr2 (nuclear factor erythroid 2-related factor 2) pathway, leading to an increased expression of cytoprotective enzymes like glutathione-S-transferase (GST) (Ma, 2013; Martínez-Huélamo et al., 2017). This results in an increased antioxidant capacity and thereby contributes to more long-lived protective effects as opposed to the direct antioxidant effects (Lee et al., 2017; Tumer et al., 2015).

Due to the physiochemical properties of the polyphenolic constituents present in PBE, i.e. aromatic rings with one or more hydroxyl groups, antioxidant activities could be demonstrated (Grimm et al., 2004; Packer et al., 1999). For example, *in vitro* studies showed that PBE has scavenging capacities and thus improves the clearance of reactive nitrogen species (RNS) and ROS such as $O_2^{\bullet-}$ and $OH^{\bullet}$ (Packer et al., 1999). Besides being important free radical scavengers, other antioxidant effects including chelation of transition metals and stimulation of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities have been outlined (Wei et al., 1997). It was demonstrated that, though catechin scavenges $O_2^{\bullet-}$ with an effectiveness similar to ascorbic acid, M1 was significantly more active than catechin or ascorbic acid (Grimm et al., 2004). Additionally, M1 concentration-dependently inhibits nitrite (NO_2^-) production and inducible nitric oxide synthase (iNOS) expression (Uhlenhut & Högger, 2012). Furthermore, *in vivo* studies demonstrated that PBE (10 mg/kg intraperitoneally (i.p.)) reduced liver and pancreas carbonyl levels, increased glutathione (GSH) levels and glutathione reductase (GR), GST, CAT, SOD and GPx activity (Berryman et al., 2004; Parveen et al., 2010, 2013) and reduces serum nitric oxide (NO) in diabetic rats (Parveen et al., 2013). Moreover, it was demonstrated that PBE can inhibit lipid peroxidation and prevent the accumulation of oxidatively damaged proteins (Sivoňová et al., 2004; Voss et al., 2009). In rat studies, PBE (20-100 mg/kg i.p. or 200 mg/kg per day orally) decreased

malondialdehyde (MDA) concentrations, DNA damage and iNOS activity while increased GSH levels were noticeable in for example kidney and skin tissues (Bayomy et al., 2016; Taner et al., 2014; Verlaet et al., 2019). Also, a number of human clinical studies investigated the antioxidant activities of PBE. Supplementation with PBE as compared to placebo prevented oxidative damage to lipids (i.e. 150 mg daily during three months in elderly significantly decreased F2-isoprostane levels by 30% (Ryan et al., 2008) and DNA (i.e. 1 mg/kg daily for one month in 6-12 years old ADHD patients significantly reduced lymphocyte 8-oxoG by generally 35% (Chovanová et al., 2009) while it significantly increased GSH levels by 27% and decreased glutathione disulfide (GSSG) levels by 22% (Dvoráková et al., 2006)). However, in elderly people, PBE (150 mg/day for three months) did not have an effect on oxidative DNA damage (Dvorakova, 2010). Finally, concerning its antioxidative effects, PBE exerts stronger biological activity as a mixture than when separated into its individual constituents, suggesting the components to have additive effects or a synergistic interaction (Yoshida et al., 2011).

Although the primary mechanism of action of polyphenols was thought to lie in their direct antioxidant effects, this is no longer relevant in the *in vivo* situation since the polyphenolic compounds in most tissues do not reach concentrations high enough to exert significant free radical scavenging effects (Fraga et al., 2019). These direct effects on antioxidant defence mechanisms might thus rather be small *in vivo* due to their low absorption rate in the GI tract (Clifford, 2004). Nevertheless, other possible biochemical and molecular mechanisms have been identified including regulation of nuclear transcription factors and modulating the synthesis of inflammatory mediators (e.g. cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin 1-beta (IL-1β)) (Fraga et al., 2019). The antioxidative activities of polyphenols have been exploited in many fields to date, such as agriculture, food, medicine, nutrition, health care, and chemicals used in daily life (Lv et al., 2021).

1.3.2.2 Immune modulating effects

Besides its antioxidative effects, PBE was also shown to exert local gut as well as systemic immune modulating effects. These anti-inflammatory mechanisms have been elucidated in a variety of *in vitro* and cell culture studies. In cell culture systems, PBE dose-dependently decreased histamine, TNF-α and interleukin 6 (IL-6) release by rat peritoneal mast cells. PBE could thus improve mast cell-mediated immediate-type

allergy (Choi & Yan, 2009). Studies using different animal models demonstrated that PBE (50 mg/kg intravenous injection or 30-100 mg/kg orally) decreased the inflammatory cell count, mucus secretion and interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 13 (IL-13) and immunoglobulin E (IgE) levels in asthmatic rats (Liu et al., 2016; Shin et al., 2013) whereas in septic and diabetic rats (10-100 mg/kg i.p.) plasma TNF-α levels decreased (Berryman et al., 2004; Parveen et al., 2010; Verlaet et al., 2019). Also, wound healing was stimulated in diabetic rats (30 mg PBE dermally) (Dogan et al., 2017) and a restored immune function as well as a prolonged survival was noticeable in mice with a diet-induced immune dysfunction (Lee et al., 2014). Another animal-based study assessed the effects of PBE on ventilation-induced lung injury of rats which typically involves excessive inflammation. It could be demonstrated that pre-treatment with one dose of 30 mg/kg PBE two days before lung injury induction reduced the production of pro-inflammatory cytokines such as TNF-α and IL-6 towards normal levels through inhibiting the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), indicating the potential of PBE as a therapeutic candidate for ventilator-induced lung injury (Xia et al., 2015).

In healthy adults, PBE supplementation (150 mg/day for five days) inhibited cyclooxygenase 2 (COX-2) and 5-lipooxygenase (5-LOX) gene expression and reduced leukotriene production in leukocytes after *ex vivo* stimulation (Canali et al., 2009). Additionally, in other *in vitro* and *ex vivo* experiments PBE was found to block NF-κB activation (Grimm et al., 2006; Packer et al., 1999) and to limit the induction of adhesion molecules including intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) (Peng et al., 2000), that play a major role in inflammation. Furthermore, PBE can inhibit upregulation of COX-1 and COX-2 and metalloproteases MMP-1, -2 and -9, with M1 being more potent than catechin (Grimm et al., 2004; Peng et al., 2012; Schäfer et al., 2006). Moreover, pro-inflammatory cytokine expression (e.g. IL-1, IL-2 an IL-8) (Cho et al., 2001; Peng et al., 2012) and acute inflammatory cell infiltration were inhibited by PBE (Peng et al., 2012). A study by Verlaet et al. (Verlaet et al., 2019) demonstrated immune modulating effects of PBE via inhibition of Toll-like receptor (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.

PBE is likely to improve inflammation and allergy symptoms (Verlaet et al., 2019). For instance, 100 mg/day PBE during six months significantly reduced the use of inhalation

corticoids, frequency of symptoms and specialist consultations in adult asthma patients, while decreasing the specific IgE titre by 15.2% (Belcaro et al., 2011). Another clinical trial demonstrated that 100 mg/day PBE for three months significantly reduced plasma C-reactive protein (CRP) levels by 71% as compared to placebo in osteoarthritis patients (Belcaro et al., 2008).

Furthermore, M1 and M2 (gut microbial biotransformation products of PBE) are believed to bind to zinc ions which are known modulators of antiviral and antibacterial immunity and therefore have regulatory properties on inflammation (Skalny et al., 2020).

1.3.2.3 Anti-cancer effects

Cancer is a leading cause of death worldwide, accounting for nearly ten million deaths in 2020, which is nearly one in six deaths. According to the WHO, many cancers can be cured if detected early and treated effectively (WHO, 2022).

PBE, and especially its procyanidin fraction, may play an important role in the prevention and treatment of cancer by inhibiting the proliferation of tumor cells, as shown already *in vitro.* According to *in vitro* research by Silva et al. (Silva et al., 2019), (+)-catechin has anti-proliferative and cytotoxic effects on pancreatic, breast and colorectal cancer cell lines. A complex of (+)-catechin and two lysines was tested in concentrations up to 1 mM and was found to dose-dependently inhibit proliferation and to stimulate apoptosis in the tested cell lines. Moreover, telomerase inhibition by (+)-catechin could also explain the anti-cancer effects of PBE. In colon adenocarcinoma and monoblastoid leukemia cells, catechins (up to 10 µM) causes telomerase shortening and chromosomal abnormalities resulting in a life-span reduction (Ghaffari et al., 2021). To extrapolate these *in vitro* into the *in vivo* setting, the rather low bioavailability of polyphenols should be taken into account.

1.3.2.4 Anti-diabetic effects

According to the International Diabetes Federation's Diabetes Atlas, type 2 diabetes mellitus is one of the world's most important public health concerns nowadays. In 2019, 400 million individuals were estimated to have diabetes and the number is expected to even rise to 700 million by 2045 (Federation, 2023). Pathophysiological alterations in β-

cell dysfunction, insulin resistance and chronic inflammation could progressively hamper proper glycemic control and lead to the development of microvascular complications (e.g. retinopathy, nephropathy, and neuropathy) and macrovascular complications (e.g., heart attack and stroke) (Nattagh-Eshtivani et al., 2022). Indeed, long-term activation of the immune system as well as high plasma levels of pro-inflammatory markers are directly linked to the development of insulin resistance (Tsalamandris et al., 2019).

Under normal physiological conditions insulin binds to the insulin receptor, located on the external surface of insulin-responsive cells. Upon insulin receptor stimulation, downstream signalling is activated thereby initiating signalling events in the cell (Nedosugova et al., 2022). Pro-inflammatory cytokines such as TNF-α stimulate the phosphorylation of insulin receptor substrate serine and threonine residues and this phosphorylation decreases the tyrosine phosphorylation of the insuline receptor substrate in response to insulin. Moreover, it reduces the ability of the insulin receptor substrate to associate with the insulin receptor, thereby inhibiting downstream signalling and insulin activity (Nattagh-Eshtivani et al., 2022; Nedosugova et al., 2022).

Several clinical studies revealed that PBE is a potential hypoglycemic agent, reducing blood glucose levels and other typical diabetes markers such as microalbuminuria. According to research work by Schäfer and Högger (Schäfer & Högger, 2007), α-glucosidase activity in the GI brush border could be inhibited by PBE, preventing the hydrolysis of carbohydrates to glucose monomers, thereby reducing the blood level of glucose. Moreover, PBE was shown to inhibit glucose transporters and to interfere with glucose absorption in enterocytes. Lastly, PBE exerts another antidiabetic action by stimulating insulin signalling pathways involving phosphatidylinositol-3-kinase (PI3K) and/or p38 mitogen-activated protein kinase (p38-MAPK) pathways. Stuard et al. (Stuard et al., 2010) demonstrated in a study of 58 patients with metabolic syndrome, that PBE significantly lowered fasting blood glucose as well as glycosylated haemoglobin (HbA1C) leading to an overall improvement of the glycemic index. More recently, in another study with 150 patients suffering from metabolic syndrome, PBE could also reduce fasting glucose status after six months of supplementation (Mármol et al., 2019). Esfahlan et al. (Navval-Esfahlan et al., 2021) also showed that after eight weeks of PBE supplementation (100 mg/day) VCAM-1 and urinary albumin-to-creatinine ratio (UACR) were lower as compared to the placebo group. A double-blind, placebo-controlled, randomised study including 77 diabetes type 2 patients also demonstrated significant lower plasma glucose levels after 12 weeks of PBE supplementation (100 mg/day) as compared to placebo (Liu

et al, 2004). In addition to its effect on sugar levels in the blood, PBE might also improve the blood lipid profile; however, results are still inconclusive. Nevertheless, a meta-analysis by Hadi et al. (Hadi et al., 2019) suggested PBE to significantly increase high density lipid (HDL) cholesterol in adult humans although no sufficient evidence could be obtained to support favourable effects of PBE on other blood lipids.

Generally, it should be noted that most studies found a lowering effect of PBE on fasting blood glucose, pre- and postprandial glycemia and HbA1C level as well as an increasing effect in insulin level. Furthermore, most animal and human studies demonstrated that PBE had a positive effect on oxidative stress parameters and inflammatory markers (Navval-Esfahlan et al., 2021).

1.3.2.5 Cardiovascular effects

PBE may also contribute to the improvement of cardiovascular (CV) risk factors such as endothelial dysfunction (which is characterised by a loss of endothelial control over vascular tone, impairment of endothelium-dependent vasorelaxation, alteration of the anticoagulant and anti-inflammatory properties leading to thrombosis and vessel wall remodelling (Widlansky et al., 2003, Seals et al., 2011)) and elevated blood pressure (BP) (Malekahmadi et al., 2019; Zhang et al., 2018). A number of clinical studies with healthy people and individuals at CV risk demonstrate the efficacy of PBE in the context of CV health problems. PBE is known for its effect on endothelial health by enhancing endothelial nitric oxide (NO) generation through stimulation of endothelial NO synthase (eNOS) from the precursor molecule L-arginine, leading to an increase in blood vessel lumen diameter and adequate tissue perfusion.

Various clinical studies with patients having no or particular comorbidities showed that PBE can improve endothelial function (Al-Abkal et al., 2021). For instance, Nishioka et al. (Nishioka et al., 2007) demonstrated that PBE augments endothelium-dependent vasodilation by an increased NO production after two weeks of daily oral administration of PBE (180 mg/day) in healthy young men as compared to placebo by measuring the forearm blood flow in response to acetylcholine (an endothelium-dependent vasodilator). Following daily intake of PBE for 14 days, the forearm blood flow in response to acetylcholine of these healthy volunteers increased up to 41% as compared to placebo whereas for sodium nitroprusside (an endothelium-independent vasodilator) vasodilation was similar before and after two weeks of treatment in the PBE and placebo

group. In an eight-week randomised, double-blind, placebo-controlled cross-over study in patients with coronary artery disease, endothelial function was assessed by measuring the flow-mediated dilatation (FMD). After eight weeks of daily supplementation with PBE (200 mg/day) FMD improved by 33% and even slightly decreased in the placebo group (Enseleit et al., 2012).

Moreover, previous meta-analyses revealed reduced systolic (with 2.54 mm Hg) and diastolic (1.76 mm Hg) BP among hypertensive patients after eight weeks of PBE supplementation as compared to placebo. Another placebo-controlled, randomised study involving patients with stage 1 hypertension were given a low molecular weight oligomeric procyanidin extract (150 mg/day) made from PBE. After five weeks of treatment a significant lower systolic BP was noticeable in the female participants as compared to placebo (Valls et al., 2016). Though the exact mechanisms behind the BP-lowering effect of PBE are not fully understood yet, Zhang et al. (Zhang et al., 2018) suggest that angiotensin converting enzyme (ACE) inhibition, NO production and antioxidant and anti-inflammatory activities are involved.

Finally, by increasing the production of endothelial NO, PBE has the ability to lower blood platelet aggregation as effectively as aspirin but without increasing the bleeding time (Al-Abkal et al., 2021). PBE has been shown to reduce platelet aggregation in smoking individuals (smoking increases blood platelet activity) to the level of non-smokers after supplementation with PBE (200 mg/day) for two months (Araghi-Niknam et al., 2000). Hence, this effect was not observed in healthy non-smokers so it could be concluded that PBE only normalise pathologically increased platelet activity but does not further decrease normal platelet function.

1.3.2.6 Neuroprotective effects

Dietary polyphenols are important active agents of neuroprotection because of their capacity to modulate and influence cellular processes including signalling, replication, cell death, redox balance and differentiation (Rana et al., 2022). After traumatic brain injury in young adult male rats, a single intravenous treatment with PBE was shown to be neuroprotective with a therapeutic window of at least four hours (Ansari et al., 2013). It should be noted that with an intravenous injection medication is directly administered to the systemic circulation and therefore, the rapid drug effects are hardly comparable with oral or i.p. given medication (Kim & Jesus, 2023).

Preliminary results from human clinical trials show promising cognitive-enhancing effects of PBE. For instance, one study found that in a group of young healthy university students, an eight-week PBE intervention (100 mg/day) resulted in improvements in sustained attention, memory and executive functioning while implementing a standardised health plan. Moreover, the PBE group also showed improvements on mood parameters (alertness, contentedness and reduced anxiety) and performed significantly better on actual university exams than the control group (Luzzi, 2011). A similar study by Belcaro and colleagues (Belcaro et al., 2013) examined the effects of a 12-week intervention comprising PBE (150 mg/day) and a healthy lifestyle plan (controlled diet and lifestyle suggestions) in young healthy professionals (aged 33 to 55 years) who showed elevated oxidative stress levels at baseline. In the PBE group, significant improvements were demonstrated on mood (alertness and attention) and cognitive parameters (episodic memory and spatial working memory) and a 30.4% reduction in plasma free radicals (considered the oxidative stress marker) with no changes in the control group.

1.4 Aim and outline of this work

Emerging scientific evidence supports the use of dietary polyphenols as an effective solution to mitigate NCDs concerns (Koch, 2019). In addition to the nutrients found in fruits and vegetables such as minerals and vitamins, there are a number of other plant-derived components that confer health benefits (Fraga et al., 2019). In fact, the majority of fruits and vegetables contain polyphenols as secondary metabolic byproducts.

During this PhD project, the main focus will be on the French Maritime Pine Bark extract (PBE) as promising polyphenol source. The wide spectrum of biological activities attributed to PBE has been extensively described in various scientific papers, including *in vitro* and *in vivo* studies. **Chapter 1** is the general introduction to this thesis in which the classification of dietary polyphenols and recent findings on the biological properties of PBE are further discussed.

Although polyphenols are gaining increasing research interest due to their potential health benefits, there is still a lack of understanding regarding the bioavailability and biotransformation of polyphenols, and incomprehension on which particular constituents or biotransformation products are responsible for the biological actions (Tresserra-Rimbau et al., 2018). **Chapter 2** starts with a brief introduction on the anatomy and physiology of the different gastrointestinal tract sites. Their effect on the bioavailability of polyphenols is discussed here and the role of the gut microbiome is highlighted. This chapter focusses on the biotransformation of PBE by using an in-house developed *in vitro* gastrointestinal dialysis model, mimicking the stomach, small intestine and colon, including fermentation by pooled human faeces. Samples collected over the time course of the biotransformation are analysed by UHPLC-ESI-QTOF-MS. Herbal extracts consist of a mixture of phenolic compounds and extensive biotransformation is to be expected. Unraveling metabolic pathways after *in vitro* biotransformation experiments generates a large number of data and is thus complex. Therefore, a metabolomics approach using multivariate data-analysis and pattern detection techniques was used to obtain a qualitative and quantitative analysis of all biotransformation products present in the extract.

Attention-Deficit Hyperactivity Disorder (ADHD) is considered to be a NCD which carries a substantial economic burden and negatively affects the quality of life including educational underachievement, difficulties with employment and relationships, and

even criminality (Loo et al., 2022; Sayal et al., 2018). Even though nowadays it is the most common mental health disorder in early childhood often persisting into adulthood, ADHD is still relatively overlooked and underdiagnosed in most countries (Sayal et al., 2018). Moreover, methylphenidate (MPH), the first-choice medication for ADHD, is linked to concerns about adverse effects and publication bias in reported efficacy (Verlaet et al., 2019). **Chapter 3** describes the ADHD aetiology including dopaminergic dysfunction, increased oxidative stress and immune disbalance and discusses the potential for nutritional approaches such as polyphenols as efficacious and safe methods for ADHD management. The aim of **Chapter 4** is to investigate the efficacy, mechanisms of action and value of PBE 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 PBE compared to MPH. Results of our phase III, randomised, active- and placebo controlled clinical trial are discussed in this chapter. The pathogenesis of ADHD is believed to be multifactorial, with a potential role for the bidirectional communication between the gut microbiome and brain development and function. It has been postulated that this 'gut-brain' axis is impaired in ADHD. Since polyphenols such as PBE are believed to modulate the gut microbiome, this potential gut microbiome modulation by PBE or MPH was also evaluated during a pilot study and findings are discussed in **Chapter 5**.

MPH use has been linked to various reported side effects including insomnia and loss of appetite. Moreover, its cardiovascular safety has been questioned in several case reports and conflicting evidence regarding a slight increase in blood pressure and accelerated heart rate has emerged over time (Jackson, 2016). Since cardiovascular diseases account for most NCD deaths per year, maintaining cardiovascular health is therefore of utmost importance. Arterial stiffness is an independent risk factor for cardiovascular morbidity and mortality and various polyphenols display protective effects towards mechanisms involved in arterial stiffness aetiology. **Chapter 6** describes the pathophysiology of arterial stiffness and highlights the underlying mechanisms contributing to it. PBE will be tested in an array of *in vitro* assays representing different mechanisms contributing to arterial stiffness and the results can be found in **Chapter 7**. Finding an alternative treatment option for ADHD without compromising cardiovascular health is of utmost importance.

Chapter 8 concludes this thesis with a critical review of the presented results followed by a few insights in imperative future research work.

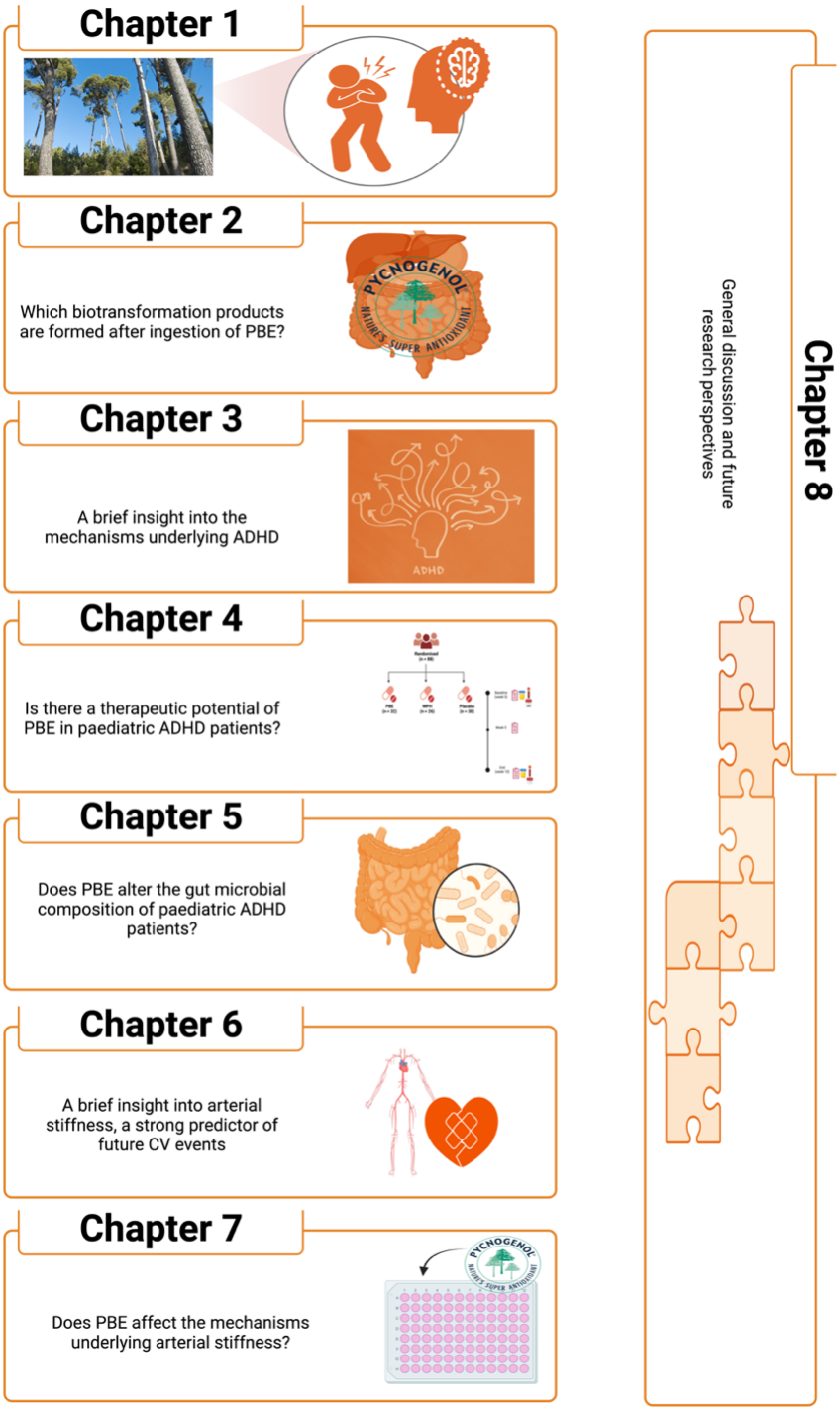

Figure 1.7: Outline of this thesis.

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Chapter 2 **Gastrointestinal biotransformation of French Maritime Pine Bark Extract**

2.1 Introduction

As mentioned earlier, the biological effects of polyphenols depend on their mechanism of action in the body which is affected by for example the bioconversion by colonic microbiota and absorption of colonic biotransformation products (Breynaert et al., 2015). For years, *in vivo* animal or clinical studies have been relied on in drug development for studying drug dosages, mechanisms of actions and the biotransformation of the given drug. Although widely applied in research lately, *in vivo* testing has some limitations and disadvantages, especially when investigating human biotransformation reactions. Firstly, these *in vivo* studies are labour-intensive, time-consuming and expensive (Verhoeckx et al., 2015). Moreover, access to the gut is rather difficult and it would require invasive techniques (Mortelé, 2021). Alternatively, in order to sample different compartments of the gut in animals, they need to be sacrificed (Venema, 2015). Additionally, inter-species variability between human and animals could affect the translatability of data (Zhang et al., 2021). Therefore, *in vitro* gastrointestinal models are considered a good, alternative approach to study the human biotransformation processes. The aim of these *in vitro* models is to mimic the different conditions in the different regions of the digestive system to obtain a good representation of the human physiological conditions. Easy access to these different compartments allows for dynamic and multiple sampling over time which can help to understand the different steps in the biotransformation of xenobiotics (substances that are normally not found *in vivo* and that are extrinsic to the metabolism of the organism (Cardona et al., 2013; Croom, 2012). Nevertheless*, in vitro* models do not fully represent the *in vivo* conditions. It is therefore extremely important to create conditions that closely resemble the *in vivo* situations to acquire a high level of physiological significance and to validate the obtained results with *in vivo* settings whenever possible (Mortelé, 2021; Verhoeckx et al., 2015).

Among the wide range of available *in vitro* gastrointestinal (GI) and fermentation models, a distinction can be made between short-term batch incubations, single stage reactors or semi-continuous systems and multi-compartmental continuous models (Venema & van den Abbeele, 2013) (Figure 2.1). The simplest and most frequently used are the short-term batch incubation models (i.e. small reactor vessels or test-tubes containing faecal suspension and substrate); however, these systems are far from physiological since they tend to over-simplify the *in vivo* situation. For a better

representation of the actual *in vivo* situation, semi-continuous systems (i.e. comprising a static bioreactor to which selected enzymes are added manually and stirred) and multi-compartmental continuous models (i.e. directly connected compartments with dynamic changes such as temperature, pH and digestive secretion) are more appropriate (Venema & van den Abbeele, 2013). Some examples of these more complex systems include: TNO-gastrointestinal model 1 (TIM-1) and 2 (TIM-2) which stimulates digestion in the upper GI tract and fermentation in the colon, respectively and the SHIME model (Simulator of the Human Intestinal Microbial Ecosystem; Ghent University; Prodigest, Belgium) which integrates the stomach (pH 2), small intestine (slightly acidic to neutral pH) and colon into one system, all kept at 37 °C (van der Auwera, 2023). Also, the gastrointestinal dialysis model with colon phase (GIDM-Colon), developed at the Laboratory of Natural Products and Food Research and Analysis- Pharmaceutical Technology (NatuRA-PT) is a continuous system and allows to study the biotransformation and absorption (passive diffusion) of an extract/compound of interest in the stomach, small intestine and colon separately in each stage and will be further discussed in detail (Breynaert et al., 2015).

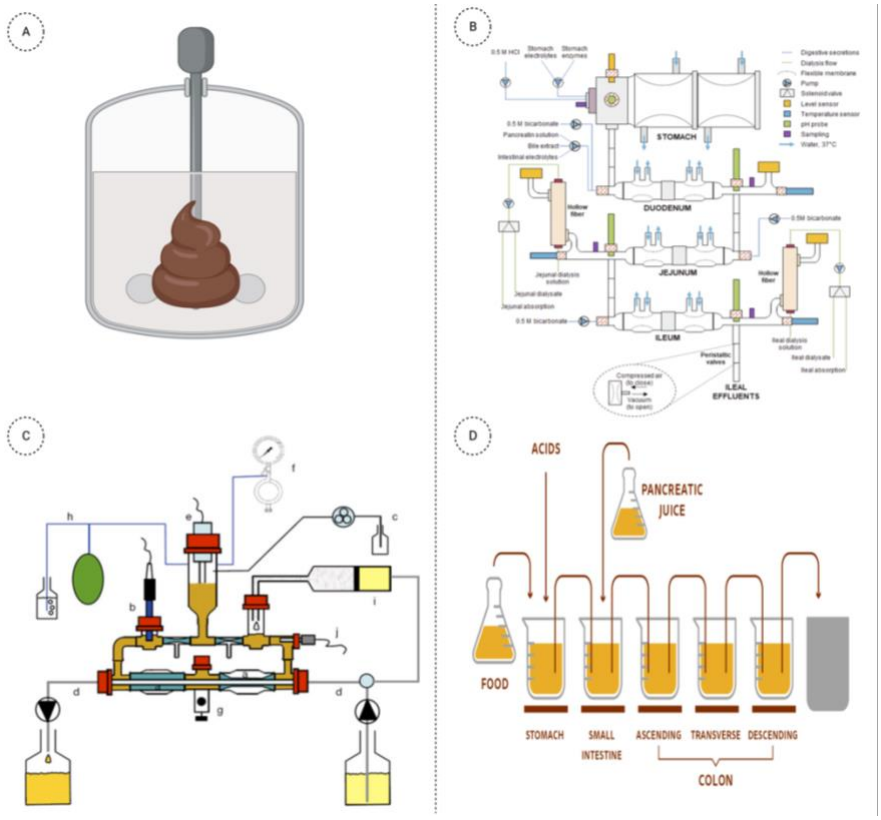

Figure 2.1: Schematic overview of the available GI models. A: Short-term batch incubation; B: TIM-1 simulating the gastric-small intestinal system; C: TIM-2 simulating the colon; D: SHIME model. Figure created in BioRender and adapted from Venema, 2015; Van de Wiele et al., 2015.

2.2 Anatomy and physiology of the gastrointestinal tract

Oral drug delivery is the most desirable administration route for patient convenience due to its noninvasive character, high patient compliance and easier formulation (does not require sterile conditions) (Sahoo et al., 2021). Nevertheless, medication administered orally encounters physical, biological and biochemical barriers which can lower the therapeutic efficacy of the drug. This bioavailability is affected by the intestinal absorption, distribution in body tissues, biotransformation processes by for instance colonic microbiota, and elimination. After oral administration, compounds follow the route of the GI tract which can be seen as a series of connected organs passing through the body from mouth to anus, all having a distinct role (Cheng et al., 2010). The mouth,

stomach, small intestine and large intestine are the most important parts of the GI tract (Figure 2.2) and will be further discussed here.

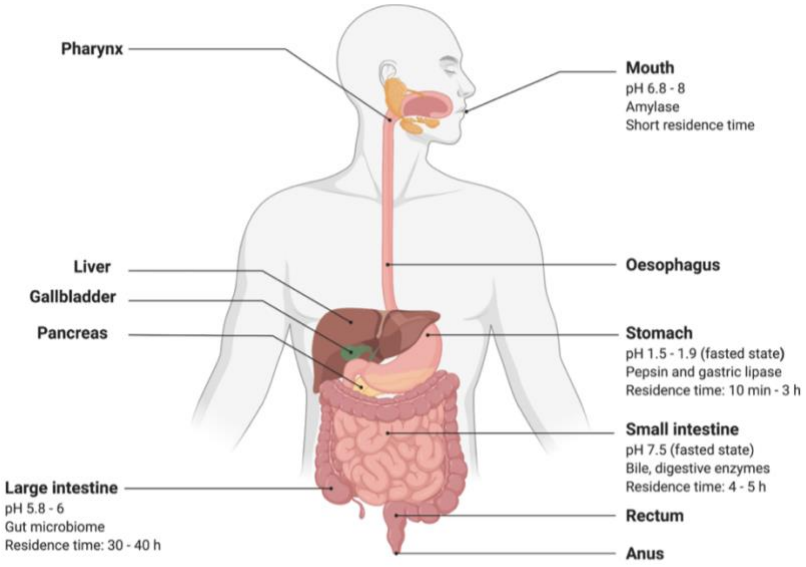

Figure 2.2: Representation of the human gastrointestinal tract and the liver, gallbladder and pancreas. The distinct conditions of each stage are depicted in the figure. Created in BioRender.

2.2.1 The mouth

The mouth is the beginning of the digestive tract where ingested materials are mixed with saliva and physically broken down by chewing (mastication) into smaller pieces that can be digested. Saliva with a pH of around 6.8 to 8, is secreted by salivary glands and contains multiple proteins and enzymes such as amylase which is responsible for the initial breakdown of polysaccharides and will lubricate the ingested material to facilitate the swallowing process (deglutition) (Maddu, 2019). Residence times of the ingested materials in the mouth differ depending on the food matrix: most food materials require mastication, which thus increases the exposure time to the saliva, while the residence time of most pharmaceuticals or food supplements will be very short as they are swallowed immediately (Vertzoni et al., 2019). The ingested materials will then be transported through the pharynx and oesophagus to the stomach according to the propulsive process of swallowing.

2.2.2 Stomach

The stomach is a muscular hollow organ which receives ingested materials (e.g. food, liquids,...) from the oesophagus and retains them for grinding and mixing with gastric fluid to obtain particles that are smaller and more soluble. Generally, the stomach is recognised to have three functions: i) storage of food, ii) digestion, and iii) protection. When food arrives from the oesophagus, the stomach relaxes and expands to hold the increased volume. It is mainly the upper half of the stomach that stores the food until it is ready to be digested, while the lower part is responsible for digestion. Without this regulation of the rate at which food enters the small intestine, the small intestine would not be capable to digest and absorb the volume presented to it, resulting in a large unabsorbed fraction excreted in the faeces. The distal part of the stomach is thus responsible for digestion by mechanical contractions and mixing the ingested material with acid, pepsin and gastric lipase resulting in a semi-solid mixture called chyme which is then gradually passed through the pylorus into the duodenum with every contraction. The gastric emptying time (the amount of time it takes for the chyme to pass on to the duodenum) can vary. For instance, it takes 10 min after consumption of a glass of water in the fasted state, whereas it lasts 3 h or more for a standardised high-caloric breakfast. This implies that food intake can highly influence the gastric residence time of xenobiotics (Mortelé, 2021; Pal et al., 2007; Simonian et al., 2005). Besides its storage function and digestion, the stomach also protects the body by destroying many of the bacteria and other pathogens that are swallowed with food. Moreover, the gastric mucosa protects itself from acid and enzymes by a mucus-bicarbonate barrier.

The gastric fluid is composed of multiple components which are (i) derived from the stomach such as gastric acid, enzymes and mucus, (ii) ingested or (iii) derived from the duodenum through reflux making the stomach an acidic environment with reported pH-values between 1.5 and 1.9 in fasted state (Mortelé, 2021). However, in a fed state, gastric pH will rise and postprandial pH values of 4.9 have been reported, dependent of the meal volume and type, which in turn could influence the disintegration and solubility of the xenobiotic (Mortelé, 2021; Simonian et al., 2005; Vertzoni et al., 2019). The two main enzymes present in the gastric fluid are pepsin and gastric lipase. Chief cells in the gastric gland secrete the inactive pepsinogen and gastric lipase. Pepsinogen, cleaved to active pepsin in the lumen of the stomach by action of H^+, is particularly responsible for the initial digestion of proteins in the stomach. The role of gastric lipase, which is co-

secreted with pepsinogen and responsible for digestion of lipids, is limited as only 10% of fat digestion takes place in the stomach (Mortelé, 2021).

In general, the gastric phase is thus responsible for digestion of proteins, the formation of chyme and the controlled entry of chyme into the small intestine where further digestion and absorption will take place, seen the limited absorption in the stomach due to the small epithelial surface and the short duration of time (maximum 4 h) that compounds are in contact with the stomach epithelium as compared to that of the small intestine.

2.2.3 The small intestine

Most absorption and digestion (the chyme entering the small intestine is only poorly digested) take place in the small intestine which can be divided into the duodenum, jejenum and ileum. It has a varying length between 3 and 5 m with typical structural properties of the intestinal wall, including the presence of villi and microvilli, hereby increasing the surface area for absorption of food nutrients, pharmaceuticals and other compounds (Collins et al., 2023). Absorption is mainly located in the duodenum and proximal part of the jejenum (Vertzoni et al., 2019). The protein digestion which already started in the stomach by pepsin, is here inactivated by the higher intestinal pH.

The intestinal epithelium and the pancreas produce digestive enzymes such as peptidases, disaccharidases and enteropeptidases anchored to the luminal side of the enterocytes which either continue protein digestion resulting in small peptides and free amino acids or carbohydrates digestion forming end products that can later be absorbed. The secreted pancreatic enzymes include amylases (for digesting starch into maltose), proteolytic enzymes and lipases and phospholipases, assisting further digestion. Non-digested food components like fibres, resistant starch, certain peptides and lipids (e.g. cholesterol (Cohn et al., 2010)) and bioactive compounds (e.g. polyphenols) are passed on to the large intestine (Breynaert et al., 2015). The small intestine transit time is relatively stable with reported mean intervals between 4 - 5 h (Hua, 2020).

2.2.4 The large intestine

The large intestine or colon is the final segment of the GI tract and is composed of the appendix, cecum, ascending, transverse, descending and sigmoid colon, rectum and the anus (Figure 2.3). The primary functions of the colon are (i) processing unabsorbed and undigested chyme, (ii) absorption of the remaining water, electrolytes and vitamins and (iii) formation and storage of faecal material until it can be evacuated by defecation. Absorption of electrolytes such as sodium, chloride and potassium by passive or active pathways will create an osmotic gradient responsible for water absorption (Azzouz & Sharma, 2021; Mortelé, 2021). The large intestine also secretes mucus, facilitating the transport of intestinal contents through the bowel. Initially it was believed that no significant digestion occurred in the large intestine; however, over the last decades multiple studies have shown that bacteria residing in the colon break down significant amounts of undigested complex carbohydrates and proteins through fermentation (Macfarlane & Macfarlane, 2011; Mortelé, 2021). The end products include lactate and short-chain fatty acids (SCFAs) such as butyric acid. Several of these are lipophilic and can thus be easily absorbed by passive diffusion. Besides these undigested macronutrients, also complex phenolic compounds will reach the large intestine where the gut microbiota will turn them into low-molecular polyphenolic structures (Swallah et al., 2020). It is only more recently that the importance of those colonic bacteria on the activity, bioavailability and/or toxicity of xenobiotics has been acknowledged (Mortelé, 2021). In fasted state, the pH of the colonic environment is reported to be around 7.8, while in the fed state, the pH will drop to approximately 5.8-6 because of enhanced bacterial fermentation, and thus SCFA formation of the indigested fibres present in the consumed meal (Mortelé, 2021).

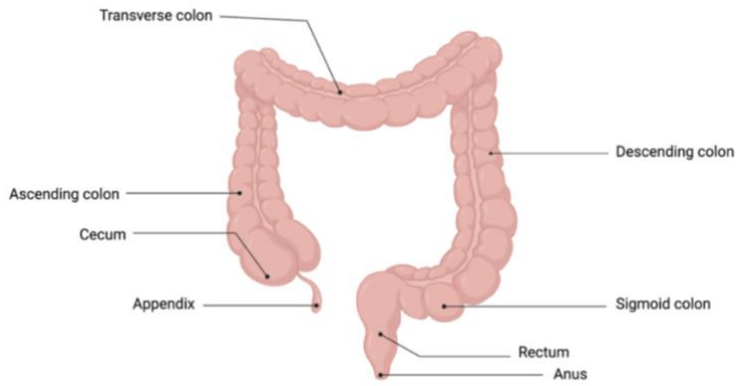

Figure 2.3: Different regions of the large intestine. Created in BioRender.

2.2.5 The gut microbiome

The adult human GI tract is seen as one of the most densely populated ecosystems on our planet, accommodating around 10^{13} microorganisms with species diversity increasing longitudinally from mouth to colon, the latter harbouring the highest amount of bacteria in the GI tract referred to as the gut microbiota (Mills et al., 2019; Venema & van den Abbeele, 2013). To illustrate this: the number of bacterial cells shifts from about 10^1 bacteria per gram of contents in the stomach, to 10^7 bacteria per gram of contents in the ileum, to 10^{12} bacteria per gram in the colon (Sekirov et al., 2010). In addition to bacteria, other microorganisms such as archaea, viruses, phages, yeast and fungi, are also present in the gut (Cani, 2018). The gut microbiome can be defined as the combined genetic material of the microorganisms living in the gut and is estimated to encode 150 times more genes than the human host genome (Jain et al., 2018; Stefanaki, 2019; Walter & Ley, 2011).

The gut microbiome of an individual is unique and substantially changes during development. However, the microbial diversity is relatively limited. The human digestive tract is composed primarily of the following eight phyla: Firmicutes (e.g. *Lactobacillus*, *Streptococcus*, *Clostridium*) and Bacteroidetes (e.g. *Bacteroides*), accounting for 90% of gut diversity, and to a lesser extent Proteobacteria (e.g. *Enterobacteriaceae*), Fusobacteria, Verrucomicrobia, Cyanobacteria, Spirochaetes and Actinobacteria (e.g. *Bifidobacterium, Corynebacteria*) (King et al., 2019; Mills et al., 2019). The gut microbiota has been shown to interact in several ways with the host, thereby contributing essential functions to its host as illustrated in Figure 2.4 (Heintz-Buschart & Wilmes, 2018). Besides the biotransformation of compounds that reach the colon, the gut microbiota executes numerous important functions, including some that the host itself is unable to perform like synthesis of vitamins K2 and B12, development and regulation of the immune and nervous system, protection against pathogenic species, maintaining a barrier function, synthesising small molecules and proteins that are taken up by the host, regulation of host fat storage and energy homeostasis (Mills et al., 2019; Mortelé, 2021; Sweeney & Morton, 2013; Valdes et al., 2018). For instance, the intestinal microbiome produces SCFAs, particularly butyrate, propionic acid and acetate derivatives, resulting from the degradation of fibres and undigested saccharides. In turn, these SCFAs serve as energy source for the colonic epithelium and the host, especially under conditions of inflammation, starvation and physical strain (Checa-Ros et al., 2021). They are also involved in the regulation of many cellular processes e.g. gene expression, proliferation

and apoptosis (van der Auwera, 2023). In addition, studies have demonstrated that vitamin K2 is an important cofactor for the carboxylation of proteins involved in the inhibition of arterial calcification. A higher vitamin K2 status has been linked to less calcification and therefore has a beneficial impact on lowering arterial stiffness, a major independent risk factor for cardiovascular complications (Diederichsen et al., 2022). Moreover, the gut microbiota is pivotal in the host's health. Indeed, changes in microbial composition has been linked to diseases including inflammatory bowel diseases and neurodevelopmental diseases.

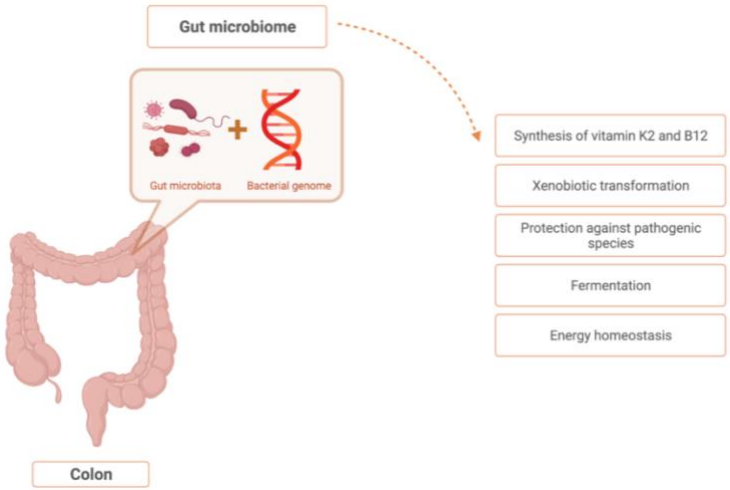

Figure 2.4: The gut microbiome, composed of the microorganisms living in the colon (gut microbiota) and their collective genome, has numerous important functions hereby influencing the hosts' physiology. Created in BioRender.

2.2.5.1 Factors influencing the composition of human gut microbiome

With the aid of next-generation sequencing, gut microbiome analyses have been applied to numeral human cohorts (Weersma et al., 2020). One important finding is the large inter-individual variability of the gut ecosystem: only a minority of the gut microbes are shared across individuals (Weersma et al., 2020). Moreover, it has been demonstrated that there is a complex interaction of the host with dietary habits, lifestyle, environmental and other factors. Throughout the past years, multiple factors including age, health status, immune system, diet and medication were shown to play a significant role on the composition of the gut microbiome (Kurilshikov et al., 2021). Twin, family

and population-based studies demonstrated that genetics can also determine gut microbiota composition (Kurilshikov et al., 2021). Due to the dynamic interactions between those factors and gut microbiota, gut microbiota display immense compositional variation between and within individuals. Indeed, besides changes in the gut microbiota between individuals, also variations within the same individual exist. These inter-individual variations are mainly due to enterotypes, body mass index (BMI) level, and external factors such as lifestyle, ethnicity and dietary and cultural habits (Rinninella et al., 2019). Some factors that modulate gut microbial composition are depicted by Figure 2.5 and will be briefly discussed below.

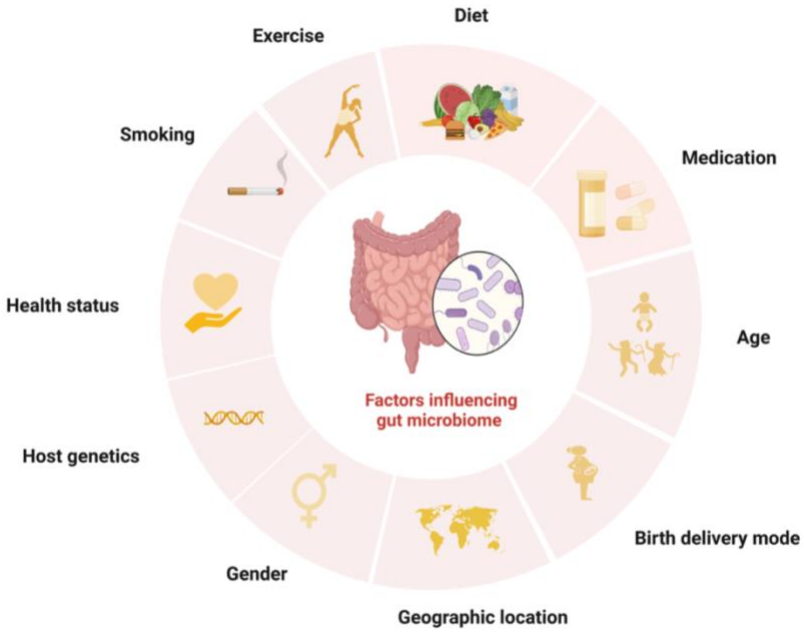

Figure 2.5: The composition of gut microbiome is influenced by multiple factors throughout life. The main modifiers include lifestyle, age, perinatal period as well as sociocultural environment. Created in BioRender.

2.2.5.1.1 Age and birth delivery method

The microbiota evolve with their host mainly during the first few years of life and even before. Moreover, the composition of the microbial community in the GI tract fluctuates throughout a lifespan since host's age significantly affects the microbial composition (Kim & Benayoun, 2020).

Until recently, it was believed children were born with a sterile gut; however, based on findings of recent studies reporting the presence of bacteria in the placenta and umbilical cord, microbial colonisation may already start *in utero* (Deering et al., 2020; Kim & Benayoun, 2020). During infancy, the gut microbiome significantly fluctuates, mainly driven by factors including delivery method, feeding, use of antibiotics and maternal diet (Kim & Benayoun, 2020). For instance, in vaginally born infants *Lactobacillus* spp. and *Prevotella* spp., microorganisms of the maternal vagina, will mainly colonise the intestines of the child whereas the intestines of infants born by cesarean section, are colonised by microorganism that resemble those of the maternal skin flora (*Streptococcum* spp*., Propionibacterium* spp*.* and *Corynebacterium* spp.) (Mortelé, 2021). In early childhood, gastrointestinal community diversity rapidly increases; however, it remains highly dependent on illness, changes in diet and other environmental factors. For instance, infants treated with antibiotics are found to have less abundance of *Lactobacilli* spp., *Bifidobacteria* spp., and *Enterococci* spp. (Nagpal et al., 2018). Overall, the microbiome composition reaches a somewhat stable structure by the age of two to three years, its profile resembling that of an adult microbiome (Kim & Benayoun, 2020; Nagpal et al., 2018; Yatsunenko et al., 2012). Nevertheless, this view is not universal and development of the microbiome may go on until puberty or even later (Deering et al., 2020). The gut microbiota profile then remains fairly constant during adulthood, although differences resulting from diet type or drug use have been described.

Upon ageing (ageing is considered to be a gradual and progressive deterioration of the integrity of multiple organs and tissues (Heinz, 2021)), a reduced bacterial diversity and metabolic capacity of the gut microbiome have been described in an elderly population (78-94 years) as compared to healthy, younger adults. For instance, in the elderly, the proportion of *Bifidobacteria* spp. and *Clostridium* spp. was declined while the proportion of *Bacteroidetes* spp. was higher as compared to young individuals (18-20 years). These differences in gut microbiota profile could be attributed to changes in lifestyle and dietary habits, a decline in general health and well-being, a weakened immune strength or use of medication (Nagpal et al., 2018).

2.2.5.1.2 Gender

Besides age-related differences in gut microbiome, also gender effects are observed. A cross-sectional study by Mueller et al. (Mueller et al., 2006) in 230 healthy subjects across four different European locations showed higher abundance levels of *Bacteroides*

spp.-*Prevotella* spp. in male as compared to female. In addition, studies in human neonates' stool samples at different timepoints during their first year of life revealed that male infants had a lower α-diversity as compared to females, which also showed higher abundance levels of *Clostridiales* (phylum Firmicutes) and lower abundance of *Enterobacteriales* (phylum Proteobacteria) (Valeri & Endres, 2021).

2.2.5.1.3 Dietary effects

Dietary habits are one of the main factors contributing to the diversity of human gut microbiota. Specific foods and dietary patters can influence the abundance of different types of bacteria in the gut, which in turn can affect human health (Mills et al., 2019; Valdes et al., 2018). For instance, a dietary pattern high in fats and mono-and oligosaccharides and low in dietary fibres (the so-called Western diet) can reshape the gut microbial ecology resulting in an enrichment of Firmicutes. Vegan and vegetarian diets on the other hand, have been linked to a greater abundance of the Bacteroidetes phylum (Jain et al., 2018; Thursby & Juge, 2017; Turnbaugh et al., 2009). Also, micronutrients (e.g. selenium) and polyphenols can promote the growth of beneficial bacterial species whereas certain food additives and alcohol negatively impact the intestinal barrier and subsequently can cause gut inflammation (Li & Zhang, 2022).

2.2.5.1.4 Medication

Also, associations between the use of specific drugs and altered microbial composition have been described. One of the major pharmaceutical drugs affecting the gut microbiota are antibiotics, affecting both pathogenic and beneficial bacteria. Although antibiotics are selectively administered to target a specific pathogenic population, many antibiotics on the market have broad-spectrum activities and will therefore also impact beneficial bacteria. Recently, long-term fluctuations in microbial populations have been reported in individuals after antibiotic use. For example, ciprofloxacin resulted in a loss of diversity and a shift in gut-microbiota composition within four days after the start of the therapy. Microbial composition started to return to initial conditions one week after the end of the treatment; however, within a period of 10 months, baseline conditions were still not met (Dethlefsen & Relman, 2011; Mortelé, 2021). Use of amoxicillin was associated with a depletion in *Lactobacillus* genus (Breynaert et al., 2015).

In addition to antibiotics, other pharmaceutical drugs such as proton pomp inhibitors (PPIs), lipid-lowering statins, laxatives, metformin, angiotensin-converting enzyme (ACE) inhibitors and beta-blockers have also been linked to changes in microbial composition

(Weersma et al., 2020). PPIs, among the most used drugs worldwide, have been prescribed to a growing number of patients to treat acid-related disorders such as peptic ulcers and gastro-oesophageal reflux (Le Bastard et al., 2018; Weersma et al., 2020). Taxonomical analyses of faecal samples of PPI users show a decrease in abundance of commensal bacteria of the intestine and an increase of bacteria from the oral cavity, more specifically an increase in the families *Enterobacteriaceae, Enterococcaceae* and *Lactobacillaceae* and a decrease in *Ruminococcaceae* and *Bifidobacteriaceae* while the shift towards typical oral bacteria is reflected by increases in the species *Rothia dentocariosa* and *Rothia mucilaginosa*, the genus *Actinomyces* and the family *Micrococcaceae* (Weersma et al., 2020). Multiple studies also reported that metformin treatment significantly increased *Escherichia coli* and lowered *Intestinobacter* abundance (Mortelé, 2021; Weersma et al., 2020). Nevertheless, it should be noted that there is a complex interaction between the use of medication, the gut microbiota and confounding factors. In fact, the use of medication by itself can indicate changes in the host's health status, which is also known to influence the gut microbial composition (Vich Vila et al., 2020).

Besides antibiotics, also probiotics can influence the microbial composition. According to the WHO probiotics are defined as "living microorganisms which when administered in adequate amounts confer health benefits on the host" (Hemarajata & Versalovic, 2013). Throughout the years, probiotics have been widely marketed and consumed, mostly as dietary supplements or functional foods. Mechanisms of probiotics include manipulation of intestinal microbial communities, suppression of pathogens, immunomodulation, stimulation of epithelial cell proliferation and differentiation and fortification of the intestinal barriers. Several trials have reported beneficial effects of probiotics (e.g. *L. johnsonni*, *Bifidobacterium breve*) on systemic immune response after oral intake, including overall health maintenance and reduction of the duration of common infections (Breynaert et al., 2015).

Following the most recent consensus statement of the International Scientific Association for Probiotics and Prebiotics (ISAPP), a prebiotic is considered to be "a substrate that is selectively utilised by host microorganisms conferring a health benefit" (Gibson et al., 2017). Fructo-oligosaccharides, galacto-oligosaccharides and trans-galactooligosaccharides are the most common prebiotics which can contribute to the health of the host (Wang et al., 2020). Compounds that can be classified as prebiotics must meet the following criteria: they are non-digestible and resistant to breakdown by

stomach acid and enzymes in the human GI tract, they can be fermented by microorganisms in the body and they stimulate growth and activity of beneficial bacteria.

Another term named “postbiotics” have emerged over the last years referring to substances derived after the microorganisms are no longer alive. These intact non-viable microbes or cell fragments can confer a health benefit on the host (Vinderola et al., 2022). Generally, postbiotics can provide similar benefits as probiotics and prebiotics.

2.2.5.1.5 Smoking

Smoking is associated with an increased morbidity and enhances the risk of developing CVD and different types of cancer (Shapiro et al., 2022). Moreover, it also results in a distinct shift in gut microbiota, perturbing the intestinal balance, which in turn is closely associated with intestinal (e.g. inflammatory bowel diseases) and extra-intestinal diseases (Yoo et al., 2020).

2.2.5.1.6 Exercise

Various studies in humans demonstrated that exercise is associated with an increased microbiome diversity. Athletes for instance show lower abundance levels of Bacteroidetes and higher abundance of Firmicutes as compared to non-athletes. Other bacterial taxa have also been reported to be altered in response to exercise (Gomaa, 2020). Moreover, research work by Barton et al. (Barton et al., 2021) provide further evidence that the human gut microbiome is affected by exercise.

2.2.5.1.7 Host genetics

Although the influence of host genetics on composition of the human gut microbiome is still not fully understood, some studies suggest that the intestinal microbiome of related humans is more similar in composition than that of unrelated individuals. Goodrich et al. (Goodrich et al., 2014) reported higher similarities in gut microbial composition for monozygotic twins compared to dizygotic twins. The heritable component was demonstrated for taxa from the phyla Firmicutes and Actinobacteria, while members of the phylum Bacteroidetes were considered as not heritable (Goodrich et al., 2014, 2017; Mortelé, 2021).

2.2.5.1.8 Geographical impact

The geographical location impacts the microbial populations due to prominent differences in dietary and other factors connected to diverse lifestyles. Therefore, the abundance of a certain taxon can vary from region to region, resulting in gut microbial differences (Cresci & Izzo, 2019).

2.2.5.1.9 Health status

The overall ecology of the gut microbiome has frequently been linked to host health status. For instance, according to an intervention study by Dao et al. (Dao et al., 2016) individuals with obesity and type 2 diabetes differ from lean and healthy individuals in their abundance levels of certain gut microbial species and microbial gene richness.

2.3 Bioavailability of polyphenols

Possible biological activities of dietary polyphenols are determined by their bioavailability, intestinal absorption and biotransformation in the GI tract, which depend on their chemical structure. Considerable differences between various types of polyphenols have been reported (Ozdal et al., 2016). To understand the beneficial effects of polyphenolic compounds, determination of their bioavailability and biotransformation is important. In general, the way these compounds are handled by the body is distinctive to that for other xenobiotics (Cardona et al., 2013). Regardless of the amount of ingested polyphenol-rich food, the bioavailability of the native polyphenols tends to be only in the nanomolar (nM) to low micromolar (μM) range (D'Archivio et al., 2007; Goszcz et al., 2017).

Although polyphenols can be found in a wide range of fruits and vegetables, it has been estimated that only 1-10% of the total polyphenol intake is found in plasma and urine samples (Nicholson et al., 2008). Bioavailability of polyphenols is determined by their rate, site and means of absorption (Goszcz et al., 2017). Direct interactions between polyphenols and other food components such as carbohydrates and proteins are also known to affect their absorption (D'Archivio et al., 2007). Moreover, most polyphenols are present in dietary sources as conjugates/derivatives (esters, glycosides (conjugated to various sugars) or polymers) which can be very complex structures with high molecular weight, therefore limiting their absorption in their native form (Manach et al.,

2004). For instance, the molecular weight of procyanidins, oligomers of catechin, epicatechin and their gallic acid esters, ranges from 500 to 20,000 g/mol (Sepúlveda et al., 2011).

After ingestion, polyphenols interact with salivary proteins and limited biotransformation processes take place in the oral cavity (Niwano et al., 2023). The majority will first pass the stomach without being absorbed, being resistant to the stomach's acidic conditions and therefore facilitating their transit to the small intestine as intact compounds (De Bruyne et al., 2019; Goszcz et al., 2017; Sepúlveda et al., 2011). Only some small phenolic acids do have low levels of bioavailability here. For example, ferulic acid is absorbed relatively faster compared to other phenolic compounds from the stomach (Alam, 2019). Though most polyphenolic compounds resist the acidic environment, it is still possible that some oligomers and polymers are cleaved into monomers such as the dimer procyanidin B2 which is partially cleaved to release epicatechin in the stomach (Zhu et al., 2002). Nevertheless, while passing through the stomach, only a few compounds will undergo hydrolysis while other numerous polyphenolic compounds remain intact and pass on through to the small intestine (Hussain et al., 2019).

In the small intestine, absorption mainly occurs in the duodenum and the proximal half of the jejunum. However, only aglycones (lacking their sugar moiety) and some glycosides can be absorbed by the intestinal mucosa (Williamson & Manach, 2005). Only a small amount of the polyphenols'intake (about 5-10%) will be absorbed in the small intestine, mostly those with monomeric and dimeric structures (Corrêa et al., 2019). To allow absorption, human digestive and microbial enzymes are needed to release native polyphenols from their sugar moiety, allowing them to pass through the intestinal barrier (Hollman & Katan, 1997; Manach et al., 2004). Here, some glycosides can be deglycosylated into their aglycon by enzymatic activity of lactase phloridizin hydrolase (LPH) or cytosolic β-glucosidase (CBG). Nevertheless, it should be noted that glycosides bound to sugars other than glucose, such as rhamnosides and rutinosides, are no substrates for these enzymes and thus not all glycosides will be biotransformed into their aglycon in the small intestine (van der Auwera, 2023). Partial deconjugation into more lipophilic aglycones by hydrolases in the lumen makes passive diffusion into enterocytes possible (Goszcz et al., 2017). Apical cell membranes of enterocytes contain microvilli, which increase the surface area of absorption and therefore facilitate absorption. Once inside the enterocytes, polyphenols can be released into the lumen by efflux

transporters (De Bruyne et al., 2019). After absorption in the small intestine, aglycones undergo biotransformation processes in the enterocytes and hepatocytes. These biotransformation products are then distributed to organs and excreted in the urine (Corrêa et al., 2019).

The remaining part of polyphenols (in the form of esters, glycosides or polymers) will pass unaltered through the small intestine towards the colon where most of the biotransformation and absorption is situated. Here, intestinal microbial enzymes as well as intestinal cell membrane hydrolases are mostly responsible for these biotransformation reactions into a wide range of low-molecular phenolic compounds, which render them available for subsequent absorption and conjugation (Hussain et al., 2019). Generally, the native compounds can undergo following major processes by the gut microbiota: hydrolysis (deglycosylation and ester hydrolysis), cleavage (C-ring cleavage, delactonisation, demethylation) and reductions (dehydroxylation and double bond reduction) (van der Auwera, 2023). The intestinal microbiota is equipped with a variety of enzymes like α-rhamnosidase, β-glucosidase, and β-glucuronidase, which are needed for hydrolysis of the sugar moiety of glycosides, resulting in aglycons, and thereby influencing the bioavailability (Corrêa et al., 2019). Additionally, gut microbiota is responsible for biotransformation of high-molecular weight polyphenolic compounds into smaller, phenolic acids. For instance, the human microbial biotransformation of quercetin has been thoroughly documented and a series of phenolic acids have been identified to date (Di Pede et al., 2020). Once absorbed, they pass through the portal circulation to the liver where these less complex polyphenolic compounds may be subjected to extensive phase I (oxidation, reduction and hydrolysis) and particularly phase II (conjugation) biotransformation reactions in the enterocytes followed by the hepatocytes, and resulting in a series of water-soluble conjugated biotransformation products (methyl, glucuronide and sulfate derivatives) which are rapidly released into the systemic circulation for further distribution to organs and excretion in urine (De Bruyne et al., 2019; Faria et al., 2014). Indeed, extensive conjugation occurs on first pass through the liver, a metabolic detoxification process that is common to many xenobiotics, acting to prevent potential toxic effects, and is followed by urinary elimination due to the increased hydrophilicity of the conjugates (D'Archivio et al., 2007). Nevertheless, the rate of absorption is quite low and the majority of ingested polyphenols is excreted in the faeces (Goszcz et al., 2017; Scalbert &Williamson, 2000).

Polyphenols can thus be found in plasma in their native intact form, their glucuronidated and/or methylated forms, and these may thus act as the true biological active agents. Besides the phase II biotransformation products, also microbial biotransformation products (i.e. gut microbial transformation of complex phenolic structures resulting into smaller, phenolic acids) can reach the bloodstream and exert biological effects (de Bruyne et al., 2019; Goszcz et al., 2017). A schematic representation of Absorption, Distribution, Metabolisation and Excretion (ADME) processes of ingested polyphenols is depicted by Figure 2.6.

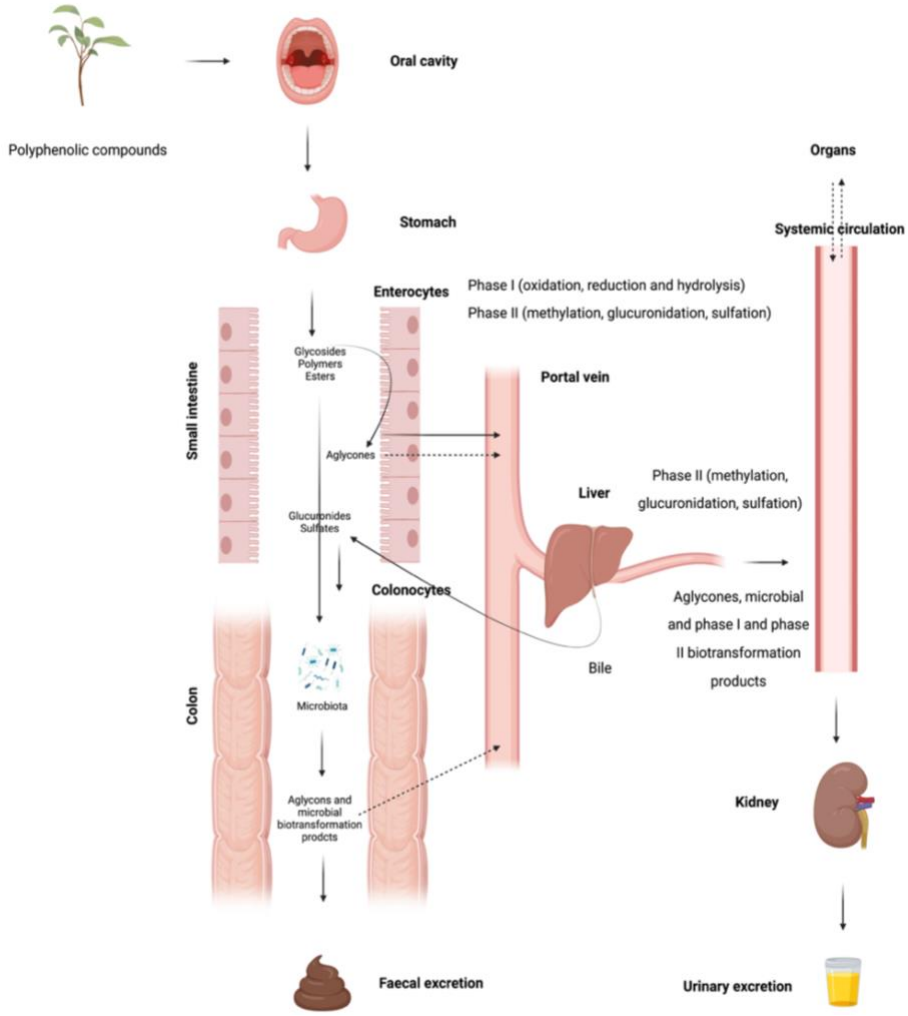

Figure 2.6: Schematic representation of absorption, biotransformation, and excretion of polyphenols in the human body. The gastrointestinal tract acts as a physical barrier, is covered by the mucosa, and is decisive for polyphenol bioavailability. Created in BioRender.

Numerous factors have been reported to affect the oral bioavailability of dietary polyphenols (see Table 2.1): phenolics related, food related, host related and other factors. Additionally, all these factors can interrelate with one another, making an interpretation of the bioavailability and thus the particular mode of action of these phenolic compounds even more difficult (Hussain et al., 2019). One of the main factors influencing the bioavailability is the chemical structure of the compound itself. Most polyphenols appear as polymers or in glycosylated forms in their food matrix which cannot be absorbed as such, but need prior hydrolysis by the intestinal enzymes or by the colonic microflora. The specific chemical structure of polyphenols as well as the type of the sugar therefore determine their rate and extent of intestinal absorption (D'Archivio et al., 2010). Also, the interaction with other compounds appears to play a role. It has been reported that several compounds (e.g. quercetin biotransformation products) show a high affinity for serum albumin, which potentially results in a longer half-life in the blood and may have consequences for the delivery of polyphenols and their biotransformation products to cells and tissues (D'Archivio et al., 2010; Manach et al., 2004). Moreover, interactions with other polyphenols with the same route of absorption may also influence the bioavailability (D'Archivio et al., 2010). Host related factors, including both intestinal and systemic factors are also known to influence bioavailability. Intestinal factors such as enzyme activity and composition of the intestinal microbiome, are probably the most important ones. Furthermore, systemic factors such as age, physiological condition and/or disorder are also known to impact bioavailability (Porrini & Riso, 2008). In addition, environmental factors such as sun exposure, rainfall and the degree of ripeness affects the plant concentrations of various polyphenols in different ways (Gómez-Rico et al., 2006; Manach et al., 2004). For example, it has been demonstrated that the concentration of phenolic compounds in extra virgin olive oil decrease with the ripeness of olive fruits (Gómez-Rico et al., 2006).

Moreover, several studies indicate a bidirectional relationship between polyphenols and gut microbiota: microbiota are responsible for biotransformation processes of polyphenols whereas in turn, parent polyphenols and their biotransformation products can influence the composition of the microbiome, by increasing the number of beneficial bacteria and hampering the increase of pathogenic bacteria (De Bruyne et al., 2019; Mari et al., 2009). This topic will be further discussed in Chapter 5.

Table 2.1: Main factors affecting the human bioavailability of dietary polyphenols. Adapted from D'Archivio et al., 2010.

Phenolics related factors	Chemical structure	Chemical structure solubility: substitution with sugars (glycosides), methyl groups, etc.
	Interaction with other compounds	Bonds with proteins (i.e. albumin) or competition with other polyphenols with a similar mechanism of absorption.
Food related factors	Food processing	Thermal treatments, cooking and other methods of culinary preparation.
	Food interaction	Food matrix: positive or negative influence on absorption
Host related factors	Dietary intake	Differences between frequency of exposure
	Absorption and metabolism	Intestinal factors (i.e., enzyme activity; intestinal transit time; colonic microflora). Systemic factors (i.e., gender and age; disorders and/or pathologies; genetics; physiological condition)
Other factors	Environmental factors	Food availability, degree of ripeness

2.4 Investigation of the biotransformation of PBE: GIDM-Colon/MS-data analysis workflow

Breynaert et al. (Breynaert et al., 2015) previously developed and validated an *in vitro* continuous flow dialysis model with colon phase (GastroIntestinal dialysis model with colon phase; GIDM-Colon) to mimic human biotransformation processes. This semi-continuous model simulates the stomach, small intestine and colon physiological conditions by using digestive enzymes and a culture of pooled human faeces respectively, thereby avoiding numerous *in vivo* studies. These faecal samples are easy to collect and represent the gut microbiota well (Hillman et al., 2017; Wahlgren et al., 2019).

The GIDM-Colon was validated using chlorogenic acid, one of the main polyphenols of the human diet, and showed a good recovery and precision (coefficient of variation < 16%). The biotransformation of chlorogenic acid has already been studied in both *in vitro* and *in vivo* conditions (Naranjo Pinta et al., 2018). According to *in vitro* research (Breynaert et al., 2015), the presence of human faecal microbiota during the colon phase of the GIDM-Colon led to the deconjugation of chlorogenic acid to caffeic acid, 3,4-dihydroxyphenyl propionic acid, 4-hydroxybenzoic acid, 3- or 4-hydroxyphenyl acetic acid, 2-methoxy-4-methylphenol and 3-phenylpropionic acid. When considering the human *in vivo* situation, it has also been demonstrated that gut microbiota may play an essential role in the biotransformation of chlorogenic acid into caffeic acid and dihydrocaffeic acid (Lu et al., 2020).

The biotransformation of PBE (Figure 2.7) was studied using this validated GIDM-Colon model as described by Breynaert et al. (Breynaert et al., 2015) and further optimised by Mortelé et al. (Mortelé, 2021). A total fermentation period of 72 h was applied, simulating a transit time of 72 h in the gut, during which samples were taken at different timepoints. Nevertheless, during all experiments, dialysis was not performed since this dialysis system is currently not compatible with our data analysis workflow due to for instance differences in pressure in between the different dialysis cells. Moreover, earlier research work by (Peeters, 2022) showed that the filter used in the dialysis cells can adsorb compounds, which can result in a loss of compounds in the solution that will be analysed. Besides PBE, also the pure compound (+)-catechin was subjected to *in vitro* gastrointestinal biotransformation to obtain an overview of its biotransformation products formed, without interference by other compounds.

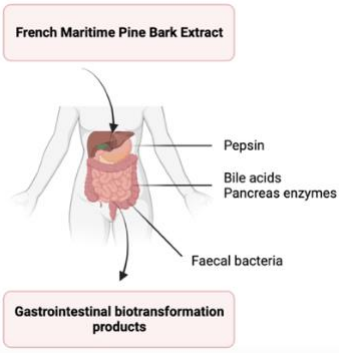

Figure 2.7: Investigation of the biotransformation processes of French Maritime Pine Bark Extract. Created in BioRender.

2.4.1 Chemicals and reagents GIDM-Colon

Sodium phosphate dibasic (Na_2HPO_4, ≥99%), sodium phosphate monobasic dihydrate ($NaH_2PO_4 \cdot 2H_2O$, ≥99%), thioglycollate broth, pepsin from porcine gastric mucosa, porcine bile extract, pancreatin from porcine pancreas, theophylline (≥99%, anhydrous), chlorogenic acid (≥95%) and (+)-catechin (≥98%) were acquired from Sigma-Aldrich (St Louis, MO, USA). French Maritime Pine Bark Extract (LOD 2.70%) was kindly offered by Horphag Research, Geneva, Switzerland. Ultrapure water (milli-Q) and sodium bicarbonate ($NaHCO_3$, >99.7%, ACS grade) were obtained from respectively Merck (Darmstadt, Germany) and Acros Organics (Geel, Belgium). Hydrochloric acid (HCl, 32 wt.% for analysis), formic acid (98–100%, Suprapur) and sodium hydroxide pellets (NaOH) were acquired from Merck (Darmstadt, Germany). UPLC-grade acetonitrile, formic acid and methanol (≥99.9%, LC– MS grade) were obtained from Fisher Scientific (Hampton, New Hampshire, USA). Nitrogen gas (N_2, AZOTE N28) and a gas mixture of hydrogen, carbon dioxide and nitrogen (5% H_2, 5% CO_2 and 90% N_2, Alphagaz Mix) were obtained from Air Liquide Belge (Liège, Belgium).

2.4.2 Materials GIDM-Colon

A shaking warm water bath from VWR (Leuven, Belgium) was used during the gastric stage of the GIDM-colon model. Another warm water bath set at 37 °C was used to mimic the small intestinal phase. A Jacomex glove box T3 from TCPS (Rotselaar, Belgium) was used to create an anaerobic environment during the colon stage (Figure 2.8).

Figure 2.8: The glove box provides an anaerobic environment containing 5% H_2, 5% CO_2 and 90% N_2. The black gloves are used to take samples from within the glove box at different timepoints during the colon stage.

2.4.3 Preparation digestive juices

To mimic human *in vivo* situation, digestive conditions and enzymes were used during the GIDM-Colon experiments (Breynaert et al., 2015). The digestive juices used during the different stages of the GIDM-Colon experiment were prepared as follows: a pepsin solution was made by dissolving 16% (*w/v*) of pepsin powder in 0.1 M HCl (622,000 FIP-U.100/mL), a pancreatin-bile mixture contained 0.4% (*w/v*) pancreatin from porcine pancreas and 0.8% (*w/v*) bile extract (porcine) dissolved in 0.1 M $NaHCO_3$ (32,000 FIP-U lipase; 143,600 FIP-U amylase; 16,400 FIP-U protease and 58.4 mmol bile/L).

2.4.4 Collection and characterisation of faecal samples

Human faecal donors were selected, 6 female and 6 male aged 20-30 years, meeting all inclusion criteria. An overview of the main inclusion criteria can be found in Table **2.2**. Eligible donors needed to be non-smoking, non-vegetarian or vegan persons with a normal defecation (no history of gastrointestinal disease or acute (gastrointestinal) disease at the moment of donation), and no intake of anti/pre/probiotics three months prior to donation. Prior ethical approval was acquired from the Ethical Committee of the University Hospital of Antwerp (reference number 20/35/444).

Table 2.2*:* Overview of the main inclusion criteria of the faecal donors.

	Young, healthy
GROUP SIZE	♀6 ♂ 6
AGE (years)	20 - 30
BMI (kg/m²)	18.5 - 25
WAIST CIRCUMFERENCE (cm)	♀< 88 ♂< 102

BMI: Body Mass Index.

Donors were asked to collect the faeces by means of a collection container (Protocult, Rochester, USA) and a paper dish holder to insert into the toilet bowl (Mortelé, 2021). After collection, faecal samples were kept at room temperature together with an

anaerocult bag from Merck (Darmstadt, Germany) and processed as soon as possible with anaerobic buffer in an anaerobic glove box (5% H_2, 5% CO_2 and 90% N_2) (Jacomex Glove (Box) T3, TCPS, Rotselaar, Belgium). For each participant, a faecal slurry suspension of 10% (*w/v*) faeces was prepared by homogenising the faecal sample with sterile phosphate buffer (0.1 M, pH 7.0, 0.58% (*w/v*) Na_2HPO_4, 1.03% (*w/v*) $NaH_2PO_4.2H_2O$, 3.45% (*v/v*) thioglycolate broth) in a stomacher (Lab-blender 400, Seward Medical, London, UK) for 3 min. Before use, the buffer solution was sonicated and autoclaved (1 bar, 121 °C). Elimination of solid food particles was done by using a sterile filter bag consisting of a full-page filter (Bagpage® R/25 400 mL, VWR (Leuven, Belgium). From each donor aliquots of 20 mL faecal slurry were stored at -80 °C until further analysis (Figure 2.9).

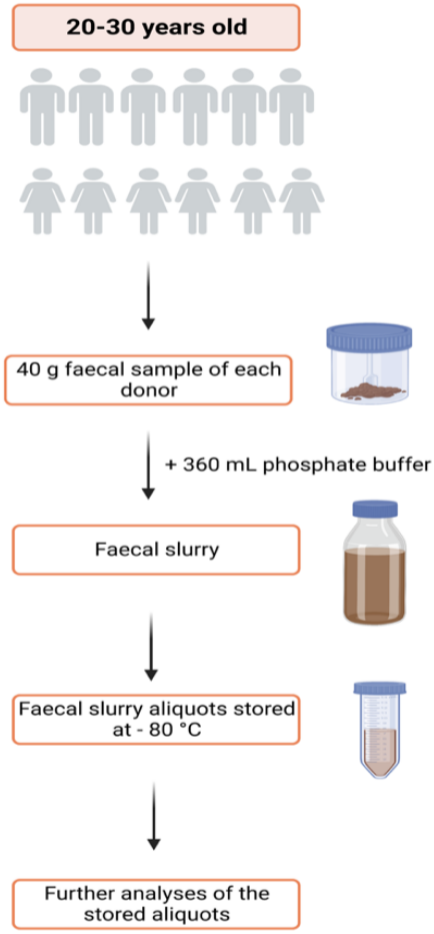

Figure 2.9: Overview of the faecal sample treatment after collection and before further analysis. Created in BioRender.

To elucidate the microbial composition of each individual faecal suspension, 16S rRNA V1-V9 gene sequencing was performed, in collaboration with the lab of prof. Lebeer (Lebeer Lab, University of Antwerp). First, DNA was isolated from faecal samples using the QIAmp DNA Stool Mini Kit (Qiagen, Venlo, Netherlands) according to the instructions of the manufacturer. The concentration of isolated DNA was assessed using a Qubit 2.0 Fluorometer (Thermo Fischer Scientific, MA, USA) with the dsDNA HS Assay Kit. Quantitative PCR (qPCR) was used for estimation of absolute bacterial, fungal and human DNA concentrations in samples after extraction. Illumina MiSeq 16S rRNA gene amplicon sequencing was performed on the extracted DNA and no less than 5 µL of each bacterial DNA sample was used to amplify the V1-V9 region of the 16S rRNA genes. Amplification of the V1-V9 gene region in the 16S rRNA was executed in triplicate with 2x KAPA HiFi HotStart Ready Mix (Kapa Biosystems, Amsterdam, The Netherlands) with the following cycling profile: 95 °C for 3 min, [95 °C for 30 sec, 62 °C for 30 sec, and 72 °C for 50 sec] x 25 and 72 °C for 10 min. Standard barcoded forward (515F) and reverse primer (806R) were used and resulting PCR products were checked on a 1% agarose gel to confirm amplification of fragments with an appropriate length (threshold set at 200 base pairs). Quality control and processing of the 16S rRNA amplicon reads were performed using the R package DADA2. Forward and reverse reads were denoised per sample using the DADA2 algorithm and the reads were then merged. These merged and denoised reads (amplicon sequence variants or ASVs) were taxonomically annotated from phylum to species level using the 16S rRNA reference database (Yoon et al., 2017) and all data handling and visualisation was performed in R using the tidyverse set of packages and the in-house developed package tidyamplicons (github.com/Swittouck/tidyamplicons).

2.4.5 Stages of the GIDM-Colon

The different phases of the biotransformation process according to the stage of the GI tract where they occur, will be discussed here below. All experiments include samples, method blanks, positive and negative controls. Samples (denoted as S1, S2, S3 in Figure 2.10) contain the compound or extract to be investigated (in this case (+)-catechin and PBE respectively) and are treated with digestive enzymes and faecal microflora (faecal slurry). During each experiment, the biotransformation of PBE (in a dose of 200 mg, reflecting an average daily intake in adults) as well as (+)-catechin, the major constituent of the procyanidin fraction in PBE, were investigated. Catechin was tested in an arbitrary dose of 25 mg, which is slightly lower than the expected theoretical amount of (+)-

catechin in PBE based on D'Andrea et al. (Table 1.1). Negative controls (NCs) were used and contain the compound or extract of interest with addition of digestive enzymes but without faecal bacteria. In these NCs, no biotransformation is observed since faecal slurry is absent; however, only chemical degradation by the digestive enzymes or due to instability of the compounds is possible. Method blanks (denoted as BL in Figure 2.10) contain digestive enzymes and faecal bacteria without addition of the compound or extract. In these method blanks bacterial biotransformation products formed during fermentation can be observed. Chlorogenic acid is used as a positive control since the gastrointestinal biotransformation of chlorogenic acid in humans has already been investigated in several studies and was used as a model compound to validate the GIDM-colon (Breynaert et al., 2015; Gonthier et al., 2006; Olthof et al., 2003). A dose of 75 mg was selected as a positive control as this approximately reflects a daily ingestion of a non-coffee drinker (Olthof et al., 2001). The different samples, method blanks, positive and negative controls were weighted and labeled accordingly. Use of a negative control allows to distinguish microbial biotransformation products from other possible compounds formed during the experiment whereas use of the positive control chlorogenic acid makes it possible to check whether known biotransformation products are formed and is therefore a good indication for the reliability of the experiment.

2.4.5.1 Gastric stage

To mimic the gastric stage all samples were dissolved in 33.5 mL milli-Q and 16.5 mL pepsin solution (19.6% protein, 622 U/mg protein) and adjusted to a pH of 2 using 6 M HCl. 2 mL of each sample solution was collected at timepoint zero (T0), thus before the start of the biotransformation. Afterwards, samples were transferred to a shaking water bath at 37 °C (120 strokes/min) for 1 h. After simulation of the gastric conditions, another 2 mL of sample solution was taken (G).

2.4.5.2 Small intestinal stage

To simulate the small intestinal stage, 50 mL milli-Q was added to the gastric digest. To adjust the pH to 7.5, a volume of 1 M $NaHCO_3$ was added to the negative control samples. The same volume was then added to the other samples using a small dialysis bag to gradually change the pH. Afterwards, samples were placed in a water bath (37 °C) and continuously stirred for 1.5 h. After 30 min of stirring, 15 mL pancreatin-bile solution was

added to each sample and stirring was continued for another hour. After the small intestinal stage 2 mL was collected from each sample solution (SI).

2.4.5.3 Colon stage

After the small intestinal phase, 50 mL sterile phosphate buffer was added to the negative control samples and the pH was adjusted to 5.8 – 6 using 1 M HCl. The same volume of 1 M HCl was also added to the other samples. Subsequently, all samples were placed in the anaerobic glove box (0.5% O_2, 35 – 37 °C), continuously stirred, with a degasser system (parafilm with holes). A faecal slurry suspension (a mix of individual faecal slurries (see Table 2.2)) was continuously stirred in the glove box for 1 h before adding 50 mL of this 10% (*v/v*) faecal slurry suspension to each sample, except for the negative controls). During the colon phase, an aliquot of each sample was taken (2 mL) at several time points: C2, C4, C6, C12, C18, C24, C30, C36, C42, C48, C54, C60, C66 and C72. C0 reflects the start of the colon stage (not used in the LC-MS analysis, only for culture analyses).

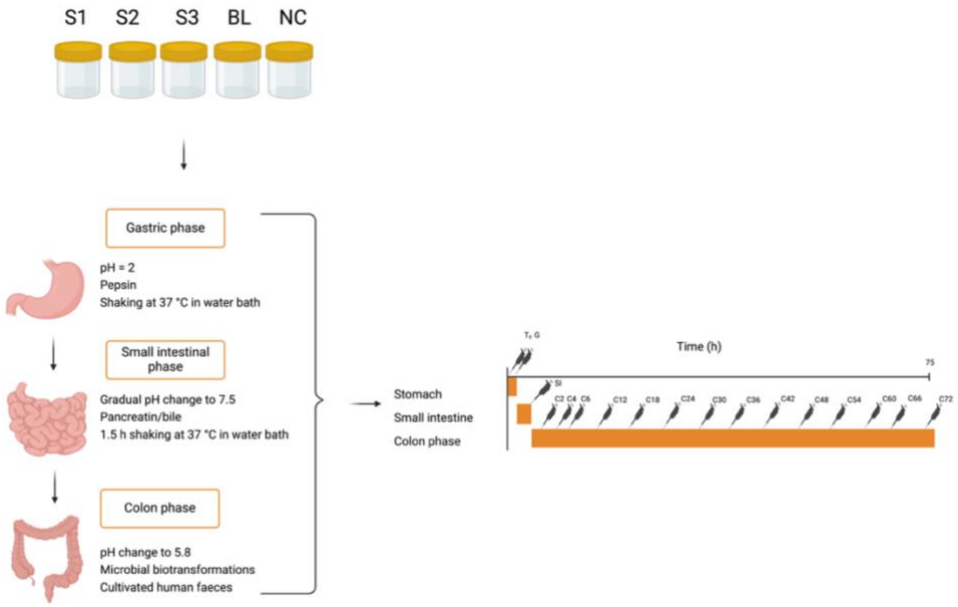

Figure 2.10: Set-up of different phases during the GIDM-Colon experiments. S1, S2 and S3 are samples with the extract or compound of interest, BL is the blank method sample and NC is the negative control used during the experiment set-up. T0 (timepoint zero), G (gastric stage), SI (small intestinal stage), C2-C72 (colon stage) are the different timepoints at which samples were taken. Created in BioRender.

2.4.6 Experimental procedures amplicon sequencing and culture analyses

At different timepoints during the colon stage (C0, C24, C30, C48 and C72) sample solutions were taken from the PBE sample, (+)-catechin and blank sample for amplicon sequencing analyses (according to the procedure as described above) to check whether PBE or (+)-catechin alters the microbial composition over time. Stacked bar plots were generated to check the microbial composition at genus taxonomical level of the different sample solutions taken during the colon stage.

The alpha diversity (α-diversity) which outlines the microbial community in individual samples with respect to its richness (number of taxonomic groups), evenness (distribution of abundances of the groups), or both was calculated with the inverse Simpson index (Oerlemans et al., 2022). Assessment of beta diversity (β-diversity; term used to describe differences in microbial composition between individuals) was performed by a dissimilarity matrix (Bray-Curtis dissimilarity) and results of the β-diversity estimates were visualised using principal coordinate analysis (PCoA). To find differences in the abundance of specific taxa between the three classes of samples (blank, (+)-catechin and PBE), a differential abundance analysis was performed. This in-house codifab-method (developed by the Laboratorium of Applied Microbiology and Biotechnology, Department of Bioscience Engineering, University of Antwerp, Belgium) allows the pairwise combination of taxa with calculation of the logratio for each sample. For each combination of taxa, the difference of their logratio between two groups of samples was expressed as the median of all pairwise differences between the two sample groups (Hodge-Lehmann estimator). Next, for each combination of taxa, a Wilcoxon rank-sum test was performed to compare their logratio between two groups of samples. The generated plot visualises the differential abundances of taxa between conditions compared to all other taxa as references.

Since PBE is known to exert antimicrobial properties in concentrations starting from 0.025% as demonstrated by (Calvo Torras et al., 2005), the effect of the extract on the viability of microorganisms was assessed. Viability of the faecal bacteria was determined by means of decimal dilution series of the samples and C0 and C72 sample solutions were sent out for automated cell count analysis while the other timepoints were visually evaluated to see if microorganisms remain present throughout the experiment. An anaerobic basal agar, Tryptic Soy Agar and blood agar were selected to support good

growth of anaerobic bacteria. A volume of 10 µL of every dilution (ranging from 10^{-3} to 10^{-10}) was plated out in triplicate under anaerobic conditions and incubated for 48 h at 37 °C. Colonies were counted after 24 h and 48 h.

2.4.7 Experimental procedures LC-MS analysis

2.4.7.1 Preparation of standard solutions

For the analysis of PBE, two standard mixtures were used. The first mixture (Cacao standard LC-MS mix) was prepared using stock solutions of the following analytes: 2-phenylethylamine (HCl), phloroglucinol (hydrate), tyramine, linalool, tryptamine, serotonin, salsolinol (HBr), theobromine, theophylline, L-tyrosine, caffeine, tryptofaan, resveratrol, kaempferol, (-)-epigallocatechin, (-)-gallocatechin, N-oleoylethanolamine, anandamide, epigallocatechin gallate and procyanidin B1. The second mix (general standard LC-MS mix) consisted of benzoic acid, p-hydroxybenzoic acid, salicylic acid, coumarin, cinnamic acid, protocatechuic acid, p-coumaric acid, vanillic acid, gallic acid, caffeic acid, ferulic acid, syringic acid, sinapic acid, apigenin, emodin, naringenin, luteolin, (+)-catechin, epicatechin, quercetin, taxifolin, isorhamnetin, chlorogenic acid, stigmasterol, β-sitosterol, quercitrin, β-carotene, procyanidin B2, rutin and tannic acid. Standard stock solutions of the analytical standards were prepared at a concentration of 1 mg/mL in UPLC-grade MeOH for each analyte separately. Serial dilutions of these solutions were prepared in MeOH:H_2O (20:80, (*v/v*)). Following dilutions were used for LC-MS analysis: 1.25 µg/mL, 625 ng/mL, 312 ng/mL, 156 ng/mL, 78 ng/mL and 39 ng/mL. Standard stock and working solutions were stored at -80 °C in the dark.

2.4.7.2 Sample preparation LC-MS analysis

At different stages of the GI tract, sample solutions (2 mL) were taken. 0.5 mL of each sample was then transferred to 1 mL methanol (MeOH) (MS grade) and centrifuged for 10 min at 3500 rpm. Supernatant (0.1 mL) was then collected, diluted 10 times with MeOH:milliQ (20:80, (*v/v*)) and stored at -20 °C until further analysis with UHPLC-ESI-QTOF-MS (Xevo® G2-XS QTOF; Waters). This sample preparation allows to avoid interference of matrix compounds in the UHPLC-HRMS chromatograms of the biotransformed samples. Addition of MeOH (MS grade) followed by centrifugation was

used to quench biotransformation reactions. Applying the 1:10 dilution rate is a compromise between sample clean-up and detection of potentially low metabolite levels which should not be overlooked.

2.4.7.3 Ultra-high-performance liquid chromatography

Ultra-high-performance liquid chromatography (UHPLC) analyses were performed on a Waters Acquity UPLC system with degasser, quaternary pump, autosampler, thermostatic column compartment and TUV detector (Waters). An Acquity UPLC BEH Shield RP18 column (100 µm × 2.1 mm, 1.7 µm; Waters) was used, operated at 40 °C. An appropriate pre-column was used to prolong the lifetime of the column. The mobile phase solvents consisted of milli-Q + 0.1% MS-grade formic acid (A) and MS-grade acetonitrile + 0.1% MS-grade formic acid (B) and the following gradient was used (min/B %): 0/2, 1/2, 14/26, 24/65, 26/100, 29/100, 31/2, 41/2. The flow rate was set at 0.4 mL/min. Raw data were processed using EmpowerTM software (Waters) and the area under the curve (AUC) was calculated.

2.4.7.4 Mass spectrometry

A quadrupole time-of-flight (QTOF) spectrometer, using electrospray ionisation (ESI), was applied to analyse the different samples taking during the GIDM experiments. For detection, accurate mass measurements were performed using a Xevo® G2-XS QTOF spectrometer (Waters) coupled with an ACQUITY LC system equipped with MassLynxTM 4.1 software. Full scan data were recorded in ESI (+) and ESI (-) mode from *m/z* 50 to 2000 and the analyser was used in sensitivity mode (approximate resolution: 22,000 FWHM). The spray voltage was set at respectively +1.5 kV or -1.0 kV for the ESI (+) and ESI (-); cone gas flow and desolvation gas flow at 50 L/h and 1000 L/h respectively; source temperature and desolvation temperature at 120 °C and 500 °C respectively. Data were also recorded using MSE in positive and negative ionisation modes, providing separate MS and MSE data. A ramp collision energy ranging from 20 V to 30 V was applied to gather supplementary structural information. For the lock mass leucine encephalin was used. The mixture with reference compounds was analysed under the same conditions as the samples taken during both GIDM experiments to allow a qualitative and semi-quantitative determination of these compounds. Throughout the analysis quality control (QC) samples were injected to observe analytical drift and assess precision. QC samples were made using a dilution of the standard solution mix (39 ng/mL). Figure 2.11 shows a total ion chromatogram (TIC) obtained for the QC mix.

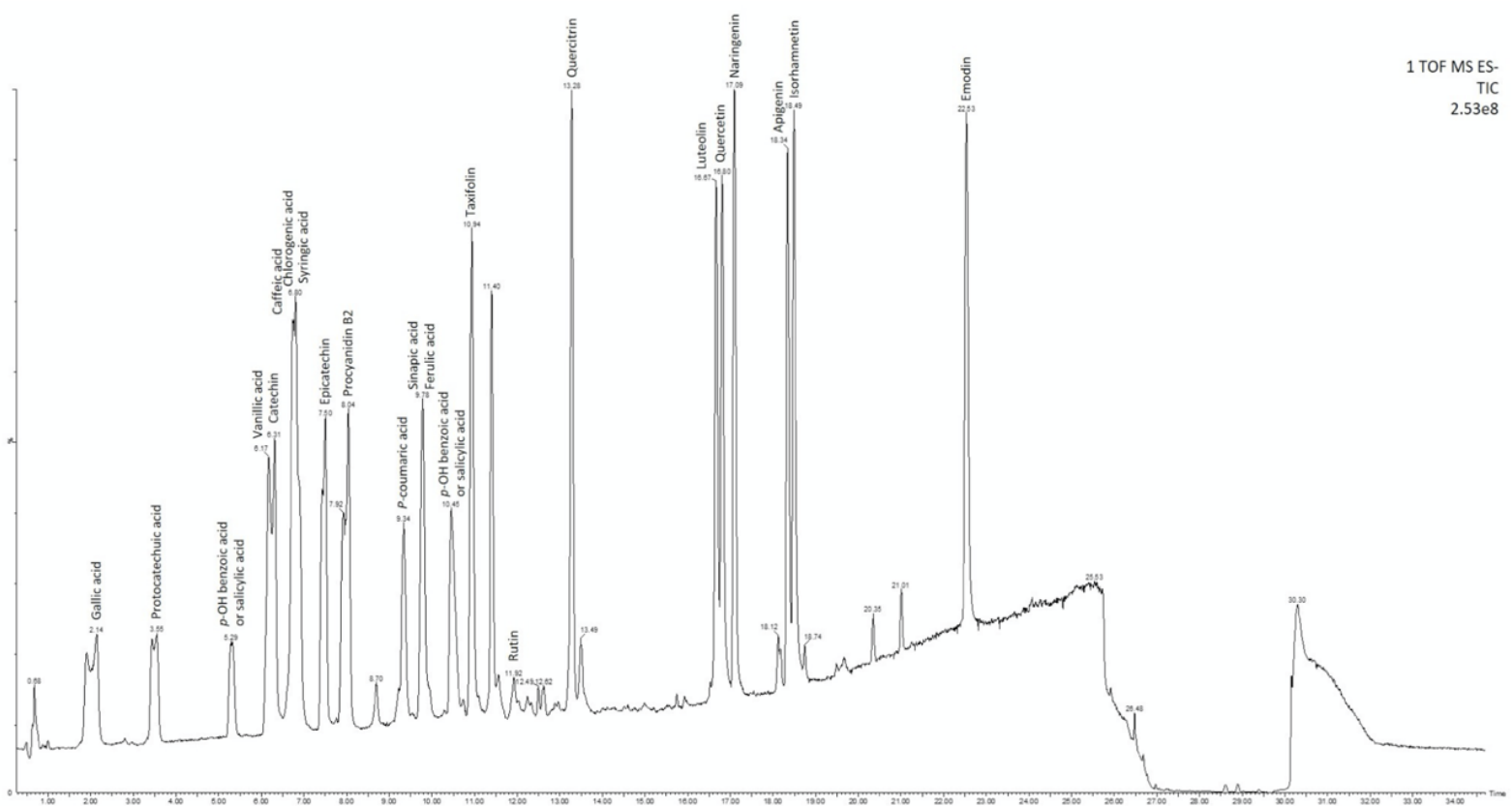

Figure 2.11: Total ion chromatogram (time in min) in negative ionisation mode obtained for the quality control mix. Adapted from Peeters, 2022

2.4.8 In-house automated data analysis workflow

The concentrations of phytochemicals and their biotransformation products exhibit continuous and fairly complex modulations throughout the experiment. During the biotransformation processes at the different stages of the GI tract, the concentration of a particular phytochemical and/or metabolite can either increase, decrease or show any combination of these two. Due to the longitudinal aspect (measurement in function of time) of the experiment, data are complex and difficult to interpret. In addition, the gastrointestinal enzymes and faecal microflora present in the samples provoke matrix interference, which makes analysis complicated (Peeters, 2022). Indeed, before biotransformation, peaks in the chromatogram can mostly be attributed to the tentatively identified compounds whereas after the gastric, small intestinal and colon phase, the chromatogram contains peaks of compounds, biotransformation products as well as matrix interferences originating from enzymes, bile salts and faecal slurry.

The number of methods available to analyse such dynamic data is limited and most of them are not specifically designed for metabolomics (Peeters, 2022; Smilde et al., 2010). Although these methods are powerful analytical tools for case vs. control experiments, they are rather difficult to apply in multiclass experiments like in this study, where samples containing a herbal extract are compared with a blank and a negative control. Therefore, a new approach is required in order to compare dynamic data of three groups to elucidate the metabolic pathway (Peeters, 2022).

To interpret the metabolic pathway of the samples studied in the GIDM-colon and to select the most interesting time profiles, an automated data analysis workflow for unbiased screening designed by Peeters et al. (Peeters, 2022) was applied for metabolomics profiling from the longitudinal UHPLC -HRMS data. Data were converted to the open source ".mzXML" format to allow XCMS processing (Stanstrup et al., 2013). The XCMS CentWave algorithm was used to convert the raw data into features via peak-picking followed by grouping, using following parameters: ppm = 10, peakwidth = c(5, 25), snthresh = 10, noise = 1000, mzdiff = 0.01, prefilter = c(3, 5000), integrate = 1. A feature is a *m/z* value and its retention time. Even though XCMS has the ability to discover features that differ between groups, it cannot take the longitudinal aspect into

account. Therefore, EDGE was used to extract significant differential profiles (Leek et al., 2006). For each feature, EDGE fits two models. The null model assumes there is no difference between the groups and fits a single cubic spline to all the data of a feature. The alternative model fits a cubic spline to each group and calculates the goodness of fit. The difference between these groups, expressed as p-values, reflects the improvement in goodness of fit. With this approach, two p-values for each feature are created: one for sample vs. blank and one for sample vs. negative control, which is non-trivial to interpret or combine. To overcome this problem, a machine learning model was constructed to obtain a single score for each feature. Moreover, by training the random forest model, the number of false positive and false negative hits can be decreased, which consecutively can be used to eliminate them from the remaining data.

To train the machine learning model and to check the quality of the significant features, a Shiny web application was constructed called tinderesting (Beirnaert et al., 2019). This application asks the user to rate the quality of the features based on whether the feature is interesting or not, by swiping right or left respectively, and uses these results to build the model in the background. This labeled training data is used to train a machine learning model, i.e. a random forest model, that is used to analyse the complete data set, rating the features in the same way as the expert. Training the random forest model thus reduces false negative and false positive hits.

Figure 2.12 gives a visual representation of the tinderesting app with a time profile for an *m/z* value of 551.227 in negative ion mode. An increase in intensity of the signal is clearly visible for the sample, which is not observed in the negative control or blank, implying that biotransformation products in the sample are formed over time. Since this time profile is only visible in the samples and not in the blanks and negative control (containing no faecal slurry), this profile is an interesting one and can be classified as such by swiping right.

Other interesting time profiles are profiles with a decrease in intensity of the signal over time in the samples (individual components present in the extract undergo biotransformation processes throughout the experiment) and intermediate profiles (newly formed biotransformation products disappear over time) (see Figure 2.13).

Figure 2.12*:* Screenshot of the tinderesting app for an *m/z* value of 551.227. Tinderesting gives a time profile (time in h) of a sample during biotransformation. The user can then rate the time profile as interesting by swiping right, or as boring by swiping left. The app allows the user to set some parameters, like the significance level or the order in which images are shown based on significance or ad random.

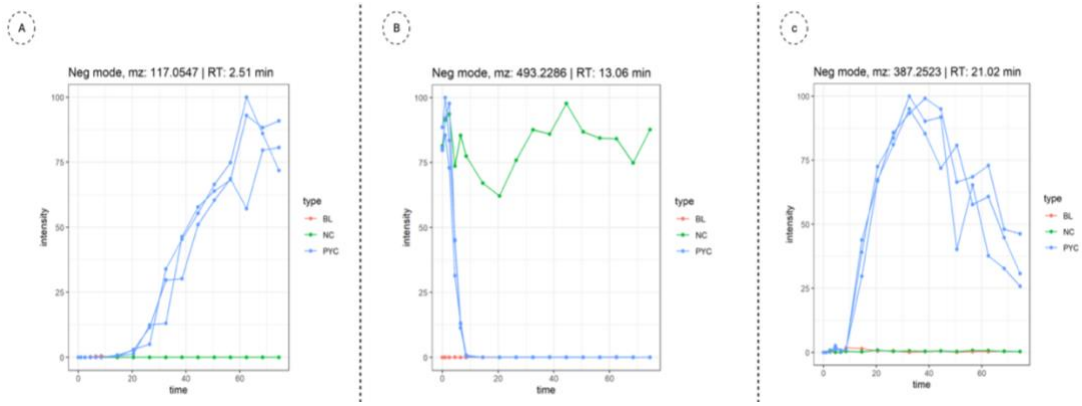

Figure 2.13*:* Features classified as interesting profiles. (A) Increase in intensity of the signal over time, (B) decrease in intensity of the signal over time and (C) intermediate profile. BL: method blank; NC: negative control; PYC: samples containing PBE; PBE: French Maritime Pine Bark Extract.

Each feature is given a single score, a tinderesting score, by the machine learning model, which provides a ranking of all features based on the difference over time between the three groups ("BL": blank; "NC": negative control and "PYC": Pycnogenol®, PBE). A maximal score of 1 is given by the model for an interesting feature, a minimal score of 0 is considered as an uninteresting feature. Features with a tinderesting score ≥ 0.8 were chosen for in-depth exploration, bearing in mind the following inclusion criteria: a reasonable intensity with good-quality MS/MS spectra and a retention time between 2 and 20 min. Various peaks in the output correspond to the same type of sample molecule, reflecting its isotope pattern. Isotopes were removed, using following parameters: IsoMass = 1.003355, IsoMassMaxDiff = 0.05, RTdiff = 10, corrThreshold = 0.8, ppm, IsoDiffThreshold = 5120. To validate whether this model can be used to adequately predict whether a time profile is a false discovery or not, a 10-fold cross validation was performed on the training dataset. To obtain a qualitative representation of all biotransformation products formed, identification of the resulting biotransformation products (assigned as interesting features) was based on the accurate mass, isotope pattern and fragmentation pattern of the product ions. A maximal mass variation between theoretical and observed mass was set at 10 ppm for parent ions and 25 ppm for product ions. Molecular formulae of the resulting metabolites were predicted by the Elemental Composition algorithm in MassLynx software based on the observed *m/z* values (Peeters, 2022). Biotransformation products were identified using the levels of confirmation as proposed by Schymanski et al. (Schymanski et al., 2014) (Table 2.3).

Table 2.3*:* Proposed identification confidence levels as proposed by Schymanski et al. (Schymanski et al., 2014).

Levels	Identification confidence	Minimum data requirements
Level 1	Confirmed structure	MS, MS^2, RT, Reference standards
Level 2	Probable structure: a) By library spectrum match b) By diagnostic evidence	MS, MS^2, Library MS^2, Experimental data
Level 3	Tentatively candidate(s)	MS, MS^2, Experimental data
Level 4	Unequivocal molecular formula	MS isotope/adduct
Level 5	Exact mass of interest	MS

MS^2 is intended to represent any form of MS fragmentation (e.g. MS^e,MS^n).

2.5 Results of the gastrointestinal biotransformation of PBE

2.5.1 Characterisation of microbial composition of individual faecal samples

The relative abundance of taxa at genus taxonomical level of the 12 individual samples are represented in Figure 2.14. Samples of these 12 individuals are denoted as PPAS-AX (for male donors with X representing the number of the donor) and PPAS-BX (for female donors). Looking at the microbial composition, *Agathobacter* (Firmicutes), *Alistipes* (Bacteroides), *Bacteroides* (Bacteroides), *Bifidobacterium* (Actinobacteria), *Cryptobacteroides* (Bacteroides), *Faecalibacterium* (Firmicutes), *Parabacteroides* (Bacteroides), *Phocaeicola* (Bacteroides) and *Prevotella* (Bacteroides) are the most dominant genera. Based on these sequencing results we could assume that all samples contain the same genera (except from PPAS-A004 which is the only one containing *Cryptobacteroides*) albeit with different abundance levels. PPAS-A004 was therefore not pooled.

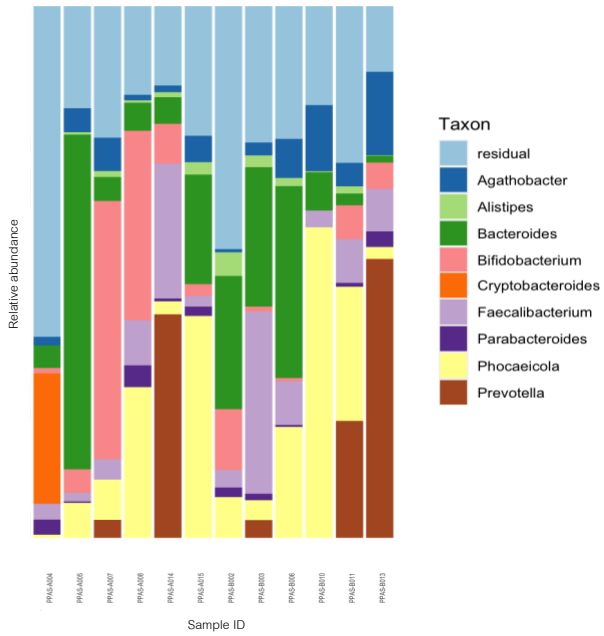

Figure 2.14: Relative abundance of taxa at genus level in the individual samples.

2.5.2 Viable cell count

The effect of the extract on viability of the faecal bacteria was assessed by comparing the method blanks (only faecal suspension, no extract or compound) and samples containing the extract (results of the cell count are not shown). Over time no significant difference in number of viable bacteria was observed for blank and sample during the *in vitro* gastrointestinal biotransformation, suggesting that the extract (addition of 300 mg PBE in the GIDM-Colon) is not toxic to faecal bacteria. These findings suggest that during the 72 h fermentation period, the number of viable faecal bacteria responsible for biotransformation processes remained stable.

2.5.3 Biotransformation of the positive control

The GIDM-Colon experiment was repeated twice and results of both experiments did not differ significantly and were thus consistent throughout both experiments. As expected, a decrease in intensity of the signal of chlorogenic acid was observed over time in both experiments and screening for biotransformation products indeed resulted in detection of several metabolites as previously reported in literature (see 2.4). According to earlier research work by Breynaert et al. (Breynaert et al., 2015) the biotransformation of chlorogenic acid is complete after 6 h of colon phase, which is in line with our findings. Four biotransformation products including quinic acid (*m/z* 191.0561 $[M-H]^-$), caffeic acid (*m/z* 179.0350 $[M-H]^-$), 3,4-dihydroxy-phenylpropionic acid (*m/z* 181.0506 $[M-H]^-$) and 3-(4-hydroxyphenyl)-propionic acid (*m/z* 165.0557 $[M-H]^-$) were found during *in vitro* gastrointestinal biotransformation in the positive control sample and were identified using standards. An overview of their structures, biotransformation pathway and biotransformation profile are depicted in Supplementary Figures S1 and S2 respectively. Identification of these biotransformation products was used to confirm adequate *in vitro* biotransformation during the GIDM-Colon experiment.

2.5.4 Biotransformation of PBE compared to (+)-catechin

It has been shown that various phenolic constituents are present in PBE such as phenolic acids (e.g. derivatives of benzoic acid, cinnamic acid, *p*-coumaric acid, caffeic acid, ferulic acid), catechin, epicatechin and taxifolin. Glycosides of phenolic acids and taxifolin have

also been identified. Nevertheless, the main constituents of PBE are procyanidins, consisting of catechin and epicatechin subunits (Rohdewald, 2005). Such a herbal extract will therefore undergo extensive biotransformation. Here, the difference between the biotransformation pattern of PBE and that of a pure compound, in this case (+)-catechin, is thoroughly explored. In general, a slower and/or incomplete biotransformation of a compound in an extract as compared to the same constituent as a pure compound can be observed, which is in line with previous findings from our research group. Indeed, as shown in Figure 2.15A, (+)-catechin as pure compound is no longer detectable in C6 samples which implicates that in the two hours following C4, (+)-catechin is fully transformed into other biotransformation products. On the contrary, (+)-catechin in the extract has a slower biotransformation rate, which can be caused by the presence of other compounds in PBE forming (+)-catechin during biotransformation, such as procyanidins. Degradation of procyanidins results in the formation of catechin and epicatechin subunits. Figure 2.15B shows that (+)-catechin in the extract is no longer present after 42 h of fermentation, which is much longer than as a pure compound.

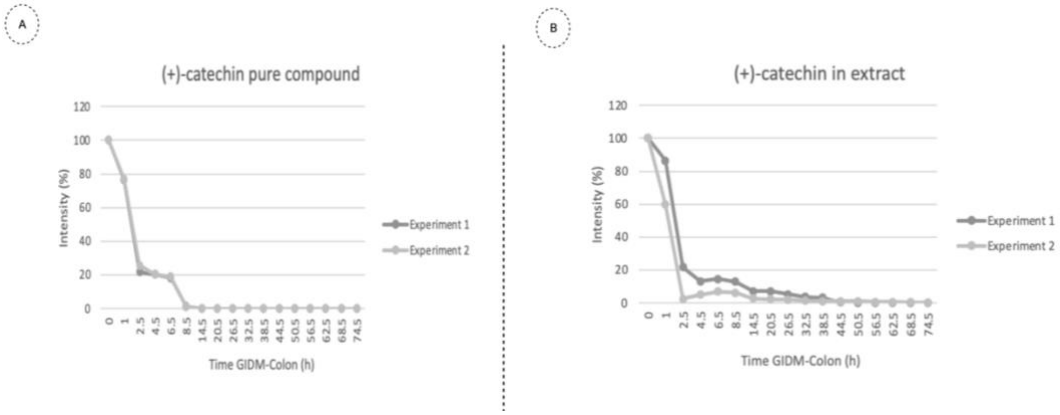

Figure 2.15: Time profiles of the biotransformation of (+)-catechin during the GIDM-Colon of the pure compound (A) and of the extract (B). The trendline in dark grey represents the average of three PBE solution samples at each timepoint in experiment 1, the trendline in light grey depicts the results of experiment 2.

Figure 2.16A shows the decrease of the procyanidin B-type content as the GIDM-Colon experiment evolves. Closer inspection of Figure 2.16A shows that for both experiments the fraction of B-type procyanidins decreases after the gastric stage and further decreases after the small intestinal stage. After the small intestinal stage, more

specifically during the first 42 h of the colon stage, B-type procyanidins are again present. Higher oligomer fractions present in the extract can reach the colon stage and can be transformed by colonic microbiota into lower molecular weight fractions, including procyanidin B-type fraction. Moreover, epicatechin subunits remain present throughout the whole experiment (Figure 2.16B). It is reasonable to believe that the slow biotransformation of epicatechin can be linked to the cleavage of the procyanidin B-type fraction.

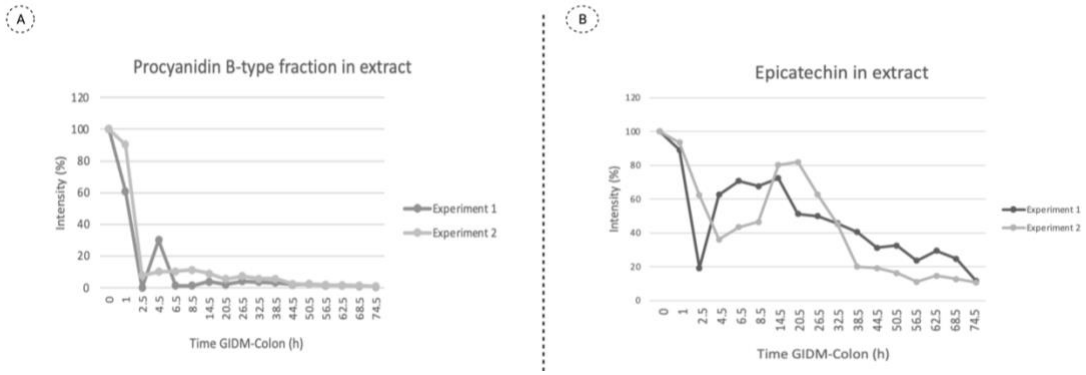

Figure 2.16: Time profiles of the biotransformation of the procyanidin B-type fraction (A) and epicatechin (B) during the GIDM-Colon. The trendline in dark grey represents the average of three PBE solution samples at each timepoint in experiment 1, the trendline in light grey depicts the results of experiment 2.

Another hypothesis is that the biotransformation of the extract is hampered by a possible antibacterial effect of the extract on the gut microbiota, resulting in an altered gut microbial composition or lower number of viable bacteria, which are normally responsible for the biotransformation processes. However, according to the results of the viable cell count (see 2.5.2), no significant difference in colony forming units was observed between the blanks and samples treated with the extract. It is therefore more likely that the differences in biotransformation reactions can be attributed to the various polyphenolic constituents present in the extract.

2.5.5 Biotransformation of PBE

The biotransformation of PBE was studied in more detail and several biotransformation products could be detected throughout both GIDM-Colon experiments for which the

results were consistent. Their structures were identified according to the proposed identification confidence levels as proposed by Schymanski et al. (Schymanski et al., 2014) according to the retention time, fragmentation pattern or as compared to reference standards when available. An overview of all identified biotransformation products can be found in Table 2.4. Generally, for most identified compounds a decrease in relative abundance is observed mainly during the colon stage, caused by bacterial degradation, suggesting formation of new biotransformation products (indicated by an increase in abundance of another compound).

2.5.5.1 Procyanidins and monomers

Procyanidins make up the largest part of the standardised PBE extract and various procyanidins with different degree of polymerisation were detected. A procyanidin B-type dimer, procyanidin B1, was confirmed by MS^2 fragmentation patterns and use of the reference standard mix. The MS^2 spectrum of procyanidin B1 (*m/z* 577.1348; $[M-H]^-$) produced ions at *m/z* 575.1182; 425.0868; 407.0775; 289.0712; 287.0545. The ion at *m/z* 425.0868 was due to a typical Retro-Diels-Alder (RDA) fragmentation with a neutral loss of 152 Da, followed by the loss of a water molecule unit (−18 Da) at *m/z* 407 [M − H – 152-18]$^-$. The ion at 289.0712 was due to quinone methide (QM) ring fission of the interflavan bond producing a loss of 288 Da and corresponds to its flavan-3-ol monomer subunits ((+)-catechin with *m/z* 289.0705 and (−)-epicatechin with *m/z* 289.0711). In addition to the procyanidin B-type dimer, a procyanidin A-type dimer (*m/z* 575.1151; $[M-H]^-$), could tentatively be identified based on the typical MS^2 fragmentation pattern. Indeed, procyanidins A-type have two hydrogen atoms less (shift of two mass units) compared to the B-type, due to the formation of an extra interflavanic linkage, and resulted in the following ions: *m/z* 449.0877; 289/0688; 285.0393. Here again, the typical elimination of 1,3,5-trihydroxybenzene ([M − H – 126]$^-$during heterocyclic ring cleavage (HRF) results in the ion detected at *m/z* 449.0877. Following QM reaction, the ion at *m/z* 289.0688 or *m/z* 285.0393 was observed (difference of 4 Da is typical for A-type procyanidins (Rue et al., 2018). Moreover, a procyanidin trimer, more specifically procyanidin C1 (*m/z* 865.1943; $[M-H]^-$), was identified by MS^2 fragmentation patterns and use of a standard mix. The fragmentation pattern showed peaks at *m/z* 695.1359; 575.1177; 451.1024; 407.0764; 289.0706. The ions at *m/z* 695.1359 were produced by the RDA mechanism followed by the loss of a water molecule $[M - H - 152 - 18]^-$. The

ion at *m/z* 577.1182 was generated by a QM [M − H − 288]⁻ cleavage between the C and D rings. The remaining ions at *m/z* 425.0868, 407.0775, and 289.0712 could be explained in the same way as for the procyanidin dimers above. The typical fragmentation pattern of the procyanidins fraction (A-type and B-type) as suggested by research work of Enomto et al. (Enomoto et al., 2020; Enomoto & Nirasawa, 2020) is depicted by Figure 2.17 and Figure 2.18, respectively.

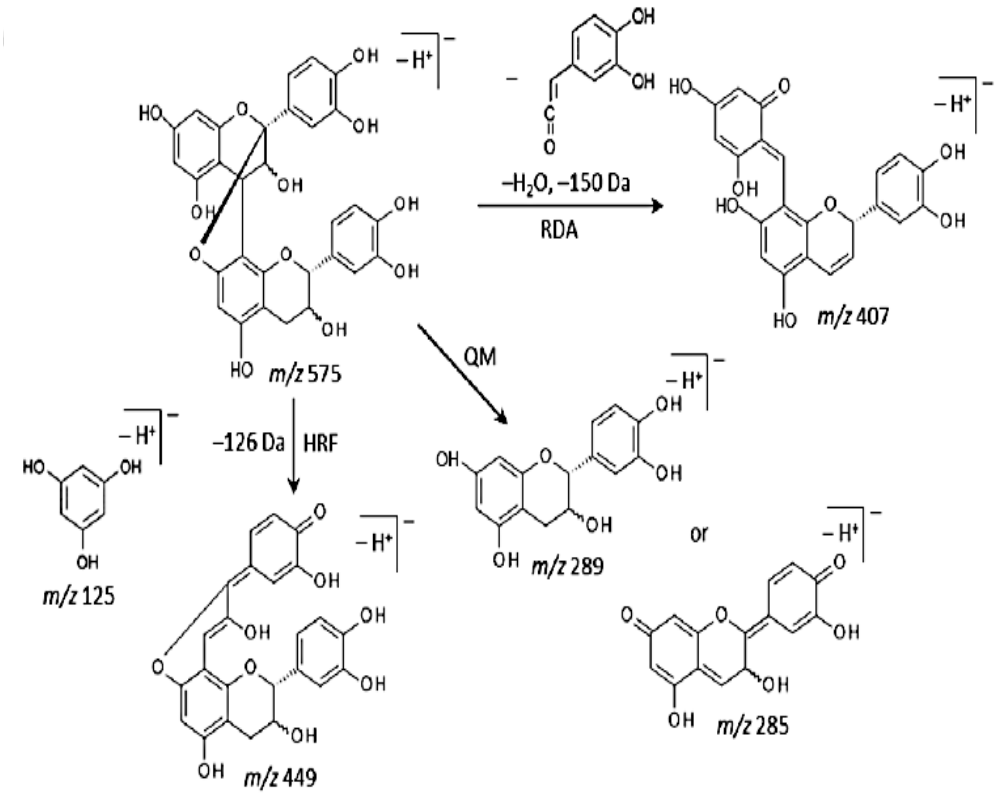

Figure 2.17: Typical biotransformation pattern of procyanidin A-type dimers. The MS^2 fragment ions found during our GIDM-Colon experiments are highlighted in yellow. HRF: heterocyclic ring fission; RDA: Retro-Diels Alder reaction; QM: Quinone methide reaction. Figure adapted from Enomoto & Nirasawa, 2020.

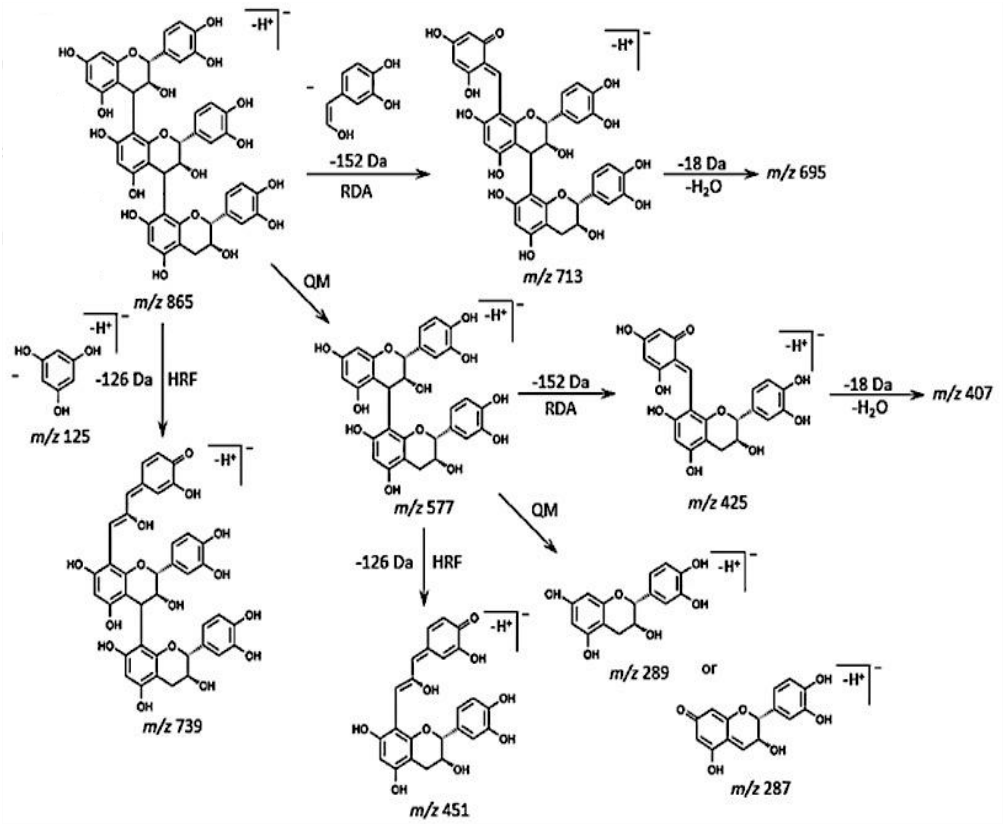

Figure 2.18: Typical biotransformation pattern of procyanidins. The MS^2 fragment ions found during our GIDM-Colon experiments are highlighted in yellow. HRF: heterocyclic ring fission; RDA: Retro-Diels Alder reaction; QM: Quinone methide reaction. Figure adapted from Enomoto et al., 2020.

In Figure 2.19, a decrease in intensity can be observed for procyanidin C1 (with a tinderesting score of 0.892), procyanidin B1 (tinderesting score of 0.974) and procyanidin A-type dimer (tinderesting score 0.842). For both the dimer (A-type and B-type) and trimer procyanidin fraction, it can be observed that biotransformation starts already in the stomach and continues during the small intestinal and colon phase, until no higher oligomeric fractions are observed anymore.

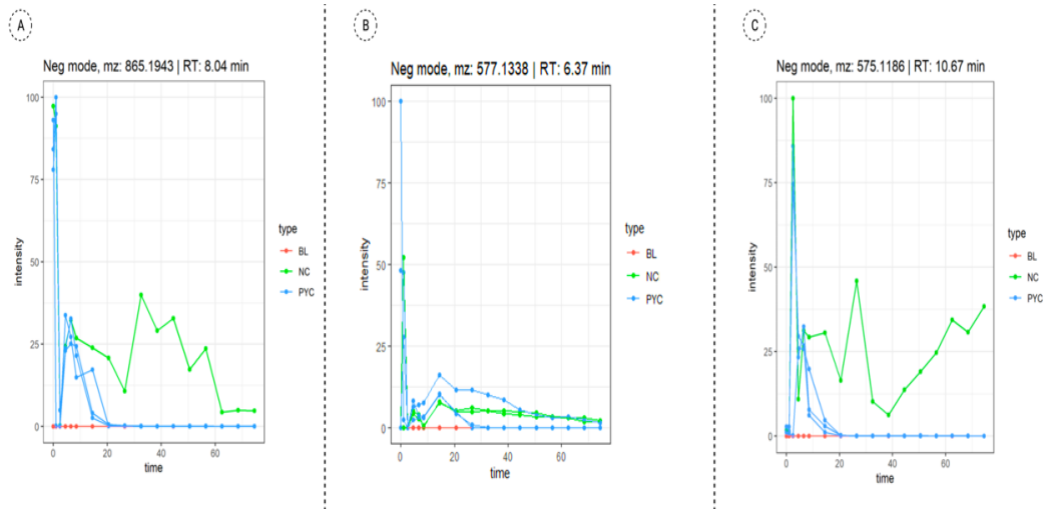

Figure 2.19: Time profiles (time in hours) of procyanidin C1 (A), procyanidin B1 (B) and procyanidin A-type dimer (C) during gastrointestinal biotransformation. The intensity (in %) is plotted on the y-axis. BL: blank sample; NC: negative control sample; PYC: PBE sample.

Next, the flavan-3-ol monomers ((+)-catechin and (-)-epicatechin, both with *m/z* 289.0705; $[M-H]^-$) were identified using standards. They were not only formed as a result of biotransformation throughout the GIDM-Colon but could also be identified at the start of the gastric stage. The MS^2 fragmentation pattern for both constituents gives ions at *m/z* 245.0810; 203.0703; 145.9296. The ion at *m/z* 245.0810 can be attributed to the loss of CO_2 moiety, the ion at *m/z* 203.0703 to the loss of $C_3H_2O_3$, the ion at *m/z* 145.9296 to the loss of $C_8H_{16}O_2$. Since (+)-catechin and (-)-epicatechin have similar chemical structures, they also exhibit a similar MS^2 fragmentation pattern and similar biotransformation profile (tinderesting score of 0.948 and 0.984 respectively, plot shown in Figure 2.20B). At the start of the GIDM-Colon, these flavan-3-ol monomers could also be found substituted to a sugar moiety in the extract, for instance a galloyl substituent as indicated by Figure 2.20A.

Moreover, other flavan-3-ol monomers could be identified using reference standards, more specifically gallocatechin and epigallocatechin (*m/z* 305.0549; $[M-H]^-$). Ions at *m/z* 167.0324 and *m/z* 137.0221 were observed for both monomers due to RDA fragmentation as depicted by Figure 2.21.

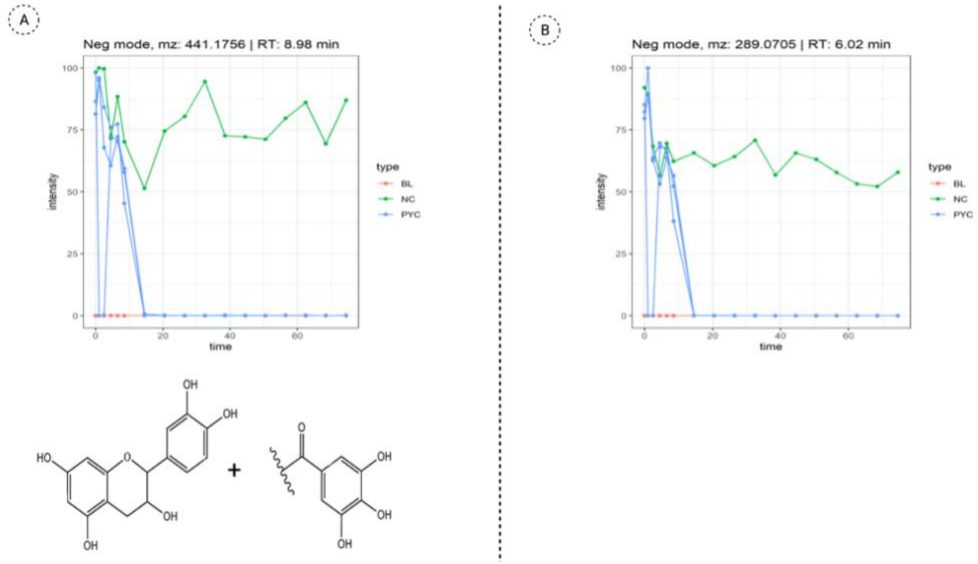

Figure 2.20: Time profiles (time in hours) of (epi)-catechin substituted with a galloyl moiety (A) and (epi)-catechin (B) during gastrointestinal biotransformation. The intensity (in %) is plotted on the y-axis. The chemical structure of (epi)-catechin and galloyl moiety is also depicted in Figure 2.24A. BL: blank sample; NC: negative control sample; PYC: PBE sample.

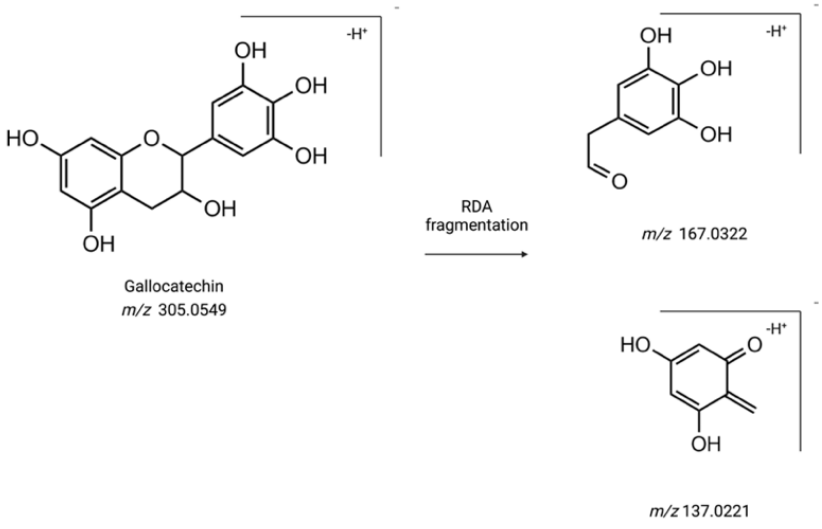

Figure 2.21: Typical biotransformation pattern following a Retro-Diels-Alder reaction (RDA) of gallocatechin.

2.5.5.2 Flavanols

Taxifolin (*m/z* 303.0497; $[M-H]^-$) was identified using a reference standard mix and showed ions at *m/z* 301.0345; 287.0552; 285.0395; 177.0185; 125.0231. The ion at *m/z* 285.0395 was due to the loss of a water molecule (−18 Da), whereas at *m/z* 177 and 125 the ions correspond to cleavage of the C ring. An *m/z* of 319.0447 could be linked to a dehydration (ring cleavage) and methylation of taxifolin. Also, a methylated taxifolin glucuronide could be retrieved, with a *m/z* 493.2286 ($[M-H]^-$). The suggested biotransformation pathway of taxifolin and the time profiles that were linked to the biotransformation of taxifolin (all features had a tinderesting score higher than 0.9) can be found in Figure 2.22.

2.5.5.3 Phenolic acids

Caffeic acid (*m/z* 179.0334; $[M-H]^-$) was identified using a reference standard mix and showed a MS^2 fragmentation pattern with ions at *m/z* 163.9412; 135.0449. The *m/z* at 163.9412 represents the loss of a hydroxyl group $([M - H - 16])^-$ and the *m/z* of 135.0449 indicates the neutral loss of a carbon dioxide $([M - H - 44])^-$. Caffeic acid underwent quick biotransformation and after 6 h of colon phase, it was no longer present (Figure 2.24). Various biotransformation products of caffeic acid (shown in Figure 2.23) were identified based on their MS^2 fragmentation patterns or by identification with a standard. 3-(3,4-dihydroxy-phenyl) propionic acid, commonly referred to as dihydrocaffeic acid was detected during the biotransformation of caffeic acid based on its MS^2 fragmentation pattern (m/z 181.0501; $[M-H]^-$) with ions at *m/z* 163.0376; 135.0420; 101.9404; 99.9254. 3-(3-Hydroxyphenyl) propionic acid (*m/z* 165.0548; $[M-H]^-$) was also identified as a microbial degradation product of caffeic acid and is formed from caffeic acid by reduction of a double bond and dehydroxylation at the C4 position. 3-(3-Hydroxyphenyl) propionic acid appeared after 6 hours in the colon phase. The MS^2 fragmentation pattern showed ions at *m/z* 145.9299; 116.9278. 3,4-Dihydroxyphenyl acetic acid (*m/z* 167.0704; $[M-H]^-$) showed a MS^2 fragmentation pattern with ions at *m/z* 145.9375; 116.9288. Besides caffeic acid, also protocatechuic acid (*m/z* 153.0187; $[M-H]^-$) could be identified based on its MS^2 fragmentation pattern and use of a reference standard. The fragmentation pattern showed ions at 145.9305; 109.0280 ($[M-H-CO_2]^-$). Phenolic glycosides could be detected in the extract. Ferulic acid 4-glucoside was identified based on the MS^2 fragmentation pattern. Ferulic acid 4-glycoside (*m/z*

355.1022; $[M-H]^-$) was deglycosylated (-176 Da; glucuronic acid) and resulted in *m/z* 179.0342, corresponding to ferulic acid.

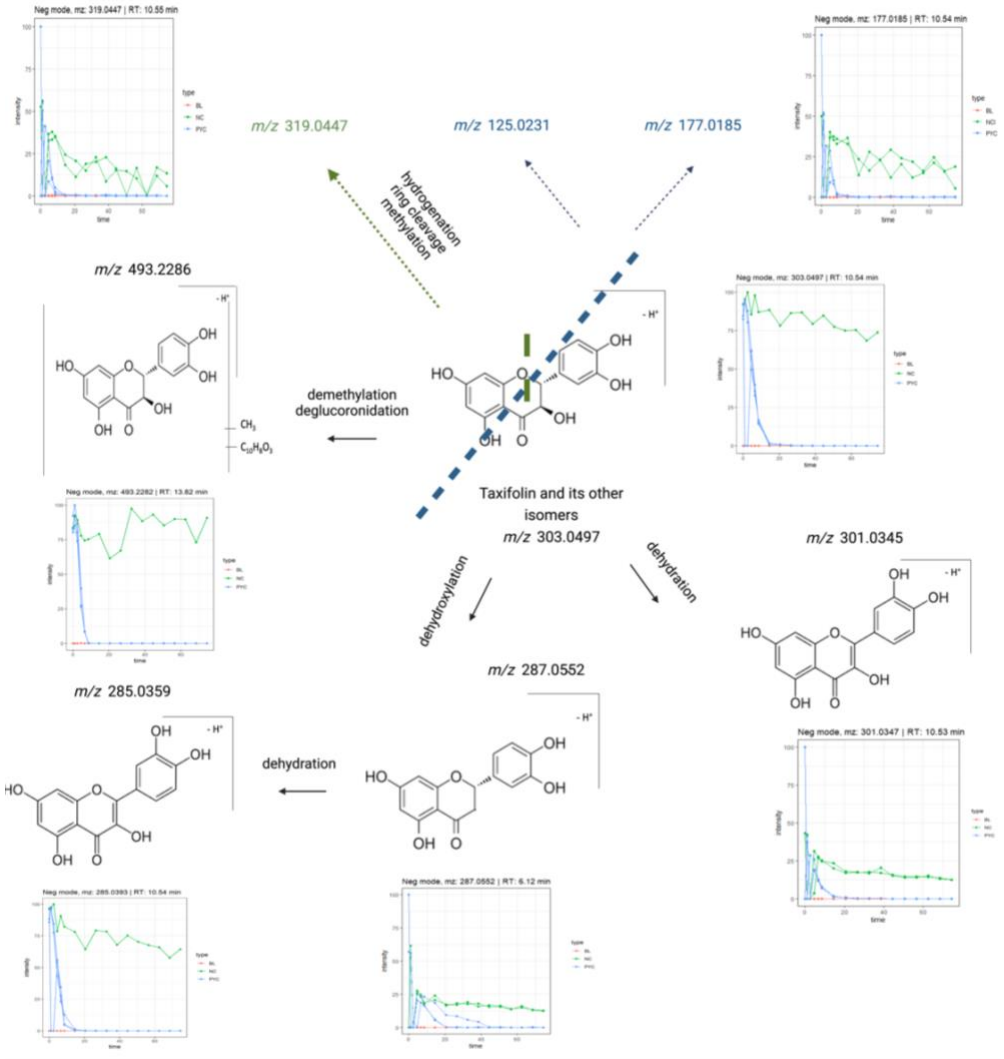

Figure 2.22: Suggested biotransformation pattern and time profiles of taxifolin and its isomers. All MS^2 fragment ions were found during our GIDM-Colon experiments. C-ring cleavage is depicted by the blue dotted line, the green dotted line indicates the dehydration (ring cleave) and methylation of taxifolin. The intensity (in %) is plotted on the y-axis. BL: blank sample; NC: negative control sample; PYC: PBE sample.

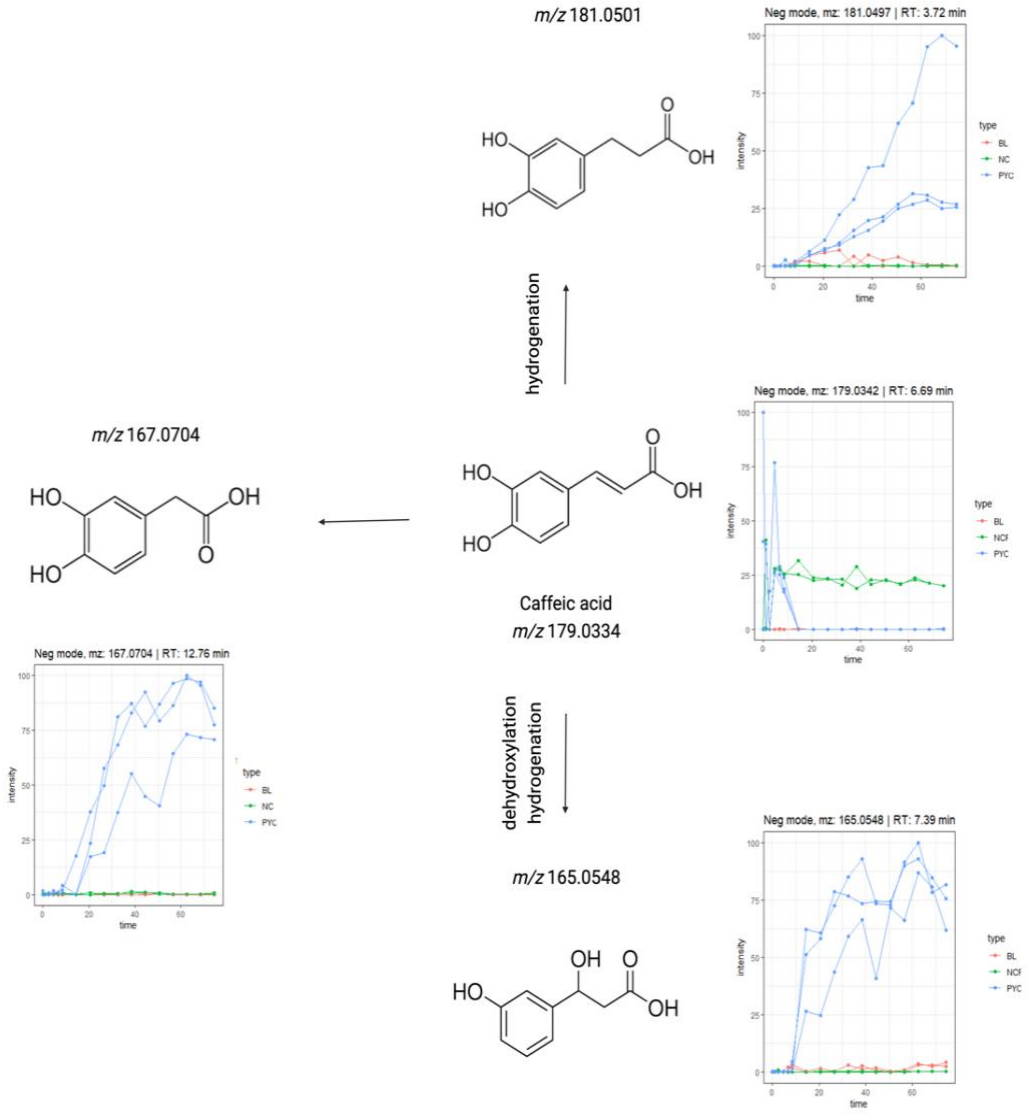

Figure 2.23: Suggested biotransformation pattern and time profiles caffeic acid. The intensity (in %) is plotted on the y-axis. BL: blank sample; NC: negative control sample; PYC: PBE sample.

Table 2.4: Overview of biotransformation products of the extract formed during GIDM-Colon experiments. Levels of confirmation are denoted as suggested by Schymanski et al., 2014 .

Compound	Molecular formula	Retention time (min)	Calculated *m/z* ($[M-H]^-$)	Experimental *m/z* ($[M-H]^-$)	MS^2 fragment ions (*m/z*)	Level of confirmation	Literature
Protocatechic acid (3,4-Dihydroxybenzoic acid)	$C_7H_6O_4$	3.44	153.0187	153.0186	145.9298; 116.9279; 109.0267 ($C_7H_6O_4$ – CO_2)	L2	(Chen et al., 2012)
Protocatechic acid 4-glucoside	$C_{13}H_{16}O_9$	9.36	315.0721	315.1227	153.0187 ($C_{13}H_{16}O_9$ – glucose)	L2	(Hong et al., 2021)
(Epi)-catechin	$C_{15}H_{14}O_6$	6.02	289.0717	289.0712	287.0546 ($C_{15}H_{14}O_6$ – 2H); 245.0810 ($C_{15}H_{14}O_6$ – CO_2); 203.0703 ($C_{15}H_{14}O_6$ - malonyl	L1	(Liu, 2021)
(Epi)-catechin substituted with galloyl moiety	$C_{22}H_{18}O_{10}$	8.98	441.0824	441.1756	289.0698 ($C_{22}H_{18}O_{10}$ – $C_7H_4O_4$) 287.0546 ($C_{22}H_{18}O_{10}$ – $C_7H_6O_4$) 279.1299 ($C_{22}H_{18}O_{10}$ – glucose)	L2	(Xu et al., 2021)
Taxifolin O-hexoside	$C_{21}H_{22}O_{12}$	9.29	465.3843	465.1032	437.1089 ($C_{21}H_{22}O_{12}$ – CO); 315.1233; 285.0399 ($-C_6H_{12}O_6$); 259.0613 ($-C_{11}H_{10}O_4$)	L2	(Ramos et al., 2022)

Table 2.4: Overview of biotransformation products of the extract formed during GIDM-Colon experiments. Levels of confirmation are denoted as suggested by Schymanski et al., 2014 (continued).

Compound	Molecular formula	Retention time (min)	Calculated *m/z* ($[M-H]^-$)	Experimental *m/z* ($[M-H]^-$)	MS^2 fragment ions (*m/z*)	Level of confirmation	Literature
Taxifolin	$C_{15}H_{12}O_7$	10.54	303.0504	303.0497	301.0345 ($C_{15}H_{12}O_7$ – 2H); 285.0395 ($C_{15}H_{12}O_7$ – H_2O); 175.0390; 125.0231 ($C_{15}H_{12}O_7$ – phloroglucinol ring)	L1	(Escobar-Avello et al., 2019)
Isorhamnetin	$C_{16}H_{12}O_7$	9.94	315.0503	315.1223	300.0277 ($C_{16}H_{12}O_7$ - CH_3) 151.1435 (A-ring cleavage)	L1	(Grati et al., 2022; Justesen, 2001)
Isorhamnetin 3-O-glucuronide	$C_{22}H_{20}O_{13}$	9.31	491.0831	491.191	315.1233 ($C_{16}H_{12}O_7$) 300.0277 ($C_{22}H_{20}O_{13}$ – $C_{10}H_8O_3$ – CH_3)	L2b	(Parejo et al., 2004)
Gallocatechin	$C_{15}H_{14}O_7$	4.03	305.0661	305.0561	167.0324; 137.0221	L1	(Singh et al., 2018)
Epigallocatechin	$C_{15}H_{14}O_7$	5.62	305.0661	305.0561	167.0324; 137.0221	L1	(Singh et al., 2018)
Hydrogenated methyl taxifolin	$C_{16}H_{16}O_7$	10.55	319.0889	319.0447	303.0513 (-CH_3-H); 285.0404 (-CH_3-H-H_2O)	L2b	(Yang et al., 2016)

Table 2.4: Overview of biotransformation products of the extract formed during GIDM-Colon experiments. Levels of confirmation are denoted as suggested by Schymanski et al., 2014 (continued).

Compound	Molecular formula	Retention time (min)	Calculated *m/z* ($[M-H]^-$)	Experimental *m/z* ($[M-H]^-$)	MS^2 fragment ions (*m/z*)	Level of confirmation	Literature
Caffeic acid	$C_9H_8O_4$	6.61	179.0344	179.0347	176.9564; 177.0177; 181.0820 ($C_9H_8O_4$ + CH_3); 163.9412 ($C_9H_8O_3$); 135.0449 ($C_9H_8O_4$ – CO_2); 116.9281	L1	(Ali et al., 2021; Gonthier et al., 2006)
Ferulic acid 4-O-glucoside	$C_{16}H_{20}O_9$	5.97	355.1034	355.1022	193.0495: $C_{16}H_{20}O_9$-$C_6H_{10}O_5$	L2b	(Hong et al., 2021; Leng et al., 2022)
Dihydrocaffeic acid	$C_9H_{10}O_4$	3.72	181.0501	181.0497	163.0376 ($C_9H_{10}O_4$ – H_2O) 135.0420; 101.9404; 99.9254	L2b	(Hong et al., 2021; Leng et al., 2022)
3-(3-hydroxyphenylpropionic acid)	$C_9H_{10}O_3$	7.39	165.0552	165.0530	145.9299; 116.9278;	L2b	(Appeldoorn, 2009)
3-(3-hydroxyphenylpropionic acid) + caffeoyl + CO	$C_{19}H_{16}O_7$	7.39	356.0896	355.9872	235.9269; 207.9308; 165.0547 ($C_9H_{10}O_3$); 145.9308; 116.9308	L3	-
3-(4-hydroxyphenylpropionic acid)	$C_9H_{10}O_3$	6.38	165.0552	165.055	147.0431; 99.9252	L2b	(Leng et al., 2022)

Table 2.4: Overview of biotransformation products of the extract and its individual constituents formed during GIDM-Colon experiments. Levels of confirmation are denoted as suggested by Schymanski et al., 2014 (continued).

Compound	Molecular formula	Retention time (min)	Calculated *m/z* ($[M-H]^-$)	Experimental *m/z* ($[M-H]^-$)	MS^2 fragment ions (*m/z*)	Level of confirmation	Literature
3,4-Dihydroxyphenylacetic acid	$C_8H_8O_4$	12.91	167.0345	167.0702	-	L2b	(Appeldoorn, 2009; Hong et al., 2021)
Rosmarinic acid	$C_{18}H_{16}O_8$	7.52	359.0772	359.0761	161.0200; 179.0344 ($C_9H_8O_4$);	L2b	(Serrano et al., 2021)
Procyanidin B1	$C_{30}H_{26}O_{12}$	6.29	517.1400	577.1348	575.1182 ($C_{30}H_{26}O_{12}$ – 2H); 425.0868 ($C_{30}H_{26}O_{12}$ – 152) 407.0775 ($C_{30}H_{26}O_{12}$ – 152-18) 289.0712 ($C_{15}H_{14}O_6$)	L1	(Escobar-Avello et al., 2019)
Methyl taxifolin glucuronide	$C_{12}H_{22}O_{13}$	13.06	493.1007	493.2286	365.1960; 315.1808; 289.0728		(Yang et al., 2016)
Procyanidin C1	$C_{45}H_{38}O_{18}$	8.02	865.2300	865.1934		L2b	(Escobar-Avello et al., 2019)
Procyanidin A1	$C_{30}H_{24}O_{12}$	10.67	576.13	575.1186	449.0877 ($C_{30}H_{24}O_{12}$ – 126); 289.0688 ($C_{15}H_{14}O_6$); 285.0393	L2b	(Enomoto et al., 2020; Enomoto & Nirasawa, 2020; Rue et al., 2018)

2.5.6 Characterisation of microbial composition of GIDM-Colon samples

To elucidate the microbial composition of the faecal suspension, 16S rRNA genome sequencing was performed. A possible effect of PBE on the bacterial composition of the faecal slurry during *in vitro* biotransformation in the GIDM-Colon was assessed by comparing method blank and samples containing the extract over time. The microbial composition of the sample solutions taken at different timepoints during the colon phase (C0, C24, C30, C48 and C72) in two identical experiments is presented in Figure 2.24. Only samples taken from PBE ("PYC"), (+)-catechin ("CAT") and blank ("Blank") are important here. Blank1 and Blank2 are *in duplo* sample solutions of the blank sample, containing only faecal samples without addition of any compound, PYC1 and PYC3 are *in duplo* sample solutions of the faecal sample to which PBE is added, whereas CAT1 and CAT2 are *in duplo* sample solution from the (+)-catechin sample. Figure 2.24 shows a similar microbial composition of the different sample solutions taken during the first (A) and second experiment (B) at the various timepoints of the colon stage. At the beginning of the colon phase (C0) the composition of blank solution samples (C0-Blank1 and C0-Blank2), PBE solution samples (C0-PYC1 and C0-PYC3) and (+)-catechin solution samples (C0-CAT1 and C0-CAT2) is quite similar for both experiments and is dominated by *Agathobacter* spp., *Blautia* spp., *Bacteroides* spp., *Bifidobacterium* spp., *Faecalibacterium* spp., *Fusicatenibacterium* spp., *Phascolarctobacterium* spp. and *Romboutia* spp.. On the contrary, the difference between microbial composition of C0 and later timepoints is very noticeable. For instance, later in the colon stage *Escheria* spp. is more abundant as compared to C0 sample solutions. Noteworthy is the absence of *Megasphaera* spp. in the samples with PBE as compared to blank samples or samples with (+)-catechin.

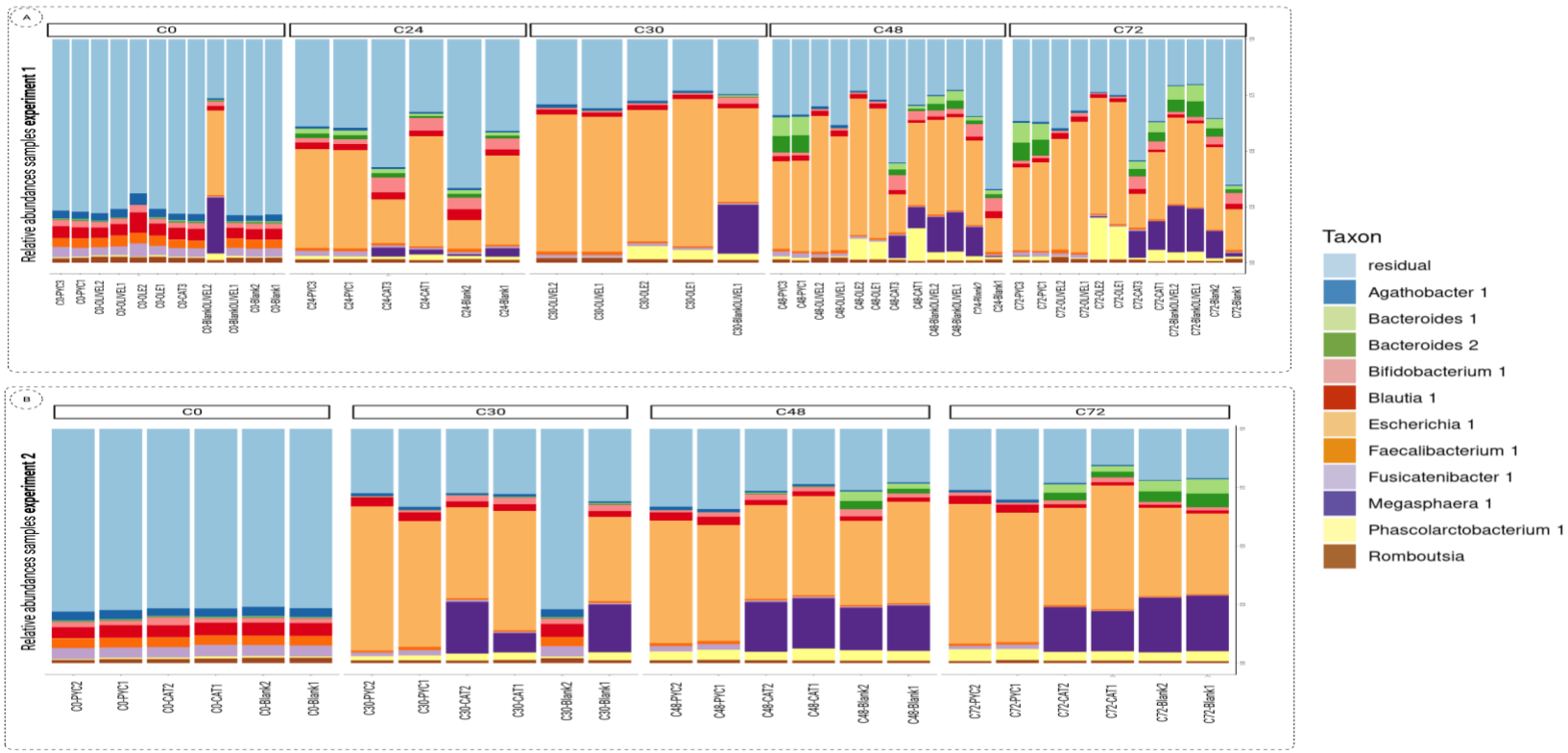

Figure 2.24: Relative abundance levels of taxa at genus level for different timepoints and test compounds during *in duplo* experiments (A and B).

Looking at the α-diversity calculated based on the inverse Simpson index (Figure 2.25), it can be noted that the α-diversity of all samples in (blank, PBE and (+)-catechin) is high at the start of the colon stage (C0) but quickly plumets as the time in the colon phase progresses (C24, C30, C48 and C72) depicted by the trendlines in the graphs. Since *Escherichia* spp. are becoming more dominant while the colon stage proceeds, making up approximately more than half of the composition of these samples, the latter becomes less diverse which implicates lower α-diversity.

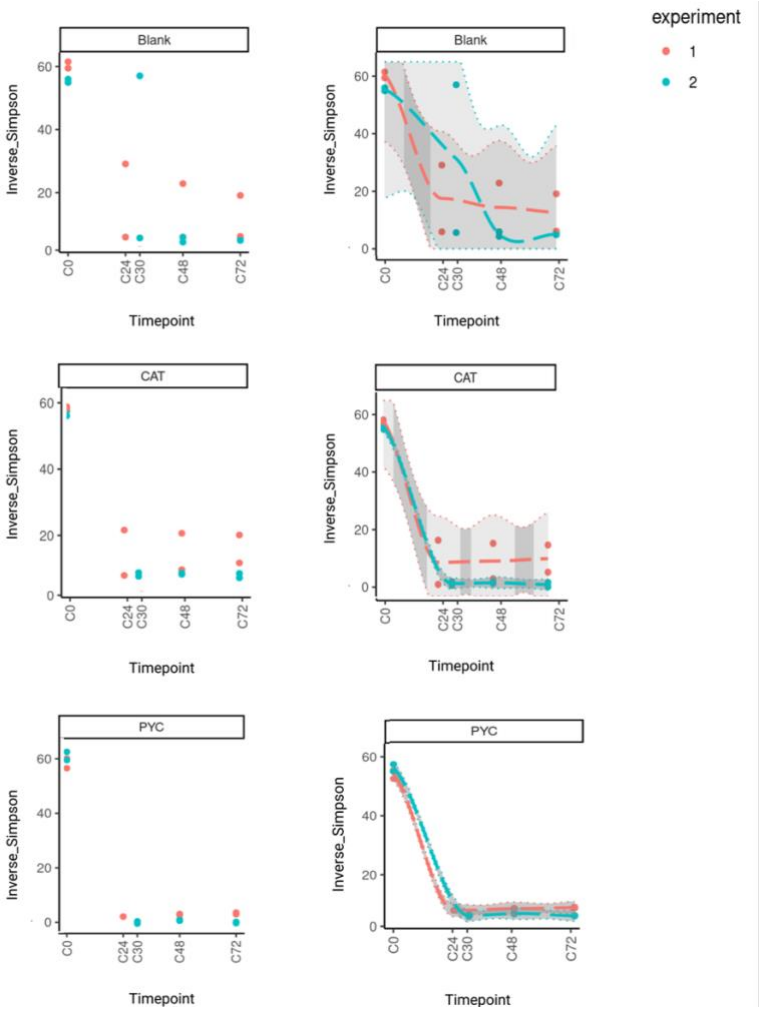

Figure 2.25: Alpha diversity calculated using the inverse Simpson index of the samples taken at different timepoints during each experiment. Samples from the first experiment are denoted in red, blue dots indicate samples taken during the second experiment.

The PCoA plots based on Bray Curtis differences were used to further reveal compositional differences between the different samples at different timepoints during the colon stage. Looking at the PCoA plot (Figure 2.26) the C0 samples cluster together in experiment 1 as well as in experiment 2, which is in line with earlier observations from the relative abundance profiles. In both experiments (+)-catechin and blank samples seem to cluster together (a little noisier in experiment 1), while PBE samples form a separate cluster. It can thus be noted that the microbial composition of the PBE samples at the different timepoints (C24, C30, C48 and C72) are more similar to each other since the datapoints are clustered but different from the microbial composition of (+)-catechin and blank samples.

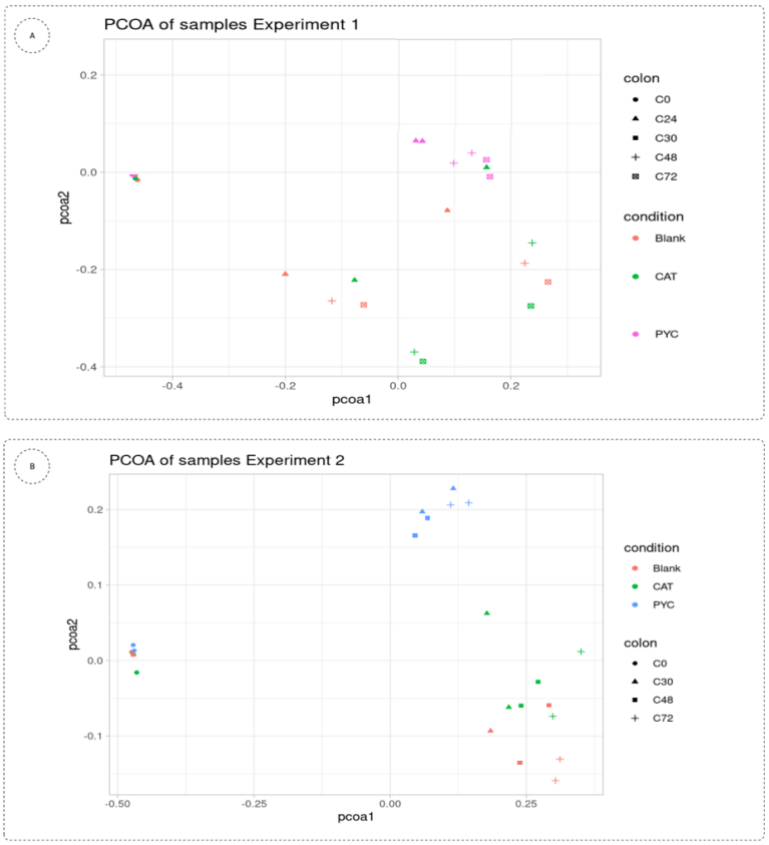

Figure 2.26: Principal coordinate analysis based on Bray-Curtis matrices of the different sample solutions taken at different timepoints during the colon phase. Each data point represents an individual sample, and the colour/shape is indicative for respectively the condition (different sample solutions) and timepoint of the colon phase (C0, C24, C30, C48 and C72).

According to the co-differential abundance plot (Figure 2.27), the largest differences in taxa abundances between PBE samples versus blank and (+)-catechin samples include bacteria of the following genera: *Fusicatenibacter* spp., *JPZU_g* spp., *Streptococcus* spp., *Bifidobacterium* spp., *Coprococcus* spp., *Agathobacter* spp., *Anaerostipes* spp. Even though based on the relative abundance levels of taxa as demonstrated in Figure 2.25 it could be observed that *Megasphaera* spp. is present in blank and (+)-catechin samples and absent in PBE samples, co-differential abundance analysis did not pick up these differences.

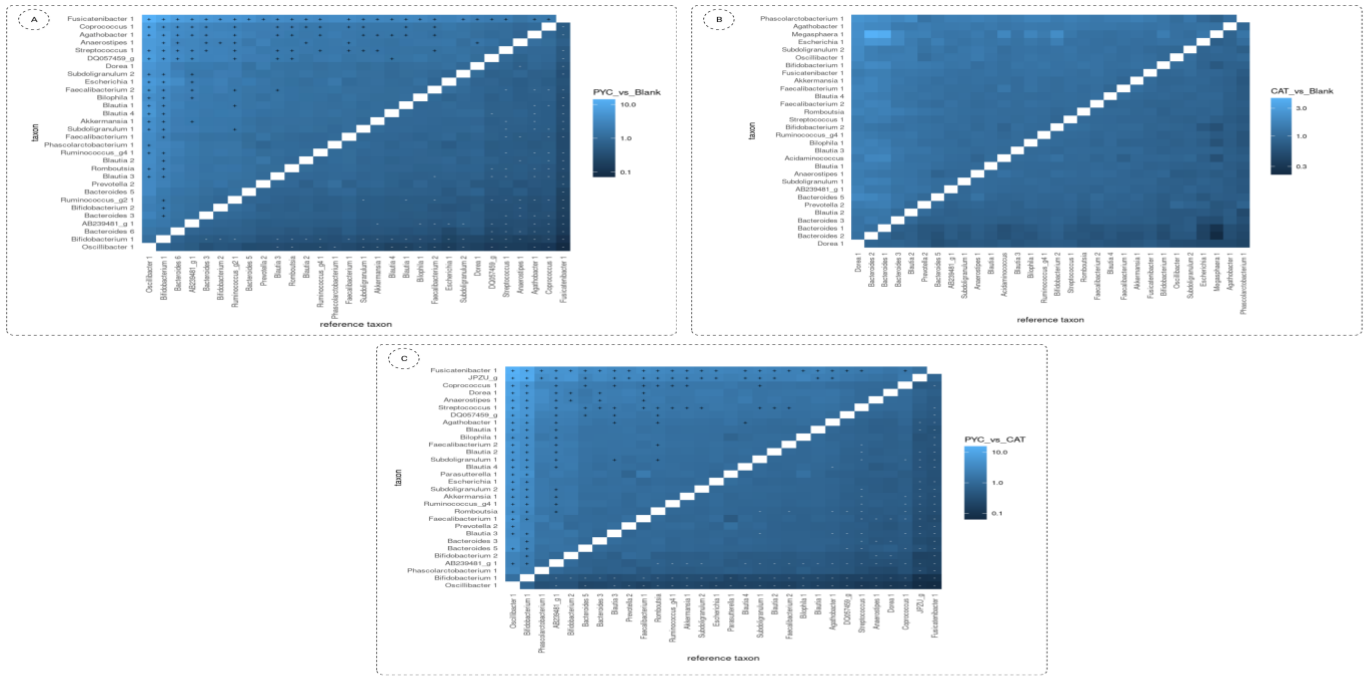

Figure 2.27: Each cell of the heatmap represents the differential abundance of a taxon when comparing the PBE samples at the different timepoints to the blank samples at the different timepoints (A), the (+)-catechin samples versus the blank samples (B) and the PBE samples to the (+)-catechin sample, relative to another taxon, on which a Wilcoxon test (+ in heatmap relates to adjusted p-values < 0.05 and - relates to adjusted p-values ≥ 0.05) was performed. The entire plot was also corrected for multiple testing. The colour represents the median of all pairwise differences of log ratios between the two groups of samples (two-sample Hodges-Lehmann estimator).

2.6 Discussion

A novel concept was used to establish metabolic pathways of, in this case, a polyphenol-rich extract: *in vitro* biotransformation via a GIDM-Colon simulation model followed by metabolic profiling. Various methodological aspects including an anaerobic environment and pH adaptation were implemented to resemble *in vivo* conditions as much as possible (Breynaert et al., 2015; Mortelé, 2021). The GIDM-Colon provides a fast and easy approach to predict enzymatic and microbial transformation processes and to detect these biotransformation products in an early stage after ingestion (Breynaert et al., 2015). Moreover, isolation and identification of metabolites from the *in vitro* GIDM is rather easy and avoids isolation from complex matrices (e.g. blood after administration to animals or humans). It also can simulate digestive conditions of various age groups and special metabolic or diseased populations which allow to compare biotransformation reactions and products formed in between these groups. It is therefore suitable for screening phases, omitting animal testing during early phases of natural product research. Furthermore, applying ESI during the LC-MS analyses of the samples collected during a GIDM-experiment, allows thermolabile and high-molecular-weight compounds to be ionized and transferred to the gas phase (Demarque et al., 2016). This analyser is an accurate mass-MS (am-MS) detector and facilitates the calculation of plausible molecular formulae of the ions and fragments, allowing the tentative identification of unknown plant metabolites without requirement of reference standards (Kind & Fiehn, 2006). Though the use of reference standards might sometimes be necessary to confirm the identification of compounds, reference standards are not always necessary for reliable identification. In fact, high resolution MS allows the identification of unknown compounds with a relatively high degree of confidence even without using reference standards. Moreover, an in-house developed automated data analysis workflow was used for unbiased screening for metabolites and shed light on the biotransformation of PBE. Although this *in vitro* model can be used to study passive diffusion when dialysis is implemented, here in this current study the model remains a simplified approach of biotransformation processes since absorption has not been taken into account and enterohepatic circulation is lacking. Extrapolating *in vitro* findings to *in vivo* conclusions therefore require caution.

Polyphenol-rich extracts and polyphenolic compounds undergo extensive biotransformation processes. To the best of our knowledge, this is one of the first studies identifying the biotransformation products of PBE by means of UHPLC-ESI-QTOF-MS analysis. Besides the well-documented individual constituents determined by HPLC analysis as described by D'Andrea et al. (D'Andrea, 2010), various other compounds (a comprehensive overview is given in Table 2.4) could be identified in the initial extract (T0 sample during the GIDM-Colon). These different polyphenolic constituents were not only found in their aglycon form, but also various glycosylated components were identified, of which the glycoconjugates were cleaved during the biotransformation.

Looking at the digestive fate of the procyanidin dimer and oligomer fractions, it can be observed that the biotransformation of B-type dimers during our experiments already starts after the gastric stage and continues after the small intestinal stage. The gastric stability of procyanidins has already been investigated in various *in vitro* studies; however, results were conflicting (Niwano et al., 2023). For instance, Kahle et al. (Kahle et al., 2011) observed an almost complete degradation of procyanidin B2 into (-)-epicatechin monomers in the gastric environment (simulated by addition of gastric fluid). In addition, other research work demonstrated that procyanidin B2 is susceptible to degradation by intestinal juice, with complete degradation occurring within two hours (Zhu et al., 2002). Also, research work from Peeters et al. (Peeters, 2022) investigating the gastrointestinal biotransformation of *Herniaria hirsuta* showed that the relative abundance of procyanidin B2 decreased over the time course of the GIDM-Colon experiment. According to previous research work by Spencer et al. (Spencer et al., 2000) on the metabolic fate of cacao procyanidins, it was observed that these procyanidin oligomers (trimer to hexamer) are unstable in an acidic environment and decompose to dimeric and monomeric units. On the contrary, other studies reveal that the procyanidin fraction remains fairly stable under gastric conditions (Niwano et al., 2023). For instance, according to Serra et al (Serra et al., 2010) dimers and trimers fraction of procyanidins are highly stable under gastric and duodenal conditions. Hence, it could be hypothesised that the stability of the procyanidins during gastric and small intestinal digestion depends on the dietary source (Chen et al., 2018). Nevertheless, structures with higher polymerisation degrees (greater than 6 monomeric forms linked to each other) are considered to be more stable in acidic conditions and do not degrade rapidly into their respective monomers. In fact, they will reach the colon where the intestinal microbiota

will break them further down (Niwano et al., 2023; Ou & Gu, 2014). A general overview of the digestive fate of orally ingested procyanidins is depicted by Figure 2.28. However, based on our *in vitro* GIDM-Colon results, a decrease in procyanidin B-type fraction (procyanidin B1 and procyanidin B2 were confirmed with a reference standard) could be observed after the gastric and small intestinal stage. During the colon stage, an increase in procyanidin B-type dimers is noticeable, most likely due the colonic biotransformation of higher oligomeric procyanidin structures. Moreover, the difference in abundance levels between (+)-catechin in the extract (no longer present after 42 hours of fermentation) and epicatechin in the extract (detectable throughout the whole GIDM-Colon experiment) can also be attributed to the cleavage of procyanidin B-type dimers. Since procyanidin B2 is comprised of a (−)-epicatechin monomer linked via a C4-C8 linkage to another (−)-epicatechin monomer whereas B1 consists of (−)-epicatechin linked via a C4-C8 linkage to (+)-catechin (Niwano et al., 2023), it is not surprising that the epicatechin fraction is higher compared to the catechin fraction when these procyanidin B-type dimers are degraded into their respective monomer fractions. Overall, our results indicate that (+)-catechin remains longer present during the GIDM-Colon experiment since this flavan-3-ol monomer is probably increased due to the degradation of oligomeric procyanidins.

During the time course of the GIDM-Colon experiment, also new biotransformation products were formed under the influence of the intestinal microflora, which were not present in the non-biotransformed extract. Various phenolic acids were identified using reference standards and/or MS2 fragmentation patterns. Most surprisingly was the absence of features that could be linked to valerolactones, the microbiota-generated biotransformation products of the procyanidins (Niwano et al., 2023). In fact, besides phenylvaleric acids, phenyl-γ-valerolactones are the major colonic biotransformation products of catechins in humans. They are absorbed in the large intestine whereafter they can undergo phase II biotransformation reactions in the liver or they can be further transformed in the gut into phenolic acids (Li, 2023). Previous research work identified δ-(3,4-dihydroxyphenyl)-γ-valerolactone and δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone, both in free form and conjugated with glucuronic acid/sulphate, as the main microbial biotransformation products (Ignacio Alonso-Esteban et al., 2022). A possible explanation for this might be the in-source fragmentation, which refers to the natural occurring phenomenon in ESI source analysis techniques where ions undergo

fragmentation within the ionisation source before entering the mass spectrometer. This dissociation of analytes during the ionisation phase can be attributed to several instrumental parameters such as the voltage applied (Xie et al., 2023). Indeed, the MS2 fragmentation spectra of some features showed *m/z* fragment values typical for phenyl-γ-valerolactones. Moreover, these γ-valerolactones can be considered as intermediate biotransformation products which can be subsequently degraded into 5-phenylvaleric acid, 3-phenylpropionic acid, phenylacetic and benzoic acids (Sánchez-Patán et al., 2011), which could be identified as increasing features throughout the GIDM-Colon experiment. In addition, testing the (+)-catechin samples with another experimental method with lower voltages, clearly resulted in MS spectra corresponding to phenyl-γ-valerolactones. In future investigations, it might thus be possible to apply another LC-MS analysis method suitable for identification of these phenyl-γ-valerolactones.

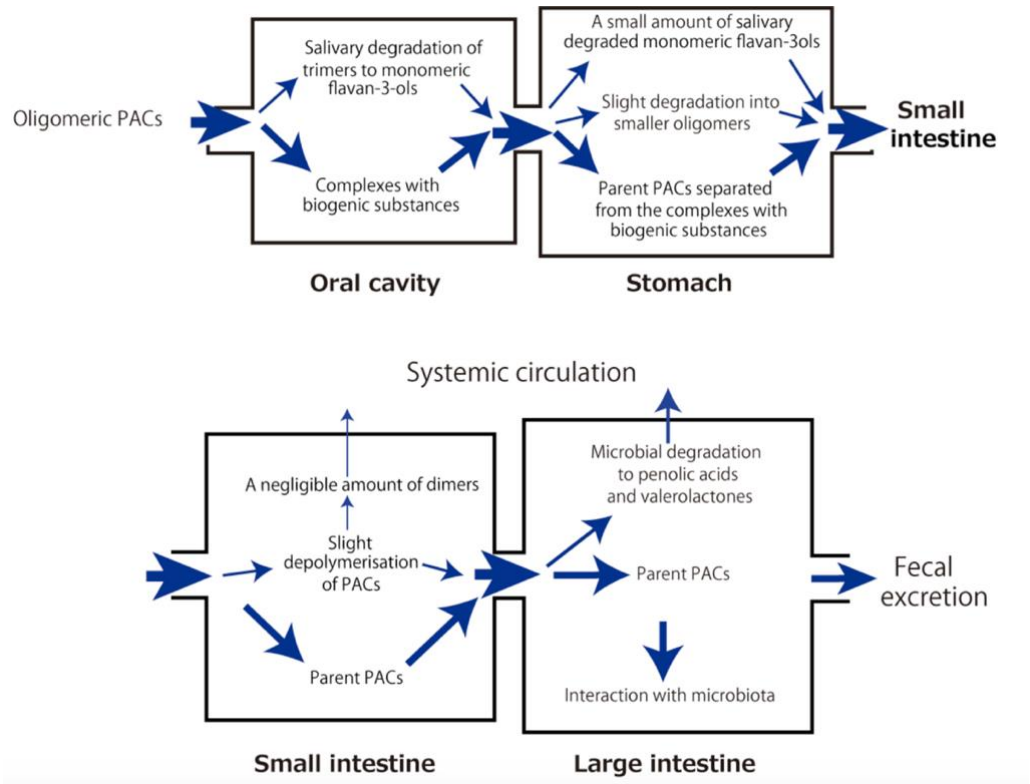

Figure 2.28: Intradigestive fate of orally ingested procyanidins as suggested by Niwano et al. PAC: proanthocyanidins. Figure adapted from Niwano et al., 2023.

Earlier research work by Mortelé et al. (Mortelé, 2021) demonstrated that no clear shifts in bacterial composition were observed over time when the faecal slurries were stored at -80 °C, which is in line with previous findings (Dorsaz et al., 2020; Kia et al., 2016; Tap et al., 2019). Nevertheless, Tap et al. (Tap et al., 2019) reported limited effects on the alpha-and beta-diversity and taxonomic composition while looking at the stability of faecal samples stored at -80 °C over a period of five years. However, these reported differences were smaller than biological variations (inter- and intrasubject) and inter-sequencing variation. Use of faecal slurries that were stored for a longer period of time after collection, did therefore not influence the microbial composition.

According to previous research work of Aguirre et al. (Aguirre et al., 2014) the use of pooled faecal samples as inoculum is beneficial for *in vitro* fermentation studies. Pooling will increase the biodiversity and therefore will lead to a more representative microbiome. Moreover, pooling will also decrease the variability in between different experiments. Until now, no consensus on the number of donors needed for the most optimal representation has been published and different protocols do exist. Studies reporting protocols for standardised faecal inocula included four (Aguirre et al. (Aguirre et al., 2015)) and six (O'Donnell et al. (O'Donnell et al., 2016)) donors (Mortelé 2021). However, in our study we included 11 donors (six female and five male) to obtain the most illustrative picture of an average human gut microbiome. By pooling we thus create a more depictive representation of the microbial composition and moreover, exclude participants who have a microbiome that is different from the included population, instead of forming a so-called extra sample.

Though there are no fixed rules on how a "normal" gut microbiome should look like, some patterns do exist. According to Ishiguro et al. (Ishiguro et al., 2018) the healthy adulthood microbiome is dominated by bacteria belonging to the families of the Lachnospiraceae and Ruminococcaceae, and to a lesser extent by members of the families *Bacteroidaceae/Prevotellaceae*. It can be observed that the relative abundance of different bacterial types at starting conditions resemble the i*n vivo* conditions. Indeed, *Agathobacter* spp., *Blautia* spp. and *Fusicatenibacter* spp. belonging to the Lachnospiraceae family and *Faecalibacterium* spp. (Ruminococcacceae) are the most abundant genera at baseline. Cultivation and supplementation with a polyhenolic extract introduces qualitative and quantitative alterations by certain bacterial strains. By

applying the plate count method to identify the number of actively growing or dividing cells in the sample, the viability of the faecal bacteria (with and without the presence of polyphenolic compounds) throughout the whole duration of the GIDM-Colon experiment could be assured. It could be demonstrated that there was no significant decrease in viable bacteria. Nevertheless, some qualitative differences could be observed.

Over time, *Escherichia* spp. were the predominant bacteria in the blank and samples containing PBE. Since *Escherichia* spp. (belonging to the family of the *Enterobacteriaceae*) are inhabitants of the GI tract, are facultative aerobe and grow at an optimum temperature of 37 °C, it is therefore not surprising that they are more abundant throughout the GIDM-Colon experiments. In turn, research has already demonstrated that *Escherichia coli* for instance, is involved in the biotransformation of polyphenolic compounds (Cardona et al., 2013). According to Torras et al. (Calvo Torras et al., 2005), PBE showed inhibitory effects against *E. coli*; however this was not observed in this study. Moreover, throughout the experiment, the appearance of *Megasphaera* spp. is noticeable as compared to baseline samples, though this is not the case for samples supplemented with PBE. The genus *Megasphaera* is a member of the phylum Firmicutes and one of the most important producers of SCFAs, especially butyrate. Supplementation of pigs with grape seed meals rich in polyphenolic compounds for 30 days, stimulated the growth of genera like *Megasphaera* spp. and *Prevotella* spp. (Grosu et al. 2020). This finding is contrary to our results and this discrepancy might be due to the short fermentation period in the presence of PBE. Also, our pilot study with 10-week PBE supplementation (Chapter 5), in which the microbiome of ADHD patients was investigated, did also not reveal higher abundance levels of *Megasphaera* spp.. Even though PBE was supplemented for a longer period of time, the study cohort was different (paediatric ADHD patients between 6-12 years old as compared to young, healthy individuals). Nevertheless, in both experimental set-ups it can be noted that the microbial composition in samples treated with PBE or patients receiving PBE, looks different from the other samples, indicated by different clusters.

In conclusion, an innovative concept was used to reveal the gastrointestinal biotransformation pathway of several compounds present in PBE. This approach could

identify various biotransformation products of PBE with distinct time profiles. Even though the *in vitro* gastrointestinal biotransformation model was optimised to approximate *in vivo* conditions as closely as possible, it remains a simplified approach to predict biotransformation as active transport, passive diffusion and enterohepatic circulation are missing. Nevertheless, the model sheds light on the enzymatic and microbial biotransformation occurring in the gastrointestinal tract, and is therefore useful for screening phases, omitting animal testing in early phases of natural product research.

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Supplementary Materials

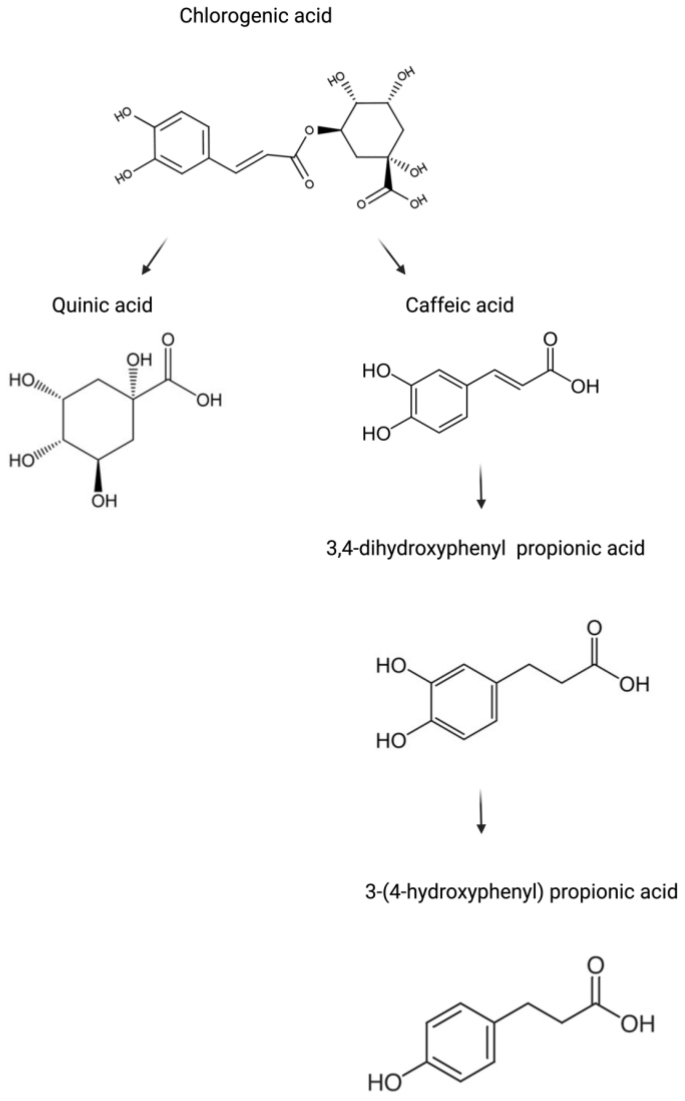

Figure S1: Biotransformation products of chlorogenic acid used as a positive control during the GIDM-Colon experiments. Quinic acid, caffeic acid, 3,4-dihydroxy-phenyl propionic acid and 3-(4-hydroxyphenyl)-propionic acid were identified using analytical standards.

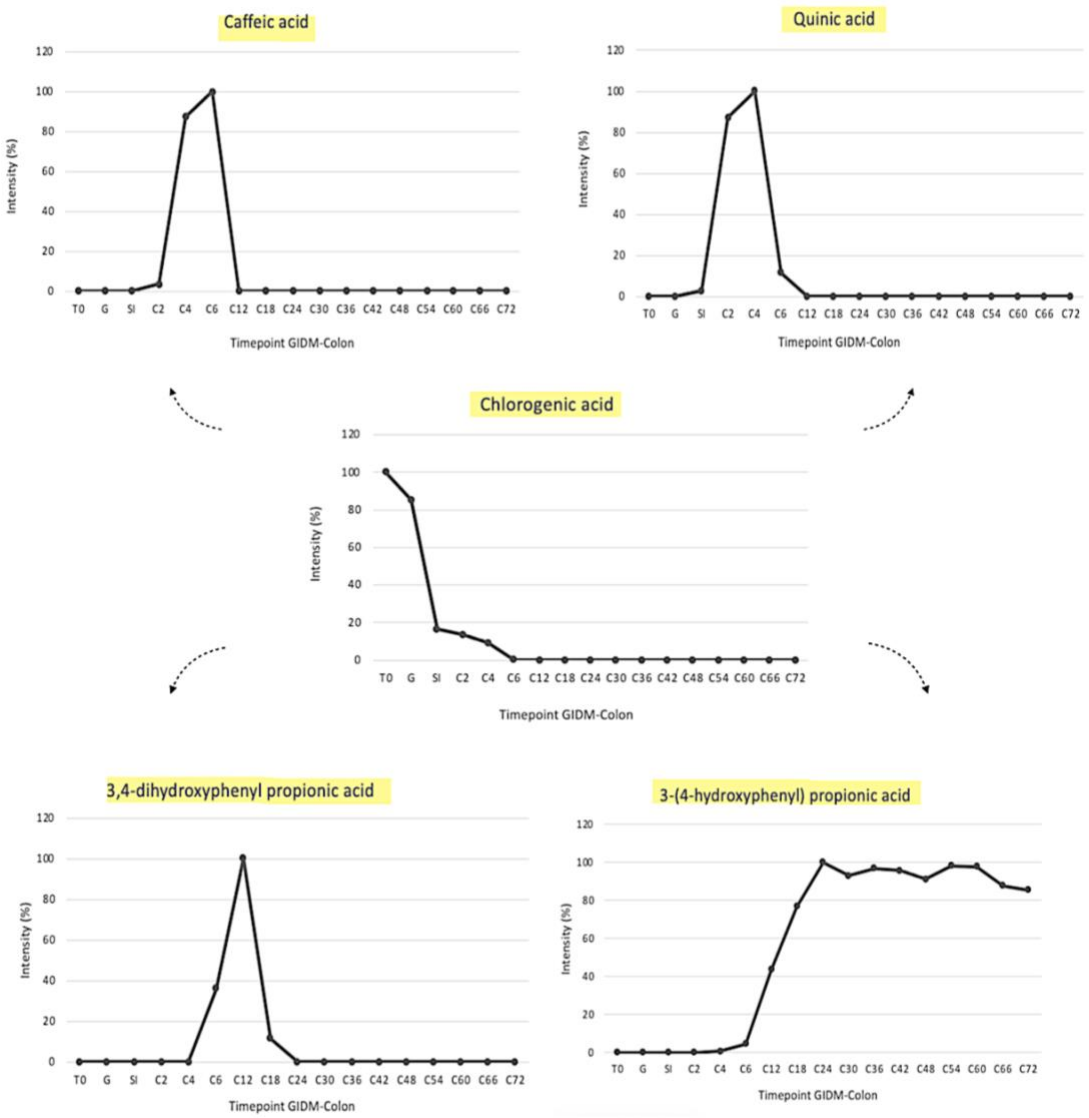

Figure S2: Biotransformation profiles of chlorogenic acid (decreasing intensity) and its biotransformation products (increasing intensity) over the time course of the GIDM-Colon experiment measured in the negative ionisation mode. Intensity can be maximal 100%.

Chapter 3 **ADHD therapy: a new approach?**

3.1 Attention-Deficit Hyperactivity Disorder

The behaviour patterns of hyperactivity, impulsivity and inattention that would eventually be defined as Attention-Deficit Hyperactivity Disorder (ADHD) have been reported for centuries. It was first described by George Still in the Coombs lectures of 1902 as a "abnormal defect in moral control in children"(Mahone & Denckla, 2017; Still, 2006). Later in the 1930s, hyperkinesis, impulsivity, learning disability and a short attention span were reported as "minimal brain damage" or "minimal brain dysfunction" due to similar behaviour in patients with central nervous system (CNS) injuries, suggesting ADHD could be a brain disorder (Spencer et al., 2007). Nowadays, the diagnosis of ADHD is made according to criteria outlined by the Diagnostic and Statistical Manual of Mental Disorders (DSM-5, 2013) of the American Psychiatric Association (APA) and is characterised as a neurodevelopmental disorder with a specific, persistent and pervasive behavioural pattern consisting of disruptive or developmentally inappropriate levels of impulsivity, hyperactivity and/or inattention (American Psychiatric Association, 2013). This diagnosis based on the DSM requires at least six out of nine ADHD symptoms (which can be divided into mainly inattention or hyperactivity-impulsivity domain) in one of the domains (five for patients aged 17 or older) and moreover, should interfere with normal functioning or development (Table 3.1).

Clinical presentation of ADHD is heterogeneous but generally three subtypes are recognised: the inattentive, hyperactive-impulsive, and a combined subtype (reflecting a combination of the two subtypes). Individuals diagnosed with ADHD differ from each other in terms of their core symptom combinations, level of impairment and comorbidities, as well as on other factors such as family background and social factors (Thapar & Cooper, 2016). Once assumed to be largely a disorder of childhood, growing evidence suggests that the symptoms of ADHD can persist into adulthood, as 60% of children with ADHD go on to have a significant impact on many aspects of the individual's wellbeing, including physical health, psychological, social, educational and occupational functioning (Faraone et al., 2006; Posner et al., 2020; Wilens et al., 2003; Wu et al., 2012). In view of its profound impact, the disorder is of high cost to both individuals and society.

Table 3.1: Characteristic symptoms of inattention and hyperactivity-impulsivity according to the DSM-5.

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.

DSM-5: Diagnostic and Statistical Manual of Mental Disorder Fifth Edition.

ADHD diagnosis is often challenging due to the substantial heterogeneity of the disorder in terms of clinical and pathophysiological aspects (da Silva et al., 2023). In fact, ADHD shows high concurrent comorbidity and overlap with other neurodevelopmental conditions and behavioural problems and more than 60% of individuals with ADHD present at least one comorbid psychiatric disorder (Jensen et al., 2001; Thapar & Cooper, 2016; Wallis et al., 2008). The most frequent comorbidities are learning disorders such as reading disorders and dyscalculia, autism spectrum disorder and tics (Drechsler et al., 2020). As such, patients are also at higher risk for depression and substance abuse (Biederman et al., 1992; Rommelse et al., 2010). Also, children diagnosed with ADHD have a predisposition to manifest behaviours commonly seen in oppositional defiant disorder (ODD) and conduct disorder (CD) including the antisocial behaviour that these children often display (Azeredo et al., 2018; Jensen & Steinhausen, 2014; Spencer et al., 2007). The presence of a comorbid disorder can affect symptom presentation, increase symptom severity, and lead to greater functional impairment (Bélanger et al., 2018).

3.1.1 Prevalence

Globally, estimates indicate that approximately 5% of children and adolescents are affected by ADHD (Song et al., 2021). Though ADHD seems to appear worldwide (Polanczyk et al., 2007), prevalence rates and reported changes in prevalence are highly variable (Song et al., 2021). For instance, ADHD prevalence estimates are higher in regions of the Middle East and North America and lower in African and Asian countries. According to Fayyad et al. (Fayyad et al., 2017) the prevalence rates in ADHD children and adolescents (aged 18 to 44 years) in the Belgian population were estimated to be 4.1%. Moreover, it was demonstrated that prevalence estimates may vary based on differences in research methodologies, the various age and gender groups being described, and changes in diagnostic criteria over time (Genro et al., 2014; Holbrook et al., 2017; Polanczyk et al., 2007). Regarding sex differences, earlier studies have demonstrated a higher ADHD prevalence in boys than in girls with an overall sex ratio boy:girl of 2:1 in children (Pérez-Crespo et al., 2020). Nevertheless, epidemiological studies suggest that ADHD is the most common childhood-onset neurocognitive behavioural disorder and one of the most prevalent chronic conditions in children (Barrio Cortes et al., 2020; Thomas et al., 2015).

3.1.2 Aetiology

ADHD is a complex and heterogeneous disorder and although its aetiology has been largely investigated over the last decades, it is still incompletely understood (Acosta et al., 2004; Bélanger et al., 2018). Combinations of genetic, neurological, and environmental factors are considered to contribute to its pathogenesis and its heterogeneous phenotype (Akutagava-Martins et al., 2016). Based on the available study findings, ADHD is presumed to be multifactorial in most cases. Genetic factors and early environmental risk factors interact in complex ways and can thus affect the structural and functional development of the brain. As for all complex disorders, no single risk factor can explain ADHD aetiology: many genetic and non-genetic (or environmental) factors contribute to the risk and no individual factor is sufficient to trigger ADHD. Moreover, the pattern of inheritance is multifactorial for most affected individuals (Banaschewski et al., 2017). Therefore, no straightforward indication can yet be given about its exact pathophysiology.

3.1.2.1 Neurological factors

Magnetic resonance imaging (MRI) is a widely used noninvasive modality for mapping *in vivo* brain changes occurring in neurological disorders (Yadav et al., 2021). According to current structural neuroimaging literature, various key brain structures are implicated in ADHD. Dysfunctional brain areas frequently implicated in the pathophysiology of ADHD are the prefrontal cortex, the anterior cingulate cortex, the parietal cortex and the basal ganglia including striatum (caudate nucleus and putamen) and globus pallidus. For instance, brain volume alterations were reported in children with ADHD compared to typically normal developing children (Wu et al., 2019). The most consistent finding is an overall reduction in total brain size and reduced dimensions of specific brain regions. Regional reductions were found in the caudate nucleus, the cerebellar vermis and the corpus callosum in ADHD patients as compared to controls (Tripp & Wickens, 2009). A disturbed white matter microstructure, reduced brain volume and grey matter density were also reported (Verlaet et al., 2019; Wu et al., 2019). A meta-analysis by Nakao et al. (Nakao et al., 2011) demonstrated that ADHD patients have a reduced overall volume of grey matter, particularly in the right caudate and lentiform nuclei. Moreover, Greven et al. (Greven et al., 2015) showed a reduction in global brain volume by 2.5% and total gray matter by 3% in ADHD patients as compared to controls. Since normal brain

development in early childhood tends to increase the grey matter volume, alterations in gray matter reflect abnormal neuronal maturation. These differences were more pronounced in children than in adults, indicating a delayed brain maturation in ADHD (Hoogman et al., 2017).

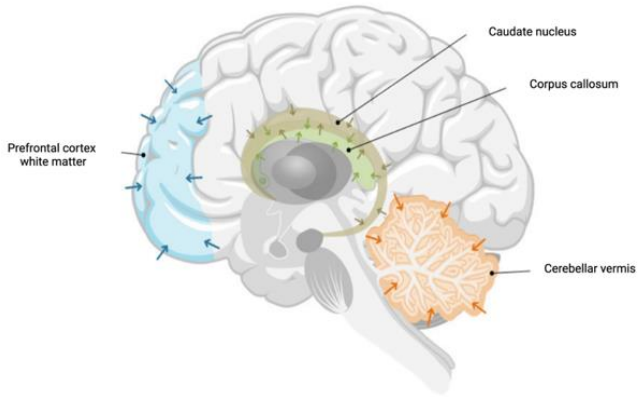

Figure 3.1: Reduced dimensions of caudate nucleus, corpus callosum, cerebellar vermis and white matter of prefrontal cortex in ADHD patients as compared to healthy controls. Reduction in size for different brain regions is represented by its respective arrow. Created in BioRender.

Besides these structural alterations, also functional changes in brain pathways have been reported in ADHD. Although the biological mechanisms underlying ADHD are not entirely clear, imaging studies have provided further supportive evidence of catecholamines and other neurotransmitter dysregulations in ADHD (Table 3.2). Catecholamines (e.g. epinephrine, norepinephrine and dopamine; monoamines derived from the amino acid tyrosine) are active in specific brain regions such as the prefrontal cerebral cortex, caudate nucleus and cerebellum and therefore involved in the regulation of cognitive processes like attention, impulse control, arousal and behaviour (Verlaet et al., 2019). Findings in animal models suggested involvement of dopaminergic and noradrenergic neurotransmission as well as involvement of serotonergic, glutamatergic and GABAergic neurotransmission systems (Faraone, 2018; Russell, 2011). For instance, higher density of dopamine transporter (DAT) or altered dopamine receptor (DRD) in brain regions mainly involved in signalling reward circuits, learning and memory and locomotor activity, have been noted in ADHD patients (da Silva et al., 2023). Also, the binding of norepinephrine with adrenergic receptors, which regulates the working memory process, is also implicated in ADHD patients. Although the serotonergic (involved in emotional regulation, sleep, mood and appetite), glutamatergic (learning and memory)

and GABAergic (motor control and behavioural inhibition) neurotransmission pathways contribute less than the dopaminergic and noradrenergic systems, they also have been implicated in ADHD (da Silva et al., 2023). In addition to the above-mentioned evidence, the involvement of neurotransmitter systems in ADHD is reinforced by the mechanisms of actions of most drugs used for its treatment.

Table 3.2: Overview of neurotransmission pathways potentially involved in ADHD based on evidence from Kessi et al., 2022; da Silva et al., 2023.

Neurotransmission pathway	Receptors	Role of the neurotransmitter	Evidence linking the system with ADHD
Dopaminergic	DRD1, DRD2, DRD3, DRD4 and DRD5, DAT1	Mediation of cognitive, motor, attentional, emotional and reward processes	Higher density of DAT and altered DRD2/DRD3 receptor availability reported in ADHD patients
Noradrenergic	Alpha and beta adrenergic receptors	Regulation of cognitive functions (e.g. working memory), arousal and alertness	ADHD is associated with decreased NET
Serotonergic	5-HT receptors	Involvement in sleep, appetite, mood and emotional regulation	Reduction in serotonergic function has been linked to the impulse ADHD subtype
Glutamatergic	Ionotropic and metabotropic glutamate receptors	Regulation of executive functions (e.g. learning and memory)	Increased glutamatergic tone and increased glutamate levels are associated with ADHD
GABAergic	GABA-A and GABA-B	Involvement in motor control and behavioural inhibition	ADHD is associated with reduced GABA levels

ADHD: Attention-Deficit Hyperactivity Disorder; DRD1,-2,-3,-4 and -5: dopamine receptor subtype 1,-2, -3, -4 and -5; GABA-A, -B: gamma aminobutyric acid subtype A, -B; NET; noradrenaline transporter; 5-HT: serotonin.

3.1.2.2 Genetic factors

Evidence from family studies (including twin and adoption studies) has suggested that ADHD is a highly hereditary disorder (the genetic contribution to ADHD is amongst the highest for psychiatric disorders) with a substantial role of genes involved (Acosta et al., 2004; Faraone & Mick, 2010). The estimated heritability of ADHD approximates 80% and suggests a remarkable effect on the aetiological background of the disorder (Balogh et al., 2022). Research work by Uchida et al. (Uchida et al., 2023) demonstrated that the offspring of ADHD parents had significantly more ADHD and associated psychiatric disorders than the offspring of healthy parents.

The usage of genome-wide association studies (GWAS) has identified many variants implicated in ADHD, including common and rare ones, that can explain more or less 40% of ADHD heritability (Boonchooduang et al., 2020). Single nucleotide polymorphisms (SNPs), copy number variations (CNVs) and microRNAs (miRNAs) have all been linked with ADHD. SNPs include common (defined as >5% population frequency) DNA sequence variants (SNPs); however, associations have only been reported when thousands of these are combined into a composite genetic risk score. More subtle chromosomal mutations, such as rare (defined as <1% frequency) deletions and CNVs, are also linked with ADHD risk. Although these are known to exert larger effects, they occur rarely (Thapar & Cooper, 2016). Also, miRNAs, responsible for post-transcriptional modulation of the genes, are involved in the regulation of some of the ADHD-related genes (Kessi et al., 2022). A close relationship between levels of circulating miRNAs and ADHD was demonstrated.

Candidate gene studies have used association methods to determine biologically relevant gene variants, by comparing cases with controls or by showing more transmission with ADHD in family studies (Biederman & Faraone, 2005; Faraone & Mick, 2010). These studies suggest that a multiplicity of candidate genes is involved, supporting the idea that biological heterogeneity might be a central factor in the clinical variability of the disorder (Corradini et al., 2009; Purper-Ouakil et al., 2011). Various genes have been reported to be associated with ADHD of which all have a significant association with neurotransmission and neurodevelopment (Figure 3.2). They can be categorised under six pathways including the dopaminergic, serotonergic, adrenergic,

cholinergic, glutaminergic and GABAergic pathway. First, genes reported in the dopaminergic pathway including dopamine receptor D1, D2 and D4 (DRD1, DRD2 and DRD4) and dopamine transporter 1 (DAT1), which codes for the main molecular target of stimulant medication appear to play a crucial role in the occurrence of ADHD (Biederman & Faraone, 2005; Cornish et al., 2005; Faraone & Mick, 2010; Kessi et al., 2022; Purper-Ouakil et al., 2011). Also, serotonergic genes were studied and polymorphisms associated with ADHD were found in tryptophan hydroxylase (TPH2), 5-hydroxytryptamine receptor 1A, 1B and 2A (HTR1A, HTR1B and HTR2A) and solute carrier family 6 member 4 (SLC6A4) (Acosta et al., 2004; Biederman & Faraone, 2005; Purper-Ouakil et al., 2011). Furthermore, genes related to the adrenergic pathway are involved including dopamine beta-hydroxylase (DBH), alpha-2A-adrenergic receptor and alpha-2C-adrenergic receptor (ADRA2A and ADRA2C). Two cholinergic genes (cholinergic receptor nicotinic alpha 4 and alpha 7 (CHRNA4 and CHRNA7)) have also been reported in ADHD. Finally, glutamic acid decarboxylase (GAD), glutamate receptor genes -1-,5,-7 and- 8 (GRM1, GRM5, GRM7 and GRM8) are also implicated. Besides, genes related to other pathways such as a gene coding for a synaptic vesicle regulating protein (SNAP-25) is important in the regulation of neurotransmission release (Wallis et al., 2008). Although various meta-analysis studies have found significant relationships in the multiple genes for common genetic variants, it is still challenging to identify ADHD risk genes due to the complex and polygenic nature of ADHD pathophysiology.

Moreover, growing evidence from genetic studies also suggests that polymorphisms in genes related to inflammatory pathways play a role in ADHD (Leffa et al., 2019). For example, SNPs in a cytokine-related gene, the ciliary neurotrophic factor receptor (CNTFR), were found. An association of ADHD with genes of the major histocompatibility complex (MHC) which are linked to various autoimmune diseases, have also been demonstrated (Aureli et al., 2008; Leffa et al., 2019; Odell et al., 1997). In a genetic association study, a preferential transmission of interleukin-1 receptor antagonist (IL-1Ra) alleles in ADHD subjects has been demonstrated (Segman et al., 2002); however, no association was found in a larger sample (Misener et al. 2004). IL-1 in the brain is involved in the differentiation of dopamine neurons and in the modulation of central monoaminergic activity (Potter et al., 1999; Segman et al., 2002). Interestingly, some IL-1Ra polymorphisms may result in altered dopaminergic neuronal differentiation during neural development, which in turn could lead to altered dopaminergic reactivity in the

pathophysiology of ADHD (Segman et al., 2002). Moreover, polymorphisms of cytokines such as IL-2, IL-6 and TNF-α have also been reported. These findings thus further support the role of inflammation and autoimmunity in ADHD.

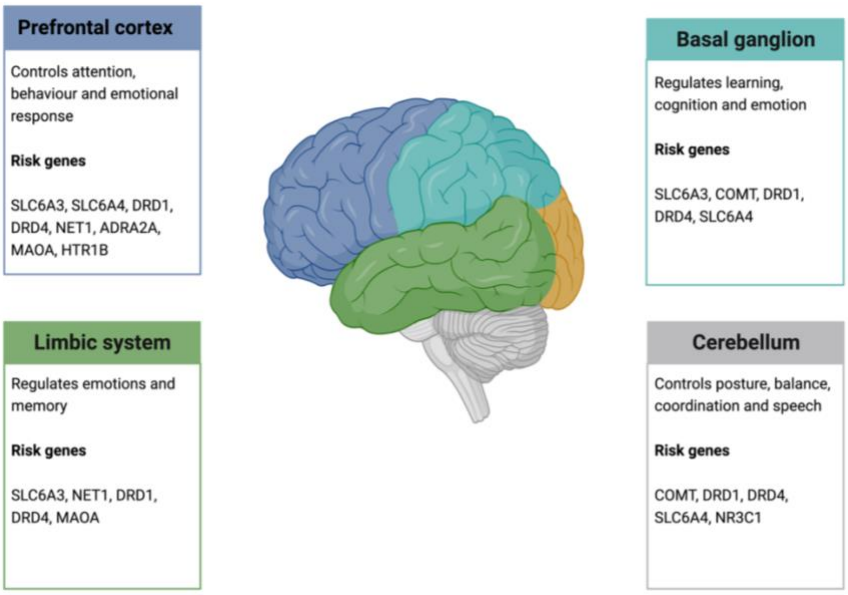

Figure 3.2: Multiple risk genes are associated with altered brain regions mainly in the prefrontal cortex, basal ganglion, limbic system and cerebellum in Attention-Deficit Hyperactivity Disorder. SLC6A3: transporter solute carrier family 6 member 3; SLC6A4: transporter solute carrier family 6 member 4; DRD1: dopamine receptor D1; DRD4: dopamine receptor D4; NET1: neuroepithelial cell transforming 1; ADRA2A: adrenoceptor alpha 2A; MAOA: monoamine oxidase A; HTR1B: 5-hydroxytryptamine receptor 1B; COMT: catechol-O-methyltransferase; NR3C1: nuclear receptor subfamily 3 group C member 1. Adapted from Kessi et al., 2022.

3.1.2.3 Environmental factors

Besides genetic factors, also environmental factors are known to be important in ADHD development (Thapar & Cooper, 2016). According to the results of observational case-control and epidemiological studies, exposure to a range of pre-, peri- and postnatal factors, environmental toxins, dietary factors, and psychosocial factors are all associated with ADHD (Thapar et al., 2013). For example, *in utero* events like maternal stress and factors impacting brain development such as toxin exposure (e.g. alcohol and tobacco), prematurity/low birth weight, encephalitis, and metabolic disorders (e.g. obesity) may

lead to an increased risk of ADHD (Banerjee et al., 2007; Talge et al., 2007; Wallis et al., 2008). Also, postnatal environmental factors such as neonatal anoxia and seizures, brain injury and exposure to air pollution or other toxins such as lead, pesticides and polychlorinated biphenyls have been considered to increase the risk of ADHD (Kuehn, 2010; Myhre et al., 2018; Purper-Ouakil et al., 2011; Thapar et al., 2013).

Other factors related to nutrition such as a consumption of food additives and reduced or elevated levels of specific micronutrients have been mentioned with respect to ADHD (Howard et al., 2011; Sinn, 2008). For example, zinc deficiency is associated with neurological dysfunction (Gower-Winter & Levenson, 2012). Zinc is an important co-factor for multiple enzymes and is required for modulating melatonin and dopamine. Moreover, low levels of copper, iron, magnesium, and omega-3 fatty acids, have been observed in ADHD patients while high levels of sugar, artificial food colourants and preservatives are linked to an increased risk of ADHD (da Silva et al., 2023).

Furthermore, some environmental exposures can also result in biological modifications, including DNA methylation, which are reversible changes in genomic function independent of the DNA sequence that has also been associated with ADHD. Therefore, environment and genetics act together in the aetiology of ADHD, making it challenging to disentangle several sources of bias/confounding (da Silva et al., 2023).

3.1.2.4 Gut microbiome

Also, deviations from the optimal assembly of the gut microbiota during early life appear to be involved in ADHD (Boonchooduang et al., 2020). The influence of alterations in gut microbiota on ADHD will be discussed in Chapter 5.

3.1.2.5 Oxidative stress and immune imbalance

Lastly, several studies suggest that alterations in oxidative status and cellular immunity may be involved as well, raising the hypothesis that ADHD could be linked to oxidative and immune dysfunctions (Ceylan et al., 2012; Corona, 2020; Verlaet et al., 2019). The involvement of oxidative stress and immunity in ADHD will be explained into further detail in the respective subchapters.

3.2 Current ADHD therapy

Early and adequate intervention can reduce the risk of negative ADHD-related outcomes in mental and physical health (da Silva et al., 2023). A wide variety of guidelines on the assessment and management of ADHD have been published over the last decade, not only for clinicians but also for patients and caregivers (Drechsler et al., 2020). All guidelines recommend a multimodal treatment approach in which a combination of psychoeducation and pharmacological therapy appears the most beneficial form of treatment.

Psychoeducation, including cognitive behavioural therapy (CBT), forms a cornerstone of the treatment and should be offered to all of those diagnosed with ADHD, as well as to their families and caregivers (Drechsler et al. 2020). Pharmacological treatment is generally only advised in case of severe symptoms, impairment that requires direct medications and when psychological and behavioural interventions are insufficient (Taylor et al. 2004).

Seen the evidence from neurobiological and neuropharmacological studies regarding dysregulation of catecholaminergic systems in frontal subcortical regions associated with ADHD symptomatology, it is straightforward that using medications working on these neurotransmitter systems can improve ADHD symptoms (da Silva et al., 2023). Clinical experience demonstrates that both stimulant and non-stimulant drugs, acting via the catecholaminergic system, are effective in alleviating ADHD symptoms. Currently the main treatment for ADHD is pharmacological, using stimulant drugs such as methylphenidate (MPH, e.g. Ritalin®, Medikinet®, Equasym®) and dexamphetamine (e.g. Dexedrine®) and non-stimulant drugs including atomoxetine (e.g. Strattera®), clonidine and guanfacine. In general, stimulant drugs present superior efficacy compared to other medications for all age populations. For patients who do not respond, do not tolerate, or present specific conditions for which stimulants are contraindicated, non-stimulant drugs are alternative FDA-approved treatment options (da Silva et al., 2023). These non-stimulant drugs were shown to be efficacious in ADHD treatment, however, due to their smaller effect sizes they are generally only recommended as second-line treatment whereas MPH is nowadays considered as the first-choice medication and is most frequently prescribed for children and adolescents with ADHD (Storebø et al., 2018).

According to the most recent regulations of the Hoge Gezondheidsraad, MPH is also in Belgium the first-choice medication for treatment of ADHD (Hoge Gezondheidsraad, 2021).

3.2.1 Mechanisms of action of methylphenidate

First synthesised in 1944, the compound nowadays known as MPH is the most used ADHD medication (Jaeschke et al., 2021). The earliest FDA-approved MPH formulations were rapidly released and absorbed into the bloodstream, quickly followed by biotransformation processes. Due to advanced drug formulation techniques, new pharmaceutical preparations with distinct pharmacokinetic profiles became available to help 'tailoring' the treatment regimens to the individual need of the patients (Jaeschke et al., 2021).

The multimodal mechanism-of-action of MPH is depicted in Figure 3.3. Firstly, there is a direct inhibition of the presynaptic dopamine transporter (DAT) especially in the prefrontal cortex and striatum, which is due to its partial similarity to the basic structure of catecholamines. The transporters are implicated in the reuptake of respectively dopamine and norepinephrine from the synaptic left to the presynaptic neuron. This MPH-induced DAT blockade leads to a decreased presynaptic reuptake of dopamine, increased concentrations of dopamine in the synaptic cleft and thereby increased neurotransmission (Engert & Pruessner, 2008; Seeman & Madras, 1998; Wilens, 2008). As suggested by positron emission tomography (PET) studies, in therapeutic doses MPH blocks more than 50% of DAT and thereby significantly increases extracellular dopamine levels. By increasing dopaminergic activity in the brain, MPH elevates the overall activity of the CNS, leading to various significant behavioural and cognitive effects (Jaeschke et al., 2021). Next to its influence on the dopamine system, MPH also increases, albeit to a lesser extent, extracellular levels of norepinephrine by blocking its reuptake (Engert & Pruessner, 2008). Growing evidence suggests that MPH not only has a high affinity for norepinephrine transporters (NET), but also interacts directly with α_2-noradrenergic receptors. In fact, after administration of MPH there is a three- to fourfold increase of dopamine and norepinephrine in the striatum as well as in the prefrontal cortex (Jaeschke et al., 2021). Besides DAT and NET blockade, MPH has been shown to increase dopamine signalling in the striatum via other mechanisms such as disinhibition of dopamine D2 autoreceptors (inhibitory receptors) on the presynaptic dopaminergic

neuron and activation of dopamine D1 receptors on the postsynaptic neuron. Dopamine autoreceptors regulate the dopamine system by providing feedback inhibition by controlling cell firing and synthesis, release and uptake of dopamine. It is postulated that MPH treatment also facilitates the release of dopamine by disinhibition of D2 autoreceptors on the presynaptic neuron (Wilens, 2008). Moreover, MPH was shown to affect the redistribution of vesicular monoamine transporter-2 (VMAT-2), which is involved in the sequestration (capturing, removal and storage) of cytoplasmic dopamine and noradrenaline, and is therefore an important regulator of neurotransmission. Through the interactions with VMAT-2, the balance between the available intra-synaptic dopamine and the intracellular pool is preserved and MPH thus protects the dopaminergic system against the ongoing 'wearing off' (by securing a substantial reserve pool of dopamine, stored in the presynaptic vesicles) (Jaeschke et al., 2021).

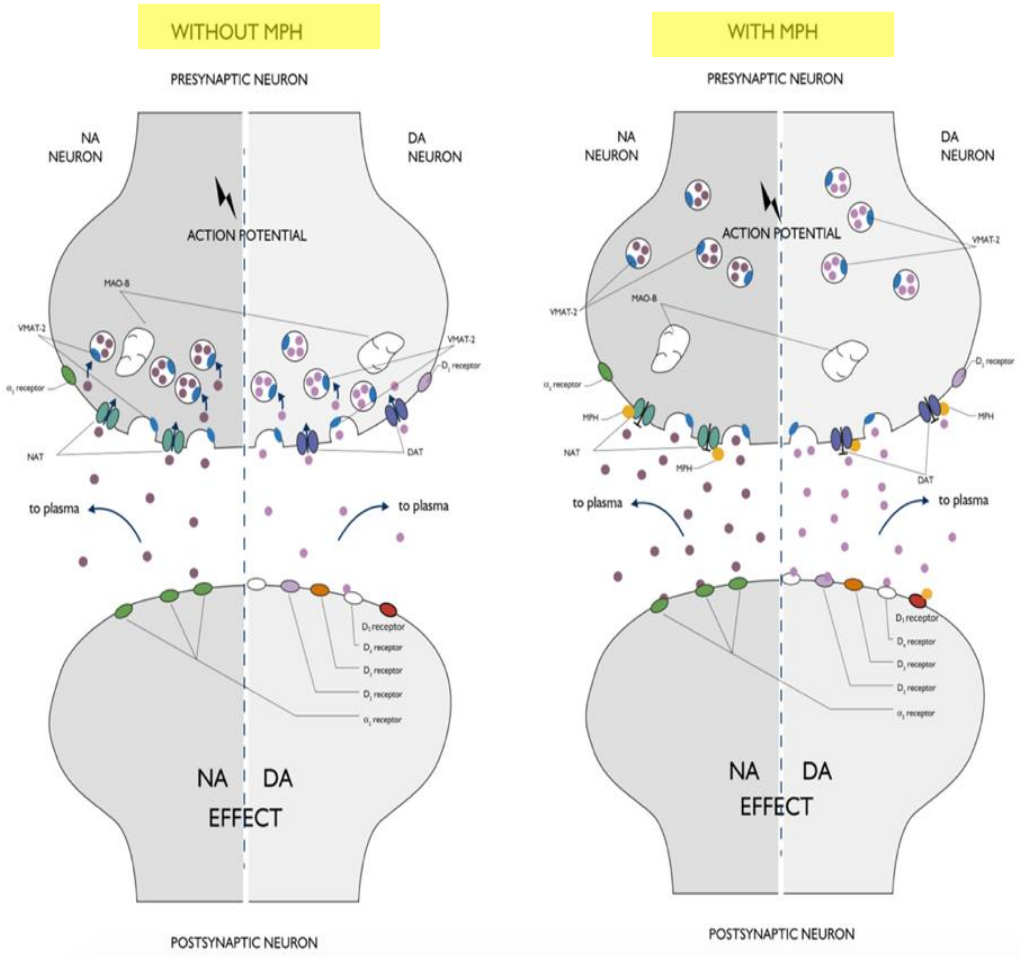

Figure 3.3*:* Mechanism of action of MPH on the dopaminergic (DA neuron) and noradrenergic (NA neuron) neurotransmission pathway, depicted at the synaptic level. Following an action potential, dopamine is released from the presynaptic neuron in the synaptic cleft. Left: a generalised striatal neuron before MPH treatment in ADHD patients with low levels of dopamine in the synaptic cleft due to reuptake by dopamine and norepinephrine transporters, which results in shorter periods of dopamine action. Right: the effect of MPH on the dopaminergic and noradrenergic system. This neuron shows an accumulation of extracellular dopamine via MPH-induced blockade of dopamine and norepinephrine transporters, via disinhibition of dopamine D2 autoreceptors on the presynaptic dopaminergic neuron and activation of dopaminergic and α2-adrenergic receptors on the post-synaptic neuron, thereby enhancing neurotransmission and dopamine action in the postsynaptic dendrite. Adapted from Jaeschke et al., 2021.

3.2.2 Side effects of methylphenidate therapy

Despite many years of clinical use and a favourable risk benefit profile, stimulant drugs remain controversial due to the concerns about side effects, long-term use and efficacy (Antshel et al., 2011; Schachter et al., 2001; Steer, 2005; Stevens et al., 2013; Storebø et al., 2015). The most reported side effects associated with MPH include headache, sleep problems and loss of appetite (Storebø et al., 2015). Concerns about cardiovascular safety, psychotic symptoms, tic disorders and drug misuse/abuse have also been reported (Antshel et al., 2011; Awudu & Besag, 2014; Stevens et al., 2013). The possible adverse cardiovascular outcomes including increases in heart rate and blood pressure will be further discussed in Chapter 6.

Additionally, still little is known about the long-term effects of psychostimulants on the developing brain, as long-term interference with neurotransmitter systems potentially causes alterations in CNS structure and functioning and might even increase the risk for diseases of the basal ganglia and cerebellum, including Parkinson's disease (Curtin et al., 2018; Konrad et al., 2007; Sadasivan et al., 2012). Moreover, there is a possibility that some ADHD patients may not respond adequately to MPH or may not tolerate it due to its side effects (Antshel et al., 2011). In fact, despite the vast number of available pharmacological therapies, the outcomes differ according to the individuals: around 80% of the patients respond well to the psychostimulants drugs whereas approximately 20% have a poor response (Kessi et al., 2022). Treatment non-adherence is therefore often high, and parents are disinclined to use MPH as only treatment option for their child (Antshel et al., 2011; Swanson, 2003). It has been demonstrated that the incidence of withdrawal from MPH varies from 6.2% to 16.2% and that adverse effects of the drug are the main reason for withdrawal (Storebø et al., 2018). Furthermore, until now, full symptom reduction in ADHD patients is yet to be achieved and the burden of ADHD-related consequences, like effects on professional performances, has not been diminished (Verlaet et al., 2019).

Alternative therapeutic options are therefore highly desired since concerns about safety and non-adherence to psychostimulants are on the rise. Due to their antioxidative and immune modulating properties, polyphenols might have potential as a therapeutic alternative (Verlaet et al., 2019).

3.3 Oxidative imbalance in ADHD

ADHD is thus a prevalent neurodevelopmental disorder that has been associated with numerous structural and functional CNS abnormalities, but also findings on neurobiological mechanisms linking genes to brain phenotypes begin to emerge (Purper-Ouakil et al., 2011). Although its exact pathophysiology, besides dopaminergic dysfunction, remains unclear, also immune and oxidant-antioxidant imbalances appear to be involved (Ceylan et al., 2010, 2012; Kawatani, et al., 2011). Before we shed light on the evidence for these imbalances, the concept of oxidative stress will be discussed first.

3.3.1 Oxidative stress

Oxidative stress is the result of a disequilibrium between oxidants and antioxidant systems, in favour of the oxidants, as depicted by Figure 3.4. It is a biological condition where an accumulation of oxidants occurs as a resultant of either production of disproportionate amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS), decreased levels of antioxidants that scavenge them, or culmination of both (Moghadas et al., 2019). High levels of oxidative stress can cause damage to e.g. lipids, proteins and DNA, thereby altering signal transduction and gene expression, inhibiting protein function and promoting cell death.

ROS including superoxide anion radical ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$) or hydrogen peroxide (H_2O_2) and RNS such as nitric oxide ($NO^{\bullet}$) are a group of highly reactive by-products produced in the cells of aerobic organisms (Hassan et al., 2022). Low physiological levels of ROS and RNS are involved in regulating gene expression and signalling pathways among other functions, and are thus required for normal cellular functioning. Formation of ROS and RNS *in vivo* can be the result of either enzymatic or non-enzymatic reactions. Furthermore, oxidant production can be generated via an exogenous or endogenous source. Exogenous sources including electromagnetic radiation, pollutants, cigarette smoke or industrial solvents and endogenous reactions including inflammation, infection or ageing can cause ROS/RNS formation. Even though the production of these ROS/RNS is inevitably associated with biotransformation and other enzymatic processes (Jelinek et al., 2021), excessive amounts of ROS/RNS and/or inefficiency of the antioxidant system to counterbalance these oxidants, can damage carbohydrates, nucleic acids,

lipids and proteins, thereby modifying their function (Moghadas et al., 2019; Valko et al., 2007).

Figure 3.4: Schematic representation of the imbalance between enzymatic and non-enzymatic antioxidants and oxidants leading to cell dysfunction or damage. Created in BioRender.

Under normal physiological conditions ROS/RNS are effectively eliminated by various antioxidant mechanisms (Jelinek et al., 2021). According to Halliwell and Gutterige's (1989) definition, an antioxidant is "any substance that, when present at low concentrations compared to those of an oxidisable substrate, significantly delays or inhibits oxidation of that substrate" (Halliwell & Gutteridge, 1990). This definition includes a broad set of both enzymatic and non-enzymatic antioxidants including oxidative enzyme inhibitors, antioxidant enzyme cofactors and protecting- and metal chelating proteins, working together to protect against tissue and cell damage (Lee, et al., 2020; Magielse et al., 2013). From a nutritional perspective a more informative distinction can be made between endogenous (if the eukaryotic cell is able to synthesise it) and exogenous (if the antioxidant needs to be ingested through the diet) antioxidant systems (Sharifi-Rad et al., 2020).

Some important enzymatic antioxidants (all endogenous) are superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (Mirończuk-Chodakowska et al., 2018). SOD is the first defence system of antioxidant enzymes and catalyses the disproportionation of the unstable superoxide anion radical ($O_2^{\bullet-}$) to the more stable hydrogen peroxide (H_2O_2), releasing molecular oxygen (O_2) (Yang et al., 2022). Three isoforms of SOD exist: SOD1 and SOD3 contain respectively copper and zinc as cofactor and the mitochondrial enzyme, SOD2, has manganese (Mn) in its reactive centre. CAT is a haem-containing intracellular enzyme and catalyses the decomposition of H_2O_2 to H_2O and O_2. It mainly acts on the dismutation reaction of H_2O_2 produced in SOD-mediated reactions (Yang et al., 2022). GPx is a selenium dependent enzyme and catalyses the reduction of H_2O_2 and peroxides (ROOH) to water (H_2O) and alcohols (ROH) respectively, while converting reduced glutathione (GSH) into oxidised glutathione (GSSG). The enzyme glutathione reductase (GR) will finally reduce the GSSG back into GSH. The interplay of the antioxidant enzymes in the organism is represented in Figure 3.5.

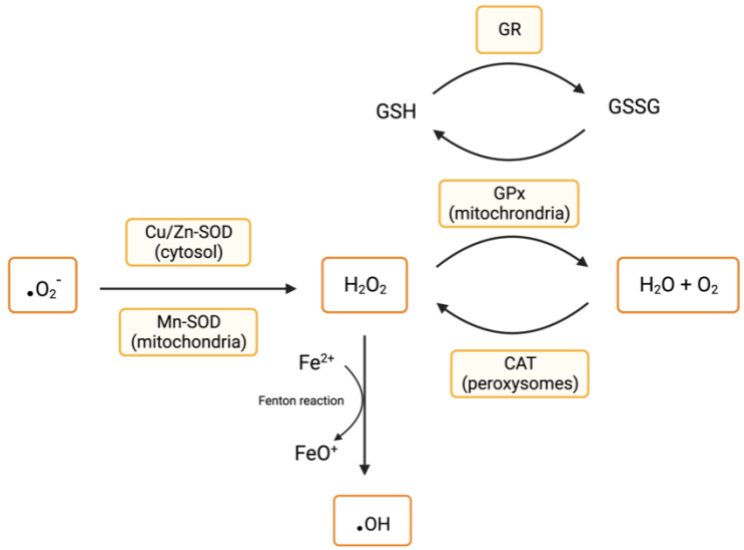

Figure 3.5: Overview of the enzymatic antioxidant defences present in a human body. The Fenton reaction results in the formation of hydroxyl radicals from hydrogen peroxide when iron (Fe^{2+}) is used as a catalyst. CAT: catalase; Cu: copper; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: reduced glutathione. GSSH: oxidised glutathione; Mn: manganese; SOD: superoxide dismutase; Zn: zinc. Created in BioRender.

GSH on the other hand, is a water-soluble tripeptide composed of the amino acids glutamic acid, cysteine and glycine, representing the most abundant intracellular thiol-containing tripeptide found in all animal tissues, plants, fungi and some microorganisms (Mirończuk-Chodakowska et al., 2018; Pastore et al., 2003). Under physiological conditions, it is mostly present in the cytoplasm in the reduced form (GSH), which is also the biologically active form (Figure 3.6). The fully oxidised form containing a disulfide between two identical GSH molecules (GSSG) represents less than 1% of the total GSH pool in the cell. In normal conditions, intracellular GSH concentrations vary between 0.1 to 10 mmol/L whereas various diseases are associated with altered levels of GSH (Potęga, 2022). The thiol group of the cysteine residue is a potent reducing agent, rendering GSH the most abundant intracellular antioxidant (Townsend et al., 2003). GSH can act directly as a potent ROS scavenger without enzymatic help, and it plays a pivotal role in reducing oxidative stress, maintaining the redox balance, enhancing metabolic detoxification and regulating the immune system (Sharifi-Rad et al., 2020).

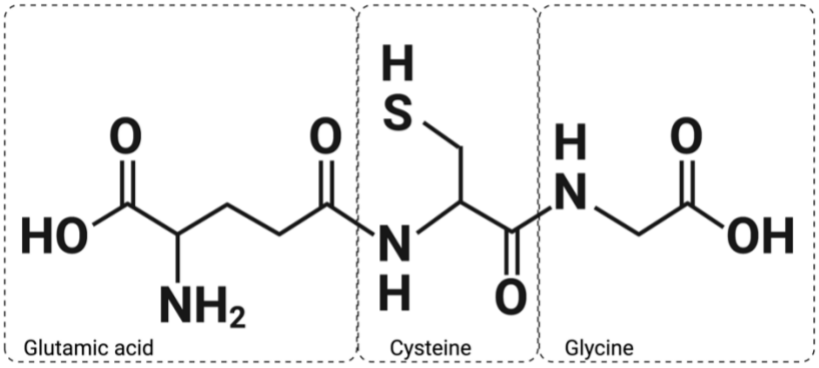

Figure 3.6: Structure of the tripeptide glutathione (GSH; reduced form). Glutamic acid is linked in a γ-peptide linkage (via its γ-carboxyl group) to cysteine, which in turn forms an α-peptide linkage with glycine. Created in BioRender.

Besides GSH, co-enzyme Q10 (coQ10) also known as ubiquinone (Figure 3.7), is an endogenously produced compound that is part of the electron transport chain within the mitochondria. It is a lipid-soluble antioxidant and, when in its active form (ubiquinone), it protects lipoproteins and lipids from radical chain reactions, peroxidation and oxidative damage by scavenging several ROS or regenerating other

antioxidants such as vitamin C or E. Afterwards, the quinone can be reduced back by various NADPH-dependent enzymatic systems. CoQ10 exists in three biologically relevant forms: the fully reduced ubiquinol ($CoQH_2$), the partially oxidised semiquinone radical (semiubiquinone) and the fully oxidised ubiquinone (Magielse et al., 2013).

In addition, exogenous antioxidants also show prominent antioxidant effects. Vitamin C and lipid soluble antioxidants such as vitamin A, vitamin E and carotenoids are potent scavengers of radical compounds (Figure 3.7) and play a pivotal role in maintaining the balance between pro-oxidant and antioxidant agents in the brain and in alleviating oxidative stress (Jiang et al., 2016). Vitamin C, also known as ascorbic acid, acts as an important free radical scavenger and exerts its antioxidant action through other mechanism (Skrzydlewska et al., 2022). Vitamin E is a lipid-soluble antioxidant essential for the development and maintenance of the human nervous system (Traber & Sies, 1996). The vitamin E family contains eight isomers, namely four tocopherols (α-, β-, γ-, and δ-tocopherol) and four tocotrienols (α-, β-, γ-, and δ-tocotrienol). The number and position of the methyl groups on the chromanol rings reflect the various isomeric forms of tocopherols and tocotrienols. None of these eight isomers can be synthesised in the human body and they must thus be supplied exogenously through the diet, mainly from vegetable oils, some oilseeds and nuts (Szewczyk et al., 2021). Due to the presence of a hydroxyl group and a hydrophobic side chain that can penetrate into biological membranes, these isomers can act as scavengers of peroxyl radicals ($ROO^{\bullet}$) and are probably the most important inhibitors of lipid peroxidation in membranes and lipoproteins (Bruno & Traber, 2006; Hermans et al., 2005; Traber & Sies, 1996). After reaction of the phenolic hydroxyl group with the peroxyl radical, corresponding organic hydroperoxide (ROOH) and vitamin E radical ($vit\text{-}E\text{-}O^{\bullet}$) are formed (Traber & Sies, 1996). The generated hydroperoxide can be detoxified via non-radical reactions and vitamin E radicals can be reduced to vitamin E by interaction with hydrogen donors, such as vitamin C (ascorbate) and GSH (Traber & Sies, 1996). RRR-α-tocopherol, the naturally occurring form of α-tocopherol (containing chiral carbons in the R-conformation at positions 2, 4', and 8'), is known to have the highest *in vivo* antioxidant activity (Brigelius-Flohé & Traber 1999; Reboul et al. 2006; Traber & Sies, 1996). It has been shown that this antioxidant activity of vitamin E isomers depends on the number of hydroxyl groups present and is in the order of $\alpha > \beta > \gamma > \delta$. Though the antioxidant activity has long been considered as the main mechanism behind the biological properties of vitamin E, it also

displays “non-antioxidant” functions such as modulation of cellular proteins with enzymatic activity and transcriptional function of genes involved in important cellular processes including cell cycle regulation, proliferation and cell death signalling or inflammation (Szewczyk et al., 2021).

Next, carotenoids are naturally occurring pigments belonging to the tetraterpene family (C40-based isoprenoid) and are mostly found in colourful fruits and vegetables, plants, algae, fungi and photosynthetic bacteria (Eggersdorfer & Wyss 2018; Milani et al., 2017). Nearly 600 carotenoids have been identified in nature, but only 50 carotenoids are found in a typical human diet and around 20 structures have been identified in human blood and tissues (Milani et al., 2017; Tan & Norhaizan, 2019). On the one hand, carotenoids can be divided into provitamin A (e.g. β-carotene, α-carotene and β-cryptoxanthin) and non-provitamin A compounds. Alternatively, carotenoids are also commonly divided into carotenes (e.g. α-carotene, β-carotene and lycopene) and xanthophylls (e.g. lutein, zeaxanthin), based on their functional groups (Milani et al., 2016). Retinyl palmitate (RP) is a retinyl ester and is the main storage form of vitamin A in the liver (O’Byrne & Blaner, 2013). β-carotene can be converted into vitamin A and is thus an important vitamin A source for humans (Grune et al., 2010). Vitamin A includes different naturally occurring compounds: retinol, retinal, retinoic acid and retinyl esters and plays a role in growth and development, immunity, maintenance of epithelial barriers and vision (McLaren & Kraemer, 2012; O’Byrne & Blaner, 2013). Important dietary sources of retinol are animal products such as dairy, liver and fish (Eggersdorfer & Wyss, 2018). Overall, numerous health benefits have been attributed to carotenoids, including immune enhancement, prevention of heart disease, inhibition of mutagenesis (cancer prevention), cellular signalling, skin protection and protection against visual disorders (Fiedor & Burda, 2014; Stahl & Sies, 2005; Zimmer et al., 2007). These beneficial health effects of carotenoids can be explained by their antioxidant potential: the presence of conjugated double bonds enables carotenoids to accept electrons from reactive species, thereby neutralising these free radicals, and thus contributing to the defence against lipid peroxidation (Fiedor & Burda, 2014; Stahl & Sies, 2005).

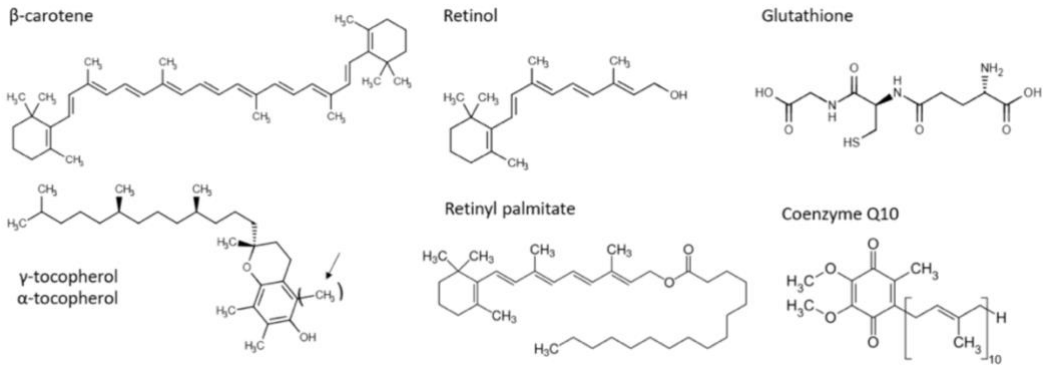

Figure 3.7*:* Chemical structures of lipid-soluble antioxidants: β-carotene, α- and γ-tocopherol (vitamin E), retinol (vitamin A), co-enzyme Q10 (coQ10), retinyl palmitate and the water-soluble tripeptide glutathione. Created in BioRender.

3.3.2 Evidence for oxidant-antioxidant imbalance in ADHD

Various reasons render the brain susceptible for oxidative stress including its high level of oxygen consumption, modest antioxidant defences and a high lipid content (Ikonomidou & Kaindl, 2011; Joseph et al., 2015). For instance, peroxidation of membrane lipids (mainly consisting of polyunsaturated fatty acids (PUFAs)) can cause alterations in the fluidity and integrity of the membrane and can cause an inactivation of membrane bound receptors and enzymes, leading to progressive neuronal damage and deterioration of normal cerebral functioning. Also, since the CNS consumes a large amount of oxygen to carry out physiological processes, metabolisation of oxygen can induce free radicals, which can negatively impact CNS functioning.

Moreover, numerous studies imply that oxidant-mediated neuronal damage plays a role in the pathophysiology of various psychiatric disorders like schizophrenia, autism, bipolar mood disorder and obsessive-compulsive disorder (OCD) (Behl et al., 2010; Ceylan et al., 2012; Chauhan & Chauhan 2006; Salim, 2014). Like ADHD, autism is a complex neurodevelopmental disorder with a strong genetic component in which a defective dopamine neurotransmission regulation plays a role. In autism patients, plasma GSH levels are found to be lower, as are cysteine and cysteinylglycine (metabolic

precursors of GSH) suggesting insufficient GSH synthesis. In addition, levels of glutathione disulphide (GSSG, oxidised glutathione) were increased (Bradstreet et al., 2010; James et al., 2004). Also, the antioxidant enzymes SOD, GPx and MDA, a biomarker of lipid peroxidation, seem implicated in the pathogenesis of schizophrenia. Moreover, patients with OCD had significantly higher MDA levels and lower antioxidant enzyme activities (Ng et al. 2008). An imbalance in the production of free radicals and antioxidant defences has thus been observed in multiple psychiatric disorders (Akif et al., 2023). These additional indications from other psychiatric disorders might further imply the association of oxidative stress with ADHD.

The relationship between ADHD and oxidative stress in children, adolescents and adults, is still being investigated (Guney et al., 2015). Although findings are still inconclusive, with some demonstrating elevated oxidative stress in ADHD patients, while others could not confirm these findings, alterations in oxidative metabolism have been reported as a significant factor in the aetiology of ADHD (Moghadas et al., 2019). A meta-analysis from Joseph et al. (Joseph et al., 2015) confirmed that ADHD is likely to be associated with oxidative stress. Changes in oxidative metabolism could thus be an important factor in ADHD pathophysiology, but the causative role has yet to be fully elucidated. It has been hypothesised that ROS can react with neuronal membrane proteins, leading to deterioration in neuronal cell membrane structure, which can also affect neuronal receptors, thereby preventing normal neurotransmitter signalling in norepinephrine and dopamine pathways (Akif et al., 2023; Oztop et al., 2012).

To make an estimation of the oxidative stress situation in ADHD patients compared to controls, several markers for oxidative damage (Table 3.3) have been investigated. For example, studies found elevated as well as reduced levels of MDA in children with ADHD (Ceylan et al., 2012; Oztop et al. 2012). A case-control study by Verlaet et al. (Verlaet et al., 2019) found a trend towards elevated MDA and 8-hydroxy-2'-deoxyguanosine (8-OHdG; a marker for oxidative DNA damage) levels in ADHD patients and more specifically, a (weak) correlation between MDA and the impulsivity score. Moreover, evidence for an increased lipid peroxidation was demonstrated by higher levels of urinary acrolein lysine as well as higher levels of exhaled ethane in ADHD patients (Kawatani et al., 2011; Ross et al., 2013). Nevertheless, some studies investigating oxidative damage in ADHD show contradictory results such as increased DNA damage in

lymphocytes, determined by 8-oxo-7,8-dihydroguanine (8-oxoG) levels, but reduced 8-OHdG levels were found in serum (Chovanová et al., 2009; Oztop et al., 2012).

In fact, when only a few biomarkers are measured, individual values may remain unchanged or decreased, even though the actual oxidant status increased or vice versa. Therefore, a study investigated the total oxidant status (TOS), and oxidative stress index (OSI) in children and adolescents with ADHD (Kul et al., 2015). It was demonstrated that TOS and OSI values were significantly higher in the patient group compared to controls. Sezen et al. (Sezen et al., 2016) have also found an increased TOS and OSI in children with ADHD. Conversely, another study did not report any changes in TOS and OSI values between patients and controls (Karababa et al., 2017).

As stated above, oxidative stress can also be due to an inadequate response of antioxidants to ROS/RNS. However, the meta-analysis by Joseph et al. (Joseph et al., 2015) concluded that ADHD is not characterised by insufficient antioxidant production as the association between ADHD and antioxidant status was not significant. Nevertheless, several studies as shown in Table 3.4, reported significantly lower plasma and saliva activity levels of the antioxidant enzymes glutathione-S transferase (GST), GPx, SOD and CAT in ADHD patients as compared to controls, suggesting a lower antioxidant activity. Some studies on the other hand reported higher GST activity in ADHD and no significant differences in SOD and CAT activity. Additionally, erythrocyte GSH levels were found to be significantly higher and GPx activity was found to be lower in ADHD patients than in controls (Ceylan et al., 2012; Verlaet et al., 2019). The total antioxidant status (TAS) was also investigated in several studies, but findings were inconsistent with higher, equal and lower TAS levels reported in paediatric ADHD as compared to control values (Guney et al., 2015; Karababa et al., 2017; Kul et al., 2015; Selek et al., 2012). Though investigations into antioxidant activity are generally inconsistent, antioxidants such as paraxonase 1 (PON-1) and SOD show somewhat consistent findings and thus hold promise as reliable markers (Bulut et al. 2013; Ceylan et al., 2012; Lopresti, 2015; Russo, 2010; Selek et al., 2008). Besides a weak response of antioxidants to higher levels of ROS/RNS, it is also believed that higher antioxidant levels might be noticeable due to a reactive increase to protect against more oxidative stress (Ceylan et al., 2010; Guney et al., 2015; Karim et al., 2011; Oztop et al., 2012; Verlaet, Breynaert, et al., 2019).

Table 3.3: Overview of levels of oxidative stress markers in ADHD patients as compared to controls investigated in previous research work.

Marker	Sample type	Compared to control	Reference
8-OHdG	Serum	↓ (4.55%; p = 0.034)	(Oztop et al., 2012)
	Urine	= (p = 0.089)	(Verlaet et al., 2019)
8-oxoG	Lymphocytes	↑(35.42%; p = 0.014)	(Chovanová et al., 2009)
MDA	Plasma	↑ (74.40%; p < 0.001)	(Ceylan et al., 2010)
	Serum	↓ (11.30%; p = 0.012)	(Oztop et al., 2012)
	Plasma	↓ (20%; p < 0.04)	(Spahis et al., 2008)
	Plasma	↑ (7.47%; p = 0.0027)	(Verlaet et al., 2019)
NO	Plasma	↑ (200%; p <0.001)	(Ceylan et al., 2010)
	Plasma	= (p = 0.589)	(Özgür et al., 2017)
	Serum	↓ (11.14%; p = 0.037)	(Tas et al., 2006)
NOS	Serum	↑ (49.54%; p < 0.001)	(Ceylan et al., 2012)
OSI	Plasma	↑ (28.36%; p = 0.005)	(Guney et al., 2015)
	Plasma	↑ (48.02%; p = 0.001)	(Kul et al., 2015)
	Serum	↑ (84.18%; p < 0.001)	(Sezen et al., 2016)
	Serum	= (p = 0.254)	(Karababa et al., 2017)
TOS	Plasma	↑(23.51%; p = 0.011)	(Guney et al., 2015)
	Plasma	↑ (32.87%; p = 0.002)	(Kul et al., 2015)
	Serum	↑ (36.40%; p < 0.001)	(Sezen et al., 2016)
	Serum	= (p =0.436)	(Karababa et al., 2017)
XO	Serum	↑ (77.78%; p < 0.001)	(Ceylan et al., 2012)

↑: 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; MDA: malondialdehyde; NO: nitric oxide; NOS: nitric oxide synthase; OSI: oxidative stress index; TOS: total antioxidant status; XO: xanthine oxidase.

Table 3.4*:* Overview of levels of antioxidants or antioxidant enzymes in ADHD patients as compared to controls.

Marker	Sample type	Compared to control	Reference
β-carotene	Plasma	= ($p > 0.05$)	(Spahis et al., 2008)
	Plasma	= ($p = 0.696$)	(Verlaet et al., 2019)
CAT	Plasma	= ($p = 0.325$)	(Çelik et al., 2013)
	Plasma	= ($p > 0.05$)	(Ceylan et al., 2010)
	Plasma	↓ (35.29%; $p < 0.001$)	(Karim et al., 2011)
	Saliva	↓ (30.23%; $p < 0.05$)	(Ruchi et al., 2011)
GPx	Plasma	↓ (25.64%; $p < 0.001$)	(Ceylan et al., 2010)
	Plasma	↓ (40.42%; $p < 0.001$)	(Karim et al., 2011)
GST	Plasma	↑(58.04%; $p = 0.001$)	(Çelik et al., 2013)
	Serum	↓ (10.39%; $p < 0.001$)	(Ceylan et al., 2012)
GST	Plasma	↓ (41.83%; $p < 0.001$)	(Karim et al., 2011)
GSH	Erythrocyte	↑ (20.16%; $p < 0.001$)	(Verlaet et al., 2019)
Retinol	Plasma	= ($p > 0.05$)	(Spahis et al., 2008)
	Plasma	= ($p = 0.772$)	(Verlaet et al., 2019)
SOD	Plasma	= ($p > 0.05$)	(Ceylan et al., 2010)
	Plasma	↓ (16.98%; $p < 0.001$)	(Karim et al., 2011)
	Serum	↓ ($p < 0.001$)	(Russo, 2010)
TAS	Plasma	↑ (173.59%; $p = 0.001$)	(Çelik et al., 2013)
	Plasma	= ($p = 0.067$)	(Guney et al., 2015)
	Saliva	↓ (30.65%; $p < 0.01$)	(Ruchi et al., 2011)
	Serum	↓ (23.16%; $p < 0.001$)	(Sezen et al., 2016)

Table 3.4: Overview of levels of antioxidants or antioxidant enzymes in ADHD patients as compared to controls (continued).

Marker	Sample type	Compared to control	Reference
Thiols	Saliva	↑ (48.49%; p < 0.001)	(Archana et al., 2012)
	Serum	↑(14.50%; p < 0.001)	(Avcil et al., 2017)
	Plasma	↓ (7.01%; p = 0.004)	(Guney et al., 2015)
	Serum	= (p = 0.510)	(Oztop et al., 2012)
α-tocopherol	Plasma	↑ (p < 0.0001)	(Spahis et al.,2008)
	Plasma	= (p = 0.425)	(Verlaet et al., 2019)
γ-tocopherol	Plasma	↑ (p < 0.04)	(Spahis et al., 2008)
	Plasma	= (p= 0.210)	(Verlaet et al., 2019)

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

In general, contradictory results might be due to methodological differences such as analytical methods, participant selection criteria or differences in analysed matrix (Verlaet et al., 2019).

3.4 Immune imbalance in ADHD

Besides oxidative stress, also neuroinflammation (inflammation involving the CNS, consisting of the brain and spinal cord) is associated with several psychiatric disorders through various mechanisms (Kerekes et al., 2021). Systematic reviews and meta-analyses found a strong association between altered inflammatory mechanisms and various psychiatric disorders including autism (Vargas et al., 2005), schizophrenia (Muller & Schwarz, 2010), bipolar disorder (Rege & Hodgkinson 2013) and depression has already been demonstrated (Leffa et al., 2019). Other than local inflammation in the CNS, peripheral or systemic inflammation was also found to be associated with ADHD (Wong, 2022). Since the 1980s, the immune system was suspected to be also involved in

ADHD due to an increased prevalence of allergic diseases in ADHD patients (Verlaet et al., 2019). Allergic reactions were believed to cause an imbalance in CNS cholinergic and adrenergic activity, resulting in ADHD symptoms in a subgroup of children (Chen et al., 2017; Verlaet et al., 2019). Moreover, over the last years, emerging evidence has supported the role of inflammation in ADHD pathophysiology (Dunn et al., 2019). For ADHD, evidence supporting a role of inflammatory mechanisms mainly comes from i) the observation that ADHD is often associated with allergic and autoimmune disorders, ii) studies evaluating biochemical markers, iii) preliminary evidence from genetic studies demonstrating associations between polymorphisms in genes associated with inflammatory pathways and ADHD, iv) emerging evidence that early life exposure to environmental factors may increase risk for ADHD via an inflammatory mechanism, and v) mechanistic evidence from animal models of maternal immune activation documenting behavioural and neural outcomes consistent with ADHD (Dunn et al., 2019; Leffa et al., 2019).

3.4.1 Comorbidities

ADHD has a high comorbidity with both type 1 helper (T_H1) and type 2 helper (T_H2) - mediated disorders and therefore with inflammation (Fasmer et al., 2011; Schmitt et al., 2009). Miyazaki et al. (Miyazaki et al., 2017) found that ADHD patients were more likely to have asthma, allergic rhinitis, atopic dermatitis and allergic conjunctivitis as compared to non-ADHD subjects from the population. Another study by Schans et al. (Schans et al., 2017) confirmed previous findings as they found a higher presence of asthma, eczema and rhinitis in ADHD patients when compared to a control population. Wang and colleagues investigated the relation between allergic conditions and ADHD risk among 216 children with ADHD and observed that rhinitis and eczema were significantly associated with an increased risk of ADHD (Wang et al., 2018).

Also, according to a population-based case-control study, a number of maternal immune-related disorders were reported to be linked to ADHD in the offspring (Wong, 2022). A prospective cohort study investigated individual and maternal history of autoimmune disease and the association with ADHD and observed an association between ADHD and a family history of thyrotoxicosis, type 1 diabetes and autoimmune hepatitis (Nielsen et al., 2017). Thus, co-occurrence of ADHD with inflammatory and

autoimmune disorders suggests a range of underlying mechanisms, though studies did not yet identify exactly which factors have a causal role in this comorbidity (Leffa et al., 2018). $CD4^+$ T cells in particular have been implicated in mediating many aspects of autoimmune and allergic associated inflammation (Skapenko et al., 2005). Different subsets of $CD4^+$ T cells exist, characterised based on the cytokines they produce, and displaying different roles in cell-mediated immunity (Abbas et al., 2019). In general, T_H1 cells are characterised by the production of pro-inflammatory cytokines such as interferon gamma (IFN-γ), interleukin 2 (IL-2), interleukin 12 (IL-12), interleukin 23 (IL-23) and TNF-α (Huang et al., 2009). T_H1 cells are known to promote cellular immunity and are involved in the development of autoimmune diseases (Skapenko et al., 2005).

On the other hand, T_H2 cells mediate humoral immunity and are involved in allergic immune responses (Skapenko et al., 2005). T_H2 cells are characterised by the production of interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6) and interleukin 10 (IL-10) (Huang et al., 2009). Growing evidence indicates that imbalances of T_H1/T_H2 cytokine production are involved in many pathological processes and play both causative and protective roles in neuronal damage (Huang et al., 2009).

3.4.2 Immune analysis

Studies investigating inflammatory biomarkers such as cytokines and specific antibodies in ADHD patients have not provided conclusive findings, most likely due to small sample sizes and a high heterogeneity among the biomarkers tested (Leffa et al., 2019). Several cytokines have been studied as possible neurochemical markers of ADHD; however, interpretation of the findings remains rather difficult. In a systematic review by Anand et al. (Anand et al., 2017) several studies measuring peripheral cytokines in ADHD subjects were reported. Although no conclusive results were found, it was hypothesised that cytokines are possible neurochemical markers of ADHD. Another study reported significant correlations between peripheral cytokine levels and ADHD symptoms: elevated interleukin 13 (IL-13) levels were associated with increased inattention symptoms while higher interleukin 16 (IL-16) levels were associated with increased hyperactivity-impulsivity symptoms (Oades et al., 2010). Moreover, two more recent studies provided further evidence on the role of cytokines in ADHD. One study revealed

significantly higher IL-6 and IL-10 levels in serum (Donfrancesco et al., 2020) while the other one found higher levels of plasma C-reactive protein (an acute phase protein produced as a response to a wide variety of inflammatory conditions) and IL-6 as well as lower levels of TNF-α in ADHD subjects (Chang et al., 2020). In addition, studies in rodents have shown that administration of cytokines like IL-1β, IL-2, and IL-6 can cause neurotransmission changes like those seen in ADHD such as increased norepinephrine and reduced dopamine levels (Anand et al. 2017).

Overall, alterations in pro- and anti-inflammatory cytokine status can influence the pathogenesis of ADHD as they can pass the blood-brain barrier (BBB) and affect synaptic plasticity and neurogenesis (McAfoose & Baune, 2009). Accordingly, cytokines can influence cognitive processes such as reaction time and working memory (Brydon et al., 2008), that can be impaired in ADHD. Furthermore, the upregulation of pro-inflammatory cytokines such as interferon gamma (IFN-γ) and TNF-α modulates tryptophan metabolism and as a result also several neurotransmitter systems such as dopaminergic transmission (Anand et al., 2017). Pro-inflammatory cytokines can also activate microglia which will produce even more pro-inflammatory cytokines that, in turn, trigger the activation of microglia. This inflammatory flow can further contribute to neuroinflammation and potentially also to the pathophysiology of ADHD (Anand et al., 2017). A study by Passarelli et al. (Passarelli et al., 2013) evaluated the role of antibodies against Purkinje cells as a possible marker of an immune response in ADHD patients. A significantly higher immunoreactivity against anti-Purkinje cell antibodies in ADHD patients when compared to controls could be demonstrated, suggesting the involvement of the immune system in the disorder.

3.4.3 Genetics

As mentioned before, ADHD is highly hereditary (Biederman & Faraone, 2005; Pelsser et al., 2009). Moreover, a growing body of evidence from genetic studies suggests that polymorphisms in genes are related to inflammatory pathways that play a role in ADHD (Leffa et al., 2019).

3.4.4 Perinatal influences

Prenatal and perinatal risk factors have been described for ADHD. Low birth weight (Pettersson et al., 2015) and exposure to maternal smoking and other substances *in utero* (Thapar et al., 2013) have been related to an increased risk of ADHD or ADHD symptoms. In addition, prenatal exposure to viral infections leading to altered dopaminergic development (e.g. measles, rubella or influenza) is also considered as a risk factor for neurodevelopmental disorders (Instanes et al., 2017; Verlaet et al., 2019). Furthermore, associations with ADHD in offspring have been found for some medical conditions of the mother, including obesity and epilepsy (Instanes et al., 2017). It has been hypothesised that ADHD may be caused by an exaggerated CNS inflammatory response in the foetus caused by maternal inflammation, such as in allergy or autoimmune disease (Instanes et al., 2017).

3.4.5 Evidence from animal studies

Lastly, evidence for an association between neuroinflammation and ADHD comes from animal models in which neurobehavioural development is characterised in offspring exposed to prenatal inflammation (Dunn et al., 2019). These studies demonstrated abnormalities in behaviour consistent with ADHD symptoms like hyperactivity, impulsivity and inattention (Dunn et al., 2019). Next to behavioural changes, also changes in brain structures in line with those observed in ADHD patients were observed after prenatal exposure to inflammation such as a reduction in cortical gray matter volume. Moreover, in addition to behavioural and structural changes, alterations in neurotransmitter systems including the dopaminergic, serotonergic, glutamatergic and GABA systems were observed similar to those seen in the ADHD population (Dunn et al., 2019).

3.5 Interconnection of oxidative and immune imbalance

Both unresolved oxidative stress and immune imbalance may thus contribute to the clinical pathology of ADHD, by injuring neuronal cells or altering neuronal development, via altered gene expression, direct DNA damage and promotion of cell death (Verlaet et al., 2019).

Genetic and environmental factors, catecholaminergic dysregulation and even medication used for the treatment of ADHD are all factors that could initiate inflammation and oxidative stress. For example, it has been shown that dopamine and norepinephrine can easily undergo oxidation, forming ROS (Napolitano et al., 2011). As stated earlier, stimulant medications increase extracellular dopamine and norepinephrine, therefore resulting in an increase in oxidative stress and consequentially causing oxidative damage (Andreazza et al., 2007; Comim et al., 2014; Corona, 2020). Additionally, oxidative stress and inflammation are strongly interconnected in a vicious cycle (Figure 3.8) (Alvarez-Arellano et al., 2020). Oxidative stress can activate pro-inflammatory pathways via NF-κB activation leading to cytokine production and lymphocyte activation and proliferation. Inflammation can in turn cause oxidative stress since activated immune cells produce ROS (Vaziri 2008).

Even though some observations indicate the involvement of oxidative changes and cellular immunity, more studies are required for a more precise conclusion. Moreover, it is important to note that ADHD is a heterogeneous disorder and individuals with ADHD may exhibit a combination of symptoms and underlying factors including neurobiological, genetic, environmental influences as well as changes in oxidative and immune status.

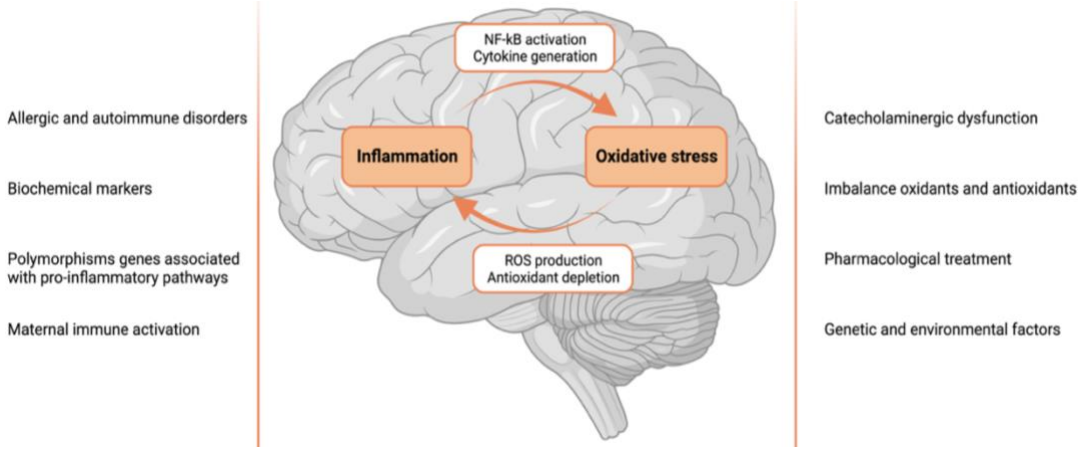

Figure 3.8*:* Schematic overview of the interplay between inflammation and oxidative stress creating a vicious cycle in the pathophysiology of ADHD. ATX: atomoxetine; MPH: methylphenidate hydrochloride; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; ROS: reactive oxygen species. Created in BioRender.

3.6 Implications for the use of polyphenols in ADHD therapy

Since there is an increasing understanding regarding the disadvantages of stimulant medication, alternative efficacious treatment options with less side effects are highly warranted (Kessi et al., 2022).

The association of oxidative and immune dysregulation with ADHD provides potential for nutritional supplements in the treatment of ADHD. Over the last years alternative therapeutic options including plant sterols, polyphenols, pre- and probiotics, vitamins, minerals and omega-3 fatty acids made their appearance in ADHD therapy (Rucklidge et al., 2009; Sarris et al., 2011; Verlaet et al., 2014). Due to their antioxidant and immune modulating properties various dietary polyphenolic extracts have been investigated and might be appropriate in ADHD therapy, although questions about *in vivo* activity remain (Fernandez-Panchon et al., 2008). Various natural products have been investigated in the context of ADHD, including PBE, *Ginkgo biloba* extract, *Hypericum perforatum* extract and *Passiflora incarnata* extract (Verlaet et al., 2019). The potential of the polyphenol-rich extract PBE in the context of ADHD treatment will be further investigated and discussed in Chapter 4.

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Chapter 4 **Clinical investigation of French Maritime Pine Bark Extract**

Weyns A-S, Verlaet A, Breynaert A, Naessens T, Fransen E, Verhelst H, et al. Clinical Investigation of French Maritime Pine Bark Extract on Attention-Deficit Hyperactivity Disorder as compared to Methylphenidate and Placebo: Part 1: Efficacy in a Randomised Trial. Journal of Functional Foods. 2022;97:105246.

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4.1 Introduction

Few studies investigated the therapeutic effect of PBE in paediatric ADHD and the therapeutic potential was already suggested before (Dvoráková et al., 2006). In a randomised double-blind placebo-controlled trial, 61 boys and girls aged 6-14 years with ADHD were treated with 1 mg/kg/day PBE (n = 44) or placebo (n = 17) for one month. Trebacticka et al. (Trebatická et al., 2006) found that PBE significantly improved hyperactivity and inattention as rated by teachers as compared to placebo. Moreover, no serious adverse events (SAEs) were reported and no changes were observed in standard blood chemistry, suggesting good tolerance. Despite the promising results, these trials had their limitations (e.g. small sample size, very small placebo group and a short supplementation period) and the exact mechanisms by which PBE improved brain function and behaviour and possibly comorbid complaints remained unclear (Trebatická et al., 2006).

Hence a phase III, randomised, double-blind, placebo and active product controlled, multicentre clinical trial was designed to elucidate the potential of PBE in ADHD patients by examining its efficacy, mechanism of action and its value as compared to the standard treatment with MPH and to placebo. Although therapeutic benefit was already suggested by a small, randomised trial and other observational studies, its efficacy and value as compared to standard therapy with MPH were still to be confirmed (Dvoráková et al., 2006; Trebatická et al., 2006; Wilson et al., 2010). The full objective of this randomised trial was to evaluate the effect of PBE on ADHD behaviour, co-morbid physical/psychiatric symptoms, immunological markers, oxidative damage and antioxidant and neurochemical status, compared to placebo and MPH (Verlaet et al., 2017).

4.2 Clinical trial set-up: methods

The full set-up of this trial has been published in detail in Verlaet et al. (Verlaet et al., 2019). Only the trial's methods will be discussed briefly below.

4.2.1 Quality control of PBE

PBE 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 for PBE (Convention USP, 2014). Quality control of the used PBE was performed to check whether PBE complies with USP requirements and specific phenolic constituents were determined to obtain more details on the composition of the studied extract. Moreover, identification of the phenolic compounds present in PBE were also determined using the Xevo® G2-XS QTOF spectrometer (Waters) coupled with an ACQUITY LC system equipped with MassLynxTM 4.1 software according to the method described in Chapter 2. The conformity of the 20 mg PBE study capsules was also assessed.

4.2.1.1 Total procyanidin content

4.2.1.1.1 Chemical and reagents

Butanol (99%, extra pure) and methanol (HPLC grade) were purchased from Acros Organics (Geel, Belgium), ferric ammonium sulphate $12H_2O$ was acquired from VWR (Leuven, Belgium) and hydrochloric acid (37%) from Fisher Chemical (Loughborough, UK). USP Maritime Pine extract reference standard (0.673 mg procyanidins/mg extract, loss on drying (LOD): 4.82%) was bought from the USP (MD, USA). PBE dry extract was kindly offered by Horphag Research, Switzerland. Capsules containing 20 mg PBE mixed with filler (175 mg microcrystalline cellulose) and lubricant (2 mg magnesium stearate) to be used in the controlled trial were produced by Qualiphar NV (Bornem, Belgium).

4.2.1.1.2 Sample and standard preparation

USP Maritime Pine extract reference standard 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 *(v/v)*) 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 room temperature (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 PBE was prepared by dissolving 125.0 mg PBE powder in 100 mL MeOH, placing it for 20 min in an ultrasonic bath, followed by centrifugation (2500 rpm, RT, 10 min) and diluting the upper layer 1:20 with MeOH *(v/v).* The test stock solution for capsules containing PBE 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 *(v/v).* 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.).

4.2.1.1.3 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 PBE (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

4.2.1.2 HPLC fingerprint

4.2.1.2.1 Chemical and reagents

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

4.2.1.2.2 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 20% MeOH. To prepare the test solution, 2 mL MeOH was added to 200.0 mg sample. After 15 min ultrasonic treatment, milli-Q was added to 10 mL volume and the solution was centrifuged (2500 rpm, RT, 10 min).

4.2.1.2.3 HPLC analysis

Samples were analysed by a High-Pressure Liquid Chromatography (HPLC) system with diode array detector (DAD) from Agilent, USA (type 1260 quaternary pump and auto-injector). Mobile phase A (0.1% H_3PO_4 in milli-Q) 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, respectively. The amount of the different components was calculated by means of a calibration curve.

4.2.1.2.4 UHPLC coupled to a quadrupole time-of-flight spectrometer

PBE was dissolved in MeOH (MS grade) and injected on a Waters Acquity UPLC BEH SHIELD RP18 column (3.0 mm × 150 mm, 1.7 µm; Waters). For detection, accurate mass measurements were done using a Xevo® G2-XS QTOF spectrometer (Waters) coupled with an ACQUITY LC system equipped with MassLynxTM 4.1 software. Individual constituents of PBE were identified using two analytical standard mixtures (Cacao standard LC-MS mix and a general standard LC-MS mix as described earlier in 2.4.7.1).

4.2.2 Ethics and registration

Ethical approval was obtained in the Belgian University Hospitals of Antwerp (UZA) (EC 15/35/365) and Ghent (UZ Ghent) (2016/0969) and Hospital Network Antwerp (ZNA) (EC

approval 4656). The trial was registered at Clinicaltrials.gov (NCT02700685, registered 18 January 2016) and EudraCT (2016-000215-32, registered 4 October 2016) (Verlaet et al., 2017).

4.2.3 Inclusion and randomisation

This was a phase III, randomised, double-blind, placebo and active product controlled, multicentre clinical trial with three parallel treatment arms (PBE, MPH and placebo). Participants from 6 to 12 years with ADHD, both diagnosed *de novo* and formerly treated, were recruited at the University Hospitals of Antwerp (UZA) and Ghent (UZ Ghent) and Hospital Network Antwerp (ZNA), as well as via general practitioners, paediatricians, speech therapists and physiotherapists. For example, a poster and a flyer were designed for hospital and doctor's waiting rooms to provide potentially interested ADHD patients and their parents with more specific information about the clinical trial. Patients were included between September 2017 and November 2020. Diagnosis was confirmed by a child neurologist or psychiatrist and written consent of the participants' legal representative was obtained before inclusion. Excluded were patients with autism spectrum disorder, psychosis, depression, inflammatory disorders, and/or use of some drugs and nutritional supplements 3 months before inclusion (Table 4.1). An additional information brochure to inform on specific details of the trial was given to the primary caregiver. Patients received all necessary contact information for the three trial centres, making sure all possible questions could be answered by a trial investigator other than their attending physician.

Following screening, enrolment by a child neurologist/psychiatrist and baseline assessments, patients were randomised, stratified by trial centre and body weight (randomisation list created by pharmacists via randomization.com with 1:1:1 allocation ratio). Patients, their parents and teachers, physicians and investigators were blind to treatment allocation. All treatments (1 or 2 oral capsules at breakfast) had an identical shape and appearance, and all were encapsulated, packaged and labelled by the same company (Qualiphar NV, Bornem, Belgium).

Treatments included 1 or 2 oral capsules at breakfast:

- MPH (Medikinet® Retard, Medice GmbH, MPH modified release): 20 or 30 mg/day if < or ≥ 30 kg, resp. Treatment started with 10 mg/day, increasing 10 mg per week.
- PBE: 20 or 40 mg/day if < or ≥ 30 kg, resp. (20 mg/day during the first two weeks).
- Placebo: excipients (microcrystalline cellulose and magnesium stearate) only.

48 patients per group were necessary based on following assumptions:

- PBE reduces teacher ADHD-RS total score by 0.75 SD after 10 weeks (Pelsser et al., 2011; Trebatická et al., 2006);
- Power of 80%, dropout of 20%;
- Two-sided testing, 0.05 significance level with Bonferroni post-hoc testing correction.

Table 4.1: Inclusion and exclusion criteria for participation.

Inclusion criteria	Exclusion criteria
1. Age 6-12 years	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, conduct disorder, tics, schizophrenia, dyskinesia, personal or family history of psychotic disorder, bipolar illness, depression or suicide attempt
3. Responsible caregiver has 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. Both 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 nay medication for longer than 1 week 3 months before inclusion
	6. Other contraindications for MPH or PBE, as defined in the Summary of Product Characteristics and Investigator's Brochure, respectively

ADHD-RS: ADHD Rating Scale; IQ: intelligence quotient; MAO: monoamine oxidase; MPH: methylphenidate hydrochloride; PBE: French Maritime Pine Bark Extract.

4.3 Clinical trial questionnaires: methods

Participant's birth date, gender, weight and height were recorded. Parents and teachers were asked to fill out several questionnaires at baseline, after 5 and 10 weeks (Table 4.2) (D'Andrea, 2010; de Vriese et al., 2005; Pelsser et al., 2010; Verlaet et al., 2017). Two reminders were sent whenever questionnaires were not received within one week after

the required date. In general, withdrawal, presence of SAEs, development of a severe and unstable disease, failure to provide follow up information or to come to a follow up appointment and intake of concurrent medication/supplements during more than one week resulted in dropouts. Dropouts have not been replaced.

Table 4.2: Questionnaires filled out by parents and teachers.

Questionnaire	Baseline	Week 5	Week 10
ADHD-RS	X	X	X
SEQ	X		X
PCQ (parents only)	X	X	X
FFQ (parents only)	X		X

ADHD-RS: ADHD-Rating Scale; FFQ: Food Frequency Questionnaire; PCQ: Physical Complaints Questionnaire; SEQ: Social-Emotional Questionnaire (SEQ).

4.3.1 ADHD Rating Scale (ADHD-RS)

The ADHD-RS was used to assess the severity of ADHD symptoms. It is an internationally accepted and validated questionnaire consisting of 9 inattention (IA) and 9 impulsivity (I) and hyperactivity (HI) questions based on the DSM, each marked out on a four-point rating scale: (0) never, (1) occasionally, (2) often and (3) very often (Döpfner et al., 2006; Trebatická et al., 2006). Some questions give a better understanding of IA while others assess HI. The total ARS score is the sum of the scores of all 18 different questions. It has been widely used in research lately, which allows comparison of results to those of previously performed trials (Dvořáková et al., 2006). Both parents and teachers were asked to fill out this questionnaire, at baseline, after 5 and 10 weeks. Parent and teacher questionnaires were analysed separately, to distinguish in between home and classroom behaviour. Comparison of all filled-in ADHD-RS will moreover provide information on differences in treatment. For instance, the extended-release formulation of MPH is effective for about 8 h, while the effect of PBE on behaviour is not expected to wear off suddenly. The reliability and validity of the ADHD-RS are considered good for the screening and diagnosis of ADHD especially when used in multimethod assessments, in combination with other behaviour rating scales and diagnostic interviews (DuPaul et al., 1998).

4.3.2 Food Frequency Questionnaire (FFQ)

The dietary habits of participants were assessed by a FFQ concerning different food groups: fruits, vegetables, cereal and potato products, dairy, meat and fish, drinks and miscellaneous. The FFQ used during the clinical trial was based upon a FFQ which evaluated dietary habits in an unpublished pilot study. This original FFQ consisted of three questions regarding meal frequency (breakfast, lunch and dinner) in addition to 60 questions on food product frequency (intake of 7 food groups taking portion size into account) which were scored using a six-point rating 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. More detailed information, such as bread type, was assessed by fifteen additional questions. This FFQ showed an acceptable validity, but an overestimation of vegetable and potato product intake was possible compared to a three-day food diary, which is seen as the golden standard to assess dietary habits. Therefore 35 questions of the original questionnaire as well as additional questions on e.g. type of milk, rice and chocolate were selected for the present FFQ. Questions concerning portion size were not taken into account. A nine-point rating scale was implemented: never (0), less than 1 day a month (1), 1-3 days a month (2), 1 day a week (3), 2-4 days a week (4), 5-6 days a week (5), once a day (6), 2-3 times a day (7) and more than 3 times a day (8). Parents were asked to fill out this questionnaire at baseline and the end of the clinical trial to assess global dietary habits and potential adaptations during the study, as well as a possible association between dietary polyphenol intake and direct effects of PBE (de Keyzer et al., 2013; de Vriese et al., 2005).

4.3.3 Social-Emotional Questionnaire (SEQ)

The SEQ assesses, besides ADHD, core symptoms of social behavioural problems, anxiety and autism. It contains 72 questions and was filled in by both parents and teachers. The questionnaire focuses on four clusters of social-emotional problems: ADHD (18 questions; ADHD score can be subdivided into a hyperactivity (H), impulsivity (I) and inattention (IA) score) and frequently occurring psychiatric comorbidities associated with ADHD, such as social behaviour problems (ODD and CD: 26 questions), anxiety (general and social anxiety; 18 questions) and autism (10 questions). Items covering the most relevant core symptoms of these four clusters were rated on a five-point scale, concerning behaviour in 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 ranked into five levels of severity, based on norm categories: normal, normal-high, subclinical, clinical and clinical-high. This latter two levels, clinical and clinical-high, can result in a diagnosis of the specific disorder. The reliability and validity of this questionnaire are good and therefore the SEQ is considered as a good tool for screening, diagnosis and treatment evaluation (Verlaet et al., 2017).

4.3.4 Physical Complaints Questionnaire (PCQ)

The 39 questions of the PCQ focus on various physical and sleep complaints, some of them frequently co-occurring with ADHD, mainly in eight different domains: pain (e.g. headache), unusual thirst or perspiration, eczema, asthma or rhinitis, skin problems, tiredness, GI problems and sleep problems (Pelsser et al., 2011). Items were rated on the same scale as the SEQ. Side effects were assessed as well, as the PCQ includes some questions on different potential AEs. The prevalence of allergies, colds and ear infections were rated in some additional questions. Allergy and atopy were assessed based both on their clinical manifestation and a physician's diagnosis of allergy in the subject and at least one first grade relative. Additionally, parents were asked whether their child experienced any illness during the trial, which illness it was, if medication was taken, and the type, dose and duration of medication intake. Although the validity and reliability are still unknown, the PCQ was already used in research on the effects of nutrition on ADHD (Pelsser et al., 2011; Verlaet et al., 2019).

4.4 Clinical trial biological samples: methods

Biological samples were collected at baseline and after ten weeks. Blood was collected in ethylenediaminetetra-acidic acid (EDTA) and serum tubes (BD, New Jersey, USA) by nursing staff at the participating hospitals. Per patient, 1 EDTA tube (12 mL) was placed on ice immediately while another EDTA tube was kept at room temperature. After centrifugation (2000 x g, 4 °C, 12 min) of cold EDTA blood, erythrocytes in phosphate buffer and plasma were aliquoted and stored frozen at -80 °C until analysis. After centrifugation (200 x g, RT, 20 min) of the RT EDTA tubes, plasma was frozen at -80 °C,

while peripheral blood mononuclear cells (PBMCs) were isolated and stored in a 20% DMSO solution for long-term storage in liquid nitrogen (-196 °C) (PBMC isolation protocol can be found in Supplementary Information). After 30 min at RT, allowing clotting of the samples, they were centrifugated (2000 x g, RT, 12 min) and stored at –80 °C until analysis. From the samples at RT, 1 EDTA tube was used to collect plasma and PBMCs, whereas the serum tube (without anticoagulant) was used to collect serum samples. Urine was collected in sterile urine containers, placed on ice immediately and stored at -80 °C until analysis. A subgroup of patients were asked to collect a faecal sample at the start and after completing the 10-week study using a Protocult collection container (Ability Building Center, Rochester, USA) and temporarily store them at -20 °C until pick-up. The samples were kept at -80 °C at our lab until further analysis. All samples were pseudonymised according to General Data Protection Regulation (GDPR) regulations and stored in the Antwerp Biobank. A short overview of all the information and analyses performed at the different timepoints during the study can be found in Table 4.3.

Table 4.3: Investigations and data acquisition during the study.

Evaluations/Interventions	Screening	Baseline	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
FFQ		X		X
PCQ		X	X	X
Pregnancy Questionnaire		X		
SEQ		X		X
Sample collection (blood, urine, faeces)		X		X

Table 4.3: Investigations and data acquisition during the study (continued).

Evaluations/Interventions	Screening	Baseline	Week 5	Week 10
GSH analysis		X		X
Lipid soluble antioxidants analysis		X		X
Antioxidant enzyme activity		X		X
Genetic 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
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.

4.4.1 Oxidative stress pathway: antioxidants and gene expression profiles of antioxidant enzymes

After hemolysis and purification, erythrocyte GSH level was analysed by a validated HPLC method (Magielse et al., 2014; Pastore et al., 2003). Plasma levels of the lipid-soluble antioxidants α- and γ-tocopherol, β-carotene, retinol and coQ10 were analysed by a validated HPLC method after extraction with hexane (Hermans et al., 2005). Both were analysed on an Agilent 1260 HPLC system (Agilent Technologies, Belgium) with an ESA-5600A CoulArray 8-channel electrochemical detector (Thermo Fisher Scientific, MA, USA). Resulting chromatograms (Figure 4.1) were analysed with CoulArray Win Software.

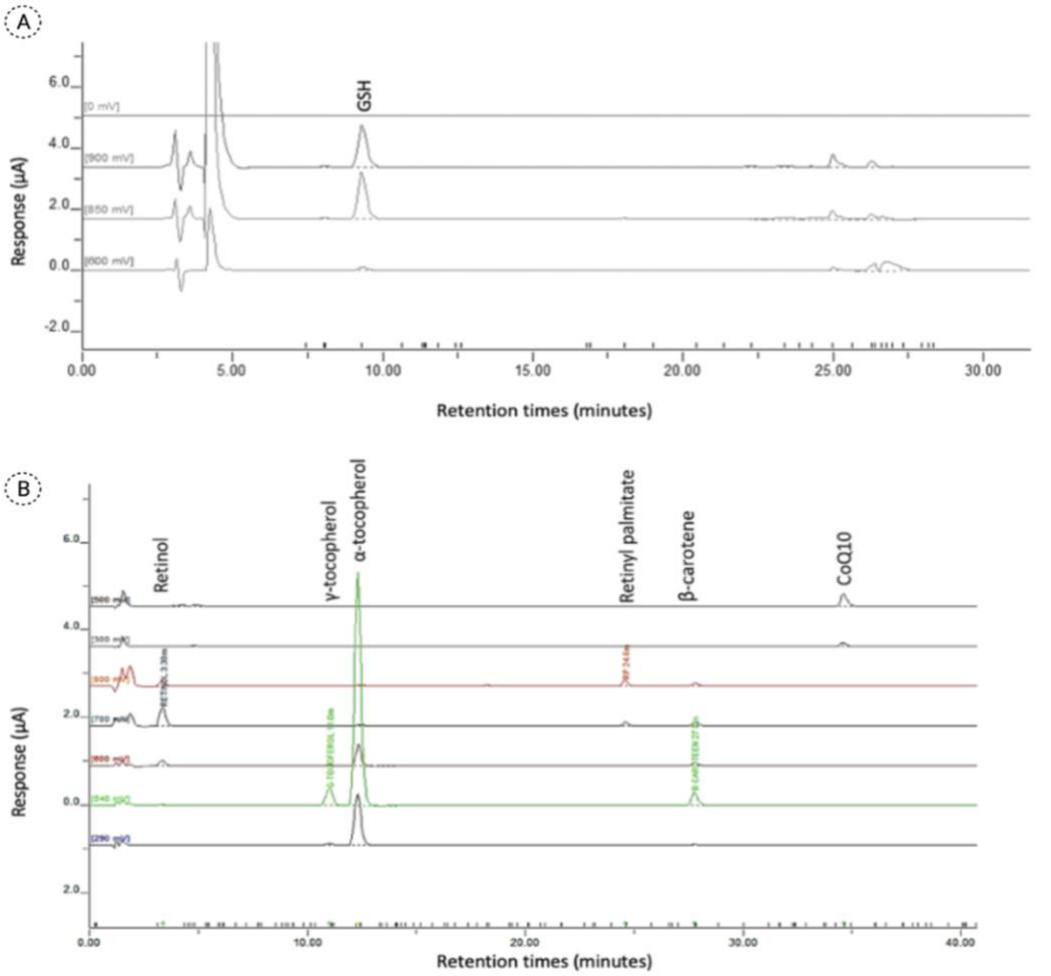

Figure 4.1: Example of chromatograms using HPLC separation and ECD detection of standard 1 (STD1). A: Resulting chromatogram from the analysis of GSH in erythrocytes. B: Chromatogram from the analysis of lipid-soluble antioxidants. Co-enzyme Q10; GSH: reduced glutathione.

Plasma CAT, SOD and GPx activities were analysed using the General Catalase Assay Kit (MBS8243260), the Glutathione Peroxidase Assay Kit (MBS841725) and the Superoxide Dismutase Colorimetric Assay Kit (WST-1 method, E-BC-K020-M), respectively for CAT and GPx activities from MyBioSource (San Diego, USA) and for SOD activities from Elabscience (Texas, USA). Each analysis was performed according to the manufacturer's instructions.

Gene expression profiles from PBMCs were analysed and quantified by real-time PCR (RT-qPCR) focusing on specific genes involved in pathways counteracting oxidative stress (GPx, CAT, SOD, XO) and the stress-related protein ApoJ. The QIAamp RNA Blood Mini kit (52304, Qiagen, Venlo, Netherlands) was used to extract total RNA from PBMCs according to the manufacturer's protocol. A Qubit RNA Broad-Range Assay Kit (10174653, Thermo Fischer Scientific, MA, USA), in combination with a Qubit Fluorometer, was used for the quantification of the isolated RNA. Total RNA (1 µg) extracted from each sample was converted into cDNA using SuperScript® II Reverse Transcriptase kit (M1705, Promega Benelux) according to the manufacturer's protocol. Next, qPCR analyses were carried out using the GoTaq qPCR master mix (A6001, Promega Benelux) according to the manufacturer's instructions. Shortly, a 25 µL reaction volume mix per sample was prepared, containing 12.5 µL GoTaq qPCR master mix, 0.4 µM forward and reverse primer and nuclease-free water. Following PCR program was applied on the Rotor-Gene Q qPCR machine (Qiagen, Venlo, Netherlands): 95 °C for 2 min, 40 cyclic denaturations (95 °C, 15 s) and annealing/extension (60 °C, 30 s), and dissociation (60–95 °C). Each sample was run in triplicate. Differential expression of each gene was done by comparing the normalised Ct values (ΔCt) of all the biological replicates (median value of the triplicates) to the average ΔCt of all baseline values (beta-actin was used as the normalisation gene after a selection based on expression stability performed in the GeNorm algorithm) from each gene of interest. By comparing the normalised expressions, it is possible to calculate the fold change of the expression of the miRNA (−ΔΔCt) of each gene of interest (Goni et al., 2009). Primers sequences are listed in Table 4.4. qPCR material was made available by the lab of prof. Wim Vanden Berghe (Protein Chemistry, Proteomics and Epigenetic Signalling, University of Antwerp).

Serum zinc level was analysed in-house by Atomic Absorption Spectroscopy (AAS; Flame AAS, Perkin-Elmer, Analyst 400) (Whitehouse et al., 1982).

Table 4.4: Sequence of primer sets used in this study.

Gene of interest	Forward primer (5'→ 3')	Reverse primer (5'→ 3')
CAT	GAGCACAGCATCCAATATTCTG	CTCATTCAGCACGTTCACATAG
SOD1	AATGTGACTGCTGACAAAGATG	GCGTTTCCTGTCTTTGTACTTT
GPX1	GTTGCCTGGAACTTTGAGAAG	CTCGATGTCAATGGTCTGGAAG
XO	TTCATTTCCGCTGATGATGTTC	ACAAGTAACCTTATCCTTCGCA
Clu/APOJ	AGATCTTGTCTGTGGACTGTTC	GTATTTCCTGGTCAACCTCTCA
BACT	CACCATTGGCAATGAGCGGTTC	AGGTCTTTGCGGATGTCCACGT

BACT: beta-actin; CAT: catalase; Clu/APOJ: clusterin/apolipoprotein J; GPX: glutathione peroxidase-1; SOD1: superoxide dismutase-1; XO: xanthin oxidase.

4.4.2 Oxidative stress pathway: oxidative damage

Oxidatively damaged fatty acids can degrade to reactive aldehydes like MDA. Plasma MDA was determined by competitive enzyme-linked immunosorbent assay ELISA (Human Malondialdehyde ELISA kit, MyBioSource, San Diego, USA). Urinary 8-OHdG, indicating oxidative DNA damage, was analysed by a competitive ELISA kit (NWK-8OHDG01, NWLSS™ Urinary 8OHdG ELISA, Northwest Life Science Specialties, Washington, USA) according to the manufacturer's protocol (Aruoma, 1998; Wu et al., 2004; Yano et al., 2009). To express urinary 8-OHdG concentration as ng/mg creatinine, urinary creatinine levels were analysed by the Creatinine Microplate Assay (CR01, Oxford Biomedical Research, Michigan, USA) according to the manufacturer's protocol.

4.4.3 Immune status

Plasma cytokine levels (IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, TNF-α, IFN-γ) were analysed by the MSD® V-PLEX Viral Panel 3 Human Kit according to the manufacturer's protocol (K15347D-1, Meso Scale Discovery, Rockville, USA). The MSD® platform to read out samples was made available by the lab of prof. Ingrid De Meester (Lab of Medical Biochemistry, University of Antwerp). Plasma antibody (IgA, IgG_{1-4}, IgE) levels were determined by the General Medical Laboratory (AML) in Antwerp.

4.4.4 Neurochemical status: the orexigenic peptide neuropeptide Y

Serum neuropeptide Y (NPY) levels were measured using a Human NPY ELISA kit (EZHNPY-25K, Merck, Darmstadt, Germany).

4.5 Outcomes of the clinical trial

4.5.1 Primary outcome

The primary outcome was the summed ADHD score of the teacher-rated ADHD-RS. Behavioural assessment by teachers is preferred as primary objective due to its higher sensitivity (Power et al., 1998; Tripp et al., 2006).

4.5.2 Secondary outcomes

Secondary outcomes for the questionnaires were the following:

- Summed ADHD score of the parent-rated ADHD-RS.
- Summed ADHD score of the teacher- and parent-rated SEQ.
- Sub scores of the teacher- and parent-rated ADHD-RS and SEQ.
- Percentage of treatment responders (≥20% reduction of total ADHD-RS score) (Buitelaar et al., 2003).
- Teacher- and parent-rated SEQ social behaviour problems, anxiety and autism scores.
- Parent-rated PCQ scores.

Also, the acceptability of PBE compared to MPH and placebo, based on adverse effects (open questions), treatment adherence (>90% ingestion as scheduled) and dropouts was investigated.

To investigate the effect of PBE as compared to placebo and MPH with regard to antioxidant levels and oxidative damage (oxidative stress pathway), the immune status and the neurochemical parameter NPY, various biomarkers were analysed at the start and the end of the study:

- GSH level, the most important intracellular antioxidant (Pastore et al., 2003).

- Plasma lipid soluble antioxidants (α- and γ-tocopherol, β-carotene, retinol and co-enzyme Q10 (coQ10)) as non-enzymatic defence mechanisms against oxidants (Conaway et al., 2013; Littarru & Tiano 2007; Molyneux et al., 2008; Naguib et al., 2003).
- Plasma antioxidant enzymes (CAT, SOD) and GPx activity.
- Gene expression of networks counteracting oxidative stress (GPx, CAT, SOD and XO) and stress-related protein APoJ.
- Serum zinc level since zinc deficiency is linked to neurological dysfunction and plays a role in oxidant status (Arnold et al., 2011; DiGirolamo & Ramirez-Zea, 2009; Lee, 2018).
- Urinary 8-OHdG, marker of oxidative DNA damage (Chovanová et al., 2009; Oztop et al., 2012).
- Plasma MDA, marker of oxidative lipid damage (Ceylan et al., 2010; Oztop et al., 2012).
- Plasma cytokines (Interleukines IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, Tumor necrosis factor TNF-α, interferon IFN-γ) and antibodies (immunoglobulins IgA, IgG_{1-4}, IgE) as markers of immune status.
- Serum NPY level.

The microbial composition of faecal samples of a subgroup of participants was analysed to see if there is a positive/prebiotic effect of PBE on microbial diversity, impact on pathogenic bacteria as compared to the participants receiving MPH and placebo (see Chapter 5).

A final objective was to investigate the acceptability of PBE compared to MPH and placebo, based on adverse effects (open questions), treatment adherence (>90% ingestion as scheduled) and dropouts.

4.6 Hypotheses

Based on the available data, it was hypothesised that:

- In ADHD therapy, PBE is more effective than placebo and not less effective than MPH.

- Compared to placebo and MPH, PBE reduces co-morbid physical and psychiatric complaints.
- Compared to placebo and MPH, PBE increases antioxidant levels, reduces oxidative damage, improves immune and neurochemical status.
- The tolerability of PBE is higher than that of MPH.

The safety of both MPH and PBE has already been investigated and demonstrated and is therefore beyond the scope of this study. Adherence and dropouts are considered important real-life indicators of the effectiveness of a treatment, based on both the achievement of positive effects and the absence of adverse effects, which make them highly valuable.

4.7 Statistics

SPSS 27.0 (IBM) and R version 4.1.1 (R core team) were used for statistical analyses (R Foundation for Statistical Computing, 2017). Data were checked for outliers and a normal distribution (Shapiro-Wilk test and QQ-plot) and presented as mean ± standard deviation (SD). The three groups were compared regarding baseline characteristics by one-way ANOVA, Chi-square test or Cochran-Armitage trend test.

During the course of this trial, parents and teachers were asked to fill out several questionnaires at different timepoints. Before statistical analyses were performed, the total score, IA score, HI and I scores of the ADHD-RS were scaled based on the number of questions answered by the summation of the individual scores of all answered questions, divided by the total amount of questions in the questionnaire, to account for missing questions (no more than two). The same scaling method to account for unanswered questions (no more than three) was applied for the SEQ and the PCQ. In case of >2 missing answers regarding a (sub)score within a patient, this (sub)score was set to missing. Participants were excluded from analyses only for those outcomes without any data available. For ADHD-RS, SEQ and combined PCQ scores, blood pressure and heart rate, the effect of treatment was modelled using linear mixed models (LMMs). Scores were entered as dependent variable. Time point (categorical), treatment and their interaction were included as fixed effects and sex as covariate. Participant ID was entered as random intercept. Individual PCQ scores were compared between start and

end of the trial within each treatment group by Cochran-Armitage trend tests. Due to lack of power, no subgroup analyses were performed. Non-inferiority of PBE compared to Medikinet® Retard is demonstrated when the difference in effect on ADHD-RS score was no more than 5 points (Berek et al., 2011; Christensen, 2007). All analyses were performed by original assigned groups. A 2-sided p-value < 0.05 was considered significant. For secondary outcomes, a stricter 2-sided p-value < 0.01 was applied to account for increased type 1 error. Bonferroni correction accounting for all secondary analyses would be overly conservative, since these do not represent independent tests. Post-hoc analysis with pairwise testing of the difference in effect between treatments was performed using Bonferroni correction for multiple testing with $p < 0.05$ considered significant.

For each biomarker of interest, the effect of treatment was investigated using linear mixed models (LMMs) on the follow-up and baseline outcomes. Only for IL-1β and IL-4 a Tobit regression model was used due to the presence of many observations below the detection limit (Twisk & Rijmen 2009). The biomarker was entered as dependent variable. Time, treatment and their interaction were entered as fixed effects. Sex, the processing time of the biological samples and time until analyses were entered as co-variates. Participant ID was entered as a random intercept to account for the dependence between observations from the same individual. The interaction between time and treatment and the corresponding p-values were used to test for a different effect between the treatment arms. In case of a significant fixed effect, post-hoc analyses were performed to check for pairwise differences in effect between the treatments. Moreover, paired-samples t-tests were conducted to specifically evaluate the impact of MPH on the different biomarkers and to assess the effect of an active treatment on NPY and weight at baseline and after 10 weeks. Since multiple hypotheses were tested, thereby increasing the possibility of a type 1 error, a multiple testing correction needs to be carried out. However, the Bonferroni correction would be too strict here, since this correction assumes independent hypothesis tests, which is not the case when testing biomarkers within the same pathway. Therefore, we evaluated the significance of the p-values within each of the two pathways using false discovery rate analysis (Hochberg, 1995). This analysis compares the observed distribution of the p-values to the uniform distribution between 0 and 1, which is expected in case all null hypotheses are true – that is, if none of the biomarkers differ between the three

treatments. A clear difference between observed and expected p-value distribution, with an enrichment of low p-values, suggests that at least some of the significant p-values represent genuine associations. To further quantify the enrichment in low p-values in both pathways, we calculated q-values as implemented in the fdr tool package (Klaus & Strimmer, 2021). In brief, observed p-values are sorted by significance. The q-value then indicates the fraction of false associations in case the p-value is declared significant. For the neurochemical parameter NPY; however, a Bonferroni correction was applied since NPY is an individual biomarker not involved in another pathway. Researchers involved in the analyses of biological samples, digitalising questionnaire answers, calculating scores and performing statistical analyses were blinded for treatment groups.

4.8 Results of the clinical trial

4.8.1 Quality control of PBE

4.8.1.1 Total procyanidin content

Based on analysis *in triplo* (denoted as test 1 to 3) on three different days (Table 4.) the average procyanidin content in PBE was determined to be 78.3 ± 3.0%.

Table 4.5: Procyanidin content (%) in PBE.

	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

PBE: French Maritime Pine Bark Extract.

4.8.1.2 HPLC fingerprint

The resulting chromatograms for reference solutions A ((+)-catechin and taxifolin) and B (caffeic and ferulic acid), determined at 280 nm can be found in Figure 4.2A and Figure

4.2B respectively. The fingerprint chromatogram of PBE determined at 280 nm as well is shown in Figure 4.2C. Mean retention times in the reference solutions were approximately 15.5 min for (+)-catechin, 21.3 min for caffeic acid, 33.1 min for taxifolin and 34.2 min for ferulic acid.

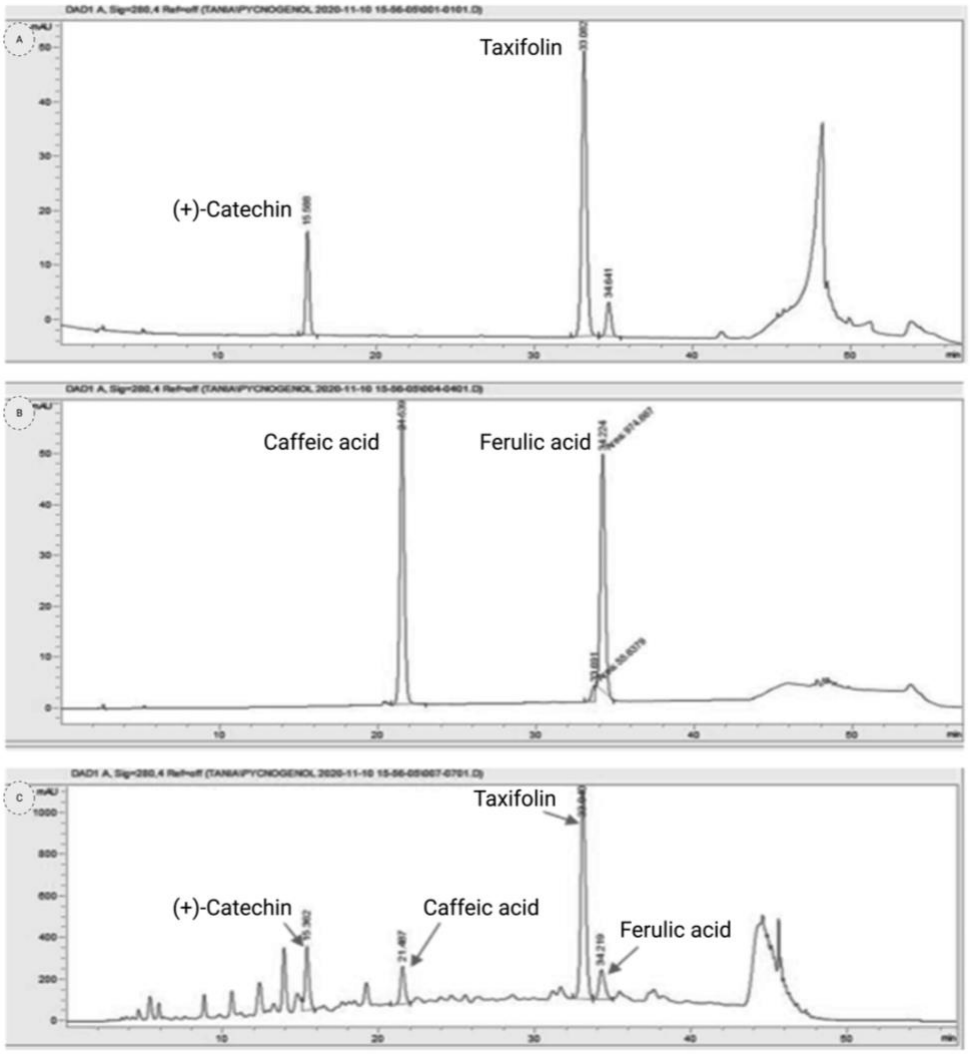

Figure 4.2: A: Reference chromatogram of (+)-catechin (peak 1) and taxifolin (peak 2) B: Reference chromatogram of caffeic acid (peak 1) and ferulic acid (peak 2) C: Chromatogram of the PBE test solution. The constituents their respective peaks are identified in the figure. Screenshot from the HPLC software.

Based on analysis *in triplo* on three different days, the average content of catechin, taxifolin, caffeic acid and ferulic acid in PBE was determined (Table 4.6).

Table 4.6: Monomer content (mean ± SD in %) in PBE.

Monomer	Content
Catechin	1.276 ± 0.031
Taxifolin	1.375 ± 0.012
Caffeic acid	0.287 ± 0.005
Ferulic acid	0.274 ± 0.003

PBE: French Maritime Pine Bark Extract; SD: standard deviation.

PBE was thus found to comply with USP requirements for Maritime Pine Extract, as its average procyanidin content 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 PBE capsules 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.

4.8.1.3 UHPLC coupled to a quadrupole time-of-flight spectrometer

The main phenolic compounds of PBE as identified in the HPLC method, were also characterised using UHPLC-ESI-QTOF-MS. Individual constituents were identified by comparing their spectra and retention times with that of corresponding standards. The peak corresponding to caffeic acid (with a theoretical *m/z* $[M-H]^-$ of 179.0344) could be identified at a RT of 6.61 as shown in Figure 4.3, with the corresponding fragmentation pattern of caffeic acid.

Ferulic acid (with a theoretical *m/z* $[M-H]^-$ of 193.0502) could be attributed to the peak at a RT of 9.34 with the corresponding fragmentation pattern as shown in Figure 4.4. Taxifolin (Figure 4.5) and (+)-catechin (Figure 4.6) were also detected from the TIC of PBE and depicted in the respective figures with their fragmentation patterns.

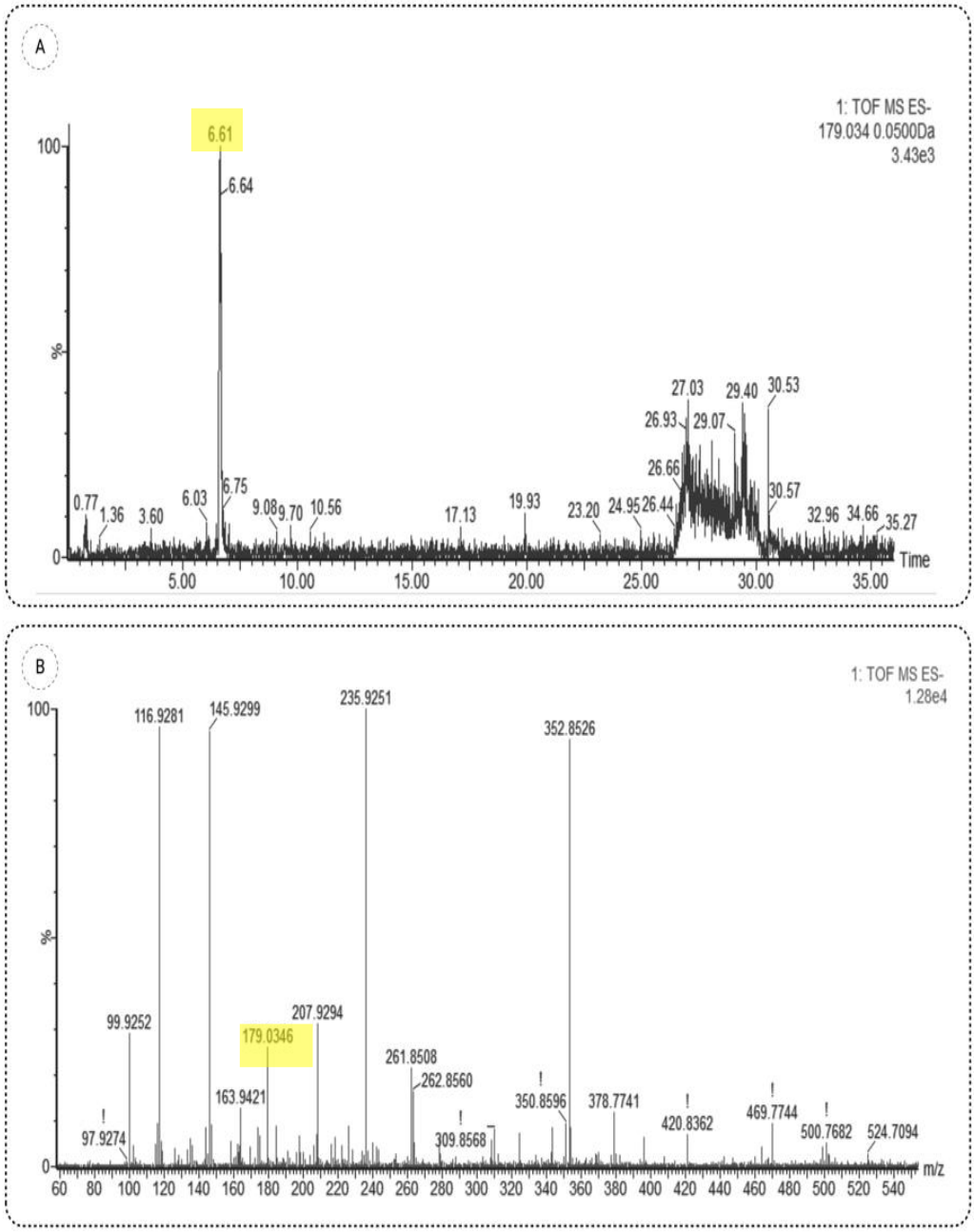

Figure 4.3: UHPLC-QTOF-HRMS data for PBE. A: Caffeic acid corresponds to the peak at RT 6.61 min; B: MS1 spectrum of the ion in which *m/z* 179.0346 corresponds to caffeic acid.

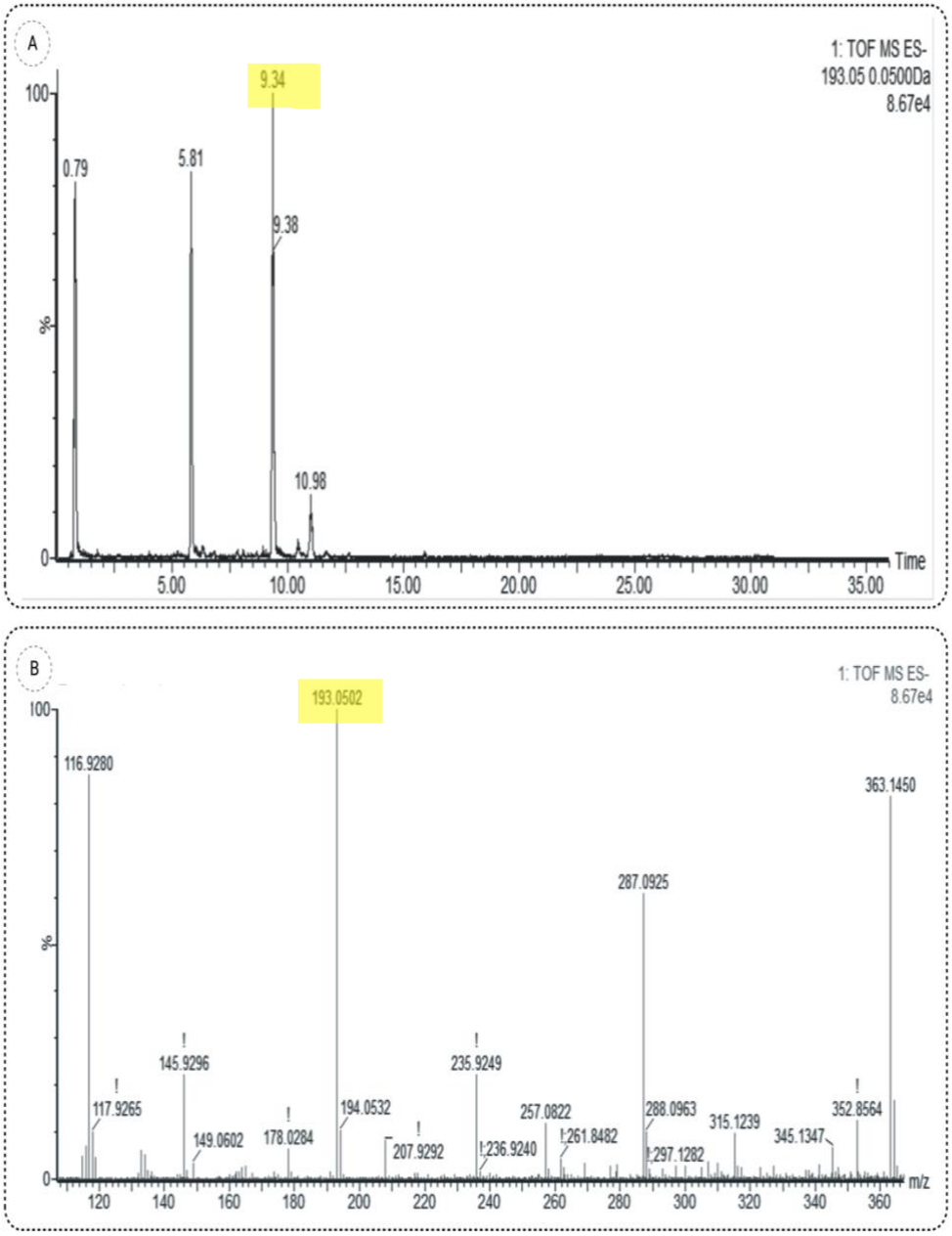

Figure 4.4: UHPLC-QTOF-HRMS data for PBE. A: ferulic acid corresponds to the peak at RT 9.34 min; B: MS1 spectrum of the ion in which *m/z* 193.0502 corresponds to ferulic acid.

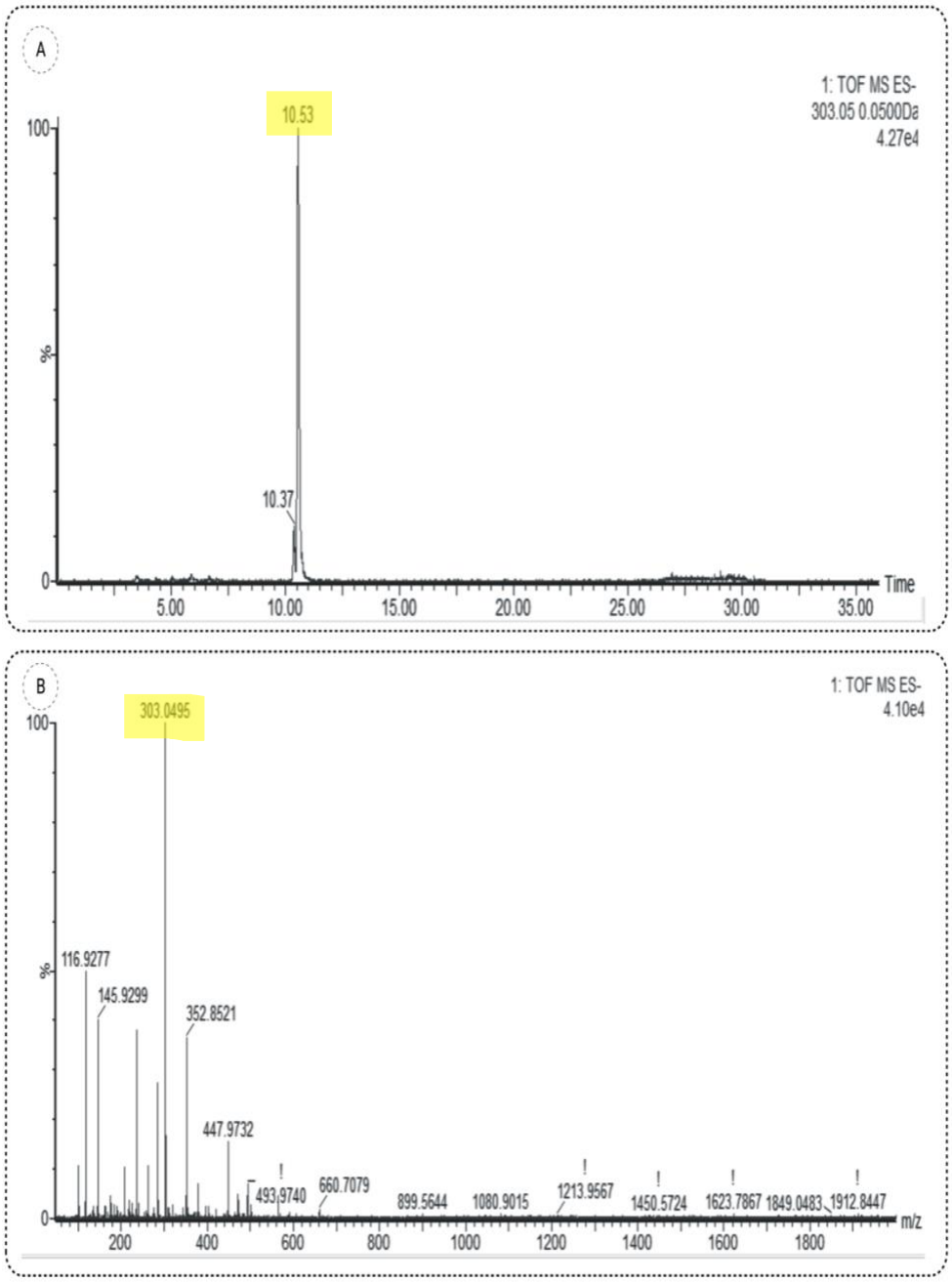

Figure 4.5: UHPLC-QTOF-HRMS data for PBE. A: taxifolin corresponds to the peak at RT 10.53 min; B: MS1 spectrum of the ion in which *m/z* 303.0495 corresponds to taxifolin.

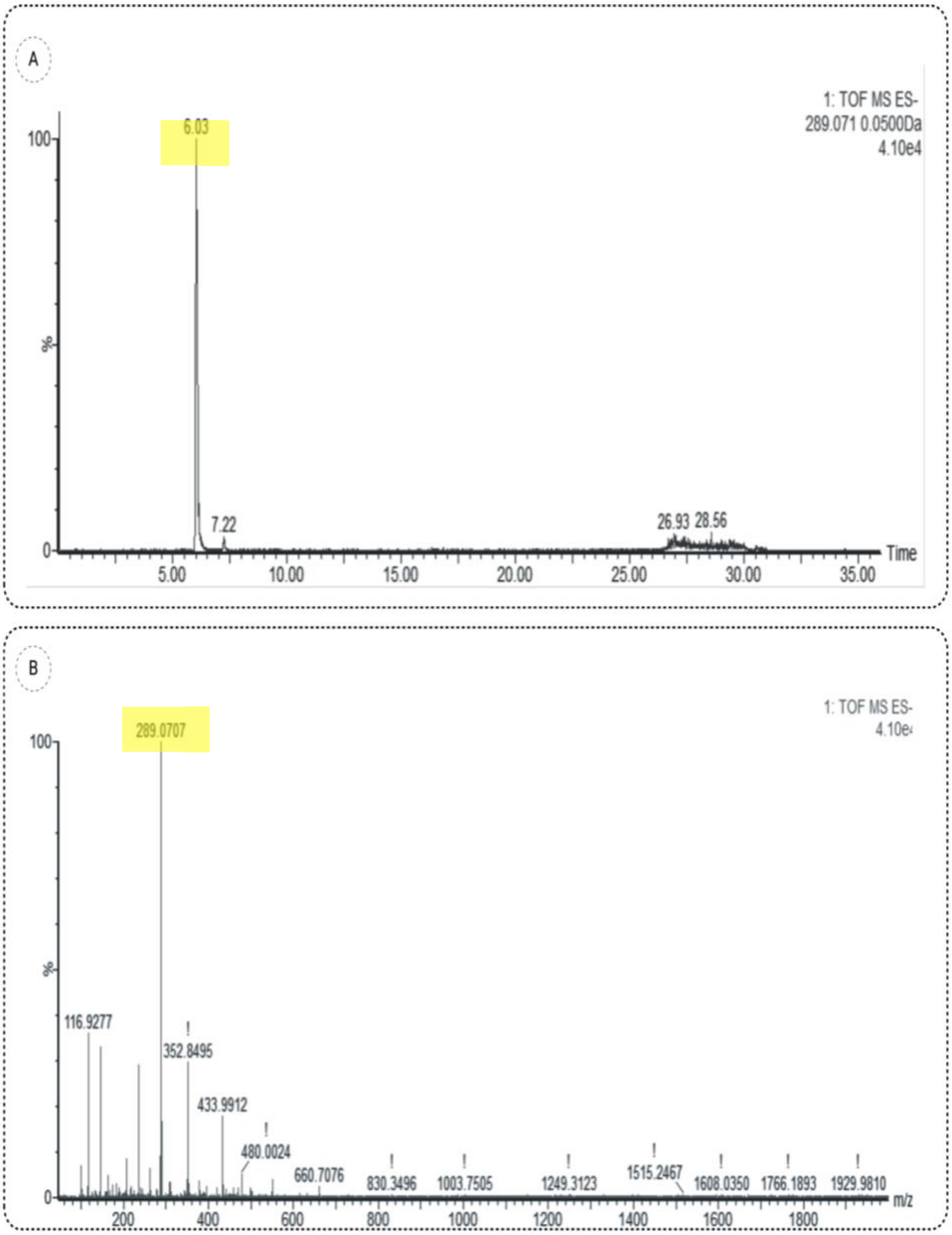

Figure 4.6: UHPLC-QTOF-HRMS data for PBE. A: MS spectrum of peak at RT 6.03 min in negative ion mode, B: MS1 spectrum of the ion in which *m/z* 289.0707 corresponds to (+)-catechin.

Moreover, with the UHPLC-QTOF-HRMS analysis it was possible to identify another polyphenolic constituent of PBE, namely procyanidin B1 as shown in Figure 4.7. In general, all constituents could thus be identified in both the HPLC fingerprint method as well as with the UHPLC-QTOF-HRMS.

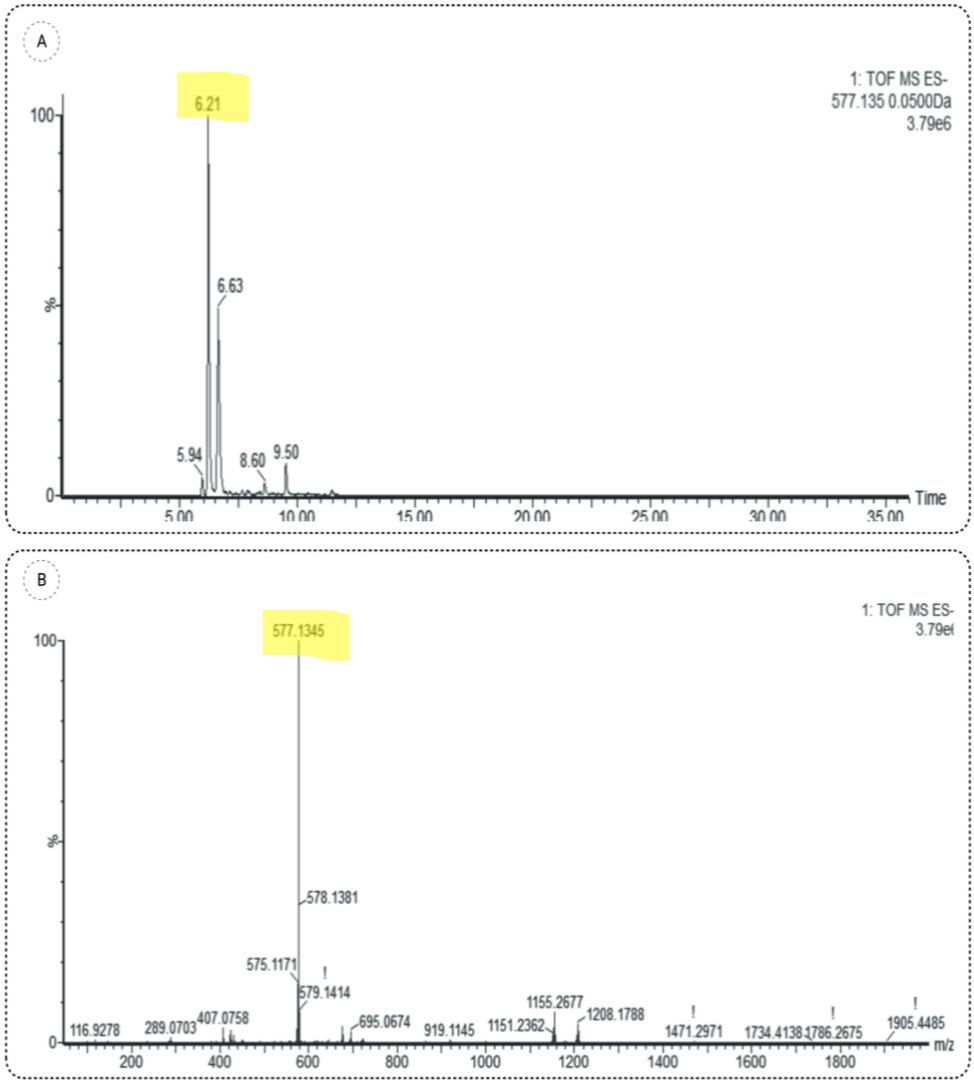

Figure 4.7: UHPLC-QTOF-HRMS data for PBE. A: Procyanidin B1 corresponds to the peak at RT 6.21 min; B: MS1 spectrum of the ion in which *m/z* 577.1345 corresponds to procyanidin B1.

4.8.2 Questionnaires

4.8.2.1 Basic characteristics

Eighty-eight ADHD patients (70% male, 89% Caucasian, mean age 10.1 years) were randomised to placebo (n = 30), PBE (n = 32) or MPH (n = 26) treatment (Figure 4.8); 76 finished their 10-week study period and 12 participants (14%; n = 5 placebo, n = 5 PBE, n = 2 MPH) dropped out (i.e., discontinued intervention and lacking further questionnaires and 10-week biological samples.

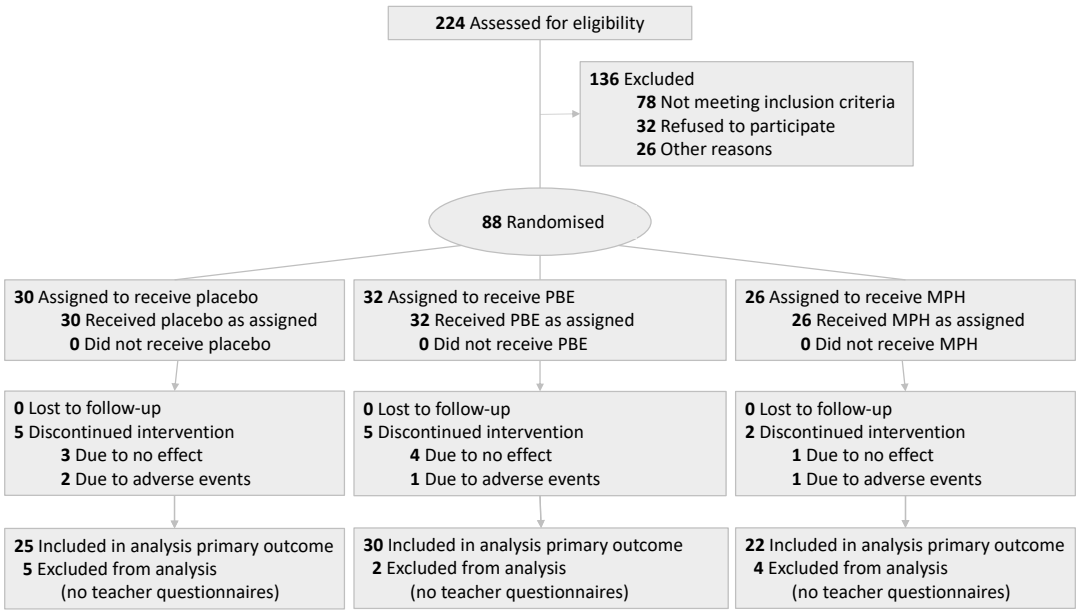

Figure 4.8: Visual representation of the randomisation of the included ADHD patients into three study cohorts. MPH: methylphenidate hydrochloride; PBE: French Maritime Pine Bark Extract.

No significant differences were found between the included 88 participants over the three treatment groups regarding the demographic variables age, height and weight (one-way ANOVA,Table 4.7). Sex ratio, compliance (an intake of >90% ingestion as scheduled, determined based on accountability of the treatment medication and self-reported adherence), general dietary habits and parents' highest educational achievement as proxy for socioeconomic status (Cochran-Armitrage trend test, data not

shown) was not different between treatment arms. The proportion of dropouts was not significantly different between groups (X^2 (1, 0.7068) = 0.401 > 0.05, Chi-Square test).

Adverse events (AEs) were asked for in the questionnaires filled out at week 5 and 10 and were defined as any undesirable sign, symptom or medical condition occurring after starting the intake of the trial supplement or medication that may impair the subject's well-being, whether considered related or not. Medical conditions present before starting the trial supplement or medication are only considered AEs if they worsen after starting the Investigational Medicinal Product (IMP) intake. Spontaneously reported AEs were recorded on the Adverse Event report form and followed carefully until they resolved. AEs reported for dropping out were anger management problems and palpitations (placebo), hospitalisation due to headache (PBE; unrelated to PBE intake since caused by a neck blockage), and sadness (MPH). Serious adverse events related to the intervention have not been observed.

Several teacher questionnaires were missing due to teachers never responding/not responding anymore, starting/ending the study during the summer holiday, covid-19 (home-schooling), or a combination of these.

Table 4.7: Baseline characteristics (mean ± SD) off all included participants (n= 88) per treatment group.

	Placebo	**PBE**	**MPH**	**P-value**
No. male/female (% male)	24/6 (80)	21/11 (66)	17/9 (65)	0.369
Age (years)	9.96 ± 1.90	10.31 ± 1.37	10.0 ± 1.73	0.660
Weight (kg)	36.21 ± 11.68	35.47 ± 9.82	34.32 ± 8.79	0.788
Height (m)	1.42 ± 0.14	1.42 ± 0.11	1.39 ± 0.10	0.664
Dose (mg/kg)	-	0.88 ± 0.03	0.78 ± 0.02	-
Compliance (%)	0.94 ± 0.24	0.99 ± 0.38	0.89 ± 0.15	-

MPH: methylphenidate hydrochloride; PBE: French Maritime Pine Bark Extract; SD: standard deviation.

4.8.2.2 ADHD Rating Scale

4.8.2.2.1 ADHD Rating Scale: Teachers ratings

Mean teacher and parent ADHD-RS scores per treatment group at baseline and follow-up are listed in Table 4.8 and graphically depicted by Figure 4.9. P-values, testing for a different effect between treatments over time, were generated by testing for interaction between time and treatment.

Regarding the teacher-rated summed ADHD-RS score (which was considered our primary outcome), and inattention and hyperactivity/impulsivity sub scores, significant differences in effects between treatments after 10 weeks were found with mean total scores at baseline and after 10 weeks being 26.07 ± 9.62 and 18.43 ± 12.57 for PBE, 24.77 ± 12.68 and 13.50 ± 11.94 for MPH, and 30.06 ± 13.53 and 28.60 ± 13.95 for placebo ($p = 0.008$).

Post-hoc analyses (Table 4.9) show that effects do not differ between the two active treatments (MPH and PBE), whereas effects of both active treatments differ from placebo for the total and hyperactivity/impulsivity score. Indeed, PBE does have a similar effect to MPH on the hyperactivity/impulsivity score. For the inattention score, only MPH shows an effect that is significantly different from placebo. Already after 5 weeks, significant differences in effects between treatments were observed. Post-hoc analyses show that the effect on inattention score differs between the two active treatments, while MPH significantly differs from placebo (total and inattention score). Although after 10 weeks, the difference in effect on total ADHD-RS score between MPH and PBE is 3.23 (no more than 5 points, based on LMM), non-inferiority cannot be demonstrated because 5 is included in the 95% confidence interval of this difference (-3.40 to 9.86).

4.8.2.2.2 ADHD Rating Scale: Parents ratings

Regarding the parent-rated summed ADHD-RS score and inattention sub score, significant differences between treatments after 10 weeks, but not after 5 weeks, were found (Table 4.8**Error! Reference source not found.**). Post-hoc analyses (Table 4.9) show that effects do not differ between the two active treatments. MPH's effects are significantly different from placebo (total score, hyperactivity/impulsivity and inattention sub scores).

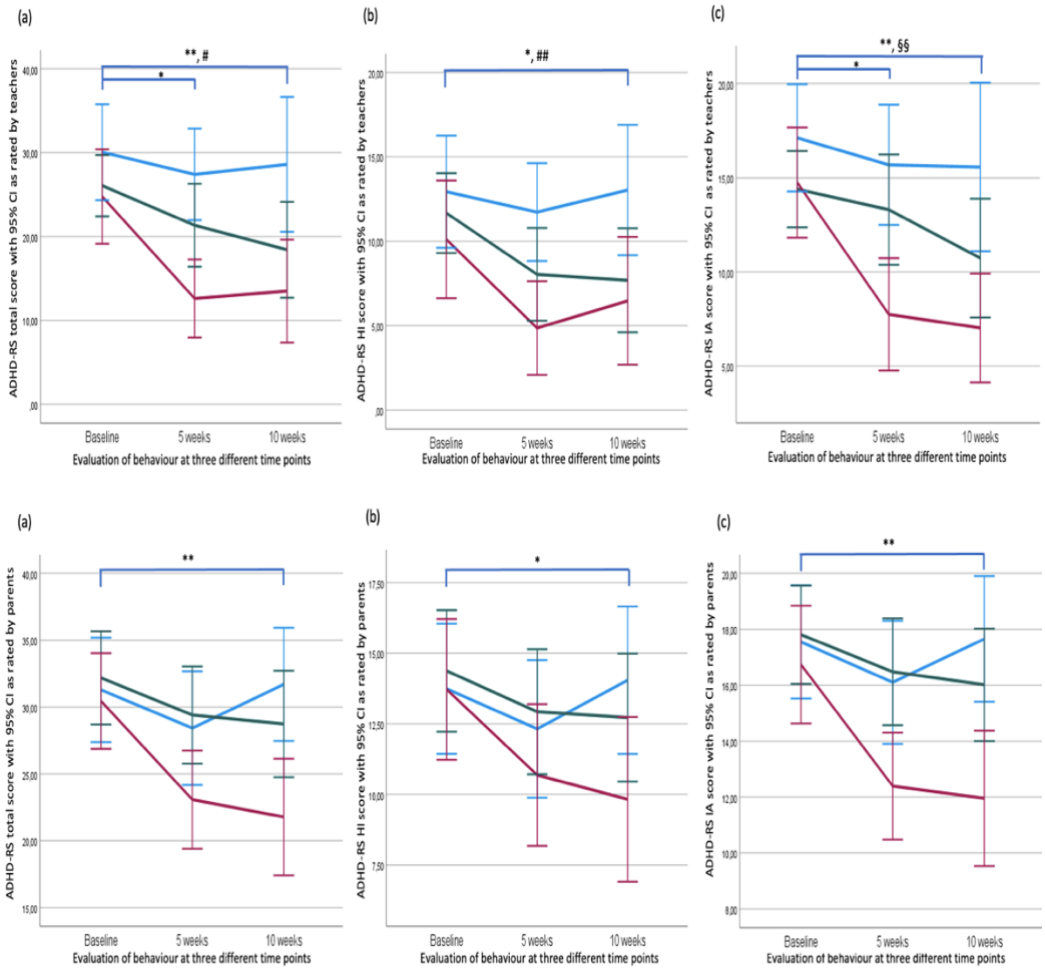

Figure 4.3: ADHD-RS total score (a) and HI (b) and IA (c) sub scores rated by teachers (upper part) and parents (lower part of the figure). Blue: placebo; green: PBE; red: MPH. *: p-value < 0.05 for the difference between placebo and MPH; **: p-value < 0.01 for the difference between placebo and MPH; #: p-value < 0.05 for the difference between placebo and PBE; ##: p-value < 0.01 for the difference between placebo and PBE; §§: p-value < 0.01 for the difference between PBE and MPH. ADHD-RS: ADHD-Rating Scale; CI: confidence interval; HI: hyperactivity/impulsivity; IA: inattention; MPH: methylphenidate hydrochloride; PBE: French Maritime Pine Bark Extract.

Table 4.8: Mean ± SD baseline and follow-up ADHD-RS scores as rated by teachers and parents.

Score	Time point	Placebo	PBE	MPH	Coefficient (SE)[a]			P-value[a]	Coefficient (SE)[b]			P-value[b]
					Intercept	Placebo[c]	PBE[c]		Intercept	Placebo[c]	PBE[c]	
					TEACHERS							
Total ADHD-RS score	Baseline	30.06 ± 13.53	26.07 ± 9.62	24.77 ± 12.68	15.04 ± 2.83	13.85 ± 3.58	8.01 ± 3.46	0.008**	17.07± 2.86	14.64 ± 3.84	3.23 ± 3.54	0.002**
	5 weeks	27.42 ± 12.94	21.35 ± 2.24	18.43 ± 12.57								
	10 weeks	28.60 ± 13.95	12.61 ± 9.36	13.50 ± 11.94								
HI sub score	Baseline	12.94 ± 7.86	11.67 ± 6.22	10.11 ± 7.87	6.55 ± 1.63	6.18 ± 2.06	2.84 ± 1.99	0.05	8.21 ± 1.67	6.55 ± 2.22	0.61 ± 2.06	0.003**
	5 weeks	11.73 ± 6.86	8.04 ± 6.80	4.86 ± 5.58								
	10 weeks	13.04 ± 6.69	7.69 ± 6.78	6.47 ± 7.37								
IA sub score	Baseline	17.13 ± 6.74	14.40 ± 5.32	14.75 ± 6.59	8.47 ± 1.60	7.69 ± 2.02	5.18 ± 1.96	0.002**	8.73 ±1.54	8.15 ±2.10	2.73 ± 1.92	0.005**
	5 weeks	15.69 ± 7.56	13.31 ± 7.25	7.75 ± 5.60								
	10 weeks	15.57 ± 7.74	10.74 ± 6.92	7.03 ± 5.62								

Table 4.8: Mean ± SD baseline and follow-up ADHD-RS scores as rated by teachers and parents (continued).

Score	Time point	Placebo	PBE	MPH	Coefficient (SE)[a]			P-value[a]	Coefficient (SE)[b]			P-value[b]
					Intercept	Placebo[c]	PBE[c]		Intercept	Placebo[c]	PBE[c]	
					PARENTS							
Total ADHD-RS score	Baseline	31.29 ± 10.27	32.19 ± 9.67	30.46 ± 8.28	22.96 ± 2.07	5.54 ± 2.66	5.91 ± 2.63	0.170	21.89 ±2.10	9.77 ±2.79	6.70±2.67	0.003*
	5 weeks	28.43 ± 10.96	29.41 ± 9.39	23.08 ± 8.68								
	10 weeks	31.70 ± 9.54	28.74 ± 10.06	21.78 ± 10.09								
HI sub score	Baseline	13.74 ± 6.07	14.38 ± 5.97	13.72 ± 5.76	10.04 ± 1.28	2.05 ± 1.64	2.33 ± 1.61	0.620	9.63 ± 1.31	4.25 ± 1.72	2.80 ± 1.65	0.040
	5 weeks	12.32 ± 6.29	12.93 ± 5.70	10.69 ± 5.94								
	10 weeks	14.05 ± 5.90	12.72 ± 5.73	9.83 ± 6.74								
IA sub score	Baseline	17.55 ± 5.32	17.81 ± 4.88	16.74 ± 4.87	12.88 ± 1.08	3.52 ± 1.23	3.61 ± 1.38	0.060	12.26 ±1.11	5.52 ± 1.48	3.89 ± 1.41	0.002**
	5 weeks	16.11 ± 5.67	16.48 ± 4.93	12.40 ± 4.54								
	10 weeks	17.66 ± 5.06	16.02 ± 5.08	11.96 ± 5.61								

[a]Coefficients and p-value of the interaction between time and treatment between 3 treatment groups, 5 weeks versus baseline, with sex as covariate (LMM). [b] Coefficients and p-value of the interaction between time and treatment between 3 treatment groups, 10 weeks versus baseline, with sex as covariate (LMM). [c]: MPH was used as reference treatment. **: p-value < 0.01. ADHD-RS: ADHD-Rating Scale; HI: hyperactivity/impulsivity; IA: inattention; MPH: methylphenidate hydrochloride; PBE: French Maritime Pine Bark Extract; SD: standard deviation

Table 4.9: Post-hoc pairwise comparisons of ADHD-RS scores as rated by teachers and parents.

Score	Comparison	Coefficient (SE)[a]		P-value[a]	Coefficient (SE)[b]		P-value[b]
		Intercept	Treatment[c]		Intercept	Treatment[c]	
			TEACHERS				
Total ADHD-RS score	Placebo vs. PBE	22.81 ± 2.52	5.97 ± 3.31	>0.990	19.95 ± 2.60	11.55 ± 3.60	0.020*
	Placebo vs. MPH	14.59 ± 3.14	14.01 ± 3.82	0.020*	16.77 ± 3.21	14.79 ± 4.11	0.004**
	PBE vs. MPH	15.52 ± 2.70	8.03 ± 3.23	0.070	17.63 ± 2.74	3.23 ± 3.33	>0.990
HI sub score	Placebo vs. PBE	9.27 ± 1.43	3.39 ± 1.87	0.160	8.68 ± 1.49	5.99 ± 2.04	0.003**
	Placebo vs. MPH	6.50 ± 1.78	6.19 ± 2.17	0.110	8.22 ± 1.85	6.55 ± 2.36	0.030*
	PBE vs. MPH	6.68 ± 1.61	2.84 ± 1.92	0.960	8.35 ± 1.67	0.60 ± 2.01	>0.990
IA sub score	Placebo vs. PBE	13.55 ± 1.42	2.57 ± 1.87	>0.990	11.28 ± 1.41	5.55 ± 1.97	0.200
	Placebo vs. MPH	8.07 ± 1.73	7.84 ± 2.11	0.013*	8.41 ± 1.69	8.29 ± 2.20	0.005**
	PBE vs. MPH	8.84 ± 1.56	5.20 ± 1.88	0.008**	9.10 ± 1.49	2.79 ± 1.83	0.200
			PARENTS				
Total ADHD-RS score	Placebo vs. PBE	29.01 ± 2.06	-0.40 ± 2.65	>0.990	28.53 ± 2.01	3.10 ± 2.67	0.170
	Placebo vs. MPH	22.22 ± 2.15	5.81 ± 2.69	0.390	21.15 ± 2.17	10.07 ± 2.80	0.006**
	PBE vs. MPH	23.40 ± 2.10	5.86 ± 2.49	0.270	22.51 ± 2.11	6.70 ± 2.64	0.160
HI sub score	Placebo vs. PBE	12.46 ± 1.22	-0.31 ± 1.57	>0.990	12.35 ± 1.21	1.48 ± 1.61	0.460
	Placebo vs. MPH	9.65 ± 1.33	2.19 ± 1.66	>0.990	9.30 ± 1.38	4.38 ± 1.76	0.050*
	PBE vs. MPH	10.26 ± 1.30	2.33 ± 1.60	>0.990	9.98 ± 1.34	2.79 ± 1.66	0.530
IA sub score	Placebo vs. PBE	16.57 ± 1.06	-0.10 ± 1.37	>0.990	16.19 ± 1.03	1.62 ± 1.36	0.140
	Placebo vs. MPH	12.51 ± 1.15	3.67 ± 1.44	0.140	11.82 ± 1.18	5.69 ± 1.53	0.005**
	PBE vs. MPH	13.09 ± 1.05	3.59 ± 1.31	0.110	12.53 ± 1.11	3.91 ± 1.40	0.120

[a]Coefficients and p-value of the interaction between time and treatment, 5 weeks versus baseline, with sex as covariate, with Bonferroni correction for multiple testing (LMM), based on scores in Table 4.7. [b]Coefficients and p-value of the interaction between time and treatment, 10 weeks versus baseline, with sex as covariate, with Bonferroni correction for multiple testing (LMM), based on scores in Table 4.7. [c]MPH was used as reference treatment (or PBE, in case of placebo vs. PBE). *: p-value < 0.05; **: p-value < 0.01. ADHD-RS: ADHD-Rating Scale; HI: hyperactivity/impulsivity; IA: inattention; MPH: methylphenidate hydrochloride; PBE: French Maritime Pine Bark Extract.

4.8.2.3 Social-Emotional Questionnaire: ADHD scores

4.8.2.3.1 Social-Emotional Questionnaire: ADHD scores rated by teachers

Based on teacher SEQ ratings, a significant difference in effect between the three treatments was only found for the hyperactivity sub score (Table 4.10, Figure 4.10). Post-hoc pairwise comparisons show that the effect on the hyperactivity score does not differ between the two active treatments, whereas the effect of both active treatments differs from the effect of placebo (Table 4.11).

4.8.2.3.2 Social-Emotional Questionnaire: ADHD scores rated by parents

Based on parent SEQ ratings, a significant difference in effect between the three treatments was found for the total, hyperactivity and inattention scores (Table 4.10, Figure 4.10). Post-hoc analyses show that the two active treatments differ significantly regarding these (sub)scores, whereas MPH differs significantly from placebo for the total and hyperactivity score (Table 4.11).

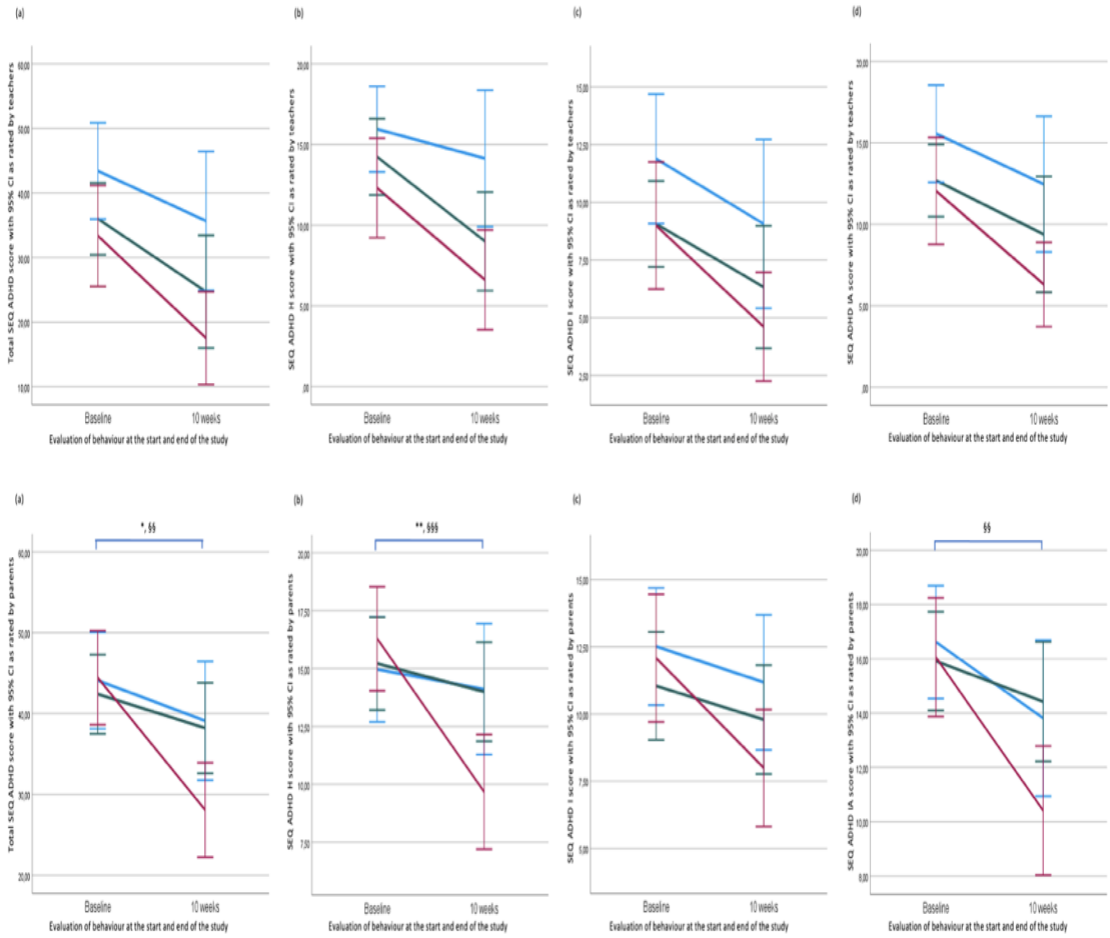

Figure 4.10*:* SEQ total score (a) and H (b), I (c) and IA (d) sub scores rated by teachers (upper part) and parents (lower part of the figure). Blue: placebo; green: PBE; red: MPH. *: p-value < 0.05 for the difference between placebo and MPH; **: p-value < 0.01 for the difference between placebo and MPH; §§: p-value < 0.01 for the difference between PBE and MPH; §§§: p-value < 0.001 for the difference between PBE and MPH. CI: confidence interval; H: hyperactivity; I: impulsivity; IA: inattention; MPH: methylphenidate hydrochloride; PBE: French Maritime Pine Bark Extract; SEQ: Social-Emotional Questionnaire.

Table 4.10: Mean ± SD baseline and follow-up SEQ ADHD scores as rated by teachers and parents.

Score	Time point	Placebo	PBE	MPH	Coefficient (SE)			P-value
					Intercept	Placebo[a]	PBE[a]	
				TEACHERS				
Total ADHD score	Baseline	43.41 ± 17.24	36.00 ± 14.61	33.36 ± 17.18	21.72 ± 4.00	18.57 ± 5.42	4.41 ± 4.99	0.07
	10 weeks	35.68 ± 18.64	24.71 ± 19.18	17.53 ± 14.46				
H sub score	Baseline	15.96 ± 6.13	14.24 ± 6.21	12.31 ± 6.78	7.96 ± 1.55	7.61 ± 2.09	1.61 ± 1.93	0.004**
	10 weeks	14.14 ± 7.33	9.00 ± 6.71	6.61 ± 6.22				
I sub score	Baseline	11.89 ± 6.49	9.07 ± 4.91	9.00 ± 6.05	5.81 ± 1.35	4.93 ± 1.83	0.94 ± 1.69	0.26
	10 weeks	9.07 ± 6.33	6.33 ± 5.83	4.61 ± 4.74				
IA sub score	Baseline	15.57 ± 6.89	12.69 ± 5.83	12.05 ± 7.21	7.87 ± 1.61	6.03 ± 2.21	1.94 ± 2.03	0.34
	10 weeks	12.46 ± 7.21	9.38 ± 7.82	6.31 ± 5.19				
				PARENTS				
Total ADHD score	Baseline	44.10 ± 15.67	42.40 ± 13.36	44.44 ± 13.75	27.87 ± 3.15	10.26 ± 4.12	9.17 ± 3.97	<0.001***
	10 weeks	39.11 ± 16.53	38.22 ± 14.15	28.09 ± 13.52				
H sub score	Baseline	14.97 ± 5.97	15.43 ± 5.56	16.29 ± 5.32	9.61 ± 1.24	4.21 ± 1.62	3.94 ± 1.56	<0.001***
	10 weeks	14.11 ± 6.38	14.00 ± 5.41	9.67 ± 5.71				
I sub score	Baseline	12.52 ± 5.72	11.05 ± 5.47	12.08 ± 5.62	7.78 ±1.19	2.85 ± 1.56	1.46 ± 1.50	0.09
	10 weeks	11.18 ± 5.66	9.80 ± 5.12	8.00 ± 5.03				
IA sub score	Baseline	16.62 ± 5.47	15.92 ± 4.95	16.06 ± 5.17	10.48 ± 1.20	3.20 ± 1.57	3.79 ± 1.51	0.003**
	10 weeks	13.82 ± 6.49	14.43 ± 5.56	10.41 ± 5.50				

Coefficients and p-values of the interaction between time and treatment between 3 treatment groups, 10 weeks versus baseline, with sex as covariate (LMM). [a]: MPH was used as standard treatment. **: p-value < 0.01. H: Hyperactivity; I: Impulsivity; IA: Inattention; MPH: Methylphenidate hydrochloride; PBE: French Maritime Pine Bark Extract; SD: Standard deviation; SEQ: Social-Emotional Questionnaire.

Table 4.11: Post-hoc pairwise comparisons of SEQ ADHD (sub)scores as rated by teachers and parents.

Score	Comparison	Coefficient (SE)		P-value
		Intercept	Treatment[a]	
		TEACHERS		
Total ADHD score	Placebo vs. PBE	25.84 ± 3.72	14.49 ± 5.16	0.120
	Placebo vs. MPH	21.29 ± 4.26	18.79 ± 5.52	0.070
	PBE vs. MPH	22.22 ± 3.98	4.54 ± 4.87	>0.990
H sub score	Placebo vs. PBE	9.45 ± 1.42	6.12 ± 1.95	0.002**
	Placebo vs. MPH	7.85 ± 1.64	7.66 ± 2.12	0.020*
	PBE vs. MPH	8.15 ± 1.58	1.62 ± 1.93	>0.990
I sub score	Placebo vs. PBE	6.74 ± 1.27	4.01 ± 1.77	0.910
	Placebo vs. MPH	5.82 ± 1.49	4.98 ± 1.92	0.280
	PBE vs. MPH	5.80 ± 1.31	0.98 ± 1.60	>0.990
IA sub score	Placebo vs. PBE	9.66 ± 1.50	4.28 ± 2.09	0.980
	Placebo vs. MPH	7.54 ± 1.71	6.16 ± 2.25	0.430
	PBE vs. MPH	8.17 ± 1.62	2.02 ± 2.00	>0.990
		PARENTS		
Total ADHD score	Placebo vs. PBE	37.05 ± 3.02	1.06 ± 3.97	>0.990
	Placebo vs. MPH	26.70 ± 3.36	10.79 ± 4.28	0.020*
	PBE vs. MPH	28.74 ± 3.04	9.16 ± 3.76	0.001**
H sub score	Placebo vs. PBE	13.55 ± 1.18	0.28 ± 1.56	>0.990
	Placebo vs. MPH	9.30 ± 1.32	4.36 ±1.68	0.002**
	PBE vs. MPH	9.85 ± 1.22	3.92 ± 1.51	<0.001***
I sub score	Placebo vs. PBE	9.25 ± 1.13	1.38 ± 1.50	>0.990
	Placebo vs. MPH	7.43 ± 1.25	3.02 ± 1.59	0.360
	PBE vs. MPH	8.03 ± 1.19	1.44 ± 1.47	0.110
IA sub score	Placebo vs. PBE	14.29 ± 1.15	-0.60 ± 1.51	0.810
	Placebo vs. MPH	9.96 ± 1.28	3.41 ± 1.63	0.130
	PBE vs. MPH	10.86 ± 1.16	3.79 ± 1.44	0.004**

Coefficients and p-values of the interaction between time and treatment with Bonferroni correction for multiple testing (LMM), based on scores in Table 4.9. [a]: [c]MPH was used as reference treatment (or PBE, in case of placebo vs. PBE). *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001. H: hyperactivity; I: impulsivity; IA: inattention; PBE: French Maritime Pine Bark Extract; MPH: methylphenidate hydrochloride; SEQ: Social-Emotional Questionnaire.

4.8.2.4 Social-Emotional Questionnaire: autism, social problem behaviour and anxiety scores

For teacher and parent ratings, no significant difference in effects between the three treatments was found regarding SEQ autism, social problem behaviour and anxiety (sub)scores (Table 4.12).

Table 4.12. Mean ± SD baseline and follow-up SEQ autism, antisocial and anxiety (sub)scores, after 10 weeks vs. baseline.

Score	Time point	Placebo	PBE	MPH	Coefficient (SE)			P-value
					Intercept	Placebo[a]	PBE[a]	
				TEACHERS				
Autism	Baseline	12.78 ±19.69	7.39 ± 4.91	7.85 ± 5.66	7.06 ± 2.73	5.32 ± 3.55	0.41 ± 3.35	0.70
	10 weeks	6.36 ± 4.62	6.29 ± 5.51	5.22 ± 5.34				
Social problem behaviour	Baseline	23.36 ±23.14	18.09 ±15.58	17.42 ±17.40	14.39±4.32	8.16 ± 5.86	0.54 ± 5.24	0.53
	10 weeks	19.65 ±24.51	9.76 ± 10.52	11.76 ±15.50				
ODD	Baseline	7.78 ± 9.40	6.24 ± 7.24	7.30 ± 8.17	6.06 ± 1.81	1.65 ± 2.42	-0.24 ± 2.26	0.57
	10 weeks	6.36 ± 0.38	3.48 ± 4.06	4.67 ± 7.40				
Aggression	Baseline	3.61 ± 4.16	2.31 ± 2.70	1.82 ± 2.67	1.86 ± 0.79	1.91 ± 1.09	0.60 ± 1.00	0.94
	10 weeks	3.39 ± 5.65	1.57 ± 3.04	1.28 ± 2.24				
Antisocial behaviour	Baseline	11.39 ± 9.48	9.05 ± 6.84	7.89 ± 6.85	6.25 ± 1.78	4.56 ± 2.41	0.26 ± 2.16	0.21
	10 weeks	9.46 ± 8.71	4.71 ± 4.36	5.47 ± 6.50				
Total fear	Baseline	12.86 ±12.03	11.72 ± 8.60	15.33 ±10.86	10.00±2.45	1.94 ± 3.30	0.34 ± 3.01	0.11
	10 weeks	7.85 ± 6.62	8.76 ± 5.89	8.74 ± 9.79				
General fear	Baseline	4.95 ± 3.99	5.69 ± 4.19	6.62 ± 4.97	3.37 ± 0.96	1.04 ± 1.34	0.87 ± 1.22	0.07
	10 weeks	3.29 ± 2.81	4.00 ± 2.61	3.22 ± 4.31				
Social fear	Baseline	3.70 ± 4.52	2.34 ± 2.73	3.98 ± 3.72	2.99 ± 0.82	-0.21 ±1.13	-0.57 ± 1.03	0.49
	10 weeks	1.64 ± 2.17	2.19 ± 3.08	2.33 ± 2.54				
Depression	Baseline	4.08 ±5.34	3.69 ± 5.09	4.76 ± 4.27	3.69 ± 1.17	0.97 ± 1.57	0.05 ± 1.45	0.18
	10 weeks	3.00 ± 3.63	2.57 ± 2.99	3.38 ± 4.60				

Table 4.12. Mean ± SD baseline and follow-up SEQ autism, antisocial and anxiety (sub)scores, after 10 weeks vs. baseline (continued).

Score	Time point	Placebo	PBE	MPH	Coefficient (SE)			P-value
					Intercept	Placebo[a]	PBE[a]	
				PARENTS				
Autism	Baseline	7.79 ± 5.68	7.68 ± 5.91	6.85 ± 4.66	4.73 ± 1.09	0.96 ± 1.42	1.25 ± 1.37	0.83
	10 weeks	5.43 ± 3.76	6.50 ± 5.04	4.37 ± 3.05				
Social problem behaviour	Baseline	26.05 ± 14.86	24.61 ±15.24	30.83 ± 17.31	22.17±3.55	0.96 ± 4.62	-0.57 ± 4.48	0.23
	10 weeks	24.20 ± 16.14	22.27 ±16.27	21.86 ± 17.58				
ODD	Baseline	10.66 ± 6.06	10.64 ± 7.86	13.81 ± 7.33	10.67±1.63	-0.81 ±2.15	-0.41 ± 2.06	0.27
	10 weeks	10.25 ± 6.99	10.48 ± 8.41	10.35 ± 7.93				
Aggression	Baseline	2.88 ± 3.18	2.48 ± 2.82	4.67 ± 4.69	3.18 ± 0.80	0.02 ± 1.05	-0.80 ± 1.01	0.10
	10 weeks	3.16 ± 4.35	2.31 ± 2.94	2.74 ± 4.08				
Antisocial behaviour	Baseline	12.52 ± 7.59	11.16 ± 5.91	12.35 ± 6.59	8.14 ± 1.46	1.91 ± 1.88	0.67 ± 1.82	0.39
	10 weeks	10.80 ± 7.42	9.31 ± 5.77	8.45 ± 6.65				
Total fear	Baseline	16.98 ± 8.74	18.92 ±12.18	20.44 ± 11.24	14.55±2.26	-2.97 ±3.04	1.62 ± 2.88	0.48
	10 weeks	11.05 ± 5.70	16.54 ±12.24	13.59 ± 9.71				
General fear	Baseline	7.07 ± 4.28	7.65 ± 5.26	8.54 ± 4.57	4.91 ± 0.99	-0.81 ±1.31	0.76 ± 1.25	0.29
	10 weeks	3.95 ± 2.80	6.24 ± 5.51	4.78 ± 3.74				
Social fear	Baseline	4.41 ± 3.70	4.61 ± 4.40	3.94 ± 2.97	3.99 ± 0.73	-0.85 ±0.99	0.73 ± 0.93	0.51
	10 weeks	2.86 ± 2.15	4.28 ± 3.77	3.35 ± 2.63				
Depression	Baseline	5.50 ± 3.55	6.66 ± 5.94	7.96 ± 6.02	5.68 ± 1.11	-1.25 ±1.49	0.09 ± 1.42	0.57
	10 weeks	4.24 ± 2.83	6.02 ± 5.77	5.46 ± 5.13				

Coefficients and p-values of the interaction between time and treatment, with sex as covariate (LMM). [a]: MPH was used as reference treatment. MPH: methylphenidate hydrochloride; ODD: opposite defiant disorder; PBE: French Maritime Pine Bark Extract; SD: standard deviation; SEQ: Social-Emotional Questionnaire.

4.8.2.5 Physical complaints and adverse effects

No significant differences in effects between the three treatments were found for combined (Table 4.13) and individual PCQ scores. Moreover, no significant changes regarding individual PCQ scores were found within treatment groups during the study period (Cochran-Armitage trend test, data not shown).

Regarding blood pressure and heart rate, no significant differences in effects between treatments were found (Table 4.14), despite a slightly increased average heart rate in the MPH group ($p = 0.1414$ after Bonferroni correction for the difference in effect between PBE and MPH).

No serious AEs related to the intervention were reported. The frequency of nonserious adverse events reported by parents after 5 and 10 weeks (open question) was significantly different between treatment groups (Table 4.15). Post-hoc analyses revealed that both after 5 and 10 weeks, significantly more adverse events were reported for participants receiving MPH than for those receiving PBE ($p = 0.004$ and $p = 0.0255$ after Bonferroni correction). Adverse effects reported for PBE were headache, dizziness, nausea and diarrhoea. Adverse effects reported for MPH were GI symptoms, reduced appetite, insomnia, headache, a feeling of tachycardia, sneezing and being emotional.

Table 4.13: Mean ± SD baseline and follow-up summed physical complaints scores between treatments, after 10 weeks vs. baseline.

Score	Time point	Placebo	PBE	MPH	Coefficient (SE)			P-value
					Intercept	Placebo [a]	PBE [a]	
Pain	Baseline	2.62 ± 1.90	2.97 ± 2.44	2.90 ± 2.14	2.50 ± 0.50	-0.66 ±0.66	-0.46 ±0.63	0.51
	10 weeks	1.91 ± 1.69	2.20 ± 2.47	2.70 ± 2.90				
Thirst/ perspiration	Baseline	1.17 ± 1.63	1.65 ± 2.90	1.67 ± 1.95	1.04 ± 0.50	0.32 ± 0.67	0.21 ± 0.64	0.48
	10 weeks	1.31 ± 2.06	1.42 ± 2.67	1.22 ± 2.15				
Asthma/ rhinitis	Baseline	2.43 ± 2.39	2.41 ± 2.31	2.43 ± 1.83	2.21 ± 0.57	0.10 ± 0.76	0.52 ± 0.73	0.67
	10 weeks	2.31 ± 3.33	1.78 ± 2.60	2.30 ± 3.00				
Skin problems	Baseline	2.21 ± 4.10	2.68 ± 2.87	2.23 ± 3.24	1.14 ± 0.68	0.65 ± 0.90	0.13 ± 0.87	0.53
	10 weeks	1.71 ± 3.24	1.81 ± 2.59	1.41 ± 2.06				
GI problems	Baseline	3.61 ± 3.59	3.68 ± 3.06	3.83 ± 2.57	2.75 ± 0.71	0.46 ± 0.94	0.00 ± 0.90	0.75
	10 weeks	2.64 ± 3.34	2.90 ± 3.12	2.68 ± 2.68				
Sleep problems	Baseline	3.36 ± 2.72	2.72 ± 2.32	4.65 ± 2.17	5.19 ± 0.48	-2.14 ±0.64	-2.69 ±0.61	0.12
	10 weeks	2.86 ± 1.74	2.02 ± 1.88	5.09 ± 2.04				

Coefficients and p-values of the interaction between time and treatment, with sex as covariate (LMM). [a]: MPH was used as reference treatment. GI: gastrointestinal; MPH: methylphenidate hydrochloride; PBE: French Maritime Pine Bark Extract; SD: standard deviation.

Table 4.14: Mean ± SD baseline and follow-up blood pressure and heart rate.

	Time point	Placebo	PBE	MPH	Coefficient (SE)			P-value
					Intercept	Placebo[a]	PBE[a]	
Systolic blood pressure, mm Hg	Baseline	108.80 ± 12.42	107.00 ±12.36	109.54 ± 9.50	115.24 ± 2.32	-5.97 ± 3.09	-3.64 ± 2.99	0.37
	10 weeks	109.52 ± 8.78	111.65 ±10.13	115.61 ± 7.73				
Diastolic blood pressure, mm Hg	Baseline	59.77 ± 8.85	60.59 ± 11.99	62.12 ± 9.97	65.75 ± 2.10	-4.72 ± 2.83	-4.91 ± 2.74	0.57
	10 weeks	61.04 ± 7.71	60.81 ± 9.51	65.70 ± 7.89				
Heart rate, beats per minute	Baseline	79 ± 12	77 ± 17	78 ± 10	85 ± 3	-4 ± 4	-10 ± 4	0.09
	10 weeks	81 ± 11	76 ± 13	86 ± 15				

P-values of the interaction between time and treatment between 3 treatment groups, 10 weeks versus baseline, with sex as covariate (LMM). [a]: MPH was used as reference treatment. MPH: methylphenidate hydrochloride; PBE: French Maritime Pine Bark Extract; SD: standard deviation.

Table 4.15: Proportion of participants with reported potential adverse events (yes/no).

Time point	Placebo	PBE	MPH	P-value
5 weeks	6/20 (23%)	3/25 (11%)	11/10 (52%)	0.004**
10 weeks	2/20 (9%)	2/24 (8%)	9/14 (39%)	0.007**

Chi square test based on "complete cases". The percentage of yes responders is calculated by dividing the amount of yes responders by the total amount of participants ** $p < 0.01$. MPH: methylphenidate hydrochloride; PBE: French Maritime Pine Bark Extract.

4.8.2.6 Percentage of treatment responders

Based on teacher (but not parent) ratings, the percentage of treatment responders was significantly different between treatment groups (Table 4.16). Post-hoc analyses on teacher ratings revealed that the percentage of treatment responders differs significantly between MPH and placebo (data not shown, $p = 0.007$ after Bonferroni correction).

Table 4.16: Proportion of treatment responders (yes/no; percentage indicates yes responders) based on teacher and parent ADHD-RS ratings.

	Placebo	PBE	MPH	P-value
Teachers	3/11 (21%)	12/8 (60%)	13/4 (76%)	0.008**
Parents	4/17 (19%)	10/17 (37%)	11/10 (52%)	0.08

Chi square test based on "complete cases" (participants with total ADHD-RS scores at baseline and 10 weeks). The percentage of yes responders is calculated by dividing the amount of yes responders by the total amount of participants; ** $p < 0.01$. ADHD-RS: ADHD-Rating Scale; MPH: methylphenidate hydrochloride; PBE: French Maritime Pine Bark Extract.

4.8.3 Biological samples analyses

4.8.3.1 Baseline characteristics

No significant differences in mean baseline concentrations for each biomarker were found between the groups (one-way ANOVA, Table 4.17).

Mean concentrations of the analysed biomarkers are listed per treatment group at baseline and after 10 weeks in Table 4.18. Test statistics and p-values of the interaction

between treatment and time are also shown in Table 4.18 In case of a significant interaction term, false discovery rate analysis was applied (Table 4.19) to account for the multitude of hypotheses tested. For the oxidative stress parameters, the qq-plot shows that the observed p-values more or less follow the expected distribution under the null hypothesis of no association. For the immunological markers, there is a slight increase in significance regarding the expected null distribution (Figure 4.11). Results of the paired samples t-test for NPY and weight assessment in the active treatment groups are listed in Table 4.20.

Table 4.17: Baseline concentration for each biomarker (mean ± SD) per treatment group.

	Placebo	PBE	MPH	P-value
GSH (µg/mL)	849.98 ± 164.89	823.15 ± 185.10	864.38 ± 128.96	0.625
Retinol (µg/mL)	0.27 ± 0.07	0.28 ± 0.07	0.26 ± 0.05	0.417
α -tocopherol µg/mL)	8.96 ± 2.43	8.95 ± 1.55	9.51 ± 2.31	0.547
γ -tocopherol (µg/mL)	0.51 ± 0.23	0.50 ± 0.19	0.49 ± 0.18	0.964
β-carotene (µg/mL)	0.35 ± 0.25	0.46 ± 0.33	0.40 ± 0.26	0.319
coQ10 (µg/mL)	0.47 ± 0.16	0.51 ± 0.17	0.54 ± 0.19	0.402
GPX (mU/mL)	131.88 ± 54.13	167.26 ± 82.64	171.88 ± 92.38	0.104
CAT (U/mL)	173.61 ± 87.10	173.36 ± 85.85	170.94 ± 81.41	0.992
SOD (U/mL)	56.71 ± 8.34	53.01 ± 8.80	56.15 ± 9.87	0.233
GPx gene (normalised value)	1.13 ± 0.62	1.20 ± 0.55	1.37 ± 1.39	0.628
CAT gene (normalised value)	1.16 ± 0.47	1.14 ± 0.58	1.03 ± 0.46	0.645
SOD gene (normalised value)	1.32 ± 0.78	1.22 ± 0.87	1.32 ± 0.79	0.861
XO gene (normalised value)	1.94 ± 2.44	1.43 ± 1.38	2.07 ± 3.78	0.624
ApoJ gene (normalised value)	1.41 ± 0.98	1.38 ± 1.24	1.38 ± 0.98	0.994
Zinc (µg/dL)	117.84 ± 27.01	112.22 ± 4.66	126.10 ± 44.14	0.301
MDA (µM)	5.46 ± 2.76	5.11 ± 2.33	3.92 ± 1.95	0.054
8-OHdG (ng/mg creatinine)	7.63 ± 3.20	9.27 ± 4.46	7.96 ± 3.44	0.208
IL-1β (pg/mL)	0.04 ± 0.02	0.06 ± 0.09	0.04 ± 0.04	0.646

Table 4.17: Baseline concentration for each biomarker (mean ± SD) per treatment group (continued).

	Placebo	PBE	MPH	P-value
IL-4 (pg/mL)	0.02 ± 0.01	0.02 ± 0.03	0.02 ± 0.01	0.790
IL-5 (pg/mL)	0.42 ± 0.42	0.38 ± 0.69	0.39 ± 0.50	0.972
IL-6 (pg/ml)	0.38 ± 0.43	0.34 ± 0.27	0.31 ± 0.28	0.729
IL-8 (pg/mL)	2.90 ± 1.06	2.87 ± 1.18	2.69 ± 1.37	0.794
IL-10 (pg/mL)	0.33 ± 0.27	0.26 ± 0.13	0,49 ± 1.04	0.336
IL-12p70 (pg/mL)	0.12 ± 0.10	0.18 ± 0.37	0.17 ± 0.17	0.683
TNF-α (pg/mL)	1.07 ± 0.34	0.91 ± 0.25	1.06 ± 0.55	0.226
IFN-γ (pg/mL)	8.74 ± 18.59	5.27 ± 5.83	11.26 ± 25.44	0.445
IgA (g/L)	1.21 ± 0.53	1.00 ± 0.37	0.91 ± 0.48	0.058
IgE (kU/L)	415.68 ± 730.53	243.66 ± 417.92	244.61 ± 489.35	0.420
IgG1 (g/L)	4.94 ± 1.15	4.91 ± 0.87	4.65 ± 1.47	0.622
IgG_2 (g/L)	1.67 ± 0.54	1.62 ± 0.71	1.55 ± 0.51	0.765
IgG_3 (g/L)	0.56 ± 0.25	0.58 ± 0.19	0.56 ± 0.26	0.940
IgG_4 (g/L)	0.36 ± 0.27	0.35 ± 0.31	0.38 ± 0.25	0.949
NPY (pg/mL)	10.03 ± 6.86	9.78 ± 7.67	11.07 ± 7.26	0.792

GSH: glutathione; coQ10: co-enzyme Q10; GPX: glutathione peroxidase; CAT: catalase; SOD: superoxide dismutase; MDA: malondialdehyde; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; IL-1β: interleukin-1 beta; IL-4: interleukin-4; IL-5: interleukin-5; IL-6: interleukin-6; IL-8: interleukin-8; IL-10: interleukin-10; IL-12p70: interleukin-12; NPY: Neuropeptide Y; TNF-α: tumor necrosis factor- alpha; IFN-γ: interferon gamma; IgA: immunoglobuline A; IgE: immunoglobuline E; IgG_1: immunoglobuline G_1; IgG_2: immunoglobuline G_2; IgG_3: immunoglobuline G_3; IgG_4: immunoglobuline G_4; MPH: methylphenidate hydrochloride; NPY: Neuropeptide Y; PBE: French Maritime Pine Bark Extract. GSH, lipid soluble antioxidants, GPX and CAT analyses were performed on respectively n=30, n=32 and n=25; SOD analysis was performed on n=30, n=31 and n=24. Gene expression analysis was carried out on n=28, n=31 and n=25. Zinc analysis was performed on n=29, n=29 and n=24. MDA concentrations were measured of n=28, n=33 and n=25. 8-OHdG analysis was carried out on n=29, n=32 and n=24. Cytokines levels were analysed in n=28, n=32 and n=25. Antibodies were measured in n=29, n=31 and n=25.

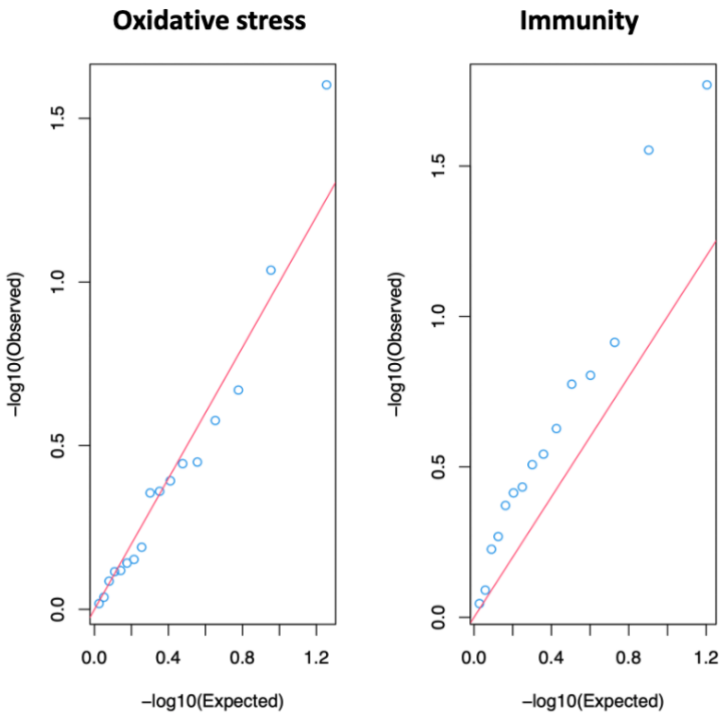

Figure 4.11*:* QQ-plots of the oxidative stress pathway and the immunity pathway. Diagonal red line: expected distribution under the null hypothesis; open circles: observed p-values. Deviations from the distribution under the null hypothesis indicates an enrichment in low p-values.

4.8.3.2 Oxidative stress pathway: antioxidants

Regarding effects on GSH levels, no significant differences in mean GSH concentrations between the treatments were found. Also no significant differences in mean plasma lipid soluble antioxidant concentrations (retinol, α- and γ-tocopherol, β-carotene and coQ10) were found between the three groups after 10 weeks (Table 4.18). LMM analyses of the measured plasma antioxidant enzymes did show a nominally significant difference in CAT activity between the groups after 10 weeks (p= 0.025) (Table 4.18). However, since multiple hypotheses are tested within one particular pathway, a multiple testing correction needs to be performed. The calculated q-value (q= 0.425) now indicates that this association is most likely to be false positive, 42.5% of this significance will be a false positive result (Table 4.19). This result is consistent with the qqplot (Figure 4.12), which

showed that the observed p-values are following the expected distribution under the null hypothesis of no association. Gene expression of the different target genes (GPX, CAT, SOD, XO and ApoJ) did not reveal any significant differences between the three groups after the study period (Table 4.18). Lastly, the LMM analysis did not reveal any significant differences in serum zinc concentrations (Table 4.18). In order to specifically evaluate the effect of MPH on oxidative stress biomarkers, paired-samples t-tests (data not shown) were performed. No significant differences in antioxidant markers could be demonstrated at the end of the 10-week period compared to the start.

4.8.3.3 Oxidative stress pathway: oxidative damage

LMM did not show any significant difference on neither MDA nor on 8-OHdG concentration between the treatment groups after 10 weeks (Table 4.18).

4.8.3.4 Immunity

In most of the analysed plasma samples, IL-1β and IL-4 levels were lower than the limit of detection of the method used. For statistical analysis of these two cytokines, a more suitable regression method, Tobit regression, was used (Twisk & Rijmen 2009). Both Tobit regression as well as the LMM analyses used for the other cytokines failed to show any significant differences in cytokine levels between the groups over time (Table 4.16). LMM analyses did; however, reveal a significant difference in IgA and IgG_2 plasma concentrations between the three treatment arms over time (p= 0.028 and p= 0.017 resp.). FDR analyses of these immunity biomarkers showed a slight enrichment of lower p-values, with the two most significant markers, IgA and IgG_2 having a q-value of 0.098 (Table 4.19). Therefore, IgA and IgG_2 have been shown, with a 90% probability, to significantly differ between the treatment arms. It could thus be assumed that after 10 weeks, increased IgA and IgG_2 concentrations in the MPH group as compared to those in the PBE group could be found. Statistical analyses of the other antibodies (IgG_1, IgG_3, IgG_4 and IgE) did not show any significant differences between the three groups after 10 weeks (Table 4.18). In particular for the MPH group, post-hoc analyses after the paired-samples t-test, to compare baseline to 10 weeks, demonstrated a q-value of 0.023 for TNF-α, IgA and IgG_2 and 0.100 for IL-5. Assuming TNF-α, IgA and IgG_2, and IL-5 are considered significant, respectively only 2% and 10% would represent false positives (data not shown).

4.8.3.5 The orexigenic neuropeptide Y and weight assessment

LMM analyses of the serum NPY levels did not show any significant differences between the treatment groups (Figure 4.12). However, results of the paired-samples t-test did reveal a significant decrease in serum NPY concentrations ($p = 0.021$) after MPH treatment for 10 weeks (Table 4.20). Moreover, a significant weight loss ($p < 0.001$) was observed in the MPH group. No significance in serum NPY concentrations was observed for the participants receiving PBE, however, a significant increase in body weight was established ($p < 0.001$).

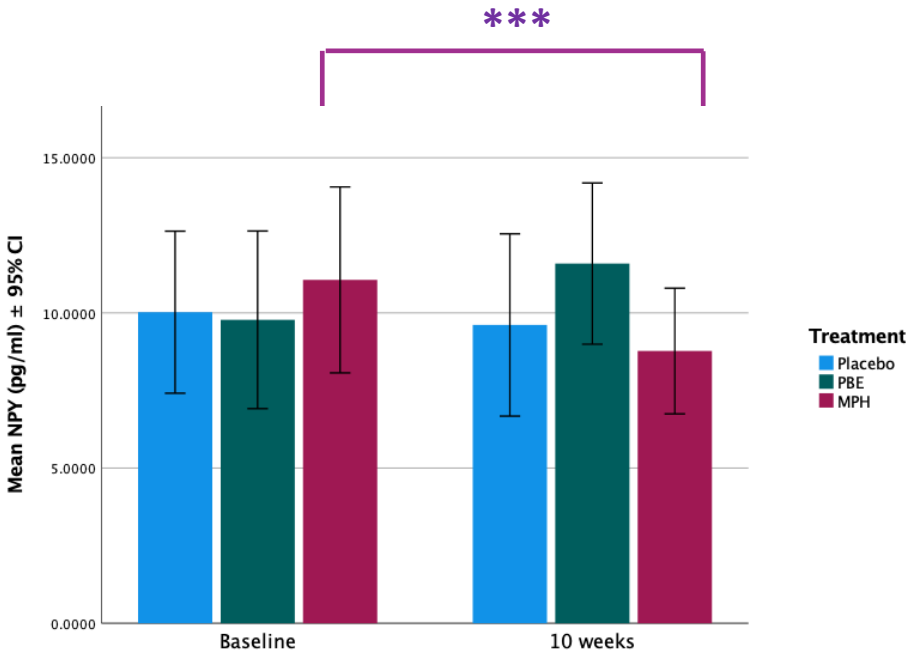

Figure 4.12: Evaluation of serum NPY levels at baseline and 10 weeks. CI: confidence interval; MPH: Methylphenidate hydrochloride; PBE: French Maritime Pine Bark Extract. ***: $p < 0.001$

Table 4.18: Baseline and follow-up concentrations ± SD of evaluated biomarkers per treatment group.

Biomarker	Time point	Placebo	PBE	MPH	Test value	P-value
GSH (µg/mL)	Baseline	849.98 ± 164.89	823.15 ± 185.10	864.38 ± 128.96	F(2, 153)= 0.438	0.646
	10 weeks	862.99 ± 134.12	777.70 ± 175.88	845.64 ± 156.85		
Retinol (µg/mL)	Baseline	0.27 ± 0.07	0.28 ± 0.07	0.26 ±0.05	F(2, 70.160)= 0.041	0.960
	10 weeks	0.27 ± 0.06	0.29 ± 0.07	0.26 ± 0.07		
α -tocopherol (µg/mL)	Baseline	8.96 ± 2.43	8.95 ± 1.55	9.51 ± 2.31	F(2, 68.330)= 0.199	0.820
	10 weeks	8.48 ± 2.09	8.77 ± 1.71	9.68 ± 2.28		
γ -tocopherol (µg/mL)	Baseline	0.51 ± 0.23	0.50 ± 0.19	0.49 ± 0.18	F(2, 73.144)= 0.353	0.704
	10 weeks	0.58 ± 0.25	0.58 ± 0.23	0.63 ± 0.23		
β-carotene (µg/mL)	Baseline	0.35 ± 0.25	0.46 ± 0.33	0.40 ± 0.26	F(2, 69.146)= 2.468	0.092
	10 weeks	0.37 ± 0.34	0.44 ± 0.37	0.30 ± 0.13		
CoQ10 (µg/mL)	Baseline	0.47 ± 0.16	0.51 ± 0.17	0.54 ± 0.19	F(2, 69.051)= 0.086	0.918
	10 weeks	0.43 ± 0.13	0.46 ± 0.14	0.50 ± 0.13		
GPX (mU/mL)	Baseline	131.88 ± 54.13	167.26 ± 82.64	171.88 ± 92.38	F(2, 69.940)= 0.915	0.405
	10 weeks	158.12 ± 47.55	184.79 ± 9.64	195.75 ± 96.12		

Table 4.18: Baseline and follow-up concentrations ± SD of evaluated biomarkers per treatment group (continued).

Biomarker	Time point	Placebo	PBE	MPH	Test value	P-value
CAT (U/mL)	Baseline	173.61 ± 87.10	173.36 ± 85.85	170.94 ± 81.41	F(2, 66.568)= 3.922	0.025*
	10 weeks	186.58 ± 72.80	132.16 ± 59.39	180.48 ± 72.49		
SOD (U/mL)	Baseline	56.71 ± 8.34	53.01 ± 8.80	56.15 ± 9.87	F(2, 68.428)= 1.039	0.359
	10 weeks	57.33 ± 6.93	58.20 ± 8.67	58.85 ± 8.01		
GPX gene (normalised value)	Baseline	1.13 ± 0.62	1.20 ±0.55	1.37 ± 1.39	F(2, 77.623)= 0.266	0.767
	10 weeks	1.09 ± 0.66	1.35 ± 0.47	1.40 ± 0.85		
CAT gene (normalised value)	Baseline	1.16 ± 0.47	1.14 ± 0.58	1.03 ± 0.46	F(2, 74.546)= 0.828	0.441
	10 weeks	1.20 ± 0.40	1.40 ± 0.62	1.33 ± 0.74		
SOD gene (normalised value)	Baseline	1.32 ± 0.78	1.22 ± 0.87	1.32 ± 0.79	F(2, 68.777)= 0.841	0.436
	10 weeks	1.38 ± 0.85	1.12 ± 0.69	1.28 ± 0.81		
XO gene (normalised value)	Baseline	1.94 ± 2.44	1.43 ± 1.38	2.07 ± 3.78	F(2, 39.932)= 1.605	0.214
	10 weeks	1.14 ± 1.06	1.91 ± 1.73	1.37 ± 1.04		
ApoJ (normalised value)	Baseline	1.41 ± 0.98	1.38 ± 1.24	1.38 ± 0.98	F(2, 69.694)= 0.327	0.722
	10 weeks	1.55 ± 1.03	1.34 ± 0.60	1.29 ± 0.62		

Table 4.18: Baseline and follow-up concentrations ± SD of evaluated biomarkers per treatment group (continued).

Biomarker	Time point	Placebo	PBE	MPH	Test value	P-value
Zinc (µg/dL)	Baseline	117.84 ± 27.01	112.22 ± 24.60	126.10 ± 44.14	F(2, 77.541)= 0.274	0.761
	10 weeks	104.00 ± 29.73	113.87 ± 31.69	116.62 ± 30.41		
MDA (µM)	Baseline	5.46 ± 2.76	5.11 ± 2.33	3.92 ± 1.95	F(2, 65.511)= 1.053	0.355
	10 weeks	4.52 ± 2.48	4.95 ± 1.98	3.94 ± 2.16		
8-OHdG (ng/mg creatinine)	Baseline	7.63 ± 3.20	9.27 ± 4.46	7.96 ± 3.44	F(2, 75.755)=1.351	0.265
	10 weeks	9.75 ± 3.51	9.05 ± 2.88	8.48 ± 2.60		
IL-1β (pg/mL)	Baseline	0.04 ± 0.02	0.06 ± 0.09	0.04 ± 0.04	Z=0.600	0.539
	10 weeks	0.05 ± 0.02	0.05 ± 0.04	0.06 ± 0.07		
IL-4 (pg/mL)	Baseline	0.02 ± 0.01	0.02 ± 0.03	0.02 ± 0.01	Z=0.187	0.900
	10 weeks	0.02 ± 0.01	0.02 ± 0.03	0.02 ± 0.01		
IL-5 (pg/mL)	Baseline	0.42 ± 0.42	0.38 ± 0.69	0.39 ± 0.50	F(2, 72.116)= 0.209	0.812
	10 weeks	0.29 ± 0.41	0.31 ± 0.28	0.25 ± 0.28		
IL-6 (pg/mL)	Baseline	0.38 ± 0.43	0.34 ± 0.27	0.31 ± 0.28	F(2, 145)= 1.003	0.369
	10 weeks	0.28 ± 0.14	0.37 ± 0.37	0.35 ± 0.34		

Table 4.18: Baseline and follow-up concentrations ± SD of evaluated biomarkers per treatment group (continued).

Biomarker	Time point	Placebo	PBE	MPH	Test value	P-value
IL-8 (pg/mL)	Baseline	2.90 ± 1.06	2.87 ± 1.18	2.69 ± 1.37	F(2, 77.227)= 0.963	0.386
	10 weeks	3.14 ± 1.21	2.86 ± 1.02	3.21 ± 2.15		
IL-10 (pg/mL)	Baseline	0.33 ± 0.27	0.26 ± 0.13	0.49 ± 1.04	F(2, 81.891)= 1.893	0.157
	10 weeks	0.55 ± 0.58	0.36 ± 0.50	0.30 ± 0.18		
IL-12p70 (pg/mL)	Baseline	0.12 ± 0.10	0.18 ± 0.37	0.17 ± 0.17	F(2, 60.998)= 0.525	0.594
	10 weeks	0.12 ± 0.11	0.19 ± 0.37	0.14 ± 0.14		
TNF-α (pg/mL)	Baseline	1.07 ± 0.34	0.91 ± 0.25	1.06 ± 0.55	F(2, 74.046)= 2.163	0.122
	10 weeks	0.91 ± 0.22	0.88 ± 0.23	0.87 ± 0.28		
IFN-γ (pg/mL)	Baseline	8.74 ± 18.59	5.27 ± 5.83	11.26 ± 25.44	F(2, 146)= 1.458	0.236
	10 weeks	7.09 ± 5.73	8.48 ± 16.95	3.97 ± 2.55		
IgA (g/L)	Baseline	1.21 ± 0.53	1.00 ± 0.37	0.91 ± 0.48	F(2, 67.847)= 3.781	0.028*
	10 weeks	1.24 ± 0.55	1.00 ± 0.37	0.99 ± 0.53		
IgE (kU/L)	Baseline	415.68 ± 730.53	243.66 ± 417.92	244.61 ± 489.35	F(2, 66.179)= 0.868	0.425
	10 weeks	441.04 ± 892.65	289.23 ± 676.60	227.91 ± 430.15		

Table 4.18: Baseline and follow-up concentrations ± SD of evaluated biomarkers per treatment group (continued).

Biomarker	Time point	Placebo	PBE	MPH	Test value	P-value
IgG_1 (g/L)	Baseline	4.94 ± 1.15	4.91 ± 0.87	4.65 ± 1.47	F(2, 68.689)= 1.833	0.168
	10 weeks	5.06 ± 1.27	4.75 ± 0.82	4.76 ± 1.39		
IgG_2 (g/L)	Baseline	1.67 ± 0.54	1.62 ± 0.71	1.55 ± 0.51	F(2, 67.492)= 4.302	0.017*
	10 weeks	1.74 ± 0.51	1.62 ± 0.69	1.64 ± 0.59		
IgG_3 (g/L)	Baseline	0.56 ± 0.25	0.58 ± 0.19	0.56 ± 0.26	F(2, 67.921)= 1.189	0.311
	10 weeks	0.59 ± 0.25	0.58 ± 0.22	0.58 ± 0.30		
IgG_4 (g/L)	Baseline	0.36 ± 0.27	0.35 ± 0.31	0.38 ± 0.25	F(2, 67.026)= 1.272	0.287
	10 weeks	0.33 ± 0.27	0.29 ± 0.22	0.37 ± 0.26		
NPY (pg/mL)	Baseline	10.03 ± 6.86	9.78 ± 7.67	11.07 ± 7.26	F(2, 73.690)= 1.304	0.278
	10 weeks	9.61 ± 6.79	11.59 ± 6.29	8.77 ± 4.80		

Test value and p-value of the interaction between time and treatment between all treatment groups, 10 weeks versus baseline with sex, processing time and time until analysis as covariates (LMM and Tobit regression for respectively IL-1β and IL-4), *: p-value < 0.05 if interaction term is significant. GSH: glutathione; coQ10: co-enzyme Q10; GPX: glutathione peroxidase; CAT: catalase; SOD: superoxide dismutase; MDA: malondialdehyde; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; IL-1β: interleukin-1 beta; IL-4: interleukin-4; IL-5: interleukin-5; IL-6: interleukin-6; IL-8: interleukin-8; IL-10: interleukin-10; IL-12p70: interleukin-12; TNF-α: tumor necrosis factor- alpha; IFN-γ: interferon gamma; IgA: immunoglobuline A; IgE: immunoglobuline E; IgG_1: immunoglobuline G_1; IgG_2: immunoglobuline G_2; IgG_3: immunoglobuline G_3; IgG_4: immunoglobuline G_4; MPH: methylphenidate hydrochloride; NPY: Neuropeptide Y; PBE: French Maritime Pine Bark Extract. Follow-up analyses of GSH, lipid soluble antioxidants, GPX and CAT were performed on respectively n=23, n=27 and n=23. SOD activity levels were measured in n=23, n=26 and n=22. Gene expression analysis was performed on n=22, n=26 and n=23. Zinc concentrations were measured in n=23, n=27 and n=24 respectively. MDA concentrations were analysed in n=21, n=27 and n=22. 8-OHdG analysis was carried out on n=22, n=27 and n=22. Cytokines analyses were performed on n=21, n=27 and n=23. Antibodies were measured in n=23, n=27 and n=24.

Table 4.19: Q-values as calculated by the fdr tool package for each biomarker of the oxidative stress and immunological pathway.

	Biomarker	P-value	Q-value
Oxidative stress pathway	**CAT (U/mL)**	0.025	0.425
	β-carotene (μg/mL)	0.092	0.782
	XO gene (normalised value)	0.214	0.833
	8-OHdG (ng/mg creatinine)	0.265	0.833
	MDA (μM)	0.355	0.833
	SOD (U/mL)	0.359	0.833
	GPX (U/mL)	0.405	0.833
	SOD gene (normalised value)	0.436	0.833
	CAT gene (normalised value)	0.441	0.833
	GSH (μg/mL)	0.646	0.929
	γ -tocopherol (μg/mL)	0.704	0.929
	ApoJ gene (normalised value)	0.722	0.929
	Zinc (μg/dL)	0.761	0.929
	GPX (normalised value)	0.767	0.929
	α -tocopherol (μg/mL)	0.820	0.929
	CoQ10 (μg/mL)	0.918	0.960
	Retinol (μg/mL)	0.960	0.960
Immunity pathway	**IgG_2 (g/L)**	0.017	0.098
	IgA (g/L)	0.028	0.098
	TNF-α (pg/mL)	0.122	0.208
	IL-10 (pg/mL)	0.157	0.224
	IgG_1 (g/L)	0.168	0.228
	IFN-γ (pg/mL)	0.236	0.247
	IgG_4 (g/L)	0.287	0.257

Table 4.19: Q-values as calculated by the fdr tool package for each biomarker of the oxidative stress and immunological pathway (continued).

Immunity pathway	Biomarker	P-value	Q-value
	IgG_3 (g/L)	0.311	0.260
	IL-6 (pg/mL)	0.369	0.267
	IL-8 (pg/mL)	0.286	0.269
	IgE (kU/L)	0.425	0.272
	IL-1β (pg/mL)	0.539	0.321
	IL-12p70 (pg/mL)	0.594	0.343
	IL-5 (pg/mL)	0.812	0.416
	IL-4 (pg/mL)	0.900	0.442

The observed p-values of the biomarkers linked to the oxidative stress and the immune pathway generated with the LMMs are sorted by significance. The q-value then indicated the fraction of false associations, in case that p-value is declared significant. GSH: glutathione; coQ10: co-enzyme Q10; GPX: glutathione peroxidase; CAT: catalase; SOD: superoxide dismutase; MDA: malondialdehyde; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; IL-1β: interleukin-1 beta; IL-4: interleukin-4; IL-5: interleukin-5; IL-6: interleukin-6; IL-8: interleukin-8; IL-10: interleukin-10; IL-12p70: interleukin-12; TNF-α: tumor necrosis factor- alpha; IFN-γ: interferon gamma; IgA: immunoglobuline A; IgE: immunoglobuline E; IgG_1: immunoglobuline G_1; IgG_2: immunoglobuline G_2; IgG_3: immunoglobuline G_3; IgG_4: immunoglobuline G_4.

Table 4.20. Baseline and follow-up concentrations ± SD of the neurochemical parameter NPY, height and weight for the active treatment groups.

Biomarker	Time point	PBE	Test value[a]	P-value[a]	MPH	Test value[b]	P-value[b]
NPY (pg/mL)	Baseline	10.65 ± 7.75	t(23)= -0.646	0.525	11.07 ± 7.26	t(23)= 2.471	0.021*
	10 weeks	11.80 ± 6.34			8.77 ± 4.80		
Height (m)	Baseline	1.42 ± 0.11	t(25)= -5.305	<0.001***	1.39 ± 0.10	t(23)= -6.699	<0.001***
	10 weeks	1.44 ± 0.11			1.41 ± 0.10		
Weight (kg)	Baseline	35.3 ± 10.02	t(26)= -3.007	0.006**	34.47 ± 9.34		
	10 weeks	36.0 ± 9.97			33.36 ± 9.25	t(22)= 6.743	<0.001***

[a]Test value and p-value of the mean difference for each parameter between the start and the end of the study for the PBE treatment group. [b]Test value and p-value of the mean difference for each biomarker between the start and the end of the study for the MPH treatment group *: if p-value < 0.05 ; ** p-value <0.01 and if interaction term is significant. [b]Test value and p-value of post-hoc pairwise comparisons with Bonferroni correction. ***: p-value <0.001; NPY: neuropeptide Y; MPH: methylphenidate hydrochloride; PBE: French Maritime Pine Bark Extract

4.9 Discussion

Most research on nutritional supplements or medication in ADHD primarily assesses effects on behaviour (Konofal et al., 2008; Rucklidge et al., 2018). This trial; however, also takes into account a broad range of immune and oxidative biomarkers, as well as the neurochemical biomarker NPY and evaluates the potential of a procyanidin-rich extract in ADHD as compared to MPH and placebo. To our knowledge, this is also the first study investigating the possible effects of MPH, considered one of the first-line treatment options in ADHD, on numerous peripheral immune and oxidative stress related biomarkers. Although 48 patients per treatment were to be included based on power calculation (n = 144 in total), the trial was ended with 88 participants due to expiry of study capsules in combination with poor inclusion during the covid-19 pandemic. Despite the fact that the dropout ratio was lower than expected (14% vs. 20%), power was too low to complete subgroup analyses. Specific differences between treatments (e.g. PBE and MPH) could therefore potentially remain unexplored.

Based upon teacher-rated ADHD-RS, the primary outcome, MPH treatment caused, as expected, significant improvement (total and inattention score, not hyperactivity/impulsivity) as compared to placebo after five weeks (Schachter et al., 2001). After 10 weeks, both PBE and MPH significantly improved the total and hyperactivity/impulsivity score, while MPH also improved inattention. PBE thus had a slower effect than MPH. This was also expected, since nutritional supplements often require weeks to months to exert an effect due to their mechanism of action, while MPH's effects can be expected promptly (Kimko et al., 1999). Earlier research for instance demonstrated that taking PBE at least five weeks before the start of the allergy season reduced allergic symptoms. It is likely that the immune modulating effects of PBE may require sufficient time to manifest noticeable symptom reduction (Wilson et al., 2010). Nevertheless, though statistical noninferiority analysis was inconclusive, both treatments were evenly matched after 10 weeks except for their effect on inattention. Improvement of ADHD behaviour at school is a desirable treatment outcome with impact on school performance, relationships and self-esteem. Since the FFQ indicates comparable baseline dietary habits (and thus polyphenol intake) between treatment groups, effects in the PBE group can be ascribed to PBE's polyphenol content.

Based on parent ratings, significant improvement on all ADHD-RS (sub)scores was found only for MPH after 10 weeks. The difference in effects between teacher and parent ratings is striking and appears to emphasize the higher sensitivity of teacher ratings (Verlaet et al., 2017). Possibly, parenting stress and exceptional focus on one child (evidenced by slightly higher baseline scores), especially during a trial focused on ADHD behaviour, affect parents' perceptions and reduce their ability to notice improvements. This might specifically be true for PBE, as its effects are expected to appear very subtle after several weeks, gradually increasing over the course of another number of weeks – as opposed to MPH's effects. This underlines the importance of parental/family psychological support and education for ADHD therapy to reach its full potential (Heath et al., 2015). Teachers might be more objective and sensitive to behavioural improvements since they are less emotionally involved, less focused on one child and can compare between children in the classroom. Nevertheless, MPH extended-release formulation is effective for about eight hours, while PBE's effect is not expected to wear off suddenly. Since study treatments were taken at breakfast, teachers, as opposed to parents, might not have noticed potential 'rebound' effects of MPH.

SEQ ratings largely confirm ADHD-RS results, thus consolidating our findings. Differences between ADHD-RS and SEQ results could be attributable to different phrasing, divergent differentiation into sub scores and the 4- vs 5-point rating scales. Moreover, our results confirm successful treatment of paediatric ADHD with PBE in an earlier controlled trial, in which improvement was also evidenced by teacher but not parent ratings (Trebatická et al., 2006).

Both PBE and MPH did not significantly affect co-occurring psychiatric and physical complaints. However, as the study population was not specifically chosen based on these conditions, potential for improvement by PBE might have been limited.

MPH frequently causes adverse effects. It is therefore important to consider both behavioural improvement and adverse effects when assessing PBE's value in ADHD therapy. Up to five times more adverse effects were reported for MPH than for PBE. Reported side effects were generally comparable to those in literature (Storebø et al., 2015; Trebatická et al., 2006). Moreover, though not statistically (but possibly clinically)

significant, a slightly increased average heart rate (but not blood pressure) was observed for those treated with MPH at the end of the trial.

Treatment adherence and proportion of dropouts can be considered indicators of treatment effectiveness, based on achievement of positive effects and absence of adverse effects. Both were not significantly different between treatments. The overall acceptability of PBE, based on adverse effects, compliance and dropouts, therefore seems at least not inferior to MPH.

Looking at the biological markers in this study, no clear evidence for an increase in antioxidant levels, reduced oxidative damage or improvement of immune status could be found after treatment with PBE as compared to the other treatment arms. In fact, in the oxidative stress pathway LMM only revealed a nominally significant reduced CAT activity after treatment with PBE as compared to placebo. This was unexpected, as multiple animal studies report upregulation of CAT activity after PBE supplementation. Nevertheless, these studies typically used high PBE concentrations, up to 100 mg/kg, as opposed to 1 mg/kg in the present trial, while the level of oxidative stress (and thus potential for improvement) in these animal models (e.g. type-2 diabetic rats) was possibly higher than in the ADHD patients participating in the present trial (Atta et al., 2020; Goel & Saxena, 2019; Lee et al., 2012; Parveen et al., 2010, 2013; Xiao et al., 2017). Moreover, lower antioxidant enzyme levels do not necessarily imply more oxidative stress. In fact, CAT can be upregulated in case of increased oxidative stress (and vice versa) in child and adolescent patients with ADHD (Ceylan et al., 2010). This has also been observed in *in vivo* models, where following administration of alcohol to rats inducing lipid peroxidation, plasma catalase levels increased. This increase could be prevented with a natural antioxidant like pineapple peel extract (Okafor et al. 2011). An observed reduction in CAT activity by PBE treatment might therefore be explained by PBE's antioxidant effects. However, in this case, this association needs to be interpreted with caution as it is possibly a false positive result demonstrated by its respective q-value (0.425). There is therefore about 43% chance of getting a false positive result whereas the probability of obtaining a true significant result is only 57%. A firm conclusion on lowered CAT activity after PBE treatment is therefore not possible.

Similarly, a slightly lower mean GSH concentration after PBE treatment can be noticed. Though this effect is not significant, this could be in line with polyphenols activating nuclear factor erythroid 2–related factor 2 (Nfr2) pathway as described before, increasing the expression of cytoprotective genes coding for i.e. glutathione-S-transferase (GST) (Ma, 2013; Martínez-Huélamo et al., 2017). Higher GST activity could lead to lower GSH concentrations by the conjugation of GSH to a wide variety of endogenous and exogenous electrophilic compounds.

Although increased lipid peroxidation has been reported for ADHD patients (Bulut et al., 2013), this could not be confirmed in this study. Indeed, no significant change in MDA levels, a lipid peroxidation biomarker could be demonstrated between the groups. Therefore, no evidence was provided that increased lipid peroxidation in patients can be improved upon treatment with PBE or with MPH (Ceylan et al., 2010; Oztop et al., 2012). Although no significant differences in 8-OHdG concentration could be observed, in the placebo group a slightly increased (although not significantly) 8-OHdG concentration could be noted, which might suggest that untreated ADHD could lead to more oxidative DNA damage. Still, a wide variety of oxidative damage biomarkers exist, of which only two were analysed in the present study. Analysis of additional markers should thus be performed in order to draw firm conclusions. Nevertheless, reasons for discrepancies between our findings and those of previous research in paediatric ADHD are unclear (Trebatická et al., 2006).

Though no strong associations were found for the oxidative stress pathway, our results suggest that the p-values observed for the immunity biomarkers are more significant than could be expected in case none of the markers would differ between the active treatment groups. The concentrations of the two most significant biomarkers, IgA and IgG_2, were found to be increased in the MPH group as compared to the PBE group after 10 weeks. Although MPH potentially increases neuroinflammation whereas PBE is known for its anti-inflammatory effects, IgA and IgG_2 are no direct markers of a neuroinflammatory status which is therefore not a plausible explanation for the increased concentrations. However, increased IgE levels under treatment with MPH were demonstrated before as well, but were not found in the present study (Auci et al.,1997).

An important limitation lies in the fact that only immune parameters were assessed in plasma whereas the relevance of the immune dysfunction and the putative immunomodulatory activities of MPH and PBE could be restricted to local tissues. Nevertheless, MPH medication does not appear to affect mucosal immunity, while decreased systemic immunity, associated with increased risk on certain infections might be restored in ADHD patients (Oliva et al. 2020). Over the past years several studies demonstrated the role of cytokines in tryptophan and dopaminergic pathways in the brain, which are implicated in ADHD (Anand et al., 2017).

Hence alterations in pro-inflammatory and anti-inflammatory cytokines might influence ADHD pathogenesis. The LMM analysis however did not reveal any significant differences in effects on plasma cytokine levels between the groups. It is important to note that this study only includes ADHD patients. Differences in cytokine levels in literature are often demonstrated between ADHD and healthy controls (Donfrancesco et al., 2020). Since in our study however, no differences in baseline cytokine levels between the groups could be demonstrated, possible small modulations could remain undetected. Also, the use of medication was an exclusion criterion of this study. Participants with intense immunological symptoms were therefore likely to be excluded. Moreover, cytokine concentrations are generally low unless in case of an active inflammation in which they are locally and transiently produced as a response to stimuli, thereby modulating the functioning of individual cells (Foster, 2001). Effects might thus have been too subtle to be detected in this study due to limited power.

In a case-control study by Verlaet et al. (Verlaet et al., 2019) in which oxidative stress and immune biomarkers between unmedicated paediatric ADHD patients and healthy controls were compared, only a significant difference in GSH levels was observed in the unmedicated paediatric ADHD group as compared to the control group. Moreover, no significance was observed for the other biomarkers, which may indicate the potential involvement of only marginal oxidative stress and immune disturbances in ADHD and might therefore be a possible explanation for our findings.

Furthermore, over the last years interest in preclinical studies investigating the role of MPH on neurological functioning is growing. Evidence from these studies suggest that (mis)use of MPH is associated with redox and energy metabolism changes in the CNS

(Foschiera et al., 2022). According to a study of Gomes et. al it was observed that MPH alters SOD activity in different brain structures like the cerebellum, prefrontal cortex in an adult rat model emphasizing that the effect of MPH on redox homeostasis is dependent on specific brain regions (Comim et al., 2014). Moreover, in an animal model of ADHD it was demonstrated that MPH causes lipid peroxidation in the brain and altered antioxidant enzymatic activities, hereby confirming that MPH can trigger oxidative stress even in an ADHD model (Comim et al., 2014; Foschiera et al., 2022). In addition to alternating activities of antioxidant enzymes in the brain, another animal study demonstrated that intraperitoneal administration of MPH (20 mg/kg) resulted in reduced levels of GSH in isolated hippocampal mitochondria (Foschiera et al., 2022; Motaghinejad et al., 2016).

Studies also pointed out that chronic treatment with MPH dose-dependently increase lipid peroxidation in the brain reflected by increasing MDA levels (Martins et al. 2006). Among the neurotoxic aspects induced by MPH, it is also necessary to highlight its inflammatory potential (Foschiera et al., 2022). A study by Schmitz et al. found that young animals after chronic treatment with MPH (2mg/kg) have an increased production of cytokines TNF-α and IL-6 (Foschiera et al., 2022) while another one demonstrated that treatment with MPH at a dose of 20 mg/kg increased the levels of pro-inflammatory cytokines TNF-α and IL-1β in the cerebellum (Raoofi et al., 2020). Unlike MPH, evidence from *in vitro*, animal and human studies suggests that PBE has neuroprotective effects and even enhances mental performance (Verlaet et al., 2019). For instance, research work by Kim et al. (Kim et al., 2020) demonstrated that PBE supplementation exerts a neuroprotective role against ischemic stroke in a gerbil model.

Despite a few studies investigating the effects of MPH on (anti)oxidative and immunological biomarkers in animal or laboratory studies, possible consequences of long-term exposure to MPH in humans are not yet known. A study by Guney et al. (Guney et al., 2015) explores the possible effects of a 12-week MPH treatment on oxidative metabolism in children and adolescents with ADHD. Pre-treatment oxidative stress index (OSI) values and plasma total oxidative status (TOS) were statistically higher than those of healthy controls suggesting an oxidative imbalance in ADHD. Moreover, plasma levels of antioxidant enzymes (paraoxonase (PON), stimulated paraoxonase (SPON), arylesterase (ARES)) and total antioxidative status (TAS) increased after 12-week

treatment with MPH whereas post-treatment OSI was significantly lower than the pre-treatment value. It could thus be demonstrated that MPH reduces oxidative stress by way of increasing the plasma antioxidant defence mechanisms in children and adolescents with ADHD. Nevertheless, there are many antioxidant enzymes in plasma whereas TAS only measures the cumulative effect of antioxidative molecules in a plasma sample (Guney et al., 2015). In our recent study however, various markers of oxidative stress status were investigated but none was reported to be significant.

From the cytokines and antibodies studied, TNF- α and IL-5 concentrations decreased while IgA and IgG_2 levels increased after 10-week MPH treatment. In the past years, some evidence has indicated that high-dose administration of MPH could play an active role in the increase of neuroinflammation factors and cytokines like IL-6, IL-1β, TNF-α in different brain regions (Raoofi et al., 2020). On the other hand, previous research work by Chuang et al. (Chuang et al., 2019) demonstrated that there were no significant differences in a series of serum cytokine levels between pre- and post-treatment with MPH in male ADHD patients aged between 6 and 12 years. To the best of our knowledge, the presented study is the first investigating the possible effects of MPH, considered one of the first-line treatment options in ADHD, on numerous peripheral oxidative stress and immunological biomarkers.

NPY is found to be one of the most appetite-stimulating neuropeptides in a large number of species and thus involved in the control of food intake (Kalra et al., 1991; Kuo et al., 2001). In the present study we could demonstrate a significant decrease in serum NPY levels for the MPH group, which can imply a loss of appetite at the end as compared to the start of the trial. Moreover, in the MPH group, a significant weight loss was also observed ($p < 0.001$) after 10 weeks treatment. This is in line with earlier research work demonstrating the effect of a two-month treatment with MPH on poor appetite and weight loss by investigating several biomolecules like ghrelin and adiponectin (Sahin et al., 2014). As far as we know, this study is the first to report the effect of MPH treatment on NPY. In addition, unlike the MPH group, patients receiving PBE underwent a moderate weight gain though no significant differences in serum NPY levels could be observed. Participants in both active treatment groups significantly increased in height, reflecting a normal growth curve for all patients, attributing the weight loss seen in the MPH group to a loss of appetite. Interestingly, this could be linked to the side effects reported for MPH.

A strength of the current trial is the active control arm MPH. In one previous trial in adult ADHD, neither MPH nor PBE outperformed placebo, possibly due to the 3-week treatment period (Tenenbaum et al., 2002). Moreover, our trial takes into account co-morbid symptoms and adverse effects, which influence the choice of therapy as well. Another strength are stricter significance limits for secondary outcomes and Bonferroni correction for post-hoc testing, which control type 1 error. Finally, LMM is an added value in case of incomplete observations (e.g., dropouts, missing questionnaires) compared to ANOVA, which is a complete case analysis. Also, validity of the proportion of responders can be questioned as this is also a complete case analysis.

A limitation of the current trial is inclusion of only 88 patients as opposed to 144 based on power calculation. Though the dropout ratio was lower than predicted and specific differences between treatments might remain undetected, striking significant differences were still found despite this reduced power.

As observed in many trials, selection bias should also be considered. It is for instance unlikely that those experiencing a high symptom burden would 'risk' a 10-week placebo treatment. Moreover, despite a solid ADHD diagnosis, very low ADHD-RS scores were reported for several participants. This underscores the subjectivity of questionnaires and leaves little opportunity for improvement. The 10-week study duration is another limitation but was chosen to limit patient burden. Moreover, compliance analysis was based on medication counts, the validity of which could be questioned, especially as this was possible for only ± 75% of the participants. Nevertheless, compliance control by blood analyses would increase participants' burden and is expensive.

In conclusion, in paediatric ADHD and especially in the primary school environment, PBE was proven to be a good alternative for MPH for those willing to wait a few weeks for effects, a fortiori when considering its almost complete lack of adverse effects as opposed to MPH. Its absence of significant behavioural effects reported by parents might be attributed to parenting stress and lower sensitivity of parents' ratings. Moreover, this trial confirmed the impact of psychostimulants such as MPH on appetite and weight. However, it is not possible to conclude that PBE increased antioxidant levels, reduced oxidative damage or improved immune status in general as compared to placebo or MPH. Possible biochemical effects on the oxidative or immunological

pathways resulting from PBE or MPH therapy are not clear yet and need to be further elucidated. For this reason, insights regarding ADHD pathophysiology and possible treatment options, especially for young children, must be further explored. Results of this study strengthen the evidence underlying 'natural' treatment options, which is highly desired by medical staff, patients and parents. These results should be confirmed by future research on long-term effects, effects on specific subgroups (e.g., dietary habits, ADHD subtype/severity) and dose ranging is indispensable to fully understand PBE's therapeutic potential.

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Supplementary material

Protocol isolation PBMC

The whole procedure should be performed in a flow cabinet!
Keep the blood-tubes always on RT, never on ice! (kick monocytes)
Maximal storage 2-3 days in the dark (12-36h, max 48h), RT. Do NOT shake, only twist (8-10x)!!
Procedure:

1. PBS and FBS: warm up to room temperature (RT)
2. Spray 70% ethanol in LAF
3. Put chloride solution in LAF
4. Leucosep tubes prefilled with separation medium: warm up to room temperature (RT)
5. Prepare PBS 10% FBS (30 mL pp) and prepare FBS 20% DMSO (1 mL pp)
6. Fill tubes carefully with anticoagulated blood (3-8 mL; all blood of 1 participant; ± 6 mL, 2 EDTA 3mL tubes); very gently (especially at beginning)
7. Centrifugate 20min at 1000 g at RT in a swinging bucket rotor, no brake
   Sequence of layers from top to bottom:
   a. Plasma
   b. enriched cell fraction (lymphocytes/monocytes/PBMCs)
   c. separation medium
   d. porous barrier
   e. separation medium
   f. pellet (RBCs and granulocytes (white layer))
8. Collect plasma up to a minimum remnant of 5-10 mm above the interphase
9. Store aliquots in -80 °C (500 µL per epp; 3x).
10. Make a smear of the buffy coat/blood on a microscope slide (for later staining and analysis of the different cells present in the blood under the microscope).
11. Harvest enriched cell fraction (lymphocytes/PBMCs) by means of pipette or by pouring the supernatant above porous barrier into another centrifugation tube. (don't pipette too close to filter!)
12. Wash the enriched cell fraction (lymphocytes/PBMCs) with 10 mL PBS (10% FBS)
13. Resuspend PBMCs by gently pipetting up and down
14. Centrifugate 12 min at 200 g, RT (with brake)
15. Remove supernatant (waste; pipette or decant).
16. Repeat washing & centrifugation twice (steps 8-10)
    (First resuspend PBMCs in volume left after step 10, then pour PBS up to 10 mL)
17. Resuspend PBMCs in small volume left
18. Estimate remaining volume (± 200-400 µL).
19. Add pure FBS up to 1 mL
20. Cool cells down (20min fridge, then 20min ice)
21. Add 1 mL FBS 20% DMSO very gently (pipette tip in solution while "mixing") and resuspend gently.
22. Store per 1000 µL in sterile cryovials, *in duplo* per participant (Overnight at -80°C (Mister Frosty))24.
23. Long-term storage in liquid nitrogen

Chapter 5 **Modulation of the gut microbiome by French Maritime Pine Bark Extract**

Weyns et al., Enhancing pediatric Attention-Deficit Hyperactivity Disorder Treatment: Exploring the Gut Microbiota Effects of French Maritime Pine Bark Extract and Methylphenidate Intervention. Under final review by Frontiers in Microbiology.

5.1 Gut microbiome and neurodevelopment

As described in Chapter 2, a rich and diverse gut microbiome is pivotal since it has profound implications on a wide range of biological functions. It does not only participate in GI functions including absorption and biotransformation of nutrients but also in non-GI functions such as the maturation of the immune and neuroendocrine systems and brain development (Checa-Ros et al., 2021). During foetal development, the initial colonisation of the gut by microbiota coincides with the development of the CNS, in a timely and coordinated manner. Emerging evidence suggests an active involvement of the microbiome and its biotransformation by-products in the regulation of early brain development from the foetal stage until childhood (Dash et al., 2022).

In fact, the gut microbiome appears to play a role in neurodevelopmental processes such as the role of the hypothalamus-pituitary-adrenal (HPA)-axis, the establishment of the BBB, neurogenesis, and maturation of microglia and myelination, which are all crucial in shaping animal behaviour and cognition as shown in Figure 5.1. Firstly, the regulation of the HPA-axis, a major neuroendocrine system forming a complex of direct influences and feedback interactions and thereby controlling reactions to stress and many other processes, is influenced by the presence of microbiota (Vagnerová et al., 2019). For example, in germ-free mice, the HPA-axis response was exaggerated and the sensitivity to negative feedback signals was reduced, whereas administration of *Bifidobacterium infantis* at an early stage reversed this response. Also, in human patients it was observed that the gut microbiome directly influences the production of immune mediators and glucocorticoids, thereby stimulating the HPA-axis (Checa-Ros et al., 2021). Secondly, the BBB which is composed of brain microvascular endothelial cells, pericytes, a continuous basement membrane and the perivascular feet of astrocytes, acts as a protective barrier for brain tissue. On the one hand, it serves as a key regulator for the entry of nutrients required for brain health and on the other hand, it prevents harmful molecules from entering into the brain, thereby maintaining brain homeostasis and providing a suitable environmental for neuronal growth (Tang et al., 2020). Even though the exact mechanisms remain unclear, a growing number of evidence suggests that gut dysbiosis (a broad term indicating the imbalance of gut microbiota associated with an unhealthy

outcome (Martinez et al., 2021)) may negatively impact BBB integrity. Furthermore, myelination in the peripheral and CNS is crucial in the regulation of motor, sensory and cognitive functions and gut microbiota regulate this critical process of myelination by controlling myelination-related gene expression in oligodendrocytes.

Since myelination takes place within a few years after birth, neonatal gut dysbiosis during early colonisation may potentially alter normal myelination by disturbing immune responses and neuronal differentiation (Keogh et al., 2021; Sarubbo et al., 2022). Also, neurogenesis which is defined as the generation of new functional neurons through the differentiation of neural stem and progenitor cells, critical for learning, memory, cognition and stress response, is negatively impacted by a disrupted gut microbiome (Sarubbo et al., 2022).

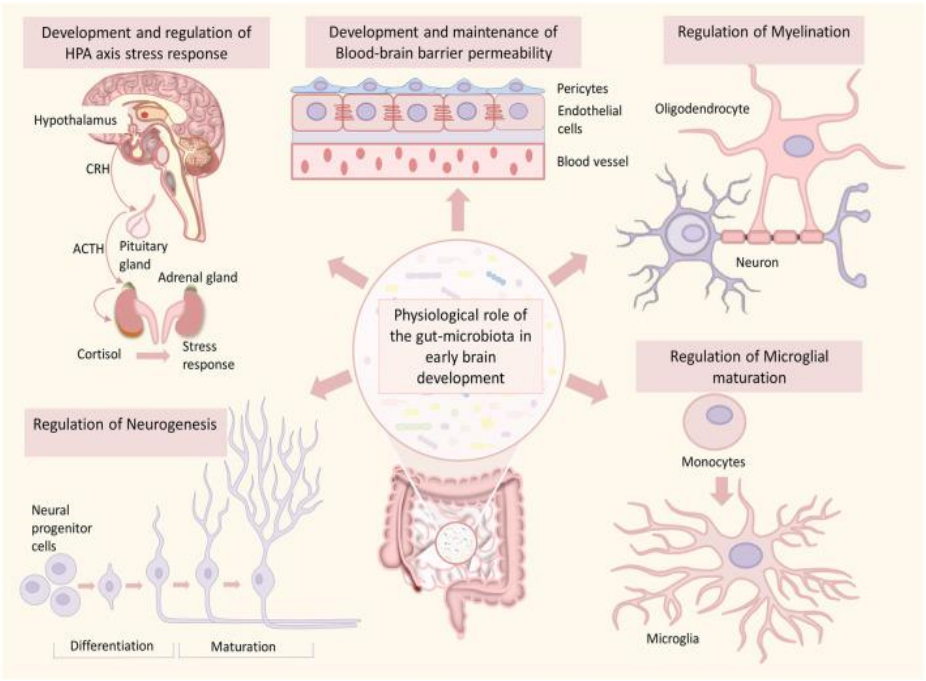

Figure 5.1: The role of the gut microbiota during normal, healthy brain development. The gut microbiome is crucial during various processes of brain development such as neurogenesis, myelination, microglial maturation, development and maintenance of blood-brain barrier integrity, development of HPA-axis and HPA-axis stress response. HPA: hypothalamus-pituitary-adrenal axis. Adapted from Dash et al., 2022.

Alterations in the aforementioned neurodevelopmental processes may thus negatively influence brain functionality, resulting in a variety of neurodevelopmental and neuropsychiatric disorders (Dash et al., 2022). Moreover, an association between gut microbiota and various neuropsychiatric conditions including autism, depression, schizophrenia and ADHD has already been observed (Boonchooduang et al., 2020). The gut microbiota appears to play a significant role herein through a complex bidirectional communication, known as the "microbiota-gut-brain-axis" (MGBA), emphasising the effect of the gut microbiota on the brain (Checa-Ros et al., 2021).

5.2 The role of the gut-brain axis in ADHD

Though ADHD is nowadays considered to be the most common neurodevelopmental disorder affecting children and adolescents, its exact aetiology and curative treatment approaches remain unclear (Boonchooduang et al., 2020; Cenit et al., 2017; Luo et al., 2019). As mentioned in Chapter 3, numerous aetiological factors are believed to be involved in ADHD: alterations in dopaminergic, adrenergic, serotonergic and cholinergic neurotransmitter systems, alterations in brain structure and functional networks, oxidative stress and inflammation as well as diverse environmental factors (Biederman & Spencer, 1999; Ceylan et al., 2012; Checa-Ros et al., 2021; Kawatani et al., 2011; Purper-Ouakil et al., 2011; Sandgren & Brummer, 2018).

Moreover, emerging evidence suggests that deviations from the optimal assembly of the gut microbiota during early life might also be involved in ADHD pathophysiology, thereby highlighting the microbiome-gut-brain bidirectional interplay. Indeed, throughout the years, it has been postulated that the GMBA is impaired in ADHD. This has been hypothesised based on the increasing number of rather small observational studies (n: ≤ 9) (Checa-Ros et al., 2021) showing a link between intestinal function, gut microbiome and the CNS, suggesting that dysbiosis in the gut could be involved in the pathophysiology of ADHD (Wang, 2022). Unfortunately, this impairment of the gut-brain axis has not been well documented yet, with only limited intervention studies associating gut microbiome modulation with clinical benefits in this patient group (Cickovski et al., 2022; Kalenik et al., 2021). However, still no clear outcome on which specific bacterial species are implicated could be obtained (Cickovski et al., 2022; Kalenik et al., 2021). Nevertheless, following mechanisms are assumed to be involved: (i) the

metabolic pathway, (ii) the vagus nerve pathway and (iii) immune pathways as illustrated in Figure 5.2. (Boonchooduang et al., 2020). Regarding the metabolic pathway, it is believed that gut microbiota can produce neurotransmitters involved in ADHD symptoms. For instance, *Bifidobacterium* spp. produce dopamine; serotonin can be produced by *Lactobacillus* spp., *Streptococcus* spp., *Escherichia* spp., *Morganella* spp., *Klebsiella* spp. and *Hafnia* spp. whereas GABA is formed by *Bifidobacterium* spp., *Lactobacillus* spp. and *Escherichia* spp. Though it has not been demonstrated that intestinal neurotransmitters can directly cross the BBB, emerging evidence suggests that gut microbiota influence host biosynthesis and catabolism of neurotransmitters (Strandwitz, 2018). For instance, *Lactobacilli* spp., *Bifidobacterium* spp., *Clostridium sporogenes* and *Clostridium bartetti* all produce tryptophan, a precursor of serotonin, which subsequently can cross the BBB, thereby affecting serotonin synthesis in the brain. These findings are in line with an animal study showing that germ-free mice have low levels of circulating serotonin (Dalile et al., 2019). In addition, gut microbiota produce SCFAs such as acetate, propionate and butyrate through the anaerobic fermentation of dietary fibres and resistant starch, thereby directly or indirectly influencing gut-brain communication and brain function (Dalile et al., 2019). SCFAs can reach the brain directly and act on antigen-presenting cells in order to decrease the inflammatory responses associated with neuroinflammation or they can act locally with intestinal epithelial cells to promote epithelial barrier function and thus gut homeostasis (Dalile et al., 2019). *Bacteroides* spp. and *Clostridiae* spp. are considered pivotal in the production of these SCFAs. Secondly, the vagus nerve pathway, consisting of afferent and efferent fibres connecting the GI tract and the parasympathetic nerve system, is reported to be involved in mood regulation. Moreover, several studies reported a link between altered vagal tone and reactivity of the parasympathetic nervous system and ADHD, implying the potential role of under-arousal of the parasympathetic pathway in ADHD pathophysiology. Lastly, gut microbiota appear to be involved in immunoregulation. Within the immune pathways, gut microbiota are partly responsible for the maturation of immune cells including macrophages, dendritic cells and microglial cells or can reinforce microglial cells by increasing tight junction protection, thereby helping the BBB to enhance brain integrity. Also, in gut dysbiosis a disproportionate concentration of pro-inflammatory cytokines (e.g. IL-13, IL-16) was observed leading to increased intestinal permeability and neuroinflammation, which in turn triggers ADHD symptoms (Boonchooduang et al., 2020).

In conclusion, all the above-mentioned pathways suggest a mechanistic link of the MGBA in ADHD patients. Nevertheless, it is still not fully understood how specific microbiome profiles are associated with this axis and ADHD profiles.

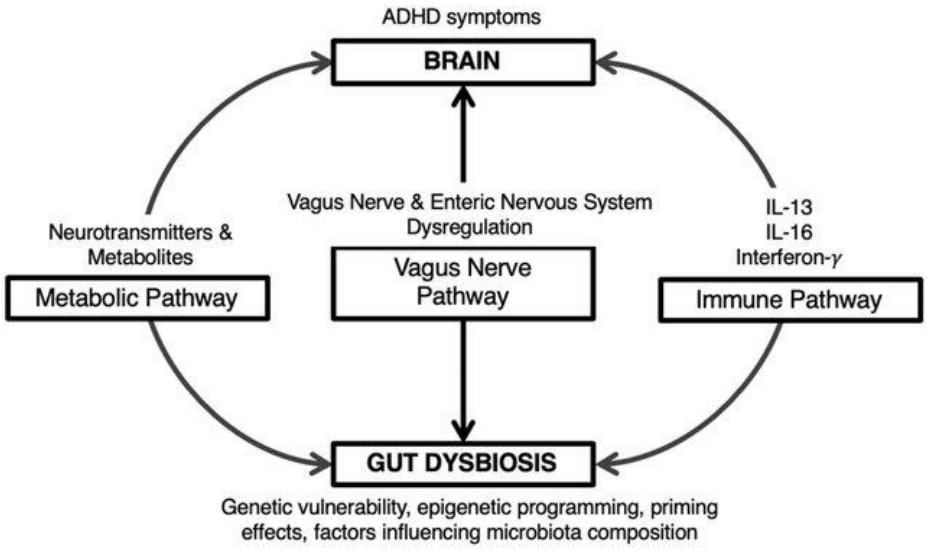

Figure 5.2: Suggested mechanisms contributing to the link between gut microbiota and ADHD: the metabolic pathway, the vagus nerve pathway and immune pathway are considered to attribute to the gut-brain axis. ADHD: Attention-Deficit Hyperactivity Disorder; IL: interleukins. Adapted from Boonchooduang et al., 2020.

Besides interfering in the catecholaminergic neurotransmission system by affecting the metabolic pathways or the expression of genes encoding for these neurotransmitter transporters, gut microbiota can also aggravate neuroinflammation and oxidative stress, both underlying factors for ADHD development, by for instance their impact on the microglia or BBB permeability (Checa-Ros et al., 2021).

5.3 Alterations in gut microbiota with ADHD

Generally, Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria represent the four dominant phyla in children and adolescents with ADHD (Boonchooduang et al., 2020). In most studies no significant differences in phyla levels were observed between

the ADHD and control groups (Jiang et al., 2018; Wang et al., 2020); however, one study found an increase in Actinobacteria and a decrease in Firmicutes in the ADHD group as compared to the non-ADHD control cohort. At family and genus levels, findings were rather inconsistent across earlier studies. According to Aarts et al. (Aarts et al., 2017), the first authors to report the impact of gut microbiota on dopaminergic pathways between young adult ADHD patients and healthy controls, a significant increase in the relative abundance of the genus *Bifidobacterium* was observed in the ADHD group as compared to controls. In turn, this increase correlated with decreased neural reward responses, considered to be one of the hallmarks of ADHD. The researchers found a significant increase in cyclohexadienyl dehydratase (CDT), an enzyme involved in the synthesis of phenylalanine which acts as a precursor of dopamine and noradrenaline (Aarts et al., 2017). Another study by Szopinska-Tokov et al. (Szopinska-Tokov et al., 2020) revealed a significant increase of a genus from the family Ruminococcaceae namely *Ruminococcaceae_UGC_004* as compared to subtreshold ADHD patients (individuals not reaching the criteria for ADHD but scoring too high to be considered as a healthy individual) and healthy controls. *Ruminococcaceae_UGC_004* was found to have sequences in common with microbial species which are able to consume the GABA neurotransmitter. Moreover, they found that the β-diversity was significantly lower among ADHD patients which in turn correlated with reported inattention scores. Prehn-Kirstensen et al. (Prehn-Kristensen et al., 2018) suggested *Neisseria* spp. and *Bacteroides* spp. to be promising ADHD-associated microbial strains. They found significant differences between ADHD patients and healthy controls due to differences in abundances of microbial taxa: at genus level, *Prevotella* and *Parabacteroides* were observed in the control group whereas *Neisseria* was present in the ADHD cohort. Another recent study found an increase in *Bifidobacterium uniformis* and *Bifidobacterium ovatus,* and a decrease in *Bifidobacterium coprocola* in ADHD children, indicating that these bacterial species might be involved in the pathophysiology of ADHD (Wang et al., 2020).

Taken together, these studies support the notion that patients with ADHD have a different microbial composition as compared to healthy controls. These gut microbiota imbalances may cause changes in the gut-brain axis and can thus influence neurotransmitter levels which contribute to the clinical presentation of ADHD (Checa-Ros et al., 2021; Wang et al., 2022.). Nevertheless, results of these studies are too

heterogeneous to draw firm conclusions regarding whether a specific microbial profile is indeed linked with ADHD. In fact, various factors may contribute to possible differences found between these studies such as diverse cultural, geographical and dietary characteristics of the study cohorts, intake of (ADHD) medication,methodological differences linked to sampling and storage or microbiome sequencing methods and the applied bioinformatics pipeline (Checa-Ros et al., 2021).

5.4 ADHD therapy and the gut microbiome

Treatment with MPH is currently the first method of choice in ADHD therapy. However, it causes side effects such as loss of appetite and sleep problems and has a significant personal, social and financial burden for patients, while evidence on long-term efficacy is still lacking (Antshel et al., 2011; Biederman & Faraone, 2005; Pelham et al., 2007; Storebø et al., 2015; Ramstad, et al., 2015). Given the new insights in the pathogenesis of AHDH, targeting the associated immune and oxidant-antioxidant imbalances or gut microbiome, could provide novel therapeutic treatment options.

Supplementation with dietary polyphenols has been linked to various health benefits including antioxidant activity and anti-inflammatory properties as suggested by both *in vitro* and *in vivo* research work (D'Andrea, 2010; Packer et al., 1999; Rohdewald, 2002). Moreover, present data suggest that dietary polyphenols could possibly act on the gut microbiota as prebiotics by specific stimulation of beneficial microbial species (e.g., enhancing the growth of bacterial families such as *Bifidobacteriaceae* and *Lactobacillaceae*) and decreasing the population of more harmful taxa (e.g., *Escherichia coli*, *Clostridium perfringens* and *Helicobacter pylori*) (Mithul Aravind et al., 2021; Singh et al., 2019.; Verlaet et al., 2019). For example, *Lactobacillus spp.* and *Bifidobacteria* spp. are two species known for their positive health effects since they enhance gut barrier function, stimulate the host immune system, prevent diarrhea or allergies, contribute to activation of provitamins, and modulate lipid metabolism (Ozdal et al., 2016). An increased abundance of these beneficial gut bacteria, displaying health benefits for the host, underlies the possible prebiotic effect of dietary polyphenols (Rodríguez-Daza et al., 2021).

A small number of clinical trials have demonstrated changes in composition of the microbiome after consumption of polyphenol-rich foods including cocoa, red wine, green tea, vegetables and fruit (Yang et al., 2020). For instance, in an intervention study in 22 healthy humans, Tzounis et al. reported increased abundance levels of *Lactobacillus* spp. and *Bifidobacterium* spp. after consumption of cocoa flavanols thereby indicating the potential prebiotic effects associated with flavanol-rich foods (Tzounis et al., 2011). Another clinical study in 30 healthy male and female volunteers showed that consumption of blueberry products (especially rich in anthocyanins (Li et al., 2017)) led to significantly increased abundances of *Lactobacillus* spp. and *Bifidobacterium* spp., which have been associated to multiple health benefits such as inhibition of gut pathogens, synthesis of vitamins and stimulation of the immune system (Lavefve et al., 2020; Molan et al., 2014). Also, consumption of red wine polyphenols significantly increased the number of *Bifidobacterium* spp. in the gut, while the quantity of *Lactobacillus* spp. was unaltered (Queipo-Ortuño et al., 2012). Furthermore, Bacteroidetes and Firmicutes are the main SCFA-producing taxa in the human gut and dysbiosis of these phyla may affect norepinephrine and dopamine biosynthesis, involved in ADHD pathophysiology, by alterations in SCFA levels (Carmichael, 2022).

Generally, the ratio of Firmicutes to Bacteroidetes (F/B-ratio) has been suggested as an important index for health status, and alterations in this ratio have been linked to pathological conditions such as obesity (increased F/B-ratio) and inflammatory bowel diseases (lower F/B-ratio) (Ley et al., 2006; Magne et al., 2020; Spychala et al., 2018; Stojanov et al., 2020). According to Wang et al. (Wang et al., 2022) a trend towards a slightly higher F/B-ratio was observed in children with ADHD as compared to healthy controls. A diet rich in polyphenols can regulate the F/B-ratio (Molinari et al., 2022). For example, earlier research by Yuan et al. (Yuan et al., 2018) demonstrated that a diet intervened with tea polyphenols rich in catechins, resulted in an increase in the number of Firmicutes and a decrease in the number of Bacteroides (higher F/B ratio). However, to the best of our knowledge, a potential prebiotic function of PBE and/or effect on F/B ratio has not yet been explored.

In this pilot study, we aimed to investigate the impact of PBE and MPH on the gut microbial composition of paediatric ADHD patients in a 10-week intervention. We particularly aimed to examine whether PBE exerts a possible prebiotic effect on gut

microbiota and whether MPH impacts the microbial composition of the gut.

5.5 Pilot study set-up: methods

5.5.1 Collection of faecal samples

A subgroup of 37 patients meeting all inclusion and exclusion criteria (see Table 4.1, Chapter 4) were asked to collect a faecal sample at baseline and after completing the 10-week study (Figure 5.3) using a Protocult collection container (Ability Building Center, Rochester, USA). No extra specific requirements were imposed prior to faecal donation. Samples were temporarily stored at -20 °C until pick-up and at the lab of Natural Products and Food Research Analysis-Pharmaceutical Technology (NatuRA-PT) samples were kept at -80 °C, without any processing until further analyses. All samples were pseudonymised according to General Data Protection Regulation (GDPR) regulations and stored in the University of Antwerp Biobank. Samples denoted as 1.X were taken at baseline, 2.X were taken after 10-week treatment. All donors were asked to fill out a validated FFQ consisting of 50 questions on different food groups to assess participants' global dietary habits throughout the study (de Vriese et al., 2005).

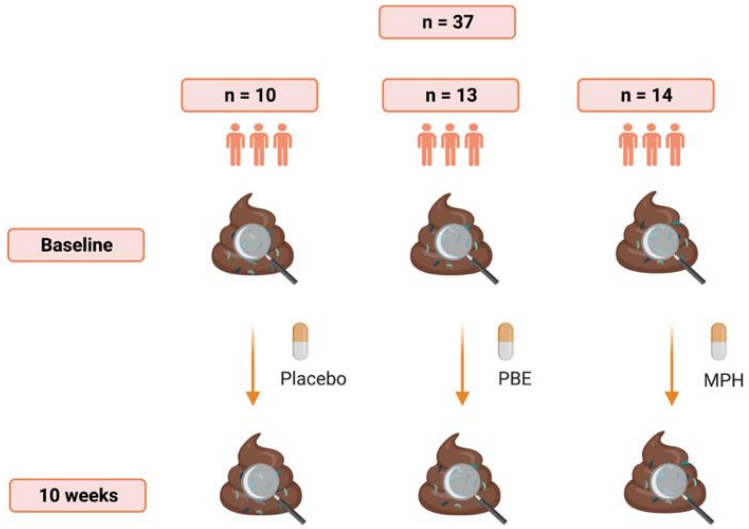

Figure 5.3: Visual representation of the pilot study set-up. PBE: French Maritime Pine Bark Extract; MPH: methylphenidate hydrochloride. Created in BioRender.

5.5.2 Microbial DNA isolation and 16S V1-V9 rRNA amplicon sequencing

The microbial composition of each individual faecal sample was investigated using 16S rRNA V1-V9 sequencing in collaboration with the lab of prof. Lebeer (Lebeer Lab, University of Antwerp). DNA isolation and sequencing was performed according to the method as described in Chapter 2 (2.4.4). A short overview of the 16S rRNA V1-V9 amplicon sequencing protocol is illustrated by Figure 5.4.

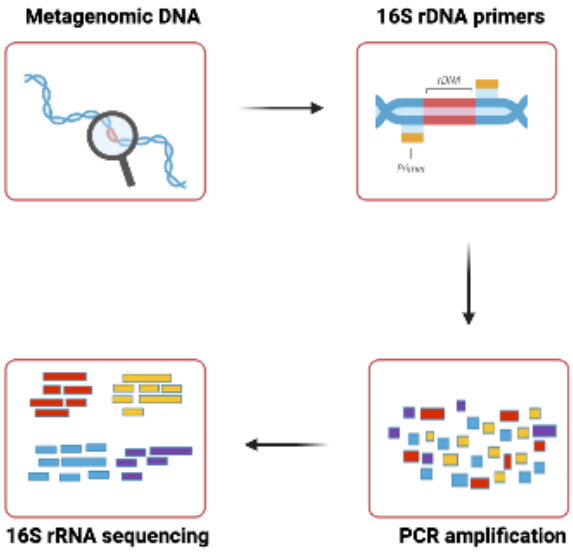

Figure 5.4: Workflow 16S rRNA V1-V9 amplicon sequencing. Created in BioRender.

5.5.3 Data import and data filtering

Sequence reads were processed using the ampliseq pipeline (Straub et al., 2020) from nf-core (Ewels et al., 2020) using the Nextflow computational workflow manager (Tommaso et al., 2017). The nfcore/ampliseq is a bioinformatics analysis pipeline used for 16S rRNA sequencing data. Processed data are uploaded from the tidyamplicons folder, a package for R, developed in-house to handle amplicon data in a tidy manner (github.com/Swittouck/tidyamplicons). Data filtering was used to remove low quality or

uninformative features in order to improve downstream statistical analyses. A first quality check was performed by the pipeline by comparing the output profiles of the positive controls (Mock communities, HM-783D, Bei Resources) to its datasheet to analyse the quality of the workflow. Next, non-bacterial amplicon sequence variants (ASVs) were removed from the dataset along with a ASVs longer than the expected 1500 base pairs. Sequencing data generated in this study are available at the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB65903.

Data from the FFQ, specific ADHD behaviour such as inattention or hyperactivity rated by the ADHD-RS, as well as significant biomarkers (CAT, NPY, IgA and IgG_2) were taken into account upon further statistical analyses (Chapter 4).

5.5.4 Statistical analyses

The relative abundance level of taxa was analysed for family and genus level and the F/B-ratio was calculated. The α-diversity which outlines the microbial community in individual samples with respect to its richness (number of taxonomic groups), evenness (distribution of abundances of the groups), or both was calculated with the inverse Simpson index (Willis, 2019). It is a common first approach to assess differences between environments and in this case, the role of a particular treatment on the microbial ecology (Nikolova et al., 2021). While α-diversity is a measure of microbiome diversity in a single sample, β-diversity is a metric that evaluates differences in microbial composition between individuals or members of different ecosystems. Assessment of β-diversity was performed by a dissimilarity matrix (Bray-Curtis dissimilarity) and results of the β-diversity estimates were visualised using principal coordinate analysis (PCoA). To analyse the association of several factors including behavioural scores, biomarkers, weight, sex and food intake on abundance levels of taxa, differential abundance analyses were also performed using the multidiffabundance R package which combines different differential abundance workflows (limma, lmclr and maaslin2) (Lebeer et al., 2022). This was done because not a single of the methods is flawless for the analysis of mixed samples such as the relative abundances retrieved in amplicon sequencing. Hence, only results with significance in two out of three methods were reported. Numbers in the differential abundance analyses indicate the number of methods for which significance

(in this case adjusted p-value < 0.01) was retrieved. In the PBE group, effect sizes smaller than 2 were left out to manage interpretability; however, in the MPH group all effect sizes were kept in the analysis since they were rather small. For other analyses a p-value < 0.05 was considered to be statistically significant.

5.6 Pilot study: results

5.6.1 High throughput V1-V9 amplicon sequencing data filtering

The relative abundance of taxa in the different kingdoms (depicted by Figure 5.5A) shows that one sample, more specifically 2.656 (10-week sample of participant 656), seems to contain an abnormal amount of Eukaryotic ASVs compared to the rest. Also sample 2.654 seems to contain an amount of Eukarya, albeit to a lesser extent. Looking into more detail at sample 2.656 (Figure 5.5B; the baseline sample 1.656 is also depicted for reference), the exact S16 sequence could not be resolved with great confidence. Nevertheless, a Basic Local Alignment Search Tool (BLAST) available at the National Center for Biotechnology Information (NCBI) was used to search for this particular sequence and results revealed that most of the hits corresponded to the *Mucorales* order, which belongs to the kingdom of the Fungi. Therefore, these reads were excluded in further analyses. Identified non-bacterial ASVs were removed from the taxon table (mitochondrial and chloroplast S16) along with any ASV longer than the expected 1500 base pairs (the entire 16s rRNA gene contains approximately 1500 base pairs). In total 452 out of 3033 identified ASVs were discarded in this step, which is a total of 14.9%.

DNA concentrations varied from around 10 to 450 ng/µL in the samples (data not shown). The number of counts (Figure 5.6A) per sample sequenced were then normalised by the concentration measured with the Qubit and plotted (Figure 5.6B). Looking at the plot, sample 1.649 has a very low read-count, while sample 1.657 has an abnormally high read-count. Although sample 1.649 has a high DNA concentration of 378 ng/µL, the multiqc report shows that this sample has an overall low level of reads prior to any filtering.

As shown in Figure 5.7 the composition of the mock community samples looks uniform over the different samples. These positive controls also seem to give similar output profiles in terms of duplicate reads and read lengths. All 20 bacteria present are identified to the genus level and 8 out of 20 could be correctly identified to the species level with an exact match in one of the ASVs. From these results we can therefore conclude that classification to the genus level with the sequencing conditions used is very likely to be correct, while sequencing errors might prevent exact matches on the species level. Only very abundant taxa could thus likely be detected on the species level.

The negative controls (blank buffer used during the sequencing library preparation) contain very little reads (maximum three) (data not shown). The positive and negative controls were then removed from the dataset before proceeding with further analyses.

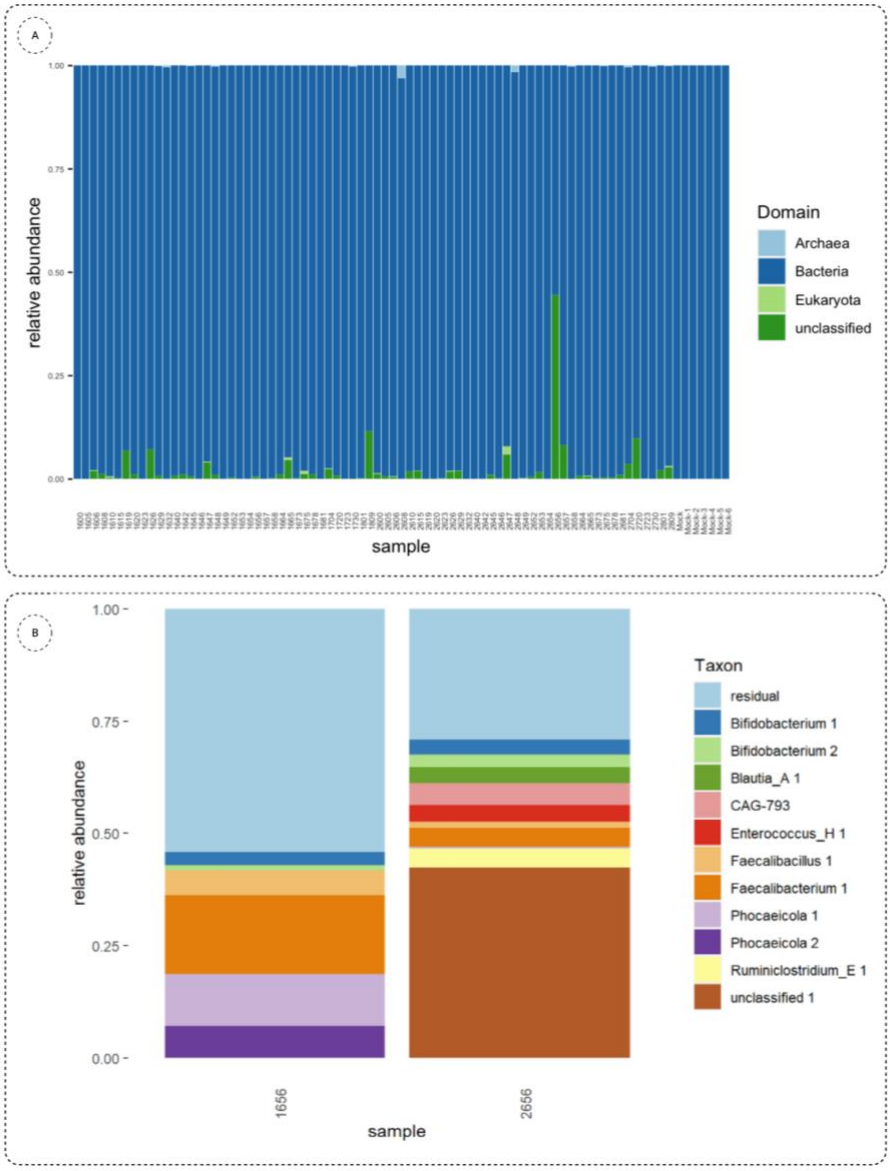

Figure 5.5: A: Stacked bar plots of the relative abundance of taxa across the different kingdoms. Light blue bars depict the kingdom of Archaea, dark blue bars represent Bacteria while light green bars are the Eukarya and dark green bars are ASVs that could not be classified. B: Further investigation of the non-bacterial ASVs possibly present in both samples represented as stacked bar plots. ASV: Amplicon Sequence Variant.

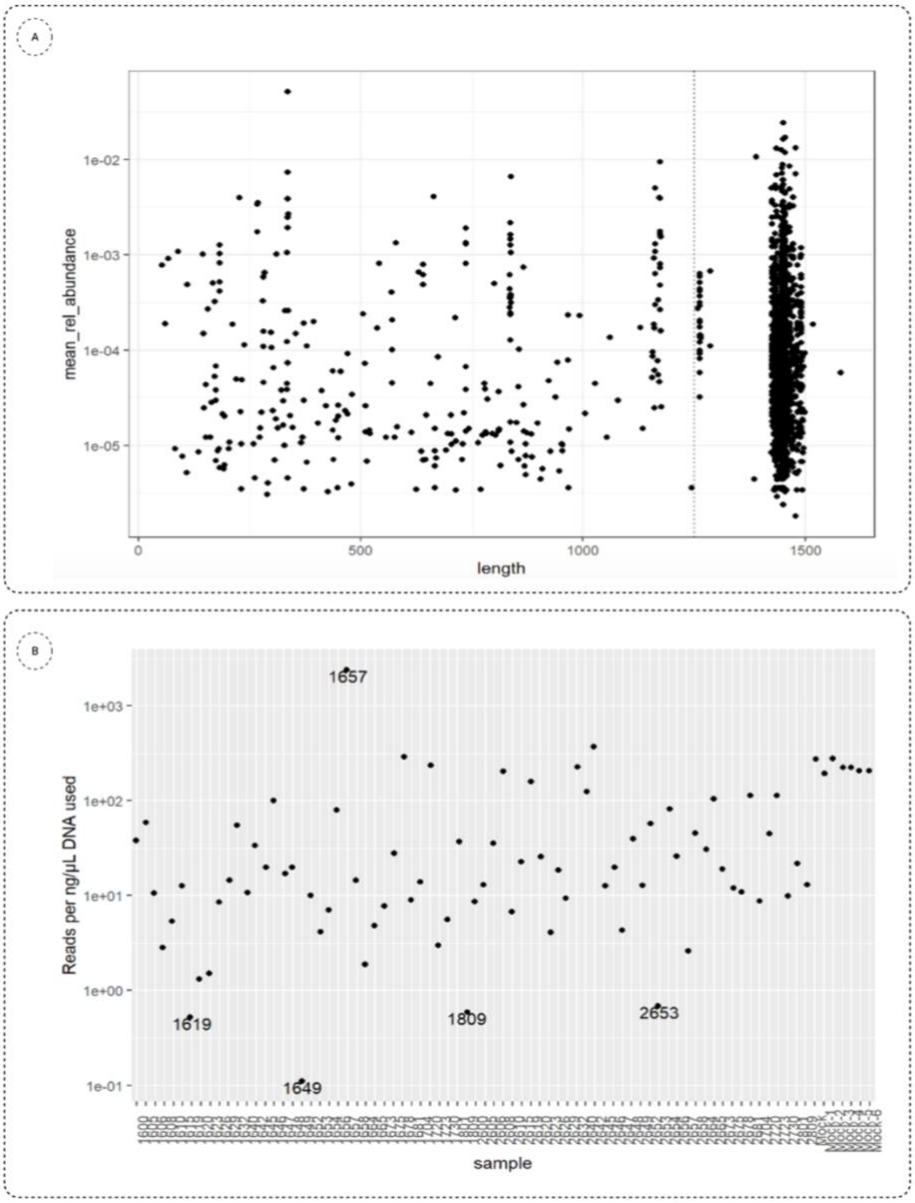

Figure 5.6: A*:* Distribution of ASV base pairs lengths. The threshold is set at 1500 base pairs. B: The amount of reads for each sample is normalised by the concentration of DNA measured with the Qubit fluorometer and plotted. Samples with lower or higher read-counts than average are depicted with their respective sample number. ASV: Amplicon Sequence Variant.

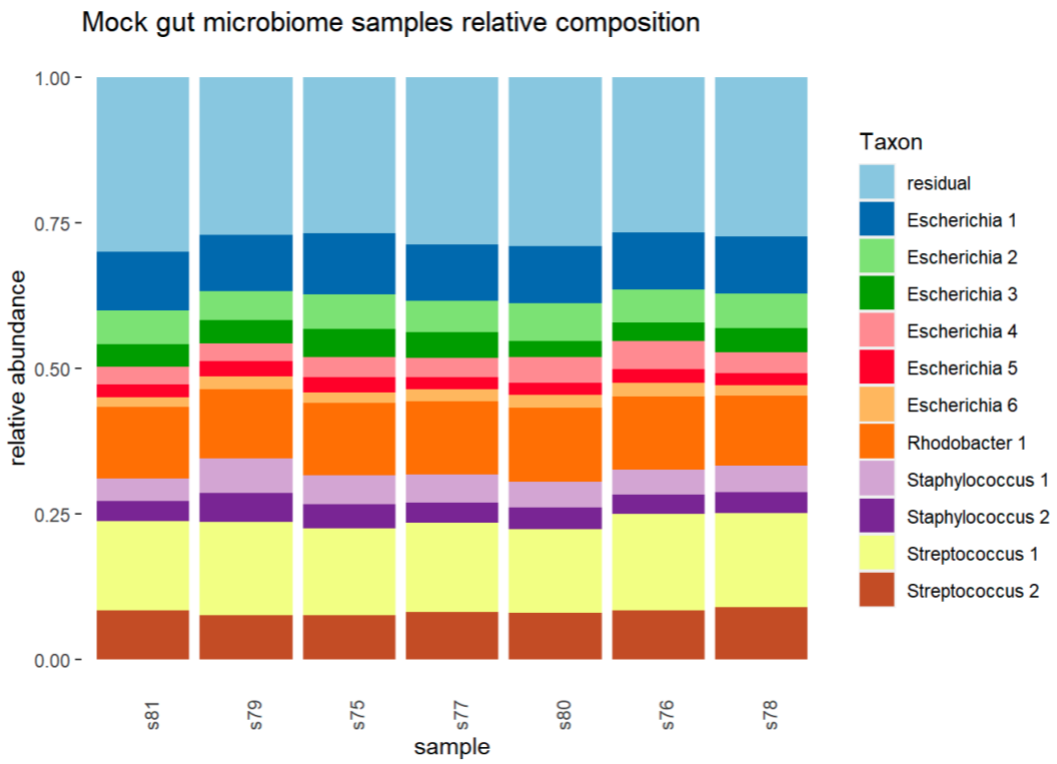

Figure 5.7: Overview of the relative composition of the mock gut microbiome samples.

5.6.2 Gut microbiome profile of treatment-naive paediatric ADHD patients

Faecal samples of 37 ADHD patients (mean age: 10.3 years, 60% male) were collected at the start and after a 10-week intervention with either PBE, MPH or placebo. These treatment groups did not differ in demographic variables (e.g. age, height and weight) nor in dietary habits at the start of the study (data not shown). However, in Figure 5.8 a striking difference in weight was observed in between the three groups after 10 weeks of treatment, namely a significant reduction of weight is noticeable in the group of participants treated with MPH (see Chapter 4).

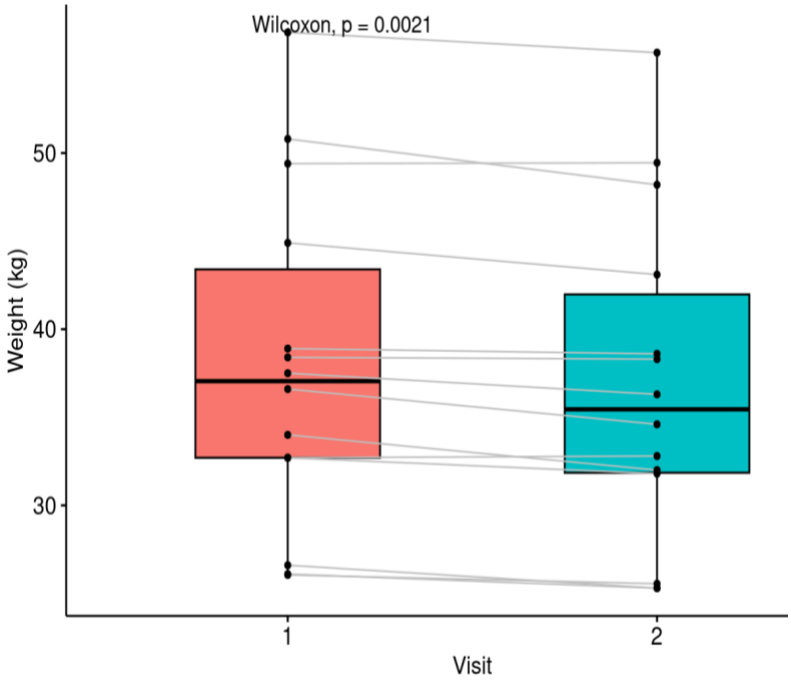

Figure 5.8*:* Weight before (visit 1) and after treatment (visit 2) with methylphenidate for 10 weeks. The Wilcoxon test was used to depict statistical differences in weight between visit 1 and 2.

Looking at the microbial composition as depicted by Figure 5.9, *Agathobacter* (Firmicutes)*, Alistipes* (Bacteroides*), Bacteroides* (Bacteroides)*, Bifidobacterium* (Actinobacteria), *Cryptobacteroides* (Bacteroides)*, Faecalibacterium* (Firmicutes)*, Gemmiger* (Proteobacteria*), Phocaelcola* (Bacteroides), *Prevotella (*Bacteroides)*, Romboutsia* (Firmicutes) and *Ruminococcus_E* (Firmicutes) showed to be the most dominant genera in our overall population. Of note, *Bifidobacterium* and *Phocaeicola* spp. show a high relative abundance and prevalence in most baseline samples (>30% relative abundance in approximately 35% and 16% of the samples, respectively).

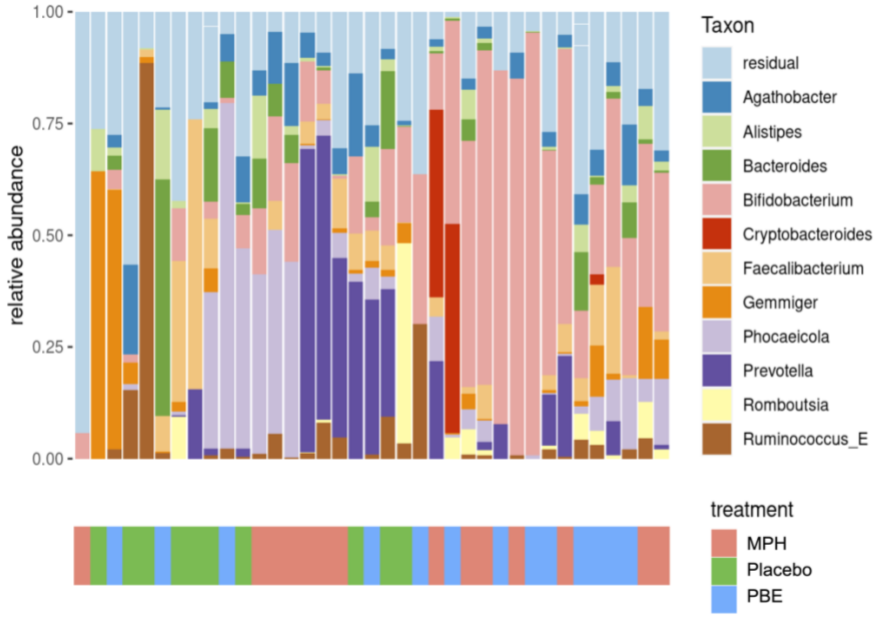

Figure 5.9: Stacked bar chart describing the baseline microbiome composition of all participants of the 10 most abundant taxa on genus taxonomical level. The panel on the right shows the genus taxonomical level in descending order from the highest relative abundance to lower relative abundances across all samples.

5.6.3 Differences in gut microbial communities between treatment groups

To investigate the impact of the three different treatments on the microbial composition of the gut between baseline and 10-week samples, the relative abundance of taxa at genus level was visualised per cohort (Figure 5.10A). Generally, only individual microbial shifts in relative abundance were observed. For example, a 0.60-fold increase in the relative abundance of *Phocaeicola* was observed after 10-weeks of MPH treatment. However, this increase was only noticeable in one participant and therefore statistical significance could not be demonstrated.

Since the literature often describes the balance between Firmicutes and Bacteroidetes and their impact on normal intestinal homeostasis, we also analysed the relative abundances of Firmicutes and Bacteroidetes phyla and their ratios for each study cohort

at baseline (visit 1) and after 10-weeks (visit 2) (Table 5.1). For the total Firmicutes relative abundance number, all Firmicutes phyla (Firmicutes_A, Firmicutes_B and Firmicutes_C) were summated. The F/B-ratio was then calculated for all samples containing Bacteroidetes (Figure 5.10B). A paired Wilcoxon test showed no significant trends in F/B ratio before and after treatment for the different treatment cohorts (Figure 5.10C).

Table 5.1: Overview of the relative abundances of Firmicutes and Bacteroidetes for each treatment group per study visit.

Treatment	Visit	Mean F/B-ratio	Relative abundance Firmicutes	Relative abundance Bacteroidetes
MPH	1	0.75	28.73%	38.22%
MPH	2	0.56	26.11%	46.48%
Placebo	1	2.68	65.29%	24.36%
Placebo	2	2.20	51.09%	23.26%
PBE	1	1.26	37.5%	29.72%
PBE	2	1.47	42.95%	29.12%

F/B: Firmicutes/Bacteroidetes ratio; MPH: methylphenidate hydrochloride; PBE: French Maritime Pine Bark Extract.

To assess the within-samples diversity, the inverse Simpson index was calculated as depicted by Figure 5.11A (results of other α-diversity indices can be found in Supplementary Materials). Though the α-diversity seems to decrease in the group treated with PBE, statistical testing with a Wilcoxon Ranked Sum test did not show a significant difference (p-value = 0.95) before and after PBE treatment (Figure 5.11B). Next, the Bray-Curtis distance matrix was calculated for the between-samples diversity and a PCoA was performed to assess β-diversity (Figure 5.11C). This two-dimensional (2D) representation gives a visual cue on how different the samples are from each other: the more distant the dots on the plot, the more different the samples are. Most samples from the different groups are scattered across the plot, but a cluster of 11 samples could be observed without any PBE treated participant's samples. Nevertheless, the goodness of fit for the PCoA is only 8.8%, meaning that a representation of this large dataset in a limited 2D visualisation is not optimal. Closer inspection of the β-diversity per cohort showed no significant difference before and after their respective treatment (Figure 5.11D).

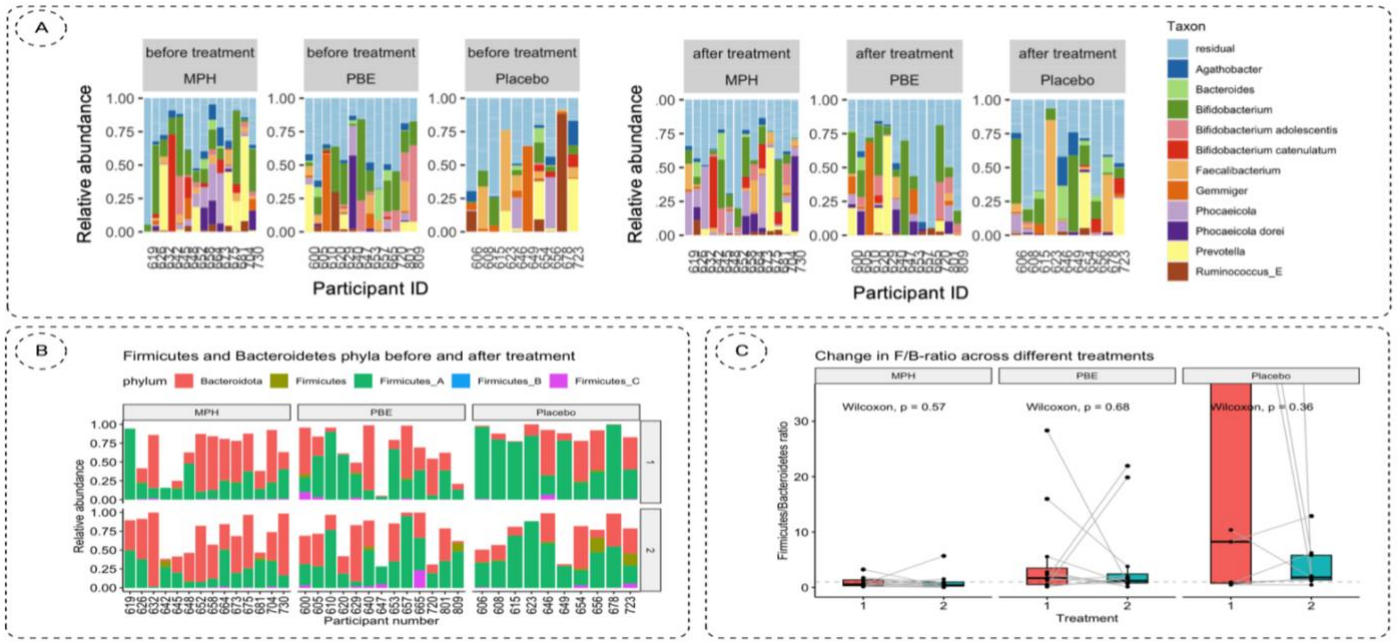

Figure 5.10: A: Relative abundances at genus level based on 16S V1-V9 rRNA gene whole genome sequencing of faecal samples at baseline (visit 1) and after 10 weeks (visit 2) to evaluate the effect of treatment on the gut microbiome. B: Relative abundances of Bacteroidetes and Firmicutes per study group on the first and second study visit. Bacteroidetes are depicted in red, Firmicutes are denoted in green, blue and red stacked bars. C: Change in Firmicutes/Bacteroidetes (F/B)-ratio across the MPH placebo and PBE group at baseline (1, red box) and 10-weeks (2, blue box). Statistical significance was tested using the paired Wilcoxon test. MPH: methylphenidate hydrochloride; PBE: French Maritime Pine Bark Extract.

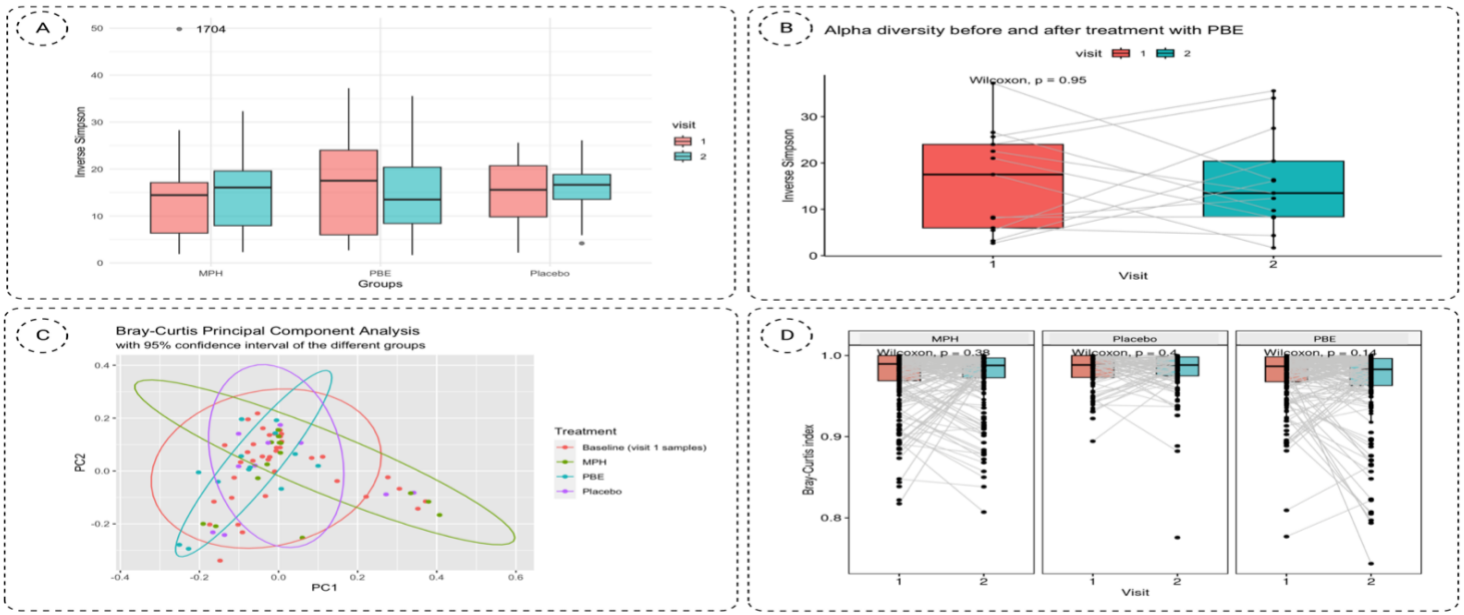

Figure 5.11: A+B: Boxplots of the α-diversity between baseline and 10-weeks samples for each study group and a close-up of the cohort treated with French Maritime Pine Bark Extract using the inverse Simpson index. C: Bray-Curtis Principal Component Analysis with 95% confidence interval of the different groups. D: Boxplots of the β-diversity between baseline and 10-weeks samples for each study group using the Bray Curtis dissimilarity index. Each boxplot represents the data range within the 1.5 interquartile range (IQR) with the median depicted as a horizontal line. Outliers are plotted as individual datapoints (outside 1.5 times the IQR above the upper quartile and below the lower quartile). All samples are depicted as dots in their respective colour.

To investigate the potential prebiotic effect of PBE, we looked at the taxa family level of participants from the PBE cohort (Figure 5.12). Looking at the relative abundance, there was one taxon less abundant after PBE treatment than before: *UBA932* (unculturable, Bacteroides*)*. This unculturable bacterial strain was found in baseline and 10-week samples of patient 653 whereas for participant 720 it is found in relatively high abundance at the first study visit. However, since these changes in abundance occur only in two samples, this effect is most likely overrated. After treatment with PBE, there is a taxon that appears more abundant namely *CAG-272* (Firmicutes) in participants 629 and 657. Particularly in the latter the relative abundance after 10-weeks of PBE treatment is higher than compared to baseline. The rest of the datapoints follow an imaginary straight line (depicted as a blue line in the graph (Figure 5.12) through the origin, thereby indicating that the relative abundance of taxa after 10-week treatment with PBE is similar to baseline. Due to the limited amount of datapoints, statistical testing would not indicate clear significant differences in taxa at family level for the PBE cohort.

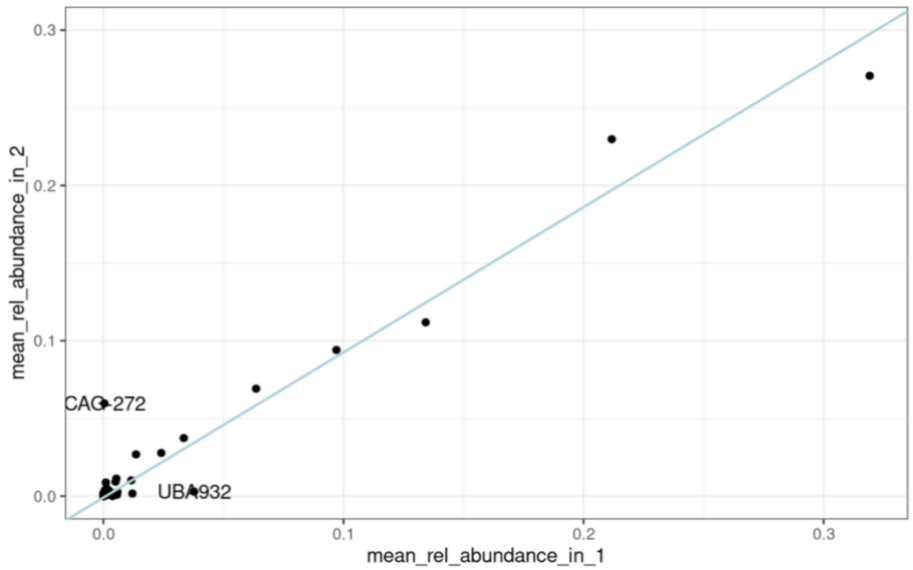

Figure 5.12: Mean relative abundance at taxa family level of PBE treated patients. Baseline visit is represented on the x-axis, 10-week visit on the y-axis. CAG-272: species belonging to Firmicutes. UBA-932: unculturable belonging to the Bacteroidales (Bacteroidetes). PBE: French Maritime Pine Bark Extract.

5.6.4 Impact of host covariates on the gut microbiome

Besides the impact of different treatments on the gut microbiome, we also aimed to explore the role of gender, biological markers and ADHD symptoms. First, it is important to mention that a steady significant reduction ($p = 0.0021$) of weight (Figure 5.8) was observed in the group treated with MPH, which could be linked to a reduced appetite as demonstrated in previous research work (Weyns et al., 2022). PERMANOVA analysis with the adonis2 function of the R package 'vegan', using 999 permutations and stratification by subject showed that treatment (placebo, MPH and PBE) of the participants (adjusted-p-value = 0.011) explained 3.94% of the variation of the distribution of taxa (data not shown). Sex (adjusted-p-value = 0.016) explained around 1.96% of the variation in microbiome and difference in weight between start and end of the trial (adjusted p-value = 0.015) 2% variation.

Analyses of the association of different factors with the microbiome and the effect sizes for these covariates on specific taxa (lower/higher abundance) are denoted in Figure 5.13A for the PBE group and in Figure 5.13B for the group treated with MPH, both compared to the placebo group. In the PBE group, the following associations between abundance levels of several taxa and the oxidative stress and immune system related biomarkers were observed: high CAT (-4.116) and IgG_2 levels (-5.692) were negatively associated with abundance levels of *Phocaeicola*, high IgG_2 levels were negatively associated (-1.654) with abundance levels of *Firmicutes bacterium CAG-41* (Firmicutes) and high IgG_2 levels (-2.388) were linked to lower abundance of *Alistipes finegoldii* (Bacteroidetes). A high weight (2.474) was linked to higher abundance levels of *Firmicutes bacterium CAG-41* (Firmicutes) as well as on the abundance of *Alistipes finegoldii* (2.737). High behavioural scores ARS-HIS (-0.534) and ARS-IAS (-0.824) were negatively associated with abundance of *Alistipes finegoldii.* Also, more chocolate intake (-2.272) was linked to lower levels of *Alistipes finegoldii,* whereas the abundance of *Intestinibacter* was positively affected by it (2.052). A male gender type (1.702) was also linked to higher levels of *Intestinibacter.* A few taxa with significantly different abundance in the MPH group were observed, but all with a rather low effect size. High levels of the immune biomarker IgA (1.136) were positively associated with the abundance levels of *Mediterraneibacter faecis.* Abundance of *Bariatricus comes* was negatively associated with high ARS-IAS (-0.613) and positively with ARS-HIS scores (1.049). Also, male gender type (0.264) was linked to higher levels of *Bariatricus comes.*

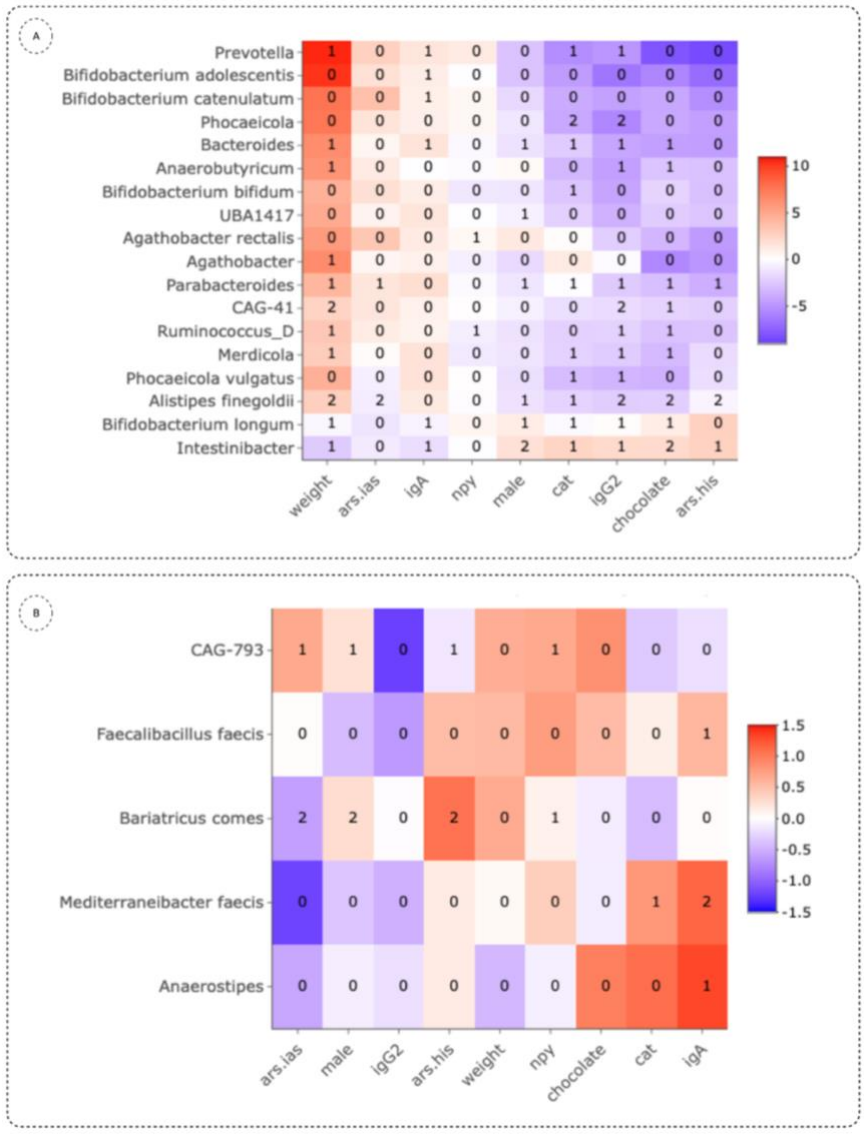

Figure 5.13: Effect sizes of different variables in the group treated with PBE (A) and MPH (B) as compared to the placebo group using three differential abundance analysis methods. Numbers in the plot indicate the number of methods that show significant (p-value < 0.01, adjusted for multiple testing) abundant taxa. ars.his: hyperactivity score given by teachers on the ADHD-Rating Scale; ars.ias: inattention score given by teachers on the ADHD-Rating Scale; Cat: catalase; IgA: immunoglobuline A; IgG_2: immunoglobuline G2, NPY: neuropeptide Y.

5.7 Discussion

Emerging evidence suggests that the gut microbiota plays a pivotal role in the gut–brain axis by influencing metabolism, inflammation, the HPA axis and neurotransmission (Jiang et al., 2018). Moreover, an association between the gut microbiota and several neuropsychiatric conditions including ADHD has been demonstrated (Boonchooduang et al., 2020; Richarte et al., 2021). Though ADHD is the most prevalent neurodevelopmental disorder in children and adolescents, current therapy with psychostimulants is far from optimal and there is still insufficient information on the correlation between the mechanisms involved and the microbiome. Nevertheless, emerging evidence suggests that dietary polyphenols could be beneficial in the treatment of oxidative stress related diseases such as ADHD due to these antioxidant and anti-inflammatory properties, while they also could exert a prebiotic effect on gut microbiota (Alves-Santos et al., 2020).

Compositional analysis revealed that *Agathobacter, Alistipes, Bacteroides, Bifidobacterium, Cryptobacteroides, Faecalibacterium, Gemmiger, Phocaeicola, Prevotella, Romboutisa* and *Rumminococcus_E* were the most abundant genera at baseline. Interestingly, *Bifidobacteria* make up most part of the overall relative abundance in our study population at baseline. Generally, at birth and during early development, bifidobacterial populations are found to be the most abundant genus and abundance levels slowly decrease considerably but remain relatively stable during adulthood, further decreasing at old age (Arboleya et al., 2016; Deering et al., 2020). Moreover, various studies have identified the genus *Bifidobacterium* as having a potential relevance to ADHD albeit with contradictory outcomes. According to a systematic review, some studies found a nominal increase in *Bifidobacterium* in ADHD compared to control subjects while others stated that the abundance of *Bifidobacterium* is reduced in ADHD (Cickovski et al., 2022.; Sukmajaya et al., 2021). This increase in *Bifidobacterium* in the ADHD cohort can occur at the expense of more developmentally appropriate bacteria (i.e. Bacteroidetes) with its dominance during childhood and possible deficiency in early life (Payen et al., 2022). Though in our study set-up no healthy controls were included, our results show high relative abundance of *Bifidobacterium* in paediatric ADHD patients which can be linked to an enhanced synthesis of the dopamine

precursor phenylalanine of which high levels have been linked to ADHD symptoms (Aarts et al., 2017). Also, at genus level, a lower abundance of *Faecalibacterium* could be observed in our baseline study population, which is in line with earlier research reporting lower levels in ADHD patients as compared to healthy controls (Soltysova et al., 2022). *Faecalibacterium* have been found to exhibit anti-inflammatory properties and increased levels of pro-inflammatory markers have been associated with ADHD. Alterations in abundance levels of *Faecalibacterium* may thus play a role in the etiology and/or symptomatology of ADHD (Schleupner & Carmichael, 2022).

Looking at phylum level, no clear trend towards altered F/B-ratio could be observed for the three study cohorts at 10-weeks compared to baseline. Since individual patients appeared to fluctuate both up and down in F/B-ratio no statistical significance was obtained. Moreover, since the F/B ratio might already be elevated at baseline in our study cohort, a significant increase in ratio as a result of a 10-week treatment might therefore remain undetected as well as a possible modulation of the F/B ratio by PBE.

Bacterial diversity analyses at family level did not reveal significant differences in any of the treatment groups. Though no statistical significance could be obtained, results of the PCoA plot suggest that there are some samples with unique factors in the populations not treated with PBE (placebo and MPH) which are absent in the samples treated with PBE. Based on our findings, we cannot conclude that PBE exerts a prebiotic effect on the gut microbiome. Nevertheless, we did notice a difference in abundance level of taxa (*UBA-932* and *CAG-272*) in some patients treated with PBE. These individuals might be responders to PBE therapy, which would be in line with our other research findings demonstrating a significant decrease in total ADHD-RS as rated by teachers and thus an improvement of ADHD behaviour (Weyns et al., 2022). Yet, qPCR should confirm these results and can maybe even highlight prebiotic effects in the other patients treated with PBE. Since the altered abundance levels of the above-mentioned taxa cannot be extrapolated to all participants treated with PBE, these results should be interpreted with caution.

Our results thus suggest that none of the treatments correspond with large scale changes in community composition of the human faecal microbiome content during this 10-week randomised trial. Previous findings of Stevens et al. (Stevens et al., 2019)

investigating micronutrient treatment consisting of a blend of vitamins, minerals, amino acids and antioxidants, also could not detect large-scale changes in microbial composition. Possibly, supplementation for 10 weeks is not long enough to observe these substantial changes in gut microbiome. In addition, apart from medication use such as antibiotic therapy, no specific inclusion criteria regarding factors with possible effects on gut microbiota, were implemented in our study. For instance, the study population was not screened for dietary habits (e.g., vegetarian or vegan) neither for defecation frequency nor lifestyle habits. Taken together the possible large differences between the individuals at the start regarding microbial composition due to differences in diet and lifestyle as well as the short supplementation period hampers to find significant results. Nevertheless, collecting personal data and information and analysis of biomarkers in blood samples, allowed us to perform an in-depth analysis of covariates. Several covariates were associated with the microbial constellation.

The impact of several environmental variables on microbial communities was investigated. Treatment showed the largest explanatory value for microbiome variation in our study. Though our analyses revealed that this effect size was rather small (covariate accounted for 3.94%) it was highly significant. Nevertheless, it only explained a small part of genus abundance variation, suggesting additional contribution from other factors such as environment or genetic background. Our findings are in line with previous research work which also showed that additional factors have a profound impact on microbiome composition (Falony et al., 2016). Though multiple factors are known to affect gut microbial composition (use of medication and/or antibiotics and consumption of pre-or probiotics) (Hasan & Yang, 2019), collection of all this valuable information is considered a strength of this study. Another strength of our study is the supplementation with a standardised extract, complying with USP requirements regarding polyphenolic constituents and procyanidin content, to make sure the bioavailability of PBE is sufficient enough to notice differences (Convention USP, 2014). Moreover, it has been demonstrated that microcrystalline cellulose does not induce microbial shifts and placebo supplementation therefore does not alter the gut microbiome itself (Deehan et al., 2022).

In general, the interactions between polyphenols and gut microbiota are reciprocal (De Bruyne et al., 2019). Research not only demonstrated that polyphenols lead to modulation of the gut microbial composition, but also that the gut microbiota plays an important role in the biotransformation of polyphenolic compounds (De Bruyne et al., 2019; Mithul Aravind et al., 2021). The gut microbiota thus contribute to the metabolism of dietary polyphenols, thereby impacting the bioavailability of both parent polyphenols and their (potentially bioactive) metabolites (Catalkaya et al., 2020; Corrêa et al., 2019). Further research is therefore imperative to improve our understanding of this two-way interaction by investigating the biotransformation processes of ingested PBE in the presence of intestinal microbiota (Breynaert et al., 2015). This longitudinal intervention study already offers some important new insights in how PBE influences microbial composition and may thus offer valuable information on which microbial strains may affect biotransformation processes of PBE.

In conclusion, only small changes in the gut microbiome of the participants of either treatment group were noticeable. This could be due to the small sample size per group and therefore a rather low power, and a high background noise since the gut microbiome of children at this age is very flexible. Moreover, weight loss was observed in the group treated with MPH (Weyns et al., 2022), thereby increasing the complexity of interpretation of the analysis. Further research involving more participants, possibly more sampling points, more inclusion criteria and more strict monitoring of covariates, is required to establish potential therapeutic efficiency of PBE in gut microbial modulation.

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Supplementary materials

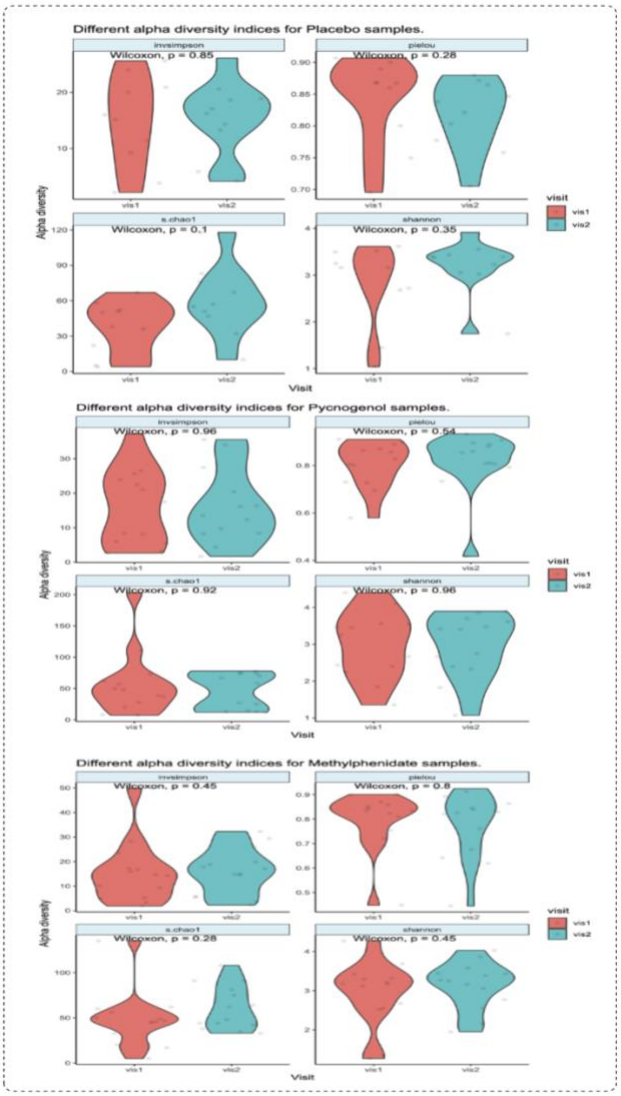

Figure S1: Overview of alpha diversity indices for the three different treatment groups (placebo, Pycnogenol (PBE) and methylphenidate). PBE: French Maritime Pine Bark Extract.

Chapter 6 **Cardiovascular health benefits of French Maritime Pine Bark Extract**

6.1 Introduction

Psychostimulants are widely considered as the first-line pharmacologic treatment for children and adolescents with ADHD, acting on the CNS to reduce ADHD symptoms, physical hyperactivity and to maintain mental focus in children and adults (Olfson et al., 2012; Torres-Acosta et al., 2020). Despite their increasing use and documented efficacy, CNS stimulants such as MPH remain controversial due to safety concerns (Tadrous et al., 2021). Besides earlier reported side effects such as insomnia or loss of appetite, there is also a substantial concern about the cardiovascular (CV) safety of ADHD medication. In fact, these compounds exert stimulant effects on the CNS by increasing the levels of noradrenaline and dopamine in the prefrontal cortex, and by activating adrenergic receptors in the heart and blood vessels leading to small increments in resting heart rate (HR), blood pressure (BP) and arterial stiffness (AS) (Kelly et al., 2014; Torres-Acosta et al., 2020).

The latter is considered to be an independent risk for CVD among adults and therefore it is reasonable to assume that altered cardiac autonomic function (activation of the sympathetic nervous system) and unfavourable haemodynamics (higher HR and BP) may put children and adolescents at high-risk for developing CVD later in life if the effects were to persist (Kelly et al., 2014).

The potential negative CV outcomes with ADHD stimulant medication use among children and adolescents have received great attention (Habel et al., 2011). Since 2010, the Food and Drug Administration (FDA) labelling of all stimulants even include a warning regarding sudden death. The labelling warns that “sudden death has been reported in association with CNS stimulant treatment at usual doses in children and adolescents with structural cardiac abnormalities or other serious heart problems” (Olfson et al., 2012). In 1958, possible adverse CV events under stimulant medication use were reported for the first time. Later on, in 1976, BP and HR were seen to be significantly increased in 27 hyperactive children after MPH therapy (Ballard et al., 1976). A longitudinal cohort study among children (n= 8,300) born in Denmark between 1990 and 1999 with a mean follow-up of 9.5 years showed an increased risk of adverse CV events including arrhythmia (23%), cerebrovascular disease (9%), hypertension (8%), ischemic heart

disease (2%), heart failure (2%) and pulmonary hypertension (<1%) in ADHD medication users as compared to non-users (Dalsgaard et al., 2014). A self-controlled case analysis using the nationwide health insurance database of 1,224 South Korean patients of 17 years or younger who have been treated with MPH for six months between 2008 and 2011, revealed a higher risk of myocardial infarction and arrhythmias (Shin et al., 2016). Lamberti et al. (Lamberti et al., 2015) also reported a significant increase in HR (from 80.5 ± 15.5 bpm to 87.7 ± 18.8 bpm) two hours after MPH therapy in paediatric ADHD patients (mean age 12.14 ± 2.6 years). According to a three-month follow-up study from Omidi et al. (Omidi et al., 2021) including 100 newly diagnosed ADHD children aged to 11 years, all on MPH treatment, no abnormal systolic, diastolic, mean arterial pressure, or significant changes in echocardiographic parameters nor QT-interval, was observed. Nevertheless, the mean of the measured variables was significantly increased. A meta-analysis of ten randomised clinical trials outlined the association between stimulants and increased HR (5.7 bpm more) and increased systolic blood pressure (2.0 mm Hg higher) (Tadrous et al., 2021). Another meta-analysis by Liang et al. (Liang et al., 2018) showed that children/adolescents and adults treated with MPH had significantly increases in post- vs. pre-treatment HR and systolic BP as compared to the placebo cohort. In a placebo-controlled, randomised clinical trial, the use of MPH was associated with a four-fold increase in the odds of developing pre-hypertension (a precursor of hypertension) in young adults who previously were normotensive (having a normal BP) and secondly, it could be linked to an increased platelet aggregation (Tadrous et al., 2021). Nevertheless, a comprehensive review including the results of 19 studies, did not reveal any statistically significant association between ADHD medication use and CVD among children and adolescents, young and middle-aged adults, or older adults (Zhang et al., 2022). Overall, it can thus be concluded that the possible effect of MPH and atomoxetine on HR and BP remain still inconclusive until now (Liang et al., 2018).

Estimates of CV safety of psychostimulants in children or adolescents cannot be directly generalised to adults affected by ADHD. Generally, compared to children, adults have accumulated more CV risk factors and, if exposed to CNS stimulants, carry even a greater burden of CV risk (Mick et al., 2013; Torres-Acosta et al., 2020). A retrospective, population-based cohort study including adults aged 25 to 64 years, using electronic health care records from four study sites, reported that although the overall CV risk was not increased in participants with a history of ADHD medication intake, adverse CV risk

events (e.g. myocardial infarction or stroke) trended to be insignificantly higher in patients who recently started taking ADHD medications (Habel et al., 2011). A meta-analysis by Mick et al. (Mick et al., 2013) reported that adults with ADHD, treated with stimulant medication showed increased BP and HR. In a population-based analysis in a cohort of paediatric and adult ADHD patients with no history of heart failure or cardiomyopathy who started treatment for ADHD with medication, the incidence of new-onset heart failure or cardiomyopathy by duration of use and age group was assessed (Mosholder et al., 2018). The highest incidence rates appeared soon after treatment initiation (days 0-90) in the 65 years of age and older patient group with one case per 10.5 person-years of follow up, or 950 cases per 10,000 person-years. In contrast, a cohort study by Schelleman et al. (Schelleman et al., 2012) could not demonstrate a causal association between MPH and risk of serious CV events in adults.

In general, as presented above, the current evidence regarding associations of stimulant drugs with CV events is mixed due to differences in age groups or sample sizes. Therefore, more research is warranted in the future (Mosholder et al., 2018). Nevertheless, a cumulative body of data suggests that ADHD medical therapy may cause elevations in HR and BP as well as other reported adverse CV effects such as arrythmia and non-ischemic cardiomyopathy (Torres-Acosta et al., 2020). Treatment with MPH in the context of ADHD could thus negatively impact CV health.

6.2 Cardiovascular health

CVDs are a broad range of disorders affecting the heart and blood vessels such as hypertension, stroke, heart failure and various other cardiac and vascular conditions. According to WHO reports, CVDs are the number one cause of death globally. In 2016 alone, around 17,900,000 people died from CVD, representing over 30% of global deaths that year (WHO, 2022). It is estimated that by 2030 this number will rise to 23 million deaths per year with stroke and heart attacks expected to cause the greatest increase in annual medical costs (Paneni et al., 2017). Maintaining CV health is therefore crucial to lower the incidence and mortality of CVD.

To reduce the burden of CVDs, several risk factors for CVD have been largely studied and have already been identified (Figure 6.1). Risk factors including unhealthy diet (e.g. diet rich in salt, fat and sugars), excessive alcohol consumption, tobacco use, hypertension and diabetes are strongly linked to growing CVD burden (Jagannathan et al., 2019). These so-called lifestyle risk factors are modifiable and prevention guidelines recommend changing these modifiable risk factors in order to decrease morbidity and mortality (Arnett et al., 2019). Controlling or eliminating them may, per se, lead to a radical decrease in CVD mortality (Timmis et al., 2022; Yusuf et al., 2020). In fact, all modifiable risk factors are included on the WHO target list to be reduced by 2025 (WHO, 2022). In addition, non-controllable risk factors including age, gender, ethnicity and genetic predisposition also play important roles in the onset of CVDs (Alves-Silva et al., 2021, Frančula-Zaninović et al., 2018).

Throughout the years, several studies pointed out the importance of another, independent predictor for developing CVD, namely arterial stiffness (AS), which emerged as a prominent marker of CV risk (De Moudt, 2022). Various CVDs like atherosclerosis, angina pectoris, stroke and myocardial infarct are often preceded by AS. Though treatment and/or prevention of AS might be beneficial to reduce CV risk and overall mortality from CVD in the population, and despite its general recognition as a relevant biomarker, its implementation has yet to become routine clinical practice (Jain et al., 2014; Lyle et al., 2017; Zieman et al., 2005). Apart from the cardiovascular risk, AS has also been associated with other diseases, including kidney problems, vascular dementia and cognitive impairment (Hughes et al., 2015; Inserra et al., 2021; Iulita et al., 2018; Liu et al., 2021).

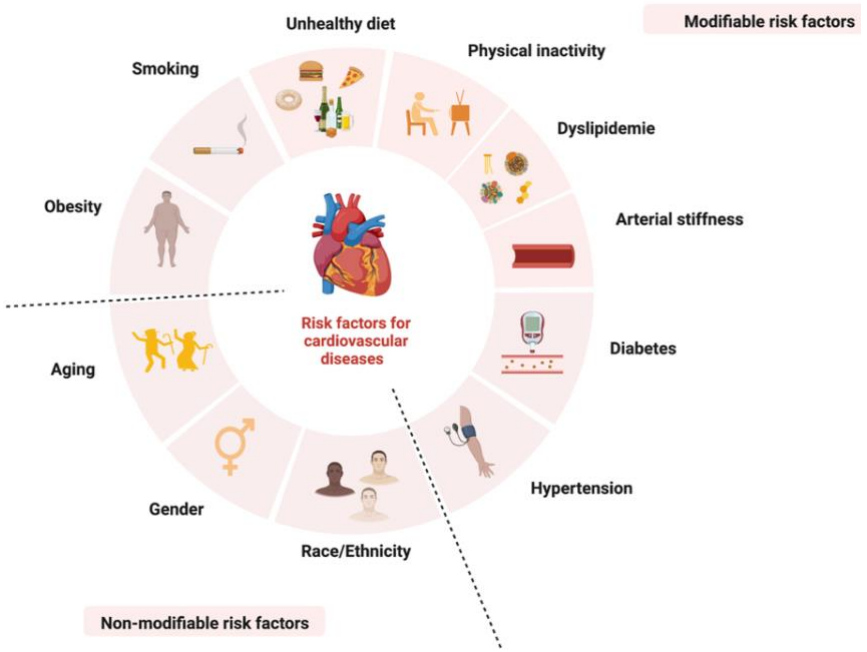

Figure 6.1: Modifiable and non-modifiable risk factors for developing cardiovascular diseases. Created in BioRender.

6.3 Arterial stiffness

6.3.1 Arteries: structure and function

Our circulatory system consists of the heart, arteries and veins. The heart acts as a pulsatile pump to maintain perfusion of all tissues, and the arteries and veins are the conduit system distributing blood from the heart to peripheral vascular beds of all vascularised tissues and organs (De Moudt 2022; Weyns, 2018). All arteries contain the following three layers: the tunica intima, the tunica media and the tunica adventitia (Figure 6.2). The tunica intima is the innermost layer and consists of a single layer of endothelial cells (EC) supported by a basal elastin layer on the luminal side of the arterial wall which is in direct contact with the blood (Lyle & Raaz, 2017). ECs ensure the release of vasodilatory mediators such as NO, synthesised by endothelial NO synthase (eNOS). The tunica media, mainly consisting of vascular smooth muscle cells (VSMCs) and elastin,

is responsible for determining vascular tone and the production of extracellular matrix (ECM) proteins. VSMCs are organised in laminar structures consisting of circumferentially oriented VSMC layers interspersed by collagen fibres which are partitioned from the surrounding VSMCs by concentric layers of elastin fibres (De Moudt, 2022). An internal elastic lamina separates the tunica media from the tunica intima. The last and outermost layer is the tunica adventitia and mostly includes fibroblasts and collagen, encapsulating the artery and connecting it to the surrounding tissues.

Arteries can be classified into two distinct types based upon their functional and morphological properties: elastic and muscular arteries (Pugsley & Tabrizchi, 2000). Elastic arteries (Figure 6.2) such as the aorta and carotid arteries, fulfill important pulse-smoothening properties of pressure waves and are particularly adapted to accommodate large changes in blood volume such as those associated with left ventricular ejections of the heart. The highly elastic nature of the walls of these blood vessels allows cushioning of the pulsatile nature of forward travelling pressure waves and ensures a homogeneous flow of blood away from the heart. This typical mechanical design of elastic arteries plays a pivotal role in maintaining normal haemodynamics in organisms with a closed circulatory system. On the other hand, muscular arteries such as femoral or mesenteric arteries have a lower elastin content and the tunica media is not organised in elastic lamina, but their walls contain a larger number of smooth muscle cells, responsible for the vascular tone and distribution of blood to all organs and tissues according to the moment-to-moment needs (Leloup, 2019).

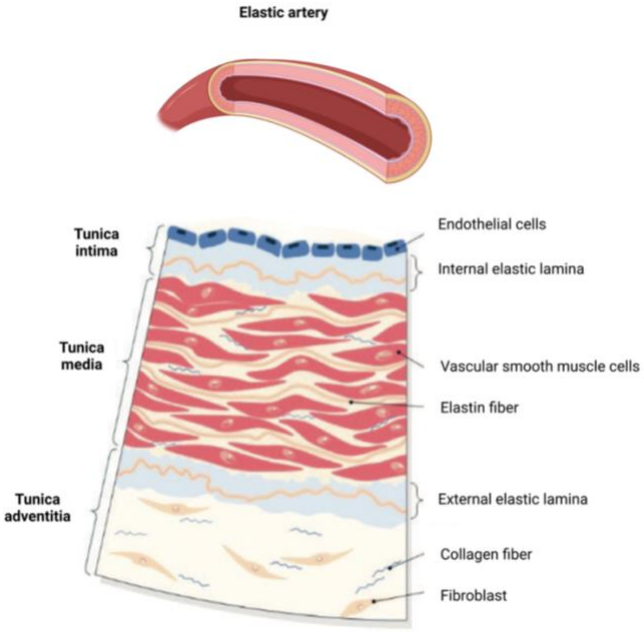

Figure 6.2: Structural cross-sectional overview of an elastic artery. Elastic arteries consist of three main layers: tunica intima, tunica media and tunica adventitia, separated by an internal and external elastic lamina. EC, VSMC and fibroblasts make up the intimal, medial, and adventitial layers, respectively. EC: endothelial cell; VSMC: vascular smooth muscle cell. Figure adapted from De Moudt, 2022.

Elastic arteries display a clear non-linear stiffness-pressure relationship, with a limited increase in stiffness (stiffness is the resistance offered by an elastic body to deformation (Chirinos et al., 2019)) in the physiological pressure range but an exponential increase at high distending pressure as shown in Figure 6.3. This phenomenon can be explained by elastin and collagen fibres, the two load-bearing ECM components of the arterial wall. At low-to-normal distending pressures, the main load-bearing component is elastin and the blood vessel is therefore highly elastic at this physiological pressure. At increasing distending pressures, collagen is slowly recruited as load-bearing material. Since collagen is very inextensible, it acts as a stiff reinforcing network in the arterial wall. In contrast, upon higher distending pressures, the compliance (the ability of the blood vessel to distend and increase volume with increasing transmural pressure) of elastic arteries decreases in a non-linear way, thereby gradually losing their ability to distend

with increasing pressure changes. This non-linear stiffness-pressure relation is thus the result of the composition of the arterial wall in which elastin bears the load at low pressure while stiffer collagen fibres are progressively recruited when the pressure increases (Chirinos et al., 2019).

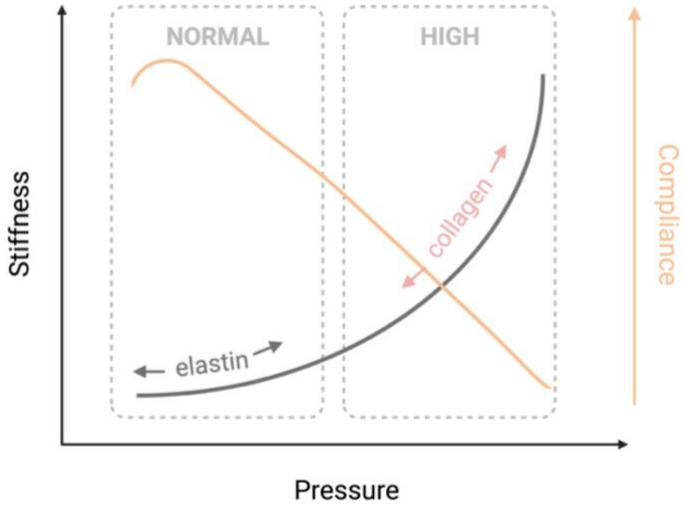

Figure 6.3: Non-linear elastic behaviour in the central arteries. In the low-to-normal pressure range, elastin is the primary load-bearing compound and responsible for a limited increase in arterial stiffness. At a higher distending pressure where collagen is the primary load bearing ECM component, there is an exponential increase at high distending pressure. Contrary, arterial compliance decreases non-linearly with increasing distending pressure. ECM: extracellular matrix. Created in BioRender.

As mentioned earlier, arterial elasticity of central arteries is of utmost importance to maintain a normal physiological function. It allows a part of the stroke volume to be stored in the elastic arteries by expanding during the systole while it is more gradually released during the diastole by passive recoil, thereby achieving a constant blood flow in the microcirculation. This “cushioning” function of the large arteries, in which the aorta serves as a compliant, dampening reservoir is known as the Windkessel phenomenon (Lacolley et al., 2017). On the contrary, increased stiffness of the large arteries reduces the capacity of the elastic arteries to cushion the pulsatile blood flow coming from the heart, with dire CV consequences (Leloup, 2019).

6.3.2 The pathophysiology of arterial stiffness

In normal physiological circumstances, the pulsatile flow generated by the pulsatile ejections from the left ventricle during every heartbeat, is thus converted into a continuous flow by dampening of the generated pressure wave by the arterial wall as illustrated in Figure 6.4. The walls can dilate and recoil as a response to these pressure changes by the local release of NO and by stretching of the arterial wall due to the presence of elastic elastin fibers. When the heart contracts, a pressure wave is generated travelling through the arteries. During the diastolic phase the generated wave will be reflected back to the heart leading to a decrease in pulse pressure (PP; the difference between the systolic and diastolic pressure) and enables perfusion of the coronaries with oxygen-rich blood during the diastolic phase. The speed at which this BP pulse propagates throughout the circulatory system is indicated by the pulse wave velocity (PWV; usually expressed in m/s) (Chirinos et al., 2019).

However, when the arteries get stiffer, they slowly lose their ability to cushion the pulsatile blood flow coming from the heart. Also, the amplitude and the reflection speed of the pressure wave will increase, resulting in an increased PWV. The reflected wave will already arrive during the late systolic phase, thereby increasing the left-ventricle afterload of the heart. Consequently, the heart needs to pump harder to overcome this increased afterload. Moreover, the early return of the reflected wave augments the systolic pressure, results in a higher PP, and leads to a decreased diastolic coronary perfusion. Apart from the effects on the heart, the transmission of the higher arterial pulsatile energy into the microcirculation can damage the capillary wall of end-organs, especially the strongly perfused brain and kidneys (Leloup, 2019; Namba et al., 2019).

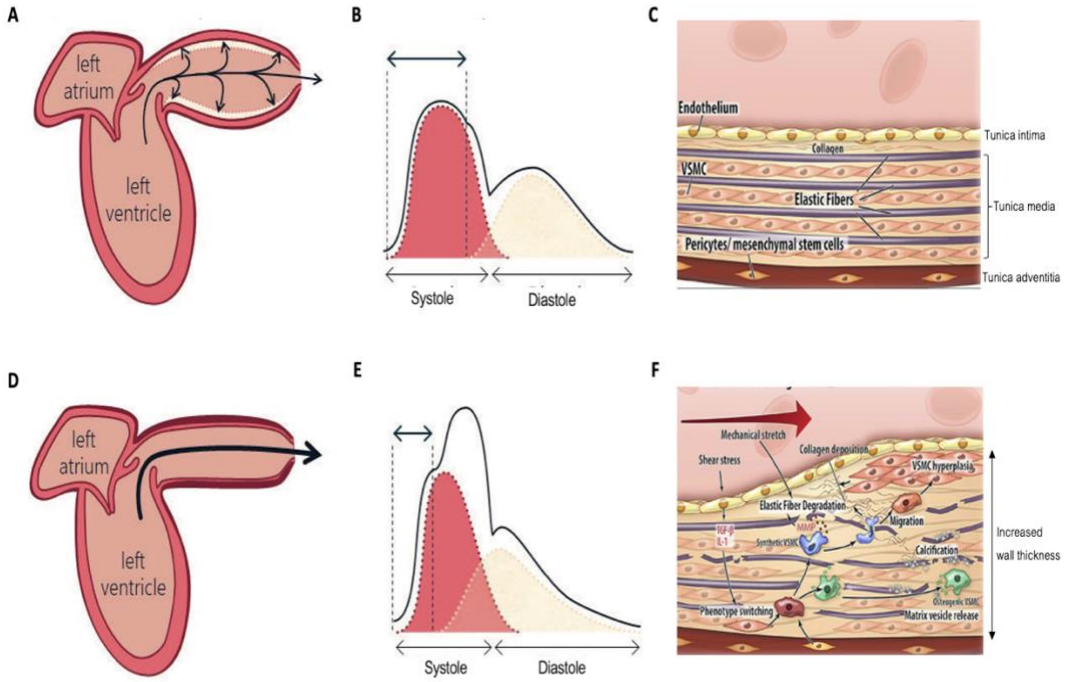

Figure 6.4: A: A healthy compliant aorta distends upon left ventricular contraction to dampen the pulsatile blood flow coming from the heart. B: The forward travelling pressure wave (red) precedes the backwards travelling wave (beige) which is a reflection of the forward travelling wave when it collides with points of resistance (healthy aorta). This reflected wave arrives back in the aorta during diastole, thereby supplying the coronary arteries with oxygenated blood. The cumulative pressure profile is represented by the black line and the delay between the forward and backward travelling waves is indicated by an arrow. C: Representation of a healthy artery with the three layers: tunica intima, tunica media and tunica adventitia. D: Increased stiffness of the arteries reduces the capacity of elastic arteries to cushion the pulsatile blood flow coming from the heart. E: Due to this impaired distension, the forward travelling wave is faster and the reflected wave already arrives during late systole in the proximal aorta (the smaller arrow indicates the early return of the backward travelling wave). The forward travelling and reflected wave overlap, resulting in an increased systolic BP and a reduced diastolic BP. F: Structural and functional change occurring in a stiffer artery such as thickening of the vessel wall, an increase in collagen deposition, a decrease in elastin content and functionality. BP: blood pressure; IL-1: interleukin 1; MMP: matrix metalloproteinases; TFG-β: transforming growth factor-beta; VSMC: vascular smooth muscle cell. Adapted from De Moudt, 2022.

6.3.3 Mechanisms underlying arterial stiffness

Upon ageing, large arteries slowly lose their powerful cushioning function and gradually become stiffer. Also, various pathological conditions impair its normal functioning and exert a negative effect on CV health (Chirinos et al., 2019). In general, the stiffness of large arteries is typically associated with alterations in the ECM, composed of a complex network of different matrix proteins, metalloproteases and glycosaminoglycans, responsible for the structural integrity of the vasculature (Ma et al., 2020). They involve interactions between the individual ECM components' integrity and structure, with passive material fatigue due to repetitive loading and unloading of the ECM fibres as an important contributing mechanism. Besides these so-called 'passive' mechanisms, also 'active' signalling pathways in ECs and VSMCs appear to contribute to the overall stiffness of the arterial wall. Arterial remodelling refers to the plethora of structural and functional changes of the vascular wall that occurs following aging, disease or injury (Figure 6.5). Indeed, ageing is linked to a decline in normal physiological function, including reduced function of the arteries (Hawes, 2020). Even though arterial remodelling due to various risk factors is considered to be a natural occurring process as we get older, early arterial remodelling is associated with haemodynamic changes and CV morbidity and mortality (Van Varik et al., 2012).

6.3.3.1 Passive contributors: alterations in ECM structure

6.3.3.1.1 Elastin

AS is often considered to be associated with degradation, cross-linking or fragmentation of elastin due to mechanical factors as well as biochemical processes such as calcification or glycation. Elastin, produced by VSMCs, is pivotal for normal elastic behaviour of large arteries (Heinz, 2021). Even though elastic fibre production is restricted to foetal life and infancy, this limited synthesis time is enough to last a lifetime in most species due to the long half-life (approximately 75 years half-life) (Wang et al., 2021). Nevertheless, under pathological conditions such as hypertension, an increase in ECM proteins including elastin has been demonstrated. In fact, these newly produced elastic fibres were shown to be less effective than those from early development and may even favour loss of wall resilience in hypertension (Arribas et al., 2006).

Generally, elastin ensures the elasticity of the vessel at the lower range of vessel stretch (low-to normal distending pressures, as shown in Figure 6.3), while collagen provides rigidity at the higher range of vessel stretch (Wahart et al., 2022). With every heartbeat, a healthy thoracic aorta of young humans dilates approximately 10% in the physiological pressure range (Boutouyrie et al., 1992). Since elastin is among the longest living proteins in the human body, millions of loading cycles throughout an organism's lifetime, make elastin vulnerable to material fatigue and fracture as a result of these repetitive cycles of stretching (Heinz, 2021). Impairments or even ruptures result in a reduced elastic fibre function and to a progressive transfer of wall stress from compliant elastic fibres to the more rigid collagen fibres (Leloup, 2019; Wahart et al., 2022).

Besides this mechanical fatigue, elastic fibre function can also be impaired by the action of members of several classes of extracellular proteinases. These elastin-cleaving proteases (the so-called elastases) belong to the following three families: serine proteinases (i.e. cathepsin G, proteinase-3 and human leukocyte elastase), matrix metalloproteinases (MMPs) and the cysteine proteinases (i.e. cathepsins K, L, S, and V). All these enzymes are capable of irreversibly damaging elastin *in vivo* (Heinz, 2021). Next to a mechanical impairment, elastin degradation also results in the formation of small soluble bioactive peptides, i.e. elastin-derived peptides (EDPs), which can for instance stimulate the proliferation and migration of VSMCs, disrupting their quiescent state and thereby promoting pathological remodelling (Le Page et al., 2019). Due to its long half-lifetime, elastin is also prone to biochemical reactions, frequently called non-enzymatic post-translational modifications, by binding of small molecules to functional groups of proteins, which result in altered structural and functional properties. For instance, glycation, in which reducing sugars or their metabolites react with a protein amino group followed by molecular rearrangements, results in the formation of advanced glycation end products (AGEs) (Wahart et al., 2022). It has been proposed that these products form permanent cross-links between collagen and elastin molecules, thereby changing normal cell-matrix interactions and leading to stiffening of the vessel wall (Lyle & Raaz, 2017).

6.3.3.1.2 Collagen

Unlike elastin, collagen is a very stiff protein (1000 times more rigid as compared to elastin) which provides structural integrity and limits vessel distension. Collagen, more specifically collagen I and III, are the most abundant in the vascular wall (Ma et al., 2020; Wang et al., 2021). Increased collagen content and destruction of the elastin fibre network is an accepted marker of passive arterial wall stiffness (Arribas et al., 2006; Harvey et al., 2016). Transforming growth factor-beta (TGF-β), a multifunctional cytokine belonging to the TGF superfamily, appears to be involved in ECM modulation by upregulating the expression and production of collagen I in VSMCs (Ma et al., 2020). Besides non-enzymatic matrix cross-linking by AGEs, enzyme-mediated cross-linking by for instance lysyl oxidase (LOX), which regulates the cross-linking of both elastin and collagen during fibre assembly, can also lead to increased stiffness in the arterial wall due to the formation of increasingly insoluble collagen fibres, which are more resistant to enzymatic activity and show progressively higher tensile strength (Cai et al., 2021).

6.3.3.1.3 Matrix metalloproteinases

In a healthy artery, ECM proteins including collagen and elastin are tightly regulated by MMPs, a group of zinc-dependent endopeptidases which regulate the degradation of ECM through their collagenolytic and elastinolytic activities (De Moudt, 2022; Harvey et al., 2016). MMPs are involved in various biological processes including tissue repair and remodelling. Under physiological conditions, there is a balance between the formation and destruction of the ECM, leading to a state of vascular homeostasis (Cai et al., 2021). Moreover, MMP activity is inhibited by endogenous tissue inhibitors of metalloproteinases (TIMPs) and vascular ECM turnover is regulated by an equilibrium between MMPs and these TIMPs. Nevertheless, dysregulation of MMP activity and MMPs/TIMPs ratio or an overexpression of MMPs can lead to impaired ECM homeostasis and the progression of several pathologies (Cai et al., 2021). For example, MMPs has been shown to be involved in the development and progression of atherosclerosis (Laronha & Caldeira, 2020). Furthermore, MMPs activation facilitates arterial remodelling during ageing (Wang et al., 2015). In fact, activated MMPs degrade collagen, elastin and other ECM proteins, resulting in a modified ECM which is associated with a pro-inflammatory micro-environment that triggers a shift of ECs and VSMCs to a more secretory, migratory, proliferative and senescent phenotype. This phenotype

switch (see 6.3.3.2) contributes to fibrosis, calcification, endothelial cell dysfunction and increased intima thickness, further impacting the vascular remodelling and arterial stiffness (Harvey et al., 2016).

6.3.3.2 Active contributors: VSMCs and endothelial dysfunction

6.3.3.2.1 Vascular Smooth Muscle Cells

Around 40-50% of the total wall volume of the large elastic arteries consists of VSMCs, which are aligned circumferentially in the tunica media and can undergo large deformations in physiological conditions and are pivotal in maintaining vessel structure and function (Leloup, 2019; Mozafari et al., 2019). Though the amount of VSMCs is lower than in muscular arteries, VSMCs still constitute a substantial component of the vessel wall of elastic arteries (De Moudt, 2022). In the capillaries, the amount of VSMCs gradually decreases and only ECs remain present (Mozafari et al., 2019).

VSMCs closely interact with the ECM and are responsible for the synthesis and regulation of different ECM proteins and can adapt to mechanical forces while ensuring adequate wall pressures (Barallobre-Barreiro et al., 2020). VSMCs display a high degree of plasticity and oscillate between two extreme phenotypes: a differentiated state often called “contractile” and a de-differentiated state often referred to as “synthetic” phenotype (Jaminon et al., 2019; Sorokin et al., 2020). VSMCs phenotype switching is usually triggered and regulated by growth factor/inhibitors, transcription factors, mechanical force, cell–cell and cell–matrix interactions, as well as a broad range of inflammatory mediators (Atkinson et al., 2023; Cao et al., 2022). Under normal, physiological conditions, VSMCs are in the contractile state which facilitates the contraction and dilation of the vasculature, and is crucial for the regulation of blood flow (Jaminon et al., 2019). These differentiated VSMCs are relatively quiescent and rich in smooth muscle contractile proteins. However, upon biological stress signals such as vascular injury or inflammation, VSMCs can de-differentiate towards a synthetic phenotype by reducing the expression of contractile proteins as well as by increasing the production of proteases which can degrade elastin and collagen and promote vessel dilation and ECM remodelling (Cao et al., 2022). The oscillation of VSMCs between these two functionally

distinct states is well-known as “phenotypic switching” and serves as a vital step in the repair process of vascular damage since the acquired proliferative and migration capacity of VSMCs allows neointima formation after vascular injury. After the injury is healed, VSMCs regain their contractile characteristics and this phenotype switching for repair is thus reversible (Cao et al., 2022). However, besides these adaptive phenotype changes under physiological conditions (e.g. during vascular injury), also pathological conditions (e.g. in atherosclerosis, hypertension) can cause a phenotypic switch which eventually can become an irreversible process. Several studies have pointed out that VSMCs phenotype switching underlies the progression of vascular diseases (Cao et al., 2022).

Next to phenotypic switches, VSMCs also play a key role in vascular calcification. It is considered to be an active process defined as a pathological mineral deposition (e.g. calcium phosphate salts) within the vessel wall, in both the tunica intima and tunica media (Jaminon et al., 2019). Though the mechanisms underlying arterial calcification are multifactorial and still incompletely understood, the transdifferentiation of VSMCs into osteoblast-like cells can acts as a trigger (Lin et al., 2022). These differentiated VSMCs subsequently produce macrocalcification deposits and calcified plaques causing a gradual decrease in arterial compliance (Durham et al., 2018; Tyson et al., 2020). In order to prevent spontaneous calcification, inhibitors of calcification are present in the circulation and in the vasculature, including fetuin-A, matrix Gla protein (MGP), osteoprotegerin, pyrophosphate and osteopontin (Bäck et al., 2018; Jaminon, 2020).

6.3.3.2.2. Endothelial dysfunction

Besides VSMCs, also endothelial function is important in arterial remodelling (Jaminon et al., 2019). As stated before, the vascular endothelium releases molecules such as NO that act in an autocrine and paracrine way to regulate the normal function and health of the vascular network (Donato et al., 2018). It activates several mechanotransduction pathways in order to maintain tissue integrity and the vascular barrier function during the rapid mechanical forces that derive from the pulsatile blood flow and vessel wall contractions (Hooglugt et al., 2022). Blood flow and wall shear stress stimulate ECs to produce NO which in turn induces relaxation of local VSMCs, leading to dilation of the vessel wall (Jaminon et al., 2019). Furthermore, besides its vasodilating properties,

endothelial NO has anti-proliferative and anti-inflammatory actions, regulates fibrinolysis as well as the coagulation pathway, thereby maintaining the homeostatic properties of the blood vessels (Cassano et al., 2022). In contrast, a dysfunctional endothelium is characterised by a reduced bioavailability of NO, an imbalance between vasoconstrictor and vasodilator factors, and is predisposed to a pro-atherogenic and pro-thrombotic phenotype (Cassano et al., 2022). Moreover, reduced endothelial NO production might influence the regulation of VSMCs tone (Chirinos et al., 2019). Under pathological conditions, ECs produce cytokines and grow factors which induce VSMCs phenotype switching from a contractile to a more synthetic state (Jaminon, 2020).

It should be noted that all these processes (both passive and active mechanisms) contributing to AS, do not occur in isolation. In fact, they often interact to promote AS. For instance, elastin fragmentation is frequently followed by collagen accumulation, whereas the generation of elastin degradation products can activate signalling pathways that promote VSMCs differentiation. Furthermore, inflammatory pathways can lead to endothelial cell dysfunction (Chirinos et al., 2019).

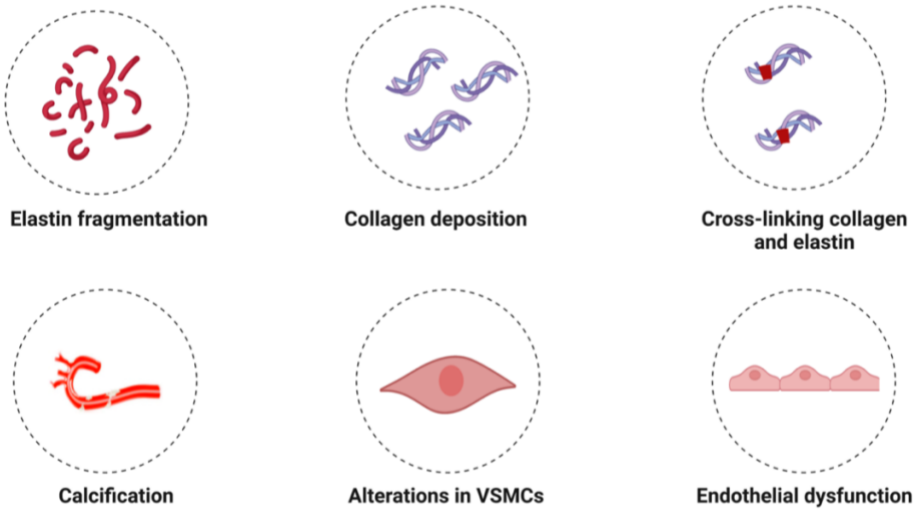

Figure 6.5: General mechanisms leading to arterial stiffness. Various processes such as elastin fragmentation, collagen deposition, collagen and elastin cross-linking, calcification, VSMCs stiffening, and phenotypic switching and endothelial dysfunction can impact arterial stiffness. VSMCs: Vascular Smooth Muscle Cells. Created in BioRender.

6.3.4 Risk factors for arterial stiffness

Various risk factors contributing to mechanisms underlying AS are listed in Figure 6.6. All CV risk factors mentioned earlier are also known risk factors of AS (Van Herreweghe, 2021). Nevertheless, ageing remains the most important one because after millions of pulse-dampening cycles over the course of a lifetime, age-related fatigue and fracture of elastin play a pivotal role in arterial ageing (Heinz, 2021).

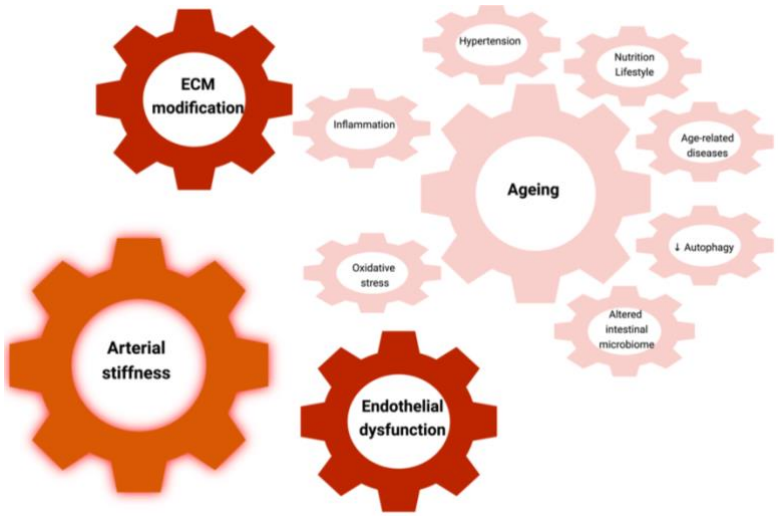

Figure 6.6: Multiple risk factors contribute to arterial stiffness. Hypertension, oxidative stress, inflammation and age-related diseases are pivotal in the structural and functional alterations leading to arterial stiffness. Created in BioRender.

Ageing often coincides with other risk factors such as metabolic syndrome, type 2 diabetes, atherosclerosis and hypertension, as well as other medical conditions such as obesity. Also, chronic inflammation and oxidative stress are key mechanisms, associated with for example smoking, high prooxidant-intake via refined foods, ROS formation and chronic inflammatory diseases (De Bruyne et al., 2019). Autophagy, which normally acts as a defence mechanism against cell death by removing damaged cellular structures, can be impaired as well (De Munck et al., 2020). Some genome-wide studies have already discovered associations between certain genetic polymorphisms and susceptibility to AS (Averta et al., 2021). All these mechanisms can play a role in the pathogenesis of AS and

targeting them may play a substantial part in its prevention and/or treatment. In this thesis, the focus will mainly be on oxidative stress and inflammation and their role as a possible target for dietary polyphenols in the prevention and/or treatment of AS.

6.3.5 Role of oxidative stress in arterial stiffness

Oxidative stress is a crucial underlying factor in health and disease. Oxidative stress is not only associated with neurodevelopmental diseases such as ADHD, but it is also a leading cause in AS and CVD (Sena et al., 2018). At physiological levels, ROS control vascular function by modulating several redox-sensitive pathways.

Oxidative stress; however, instigates endothelial dysfunction and inflammation, thereby affecting vascular cell functioning (Hendrickx et al., 2021). Oxidative stress can result in an uncoupling of eNOS or can cause oxidative damage to proteins, lipids and DNA of vascular cells. Besides these functional alterations, oxidative stress may also initiate structural changes leading to AS by stimulating hyperplasia of VSMCs and increase collagen synthesis (Ismaeel et al., 2018).

6.3.5.1 eNOS uncoupling

As stated before, NO is a key molecule that significantly influences the physiology of the vascular endothelium (Janaszak-Jasiecka et al., 2023). Since it is the main vasorelaxant agent of the arterial system, endothelial NO is tightly regulated (Figure 6.7) (De Moudt, 2022). In endothelial cells, NO is produced by eNOS, one of the three nitric oxide synthases (NOS) present in human tissues, besides neuronal NOS (nNOS) which is primarily expressed in neurons, and inducible NOS (iNOS), expressed in several cell types (mainly in immune system cells) during infection or inflammation (Janaszak-Jasiecka et al., 2023). eNOS utilises L-arginine and L-citrulline to produce NO. However, under pathological conditions such as oxidative stress, eNOS becomes dysfunctional and produces highly reactive superoxide radical anions $O_2^{\bullet-}$ rather than NO. The presence of both functional and uncoupled eNOS in the cell can result in the concomitant production of NO and $O_2^{\bullet-}$ in the close vicinity. Moreover, NO can react with $O_2^{\bullet-}$ leading to the formation of peroxynitrite ($ONOO^-$) and thus resulting in even more ROS production.

eNOS uncoupling is thought to be one of the major causes underlying endothelial dysfunction observed in the pathogenesis of AS and CVD. Therefore, the mechanisms leading to eNOS uncoupling are considered promising therapeutic targets (Janaszak-Jasiecka et al., 2023; Lee & Im, 2021).

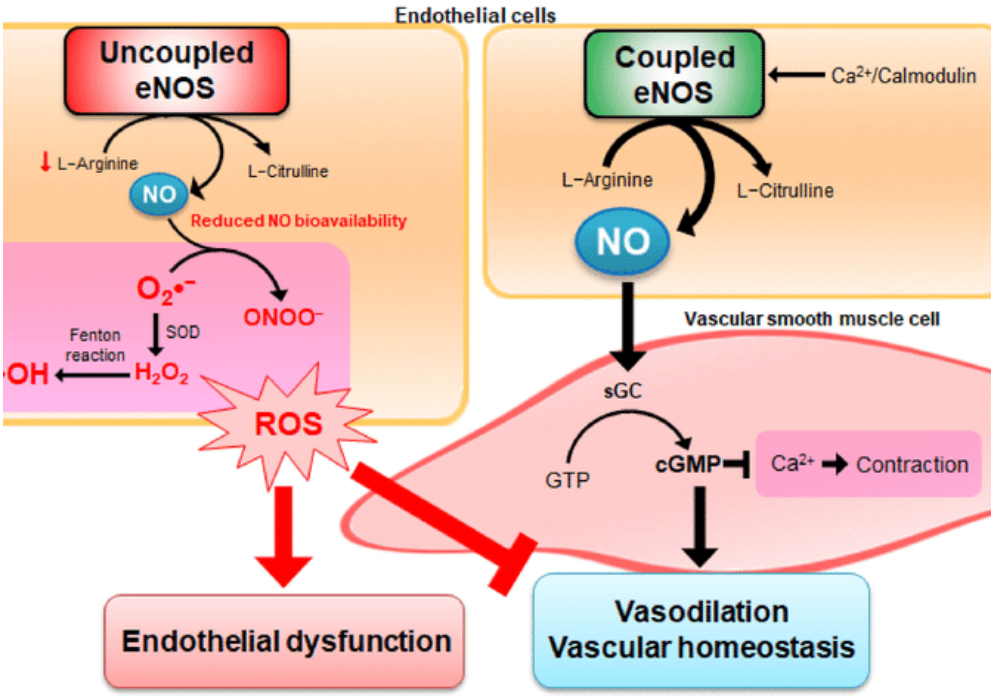

Figure 6.7: Endothelial dysfunction and eNOS uncoupling. Coupled eNOS utilises L-arginine to produce NO and L-citrulline. NO is important in the vasodilation of VSMCs to maintain vascular homeostasis. Under pathological conditions, eNOS becomes dysfunctional and produces $O_2^{\bullet-}$ rather than NO. $O_2^{\bullet-}$ are dismutated to form H_2O_2 by SOD and are used to generate $OH^{\bullet}$ by the interaction of Fe^{2+} and H_2O_2 (Fenton reaction). Moreover, the interaction between NO and $O_2^{\bullet-}$ leads to the formation of $ONOO^-$ and thus results in more ROS production with subsequent endothelial dysfunction. eNOS: endothelial nitric oxide; Fe^{2+}: ferrous ion; H_2O_2 : hydrogen peroxide; NO: nitric oxide; $O_2^{\bullet-}$: superoxide anion; $ONOO^-$: peroxynitrite. Adapted from Lee & Im, 2021.

eNOS activity can be regulated by post-translational modifications including phosphorylation, which can occur at multiple sites including serine, threonine and tyrosine residues (Eroglu et al., 2019). Depending on the site of phosphorylation, it can

either result in eNOS activation or in inhibition of eNOS activity. Phosphorylation of eNOS at serine 1177 (S1177) is the most extensively studied eNOS phosphorylation site and results in activation of eNOS whereas the activity of eNOS decreases with phosphorylation at threonine (Thr495) (Erdek et al., 2022).

6.3.5.2 Dysregulation of antioxidant defence mechanisms

AS is not only characterised by an increased ROS generation, but also by dysregulated antioxidant defence mechanisms (El Assar et al., 2022). In the vascular wall, the primary antioxidant defence systems to neutralise ROS are for example SOD, CAT, GPx and HO (Sena et al., 2018). In addition, the nuclear factor erythroid 2–related factor 2 (Nrf2) antioxidant defence pathway as illustrated in Figure 6.8 is also pivotal in regulating the oxidative stress response in cells (Arefin et al., 2020).

Nrf2 belongs to the family of transcription factors that activate a battery of cytoprotective genes through the antioxidant respons elements (ARE), participating in biotransformation, antioxidant and inflammation reactions (Martínez-Huélamo et al., 2017). By activating protective proteins, Nrf2 helps to prevent, deactivate and repair oxidative stress and oxidative damage. Under basal conditions, Nrf2 is maintained inactive in the cytoplasm bound to the Kelch-like ECH-associated protein 1 (Keap1), a receptor of electrophilic compounds and stimulator of Nrf2 ubiquitination and proteosomal degradation (Arefin et al., 2020). Keap1 is thus associated with Nrf2, forming a complex anchored by actin in the cytoplasm, and negatively regulates the function of Nrf2. Nrf2 activity can be induced by several mechanisms of which the Keap1 dissociation by oxidative stress or by the covalent modification of the thiol groups of Keap1 are the best known (Martínez-Huélamo, et al., 2017). Indeed, upon oxidative stress, Nrf2 dissociates from Keap1 and is quickly translocated to the nucleus where Nrf2 heterodimerises with musculoaponeurotic fibrosarcoma (Maf) protein and binds to the ARE, to activate the transcription of antioxidant genes (Ngo & Duennwald, 2022). These antioxidant enzymes and phase II antioxidant enzymes such as SOD and heme oxygenase 1 (HO-1) remove ROS and thereby counteract oxidative stress and facilitate anti-inflammatory, anti-apoptosis and other cell protective mechanisms (Zhang et al., 2021). In addition to Keap1-dependent regulation, Nrf2 can be phosphorylated by several kinases as shown in Figure 6.8. For instance, protein kinase C (PKC) phosphorylates Nrf-

2, followed by dissociation of Nrf2 from Keap1, which promotes nuclear translocation of Nrf2.

HO-1, an endogeneous antioxidant regulated by Nrf2, is an important inducible stress response protein that acts as the key-limiting enzyme in the degradation of heme into CO, Fe^{2+}, and biliverdin. Subsequently, biliverdin is converted to bilirubin by biliverdin reductase. Both biliverdin and bilirubin act as antioxidants by scavenging ROS. CO functions in the signal transduction by vasodilating the blood vessels, and by producing anti-inflammatory cytokines. Though Fe^{2+} exerts a pro-oxidant activity, activation of HO-1 upregulates ferritin expression which binds Fe^{2+} and thereby detoxifies this pro-oxidant effect (Chiang et al., 2021).

Seen its pivotal role in oxidative stress regulation and additional roles in many other cellular processes, aberrant Nrf2 expression has been associated with numerous pathologies including CVDs (Zhao et al., 2021). Moreover, since Nrf2 acts a major modulator of antioxidant defence, it is also closely associated with AS and is reported to play an antagonistic role in its development (Wu et al., 2022). In fact, emerging evidence points out the anti-oxidative potential of HO-1 to protect ECs from oxidative stress-induced injury and to inhibit vessel remodelling and endothelial dysfunction. Since the activation and transcription of Nrf2 is crucial for HO-1 expression, Nrf2/HO-1 signalling activation would be beneficial to protect ECs from oxidative stress-induced injury and thereby controlling AS (Zhang et al., 2021).

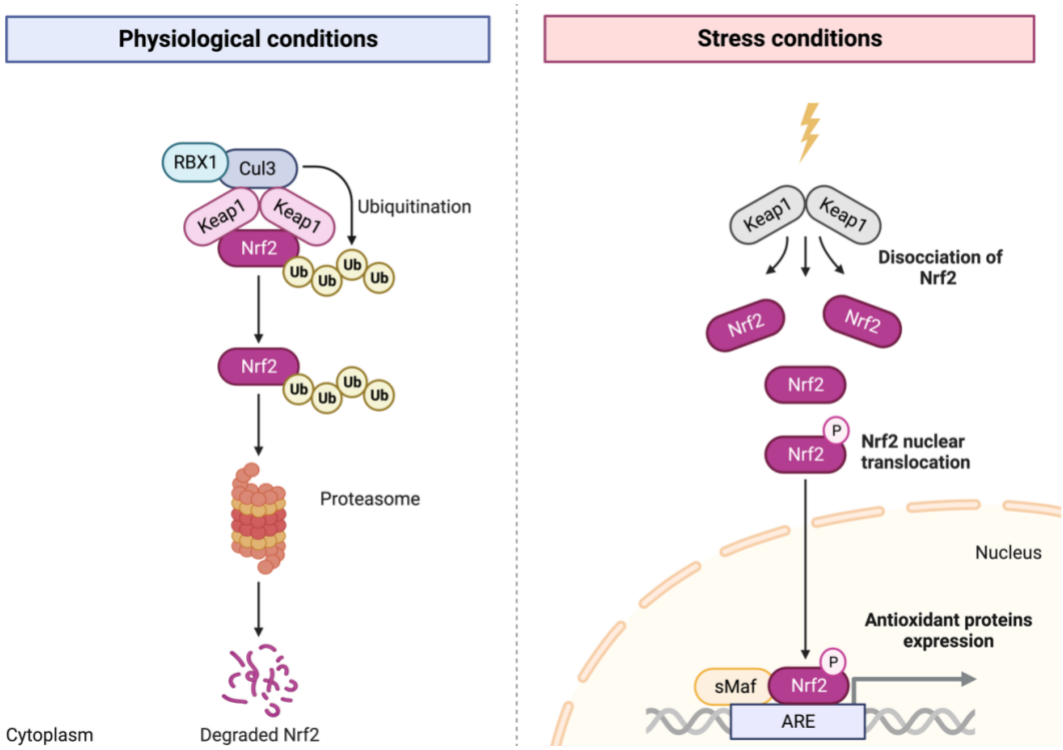

Figure 6.8: Nrf2/ARE signalling pathway. Under physiological conditions, Keap1 forms a complex with Nrf2 in the cytoplasm and ubiquitinated Nrf2 promotes proteolysis of Nrf2 by proteosomes. Under stress conditions Nrf2 dissociates from the Keap/Nrf2 complex, translocates into the nucleus where it combines with Maf and ARE to initiate transcription of downstream genes such as HO-1. ARE: antioxidant response elements; HO-1: heme oxygenase 1; Maf: musculoaponeurotic fibrosarcoma; Nrf2: nuclear factor erythroid 2–related factor 2; P-Nrf2: phosphorylated Nrf2; Ub: ubiquitinated. Figure created in BioRender.

6.3.6 Role of inflammation in arterial stiffness

Though inflammation is a physiological response of the organism to harmful stimuli, it becomes deleterious when the pro-inflammatory process becomes persistent (Ngo & Duennwald, 2022). An inflammatory state, characterised by the presence of immune cells such as macrophages, a highly oxidative environment and upregulation of the expression of pro-inflammatory cytokines, creates a condition in which the vessel wall can undergo changes leading to AS (Jain et al., 2014). In fact, epidemiological data from

several studies have already demonstrated the importance of inflammation in CVD and moreover, pointed out its role in the regulation of AS (Maki-Petaja & Wilkinson, 2022).

There is growing evidence identifying the role of inflammation in AS; however, the mechanisms behind inflammation induced AS are still incompletely understood. Some of these mechanisms could induce rapid changes in the stiffness of large arteries through functional effects on the vessel wall, whereas others link inflammation with structural changes in the arterial wall (Jain et al., 2014). Nevertheless, there is no absolute distinction since inflammation induced structural and functional alterations are likely to be interconnected (Jain et al., 2014).

6.3.6.1 Structural modifications in the arterial wall

Inflammation can induce structural modifications in the arterial wall through the breakdown of elastin, proliferation and phenotypic switching of VSMCs, and alterations in the ECM composition. As mentioned earlier, fragmentation of elastin leads to a greater transfer of load to the less compliant collagen fibres, resulting in stiffer arteries. Though this elastin fracture can be considered as a passive mechanism resulting from the multiple pulsations throughout a lifespan, it can also be caused by proteolytic enzymes (i.e. MMPs) activated during inflammatory conditions (Jain et al., 2014). For instance, MMP-2 is produced constitutively by ECs and VSMCs at physiological conditions and its enzymatic activity is not significant due to a tight counterbalance with TIMPs. However, ROS and pro-inflammatory stimuli can alter this equilibrium as shown in Figure 6.9. Pro-inflammatory cytokines such as IL-1 can activate macrophages and neutrophils, who in turn produce MMP-1, MMP-7 and MMP-9 leading to elastin degradation. Similar to these MMPs, MMP-12 can also degrade elastin and activate MMP-2 and MMP-3, which subsequently leads to the degradation of other ECM proteins (Wu et al., 2003). Another important enzyme is lysyl oxidase (LOX), which serve an important role in the formation of the ECM by modulating the crosslinking of elastin and collagen (Lyle & Raaz, 2017). According to Rodriguez et al. (Rodríguez et al., 2008) pro-inflammatory TNF-α down-regulates LOX levels, thereby altering the structural composition of the vessel wall.

Inflammatory reactions may also change the ECM structure. The ECM consists of fibrous proteins and glycoproteins which are embedded in a hydrated ground substance of prostaglandins (PGs), proteins with a glycosaminoglycan (CAG) chain attached to it (Maki-Petaja & Wilkinson, 2022). These PGs exert various roles within the ECM including hydration, filtration as well as modulating inflammatory processes. During inflammation CAGs such as hyaluronan accumulate in the tunica intima. Tissue enriched with CAGs traps water and swells, thereby increasing the water content of the vessel wall, allowing the ECM to resist compression forces. As a result, the arterial wall becomes stiffer (Figure 6.9) (Jain et al., 2014) .

Moreover, besides oxidative stress markers, also cytokines can induce a phenotypic transformation of VSMCs into osteoblast-like cells, leading to calcification of the vessel wall. Fetuin-A for instance, which under normal physiological conditions acts as an endogenous inhibitor of vascular calcification, is downregulated during inflammation (Chekol Abebe et al., 2022).

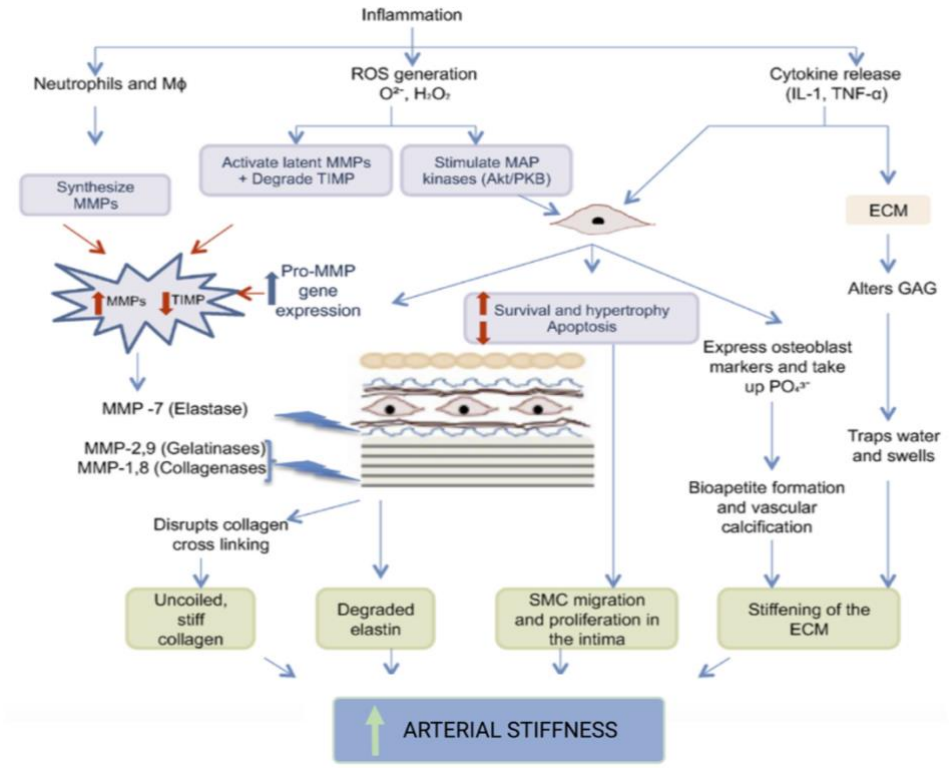

Figure 6.9: Possible mechanisms by which inflammation can induce structural changes in the vessel wall contributing to increased vascular stiffness. Mφ: macrophage; CRP: C reactive protein; ROS: reactive oxygen species; $O_2^{\bullet -}$:superoxide; H_2O_2: hydrogen peroxide; Akt/PKB: serine threonine kinase/protein kinase B; SMC: smooth muscle cell; MMP: matrix metalloproteinase; TIMP: tissue inhibitor of matrix metalloproteinase; BM: basement membrane; ECM: extracellular matrix; PO_4^{3-}: phosphate; GAG: Glycosaminoglycan; TNF-α: tumor necrosis factor α; IL-1: Interleukin-1. Adapted from Jain et al. 2014.

6.3.6.2 Functional modification in the arterial wall

NO is pivotal in endothelial functioning and inflammatory cytokines can impair the vasodilatory response by reducing the bioavailability of NO (Jain et al., 2014). Indeed, inflammatory cytokines can cause eNOS uncoupling, resulting in a decreased NO production and bioavailability. Additionally, C-reactive protein (CRP) has been shown to decrease eNOS expression, thereby decreasing NO production (Jain et al., 2014).

Apart from inflammatory cytokines, other enzymes and mediators may also influence inflammation-induced AS. For instance, cyclooxygenase 2 (COX-2) derived prostaglandins were shown to play a role in all of the aforementioned functional and structural changes by amplifying the effects of other mediators such as cytokines, and therefore perpetuating the inflammatory process (Mozos et al., 2017).

6.3.6.3 The cyclooxygenase pathway in inflammation and cardiovascular health

There are various risk factors contributing to the pathogenesis of CVDs. Among them, the arachidonic acid (AA) pathway is pivotal in CV health regulation (Beccacece et al., 2023; Rytz et al., 2023). In fact, the AA pathway is the most essential metabolic precursor for various inflammatory pathways (Attiq et al., 2018).

AA is a ω-6 PUFA abundantly found in cell membrane phospholipids and is mainly obtained through the diet (Beccacece et al., 2023). AA can be metabolised by three distinct classes of enzymes, i.e., COXs, LOXs, and cytochrome P450 (CYP) enzymes (ω-hydroxylases and epoxygenases) to generate a broad spectrum of biologically active fatty acid mediators (Wang et al., 2021). Here, the main focus will be on the cyclooxygenases (COXs), the main pathway in the conversion of AA to prostanoids, a subclass of eicosanoids including prostaglandins (i.e. PGE_2, PGD_2, $PGF_{2\alpha}$), thromboxanes (TXA_2) and prostacyclins (PGI_2) (Nørregaard et al., 2015; van der Auwera, 2023). The COX enzymes synthesise prostanoids, starting from AA, in three steps as depicted by Figure 6.10. First, AA is mobilised from cell membrane phospholipids by phospholipase A_2. The bioavailability of AA is thus tightly linked to phospholipase A_2 activity since it is responsible for cleaving AA from cell membranes, and is considered to be the rate-limiting step influencing subsequent synthesis of PGs (Beccacece et al., 2023). Lastly, COX generates an unstable PGG_2 by the cyclooxygenase reaction, which immediately converts into PGH_2 through the peroxidase reaction. In turn, PGH_2 serves as a substrate for a series of tissue specific isomerases and synthases, producing four signalling PG products (PGE_2, PGI_2, $PGF_{2\alpha}$, and PGD_2) or TXA_2 (Rouzer & Marnett, 2020). These two activities occur at different, but interactive sites within the COX protein. The prostanoids afterwards act via specific G-protein-coupled receptors (GPCRs) and exert multiple and sometimes opposing effects throughout the body (van der Auwera, 2023). Indeed, the

effect of a particular PG can even differ according to the tissue in which it is synthesised or the receptor to which it binds (Rang et al., 2016). Besides AA, COX can also use other substrates than AA, such as unsaturated fatty acids like eicosapentaenoic acid, a nutritionally essential ω6-fatty acid present in for example oily fish (Rouzer & Marnett, 2020).

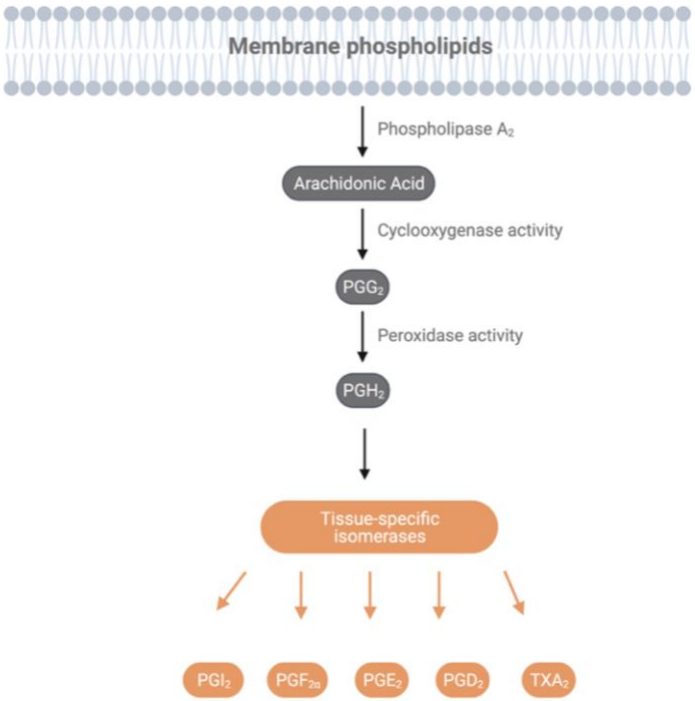

Figure 6.10: Prostaglandin (PG) biosynthetic pathway by cyclooxygenase activity of arachidonic acid. Arachidonic acid is mobilised from membrane phospholipids by phospholipase A_2 activity. Bis-dioxygenation and cyclisation of arachidonic acid at the cyclooxygenase active site of COX-1 or COX-2 yields the unstable PGG_2. Reduction of the 15-hydroperoxyl group of PGG_2 at the peroxidase active site of COX-1 or COX-2 results in the PGH_2, which serves as a substrate for different tissue-specific isomerases and synthases, producing four signalling PG products (PGE_2, PGI_2, $PGF_{2\alpha}$, and PGD_2) or thromboxane A_2 (TXA_2). Created in BioRender.

There are two distinct COX isoforms, COX-1 and COX-2, and their structural homology is about 60%, both having a cyclooxygenase and peroxidase active site in their catalytic

domain (Mitchell et al., 2021; Wang et al., 2021). More recently, a splice variant of COX-1, COX-3 was discovered (Biswas et al., 2023). However, COX-3 inhibition and its mechanism in humans is not fully elucidated yet (van der Auwera, 2023). Only COX-1 and COX-2 will therefore be discussed in this thesis. Each isoform possesses similar activities, but mostly differ in expression characteristics and inhibition profiles by non-steroidal anti-inflammatory drug (NSAIDs) (van der Auwera, 2023). COX-1 is constitutively expressed in most cells and found ubiquitously throughout the body and functions mostly as a homeostatic regulator, synthesising prostanoids subserving housekeeping functions (Wang et al., 2021). COX-2 on the other hand, is induced by inflammatory stimuli (e.g. pro-inflammatory cytokines such as IL-1 and IL-6) , hormones and growth factors, and acts as the more important source of prostanoids formation in inflammation and proliferative diseases (Attiq et al., 2018; Wang et al., 2021). Though COX-1 is often referred to as a "housekeeping" enzyme due to its constitutive role in human physiology, recent COX-1 knock out mice studies have shown that COX-1 is also important in the development and progression of inflammation. In fact, these studies revealed that COX-1 derived PGs initiate the inflammatory acute phase whereas COX-2 related PGs are responsible for maintaining the inflammatory response for several hours (because of the prior need for upregulation) (Attiq et al., 2018). PGE_2, responsible for maintaining various physiological functions in the body, is the main PG in this process. For instance, during inflammation higher levels of COX-2 mediated PGE_2 are responsible for the inflammatory signs and symptoms (e.g. redness, heat, swelling, pain and loss of function). PGE_2 elevates the blood flow towards the site of inflammation through its vasodilatory effects and increases vascular permeability. It also induces pain via sensitising the nerve ending in the central and peripheral nervous system (Attiq et al., 2018).

Since inflammation is pivotal in the development of various diseases and disorders, and COX are the main mediators of inflammation by catalising the first step of AA metabolisation and PG synthesis, COX inhibitors might be a good therapeutic target in inflammatory diseases (Ferrer et al., 2018). NSAIDs block the biosynthesis of PGs through inhibition of COX enzymes (Attiq et al., 2018). NSAIDs are amongst the most commonly used medications worldwide, either by prescription or over-the-counter, with more than 30 million people worldwide using these drugs daily because of their efficient anti-inflammatory, analgesic and antipyretic properties (Conaghan, 2012). Though all NSAIDs

share similarities in their mechanism of action, they slightly differ in the way they interact with the COX enzyme and show differences in their degree of selectivity for the two COX-isoenzymes (van der Auwera, 2023). A distinction can be made between non-selective COX-1 and COX-2 inhibitors (e.g. ibuprofen and indomethacin), preferential COX-2 inhibitors (e.g. meloxicam) and selective COX-2 inhibitors ("-coxibs" such as celocoxib) (van der Auwera, 2023).

Notwithstanding the fact that NSAIDs are widely used to treat pain, fever, and other inflammatory processes, NSAIDs have been associated with adverse and sometimes serious side effects (Rahman et al., 2022). Regarding the most common side effects, it is important to look at their COX selectivity. NSAIDs with a selectivity towards COX-1, cause more GI side effects. Since COX-1 derived PGE_2 and PGI_2 play a protective role in the gastric mucosa (i.e. decrease in gastric acid secretion and enhanced thickness of the mucus layer), blocking COX-1 increases the risk of GI side effects, ranging from mild irritation to more severe adverse events such as bleeding and perforation (van der Auwera, 2023). These GI complications were the driven force in the development of COX-2 selective NSAIDs. Despite their initial success as "safer NSAIDs" by avoiding GI complications, concerns were raised about their CV safety including atherosclerosis, hypertension, heart failure and sudden cardiac death (Chen et al., 2021; Grosser et al., 2017). The preferential inhibition of COX-2 holds greater risks for CV events due to the imbalance between TXA_2 (which acts as a vasoconstrictor and exerts prothrombotic effects) and PGI_2 (an inhibitor of platelet aggregation and a vasodilator) (van der Auwera, 2023). Due to the higher concentration of COX-2 in the vascular endothelium of the kidney, selective COX-2 inhibition leads to inhibition of PG production. This results in a compromised kidney function, increased sodium retention and oedema, contributing to heart and kidney failure (Ribeiro et al., 2022). Nevertheless, various studies investigating the CV safety of COX-2 selective inhibitors and non-selective COX inhibitors (both in populations with mixed baseline CV risk and in patients with elevated baseline risk), revealed similar harmful CV effects for COX-2 selective as well as for non-selective COX-inhibitors.

In conclusion, most of the NSAIDs (except for aspirin, since it prevents rather than triggers CV events (van der Auwera, 2023)) may increase CV risk or have other major side

effects when used chronically (Patil et al., 2015). Hence, it would be beneficial to identify alternative therapeutic options with anti-inflammatory properties but less side effects. Over the years, plant polyphenols have already been proven to interfere with inflammatory signalling pathways and transcription factors and are therefore promising sources of new anti-inflammatory drugs (Goszcz et al., 2017). In light of the prevention of CVDs, it would thus be useful to further investigate polyphenols since they exert anti-inflammatory properties. Moreover, understanding the exact mechanisms by which these polyphenols interfere with signalling pathways is pivotal in view of the development of future therapies.

6.3.6.4 The role of NF-κB in inflammation

Nuclear factor-kappa beta (NF-κB) is considered to be the chief regulator of the immune system as well as the inflammatory response (Patil et al., 2015). It is a small family of inducible transcription factors ubiquitously present in nearly all mammalian cells (van der Auwera, 2023). Besides its involvement in inflammatory and immune responses, NF-κB participates in the regulation of various other genes related to cell survival, proliferation and differentiation (Giuliani et al., 2018). Due to its role in various biological processes, a disruption in NF-κB signalling can have deleterious effects on human health (Mendez et al., 2020). Indeed, emerging evidence suggest that a dysregulated NF-κB activity is linked to inflammation-related diseases and as well as cancers (Yu et al., 2020).

Simply stated, the mechanism underlying NF-κB transcription can be described as follows (Figure 6.11). In normal physiological conditions, NF-κB is present as a heterodimer bound to one of the eight inhibitory proteins of NF-κB (IκBs), retaining it inactive in the cytoplasm (Giuliani et al., 2018). In order to activate the NF-κB pathway, the NF-κB protein needs to be separated from its inhibitor (van der Auwera, 2023). When cells receive intracellular or extracellular stimulation, downstream signalling cascades are triggered, leading to the activation of the IκB kinase (IKK) complex, consisting of three subunits, IKKα, IKKβ and IKKγ (Liu et al., 2022). The activated IKK phosphorylates the inhibitory IκB, resulting in polyubiquitination and leading to proteasomal degradation of IκB. As a result, the free NF-κB dimer is translocated to the nucleus. Here, it binds to the promotor region of target genes and stimulates their

transcription, including pro-inflammatory cytokines, chemokines, adhesion molecules as well as inducible pro-inflammatory enzymes (COX-2 and iNOS) which exacerbate and perpetuate the inflammatory process (Karunaweera et al., 2015). Once the inflammatory triggers are cleared and inflammation is no longer needed, IKK is deactivated and the IκBs accumulate again and remove NF- κB from the DNA back to the cytoplasm (Liu et al., 2022). The first identified and most abundant NF-κB dimer is the p50/p65 heterodimer. Furthermore, various other combinations have been described including homodimers (e.g. p65/p65 and p50/50) and heterodimers (e.g. p65/p52) (Giuliani et al., 2018).

There are two different signalling pathways involved in NF-κB activation: the classical or canonical pathway and the alternative or non-canonical pathway (Yu et al., 2020). The canonical pathway is the most common signalling pathway in the activation of NF-κB and is triggered by diverse pro-inflammatory stimuli, such as inflammatory cytokines and B-cell and T-cell receptor signals. It also activates the most frequent NF-κB dimers, formed by p65, p50, c-Rel and RelB subunits. The non-canonical pathway on the other hand, is activated by a select group of stimuli such as B-cell activating factor (BAFF) and manly targets the activation of the p52/RelB heterodimer complex (Giuliani et al., 2018; van der Auwera, 2023). The canonical pathway for NF-κB is thus triggered by several stimuli, transducing a quick and transient transcriptional activity to regulate a rapid and reversible inflammatory response. On the contrary, since the non-canonical NF-κB pathway requires stimulation through TNF receptor family members and its activation involves protein synthesis, the kinetics are rather slow but persistent, according to its biological functions in immune homeostasis and immune response (Yu et al., 2020).

Since NF-κB signalling pathway is involved in diverse biological processes and dysregulation of NF-κB can lead to autoimmune diseases and chronic inflammation, NF-κB could be an interesting target for therapy by interfering at various levels of regulation (Guan et al., 2022). More than 700 inhibitors of the NF-κB activation pathway have been described and they all can target one of the specific steps in the pathway. Polyphenols for instance, have been documented to counteract the NF-κB signaling pathway and their anti-inflammatory effects are mostly attributed to the inhibition of the canonical

NF-κB pathway (Khan et al., 2019). Polyphenols can inhibit the phosphorylation of kinases, thereby preventing the translocation of NF-κB to the nucleus. Moreover, they can influence the NF-κB transcriptional activity. Lastly, the expression of pro-inflammatory mediators regulated by NF-κB can be inhibited (Khan et al., 2019). For instance, it has been demonstrated that curcumin blocks the translocation of p65 subunits to the nucleus by suppression of the phosphorylation and thus degradation of IκB. In addition, catechin has been shown to suppress the NF-κB signaling pathway in allergic rhinitis mice by suppressing the degradation of IκB and inhibiting nuclear translocation of NF-κB (Khan et al., 2019). Besides its major regulating role in inflammation processes, NF-κB is also a sensor of oxidative stress (Guan et al., 2022).

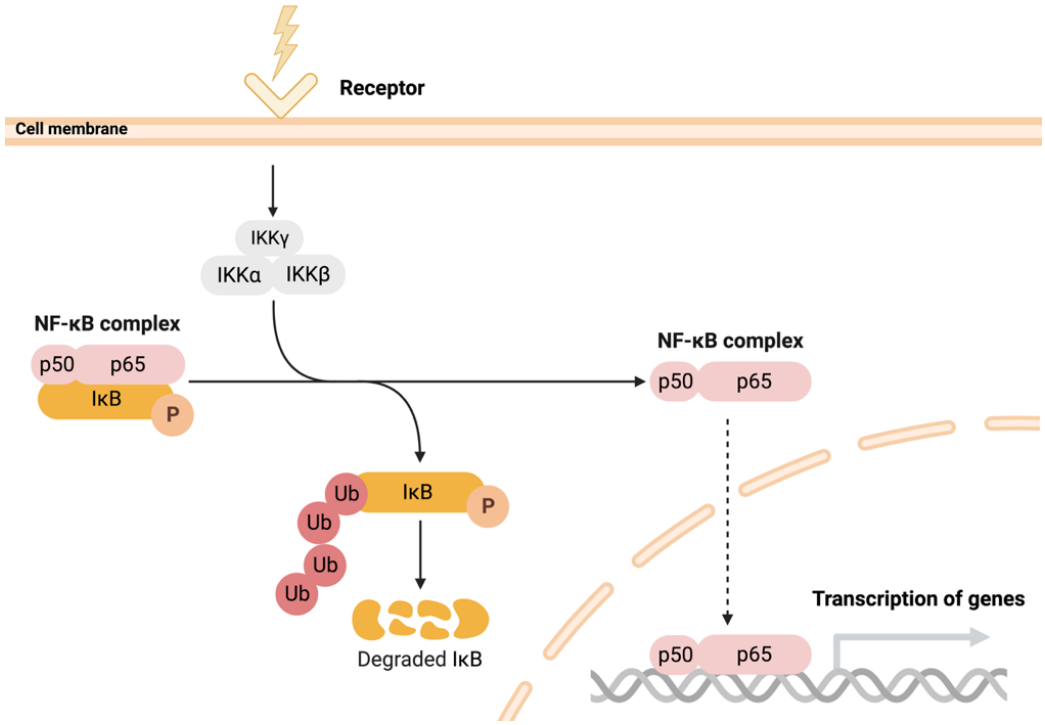

Figure 6.11: Schematic overview of NF-κB activation via the classical or canonical pathway, in which the p50/p65 heterodimer is the most common signal. IKK: IκB kinase consisting of IKKα, IKKβ and IKKγ; P: phosphorylation; Ub: ubiquitination. Created in BioRender.

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Chapter 7 The role of French Maritime Pine Bark Extract in arterial stiffness related mechanisms

7.1 Introduction

AS is an important risk factor for CV morbidity and mortality. Various mechanisms including reduced collagen/elastin ratio, calcification, endothelial dysfunction, oxidative stress and inflammation may contribute to the pathophysiology of AS (Lacolley et al., 2020). Since it is a good predictor for later CV events, early detection of AS helps in initiating preventive strategies and possible treatment options to reduce its health care burden (Kim, 2023). Since structural remodelling of the ECM of the arterial wall is little reversible, it is pivotal to evaluate the impact of prevention of increasing stiffness (Humphrey, 2021). Additionally, it is evident that preventing AS is easier than reversing it. Given the various biological mechanisms underlying arterial stiffness, it is challenging to pinpoint the exact cause retrospectively.

Most CVDs can be prevented by addressing behavioural risk factors of which dietary factors make the largest contribution. Dietary polyphenols display various biological effects and may interact with several mechanisms involved in AS aetiology. The polyphenol-rich extract PBE will be tested in an array of *in vitro* assays focused on inflammation and oxidative stress related endothelial dysfunction and thereby contributing to AS. Nevertheless, it should be noted that these *in vitro* tests only highlight some active mechanisms underlying arterial stiffness. As indicated in Chapter 6, there are also passive mechanisms (e.g. calcification) which have not been taken into account during this study.

7.2 *In vitro* assays: methods

7.2.1 COX-1 and COX-2 catalysed PGE_2 biosynthesis inhibition assay

7.2.1.1 Materials

Ethanol ≥99.8% (analytical reagent grade), formic acid (98/100%), hydrochloric acid 37% and methanol ≥99.8% (HPLC grade) were purchased from Thermo Scientific Fischer

(MA,USA). All milli-Q water was obtained using a Milli-Q® Reference system from Merck (Darmstadt, Germany). DMSO for spectroscopy (dimethyl sulfoxide, Uvasol®) and Titriplex III ($Na_2EDTA.2H_2O$, for analysis) were purchased from VWR (Leuven, Belgium). Tween 20, Trizma® base ≥99.9% (Tris), (-)- epinephrine-(+)-bitartrate salt, haematin (porcine), DDC ≥99% (ACS reagent) and celecoxib (100%, HPLC grade) were all purchased from Sigma-Aldrich (St. Louis, USA). The enzymes COX-1 (ovine) and COX-2 (human recombinant) as well as arachidonic acid were acquired from Sanbio (Uden, Netherlands). Indomethacin (≥99%, TLC) was purchased from MP Biomedicals Europe (Eschwege, Germany). PBE was kindly offered by Horphag Research (Geneva, Switzerland). (+)-catechin (99%), taxifolin (95.2%), caffeic acid (98.5%), ferulic acid (99%), (-)-epicatechin (100%) and tyrosol (99.8%) were all purchased from Sigma-Aldrich (St. Louis, USA). Procyanidin B1 (96.1%) was purchased from Extrasynthese (Genay, France).

A competitive ELISA kit from Enzo Life Sciences (New York, USA) was used for quantitative, colourimetric determination of PGE_2. The kit included: a 96-well plate coated with goat anti-mouse IgG antibodies, PGE_2-specific mouse monoclonal antibody, enzyme conjugate (PGE_2 conjugated with alkaline phosphatase), PGE_2 standard solution, pNpp-substrate (p-nitrophenyl phosphate), EIA assay (Tris, saline, proteins, sodium azide) and wash (Tris, saline, detergents) buffer concentrates and a stop solution (trisodium phosphate, water). A Synergy H1 microplate reader (BioTek, VT, USA) was used to measure the optical density at 405 nm.

7.2.1.2 Method

Samples and standards were analysed *in duplo* during one assay. The assay (schematically depicted by Figure 7.1) was repeated on another day to obtain at least four results per sample in total. Generally, the same method was used for the COX-1 and COX-2 inhibition assay; however, there were some slight differences between the two procedures. The basis of this assay is the formation of PGE_2 out of AA by COX-1 or COX-2 in cell-free conditions (no cells were used during these experiments). Certain co-factors are added to the reaction mixture to maximise the amount of PGE_2 synthesised during the assay. When an inhibitor of a particular COX-enzyme is added to the mixture, it will bind to it and will decrease the amount of PGE_2 that can be formed. When the reaction is stopped, the amount of PGE_2 formed is determined by means of a selective ELISA. The

effect of the inhibitor is then reported as an inhibition percentage, calculated as the amount of PGE_2 synthesised in the presence of a test compound compared to the amount formed in the negative control condition (the solvent (DMSO) alone). The method was validated by the research group NatuRA-PT and Graz University prior to this PhD thesis. Firstly, an incubation step was carried out in a clear 96-well microtiter plate, during which the PGE_2 was formed. A TRIS/HCl-buffer (pH 8.0 (optimal pH for PGE_2 formation (Noreen et al., 1998)) was prepared. An amount of 20 µL of this buffer was pipetted in each well for the COX-1 assay, or 10 µL for the COX-2 assay together with 10 µL of a 1mM Na_2EDTA solution in TRIS buffer (50 µM/well, co-factor for COX-2). Then, 50 µL of a 72 mM adrenaline bitartrate solution in TRIS buffer (18 mM/well) was added to each well (co-factor, stimulates PG synthesis (Noreen et al., 1998)). 10 µL of each sample solution was pipetted into the according well. Indomethacin (IC_{50} 1.25 µM / 0.45 µg/mL) or celecoxib (IC_{50} 2.5 µM / 0.95 µg/mL) was used as a positive control in respectively the COX-1 and COX-2 assay. COX-1 or COX-2 enzyme was diluted in TRIS assay buffer (2U/mL) and 100 µL of this dilution was pipetted in each well (0.2 U/well). 10 µL of a 100 µM haematin solution in TRIS buffer (5µM/well) was added (prosthetic group is essential for catalytic activity (Noreen et al., 1998)) and after five minutes of pre-incubation at RT 10 µL of a 100 µM AA solution in ethanol (5 µM/well) was added to initiate the reaction. It was then covered and incubated (37.0 °C, 20 min, medium shake on a shaker). 10 µL of a 10% formic acid solution in milli-Q was added to each well to stop the reaction afterwards. The PGE_2 formed in the first clear well plate was diluted in a second clear 96-well plate before the start of the ELISA assay to avoid saturation of the ELISA plate.

This dilution was made in EIA buffer: therefore a 1:10 dilution of the supplied EIA assay buffer concentrate was prepared in milli-Q. A 1:20 dilution of the samples was made for the COX-1 inhibition assay and a 1:40 dilution for the COX-2 inhibition assay. The ELISA assay was carried out according to the supplier's manual. The PGE_2 standard was thawed and a dilution series was prepared in EIA assay buffer. An amount of 100 µL of diluted PGE_2 sample or standard was pipetted in the corresponding well on the ELISA plate. Afterwards, 50 µL of enzyme conjugate was pipetted into each well, except for the total activity and blank wells. 50 µL of monoclonal antibody solution was pipetted into each well except for the total activity, blank and non-specific binding wells. The plate was then

incubated for the first time (RT, 2 h, ~ 550 rpm) and subsequently washed for three times with wash buffer (1:20 dilution of wash buffer concentrate in milli-Q). Enzyme conjugate (5 µL) was added to the total activity wells, after which 200 µL of pNpp-substrate was pipetted into each well. The plate was then covered and incubated again (RT, in the dark, 1 h). 50 µL of stop solution was added to each well and optical densities were measured at 405 nm using the Synergy microplate reader. Inhibition of COX refers to the reduction in PGE_2 formation in comparison to a blank vehicle control run without inhibitor (DMSO). DMSO did not exceed 2.5% in the final test solutions.

Analysis of the results was performed using Microsoft Excel (Microsoft, USA) and GraphPad Prism (GraphPad Software, USA). IC_{50}-values were calculated using a "log(inhibitor) vs. normalised response – variable slope" model to fit the data. Differences in percentage inhibition between DMSO and possible inhibitors were evaluated using a one-way ANOVA. Post-hoc analysis was performed using Dunnett correction. Normality of the data was assessed before performing a one-way ANOVA using the Shapiro-Wilk test. All p-values < 0.05 were considered significant. The extract concentrations are expressed µg/mL, while the concentrations of pure compounds are stated in µM. Due to the complex nature of the extract, comprising several individual polyphenolic compounds, expressing its concentration in micromolar is challenging and therefore maintained in µg/mL.

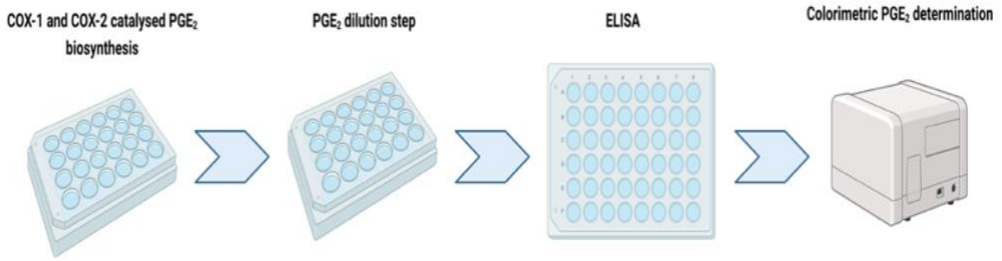

Figure 7.1: Workflow of the COX-1 and COX-2 catalysed PGE_2 biosynthesis inhibition assay which is performed in several steps: COX-1 and COX-2 catalysed PGE_2 biosynthesis, a PGE_2 dilution step to avoid saturation of the ELISA plate and a competitive ELISA with colourimetric read-out of PGE_2 at 405 nm. COX-1: cyclooxygenase 1; COX-2: cyclooxygenase 2; ELISA: Enzyme-Linked Immunosorbent Assay; PGE_2: prostaglandin E2. Created in BioRender.

7.2.2 NF-κB assay

7.2.2.1 Materials

An adherent type of mouse fibroblast cell line, L929 cells, were transfected with the recombinant NF-κB dependent reporter gene construct $p(IL6\kappa B)_3$50hu.IL6P-Luc (kindly offered by the lab of prof. Wim Vanden Berghe, Protein Chemistry, Proteomics and Epigenetic Signalling, University of Antwerp) and grown at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with 1% Penicillin/Streptomycin, Trypsin/Ethylenediamine tetra-acetic acid (EDTA) (0.05%), and 10% heat-inactivated foetal bovine serum (FBS), all purchased from Life Sciences (Bedford, UK). The cells were kept in a humidified incubator containing 5% CO_2. Dexamethasone was purchased from Merck (Darmstadt, Germany) and recombinant mouse TNF-α was purchased from Thermo Scientific Fisher (5.0×10^7 - 2.0×10^8 units/mg; MA, USA). A luciferase assay was purchased from Promega Benelux (E4030) and a Glomax plate reader (available at the lab of prof. Paul Cos, Laboratory of Microbiology, Parasitology and Hygiene, University of Antwerp) was used for read-outs. Cell viability was measured with the colourimetric 3-(4, 5-dimethylthiozol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (ATCC, VA, USA) to test the viability of the cells after treatment with different polyphenolic compounds at a concentration of 1 mg/mL extract and 200 µM pure compound. PBE (Loss on Drying (LOD) 2.70%) was kindly offered by Horphag Research (Geneva, Switzerland). The individual compounds ((+)-catechin (99 %), taxifolin (95.2 %), caffeic acid (98.5%), tyrosol (99.8%) and 3-hydroxytyrosol (99%) and ferulic acid (99%)) were purchased from Sigma Aldrich (St. Louis, USA). Procyanidin B1 (96.1%) was purchased from Extrasynthese (Genay, France). The extract and compounds were dissolved in DMSO (Merck, Darmstadt, Germany) and attention was paid to keep the final concentration of DMSO in each well below 1% to avoid toxicity (by further diluting the stock solution with PBS and DMEM) to obtain the desired concentration.

7.1.2.2. Method

The cells were kept in a humidified atmosphere containing 5% CO_2 at 37 °C. At 80% confluence, cells were plated in 24-well plates (100 000 cells/well) and incubated for 24

h before testing. The next day, the seeded L929 cells were pre-incubated with different concentrations of polyphenolic test compounds at 37 °C for 1 h, subsequently followed by stimulation with 10 ng/mL TNF-α for an additional 3 h (37 °C) to provoke an inflammation reaction. Afterwards the reporter quantification was carried out following the manufacturer's instructions from Promega. In brief, after aspirating the cell medium, the L929 cells were lysed with reporter lysis buffer, which requires one single freeze-thaw cycle to achieve complete lysis. Lysates were vortexed, centrifuged at 12 000 g for 15 sec and stored at -80 °C until further analysis. Afterwards, 20 µL of cell lysate was pipetted manually per well in a black 96-well plate, followed by the automatic addition of 100 µL Luciferase Assay Reagent per well in the GloMax 96 Microplate Luminometer. The produced light was measured for a period of 10 s after a delay time of 2 s.

NF-κB activation was thus measured using a non-viral vector system that encodes the firefly luciferase reporter gene. This reporter gene is only transcribed when the NF-κB signalling pathway is active. Inflammatory signals including TNF-α trigger NF-κB activation which involves nuclear translocation of the NF-κB transcription factors. The reporter gene will be transcribed, like endogenous NF-κB targets, proportionally to the magnitude of NF-κB activation and the time persistence of the induction signal. Expression of luciferase is dependent on the activation of NF-κB, which occurs following cell stimulation. Monitoring the bioluminescence light output by the cells is proportional to the amount of luciferase present in the cell lysate and serves as a measure of NF-κB activity *in vitro.* Dexamethasone (1 µM) was used as a reference inhibitor of NF-κB driven transcription. The degree of NF-κB induction initiated by TNF-α was investigated for PBE and its polyphenolic constituents by comparing their luciferase activity with the luciferase activity of L929 cells with addition of only TNF-α (100% luciferase activity and thus 100% NF-κB activity). The data were expressed as mean ± SD. Significantly different luciferase activity as compared to the TNF-α control group was tested using a one-way ANOVA test, followed by a Tukey test, using Graphpad Prism. The results were considered statistically significant at $p < 0.05$.

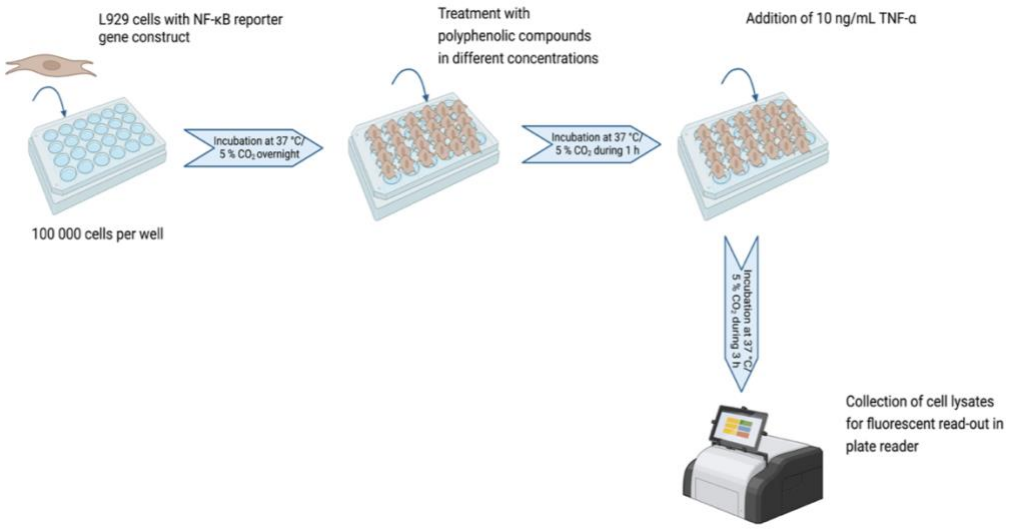

Figure 7.2: Workflow of the NF-κB experiment. Created in BioRender.

7.2.3 Infrared Western blotting to determine expression levels of proteins

7.2.3.1 Materials

Human Aortic Endothelial Cells (HAoECs) and Human Aortic Smooth Muscle Cells (HAoSMCs) were purchased from Merck (Darmstadt, Germany). Endothelial cell growth medium (ECGM) for HAoECs and Smooth muscle cell growth medium (SMCGM) for HAoSMCs were acquired from Cell Applications (CA, USA). Dulbecco's phosphate-buffered saline (DPBS), Penicillin-Streptomycin (P/S), Trypsin/Ethylenediamine tetra-acetic acid (EDTA) (0.05%), and Fetal bovine serum (FBS) were all purchased from Gibco (London, UK). Trypan blue solution was purchased from Life Sciences (Bedford, UK). A water bath set at 37 °C was used to warm up the reagents used for the cell cultures. Cell viability was measured with the colourimetric 3-(4, 5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (ATCC, VA, USA) to test the viability of the cells

after treatment with different polyphenolic compounds at a concentration of 1 mg/mL extract and 200 µM pure compound. PBE (Loss on Drying (LOD) 2.70%) was kindly offered by Horphag Research (Geneva, Switzerland). The individual compounds ((+)-catechin (99%), taxifolin (95.2%), caffeic acid (98.5%), tyrosol (99.8%) and 3-hydroxytyrosol (99%) and ferulic acid (99%)) were purchased from Sigma Aldrich (St. Louis, USA). Procyanidin B1 (96.1%) was purchased from Extrasynthese (Genay, France). The extract and compounds were dissolved in DMSO (Merck, Darmstadt, Germany) and attention was paid to keep the final concentration of DMSO in each well below 1% to avoid toxicity.

All the following materials needed for the infrared Western blotting were kindly provided by the lab of Physiopharmacology at the University of Antwerp. Laemmli sample buffer was purchased from Bio-Rad (CA, USA) and β-mercaptoethanol was purchased from Merck (Darmstadt, Germany). Immobilon-FL membranes were bought from Merck (Darmstadt, Germany). Bolt 4-12% gels were purchased from Life Technologies (MD, USA). Odyssey Blocking Buffer was acquired from LI-COR Biosciences (NE, USA). PageRuler Prestained Protein Ladder was bought from Thermo Fischer Scientific (MA, USA). Mouse anti-p-eNOS (Ser^{1177}, 612392) and mouse anti-p-eNOS (Thr^{495}, 612706) monoclonal antibodies and rabbit anti-eNOS polyclonal antibody (3365612) were purchased from BD Biosciences (NJ, USA). Rabbit anti-Nrf2 polyclonal antibody (00102781) was purchased from Proteintech, USA. Rabbit anti-HO-1 antibody (ADI-SPA-896) was bought from Enzo life sciences (NY, USA). A recombinant rabbit anti-MMP12 antibody (ab52897) and mouse anti-β-actin (Ab8226) were purchased from Abcam (Cambridge, UK). Fluorescently labelled secondary antibodies anti-rabbit (IgG926-3221) and anti-mouse (IgG926-68070) were purchased from LI-COR Biosciences (NE, USA).

7.2.3.2 Method

HAoECs and HAoSMCs were maintained in respectively ECGM and SMCGM both supplemented with 1% penicillin-streptomycin at 37 °C in a humidified incubator set at 5% CO_2. Growth medium of both cultures was routinely changed. Cells were subcultured

at 80% confluency by disassociation with 0.05% trypsin and 0.02% EDTA. Cells were counted using tryptan blue solution on a counter plate under the microscope. Cells were plated out in sterile well plates in a concentration of approximately 100 000 cells per well and incubated at 37 °C overnight. Different concentrations of test compounds were pipetted in each well and incubated at 37 °C for 6 h. For some experiments 100 µM H_2O_2 was added (during 30 min before starting treatment with polyphenolic compound or during 6 h together with the polyphenolic compound) to induce oxidative stress. Afterwards, cells were collected in 75 µL Laemmli sample buffer (supplemented with 5% β-mercaptoethanol), heat denatured for 5 min at 100 °C on a heat block and stored in Eppendorf tubes at -20 °C (Figure 7.3). HAoSMCs of passage numbers 1 to 8 and HAoECs of passage numbers 1 to 4 were used for the experiments without oxidative stress induction. For the stimulated cell experiments, HAoSMCs and HAoECS with passage numbers 5 to 7 were used. A MTT assay was performed to determine cytotoxicity of all tested compounds on HAoECs and HAoSMCs viability.

Samples were heat denatured for 5 min at 100 °C and loaded on Bolt 4-12% gels. After gel electrophoresis, proteins were transferred to Immobilon-FL membranes according to standard procedures and incubated for 1 h in Odyssey Blocking Buffer. Next, membranes were incubated at 4 °C overnight with the following primary antibodies: rabbit anti-eNOS, mouse anti-PeNOS(Ser1177), mouse anti-ePNOS(Thr495), rabbit anti-Nrf2 polyclonal antibody, rabbit anti-HO-1 antibody and rabbit recombinant anti-MMP12 antibody. Both the MMP-12 (propeptide) and the MMP-12 (active form) were targeted by the MMP-12 antibody. β-actin (mouse or rabbit) and Total Protein Stain (TPS) were used as an internal control to normalise protein expression. Finally, membranes were incubated with fluorescently labelled secondary antibodies (rabbit and mouse) to allow infrared detection and quantification on an Odyssey SA instrument (Li-COR Biosciences, USA). At least two western blots were performed for the extract and each individual compound. A schematic overview is depicted by Figure 7.4.

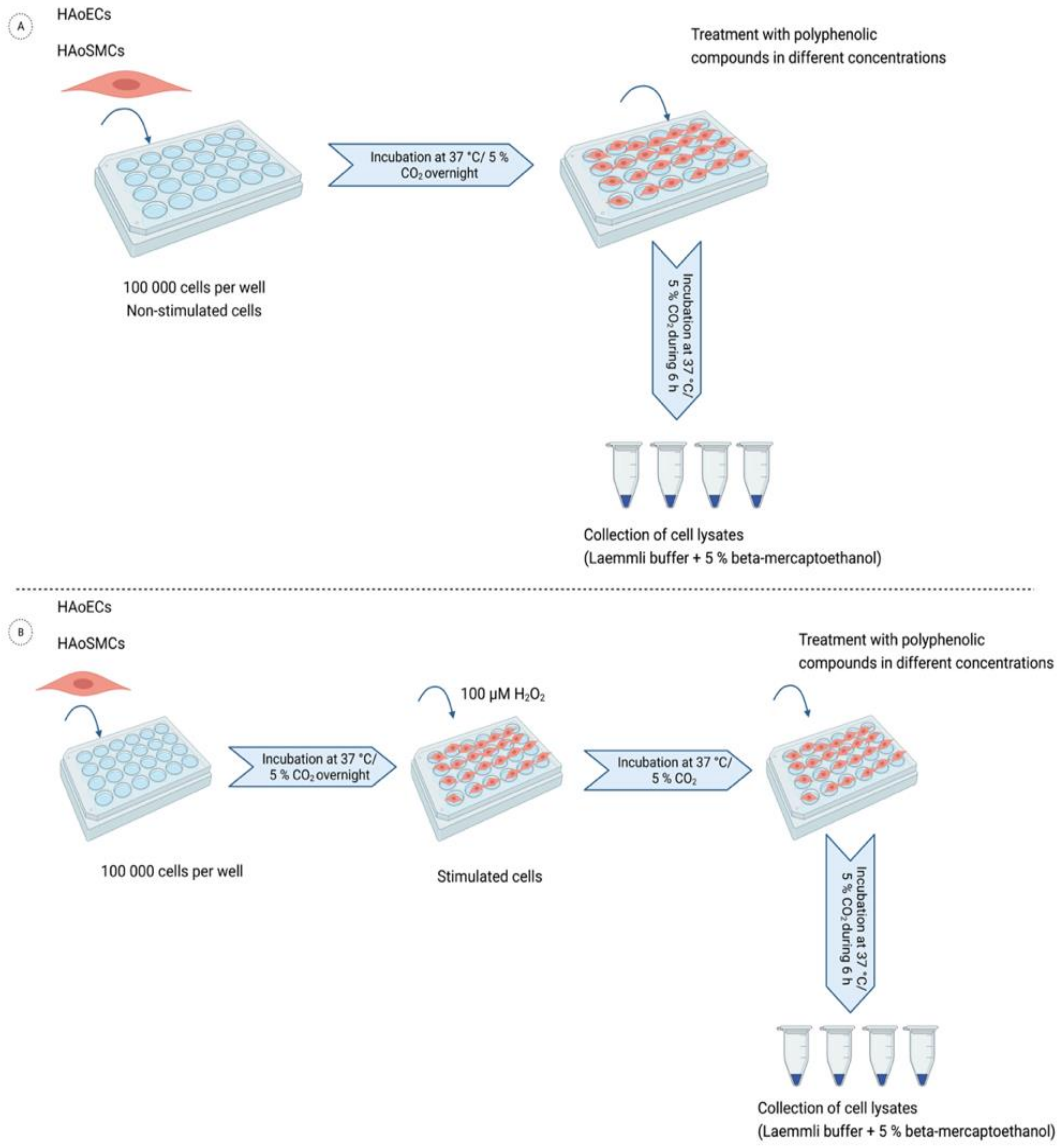

Figure 7.3: Plating out of HAoECs and HAoSMCs and treatment of these cells with polyphenolic compounds. Cells were kept in the incubator set at 37 °C and 5% CO_2. Cell lysates were collected in Laemmli buffer supplemented with 5% β-mercapthoethanol. HAoECs: Human Aortic Endothelial Cells; HAoSMCs: Human Aortic Smooth Muscle Cells. Created in BioRender.

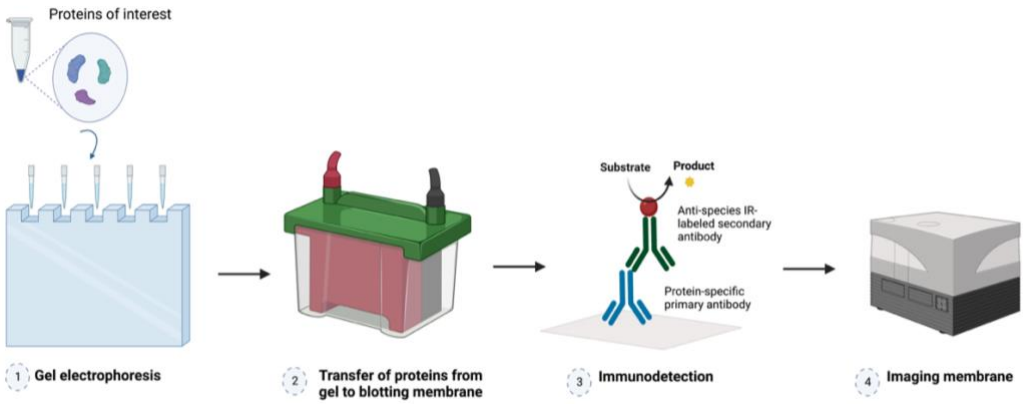

Figure 7.4*:* Visualisation of the Western blot procedure. Samples collected in Laemmli buffer were loaded on a 4-12% gel and gel electrophoresis was started. Afterwards, proteins were transferred to a blotting membrane. An antibody mixture was then added to the membrane and afterwards quantification of protein expression was done by means of infrared detection using an Odyssey instrument. Created in BioRender.

Graphpad Prism (GraphPad Software, USA) and SPSS 28.0 (IBM) were used for statistical analyses. Normalised signals were calculated by comparing each target protein to an internal loading control, in this case, β-actin. The fold change and log2 fold change were calculated by LI-COR software (Biosciences, USA) and Excel (software) respectively. Data were also checked for outliers. The residuals of the model were tested for normal distribution (Q-Q plots and Shapiro-Wilk test) and homoscedasticity (residuals vs predicted values). For each protein of interest, the effect of treatment was investigated using linear mixed models (LMM). The normalised signal of the protein of interest was entered as the dependent variable. Treatment was entered as a fixed independent variable and blot and passage number of the cells used were entered as random effects. Each treatment group was compared to a control group (untreated cells, only medium added). In case of a significant fixed effect, post-hoc analyses were performed to check for pairwise differences in effect between the treatments. Since multiple hypotheses were tested, thereby increasing the possibility of a type 1 error, a multiple testing correction, in this case, Bonferroni, was carried out. Differences were accepted and considered statistically significant when $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

7.3 Results and discussion *in vitro* assays

7.3.1 COX-1 and COX-2 catalysed PGE_2 biosynthesis inhibition assay

Each percentage is the mean of at least four assays represented as the mean ± standard error of the mean (SEM). Due to variability of the assay, a lower limit of -20% inhibition was used and all percentages below this value were equated to -20%. Percentages below 0% inhibition were labelled as having "no effect", as induction of PGE_2 synthesis was not possible in the context of this assay. All datasets were normally distributed according to the Shapiro-Wilk test. An assay was considered invalid when the positive control showed a percentage inhibition outside of the 40 - 60% range (which was considered the normal inhibition range for the positive controls).

7.3.1.1 PBE

Results from the COX-1 (PBE tested in the range of 1-100 µg/mL) and COX-2 (PBE tested in the range of 20 – 400 µg/mL) inhibition assay are shown in Table 7.1 and Table 7.2 as well as in Figure 7.5 and Figure 7.6. The inhibitory effect of PBE on COX-1 was clearly visible and concentration-dependent. An enzyme-inhibition curve was plotted, resulting in an IC_{50}-value of 9.035 µg/mL (R^2 of 0.8879). The reported COX-1 inhibition by 1.0 µg/mL PBE is negligible since 0% falls within its SEM and inhibition by 2.5 µg/ml was not statistically significant. PBE showed significant inhibitory effects on COX-2 as well; however, no concentration-dependent effect could be observed. Mean inhibition of COX-2 was 33.13 ± 3.99% between the concentrations of 20 and 200 µg/mL, with a sharp rise in inhibition to 88.09 ± 2.62% with a concentration of 400 µg/mL. However, a COX-2 enzyme-inhibition curve could not be plotted and therefore it was not possible to calculate an IC_{50}.

Table 7.1: COX-1 inhibition assay results of PBE tested in different concentrations denoted as a percentage which represents the mean of at least four assays ± SEM.

Concentration PBE (µg/mL)	COX-1 inhibition ± SEM (%)
1.0	1.20 ± 2.84
2.5	18.08 ± 3.61
5.0	41.89 ± 3.41
10.0	57.13 ± 3.46
15.0	60.24 ± 7.28
20.0	65.06 ± 2.38
22.5	70.92 ± 2.19
25.0	67.97 ± 0.42
27.5	74.68 ± 2.56
30.0	78.73 ± 3.25
35.0	78.32 ± 3.12
50.0	85.08 ± 1.01
75.0	86.77 ± 1.03
100.0	72.33 ± 2.16
	IC_{50}-value = 9.035 µg/mL

COX-1: cyclooxygenase 1; IC_{50}-value: *c*oncentration of a compound that gives half-maximal response*;* PBE: French Maritime Pine Bark Extract; SEM: standard error of the mean.

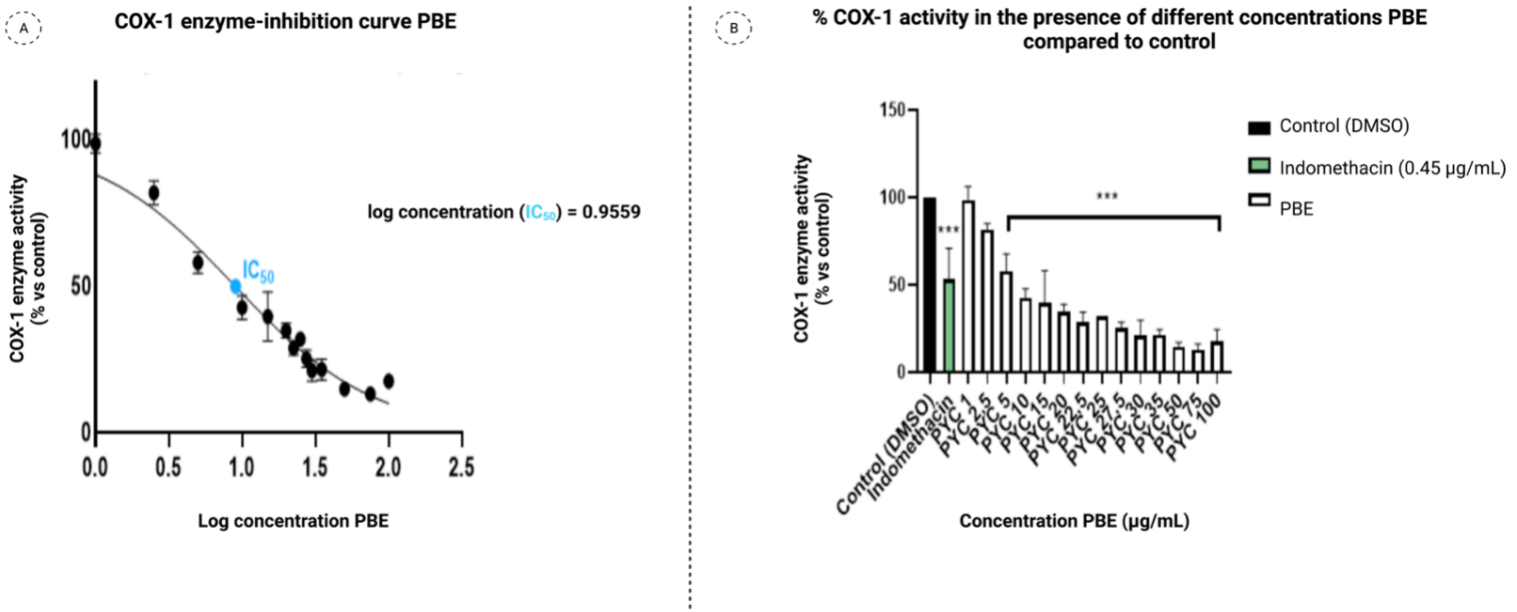

Figure 7.5: A: A COX-1 enzyme-inhibition curve of PBE was calculated with a R^2 value of 0.88779. B: Bar plots indicating the COX-activity in the presence of different concentrations of PBE (colourless bars) expressed in percentages as compared to a control condition (DMSO, represented by the black bars). Indomethacin was used as a positive control (green bars; IC_{50} of 0.45 µg/mL corresponds to 1.25 µM). Inhibition was significant up from 5.0 µg/mL (*** $p < 0.001$). COX-1: cyclooxygenase 1; DMSO: dimethyl sulfoxide; PBE/PYC: French Maritime Pine Bark Extract.

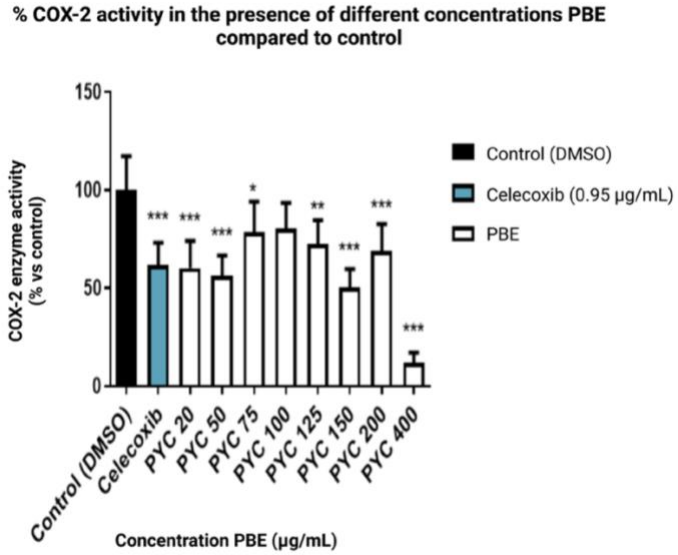

Figure 7.6: Bar plot of the COX-2 enzyme-inhibition assay results of PBE (depicted by colourless bars) as compared to control (DMSO, black bar). Inhibition was significant though not concentration-dependent. Celecoxib was used as a positive control (turquoise bars; IC_{50} of 0.95 µg/mL corresponds to 2.50 µM. *** $p < 0.001$ ** $p < 0.01$ * $p < 0.05$. COX-2: cyclooxygenase 2; DMSO: Dimethylsulfoxide; PBE/PYC: French Maritime Pine Bark Extract.

Table 7.2: COX-2 inhibition assay results of PBE tested in different concentrations denoted as a percentage which represents the mean of at least four assays ± SEM.

Concentration PBE (µg/mL)	COX-2 inhibiton ± SEM (%)
20	1.20 ± 2.84
50	18.08 ± 3.61
75	41.89 ± 3.41
100	57.13 ± 3.46
125	60.24 ± 7.28
150	65.06 ± 2.38
200	70.92 ± 2.19
400	67.97 ± 0.42

COX-2: cyclooxygenase 2; PBE: French Maritime Pine Bark Extract; SEM: standard error of the mean.

7.3.1.2 Individual constituents of PBE

Then, some interesting polyphenolic constituents of PBE were tested separately to see which ones were responsible for the inhibitory effects (possible ranges between 1 and 600 µM in both assays). Results of all compounds that showed inhibitory effects on any of the two isozymes are shown in Figure 7.7 (+)-catechin, caffeic acid and ferulic acid showed concentration-independent inhibition of COX-1, but none of them reached statistical significance. (+)-catechin only inhibited COX-1 in concentrations of 100 µM and more, with a mean inhibition of 24.49 ± 4.92%. Mean inhibition of COX-1 was 14.48 ± 3.45% for caffeic acid and 17.86 ± 4.74% for ferulic acid. Epicatechin and taxifolin did not show any inhibition of COX-1 in our assay, regardless of the concentration (results not shown in Figure 7.7). None of the polyphenols except for caffeic acid had any inhibitory effect on COX-2: caffeic acid showed a small inhibitory effect of 5.51 ± 1.71% in concentrations above 20 µM, but this was not significant.

Another assay was performed to evaluate the effects of the oligomeric fraction of PBE. Since procyanidin B1 is the most common dimer in PBE, it was chosen as the reference substance for this group of compounds. No assays were performed with higher oligomeric fractions. It could be demonstrated that procyanidin B1 inhibited both COX-1 and COX-2. COX-1 inhibition was concentration-dependent (Table 7.3), but not all concentrations reached statistically significant inhibition levels. The inhibition curve (R^2 value of 0.7273) and IC_{50}-value of 525.9 µM are shown in Figure 7.8 and Table 7.3. In fact, this generated enzyme-inhibition curve of procyanidin B1 for COX-1 has a suboptimal R^2-value (for accurate IC_{50} estimations, an R^2 of ≥ 0.8000 is usually preferred (Sebaugh, 2011) and a low number of measuring points with none above the suspected IC_{50}-value. Our results are thus not ideal. COX-2 inhibition was only observed from 100 µM onward and was concentration-independent: mean inhibition by procyanidin B1 was 15.22 ± 0.69% between 100 – 400 µM (400 µM PBE was statistically significant; $p < 0.05$). Results for COX-2 are shown in Figure 7.8 as well.

Table 7.3: COX-1 inhibition assay results of procyanidin B1 tested in different concentrations denoted as a percentage which represents the mean of at least four assays ± SEM.

Concentration procyanidin B1 (µM)	COX-1 inhibition ± SEM (%)
20	10.69 ± 5.96
50	16.61 ± 1.39
300	36.44 ± 4.75
400	48.89 ± 9.74
	IC_{50}-value = 525.9 µM

COX-1: cyclooxygenase 1; PBE: French Maritime Pine Bark Extract; SEM: standard error of the mean.

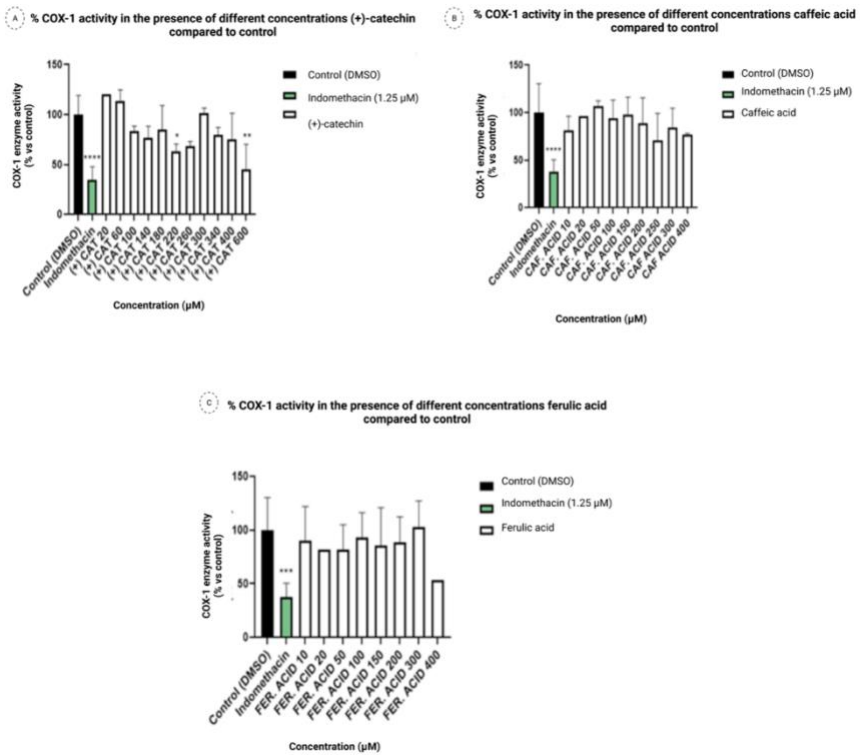

Figure 7.7: A: COX-1 inhibition assay results for procyanidin B1 and COX-1 inhibition assay results for (+)-catechin (B), caffeic acid (C) and ferulic acid (D). Indomethacin and celecoxib were used as positive controls in respectively the COX-1 and COX-2 inhibition assay. *** $p < 0.001$ ** $p < 0.01$ * $p < 0.05$

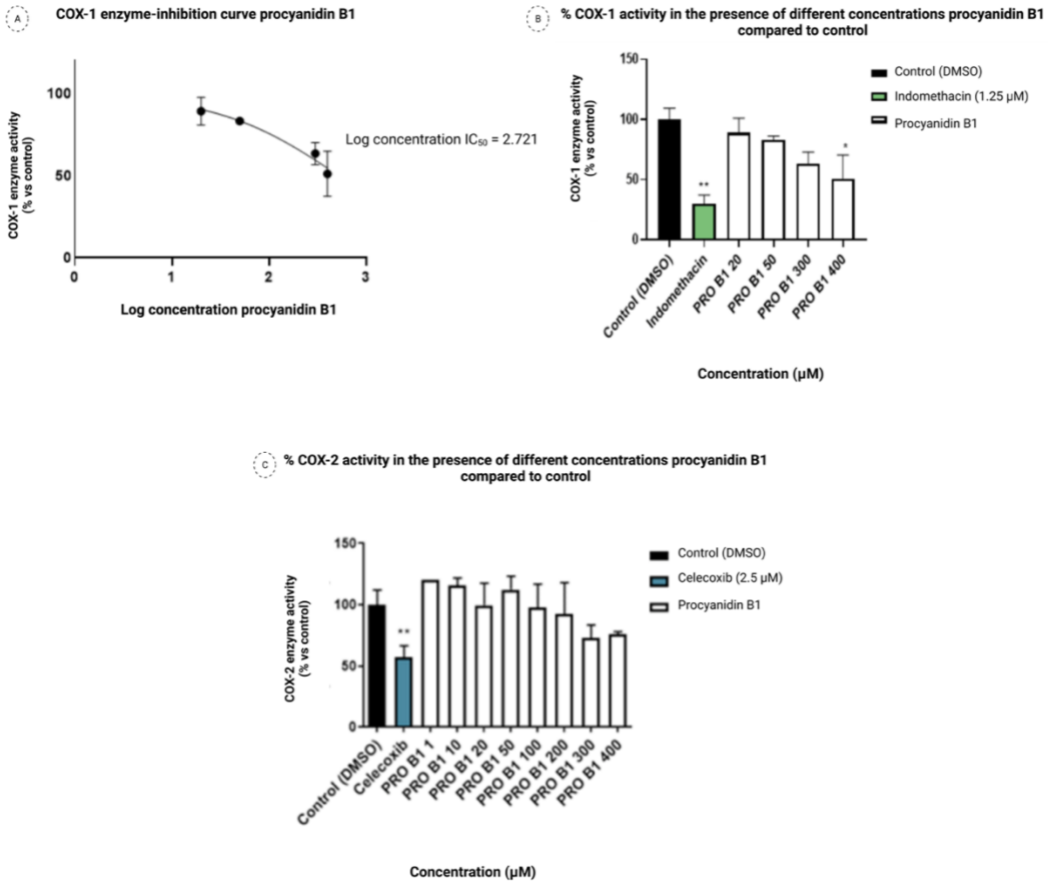

Figure 7.8: A: A COX-1 enzyme-inhibition curve for procyanidin B1 was plotted with a R^2 of 0.7273. Only inhibition by 400 µM procyanidin B1 was significant. Indomethacin was used as a positive control. C: COX-2 inhibition assay results for procyanidin B1. Celecoxib was used as a positive control. ** $p < 0.01$ * $p < 0.05$

7.3.1.3 Mixture of individual polyphenolic constituents

The findings for the individual constituents did not explain the concentration-dependent effects of PBE on COX-1. Therefore, a mixture of the different individual polyphenolic compounds was prepared, respecting the percentage distribution found in the HPLC-

DAD fingerprint assay of PBE (Chapter 4) or as reported by D'Andrea et al. (D'Andrea, 2010) to see whether it would generate similar inhibitory effects on COX-1 as PBE itself. This mixture consisted of 377 µg/mL (+)-catechin, 43 µg/mL taxifolin, 39 µg/mL caffeic acid, 12 µg/mL ferulic acid and 5 µg/mL epicatechin which is the equivalent of an extract of 2 mg/mL PBE. Results showed that the mixture only inhibited COX-1 starting from a concentration equivalent to 10 µg/mL PBE. The highest tested concentration of 100 µg/mL extract-equivalent only resulted in an inhibition of 19.63 ± 8.82%, which did not reach statistical significance. Also, all individual polyphenolic constituents were tested in the concentration in which they are present in PBE 9.035 µg/mL. None of them showed any COX-1 inhibition in these concentrations.

7.3.1.4 Discussion COX-1 and COX-2 catalysed PGE_2 biosynthesis inhibition assay

In our study, PBE clearly showed inhibitory effects on COX-1 and COX-2. This was to be expected: a study from Schäfer et al. (Schäfer et al., 2006) showed that COX-1 and COX-2 activity was inhibited by plasma of human volunteers after they ingested 200 mg of PBE. They reported non-selective inhibition of both COX-1 and, albeit to a lesser extent, of COX-2, which is in line with our own observations. However, it should be noted that the plasma used by Schäfer et al. is a human sample: it cannot be ruled out that some of the observed effects can be attributed to PBE biotransformation products formed *in vivo* after ingestion. Effects from other plasma components were excluded by comparing the inhibition with that of basal plasma, taken from the same volunteers after a 24 hours polyphenol-free diet and before intake of PBE (Schäfer et al., 2006).

In our study, the IC_{50} of PBE for COX-1 was 9.035 µg/mL. Compared to the IC_{50} of a classic NSAID indomethacin (0.45 µg/mL), it is clear that indomethacin is still approximately twenty times more potent at inhibiting COX-1 than PBE. An IC_{50} for PBE on COX-2 could not be calculated, but the 50 % inhibition point is expected to be between 200 and 400 µg/mL, looking at our results. Since celecoxib has an IC_{50} of 0.95 µg/mL, this effect of PBE on COX-2 is of marginal value. Further experiments showed that none of the individual constituents were observed as responsible for the effects seen when testing PBE. Although inhibition of COX-1 by (+)-catechin, caffeic acid and ferulic acid and of COX-2 by caffeic acid were observed, none of these were strong enough to account for the

effects of the extract. Moreover, the difference between (+)-catechin and epicatechin in inhibitory effects on COX-1 is remarkable, considering they only differ in the stereochemistry of one hydroxyl group. This suggests that this group plays an essential role in the binding interactions with the enzyme. Even though (+)-catechin and epicatechin are epimers, differences in biological activities have been reported so far in literature. For instance, *in vitro* testing of their antioxidant capacity showed that epicatechin is slightly more effective than (+)-catechin (Braicu et al., 2011).

Testing the individual constituents in the concentrations in which they are present in PBE 9.035 µg/mL, showed that none of the individual compounds had inhibitory effects. According to this data, we can infer that none of the individual polyphenols are responsible for the inhibition of COX-1 by PBE. However, when tested in a mixture (in a ratio comparable to the one in PBE), a small fraction of the inhibitory effect of PBE was observed. This observation may support the hypothesis of synergy between the different polyphenols. Though the effect of the mixture was greater than the sum of the inhibitory effects of the individual polyphenols, the observed effect of the mixture was still lower than the effect of the corresponding PBE. Therefore, it is not possible to attribute all of PBE's inhibitory potential to synergic effects of the separately tested compounds.

The literature on COX inhibition by these polyphenols is confounding: several studies have already been published, but there is no consensus. For example, IC_{50}-values of (+)-catechin reported in the literature vary between 40 and 943 µM (Noreen et al., 1998). A study by Takahashi and Miyazawa (Takahashi & Miyazawa, 2012) reported COX-2 selective inhibition by caffeic acid with an IC_{50} of 129.7 ± 2.8 µM. Another study reported COX-2 selectiveness as well, but with an IC_{50} of approximately 170 µM (Jayaprakasam et al., 2006). In the same study, they also tested ferulic acid and reported no COX-1 and COX-2 inhibition (Jayaprakasam et al., 2006). On the contrary, a study from Nile et al. (Hariram Nile et al., 2016) screened ferulic acid related compounds for their COX-2 inhibition and observed inhibitory effects by both caffeic and ferulic acid, with an IC_{50} of respectively 346.9 and 335.8 µM. Lastly, Silva et al. (Silva et al., 2015) reported caffeic acid to have a mean inhibitory effect of 15.5 ± 2.1% on COX-2 in a concentration of 100 µM (our result at 100 µM: 8.86 ± 2.0%). Other studies did confirm our findings: Ribeiro et al. (Ribeiro et al., 2015) demonstrated that taxifolin did not inhibit either of the COX-enzymes, which is in line with our data. Procyanidin B1 did show inhibitory effects in

both COX-1 and COX-2 inhibition assays. Both PBE and procyanidin B1 showed concentration-dependent inhibition of COX-1 and concentration-independent inhibition of COX-2. Nevertheless, the IC_{50}-value of 525.9 µM for procyanidin B1 on COX-1 should be interpreted with caution since the enzyme-inhibition curve is not perfectly accurate (does not meet the quality demands that are usually maintained for them). Based on our data the IC_{50} will be closer to 400 µM, considering the mean inhibition of 48.89 ± 0.28% found in our experiments. In fact, it is common in IC_{50} calculation to obtain an estimate that is higher than the actual value if there are no measurements in the upper plateau of the curve (Sebaugh, 2011). Nevertheless, procyanidin B1 certainly did not result in enough inhibition in either of the assays to account for the inhibitory effects of PBE. It is possible that there is an additional synergistic effect of procyanidin B1 and the other polyphenols, or that other constituents of PBE which we have not yet tested, are responsible for the observed inhibition. For instance, procyanidin B3 is another dimer commonly found in PBE and there are also higher oligomers including trimers and tetramers as well as still unknown components such as "Pic X" (D'Andrea, 2010). Possibly, some of these account for the remaining inhibitory activity *in vitro*.

Concentration-independent inhibition was observed in several assays we performed. This could be due to uncompetitive inhibition. In this type of kinetic behaviour, the inhibitor only binds the enzyme-substrate complex instead of competing with the substrate for binding of the active site, which is what happens during competitive, concentration-dependent inhibition. Inhibition is therefore not only determined by the amount of inhibitor present, but also by the concentration of substrate. In our method the same concentration of AA was used every time (5 µM), so it is to be expected that uncompetitive inhibitors would give approximately the same percentage of inhibition in every concentration. In practice, this type of inhibition is only observed for enzymes having two or more substrates (Nelson & Cox, 2008). COX fits this requirement, having both multiple possible fatty acids and a cellular hydroperoxide as substrates (Smith & Murphy, 2002). According to the kinetic studies performed by Takahashi and Miyazawa (Takahashi & Miyazawa, 2012) caffeic acid is an uncompetitive inhibitor of COX-2. It is therefore not improbable that other polyphenolic compounds could also be uncompetitive inhibitors. Moreover, mixed forms of inhibition do exist and this could be a possible explanation for the tested polyphenolic compounds to not show fully concentration-dependent inhibition (Patrick, 2017). These hypotheses should be

confirmed with kinetic studies on for example ferulic acid and (+)-catechin. Furthermore, it should be noted that there are several assays described to determine COX inhibition: several studies use different, cell-based methods and differences in the results could originate therefrom, as reported by Brooks et al. (Brooks et al., 1999).

There is emerging evidence for the treatment of inflammatory symptoms with PBE supplementation *in vivo* (Schäfer et al., 2006). After oral intake (100-300 mg per day, dosing different between studies) PBE showed cardiovascular and anti-inflammatory effects in several clinical trials (D'Andrea, 2010; Grimm et al., 2006). As mentioned earlier, *ex vivo* COX-inhibition by human volunteer plasma was already demonstrated (Schäfer et al., 2006). This study shows that the plasma concentrations of the COX-inhibiting compounds were high enough to be effective after administration of 200 mg PBE during five days to five healthy humans. These findings thus suggest that COX inhibition with PBE is possible *in vivo*. Nevertheless, the pharmacokinetic study from Grimm et al. (Grimm et al., 2006) reported plasma concentrations of the polyphenolic compounds tested here to be in the ng/mL range. All polyphenolic constituents showing inhibitory effects in our assay only did so in the µg/mL or µM range. It is therefore impossible that the *in vivo* inhibitory effects could be attributed to the COX inhibition of the tested parent polyphenols in our study. In the study of Grimm et al. (Grimm et al., 2006) ten unknown compounds were found in the plasma after oral ingestion as well. Moreover, the aforementioned polyphenolic constituents are present as conjugates from phase II biotransformation reactions *in vivo*. It is plausible, considering our results, that these conjugates and other compounds such as the colon biotransformation products are responsible for the COX-inhibiting effects of PBE *in vivo.* Generally, taking into account the relatively high concentration at which inhibition started and the plasma concentrations found in literature, significant COX inhibition will be hard to achieve with the tested polyphenolic compounds *in vivo*.

7.3.2 NF-κB assay

7.3.2.1 Results

As described earlier, the transcription factor NF-κB exerts a crucial role in the inflammatory response in both normal and pathological conditions, and serves therefore as an interesting target of therapy. PBE and its individual polyphenolic compounds were tested for their potential to inhibit NF-κB mediated cell signaling transduction that has been induced by TNF-α. Expression of luciferase is dependent on the activation of NF-κB which occurs following cell stimulation with TNF-α. Higher luciferase activity is thus correlated with more NF-κB activation.

Cytotoxic effects of PBE and its polyphenolic compounds were first evaluated by an MTT colorimetric assay. Neither of the compounds significantly affected the cell viability as compared to control (L929 cells in DMEM). For each compound, a cell viability greater than 80% was maintained, suggesting that none of them was toxic towards L929 cells within the dose range applied in this study (data not shown).

Figure 7.9 compiles the data from three different experiments. Dexamethasone (1 μM), used as a positive control during the assay, significantly inhibited NF-κB activation (lower luciferase activity) (p-value < 0.001). According to our results, L929 cells treated with PBE in a concentration of 20 μg/mL clearly inhibited (p-value < 0.05) the NF-κB reporter gene induction by TNF-α compared to the stimulated control (Figure 7.9A). No other, lower concentrations of PBE did show significant inhibition. Looking at the individual compounds of PBE, a significant lower luciferase activity was noticeable for procyanidin B1 (20 μM) and caffeic acid (20 μM), with a p-value < 0.0001 and a p-value < 0.05 respectively.

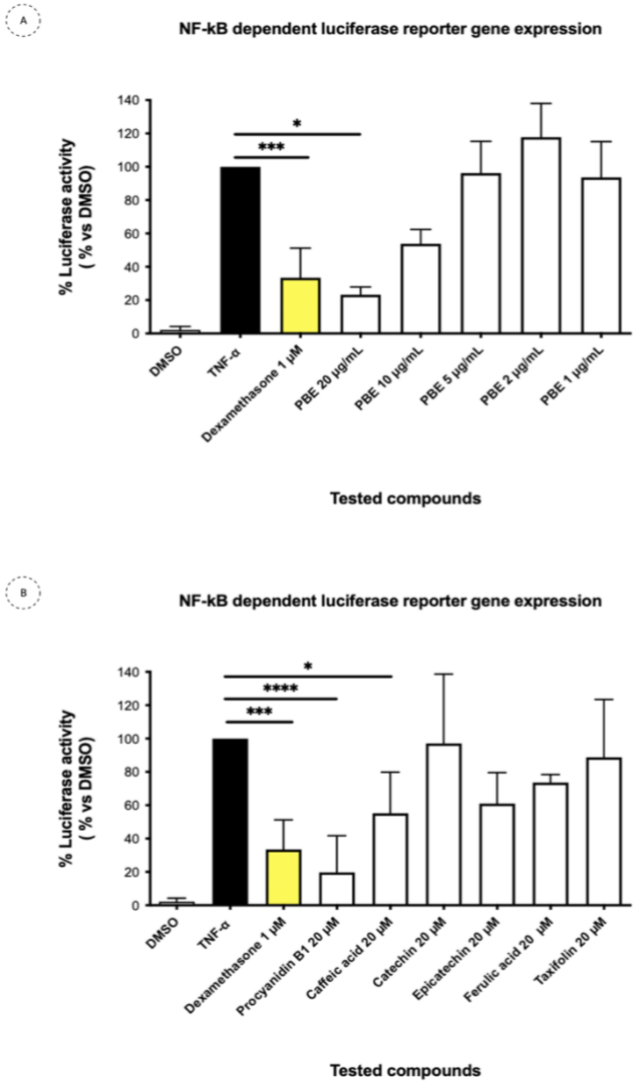

Figure 7.9*:* NF-κB dependent luciferase reporter gene expression measured by the luciferase receptor assay (luciferase activity is represented as a percentage compared to the control DMSO). Addition of TNF-α results in an activation of NF-κB which is linked to the expression of luciferase that can be quantified via a bioluminescence assay. Dexamethasone was used as a reference NF-κB inhibitor. The graphs depict compiled data of at least three independent experiments. P-values are expressed as **** p-value < 0.0001, ***p-value < 0.001 and * p-value < 0.05 as compared to control stimulated with TNF-α.

An IC_{50}-value could only be calculated for PBE (IC_{50} = 8.503 µg/mL) (Figure 7.10) since procyanidin B1 and caffeic acid both resulted in a dose-response curve with unstable parameters and therefore, the IC_{50}-value could not be calculated in GraphPad.

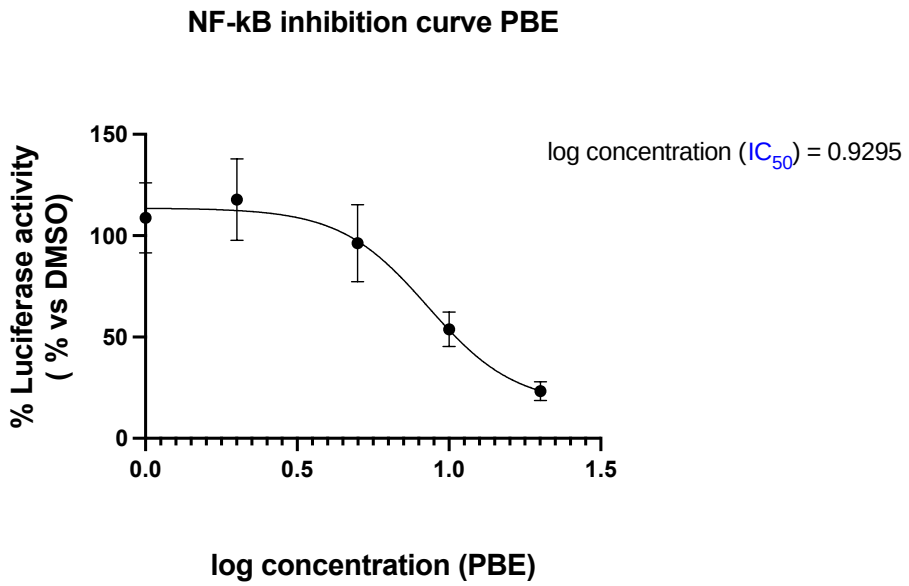

Figure 7.10: NF-κB inhibition curve for PBE. A NF-κB inhibition curve was calculated with a R^2 value of 0.8853. DMSO: dimethyl sulfoxide; PBE: French Maritime Pine Bark Extract.

7.3.2.2 Discussion NF-κB assay

According to earlier research work by Di Lorenzo et al. (Di Lorenzo et al., 2013) green and black tea extracts inhibit TNF-α induced NF-κB driven transcription *in vitro* in human adenocarcinoma cells transfected with a plasmid containing the luciferase reporter gene under the control of NF-κB promoter, and the effect on this inflammatory cellular response is closely linked to their total catechin content. They demonstrated a strong correlation between catechin content and inhibition of NF-κB driven transcription. Even though in our study no significant lower luciferase activity could be observed for (+)-catechin and epicatechin, PBE (20 µg/mL) and its main polyphenolic constituent procyanidin B1 (20 µM) did show a significantly lower NF-κB driven transcription than

L929 cells only treated with TNF-α. Nevertheless, procyanidins (composed of catechin and epicatechin subunits linked to each other) and especially procyanidin B1, which constitute the main fraction of PBE, significantly decreased NF-κB driven transcription in our study. Even though it was previously reported that procyanidin B-type dimers undergo extensive biotransformation (Chapter 2), some studies detected procyanidin B1 in human serum samples after intake of a procyanidin-rich extract (Sano et al., 2003). Research work by Park et al. (Park & Hong, 2016) showed that the monomeric flavonoids (catechin, epicatechin and taxifolin) counteracted IFN-γ induced NF-κB activation (IFN-γ stimulation of RAW 264.7 macrophages resulted in a nearly two-fold increase of the luciferase activity). Moreover, pretreatment of macrophages with procyanidin B1 and procyanidin B2 led to a slight decrease in the luciferase activity. In agreement with the above, Jurkat T cells pretreated with procyanidin B1 or B2 showed a reduced NF-κB-dependent gene expression as compared to cells treated with TNF-α alone (Mackenzie et al., 2004). Nevertheless, it should be noted that procyanidins are not absorbed as such *in vivo*, since they undergo biotransformation processes by the gut microbiome as described by *in vivo* studies (Gado et al., 2023). Nevertheless, earlier research work did observe low dimeric plasma concentrations (Sano et al., 2003).

Regarding the PBE extract, Fan et al. (Fan et al., 2015) demonstrated that PBE (50 μg/mL) suppressed lipopolysaccharide induced NF-κB activation in BV2 microglia. These results are consistent with our findings showing that PBE in a concentration of 20 μg/mL significantly reduces NF-κB activation. Also, results from an animal-based study in which the effects of PBE on ventilator-induced lung injury of rats were investigated, demonstrated that the production of pro-inflammatory cytokines, such as TNF-α and IL-1β was reduced towards normal levels through the inhibition of NF-κB activation after administration of PBE (Xia et al., 2015).

The NF-κB dependent luciferase reporter assay was investigated using L929 mouse fibroblasts, after prior transfection with the recombinant NF-κB dependent reporter gene construct p(IL6κB)350hu.IL6P-Luc. Initially our aim was to test the effects on TNF-induced NF-κB activation in human EA.hy.926 endothelial cells (hybrid of human umbilical vein cells (HUVECs) with the human epithelial cell line A549), transfected with the recombinant NF-κB dependent reporter gene, since upregulation of cell adhesion

molecules by NF-κB on endothelial cells alters the adhesive property of the vasculature and causes uncontrolled infiltration of leukocytes into the inflamed tissue (Balwani et al., 2012). Nevertheless, a vast body of evidence confirms that fibroblasts produce collagen and various other proteins that make up the ECM (Lendahl et al., 2022). Therefore, extrapolation from our results is still possible in order to get a better understanding of the role of NF-κB in the blood vessel wall.

7.3.3 Infrared Western blotting to determine expression levels of proteins

7.3.3.1 Results of HO-1 expression levels in non-stimulated and stimulated HAoECs

Non-stimulated (no addition of H_2O_2) HAoECs and stimulated HAoECs (addition of H_2O_2 to induce oxidative stress in cell culture) were treated with different concentrations of PBE and its polyphenolic constituents. Only the compounds that resulted in significant altered expression levels of HO-1 as compared to untreated cells, are depicted using log2 fold change graphs and Western blots (Figure 7.11). An overview of all the tested compounds (both significant and non-significant results can be found in Supplementary Table S1).

Considering the fact that stimulation of HO-1 inhibits excessive oxidation of for instance proteins by scavenging free radicals, it would therefore be beneficial in protecting ECs from oxidative stress-induced injury. Indeed, a significantly higher expression of HO-1 was observed in HAoECs treated with 80 µg/mL PBE as compared to untreated, unstimulated cells ($p = 0.0023$). In HAoECs stimulated with H_2O_2 for 30 min and afterwards treated with 80 µg/mL PBE, no consistent results could be obtained for HO-1 expression levels. On the contrary, HAoECs stimulated with H_2O_2 during 6 h in the presence of 80 µg/mL PBE clearly showed significantly higher HO-1 expression levels as compared to untreated, stimulated (6 h) cells ($p = 0.048$).

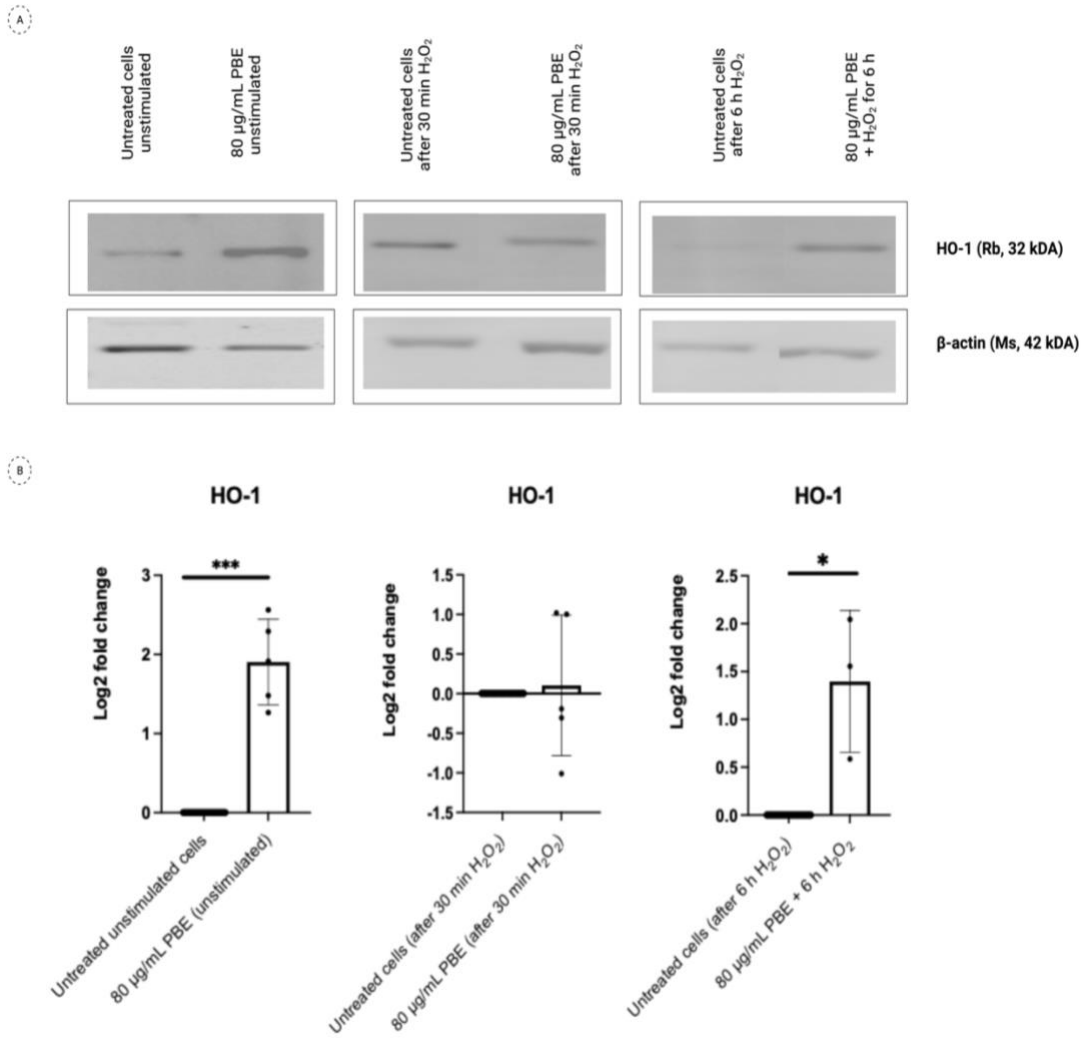

Figure 7.11: Effect of PBE (80 µg/mL) on HO-1 expression levels. Different test conditions are depicted by their respective Western blot results (A) and are graphically depicted using a log2 fold change (B). A significant increase in HO-1 expression was observed for unstimulated HAoECs treated with 80 µg/mL PBE as compared to untreated, unstimulated cells and for H_2O_2-stimulated HAoECs (6 h) in the presence of 80 µg/mL PBE as compared to their respective controls. β-actin was used as a loading control. HO-1: heme oxygenase 1; HAoECs: Human Aortic Endothelial Cells; Rb: rabbit; PBE: French Maritime Pine Bark Extract. ***: p-value < 0.001; *: p-value < 0.05. LMM with Bonferroni correction.

7.3.3.2 Results of Nrf2 expression levels in non-stimulated and stimulated HAoECs

Higher Nrf2 expression removes ROS and facilitates antioxidative and anti-inflammatory cell protective processes. Nevertheless, for none of the polyphenolic compounds nor for PBE, it was possible to observe significantly altered Nrf2 expression levels as compared to untreated cells, not for unstimulated HAoECs or stimulated HAoECs, as shown in Supplementary Table S1.

7.3.3.3 Results of eNOS and phosphorylated eNOS expression levels in non-stimulated and stimulated HAoECs

eNOS is known to be phosphorylated at multiple sites such as Ser1777 which is associated with increased enzymatic activity of eNOS and Thr495, linked to a decreased enzyme activity (Serreli & Deiana, 2023). Expression levels of eNOS and phosphorylated eNOS (phosphorylation at Ser1177 and Thr495) were analysed in HAoECs (both unstimulated and stimulated) treated with different concentrations of PBE and its polyphenolic compounds. Throughout the Western blot experiments, no expression of eNOS was visible on the blots (also not for untreated cells). Since no clear bands indicating eNOS expression could be observed, log2 fold change graphs were not generated. Also, stimulated HAoECs did not show eNOS expression.

Statistical analyses revealed significantly altered expression levels of PeNOS(Ser1177) in unstimulated HAoECs treated with 80 µg/mL PBE ($p = 0.0052$). Also stimulation with H_2O_2 for 30 min followed by addition of 80 µg/mL PBE, significantly increased PeNOS(Ser1177) expression levels in HAoECs ($p= 0.027$) as shown in Figure 7.12. Treatment with PBE in the presence of H_2O_2 during 6 h did not show clear changes in PeNOS(Ser1177) expression levels since there was only 1 measurement.

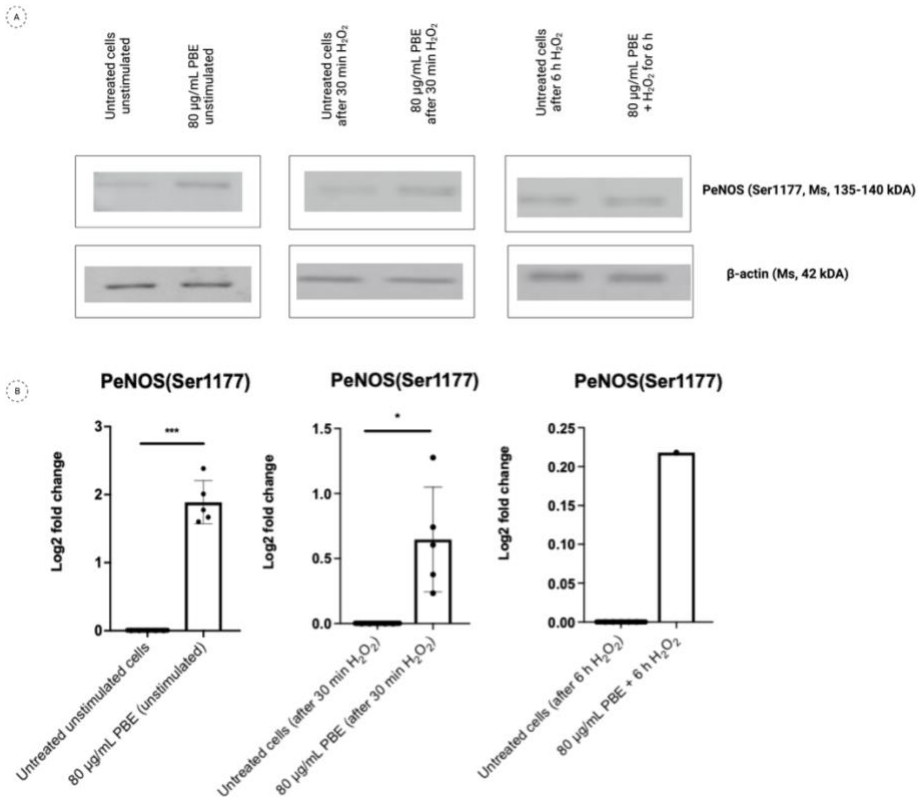

Figure 7.12: Effect of PBE (80 µg/mL) on PeNOS(Ser1177) expression levels. Different test conditions are depicted by their respective Western blot results (A) and are graphically depicted using a log2 fold change (B). A statistically non-significant increase in PeNOS(Ser1177) expression was observed for unstimulated HAoECs treated with 80 µg/mL PBE as compared to untreated, unstimulated cells and for stimulated HAoECs (30 min with H_2O_2) in the presence of 80 µg/mL PBE as compared their respective controls. β-actin was used as a loading control. HAoECs: Human Aortic Endothelial Cells; kDA: kilo Dalton; Ms: mouse; PBE: French Maritime Pine Bark Extract; PeNOS(Ser1177): phosphorylated endothelial nitric oxide synthase. ***: p-value < 0.001; *: p-value < 0.05. LMM with Bonferroni correction.

In Figure 7.13 the expression levels of PeNOS(Thr495) in unstimulated and stimulated HAoECs treated with 80 µg/mL PBE is represented using log2 fold change graphs and

Western blots. According to statistical analyses, no significant altered PeNOS(Thr495) could be observed. However, looking at the Western blots and the individual datapoints on the log2 fold change graph, a statistically non-significant decreased expression of PeNOS(Thr495) was noticeable for unstimulated HAoECs treated with 80 µg/mL PBE as compared to untreated cells, and for simulated HAoECs (30 min H_2O_2) as compared to untreated, stimulated (30 min H_2O_2) cells. In fact, in the conditions where PBE was added, blots did not show a clear band for PeNOS(Thr495) whereas the β-actin expression levels were normal. This suggests that PBE could indeed reduce PeNOS(Thr495) expression in our experiments.

The ratio PeNOS(Ser1177) to PeNOS(Thr495) was calculated (data not shown) to assess the overall effect of PBE on eNOS activation; however, LMM analysis did not reveal statistical significance ($p = 0.054$).

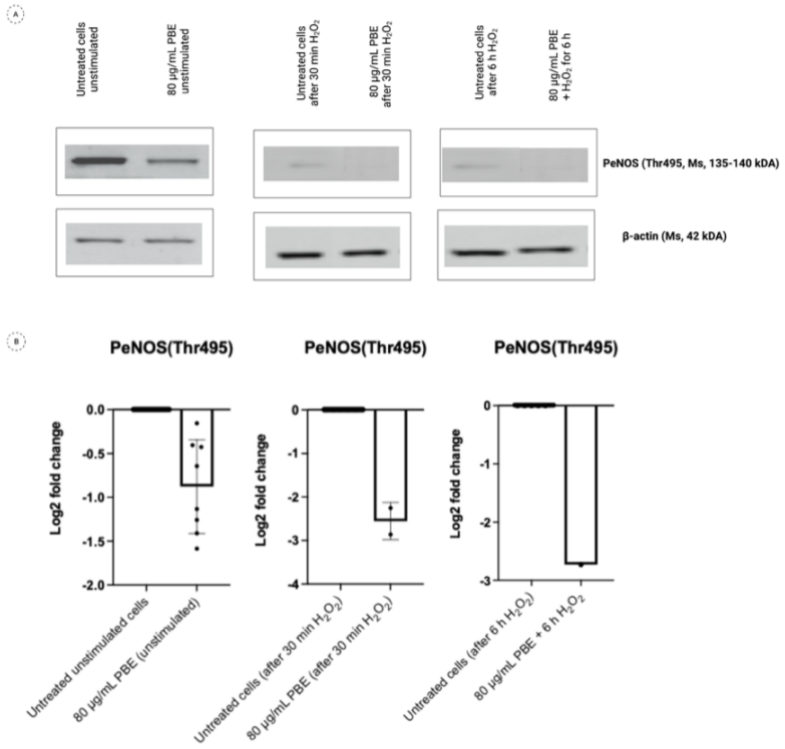

Figure 7.13: Effect of PBE (80 µg/mL) on PeNOS(Thr495) expression levels. Different test conditions are depicted by their respective Western blot results (A) and are graphically depicted using a log2 fold change (B). A statistically non-significant decrease in PeNOS(Thr495) expression was observed for unstimulated HAoECs treated with 80 µg/mL PBE as compared to untreated, unstimulated cells and for stimulated HAoECs (30 min with H_2O_2) in the presence of 80 µg/mL PBE as compared to their respective controls β-actin was used as a loading control. HAoECs: Human Aortic Endothelial Cells; kDA: kilo Dalton; Ms: mouse; PBE: French Maritime Pine Bark Extract; PeNOS(Thr495): phosphorylated endothelial nitric oxide synthase.

7.3.3.4 Results of HO-1 expression levels in non-stimulated and stimulated HAoSMCs

Expression levels of HO-1 were also assessed in non-stimulated and stimulated HAoSMCs. An overview of all tested compounds with their respective concentrations on

expression levels of different proteins including HO-1 can be found in Supplementary Table S2. Figure 7.14 represents the expression levels of HO-1 in unstimulated HAoMSCs after treatment with 80 µg/mL PBE, and in stimulated HAoSMCs (both 30 min H_2O_2 and 6 h H_2O_2) treated with 80 µg/mL PBE as compared to untreated, unstimulated and untreated, stimulated respectively. Even though statistical analyses did not reveal significantly altered expression levels of HO-1 in HAoSMCs treated with 80 µg/mL PBE, altered HO-1 expression was noticeable (albeit with variable results) by looking at the log2 fold change graphs and the intensity of the bands on the Western blots itself (Figure 7.14). Indeed, unstimulated HAoSMCs treated with 80 µg/mL PBE showed a non-significant higher HO-1 expression as compared to untreated, unstimulated cells. Moreover, stimulation with H_2O_2 for 30 min followed by addition of 80 µg/mL PBE non-significantly increased HO-1 expression levels in HAoSMCs. Treatment with 80 µg/mL PBE in the presence of H_2O_2 during 6 h also seems to increase the expression of HO-1; however, there is only one measurement here.

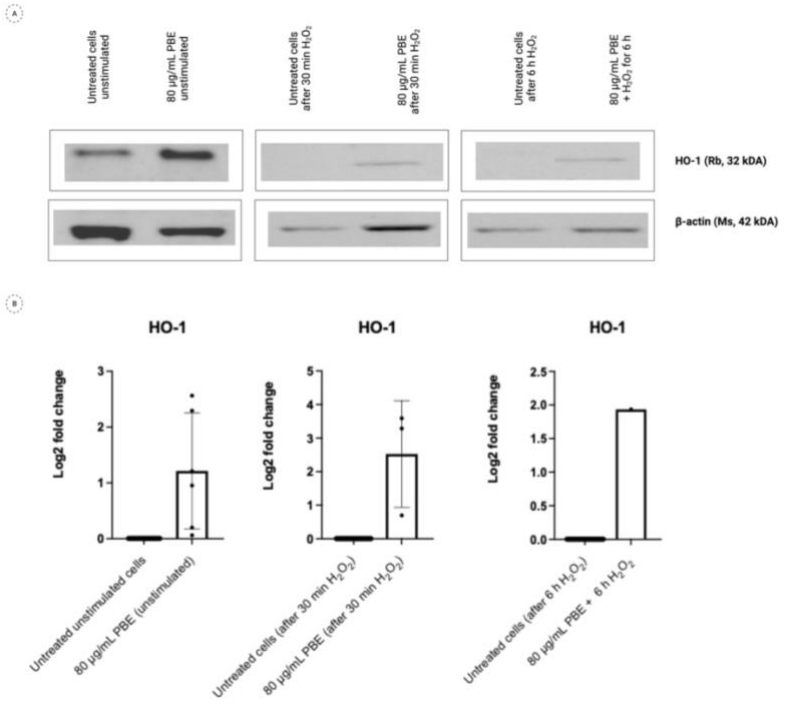

Figure 7.14: Effect of PBE (80 µg/mL) on HO-1 expression levels. Different test conditions are depicted by their respective Western blot results (A) and are graphically depicted using a log2 fold change (B). A statistically non-significant increase in HO-1 expression was observed for unstimulated HAoSMCs treated with 80 µg/mL PBE as compared to untreated, unstimulated cells and for stimulated HAoSMCs (30 min with H_2O_2) in the presence of 80 µg/mL PBE as compared to their respective controls. β-actin was used as a loading control. HO-1: heme oxygenase 1; HAoSMCs: Human Aortic Smooth Muscle Cells; kDA: kilo Dalton; Ms: mouse; PBE: French Maritime Pine Bark Extract; Rb: rabbit.

7.3.3.5 Results of Nrf-2 expression levels in non-stimulated and stimulated HAoSMCs

As shown in Figure 7.15 unstimulated HAoSMCs treated with 80 µg/mL PBE did show a non-significant higher Nrf2 expression as compared to untreated, unstimulated cells. Stimulation with H_2O_2 for 30 min followed by addition of 80 µg/mL PBE also non-significantly increased Nrf2 expression levels in HAoSMCs as well as treatment with 80

µg/mL PBE in the presence of H_2O_2 during 6 h. The increased Nrf2 expression is much smaller after 6 h H_2O_2 stimulation than after 30 min of H_2O_2. Even though statistical analyses did not reveal significance for Nrf2 expression levels in HAoSMCs treated with 80 µg/mL PBE, a trend towards altered Nrf2 expression could be observed.

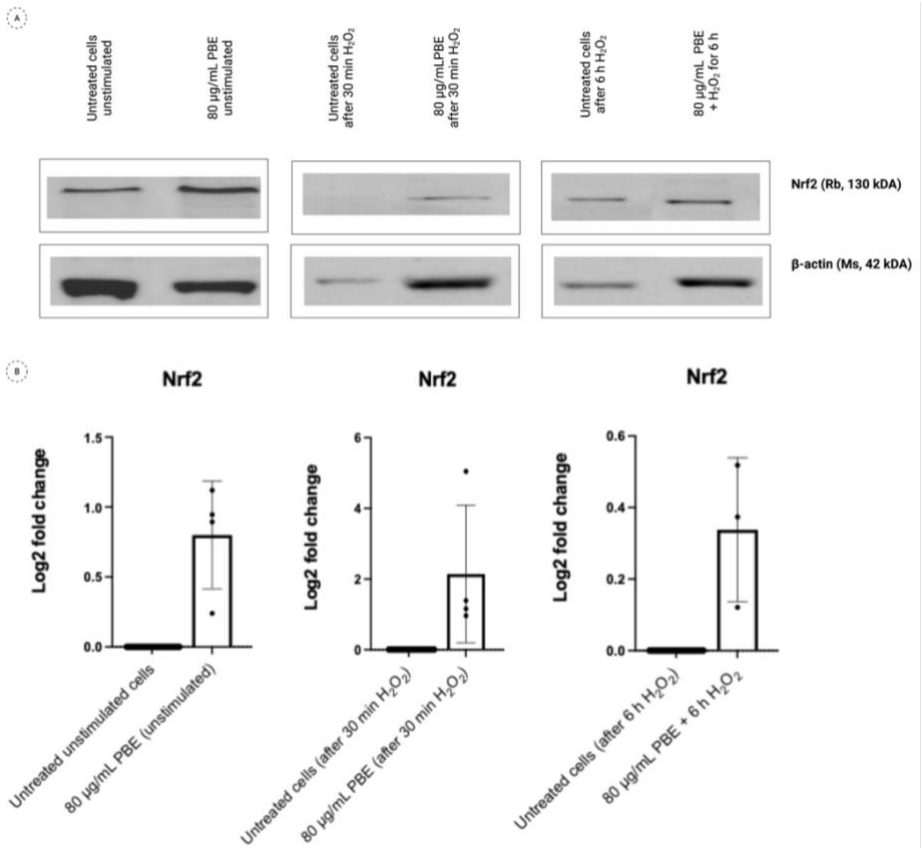

Figure 7.15: Effect of PBE (80 µg/mL) on Nrf2 expression levels. Different test conditions are represented by their respective Western blot results (A) and are graphically depicted using a log2 fold change (B). A statistically non-significant increase in Nrf2 expression was observed for unstimulated HAoSMCs treated with 80 µg/mL PBE as compared to untreated, unstimulated cells and for stimulated HAoSMCs (30 min with H_2O_2 and 6 h H_2O_2) in the presence of 80 µg/mL PBE as compared to their respective controls. β-actin was used as a loading control. HAoSMCs: Human Aortic Smooth Muscle Cells; kDA: kilo Dalton; Ms: mouse; Nrf2: nuclear factor erythroid 2–related factor 2; PBE: French Maritime Pine Bark Extract; Rb: rabbit.

7.3.3.6 Results of MMP-12 expression levels in non-stimulated and stimulated HAoSMCs

Since elevated levels of MMP-12, are responsible for cleavage of elastin, MMP levels should be reduced in order to have a protective role against the underlying mechanisms of AS. Figure 7.16 shows the log2 fold change graphs and Western blots for the MMP-12 expression levels (propeptide and active form) in unstimulated HAoSMCs treated with 80 µg/mL PBE. No significance could be obtained since there is a large variability within the datapoints and no firm conlcusions could be made. Nevertheless, on the log2 fold change graphs and the Western blots, a trend towards ecreased expression of both MMP-12 forms (propeptide and active form) was noticeable. In stimulated HAoSMCs (30 min H_2O_2 and after 6 h of H_2O_2) a clear expression of MMP-12 was no longer observed. Therefore, no log2 fold change graphs were generated.

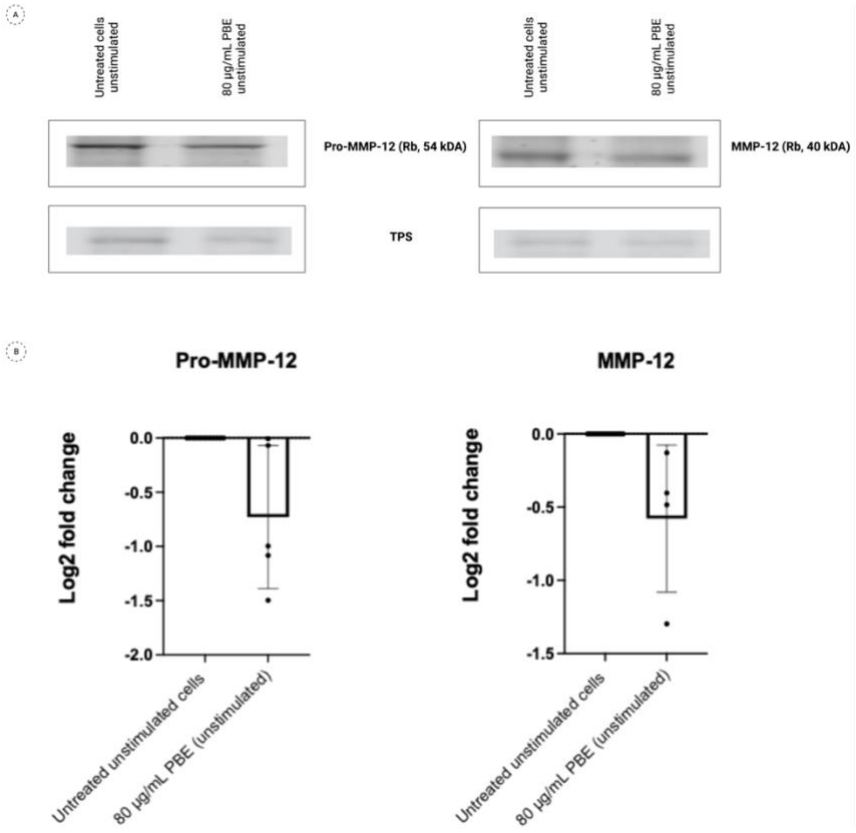

Figure 7.16: Effect of PBE (80 µg/mL) on MMP-12 expression levels. Different test conditions are represented by their respective Western blot results (A) and are graphically depicted using a log2 fold change (B). A statistically non-significant decrease in MMP-12 expression (propeptide) and active form was observed for unstimulated HAoSMCs treated with 80 µg/mL PBE as compared to untreated, unstimulated cells. TPS was used as a loading control. MMP-12: matrix metalloprotease 12; HAoSMCs: Human Aortic Smooth Muscle Cells; kDA: kilo Dalton; PBE: French Maritime Pine Bark Extract; Rb: rabbit; TPS: Total Protein Stain.

7.3.3.7 Discussion Western blot data

According to our Western blot data, PBE significantly enhanced the HO-1 expression in HAoECs whereas in HAoSMCs a trend towards elevated expression of HO-1 was noticeable after treatment with PBE. Moreover, after induction of oxidative stress by

addition of H_2O_2, a significantly higher expression was observed in HAoECs as well as a non-significant elevated expression in HAoSMCs. These findings broadly support the work of Shin et al. (Shin et al., 2013) who also observed an enhanced HO-1 expression after addition of PBE in LPS-stimulated RAW264.7 cells. Another study by Li et al. (Li et al., 2021) investigating the effects of tea polyphenols (which contain epicatechin as polyphenolic constituent) revealed that these polyphenols can upregulate HO-1 expression and thereby reverse oxidative stress. Indeed, HO-1 can be induced by various stimuli linked to oxidative stress conditions and is considered to be a stress response protein, and enhanced HO-1 production can therefore provide cellular protection against oxidative injury (Bellner et al., 2020; Chiang et al., 2021). Taking into account the low basal levels of HO-1 in untreated endothelial and smooth muscle cells (Li Volti et al., 2002), our results thus show an altered expression of HO-1 (significantly and non-significantly higher in the cells treated with PBE.

Previous studies have reported that tea polyphenols and certain polyphenolic constituents enhance the activity of HO-1 by activating the Nrf2 signalling pathway (Li et al., 2021). Increasing evidence shows that plant-derived polyphenols are natural activators of Nrf2, thereby driving downstream antioxidant target genes. Indeed, it has been demonstrated that the activation of Nrf2 is linked to higher expression levels of downstream proteins including HO-1 (Chen et al., 2022). For instance, catechins from olives have been shown to activate this Keap/Nrf2/ARE signaling pathway and thereby providing a protective effect during oxidative stress conditions (Zenkov et al., 2016). Even though HO-1 expression seemed to be increased in HAoECs and HAoSMCs (after addition of H_2O_2 as well as in non-stimulated conditions), firm evidence of enhanced Nrf2 expression is lacking. Since our results only show an increase in Nrf2 expression (statistically not significant) for HAoSMCs, it is difficult to directly link the enhanced HO-1 expression to activation of Nrf2. A plausible explanation of the increase in HO-1 expression might lie in the fact that HO-1 expression levels can be triggered by a variety of stimuli, of which the Nrf2 signaling pathway is only one of them (Funes et al., 2020). Flavonoids for example can modulate various signalling cascades such as the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (De Bruyne et al., 2019).

Sufficient production and bioavailability of eNOS-derived NO is pivotal for the maintenance of a healthy endothelium, whereas a reduced eNOS availability results in

endothelial dysfunction (De Bruyne et al., 2019). In this study, no expression levels of eNOS were observed during the Western blot experiments; however, the most striking was the substantial difference in expression levels between different phosphorylation sites of eNOS. A clear significant increase in PeNOS(Ser1177) was observed for unstimulated and stimulated (30 min H_2O_2) HAoECS treated with 80 µg/mL PBE. On the contrary, a statistically non-significant decrease was noticeable in PeNOS(Thr495) status for unstimulated and stimulated (30 min H_2O_2) HAoECS treated with 80 µg/mL PBE. Indeed, the regulation of eNOS involves various pathways including post-translational modifications such as phosphorylation, caused upstream by the activation of PI3k/Akt or for example mitogen activated protein kinase (MAPK).According to earlier research work by Kwak et al. (Kwak et al., 2009), Western blot analyses revealed that 3 µg/mL PBE already increased PeNOS(Ser1177) expression in aortic segments. Another study showed that catechins from green tea activate dephosphorylation at Thr495, and thus enhance the activity of eNOS (Forte et al., 2016). Even though the effect of PBE on phosphorylation of eNOS(Thr495) expression levels has not yet been described in literature, our findings demonstrate that PBE lowered the phosphorylation at the inhibitory site Thr495, leading to increased eNOS activity and enhanced NO production. Even though the overall effect of PBE (depicted by the ratio of PeNOS(Ser1177) to PeNOS(Thr495) on the activation of eNOS did not reach statistical significance, our findings suggest that PBE can increase eNOS activity and therefore augments NO production.

Another finding from this study is the potential of PBE and some of its individual constituents to decrease MMP-12 expression levels in non-stimulated HAoSMCs, albeit non-significantly. Since MMP-12 is responsible for the breakdown of extracellular matrix components and its activity is linked to vascular remodelling, inhibition of its activity may exert potential cardiovascular health benefits (Atkinson et al., 2023). A possible explanation for the decreased MMP-12 activity might be due to the downregulated expression of for instance NF-κB. Indeed, previous studies have demonstrated that the activity and expression of these transcription factors is downregulated by dietary polyphenols, and that it might be an important underlying anti-MMP mechanism.

Overall, the individual constituents did not result in significantly higher HO-1, Nrf2, and PeNOS(Ser1177) expression levels nor in a significant decreased expression of

PeNOS(Thr495 and MMP-12, in both tested cell lines. These results further support the idea that the effect of the extract is larger than the effect of its individual polyphenolic compounds.

In conclusion, we aimed to investigate the expression of several proteins, related to regulatory mechanisms underlying arterial stiffness, in HAoECs and HAoSMCs under unstimulated and H_2O_2 stimulated conditions. While our results provide valuable insights, it is important to acknowledge the limitations of this study such as the small sample size for some proteins.

7.4 General discussion

Inflammation is the immune system's response to infection and injury and has been implicated in the pathogenesis of NCDs. Prostaglandins are pivotal in the generation of the inflammatory response. Prostaglandin production mainly depends on the activity of COX-1 and COX-2 enzymes, both contributing to the prostanoid release during inflammation. Suppressing the generation of prostaglandins from arachidonic acid by these COX-enzymes, and NF-κB pathway inhibition, can be important anti-inflammatory mechanisms. In this research work, the anti-inflammatory properties of PBE and its individual constituents were evaluated in *in vitro* assays, focusing on COX and NF-κB. Their anti-inflammatory potential was evaluated in order to investigate their role and mechanism of action in the prevention and treatment of AS, considered to be an important risk factor for CVD that often precedes its development.

Our results demonstrate that COX inhibition is, at least in part, responsible for the anti-inflammatory mechanisms of PBE since it showed *in vitro* inhibitory effects on COX-1 and/or COX-2, albeit with a different profile. Inhibitory effects could be observed for PBE (with an IC_{50} of 9.035 µg/mL on COX-1) and its constituents procyanidin B1 (IC_{50} of 525.9 µM on COX-1) and caffeic acid, which inhibited both COX-1 and COX-2. Ferulic acid and (+)-catechin showed selective but weak COX-2 inhibition. In addition, a synergistic effect was observed between the individual polyphenolic compounds, which could only partly account for the effects of PBE itself. Moreover, PBE (20 µg/mL) showed a clear, significant inhibition on the NF-κB reporter gene induction by TNF-α in L929 cells, compared to the stimulated control (only TNF-α) reaching an IC_{50} of 8.503 µg/mL. Besides the extract, also some of its individual constituents were shown to significantly inhibit the NF-κB reporter gene induction by TNF-α (procyanidin B1 and caffeic acid both in a concentration of 20 µM). The Western blot data presented herein aimed to investigate the expression of several proteins in HAoECs and HAoSMCs under unstimulated and H_2O_2 stimulated conditions. Our results aim to shed light on the regulatory mechanisms underlying arterial stiffness. Even though these results provide

valuable insights, it is important to acknowledge the limitations of this study such as the small number of observations for some proteins.

Interpreting our *in vitro* data and extrapolating it to the *in vivo* situation requires caution. Indeed, these *in vitro* assays do not take into consideration important *in vivo* aspects such as bioavailability, sites of distribution within the body and the biotransformation of the compounds (Mülek et al., 2017). In view of the relatively high concentrations at which inhibition or altered protein inhibition start, and taking into account the plasma concentrations found in literature, significant COX/NF-κB inhibition or altered protein expression of these parent compounds themselves, will not be relevant *in vivo*. Nevertheless, while evidence from *in vitro* experiments does not necessarily prove its *in vivo* activity, our findings do support and provide a rationale for the use of polyphenols to target mechanisms underlying arterial stiffness. A first step to extrapolate our *in vitro* data into a more clinically relevant situation, might be the implementation of an *ex-vivo* evaluation of aortic stiffness and vascular reactivity. The use of an organ bath (developed in the Lab of Physiopharmacology, University of Antwerp) allows to test the potential effects of PBE on mechanisms underlying arterial stiffness. Furthermore, animal experiments using animal models of CV ageing and disease can be performed using *in vivo* measurements of aortic PWV and peripheral BP after treatment with PBE.

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Supplementary materials

Table S1 : Overview of the tested PBE extract and polyphenolic constituents and their effect on the expression levels of various proteins in HAoECs using Western Blot analyses.

Tested conditions	Tested compound	Protein of interest	Number of analyses	Effect	Significance
Unstimulated HAoECs	80 µg/mL PBE	HO-1	n = 5	↑	$p \leq 0.05$
		Nrf2	n = 5	↑ and ↓	ns
		eNOS	n = 4	-	-
		PeNOS(Ser1177)	n = 4	↑	$p \leq 0.001$
		PeNOS(Thr495)	n = 8	↓	ns
Unstimulated HAoECs	60 µg/mL PBE	HO-1	n = 3	↑ and ↓	ns
		Nrf2	n = 3	↑ and ↓	ns
		eNOS	n = 4	-	-
		PeNOS(Ser1177)	n = 6	↑ and ↓	ns
		PeNOS(Thr495)	n = 8	↓	ns
Unstimulated HAoECs	40 µg/mL PBE	HO-1	n = 2	↓	ns
		Nrf2	n = 3	↑ and ↓	ns
		eNOS	n = 3	-	-
		PeNOS(Ser1177)	n = 3	↑ and ↓	ns
		PeNOS(Thr495)	n = 5	↑ and ↓	ns
Unstimulated HAoECs	20 µg/mL PBE	HO-1	n = 3	↓	ns
		Nrf2	n = 4	↑ and ↓	ns
		eNOS	n = 3	-	-
		PeNOS(Ser1177)	n = 6	↑ and ↓	ns
		PeNOS(Thr495)	n = 8	↑ and ↓	ns
Unstimulated HAoECs	20 µM (+)-catechin	HO-1	n = 3	↓	ns
		Nrf2	n = 3	↑ and ↓	ns
		eNOS	n = 3	-	-
		PeNOS(Ser1177)	n = 3	↑ and ↓	ns

Table S1 : Overview of the tested PBE extract and polyphenolic constituents and their effect on the expression levels of various proteins in HAoECs using Western Blot analyses (continued).

Tested conditions	Tested compound	Protein of interest	Number of analyses	Effect	Significance
Unstimulated HAoECs	20 µM (+)-catechin	PeNOS(Thr495)	n = 4	↓	ns
Unstimulated HAoECs	20 µM epicatechin	HO-1	n = 2	↓	ns
		Nrf2	n = 2	↓	ns
		eNOS	n = 3	-	-
		PeNOS(Ser1177)	n = 3	↑	ns
		PeNOS(Thr495)	n = 4	↑ and ↓	ns
Unstimulated HAoECs	20 µM procyanidin B1	HO-1	n = 2	↓	ns
		Nrf2	n = 2	↑	ns
		eNOS	n = 2	-	-
		PeNOS(Ser1177)	n = 3	↓	ns
		PeNOS(Thr495)	n = 4	↑ and ↓	ns
Unstimulated HAoECs	20 µM caffeic acid	HO-1	n = 2	↓	ns
		Nrf2	n = 2	↓	ns
		eNOS	n = 2	-	-
		PeNOS(Ser1177)	n = 2	↓	ns
		PeNOS(Thr495)	n = 2	↓	ns
Unstimulated HAoECs	20 µM ferulic acid	HO-1	n = 2	↑ and ↓	ns
		Nrf2	n = 2	↑	ns
		eNOS	n = 2	-	-
		PeNOS(Ser1177)	n = 2	↑	ns
		PeNOS(Thr495)	n = 4	↓	ns
Unstimulated HAoECs	20 µM taxifolin	HO-1	n = 2	↑ and ↓	ns
		Nrf2	n = 2	↑	ns
		eNOS	n = 2	-	-
		PeNOS(Ser1177)	n = 2	↓	ns
		PeNOS(Thr495)	n = 3	↓	ns

Table S1 : Overview of the tested PBE extract and polyphenolic constituents and their effect on the expression levels of various proteins in HAoECs using Western Blot analyses (continued).

Tested conditions	Tested compound	Protein of interest	Number of analyses	Effect	Significance
Stimulated (30 min H_2O_2) HAoECs	80 µg/mL PBE	HO-1	n = 5	↑ and ↓	ns
		Nrf2	n = 2	↑	ns
		eNOS	n = 2	-	-
		PeNOS(Ser1177)	n = 5	↑	$p \leq 0.05$
		PeNOS(Thr495)	n = 2	↓	ns
Stimulated (30 min H_2O_2) HAoECs	40 µg/mL PBE	HO-1	n = 2	↑ and ↓	ns
		Nrf2	n = 2	↑	ns
		eNOS	n = 2	-	-
		PeNOS(Ser1177)	n = 2	↑	ns
		PeNOS(Thr495)	n = 2	↓	ns
Stimulated (6 h H_2O_2) HAoECs	80 µg/mL PBE	HO-1	n = 3	↑	$p \leq 0.001$
		Nrf2	n = 3	↑	ns
		eNOS	n = 2	-	-
		PeNOS(Ser1177)	n = 1	↑	ns
		PeNOS(Thr495)	n = 1	↓	ns
Stimulated (6 h H_2O_2) HAoECs	40 µg/mL PBE	HO-1	n = 2	↑ and ↓	ns
		Nrf2	n = 2	↓	ns
		eNOS	n = 2	-	-
		PeNOS(Ser1177)	n = 2	↑ and ↓	ns
		PeNOS(Thr495)	n = 2	↓	ns

eNOS: endothelial nitric oxide synthase; HAoECs: Human Aortic Endothelial Cells; HO-1: heme oxygenase 1; H_2O_2: hydrogen peroxide; Nrf2: nuclear factor erythroid 2–related factor 2; ns: not significant; PBE: French Maritime Pine Bark Extract; PeNOS(Thr495): phosphorylated endothelial nitric oxide synthase; PeNOS(Ser1177): phosphorylated endothelial nitric oxide synthase; -: not measured.

Table S2 : Overview of the tested PBE extract and polyphenolic constituents and their effect on the expression levels of various proteins in HAoSMCs using Western Blot analyses.

Tested conditions	Tested compound	Protein of interest	Number of analyses	Effect	Significance
Unstimulated HAoSMCs	80 µg/mL PBE	HO-1	n = 6	↑	ns
		Nrf2	n = 4	↑ and ↓	ns
		MMP-12	n = 5	↓	ns
Unstimulated HAoSMCs	60 µg/mL PBE	HO-1	n = 2	↓	ns
		Nrf2	n = 3	↓	ns
		MMP-12	n = 2	↓	ns
Unstimulated HAoSMCs	40 µg/mL PBE	HO-1	n = 4	↓	ns
		Nrf2	n = 3	↓	ns
		MMP-12	n = 3	↓	ns
Unstimulated HAoSMCs	20 µg/mL PBE	HO-1	n = 4	↓	ns
		Nrf2	n = 3	↓	ns
		MMP-12	n = 3	↓	ns
Unstimulated HAoSMCs	20 µM (+)-catechin	HO-1	n = 4	↓	$p \leq 0.05$
		Nrf2	n = 6	↓	ns
		MMP-12	n = 4	↓	ns
Unstimulated HAoSMCs	20 µM epicatechin	HO-1	n = 4	↑ and ↓	ns
		Nrf2	n = 6	↓	ns
		MMP-12	n = 5	↓	ns
Unstimulated HAoSMCs	20 µM procyanidin B1	HO-1	n = 4	↓	$p \leq 0.01$
		Nrf2	n = 6	↓	ns
		MMP-12	n = 7	↓	$p \leq 0.05$
Unstimulated HAoSMCs	20 µM caffeic acid	HO-1	n = 2	↓	ns
		Nrf2	n = 3	↓	ns
		MMP-12	n = 3	↓	ns

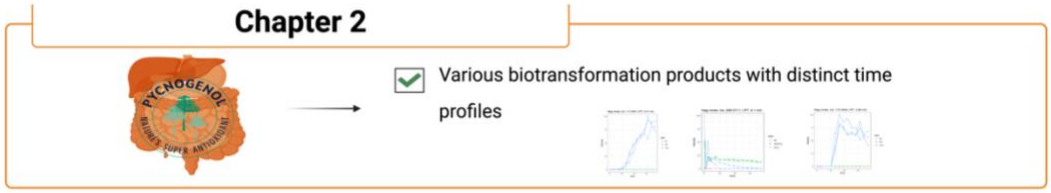
Table S2 : Overview of the tested PBE extract and polyphenolic constituents and their effect on the expression levels of various proteins in HAoSMCs using Western Blot analyses (continued).

Tested conditions	Tested compound	Protein of interest	Number of analyses	Effect	Significance
Unstimulated HAoSMCs	20 µM ferulic acid	HO-1	n = 4	↑ and ↓	ns
		Nrf2	n = 5	↓	$p \leq 0.01$
		MMP-12	n = 6	↓	ns
Unstimulated HAoSMCs	20 µM taxifolin	HO-1	n = 2	↓	ns
		Nrf2	n = 4	↓	ns
		MMP-12	n = 3	↓	ns
Stimulated (30 min H_2O_2) HAoSMCs	80 µg/mL PBE	HO-1	n = 3	↑	ns
		Nrf2	n = 4	↑	ns
		MMP-12	-	-	-
Stimulated (30 min H_2O_2) HAoSMCs	40 µg/mL PBE	HO-1	n = 3	↑	ns
		Nrf2	n = 4	↑	ns
		MMP-12	n = 2	-	-
Stimulated (6 h H_2O_2) HAoSMCs	80 µg/mL PBE	HO-1	n = 1	↑	ns
		Nrf2	n = 3	↑	ns
		MMP-12	-	-	-
Stimulated (6 h H_2O_2) HAoSMCs	40 µg/mL PBE	HO-1	n = 2	↑	ns
		Nrf2	n = 4	↑ and ↓	ns
		MMP-12	-	-	-

HAoSMCs: Human Aortic Smooth Muscle Cells; HO-1: heme oxygenase 1; H_2O_2: hydrogen peroxide; MMP-12: matrix metalloprotease 12; nuclear factor erythroid 2–related factor 2; ns: not significant; PBE: French Maritime Pine Bark Extract; -: not measured.

Chapter 8 General discussion and research perspectives

Non-communicable diseases continue to be an important health problem worldwide, with a substantial morbidity and mortality. Polyphenols and their impact on human health have attracted great interest over the last decades. Notably due to their antioxidant and anti-inflammatory effects, polyphenols might be beneficial in the treatment of these non-communicable diseases such as ADHD and cardiovascular diseases. For this thesis, French Maritime Pine Bark Extract (PBE) was selected as an interesting polyphenol source, originating from the outer bark of the French maritime pine (*Pinus pinaster*). It is standardised to contain 70 ± 5% procyanidins and has antioxidant and immune modulating properties.

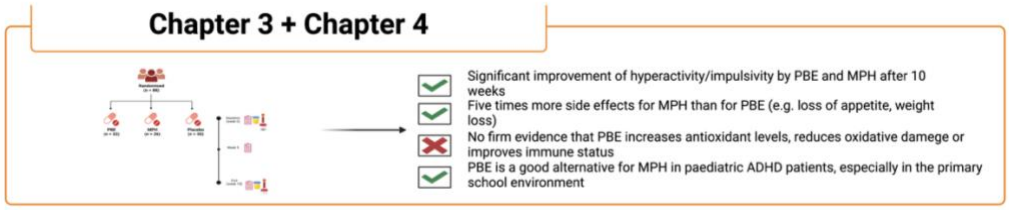


Since the biological effects of polyphenols, or PBE in particular, depend on the bioavailability, the rate of absorption, and the biotransformation within the body, understanding these dynamics is pivotal for revealing the full potential of PBE in promoting human health.

An in-house developed workflow was applied to reveal the gastrointestinal biotransformation of various compounds present in PBE, namely *in vitro* biotransformation via a gastrointestinal simulation model followed by metabolomics profiling. This automated data analysis strategy can replace laborious human revision processing in an unbiased manner, allowing rapid scoring of the generated large amounts of data. To the best of our knowledge, this is one of the first studies exploring the metabolic fate of PBE using an *in vitro* model. Generally, substantial biotransformation of glycosylated compounds in the gastrointestinal tract was observed

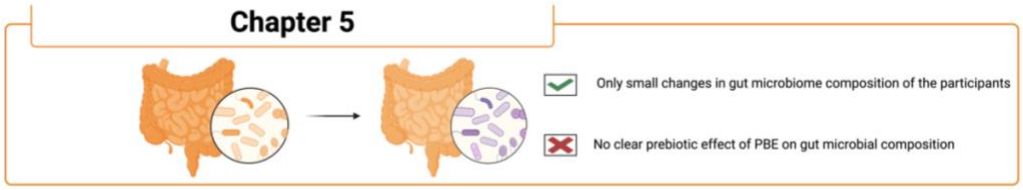
and a rich diversity of polyphenolic constituents was (tentatively) identified. Contrary to expectations, this study was not able to identify valerolactones as such. In fact, flavan-3-ols (including catechin and procyanidins, which are omnipresent in the extract) are expected to be biotransformed by colonic microbiota into phenolic acids and phenyl-γ-valerolactones (Angelino et al., 2019). According to previous research, δ-(3,4-dihydroxy-phenyl)-γ-valerolactone is the major microbial biotransformation product of PBE and has been detected in the plasma of healthy volunteers within 14 hours after oral administration (Sahebkar, 2014). However, with the applied LC/MS analysis valerolactones were not detected. Further investigations, in which another LC/MS method with different instrumental settings is applied, is needed to develop a more comprehensive overview of the biotransformation of PBE.

The GIDM-Colon is considered to be a highly valuable tool to mimic *in vivo* conditions as much as possible. Even though it sheds light on the enzymatic and microbial biotransformation processes taking place in the gastrointestinal tract, it is important to bear in mind that the *in vitro* model in the way it is used here, remains a simplified approach to predict biotransformation since active transport, passive diffusion and enterohepatic circulation are missing. To develop a full picture of the biotransformation processes taking place, additional studies will be needed. For instance, epithelial Caco-2 cell absorption can be used to simulate the intestinal barrier *in vitro*. Additionally, implementation of hepatic cells (microsomes or S9 fractions) can give insight in phase I and phase II biotransformation processes occurring in the liver. Moreover, since large inter- and intraindividual variability is observed in the human gut microbiota, further investigations involving different groups of pooled faeces are required to compare the effect on the biotransformation of a polyphenolic extract (e.g. young versus old population). Finally, further i*n vitro* testing with the identified biotransformation products is needed to elucidate PBE's full potential in the treatment of NCDs by interfering with for instance oxidative stress and inflammation. Hence, to attribute *in vitro* activity to one specific biotransformation product, a pure compound should be available for purchase and needs to be tested further.

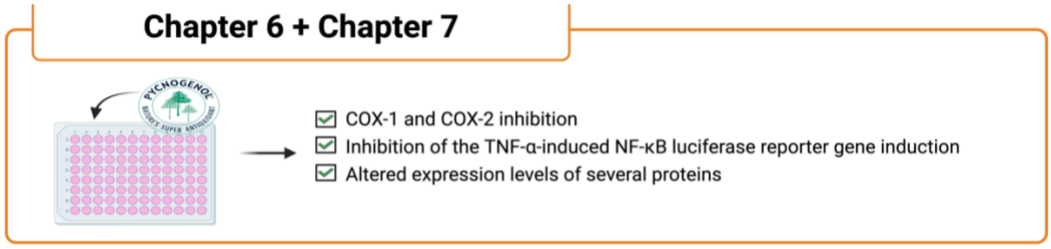


In a second part, the potential of nutritional approaches, such as polyphenols, as efficacious and safe methods for ADHD management, is explored. We conducted a 10-week randomised, double-blind, placebo and active controlled clinical trial investigating the effect of PBE and methylphenidate hydrochloride (MPH) on ADHD. Since ADHD is one of the most prevalent chronic paediatric conditions and constitutes a major health problem, and stimulant drugs like MPH often cause side effects, alternative treatment options are highly warranted. The pathogenesis of ADHD is believed to be multifactorial and has been associated with numerous structural and functional central nervous system abnormalities, findings on neurobiological mechanisms linking genes to brain phenotypes, immune and oxidant-antioxidant imbalances, as well as a potential role for the bidirectional communication between the gut microbiome and brain development and function. In fact, therapeutic benefit of PBE in paediatric ADHD was already suggested before. The full objective of this randomised trial was to evaluate the effect of PBE in paediatric (aged 6-12 years) ADHD patients on behaviour, co-morbid physical/psychiatric symptoms, and a broad range of innovative immunological markers, oxidative damage and antioxidant and neurochemical status, as compared to placebo and methylphenidate. According to the behavioural assessments of the 88 included paediatric ADHD patients filled out by the teachers, considered to be the primary outcome of the study, there was a significant improvement of the hyperactivity/impulsivity by PBE and MPH after 10 weeks compared to placebo. However, adverse effects were reported five times more for MPH than for PBE. Indeed, a loss of appetite (reflected by the neurochemical parameter neuropeptide Y (NPY)) and weight loss was observed for MPH, whereas no differences in NPY concentrations and a significant weight gain was established for PBE. The present study is the first to report the effect of MPH treatment on NPY. The weight loss seen in the MPH group can be attributed to a loss of appetite since participants in both active treatment groups significantly increased in height, reflecting a normal growth curve for all patients. Firm

evidence that PBE increases antioxidant levels, reduces oxidative damage and improves immune status in general as compared to placebo or MPH, could not be obtained in this study. The potential involvement of only marginal oxidative stress and immune disturbances in ADHD might therefore be a possible explanation for these findings. Nevertheless, based on our results, it could be concluded that PBE is a good alternative for MPH in paediatric ADHD and especially in the primary school environment, especially seen its almost complete lack of adverse effects. As a continuation of this clinical trial, future research could explore the effects of a combination therapy involving MPH and PBE. Combining both may potentially allow for a reduction in the dosage of MPH, consequently minimising side effects. Alternatively, maintaining the same dose of MPH in the presence of PBE might prevent the occurrence of side effects.



Besides their antioxidant and anti-inflammatory effects, polyphenols are believed to modulate gut microbial composition, which render them good candidates for ADHD therapy. In our pilot study (n = 35) our aim was to provide insights on the alterations in gut microbial composition after the three different treatments in ADHD patients. Looking at the gut microbial composition of the included ADHD patients, the high relative abundance of *Bifidobacteria* among all patients in this study cohort was remarkable. Moreover, subtle changes were noticeable and some limited compositional changes could be observed in the PBE treatment group, despite the small sample size used to evaluate microbial composition. Further research involving more participants, a longer supplementation period and possibly more sampling points is required to establish potential therapeutic efficiency of PBE in gut microbial modulation.



As mentioned earlier, several side effects have been reported with MPH use such as insomnia and loss of appetite. Furthermore, concerns about its cardiovascular safety have been raised. Besides, cardiovascular diseases are among the top four of most prevalent non-communicable diseases and maintaining cardiovascular health is therefore imperative. According to epidemiological studies aortic stiffness precedes the onset of hypertension and is thus considered to be a good predictor of cardiovascular morbidity and mortality. Because polyphenols are known to interact with mechanisms involved in arterial stiffness, they could constitute an interesting alternative option to target arterial stiffness. In a second part of this PhD thesis, PBE was tested in an array of *in vitro* assays representing different mechanisms underlying arterial stiffness. On the one hand, the activity was evaluated with *in vitro* anti-inflammatory assays, focusing on cyclooxygenase and NF-κB. Our investigations have found that PBE clearly exerted anti-inflammatory potential, which is in line with the findings from literature. The non-biotransformed PBE significantly inhibited the activity of both COX isoenzymes: PBE inhibited COX-1 in a dose-dependent manner whereas no clear dose-dependent inhibition was observed for the COX-2 enzyme. However, the calculated IC_{50} values are 10- to 100-fold higher compared to those of the known non-steroidal anti-inflammatory drugs (NSAIDs). Moreover, inhibition of the TNF-α-induced NF-κB luciferase reporter gene induction in L929 cells was also seen for PBE, again with an IC_{50} value in the micromolar range. Nevertheless, considerably more work will need to be done to unravel this NF-κB inhibition more in detail. To check for instance the effect of PBE on IκBα phosphorylation or p65 nuclear translocation by means of Enzyme-Linked Immuno Sorbent Assay. Indeed, the ratio of phosphorylated NF-κB to total NF-κB is a critical parameter in understanding the activation status of NF-κB signalling (Wang et al., 2020). Furthermore, the ability of NF-κB to bind to DNA consensus sequences, which is pivotal in its activation and subsequent transcriptional activity, could also be measured (Zhou et al., 2022).

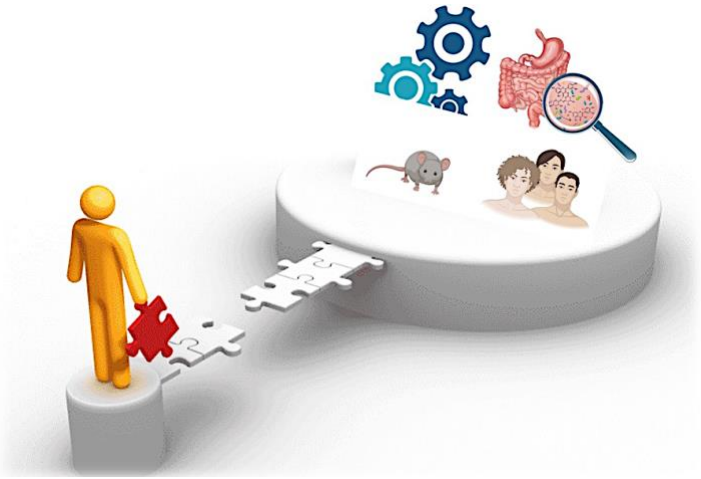
Taken into account the moderate inhibition of PBE (and only at higher concentrations) in COX-1 and COX-2 in *in vitro* assays, it should be clear that PBE will be less potent as compared to the marketed NSAIDs. Even though NSAIDs are one of the most used drugs to attenuate pain and inflammation, NSAIDs are responsible for serious side effects such as gastrointestinal (COX-1 selective inhibitors) and cardiovascular (COX-2 selective inhibitors) complications. In fact, the search for these COX-2 selective inhibitors, unraveled a cardiovascular protective effect of COX-2 by generation of PGI_2. This encourages the need for new therapeutic drugs that overcome the reported side effects of the currently known COX-inhibitors. Taken together the fact that NSAIDs are very powerful drugs with various serious side effects, might still offer potential for the less potent PBE with much less adverse effects, allowing a much longer supplementation period. Besides COX enzymes, lipoxygenase (LOX) enzymes are also essential in the conversion of arachidonic acid to eicosanoids, responsible for initiation of immunological responses and inflammation reactions (Mukhopadhyay et al., 2023). Eicosanoids are released by the activation of 5-lipoxygenase (5-LOX), an isozyme of LOX, and leukotriene B_4 is an end-product of the 5-LOX pathway. Leukotriene B_4 is considered to be a mediator of atherosclerosis and cardiovascular diseases and inhibiting the 5-LOX pathway may therefore mitigate the abovementioned side effects of selective COX-1 and COX-2 inhibitors (Rudrapal et al., 2023). By co-inhibiting COX (COX-1 and COX-2) and 5-LOX, desired anti-inflammatory activity can be achieved by preserving the drugs' primary effectiveness against COX and COX-2 in particular, while reducing the cardiovascular and gastrointestinal side effects. Dual COX/5-LOX inhibitors offer thus therapeutic benefits over NSAIDs and emerging research revealed that various classes of natural products (e.g. flavonoids) show potent inhibition against both COX and 5-LOX isoforms (Mukhopadhyay et al., 2023; Rudrapal et al., 2023). Investigating the potential inhibition of the 5-LOX pathway by PBE would therefore be a fruitful area for further research work.

Looking at the Western blot experiments, revealing the expression levels of various proteins linked to mechanisms underlying arterial stiffness, it can be observed that PBE can alter these expression levels. For instance, PBE significantly enhanced the HO-1 expression in HAoECs (in unstimulated and in stimulated conditions) whereas it non-significantly increased the HO-1 expression in HAoSMCs. Based on these findings, PBE

seems to upregulate HO-1 expression and can protect cells from oxidative stress conditions. Of interest here is also the increased (albeit not statistically significant) expression of the activating phosphorylation side of eNOS (PeNOS(Ser1177)) and a non-significantly decreased expression of the inhibitory phosphorylation side of PeNOS(Thr495)). Together these results provide important insight in the possible effect of PBE on increased eNOS activity and thereby enhanced NO production for maintenance of a healthy endothelium. The insights gained from these *in vitro* studies contribute to our understanding of the mechanisms underlying arterial stiffness. However, it is important to acknowledge that these *in vitro* tests specifically target a limited number of active mechanisms that contribute to AS, and do not take into account passive processes such as calcification.

Generally, evidence from *in vitro* experiments does not necessarily prove its *in vivo* activity. For instance, taking into account the relative high concentration at which COX-inhibition and NF-κB inhibition started, and the plasma concentrations for polyphenolic compounds found in literature (often ranging in the nanomolar range), significant COX-inhibition and NF-κB inhibition will be hard to achieve with the tested polyphenolic compounds *in vivo*. Indeed, many polyphenols have a lower bioavailability since they are not easily absorbed and therefore, the plasma concentrations are typically low. Hence, any single mechanism cannot explain all of polyphenols' *in vivo* activity, and probably multiple cellular mechanisms are involved in their *in vivo* activity. More research could thus shed light on the extract's anti-inflammatory properties to elucidate the true mechanisms of action against inflammation. One possible explanation for the low doses at which polyphenolic compounds already exert an anti-inflammatory response, might be by altering gene expression. Recently, polyphenolic compounds are investigated for their possible effect on epigenetic mechanisms, such as DNA methylation and post-translational histone modification, regulating the gene expression of molecules involved in inflammatory processes (Číž et al., 2020). Several studies have shown strong evidence that natural compounds can regulate gene expression by targeting different stages of the epigenetic machinery (Arora et al., 2019). A randomised controlled clinical trial ("18-month DIRECT-PLUS") for example investigated the beneficial effect of a green Mediterranean diet, richer in polyphenols and lower in meat as compared to other healthy lifestyle changes (Yaskolka Meir et al., 2023). Researchers noticed that the diets with increased polyphenol intake were inversely associated with biological ageing,

measured by DNA methylation which is highly correlated with chronological age (Yaskolka Meir et al., 2023). Also, it was recently observed that procyanidins are potent and beneficial modulators of the epigenetic machinery (Nie & Stürzenbaum, 2019). In addition, the regulation of the epigenetic mechanisms can in turn influence NF-κB signaling. For instance, dietary polyphenolic compounds can modulate NF-κB mediated inflammation through histone acetyltransferase (HAT) inhibition or histone deacetylase (HDAC) activation, responsible for NF-κB mediated transcriptional activation and repression of NF-κB gene transcription respectively. Looking into more detail at the epigenetic machinery can be useful to further elucidate the effect of PBE on NF-κB mediated inflammation.



To conclude, based on the results of the clinical trial, PBE was proven to be a good alternative for MPH in the context of ADHD. Results of the preliminary *in vitro* activity testing also demonstrated the potential of the extract in targeting the mechanisms underlying arterial stiffness. Even though our research strengthens the evidence for natural treatment options, further research is necessary. For instance, it would be useful to untangle the complete mechanism of action of PBE. Another interesting path is to combine the GIDM-Colon with Caco-2 cells to simulate the intestinal barrier or to extend the model with the metabolism reaction in the liver to give a more complete picture. Afterwards, the bioavailable biotransformation products should undergo extensive *in vitro* and *in vivo* testing to elucidate the possible role of PBE in maintaining cardiovascular health.

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Summary

Non-communicable diseases (NCDs) including neurodevelopmental diseases such as Attention-Deficit Hyperactivity Disorder and cardiovascular diseases, represent a growing global health problem that not only impact the quality of life but also result in a substantial economic burden. Even though the prevention of NCDs is considered to be a global challenge and highly prioritised by the World Health Organisation, the integration of a succesfull preventive strategy for NCDs is still not implemented in the Belgian health care system. Nevertheless, emerging evidence suggests that a high-quality diet with an increased consumption of fruits, vegetables, whole grains, and seeds is linked to a lower risk of NCDs since these foods are rich in bioactive agents such polyphenolic compounds. Indeed, polyphenols have been found in nature and in various dietary sources such as fruits, vegetables, whole grains and drinks including coffee, tea and wine. Moreover, polyphenols have been continuously explored for their beneficial health effects (e.g. antioxidant and anti-inflammatory properties) and could therefore be helpful to alleviate NCD's burden which is often linked to a state of oxidative stress and/or inflammation.

In this PhD thesis, a patented polyphenol-rich extract from the bark of the French Maritime Pine (PBE; *Pinus pinaster,* Pycnogenol®) was investigated as a potential novel treatment option for NCDs due to its antioxidant and anti-inflammatory activities. Firstly, the potential role of PBE was evaluated in the context of Attention-Deficit Hyperactivity Disorder, a prevalent neurodevelopmental disorder in which dopaminergic dysfunction, immune and oxidant-antioxidant imbalances appear to be involved. Moreover, methylphenidate, the first-choice medication for Attention-Deficit Hyperactivity Disorder, is linked to adverse effects including loss of appetite or insomnia. Though therapeutic benefit of PBE in paediatric Attention-Deficit Hyperactivity Disorder patients was already suggested before, its efficacy and value as compared to standard treatment with methylphenidate were to be confirmed. Therefore, a 10-week double-blind, randomised, placebo and active control group clinical trial was conducted to evaluate the effect of PBE on behaviour, co-morbid physical/psychiatric symptoms, immunological markers, oxidative damage and antioxidant and neurochemical status,

compared to placebo and methylphenidate. Our results show that both methylphenidate and PBE significantly improved hyperactivity/impulsivity after 10 weeks and prove that PBE is a good alternative for methylphenidate, especially for those willing to wait a few weeks for effects. Besides, loss of appetite, indicated by the neurochemical parameter neuropeptide Y, and weight loss were observed for methylphenidate, whereas a significant weight gain was established for PBE. To the best of our knowledge, this is the first study to elucidate the impact of MPH and PBE on neuropeptide Y. Firm evidence that PBE increases antioxidant levels, reduces oxidative damage and improves immune status in general as compared to placebo or MPH could not be obtained. One possible explanation for these results could be the contribution of minor oxidative stress and immune disturbances in ADHD. Next to these primary research questions, a potential prebiotic effect of PBE was explored in a pilot study by investigating its impact on the gut microbiota via amplicon sequencing of the full length 16s rRNA ribosomal subunit (V1-V9). Even though no clear prebiotic effects in the patients treated with PBE could be observed, subtle changes and limited compositional changes were noticeable.

Secondly, the therapeutic potential of PBE in cardiovascular diseases was looked at, and more specifically in the context of arterial stiffness, which refers to the reduced ability of the arteries to expand and contract in response to blood pressure changes. It is a pivotal physiological feature of the arteries and is closely related to overall cardiovascular health. Ongoing research is focused on understanding the mechanisms underlying arterial stiffness and developing new treatment options to mitigate its effects. Both oxidative stress and inflammation appear to play a role in arterial stiffness and can lead to structural and functional changes in the arterial wall, impair endothelial function, and thus promote the development of cardiovascular diseases. Managing oxidative stress and inflammation through for instance supplementation with a polyphenol-rich extract might reduce the risk of arterial stiffness and associated complications. PBE and its individual polyphenolic constituents were tested in an array of *in vitro* assays on oxidative and inflammatory mechanisms contributing to arterial stiffness aetiology. Cyclooxygenases, cyclooxygenase-1 and cyclooxygenase-2 catalyse the first two steps in prostaglandin biosynthesis from arachidonic acid, and thereby play an important role in pain, fever and inflammation. PBE significantly inhibited both cyclooxygenase enzymes in a cell-free assay, with a preference for cyclooxygenase-1

compared to cyclooxygenase-2. Nuclear factor κB (NF-κB) is an oxidative stress-related transcription factor, crucial in the regulation of various inflammatory diseases and arterial stiffness. PBE extract showed significant inhibitory activity in a NF-κB luciferase assay on L929 cells. Expression levels of several proteins (e.g. endothelial nitric oxide synthase, matrix metalloprotease, nuclear factor erythroid 2-related factor 2) involved in mechanisms underlying arterial stiffness were assessed in human aortic smooth muscle and human aortic endothelial cells using Western Blot technique. It could be demonstrated that PBE indeed alters expression levels of several proteins. Results of these *in vitro* assays increase our understanding regarding the influence of PBE or its individual constituents on several fundamental pathways underlying arterial stiffness.

One should bear in mind that extrapolation from *in vitro* findings to *in vivo* conclusions is not straightforward and requires caution. In fact, in view of the various phenolic constituents present in the extract, extensive biotransformation after oral intake and before absorption can be expected. This necessitates the identification of the intestinal biotransformation products formed after ingestion. PBE was therefore subjected to *in vitro* gastrointestinal biotransformation, which mimics the gastric, small intestinal and colonic phase, including faecal fermentation using a culture of pooled human faeces in an anaerobic environment. Samples were taken before, during and after biotransformation (with the endpoint at 72 h of colon phase) and analysed with UHPLC-ESI-QTOF-MS. These analyses generate dynamic and complex data and an in-house automated data analysis workflow for multiclass longitudinal data was used to screen interesting biotransformation profiles in an unbiased manner. Extensive biotransformation of PBE was observed and various biotransformation products could be identified. Even though PBE is rich in flavan-3-ols, the expected microbial biotransformation products valerolactones were not detected in the biotransformation experiment.

Samenvatting

Niet-overdraagbare aandoeningen, waaronder neurologische ontwikkelingsstoornissen zoals aandachtstekortstoornis met hyperactiviteit (ADHD) en cardiovasculaire aandoeningen, vertegenwoordigen een groeiend mondiaal gezondheidsprobleem dat niet alleen de kwaliteit van het leven beïnvloedt maar ook resulteert in een aanzienlijke economische last. Hoewel de preventie van niet-overdraagbare ziekten wordt beschouwd als een wereldwijde uitdaging en hoog op de prioriteitenlijst staat van de Wereldgezondheidsorganisatie, ontbreekt de implementatie van een succesvolle preventiestrategie voor dergelijke aandoeningen nog steeds in ons Belgisch gezondheidssysteem. Desalniettemin is er meer en meer bewijs dat een hoogwaardig dieet met een verhoogde inname van fruit, groenten, granen en zaden, gelinkt wordt aan een verlaagd risico op niet-overdraagbare aandoeningen aangezien deze rijk zijn aan bioactieve componenten zoals polyfenolische componenten. Polyfenolen zijn inderdaad terug te vinden in de natuur en in talrijke, belangrijke voedingsbronnen waaronder fruit, groenten en dranken zoals koffie, thee en wijn. Bovendien worden er gunstige gezondheidseffecten toegeschreven aan polyfenolen zoals antioxiderende en onstekingsremmende eigenschappen en kunnen ze daarom fungeren als mogelijke oplossing om de gezondheidslast van niet-overdraagbare aandoeningen te verminderen aangezien deze vaak geassocieerd worden met een toestand van oxidatieve stress en/of ontsteking.

In dit proefschrift werd een gepatenteerd, polyfenolrijk extract van de schors van de Franse maritieme pijnboom (*Pinus pinaster*, Pycnogenol®) onderzocht als mogelijke nieuwe behandeling voor niet-overdraagbare aandoeningen vanwege zijn antioxiderende en ontstekingsremmende eigenschappen. Allereerst werd de potentiële rol van het extract geëvalueerd in de behandeling van ADHD, een veelvoorkomende neurologische ontwikkelingsstoornis waarbij dopaminerge dysfunctie, immuun en oxidant-antioxidant verstoringen, betrokken blijken te zijn. Bovendien is methylfenidaat, het eerstelijnsmedicijn voor ADHD, gelinkt aan bijwerkingen zoals een verlies van eetlust of slapeloosheid. Hoewel eerder al een therapeutisch voordeel van het extract werd

aangetoond bij pediatrische ADHD patienten, moest de werkzaamheid en waarde van het extract nog worden vergeleken met de standaardbehandeling methylfenidaat. Daarom werd een 10 weken durende dubbelblinde, gerandomiseerde klinische studie met placebo en actieve controle groep uitgevoerd om het effect van het extract op gedrag, comorbide fysieke/psychiatrische symptomen, immunologische biomerkers, oxidatieve schade en antioxidant- en neurochemische status te evalueren, in vergelijking met placebo en methylfenidaat. Onze resultaten bevestigen dat zowel methylfenidaat als het extract de hyperactiviteit en impulsiviteit significant gaan verbeteren na 10 weken en het extract kan daarom beschouwd worden als een goed alternatief voor methylfenidaat, zeker voor diegenen die bereid waren enkele weken te wachten alvorens verbetering zichtbaar werd. Bovendien werd bij methylfenidaat een verlies van eetlust aangetoond door de neurochemische parameter neuropeptide Y, en gewichtsverlies waargenomen, terwijl een significante gewichtstoename werd vastgesteld voor de groep behandeld met extract. Naar ons beste weten is dit het eerste onderzoek die het effect van methylfenidaat en het extract onderzoekt op neuropeptide Y. Overtuigend bewijs dat het extract de antioxidant niveaus verhoogt, oxidative schade vermindert en de immuunstatus verbetert in vergelijking met placebo en methylfenidaat, ontbrak echter. Een mogelijke verklaring voor deze resultaten zou de bijdrage zijn van slechts beperkte oxidatieve stress en verstoringen in het immuunsysteem bij ADHD. Naast deze primaire onderzoeksvragen werd ook het mogelijke probiotische effect van het extract in een pilootstudie onderzocht, waarbij de invloed op de darmmicrobiota bestudeerd werd via amplicon-sequencing van het volledige 16S rRNA ribosoom (V1-V9 regio). Hoewel er geen duidelijke prebiotische effecten waargenomen werden bij de groep die het extract kreeg, waren er subtiele veranderingen en beperkte veranderingen in samenstelling van het microbioom merkbaar.

Vervolgens werd het therapeutische potentieel van het extract bij cardiovasculaire aandoeningen onderzocht, en meer specifiek in het kader van arteriële stijfheid, wat verwijst naar het verminderde vermogen van de bloedvaten om uit te zetten en samen te trekken als reactie op veranderingen in de bloeddruk. Echter is dit een cruciale fysiologische eigenschap van de bloedvaten die in nauw verband staat met de algehele cardiovasculaire gezondheid. Lopend onderzoek richt zich op het begrijpen van de onderliggende mechanismen van arteriële stijfheid en op het ontwikkelen van nieuwe

behandelingsmogelijkheden hiertegen. Zowel oxidatieve stress als ontstekingsreacties lijken een rol te spelen bij arteriële stijfheid en kunnen leiden tot structurele en functionele veranderingen in de vaatwand, de endotheelfunctie aantasten en zo de ontwikkelingen van cardiovasculaire aandoeningen in de hand werken. Oxidatieve stress en inflammatie onder controle houden door bijvoorbeeld suppletie met een polyfenolrijk extract kan het risico op arteriële stijfheid en de daarmee samenhangende complicaties verminderen. Het extract en zijn individuele bestanddelen werden getest in een reeks *in vitro* assays van oxidatieve- en ontstekingsmechanismen die aan de basis liggen van het ontstaan van arteriële stijfheid. Cyclooxygenases, cyclooxygenase-1 en cyclooxygenase-2 katalyseren de eerste twee stappen in de biosynthese van prostaglandinen uit arachidonzuur, en spelen zo een belangrijke rol in pijn, koorts en onstekingsreacties. Het extract remde significant beide cyclooxygenase enzymen in een celvrije assay, met een voorkeur voor cyclooxygenase-1 ten opzichte van cyclooxygenase-2. Nuclear-factor κB is een oxidatieve stress-gerelateerde transcriptiefactor, cruciaal voor de regulatie van verscheidene inflammatoire aandoeningen en arteriële stijfheid. Het extract vertoonde een significante remmende activiteit in een NF-κB luciferase assay op L929 cellen. De expressieniveaus van verschillende eiwitten (bijvoorbeeld endotheliale stikstofmonoxide synthase, matrix metalloprotease, nucleaire factor erytroïde 2-gerelateerde factor 2) die betrokken zijn bij mechanismen die aan de basis liggen van arteriële stijfheid, werden beoordeeld in humane aorta gladde spiercellen en humane aorta endotheelcellen met behulp van Western Blot techniek. Er kon worden aangetoond dat het extract inderdaad zorgt voor een verschil in expressieniveau van bepaalde eiwitten. De resultaten van deze *in vitro* assays vergroten onze kennis met betrekking tot de invloed van het extract en zijn individuele bestanddelen op de verschillende fundamentele paden die aan de basis liggen van arteriële stijfheid.

Echter is het belangrijk te bedenken dat extrapolatie van deze *in vitro* bevindingen naar *in vivo* conclusies niet eenvoudig en rechtlijnig is. Gezien de verschillende fenolische bestanddelen aanwezig in het extract, is een uitgebreide biotransformatie na orale inname en voor absorptie niet ondenkbaar. Identificatie van de verscheidene, gevormde biotransformatieproducten na inname is daarom ook belangrijk. Het extract werd daarom onderworpen aan *in vitro* gastrointestinale biotransformatie die de maag, dunne darm en dikke darm nabootst, inclusief fermentatie met behulp van

gecollecteerde humane stoelgangstalen in een anaërobe omgeving. Staalname gebeurde voor, tijdens en na biotransformatie (met als eindpunt 72 uur in de dikke darm) en analyse gebeurde met behulp van UHPLC-ESI-QTOF-MS. Deze analyses genereren dynamische en complexe gegevens, waarvoor een in-house ontwikkelde geautomatiseerde gegevensanalyse workflow voor longitudinale data werd gebruikt om interessante biotranformatie profielen op een onafhankelijke manier te screenen. Er werd een uitgebreide biotransformatie van PBE waargenomen en verschillende biotransformatieproducten konden worden geïdentificeerd. Desondanks dat het extract rijk is aan flavan-3-olen, werden de te verwachte microbiële biotransformatieproducten, de valerolactonen, niet gedetecteerd in het experiment.

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Scientific curriculum vitae

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Education

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Scientific experience

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Sep 2018 – Dec 2018	Master thesis "Verhoogde compliantie van aorta segmenten bij muizen na hoge cyclische stretch: simuleren van acute fysieke activiteit aan hoge intensiteit"
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Scientific publications

Papers in peer-reviewed journals

2023	Weyns et al. Impact of French Maritime Pine Bark Extract and Methylphenidate on the Gut Microbiota of paediatric Attention-Deficit Hyperactivity Disorder Patients Under review in *Frontiers in Microbiology*
2023	Cédric Neutel, Anne-Sophie Weyns et al. Increasing Pulse Pressure Ex Vivo, Mimicking Acute Physical Exercise, Induces Smooth Muscle Cell-Mediated De-Stiffening of Murine Aortic Segments. *Communications Biology* 2023.
Sep 2022	Weyns et al. Clinical Investigation of French Maritime Pine Bark Extract on Attention-Deficit Hyperactivity Disorder as Compared to Methylphenidate and Placebo : Part 1 : Efficacy in a Randomised Trial. *J Funct Foods* 2022, *97*, 105246.
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Oral presentations

May 2023	Weyns et al. Clinical Investigation of French Maritime Pine Bark Extract on Attention-Deficit Hyperactivity Disorder as Compared to Methylphenidate and Placebo: Efficacy in a Randomised Trial. 4th World Congress on Mental Health, May 22-23, London, UK.
April 2023	Weyns et al. Clinical Investigation of French Maritime Pine Bark Extract on Attention-Deficit Hyperactivity Disorder as Compared to Methylphenidate and Placebo: Efficacy in a Randomised Trial. 21st Forum of Pharmaceutical Sciences, April 28, Blankenberge, Belgium.
May 2022	Weyns et al. Effect of French Maritime Pine Bark Extract on Attention-Deficit Hyperactivity Disorder: a randomised clinical trial. Departmental research Day, May 11, Blankenberge, Belgium.
Sep 2021	Weyns et al. Effect of French Maritime Pine Bark Extract on Attention-Deficit Hyperactivity Disorder: a randomised clinical trial. Polyphenol Applications 2021, Sep 22-24, virtual.

Poster presentations

Sep 2022	Weyns et al. Evaluation of beneficial effects of French Maritime Pine Bark Extract on gut microbiota as compared to methylphenidate and placebo in paediatric Attention-Deficit Hyperactivity Disorder. Polyphenols Application 2022, Sep 28-30, Valencia, Spain.
Sep 2022	Lauwers S., Weyns et al. In vitro gastrointestinal biotransformation of polyphenolic rich extracts and identification of their metabolites: an in-house developed workflow. Polyphenols Application 2022, Sep 28-30, Valencia, Spain.

Interview

Mar 2023	Weyns A.S. Pycnogenol®: dubbelblind, gerandomiseerd onderzoek bevestigt de positieve effecten bij kinderen met ADHD. Maart/April 2023, De medische Referentie, België.