Faculteit Farmaceutische, Biomedische en Diergeneeskundige Wetenschappen

Departement Biomedische Wetenschappen

Targeted search for genetic causes of the Prader Willi *like* phenotype

---

Gerichte zoektocht naar genetische oorzaken van het Prader Willi *like* fenotype

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

Ellen GEETS

Promotor: Prof. Dr. Wim Van Hul

Antwerpen, 2018

MEMBERS OF THE JURY

Prof. Dr. Wim Van Hul *(promotor)*
Department of Medical Genetics, University of Antwerp and University Hospital of Antwerp, Belgium

Prof. Dr. Kristel Sleegers *(chairman)*
VIB Department of Molecular Genetics, University of Antwerp, Belgium

Prof. Dr. Bart Loeys
Department of Medical Genetics, University of Antwerp and University Hospital of Antwerp, Belgium

Dr. Marcel Twickler
Department of Endocrinology, Diabetology and Metabolic Diseases, Antwerp University Hospital, Belgium

Prof. Dr. Bart Van der Auwera
Diabetes Research Center, Brussels Free University, Belgium

Dr. Marelise Eekhoff
Department of Internal Medicine-Diabetes Center, VU University Medical Center, Amsterdam, the Netherlands

TABLE OF CONTENTS

Table of contents

SUMMARY/SAMENVATTING

Summary

Defined as abnormal or excessive fat accumulation due to an imbalance between energy intake and expenditure, obesity has become a chronic disorder reaching epidemic proportions. In 1973, Coleman and Hummel demonstrated for the first time that genetic factors are involved in the disease. Later on, the heritability of obesity has been estimated between 40 – 70%. However, as obesity is extremely heterogeneous only 2.7% of this heritability could be explained to date.

Over the years, various forms of obesity have been reported including monogenic, complex and syndromic obesity. The Prader Willi syndrome (PWS), caused by a paternal defect in the imprinted region 15q11.2 – q13, is with its prevalence of 1 in 10,000 – 30,000 individuals the most common form of syndromic obesity. Once the clinical diagnosis of PWS cannot be confirmed molecularly, patients are classified as Prader Willi *like* (PWL). In literature, the PWL phenotype has mostly been associated with deletions on chromosome 6q. By investigating the genetic architecture in this specific study population of syndromic patients more in detail, we ultimately want to contribute to the unravelment of the genetic background of obesity.

In the first part of this thesis we wanted to elucidate the role of *SIM1* and *MRAP2* in the pathogenesis of PWL and the obesity phenotype seen in these patients. To do so, we decided to perform copy number variation analysis with the use of an array-based genome-wide technology followed by mutation analysis of both genes. This lead to the identification of one possible interesting variant, p.P352S, in the *SIM1* gene and another possible interesting variant, p.A40S, in the *MRAP2* gene. Further functional analysis of both variants would be useful to elucidate their role in the pathogenesis of obesity. With this research we did confirm the results of previous studies that *SIM1* and *MRAP2* could play a role in the PWL phenotype albeit, in our case, in a limited number of patients.

In the second part the role of *MCHR2* in PWL and obesity was examined. With the use of copy number variation and mutation analysis of the gene we were not able to identify any gene-harboring deletions nor variants. We only identified a significantly higher prevalence of the c.228T>C C allele in our PWL cohort compared to previously published results and controls of the ExAC Database. Consequently, we suggested that *MCHR2* is not a major contributor to human obesity nor the PWL phenotype.

Due to the rather negative results of the previous two parts, we decided to look beyond the 6q region and hypothesized that a reduction in *OXT* or even *OXTR* signaling could be present in PWL patients due to a mutation or deletion in one or both genes. The structural and mutation screen, however, only revealed known intronic (*OXT* and *OXTR*) or (non-)synonymous variants (*OXTR*) and, as for *MCHR2*, both genes did not show a major involvement in PWL patients. Consequently, we proposed that the hypothesized OXT or OXTR signaling deficiency in PWL patients could be mediated by another gene upstream of *OXT* in the hypothalamic leptin-melanocortin signaling pathway, i.e. the *POU3F2* gene. Therefore, we screened the *POU3F2* gene in our cohort of PWL patients. Copy number variation analysis did not display any structural variation encompassing the *POU3F2* gene. However, mutation analysis resulted in the identification of one previously unreported variant, p.H255R, in one PWL patient. Further mutation screening of obese children and adolescents revealed two additional interesting variants, p.G85R and p.G352V. Unfortunately, preliminary results of functional experiments for p.H255R and p.G352V do not gave a clear indication whether the mutations have an effect on the signaling pathway so further functional research is still needed.

In conclusion, we can state that we identified possible disease causing mutations and one new interesting gene, *POU3F2*, in the pathogenesis of PWL and the obesity phenotype. These results can help in the unravelment of the underlying mechanisms of energy metabolism and ultimately into accurate therapeutic treatment.

Samenvatting

Obesitas, gedefinieerd als een overmaat van vetweefsel ten gevolge van een verstoord evenwicht tussen energieopname en –verbruik, is een chronische aandoening die momenteel reeds epidemische proporties aanneemt. In 1973 toonden Coleman en Hummel voor het eerst aan dat genetische factoren betrokken zijn bij de ziekte. Later werd de heritabiliteit van obesitas dan ook geschat tussen 40 en 70%. Aangezien de genetica van obesitas extreem heterogeen is, kon er tot op heden slechts 2,7% van deze heritabiliteit verklaard worden.

Over de jaren heen werden verschillende vormen van obesitas beschreven waaronder monogene, complexe en syndromale obesitas. Het Prader Willi syndroom (PWS), veroorzaakt door een paternaal defect ter hoogte van de imprinting regio 15q11.2 - q13, is met zijn prevalentie van 1 op 10.000 - 30.000 individuen de meest voorkomende vorm van syndromale obesitas. Wanneer de klinische diagnose van PWS niet moleculair bevestigd kan worden, worden deze patiënten ingedeeld als Prader Willi *like* (PWL). In de literatuur werd het PWL fenotype reeds geassocieerd met deleties op chromosoom 6q. In deze thesis willen we de genetische architectuur in deze specifieke studiepopulatie van syndromale patiënten meer in detail onderzoeken en zo uiteindelijk bijdragen tot de ontrafeling van de genetische achtergrond van obesitas.

In het eerste gedeelte van deze thesis wilden we de rol van *SIM1* en *MRAP2* ophelderen in de pathogenese van PWL en het obesitas fenotype dat we zien in deze patiënten. Om dit te verwezenlijken, voerden we een genoomwijde microarray analyse uit gevolgd door een mutatie-analyse van beide genen. Dit leidde tot de identificatie van één mogelijk interessante variant in *SIM1*, p.P352S, en een andere mogelijk interessante variant in *MRAP2*, p.A40S. Verdere functionele analyse van beide varianten zou nuttig zijn om hun rol in de pathogenese van obesitas te verduidelijken. Met dit onderzoek hebben we de resultaten van vorige studies

bevestigd, namelijk dat *SIM1* en *MRAP2* een rol spelen in het PWL fenotype ook al is het, in ons geval, in een beperkt aantal patiënten.
In het tweede gedeelte werd de rol van *MCHR2* in PWL en obesitas onderzocht. We zijn er niet in geslaagd om structurele deleties of varianten te identificeren met behulp van *copy number* variatie en mutatie-analyse. We identificeerden enkel een significant hogere prevalentie van het c.228T>C C allel in onze PWL cohorte wanneer we deze vergeleken met eerder gepubliceerde resultaten en met controles uit de ExAC database. Bijgevolg toonden we aan dat *MCHR2* geen grote bijdrage levert aan humane obesitas of het PWL fenotype.

Door de eerder negatieve resultaten van de eerste twee studies, beslisten we om verder te kijken dan de 6q regio. We stelden de hypothese dat een vermindering in *OXT* of zelfs *OXTR* signalering door een mutatie of deletie van één van deze genen aanwezig zou kunnen zijn in PWL patiënten. Echter toonde de structurele en mutatie-analyse van de genen enkel gekende intronische (*OXT* en *OXTR*) en (niet-) synonieme varianten (*OXTR*) aan. Beide genen zijn, net als *MCHR2*, dus niet betrokken in de pathogenese van PWL.
Daarnaast stelden we dat onze hypothese van *OXT* of *OXTR* deficiëntie in PWL patiënten gemedieerd zou kunnen worden door een ander gen gelinkt aan *OXT* in de leptine melanocortine signaaltransductie in de hypothalamus, namelijk het *POU3F2* gen. In het laatste gedeelte onderzochten we bijgevolg het *POU3F2* gen in onze cohorte van PWL patiënten. *Copy number* variatie analyse toonde geen enkele structurele variatie aan ter hoogte van het *POU3F2* gen. Anderzijds leidde mutatie-analyse wel tot de identificatie van een niet eerder gerapporteerde variant, p.H255R, in één PWL patiënt. Verder onderzoek van obese kinderen en adolescenten legde nog twee bijkomende interessante varianten bloot, p.G85R en p.G352V. Preliminaire resultaten van functionele experimenten uitgevoerd voor p.H255R en p.G352V toonden helaas geen duidelijkheid of de mutaties een effect hebben op de signaaltransductie. Verder functioneel onderzoek is daardoor nog steeds noodzakelijk.

Hiermee kunnen we concluderen dat we mogelijk ziekteveroorzakende mutaties hebben geïdentificeerd en één nieuw interessant gen, *POU3F2*, in de pathogenese van PWL en het obesitas fenotype zichtbaar in deze patiënten. Deze resultaten kunnen helpen in het blootleggen van de onderliggende mechanismen van energiemetabolisme en uiteindelijk leiden tot accurate therapeutische behandelingen.

INTRODUCTION

CHAPTER 1

CLINICAL, MOLECULAR GENETICS AND THERAPEUTIC ASPECTS OF SYNDROMIC OBESITY.

Ellen Geets[1], Marije EC Meuwissen[1], Wim Van Hul[1]

[1]Department of Medical Genetics, University of Antwerp, Antwerp, Belgium

Submitted in Clinical Genetics

Abstract

The prevalence of obesity is more than doubled since 1980 and continues to increase. As obesity can contribute to the development of additional comorbidities such as type II diabetes mellitus, cardiovascular disease and different types of cancer, it has become a major health problem worldwide. Twin and adoption studies indicated a heritability of body mass index (BMI) between 40 and 70%, however for most individuals an interplay between genetic and environmental factors lead to an obesity phenotype. In addition, to date, more than 25 different syndromic forms of obesity are known in which one (monogenic) or multiple (polygenic) genes are involved. This review gives an overview of the different forms and focuses more in detail on six obesity syndromes: Prader Willi syndrome (PWS) and the Prader Willi *like* (PWL) phenotype, Bardet-Biedl syndrome (BBS), Alström syndrome (AS), Wilms tumor, Aniridia, Genitourinary malformations and mental Retardation (WAGR) syndrome and 16p11.2 (micro)deletions. Years of research provided plenty of information on the molecular genetics of these disorders and the obesity phenotype leading to a more individualized treatment of the symptoms, however, a lot of questions still remain unanswered. As these obesity syndromes have different signs and symptoms in common, it makes it difficult to accurately diagnose patients which may result in inappropriate treatment of the disease. Therefore, the big challenge for clinicians and scientists is to more clearly differentiate all syndromic forms of obesity to provide conclusive genetic explanations and eventually deliver accurate genetic counseling and treatment instead of only treating the patient's symptoms. In addition, further delineation of the (functions of the) underlying genes with the use of array- or next generation sequencing (NGS)-based technology will be helpful to unravel the mechanisms of energy metabolism in the general population.

1. Introduction

Obesity is a metabolic disorder characterized by an excess of adipose tissue. As illustrated in table 1 this increase can be caused by several factors.

Table 1. Possible causes of obesity.
For each category specific causes are described (depicted from Pervez and Ramonaledi 2017[1]).

CATEGORY	SPECIFIC CAUSE
Psychological causes	o Stress leading to comfort eating o Perceptions/choices of food o Consumption patterns o Eating disorders
Biological and physiological causes	o Genetic susceptibility o Disturbance of regulation of hunger and satiety feelings o Hormonal of metabolic disorders such as hypothyroidism or diabetes
Lifestyle causes	o Sedentary lifestyle/lack of exercise o Diet rich in dense fat, sugars, salt and alcohol
Socioeconomic causes	o Culture/education o Peer pressure o Employment/income o In some areas, lack of food choice
Other causes	o Medications such as antipsychotics, steroids and antidepressants o Stopping smoking

Since 1980, the prevalence of obesity is more than doubled. World Health Organisation (WHO) figures of 2014 showed that 58.6% of adults are considered overweight (body mass index (BMI) = 25.0 – 29.9 kg/m²) and even 23% is obese (BMI ≥ 30 kg/m²) in Europe. The problem also affects children in whom 41 million under the age of 5 were overweight or obese in 2014 worldwide. If current trends continue, the number of overweight or obese infants and young children will increase to 70 million by 2025[2]. This high prevalence is alarming as obesity can contribute to several adverse consequences on health like cardiovascular disease, type II diabetes mellitus

(T2DM) and various types of cancer[2, 3]. Due to the increased risk of developing the abovementioned comorbidities and its high, still increasing, prevalence, obesity has a major impact on global health and consequently represents a significant expenditure of national health-care budgets. Moreover, obese individuals accrued medical costs approximately 30% higher than their normal weight peers[4].

In 1973 Coleman and Hummel demonstrated for the first time that, to a certain extent, genetic factors are responsible for the familial aggregation of obesity[5]. Several years later twin and adoption studies confirmed this and estimated the heritability of BMI at 40 – 70%[6-8]. In most cases, a combination of genetic and environmental factors such as a high-fat diet and sedentary lifestyle causes the development of obesity, which is called common or complex obesity. To date, genome-wide association studies (GWAS) identified 97 loci related to complex obesity that account for approximately 2.7% of BMI variation[9] (Figure 1). On the other hand, an even more severe and rare form of obesity is monogenic (non-syndromic) obesity; individuals carry mutations in a single gene that cause the obesity phenotype (Figure 1). Most of the genes affected in monogenic obesity are part of the leptin-melanocortin signaling pathway in the hypothalamus which has an important function in maintaining energy homeostasis[10]. In the European population heterozygous mutations in the *melanocortin-4 receptor* (*MC4R*) gene, coding for a G protein-coupled receptor that plays an important role in this signaling pathway, are the most common causes of monogenic forms of obesity and cumulates to approximately 2-5%[11].

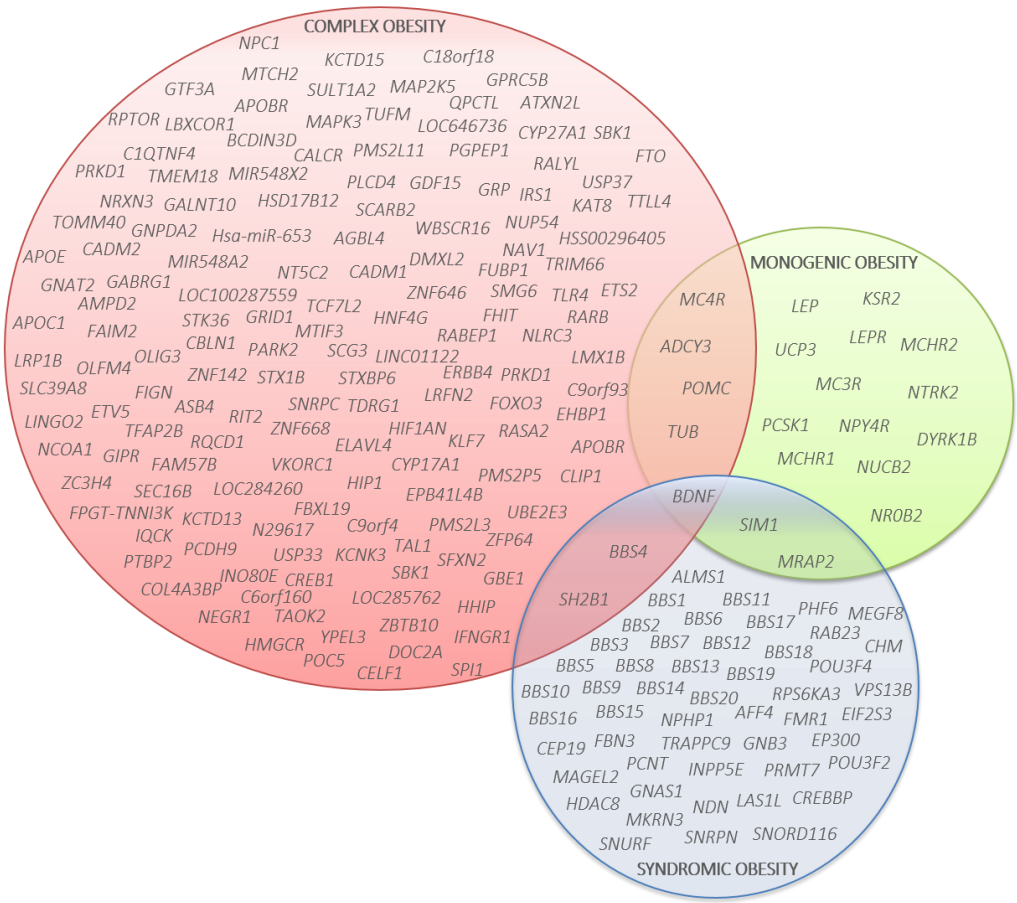


Figure 1. Diagram showing an overview of known genes associated with monogenic, complex and/or syndromic obesity.

1.1. Syndromic obesity

Syndromic obesity refers to obesity occurring in the context of a distinct set of associated clinical phenotypes such as intellectual disability (ID), dysmorphic features and organ-specific developmental abnormalities[12, 13]. Up to now over 25 syndromic forms of obesity have been identified[14]. As shown in figure 1 and table 2, syndromic obesity is extremely heterogeneous[15] and different molecular mechanisms underlie the various syndromes. In case of 'pleiotropy' one single gene is responsible for the presence of two or more distinct and seemingly unrelated traits in the patient[16]. Secondly, a contiguous gene syndrome (CGS) is a clinical phenotype caused by the absence of a contiguous set of genes with each of the genes underlying one of the

unrelated clinical features. The segmental aneuploidy syndrome is a special type of CGS usually originated from non-allelic homologous recombination between low copy repeats that flank the region. A good example of this is the Prader Willi syndrome (PWS)[17].

In table 2 the most important aspects of all syndromic forms of obesity are described. In addition, six syndromes were selected for a more detailed discussion. The selection was made in order to illustrate frequent causes, phenotypic overlap, both monogenic causes as well as contiguous gene effects and different types of molecular mechanisms underlying obesity. PWS and Bardet-Biedl syndrome (BBS) were selected since they are the most prevalent (non-X-linked) obesity syndromes. The Prader Willi *like* (PWL) phenotype and 16p11.2 (micro)deletions were included due to their clinical similarity with PWS, therefore being important differential diagnostic considerations. Additionally, we also choose Alström syndrome (AS) due to its phenotypic overlap with BBS. Finally, Wilms tumor, Aniridia, Genitourinary malformations and mental Retardation (WAGR) syndrome was selected as it is an excellent example of a CGS (Table 3).

Table 2. Overview of the prevalence, inheritance, the responsible gene(s) or chromosomal region and most important cardinal and additional clinical features of known obesity syndromes.

DISEASE	PREVALENCE	INHERITANCE	GENE(S) OR REGION	CARDINAL CLINICAL FEATURES	ADDITIONAL CLINICAL FEATURES
Alström Syndrome (AS)[18-21]	1-9 in 1.000.000	Autosomal recessive	*ALMS1* (mutations)	Obesity, cone-rod dystrophy, renal anomalies, progressive sensorineural hearing impairment, male hypogonadism/female hyperandrogenism, adult short stature, T2DM, and dilated or restrictive cardiomyopathy, shortened life expectancy (40 – 50 years).	/
Bardet-Biedl Syndrome (BBS)[22-31]	1 in 13.500 (Israel and Arab counties) 1 in 160.000 (Switzerland)	Autosomal/ Oligogenic recessive	*BBS1 – BBS20*, *NPHP1*, *FBN3* and *CEP19* (mutations)	Obesity, cone-rod dystrophy, postaxial polydactyly, cognitive impairment, hypogenitalism and renal abnormalities, shortened life expectancy (men = 43 years, women = 46 years).	Speech deficits, olfaction disorders (anosmia or hyposmia), psychiatric problems, T2DM and ataxia or impaired coordination.
Borjeson-Forssman-Lehmann Syndrome[32-34]	< 1 in 1.000.000	X-linked recessive	*PHF6* (mutations)	Mild to severe ID, hypogonadism, hypometabolism, obesity with marked gynaecomastia, facial dysmorphism, hypotonia, tapered fingers, broad shortened toes, small genitalia and short stature.	Microcephaly or macrocephaly and epileptic seizures.

Carpenter Syndrome (CS)[35-39]	< 1 in 1.000.000	Autosomal recessive	*RAB23* and *MEGF8* (mutations)	Craniofacial features, mild to moderate ID, increased birth weight (90% of CS patients obese later in life), central nervous system abnormalities, dental problems, cardiovascular malformations, foot and hand syndactyly/brachydactyly, hip, knee, and ankle deformities and clinodactyly, undescended or underdeveloped testicles, shortened life expectancy but extremely variable.	Omphalocele and umbilical hernia.
Choroideremia-deafness-obesity Syndrome or Ayazi Syndrome[40-43]	< 1 in 1.000.000	X-linked recessive	Deletion of Xq21 including at least the CHM and POU3F4 genes	Choroideremia (Males: progressive nyctalopia and eventual central blindness; Females: retinal changes), obesity, moderate ID and congenital mixed (sensorineural and conductive) deafness.	/
Coffin-Lowry Syndrome[44-47]	1 in 50.000 – 100.000	X-linked dominant and *de novo* cases	*RPS6KA3* (mutations)	Growth and psychomotor retardation, hypotonia, hyperlaxity of joints, characteristic facial and digital abnormalities, progressive skeletal alterations, microcephaly, (severe) cognitive deficiency, impaired speech development, shortened life expectancy.	Obesity, psychiatric illness (depression, psychotic behavior and schizophrenia), sensorineural hearing deficit, mitral regurgitation, seizures.

Cognitive impairment-coarse facies-heart defects-obesity-pulmonary involvement-short stature-skeletal dysplasia (CHOPS) Syndrome[48-50]	N.A.	Autosomal dominant	*AFF4* (mutations)	Cognitive impairment, coarse facies, heart defects, obesity, pulmonary involvement and short stature and skeletal dysplasia.	/
Cohen Syndrome[51-54]	N.A.	Autosomal recessive	*VPS13B* (deletion or mutation)	Facial dysmorphism, microcephaly, truncal obesity, ID, progressive retinopathy, and intermittent congenital neutropenia.	Childhood hypotonia, joint laxity, cheerful disposition.
Colobomatous microphtalmia-obesity-hypogenitalism ID Syndrome[55, 56]	< 1 in 1.000.000	Autosomal dominant	N.A.	Colobomatous microphthalmia, obesity, hypogonadism/genitalism and ID.	/
16p11.2 (micro)deletion Syndrome (220 kb, 593 kb and 1.7 Mb deletion)[57-62]	N.A.	Autosomal dominant	Deletion of 16p11.2 (*SH2B1*)	Severe early-onset obesity, developmental delay (DD), autism spectrum disorders (ASD), schizophrenia, neuropsychiatric disorders.	ID, hypotonia, epilepsy, behavioral problems, speech articulation abnormalities.

Fragile X Syndrome[63-68]	1 in 4.000 – 5.000	X-linked dominant	*FMR1* ((pre)mutations)	Mild to severe ID, cognitive and developmental impairment (e.g. delayed or absent speech), physical features including macroorchidism and facial dysmorphisms (elongated face, prominent ears and forehead), behavioral problems (hyperactivity, impulsivity, attention problems, anxiety, mood lability, autistic features, attention deficit hyperactivity disorder (ADHD)), obesity, otitis media, hyperextensible finger joints.	Recurrent otitis, (childhood) seizures, pes planus, strabismus, mitral valve prolapse, scoliosis.
Hydrocephalus-obesity-hypogonadism or Sengers-Hamel-Otten Syndrome[69]	< 1 in 1.000.000	X-linked recessive	N.A.	Congenital hydrocephalus, centripetal obesity, hypogonadism, intellectual deficit and short stature.	/
ID-obesity-brain malformations-facial dysmorphism Syndrome[70, 71]	< 1 in 1.000.000	Autosomal recessive	*TRAPPC9* (mutations)	Obesity, hypotonia, microcephaly, moderate to severe ID, brain abnormalities.	Peculiar facial appearance, epilepsy.

ID-obesity-prognathism-eye and skin anomalies syndrome or MOMES Syndrome[72-75]	< 1 in 1.000.000	Autosomal recessive	4q35.1-qter deletion and 5pter- 5p14.3 duplication	ID, speech delay, obesity, macrocephaly, maxillary hypoplasia, mandibular prognathism, crowding of teeth, ocular anomalies (blepharophimosis, blepharoptosis, decreased visual acuity, abducens palsy, hyperopic astigmatism and accommodative esotropia), chronic atopic dermatitis, lateral deviation of the great toes and cone-shaped epiphyses (toes 2, 3 and 4)	/
ID-seizures-macrocephaly-obesity Syndrome[76-81]	< 1 in 1.000.000	N.A.	der(8)t(8;12)(p23.1;p13.31) (*GNB3*)	ID, DD, obesity, seizures, hypotonia, dysmorphic features, macrocephaly, eczema, poor coordination, ocular problems, social personality.	Abnormal gait, dental/palate abnormalities, hypertelorism, scoliosis.
X-linked ID-epileptic seizures-hypogenitalism-microcephaly-obesity syndrome or MEHMO Syndrome[82-87]	< 1 in 1.000.000	X-linked recessive Mitochondrial inheritance	*EIF2S3* (mutations)	ID, DD, severe postnatal growth delay, seizures, hypogonadism and -genitalism, microcephaly and infancy-onset obesity, life expectancy is less than 2 years.	Characteristic facies, diabetes, hypertonia and hyperreflexia, nystagmus, agitated and irritable behavioral pattern.

Microcephalic osteodysplastic primordial dwarfism (MOPD) type II or Majewski osteodysplastic primordial dwarfism type II[88-91]	N.A.	Autosomal recessive	*PCNT* (mutations)	Severe intrauterine and postnatal growth retardation, disproportionate short stature (adult height < 100cm), skeletal abnormalities, microcephaly, dysmorphic features (e.g.: retrognathia, upward-slanting palpebral fissures, prominent nose, …), truncal obesity, ID, scoliosis, unusual pigmentation (e.g. café-au-lait spots), high-pitched voice, shortened life expectancy (30 years).	Severe microdontia, rootless molars, malformation of mandibular premolars, narrow chest, increased susceptibility to infections, aneurysms, Moya Moya disease, elevated platelet counts, vascular anomalies (can affect neurovasculature in childhood and renal and coronary arteries in adulthood), T2DM.
Macrocephaly-obesity-mental disability-ocular abnormalities (MOMO) Syndrome or Macrosomia-obesity-macrocephaly-ocular abnormalities Syndrome[92-98]	< 1 in 1.000.000	Autosomal dominant	Balanced reciprocal translocation (16;20)(q21;p11.2) (*LINC00237*?)	Macrocephaly, obesity, overgrowth, ID and ocular abnormalities (retinal coloboma and nystagmus), macrosomia, downslanting palpebral fissures, hypertelorism, broad nasal root, high and broad forehead and delayed bone maturation.	Behavioral disorders (aggressiveness, self-mutilation, excessive shyness), short stature, recurvation of femur or straight femur, autism, developmental issues, clavicular pseudoarthrosis.
Mental retardation (ID)-truncal obesity-retinal dystrophy-micropenis (MORM) Syndrome[99-101]	N.A.	Autosomal recessive	*INPP5E* (mutations)	Moderate ID, truncal obesity, congenital nonprogressive retinal dystrophy, micropenis	/

Prader Willi Syndrome[102-108]	1 in 10.000 – 30.000	Familial inheritance as well as *de novo* cases	15q11.2 – q13 (imprinting region)	Severe neonatal hypotonia, feeding difficulties followed by hyperphagia and excessive weight gain, DD, hypogonadism, ID, characteristic facial features (almond-shaped eyes, a thin upper lip, downturned corners of the mouth and/or a narrow face), behavioral problems (e.g. temper tantrums, stubbornness, skin picking), small hands and feet, short stature.	T2DM, sleep abnormalities, apnea, hypothyroidism, delayed language development, respiratory infections, hypopigmentation of hair, eyes, and skin, strabismus, hip dysplasia, scoliosis, psychosis.
Prader Willi *like* Syndrome[107, 109-112]	N.A.	Familial inheritance as well as *de novo* cases	1p36.3, 2p21, 3p26.3, 6q (*SIM1*, *MRAP2*, *POU3F2*), 9q34, 10q26, 12q, maternal uniparental disomy (UPD) of chromosome 14 and X, heterozygous truncating mutations in *MAGEL2*	see Prader-Willi Syndrome	see Prader-Willi Syndrome + heart defects, neurological defects (seizures, hearing loss).

Pseudohypoparathyroidism (PHP) with Albright hereditary osteodystrophy (AHO)[113-121]	N.A.	Autosomal dominant	*GNAS1* (mutations or duplication)	Resistance to parathyroid hormone, thyroid-stimulating hormone, growth hormone–releasing hormone and gonadotropins, AHO: short stature, obesity, dysmorphic features (round face, nystagmus, low nasal bridge, short neck), hypocalcaemia, subcutaneous ossifications, brachydactyly (hands/feet), osteoporosis, hypogonadism, calcified choroid plexus, hypocalcemic tetany.	ID, mild DD.
Rubinstein-Taybi Syndrome or Broad Thumb-Hallux Syndrome[122-128]	1 in 100.000 – 125.000	Autosomal dominant as well as *de novo* cases	*CREBBP* and *EP300* (deletions or mutations)	Craniofacial features (microcephaly, low anterior hairline, downslanted palpebral fissures, broad nasal bridge/beaked nose, low hanging columella, high palate, grimacing smile, talon cusps), hypotonia, broad and often angulated thumbs and great toes, short stature, gastroesophageal reflux, moderate to severe ID, obesity after puberty, speech delay, delayed bone age, strabismus, cryptorchidism, recurrent infections, shortened life expectancy in children with heart defects.	Behavioral and psychiatric problems, intrauterine growth retardation, increased fractures, orthopedic problems (scoliosis, kyphosis, lordosis), renal malformations, congenital heart defects, vascular anomalies, hirsutism, nocturnal obstructive apnea, increased risk of benign and malignant tumors, increased risk of leukemia, seizures.

Short stature-brachydactyly-obesity-global DD Syndrome[129-131]	< 1 in 1.000.000	Autosomal recessive	*PRMT7* (mutations)	Short stature, obesity, symmetrical shortening of the digits and posterior metacarpals and metatarsals, global DD, mild ID	/
Diploid Triploid Mosaicism[132-138]	N.A.	Mosaicism	N.A.	DD, ID, learning difficulties, seizures, hearing loss, depression, short stature, truncal obesity, hypotonia, syndactyly or camptodactyly, facial features (small chin or lower jaw, broad/prominent forehead, small mouth, low set ears), scoliosis, genital anomalies, patches or streaks of darker or lighter skin, shortened life expectancy.	/
Wilms tumor, Aniridia, Renitourinary malformations and mental Retardation (WAGR) Syndrome[139-148]	1 in 1.000.000	Autosomal dominant	Deletion of 11p13-p14.1 (*WT1*, *PAX6* and *BDNF*)	Wilms tumor, aniridia, genitourinary malformations (hypospadias, cryptorchidism), ID.	Obesity or severe hyperphagia, renal problems, cardiopulmonary defects, behavioral difficulties like autism.
Wilson-Turner or X-linked ID-gynaecomastia-obesity Syndrome[149-152]	N.A.	X-linked recessive	*LAS1L* and *HDAC8* (mutations)	Severe ID, dysmorphic facial features, hypogonadism, short stature, truncal obesity, gynaecomastia, speech impairment, tapering fingers, behavioral problems (e.g. emotional lability), small feet.	Seizures.

Table 3. Overview of prevalence, clinical features, responsible gene(s) or chromosomal region, molecular analysis and treatment of the six selected syndromic obesity forms: Prader Willi syndrome (PWS) and the Prader Willi *like* (PWL) phenotype, Bardet-Biedl syndrome (BBS), Alström syndrome (AS), Wilms tumor, Aniridia, Genitourinary malformations and mental Retardation (WAGR) syndrome and 16p11.2 (micro)deletions.

DISEASE	PREVALENCE	CLINICAL FEATURES	GENE(S) OR REGION	MOLECULAR ANALYSIS	TREATMENT
Prader Willi Syndrome Prader Willi *like* phenotype	PWS: 1 in 10.000 – 30.000 PWL: N.A.	Main features: Severe neonatal hypotonia, feeding difficulties followed by hyperphagia and excessive weight gain, DD, hypogonadism, ID, characteristic facial features (almond-shaped eyes, a thin upper lip, downturned corners of the mouth and/or a narrow face), behavioral problems (e.g. temper tantrums, stubbornness, skin picking), small hands and feet, short stature. Additional features: T2DM, sleep abnormalities, apnea, hypothyroidism, delayed language development, respiratory infections, hypopigmentation of hair, eyes, and skin, strabismus, hip dysplasia, scoliosis, psychosis, heart defects, seizures, hearing loss.	Prader Willi syndrome: 15q11.2 – q13 (imprinting region) Prader Willi *like* phenotype: 1p36.3, 2p21, 3p26.3, 6q (*SIM1*, *MRAP2*, *POU3F2*), 9q34, 10q26, 12q, maternal UPD of chromosome 14 and X, heterozygous truncating mutations in *MAGEL2*	Present: Methylation-Specific-Multiplex Ligation-dependent Probe Amplification analysis (MS-MLPA) in combination with karyotyping, Fluorescence In Situ Hybridization (FISH) or chromosomal microarray, DNA polymorphism analysis, microsatellite analysis or sequencing the 4.3kb smallest PWS region.	○ Nutritional management: Low calorie diet and/or protein diet, daily physical activity, locking kitchen, refrigerator and/or cupboards ○ Recombinant Human Growth Hormone (rhGH) ○ Intranasal oxytocin ○ Hormonal replacement therapy ○ Serotonin reuptake inhibitors
Bardet-Biedl Syndrome	1 in 13.500 (Israel and Arab counties) 1 in 160.000 (Switzerland)	Main features: Obesity, cone-rod dystrophy, postaxial polydactyly, cognitive impairment, hypogenitalism and renal abnormalities. Additional features: Speech deficits, olfaction disorders (anosmia or hyposmia), psychiatric problems, T2DM and ataxia or impaired coordination.	*BBS1 – BBS20*, *NPHP1*, *FBN3* and *CEP19* (mutations)	Past: Restriction enzyme digests and/or amplification-refractory mutation system (ARMS) assays. Present/Future: Targeted high-throughput sequencing such as Next Generation Sequencing (NGS) panels.	○ Nutritional management: Low calorie and/or protein diet, daily physical activity and behavioral therapies ○ Low vision aids and mobility training or even subretinal injection ○ Removal of the accessory digit(s) ○ Hormonal replacement therapy ○ Renal transplantation

Alström Syndrome	1-9 in 1.000.000	Main features: Obesity, cone-rod dystrophy, renal anomalies, progressive sensorineural hearing impairment, male hypogonadism/ female hyperandrogenism, adult short stature, T2DM and dilated or restrictive cardio-myopathy.	*ALMS1* (mutations)	Past: Mutation analysis of the gene hotspots (exon 8, 10 and 16) in *ALMS1*. Present/Future: Array- or NGS-based technology.	○ Nutritional management: Low calorie and/or protein diet, daily physical activity ○ RhGH ○ Red-orange tinted lenses ○ Angiotensin-converting-enzyme (ACE) inhibitors or renal transplantation ○ Myringotomy ○ Metformin and Rosiglitazone ○ Cardiac transplantation
WAGR Syndrome	1 in 1.000.000	Main features: Wilms tumor, aniridia, genitourinary malformations (hypospadias, cryptorchidism), ID. Additional features: Obesity or severe hyperphagia, renal problems, cardiopulmonary defects, behavioral difficulties.	Deletion of 11p13-p14.1 (*WT1*, *PAX6* and *BDNF*)	Present: FISH, MLPA, Multiplex Amplicon Quantification (MAQ), Chromosomal microarray (Single-nucleotide polymorphism (SNP)-based array or array Comparative Genomic Hybridization (CGH)) or high-resolution cytogenetics.	○ Chemotherapy, radiotherapy or even nephrectomy ○ Colored, tinted contact lenses or implantation of an artificial iris-intraocular lens ○ Surgical intervention for genitourinary malformations ○ Nutritional management: Low calorie and/or protein diet, daily physical activity ○ ACE inhibitors

16p11.2 ***593 kb deletion*** ***220 kb deletion*** ***1.7 Mb deletion***	N.A.	<u>Main features:</u> ASD, schizophrenia, neuropsychiatric disorders <u>Additional features:</u> Severe early-onset obesity, DD, ID, hypotonia, epilepsy, behavioral problems, speech articulation abnormalities. <u>Main features:</u> Severe early-onset obesity, DD. Combination of features seen in 593 kb deletion and 220 kb deletion	Deletion of 16p11.2 (*SH2B1*)	<u>Present:</u> FISH, MLPA, MAQ, Chromosomal microarray (SNP-based array or array CGH) or high-resolution cytogenetics.	○ Neuropsychological testing ○ Nutritional management: controlling food intake and physical activity ○ Epileptic medication ○ Behavioral strategies and habilitative therapies ○ Didactic, naturalistic behavioral methodologies and developmental-pragmatic approaches

2. Prader Willi syndrome (PWS)

PWS is the most common form of syndromic obesity with a prevalence of 1 in 10.000 – 30.000 live births. It is mainly characterized by severe neonatal hypotonia, feeding difficulties followed by hyperphagia and excessive weight gain (Table 1: 'Disturbance of regulation of hunger and satiety feelings'), hypogonadism and ID with an average IQ of 65. Characteristic facial features are almond-shaped eyes, a thin upper lip, downturned corners of the mouth and/or a narrow face. Also behavioral problems (e.g. temper tantrums, stubbornness and skin picking), sleep abnormalities, small hands and feet and short stature are frequently described features[102-106].

In order to clinically diagnose PWS, Holm and colleagues developed a scoring system based on the abovementioned features. Characteristics were divided into three groups: major criteria (1 point), minor criteria (0.5 point) and supportive criteria (no points). When younger than three years of age, five points (at least four of them major) are required to diagnose PWS. Eight points (at least five of them major) are needed when 3 years or older[153]. Gunay-Aygun and colleagues later modified these clinical criteria to help identify appropriate patients for further diagnostic testing of PWS and which criteria are characteristic at different ages[103].

In spite of the accuracy of this clinical scoring system, a DNA methylation analysis is always performed to confirm the PWS diagnosis. PWS is caused by the absence of expression of the paternal genes (*small nuclear ring finger* (*SNURF*), *small nuclear ribonucleoprotein polypeptide N* (*SNRPN*), *makorin ring finger protein 3* (*MKRN3*), *MAGE family member 2* (*MAGEL2*) and *necdin MAGE family member* (*NDN*)) on the imprinting region 15q11.2 – q13 due to a paternal deletion (70-75%), maternal UPD (20-25%) or an imprinting defect of the critical region (1-3%)[102, 104]. Two main deletion types, I and II, are described. The type I deletion, occurring in approximately 40% of patients, is located between breakpoint (BP) 2 and BP3 with a mean size of 6.6 Mb. Deletion type II occurs in the remaining 60% of patients spanning a region of 5.3 Mb between BP2 and BP3[154, 155] (Figure 2).

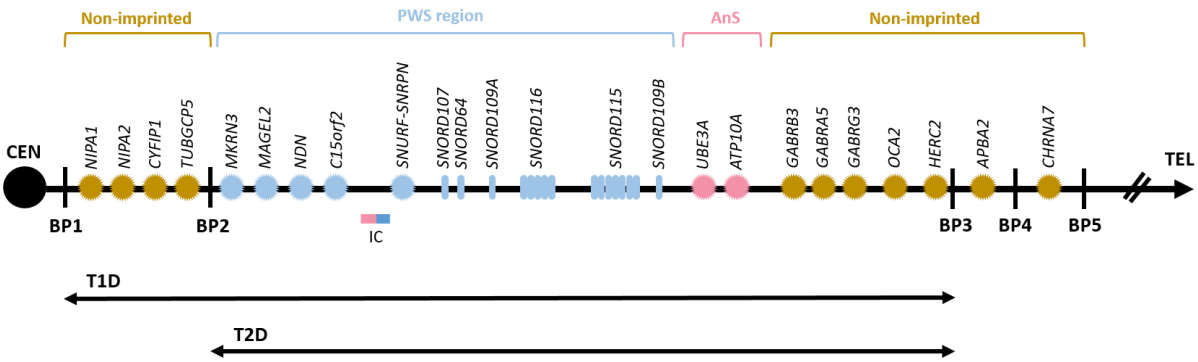


Figure 2. Schematic representation of chromosomal region 15q11.2 – q13.
The region involved in Prader Willi syndrome (PWS) is depicted in blue. The type I deletion (T1D), occurring in approximately 40% of patients, is located between breakpoint (BP) 1 and BP3 with a mean size of 6.6 Mb. Deletion type II (T2D) occurs in the remaining 60% of patients spanning a region of 5.3 Mb between BP2 and BP3. AnS = Angelman syndrome, IC = Imprinting center, CEN = centromere, TEL = telomere.

MS-MLPA targeting the 5' CpG island of the *SNURF-SNRPN* locus is the gold standard technique for diagnosing PWS in all three molecular genetic classes, however, it won't identify the genetic subtype[106]. Therefore, to diagnose deletions, additional cytogenetic analyses such as FISH can be performed, however nowadays chromosomal microarrays are routinely used in clinical genetics and have replaced the FISH analysis in most centers[155, 156]. If MS-MLPA shows an abnormal methylation pattern but it does not indicate a paternal deletion, additional microsatellite analysis can be performed in order to characterize UPD (heterodisomy or isodisomy) or imprinting defects. If this technique shows a biparental pattern, a mutation (0.85 – 0.9%) or microdeletion (0.1 – 0.15%) in the imprinting center is present. Testing for the presence of a microdeletion is important, as the recurrence risk can be 50% when the father also harbors the deletion[156]. This can be done with the use of the MS-MLPA assay or sequencing the 4.3kb smallest region of overlap for the PWS imprinting center[155, 157, 158]. In extremely rare cases a(n) (un)balanced translocation (0.1%) can be the cause of PWS in an individual. When the breakpoint of the translocation is located in the 15q11.2 – q13 PWS region, it can be indicated with the use of the karyotype of

the patient in association with the MS-MLPA results or using FISH analysis[105, 156] (Figure 3).

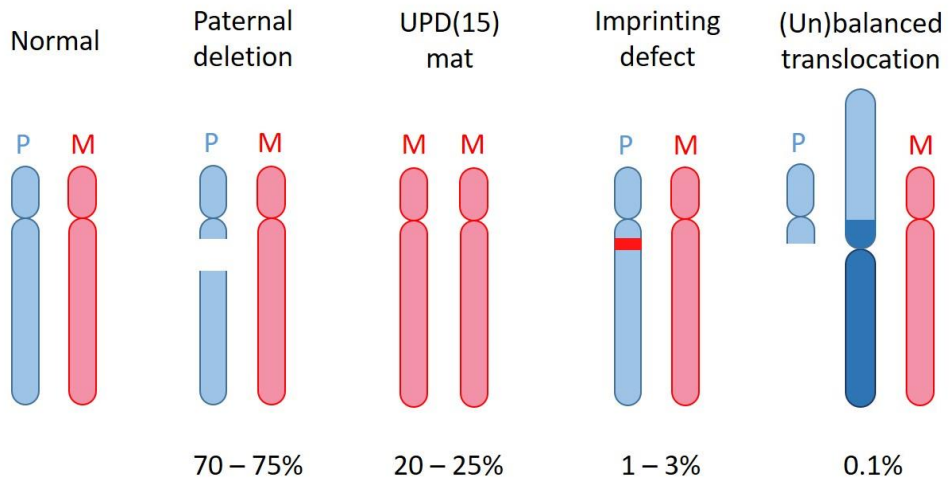


Figure 3. Ideograms showing possible causes of chromosomal abnormalities in Prader Willi syndrome.
Blue = paternal chromosome 15, pink = maternal chromosome 15. UPD(15)mat = maternal uniparental disomy of chromosome 15.

An additional advantage of the MS-MLPA technique is that it can differentiate PWS from Angelman Syndrome (AnS) (1 in 15.000 – 20.000), a condition genetically related to PWS[106, 159, 160]. In contrary to PWS, AnS patients show an absence of expression of the maternal genes *ubiquitin protein ligase E3A* (*UBE3A*) and *ATPase Phospholipid Transporting 10A (Putative)* (*ATP10A*) on the imprinting region 15q11.2 – q13. Later, the disruption of the *UBE3A* gene is identified as the cause of AnS due to a deletion, paternal UPD, imprinting defects or intragenic mutations[160, 161]. Characteristic features of AnS are severe DD, hypotonia in infancy, jerky movements, seizures and a happy disposition[162, 163].

Because of hypotonia, AnS and Fragile X Syndrome need to be considered as a differential diagnosis of PWS in infants. Other differential diagnoses are Bardet-Biedl syndrome (BBS), Cohen Syndrome, Alström Syndrome (AS), Borjeson-Forssman-Lehmann syndrome, Prader Willi *like* (PWL) syndrome and 16p11.2 (micro)deletions.

Which gene effectively is responsible for the PWS phenotype is not clear yet. Initially *SNRPN* was designated as a strong candidate gene, however, further research of two balanced translocations, though a rare cause of PWS, excluded the gene as a primary candidate[164-166]. Located within the introns of very long transcripts extending downstream of *SNRPN* are clusters of C/D box-containing small nucleolar RNA (snoRNA) genes of which the expression (especially in the brain) is controlled by the SNRPN promoter[166-168]. Consequently, a significant role for the snoRNA genes in the PWS phenotype emerged. In 2008, Sahoo and colleagues identified for the first time a microdeletion of SNORD116 and a substantial segment of the SNORD115 cluster in a patient with PWS characteristics[168]. Since previously identified deletions of the SNORD115 cluster did not show a clear PWS phenotype with paternal inheritance, the SNORD116 cluster was designated as the most likely candidate to cause PWS[169, 170]. One year later, a research group in the United Kingdom confirmed the role of the SNORD116 cluster in PWS and particular in human energy homeostasis, growth and reproduction[171]. Even more recently, Bieth and colleagues identified the first restricted deletion of the SNORD116 cluster in a patient with PWS features increasing the contributing role of SNORD116 in the pathogenesis of PWS[172]. Up to now, four research groups also studied a PWS mouse mutant line which carries a Snord116 deletion[173-176]. Heterozygous mice exhibiting a paternally inherited deletion show postnatal growth retardation and hyperphagia[173, 174]. Mice with a biallelic deletion of Snord116 have low birth weight with an increase in body weight, energy expenditure and hyperphagia in early adulthood[176]. Overall, both mice models show important characteristics of the PWS phenotype, supporting a significant role for SNORD116 in PWS pathogenesis[166].

Therapeutically, there are no opportunities yet to address the primary cause of PWS, however symptoms can be treated. In order to lower the risk of developing obesity and its comorbidities, nutritional management is crucial. In general, a well-balanced, low calorie diet together with daily physical activity is a fundamental therapy. From early childhood (age of 2 years) PWS patients also tend to sneak and hoard food with

the result that locking the kitchen, refrigerator and/or cupboards is necessary in order to control their dietary intake[102, 105, 177]. Another promising therapy is the administration of recombinant human growth hormone (rhGH). Besides its beneficial effect on weight gain (decrease in adipose tissue and increase in lean body mass), rhGH also normalizes the patient's height and improves respiratory function and physical activity[105, 106, 178, 179]. The effects of liraglutide, a glucagon-like peptide (GLP)-1 analog approved by the United States Food and drug Administration and the European Medicines Agency for weight management in obese adults, were also investigated in PWS individuals with T2DM. Liraglutide therapy leads to a decrease in plasma ghrelin levels and BMI, however, the long-term effects of such treatment in individuals with PWS are uncertain and further studies on a larger number of patients are required[180-182]. De Waele and colleagues also examined the effect of long-acting octreotide treatment in PWS. As for liraglutide a decrease in ghrelin concentrations was noticed after octreotide administration for 16 weeks, however it did not affect weight, appetite or compulsive behavior towards food in the PWS subjects[183]. Another still investigational therapy is the administration of intranasal oxytocin (OXT) to treat hyperphagia. Despite contradictory results of different clinical trials so far, a promising effect on food-related behavior was seen in children with PWS younger than 11 years of age[184-186]. This hormone shows a high expression in the hypothalamus and, as seen in functional magnetic resonance imaging scans, this brain region also shows a significant delay in activation in PWS patients[187] so further research of the effects of intranasal OXT administration on the hypothalamic activity is still necessary. In addition, different studies in whole-room indirect calorimeters also indicated a significant reduction in total, resting, sleep and activity energy expenditure in PWS patients compared to age-, sex- and BMI-matched subjects. This decrease can be attributed to reduced activity but also to lower energy utilization due to reduced lean body mass which primarily consists of muscle[188, 189]. These results highlight the importance of daily physical activity in PWS patients.

Important to mention, restrictive bariatric surgery is not recommended in PWS as it is associated with high complication rates and even mortality[190, 191]. Also in a case report Fonkalsrud and Bray describe the limited success of a truncal vagotomy to treat obesity in PWS patients as the subject initially lost 29 kg but regained most of this in almost a year time due to almost continuous ingestion of small quantities of food[192]. Other therapies that may benefit PWS patients are the use of serotonin reuptake inhibitors to manage behavioral disorders, especially obsessive-compulsive symptoms and a hormonal replacement therapy in order to produce adequate secondary sexual characteristics at puberty. The latter, however, is somewhat controversial due to the effects of i.e. testosterone replacement on behavior[102, 177, 193].

Noteworthy, recently Kim and colleagues reported a first epigenetics-based therapy for PWS. In a PWS mice model, they restored the expression from the maternal copies of PWS-associated genes by targeting the histone methyltransferase G9a with the use of two small molecules UNC0638 and UNC0642. Administration of UNC0642 resulted in the improvement of survival and growth of the pups. However, the effect of the drug on other disease symptoms such as hyperphagia and obesity still needs to be evaluated[194].

As an additional remark, since the genes and pathways disrupted in PWS are also highly conserved in zebrafish, in the future zebrafish could also serve as a promising model for high-throughput screening in order to quickly identify and deliver potential curative pharmacotherapies for PWS patients[195].

2.1. Prader Willi like (PWL)

Patients with a PWL phenotype have similar clinical characteristics as PWS individuals but occasionally show heart defects or neurological defects like seizures or hearing loss[110, 196, 197]. Consequently, MS-MLPA and/or additional techniques are always performed in order to exclude PWS in these patients. Once the clinical diagnosis of PWS cannot be confirmed molecularly, the patients are considered PWL. Clinically but also genetically the PWL phenotype is heterogeneous. In literature the phenotype has

already been associated with copy number variations (CNVs) on different chromosomes such as 1p36.3[198], 2p21[199], 3p26.3[200, 201], 6q[110, 157, 196, 197, 200, 202-213], 9q34[214], 10q26[215], 12q[215] and X[215] as well as maternal UPD of chromosome 14[215]. Therefore, the use of chromosomal microarrays is recommended in order to identify any of these CNVs. Noteworthy, deletions at chromosome 6q are most frequently reported. In 1988, Turleau and colleagues linked the PWL phenotype with a 6q deletion for the first time[216]. Several years later, similar reports appeared which enlarged the role of chromosome 6q in the PWL phenotype[110, 157, 196, 197, 204, 205, 208, 209, 211-213, 217]. In these studies most 6q deletions encompass *Single-minded 1* (*SIM1*) which is part of the leptin-melanocortin signaling pathway that regulates energy homeostasis. This ultimately led to the identification of haploinsufficiency of the *SIM1* gene as a possible cause for obesity in these patients[110, 157, 196, 197, 205, 208, 209, 213]. This hypothesis was substantiated further in an eighteen month old girl with early-onset severe obesity who presented with a *de novo* balanced translocation between chromosome 1p22.1 and 6q16.2 separating the 5' promoter region and basic helix-loop-helix (bHLH) domain from the 3' PER-ARNT-SIM (PAS) region and putative transcriptional regulating domains of the *SIM1* gene[218]. In addition, different research groups, including our own, reported loss-of-function point mutations and variants in *SIM1* in obese individuals with or without PWL features, indicating a possible contribution of the *SIM1* gene in the development of obesity and the PWL syndrome[219-222]. Moreover, Wentzel and colleagues identified two interstitial deletions of 8.73 and 4.50 Mb at chromosome 6q14.1 – q15 in two children with a PWL phenotype[223]. The overlapping region of 4.2 Mb encompasses the *Melanocortin 2 Receptor Accessory Protein 2* (*MRAP2*) gene. By sequencing the coding regions and intron/exon boundaries of the gene, Asai *et al.* also revealed four rare heterozygous variants in four severely obese children of which one (E24X) was clearly disruptive[224]. In contrast to this, own CNV and mutation analysis in a cohort of 109 PWL patients only showed a limited involvement of copy number and sequence variation in *SIM1* and *MRAP2* in the PWL phenotype[225]. More recently, Kasher and colleagues also

identified small deletions encompassing *POU Class 3 Homeobox 2* (*POU3F2*), and not *SIM1*, at chromosome 6q in ten individuals from six families with DD, ID, neonatal hypotonia, susceptibility to obesity and hyperphagia, characteristics resembling the PWL phenotype. In addition, with the use of morpholino and mutant zebrafish models, they showed that the gene lies downstream of *SIM1* in the leptin-melanocortin signaling pathway which indicates that *POU3F2* also has a potentially interesting role in the obesity phenotype of PWL patients[226]. In extremely rare cases, heterozygous truncating mutations in *MAGE Family Member L2* (*MAGEL2*), occurring on the paternal allele, are reported in individuals with features resembling the PWS/PWL phenotype which is called the Schaaf-Yang syndrome[227, 228]. Also the Magel2-null mouse model indicates a role in PWS and obesity[229]. However, in contrast to this findings, Buiting and colleagues identified two deletions including *MAGEL2* and not the *SNRPN/SNORD116* locus in two patients who do not have PWS or PWL characteristics and consequently concluded that the role of *MAGEL2* in PWS/PWL still remains unclear[230, 231].

As for PWS, PWL therapy is mainly focused on the treatment of the symptoms. As these symptoms are largely comparable, the corresponding treatment of both phenotypes is similar. In addition, depending on the patient's phenotype, specific treatment of epilepsy or cardiovascular abnormalities can be indicated. In case of hearing loss a cochlear hearing device implantation could be a solution.

3. Bardet-Biedl syndrome (BBS)

BBS is a pleiotropic recessive disorder belonging to the family of ciliopathies with a prevalence of 1 in 13.500 (Israel and Arab counties) to 1 in 160.000 (Switzerland) individuals. The big difference in prevalence can be attributed to a high rate of consanguinity in the first population group[22, 23, 232]. Apart from obesity, BBS patients are primarily characterized by cone-rod dystrophy, postaxial polydactyly, cognitive impairment, hypogenitalism and renal abnormalities. Secondary features are speech deficits, olfaction disorders (anosmia or hyposmia), psychiatric problems, T2DM and

ataxia/impaired coordination[22-26]. At birth, several major diagnostic clues such as the ophthalmological features and obesity are not noticeable yet, however they appear and progressively worsen during the first and second decade of life[26]. E.g.: For children with BBS the visual prognosis is poor. At age seven to eight years, patients usually develop night blindness which eventually leads to legal blindness by the age of 20 years in more than 60% of patients[22, 233, 234].

As for PWS, BBS is also diagnosed based on clinical findings. BBS diagnosis is assigned to patients bearing at least four primary criteria. When only three major features are present, two secondary criteria are required to confirm the presence of the disorder[26, 234, 235].

A substantial clinical overlap exists with other ciliopathies like AS and McKusick-Kaufman syndrome. Due to the evolving phenotype of BBS in time, the clinical overlap with other disorders, and the increasing possibilities of large-scale genetic testing, molecular diagnostics is becoming more important in establishing a diagnosis of BBS. To date, 23 genes (*BBS1 – BBS20*, *Nephrocystin 1* (*NPHP1*), *Fibrillin 3* (*FBN3*)[29] and *Centrosomal Protein 19* (*CEP19*)[30]) have been identified to be associated with BBS and which are all involved in primary cilia functioning[26]. Most BBS cases can be attributed to mutations in *BBS1* and *BBS10* accounting for 23.2% and 20% in the populations of Europe and North America[25, 236, 237]. Disease-causing mutations in other BBS genes have a prevalence below 10% and even in most cases ≤5% (overview in Suspitsin *et al.* 2016[26]). Early studies suggested a classical mode of autosomal recessive inheritance for BBS in which a biallelic mutation in one of the affected genes gives rise to the disorder. However, as the years proceeded, the genetics of BBS became more complex. Katsanis *et al.* was the first to propose a 'triallelic inheritance' to manifest the phenotype. They identified a homozygous *BBS6* mutation in combination with a heterozygous *BBS2* mutation in one BBS family and two heterozygous *BBS2* nonsense mutations and a *BBS6* nonsense mutation in BBS patients in three other families[238]. This triallelic model was supported by some research groups[239-242], as others do not underpin the triallelic inheritance

hypothesis[243-248]. In addition, cases are reported in which even healthy individuals carry a biallelic mutation in one of the known BBS genes which suggests incomplete penetrance for at least some genes and/or types of mutations[26, 238, 241, 249]. The only way to achieve molecular confirmation of BBS is the use of clinically available tests. In the past, the most frequently reported mutations, such as p.M390R in *BBS1* and p.C91Lfs*5 in *BBS10*, were identified with the use of simple screening methods such as restriction enzyme digests and/or ARMS assays[250]. Nowadays targeted high-throughput sequencing, such as NGS panels, offer the most feasible and effective approach to provide high diagnostic yields as previously used mutation screening techniques are too time-consuming and expensive due to the broad genetic locus heterogeneity of the disease[22, 251-254]. To date, a clear genotype-phenotype correlation is not yet identified in BBS, however, patients with *BBS1* mutations do show a milder phenotype compared to patients harboring mutations in other BBS genes[242, 255].

BBS therapy mainly exist of the treatment of the patient's symptoms. Polydactyly can be surgically corrected. In most children this happens within the first two years of age. Secondly, the management of obesity is initiated at an early age with the use of a low calorie and/or protein diet, exercise and behavioral therapies[22]. One study also discovered a positive effect of the diet on renal function, however it only may slow the progression of renal failure[256]. Due to the high incidence of renal malformations and abnormal renal function that can lead to end-stage renal disease (ESRD), renal function must be monitored closely. In case of ESRD a renal dialysis and/or renal transplantation are warranted[22]. Thirdly, up till now researchers are unable to prevent the development of blindness in BBS patients. With the use of low vision aids and mobility training they are being prepared to progressive visual loss and possibly blindness by a specialist. However, an experimental set-up of subretinal injection of BBS-containing adenovirus constructs in mice demonstrated encouraging results so far[22, 26]. In case of abnormal gonadotropin or sex hormone levels at puberty, a

hormonal replacement therapy in order to produce adequate secondary sexual characteristics can be used in BBS patients[22].

4. Alström syndrome (AS)

AS was first described in 1959[257] and is, as BBS, included in the ciliopathies group[258]. It is a rare autosomal recessive disorder with a prevalence estimated at one to nine cases per 1.000.000 individuals. To date, approximately 950 cases are described worldwide[258, 259]. AS shows a wide clinical variability even within the same family. It does share some features with BBS such as obesity, cone-rod dystrophy, renal anomalies, male hypogonadism/female hyperandrogenism and adult short stature, suggesting BBS as an important differential diagnostic consideration. Additionally, AS is characterized by progressive sensorineural hearing impairment, T2DM and dilated or restrictive cardiomyopathy[18, 19]. A clinical distinction between AS and BBS can be made based on the timing of the onset of visual problems and the presence of postaxial polydactyly. AS patients usually show vision difficulties before the age of two years, whereas in BBS this appears on average 6.5 years later. Polydactyly, which is common in BBS, has not been described in AS[18]. However, since both ciliopathies show considerable phenotypic overlap, especially in early infancy, molecular techniques are crucial to clearly differentiate AS from BBS[252, 260, 261].

AS is caused by homozygous or compound heterozygous mutations in *Alström syndrome protein 1* (*ALMS1*) on chromosome 2p13[20]. Recently, however, Ozantürk and colleagues also reported triallelic mutations in *ALMS1* in Turkish AS cases who incredibly did not show more severe characteristics[262]. To date, more than 200 mutations have been reported of which exon 8, 10 and 16 are the three big hotspots for *ALMS1* mutations (overview Supplemental S1 Table in Marshall *et al.* 2015[263]). The mutations with the largest impact on the ALMS1 protein are frameshift or nonsense mutations occurring downstream of exon 7 resulting in an early termination of ALMS1 and subsequently a non-functional protein[258, 263]. In addition, also splice-

site mutations[264], deletions[265, 266], one *Alu* transposon insertion[267] and one balanced translocation[268] have been identified in *ALMS1*.

As ALMS1 localizes to centrosomes and basal bodies of ciliated cells, a role in microtubule organization, intracilia transport, endosome recycling and cell cycle regulations is suggested[18, 262, 269, 270]. Secondly, it is also hypothesized that ALMS1 could play a role in β-cell function and/or peripheral insulin signaling pathways since AS patients are more likely to develop T2DM in contrast to BBS patients despite the fact that obesity levels are equivalent in both syndromes[271, 272]. Unfortunately, up till now the precise molecular mechanism causing the AS phenotype has not been fully elucidated[263].

In the past, the first step in the molecular diagnosis of AS in patients was to perform mutation analysis of the gene hotspots (exon 8, 10 and 16) in *ALMS1*. When this approach did not reveal any mutation, an array-based technology, which includes a set of known mutations in *ALMS1*, was often carried out[258, 260]. Nevertheless, as the NGS methodology is rapidly growing and gradually replacing above-mentioned technologies, whole-exome and whole-genome sequencing are nowadays the most widely used approaches to identify the causal mutation and secondly also exclude mutations in other genes in the differential diagnosis[258, 273, 274].

As for PWS and BBS, the only therapy for AS patients is restricted to the management of the clinical symptoms and the improvement of the quality of life[258]. Between birth and age 15 months most patients become very sensitive to light (photodysphoria) which can be reduced with the use of red-orange tinted lenses. However, almost all patients have decreasing visual accuracy with loss of light perception around the age of 20 years, although the progression rate is variable. Secondly, weight gain can be controlled with the use of a healthy, reduced calorie diet and regular exercise taking into account the vision limitations of the patient[18]. Also beneficial effects of rhGH on body composition and liver fat content were reported in a 15-year-old AS patient[275]. Urinary analysis for proteinuria is warranted in order to detect decreased renal function. In this case, ACE inhibitors are often prescribed, which are also used for the

treatment of the dilated or restrictive cardiomyopathy. Unfortunately, in select cases a renal and/or cardiac transplantation is the only viable therapeutic option[18, 276]. Myringotomy, a surgical procedure in which a tiny incision is created in the tympanic membrane, or the implantation of an cochlear hearing device can improve the hearing impairment in the patients. Lastly, when T2DM is present a treatment as in the general population is recommended[18], though valuable results are already obtained with a combined therapy comprising a high dose of metformin and rosiglitazone[277].

5. Wilms tumor, Aniridia, Genitourinary malformations and mental Retardation (WAGR) syndrome

WAGR syndrome, first described in 1964 by Miller *et al.*[278], is an autosomal dominant disorder with an estimated prevalence of 1 in 1.000.000 individuals[147, 279]. The main characteristics are stated in the acronym: Wilms tumor (an embryonic malignancy of the kidney), aniridia, genitourinary malformations and mental retardation or ID. Obesity or severe hyperphagia has also been described in a subgroup of these patients, in which case the condition is often called WAGRO[139-145]. Secondary features often seen in WAGR patients are renal problems, cardiopulmonary defects and behavioral difficulties like autism[143, 148]. Due to the specific combination of manifestations, the differential diagnosis of WAGR is very limited.

WAGR is caused by a *de novo* heterozygous deletion on chromosome 11p13 in which haploinsufficiency of *Wilms tumor 1* (*WT1*) and *paired box 6* (*PAX6*) is responsible for the core features of the disorder[279]. Evidence is shown that a deletion of *WT1* is associated with an increased risk of Wilms tumor, genitourinary anomalies and nephropathies, whereas *PAX6* deletions give rise to eye abnormalities[143, 280]. There is also a possibility that *PAX6* deficiency results in brain and pancreas defects[281-284]. Both genes are also located at approximately 4 Mb from the *Brain-derived Neurotrophic Factor* (*BDNF*) gene at chromosome 11p14.1[145, 279]. More than half the WAGR patients exhibit a deletion also including this gene who notably all show childhood obesity compared to only 20% of the patients without a *BDNF*

encompassing deletion[145] (Figure 4). Consequently, it is suggested that *BDNF* is a key player in the obesity phenotype seen in WAGR patients and even in overall energy homeostasis in humans. BDNF encodes a member of the nerve growth factor family which is involved in neuronal proliferation, differentiation and survival of specific neuronal populations[10, 285, 286]. Its role in energy homeostasis has extensively been investigated resulting in the development of different *Bdnf* animal models exhibiting an obese phenotype and marked hyperphagia[287-290]. In addition, since its neural function, the protein is widely distributed in the central nervous system including the hypothalamus[10, 279]. It is believed that BDNF functions within the ventromedial hypothalamic leptin-melanocortin signaling pathway as a downstream target of MC4R and, as a result, is an important effector in the control of energy balance[291, 292]. On the other hand, a recent study determined more severe neurocognitive impairments in WAGR syndrome patients with *BDNF* haploinsufficiency which shows that BDNF could also be involved in cognitive functioning[293].

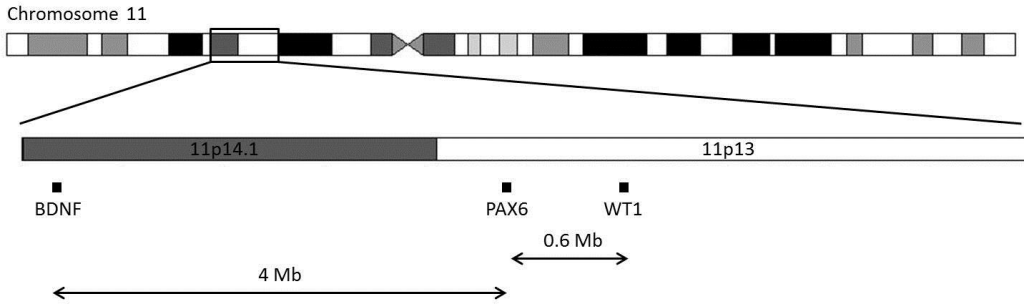


Figure 4. Schematic representation of the critical region of Wilms tumor, Aniridia, Genitourinary malformations and mental Retardation (WAGR) syndrome on chromosome 11p.
WAGR syndrome is caused by a *de novo* heterozygous deletion of *Wilms tumor 1* (*WT1*) and *paired box 6* (*PAX6*) on chromosome 11p13. More than half the WAGR patients exhibit a deletion also including the *Brain-derived Neurotrophic Factor* (*BDNF*) gene located at approximately 4 Mb from *WT1* and *PAX6* at chromosome 11p14.1. Notably, these patients all show childhood obesity compared to only 20% of the patients without a *BDNF* encompassing deletion.

Genetic testing for WAGR syndrome is typically performed in children presented with aniridia[143]. To identify large deletions or translocations of chromosome 11p13, high-resolution cytogenetics is an appropriate method[147, 294]. Small deletions, however, cannot be recognized with this technique. In this case, FISH, MLPA or array CGH are more suitable[143, 145, 280, 294].

Once genetic tests confirm the diagnosis of WAGR syndrome, ultrasound screening for Wilms tumor is initiated every three months until the age of five to six years to allow early detection[143, 294, 295]. The prevalence of Wilms tumor in WAGR patients is estimated between 45%[296] and 57%[143]. Depending on the stage of the tumor, patients may require chemotherapy, radiotherapy or even nephrectomy (surgical removal of the kidney). Other renal problems are also frequently reported in which proteinuria or mild hypertension can be an early indication of renal insufficiency and subsequently can be treated with ACE inhibitors[143, 294]. Aniridia should be monitored by an expert ophthalmologist[294, 297]. Colored, tinted contact lenses can be worn to reduce light sensitivity and restore a more normal appearance of the eye, however, due to poor ocular surface and reduced tear production, difficulties can be experienced[297]. That's why implantation of an artificial iris-intraocular lens is nowadays suggested[297, 298]. In case of gonadal dysgenesis, prophylactic removal of the gonads is warranted to prevent the occurrence of gonadoblastoma[294].

6. 16p11.2 (micro)deletions

Due to the presence of flanking homologous segmental duplications, the 16p region is particularly prone to rearrangements leading to copy number changes[57]. The recurrent proximal ~593 kb deletions (and duplications) are among the most frequent genetic etiologies of ASD[299-301], schizophrenia[302] and neuropsychiatric disorders[303]. The prevalence is estimated at approximately 1% in all ASD cases[299]. In addition, the microdeletion syndrome also cosegregates with severe early-onset obesity[58, 62], DD[304], ID[305, 306], hypotonia[307], epilepsy[304, 306-308], behavioral problems[111, 304] and even a high prevalence of speech articulation abnormalities[111, 307]. On the contrary,

patients harboring a ~593 kb microduplication share some characteristics with the deletion phenotype, however, confer an increased risk for being underweight indicating that haploinsufficiency and triplosensitivity at the 16p11.2 locus clearly has opposite effects on BMI. To date, no particular candidate gene located within the 593kb region is pointed forward to clarify the obesity/underweight phenotype[309]. In addition, larger deletions of 1.7 Mb encompassing the 593 kb proximal deletion are also reported in patients harboring similar characteristics[58, 62, 310, 311] supplemented with or without secondary features such as dysmorphism, heart defects[311] or Hirschsprung disease[312]. Noteworthy, this 1.7 Mb deletion also includes a second 220 kb distal deletion which in literature mainly is associated with severe early-onset obesity as well as obesity with DD[57-60] (Figure 5). Additionally, dysmorphic features, behavioral problems, seizures, speech and motor delays and autism are also commonly reported[57, 313].

In case of obesity in association with DD or ID, PWS or PWL should be considered as a differential diagnosis. However, as nowadays genome-wide microarray analysis is a routine diagnostic screening method, 16p11.2 (micro)deletions are unlikely to be missed. Therefore extensive differential diagnosis is useless.

All four CNVs (593 kb deletion, 593 kb duplication, 1.7 Mb deletion and 220 kb deletion) are inherited in an autosomal dominant matter, either due to a *de novo* event or inherited from one of the (even healthy) parents[57, 58, 310, 314].

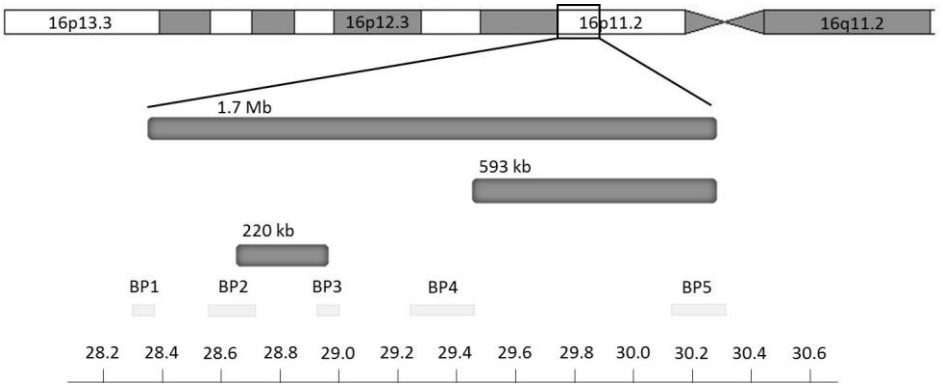


Figure 5. Schematic representation of the proximal (593 kb), distal (220 kb) and overlapping 1.7 Mb copy number variations on chromosome 16p11.2.

For the distal 16p11.2 deletion (and the overlapping 1.7 Mb deletion) there is increasing evidence that haploinsufficiency of the *Sarcoma* (*Src*) *homology 2B adaptor protein 1* (*SH2B1*) gene could induce the obesity phenotype seen in the patients[309] as other genes within the 220 kb region might clarify the presence of the additional features[315]. Previous research in a yeast two-hybrid system demonstrated that SH2B1 is a Janus kinase 2 (JAK2)-binding protein[316]. Later on, the same research group showed that, in response to the binding of leptin to its receptor, SH2B1 not only binds to JAK2 but also promotes its activation[317, 318] indicating that SH2B1 could act as a positive regulator of leptin signaling and consequently might be implicated in energy homeostasis[315]. This hypothesis was substantiated further with the generation of a *SH2B1* deficient mouse model in which a significant decrease in leptin signaling in the hypothalamus was indicated. The *SH2B1*$^{-/-}$ mice also clearly developed an obesity phenotype with hyperphagia, hyperlipidemia, hyperglycemia, hyperleptinemia, hyperinsulinemia, hepatic steatosis and glucose intolerance[319, 320]. Further genetic evidence appeared in 2009 when GWAS reported an association of the A484T variant (rs7498665) in *SH2B1* with BMI which could also be replicated in our own Belgian cohort[59, 321, 322]. Published mutation analyses of *SH2B1* in both obese and lean study populations, however, show conflicting results. Two research groups identified potentially causative mutations in approximately 1% of severe obese subjects whereas no private variants were identified in lean control populations[323-325]. Own research, on the other hand, showed an equal presence of rare nonsynonymous *SH2B1* variations in both lean and obese individuals[315] which was also seen in a Chinese study population[326]. Both concluded that it seems unlikely that *SH2B1* variants do confer risks for obesity, however, further research is necessary to clarify the contradictory findings.

As for the WAGR syndrome, genetic screening for a 16p11.2 microdeletion in patients can include chromosomal microarray (SNP-based array or array CGH) or targeted deletion analysis using FISH, MLPA or MAQ[57, 313-315].

Noteworthy, the deletion patients (either with a 220 kb or 593 kb deletion) and duplication patients (either with a 220 kb or 593 kb duplication) share a common phenotype. Due to this phenomenon, Loviglio and colleagues suggested a genomic interaction between the distal (220 kb) and proximal (593 kb) region and in fact observed a complex chromatin looping between the genes located in the 220 kb region and those mapped to the proximal region. This observation indicates that the Chromosome Conformation Capture technique can be proposed as a new and effective tool to identify genes and/or pathways that are perturbed in patients harboring for example an overlapping obesity phenotype[327].

Therapeutically, as for the foregoing disorders, treatment should target the specific features identified. Before excessive weight gain begins, controlling food intake and physical activity at an early age is essential in order to avoid the development of obesity. A neurologist is seen when epilepsy is suspected who will prescribe epileptic medication based on the patient's age, seizure type, electroencephalography (EEG) and side effect profiles[314]. For the management of autism spectrum disorders, different educational interventions such as behavioral strategies and habilitative therapies are used. In addition, to improve communication skills and speech deficits in patients with 16p11.2 microdeletions, different didactic methodologies and developmental-pragmatic approaches are effective. A visit to a speech-language therapist is usually an appropriate first step[328].

7. Conclusions and future perspectives

Due to its increasing prevalence and the associated comorbidities, obesity has become a major health problem worldwide. As shown in this review, several genes/chromosomal regions are associated with energy homeostasis. Many years of research already resulted in a lot of information on the molecular genetics of these disorders and the obesity phenotype, however, as discussed in detail for the six selected syndromes, treatment of all syndromic forms is only restricted to the management of the patient's symptoms. Due to the important phenotypic overlap

between various obesity syndromes, it remains very difficult to make an appropriate syndromic diagnosis solely based on clinical phenotype. A good example of this is the differentiation between PWS and the PWL phenotype. So far, a clear difference between both phenotypes on clinical level is not yet made[155, 215]. In addition, signs and symptoms of PWS and PWL are also common to other syndromes such as BBS, AS, 16p11.2 (micro)deletions but also Fragile X syndrome, Cohen syndrome and even Temple syndrome[329, 330] which makes it even more difficult to determine in which circumstances the MS-MLPA analysis for PWS diagnosis should be performed. A correct and fast diagnosis, however, is of great importance. An update in the phenotypic and genetic information of these patients is therefore of great use to recognize the appropriate syndrome in an individual[155, 215]. In general, early diagnosis of an obesity syndrome is important as this would result in the delivery of accurate genetic counseling for parents. This will help in a better understanding of their child's condition. In addition, it can influence family planning and can help in the identification of other family members at risk of having a similar genetic condition. Furthermore, when a patient can be diagnosed with a syndromic form of obesity early in life, appropriate screening, follow-up and treatment of other associated medical problems can be offered. Examples are follow-up of renal function or ophthalmological problems in BBS or AS. Also, early observation of eating behavior of a patient is important as this would make it possible to intervene in time and even refer the patient to a specialized team.

Lastly, further delineation of (the functions of) the underlying genes will be helpful to unravel the mechanisms of energy metabolism in the general population as well[23]. Previous research mostly focused on simple screening methods like Sanger sequencing and FISH, however, due to the extremely heterogeneous character of the different syndromic obesity forms, these techniques are becoming too expensive and time-consuming to screen for disease-causing mutations. Yet nowadays, with the advent of automated DNA sequencing instruments, the NGS technology provides a new method for molecular diagnosis allowing the sequencing of gene panels, whole

exomes and even whole genomes[15, 331]. Furthermore, also chromosomal microarrays are routinely utilized in most clinical genetics centers and are already replacing the previously used FISH analysis[155, 156]. To conclude, syndromic obesity is likely to be a monogenic or contiguous gene disorder. Due to its great heterogeneity, nowadays molecular diagnostics is increasingly important in order to make a fast and accurate diagnosis, since an appropriate diagnosis based solely on clinical signs is difficult. Chromosomal microarrays and NGS technology are being introduced as important diagnostic methods. In the near future, it will even be possible to use whole-genome sequencing in order to detect both genomic mutations as well as translocations. However, understanding the clinical phenotypes still remains crucial for accurate counseling of families coping with syndromic obesity and appropriate follow-up of patients.

References

1. Pervez H, R.S., *Nurses' attitudes towards obese patients: a review of the literature.* Nursing Times [online], 2017. **113**(2): p. 42-45.
2. (WHO), W.H.O. *World Health Organisation (WHO)*. 2016 October; Available from: http://www.who.int/en/.
3. Moore, S.C., et al., *Association of Leisure-Time Physical Activity With Risk of 26 Types of Cancer in 1.44 Million Adults.* JAMA Intern Med, 2016. **176**(6): p. 816-25.
4. Withrow, D. and D.A. Alter, *The economic burden of obesity worldwide: a systematic review of the direct costs of obesity.* Obes Rev, 2011. **12**(2): p. 131-41.
5. Coleman, D.L. and K.P. Hummel, *The influence of genetic background on the expression of the obese (Ob) gene in the mouse.* Diabetologia, 1973. **9**(4): p. 287-93.
6. Stunkard, A.J., T.T. Foch, and Z. Hrubec, *A twin study of human obesity.* JAMA, 1986. **256**(1): p. 51-4.
7. Maes, H.H., M.C. Neale, and L.J. Eaves, *Genetic and environmental factors in relative body weight and human adiposity.* Behav Genet, 1997. **27**(4): p. 325-51.
8. Luke, A., et al., *Heritability of obesity-related traits among Nigerians, Jamaicans and US black people.* Int J Obes Relat Metab Disord, 2001. **25**(7): p. 1034-41.
9. Locke, A.E., et al., *Genetic studies of body mass index yield new insights for obesity biology.* Nature, 2015. **518**(7538): p. 197-206.
10. Zegers, D., et al., *Monogenic and Complex Forms of Obesity: Insights from Genetics Reveal the Leptin-Melanocortin Signaling Pathway as a Common Player.* Critical Reviews in Eukaryotic Gene Expression, 2012. **22**(4): p. 325-343.
11. Hinney, A., A.L. Volckmar, and N. Knoll, *Melanocortin-4 receptor in energy homeostasis and obesity pathogenesis.* Prog Mol Biol Transl Sci, 2013. **114**: p. 147-91.
12. Mutch, D.M. and K. Clement, *Unraveling the genetics of human obesity.* PLoS Genet, 2006. **2**(12): p. e188.
13. Ichihara, S. and Y. Yamada, *Genetic factors for human obesity.* Cell Mol Life Sci, 2008. **65**(7-8): p. 1086-98.
14. Chung, W.K., *An overview of mongenic and syndromic obesities in humans.* Pediatr Blood Cancer, 2012. **58**(1): p. 122-8.
15. Albuquerque, D., et al., *Current review of genetics of human obesity: from molecular mechanisms to an evolutionary perspective.* Mol Genet Genomics, 2015. **290**(4): p. 1191-221.
16. He, X. and J. Zhang, *Toward a molecular understanding of pleiotropy.* Genetics, 2006. **173**(4): p. 1885-91.

17. Strachan, T.a.R., A., *Human Molecular Genetics 4th edition*. 2011, New York: Garland Science, Taylor & Francis Group.
18. Marshall, J.D., et al., *Alstrom Syndrome*, in *GeneReviews(R)*, R.A. Pagon, et al., Editors. 1993: Seattle (WA).
19. Marshall, J.D., et al., *New Alstrom syndrome phenotypes based on the evaluation of 182 cases.* Arch Intern Med, 2005. **165**(6): p. 675-83.
20. *OMIM: Alstrom syndrome; ALMS*. [cited 2016 October]; Available from: http://www.omim.org/entry/203800.
21. *Orpha.net: Alström Syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?Lng=GB&Expert=64.
22. Forsythe, E. and P.L. Beales, *Bardet-Biedl Syndrome*, in *GeneReviews(R)*, R.A. Pagon, et al., Editors. 1993: Seattle (WA).
23. Gunay-Aygun, M., S.B. Cassidy, and R.D. Nicholls, *Prader-Willi and other syndromes associated with obesity and mental retardation.* Behav Genet, 1997. **27**(4): p. 307-24.
24. Braun, J.J., et al., *Olfaction evaluation and correlation with brain atrophy in Bardet-Biedl syndrome.* Clin Genet, 2014. **86**(6): p. 521-9.
25. Khan, S.A., et al., *Genetics of human Bardet-Biedl syndrome, an updates.* Clin Genet, 2016. **90**(1): p. 3-15.
26. Suspitsin, E.N. and E.N. Imyanitov, *Bardet-Biedl Syndrome.* Mol Syndromol, 2016. **7**(2): p. 62-71.
27. *OMIM: Bardet-Biedl Syndrome 1; BBS1*. [cited 2017 March]; Available from: https://omim.org/entry/209900.
28. *Orpha.net: Bardet-Biedl Syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?Expert=110.
29. Wang, Y., et al., *FBN3 gene involved in pathogenesis of a Chinese family with Bardet-Biedl syndrome.* Oncotarget, 2017. **8**(49): p. 86718-86725.
30. Yildiz Bolukbasi, E., et al., *Homozygous mutation in CEP19, a gene mutated in morbid obesity, in Bardet-Biedl syndrome with predominant postaxial polydactyly.* J Med Genet, 2017.
31. Riise, R., *The cause of death in Laurence-Moon-Bardet-Biedl syndrome.* Acta Ophthalmol Scand Suppl, 1996(219): p. 45-7.
32. Jahani-Asl, A., et al., *Pathogenesis of Borjeson-Forssman-Lehmann syndrome: Insights from PHF6 function.* Neurobiol Dis, 2016. **96**: p. 227-235.
33. *OMIM: Borjeson-Forssman-Lehmann Syndrome; BFLS*. [cited 2017 March].
34. *Orpha.net: Borjeson-Forssman-Lehmann syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?Lng=GB&Expert=127.
35. Twigg, S.R., et al., *Mutations in multidomain protein MEGF8 identify a Carpenter syndrome subtype associated with defective lateralization.* Am J Hum Genet, 2012. **91**(5): p. 897-905.
36. Kadakia, S., et al., *Carpenter syndrome: a review for the craniofacial surgeon.* J Craniofac Surg, 2014. **25**(5): p. 1653-7.

37. *OMIM: Carpenter Syndrome 1; CRPT1*. [cited 2017 March]; Available from: https://omim.org/entry/201000.
38. *OMIM: Carpenter Syndrome 2; CRPT2*. [cited 2017 March]; Available from: https://omim.org/entry/614976.
39. *Orpha.net: Carpenter syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?Lng=GB&Expert=65759.
40. Ayazi, S., *Choroideremia, obesity, and congenital deafness.* Am J Ophthalmol, 1981. **92**(1): p. 63-9.
41. Merry, D.E., et al., *Choroideremia and deafness with stapes fixation: a contiguous gene deletion syndrome in Xq21.* Am J Hum Genet, 1989. **45**(4): p. 530-40.
42. *OMIM: Choroidemia, Deafness and mental retardation*. [cited 2017 March]; Available from: https://omim.org/entry/303110.
43. *Orpha.net: Choroideremia-deafness-obesity syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?lng=EN&Expert=1435.
44. Pereira, P.M., et al., *Coffin-Lowry syndrome.* Eur J Hum Genet, 2010. **18**(6): p. 627-33.
45. Hunter, A.G., *Coffin-Lowry syndrome: a 20-year follow-up and review of long-term outcomes.* Am J Med Genet, 2002. **111**(4): p. 345-55.
46. *OMIM: Coffin-Lowry Syndrome; CLS*. [cited 2017 March]; Available from: https://omim.org/entry/303600.
47. *Orpha.net: Coffin-Lowry syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?Expert=192.
48. Izumi, K., et al., *Germline gain-of-function mutations in AFF4 cause a developmental syndrome functionally linking the super elongation complex and cohesin.* Nat Genet, 2015. **47**(4): p. 338-44.
49. *OMIM: CHOPS Syndrome; CHOPS*. [cited 2017 March]; Available from: https://omim.org/entry/616368.
50. *Orpha.net: Cognitive impairment-coarse facies-heart defects-obesity-pulmonary involvement-short stature-skeletal dysplasia syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?lng=EN&Expert=444077.
51. Duplomb, L., et al., *Cohen syndrome is associated with major glycosylation defects.* Hum Mol Genet, 2014. **23**(9): p. 2391-9.
52. Norio, R., *Finnish Disease Heritage I: characteristics, causes, background.* Hum Genet, 2003. **112**(5-6): p. 441-56.
53. *OMIM: Cohen Syndrome; COH1*. [cited 2017 March]; Available from: https://omim.org/entry/216550.
54. *Orpha.net: Cohen syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?Expert=193.
55. Verloes, A., et al., *Coloboma, mental retardation, hypogonadism, and obesity: critical review of the so-called Biemond syndrome type 2, updated nosology,*

and delineation of three "new" syndromes. Am J Med Genet, 1997. **69**(4): p. 370-9.
56. *Orpha.net: Colobomatous microphthalmia-obesity-hypogenitalism-intellectual disability syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?lng=EN&Expert=363741.
57. Bachmann-Gagescu, R., et al., *Recurrent 200-kb deletions of 16p11.2 that include the SH2B1 gene are associated with developmental delay and obesity.* Genet Med, 2010. **12**(10): p. 641-7.
58. Bochukova, E.G., et al., *Large, rare chromosomal deletions associated with severe early-onset obesity.* Nature, 2010. **463**(7281): p. 666-70.
59. Thorleifsson, G., et al., *Genome-wide association yields new sequence variants at seven loci that associate with measures of obesity.* Nat Genet, 2009. **41**(1): p. 18-24.
60. *OMIM: CHROMOSOME 16p11.2 DELETION SYNDROME, 220-KB*. [cited 2016 October]; Available from: http://omim.org/entry/613444.
61. *Orpha.net: Distal 16p11.2 microdeletion syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?lng=EN&Expert=261222.
62. Walters, R.G., et al., *A new highly penetrant form of obesity due to deletions on chromosome 16p11.2.* Nature, 2010. **463**(7281): p. 671-5.
63. Raspa, M., et al., *Obesity, food selectivity, and physical activity in individuals with fragile X syndrome.* Am J Intellect Dev Disabil, 2010. **115**(6): p. 482-95.
64. McLennan, Y., et al., *Fragile x syndrome.* Curr Genomics, 2011. **12**(3): p. 216-24.
65. Bagni, C., et al., *Fragile X syndrome: causes, diagnosis, mechanisms, and therapeutics.* J Clin Invest, 2012. **122**(12): p. 4314-22.
66. Braat, S. and R.F. Kooy, *Insights into GABA(A)ergic system deficits in fragile X syndrome lead to clinical trials.* Neuropharmacology, 2015. **88**: p. 48-54.
67. *OMIM: Fragile X mental retardation syndrome*. [cited 2017 March]; Available from: https://omim.org/entry/300624.
68. *Orpha.net: Fragile X syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?Expert=908.
69. *Orpha.net: Hydrocephalus-obesity-hypogonadism syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?lng=en&Expert=2183.
70. Marangi, G., et al., *TRAPPC9-related autosomal recessive intellectual disability: report of a new mutation and clinical phenotype.* Eur J Hum Genet, 2013. **21**(2): p. 229-32.
71. *Orpha.net: Intellectual disability-obesity-brain malformations-facial dysmorphism syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?lng=EN&Expert=352530.
72. Kantaputra, P.N., J. Kunachaichote, and P. Patikulsila, *Mental retardation, obesity, mandibular prognathism with eye and skin anomalies (MOMES*

syndrome): a newly recognized autosomal recessive syndrome. Am J Med Genet, 2001. **103**(4): p. 283-8.

73. van Haelst, M.M., et al., *Obesity syndrome, MOMES caused by deletion-duplication (4q35.1 del and 5p14.3 dup).* Am J Med Genet A, 2009. **149A**(4): p. 833-4.
74. *OMIM: Mental retardation, obesity, mandibular prognathism, and eye and skin anomalies*. [cited 2017 March]; Available from: https://omim.org/entry/606772.
75. *Orpha.net: Intellectual disability-obesity-prognathism-eye and skin anomalies syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/Disease_Search.php?lng=EN&data_id=22715.
76. Segel, R., et al., *The natural history of trisomy 12p.* Am J Med Genet A, 2006. **140**(7): p. 695-703.
77. Ou, Z., et al., *Observation and prediction of recurrent human translocations mediated by NAHR between nonhomologous chromosomes.* Genome Res, 2011. **21**(1): p. 33-46.
78. Margari, L., et al., *Molecular cytogenetic characterization and genotype/phenotype analysis in a patient with a de novo 8p23.2p23.3 deletion/12p13.31p13.33 duplication.* Am J Med Genet A, 2012. **158A**(7): p. 1713-8.
79. Goldlust, I.S., et al., *Mouse model implicates GNB3 duplication in a childhood obesity syndrome.* Proc Natl Acad Sci U S A, 2013. **110**(37): p. 14990-4.
80. D'Angelo, C.S., et al., *Two New Cases of 1p21.3 Deletions and an Unbalanced Translocation t(8;12) among Individuals with Syndromic Obesity.* Mol Syndromol, 2015. **6**(2): p. 63-70.
81. *Orpha.net: Intellectual disability-seizures-macrocephaly-obesity syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?lng=en&Expert=369950.
82. Steinmuller, R., D. Steinberger, and U. Muller, *MEHMO (mental retardation, epileptic seizures, hypogonadism and -genitalism, microcephaly, obesity), a novel syndrome: assignment of disease locus to xp21.1-p22.13.* Eur J Hum Genet, 1998. **6**(3): p. 201-6.
83. DeLozier-Blanchet, C.D., C.A. Haenggeli, and A. Bottani, *MEHMO, a novel syndrome: assignment of disease locus to Xp21.1-p22.13. Mental retardation, epileptic seizures, hypogonadism and genitalism, microcephaly, obesity.* Eur J Hum Genet, 1999. **7**(6): p. 621-2.
84. Leshinsky-Silver, E., et al., *MEHMO (Mental retardation, Epileptic seizures, Hypogenitalism, Microcephaly, Obesity): a new X-linked mitochondrial disorder.* Eur J Hum Genet, 2002. **10**(4): p. 226-30.
85. *OMIM: Mental retardation, epileptic seizures, hypogonadism and hypogenitalism, microcephaly and obesity; MEHMO*. [cited 2017 March]; Available from: https://omim.org/entry/300148.

86. *Orpha.net: MEHMO syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/Disease_Search.php?lng=EN&data_id=11672.
87. Skopkova, M., et al., *EIF2S3 Mutations Associated with Severe X-Linked Intellectual Disability Syndrome MEHMO.* Hum Mutat, 2017. **38**(4): p. 409-425.
88. Majewski, F. and T.O. Goecke, *Microcephalic osteodysplastic primordial dwarfism type II: report of three cases and review.* Am J Med Genet, 1998. **80**(1): p. 25-31.
89. Hall, J.G., et al., *Majewski osteodysplastic primordial dwarfism type II (MOPD II): natural history and clinical findings.* Am J Med Genet A, 2004. **130A**(1): p. 55-72.
90. *OMIM: Microcephalic, osteodysplastic primordial dwarfism, type II; MOPD2*. [cited 2017 March]; Available from: https://omim.org/entry/210720.
91. *Orpha.net: Microcephalic osteodysplastic primordial dwarfism type II*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?Lng=EN&Expert=2637.
92. Moretti-Ferreira, D., et al., *Macrosomia, obesity, macrocephaly and ocular abnormalities (MOMO syndrome) in two unrelated patients: delineation of a newly recognized overgrowth syndrome.* Am J Med Genet, 1993. **46**(5): p. 555-8.
93. Zannolli, R., et al., *MOMO syndrome: a possible third case.* Clin Dysmorphol, 2000. **9**(4): p. 281-4.
94. Giunco, C.T., et al., *MOMO syndrome associated with autism: a case report.* Genet Mol Res, 2008. **7**(4): p. 1223-5.
95. Wallerstein, R. and R.D. Sugalski, *A new case of MOMO syndrome.* Clin Dysmorphol, 2010. **19**(1): p. 1-4.
96. *OMIM: MOMO Syndrome*. [cited 2017 March]; Available from: https://omim.org/entry/157980.
97. *Orpha.net: MOMO syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/Disease_Search.php?lng=EN&data_id=2364.
98. Vu, P.Y., et al., *A homozygous balanced reciprocal translocation suggests LINC00237 as a candidate gene for MOMO (macrosomia, obesity, macrocephaly, and ocular abnormalities) syndrome.* Am J Med Genet A, 2012. **158A**(11): p. 2849-56.
99. Hampshire, D.J., et al., *MORM syndrome (mental retardation, truncal obesity, retinal dystrophy and micropenis), a new autosomal recessive disorder, links to 9q34.* Eur J Hum Genet, 2006. **14**(5): p. 543-8.
100. *OMIM: Mental retardation, truncal obesity, retinal dystrophy, and micropenis syndrome; MORMS*. [cited 2017 March]; Available from: https://omim.org/entry/610156.
101. *Orpha.net: MORM syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?lng=EN&Expert=75858.

102. Driscoll, D.J., et al., *Prader-Willi Syndrome*, in *GeneReviews(R)*, M.P. Adam, et al., Editors. 1993: Seattle (WA).
103. Gunay-Aygun, M., et al., *The changing purpose of Prader-Willi syndrome clinical diagnostic criteria and proposed revised criteria.* Pediatrics, 2001. **108**(5): p. E92.
104. Buiting, K., *Prader-Willi syndrome and Angelman syndrome.* Am J Med Genet C Semin Med Genet, 2010. **154C**(3): p. 365-76.
105. Elena, G., et al., *Prader-willi syndrome: clinical aspects.* J Obes, 2012. **2012**: p. 473941.
106. Angulo, M.A., M.G. Butler, and M.E. Cataletto, *Prader-Willi syndrome: a review of clinical, genetic, and endocrine findings.* J Endocrinol Invest, 2015. **38**(12): p. 1249-63.
107. *OMIM: Prader-Willi Syndrome; PWS*. [cited 2017 March]; Available from: https://omim.org/entry/176270.
108. *Orpha.net: Prader-Willi syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?Expert=739.
109. Faivre, L., et al., *Deletion of the SIM1 gene (6q 16.2) in a patient with a Prader-Willi-like phenotype.* Journal of Medical Genetics, 2002. **39**(8): p. 594-596.
110. Bonaglia, M.C., et al., *Detailed phenotype-genotype study in five patients with chromosome 6q16 deletion: narrowing the critical region for Prader-Willi-like phenotype.* Eur J Hum Genet, 2008. **16**(12): p. 1443-9.
111. Rosenfeld, J.A., et al., *Speech delays and behavioral problems are the predominant features in individuals with developmental delays and 16p11.2 microdeletions and microduplications.* J Neurodev Disord, 2010. **2**(1): p. 26-38.
112. *Orpha.net: Prader-Willi-like syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?Lng=EN&Expert=398073.
113. Fitch, N., *Albright's hereditary osteodystrophy: a review.* Am J Med Genet, 1982. **11**(1): p. 11-29.
114. Wilson, L.C. and R.C. Trembath, *Albright's hereditary osteodystrophy.* J Med Genet, 1994. **31**(10): p. 779-84.
115. Simon, A., et al., *Pseudohypoparathyroidism type Ia. Albright hereditary osteodystrophy: a model for research on G protein-coupled receptors and genomic imprinting.* Neth J Med, 2000. **56**(3): p. 100-9.
116. Mantovani, G., *Clinical review: Pseudohypoparathyroidism: diagnosis and treatment.* J Clin Endocrinol Metab, 2011. **96**(10): p. 3020-30.
117. Elli, F.M., et al., *Quantitative analysis of methylation defects and correlation with clinical characteristics in patients with pseudohypoparathyroidism type I and GNAS epigenetic alterations.* J Clin Endocrinol Metab, 2014. **99**(3): p. E508-17.
118. Kottler M-L., L.A., Carel J-C. *Albright Hereditary osteodystrophy. Orphanet encyclopedia. January 2004*. Available from: https://www.orpha.net/data/patho/GB/uk-AHO.pdf.

119. *OMIM: Pseudohypoparathyroidism, Type Ia; PHP1A*. [cited 2017 March]; Available from: https://omim.org/entry/103580.
120. *Orpha.net: Pseudohypoparathyroidism with Albright hereditary osteodystrophy*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?lng=EN&Expert=457059.
121. Suzuki, E., et al., *A de novo 50-bp GNAS Intragenic Duplication in a Patient with Pseudohypoparathyroidism Type 1a.* Cytogenet Genome Res, 2018.
122. Petrij, F., et al., *Rubinstein-Taybi syndrome caused by mutations in the transcriptional co-activator CBP.* Nature, 1995. **376**(6538): p. 348-51.
123. Roelfsema, J.H., et al., *Genetic heterogeneity in Rubinstein-Taybi syndrome: mutations in both the CBP and EP300 genes cause disease.* Am J Hum Genet, 2005. **76**(4): p. 572-80.
124. Stevens, C.A., *Rubinstein-Taybi Syndrome*, in *GeneReviews(R)*, R.A. Pagon, et al., Editors. 1993: Seattle (WA).
125. Spena, S., C. Gervasini, and D. Milani, *Ultra-Rare Syndromes: The Example of Rubinstein-Taybi Syndrome.* J Pediatr Genet, 2015. **4**(3): p. 177-86.
126. *OMIM: Rubinstein-Taybi Syndrome 1; RSTS1*. [cited 2017 March]; Available from: https://omim.org/entry/180849.
127. *OMIM: Rubinstein-Taybi Syndrome 2; RSTS2*. [cited 2017 March]; Available from: https://omim.org/entry/613684.
128. *Orpha.net: Rubinstein-Taybi syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?Expert=783.
129. Akawi, N., et al., *Discovery of four recessive developmental disorders using probabilistic genotype and phenotype matching among 4,125 families.* Nat Genet, 2015. **47**(11): p. 1363-9.
130. Jeong, H.J., et al., *Prmt7 Deficiency Causes Reduced Skeletal Muscle Oxidative Metabolism and Age-Related Obesity.* Diabetes, 2016. **65**(7): p. 1868-82.
131. *Orpha.net: Short stature-brachydactyly-obesity-global developmental delay syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?lng=NL&Expert=464288.
132. van de Laar, I., et al., *Diploid/triploid mosaicism in dysmorphic patients.* Clin Genet, 2002. **62**(5): p. 376-82.
133. Rittinger, O., et al., *The changing phenotype in diploid/triploid mosaicism may mimic genetic syndromes with aberrant genomic imprinting: follow up in a 14-year-old girl.* Eur J Med Genet, 2008. **51**(6): p. 573-9.
134. Boonen, S.E., et al., *Diploid/triploid mosaicism: a rare event or an under-diagnosed syndrome?* Eur J Med Genet, 2011. **54**(3): p. 374-5.
135. Jewell, R., et al., *Phenotypic features of diploid/triploid mosaicism in an adult.* Clin Dysmorphol, 2014. **23**(2): p. 56-9.
136. *Unique: Understanding chromosome disorders: Diploid Triploid Mosaicism*. [cited 2017 March]; Available from: http://www.rarechromo.org/information/Other/Diploid%20Triploid%20Mosaicism%20FTNW.pdf.

137. *Rarediseases.org: Triploidy*. [cited 2017 March]; Available from: https://rarediseases.org/rare-diseases/triploidy/.
138. *Orpha.net: Triploidy*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?lng=en&Expert=3376.
139. Marlin, S., et al., *Obesity: a new feature of WAGR (del 11p) syndrome.* Clin Dysmorphol, 1994. **3**(3): p. 255-7.
140. Tiberio, G., M.C. Digilio, and A. Giannotti, *Obesity and WAGR syndrome.* Clin Dysmorphol, 2000. **9**(1): p. 63-4.
141. Amor, D.J., *Morbid obesity and hyperphagia in the WAGR syndrome.* Clin Dysmorphol, 2002. **11**(1): p. 73-4.
142. Gul, D., et al., *Third case of WAGR syndrome with severe obesity and constitutional deletion of chromosome (11)(p12p14).* Am J Med Genet, 2002. **107**(1): p. 70-1.
143. Fischbach, B.V., et al., *WAGR syndrome: a clinical review of 54 cases.* Pediatrics, 2005. **116**(4): p. 984-8.
144. Jung, R., et al., *Clinical, cytogenetic and molecular characterization of a patient with combined succinic semialdehyde dehydrogenase deficiency and incomplete WAGR syndrome with obesity.* Mol Genet Metab, 2006. **88**(3): p. 256-60.
145. Han, J.C., et al., *Brain-derived neurotrophic factor and obesity in the WAGR syndrome.* N Engl J Med, 2008. **359**(9): p. 918-27.
146. *OMIM: Wilms tumor, aniridia, genitourinary anomalies and mental retardation syndrome; WAGR*. [cited 2017 March]; Available from: https://omim.org/entry/194072.
147. *Orpha.net: WAGR syndrome*. [cited 2016 October]; Available from: http://www.orpha.net/consor/cgi-bin/Disease_Search_Simple.php?lng=EN&diseaseGroup=WAGR.
148. Justice, E.D., S.J. Barnum, and T. Kidd, *The WAGR syndrome gene PRRG4 is a functional homologue of the commissureless axon guidance gene.* PLoS Genet, 2017. **13**(8): p. e1006865.
149. Wilson, M., et al., *New X-linked syndrome of mental retardation, gynecomastia, and obesity is linked to DXS255.* Am J Med Genet, 1991. **40**(4): p. 406-13.
150. Hu, H., et al., *X-exome sequencing of 405 unresolved families identifies seven novel intellectual disability genes.* Mol Psychiatry, 2016. **21**(1): p. 133-48.
151. *OMIM: Wilson-Turner X-linked mental retardation syndrome; WTS*. [cited 2017 March]; Available from: https://omim.org/entry/309585.
152. *Orpha.net: Wilson-Turner syndrome*. [cited 2017 March]; Available from: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?Lng=GB&Expert=3459.
153. Holm, V.A., et al., *Prader-Willi syndrome: consensus diagnostic criteria.* Pediatrics, 1993. **91**(2): p. 398-402.
154. Butler, M.G., et al., *Array comparative genomic hybridization (aCGH) analysis in Prader-Willi syndrome.* Am J Med Genet A, 2008. **146A**(7): p. 854-60.

155. Cheon, C.K., *Genetics of Prader-Willi syndrome and Prader-Will-Like syndrome.* Ann Pediatr Endocrinol Metab, 2016. **21**(3): p. 126-135.
156. Cassidy, S.B., et al., *Prader-Willi syndrome.* Genet Med, 2012. **14**(1): p. 10-26.
157. Izumi, K., et al., *Endocrine phenotype of 6q16.1-q21 deletion involving SIM1 and Prader-Willi syndrome-like features.* Am J Med Genet A, 2013. **161A**(12): p. 3137-43.
158. Ohta, T., et al., *Imprinting-mutation mechanisms in Prader-Willi syndrome.* Am J Hum Genet, 1999. **64**(2): p. 397-413.
159. Clayton-Smith, J., et al., *Difference in methylation patterns within the D15S9 region of chromosome 15q11-13 in first cousins with Angelman syndrome and Prader-Willi syndrome.* Am J Med Genet, 1993. **47**(5): p. 683-6.
160. Jiang, Y., et al., *Genetics of Angelman syndrome.* Am J Hum Genet, 1999. **65**(1): p. 1-6.
161. Glenn, C.C., et al., *Genomic imprinting: potential function and mechanisms revealed by the Prader-Willi and Angelman syndromes.* Mol Hum Reprod, 1997. **3**(4): p. 321-32.
162. Angelman, H., *'Puppet' children. A report of three cases. .* Dev Med Child Neurol 1965. **7**: p. 681–688.
163. Williams, C.A., D.J. Driscoll, and A.I. Dagli, *Clinical and genetic aspects of Angelman syndrome.* Genet Med, 2010. **12**(7): p. 385-95.
164. Schulze, A., et al., *Exclusion of SNRPN as a major determinant of Prader-Willi syndrome by a translocation breakpoint.* Nat Genet, 1996. **12**(4): p. 452-4.
165. Wirth, J., et al., *A translocation breakpoint cluster disrupts the newly defined 3' end of the SNURF-SNRPN transcription unit on chromosome 15.* Hum Mol Genet, 2001. **10**(3): p. 201-10.
166. Duker, A.L., et al., *Paternally inherited microdeletion at 15q11.2 confirms a significant role for the SNORD116 C/D box snoRNA cluster in Prader-Willi syndrome.* Eur J Hum Genet, 2010. **18**(11): p. 1196-201.
167. Runte, M., et al., *The IC-SNURF-SNRPN transcript serves as a host for multiple small nucleolar RNA species and as an antisense RNA for UBE3A.* Hum Mol Genet, 2001. **10**(23): p. 2687-700.
168. Sahoo, T., et al., *Prader-Willi phenotype caused by paternal deficiency for the HBII-85 C/D box small nucleolar RNA cluster.* Nat Genet, 2008. **40**(6): p. 719-21.
169. Burger, J., et al., *Familial interstitial 570 kbp deletion of the UBE3A gene region causing Angelman syndrome but not Prader-Willi syndrome.* Am J Med Genet, 2002. **111**(3): p. 233-7.
170. Runte, M., et al., *Exclusion of the C/D box snoRNA gene cluster HBII-52 from a major role in Prader-Willi syndrome.* Hum Genet, 2005. **116**(3): p. 228-30.
171. de Smith, A.J., et al., *A deletion of the HBII-85 class of small nucleolar RNAs (snoRNAs) is associated with hyperphagia, obesity and hypogonadism.* Hum Mol Genet, 2009. **18**(17): p. 3257-65.
172. Bieth, E., et al., *Highly restricted deletion of the SNORD116 region is implicated in Prader-Willi Syndrome.* Eur J Hum Genet, 2015. **23**(2): p. 252-5.

173. Skryabin, B.V., et al., *Deletion of the MBII-85 snoRNA gene cluster in mice results in postnatal growth retardation.* PLoS Genet, 2007. **3**(12): p. e235.
174. Ding, F., et al., *SnoRNA Snord116 (Pwcr1/MBII-85) deletion causes growth deficiency and hyperphagia in mice.* PLoS One, 2008. **3**(3): p. e1709.
175. Lassi, G., et al., *Deletion of the Snord116/SNORD116 Alters Sleep in Mice and Patients with Prader-Willi Syndrome.* Sleep, 2016. **39**(3): p. 637-44.
176. Qi, Y., et al., *Snord116 is critical in the regulation of food intake and body weight.* Sci Rep, 2016. **6**: p. 18614.
177. Irizarry, K.A., et al., *Prader Willi Syndrome: Genetics, Metabolomics, Hormonal Function, and New Approaches to Therapy.* Adv Pediatr, 2016. **63**(1): p. 47-77.
178. Myers, S.E., et al., *Sustained benefit after 2 years of growth hormone on body composition, fat utilization, physical strength and agility, and growth in Prader-Willi syndrome.* J Pediatr, 2000. **137**(1): p. 42-9.
179. Burman, P., E.M. Ritzen, and A.C. Lindgren, *Endocrine dysfunction in Prader-Willi syndrome: a review with special reference to GH.* Endocr Rev, 2001. **22**(6): p. 787-99.
180. Senda, M., et al., *The glucagon-like peptide-1 analog liraglutide suppresses ghrelin and controls diabetes in a patient with Prader-Willi syndrome.* Endocr J, 2012. **59**(10): p. 889-94.
181. Cyganek, K., et al., *Liraglutide therapy in Prader-Willi syndrome.* Diabet Med, 2011. **28**(6): p. 755-6.
182. Fintini, D., et al., *Use of GLP-1 receptor agonists in Prader-Willi Syndrome: report of six cases.* Diabetes Care, 2014. **37**(4): p. e76-7.
183. De Waele, K., et al., *Long-acting octreotide treatment causes a sustained decrease in ghrelin concentrations but does not affect weight, behaviour and appetite in subjects with Prader-Willi syndrome.* Eur J Endocrinol, 2008. **159**(4): p. 381-8.
184. Einfeld, S.L., et al., *A double-blind randomized controlled trial of oxytocin nasal spray in Prader Willi syndrome.* Am J Med Genet A, 2014. **164A**(9): p. 2232-9.
185. Kuppens, R.J., S.H. Donze, and A.C. Hokken-Koelega, *Promising effects of oxytocin on social and food-related behaviour in young children with Prader-Willi syndrome: a randomized, double-blind, controlled crossover trial.* Clin Endocrinol (Oxf), 2016.
186. *ClinicalTrials.gov: A service of the U.S. National Institutes of Health*. [cited 2016 October]; Available from: https://clinicaltrials.gov.
187. Shapira, N.A., et al., *Satiety dysfunction in Prader-Willi syndrome demonstrated by fMRI.* J Neurol Neurosurg Psychiatry, 2005. **76**(2): p. 260-2.
188. Butler, M.G., et al., *Energy expenditure and physical activity in Prader-Willi syndrome: comparison with obese subjects.* Am J Med Genet A, 2007. **143A**(5): p. 449-59.

189. Alsaif, M., et al., *Energy Metabolism Profile in Individuals with Prader-Willi Syndrome and Implications for Clinical Management: A Systematic Review.* Adv Nutr, 2017. **8**(6): p. 905-915.
190. Papavramidis, S.T., E.V. Kotidis, and O. Gamvros, *Prader-Willi syndrome-associated obesity treated by biliopancreatic diversion with duodenal switch. Case report and literature review.* J Pediatr Surg, 2006. **41**(6): p. 1153-8.
191. Scheimann, A.O., et al., *Critical analysis of bariatric procedures in Prader-Willi syndrome.* J Pediatr Gastroenterol Nutr, 2008. **46**(1): p. 80-3.
192. Fonkalsrud, E.W. and G. Bray, *Vagotomy for treatment of obesity in childhood due to Prader-Willi syndrome.* J Pediatr Surg, 1981. **16**(6): p. 888-9.
193. Hellings, J.A. and J.K. Warnock, *Self-injurious behavior and serotonin in Prader-Willi syndrome.* Psychopharmacol Bull, 1994. **30**(2): p. 245-50.
194. Kim, Y., et al., *Targeting the histone methyltransferase G9a activates imprinted genes and improves survival of a mouse model of Prader-Willi syndrome.* Nat Med, 2017. **23**(2): p. 213-222.
195. Spikol, E.D., et al., *Zebrafish Models of Prader-Willi Syndrome: Fast Track to Pharmacotherapeutics.* Diseases, 2016. **4**(1).
196. Faivre, L., et al., *Deletion of the SIM1 gene (6q16.2) in a patient with a Prader-Willi-like phenotype.* J Med Genet, 2002. **39**(8): p. 594-6.
197. Rosenfeld, J.A., et al., *Genotype-phenotype correlation in interstitial 6q deletions: a report of 12 new cases.* Neurogenetics, 2012. **13**(1): p. 31-47.
198. Tsuyusaki, Y., et al., *1p36 deletion syndrome associated with Prader-Willi-like phenotype.* Pediatr Int, 2010. **52**(4): p. 547-50.
199. Martens, K., et al., *PREPL: a putative novel oligopeptidase propelled into the limelight.* Biol Chem, 2006. **387**(7): p. 879-83.
200. D'Angelo, C.S., et al., *Obesity with associated developmental delay and/or learning disability in patients exhibiting additional features: report of novel pathogenic copy number variants.* Am J Med Genet A, 2013. **161A**(3): p. 479-86.
201. Ben-Abdallah-Bouhjar, I., et al., *Chromosomal microarray analysis of functional Xq27-qter disomy and deletion 3p26.3 in a boy with Prader-Willi like features and hypotonia.* Eur J Med Genet, 2012. **55**(8-9): p. 461-5.
202. Turleau, C., et al., *6q1 monosomy: a distinctive syndrome.* Clin Genet, 1988. **34**(1): p. 38-42.
203. Villa, A., et al., *De novo interstitial deletion q16.2q21 on chromosome 6.* Am J Med Genet, 1995. **55**(3): p. 379-83.
204. Gilhuis, H.J., et al., *Interstitial 6q deletion with a Prader-Willi-like phenotype: a new case and review of the literature.* Eur J Paediatr Neurol, 2000. **4**(1): p. 39-43.
205. Varela, M.C., et al., *A new case of interstitial 6q16.2 deletion in a patient with Prader-Willi-like phenotype and investigation of SIM1 gene deletion in 87 patients with syndromic obesity.* Eur J Med Genet, 2006. **49**(4): p. 298-305.
206. Klein, O.D., et al., *Interstitial deletions of chromosome 6q: genotype-phenotype correlation utilizing array CGH.* Clin Genet, 2007. **71**(3): p. 260-6.

207. Wang, J.C., et al., *A 5-Mb microdeletion at 6q16.1-q16.3 with SIM gene deletion and obesity.* Am J Med Genet A, 2008. **146A**(22): p. 2975-8.
208. Spreiz, A., et al., *Phenotypic variability of a deletion and duplication 6q16.1 --> q21 due to a paternal balanced ins(7;6)(p15;q16.1q21).* Am J Med Genet A, 2010. **152A**(11): p. 2762-7.
209. Vignoli, A., et al., *Interstitial 6q microdeletion syndrome and epilepsy: a new patient and review of the literature.* Am J Med Genet A, 2013. **161A**(8): p. 2009-15.
210. El Khattabi, L., et al., *Incomplete penetrance and phenotypic variability of 6q16 deletions including SIM1.* Eur J Hum Genet, 2015. **23**(8): p. 1010-8.
211. Stein, C.K., et al., *Interstitial 6q deletion and Prader-Willi-like phenotype.* Clinical Genetics, 1996. **49**(6): p. 306-310.
212. Le Caignec, C., et al., *Interstitial 6q deletion: clinical and array CGH characterisation of a new patient.* Eur J Med Genet, 2005. **48**(3): p. 339-45.
213. Klein, O.D., et al., *Interstitial deletions of chromosome 6q: genotype-phenotype correlation utilizing array CGH.* Clinical Genetics, 2007. **71**(3): p. 260-266.
214. Cormier-Daire, V., et al., *Cryptic terminal deletion of chromosome 9q34: a novel cause of syndromic obesity in childhood?* J Med Genet, 2003. **40**(4): p. 300-3.
215. Rocha, C.F. and C.L. Paiva, *Prader-Willi-like phenotypes: a systematic review of their chromosomal abnormalities.* Genet Mol Res, 2014. **13**(1): p. 2290-8.
216. Turleau, C., et al., *6q1 Monosomy - a Distinctive Syndrome.* Clinical Genetics, 1988. **34**(1): p. 38-42.
217. Villa, A., et al., *De-Novo Interstitial Deletion Q16.2q21 on Chromosome-6.* American Journal of Medical Genetics, 1995. **55**(3): p. 379-383.
218. Holder, J.L., N.F. Butte, and A.R. Zinn, *Profound obesity associated with a balanced translocation that disrupts the SIM1 gene.* Human Molecular Genetics, 2000. **9**(1): p. 101-108.
219. Bonnefond, A., et al., *Loss-of-function mutations in SIM1 contribute to obesity and Prader-Willi-like features.* J Clin Invest, 2013. **123**(7): p. 3037-41.
220. Ramachandrappa, S., et al., *Rare variants in single-minded 1 (SIM1) are associated with severe obesity.* J Clin Invest, 2013. **123**(7): p. 3042-50.
221. Montagne, L., et al., *Identification of Two Novel Loss-of-Function SIM1 Mutations in Two Overweight Children with Developmental Delay.* Obesity, 2014. **22**(12): p. 2621-2624.
222. Zegers, D., et al., *Mutation screen of the SIM1 gene in pediatric patients with early-onset obesity.* Int J Obes (Lond), 2014. **38**(7): p. 1000-4.
223. Wentzel, C., et al., *Interstitial Deletions at 6q14.1-q15 Associated with Obesity, Developmental Delay and a Distinct Clinical Phenotype.* Mol Syndromol, 2010. **1**(2): p. 75-81.
224. Asai, M., et al., *Loss of function of the melanocortin 2 receptor accessory protein 2 is associated with mammalian obesity.* Science, 2013. **341**(6143): p. 275-8.

225. Geets, E., et al., *Copy number variation (CNV) analysis and mutation analysis of the 6q14.1-6q16.3 genes SIM1 and MRAP2 in Prader Willi like patients.* Mol Genet Metab, 2016. **117**(3): p. 383-8.
226. Kasher, P.R., et al., *Small 6q16.1 Deletions Encompassing POU3F2 Cause Susceptibility to Obesity and Variable Developmental Delay with Intellectual Disability.* Am J Hum Genet, 2016. **98**(2): p. 363-72.
227. Schaaf, C.P., et al., *Truncating mutations of MAGEL2 cause Prader-Willi phenotypes and autism.* Nat Genet, 2013. **45**(11): p. 1405-8.
228. Soden, S.E., et al., *Effectiveness of exome and genome sequencing guided by acuity of illness for diagnosis of neurodevelopmental disorders.* Sci Transl Med, 2014. **6**(265): p. 265ra168.
229. Bischof, J.M., C.L. Stewart, and R. Wevrick, *Inactivation of the mouse Magel2 gene results in growth abnormalities similar to Prader-Willi syndrome.* Hum Mol Genet, 2007. **16**(22): p. 2713-9.
230. Buiting, K., et al., *Clinical phenotypes of MAGEL2 mutations and deletions.* Orphanet J Rare Dis, 2014. **9**: p. 40.
231. Kanber, D., et al., *A paternal deletion of MKRN3, MAGEL2 and NDN does not result in Prader-Willi syndrome.* Eur J Hum Genet, 2009. **17**(5): p. 582-90.
232. M'Hamdi, O., I. Ouertani, and H. Chaabouni-Bouhamed, *Update on the genetics of bardet-biedl syndrome.* Mol Syndromol, 2014. **5**(2): p. 51-6.
233. Klein, D. and F. Ammann, *The syndrome of Laurence-Moon-Bardet-Biedl and allied diseases in Switzerland. Clinical, genetic and epidemiological studies.* J Neurol Sci, 1969. **9**(3): p. 479-513.
234. Beales, P.L., et al., *New criteria for improved diagnosis of Bardet-Biedl syndrome: results of a population survey.* J Med Genet, 1999. **36**(6): p. 437-46.
235. Putoux, A., et al., *Phenotypic variability of Bardet-Biedl syndrome: focusing on the kidney.* Pediatr Nephrol, 2012. **27**(1): p. 7-15.
236. Katsanis, N., *The oligogenic properties of Bardet-Biedl syndrome.* Hum Mol Genet, 2004. **13 Spec No 1**: p. R65-71.
237. Stoetzel, C., et al., *BBS8 is rarely mutated in a cohort of 128 Bardet-Biedl syndrome families.* J Hum Genet, 2006. **51**(1): p. 81-4.
238. Katsanis, N., et al., *Triallelic inheritance in Bardet-Biedl syndrome, a Mendelian recessive disorder.* Science, 2001. **293**(5538): p. 2256-9.
239. Katsanis, N., et al., *BBS4 is a minor contributor to Bardet-Biedl syndrome and may also participate in triallelic inheritance.* Am J Hum Genet, 2002. **71**(1): p. 22-9.
240. Badano, J.L., et al., *Heterozygous mutations in BBS1, BBS2 and BBS6 have a potential epistatic effect on Bardet-Biedl patients with two mutations at a second BBS locus.* Hum Mol Genet, 2003. **12**(14): p. 1651-9.
241. Beales, P.L., et al., *Genetic interaction of BBS1 mutations with alleles at other BBS loci can result in non-Mendelian Bardet-Biedl syndrome.* Am J Hum Genet, 2003. **72**(5): p. 1187-99.

242. Hjortshoj, T.D., et al., *Bardet-Biedl syndrome in Denmark--report of 13 novel sequence variations in six genes.* Hum Mutat, 2010. **31**(4): p. 429-36.
243. Fauser, S., M. Munz, and D. Besch, *Further support for digenic inheritance in Bardet-Biedl syndrome.* J Med Genet, 2003. **40**(8): p. e104.
244. Mykytyn, K., et al., *Evaluation of complex inheritance involving the most common Bardet-Biedl syndrome locus (BBS1).* Am J Hum Genet, 2003. **72**(2): p. 429-37.
245. Hichri, H., et al., *Testing for triallelism: analysis of six BBS genes in a Bardet-Biedl syndrome family cohort.* Eur J Hum Genet, 2005. **13**(5): p. 607-16.
246. Laurier, V., et al., *Pitfalls of homozygosity mapping: an extended consanguineous Bardet-Biedl syndrome family with two mutant genes (BBS2, BBS10), three mutations, but no triallelism.* Eur J Hum Genet, 2006. **14**(11): p. 1195-203.
247. Smaoui, N., et al., *Screening of the eight BBS genes in Tunisian families: no evidence of triallelism.* Invest Ophthalmol Vis Sci, 2006. **47**(8): p. 3487-95.
248. Abu-Safieh, L., et al., *In search of triallelism in Bardet-Biedl syndrome.* Eur J Hum Genet, 2012. **20**(4): p. 420-7.
249. Estrada-Cuzcano, A., et al., *BBS1 mutations in a wide spectrum of phenotypes ranging from nonsyndromic retinitis pigmentosa to Bardet-Biedl syndrome.* Arch Ophthalmol, 2012. **130**(11): p. 1425-32.
250. Billingsley, G., C. Deveault, and E. Heon, *BBS mutational analysis: a strategic approach.* Ophthalmic Genet, 2011. **32**(3): p. 181-7.
251. Janssen, S., et al., *Mutation analysis in Bardet-Biedl syndrome by DNA pooling and massively parallel resequencing in 105 individuals.* Hum Genet, 2011. **129**(1): p. 79-90.
252. Redin, C., et al., *Targeted high-throughput sequencing for diagnosis of genetically heterogeneous diseases: efficient mutation detection in Bardet-Biedl and Alstrom syndromes.* J Med Genet, 2012. **49**(8): p. 502-12.
253. Gonzalez-Del Pozo, M., et al., *Deciphering intrafamilial phenotypic variability by exome sequencing in a Bardet-Biedl family.* Mol Genet Genomic Med, 2014. **2**(2): p. 124-33.
254. Ece Solmaz, A., et al., *Targeted multi-gene panel testing for the diagnosis of Bardet Biedl syndrome: Identification of nine novel mutations across BBS1, BBS2, BBS4, BBS7, BBS9, BBS10 genes.* Eur J Med Genet, 2015. **58**(12): p. 689-94.
255. Daniels, A.B., et al., *Genotype-phenotype correlations in Bardet-Biedl syndrome.* Arch Ophthalmol, 2012. **130**(7): p. 901-7.
256. Dervisoglu, E., et al., *Obesity control and low protein diet preserve or even improve renal functions in Bardet-Biedl syndrome: a report of two cases.* Med Sci Monit, 2011. **17**(1): p. CS12-14.
257. Alstrom, C.H., et al., *Retinal degeneration combined with obesity, diabetes mellitus and neurogenous deafness: a specific syndrome (not hitherto described) distinct from the Laurence-Moon-Bardet-Biedl syndrome: a clinical,*

endocrinological and genetic examination based on a large pedigree. Acta Psychiatr Neurol Scand Suppl, 1959. **129**: p. 1-35.
258. Alvarez-Satta, M., S. Castro-Sanchez, and D. Valverde, *Alstrom syndrome: current perspectives.* Appl Clin Genet, 2015. **8**: p. 171-9.
259. *Orpha.net: Alström syndrome*. [cited 2016 October]; Available from: http://www.orpha.net/consor/cgi-bin/Disease_Search_Simple.php?lng=EN&diseaseGroup=Alstrom.
260. Pereiro, I., et al., *Arrayed primer extension technology simplifies mutation detection in Bardet-Biedl and Alstrom syndrome.* Eur J Hum Genet, 2011. **19**(4): p. 485-8.
261. Aliferis, K., et al., *Differentiating Alstrom from Bardet-Biedl syndrome (BBS) using systematic ciliopathy genes sequencing.* Ophthalmic Genet, 2012. **33**(1): p. 18-22.
262. Ozanturk, A., et al., *The phenotypic and molecular genetic spectrum of Alstrom syndrome in 44 Turkish kindreds and a literature review of Alstrom syndrome in Turkey.* J Hum Genet, 2015. **60**(1): p. 1-9.
263. Marshall, J.D., et al., *Alstrom Syndrome: Mutation Spectrum of ALMS1.* Hum Mutat, 2015. **36**(7): p. 660-8.
264. Sanyoura, M., et al., *A novel ALMS1 splice mutation in a non-obese juvenile-onset insulin-dependent syndromic diabetic patient.* Eur J Hum Genet, 2014. **22**(1): p. 140-3.
265. Bond, J., et al., *The importance of seeking ALMS1 mutations in infants with dilated cardiomyopathy.* J Med Genet, 2005. **42**(2): p. e10.
266. Lee, N.C., et al., *Caloric restriction in Alstrom syndrome prevents hyperinsulinemia.* Am J Med Genet A, 2009. **149A**(4): p. 666-8.
267. Taskesen, M., et al., *Novel Alu retrotransposon insertion leading to Alstrom syndrome.* Hum Genet, 2012. **131**(3): p. 407-13.
268. Hearn, T., et al., *Mutation of ALMS1, a large gene with a tandem repeat encoding 47 amino acids, causes Alstrom syndrome.* Nat Genet, 2002. **31**(1): p. 79-83.
269. Collin, G.B., et al., *Alms1-disrupted mice recapitulate human Alstrom syndrome.* Hum Mol Genet, 2005. **14**(16): p. 2323-33.
270. Jagger, D., et al., *Alstrom Syndrome protein ALMS1 localizes to basal bodies of cochlear hair cells and regulates cilium-dependent planar cell polarity.* Hum Mol Genet, 2011. **20**(3): p. 466-81.
271. Girard, D. and N. Petrovsky, *Alstrom syndrome: insights into the pathogenesis of metabolic disorders.* Nat Rev Endocrinol, 2011. **7**(2): p. 77-88.
272. Lodh, S., et al., *Differential effects on beta-cell mass by disruption of Bardet-Biedl syndrome or Alstrom syndrome genes.* Hum Mol Genet, 2016. **25**(1): p. 57-68.
273. Katagiri, S., et al., *Whole-exome sequencing identifies a novel ALMS1 mutation (p.Q2051X) in two Japanese brothers with Alstrom syndrome.* Mol Vis, 2013. **19**: p. 2393-406.

274. Long, P.A., J.M. Evans, and T.M. Olson, *Exome sequencing establishes diagnosis of Alstrom syndrome in an infant presenting with non-syndromic dilated cardiomyopathy.* Am J Med Genet A, 2015. **167A**(4): p. 886-90.
275. Tai, T.S., S.Y. Lin, and W.H. Sheu, *Metabolic effects of growth hormone therapy in an Alstrom syndrome patient.* Horm Res, 2003. **60**(6): p. 297-301.
276. Goerler, H., et al., *Heart-lung transplantation in a 14-year-old boy with Alstrom syndrome.* J Heart Lung Transplant, 2007. **26**(11): p. 1217-8.
277. Sinha, S.K., et al., *Effect of metformin and rosiglitazone in a prepubertal boy with Alstrom syndrome.* J Pediatr Endocrinol Metab, 2007. **20**(9): p. 1045-52.
278. Miller, R.W., J.F. Fraumeni, Jr., and M.D. Manning, *Association of Wilms's Tumor with Aniridia, Hemihypertrophy and Other Congenital Malformations.* N Engl J Med, 1964. **270**: p. 922-7.
279. Han, J.C., *Rare Syndromes and Common Variants of the Brain-Derived Neurotrophic Factor Gene in Human Obesity.* Prog Mol Biol Transl Sci, 2016. **140**: p. 75-95.
280. Xu, S., et al., *Characterization of 11p14-p12 deletion in WAGR syndrome by array CGH for identifying genes contributing to mental retardation and autism.* Cytogenet Genome Res, 2008. **122**(2): p. 181-7.
281. Glaser, T., et al., *PAX6 gene dosage effect in a family with congenital cataracts, aniridia, anophthalmia and central nervous system defects.* Nat Genet, 1994. **7**(4): p. 463-71.
282. Yasuda, T., et al., *PAX6 mutation as a genetic factor common to aniridia and glucose intolerance.* Diabetes, 2002. **51**(1): p. 224-30.
283. Mitchell, T.N., et al., *Polymicrogyria and absence of pineal gland due to PAX6 mutation.* Ann Neurol, 2003. **53**(5): p. 658-63.
284. Talamillo, A., et al., *Pax6 regulates regional development and neuronal migration in the cerebral cortex.* Dev Biol, 2003. **255**(1): p. 151-63.
285. Barde, Y.A., *The nerve growth factor family.* Prog Growth Factor Res, 1990. **2**(4): p. 237-48.
286. McAllister, A.K., *Neurotrophins and neuronal differentiation in the central nervous system.* Cell Mol Life Sci, 2001. **58**(8): p. 1054-60.
287. Lapchak, P.A. and F. Hefti, *BDNF and NGF treatment in lesioned rats: effects on cholinergic function and weight gain.* Neuroreport, 1992. **3**(5): p. 405-8.
288. Lyons, W.E., et al., *Brain-derived neurotrophic factor-deficient mice develop aggressiveness and hyperphagia in conjunction with brain serotonergic abnormalities.* Proc Natl Acad Sci U S A, 1999. **96**(26): p. 15239-44.
289. Rios, M., et al., *Conditional deletion of brain-derived neurotrophic factor in the postnatal brain leads to obesity and hyperactivity.* Mol Endocrinol, 2001. **15**(10): p. 1748-57.
290. Unger, T.J., et al., *Selective deletion of Bdnf in the ventromedial and dorsomedial hypothalamus of adult mice results in hyperphagic behavior and obesity.* J Neurosci, 2007. **27**(52): p. 14265-74.
291. Xu, B., et al., *Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor.* Nat Neurosci, 2003. **6**(7): p. 736-42.

292. Bariohay, B., et al., *Brain-derived neurotrophic factor/tropomyosin-related kinase receptor type B signaling is a downstream effector of the brainstem melanocortin system in food intake control.* Endocrinology, 2009. **150**(6): p. 2646-53.
293. Han, J.C., et al., *Association of brain-derived neurotrophic factor (BDNF) haploinsufficiency with lower adaptive behaviour and reduced cognitive functioning in WAGR/11p13 deletion syndrome.* Cortex, 2013. **49**(10): p. 2700-10.
294. Clericuzio, C., et al., *Clinical utility gene card for: WAGR syndrome.* Eur J Hum Genet, 2011. **19**(4).
295. Dome, J.S. and V. Huff, *Wilms Tumor Predisposition*, in *GeneReviews(R)*, R.A. Pagon, et al., Editors. 1993: Seattle (WA).
296. Muto, R., et al., *Prediction by FISH analysis of the occurrence of Wilms tumor in aniridia patients.* Am J Med Genet, 2002. **108**(4): p. 285-9.
297. Hingorani, M. and A. Moore, *Aniridia*, in *GeneReviews(R)*, R.A. Pagon, et al., Editors. 1993: Seattle (WA).
298. De Grande, V., et al., *Artificial iris-intraocular lens implantation for traumatic aniridia and aphakia assisted by silicone oil retention sutures.* J Cataract Refract Surg, 2012. **38**(11): p. 2045-8.
299. Weiss, L.A., et al., *Association between microdeletion and microduplication at 16p11.2 and autism.* N Engl J Med, 2008. **358**(7): p. 667-75.
300. Kumar, R.A., et al., *Recurrent 16p11.2 microdeletions in autism.* Hum Mol Genet, 2008. **17**(4): p. 628-38.
301. Marshall, C.R., et al., *Structural variation of chromosomes in autism spectrum disorder.* Am J Hum Genet, 2008. **82**(2): p. 477-88.
302. McCarthy, S.E., et al., *Microduplications of 16p11.2 are associated with schizophrenia.* Nat Genet, 2009. **41**(11): p. 1223-7.
303. Zufferey, F., et al., *A 600 kb deletion syndrome at 16p11.2 leads to energy imbalance and neuropsychiatric disorders.* J Med Genet, 2012. **49**(10): p. 660-8.
304. Shinawi, M., et al., *Recurrent reciprocal 16p11.2 rearrangements associated with global developmental delay, behavioural problems, dysmorphism, epilepsy, and abnormal head size.* J Med Genet, 2010. **47**(5): p. 332-41.
305. Bijlsma, E.K., et al., *Extending the phenotype of recurrent rearrangements of 16p11.2: deletions in mentally retarded patients without autism and in normal individuals.* Eur J Med Genet, 2009. **52**(2-3): p. 77-87.
306. Mullen, S.A., et al., *Copy number variants are frequent in genetic generalized epilepsy with intellectual disability.* Neurology, 2013. **81**(17): p. 1507-14.
307. Steinman, K.J., et al., *16p11.2 deletion and duplication: Characterizing neurologic phenotypes in a large clinically ascertained cohort.* Am J Med Genet A, 2016. **170**(11): p. 2943-2955.
308. Olson, H., et al., *Copy number variation plays an important role in clinical epilepsy.* Ann Neurol, 2014. **75**(6): p. 943-58.

309. D'Angelo, C.S. and C.P. Koiffmann, *Copy number variants in obesity-related syndromes: review and perspectives on novel molecular approaches.* J Obes, 2012. **2012**: p. 845480.
310. Hanson, E., et al., *Cognitive and behavioral characterization of 16p11.2 deletion syndrome.* J Dev Behav Pediatr, 2010. **31**(8): p. 649-57.
311. Ballif, B.C., et al., *Discovery of a previously unrecognized microdeletion syndrome of 16p11.2-p12.2.* Nat Genet, 2007. **39**(9): p. 1071-3.
312. Sampson, M.G., et al., *Evidence for a recurrent microdeletion at chromosome 16p11.2 associated with congenital anomalies of the kidney and urinary tract (CAKUT) and Hirschsprung disease.* Am J Med Genet A, 2010. **152A**(10): p. 2618-22.
313. Barge-Schaapveld, D.Q., et al., *The atypical 16p11.2 deletion: a not so atypical microdeletion syndrome?* Am J Med Genet A, 2011. **155A**(5): p. 1066-72.
314. Miller, D.T., et al., *16p11.2 Recurrent Microdeletion*, in *GeneReviews(R)*, R.A. Pagon, et al., Editors. 1993: Seattle (WA).
315. Aerts, E., et al., *Genetic and structural variation in the SH2B1 gene in the Belgian population.* Mol Genet Metab, 2015. **115**(4): p. 193-8.
316. Rui, L., et al., *Identification of SH2-Bbeta as a substrate of the tyrosine kinase JAK2 involved in growth hormone signaling.* Mol Cell Biol, 1997. **17**(11): p. 6633-44.
317. Duan, C., M. Li, and L. Rui, *SH2-B promotes insulin receptor substrate 1 (IRS1)- and IRS2-mediated activation of the phosphatidylinositol 3-kinase pathway in response to leptin.* J Biol Chem, 2004. **279**(42): p. 43684-91.
318. Li, Z., et al., *SH2B1 enhances leptin signaling by both Janus kinase 2 Tyr813 phosphorylation-dependent and -independent mechanisms.* Mol Endocrinol, 2007. **21**(9): p. 2270-81.
319. Ren, D., et al., *Identification of SH2-B as a key regulator of leptin sensitivity, energy balance, and body weight in mice.* Cell Metab, 2005. **2**(2): p. 95-104.
320. Duan, C., et al., *Disruption of the SH2-B gene causes age-dependent insulin resistance and glucose intolerance.* Mol Cell Biol, 2004. **24**(17): p. 7435-43.
321. Willer, C.J., et al., *Six new loci associated with body mass index highlight a neuronal influence on body weight regulation.* Nat Genet, 2009. **41**(1): p. 25-34.
322. Beckers, S., et al., *Replication of the SH2B1 rs7498665 association with obesity in a Belgian study population.* Obes Facts, 2011. **4**(6): p. 473-7.
323. Doche, M.E., et al., *Human SH2B1 mutations are associated with maladaptive behaviors and obesity.* J Clin Invest, 2012. **122**(12): p. 4732-6.
324. Volckmar, A.L., et al., *Mutation screen in the GWAS derived obesity gene SH2B1 including functional analyses of detected variants.* BMC Med Genomics, 2012. **5**: p. 65.
325. Pearce, L.R., et al., *Functional characterization of obesity-associated variants involving the alpha and beta isoforms of human SH2B1.* Endocrinology, 2014. **155**(9): p. 3219-26.

326. Zheng, Z., et al., *Screening for coding variants in FTO and SH2B1 genes in Chinese patients with obesity.* PLoS One, 2013. **8**(6): p. e67039.
327. Loviglio, M.N., et al., *Chromosomal contacts connect loci associated with autism, BMI and head circumference phenotypes.* Mol Psychiatry, 2016.
328. Myers, S.M., C.P. Johnson, and D. American Academy of Pediatrics Council on Children With, *Management of children with autism spectrum disorders.* Pediatrics, 2007. **120**(5): p. 1162-82.
329. Cassidy, S.B. and D.J. Driscoll, *Prader-Willi syndrome.* Eur J Hum Genet, 2009. **17**(1): p. 3-13.
330. Severi, G., et al., *New patients with Temple syndrome caused by 14q32 deletion: Genotype-phenotype correlations and risk of thyroid cancer.* Am J Med Genet A, 2016. **170A**(1): p. 162-9.
331. Marian, A.J., *Molecular genetic studies of complex phenotypes.* Transl Res, 2012. **159**(2): p. 64-79.

CHAPTER 2

THE ROLE OF SIM1, MRAP2, MCHR2, OXT, OXTR AND POU3F2 IN THE LEPTIN-MELANOCORTIN SIGNALING PATHWAY

---

1. Introduction

Obesity is a chronic disease defined as an excess of adipose tissue as a result of an imbalance between energy intake and energy expenditure. Although environmental factors such as the availability of (high-fat) nutrition and today's sedentary lifestyle contribute to the current global increase in the prevalence of obesity, yet the susceptibility to the development of obesity in an individual is largely determined by genetic predisposition[1]. In 1973, Coleman and Hummel demonstrated for the first time that genetic factors play a role in the familial aggregation of obesity[2]. Several years later, twin and adoption studies confirmed this and estimated the heritability of BMI at 40 – 70%[3-5]. Subsequently, a combination of linkage analyses[6] and mouse models[7, 8] led to the identification of disease causing loci and genes that are mostly part of the leptin-melanocortin signaling pathway in the hypothalamus and in this way involved in the pathogenesis of obesity.

2. Leptin-melanocortin signaling pathway

The hypothalamic leptin-melanocortin signaling pathway (Figure 1) plays an especially important role in the maintenance of energy homeostasis and the control of food intake and body weight with the leptin hormone and the two G protein-coupled receptors, melanocortin-3 receptor (MC3R) and MC4R, as central players[9, 10]. In brief, leptin (LEP) is secreted by adipocytes in proportion to the amount of body fat and circulates to the brain where it binds to its receptors in the arcuate nucleus (ARC) of the hypothalamus[11, 12]. Subsequently, this leads to the stimulation of the anorexigenic neurons which express pro-opiomelanocortin (POMC) and cocaine and amphetamine related transcript (CART)[13, 14] and to an inhibitory effect on the orexigenic neurons co-expressing agouti-related peptide (AgRP) and neuropeptide Y (NPY)[15]. AgRP has an antagonistic effect on MC4R expressed on the transmembrane of the paraventricular nuclei (PVN) of the hypothalamus ultimately leading to an increase in food intake[16]. On the contrary, POMC is cleaved into the biologically active proteins α-, β-, and γ-melanocyte stimulating hormones (α-MSH, β-MSH and γ-MSH) by pro-protein

convertase subtilisin/kexin type 1 (PCSK1)[17]. α-MSH on its turn stimulates MC3R and MC4R leading to a decrease of feed efficiency and food intake, respectively[18-20]. Due to the complementary action of leptin on the catabolic and anabolic neurons, the energy homeostasis is maintained in healthy individuals. However, disruption of this well-balanced system can lead to an excess of adipose tissue of which the two most illustrious examples are congenital leptin and MC4R deficiency.

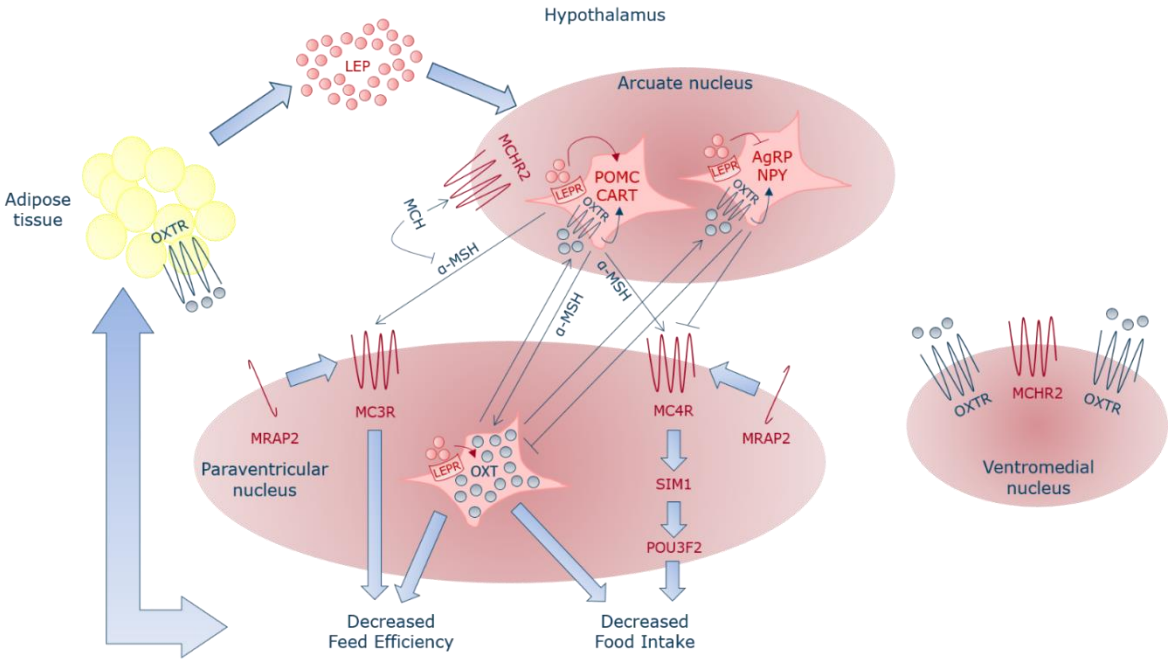


Figure 1. Schematic representation of part of the leptin-melanocortin pathway and the role of SIM1, MRAP2, MCHR2, OXT, OXTR and POU3F2 in the arcuate nucleus, paraventricular nucleus and ventromedial nucleus of the hypothalamus.

Leptin (LEP) is secreted by adipocytes in proportion to the amount of body fat. Through binding to its receptors (LEPR) it has an agonistic effect on pro-opiomelanocortin (POMC)/cocaine and amphetamine related transcript (CART) neurons and an antagonistic effect on agouti-related protein (AgRP)/neuropeptide Y (NPY) neurons in the hypothalamic arcuate nucleus (ARC). Subsequently, POMC is cleaved into different biologically active proteins of which the α-melanocyte stimulating hormone (α-MSH) has a stimulatory effect on the melanocortin 3 receptor (MC3R) and melanocortin 4 receptor (MC4R) expressed on the transmembrane of the paraventricular nucleus (PVN). This ultimately leads to 1) a decrease of feed efficiency and 2) a decrease in food intake through single-minded 1 (SIM1) and POU Class 3 Homeobox 2 (POU3F2). On the contrary, AgRP inhibits MC4R and subsequently induces an increase in food intake.

In addition, LEP activates oxytocin (OXT) neurons in the paraventricular nucleus after LEPR binding. LEP also regulates OXT neurons indirectly through the stimulation of POMC neurons which on their turn secrete α-MSH. In response to that, OXT neurons secrete a large amount of peptide from their dendrites and binds to its receptors (OXTR) stimulating POMC and AgRP neurons in the arcuate nucleus, the ventromedial nucleus (VMN) and adipocytes initiating dissimilar effects on the signaling pathway.
For melanocortin 2 receptor accessory protein 2 (MRAP2), most *in vitro* studies indicate a stimulating effect of the protein on MC3R and MC4R signaling on the PVN transmembrane. To date the functional role of melanin-concentrating hormone receptor 2 (MCHR2) is not yet known, however it does show an expression on the transmembrane of the ARC and VMN with a high binding affinity for melanin-concentrating hormone (MCH) indicating a role of MCHR2 in the control of feeding behaviors and energy metabolism.
This hypothalamic signaling pathway ultimately functions as an endocrine feedback loop maintaining energy homeostasis.

2.1. Congenital leptin deficiency

Congenital leptin deficiency is an extremely rare monogenic form of obesity arising in consanguineous families caused by a homozygous deletion or mutation in the *LEP* gene. Patients with this condition characteristically represent with severe, early-onset obesity, hyperphagia, hypogonadotrophic hypogonadism, thyroid dysfunction and low leptin levels due to misfolding and degradation of the mutant protein leading to a severely impaired leptin secretion[21-27]. The phenotype is very similar to the phenotype seen in the C57BL/6J *ob/ob* mouse firstly described in 1994 by Zhang and colleagues. These mice display a nonsense mutation at codon 105 in the *obese* (*ob*) gene and consequently lack the leptin protein[7].
To date, less than 100 patients worldwide (mainly of Pakistani or Turkish origin) are diagnosed with congenital leptin deficiency[28], though the phenotype can be reversed by the administration of recombinant human leptin (Metreleptin)[22, 29-31] (Figure 2).

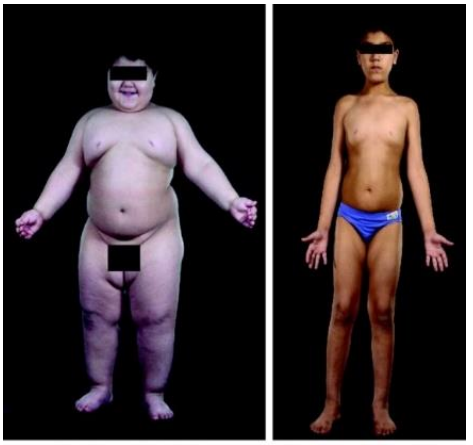


Figure 2. Effect of the administration of recombinant human leptin to a patient with congenital leptin deficiency (depicted from Farooqi and O'Rahilly 2005[30]).

2.2. MC4R deficiency

Heterozygous mutations in the *MC4R* gene are the most common causes of monogenic forms of obesity, with a frequency of approximately 2-5% in (extremely) obese individuals[32]. Also homozygous *MC4R* mutation carriers presenting with a more severe obesity phenotype are described in literature, however this is less common[1, 33-36]. Compared to their heterozygous littermates, *Mc4r*$^{-/-}$ mice also display a more severe phenotype with severe obesity, hyperphagia, increased linear growth, hyperinsulinemia, hypometabolism and hyperglycemia[20, 37]. In patients with *MC4R* deficiency these first three characteristics are also frequently described[1, 9].

In 1998, Yeo *et al.*[38] and Vaisse *et al.*[39] reported the first two heterozygous frameshift mutations in *MC4R* in two severely obese children leading to a premature stop codon and subsequently a dysfunctional receptor. To date, however, already 166 different nonsynonymous, nonsense, deletion, and frameshift *MC4R* mutations have been reported in literature[32].

3. Role of *SIM1* in the leptin-melanocortin signaling pathway

Single-minded 1 or SIM1 is a member of the bHLH-PAS (basic helix-loop-helix Per-Arnt-Sim) family of transcription factors comprising one bHLH domain, two PAS domains, one PAS associated C-terminal (PAC) domain and a C-terminal domain with the nuclear localization signal (NLS)(Figure 3).

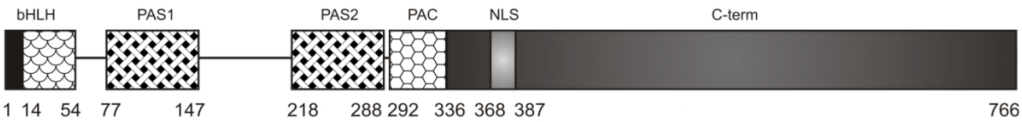


Figure 3. Schematic representation of the protein structure of SIM1 (depicted from Zegers *et al.* 2014[40]).

In the late 90s, Michaud and colleagues created a *Sim1* mouse model to investigate its role in the hypothalamus and discovered that *Sim1* RNA and protein are expressed in the PVN from the early stages of their development to the neonatal period. In addition, they also noticed that $Sim1^{-/-}$ mice die shortly after birth due to the lack of a PVN showing that *Sim1* is essential for the development of the hypothalamic PVN in mice. On the other hand, heterozygous *Sim1* ($Sim1^{+/-}$) mice are viable, but show early-onset obesity, hyperphagia, hyperinsulinemia, hyperleptinemia and increased linear growth, a phenotype resembling *Mc4r* deficiency, which indicates a possible role of *Sim1* in obesity[40-42]. Additionally, Kublaoui *et al.* suggested that, in response to melanocortin signaling, *Sim1*-expressing PVN neurons act downstream of Mc4r regulating food intake and not energy expenditure[43] (Figure 1). They confirmed their hypothesis with the generation of transgenic mice overexpressing human *SIM1*. Overexpression of *SIM1* completely rescues the hyperphagia and partially rescues the obesity seen in agouti yellow mice, which show a disruption in melanocortin signaling[44]. More recently, Tolson and colleagues supported the role of *SIM1* in the leptin-melanocortin signaling pathway with the breeding of conditional *Sim1* heterozygous and (viable) homozygous mice in which they deleted *Sim1* postnatally in the central nervous system (CNS). In addition to a dosage-dependent effect of *Sim1* on

obesity, they also noticed a remarkable decrease in hypothalamic oxytocin (Oxt) and PVN Mc4r mRNA. This indicates that the hyperphagic obesity seen in the *Sim1* deficient mice may be attributable to changes in the leptin-melanocortin-oxytocin pathway[45].

4. Role of MRAP2 in the leptin-melanocortin signaling pathway

Melanocortin 2 receptor accessory protein 2 or MRAP2 is a single-pass membrane accessory protein expressed in the PVN of the hypothalamus (Figure 1) which first was identified as a unique homologue of MRAP by Chan and colleagues in 2009 (Figure 4).

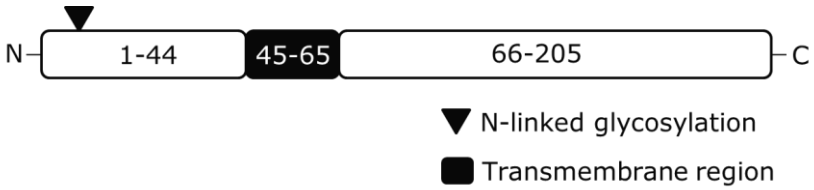


Figure 4. Schematic representation of the protein structure of MRAP2.

Co-immunoprecipitation assays show that MRAP2 can interact with all five G protein-coupled receptors (GPCRs) of the melanocortin receptor (MCR) family (MC1R-MC5R) and performs a modulating role in GPCR trafficking, ligand binding and signaling to the cell surface[46]. To date, results of *in vitro* studies investigating the effect of MRAP2 on the two important energy homeostasis MCRs, MC3R and MC4R, are very conflicting[46-52]. However, most recent findings do hypothesize a stimulating effect of MRAP2 on signaling of the two MCRs[48, 49]. *In vivo* experiments in zebrafish confirm its stimulating effect on MC4R as the zebrafish ortholog of MRAP2, zMRAP2b, enhances the responsiveness of MC4R to α-MSH once the zebrafish begins feeding[49]. In addition to this, it is demonstrated that fasting severely increases central zMRAP2b expression which makes it plausible that zMRAP2b decreases the constitutive activity of MC4R during fasting periods, driving the animal towards a positive energy balance[53]. Asai and colleagues also showed an activation of the expression of *Mrap2* in mice proximal to weaning which in turn increases the sensitivity of MC4R to α-MSH. Furthermore,

they also generated mice with whole body ($Mrap2^{-/-}$) or brain-specific targeted ($Sim1^{Cre}$::$Mrap2^{flox/flox}$) knock-out of the gene. These mice appeared normal at birth, but gradually became extremely obese on regular chow diet and showed age-dependent hyperphagia[48]. All these findings indicate that 1) inactivation of the *MRAP2* gene in mice can induce obesity and 2) *MRAP2* controls the signaling of MC3/4R in the leptin-melanocortin pathway and subsequently plays a role in energy homeostasis.

5. Role of MCHR2 in the leptin-melanocortin signaling pathway

Melanin-concentrating hormone receptor 2 or MCHR2 is, like MCHR1, a G protein-coupled receptor for melanin-concentrating hormone (MCH) with high expression levels in the brain, including the arcuate and ventromedial nucleus (VMN) of the hypothalamus[54, 55] (Figure 1). To date the functional role of MCHR2 is not well defined due to the lack of animal models. In fact, *MCHR2* is only expressed in higher animals such as primates and carnivores and not in rodents and related species like hamsters, guinea pigs or rabbits which are mainly used for animal experiments[56]. However, a role of *MCHR2* in the control of feeding behaviors and energy metabolism is suggested as the neuropeptide MCH, for which it has a high binding affinity, is involved in these processes[57]. In the past, different animal experiments already showed that nutritional status regulates the expression of MCH. For instance, an increase in MCH expression is seen in C57BL/6J *+/+*, C57BL/6J *ob/+* and C57BL/6J *ob/ob* mice after 24 hours of fasting. In addition, also plasma leptin concentrations influence MCH expression as MCH mRNA is overexpressed in the hypothalamus of hyperphagic and obese leptin-deficient *ob/ob* mice[58, 59]. Chronic or acute injection of MCH, either intracerebroventricular or directly into the PVN or ARC, leads to an increase in food intake (and body weight) in mice and rats[60-63]. Furthermore, transgenic mice overexpressing *MCH* in the hypothalamus develop obesity and insulin resistance[64] whereas mice lacking the *MCH* gene are hypophagic and lean[65]. MCH

also acts as a functional antagonist of α-MSH indicating an orexigenic role for *MCH* in the leptin-melanocortin pathway.

As for *MCH*, deletion of *MCHR1* in mice also results in a lean phenotype[66, 67]. Consistent with this, a decrease in food intake leading to weight loss was noticed in rodents after administration of MCHR1 antagonists[68, 69]. Because the distribution of MCHR2 in the primate brain nearly overlaps that of MCHR1[70], MCHR2 shows 38% homology with MCHR1[56] and *MCHR2* is not expressed in rodents, Chee and colleagues generated a MCHR1R2 transgenic mouse model to investigate the effect of MCHR2 on MCHR1 and its potential contribution to energy balance. When fed a high-fat diet, MCHR1R2 mice showed lower food intake compared to their WT littermates only expressing MCHR1 which developed obesity. This suggests that MCHR2 indeed plays a role in energy metabolism and can attenuate the endogenous actions of MCHR1 in the CNS and subsequently the leptin-melanocortin signalling cascade[71].

6. Role of OXT and OXTR in the leptin-melanocortin signaling pathway

Oxytocin or OXT is a nine-amino acid neurohypophysial hormone predominantly known from its peripheral functions including the stimulation of uterine contraction during labor and milk ejection. However, the nonapeptide is mainly synthesized in the CNS within both the magnocellular and parvocellular neurons of the PVN as well as by the magnocellular neurons of the supraoptic nucleus (SON) in the hypothalamus indicating a possible role in the regulation of metabolic homeostasis. In addition, OXT release also occurs locally in the PVN and SON via somatodendrites. Moreover, OXT neurons abundantly express leptin receptors meaning that binding of LEP leads to activation of these neurons in the PVN and SON. LEP also regulates OXT neurons indirectly through the stimulation of POMC neurons which on their turn secrete α-MSH. In response to that, OXT neurons secrete a large amount of peptide from their dendrites[72-74] (Figure 1).

To date, only one oxytocin receptor (OXTR) has been identified. OXTR is a class I GPCR functionally coupled to the Gαq class GTP binding proteins that stimulates the activity

of phospholipase C. It shows a wide expression within brain regions as well as various peripheral tissues including adipocytes[75]. For example, the ventromedial hypothalamus expresses a high density of OXTRs (Figure 1). Besides their expression in the hypothalamic region, additional evidence for the role of OXT and OXTR in the leptin-melanocortin signaling pathway and obesity comes from different mouse models. As previously mentioned, Kublaoui and colleagues identified a decrease by 80% of the *Oxt* mRNA and peptide levels in *Sim1* deficient mice and demonstrated that the transcription factor controls the expression of *OXT*[76]. Furthermore, $Oxt^{-/-}$ mice as well as $Oxtr^{-/-}$ mice develop late-onset obesity without any difference in food intake or motor activity [77, 78]. Noteworthy, also patients with Prader Willi syndrome (PWS) (main features: obesity, intellectual disability, hypotonia) show a significant reduction in the number of OXT-producing neurons (42%) in the PVN of the hypothalamus[79]. This leads to the speculation that OXT can be considered as an anorexigenic peptide and that a reduction in *OXT* or *OXTR* signaling might contribute to the development of obesity.

7. Role of POU3F2 in the leptin-melanocortin signaling pathway

POU Class 3 Homeobox 2 or POU3F2 belongs to the class III POU family of transcription factors and is predominantly expressed in the hypothalamus. Members of the family share a highly homologous POU domain and bind to the octameric DNA sequence 5'-ATGCAAAT-3'[80]. In 1995, two different research groups generated homozygous null and mutant *Brn2* (*Pou3f2*) mice and noticed that all homozygous animals died within 10 days after birth, possibly because of a complete deficiency of the neurons of the PVN and the SON in the hypothalamus[81, 82]. Nakai *et al.* also investigated heterozygous *Brn2* mice in which they noticed that the levels of *OXT* expression in the hypothalamus were half these of wild-type mice suggesting that *Brn2* plays an essential role in the determination and development of the PVN and SON neuronal lineages in the hypothalamus[81]. A couple of years later, Michaud and colleagues determined that *Brn2* functions within the same pathway as *Sim1* (i.e. the

leptin-melanocortin pathway). They noticed that all hypothalamic lineages reported to be affected by the loss of *Brn2* are also affected in *Sim1*-deficient mice[41]. Recently, with the use of morpholino (MO) and mutant zebrafish models, Kasher *et al.* confirmed the findings of Michaud and colleagues that *SIM1* acts upstream of *POU3F2* in the leptin-melanocortin signaling pathway (Figure 1). *Pou3f2a* and *Pou3f2b*, two orthologs of the human *POU3F2*, expression levels showed a significant reduction in *Sim1* morphants. Furthermore, a significant decrease in *Oxt* expression was noticed in MO-mediated knockdown of *Pou3f2a* and/or *Pou3f2b* in zebrafish[80]. As previous research in *Sim1* haploinsufficient mice also showed a decrease in *Oxt* expression[41], this suggests a role of *POU3F2* in the regulation of the expression of *OXT* in the hypothalamus downstream of *SIM1* and subsequently indicates *POU3F2* as an interesting player in the leptin-melanocortin-oxytocin signaling pathway.

In conclusion, a lot of functional studies in combination with animal models indicated an important role for the leptin-melanocortin-oxytocin pathway, as shown in figure 1, in the regulation of food intake and energy expenditure and consequently the pathogenesis of obesity.

References

1. Farooqi, I.S., et al., *Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene.* N Engl J Med, 2003. **348**(12): p. 1085-95.
2. Coleman, D.L. and K.P. Hummel, *The influence of genetic background on the expression of the obese (Ob) gene in the mouse.* Diabetologia, 1973. **9**(4): p. 287-93.
3. Stunkard, A.J., T.T. Foch, and Z. Hrubec, *A twin study of human obesity.* JAMA, 1986. **256**(1): p. 51-4.
4. Maes, H.H., M.C. Neale, and L.J. Eaves, *Genetic and environmental factors in relative body weight and human adiposity.* Behav Genet, 1997. **27**(4): p. 325-51.
5. Luke, A., et al., *Heritability of obesity-related traits among Nigerians, Jamaicans and US black people.* Int J Obes Relat Metab Disord, 2001. **25**(7): p. 1034-41.
6. Rankinen, T., et al., *The human obesity gene map: the 2005 update.* Obesity (Silver Spring), 2006. **14**(4): p. 529-644.
7. Zhang, Y., et al., *Positional cloning of the mouse obese gene and its human homologue.* Nature, 1994. **372**(6505): p. 425-32.
8. Tartaglia, L.A., et al., *Identification and expression cloning of a leptin receptor, OB-R.* Cell, 1995. **83**(7): p. 1263-71.
9. Zegers, D., et al., *Monogenic and Complex Forms of Obesity: Insights from Genetics Reveal the Leptin-Melanocortin Signaling Pathway as a Common Player.* Critical Reviews in Eukaryotic Gene Expression, 2012. **22**(4): p. 325-343.
10. Beckers, S., et al., *The role of the leptin-melanocortin signalling pathway in the control of food intake.* Crit Rev Eukaryot Gene Expr, 2009. **19**(4): p. 267-87.
11. Maffei, M., et al., *Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects.* Nat Med, 1995. **1**(11): p. 1155-61.
12. Considine, R.V., et al., *Serum immunoreactive-leptin concentrations in normal-weight and obese humans.* N Engl J Med, 1996. **334**(5): p. 292-5.
13. Elias, C.F., et al., *Leptin activates hypothalamic CART neurons projecting to the spinal cord.* Neuron, 1998. **21**(6): p. 1375-85.
14. Cheung, C.C., D.K. Clifton, and R.A. Steiner, *Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus.* Endocrinology, 1997. **138**(10): p. 4489-92.
15. Hahn, T.M., et al., *Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons.* Nat Neurosci, 1998. **1**(4): p. 271-2.
16. Mutch, D.M. and K. Clement, *Unraveling the genetics of human obesity.* PLoS Genet, 2006. **2**(12): p. e188.
17. Benjannet, S., et al., *PC1 and PC2 are proprotein convertases capable of cleaving proopiomelanocortin at distinct pairs of basic residues.* Proc Natl Acad Sci U S A, 1991. **88**(9): p. 3564-8.

18. Butler, A.A., et al., *A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse.* Endocrinology, 2000. **141**(9): p. 3518-21.
19. Chen, A.S., et al., *Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass.* Nat Genet, 2000. **26**(1): p. 97-102.
20. Huszar, D., et al., *Targeted disruption of the melanocortin-4 receptor results in obesity in mice.* Cell, 1997. **88**(1): p. 131-41.
21. Strobel, A., et al., *A leptin missense mutation associated with hypogonadism and morbid obesity.* Nat Genet, 1998. **18**(3): p. 213-5.
22. Gibson, W.T., et al., *Congenital leptin deficiency due to homozygosity for the Delta133G mutation: report of another case and evaluation of response to four years of leptin therapy.* J Clin Endocrinol Metab, 2004. **89**(10): p. 4821-6.
23. Mazen, I., et al., *A novel homozygous missense mutation of the leptin gene (N103K) in an obese Egyptian patient.* Mol Genet Metab, 2009. **97**(4): p. 305-8.
24. Montague, C.T., et al., *Congenital leptin deficiency is associated with severe early-onset obesity in humans.* Nature, 1997. **387**(6636): p. 903-8.
25. Fischer-Posovszky, P., et al., *A new missense mutation in the leptin gene causes mild obesity and hypogonadism without affecting T cell responsiveness.* J Clin Endocrinol Metab, 2010. **95**(6): p. 2836-40.
26. Fatima, W., et al., *Leptin deficiency and leptin gene mutations in obese children from Pakistan.* Int J Pediatr Obes, 2011. **6**(5-6): p. 419-27.
27. Saeed, S., et al., *High prevalence of leptin and melanocortin-4 receptor gene mutations in children with severe obesity from Pakistani consanguineous families.* Mol Genet Metab, 2012. **106**(1): p. 121-6.
28. Huvenne, H., et al., *Rare Genetic Forms of Obesity: Clinical Approach and Current Treatments in 2016.* Obes Facts, 2016. **9**(3): p. 158-73.
29. Farooqi, I.S., et al., *Effects of recombinant leptin therapy in a child with congenital leptin deficiency.* N Engl J Med, 1999. **341**(12): p. 879-84.
30. Farooqi, I.S. and S. O'Rahilly, *Monogenic obesity in humans.* Annu Rev Med, 2005. **56**: p. 443-58.
31. Chou, K. and C.M. Perry, *Metreleptin: first global approval.* Drugs, 2013. **73**(9): p. 989-97.
32. Hinney, A., A.L. Volckmar, and N. Knoll, *Melanocortin-4 receptor in energy homeostasis and obesity pathogenesis.* Prog Mol Biol Transl Sci, 2013. **114**: p. 147-91.
33. Farooqi, I.S., et al., *Dominant and recessive inheritance of morbid obesity associated with melanocortin 4 receptor deficiency.* J Clin Invest, 2000. **106**(2): p. 271-9.
34. Kobayashi, H., et al., *A Novel homozygous missense mutation of melanocortin-4 receptor (MC4R) in a Japanese woman with severe obesity.* Diabetes, 2002. **51**(1): p. 243-6.

35. Lubrano-Berthelier, C., et al., *A homozygous null mutation delineates the role of the melanocortin-4 receptor in humans.* J Clin Endocrinol Metab, 2004. **89**(5): p. 2028-32.
36. Dubern, B., et al., *Homozygous null mutation of the melanocortin-4 receptor and severe early-onset obesity.* J Pediatr, 2007. **150**(6): p. 613-7, 617 e1.
37. Ste Marie, L., et al., *A metabolic defect promotes obesity in mice lacking melanocortin-4 receptors.* Proc Natl Acad Sci U S A, 2000. **97**(22): p. 12339-44.
38. Yeo, G.S., et al., *A frameshift mutation in MC4R associated with dominantly inherited human obesity.* Nat Genet, 1998. **20**(2): p. 111-2.
39. Vaisse, C., et al., *A frameshift mutation in human MC4R is associated with a dominant form of obesity.* Nat Genet, 1998. **20**(2): p. 113-4.
40. Zegers, D., et al., *Mutation screen of the SIM1 gene in pediatric patients with early-onset obesity.* International Journal of Obesity, 2014. **38**(7): p. 1000-1004.
41. Michaud, J.L., et al., *Development of neuroendocrine lineages requires the bHLH-PAS transcription factor SIM1.* Genes & Development, 1998. **12**(20): p. 3264-3275.
42. Michaud, J.L., et al., *Sim1 haploinsufficiency causes hyperphagia, obesity and reduction of the paraventricular nucleus of the hypothalamus.* Human Molecular Genetics, 2001. **10**(14): p. 1465-1473.
43. Kublaoui, B.M., et al., *Sim1 haploinsufficiency impairs melanocortin-mediated anorexia and activation of paraventricular nucleus neurons.* Mol Endocrinol, 2006. **20**(10): p. 2483-92.
44. Kublaoui, B.M., et al., *SIM1 overexpression partially rescues agouti yellow and diet-induced obesity by normalizing food intake.* Endocrinology, 2006. **147**(10): p. 4542-4549.
45. Tolson, K.P., et al., *Postnatal Sim1 Deficiency Causes Hyperphagic Obesity and Reduced Mc4r and Oxytocin Expression.* Journal of Neuroscience, 2010. **30**(10): p. 3803-3812.
46. Chan, L.F., et al., *MRAP and MRAP2 are bidirectional regulators of the melanocortin receptor family.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(15): p. 6146-6151.
47. Ramachandrappa, S., et al., *The melanocortin receptors and their accessory proteins.* Front Endocrinol (Lausanne), 2013. **4**: p. 9.
48. Asai, M., et al., *Loss of Function of the Melanocortin 2 Receptor Accessory Protein 2 Is Associated with Mammalian Obesity.* Science, 2013. **341**(6143): p. 275-278.
49. Sebag, J.A., et al., *Developmental Control of the Melanocortin-4 Receptor by MRAP2 Proteins in Zebrafish.* Science, 2013. **341**(6143): p. 278-281.
50. Cerda-Reverter, J.M., et al., *Involvement of melanocortin receptor accessory proteins (MRAPs) in the function of melanocortin receptors.* Gen Comp Endocrinol, 2013. **188**: p. 133-6.

51. Sebag, J.A. and P.M. Hinkle, *Regulation of G protein-coupled receptor signaling: specific dominant-negative effects of melanocortin 2 receptor accessory protein 2.* Sci Signal, 2010. **3**(116): p. ra28.
52. Novoselova, T.V., et al., *Melanocortin receptor accessory proteins in adrenal gland physiology and beyond.* J Endocrinol, 2013. **217**(1): p. R1-11.
53. Josep Agulleiro, M., et al., *Melanocortin 4 receptor becomes an ACTH receptor by coexpression of melanocortin receptor accessory protein 2.* Mol Endocrinol, 2013. **27**(11): p. 1934-45.
54. An, S., et al., *Identification and characterization of a melanin-concentrating hormone receptor.* Proc Natl Acad Sci U S A, 2001. **98**(13): p. 7576-81.
55. Sailer, A.W., et al., *Identification and characterization of a second melanin-concentrating hormone receptor, MCH-2R.* Proc Natl Acad Sci U S A, 2001. **98**(13): p. 7564-9.
56. Tan, C.P., et al., *Melanin-concentrating hormone receptor subtypes 1 and 2: species-specific gene expression.* Genomics, 2002. **79**(6): p. 785-92.
57. Pissios, P. and E. Maratos-Flier, *Melanin-concentrating hormone: from fish skin to skinny mammals.* Trends Endocrinol Metab, 2003. **14**(5): p. 243-8.
58. Qu, D., et al., *A role for melanin-concentrating hormone in the central regulation of feeding behaviour.* Nature, 1996. **380**(6571): p. 243-7.
59. Elliott, J.C., et al., *Increases in melanin-concentrating hormone and MCH receptor levels in the hypothalamus of dietary-obese rats.* Brain Res Mol Brain Res, 2004. **128**(2): p. 150-9.
60. Rossi, M., et al., *Melanin-concentrating hormone acutely stimulates feeding, but chronic administration has no effect on body weight.* Endocrinology, 1997. **138**(1): p. 351-5.
61. Rossi, M., et al., *Investigation of the feeding effects of melanin concentrating hormone on food intake--action independent of galanin and the melanocortin receptors.* Brain Res, 1999. **846**(2): p. 164-70.
62. Della-Zuana, O., et al., *Acute and chronic administration of melanin-concentrating hormone enhances food intake and body weight in Wistar and Sprague-Dawley rats.* Int J Obes Relat Metab Disord, 2002. **26**(10): p. 1289-95.
63. Gomori, A., et al., *Chronic intracerebroventricular infusion of MCH causes obesity in mice. Melanin-concentrating hormone.* Am J Physiol Endocrinol Metab, 2003. **284**(3): p. E583-8.
64. Ludwig, D.S., et al., *Melanin-concentrating hormone overexpression in transgenic mice leads to obesity and insulin resistance.* J Clin Invest, 2001. **107**(3): p. 379-86.
65. Shimada, M., et al., *Mice lacking melanin-concentrating hormone are hypophagic and lean.* Nature, 1998. **396**(6712): p. 670-4.
66. Chen, Y., et al., *Targeted disruption of the melanin-concentrating hormone receptor-1 results in hyperphagia and resistance to diet-induced obesity.* Endocrinology, 2002. **143**(7): p. 2469-77.

67. Marsh, D.J., et al., *Melanin-concentrating hormone 1 receptor-deficient mice are lean, hyperactive, and hyperphagic and have altered metabolism.* Proc Natl Acad Sci U S A, 2002. **99**(5): p. 3240-5.
68. MacNeil, D.J. and M.A. Bednarek, *MCH receptor peptide agonists and antagonists.* Peptides, 2009. **30**(11): p. 2008-13.
69. Chung, S., et al., *Recent updates on the melanin-concentrating hormone (MCH) and its receptor system: lessons from MCH1R antagonists.* J Mol Neurosci, 2011. **43**(1): p. 115-21.
70. Mori, M., et al., *Cloning of a novel G protein-coupled receptor, SLT, a subtype of the melanin-concentrating hormone receptor.* Biochem Biophys Res Commun, 2001. **283**(5): p. 1013-8.
71. Chee, M.J., et al., *Expression of melanin-concentrating hormone receptor 2 protects against diet-induced obesity in male mice.* Endocrinology, 2014. **155**(1): p. 81-8.
72. Maejima, Y., et al., *Oxytocinergic circuit from paraventricular and supraoptic nuclei to arcuate POMC neurons in hypothalamus.* FEBS Lett, 2014. **588**(23): p. 4404-12.
73. Altirriba, J., A.L. Poher, and F. Rohner-Jeanrenaud, *Chronic Oxytocin Administration as a Treatment Against Impaired Leptin Signaling or Leptin Resistance in Obesity.* Front Endocrinol (Lausanne), 2015. **6**: p. 119.
74. Sabatier, N., G. Leng, and J. Menzies, *Oxytocin, feeding, and satiety.* Front Endocrinol (Lausanne), 2013. **4**: p. 35.
75. Gimpl, G. and F. Fahrenholz, *The oxytocin receptor system: structure, function, and regulation.* Physiol Rev, 2001. **81**(2): p. 629-83.
76. Kublaoui, B.M., et al., *Oxytocin deficiency mediates hyperphagic obesity of Sim1 haploinsufficient mice.* Molecular Endocrinology, 2008. **22**(7): p. 1723-34.
77. Camerino, C., *Low sympathetic tone and obese phenotype in oxytocin-deficient mice.* Obesity (Silver Spring), 2009. **17**(5): p. 980-4.
78. Takayanagi, Y., et al., *Oxytocin receptor-deficient mice developed late-onset obesity.* Neuroreport, 2008. **19**(9): p. 951-5.
79. Swaab, D.F., J.S. Purba, and M.A. Hofman, *Alterations in the hypothalamic paraventricular nucleus and its oxytocin neurons (putative satiety cells) in Prader-Willi syndrome: a study of five cases.* J Clin Endocrinol Metab, 1995. **80**(2): p. 573-9.
80. Kasher, P.R., et al., *Small 6q16.1 Deletions Encompassing POU3F2 Cause Susceptibility to Obesity and Variable Developmental Delay with Intellectual Disability.* Am J Hum Genet, 2016. **98**(2): p. 363-72.
81. Nakai, S., et al., *The POU domain transcription factor Brn-2 is required for the determination of specific neuronal lineages in the hypothalamus of the mouse.* Genes Dev, 1995. **9**(24): p. 3109-21.
82. Schonemann, M.D., et al., *Development and survival of the endocrine hypothalamus and posterior pituitary gland requires the neuronal POU domain factor Brn-2.* Genes Dev, 1995. **9**(24): p. 3122-35.

OBJECTIVES

Objectives

To date, obesity has become a major health burden worldwide. Since 1975 its prevalence even nearly tripled. Moreover, it contributes to several adverse complications leading to a significant expenditure of national health-care budgets. Already several years ago, different twin and adoption studies estimated the heritability of BMI between 40 – 70%. Though, to date only 2.7% has been explained, indicating that the genetics of obesity is extremely complex and difficult to decipher. In order to generate new insight into the genetic background of the disorder, both monogenic and syndromic forms of obesity are interesting to investigate. Therefore we decided to use a specific cohort of patients with syndromic obesity, i.e. individuals with a Prader Willi *like* (PWL) phenotype. We screened the patient database of the Department of Medical Genetics over the last 25 years for individuals with a putative diagnosis of Prader Willi syndrome (PWS) or PWL for whom the clinical diagnosis of PWS could be excluded molecularly. All patients from the study population already showed obesity at a young age, indicating that genetic factors must be involved in the development of obesity in these patients. In addition, as in this research we are looking for rare variants and rare copy number variations (CNVs) in order to contribute to the missing heritability of obesity ('common disease, rare variant' hypothesis), the study of our syndromic cohort could shed light to interesting genes which could eventually aid in the elucidation of the more common and complex obesity.

In literature the PWL phenotype has already been associated with CNVs on different chromosomes such as 1p36.3, 2p21, 6q, 9q34 and maternal uniparental disomy of chromosome 14. Therefore we first performed a **genome-wide microarray analysis** in order to search for (new) important chromosomal regions in our PWL cohort (Figure 1, objective 1).

As deletions at chromosome 6q were most frequently described, we also wanted to **clarify the role of chromosome 6q in PWL patients and their obesity phenotype** (Figure

1, objective 2). Consequently, we performed mutation analysis of three interesting genes in the 6q region: *SIM1*, *MRAP2* and *MCHR2* (Table 1). For *MRAP2* an additional cohort of severely overweight or obese children and adolescents was screened.

Secondly, due to its link with *SIM1* in the leptin-melanocortin signaling pathway and its possible use as a therapeutic target in obese individuals, CNV and mutation analysis was also performed for *OXT* and *OXTR* in the 109 PWL patients (Figure 1, objective 2) (Table 1). However, as this research resulted in rather negative results, we suggested that the hypothesized *OXT* or *OXTR* signaling deficiency could be mediated by another gene upstream of *OXT* in the leptin-melanocortin signaling pathway. Subsequently, mutation analysis of *POU3F2* was performed in the PWL cohort (Table 1). In addition, also the cohort of obese children and adolescents was screened for mutations in the gene. The pathogenic relevance of two identified variants was investigated further with the use of two different functional studies (Figure 1, objective 2).

This research can eventually lead to an improvement of diagnosing PWL patients and clearly differentiating the PWL phenotype from other syndromic forms of obesity. Additionally, it can produce new insights into the genetic background of obesity, unravel the underlying mechanisms of energy metabolism and ultimately into accurate therapeutic treatment.

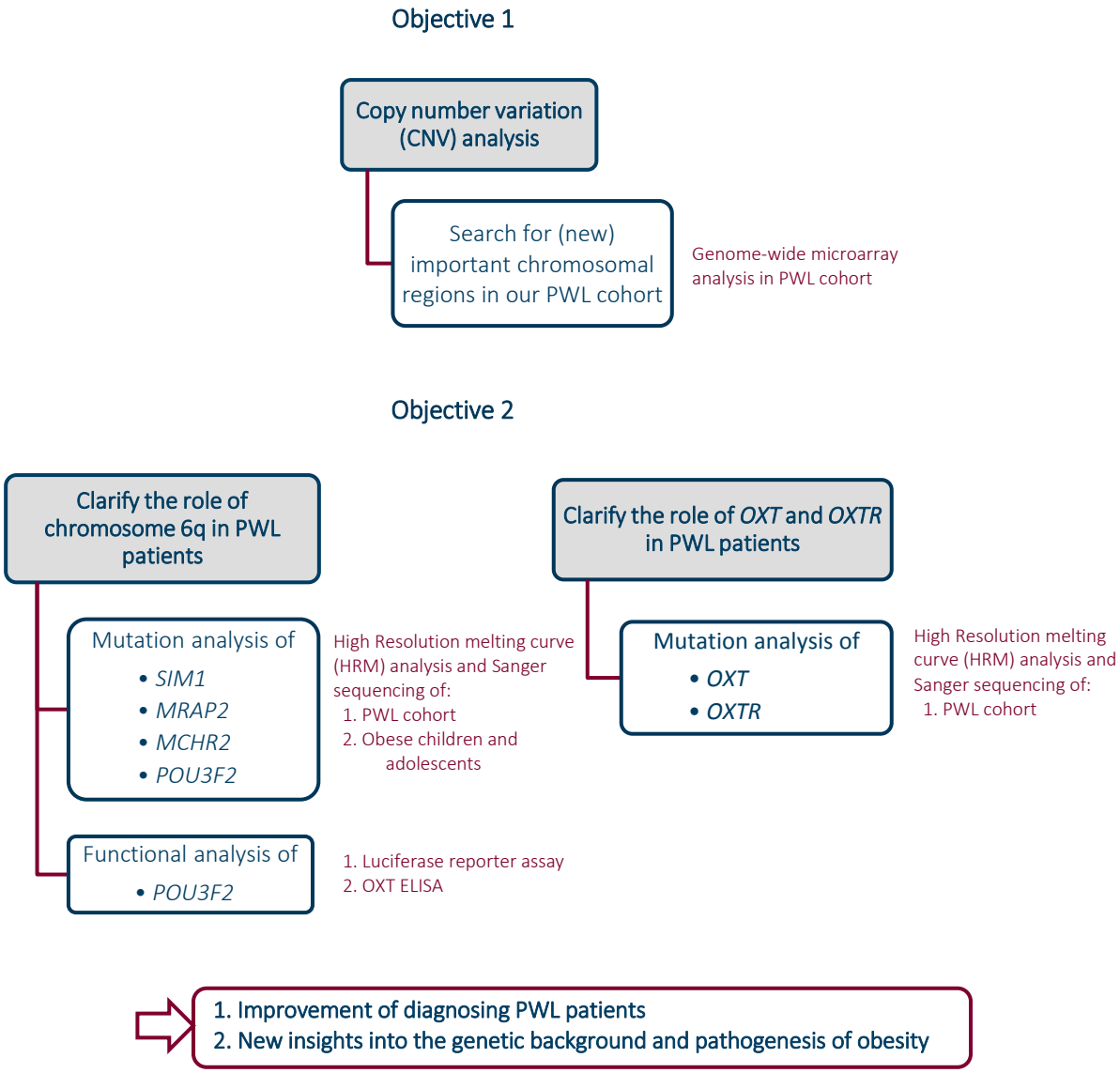


Figure 1. Schematic representation of the objectives.

Table 1. Overview of the six investigated genes and their chromosomal position, importance in the leptin-melanocortin signaling pathway and main phenotypic features of investigated animal models.

GENE	CHROMOSOMAL REGION	LEPTIN-MELANOCORTIN SIGNALING PATHWAY	ANIMAL MODELS
SIM1	6q16.3	Transcription factor downstream MC4R in paraventricular nucleus (PVN)	• *Sim1*$^{-/-}$ mice: - Lethal due to absence of PVN • *Sim1*$^{+/-}$ mice: - Early-onset obesity - Hyperphagia - Increased linear growth • Postnatal *Sim1* deficient mice: - Obesity - Hyperphagia - Decrease in Oxt and Mc4r mRNA
MRAP2	6q14.2	Accessory protein for MC3R and MC4R in PVN	• *Mrap2*$^{-/-}$ and *Sim1*^{Cre}::*Mrap2*$^{flox/flox}$ mice: - Become extremely obese on regular chow diet - Age-dependent hyperphagia • *Mrap2*$^{+/-}$ mice: - Significantly heavier than WT
MCHR2	6q16.2	G-protein coupled receptor (GPCR) of melanin-concentrating hormone in arcuate nucleus (ARC) and ventromedial nucleus (VMN)	• *MCHR1R2* transgenic mice: - Lower food intake when fed high-fat diet
OXT	20p13	Neurohypophysial hormone in PVN and supraoptic nucleus	• *Oxt*$^{-/-}$ mice: - Late-onset obesity

OXTR	3p25.3	GPCR for OXT in ARC, VMN and adipocytes	• *Oxtr*$^{-/-}$ mice: - Late-onset obesity
POU3F2	6q16.1	Transcription factor downstream SIM1 and upstream OXT in PVN	• *Pou3f2*$^{-/-}$ mice: - Lethal due to deficiency of PVN and SON neurons • *Pou3f2*$^{+/-}$ mice: - Oxt expression ½ of WT • Morpholino-mediated knockdown of *Pou3f2a* and/or *Pou3f2b* in zebrafish models: - Decrease in Oxt expression

RESULTS

CHAPTER 3

COPY NUMBER VARIATION (CNV) ANALYSIS AND MUTATION ANALYSIS OF THE 6Q14.1 – 6Q16.3 GENES SIM1 AND MRAP2 IN PRADER WILLI LIKE PATIENTS.

Ellen Geets[1], Doreen Zegers[1], Sigri Beckers[1], An Verrijken[2], Guy Massa[3], Kim Van Hoorenbeeck[4], Stijn Verhulst[4], Luc Van Gaal[2], Wim Van Hul[1]

[1]Department of Medical Genetics, University of Antwerp, Antwerp, Belgium
[2]Department of Endocrinology, Diabetology and Metabolic Diseases, Antwerp University Hospital, Antwerp, Belgium
[3]Department of Paediatrics, Jessa Hospital, Hasselt, Belgium
[4]Department of Paediatrics, Antwerp University Hospital, Antwerp, Belgium

Molecular Genetics and Metabolism, Volume 117, Issue 3: 383-388, 2016.

Abstract

Background: Prader-Willi syndrome (PWS), caused by a paternal defect on 15q11.2 – q13, is the most common form of syndromic obesity. However, patients clinically diagnosed with PWS do not always show this defect on chromosome 15q and are therefore molecularly categorized as Prader Willi *like* (PWL). Deletions at 6q14.1– q16.3 encompassing *MRAP2* and *SIM1* were reported in some individuals with a PWL phenotype. In addition, a few mutations in *SIM1* and *MRAP2* were also previously identified in cohorts of obese individuals. Therefore, we decided to perform copy number variation analysis of the 6q14.1– 6q16.3 region followed by mutation analysis of *SIM1* and *MRAP2* in a PWL cohort.

Methods: A genome-wide microarray analysis was performed in a group of 109 PWL patients. Next, we screened 94 PWL patients for mutations in *SIM1* and *MRAP2* using high-resolution melting curve analysis and Sanger sequencing. Additionally, 363 obese children and adolescents were screened for mutations in *MRAP2*.

Results: No gene harboring deletions were identified at the 6q14.1 – q16.3 region in the 109 PWL patients. *SIM1* mutation analysis resulted in the identification of one very rare nonsynonymous variant p.P352S (rs3734354). Another rare nonsynonymous variant, p.A40S, was detected in the *MRAP2* gene. No variants were identified in the 363 obese individuals.

Conclusions: In contrast to literature reports, no gene harboring deletions were identified in the *SIM1* and *MRAP2* regions in our PWL cohort. Secondly, taking into account their very low minor allele frequencies in public sequencing databases and the results of *in silico* prediction programs, further functional analysis of p.P352S found in *SIM1* and p.A40S found in *MRAP2* is useful. This would provide further support for a possible role of *SIM1* and *MRAP2* in the pathogenesis of the PWL phenotype albeit in a limited number of patients.

Keywords: *MRAP2*; Microarray; Mutation analysis; Obesity; Prader Willi *like*; *SIM1*

1. Introduction

Obesity is a chronic condition defined as an excess of adipose tissue as a result of an imbalance between energy intake and expenditure. It constitutes a major health problem, partly due to the increasing prevalence and secondly because of its associated morbidity. A recent meta-analysis of twenty major studies in the United States, Australia and Sweden showed that morbid obesity is associated with substantially elevated rates of total mortality, with most of the excess deaths due to heart disease, cancer and type II diabetes mellitus. Also the life expectancy of individuals with morbid obesity reduces by 6.5 years compared to individuals with normal BMI[1]. Due to the increased risk of developing the abovementioned conditions and the high prevalence, obesity has a major impact on global health and consequently represents a significant expenditure of national health-care budgets[2]. At present, no effective therapies are available, making the unravelment of the different pathways influencing the development of obesity increasingly important. Considerable attention has focused on the leptin-melanocortin signaling pathway in the hypothalamus which has an especially important function in maintaining energy homeostasis[3]. Mutations in genes that disrupt the proteins in this pathway cause early-onset, severe obesity in humans worldwide. Mutations in the *melanocortin-4 receptor* (*MC4R*) gene, coding for a G protein-coupled receptor (GPCR) that plays an important role in leptin-melanocortin signaling, are the most common causes of monogenic forms of obesity, with a frequency of approximately 2-5%[4].

In contrast to the rare monogenic form of obesity which is caused by a mutation in one of the known disease causing genes, complex obesity is far more common and caused by a combination of genetic and environmental factors. Lastly, syndromic forms of obesity are also described with Prader-Willi syndrome (PWS) being most common with a prevalence of 1 in 10.000 – 30.000 individuals. It is mainly characterized by obesity, neonatal hypotonia, hypogonadism, intellectual disability and characteristic facial features like almond-shaped eyes and downturned corners of the mouth. PWS is caused by the absence of the expression of the paternal genes on

the imprinting region 15q11.2 – q13 due to a paternal deletion (70-75%), maternal uniparental disomy (20-25%) or an imprinting defect of the critical region (1-3%)[5]. Furthermore, patients with a Prader Willi *like* (PWL) phenotype share clinical characteristics with PWS, but do not show the defect on chromosome 15q11.2 – q13. Occasionally these patients show heart defects or neurological defects like seizures or hearing loss[6-8]. In patients with suspected PWS or a PWL phenotype deletions at chromosome 6q16.3 were reported in a small percentage. This ultimately led to the identification of haploinsufficiency of the *SIM1* gene at 6q16.3 as a possible cause for the presence of obesity in these patients[6-13]. This hypothesis was substantiated further in an eighteen month old girl with early-onset severe obesity who presented with a *de novo* balanced translocation between chromosome 1p22.1 and 6q16.2 separating the 5' promotor region and basic helix-loop-helix (bHLH) domain from the 3' PER-ARNT-SIM (PAS) region and putative transcriptional regulating domains of the *SIM1* gene[14]. More recently, different research groups, including our own, reported loss-of-function point mutations and variants in *SIM1* in obese individuals with or without PWL features, indicating a possible contribution of the *SIM1* gene in the development of obesity and the PWL syndrome[15-18]. Additionally, deletions in other regions of chromosome 6q are also described in individuals with the PWL phenotype[7, 19] which creates the presumption that besides *SIM1* other genes located on chromosome 6q could be involved in PWL and the obesity phenotype. Recent research in human cohorts with severe early-onset obesity (Genetics of Obesity Study (GOOS) and The Swedish obese children's cohorts) revealed the identification of four rare heterozygous variants in *MRAP2*, located at chromosome 6q14.2, in four unrelated, nonsyndromic, severely obese individuals of which one (E24X) was clearly disruptive[20]. Consequently, *MRAP2* is thought to be a good candidate gene to explain the presence of obesity in patients with a PWL phenotype or even cases of nonsyndromic obesity. Therefore, we decided to first perform copy number variation (CNV) analysis followed by mutation analysis of both the *SIM1* and the *MRAP2* gene in a cohort of PWL patients.

2. Materials and methods

2.1. Study populations

The genome-wide microarray analysis was initially performed in a cohort of 109 patients who were referred to our Department of Medical Genetics (University of Antwerp, Antwerp, Belgium) over the last 25 years with a putative diagnosis of PWS or PWL syndrome. The cohort was recruited through collaboration with various Antwerp Hospital Network (ZNA) clinics, including the Queen Paola Children's Hospital and the Middelheim Hospital Antwerp, and the Antwerp University Hospital. All the selected patients were reported with at least a combination of obesity and developmental delay or intellectual disability. In some cases also behavioral problems (32/109) and hypotonia (20/109) were identified. The clinical diagnosis of PWS could be excluded molecularly with the use of a methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)[21]. Mutation analysis of *MC4R*, performed as described earlier[22], showed no obesity-related mutations in any of the patients. Table 1 shows an overview of the population characteristics of the 109 PWL patients which is based on obtained data from the corresponding physicians. For 94 out of 109 patients DNA was available for extra mutation screening of *SIM1* and *MRAP2*.

In addition, *MRAP2* mutation analysis was also performed in a cohort of 363 obese children and adolescents which were recruited from the paediatrics departments of the Antwerp University Hospital and the Jessa Hospital in Hasselt (Table 2). This group includes 171 boys and 192 girls with a mean age of 11.3 ± 3.5 years and a mean body mass index (BMI) z-score of 2.6 ± 0.6.

This study protocol was approved by the local ethics committee and all participants gave their written informed consent.

Table 1. Population characteristics of the PWL cohort for which genome-wide microarray analysis was performed.

Parameters	Prader Willi *like* patients
N	109
Age (years)	11.5 ± 8.4
Male/Female	70/39
15q11.2-13 defect	No
Obesity	109/109
Developmental delay/Intellectual disability	109/109
Behavioral problems	32/109
Hypotonia	20/109
Small hands/feet	14/109
Hypogonadism	12/109
Seizures	9/109
Short stature	5/109
Hearing loss	1/109

Mean value ± standard deviation of mean is given.

Table 2. Population characteristics of the obese children and adolescents cohort screened for the *MRAP2* gene.

Parameters	Obese children and adolescents
N	363
Age (years)	11.3 ± 3.5
Male/Female	171/192
BMI (kg/m²)	30.5 ± 5.7
BMI z-score	2.6 ± 0.6

Mean value ± standard deviation of mean is given.

2.1.1. Anthropometry

For the obese children and adolescents, height was measured to the nearest 0.1 cm and body weight was determined to the nearest 0.1 kg on a digital scale. BMI was computed as weight (in kilograms) over length (in meters) squared and the BMI z-scores were calculated based on the Flemish Growth Curves 2004[23]. Using these age- and sex-specific BMI growth curves, the cut-off values for overweight and obesity are defined as the percentile lines on the chart that cross BMI 25 kg/m² and 30 kg/m², respectively, at the age of 18 years.

2.2. Genome-wide microarray analysis

For the genome-wide microarray analysis two different BeadChips were used. Initially, 31 samples were analyzed with the HumanCytoSNP-12 BeadChip (298.563 Single Nucleotide Polymorphisms (SNPs); Illumina, San Diego, CA, USA). Subsequently, the analysis of the remaining 78 patient samples was performed on a more dense HumanOmniExpress-24 BeadChip (713.014 SNPs; Illumina, San Diego, CA, USA). Both BeadChips contain a sufficient amount of SNPs within both genes (3 and 19 SNPs in *MRAP2* and 13 and 17 SNPs in *SIM1*, respectively) to detect CNVs when present.

Overall, genomic DNA from the patients was isolated from whole blood according to standard procedures[24]. 200 ng of this DNA was denatured and isothermally amplified in an overnight step at 37°C in an Illumina Hybridization Oven (Illumina, San Diego, CA, USA). The amplified product is then enzymatically fragmented using end-point fragmentation to prevent over-fragmentation. After isopropanol precipitation and DNA resuspension in hybridization buffer, the amplified and fragmented DNA samples are applied to the BeadChip and incubated at 37°C for 16 to 24 hours. During this hybridization samples anneal to locus-specific 50-mer oligonucleotides which are linked to one of up to 713,014 bead types (HumanOmniExpress-24). One bead type corresponds to each allele per SNP locus. Next, allelic specificity is conferred by enzymatic base extension and subsequently products are fluorescently stained in capillary flow-through chambers. Through the single-base extension of the oligonucleotides, using the captured DNA as template, detectable labels are incorporated dependent on the SNP involved. The intensities of the products' fluorescence are detected using the iScan Reader (Illumina, San Diego, CA, USA) and eventually these raw data are processed using the Illumina GenomeStudio software and analyzed further by the in-house CNV-Webstore software[25].

2.3. Mutation analysis

Mutation analysis of the *SIM1* gene was performed as previously described[15]. Screening of all coding exons and intron-exon boundaries of the *MRAP2* gene was executed on genomic DNA isolated from whole blood according to standard procedures[24]. *MRAP2* (GenBank NM_ 138409.2) has four exons of which three are coding. We designed four PCR amplicons in order to perform mutation screening of all three coding exons and their intron-exon boundaries by high-resolution melting curve analysis (HRM) in combination with Sanger sequencing. HRM, which is accomplished using the Lightcycler LC480 Real-Time PCR System (Roche, Penzberg, Germany), is a highly specific and sensitive technique with high sample throughput[26]. The amplification of the four amplicons was achieved either by a standard or a touchdown

PCR protocol. Real-time PCR was performed in a total reaction volume of 10 µl. The amplification mixture included 10 ng of template DNA, 1.5 mM $MgCl_2$, 0.15 mM dNTP's, 500 nM primers, 0.015 U/µl GoTaq (Promega Corporation, Madison, WI, USA), 1x GoTaq buffer and the saturating dye LCGreen Plus (Idaho Technology, Salt Lake City, UT, USA) in a 0.5x concentration. After amplification, DNA samples were denatured at 95°C, rapidly reannealed at 40°C and subsequently heated from 70°C – 90°C measuring fluorescence constantly. For DNA samples with melting patterns deviating from wild-type, Sanger sequencing was performed with ABI BigDye Terminator v3.1 Cycle Sequencing kits on an ABI Prism Genetic Analyzer 3130xl (Applied Biosystems Inc, Foster City, CA, USA) in order to confirm and characterize putative sequence variations. Primer sequences for HRM and sequencing are available upon request.

3. Results

3.1. Genome-wide microarray analysis

An overview of the CNVs identified at the 6q14.1 – 6q16.3 region in the 109 PWL patients is shown in table 3. At this region a total of seven CNVs were found in eight different PWL patients. Four of them are located at the 6q14.1 region, three heterozygous deletions and one heterozygous duplication. The heterozygous deletion with a maximum size of 41,170 bp was identified in two unrelated PWL patients. The three heterozygous deletions at chromosome 6q15, 6q16.1 and 6q16.3 were each found in one PWL individual. In contrast to the reported CNVs in literature, no genes were located in or near these deletions or duplication. In addition, the CNVs identified in our PWL cohort were already described previously in healthy control samples (Database of Genomic Variance (DGV)[27]) and 1137 in-house healthy controls.

Table 3. HumanOmniExpress-24 / HumanCytoSNP-12 microarray results: Overview of identified copy number variations (CNVs) at the 6q14.1 – 6q16.3 region in 109 PWL patients.

Chromosomal position	Min. region	Max. region	CNV	Number of patients	Genes
6q14.1	77,315,788-77,322,488 (6,701 bp)	77,299,933-77,324,522 (24,590 bp)	Het. Del.	1	0
6q14.1	77,315,788-77,326,802 (11,015 bp)	77,299,933-77,327,833 (27,901 bp)	Het. Del.	1	0
6q14.1	77,315,788-77,326,802 (11,015 bp)	77,299,933-77,341,104 (41,170 bp)	Het. Del.	2	0
6q14.1	81,463,045-81,593,880 (130,835 bp)	81,460,295-81,597,546 (137,252 bp)	Het. Dup.	1	0
6q15	91,981,841-92,119,129 (137,289 bp)	91,978,768-92,132,799 (154,032 bp)	Het. Del.	1	0
6q16.1	94,624,568-94,640,147 (15,580 bp)	94,603,085-94,646,136 (43,050 bp)	Het. Del.	1	0
6q16.3	104,566,964-104,579,138 (12,175 bp)	104,560,332-104,587,040 (26,707 bp)	Het. Del.	1	0

Bp = Base pairs, Het. Del. = Heterozygous deletion, Het. Dup. = heterozygous duplication, CNV = Copy number variation

3.2. Mutation analysis of SIM1

Screening of the coding region of *SIM1* in 561 severely overweight and obese children and adolescent has been performed previously by our research group[15]. Additional screening of 94 PWL patients led to the identification of three earlier reported rare synonymous variants, c.171A>T (p.P57P), c.624G>A (p.V208V) and c.1959C>T (p.T653T, rs41318041). Only the latter SNP has already been repeatedly reported in the Exome Aggregation Consortium (ExAC) Browser (Beta) (Accessed on the 13th of November 2015)[28]. In addition, two common nonsynonymous SNPs, rs3734354 (p.P352T) and rs3734355 (p.A371V), were identified in either a heterozygous (10.6%

(20/94)) or homozygous (3.2% (3/94)) state and are in complete linkage disequilibrium (LD) with each other. Furthermore, the rare variant rs3734354 resulting in the amino-acid substitution p.P352S (Minor allele frequency (MAF) in an European Non-Finnish population = 0.03% (ExAC)) has also been identified in one of our 94 PWL patients. *In silico* analysis using a set of three different prediction programs (PolyPhen-2[29], Mutationtaster[30] and SIFT[31]) showed conflicting results. However Mutationtaster categorized the variant as 'disease causing'. Finally, an intronic variant near exon six (c.544-15A>C, rs3734353), which was associated with various adiposity measures previously[32], was detected in heterozygous and homozygous state in 32 (17.0%) and 7 (7.4%) PWL patients, respectively. An overview of the identified nonsynonymous coding variants found in *SIM1* in our PWL cohort is shown in table 4.

3.2.1. p.P352S variant

The patient, in whom we discovered the p.P352S variant, is a ten-year-old boy presenting with early-onset extreme obesity, intellectual disability and behavior and learning deficits. In addition, the patient shows small hands and feet and an acceleration in bone age. He was clinically diagnosed with PWS although this could not be confirmed molecularly as no aberration on chromosome 15q11.2 – q13 was detected with MS-MLPA. Results from the genome-wide HumanOmniExpress-24 analysis also show no deletions at the 6q14.1 – q16.3 region. As no family members were willing to cooperate for further research, it was not possible to determine whether the variant segregates with obesity in this family.

3.3. Mutation analysis of MRAP2

Mutation analysis of the *MRAP2* gene in both the PWL and the obese children and adolescents cohort led to the identification of various sequence variants. We found two common synonymous variants, c.603C>T (p.H201H, rs2875382) and c.477C>T (p.N159N, rs9449776), either in heterozygous or homozygous state in both study populations. We identified the heterozygous p.H201H variant in 18.6% of the PWL

cohort and in 16.1% of the obese children and adolescents. The homozygous form was found in 3.2% of PWL patients and 4.4% of the obese cohort. The heterozygous and homozygous p.N159N variant, on the other hand, was found in 9.0% (PWL), 8.1% (obese children and adolescents) and 0% (PWL), 0.6% (obese children and adolescents), respectively. This SNP is in complete LD with the intronic variant rs9449777.

Additionally, a total of two rare nonsynonymous coding variants were detected (Table 5). The c.374G>A (p.R125H, rs115655382) was found in two PWL patients (MAF = 1.1%) and in two obese children and adolescents (MAF = 0.3%). *In silico* analysis using PolyPhen-2, Mutationtaster and SIFT, however, showed that this variant is predicted to be benign by all programs. The final variant, c.118G>T, which results in the substitution of an alanine to a serine on position 40 (p.A40S), was identified in only one PWL patient and is shown to be (probably) damaging by *in silico* analysis. In addition, the variant is reported in only one European (Non-Finnish) individual in the ExAC database. Since this database cannot release phenotype information about the screened individuals, no conclusions regarding causality of this variant can be drawn. Finally, an intronic splice site variant c.127+7T>G (rs112932891) was identified in 0.5% of PWL patients and 1.2% of the obese children's cohort. These MAFs are comparable to those reported in the ExAC database.

3.3.1. p.A40S variant

The patient harboring the p.A40S variant is a ten-year-old boy who suffers from overall obesity in combination with intellectual disability. The clinically diagnosed PWS could not be confirmed molecularly with MS-MLPA and CNV analysis of the 6q14.1 – q16.3 region also showed no deletions. As no further family data were available, it was not possible to determine whether the variant segregates with obesity in this family.

Table 4. Identified nonsynonymous coding variants in *SIM1* in 94 PWL patients.

For each variant the minor allele frequency (MAF) is calculated in percentage and compared with the MAF reported in the Exome Aggregation Consortium (ExAC) Browser (Beta) (Accessed on the 13th of November 2015). *In silico* analysis data are shown for all variants.

SIM1						
Amino acid substitution	Nucleotide substitution	MAF (%) PWL cases (N = 94)	MAF (%) ExAC (European (Non-Finnish))	Polyphen-2	Mutationtaster	SIFT
p.A371V	c.1112C>T	13.8	14.1	benign	polymorphism	tolerated
p.P352T	c.1054C>A	13.8	14.2	benign	polymorphism	tolerated
p.P352S	c.1054C>T	0.5	0.03	benign	disease causing	tolerated

Table 5. Identified nonsynonymous coding variants in *MRAP2* in 94 PWL patients and 363 obese children and adolescents.

For each variant the minor allele frequency (MAF) is calculated in percentage and compared with the MAF reported in the Exome Aggregation Consortium (ExAC) Browser (Beta) (Accessed on the 13th of November 2015). *In silico* analysis data are shown for all variants.

MRAP2							
Amino acid substitution	Nucleotide substitution	MAF (%) PWL cases (N = 94)	MAF (%) Obese cases (N = 363)	MAF (%) ExAC (European (Non-Finnish))	Polyphen-2	Mutationtaster	SIFT
p.R125H	c.374G>A	1.1	0.3	0.13	benign	polymorphism	tolerated
p.A40S	c.118G>T	0.5	0	0.0015	probably damaging	disease causing	damaging

4. Discussion

Formerly, the Prader Willi *like* (PWL) phenotype has been associated with deletions at chromosome 6q14.1 – q15 (*MRAP2*) and 6q16.2 – q16.3 (*SIM1*) which creates the hypothesis that the 6q14.1 – q16.3 region could be involved in the pathogenesis of the PWL phenotype and more specifically in obesity. Wentzel *et al.* used array comparative genomic hybridization to identify two interstitial deletions at chromosome 6q14.1 – q15 of 8.73 and 4.50 Mb in two children with obesity and developmental delay. In the 4.2 Mb region of overlap the *MRAP2* gene was located[19]. As mentioned earlier, the identification of a *de novo* balanced translocation between chromosome 1p22.1 and 6q16.2 (*SIM1*) in an eighteen month old girl with early-onset severe obesity led to the identification of haploinsufficiency of the *SIM1* gene as a possible cause of obesity[14]. Subsequently, 16 case reports of PWL patients presenting with deletions encompassing the *SIM1* gene appeared in literature[6-13]. In our genome-wide microarray analysis, no gene harboring deletions could be identified at the 6q14.1 – q16.3 region in our study population of 109 PWL patients. This outcome is in line with the study conducted by Varela and colleagues in which they screened 87 PWL patients and merely one patient harbored a deletion at the *SIM1* gene[9]. Consequently we started mutation analysis of the two interesting genes located in the 6q14.1 – q16.3 region.

Screening of *SIM1* in the PWL cohort is not only interesting because of the reported *SIM1* deletions, but also for its involvement in (1) the development and function of the paraventricular nucleus of the hypothalamus and (2) the leptin-melanocortin signaling pathway (Figure 1). The hypothesis that SIM1 plays a role downstream of MC4R in this pathway to control food intake is also supported by different mouse models[33-35]. In addition, several research groups (including our own) described loss-of-function point mutations and variants in *SIM1* in cohorts of obese individuals. In 2013, Bonnefond and colleagues[16] identified four rare variants in a cohort of 44 children with PWL features (including severe obesity). A year later, the same research group described two novel *SIM1* mutations in a cohort of 283 overweight children with developmental

delay[18]. None of these variants were found in one of our 94 PWL patients. They also discovered four other variants in a cohort of 568 morbidly obese adults[16] which were different to the four variants identified in our own cohort of 561 obese children and adolescents[15]. Ramachandrappa *et al.* also screened the coding region of *SIM1* in 2,100 patients with severe, early-onset obesity and identified thirteen variants. Like Bonnefond *et al.*, they concluded that the identified variants in *SIM1* segregate with obesity but with variable penetrance[16, 17]. Overall, in literature a possible causative mutation in the *SIM1* gene could be identified in less than 2% of all screened PWL patients reported.

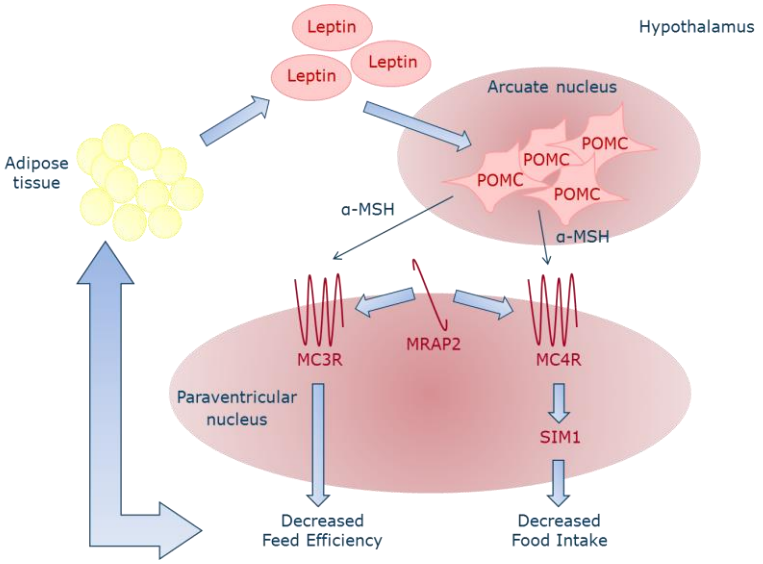


Figure 1. Schematic view of a part of the leptin-melanocortin signaling pathway and the possible roles of MRAP2 and SIM1.

Leptin is secreted by adipose tissue in proportion to the amount of body fat, binds to its receptors in the arcuate nucleus of the hypothalamus and stimulates the anorectic neurons expressing POMC. Next, POMC is cleaved in different biologically active proteins of which α-MSH is the most important. This melanocortin stimulates MC3R and MC4R in the paraventricular nucleus and regulates energy homeostasis by decreasing feed efficiency and food intake. The proposed stimulating effect of the single-pass membrane MRAP2 on MC3R and MC4R signaling in the paraventricular nucleus of the hypothalamus and the downstream role of SIM1 still needs to be fully elucidated. POMC = pro-opiomelanocortin, α-MSH = alfa-melanocyte stimulating hormone, MC3R = melanocortin-3 receptor, MC4R = melanocortin-4 receptor, MRAP2 = melanocortin-2 receptor accessory protein 2, SIM1 = single-minded 1.

Comparing these results with our own mutation analysis in a cohort of 94 PWL patients, we identified one interesting variant (rs3734354) resulting in the amino-acid substitution p.P352S in one PWL patient. Prediction programs regarding this variant are very conflicting and the substitution was reported in merely 19/66010 alleles in the ExAC database. As this database cannot release phenotype information about the screened individuals, no conclusions regarding pathogenicity of this variant can be drawn. Further research of p.P352S is necessary to determine its possible causative effect.

Further investigation into the role of the two common nonsynonymous coding SNPs, rs3734354 (p.P352T) and rs3734355 (p.A371V), was performed by several research groups[32, 36, 37]. Two research groups observed that homozygosity for the p.P352T/p.A371V haplotype is associated with higher BMI in white males[32, 36]. The homozygous p.P352T and p.A371V variants in our PWL cohort were also identified in three white males, which might be involved in the development of their obesity.

Next to these nonsynonymous coding variants also the intronic variant rs3734353 (c.544-15A>C) was frequently identified in our PWL cohort. In 32 patients (17.0%) the variant was found heterozygously and in 7 patients (7.4%) homozygously. Significant association of, predominantly, the homozygous form of this SNP with various adiposity measures was previously described in two different study populations (black males[32] and Pima Indians[38]). However, this could not be replicated in a French Caucasian case/control cohort of 602 unrelated severely obese children, 673 unrelated morbidly obese adults, and 1,395 unrelated non-obese control subjects[38]. In addition, the MAF of the homozygous rs3734353 (7.4%) variant in our cohort is comparable to the MAF found in the ExAC database (8.0%).

Due to the modulating role of MRAP2 in GPCR trafficking, ligand binding and signaling to the cell surface and its expression in the paraventricular nucleus of the hypothalamus, it is thought to be an accessory protein for MC3R and MC4R, two key players in the leptin-melanocortin signaling pathway and consequently energy

homeostasis[39] (Figure 1). Based on *in vitro* studies it is hypothesized that MRAP2 would play a stimulating effect on signaling of these two melanocortin receptors[20, 39]. Furthermore, Asai *et al.* also indicated that inactivation of the *MRAP2* gene in mice can induce obesity. According to that study, mice with overall or brain-specific targeted ($Sim1^{Cre}$::$Mrap2^{flox/flox}$) knock-out of the gene become extremely obese on regular chow diet and show age-dependent hyperphagia[20].

The same research group performed mutation analysis in 864 severe early-onset obese individuals of the Genetics of Obesity Study (GOOS) cohort (N = 488) and the Swedish obese children's cohort (N = 376). They identified four rare heterozygous variants in four unrelated, nonsyndromic, severely obese individuals. Two of these variants were predicted to be possibly damaging by *in silico* analysis (p.N88Y and p.R125C), but the p.E24X variant, found in a 19-year-old male subject with a BMI of 63 kg/m², is clearly disruptive[20]. This variant is located in exon 2 of the *MRAP2* gene just like the (probably) damaging variant we identified in our PWL patient, p.A40S. Additionally, it is plausible that the substitution to the serine amino acid (AA) can result in an alteration of the protein structure of MRAP2. Finally, the AA substitution also occurs close to the transmembrane domain (AA 45-65), which could have an effect on the expression of MRAP2 on the membrane or on its stimulating effect on MC3R and MC4R signaling. Further functional analysis is necessary to investigate these effects and to indicate a possible role of *MRAP2* in the pathogenesis of the PWL phenotype, albeit in a limited number of patients as for the *SIM1* gene.

5. Conclusion

In conclusion, comparing the previously published CNV and mutation analysis results with the screening performed in our own cohort of 109 PWL patients, it is suggested that the association of the 6q14.1 – q16.3 genes *SIM1* and *MRAP2* with the PWL phenotype is rather limited.

Disclosure statement: The authors declare no conflict of interest.

Acknowledgements

This study was supported by an Interuniversity Attraction Pole Project (Phase VII project 43, BELSPO) and a TOP project from the University of Antwerp to WVH. EG designed the study, carried out experiments and wrote the manuscript. DZ, SB and WVH contributed to the discussion. AV, GM, KVH, SV and LVG recruited and phenotyped subjects. WVH designed the study and wrote the manuscript. All authors read and approved the final version of the manuscript.

References

1. Kitahara, C.M., et al., *Association between class III obesity (BMI of 40-59 kg/m2) and mortality: a pooled analysis of 20 prospective studies.* PLoS Med, 2014. **11**(7): p. e1001673.
2. Kopelman, P.G., *Obesity as a medical problem.* Nature, 2000. **404**(6778): p. 635-643.
3. Zegers, D., et al., *Monogenic and Complex Forms of Obesity: Insights from Genetics Reveal the Leptin-Melanocortin Signaling Pathway as a Common Player.* Critical Reviews in Eukaryotic Gene Expression, 2012. **22**(4): p. 325-343.
4. Hinney, A., A.L. Volckmar, and N. Knoll, *Melanocortin-4 receptor in energy homeostasis and obesity pathogenesis.* Prog Mol Biol Transl Sci, 2013. **114**: p. 147-91.
5. Driscoll, D.J., et al., *Prader-Willi Syndrome*, in *GeneReviews(R)*, M.P. Adam, et al., Editors. 1993: Seattle (WA).
6. Bonaglia, M.C., et al., *Detailed phenotype-genotype study in five patients with chromosome 6q16 deletion: narrowing the critical region for Prader-Willi-like phenotype.* Eur J Hum Genet, 2008. **16**(12): p. 1443-9.
7. Rosenfeld, J.A., et al., *Genotype-phenotype correlation in interstitial 6q deletions: a report of 12 new cases.* Neurogenetics, 2012. **13**(1): p. 31-47.
8. Faivre, L., et al., *Deletion of the SIM1 gene (6q16.2) in a patient with a Prader-Willi-like phenotype.* J Med Genet, 2002. **39**(8): p. 594-6.
9. Varela, M.C., et al., *A new case of interstitial 6q16.2 deletion in a patient with Prader-Willi-like phenotype and investigation of SIM1 gene deletion in 87 patients with syndromic obesity.* Eur J Med Genet, 2006. **49**(4): p. 298-305.
10. Klein, O.D., et al., *Interstitial deletions of chromosome 6q: genotype-phenotype correlation utilizing array CGH.* Clinical Genetics, 2007. **71**(3): p. 260-266.
11. Spreiz, A., et al., *Phenotypic variability of a deletion and duplication 6q16.1 --> q21 due to a paternal balanced ins(7;6)(p15;q16.1q21).* Am J Med Genet A, 2010. **152A**(11): p. 2762-7.
12. Izumi, K., et al., *Endocrine phenotype of 6q16.1-q21 deletion involving SIM1 and Prader-Willi syndrome-like features.* Am J Med Genet A, 2013. **161A**(12): p. 3137-43.
13. Vignoli, A., et al., *Interstitial 6q microdeletion syndrome and epilepsy: a new patient and review of the literature.* Am J Med Genet A, 2013. **161A**(8): p. 2009-15.
14. Holder, J.L., N.F. Butte, and A.R. Zinn, *Profound obesity associated with a balanced translocation that disrupts the SIM1 gene.* Human Molecular Genetics, 2000. **9**(1): p. 101-108.
15. Zegers, D., et al., *Mutation screen of the SIM1 gene in pediatric patients with early-onset obesity.* Int J Obes (Lond), 2014. **38**(7): p. 1000-4.
16. Bonnefond, A., et al., *Loss-of-function mutations in SIM1 contribute to obesity and Prader-Willi-like features.* J Clin Invest, 2013. **123**(7): p. 3037-41.

17. Ramachandrappa, S., et al., *Rare variants in single-minded 1 (SIM1) are associated with severe obesity.* J Clin Invest, 2013. **123**(7): p. 3042-50.
18. Montagne, L., et al., *Identification of Two Novel Loss-of-Function SIM1 Mutations in Two Overweight Children with Developmental Delay.* Obesity, 2014. **22**(12): p. 2621-2624.
19. Wentzel, C., et al., *Interstitial Deletions at 6q14.1-q15 Associated with Obesity, Developmental Delay and a Distinct Clinical Phenotype.* Mol Syndromol, 2010. **1**(2): p. 75-81.
20. Asai, M., et al., *Loss of Function of the Melanocortin 2 Receptor Accessory Protein 2 Is Associated with Mammalian Obesity.* Science, 2013. **341**(6143): p. 275-278.
21. Bittel, D.C., N. Kibiryeva, and M.G. Butler, *Methylation-specific multiplex ligation-dependent probe amplification analysis of subjects with chromosome 15 abnormalities.* Genet Test, 2007. **11**(4): p. 467-75.
22. Beckers, S., et al., *Identification and functional characterization of novel mutations in the melanocortin-4 receptor.* Obes Facts, 2010. **3**(5): p. 304-11.
23. Roelants, M., R. Hauspie, and K. Hoppenbrouwers, *References for growth and pubertal development from birth to 21 years in Flanders, Belgium.* Annals of Human Biology, 2009. **36**(6): p. 680-694.
24. Miller, S.A., D.D. Dykes, and H.F. Polesky, *A Simple Salting out Procedure for Extracting DNA from Human Nucleated Cells.* Nucleic Acids Research, 1988. **16**(3): p. 1215-1215.
25. Vandeweyer, G., et al., *CNV-WebStore: online CNV analysis, storage and interpretation.* BMC Bioinformatics, 2011. **12**: p. 4.
26. Reed, G.H. and C.T. Wittwer, *Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis.* Clinical Chemistry, 2004. **50**(10): p. 1748-1754.
27. MacDonald, J.R., et al., *The Database of Genomic Variants: a curated collection of structural variation in the human genome.* Nucleic Acids Res, 2014. **42**(Database issue): p. D986-92.
28. *Exome Aggregation Consortium (ExAC), Cambridge, MA*. [cited 2015 April]; Available from: http://exac.broadinstitute.org.
29. Adzhubei, I.A., et al., *A method and server for predicting damaging missense mutations.* Nat Methods, 2010. **7**(4): p. 248-9.
30. Schwarz, J.M., et al., *MutationTaster2: mutation prediction for the deep-sequencing age.* Nat Methods, 2014. **11**(4): p. 361-2.
31. Ng, P.C. and S. Henikoff, *Predicting deleterious amino acid substitutions.* Genome Res, 2001. **11**(5): p. 863-74.
32. Swarbrick, M.M., et al., *Replication and Extension of Association Between Common Genetic Variants in SIM1 and Human Adiposity.* Obesity, 2011. **19**(12): p. 2394-2403.
33. Michaud, J.L., et al., *Sim1 haploinsufficiency causes hyperphagia, obesity and reduction of the paraventricular nucleus of the hypothalamus.* Human Molecular Genetics, 2001. **10**(14): p. 1465-1473.

34. Tolson, K.P., et al., *Postnatal Sim1 Deficiency Causes Hyperphagic Obesity and Reduced Mc4r and Oxytocin Expression.* Journal of Neuroscience, 2010. **30**(10): p. 3803-3812.
35. Kublaoui, B.M., et al., *SIM1 overexpression partially rescues agouti yellow and diet-induced obesity by normalizing food intake.* Endocrinology, 2006. **147**(10): p. 4542-4549.
36. Hung, C.C.C., et al., *Studies of the SIM1 gene in relation to human obesity and obesity-related traits.* International Journal of Obesity, 2007. **31**(3): p. 429-434.
37. Ghoussaini, M., et al., *Analysis of the SIM1 Contribution to Polygenic Obesity in the French Population.* Obesity, 2010. **18**(8): p. 1670-1675.
38. Traurig, M., et al., *Common Variation in SIM1 Is Reproducibly Associated With BMI in Pima Indians.* Diabetes, 2009. **58**(7): p. 1682-1689.
39. Chan, L.F., et al., *MRAP and MRAP2 are bidirectional regulators of the melanocortin receptor family.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(15): p. 6146-6151.

CHAPTER 4

DNA SEQUENCING AND COPY NUMBER VARIATION ANALYSIS OF MCHR2 IN A COHORT OF PRADER WILLI LIKE (PWL) PATIENTS

Ellen Geets[1], Evi Aerts[1], An Verrijken[2,3], Kim Van Hoorenbeeck[4], Stijn Verhulst[4], Luc Van Gaal[2,3], Wim Van Hul[1]

[1]Department of Medical Genetics, University of Antwerp, Antwerp, Belgium
[2]Department of Endocrinology, Diabetology and Metabolic Diseases, Antwerp University Hospital, Antwerp, Belgium
[3] Laboratory of Experimental Medicine and Paediatrics, Faculty of Medicine and Health Sciences, University of Antwerp, Antwerp, Belgium.
[4]Department of Paediatrics, Antwerp University Hospital, Antwerp, Belgium

Obesity Research and Clinical Practice, 2017, in press, doi: 10.1016/j.orcp.2017.10.001

Abstract

Background: Prader Willi Syndrome (PWS) is a syndromic form of obesity caused by a chromosomal aberration on chromosome 15q11.2 – q13. Patients with a comparable phenotype to PWS not carrying the 15q11.2 – q13 defect are classified as Prader Willi *like* (PWL). In literature, PWL patients do frequently harbor deletions at 6q16, which led to the identification of the *single-minded 1* (*SIM1*) gene as a possible cause for the presence of obesity in these patients. However, our previous work in a PWL cohort showed a rather limited involvement of *SIM1* in the obesity phenotype. In this paper, we investigated the causal role of the *melanin-concentrating hormone receptor 2* (*MCHR2*) gene in PWL patients, as most of the reported 6q16 deletions also encompass this gene and it is suggested to be active in the control of feeding behavior and energy metabolism.

Methods: Copy number variation analysis of the *MCHR2* genomic region followed by mutation analysis of *MCHR2* was performed in a PWL cohort.

Results: Genome-wide microarray analysis of 109 patients with PWL did not show any gene harboring deletions on chromosome 6q16. Mutation analysis in 92 patients with PWL demonstrated three *MCHR2* variants: p.T47A (c.139A>G), p.A76A (c.228T>C) and c.*16A>G. We identified a significantly higher prevalence of the c.228T>C C allele in our PWL cohort compared to previously published results and controls of the ExAC Database.

Conclusion: Overall, our results are in line with some previously performed studies suggesting that *MCHR2* is not a major contributor to human obesity and the PWL phenotype.

Keywords: Prader Willi *like*; obesity; *MCHR2*; microarray; mutation analysis

1. Introduction

Since 1980, the prevalence of obesity is more than doubled. The disease also contributes to several adverse complications like cardiovascular disease, type II diabetes mellitus and different types of cancer[1]. Due to the increased risk of developing these comorbidities and the high, still increasing, prevalence, obesity has become a major health burden worldwide and consequently represents a significant expenditure of national health-care budgets[2].

Prader Willi syndrome (PWS) is the most common form of syndromic obesity with a prevalence of 1 in 10,000 – 30,000 individuals. The disease is mainly characterized by feeding difficulties followed by hyperphagia and excessive weight gain, intellectual disability, severe neonatal hypotonia, hypogonadism, characteristic facial features like almond-shaped eyes and downturned corners of the mouth and behavioral problems. Also small hands and feet and short stature are frequently described features. PWS is caused by the absence of expression of the paternal genes from the imprinted chromosomal region 15q11.2 – q13. Imprinting refers to an epigenetic process which leads to parent-of-origin specific gene expression during development. As a consequence some genes from the 15q11.2 – q13 region only show expression for the paternal gene copy, others only for the maternal copy. In case of PWS, normal expression of the paternal genes on chromosome 15q11.2 – q13 is lost due to the presence of a paternal deletion (70-75%), maternal uniparental disomy (20-25%) characterized by two maternal copies and lacking of a paternal copy or an imprinting defect of the critical region (1-3%)[3].

However, once a clinical diagnosis of PWS cannot be confirmed by molecular analysis of the 15q11.2 – q13 locus with the use of methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), these patients are classified as Prader Willi *like* (PWL). Clinically but also genetically the PWL phenotype is heterogeneous as it has already been associated with copy number variations (CNVs) on different chromosomes such as 1p36.3[4], 2p21[5], 3p26.3[6, 7], 6q[6, 8-20], 9q34[21], 10q26[22], 12q[22] and X[22] as well as maternal uniparental disomy of chromosome 14[22]. Noteworthy,

PWL (and subsequently PWS) also shares clinical characteristics (obesity and intellectual disability) with the chromosome 16p11.2 deletion syndrome, which has been reported as a cause of obesity[23].

In literature, deletions at chromosome 6q are most frequently reported in association with PWL which led to the suggestion of haploinsufficiency (presence of only one active copy) of the *single-minded 1* (*SIM1*) gene at 6q16.3 as a possible cause for the presence of obesity in these patients[6, 8-20]. However, our own research showed a limited involvement of copy number and sequence variation in *SIM1* in the PWL phenotype[24]. In addition, most reported deletions in the 6q region in patients with PWL also encompass the *melanin-concentrating hormone receptor 2* (*MCHR2*) gene. *MCHR2*, located at chromosome 6q16.2 and within 1 Megabase (Mb) of *SIM1*, encodes a G protein-coupled receptor for melanin-concentrating hormone (MCH). MCH is a neuropeptide that plays an important role in the control of feeding behavior and energy metabolism, which ultimately also indicates a possible involvement of MCHR2 in these processes [14, 25, 26]. To date, little is known about the physiological role of MCHR2 as *MCHR2* is only expressed in higher animals such as primates and carnivores and not in rodents which are mainly used for animal experiments[27]. However, mice overexpressing *MCH* in the hypothalamus develop obesity and insulin resistance[28] whereas mice lacking the *MCH* gene are hypophagic and lean[29]. MCH also acts as a functional antagonist of the α-melanocyte stimulating hormone (α-MSH) in the leptin-melanocortin pathway in the hypothalamus which is a key pathway in maintaining energy homeostasis[30]. Strikingly, *MCHR2* is also highly expressed in the brain, including the arcuate and ventromedial nucleus of the hypothalamus[31, 32].

This all suggests that MCHR2 might also play a role in the leptin-melanocortin pathway and therefore we hypothesize that PWL might be a contiguous gene syndrome and that CNVs involving *MCHR2* may explain the presence of obesity in patients with a PWL phenotype and consequently could also be a contributor to human obesity in general. Alternatively, mutations in *MCHR2* could have a pleiotropic effect resulting in the PWL phenotype.

2. Materials and methods

2.1. Study populations

Over the last 25 years a cohort of 109 patients who were referred to our Department of Medical Genetics (University of Antwerp and University Hospital Antwerp, Antwerp, Belgium) with a putative diagnosis of PWS or PWL syndrome was ascertained. All selected patients were reported with at least a combination of obesity and developmental delay or intellectual disability. In some cases also behavioral problems (32/109) and hypotonia (20/109) were identified. Table 1 shows an overview of the population characteristics of the 109 PWL patients which is based on obtained data from the corresponding physicians. To exclude the most common cause of obesity, the patients were screened for causal mutations in the *melanocortin 4 receptor* (*MC4R*) gene as described earlier[33]. No mutations were identified in any of the patients. The clinical diagnosis of PWS could be excluded molecularly with the use of MS-MLPA[34] as no defect could be identified in the 15q11.2 – q13 region. Unfortunately, grades of obesity, developmental delay/intellectual disability and hypotonia of our patients with PWL are not known, thus the patients could not be grouped based on their clinical features.

This study protocol was approved by the local ethics committee and all participants gave their written informed consent.

Table 1. Population characteristics of the PWL cohort for which genome-wide microarray analysis was performed.

Parameters	Prader Willi *like* patients
N	109
Age (years)	11.5 ± 8.4
Male/Female	70/39
15q11.2-13 defect	No
Obesity	109/109
Developmental delay/Intellectual disability	109/109
Behavioral problems	32/109
Hypotonia	20/109
Small hands/feet	14/109
Hypogonadism	12/109
Seizures	9/109
Short stature	5/109
Hearing loss	1/109

Mean value ± standard deviation of mean is given

2.2. Genome-wide microarray analysis

A genome-wide microarray analysis approach was performed on the 109 patients to identify CNVs encompassing the *MCHR2* gene. As described previously, two different BeadChips were used[24].

Overall, genomic DNA from the patients was isolated from whole blood according to standard procedures[35]. 200 ng of this DNA was denatured and isothermally amplified in an overnight step at 37°C in an Illumina Hybridization Oven (Illumina, San Diego, CA,

USA). The amplified product is then enzymatically fragmented using end-point fragmentation to prevent over-fragmentation. After isopropanol precipitation and DNA resuspension in hybridization buffer, the amplified and fragmented DNA samples are applied to the BeadChip and incubated at 37°C for 16 to 24 hours. During this hybridization samples anneal to locus-specific 50-mer oligonucleotides which are linked to one of up to 713,014 bead types (HumanOmniExpress-24). One bead type corresponds to each allele per SNP locus. Next, allelic specificity is conferred by enzymatic base extension and subsequently products are fluorescently stained in capillary flow-through chambers. Through the single-base extension of the oligonucleotides, using the captured DNA as template, detectable labels are incorporated dependent on the SNP involved. The intensities of the products' fluorescence are detected using the iScan Reader (Illumina, San Diego, CA, USA) and eventually these raw data are processed using the Illumina GenomeStudio software and analyzed further by the in-house CNV-Webstore software[36].

2.3. Mutation analysis

For 92 out of the 109 patients, sufficient DNA was available for mutation screening of all coding exons and intron-exon boundaries of the *MCHR2* gene. The genomic DNA was isolated from whole blood according to standard procedures[35]. *MCHR2* has two different transcripts (GenBank NM_001040179.1 and NM_032503.2) encoding one protein isoform. It consists of six exons of which five are coding. We designed six PCR amplicons in order to perform mutation screening of all five coding exons and their intron-exon boundaries by high-resolution melting curve analysis (HRM) in combination with Sanger sequencing. HRM, which is accomplished using the Lightcycler LC480 Real-Time PCR System (Roche, Penzberg, Germany), is a highly specific and sensitive technique with high sample throughput[37]. The amplification of the six amplicons was achieved either by a standard or a touchdown PCR protocol. Real-time PCR was performed in a total reaction volume of 10 µl. The amplification mixture included 10 ng of template DNA, 1.5 mM $MgCl_2$, 0.15 mM dNTP's, 500 nM

primers, 0.015 U/µl GoTaq (Promega Corporation, Madison, WI, USA), 1x GoTaq buffer and the saturating dye LCGreen Plus (Idaho Technology, Salt Lake City, UT, USA) in a 0.5x concentration. After amplification, DNA samples were denatured at 95°C, rapidly reannealed at 40°C and subsequently heated from 70°C – 90°C measuring fluorescence constantly. For DNA samples with melting patterns deviating from wild-type, Sanger sequencing was performed with ABI BigDye Terminator v3.1 Cycle Sequencing kits on an ABI Prism Genetic Analyzer 3130xl (Applied Biosystems Inc, Foster City, CA, USA) in order to confirm and characterize putative sequence variations. Primer sequences for HRM and sequencing are available upon request.

3. Results

3.1. Genome-wide microarray analysis

Genome-wide microarray analysis of 109 patients with PWL resulted in some CNVs but did not show any structural variation encompassing the *MCHR2* gene (table 2). In one PWL patient a heterozygous deletion with a maximum size of 26,707 base pairs (bp) was identified at approximately 4 Mb distance from *MCHR2* (figure 1). This deletion is benign as no genes were located in this deletion and the CNV was already described previously in healthy control samples in the Database of Genomic Variance (DGV)[38].

Table 2. Genome-wide microarray results: Overview of identified copy number variations (CNVs) associated with the PWL phenotype in 109 patients with PWL.

Chromosomal region	Copy number variation	Size (bp*)	# patients(**)
1p36.33 (chr1:742,286-781,258)	heterozygous deletion	38,971	1
1p36.33 (chr1:1,227,897-1,246,972)	heterozygous deletion	19,074	1$^{\Delta}$
1p36.33 (chr1:1,258,246-1,295,323)	heterozygous deletion	37,076	2$^{\Delta,\square}$
1p36.33 (chr1:1,385,211-1,425,700)	heterozygous deletion	40,489	1
1p36.33 (chr1:1,598,908-1,916,587)	heterozygous deletion	317,679	1
1p36.33 (chr1:1,615,348-1,680,219)	heterozygous deletion	64,870	2
1p36.33 (chr1:1,647,686-1,664,124)	homozygous deletion	16,438	1
2p21 (chr2:44,578,898-44,757,217)	heterozygous deletion	178,320	1
3p26.3 (chr3:330,037-337,727)	heterozygous deletion	7,690	1
6q14.1 (chr6:77,299,933-77,324,522)	heterozygous deletion	24,590	1
6q14.1 (chr6:77,299,933-77,327,833)	heterozygous deletion	27,901	1
6q14.1 (chr6:77,299,933-77,341,104)	heterozygous deletion	41,170	2^
6q14.1 (chr6:81,460,295-81,597,546)	heterozygous duplication	137,252	1
6q15 (chr6:91,978,768-92,132,799)	heterozygous deletion	154,032	1
6q16.1 (chr6:94,603,083-94,646,136)	Heterozygous deletion	43,050	1°
6q16.3 (chr6:104,560,332-104,587,040)	heterozygous deletion	26,707	1
9q34.3 (chr9:139,552,461-139,570,617)	heterozygous deletion	18,156	1$^{\square}$
9q34.3 (chr9:139,921,814-139,930,945)	heterozygous deletion	9,131	1$^{\square}$
9q34.3 (chr9:140,301,447-140,329,141)	heterozygous deletion	27,694	1$^{\square}$

9q34.3 (chr9:139,995,065-140,218,978)	heterozygous deletion	223,912	1
16p11.2 (chr16:28,484,556-30,192,561)	heterozygous deletion	1,708,006	1
16p11.2 (chr16:28,825,605-29,043,450)	heterozygous deletion	217,846	2
16p11.2 (chr16:29,595,483-30,198,151)	heterozygous deletion	602,669	2
Xp22.33 (chrX:62,321-2,697,868)	heterozygous duplication	2,635,548	1°
Xp22.33 (chrX:704,713-725,756)	heterozygous duplication	21,042	1
Xp22.33 (chrX:1,382,562-1,497,530)	heterozygous duplication	114,967	1
Xp22.33 (chrX:1,478,557-1,504,864)	heterozygous duplication	26,306	1×
Xp22.33 (chrX:3,438,945-3,656,805)	heterozygous duplication	217,860	1
Xp22.11 (chrX:22,950,303-23,010,125)	homozygous deletion	59,822	1
Xp22.11 (chrX:23,063,058-23,125,967)	heterozygous deletion	62,908	1^
Xp11.23 (chrX:47,861,829-48,044,615)	homozygous deletion	182,785	1
Xq22.1 (chrX:99,849,017-99,870,208)	homozygous deletion	21,190	1
Xq23 (chrX:111,704,851-111,751,715)	homozygous deletion	46,863	1×
Xq28 (chrX:151,489,659-152,105,473)	heterozygous duplication	615,813	1
Xq28 (chrX:154,939,018-155,236,747)	heterozygous duplication	297,730	1°
Xq28 (chrX:155,077,792-155,104,608)	heterozygous duplication	26,815	1

*bp = base pairs, ** = number of patients, Δ = copy number variations were found in the same patient (patient A), □ = copy number variations were found in the same patient (patient B), ^ = copy number variations were found in the same patient (patient C), ° = copy number variations were found in the same patient (patient D), × = copy number variations were found in the same patient (patient E).

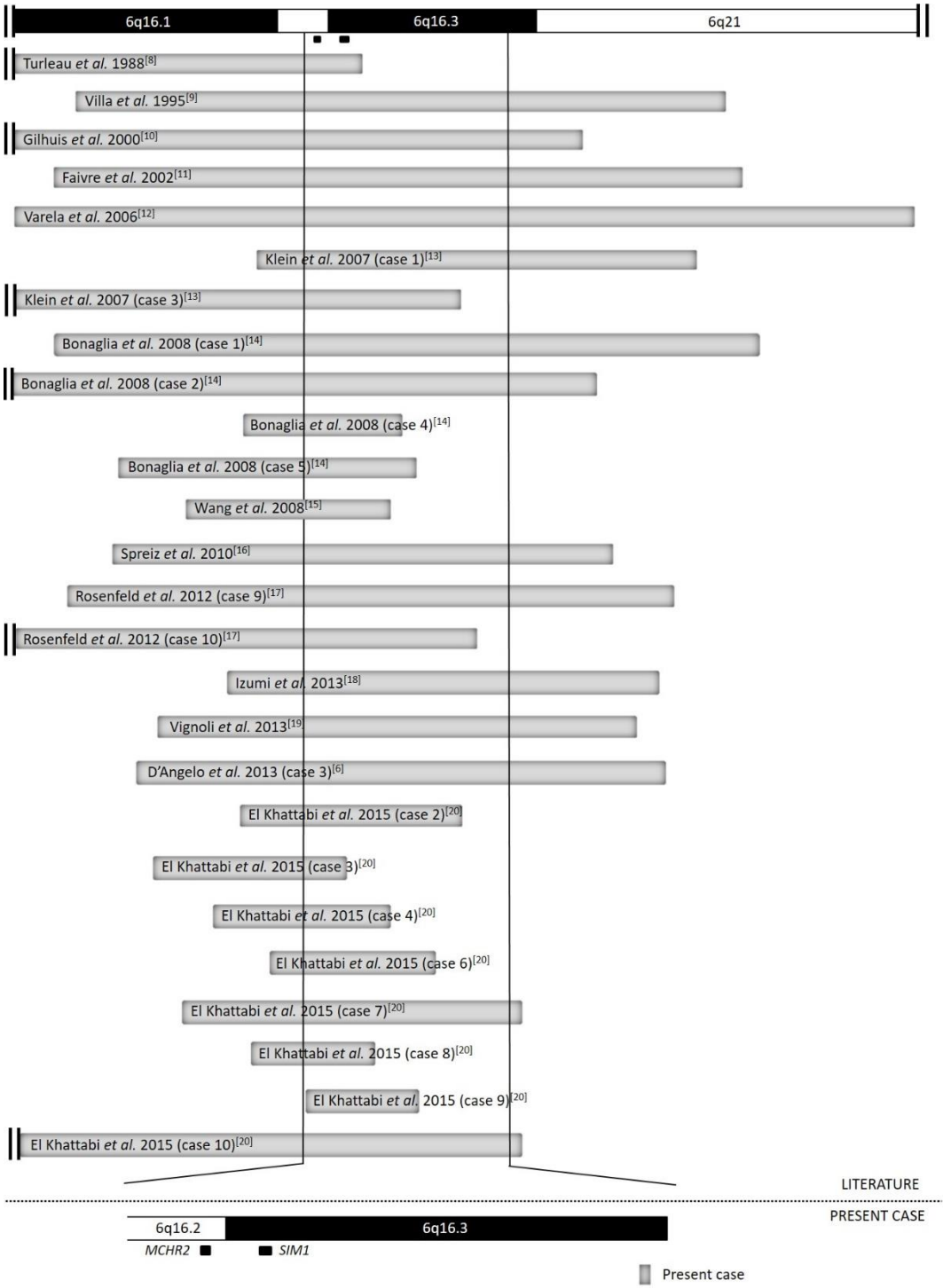


Figure 1. Schematic overview of 6q deletions in Prader Willi *like* (PWL) patients encompassing *SIM1* and *MCHR2* reported in literature supplemented with our identified deletion in one PWL patient located at approximately 4 Mb distance from *MCHR2* with a maximum size of 26,707 base pairs.

3.2. Mutation analysis of MCHR2

Sequence analysis of the *MCHR2* gene in 92 patients with PWL led to the identification of a rare nonsynonymous variant c.139A>G (p.T47A, rs199555524), a synonymous variant c.228T>C (p.A76A, rs74531963) and a 3' UTR variant (c.*16A>G). The rare variant rs199555524 resulting in the amino-acid substitution p.T47A has been reported in an European Non-Finnish population in the Exome Aggregation Consortium (ExAC) Browser (Beta) with a low minor allele frequency (MAF) of 0.06% (Accessed on the 11th of May 2016). *In silico* analysis using a set of four different prediction programs (PolyPhen-2[39], Mutationtaster[40], SIFT[41] and SNPs&GO[42]) showed conflicting results ranging from 'benign' to 'deleterious'. The patient, in whom we discovered the p.T47A variant, is a nine-year-old girl presenting increasing obesity in combination with learning deficits. As no further family data nor DNA were available, it was not possible to determine whether the variant segregates with obesity in this family or whether it might be a *de novo* variant not present in the parents.

The synonymous variant p.A76A was identified in either heterozygous (12.5% (23/92)) or homozygous (3.3% (3/92)) state in a total of 26 patients with PWL (Supplementary table 1). Using Pearson's chi square analysis we compared these results with the MAFs in the ExAC Database (8.1% and 0.95% respectively). We detected a significant difference for the heterozygous variant (p = 0.04) and a trend towards significance (p = 0.08) for the homozygous variant.

Supplementary table 1. Overview of gender, age and clinical characteristics of the 26 patients harboring a heterozygous or homozygous p.A76A variant.

Patient	p.A76A variant	Gender (M/F*)	Age (years)	Obesity	ID/DD°	Additional characteristics
1	heterozygous	M	10.7	+	+	hypogonadism
2	heterozygous	M	12.3	+	+	/
3	heterozygous	M	39.2	+	+	/
4	heterozygous	F	13.0	+	+	/
5	homozygous	M	12.0	+	+	hyperphagia

6	heterozygous	F	36.6	+	+	/
7	heterozygous	M	18.9	+	+	behavioral problems
8	heterozygous	F	5.9	+	+	dysmorphic features, ADHD[×]
9	heterozygous	M	11.3	+	+	behavioral problems
10	heterozygous	M	4.9	+	+	/
11	heterozygous	M	12.1	+	+	pronounced appetite
12	heterozygous	M	8.0	+	+	hypogonadism, hyperphagia, small hands and feet
13	heterozygous	M	10.3	+	+	seizures, aggressive
14	heterozygous	F	8.3	+	+	/
15	homozygous	M	12.8	+	+	behavioural problems, dysmorphic features, tics
16	heterozygous	M	1.9	+	+	hypogonadism
17	heterozygous	F	4.6	+	+	speech problems
18	heterozygous	M	14.0	+	+	ADHD
19	heterozygous	F	31.0	+	+	behavioural problems, automutilation, hirsutism
20	heterozygous	M	7.5	+	+	small stature, aggressive
21	heterozygous	M	6.5	+	+	pronounced appetite, tantrums, brachydactyly, partial syndactyly of toes
22	homozygous	M	17.8	+	+	small stature, dysmorphic features, pronounced appetite, small hands and feet
23	heterozygous	F	9.7	+	+	small hands, ADHD, tics
24	heterozygous	M	9.8	+	+	/
25	heterozygous	F	0.3	+	+	/
26	heterozygous	F	31.8	+	+	pronounced appetite

*M = Male, F = Female; °ID = Intellectual disability, DD = Developmental delay; [×]ADHD = Attention Deficit Hyperactivity Disorder

Finally, the 3'UTR variant c.*16A>G was detected in heterozygous state in a nineteen-year-old male who suffers from obesity in combination with intellectual disability. The variant is not yet reported in the ExAC Database. Since the 3'UTR region plays a role in

translation efficiency, localization, and stability of the mRNA, we investigated the c.*16 position for RNA binding protein and microRNA (miRNA) binding sites, conservation and the presence of AU-rich elements (AREs) with the use of The Atlas of UTR Regulatory Activity (AURA) version 2.4.2. A thin grey bar spanning the entire UTR demonstrated the presence of a binding site for miRNA 'hsa-miR-335-5p', however the precise location of binding is not known[43]. In addition, mutation screening of the lean brother and mother with obesity showed that the variant was also present in the lean brother indicating that the variant does not segregate with obesity in this family and in all probability does not explain the presence of obesity or PWL in the patient. An overview of the identified variants found in *MCHR2* in our PWL cohort is shown in table 3.

Table 3. Identified variants in *MCHR2* in 92 patients with PWL.

For each variant the minor allele frequency (MAF) is calculated in percentage and compared with the MAF reported in the Exome Aggregation Consortium (ExAC) Browser (Beta) (Accessed on the 11th of May 2016). *In silico* analysis data are shown for the nonsynonymous variant.

MCHR2							
Amino acid substitution	Nucleotide substitution	MAF (%) PWL cases (N = 92)	MAF (%) ExAC (European (Non-Finnish))	Polyphen-2	Mutationtaster	SIFT	SNPs&GO
p.T47A	c.139A>G	0.5	0.06	Benign	Polymorphism	Deleterious	Neutral
p.A76A	c.228T>C	15.8	9.1	/	/	/	/
/	c.*16A>G	0.5	0	/	/	/	/

4. Discussion

The phenotypic aspects of our 109 patients led to the initial clinical suspicion of PWS. This diagnosis was ruled out since no genetic defect could be identified at chromosome 15 and therefore the patients were classified as PWL. As mentioned in the introduction, not only clinically but also genetically the PWL phenotype is heterogeneous and has in literature been associated with CNVs on different chromosomes such as 1p36.3, 2p21, 3p26.3, 6q, 9q34, 10q26, 12q and X as well as maternal uniparental disomy of chromosome 14. For 1p36.3, we did identify seven different deletions in a total of nine patients with PWL. However, these CNVs have a maximum size of 317 kilobases (kb) and all but one (the 16 kb homozygous deletion) were already described previously in healthy control samples in DGV. In addition, the ones reported in literature are also much larger with a size of 4 to 6 Mb[4]. In one patient with PWL we identified a heterozygous deletion at 2p21 of approximately 180 kb encompassing the *prolyl endopeptidase-like* (*PREPL*) and *Calmodulin-Lysine N-Methyltransferase* (*CAMKMT*) genes though the CNVs reported in literature are either homozygous or compound heterozygous[5]. Noteworthy, this patient also harbored the disease causing mutation p.S127L in *MC4R*. One small deletion of 7,690 bp at 3p26.3 (*Cell adhesion molecule L1 like* (*CHL1*) gene) was found in one patient. However, D'Angelo *et al.*[6] and Ben-Abdallah-Bouhjar *et al.*[7] reported disease causing deletions of 1.2 Mb and 1.05 Mb respectively in two patients with a PWL phenotype. A similar situation is true for 9q34 where reported CNVs are a couple of Mb in size[21] whereas the ones identified in two of our patients have a maximum size of only 223 kb. As reviewed by Rocha and Paiva[22], different alterations on chromosome X have been associated with the PWL phenotype, however none of these were identified in our population. Nevertheless, one patient harboured a 2.6 Mb duplication at Xp22.33 encompassing 24 genes, whereas in another patient a smaller duplication of 217 kb at Xp22.33 was found. However, both CNVs did not overlap. The patient with the 2.6 Mb duplication also harboured a 297 kb duplication at Xq28. Lastly, a duplication of 19 genes at Xq28 with a maximum size of 615 kb was identified in one of our other

patients with PWL. To our knowledge all four CNVs were not yet reported in association with the PWL phenotype previously. We did not identify any structural variations on chromosome 10q26, 12q subtelomere deletions nor maternal uniparental disomy of chromosome 14. Noteworthy, we also noticed the presence of one unique and two recurrent 16p11.2 deletions in a total of five patients with PWL. In literature, 16p11.2 deletions have already been associated with obesity and intellectual disability and would in all probability explain the phenotype in these patients[23]. In two patients a 593kb deletion was found. Two others harboured a 220kb deletion encompassing the *Sarcoma* (*Src*) *homology 2B adaptor protein 1* (*SH2B1*) gene and in one patient a larger deletion of 1.7Mb overlapping both deletions was identified.

Still, deletions of *SIM1* at 6q16.3 are most frequently reported in association with PWL and have been proposed as a possible cause for the presence of obesity in these patients. However our own CNV and mutation analysis of *SIM1* showed a limited involvement of the gene in the PWL phenotype[24]. In addition, the *MCHR2* gene is also located in most reported deletions in the 6q region discovered in patients with PWL. In combination with its receptor activity for MCH, *MCHR2* could possibly be involved in the control of feeding behavior and energy metabolism[14, 25, 26]. Other research groups also noticed that *MCHR2* is an interesting candidate gene for human obesity. In 2004, Gibson and colleagues performed mutation-analysis of the *MCHR2* gene among 106 subjects with severe early onset obesity and a history of hyperphagia. However, they only identified two rare noncoding variants in *MCHR2* indicating that mutations in *MCHR2* are not commonly found in humans with severe early onset obesity[44]. Ghoussaini *et al.*, on the other hand, performed case control and family-based studies. They identified an association with childhood obesity for a variant in the promoter region (-38,245 ATG A/G) and the p.A76A T allele comparing their frequencies in obese and non-obese individuals. Additional research of 645 trios of obese children and their parents further supported the p.A76A T/C association, showing an overtransmission of the at risk T allele to children with obesity[25]. Conversely, in our

study, we noticed a significantly higher presence of the C allele in the PWL cohort compared to controls of the ExAC Database. In addition, in contrary to their association study, Ghoussaini and colleagues were not able to replicate the association of the p.A76A T/C with childhood obesity in an independent French case-control study encompassing 1573 subjects[25]. If we combine all results regarding the p.A76A variant, it is not clear whether a real association with human obesity of either the T or C allele is present and additional research will be necessary to elucidate these contradictory results.

Lastly, a study performed in New Jersey identified a total of eight polymorphisms in *MCHR2* in a cohort of white and African-American individuals (n = 91) of which four were located in coding regions. Two of these coding SNPs produce amino acid substitutions (p.R63K and p.R152Q). Functional analysis was performed for both variants to determine the effect of the presence of the variant on the binding and intracellular signaling characteristics of the MCHR2 receptor. However, no alteration in function was observed compared to wild-type[45].

Noteworthy is that none of these research groups identified the p.T47A variant we identified in a nine-year-old girl with obesity in combination with learning deficits. However, as no family members were willing to cooperate for further DNA analysis, segregation of the variant with obesity in this family could not be determined. In addition, only one prediction program (SIFT) categorized the variant as 'deleterious' indicating that further research of this variant would be less interesting.

Overall, our results confirm the conclusion of three previously performed studies that *MCHR2* is not a major contributor to human obesity or PWL. Further research of the p.A76A variant in either individuals with obesity or a PWL cohort would be interesting to clarify the contradictory findings.

Our data generated with the micro-array analysis support the heterogeneity of molecular mechanisms involved in the PWL phenotype. Previous studies reported

already more than 20 different CNVs in PWL patients, most of them found in only a few patients. Our study could not provide strong support for involvement for any of these including the most common CNV on chromosome 6q16. This is probably due to the heterogeneous character of the PWL phenotype. Our PWL cohort is recruited over the past 25 years in which all patients are reported with at least a combination of obesity and developmental delay/intellectual disability and in some cases also hypotonia or behavioral problems. However, obesity, developmental delay, intellectual disability, behavioral problems and/or hypotonia are relatively frequent features in the general population. Also other syndromic forms of obesity are associated with developmental delay or intellectual disability in combination with obesity including the 16p11.2 deletion syndrome. This leads to the fact that the risk for misdiagnosing PWL patients is relatively high and explains why 5 out of 109 patients of our cohort harbored a 16p11.2 deletion. These results show that there is an urgent need to differentiate all syndromic forms of obesity more clearly in order to correctly diagnose the patients.

Conflict of Interest statement: The authors declare no conflict of interest.

Acknowledgements

This study was supported by an Interuniversity Attraction Pole Project (Phase VII project 43, BELSPO) and a TOP project from the University of Antwerp to WVH. EG designed the study, carried out experiments and wrote the manuscript. EA and WVH contributed to the discussion. AV, KVH, SV and LVG recruited and phenotyped subjects. WVH designed the study and wrote the manuscript. All authors read and approved the final version of the manuscript.

References

1. (WHO), W.H.O. *World Health Organisation (WHO)*. 2016 June; Available from: http://www.who.int/en/.
2. Kopelman, P.G., *Obesity as a medical problem.* Nature, 2000. **404**(6778): p. 635-643.
3. Driscoll, D.J., et al., *Prader-Willi Syndrome*, in *GeneReviews(R)*, R.A. Pagon, et al., Editors. 1993: Seattle (WA).
4. Tsuyusaki, Y., et al., *1p36 deletion syndrome associated with Prader-Willi-like phenotype.* Pediatr Int, 2010. **52**(4): p. 547-50.
5. Martens, K., et al., *PREPL: a putative novel oligopeptidase propelled into the limelight.* Biol Chem, 2006. **387**(7): p. 879-83.
6. D'Angelo, C.S., et al., *Obesity with associated developmental delay and/or learning disability in patients exhibiting additional features: report of novel pathogenic copy number variants.* Am J Med Genet A, 2013. **161A**(3): p. 479-86.
7. Ben-Abdallah-Bouhjar, I., et al., *Chromosomal microarray analysis of functional Xq27-qter disomy and deletion 3p26.3 in a boy with Prader-Willi like features and hypotonia.* Eur J Med Genet, 2012. **55**(8-9): p. 461-5.
8. Turleau, C., et al., *6q1 monosomy: a distinctive syndrome.* Clin Genet, 1988. **34**(1): p. 38-42.
9. Villa, A., et al., *De novo interstitial deletion q16.2q21 on chromosome 6.* Am J Med Genet, 1995. **55**(3): p. 379-83.
10. Gilhuis, H.J., et al., *Interstitial 6q deletion with a Prader-Willi-like phenotype: a new case and review of the literature.* Eur J Paediatr Neurol, 2000. **4**(1): p. 39-43.
11. Faivre, L., et al., *Deletion of the SIM1 gene (6q16.2) in a patient with a Prader-Willi-like phenotype.* J Med Genet, 2002. **39**(8): p. 594-6.
12. Varela, M.C., et al., *A new case of interstitial 6q16.2 deletion in a patient with Prader-Willi-like phenotype and investigation of SIM1 gene deletion in 87 patients with syndromic obesity.* Eur J Med Genet, 2006. **49**(4): p. 298-305.
13. Klein, O.D., et al., *Interstitial deletions of chromosome 6q: genotype-phenotype correlation utilizing array CGH.* Clin Genet, 2007. **71**(3): p. 260-6.
14. Bonaglia, M.C., et al., *Detailed phenotype-genotype study in five patients with chromosome 6q16 deletion: narrowing the critical region for Prader-Willi-like phenotype.* Eur J Hum Genet, 2008. **16**(12): p. 1443-9.
15. Wang, J.C., et al., *A 5-Mb microdeletion at 6q16.1-q16.3 with SIM gene deletion and obesity.* Am J Med Genet A, 2008. **146A**(22): p. 2975-8.
16. Spreiz, A., et al., *Phenotypic variability of a deletion and duplication 6q16.1 --> q21 due to a paternal balanced ins(7;6)(p15;q16.1q21).* Am J Med Genet A, 2010. **152A**(11): p. 2762-7.
17. Rosenfeld, J.A., et al., *Genotype-phenotype correlation in interstitial 6q deletions: a report of 12 new cases.* Neurogenetics, 2012. **13**(1): p. 31-47.

18. Izumi, K., et al., *Endocrine phenotype of 6q16.1-q21 deletion involving SIM1 and Prader-Willi syndrome-like features.* Am J Med Genet A, 2013. **161A**(12): p. 3137-43.
19. Vignoli, A., et al., *Interstitial 6q microdeletion syndrome and epilepsy: a new patient and review of the literature.* Am J Med Genet A, 2013. **161A**(8): p. 2009-15.
20. El Khattabi, L., et al., *Incomplete penetrance and phenotypic variability of 6q16 deletions including SIM1.* Eur J Hum Genet, 2015. **23**(8): p. 1010-8.
21. Cormier-Daire, V., et al., *Cryptic terminal deletion of chromosome 9q34: a novel cause of syndromic obesity in childhood?* J Med Genet, 2003. **40**(4): p. 300-3.
22. Rocha, C.F. and C.L. Paiva, *Prader-Willi-like phenotypes: a systematic review of their chromosomal abnormalities.* Genet Mol Res, 2014. **13**(1): p. 2290-8.
23. *OMIM: CHROMOSOME 16p11.2 DELETION SYNDROME, 220-KB*. [cited 2017 May]; Available from: https://www.omim.org/entry/613444.
24. Geets, E., et al., *Copy number variation (CNV) analysis and mutation analysis of the 6q14.1-6q16.3 genes SIM1 and MRAP2 in Prader Willi like patients.* Mol Genet Metab, 2016. **117**(3): p. 383-8.
25. Ghoussaini, M., et al., *Genetic study of the melanin-concentrating hormone receptor 2 in childhood and adulthood severe obesity.* J Clin Endocrinol Metab, 2007. **92**(11): p. 4403-9.
26. Pissios, P. and E. Maratos-Flier, *Melanin-concentrating hormone: from fish skin to skinny mammals.* Trends Endocrinol Metab, 2003. **14**(5): p. 243-8.
27. Tan, C.P., et al., *Melanin-concentrating hormone receptor subtypes 1 and 2: species-specific gene expression.* Genomics, 2002. **79**(6): p. 785-92.
28. Ludwig, D.S., et al., *Melanin-concentrating hormone overexpression in transgenic mice leads to obesity and insulin resistance.* J Clin Invest, 2001. **107**(3): p. 379-86.
29. Shimada, M., et al., *Mice lacking melanin-concentrating hormone are hypophagic and lean.* Nature, 1998. **396**(6712): p. 670-4.
30. Gonzalez, M.I., S. Vaziri, and C.A. Wilson, *Behavioral effects of alpha-MSH and MCH after central administration in the female rat.* Peptides, 1996. **17**(1): p. 171-7.
31. An, S., et al., *Identification and characterization of a melanin-concentrating hormone receptor.* Proc Natl Acad Sci U S A, 2001. **98**(13): p. 7576-81.
32. Sailer, A.W., et al., *Identification and characterization of a second melanin-concentrating hormone receptor, MCH-2R.* Proc Natl Acad Sci U S A, 2001. **98**(13): p. 7564-9.
33. Beckers, S., et al., *Identification and functional characterization of novel mutations in the melanocortin-4 receptor.* Obes Facts, 2010. **3**(5): p. 304-11.
34. Bittel, D.C., N. Kibiryeva, and M.G. Butler, *Methylation-specific multiplex ligation-dependent probe amplification analysis of subjects with chromosome 15 abnormalities.* Genet Test, 2007. **11**(4): p. 467-75.

35. Miller, S.A., D.D. Dykes, and H.F. Polesky, *A Simple Salting out Procedure for Extracting DNA from Human Nucleated Cells.* Nucleic Acids Research, 1988. **16**(3): p. 1215-1215.
36. Vandeweyer, G., et al., *CNV-WebStore: online CNV analysis, storage and interpretation.* BMC Bioinformatics, 2011. **12**: p. 4.
37. Reed, G.H. and C.T. Wittwer, *Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis.* Clinical Chemistry, 2004. **50**(10): p. 1748-1754.
38. MacDonald, J.R., et al., *The Database of Genomic Variants: a curated collection of structural variation in the human genome.* Nucleic Acids Res, 2014. **42**(Database issue): p. D986-92.
39. Adzhubei, I.A., et al., *A method and server for predicting damaging missense mutations.* Nat Methods, 2010. **7**(4): p. 248-9.
40. Schwarz, J.M., et al., *MutationTaster2: mutation prediction for the deep-sequencing age.* Nat Methods, 2014. **11**(4): p. 361-2.
41. Ng, P.C. and S. Henikoff, *Predicting deleterious amino acid substitutions.* Genome Res, 2001. **11**(5): p. 863-74.
42. *SNPs&GO*. [cited 2016 June]; Available from: http://snps.biofold.org/snps-and-go/snps-and-go.html.
43. Dassi, E., et al., *AURA 2: Empowering discovery of post-transcriptional networks.* Translation (Austin), 2014. **2**(1): p. e27738.
44. Gibson, W.T., et al., *Melanin-concentrating hormone receptor mutations and human obesity: functional analysis.* Obes Res, 2004. **12**(5): p. 743-9.
45. Hawes, B.E., et al., *Identification and characterization of single-nucleotide polymorphisms in MCH-R1 and MCH-R2.* Obes Res, 2004. **12**(8): p. 1327-34.

CHAPTER 5

STRUCTURAL AND MUTATION SCREENING INDICATES NO MAJOR INVOLVEMENT OF OXT AND OXTR IN PRADER WILLI LIKE PATIENTS

Ellen Geets[1], Evi Aerts[1], An Verrijken[2,3], Kim Van Hoorenbeeck[4], Stijn Verhulst[4], Luc Van Gaal[2,3], Wim Van Hul[1]

[1]Department of Medical Genetics, University of Antwerp, Antwerp, Belgium
[2]Department of Endocrinology, Diabetology and Metabolic Diseases, Antwerp University Hospital, Antwerp, Belgium
[3] Laboratory of Experimental Medicine and Paediatrics, Faculty of Medicine and Health Sciences, University of Antwerp, Antwerp, Belgium.
[4]Department of Paediatrics, Antwerp University Hospital, Antwerp, Belgium

Submitted in Endocrine Research

Abstract

Background: Literature study showed an interesting role of *oxytocin* (*OXT*) and *oxytocin receptor* (*OXTR*) in energy homeostasis and a link with the Prader Willi Syndrome (PWS). Apparently, PWS patients, sharing clinical characteristics with Prader Willi *like* (PWL) patients, show a lower amount of oxytocin-producing neurons (42%) in the hypothalamus leading to speculation that a reduction in *OXT* signaling might contribute to the presence of overweight/obesity in these patients. Consequently, we hypothesized that a reduction in *OXT* or even *OXTR* signaling could also be present in PWL patients possibly due to a mutation or deletion in one or both genes.

Methods: Genome-wide microarray analysis was performed in a group of 109 PWL patients. Subsequently, we screened 91 PWL patients for mutations in *OXT* and *OXTR* using high-resolution melting curve analysis and Sanger sequencing.

Results: No structural variations encompassing *OXT* or *OXTR* were identified in our 109 PWL patients. Additional mutation analysis only revealed known intronic (*OXT* and *OXTR*) or (non)synonymous variants (*OXTR*).

Conclusion: Our copy number variation and mutation analysis showed no major involvement of *OXT* and *OXTR* in PWL patients. Additional research upstream of *OXT* in the hypothalamic leptin-melanocortin signaling pathway would be an interesting next step.

Keywords: Microarray; mutation analysis; obesity; *Oxytocin*; *Oxytocin receptor;* Prader Willi *like*

1. Introduction

Prader Willi syndrome (PWS), the most common form of syndromic obesity with a prevalence of 1 in 10.000 – 30.000 individuals, is characterized by obesity, neonatal hypotonia, hypogonadism, intellectual disability and characteristic facial features like almond-shaped eyes and downturned corners of the mouth. It is caused by the absence of expression of the paternal genes on the imprinting region 15q11.2 – q13 due to a paternal deletion (70-75%), maternal uniparental disomy (20-25%) or an imprinting defect of the critical region (1-3%)[1]. In rare cases also a balanced or unbalanced translocation could be responsible for the PWS phenotype[2]. However, not all patients with PWS characteristics show this defect on chromosome 15 and are therefore classified as Prader Willi *like* (PWL). For several years, the PWL phenotype is mainly associated with deletions at chromosome 6q16.1 - 6q16.3 ultimately leading to the identification of haploinsufficiency of the *SIM1* gene as a possible cause for the presence of obesity in these patients[3-10]. However, our own research showed a rather limited involvement of structural and genetic variation in *SIM1* with the PWL phenotype[11]. Also mutation and copy number variation (CNV) screening of other genes, *MRAP2* and *MCHR2*, located at the 6q region in close proximity to *SIM1* did only show a few variants with unknown influence[11, 12]. For this reason we decided to look beyond chromosome 6q and performed further literature study. This search revealed a possible interesting role of *oxytocin* (*OXT*) and *oxytocin receptor* (*OXTR*) in the regulation of appetite. Both genes are expressed in the hypothalamus, a region in which the leptin-melanocortin signaling pathway, an important pathway in energy homeostasis, is active[13, 14]. In fact, in this pathway α-melanocyte-stimulating hormone (α-MSH), besides being an important stimulator of the melanocortin 4 receptor (MC4R), also induces dendritic secretion of oxytocin[15]. Even more interesting is that PWS patients show a significant reduction in the number of oxytocin-producing neurons (42%) in the paraventricular nucleus (PVN) of the hypothalamus leading to speculation that a reduction in *OXT* signaling might contribute to the presence of obesity in these patients[16]. Moreover, also mouse models show a role of *OXT* and

OXTR in obesity. Kublaoui and colleagues identified a decrease by 80% of the Oxt mRNA and peptide levels in *Sim1* deficient mice[17] and $Oxt^{-/-}$ as well as $Oxtr^{-/-}$ mice develop late-onset obesity without any difference in food intake or motor activity[18, 19]. Noteworthy is that recent studies indicate OXT as a possible medicine to treat obesity. Chronic subcutaneous or intranasal administration of OXT decreases food intake leading to the reversal of the obese phenotype in diet-induced obese mice, rats as well as non-human primates[20-23]. E.g., treating *Sim1* deficient mice with Oxt decreases food intake and weight gain. Administration of an Oxtr antagonist, on the other hand, showed a hypersensitive reaction of the $Sim1^{+/-}$ mice to the orexigenic effect[17]. Treatment of prediabetic obese humans with chronic subcutaneous or intranasal OXT also led to effective body weight loss[23, 24]. Interestingly, Wheeler *et al.* also identified CNVs disrupting the *OXTR* gene in patients with extreme early-onset obesity and developmental delay[25].

Therefore, we hypothesized that a reduction in *OXT* or *OXTR* signaling is present in PWL patients possibly due to the presence of a mutation or deletion at *OXT* or *OXTR*. Subsequently, we decided to be the first to perform CNV analysis followed by mutation analysis of both genes in a cohort of PWL patients.

2. Material and methods

2.1. Study populations

A genome-wide microarray analysis was initially performed in a cohort of 109 patients who were referred to our Department of Medical Genetics (University of Antwerp, Antwerp, Belgium) over the last 25 years with a putative diagnosis of PWS or PWL syndrome from several hospitals from the Antwerp area. All selected patients were reported with at least a combination of obesity and developmental delay or intellectual disability. In some cases also behavioral problems (32/109) and hypotonia (20/109) were identified. The clinical diagnosis of PWS could be excluded molecularly with the use of a methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)[26] and mutation analysis of *MC4R*, performed as described

earlier[27], showed no mutations in any of the patients. Table 1 shows an overview of the population characteristics of the 109 PWL patients which is based on obtained data from the corresponding physicians. For 91 out of 109 patients sufficient DNA was available for mutation screening of *OXT* and *OXTR*.

This study protocol was approved by the local ethics committee and all participants gave their written informed consent.

Table 1. Population characteristics of the PWL cohort for which genome-wide microarray analysis was performed.

Parameters	Prader Willi *like* patients
N	109
Age (years)	11.5 ± 8.4
Male/Female	70/39
15q11.2-13 defect	No
Obesity	109/109
Developmental delay/Intellectual disability	109/109
Behavioral problems	32/109
Hypotonia	20/109
Small hands/feet	14/109
Hypogonadism	12/109
Seizures	9/109
Short stature	5/109
Hearing loss	1/109

Mean value ± standard deviation of mean is given

2.2. Genome-wide microarray analysis

A genome-wide microarray analysis approach was used to identify CNVs encompassing the *OXT* and *OXTR* gene. As described previously, two different BeadChips were used[11].

Overall, genomic DNA from the patients was isolated from whole blood according to standard procedures[28] of which 200 ng was denatured and isothermally amplified in an overnight step at 37°C in an Illumina Hybridization Oven (Illumina, San Diego, CA, USA). Then, using end-point fragmentation to prevent over-fragmentation, the amplified product is enzymatically fragmented. Subsequently, the amplified and fragmented DNA samples are applied to the BeadChip and incubated at 37°C for 16 to 24 hours after isopropanol precipitation and DNA resuspension in hybridization buffer. During the hybridization, samples anneal to locus-specific 50-mer oligonucleotides which are linked to one of up to 713,014 bead types (HumanOmniExpress-24). One bead type corresponds to each allele per SNP locus. Next, allelic specificity is conferred by enzymatic single-base extension and subsequently products are fluorescently stained in capillary flow-through chambers. Detectable labels are incorporated dependent on the SNP involved, using the captured DNA as template. With the use of the iScan Reader (Illumina, San Diego, CA, USA) the intensities of the products' fluorescence are detected and eventually processed using the Illumina GenomeStudio software and analyzed further by the in-house CNV-Webstore software[29].

2.3. Mutation analysis

Screening of all coding exons and intron-exon boundaries of the *OXT* (Genbank NM_000915.3) and *OXTR* (Genbank NM_000916.3) genes was executed on genomic DNA isolated from whole blood according to standard procedures[28]. *OXT* consists of three coding exons and *OXTR* consists of four exons of which two are coding. Respectively, we designed three and four PCR amplicons in order to perform mutation screening of all coding exons and the intron-exon boundaries of both genes by high-

resolution melting curve analysis (HRM) in combination with Sanger sequencing. HRM is a highly specific and sensitive technique with high sample throughput which is accomplished using the Lightcycler LC480 Real-Time PCR System (Roche, Penzberg, Germany)[30]. The amplification of the seven amplicons was achieved either by a standard or a touchdown PCR protocol. Real-time PCR was performed in a total reaction volume of 10 µl. The amplification mixture included 10 ng of template DNA, 1.5 mM $MgCl_2$, 0.15 mM dNTP's, 500 nM primers, 0.015 U/µl GoTaq (Promega Corporation, Madison, WI, USA), 1x GoTaq buffer and the saturating dye LCGreen Plus (Idaho Technology, Salt Lake City, UT, USA) in a 0.5x concentration. After amplification, DNA samples were denatured at 95°C, rapidly reannealed at 40°C and subsequently heated from 70°C – 90°C measuring fluorescence constantly. Sanger sequencing was performed with ABI BigDye Terminator v3.1 Cycle Sequencing kits on an ABI Prism Genetic Analyzer 3130xl (Applied Biosystems Inc, Foster City, CA, USA) for DNA samples with melting patterns deviating from wild-type in order to confirm and characterize putative sequence variations. Primer sequences for HRM and sequencing are available upon request.

3. Results

3.1. Genome-wide microarray analysis

Genome-wide microarray analysis of 109 PWL patients did not show any structural variation encompassing the *OXT* or *OXTR* gene.

3.2. Mutation analysis of OXT

Mutation analysis of the *OXT* gene in 91 PWL patients only led to the identification of five intronic variants rs528977569 (c.120+46del), rs144102485 (c.120+131C>T), rs6139004 (c.120+145A>G), rs111869749 (c.121-137A>G) and rs34097556 (c.322+27_322+28insA). The first variant was identified in three out of 91 PWL patients with a corresponding minor allele frequency (MAF) of 1.6%. The

c.120+131C>T variant was detected in heterozygous state in 17 patients (MAF = 9.3%). With the use of a two-tailed Fisher's exact test, we identified that the MAFs of both variants were not significantly higher in our population compared with the MAFs reported in the European population in the Genome Aggregation Database (gnomAD) Browser Beta[31] and the 1000 Genomes Project[32] (Accessed on the 3th of January 2018). The rs6139004 variant was identified in either a heterozygous (30/91) or homozygous (6/91) state in a total of 36 PWL patients resulting in a MAF of 23.1%. c.121-137A>G was found in 17 patients of which one time in a homozygous state (MAF = 9.9%). Finally, the insertion of an adenine (A) intronically between position 322+27 and 322+28 was also noticed. We detected the heterozygous c.322+27_322+28insA variant in 29 out of 91 PWL patients and the homozygous form in three patients with a resulting MAF of 19.2%. The MAFs of the latter three variants are comparable to those reported in the gnomAD and 1000 Genomes databases. An overview of the identified variants found in *OXT* in our PWL cohort is shown in table 2.

Table 2. Identified variants in *OXT* in 91 PWL patients.
For each variant the minor allele frequency (MAF) is calculated in percentage and compared with the MAF reported in the European population in the Genome Aggregation Database (gnomAD) Browser Beta or the Database of Short Genetic Variation (dbSNP), 1000 Genomes (Accessed on the 3th of January 2018).

OXT

Rs-number	Nucleotide substitution	MAF (%) PWL cases (N = 91)	MAF (%) gnomAD (European (Non-Finnish))	MAF (%) 1000 Genomes (European)
rs528977569	c.120+46del	1.6	0.5	0.8
rs144102485	c.120+131C>T	9.3	6.9	6.9
rs6139004	c.120+145A>G	23.1	20.6	20.3
rs111869749	c.121-137A>G	9.9	7.1	9.2
rs34097556	c.322+27_322+28insA	19.2	17.1	16.3

3.3. Mutation analysis of OXTR

Mutation screening of all coding exons and intron-exon boundaries of *OXTR* in 91 PWL patients revealed various sequence variants. We identified two common intronic variants, c.-142-35G>T (rs237913) and c.-135T>C (rs237911), either in heterozygous (18.1% and 10.4%) or homozygous (14.3% and 1.1%) state. Additionally, we found two common synonymous variants, p.N57N (c. c.171T>C, rs2228485) and p.N230N (c.690C>T, rs237902), and one rare synonymous variant p.S368S (c.1104G>A, rs148736817). The heterozygous p.N57N variant was identified in 18.1% of our PWL cohort and the homozygous form in 2.2%. The heterozygous and homozygous p.N230N variant, on the other hand, was found in 20.9% and 16.5% respectively. The p.S368S was detected heterozygously in only one PWL patient with a corresponding MAF of 0.5%. MAFs of both intronic and all synonymous variants are comparable to those reported in the gnomAD and the 1000 Genomes databases. Also two nonsynonymous variants were identified, p.A218T (c.652G>A, rs4686302) and p.A238T (c.712G>A, rs61740241). Both variants were found in a heterozygous state with a MAF of 8.8% and 3.3% respectively. *In silico* analysis using PolyPhen-2[33], Mutationtaster[34] and SIFT[35], however, showed that this variants are predicted to be benign by all programs. In addition, both variants encompass similar MAFs compared to the public databases gnomAD and 1000 Genomes. Table 3 shows an overview of all variants identified in the *OXTR* gene in our PWL cohort.

Table 3. Identified variants in *OXTR* in 91 PWL patients.

For each variant the minor allele frequency (MAF) is calculated in percentage and compared with the MAF reported in the European population in the Genome Aggregation Database (gnomAD) Browser Beta or the Database of Short Genetic Variation (dbSNP), 1000 Genomes (Accessed on the 3th of January 2018). *In silico* analysis data are shown for the nonsynonymous variants. *Combined Annotation Dependent Depletion (CADD) – PHRED score.

OXTR

Amino acid substitution	Nucleotide substitution	MAF (%) PWL cases (N = 91)	MAF (%) gnomAD (European (Non-Finnish))	MAF (%) 1000 Genomes (European)	Polyphen-2	Mutationtaster	SIFT	CADD – PHRED*
/	c.-142-35G>T	32.4	27.2	28.5	/	/	/	/
/	c.-135T>C	11.5	17.1	14.8	/	/	/	/
p.N57N	c.171T>C	20.3	24.6	24.0	/	/	/	/
p.A218T	c.652G>A	8.8	12.1	11.8	Benign	Polymorphism	Tolerated	17.5
p.N230N	c.690C>T	37.4	34.2	32.7	/	/	/	/
p.A238T	c.712G>A	3.3	3.2	2.8	Benign	Polymorphism	Tolerated	8.1
p.S368S	c.1104G>A	0.5	0.3	0.4	/	/	/	/

4. Discussion

As our previous mutation and CNV analysis of the 6q region encompassing the *SIM1*, *MRAP2* and *MCHR2* genes in 109 PWL patients did not reveal an involvement with obesity[11, 12], we decided to look beyond this region and searched for other interesting genes. This literature study revealed an interesting role of *OXT* and *OXTR* in energy homeostasis, since 1) both genes are expressed in a region where the leptin-melanocortin signaling pathway is active[13, 14], 2) mouse models deficient in *Oxt* or *Oxtr* show late-onset obesity without any difference in food intake or motor activity[18, 19] and, even more interesting, 3) OXT is indicated as a possible treatment for obesity in animal models[20-23] as well as in prediabetic obese humans[23, 24]. Also a marked deficiency in oxytocin-producing neurons (42%) in the PVN of the hypothalamus is detected in PWS patients speculating that *OXT* deficiency could play a role in the obesity phenotype present in these patients. As PWL patients share clinical characteristics with PWS we hypothesized that a reduction in *OXT* or even *OXTR* signaling could also be present in PWL patients due to a mutation or deletion in one of the genes. Unfortunately, our CNV and mutation analysis of *OXT* and *OXTR* in 109 PWL patients did not reveal any structural variations in or near both genes and only known intronic (*OXT* and *OXTR*) or (non-)synonymous variants (*OXTR*) were discovered with the use of Sanger sequencing, indicating that both genes are no major contributors to human obesity in PWL patients.

Also striking is that, in contrast to the positive effects of OXT administration (intranasal or subcutaneous) in diet-induced obese animal models and prediabetic obese humans on body weight, a double-blind randomized controlled trial of oxytocin nasal spray in twenty-two Australian PWS patients was unable to demonstrate a beneficial effect. The authors dedicated the lack of effect to a deficiency of the hypothalamic oxytocin-producing neurons to exogenously administered oxytocin due to the fact that PWS patients have a significant reduction in the number of these neurons in the PVN[36]. However, almost all participants in this study showed additional characteristics as for example depression, schizophrenia, psychosis and behavioral disorders which could be

potential confounding factors and which is why patients with these characteristics are excluded in an ongoing clinical OXT trial in PWS patients[36, 37]. A possible additional reason for the lack of effect on food intake is the age category (12 – 30 years) in which Einfeld and colleagues performed their research. A recent study also investigated the effects of oxytocin in PWS patients on hyperphagia and only found a promising effect on food-related behavior in children with PWS younger than 11 years of age and not in those older than 11 years of age[38].

Also possible is that the hypothesized *OXT* or *OXTR* signaling deficiency in PWL patients could be mediated by (an)other gene(s) linked to the leptin-melanocortin-oxytocin pathway. Recently, Kasher and colleagues identified small deletions encompassing *POU3F2* in ten individuals from six families with developmental delay, intellectual disability, neonatal hypotonia, susceptibility to obesity and hyperphagia, characteristics resembling the PWL phenotype. With the use of morpholino (MO) and mutant zebrafish models, they showed that the gene lies downstream of *SIM1* in the leptin-melanocortin signaling pathway as *Pou3f2a* and *Pou3f2b* (two orthologs of the human *POU3F2)* expression levels in *Sim1* morphants showed a significant reduction. In addition, they also identified a significant decrease in *Oxt* expression in MO-mediated knockdown of *Pou3f2a* and/or *Pou3f2b* in zebrafish. Bearing in mind that *Oxt* expression is also decreased in *Sim1* haploinsufficient mice[17], this indicates an important role of *POU3F2* in regulating the expression of *OXT* in the hypothalamus as a downstream target for *SIM1*[39]. Finally, *POU3F2* is also located on the long arm of chromosome 6 (6q16.1) at approximately 1.5 Mb from *SIM1* which makes it an even more interesting gene for further analysis in the PWL phenotype.

In conclusion, since our CNV and mutation analysis results showed no major contribution of *OXT* and *OXTR* in the obese phenotype in our PWL patients, further screening of the *POU3F2* gene would be interesting due to its demonstrated role downstream of *SIM1* in the leptin-melanocortin signaling pathway in the regulation of *OXT* expression.

Conflict of Interest statement: The authors declare no conflict of interest.

Acknowledgements

This study was supported by an Interuniversity Attraction Pole Project (Phase VII project 43, BELSPO) and a TOP project from the University of Antwerp to WVH. EG designed the study, carried out experiments and wrote the manuscript. EA and WVH contributed to the discussion. AV, KVH, SV and LVG recruited and phenotyped subjects. WVH designed the study and wrote the manuscript. All authors read and approved the final version of the manuscript.

References

1. Driscoll, D.J., et al., *Prader-Willi Syndrome*, in *GeneReviews(R)*, M.P. Adam, et al., Editors. 1993: Seattle (WA).
2. Elena, G., et al., *Prader-willi syndrome: clinical aspects.* J Obes, 2012. **2012**: p. 473941.
3. Bonaglia, M.C., et al., *Detailed phenotype-genotype study in five patients with chromosome 6q16 deletion: narrowing the critical region for Prader-Willi-like phenotype.* Eur J Hum Genet, 2008. **16**(12): p. 1443-9.
4. Rosenfeld, J.A., et al., *Genotype-phenotype correlation in interstitial 6q deletions: a report of 12 new cases.* Neurogenetics, 2012. **13**(1): p. 31-47.
5. Faivre, L., et al., *Deletion of the SIM1 gene (6q16.2) in a patient with a Prader-Willi-like phenotype.* J Med Genet, 2002. **39**(8): p. 594-6.
6. Varela, M.C., et al., *A new case of interstitial 6q16.2 deletion in a patient with Prader-Willi-like phenotype and investigation of SIM1 gene deletion in 87 patients with syndromic obesity.* Eur J Med Genet, 2006. **49**(4): p. 298-305.
7. Klein, O.D., et al., *Interstitial deletions of chromosome 6q: genotype-phenotype correlation utilizing array CGH.* Clinical Genetics, 2007. **71**(3): p. 260-266.
8. Spreiz, A., et al., *Phenotypic variability of a deletion and duplication 6q16.1 --> q21 due to a paternal balanced ins(7;6)(p15;q16.1q21).* Am J Med Genet A, 2010. **152A**(11): p. 2762-7.
9. Izumi, K., et al., *Endocrine phenotype of 6q16.1-q21 deletion involving SIM1 and Prader-Willi syndrome-like features.* Am J Med Genet A, 2013. **161A**(12): p. 3137-43.
10. Vignoli, A., et al., *Interstitial 6q microdeletion syndrome and epilepsy: a new patient and review of the literature.* Am J Med Genet A, 2013. **161A**(8): p. 2009-15.
11. Geets, E., et al., *Copy number variation (CNV) analysis and mutation analysis of the 6q14.1-6q16.3 genes SIM1 and MRAP2 in Prader Willi like patients.* Mol Genet Metab, 2016. **117**(3): p. 383-8.
12. Geets, E., et al., *DNA sequencing and copy number variation analysis of MCHR2 in a cohort of Prader Willi like (PWL) patients.* Obes Res Clin Pract, 2017.
13. Gimpl, G. and F. Fahrenholz, *The oxytocin receptor system: structure, function, and regulation.* Physiol Rev, 2001. **81**(2): p. 629-83.
14. van der Klaauw, A.A. and I.S. Farooqi, *The hunger genes: pathways to obesity.* Cell, 2015. **161**(1): p. 119-32.
15. Sabatier, N., G. Leng, and J. Menzies, *Oxytocin, feeding, and satiety.* Front Endocrinol (Lausanne), 2013. **4**: p. 35.
16. Swaab, D.F., J.S. Purba, and M.A. Hofman, *Alterations in the hypothalamic paraventricular nucleus and its oxytocin neurons (putative satiety cells) in Prader-Willi syndrome: a study of five cases.* J Clin Endocrinol Metab, 1995. **80**(2): p. 573-9.

17. Kublaoui, B.M., et al., *Oxytocin deficiency mediates hyperphagic obesity of Sim1 haploinsufficient mice.* Mol Endocrinol, 2008. **22**(7): p. 1723-34.
18. Takayanagi, Y., et al., *Oxytocin receptor-deficient mice developed late-onset obesity.* Neuroreport, 2008. **19**(9): p. 951-5.
19. Camerino, C., *Low sympathetic tone and obese phenotype in oxytocin-deficient mice.* Obesity (Silver Spring), 2009. **17**(5): p. 980-4.
20. Deblon, N., et al., *Mechanisms of the anti-obesity effects of oxytocin in diet-induced obese rats.* PLoS One, 2011. **6**(9): p. e25565.
21. Maejima, Y., et al., *Peripheral oxytocin treatment ameliorates obesity by reducing food intake and visceral fat mass.* Aging (Albany NY), 2011. **3**(12): p. 1169-77.
22. Zhang, H., et al., *Treatment of obesity and diabetes using oxytocin or analogs in patients and mouse models.* PLoS One, 2013. **8**(5): p. e61477.
23. Blevins, J.E. and D.G. Baskin, *Translational and therapeutic potential of oxytocin as an anti-obesity strategy: Insights from rodents, nonhuman primates and humans.* Physiol Behav, 2015. **152**(Pt B): p. 438-49.
24. Lawson, E.A., et al., *Oxytocin reduces caloric intake in men.* Obesity (Silver Spring), 2015. **23**(5): p. 950-6.
25. Wheeler, E., et al., *Genome-wide SNP and CNV analysis identifies common and low-frequency variants associated with severe early-onset obesity.* Nat Genet, 2013. **45**(5): p. 513-7.
26. Bittel, D.C., N. Kibiryeva, and M.G. Butler, *Methylation-specific multiplex ligation-dependent probe amplification analysis of subjects with chromosome 15 abnormalities.* Genet Test, 2007. **11**(4): p. 467-75.
27. Beckers, S., et al., *Identification and functional characterization of novel mutations in the melanocortin-4 receptor.* Obes Facts, 2010. **3**(5): p. 304-11.
28. Miller, S.A., D.D. Dykes, and H.F. Polesky, *A Simple Salting out Procedure for Extracting DNA from Human Nucleated Cells.* Nucleic Acids Research, 1988. **16**(3): p. 1215-1215.
29. Vandeweyer, G., et al., *CNV-WebStore: online CNV analysis, storage and interpretation.* BMC Bioinformatics, 2011. **12**: p. 4.
30. Reed, G.H. and C.T. Wittwer, *Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis.* Clinical Chemistry, 2004. **50**(10): p. 1748-1754.
31. Lek, M., et al., *Analysis of protein-coding genetic variation in 60,706 humans.* Nature, 2016. **536**(7616): p. 285-91.
32. Genomes Project, C., et al., *A map of human genome variation from population-scale sequencing.* Nature, 2010. **467**(7319): p. 1061-73.
33. Adzhubei, I.A., et al., *A method and server for predicting damaging missense mutations.* Nat Methods, 2010. **7**(4): p. 248-9.
34. Schwarz, J.M., et al., *MutationTaster2: mutation prediction for the deep-sequencing age.* Nat Methods, 2014. **11**(4): p. 361-2.
35. Ng, P.C. and S. Henikoff, *Predicting deleterious amino acid substitutions.* Genome Res, 2001. **11**(5): p. 863-74.

36. Einfeld, S.L., et al., *A double-blind randomized controlled trial of oxytocin nasal spray in Prader Willi syndrome.* Am J Med Genet A, 2014. **164A**(9): p. 2232-9.
37. *ClinicalTrials.gov: A service of the U.S. National Institutes of Health*. [cited 2016 October]; Available from: https://clinicaltrials.gov.
38. Kuppens, R.J., S.H. Donze, and A.C. Hokken-Koelega, *Promising effects of oxytocin on social and food-related behaviour in young children with Prader-Willi syndrome: a randomized, double-blind, controlled crossover trial.* Clin Endocrinol (Oxf), 2016.
39. Kasher, P.R., et al., *Small 6q16.1 Deletions Encompassing POU3F2 Cause Susceptibility to Obesity and Variable Developmental Delay with Intellectual Disability.* Am J Hum Genet, 2016. **98**(2): p. 363-72.

CHAPTER 6

COPY NUMBER VARIATION AND MUTATION ANALYSIS INDICATE A POSSIBLE INTERESTING ROLE OF POU3F2 IN THE PRADER WILLI LIKE PHENOTYPE.

Ellen Geets[1], Evi Aerts[1], An Verrijken[2,3], Sara Diels[1], Guy Massa[4], Kim Van Hoorenbeeck[5], Stijn Verhulst[5], Luc Van Gaal[2,3], Wim Van Hul[1]

[1]Department of Medical Genetics, University of Antwerp, Antwerp, Belgium
[2]Department of Endocrinology, Diabetology and Metabolic Diseases, Antwerp University Hospital, Antwerp, Belgium
[3] Laboratory of Experimental Medicine and Paediatrics, Faculty of Medicine and Health Sciences, University of Antwerp, Antwerp, Belgium.
[4]Department of Paediatrics, Jessa Hospital, Hasselt, Belgium
[5]Department of Paediatrics, Antwerp University Hospital, Antwerp, Belgium

Unpublished results

Abstract

Background: Prader-Willi syndrome (PWS), caused by a paternal defect on 15q11.2 – q13, is the most common form of syndromic obesity. Once a clinical diagnosis of PWS cannot be confirmed molecularly with the use of MS-MLPA or additional techniques, patients are classified as Prader Willi *like* (PWL). Deletions at 6q14.1 – q16.3 encompassing *SIM1* were reported in some individuals with a PWL phenotype. More recently, Kasher and colleagues also identified small deletions encompassing *POU3F2*, and not *SIM1*, in ten individuals from six families with developmental delay, intellectual disability, neonatal hypotonia, susceptibility to obesity and hyperphagia, characteristics resembling the PWL phenotype. In addition, with the use of morpholino and mutant zebrafish models, they showed that the gene lies downstream of *SIM1* in the leptin-melanocortin signaling pathway which indicates that *POU3F2* also has a potentially interesting role in the obesity phenotype of PWL patients. Consequently, we decided to perform copy number variation (CNV) analysis of the *POU3F2* region in a PWL cohort followed by mutation and functional analysis of the gene in both a PWL cohort and obese children and adolescents.

Methods: A genome-wide microarray analysis was performed in a group of 109 PWL patients. Next, we screened 95 PWL patients and 459 obese children and adolescents for mutations in *POU3F2* using high-resolution melting curve analysis and Sanger sequencing. For the interesting variants a dual luciferase reporter assay and oxytocin (OXT) ELISA was performed.

Results: CNV analysis of 109 PWL patients did not show any structural variation encompassing the *POU3F2* gene. The mutation screening resulted in the identification of three interesting nonsynonymous variants: a previously unreported variant p.H255R (c.764A>G) in one PWL patient and two variants, p.G85R (c.253G>A) and p.G352V (c.1055G>T), in two obese adolescents. The latter was also not previously described in the gnomAD database. For the luciferase reporter assay a decrease in luciferase activity was measured in the presence of WT POU3F2 compared to the

negative control. The OXT ELISA revealed an OXT serum level of 223.2 pg/ml in the patient harboring the p.G352V variant.

Conclusions: In contrast to Kasher and colleagues, no gene harboring deletions were identified in the *POU3F2* region in our PWL cohort. On the contrary, three interesting variants, p.G85R, p.H255R and p.G352V, are identified. The preliminary results of neither the luciferase reporter assay nor the OXT ELISA resulted in an indication whether the variants have an effect on the signaling pathway. Further functional investigation is necessary in order to provide further support for a possible role of *POU3F2* in the pathogenesis of the PWL phenotype and the obesity phenotype seen in the patients.

Keywords: Obesity; Prader Willi *like*; *POU3F2*; structural and mutation analysis

1. Introduction

Prader Willi syndrome (PWS), caused by a paternal defect on the imprinting region 15q11.2 – 15q13, is the most common form of syndromic obesity with a prevalence of 1 in 10.000 – 30.000 individuals[1]. The phenotype of patients with a Prader Willi *like* (PWL) phenotype resembles that of PWS, however, they do not carry the defect on chromosome 15. To date, genetic research of the PWL phenotype shows an extremely heterogeneous pattern, nevertheless deletions at chromosome 6q16.3 encompassing the *single-minded 1* (*SIM1*) gene are most frequently reported[2-4]. Own structural analysis of the *SIM1* chromosomal region of 109 PWL patients did however not confirm this finding[5]. Recent research of Kasher and colleagues also showed the presence of small deletions in the same chromosomal region harboring *POU Class 3 Homeobox 2* (*POU3F2*), but not *SIM1*, in ten individuals with characteristics resembling the PWL phenotype. POU3F2 is a class III POU transcription factor with a high affinity for the octameric DNA sequence 5'-ATGCAAAT-3'[6]. As *SIM1*, it shows a high expression in the paraventricular nucleus (PVN) of the hypothalamus, a region in which an important pathway in energy homeostasis (i.e. the leptin-melanocortin signaling pathway) is active[7]. With the generation of $Brn2^{-/-}$ (=$Pou3f2^{-/-}$) and $Brn2^{+/-}$ (=$Pou3f2^{+/-}$) mice models, two different research groups indeed identified *Pou3f2* as an important player in the development of the hypothalamic PVN. Compared to $Sim^{-/-}$ mice that die shortly after birth due to the lack of the PVN, homozygous $Brn2^{-/-}$ mice also decease within 10 days after birth most probably attributable to complete deficiency of the PVN and even the supraoptic nuclei of the hypothalamus[7-9]. With the use of a *Sim1*-deficient mice model and morpholino and mutant zebrafish models, Michaud *et al.* and Kasher *et al.* even discovered that *POU3F2* functions downstream of *SIM1* along this leptin-melanocortin cascade indicating a possible interesting role of *POU3F2* in the obesity phenotype of PWL patients[6, 7].

Consequently, we decided to perform copy number variation (CNV) analysis followed by mutation screening of the *POU3F2* gene in a PWL cohort of 109 patients and 459

obese children and adolescents. For two interesting variants functional studies were conducted.

2. Material and methods

2.1. Study populations

A genome-wide microarray analysis was initially performed in a cohort of 109 patients who were referred from several hospitals from the Antwerp area to our Department of Medical Genetics (University of Antwerp, Antwerp, Belgium) over the last 25 years with a putative diagnosis of PWS or PWL syndrome. All selected patients were reported with at least a combination of obesity and developmental delay or intellectual disability. In some cases also behavioral problems (32/109) and hypotonia (20/109) were identified (Table 1). The clinical diagnosis of PWS could be excluded molecularly with the use of a methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)[10]. In addition, mutation analysis of *MC4R*, performed as described earlier[11], showed no mutations in any of the patients. Table 1 shows an overview of the population characteristics of the 109 PWL patients which is based on obtained data from the corresponding physicians. For 95 out of 109 patients, sufficient DNA was available for mutation screening of POU3F2.

An additional *POU3F2* mutation screen was performed in a cohort of 459 obese children and adolescents which were recruited from the paediatrics departments of the Antwerp University Hospital and the Jessa Hospital in Hasselt (Table 2). This group includes 208 boys and 251 girls with a mean age of 11.4 ± 3.7 years and a mean body mass index (BMI) z-score of 2.6 ± 0.5.

This study protocol was approved by the local ethics committee and all participants gave their written informed consent.

Table 1. Population characteristics of the PWL cohort for which genome-wide microarray analysis was performed.

Parameters	Prader Willi *like* patients
N	109
Age (years)	11.5 ± 8.4
Male/Female	70/39
15q11.2-13 defect	No
Obesity	109/109
Developmental delay/Intellectual disability	109/109
Behavioral problems	32/109
Hypotonia	20/109
Small hands/feet	14/109
Hypogonadism	12/109
Seizures	9/109
Short stature	5/109
Hearing loss	1/109

Mean value ± standard deviation of mean is given

Table 2. Population characteristics of the obese children and adolescents cohort screened for the *POU3F2* gene.

Parameters	Obese children and adolescents
N	459
Age (years)	11.4 ± 3.7
Male/Female	208/251
BMI (kg/m²)	31.0 ± 6.3
BMI z-score	2.6 ± 0.5

Mean value ± standard deviation of mean is given.

2.1.1. Anthropometry

For the obese children and adolescents, height was measured to the nearest 0.1 cm and body weight was determined to the nearest 0.1 kg on a digital scale. Body mass index (BMI) was computed as weight (in kilograms) over length (in meters) squared and the BMI z-scores were calculated based on the Flemish Growth Curves 2004[11]. Using these age- and sex-specific BMI growth curves, the cut-off values for overweight and obesity are defined as the percentile lines on the chart that cross BMI 25 kg/m² and 30 kg/m², respectively, at the age of 18 years.

2.2. Genome-wide microarray analysis

A genome-wide microarray analysis approach was used to identify CNVs encompassing the *POU3F2* gene. Two different BeadChips were used and the protocol was followed according to manufacturer's recommendations as described previously[5].

2.3. Mutation analysis

Screening of the *POU3F2* coding exon with its intron-exon boundaries (Genbank NM_005604.3) was executed on genomic DNA isolated from whole blood according to

standard procedures[12]. Respectively, we designed three amplicons. Due to high GC-content of the *POU3F2* gene and the presence of a Single Nucleotide Polymorphism (SNP) with an allele frequency of 85.4% in the European Non-Finnish population in the Genome Aggregation Database (gnomAD) Browser Beta[13] (rs195860, c.627G>T, p.G209G), we performed direct sequencing of the first two amplicons. Respectively, a 3x Enhanced and a touchdown PCR protocol supplemented with betaine were used in order to obtain amplification. Mutation screening for the third amplicon was achieved with the use of the highly specific and sensitive high-resolution melting curve analysis (HRM) technique using the Lightcycler LC480 Real-Time PCR System (Roche, Penzberg, Germany)[14] in combination with Sanger sequencing. A touchdown PCR protocol was used for amplification. Real-time PCR was performed in a total reaction volume of 10 µl. The amplification mixture included 10 ng of template DNA, 1.5 mM $MgCl_2$, 0.15 mM dNTP's, 500 nM primers, 0.015 U/µl GoTaq (Promega Corporation, Madison, WI, USA), 1x GoTaq buffer and the saturating dye LCGreen Plus (Idaho Technology, Salt Lake City, UT, USA) in a 0.5x concentration. After amplification, DNA samples were denatured at 95°C, rapidly reannealed at 40°C and subsequently heated from 70°C – 90°C measuring fluorescence constantly. Sanger sequencing was performed with ABI BigDye Terminator v3.1 Cycle Sequencing kits on an ABI Prism Genetic Analyzer 3130xl (Applied Biosystems Inc, Foster City, CA, USA) for DNA samples with melting patterns deviating from wild-type in order to confirm and characterize putative sequence variations. Primer sequences for HRM and sequencing are available upon request.

2.4. POU3F2 construct and mutagenesis

Wild type (WT) human *POU3F2* (Genbank NM_005604.2) cloned into a pCMV6-XL4 vector was obtained from Origene Technologies (SC116625). The variant identified in the PWL patient (p.H255R) and the variant found in an obese child and predicted to be damaging by all prediction programs (p.G352V) were individually introduced in the WT *POU3F2* vector by Mutagenex Inc. (Suwanee, Georgia, USA). The POU3F2 WT insert

and the presence of variants were checked and confirmed by Sanger sequencing using an ABI Prism Genetic Analyzer 3130xl (Applied Biosystems Inc, Foster City, CA, USA).

2.5. Dual luciferase reporter assay

In order to monitor the transcription factor activity in cells transiently transfected with WT or mutant *POU3F2*, a dual luciferase reporter assay is conducted. Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with fetal bovine serum (10%, Invitrogen) and penicillin/streptomycin (1%). Next, these cells were plated at a density of 0.2 x 10^5 cells per well in a 96-well plate and transfected using Fugene6 (Roche, Penzberg, Germany) with 25 ng pGL2-promoter-2XMORE firefly luciferase construct (Addgene, Cambridge, USA), 2.5 ng pRL-TK renilla luciferase construct and 2 or 4 ng WT/mutant *POU3F2* pCMV6-XL4 or empty pcDNA3.1 construct per well. 48 hours post-transfection cells were lysed and assayed for luciferase activity using the Dual-Luciferase Assay System (Promega, Madison, WI) on a Glomax-multi Detection System (Turner Biosystems, Sunny-vale, CA). The ratio of firefly and renilla luciferase measurements was calculated and compared between the different conditions. Each transfection was carried out in triplicate and repeated in three separate experiments with similar results. Comparison between two measurements for a single experiment was performed using a two-tailed Student's *t*-test. Values of $P < 0.05$ were considered significant.

2.6. Oxytocin (OXT) enzyme-linked immunosorbent assay (ELISA)

The serum sample from the patient with the p.G352V variant (female) was collected at 10:00am. Accordingly serum samples from three control subjects (2 females, 1 male) were collected at 11:30 am. All samples were stored at -20°C. Oxytocin (OXT) levels in the serum were measured with an OXT ELISA kit (Catalog number: ADI-900-153, Enzo Life Sciences, Farmingdale, New York, USA). Before performing the ELISA, a solid phase extraction of the serum samples was conducted to eliminate background interference.

In brief, 250 µl of 0.1% trifluoroacetic acid (TFA) in H_2O was added to 250 µl of each serum sample and centrifuged at 4°C for 18 minutes at 16,100 g. Subsequently the supernatant was saved. During the centrifugation, 200 mg C18 Sep-Pak columns (Waters Corporation, Milford, Massachusetts, USA) were equilibrated with 1 ml 99.8% acetonitrile followed by 12 ml of 0.1% TFA-H_2O. After applying the supernatant to the column, it is washed with 12 ml of 0.1% TFA-H_2O where-after the flow-through was discarded. To slowly elute the supernatant, 3mL of a solution comprised of 95% acetonitrile and 5% of 0.1% TFA-H_2O was applied to the column. Next, under cold temperature the eluent was evaporated to dryness with the use of a vacuum centrifuge and stored at -20°C. Before starting the assay according to the manufacturer's protocol, samples were reconstituted with assay buffer provided with the kit. Finally, the optical density was read at 405 nm using a Victor 1420 fluorimeter (PerkinElmer Life Sciences, Groningen, the Netherlands).

3. Results

3.1. Genome-wide microarray analysis

Genome-wide microarray analysis of 109 PWL patients did not show any structural variation encompassing the *POU3F2* gene.

3.2. Mutation analysis of POU3F2

Mutation screening of the *POU3F2* coding exon and its intron-exon boundaries in 95 PWL patients led to the identification of one common synonymous variant c.627T>G (p.G209G, rs195860), one rare synonymous variant c.222T>G (p.G74G), one in-frame insertion c.423_424insCAG (p.Gln149_Arg150insGln, rs564719242) and one nonsynonymous variant c.764A>G (p.H255R). The c.627T>G variant was identified in either a heterozygous (minor allele frequency (MAF) = 16.8%) or homozygous (MAF = 4.2%) state in a total of 36 patients. The rare p.G74G variant was found

heterozygously in only one PWL patient with a corresponding MAF of 0.5% and was not yet previously described in the gnomAD database.

The heterozygous in-frame insertion was detected in five patients with a MAF (2.6%) not significantly different from that reported in the gnomAD database (1.6%). Finally, the c.764A>G variant, which results in the substitution of an histidine to an arginine on position 255 (p.H255R), was identified heterozygously in only one PWL patient (MAF = 0.5%). The variant was not yet reported in the gnomAD database and, although prediction programs show conflicting results, both Polyphen-2[15] and Mutationtaster[16] indicate it as (possibly) damaging. The patient harboring the p.H255R variant is a seven-year-old girl presenting with obesity, mild intellectual disability and short stature. Mutation screening of additional family members did show that the variant was inherited from the obese mother, but was not present in the obese father and two obese sisters.

Additional mutation analysis of 459 obese children and adolescents resulted in the identification of six synonymous, three nonsynonymous, one in-frame insertion and one 3'UTR variant. One of the six synonymous variants is the c.627T>G variant, detected either in heterozygous or homozygous state in the study population. The heterozygous p.G209G variant was identified in 13.3% of the population whereas the homozygous form was found in 2.6%. All five other heterozygous synonymous variants, c.72C>T (p.P24P, rs766738537), c.90C>T (p.G30G), c.234G>A (p.G78G), c.798C>T (p.T266T) and c.1074G>T (p.R358R) were found in only one individual with a corresponding MAF of 0.1%. The latter four variants were not yet reported in the gnomAD database, whereas the c.72C>T variant was identified in only one European (Non-Finnish) individual (MAF = 0.0009%). Also the three nonsynonymous variants were found in heterozygous state in only one patient representing a MAF of 0.1%. For the c.253G>A (G85R, rs561021011) prediction programs show conflicting results, but both Polyphen-2 and SNPs&Go[17] indicate the variant as (probably) damaging. In addition, the MAF reported in gnomAD for this variant is 0.06% which is not

significantly different from the MAF identified in our study population. Although it was not yet described in the gnomAD database, *in silico* analysis for the c.678C>A (p.D226E) variant using PolyPhen-2, Mutationtaster, SIFT[18] and SNPs&GO, showed that it is predicted to be benign by all programs. The last nonsynonymous variant c.1055G>T (p.G352V) was also not previously reported, however, for this variant all four prediction programs indicate a probably damaging effect. The patient in whom we discovered the p.G352V variant, is a sixteen-year-old girl with obesity (BMI = 36.3 kg/m², BMI z-score = 2.88) and persistent primary nocturnal enuresis. As no family members were willing to cooperate for further research, it was not possible to determine whether the variant segregates with obesity in this family.

As for the PWL cohort, we also identified the heterozygous in-frame insertion c.423_424insCAG in our obese cases with a corresponding MAF of 1.0% which is comparable to that reported in the gnomAD database. Finally, the 3'UTR variant c.*27C>T was detected in heterozygous state in a 7,5-year-old girl with a BMI of 33.0 kg/m² and a BMI z-score of 3.33 (MAF = 0.1%). GnomAD describes a MAF of 0.02% for this variant. Using Pearson's chi square analysis we compared both MAFs, however we did not find a statistically significant difference.

An overview of the identified variants found in *POU3F2* in both study populations with the corresponding MAFs and *in silico* prediction analyses is shown in table 3 and 4.

Table 3. Identified variants in *POU3F2* in 95 PWL patients.

For each variant the minor allele frequency (MAF) is calculated in percentage and compared with the MAF reported in the Non-Finnish European population in the Genome Aggregation Database (gnomAD) Browser Beta (Accessed on the 26th of October 2017). *In silico* analysis data are shown for the nonsynonymous variant.

POU3F2							
Amino acid substitution	Nucleotide substitution	MAF (%) PWL cases (N = 95)	MAF (%) gnomAD (European (Non-Finnish))	Polyphen-2	Mutationtaster	SIFT	SNPs&Go
p.G74G	c.222T>G	0.5	/	/	/	/	/
p.Gln149_Arg150insGln	c.423_424insCAG	2.6	1.6	/	polymorphism	/	/
p.G209G	c.627T>G	21.0	14.6	/	/	/	/
p.H255R	c.764A>G	0.5	/	Possibly damaging	Disease causing	Tolerated	Neutral

Table 4. Identified variants in *POU3F2* in 459 obese children and adolescents.

For each variant the minor allele frequency (MAF) is calculated in percentage and compared with the MAF reported in the Non-Finnish European population in the Genome Aggregation Database (gnomAD) Browser Beta (Accessed on the 26th of October 2017). *In silico* analysis data are shown for the nonsynonymous variants.

POU3F2							
Amino acid substitution	Nucleotide substitution	MAF (%) Obese cases (N = 459)	MAF (%) gnomAD (European (Non-Finnish))	Polyphen-2	Mutationtaster	SIFT	SNPs&Go
p.P24P	c.72C>T	0.1	0.0009	/	/	/	/
p.G30G	c.90C>T	0.1	/	/	/	/	/
p.G78G	c.234G>A	0.1	/	/	/	/	/
p.G85R	c.253G>A	0.1	0.06	Probably damaging	Polymorphism	Tolerated	Disease
p.Gln149_Arg150insGln	c.423_424insCAG	1.0	1.6	/	Polymorphism	/	/
p.G209G	c.627T>G	15.9	14.6	/	/	/	/
p.D226E	c.678C>A	0.1	/	Benign	Polymorphism	Tolerated	Neutral
p.T266T	c.798C>T	0.1	/	/	/	/	/
p.G352V	c.1055G>T	0.1	/	Probably damaging	Disease causing	Damaging	Disease
p.R358R	c.1074G>T	0.1	/	/	/	/	/
/	c.*27C>T	0.1	0.02	/	/	/	/

3.3. Dual luciferase reporter assay

In order to investigate the effect of the mutants p.H255R and p.G352V on the transcription factor activity of POU3F2 a dual luciferase reporter assay was performed (Figure 1). Preliminary results from three independent experiments are depicted in figure 2. Compared to the negative control (= empty pcDNA3.1 construct) the luciferase activity is reduced when HEK cells are transfected with 25 ng 2xMORE firefly luciferase, 2.5 ng pRL-TK and 2 ng POU3F2 WT. An even bigger drop is seen when 4 ng POU3F2 WT is added. The same pattern is visible in the presence of 2 or 4 ng POU3F2 H255R. With the use of a two-tailed Student's *t*-test no significant difference was measured between POU3F2 WT and POU3F2 H255R (2 ng: p = 0.74, 4 ng: p = 0.11). On the contrary, when we compare the results of 2 ng POU3F2 WT with the results of 2 ng POU3F2 G352V, POU3F2 G352V shows a significantly stronger inhibitory effect (p = 0.007). This significant difference disappeared when 4 ng was added (p = 0.15).

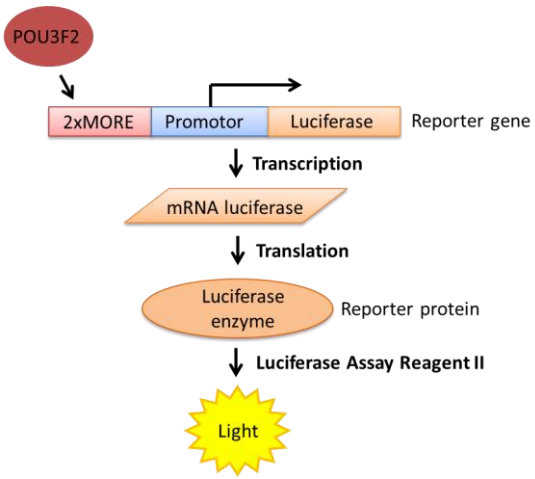


Figure 1. Schematic overview of the dual luciferase reporter assay to investigate the effect of p.H255R and p.G352V on the transcription factor activity of POU3F2.
The 2xMORE response element harboring the DNA sequence ATGCAT two times is cloned upstream of the firefly luciferase gene. Binding of POU3F2 to its response element will activate the promoter and the luciferase will be transcribed and translated into luciferase protein. With the use of the dual luciferase reporter assay the amount of luciferase generated can be converted in light production and subsequently the ratio of firefly to renilla luciferase will be a reference for the POU3F2 transcriptional activity.

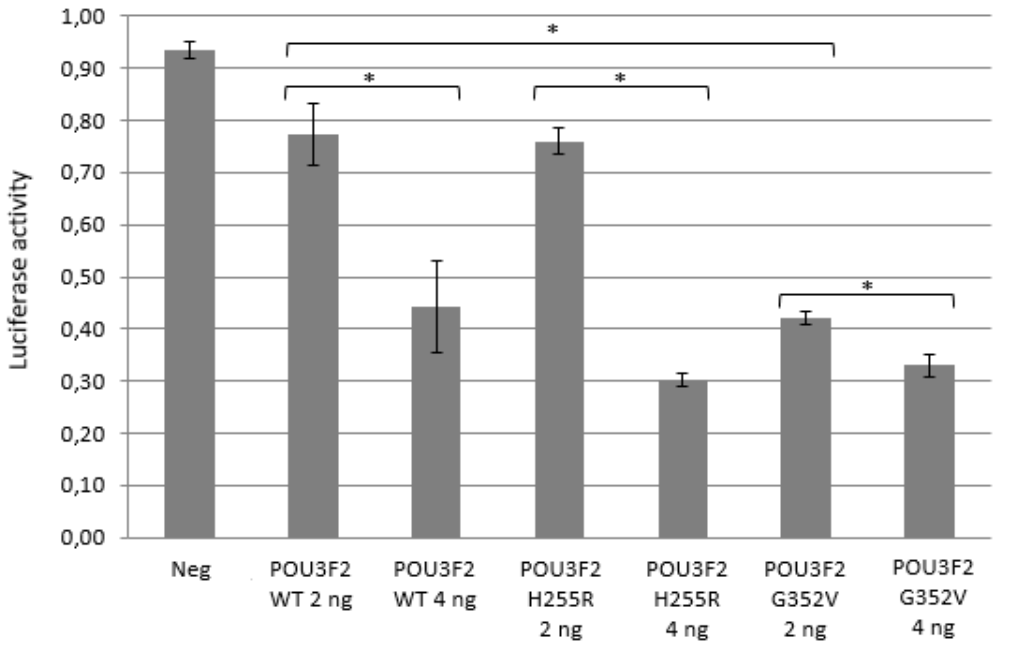
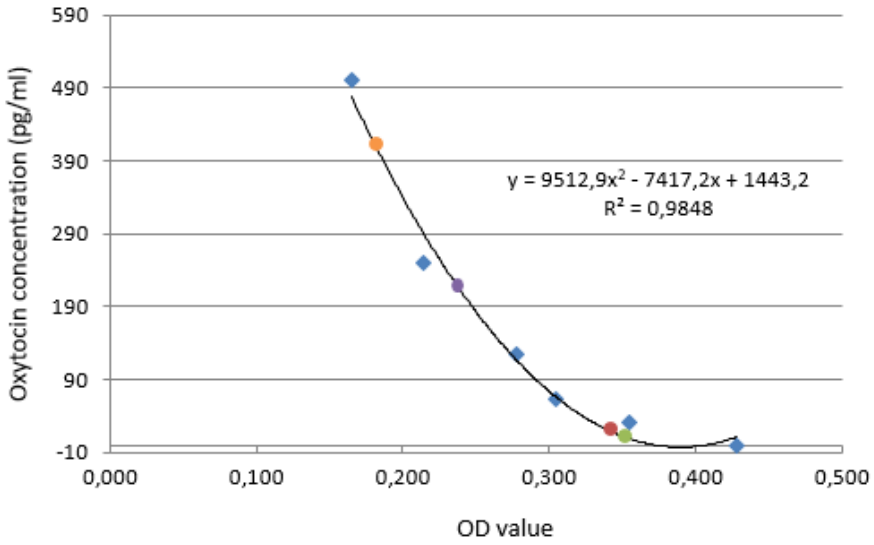


Figure 2. Dual luciferase reporter assay to investigate the transcription factor activity of POU3F2 WT, H255R and G352V.

HEK293T cells are transiently transfected with pGL2-promoter-2XMORE, pRL-TK and WT/mutant POU3F2 (2 or 4 ng). The ratio of firefly and renilla luciferase measurements was calculated and compared to the luciferase activity of HEK293T cells transfected with empty pcDNA3.1. Bars represent mean value ± standard deviation. Each transfection was carried out in triplicate and repeated in three separate experiments with similar results. *P-value < 0.05.

3.4. OXT ELISA

In order to measure OXT levels in serum samples from three controls (2 females and 1 male) and the patient harboring the p.G352V variant (female), an OXT ELISA was conducted (Figure 3). For the male control an OXT level of 8.6 pg/ml was measured in the serum. For one female control a comparable concentration was defined (15.8 pg/ml). On the contrary, the second female control showed an OXT concentration of 415.6 pg/ml in her serum. Finally, the OXT concentration in the serum of the patient harboring the p.G352V variant was determined on 223.2 pg/ml.



Sample	OXT concentration (pg/ml)
Male control	8.6
Female control 1	15.8
Female control 2	415.6
Patient	223.2

Figure 3. Oxytocin (OXT) Enzyme-linked immunosorbent assay (ELISA): Standard curve and corresponding OXT concentrations of three controls and one patient sample.
In order to eliminate the effect of potentially interacting molecules, first a solid phase extraction of the serum samples was conducted. Next the ELISA was executed according to the manufacturer's protocol. The measured OXT levels for the male control and the two female controls are depicted as a green, red and orange dot respectively. The purple dot represents the OXT level of the patient harboring the p.G352V variant.

4. Discussion

Different research groups already associated the Prader Willi *like* (PWL) phenotype with deletions on the 6q chromosome encompassing the *SIM1* gene leading to the suggestion that haploinsufficiency of *SIM1* could explain the obesity phenotype in these patients[2-4]. However, not all reported deletions on chromosome 6q identified in PWL patients do harbor *SIM1*[4, 6, 19] and own additional research only showed limited association of the gene with the PWL phenotype[5]. Recent findings of Kasher

and colleagues identified *POU3F2* as an interesting gene in the PWL phenotype[6]. In addition, also different mouse and zebrafish models showed an expression of the gene in the hypothalamic PVN region playing a role in the development of the PVN downstream of *SIM1* in the leptin-melanocortin signaling pathway[6, 8, 9, 20].

With the use of an array comparative genomic hybridization and metaphase fluorescent in situ hybridization Kasher *et al.* also identified 6q16.1 deletions encompassing *POU3F2*, but not *SIM1*, in ten individuals (six males and four females) from six different families with variable developmental delay, intellectual disability (ID) and susceptibility to obesity and hyperphagia. However, we did not identify any *POU3F2* deletion in our PWL study population, despite the fact that with the arrays used we would detect these small deletions. They did not find any *POU3F2* mutations in a cohort of more than 4,000 trios with ID and obesity, whereas we, although accurate sequencing of the extremely GC-rich region of *POU3F2* was very challenging, did found one interesting mutation, p.H255R, in our PWL cohort of 95 patients and two interesting mutations, p.G85R and p.G352V, in a cohort of 459 obese children and adolescents. Both the p.H255R variant, found in a seven-year-old girl presenting with obesity, mild intellectual disability and short stature, and the p.G352V variant, identified in a sixteen-year-old girl with obesity and persistent primary nocturnal enuresis, are not yet previously described in the gnomAD database. Hitherto, the p.G85R variant was detected in 10/15982 individuals of the Non-Finnish European population. In addition, the Combined Annotation Dependent Depletion (CADD) – PHRED score was also calculated for these variants with a score of 4.65 (p.H255R), 27.5 (p.G352V) and 21.1 (p.G85R) respectively[21]. The high score of the two latter variants can be attributed to the high conservation of the amino acids through evolution[22]. Taking into account their absence in the public sequencing database gnomAD and the results of *in silico* prediction programs, we decided to perform further functional analysis of p.H255R (found in a PWL patient) and the p.G352V (found in an obese girl) in order to provide further support for a possible role of *POU3F2* in the pathogenesis of PWL and the obesity phenotype seen in these patients.

However, the results of the dual luciferase reporter assay and OXT ELISA were rather unexpected. For the luciferase reporter assay an increase in luciferase activity was expected after the addition of POU3F2 WT compared to the negative control. On the contrary, a decrease was seen and this decrease was even higher when the concentration of POU3F2 WT increased. We hypothesize that this decrease might be attributed to a competition between the endogeneously expressed POU3F2 in the HEK293T cells and the protein encoded by the POU3F2 WT construct. The latter might have a decreased capacity to bind its response element resulting in a lower luciferase activity. Noteworthy, no significantly stronger inhibitory effect was noticed for the mutant found in the PWL patient (p.H255R), however, a clear significant difference was seen between WT and p.G352V when 2 ng was added ($p = 0.007$). In the future it is worthwhile to perform the same experiment in another cell type without endogeneously expressed POU3F2 in order to 1) avoid the effect of endogeneous POU3F2 on the POU3F2 construct and 2) to see whether the significant difference between WT and p.G352V is kept.

In the OXT ELISA we wanted to determine the OXT serum level in the patient harboring the p.G352V variant and compare it to three controls. In the presence of the mutant we expect a decrease in POU3F2 expression leading to a decrease in OXT level in the serum. As visible in figure 3, the corresponding OXT concentration of the patient is located somewhere in the middle of the control concentrations, however OXT levels of the three control samples are also very variable. It is possible that the mutation in POU3F2 does not have an effect on the serum OXT levels, however a clear conclusion cannot be drawn yet. In general, oxytocin is very labile which could explain the big difference in OXT levels of the three control samples. Secondly, the amount of oxytocin a sample has at the time of measurement may not represent the amount of oxytocin at the time of storage. Noteworthy, the serum sample of the patient was stored at -20°C for almost six months before the extraction protocol was executed whereas control samples were already processed after a couple of days. In conclusion,

different independent experiments are still needed before a clear conclusion regarding the effect of the p.G352V POU3F2 variant on serum OXT level can be drawn.

Disclosure statement: The authors declare no conflict of interest.

Acknowledgements

This study was supported by an Interuniversity Attraction Pole Project (Phase VII project 43, BELSPO) and a TOP project from the University of Antwerp to WVH. EG designed the study, carried out experiments and wrote the manuscript. EA, SD and WVH contributed to the discussion. AV, GM, KVH, SV and LVG recruited and phenotyped subjects. WVH designed the study and wrote the manuscript. All authors read and approved the final version of the manuscript.

References

1. Driscoll, D.J., et al., *Prader-Willi Syndrome*, in *GeneReviews(R)*, M.P. Adam, et al., Editors. 1993: Seattle (WA).
2. D'Angelo, C.S., et al., *Obesity with associated developmental delay and/or learning disability in patients exhibiting additional features: report of novel pathogenic copy number variants.* Am J Med Genet A, 2013. **161A**(3): p. 479-86.
3. Bonaglia, M.C., et al., *Detailed phenotype-genotype study in five patients with chromosome 6q16 deletion: narrowing the critical region for Prader-Willi-like phenotype.* Eur J Hum Genet, 2008. **16**(12): p. 1443-9.
4. Rosenfeld, J.A., et al., *Genotype-phenotype correlation in interstitial 6q deletions: a report of 12 new cases.* Neurogenetics, 2012. **13**(1): p. 31-47.
5. Geets, E., et al., *Copy number variation (CNV) analysis and mutation analysis of the 6q14.1-6q16.3 genes SIM1 and MRAP2 in Prader Willi like patients.* Mol Genet Metab, 2016. **117**(3): p. 383-8.
6. Kasher, P.R., et al., *Small 6q16.1 Deletions Encompassing POU3F2 Cause Susceptibility to Obesity and Variable Developmental Delay with Intellectual Disability.* Am J Hum Genet, 2016. **98**(2): p. 363-72.
7. Michaud, J.L., et al., *Development of neuroendocrine lineages requires the bHLH-PAS transcription factor SIM1.* Genes Dev, 1998. **12**(20): p. 3264-75.
8. Nakai, S., et al., *The POU domain transcription factor Brn-2 is required for the determination of specific neuronal lineages in the hypothalamus of the mouse.* Genes Dev, 1995. **9**(24): p. 3109-21.
9. Schonemann, M.D., et al., *Development and survival of the endocrine hypothalamus and posterior pituitary gland requires the neuronal POU domain factor Brn-2.* Genes Dev, 1995. **9**(24): p. 3122-35.
10. Bittel, D.C., N. Kibiryeva, and M.G. Butler, *Methylation-specific multiplex ligation-dependent probe amplification analysis of subjects with chromosome 15 abnormalities.* Genet Test, 2007. **11**(4): p. 467-75.
11. Roelants, M., R. Hauspie, and K. Hoppenbrouwers, *References for growth and pubertal development from birth to 21 years in Flanders, Belgium.* Annals of Human Biology, 2009. **36**(6): p. 680-694.
12. Miller, S.A., D.D. Dykes, and H.F. Polesky, *A Simple Salting out Procedure for Extracting DNA from Human Nucleated Cells.* Nucleic Acids Research, 1988. **16**(3): p. 1215-1215.
13. Lek, M., et al., *Analysis of protein-coding genetic variation in 60,706 humans.* Nature, 2016. **536**(7616): p. 285-91.
14. Reed, G.H. and C.T. Wittwer, *Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis.* Clinical Chemistry, 2004. **50**(10): p. 1748-1754.
15. Adzhubei, I.A., et al., *A method and server for predicting damaging missense mutations.* Nat Methods, 2010. **7**(4): p. 248-9.

16. Schwarz, J.M., et al., *MutationTaster2: mutation prediction for the deep-sequencing age.* Nat Methods, 2014. **11**(4): p. 361-2.
17. *SNPs&GO*. [cited 2016 June]; Available from: http://snps.biofold.org/snps-and-go/snps-and-go.html.
18. Ng, P.C. and S. Henikoff, *Predicting deleterious amino acid substitutions.* Genome Res, 2001. **11**(5): p. 863-74.
19. Wentzel, C., et al., *Interstitial Deletions at 6q14.1-q15 Associated with Obesity, Developmental Delay and a Distinct Clinical Phenotype.* Mol Syndromol, 2010. **1**(2): p. 75-81.
20. Michaud, J.L., et al., *Development of neuroendocrine lineages requires the bHLH-PAS transcription factor SIM1.* Genes & Development, 1998. **12**(20): p. 3264-3275.
21. Kircher, M., et al., *A general framework for estimating the relative pathogenicity of human genetic variants.* Nat Genet, 2014. **46**(3): p. 310-5.
22. Ashkenazy, H., et al., *ConSurf 2016: an improved methodology to estimate and visualize evolutionary conservation in macromolecules.* Nucleic Acids Res, 2016. **44**(W1): p. W344-50.

DISCUSSION

Discussion

The aim of this PhD research was to investigate the involvement of six selected candidate genes of the leptin-melanocortin signaling pathway more into detail in the context of PWL and obesity. Therefore, we performed both CNV and mutation analysis in a specific cohort of syndromic patients with a PWL phenotype in order to look for rare variants explaining the imbalance of energy metabolism in these patients. As we choose for a genome-wide microarray approach, (new) important chromosomal regions in our PWL cohort could also be detected. By doing so, this study can contribute to the elucidation of the underlying mechanisms and genes involved in the PWL phenotype and eventually create new insights in the genetic background and the pathogenesis of obesity.

INVESTIGATION OF THE ROLE OF CHROMOSOME 6Q IN PWL PATIENTS AND THEIR OBESITY PHENOTYPE

1. SIM1

In different case reports, haploinsufficiency of *SIM1* at chromosome 6q16.3 has been put forward as the cause of the presence of obesity in PWL patients. Also previous mutation analyses by us and others of the *SIM1* gene in different obese individuals with or without PWL features, indicated *SIM1* as a possible interesting candidate gene. On top of that, animal models showed that SIM1 is involved in the development and function of the hypothalamic PVN as a downstream player of MC4R in the leptin-melanocortin signaling pathway. Subsequently, we first started CNV analysis of our cohort of 109 PWL patients. Genome-wide microarray analysis did not indicate any gene harboring deletion at the 6q16.3 region. However, this outcome was in line with the study conducted by Varela *et al.* in which they screened 87 PWL patients and merely one patient harbored a deletion of the *SIM1* gene. Consequently, we performed mutation analysis of the PWL patients for which enough DNA was available (94). In one patient, we identified an interesting variant, p.P352S. Further functional

screening of this variant is an interesting next step to investigate its possible causative effect. Two common nonsynonymous SNPs (p.P352T and p.A371V), which are in complete LD with each other, were also repeatedly found in our PWL cohort. In three PWL patients we even identified both variants in a homozygous state. Two research groups indicated an association of the homozygous p.P352T/p.A371V haplotype with higher BMI in white males. As the homozygous variants in our PWL cohort were also found in three white males, these variants could be involved in the development of their obesity.

Additionally, we also identified the intronic variant c.544-15A>C heterozygously in 17.0% and homozygously in 7.4% of our PWL patients. Previous research in black males and Pima Indians indicated a significant association of, predominantly, the homozygous SNP with various adiposity measures. However, these findings could not be replicated in a Caucasian case/control study population.

Overall, in literature a possible causative mutation in the *SIM1* gene could be identified in less than 2% of all screened PWL patients reported so far. Additionally, taking into account our own single nucleotide and structural variation results, we concluded that the association of the 6q16.3 (*SIM1*) region with the PWL phenotype is rather limited.

2. *MRAP2*

In 2010, Wentzel and colleagues reported two patients with an interstitial deletion at chromosome 6q14.1 – q15 encompassing *MRAP2* but not *SIM1*. However, both patients did show strikingly similar symptoms to those reported for PWL patients with *SIM1* deletions. Together with its role as a stimulating accessory protein for MC3R and MC4R in the leptin-melanocortin signaling pathway and animal models showing extremely obese mice when the gene is knocked out, further investigation of *MRAP2* in a PWL cohort is interesting and could support it as a good candidate gene for the presence of obesity in patients with a PWL phenotype or even cases of nonsyndromic obesity.

As for *SIM1*, copy number variation analysis of the *MRAP2* region did not show any gene harboring deletions. Though, mutation screening of the gene in a cohort of 94 PWL patients demonstrated two rare nonsynonymous variants, p.R125H and p.A40S. The first variant was identified in two patients, however *in silico* analysis using PolyPhen-2, Mutationtaster and SIFT predicted the variant as being benign. On the other hand, p.A40S, found in a ten-year-old boy who suffers from overall obesity in combination with intellectual disability, was predicted as (probably) damaging by the same prediction programs. Because the substitution to a serine amino acid occurs close to the transmembrane domain of the protein (amino acid 45-65), this could result in an alteration of the protein structure of MRAP2 and ultimately have an effect on the expression of MRAP2 on the transmembrane or on its stimulating effect on MC3R and MC4R signaling. Subsequently, we decided to perform an additional mutation analysis of 363 extremely overweight and obese children and adolescents. This, however, only led to the identification of the benign p.R125H variant in two patients. To conclude, further functional analysis of the p.A40S is necessary to investigate its potentially damaging effect and to indicate a possible role of *MRAP2* in the pathogenesis of the PWL phenotype, albeit in a limited number of patients as for the *SIM1* gene.

3. MCHR2

Own investigation of the *SIM1* chromosomal region did not indicate a major involvement of the gene in the PWL phenotype. However, most of the 6q deletions reported in literature in patients with a PWL phenotype also encompass the *MCHR2* gene located within 1 Mb of *SIM1* (Figure 1).

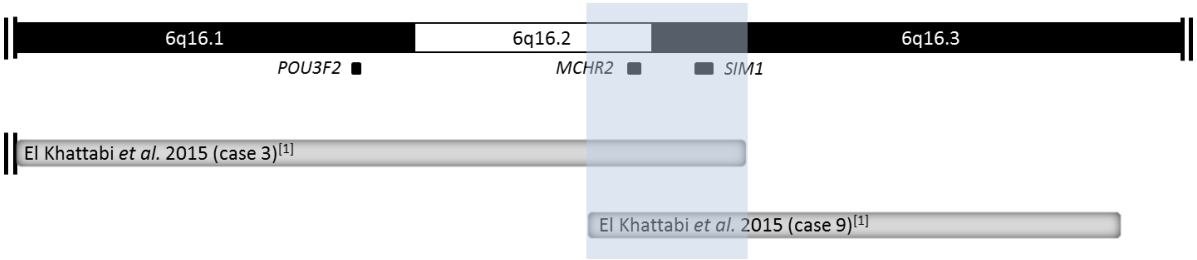


Figure 1. Schematic representation of the two key deletions[1] defining the minimal critical region on chromosome 6q associated with the Prader Willi *like* phenotype.
The region (indicated in blue) encompasses both the *MCHR2* and *SIM1* gene. An extended figure can be found in chapter 4 on page 149.

MCHR2 encodes a G protein-coupled receptor for MCH, an important neuropeptide in the control of feeding behavior and energy metabolism in the hypothalamus, indicating a possible involvement of MCHR2 in these processes as well. Subsequently, in a first hypothesis we suggested that PWL might be a contiguous gene syndrome and that CNVs involving *MCHR2* may explain the presence of obesity in patients with the phenotype. Consequently *MCHR2* could also contribute to human obesity in general. However, structural variation analysis only demonstrated a benign 26 kb deletion encompassing no genes at approximately 4 Mb of the *MCHR2* gene in one PWL patient.

In a second hypothesis we suggested that mutations in *MCHR2* could have a pleiotropic effect and on their turn result in the PWL phenotype. Mutation analysis in 92 PWL patients led to the identification of one rare nonsynonymous variant, p.T47A, one synonymous variant, p.A76A, and one 3' UTR variant, c.*16A>G. The latter was identified in a nineteen-year-old male who suffers from obesity in combination with intellectual disability. Also his lean brother harbors the variant indicating that the variant does not segregate with obesity in the family and in all probability does not explain the presence of obesity or PWL in the patient. The synonymous variant was found in 15.8% of our PWL patients with a significantly higher presence of the C allele compared to controls of the ExAC Database. On the contrary, another study showed an association with childhood obesity for the p.A76A T allele. However, they were not

able to replicate this association in an independent Caucasian study. Altogether, it is not clear whether a real association with human obesity of the T or C allele is present and additional research in either individuals with obesity or a PWL cohort would be interesting to clarify these contradictory findings. Lastly, the p.T47A variant was found in a nine-year-old girl with obesity and learning deficits. Segregation of the variant could not be determined in the family but, as only one prediction program indicated the variant as 'deleterious', further research of the variant would be less interesting.
Overall, our results confirm the conclusion of three previously performed mutation analyses by Hawes *et al.*, Gibson *et al.* and Goussaini *et al.* that *MCHR2* is not a major contributor to human obesity or PWL.

Investigation of the role of OXT and its receptor in PWL patients and their obesity phenotype

Our previous results on the 6q region did not indicate a major involvement of *SIM1*, *MRAP2* and *MCHR2* in the PWL phenotype and the obesity seen in these patients. Therefore, we decided to look beyond this chromosomal region and searched for other interesting genes. Already in 1995, Swaab and colleagues detected a significant deficiency in the number of oxytocin-producing neurons (42%) in the PVN of PWS patients revealing a possible involvement of *OXT* in the PWS phenotype. Extended research of expression patterns and mouse models ultimately indicated an interesting role of *OXT* and its receptor (*OXTR*) in energy homeostasis speculating that *OXT* or *OXTR* deficiency could play a role in the obesity phenotype present in PWS patients. (Ongoing) trials in animal models, prediabetic obese humans and PWS patients even indicate OXT as a possible medicine to treat obesity. Subsequently, as PWL patients share clinical characteristics with PWS we hypothesized that a reduction in *OXT* or *OXTR* signaling could also be present in PWL patients due to the presence of a mutation or deletion at *OXT* or *OXTR* and decided to be the first to perform CNV analysis followed by mutation analysis of both genes in a cohort of 109 PWL patients. Unfortunately, these experiments did not reveal any structural variations in or near

both genes and only led to the identification of known intronic (*OXT* and *OXTR*) or (non)synonymous variants (*OXTR*). However, we do hold on to our hypothesis that *OXT* or *OXTR* signaling deficiency is present in PWL patients presumably driven by another gene upstream of *OXT* in the leptin-melanocortin-oxytocin pathway i.e. *POU3F2*. Noteworthy, this gene is also located at chromosome 6q at approximately 1.5 Mb distance from *SIM1* which makes it an even more interesting for further analysis in the PWL phenotype.

INVESTIGATION OF THE ROLE OF POU3F2 IN PWL PATIENTS AND THEIR OBESITY PHENOTYPE

With the use of different zebrafish models Kasher and colleagues recently demonstrated a role for *POU3F2* as a regulator of *OXT* expression downstream of *SIM1* in the hypothalamic leptin-melanocortin signaling pathway. They also reported small deletions of *POU3F2*, and not *SIM1*, in ten individuals from six different families with characteristics resembling the PWL phenotype. Accordingly, we performed CNV analysis of our 109 PWL patients but did not identify any structural variation encompassing the *POU3F2* gene. On the contrary, mutation screening of the gene resulted in the identification of two synonymous variant, one in-frame insertion and one interesting nonsynonymous variant, p.H255R. This variant was found in a seven-year-old girl presenting with obesity, mild intellectual disability and short stature which was inherited from her obese mother. It was not previously described in the gnomAD database and two prediction programs indicate the variant as possibly damaging. Subsequently, a cohort of 459 obese children and adolescents was screened which resulted in five additional synonymous, three nonsynonymous and one 3'UTR variants. The p.D226E was identified in one PWL patient and was not previously reported in gnomAD. However, four different prediction programs predicted it to be benign making it less interesting for further research. For the second nonsynonymous variant, p.G85R, *in silico* analysis showed conflicting results, but two prediction programs indicate it as probably damaging. Also with its CADD – PHRED score of 21.1 it is categorized among the 1% most deleterious substitutions in the

human genome. In contrast, the MAF reported in gnomAD is not significantly different from the MAF identified in our study population. Finally, the p.G352V variant, discovered in a sixteen-year-old girl with obesity and persistent primary nocturnal enuresis, is absent in the gnomAD database. All prediction programs indicate a probably damaging effect for the variant with even a higher CADD – PHRED score than that reported for the p.G85R variant (27.5). Taking into account these scores, their absence in the public sequencing database gnomAD and the results of *in silico* prediction programs, we decided to perform further functional analysis of p.H255R (found in one PWL patient) and p.G352V (found in an obese girl). However, preliminary results of the luciferase reporter assay and the OXT ELISA did not clearly indicate whether the variants have an effect on the signaling pathway. Further functional investigation is still necessary in order to provide further support for a possible role of *POU3F2* in the pathogenesis of the PWL phenotype and the obesity phenotype seen in the patients.

OVERVIEW OF MICROARRAY AND MUTATION ANALYSES IN PWL COHORT

Table 1 shows an overview of the identified CNVs associated with the PWL phenotype together with important findings from the mutation analyses of *MC4R*, *SIM1*, *MRAP2*, *MCHR2*, *OXT*, *OXTR* and *POU3F2* for the entire PWL cohort. At a first glance, we noticed the presence of one unique and two recurrent 16p11.2 deletions in a total of five PWL patients. Two individuals harbored the proximal 593 kb deletion whereas in two other patients the distal 220 kb deletion encompassing the *Sarcoma* (*Src*) *homology 2B adaptor protein 1* (*SH2B1*) gene was found. One other patient was identified with a larger 1.7 Mb deletion overlapping the distal and proximal CNVs. In literature, 16p11.2 deletions have already been associated with obesity and intellectual disability (see chapter 1) and would in all probability explain the phenotype in these patients[2]. Secondly, patient 33, a 3.5 year old girl with obesity and ID, harbored a heterozygous deletion at 2p21 of approximately 180 kb encompassing the *prolyl endopeptidase-like* (*PREPL*) and *Calmodulin-Lysine N-*

Methyltransferase (*CAMKMT*) genes together with the p.S127L *MC4R* mutation. Though all PWL associated CNVs at chromosome 2p21 reported in literature are either homozygous or compound heterozygous[3], this result in the first place points to the importance of *MC4R* screening in obese individuals as the *MC4R* mutation found in this patient is a known disease causing variant and would in all probability explain the obesity phenotype seen in this girl. A third example is patient 76, a 17 year old male diagnosed with obesity, ID, short stature, small hands and feet and dysmorphic features. This male harbors a 41 kb 6q14.1 deletion (chr6:77,299,933-77,341,104) together with the heterozygous variants p.P352T and p.A371V in *SIM1* and the homozygous p.A76A variant in *MCHR2*. For each variation on its own no clear association with PWL or the obesity phenotype is demonstrated, however for this patient it could be possible that an oligogenic pattern emerges. All identified variants are located in the 6q region and could have a long-range effect on each other (or other genes/regions) which highlights the importance for the investigation of gene-gene or even gene-protein interactions in the future (see page 225). In addition, also topologically associating domains (TADs) and their boundaries emerge as a fundamental structural unit. Recent studies highlight the potential of CNVs to interfere with the TAD structure by disrupting or repositioning boundaries and consequently cause a disease[4]. A topological domain is located from position 76,543,280 to 78,183,281 on chromosome 6[5, 6], however, as the precise mapping of the boundary regions at this position is not yet done, it is not clear whether the identified CNV in our patient could interfere with the TAD structure. Also for the patient harboring the heterozygous variants p.P352T and p.A371V in *SIM1* and the 26 kb 6q16.3 deletion (chr6:104,560,332-104,587,040) explained into detail in chapter 4, the importance of these structures should be bared in mind (patient 89). Also for this region the TAD boundary locations are not yet determined, but a TAD is found in the neighborhood of the CNV (103,293,307-104,973,307)[5, 6].

A fifth example is patient 83, a 9.5 year old boy with obesity and ID who harbors the p.A40S variant in *MRAP2*, the heterozygous variants p.P352T and p.A371V in *SIM1* and

two CNVs on chromosome X (a 26 kb duplication on Xp22.33 (chrX:1,478,557-1,504,864) and a 46 kb homozygous deletion on Xq23 (chrX:111,704,851-111,751,715)). Although both CNVs were already previously described in 1137 in-house healthy controls, there is still a possibility that they play a specific role in the phenotype of this boy. At the position of the 26 kb duplication, no TAD is reported, however one is located at the position of the homozygous deletion from position 111,033,344-111,953,344. In addition, further functional analysis of the p.A40S variant found in *MRAP2* could also create more insight into its role in the obesity phenotype seen in this patient.

The last example is a 5 year old girl with obesity, ID and behavioral problems who showed a 37 kb deletion on chromosome 1p36.33 and three deletions on chromosome 9q34 (patient 107). As we take a closer look to the chromosomal positions of the three 9q34 deletions, these are small CNVs (18 kb, 9 kb and 27 kb) that are located very close to each other. Consequently, there is a possibility that these small CNVs form a bigger deletion together ranging from position 139,552,461 to 140,329,141 overlapping a region of 776 kb. An independent MAQ or MLPA analysis for this region is necessary in order to confirm this hypothesis. Although the reported 9q34 CNVs in literature in PWL individuals are a couple of Mb in size[7], the 776 kb deletion together with the 1p36.33 deletion could still be involved in the development of obesity in our PWL patient. However, to date both CNVs are classified as variations with unknown significance.

Table 1. Overview of CNVs associated with the PWL phenotype and important results from mutation analysis of *MC4R*, *SIM1*, *MRAP2*, *MCHR2*, *OXT*, *OXTR* and *POU3F2* for the entire PWL cohort. / = No mutation analysis is performed for this patient. - = No important variants were identified for this patient. Het. = heterozygous. Hom. = homozygous.

Patient	Microarray results associated with the Prader Willi *like* phenotype	*MC4R* mutation analysis	*SIM1* mutation analysis	*MRAP2* mutation analysis	*MCHR2* mutation analysis	*OXT* mutation analysis	*OXTR* mutation analysis	*POU3F2* mutation analysis
1	Xp22.33 duplication (217 kb)	-	-	-	-	-	-	-
2	-	-	-	-	p.A76A (het.)	-	-	-
3	1p36.33 deletion (317 kb)	p.I251L polymorphism	-	-	-	-	-	-
4	-	-	-	-	p.A76A (het.)	-	-	-
5	-	-	-	-	p.A76A (het.)	-	p.A238T	-
6	-	-	p.P57P	-	p.A76A (het.)	-	p.A238T	-
7	Xp22.11 homozygous deletion (59 kb)	-	-	-	p.A76A (hom.)	-	-	-
8	16p11.2 deletion (602 kb)	-	-	-	p.A76A (het.)	-	-	p.Q149_R150insQ
9	-	-	p.T653T	-	p.A76A (het.)	-	-	-
10	Xq22.1 homozygous deletion (21 kb)	-	-	-	/	/	/	/
11	3p26.3 deletion (7.7 kb)	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	p.A238T	p.Q149_R150insQ
14	-	-	-	-	-	-	-	-
15	-	-	p.P352T (het.), p.A371V (het.)	-	-	-	-	p.Q149_R150insQ
16	6q14.1 deletion (24 kb)	-	-	-	-	-	-	-
17	Xp11.23 homozygous deletion (182 kb)	-	p.P352T (het.), p.A371V (het.)	-	-	-	p.A218T	-
18	6q14.1 duplication (137 kb)	-	-	-	-	-	-	-
19	1p36.33 deletion (40 kb)	-	-	-	-	-	-	-
20	1p36.33 homozygous deletion (16 kb)	p.I251L polymorphism	-	-	-	-	-	-
21	-	-	p.P352T (het.), p.A371V (het.)	-	/	-	-	-
22	-	-	-	-	p.A76A (het.)	-	-	-
23	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-
26	-	-	-	-	p.A76A (het.)	-	-	-
27	-	-	-	p.R125H	-	-	-	-
28	-	-	p.P352T (het.), p.A371V (het.)	-	-	-	-	-
29	-	-	p.P352T (hom.), p.A371V (hom.)	-	p.A76A (het.)	-	-	-
30	6q14.1 deletion (27 kb)	-	-	-	-	-	p.A218T	-
31	-	-	-	-	-	-	-	-
32	-	-	-	-	-	-	p.A218T	-
33	2p21 deletion (178 kb)	p.S127L mutation	/	/	/	/	/	/
34	-	-	-	-	/	/	/	-
35	-	-	-	-	-	-	-	-
36	6q15 deletion (154 kb)	-	p.T653T	-	-	-	-	-
37	-	-	-	-	-	-	-	p.Q149_R150insQ
38	-	-	-	-	-	-	-	-
39	-	-	p.P352S	-	-	/	/	-
40	-	p.V103I polymorphism	p.P352T (het.), p.A371V (het.)	-	-	-	p.A238T	-

Table 1 continued

Patient	Microarray results associated with the Prader Willi *like* phenotype	*MC4R* mutation analysis	*SIM1* mutation analysis	*MRAP2* mutation analysis	*MCHR2* mutation analysis	*OXT* mutation analysis	*OXTR* mutation analysis	*POU3F2* mutation analysis
41	-	-	-	p.R125H	p.A76A (het.)	-	p.A218T	-
42	-	-	-	-	-	-	p.S368S	-
43	-	-	-	-	p.A76A (het.)	-	-	-
44	-	-	-	-	-	-	-	-
45	-	-	-	-	-	-	-	-
46	-	-	p.P352T (hom.), p.A371V (hom.)	-	-	-	-	-
47	16p11.2 deletion (1.7 Mb)	-	-	-	-	-	-	-
48	-	-	-	-	p.A76A (het.)	-	-	-
49	-	-	p.P352T (het.), p.A371V (het.)	-	-	-	-	-
50	-	-	p.P352T (het.), p.A371V (het.)	-	-	-	-	-
51	-	-	p.P352T (het.), p.A371V (het.)	-	-	-	p.A218T	-
52	16p11.2 deletion (217 kb)	-	-	-	p.A76A (het.)	-	p.A218T	-
53	-	-	p.V208V	-	-	-	p.A218T	-
54	1p36.33 deletion (11 kb) 1p36.33 deletion (24 kb)	-	-	-	p.A76A (hom.)	-	-	-
55	-	-	-	-	p.A76A (het.)	-	p.A218T	-
56	-	-	-	-	-	/	/	-
57	16p11.2 deletion (217 kb)	-	-	-	-	-	-	p.G74G
58	-	-	p.P352T (het.), p.A371V (het.)	-	p.T47A	-	p.A218T	-
59	-	-	-	-	p.A76A (het.)	-	-	-
60	-	-	p.P352T (het.), p.A371V (het.)	-	-	-	-	-
61	-	-	-	-	-	-	-	-
62	-	-	-	-	-	-	p.A218T	-
63	-	-	-	-	-	-	-	-
64	-	-	p.P352T (het.), p.A371V (het.)	-	-	-	-	-
65	-	-	-	-	-	-	p.A218T	-
66	-	-	-	-	p.A76A (het.)	/	/	-
67	-	-	-	-	-	-	-	-
68	-	-	-	-	-	-	p.A218T	-
69	9q34.3 deletion (223 kb)	-	p.P352T (het.), p.A371V (het.)	-	-	-	-	-
70	-	-	-	-	-	-	-	-
71	Xq28 duplication (615 kb)	-	-	-	p.A76A (het.)	-	-	-
72	-	-	-	-	-	-	-	-
73	-	-	-	-	p.A76A (het.)	-	-	p.H255R
74	-	-	p.P352T (het.), p.A371V (het.)	-	p.A76A (het.)	-	-	-
75	16p11.2 deletion (602 kb)	-	-	-	-	-	-	-
76	6q14.1 deletion (41 kb)	-	p.P352T (het.), p.A371V (het.)	-	p.A76A (hom.)	-	-	-
77	6q14.1 deletion (41 kb) Xp22.11 deletion (62 kb)	-	p.P352T (het.), p.A371V (het.)	-	p.A76A (het.)	-	-	-
78	1p36.33 deletion (40 kb)	-	p.P352T (het.), p.A371V (het.)	-	-	-	p.A238T	-
79	-	-	p.P352T (hom.), p.A371V (hom.)	-	-	-	-	-

Table 1 continued

Patient	Microarray results associated with the Prader Willi *like* phenotype	*MC4R* mutation analysis	*SIM1* mutation analysis	*MRAP2* mutation analysis	*MCHR2* mutation analysis	*OXT* mutation analysis	*OXTR* mutation analysis	*POU3F2* mutation analysis
80	-	-	-	-	c.*16A>G	-	-	-
81	Xp22.33 duplication (21 kb)	-	p.P352T (het.), p.A371V (het.)	-	-	-	-	-
82	-	-	p.P352T (het.), p.A371V (het.)	-	-	-	-	-
83	Xp22.33 duplication (26 kb) Xq23 homozygous deletion (46 kb)	-	p.P352T (het.), p.A371V (het.)	p.A40S	-	-	-	-
84	-	-	-	-	-	-	p.A218T	-
85	-	-	-	-	-	-	p.A218T	p.Q149_R150insQ
86	Xq28 duplication (26 kb)	-	-	-	p.A76A (het.)	-	-	-
87	1p36.33 deletion (64 kb)	-	-	-	-	-	-	-
88	Xp22.33 duplication (114 kb)	-	-	c.127+7T>G	p.A76A (het.)	-	p.A218T, p.A238T	-
89	6q16.3 deletion (26 kb)	-	p.P352T (het.), p.A371V (het.)	-	-	-	-	-
90	1p36.33 deletion (64 kb)	-	-	-	-	-	-	-
91	-	-	-	-	-	-	-	-
92	-	-	-	-	-	-	-	-
93	-	-	-	-	-	-	-	-
94	-	-	-	-	-	-	-	-
95	-	-	-	-	p.A76A (het.)	-	-	-
96	-	-	/	/	/	-	p.A218T	-
97	-	-	/	/	-	-	-	-
98	-	-	/	/	/	/	/	/
99	-	-	/	/	/	/	/	/
100	-	-	/	/	/	/	/	/
101	-	-	/	/	/	/	/	/
102	6q16.1 deletion (43 kb) Xp22.33 duplication (2.6 Mb) Xq28 duplication (297 kb)	-	/	/	/	/	/	/
103	-	-	/	/	/	/	/	/
104	-	-	/	/	/	/	/	/
105	-	-	/	/	/	/	/	/
106	-	-	/	/	/	/	/	/
107	1p36.33 deletion (37 kb) 9q34.3 deletion (18 kb) 9q34.3 deletion (9 kb) 9q34.3 deletion (27 kb)	-	/	/	/	/	/	/
108	-	-	/	/	/	/	/	/
109	-	-	/	/	/	/	/	/
110	-	-	/	/	/	/	/	/

CONCLUSIONS

In this thesis, we investigated the involvement of six genes in the etiology of PWL and subsequently the obesity phenotype of these patients. We identified some possible disease causing variants and one interesting new gene: *POU3F2*. In order to elucidate the real causality of these variants and their contribution to obesity, further functional analysis is still necessary. This can result into a better understanding of the genes and the pathways they are involved in. In addition, the involvement of long-range gene-gene or gene-protein interactions and the interference of identified CNVs with the TAD structure should be considered. Subsequently this can lead to the identification of new genes and possible interesting therapeutic targets for obesity and ultimately to accurate treatment of the PWL phenotype and obesity in general.

PITFALLS OF OUR STUDY

Throughout the years, the PWL phenotype has already been associated with more than 20 different CNVs of which a deletion of chromosome 6q was most frequently reported. This already indicates the extremely heterogeneous character of the disorder. With the use of the genome-wide microarray approach, we also confirmed this heterogeneity in our study as 1) we were not able to provide strong support for involvement for any of the previously reported CNVs including the most common one on chromosome 6q16.3 and 2) we even noticed the presence of obesity associated 16p11.2 deletions in five out of the 109 PWL patients. This can be related to the inclusion criteria that were used for our PWL cohort at the start of this thesis. All individuals who were referred to our Department of Medical Genetics (University of Antwerp and University Hospital Antwerp, Antwerp, Belgium) with suspected PWS (i.e. a combination of obesity and developmental delay/intellectual disability and occasionally hypotonia or behavioral problems) over the past 25 years were enrolled in the study. As these inclusion criteria are not that strict, the presence of 16p deletions in our study population could be expected as in literature 16p11.2 deletions have already been associated with obesity and intellectual disability and explains, in all

probability, the phenotype in these patients. In addition, all the features are also quite frequently described in the general population leading to a relatively high risk for misdiagnosing PWL patients. These results show that accurate phenotyping of PWL patients or even a creation of different subgroups in the PWL phenotype is required. Although the improved resolution of the array-based technology already provided more accurate genotype – phenotype correlations, many more PWL cases are needed to be able to subdivide the patients in different groups as to date the heterogeneous character, even within patients with the same structural variation, impedes this. Eventually this could lead to a better delineation of candidate genes responsible for the obesity phenotype seen in these patients and to a clear differentiation from PWS and other obesity syndromes.

MISSING HERITABILITY

In the past, linkage analyses in combination with mouse models led to the identification of causal genes for monogenic obesity. One of these genes is *LEP* encoding leptin. Congenital leptin deficiency, due to a homozygous deletion or mutation in the gene, is the only treatable form of monogenic obesity. However it is extremely rare. On the contrary, mutations in *MC4R* are the most common causes of monogenic obesity, with a frequency of approximately 2-5%[8]. Recently, our own research group also brought forward *NPY4R* as a new risk factor for obesity as genetic and structural variation in the gene (at least partially) explained the obesity in respectively 0.6% and 3.7% of the study population which are prevalences close to those reported for *MC4R*[9]. Other identified causal genes for monogenic obesity are all implicated in the hypothalamic leptin-melanocortin signaling pathway but only explain a small fraction of the heritability of obesity.

However, in most cases obesity is a combination of genetic and environmental factors. Twin and adoption studies estimated the contribution of genetic variants at 40-70%. To date, genome-wide association studies (GWAS) and meta-analyses of BMI identified 97 loci accounting for approximately 2.7% of this heritability[10]. The

underlying rationale for GWAS is the 'common disease, common variant' hypothesis, meaning that it focuses on variants with a MAF ≥ 0.05, i.e. SNPs present in 5% or more of the population. Less common variants (0.005 < MAF < 0.05) and even rare variants (MAF < 0.005) cannot be captured by the current GWA genotyping arrays, however as visible from the explained heritability so far, it can be argued that the missing heritability needs to be found in the more rare variants or even rare deletions or duplications ('common disease, rare variant' hypothesis)[11]. Subsequently, in our study we searched for rare SNVs and CNVs in a specific cohort of patients with syndromic obesity. In order to do so, we first performed a genome-wide microarray analysis. This is a valuable tool to identify rare CNVs in patients with extreme and different phenotypic features that can aid in the elucidation of more common diseases. Secondly, the primary technology for the detection of rare SNVs is sequencing. In our study, we decided to go for a candidate gene approach using the HRM technique in combination with Sanger sequencing. The HRM technique is highly sensitive and specific and, due to the high sample throughput, low in cost[12]. As our genes of interest were rather small this was an efficient method. However, these simple screening techniques are becoming too expensive and time-consuming to screen for disease-causing mutations especially in highly heterogeneous disorders. Yet nowadays, with the advent of automated DNA sequencing instruments, the Next Generation Sequencing (NGS) technology provides a new method for molecular diagnosis.

1. Identification of rare variants by Next Generation Sequencing (NGS)

In contrast to conventional capillary-based sequencing, the NGS technology is able to process millions of sequence reads in parallel which makes it possible to sequence gene panels, whole exomes and even whole genomes[13, 14]. Consequently, in 2010 the UK10K project was established harboring sequencing results of 2000 exomes of deeply phenotyped individuals with severe, early-onset obesity. Due to the high sample set the study's power to identify rare variants linked to the disease increases, leading to a

clearer overview of genetic variation in obesity. As these data are available for researchers worldwide, this greatly empowers the genetic research for causal genes for obesity[15].

During my PhD we also performed whole-exome sequencing for four patients with a PWL phenotype. However, after accurate filtering a lot of variants with unknown significance remained and subsequently we could not identify the possible gene(s) explaining the obesity phenotype in any of these patients. This can probably be attributed to the limited information available for the patients as the features of PWL patients (obesity and intellectual disability/developmental delay) are also frequently described in the general population. In the future, screening of a group of patients with a more extreme and the same detailed phenotype can aid in better variant selection leading to a higher chance to indicate the possible disease-causing one(s). Also for the four patients no DNA of additional family members was available. As co-segregation is one of the key hallmarks of a pathogenic mutation, recruitment of either parents or siblings is a plus but however very challenging in many cases.

2. Epigenetics

As it is very unlikely that rare variants will explain the missing heritability completely, in the last few years epigenetic regulation of gene expression emerged as a potential factor explaining individual differences in obesity risk[13, 16]. Epigenetics has to date been defined as 'the study of changes in gene function that are mitotically and/or meiotically heritable but do not entail a change in DNA sequence'[17]. Subsequently these changes are reversible[18]. The major epigenetic markers include DNA methylation, histone modifications which could induce chromatin alterations, microRNA and genomic imprinting. For example, in case of Prader Willi syndrome correct imprinting of the chromosome 15q11.2 – q13 region could not be maintained. In healthy individuals, imprinting caused by DNA methylation virtually silences either the paternally or maternally inherited copies of the genes at this chromosomal region

and only the other copies of the genes are expressed. In patients with PWS, however, the expression of the paternal genes is lost due to a defect (deletion, maternal uniparental disomy or imprinting defect) of this imprinted region causing the corresponding obesity phenotype. The role of epigenetics in this obesity syndrome with a rather high prevalence (1 in 10.000 – 30.000 live births) points to the importance for further research of epigenetic regulation of genes involved in obesity. In addition, further research of children born around the time of the Dutch famine in 1944 – 1945 also revealed the importance of epigenetics in obesity as these studies discovered an association between restricted maternal nutrition and obesity of the child later in life[19-21]. Lastly, also different candidate-gene studies demonstrate the role of epigenetics (especially DNA methylation) in obesity. These studies indicated for example an aberrant methylation pattern for *LEP* (hypomethylation)[22] and *POMC* (hypermethylation)[23], two key players of the leptin-melanocortin signaling pathway.
Importantly, although the genome of an individual is largely stable, the epigenome is not. It has the potential to be reversibly modified by exposure to a range of environmental and nutritional factors. Recent technological advances in epigenome profiling already led to an increase in large-scale epigenome studies exploring the relationship between the environment, the epigenome and complex diseases. In addition, recently also EpiSCOPE (Epigenome Study Consortium for Obesity primed in the Perinatal Environment) is founded by four Australian institutions with the aim to add valuable new information to our understanding of the role of epigenetics in the etiology of obesity[18].

3. *Gene-gene, gene-environment, gene-protein interactions and topologically associating domains (TADs)*

Last mechanisms which could possibly explain the missing heritability in obesity are gene-gene, gene-environment, gene-protein interactions and topologically associating domains (TADs). For example, there is a possibility that a specific genetic factor operates through a complex network of multiple genes and/or environmental factors

and when investigating this genetic factor in isolation the fear exists that the actual effect is missed because the potential interaction with the other (unknown) factors is not included. Detection of these gene-gene interactions in GWAS with the use of statistical and computational methods is becoming extremely popular, however challenging due to the complexity of these gene networks[24]. In case of gene-protein interactions a protein, e.g. a transcription factor, binds a molecule of DNA and regulates the expression of the corresponding gene or promoter. Interactions can be accomplished through electrostatic interactions (salt bridges), dipolar interactions (hydrogen bonding, H-bonds), entropic effects (hydrophobic interactions) or dispersion forces (base stacking). Lastly, as already mentioned previously, the study of TADs and their boundary regions is really emerging as they are critical for correct gene expression and their disruption due to CNVs can cause diseases[4]. In order to study the gene-gene and gene-protein interactions, different laboratory techniques are available however the chromatin immunoprecipitation (ChIP) assays (ChIP-Seq and ChIP-on-chip) are extremely powerful and versatile techniques[25]. In addition, different chromosome conformation capture techniques such as 3C, 4C and Hi-C have enabled scientist to study the 3D organization of the genome and consequently to identify different TADs[4]. Especially due to its heterogeneous character, all these complex mechanisms should definitely be considered in the PWL phenotype.

FUTURE PERSPECTIVES: THERAPEUTIC AGENTS ORIGINATED FROM GENETIC FINDINGS

As obesity has become a chronic disease reaching epidemic proportions, different pharmaceutical companies focus on the production of pharmatherapeutic agents which are effective in inducing weight loss but also in maintaining weight loss achieved by lifestyle measures.

For patients with monogenic obesity due to a *LEP* mutation resulting in congenital leptin deficiency, Amylin Pharmaceutics produced Metreleptin, a synthetic analogue of the leptin hormone. Daily subcutaneous injection of the analogue completely

reverses the phenotype and since 2014 has been approved by the Food and Drug Administration (FDA) for the treatment of congenital leptin deficiency[26, 27].

Another potentially interesting target in the leptin-melanocortin signaling pathway in the development of obesity drugs is MC4R. As activation of MC4R causes dramatic weight loss in rodent models and mutations in humans are associated with obesity, pharmaceutical companies such as Rhythm Pharmaceuticals initially started with the development of MC4R agonists[28]. Although administration of different MC4R agonists resulted in the production of different adverse effects including increased heart rate and blood pressure, clinical trials of one agonist, Setmelanotide, show promising results. Setmelanotide is a highly-selective MC4R agonist inducing a reduction in appetite and an increase of resting energy expenditure in obese individuals[29]. It recently also proved its effect in two patients with POMC deficiency[30]. However, long-term effects still need to be investigated. Noteworthy, also phase II studies evaluating the efficacy and safety of Setmelanotide on weight and eating behaviors in PWS patients are ongoing. This trial can assess the effect of Setmelanotide as a 'replacement therapy' for the treatment of the severe obesity and hyperphagia seen in PWS patients[31].

Recent studies also indicate that OXT administration can work to exert anti-obesity effects. Pilot studies in which intranasal OXT is delivered to prediabetic obese humans led to effective weight loss and reversal of related prediabetic changes in the patients[32]. In addition, the effects of the oxytocin nasal spray are also under investigation in PWS patients to treat hyperphagia. Despite contradictory results of different clinical trials, a promising effect on food-related behavior was seen in children with PWS younger than 11 years of age[33]. Further clinical trials in obese individuals with and without the PWS phenotype are still in progress but based on the results so far, OXT administration can be a very promising therapeutic for obesity and/or PWS.

In conclusion, the overall objective of genetic obesity studies is to improve the knowledge of the underlying mechanisms of energy metabolism and identify interesting genes explaining a part of the missing heritability which could eventually be used as an interesting new target for future therapeutic treatment.

By investigating a specific cohort of patients with syndromic obesity, we tried to generate new insights into the genetic background of PWL and the obesity phenotype seen in these patients. Although our research only led to the identification of a few variants with a possible damaging effect, we are convinced we contributed to either PWL and obesity research. Especially further functional analysis of the interesting variants found in *POU3F2* would provide further support for a possible role of the gene in the pathogenesis of PWL and their obesity phenotype. Eventually POU3F2 could also be used as a potential therapeutic target for obesity in the future.

References

1. Khattabi, L.E., et al., *Incomplete penetrance and phenotypic variability of 6q16 deletions including SIM1.* Eur J Hum Genet, 2014.
2. Zegers, D., et al., *Mutation screen of the SIM1 gene in pediatric patients with early-onset obesity.* Int J Obes (Lond), 2014. **38**(7): p. 1000-4.
3. Martens, K., et al., *PREPL: a putative novel oligopeptidase propelled into the limelight.* Biol Chem, 2006. **387**(7): p. 879-83.
4. Lupianez, D.G., M. Spielmann, and S. Mundlos, *Breaking TADs: How Alterations of Chromatin Domains Result in Disease.* Trends Genet, 2016. **32**(4): p. 225-37.
5. Dixon, J.R., et al., *Topological domains in mammalian genomes identified by analysis of chromatin interactions.* Nature, 2012. **485**(7398): p. 376-80.
6. *ENCODE: Encyclopedia of DNA Elements*. [cited 2018 January].
7. Cormier-Daire, V., et al., *Cryptic terminal deletion of chromosome 9q34: a novel cause of syndromic obesity in childhood?* J Med Genet, 2003. **40**(4): p. 300-3.
8. Hinney, A., A.L. Volckmar, and N. Knoll, *Melanocortin-4 receptor in energy homeostasis and obesity pathogenesis.* Prog Mol Biol Transl Sci, 2013. **114**: p. 147-91.
9. Aerts, E., et al., *CNV analysis and mutation screening indicate an important role for the NPY4R gene in human obesity.* Obesity (Silver Spring), 2016. **24**(4): p. 970-6.
10. Locke, A.E., et al., *Genetic studies of body mass index yield new insights for obesity biology.* Nature, 2015. **518**(7538): p. 197-206.
11. Manolio, T.A., et al., *Finding the missing heritability of complex diseases.* Nature, 2009. **461**(7265): p. 747-53.
12. Reed, G.H. and C.T. Wittwer, *Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis.* Clinical Chemistry, 2004. **50**(10): p. 1748-1754.
13. Albuquerque, D., et al., *Current review of genetics of human obesity: from molecular mechanisms to an evolutionary perspective.* Mol Genet Genomics, 2015. **290**(4): p. 1191-221.
14. Marian, A.J., *Molecular genetic studies of complex phenotypes.* Transl Res, 2012. **159**(2): p. 64-79.
15. *UK10K Project*. [cited 2017 November]; Available from: https://www.uk10k.org.
16. Campion, J., F.I. Milagro, and J.A. Martinez, *Individuality and epigenetics in obesity.* Obes Rev, 2009. **10**(4): p. 383-92.
17. Dupont, C., D.R. Armant, and C.A. Brenner, *Epigenetics: definition, mechanisms and clinical perspective.* Semin Reprod Med, 2009. **27**(5): p. 351-7.
18. van Dijk, S.J., et al., *Epigenetics and human obesity.* Int J Obes (Lond), 2015. **39**(1): p. 85-97.

19. Roseboom, T., S. de Rooij, and R. Painter, *The Dutch famine and its long-term consequences for adult health.* Early Hum Dev, 2006. **82**(8): p. 485-91.
20. Kyle, U.G. and C. Pichard, *The Dutch Famine of 1944-1945: a pathophysiological model of long-term consequences of wasting disease.* Curr Opin Clin Nutr Metab Care, 2006. **9**(4): p. 388-94.
21. van Abeelen, A.F., et al., *Postnatal acute famine and risk of overweight: the dutch hungerwinter study.* Int J Pediatr, 2012. **2012**: p. 936509.
22. Obermann-Borst, S.A., et al., *Duration of breastfeeding and gender are associated with methylation of the LEPTIN gene in very young children.* Pediatr Res, 2013. **74**(3): p. 344-9.
23. Kuehnen, P., et al., *An Alu element-associated hypermethylation variant of the POMC gene is associated with childhood obesity.* PLoS Genet, 2012. **8**(3): p. e1002543.
24. Hu, J.K., X. Wang, and P. Wang, *Testing gene-gene interactions in genome wide association studies.* Genet Epidemiol, 2014. **38**(2): p. 123-34.
25. *Thermofisher*. [cited 2017 November]; Available from: www.thermofisher.com.
26. Chou, K. and C.M. Perry, *Metreleptin: first global approval.* Drugs, 2013. **73**(9): p. 989-97.
27. *Food and Drug Administration*. [cited 2017 November]; Available from: https://www.fda.gov/.
28. Kievit, P., et al., *Chronic treatment with a melanocortin-4 receptor agonist causes weight loss, reduces insulin resistance, and improves cardiovascular function in diet-induced obese rhesus macaques.* Diabetes, 2013. **62**(2): p. 490-7.
29. Chen, K.Y., et al., *RM-493, a melanocortin-4 receptor (MC4R) agonist, increases resting energy expenditure in obese individuals.* J Clin Endocrinol Metab, 2015. **100**(4): p. 1639-45.
30. Kuhnen, P., et al., *Proopiomelanocortin Deficiency Treated with a Melanocortin-4 Receptor Agonist.* N Engl J Med, 2016. **375**(3): p. 240-6.
31. *Rhythm Pharmaceuticals*. [cited 2017 November]; Available from: http://www.rhythmtx.com/.
32. Zhang, H., et al., *Treatment of obesity and diabetes using oxytocin or analogs in patients and mouse models.* PLoS One, 2013. **8**(5): p. e61477.
33. Kuppens, R.J., S.H. Donze, and A.C. Hokken-Koelega, *Promising effects of oxytocin on social and food-related behaviour in young children with Prader-Willi syndrome: a randomized, double-blind, controlled crossover trial.* Clin Endocrinol (Oxf), 2016. **85**(6): p. 979-987.

LIST OF ABBREVIATIONS

List of abbreviations

AA = Amino acid

ACE = Angiotensin-converting-enzyme

ADHD = Attention deficit hyperactivity disorder

AFF4 = AF4/FMR2 family member 4

AgRP = Agouti-related peptide

AHO = Albright hereditary osteodystrophy

ALMS1 = Alstrom syndrome protein 1

α-MSH = α-melanocyte stimulating hormone

AnS = Angelman syndrome

ARC = Arcuate nucleus

AREs = AU-rich elements

ARMS = Amplification-refractory mutation system

AS = Alström syndrome

ASD = Autism spectrum disorders

ATP10A = ATPase phospholipid transporting 10A (putative)

AURA = Atlas of UTR regulatory activity

BBS = Bardet-Biedl syndrome

BBS1 = Bardet-Biedl syndrome 1

BBS2 = Bardet-Biedl syndrome 2

BBS6 = Bardet-Biedl syndrome 6

BBS10 = Bardet-Biedl syndrome 10

BBS20 = Bardet-Biedl Syndrome 20

BDNF = Brain-derived neurotrophic factor

bHLH = Basic helix-loop-helix

bHLH-PAS = Basic helix-loop-helix Per-Arnt-Sim

β-MSH = β-melanocyte stimulating hormone

BMI = Body mass index

bp = base pairs

BP = Breakpoint

BRN2 = POU3F2

CADD = Combined annotation dependent depletion

CAMKMT = Calmodulin-lysine N-methyltransferase

CART = Cocaine and amphetamine related transcript

CEN = Centromere

CEP19 = Centrosomal Protein 19

CGH = Comparative genomic hybridization

CGS = Contiguous gene syndrome

CHL1 = Cell adhesion molecule L1 like

CHM = Choroideremia (Rab escort protein 1)

CHOPS = Cognitive impairment-coarse facies-heart defects-obesity-pulmonary involvement-short stature-skeletal dysplasia

CNS = Central nervous system

CNV = Copy number variation

CREBBP = CREB binding protein

CS = Carpenter syndrome

DNA = Deoxyribonucleic acid

dNTPs = Deoxynucleotides

EEG = electroencephalography

EIF2S3 = Eukaryotic Translation Initiation Factor 2 Subunit Gamma

EP300 = E1A binding protein P300

ESRD = End-stage renal disease

ExAC = Exome aggregation consortium

FBN3 = Fibrillin 3

FISH = Fluorescence in situ hybridization

FMR1 = Fragile X mental retardation 1

γ-MSH = γ-melanocyte stimulating hormone

GLP-1 = glucagon-like peptide -1

GNAS1 = Guanine nucleotide-binding protein G(s) subunit alpha

GNB3 = Guanine nucleotide binding protein (G Protein), beta polypeptide 3

gnomAD = Genome aggregation database

GOOS = Genetics of obesity study

GPCR = G protein-coupled receptor

GTP = Guanosine-5'-triphosphate

GWAS = Genome-wide association studies

Het. Del. = Heterozygous deletion

Het. Dup. = Heterozygous duplication

HRM = High-resolution melting curve analysis

IC = Imprinting center

ID = Intellectual disability

IQ = Intelligence quotient

INPP5E = Inositol polyphosphate-5-phosphatase E

JAK2 = Janus kinase 2

kb = kilobase

LAS1L = LAS1 like, ribosome biogenesis factor

LD = Linkage disequilibrium

LEP = Leptin

MAF = Minor allele frequency

MAGEL2 = MAGE family member 2

MAQ = Multiplex Amplicon Quantification

Mb = Megabase

MCH = Melanin-concentrating hormone

MCHR1 = Melanin-concentrating hormone receptor 1

MCHR1R2 = Melanin-concentrating hormone receptor 1 receptor 2

MCHR2 = Melanin-concentrating hormone receptor 2

MCR = Melanocortin receptor

MC3R = Melanocortin 3 receptor

MC4R = Melanocortin 4 receptor

MEGF8 = Multiple epidermal growth factor like domains 8

MEHMO = X-linked mental retardation (intellectual disability)-epileptic seizures-hypogenitalism-microcephaly-obesity

$MgCl_2$ = Magnesium chloride

miRNA = Micro ribonucleic acid

µl = Microliter

MKRN3 = Makorin ring finger protein 3

mM = Millimolar

MO = Morpholino

MOMES = Mental retardation (intellectual disability)-obesity-prognathism-eye and skin anomalies

MOMO = Macrocephaly-obesity-mental disability-ocular abnormalities

MOPD = Microcephalic osteodysplastic primordial dwarfism

MORM = Mental retardation (intellectual disability)-truncal obesity-retinal dystrophy-micropenis

MRAP2 = Melanocortin 2 receptor accessory protein 2

mRNA = Messenger ribonucleic acid

MS-MLPA = Methylation-specific-multiplex ligation-dependent probe amplification analysis

NDN = Necdin MAGE family member

ng = Nanogram

NGS = Next generation sequencing

NLS = Nuclear localization signal

nM = Nanomolar

NPHP1 = Nephrocystin 1

NPY = Neuropeptide Y

Ob = Obese

OXT = Oxytocin

OXTR = Oxytocin receptor

PAC = Per-Arnt-Sim associated C-terminal

PAS = Per-Arnt-Sim

PAX6 = Paired box 6

PCNT = Pericentrin

PCR = Polymerase chain reaction

PCSK1 = Pro-protein convertase subtilisin/kexin type 1

PHF6 = PHD finger protein 6

PHP = Pseudohypoparathyroidism

POMC = Pro-opiomelanocortin

POU3F2 = POU Class 3 Homeobox 2

Pou3f2a = Zebrafish ortholog a of POU3F2

Pou3f2b = Zebrafish ortholog b of POU3F2

POU3F4 = POU Class 3 Homeobox 4

PREPL = Prolyl endopeptidase-like

PRMT7 = Protein arginine methyltransferase 7

PVN = Paraventricular nucleus

PWL = Prader Willi *like*

PWS = Prader Willi syndrome

RAB23 = RAB family small GTP binding protein RAB 23

rhGH = Recombinant human growth hormone

RNA = Ribonucleic acid

RPS6KA3 = Ribosomal protein S6 kinase A3

SH2B1 = Sarcoma (Src) homology 2B adaptor protein 1

SIM1 = Single-minded 1

SNORD115 = Small nucleolar RNA, C/D box 115

SNORD116 = Small nucleolar RNA, C/D box 116

snoRNA = Small nucleolar RNA

SNP = Single-nucleotide polymorphism

SNRPN = Small nuclear ribonucleoprotein polypeptide N

SNURF = Small nuclear ring finger

SON = Supraoptic nucleus

TADs = Topologically associating domains

TEL = Telomere

TRAPPC9 = Trafficking protein particle complex 9

T1D = Type I deletion

T2D = Type II deletion

T2DM = Type II diabetes mellitus

UBE3A = Ubiquitin protein ligase E3A

UPD = Uniparental disomy

UTR = Untranslated region

VMN = Ventromedial nucleus

VPS13B = Vacuolar protein sorting 13 homolog B

WAGR = Wilms tumor, aniridia, genitourinary malformations and mental retardation

WAGRO = Wilms tumor, aniridia, genitourinary malformations, mental retardation and obesity

WHO = World health organisation

WT = Wild type

WT1 = Wilms tumor 1

zMRAP2b = Zebrafish ortholog of MRAP2

ZNA = Antwerp hospital network

CURRICULUM VITAE

Minderhoutsestraat 5 bus 10
2320 Hoogstraten
0479 30 18 24
ellengeets@hotmail.com
°18 januari 1989, Turnhout

Ellen Geets

Profile

- ✓ PhD in Biomedical Sciences
- ✓ Both independent and team player
- ✓ Communication and organizational skills
- ✓ Social
- ✓ Eager to learn

Academic career and education

ACADEMIC CAREER

2013 – Present | **PHD IN SCIENCE: BIOMEDICAL SCIENCES**

Department of Medical Genetics, University of Antwerp

PhD dissertation: "Targeted search for genetic causes of the Prader Willi *like* phenotype"

- *Promotor: Prof. Dr. Wim Van Hul*

Optional courses:

- Leadership and teamwork
- Introductory course to Next Generation Sequencing
- Basic principles of statistics
- Applied communication

EDUCATION

2011 – 2013 | **MASTER OF SCIENCE IN BIOMEDICAL SCIENCES**

University of Antwerp

Specialization: Molecular and Cellular Biomedical Sciences

Optional courses:

- Strategic Business Communication
- Ecology, epidemiology and combatting tropical diseases
- Toxicological working mechanisms

Master dissertation: "Research of the role of LRP4 in the canonical WNT pathway"

- *Promotor: Prof. Dr. Wim Van Hul*

Research internship: "Research of the role of *GFI1B* and *TGFBR3* in Gray Platelet Syndrome"

- *Promotors: Prof. Dr. Bart Loeys and Prof. Dr. Lut Van Laer*

Great distinction

2007 – 2011	**BACHELOR OF SCIENCE IN BIOMEDICAL SCIENCES** University of Antwerp **Bachelor dissertation: "Passive smoking: Prevention and legislation"** **Distinction**
2001 – 2007	**SCIENCE - MATHEMATICS** Klein Seminarie, Hoogstraten

Additional scientific training

Oct 2012	**LABORATORY ANIMAL SCIENCE, FELASA TYPE C** University of Antwerp

Internships

Nov '12-May '13	**LAB WORK** Department of Medical Genetics, University of Antwerp ▫ Polymerase Chain Reaction, Sanger sequencing ▫ Cell culture, functional studies (Western blot, Luciferase Reporter Assays) English **Great distinction**
May - June '12	**LAB WORK** Department of Medical Genetics, University of Antwerp ▫ Primer design, Polymerase Chain Reaction, Sanger sequencing ▫ Cell culture English **Great distinction**

Holiday work

July/Sept. 2012	**LAB WORK** Department of Medical Genetics, University of Antwerp ▫ Polymerase Chain Reaction, Sanger sequencing

2008 - 2013	**HORECA**
	De Vredesboom, Meer (Weekend work) ▫ Take charge of the hall staff

Language skills

	Speaking	*Understanding*	*Writing*
Dutch	Native language	Native language	Native language
English	Very good	Very good	Good

Grants

FWO Travel Grant - The European Obesity Summit 2016, Göteborg, Sweden, 1-4 June 2016.

Educational activities

Assistance internship: *"Mutatie-analyse van het MAGEL2 gen in Prader-Willi like patiënten"* by Lena Duchateau, 2nd Master in Biochemistry and Biotechnology, University of Antwerp, Belgium, Academic year 2016 - 2017.

Assistance internship: *"Mutatie-analyse van POU3F2 in obese kinderen en adolescenten"* by Pien Barendsen, 1st Master in Biomedical Sciences, University of Antwerp, Belgium, Academic year 2015 - 2016.

Assistance Bachelor Thesis: *"Syndromale vormen van obesitas"* by Lisa Van Nieuwenhuysen, 3rd Bachelor in Biomedical Sciences, University of Antwerp, Belgium, Academic year 2015 - 2016.

Assistance internship: *"LEP and MCHR2 mutation analysis in patients with Prader Willi like phenotype"* by Ruben François, 2nd Master in Biochemistry and Biotechnology, University of Antwerp, Belgium, Academic year 2015 – 2016.

Assistance Honours College: *"Genetisch onderzoek naar de rol van POU3F2 in patiënten met het Prader Willi like (PWL) fenotype"* by Matthias Govaerts, 2nd Bachelor Biochemistry and Biotechnology, University of Antwerp, Belgium. Academic year 2014 - 2015.

Assistance internship: *"Mutation analysis of MC4R in Prader Willi like patients"* by Habib Sayaf, 2nd master in Molecular Biology, Vrij Universiteit Brussel, Belgium, Academic year 2013 - 2014.

Publication list

Geets E, Aerts E, Verrijken A, Diels S, Van Hoorenbeeck K, Verhulst S, Van Gaal L, Van Hul W. "*Copy number variation and mutation analysis indicate a possible interesting role of POU3F2 in the Prader Willi like phenotype."* In preparation.

Geets E, Aerts E, Verrijken A, Van Hoorenbeeck K, Verhulst S, Van Gaal L, Van Hul W. *"Structural and mutation screening indicates no major involvement of OXT and OXTR in Prader Willi like patients."* Submitted in Endocrine Research.

Geets E, Meuwissen MEC, Van Hul W. *"Clinical, molecular genetics and therapeutic aspects of syndromic obesity."* Submitted in Clinical Genetics.

Geets E, Aerts E, Verrijken A, Van Hoorenbeeck K, Verhulst S, Van Gaal L, Van Hul W. *"DNA sequencing and copy number variation analysis of MCHR2 in a cohort of Prader Willi like (PWL) patients."* Obes Res Clin Pract. 2017. In Press. doi: 10.1016/j.orcp.2017.10.001.

Aerts E, **Geets E**, Sorber L, Beckers S, Verrijken A, Massa G, Van Hoorenbeeck K, Verhulst SL, Van Gaal LF, Van Hul W. *"Evaluation of a Role for NPY and NPY2R in the Pathogenesis of Obesity by Mutation and Copy Number Variation Analysis in Obese Children and Adolescents."* Ann Hum Genet. 2017 Aug 31. doi: 10.1111/ahg.12211.

Fijalkowski I, **Geets E**, Steenackers E, Van Hoof V, Ramos FJ, Mortier G, Fortuna AM, Van Hul W, Boudin E. *"A Novel Domain-Specific Mutation in a Sclerosteosis Patient Suggests a Role of LRP4 as an Anchor for Sclerostin in Human Bone."* J Bone Miner Res. 2016 Apr;31(4):874-81. doi: 10.1002/jbmr.2782.

Geets E, Zegers D, Beckers S, Verrijken A, Massa G, Van Hoorenbeeck K, Verhulst S, Van Gaal L, Van Hul W. *"Copy number variation (CNV) analysis and mutation analysis of the 6q14.1-6q16.3 genes SIM1 and MRAP2 in Prader Willi like patients."* Mol Genet Metab. 2016 Mar;117(3):383-8. doi: 10.1016/j.ymgme.2016.01.003.

Congressional contributions

ORAL PRESENTATIONS

Belgian Association for the study of Obesity (BASO) Free communications Meeting 2018, Antwerp, Belgium

"Structural and mutation screening indicates a possible interesting role of POU3F2 in Prader Willi like patients."

Belgian Medical Genomics Initiative (BeMGI) – Annual Meeting 2016, Brussels, Belgium

"Combination of CNV analysis and mutation screening indicates an important role for the NPY4R gene in human obesity"

Belgian Association for the study of Obesity (BASO) Free communications Meeting 2016, Brussels, Belgium

"Copy Number Variation (CNV) and mutation analysis of the 6q14.1 – 6q16.3 genes SIM1 and MRAP2 in Prader Willi like patients"

Belgian Association for the study of Obesity (BASO) Free communications Meeting 2015, Brussels, Belgium

"Mutation analysis of the MRAP2 gene in Prader Willi like patients and obese children and adolescents"

POSTER PRESENTATIONS

Belgian Society of Human Genetics (BeSHG) – Annual Meeting 2018, Ghent, Belgium
"Copy number variation and mutation analysis indicate a possible interesting role of POU3F2 in the Prader Willi like phenotype."

American Society of Human Genetics (ASHG) – Annual Meeting 2017, Orlando, Florida, United States of America
"Copy number variation and mutation analysis indicate a possible interesting role of POU3F2 in the Prader Willi like phenotype."

European Obesity Summit (EOS) 2016, Göteburg, Sweden
"Copy Number Variation (CNV) and mutation analysis of the 6q14.1 – 6q16.3 genes SIM1 and MRAP2 in Prader Willi like patients."

Belgian Society of Human Genetics (BeSHG) & NVHG First Joint Meeting 2016, Leuven, Belgium
"Copy Number Variation (CNV) and mutation analysis of the 6q14.1 – 6q16.3 genes SIM1 and MRAP2 in Prader Willi like patients."

Belgian Medical Genomics Initiative (BeMGI) – Annual Meeting 2016, Brussels, Belgium
"Copy Number Variation (CNV) and mutation analysis of the 6q14.1 – 6q16.3 genes SIM1 and MRAP2 in Prader Willi like patients."

European Society of Human Genetics (ESHG) – Annual Meeting 2015, Glasgow, Scotland
"Mutation analysis of the MRAP2 gene in Prader Willi like patients and obese children and adolescents."

Belgian Medical Genomics Initiative (BeMGI) – Annual Meeting 2015, Ghent, Belgium
"Mutation analysis of the MRAP2 gene in Prader Willi like patients and obese children and adolescents."

Belgian Society of Human Genetics (BeSHG) – Annual Meeting 2015, Charleroi, Belgium
"Mutation analysis of the MRAP2 gene in Prader Willi like patients and obese children and adolescents."

DANKWOORD

In dit laatste gedeelte zou ik heel graag de kans willen grijpen om alle mensen te bedanken die rechtstreeks of onrechtstreeks mee hebben geholpen aan het verwezenlijken van deze doctoraatsthesis.

Allereerst zou ik graag mijn promotor, Prof. Dr. Wim Van Hul, willen bedanken om mij de kans te geven om na mijn masterthesis een doctoraat te starten in zijn onderzoeksgroep. Wim, dat allereerste jaar verliep al niet zonder slag of stoot. Na tweemaal er niet in te slagen om een IWT-beurs te behalen en daarnaast nog eens in het ziekenhuis terecht te komen waardoor ik mijn allereerste Europese congres op het laatste nippertje niet meer kon bijwonen, gaf je me toch nog de kans om dit doctoraat ook daadwerkelijk af te werken en daar ben ik je enorm dankbaar voor. Bedankt ook voor je vertrouwen en de kansen die je me gaf om nieuwe technieken aan te leren en het enthousiasme dat je toonde voor mijn werk. Hiernaast wil ik je ook oprecht bedanken voor je enorme medeleven en begrip, niet enkel in dat eerste jaar tijdens mijn ziekte, maar ook toen ik vroeger dan normaal uitviel wegens de geboorte van Elise en in de tijden dat ze ziek was. Ik kon me geen betere promotor voorstellen.

Ik ben ook Prof. Dr. Geert Mortier enorm dankbaar voor zijn inbreng en suggesties tijdens de botvergaderingen en in het bijzonder ook voor de kans die hij mij gegeven heeft om mijn doctoraat op een goede manier af te werken.

Hiernaast zou ik ook graag de leden van de jury, Prof. Dr. Kristel Sleegers, Prof. Dr. Bart Loeys, Dr. Marcel Twickler, Prof. Dr. Bart Van der Auwera en Dr. Marelise Eekhoff, willen bedanken voor hun kritische kijk op dit proefschrift en hun opmerkingen en suggesties die deze thesis enkel maar beter hebben gemaakt.

Ook zou ik mijn oprechte dank willen betuigen aan alle artsen die ons de afgelopen 25 jaar bloedstalen hebben bezorgd van Prader Willi *like* patiënten. Daarnaast zijn de bloedstalen van kinderen en adolescenten met obesitas ook van groot belang geweest

voor deze doctoraatsthesis. Hiervoor wil ik dan ook **Dr. Kim Van Hoorenbeeck, Prof. Dr. Stijn Verhulst, Prof. Dr. Guy Massa en Dr. Martine Docx** enorm bedanken. Het was een zeer aangename samenwerking met jullie.

Ook zou ik graag een woordje willen richten aan **Prof. Dr. Luc Van Gaal** en **Dr. An Verrijken**. Bedankt om steeds de tijd te nemen om de manuscripten kritisch door te nemen. An, ook bedankt voor je leuke en vooral lieve mailtjes om te vragen hoe het met Elise was toen het wat minder ging.

Ik ben de afgelopen jaren ook terecht gekomen in een ontzettend leuke onderzoeksgroep: De **BoOb groep**.

Evi, mijn mede-obesitas maatje (die ook zo dun is als ik :-P), wat ben ik toch zo enorm dankbaar om jou te leren kennen en met jou te mogen samenwerken. Je hebt echt enorm veel betekend voor mij. Niet enkel werkgerelateerd, maar ook op privévlak. Het afgelopen jaar gingen we samen en voor onze eerste keer naar het verre Amerika om het ASHG congres bij te wonen. Al hadden we die eerste keer in Amerika wel wat anders voorgesteld toen we er aan kwamen, het was uiteindelijk toch een enorm leuke ervaring geweest. Ik ben ontzettend blij dat ik dit samen met jou mocht doen. Ook bedankt Evi, voor de vele leuke babbels en lieve mailtjes, berichtjes, appjes wanneer ik het even niet meer zag zitten of frustraties had om de één of andere reden. Spijtig genoeg konden we veel ervaring uitwisselen over de longproblemen bij onze dochters, maar gelukkig gaat het met beide nu helemaal goed ☺. Tot slot ook een welgemeende dankjewel voor de vele babyspullen en zwangerschapskleren. Evi, Je bent echt een vriendin voor het leven geworden. Ik wil er daarom ook alles aan doen om, ondanks het feit dat we nu beiden onze eigen weg (zijn) (ge)gaan en we ver van elkaar wonen, dit contact zeker in stand te houden.

Sara en Silke, samen met Evi maakten jullie onze kant van de bureau compleet. **Sara**, je blijft misschien helemaal alleen over als doctoraatsstudent in het obesitas-onderzoek, maar ik ben er van overtuigd dat je dit, met je super motivatie en je sterke karakter,

geweldig gaat doen. Ik ben ook enorm blij om jou te leren kennen, al had ik er toch graag wat meer willen zijn voor je bij de opstart van je doctoraat en de voorbereiding van je IWT. En Sara, dat obesitas-genpanel gaat helemaal goed komen ;-). **Silke,** de weledelgeleerde PowerPoint-queen ☺! Ik vond het superleuk om samen een team te vormen toen je je eerste WES-prep ging uitvoeren. Hiernaast wil ik je ook ontzettend bedanken voor je enorm enthousiasme, je interesse in mijn werk, je doorzettingsvermogen, de vele lachmomenten en toch ook de serieuzere momenten toen we ons toch echt wel moesten concentreren op ons werk. Het was superleuk om je als buurvrouw te hebben in de bureau ☺. **Raphaël**, onze (hyper)actieveling ;-), ook jij enorm bedankt voor je enthousiasme, de 'high-fives' en te zorgen voor de nodige koeken, fruit, snoep (en voor sommigen koffie) wanneer deze nodig waren.

Ook wil ik een speciaal woordje richten aan **Igor en Eveline**. Hartelijk bedankt voor de ontzettend goede begeleiding tijdens mijn masterthesis. Ik heb door jullie enorm veel kennis verworven omtrent Western Blot, ELISA en de Luciferase Reporter Assay die ik goed kon gebruiken tijdens mijn doctoraat. En ook jij, **Gretl**, enorm bedankt voor al je tips om de functionele studies van *POU3F2* toch proberen te laten werken.

Ellen, of beter bekend als Ellen S (of ik eigenlijk als Ellen G ;-)), jou wil ik speciaal bedanken voor alle experimenten die je hebt uitgevoerd voor me toen ik zwanger was. Zonder jouw hulp was dit alles niet gelukt binnen deze termijn.

Esther, al hoor je officieel niet bij de BoOb groep, toch ben ik enorm blij dat je deel uitmaakte van onze bureau. Je was een ontzettend lieve collega en jouw gedrevenheid gaat je nog enorm ver brengen.

Hartelijk bedankt allemaal voor jullie enorme steun en vriendschap de afgelopen jaren. Ik ga deze toffe bureau (en onze lunches) echt ontzettend hard missen.

Nele, Jean, Hanne, Ilse VdW en Ilse L, in 2013 zijn we samen in het doctoraat-avontuur gesprongen en ik kan toch stellen dat we het er allemaal goed vanaf hebben gebracht. Enkele van ons zijn er intussen al in geslaagd hun doctoraat te behalen en voor de anderen staat dit in de nabije toekomst gepland. **Nele en Jean**, jullie heb ik

voornamelijk beter leren kennen tijdens onze masterthesis in het CMG. Ik ben dan ook blij dat ik samen met jullie kon starten aan ons doctoraat zodat we in het begin en nu ook aan het einde toch steeds bij elkaar terecht konden voor bepaalde (stomme) praktische zaken, tips in het labo of gewoon een luchtige babbel. Hanne, aan jouw enthousiasme en (hyper)activiteit kan niemand tippen! Voor jouw doorzettingsvermogen, voornamelijk aan het einde van je doctoraat, heb ik enorme bewondering. Met deze ingesteldheid komt jouw verdediging binnen een kleine twee weken dik in orde. Ilse VdW, aan het begin van ons doctoraat was je een beetje stil en terughoudend wat ik absoluut kan begrijpen tussen zo een hoop Belgen ;-), maar dat veranderde gelukkig vrij snel. Jouw kennis over letterlijk ALLES slaat me nog steeds met verstomming. Je bent echt een pientere, gedreven meid die enorm gepassioneerd is door de genetica en voornamelijk de neurologie. Er is dan ook geen twijfel mogelijk dat de postdoc-positie waarvan je droomt er zeker van gaat komen. Ilse L, we moesten zowat tegelijkertijd de analyses van onze genoomwijde microarray data verwerken en hierbij was je toch wel een enorme hulp, dankjewel daarvoor.
Ik vond het ontzettend leuk dat jullie deel uitmaakten van dit avontuur, dat ik jullie mocht leren kennen en de vele etentjes en drankjes met jullie kon delen.

Jean, Ilse VdW en Manou, mijn carpoolmatties, wat hebben we samen vele uren doorgebracht in de auto. Al leed dit regelmatig tot frustraties doordat er toch weer iemand besliste om op iemand anders te rijden en wij daardoor weeral in de file stonden, toch relativeerden we (meestal) de situatie en werd er vooral veel gelachen. Op de terugrit was het ook altijd fijn om even de gebeurtenissen (en soms ook ergernissen) van die dag met elkaar te delen en deze wat te filteren zodat onze wederhelft toch niet zot werd van onze verhalen ;-). Ladies, ik vond het echt enorm fijn om deze tijd met jullie te delen, ik kon me geen betere carpoolmatties inbeelden en ik blijf graag op de hoogte van jullie verdere toekomstplannen.

Hiernaast zijn er nog een heleboel andere collega's van zowel DNA diagnostiek als Research die heel wat betekend hebben tijdens mijn doctoraat en die een woordje van dank verdienen. Edwin, Kathleen en Mia, bedankt om me de praktische zaken van de SNP arrays aan te leren. Edwin, in het bijzonder wil ik jou nog extra bedanken om me wegwijs te maken in de analyse van de data van deze SNP arrays. Zonder jou was dit nooit gelukt. Kathleen, jou wil ik ook nog bedanken voor de vele lieve mailtjes en babbels in de wandelgangen. Nils, Charlotte en Els, samen met Marieke en Elyssa vormden we een geruime tijd het 'ABI-team'. Bedankt allemaal voor deze leuke samenwerking en om in te springen voor me wanneer dit nodig was. En jij, Nils, enorm bedankt om me alles van de Xiril robot en de ABI sequencers aan te leren. Geert, de postdoc van de frax-groep, maar eigenlijk de bioinformatica-man van iedereen in het centrum. Bedankt voor al je hulp bij de analyse van mijn WES-data en om me te helpen met stomme vragen zoals *'hoe moet ik nu weer die file importeren in IGV?'* of *'kan je eens nagaan wat deze error juist wil betekenen?'*. Ann en Lizzy, jullie wil ik in het bijzonder bedanken voor de praktische ondersteuning gaande van de declaratie van bepaalde onkosten tot een badge die weer niet werkt. Arvid, ook jij bedankt dat je deur altijd open stond en dat ik telkens bij je terecht kon met zowel kleine als grote problemen.

Hiernaast wil ik ook graag alle andere (ex-)CMGers bedanken voor de enorm aangename werksfeer, de leuke lunchpauzes, de gezellige babbels en van velen ook de superlieve mailtjes, berichtjes en babybezoekjes.

Evelyn, Sofie, Kim, Evelien B., Katrien, Lien, Nele en Femke, of beter bekend als de PP's (you know what I mean ;-)). De Katholieke Landelijke Jeugd (KLJ) heeft ons eigenlijk allemaal samen gebracht. Ook al zijn de +16-activiteiten de laatste jaren wat geminderd (lees: er zijn er eigenlijk geen meer ;-)), toch bleven we contact houden met elkaar en daar ben ik enorm blij om. Ik weet dat ik door de geboorte van Elise en de drukte bij de afwerking van mijn doctoraat er spijtig genoeg niet in geslaagd ben

om bij elk feestje, etentje of cafébezoekje aanwezig te zijn, maar ik ga mijn uiterste best doen om er vanaf nu terug vaker bij te zijn ☺. Ik wil jullie ook nog speciaal bedanken voor jullie steun en interesse in mijn werk ook al begrepen (de meesten van) jullie er geen snars van :-P.

Evelien E., jou heb ik leren kennen tijdens mijn weekendwerk in de Vredesboom en je bent vanaf toen een enorm goede vriendin geworden. Ik wil je bedanken voor de interesse die je toonde in mijn job en de vele *'hoe gaat het?'* en *'komt allemaal goed!'* berichtjes. Ook in de tijden dat het niet goed ging met Elise was je er voor mij en daar wil ik je nog eens speciaal voor bedanken.

Caroline, wij kennen elkaar al vanaf het middelbaar, maar ondanks het feit dat we beiden iets totaal anders zijn gaan studeren, zijn we elkaar niet uit het oog verloren en gelukkig maar. Ook jou wil ik graag bedanken voor je interesse, je baby-tips en de leuke babbels tijdens onze driemaandelijkse bezoekjes aan elkaar.

Ma en pa, bedankt om me in 2007 de kans te geven om verder te studeren in de Biomedische Wetenschappen. Ook al leek het jullie in eerste instantie geen goed idee om op kot te gaan, na enkele maanden werd het jullie (gelukkig!) duidelijk dat de busverbinding Minderhout – Wilrijk toch niet echt evident was. Een dikke 10 jaar later heeft me dit uiteindelijk hier gebracht en dat was zeker niet mogelijk zonder jullie grenzeloze steun en geloof in mij. Met elk probleem (en vooral met printerproblemen :-P) kon ik steeds bij jullie terecht. De laatste maanden heb ik het enorm druk gehad met het afwerken van deze doctoraatsthesis en uiteraard ook het zoeken naar een nieuwe job, waardoor ik niet altijd de tijd had om er voor Elise te zijn. Gelukkig kon ik steeds op jullie, maar ook op Stan, Kristel, Ellen en Frank, rekenen als we weer maar eens oppas nodig hadden. Ontzettend bedankt hiervoor! Ik ben echt enorm trots om de dochter te zijn van zo'n fantastische ouders en ik hoop dat ik ook zo'n mooi voorbeeld kan zijn voor Elise.

Kevin (a.k.a. gaajzies) en **Sven** (a.k.a. Svenson), mijn allerliefste grote broers. Opgroeien met zo'n twee toppers is al een voorrecht, maar ik wil jullie vooral bedanken voor de vele ontspannende momenten op familiefeestjes, babyborrels, verjaardagfeestjes, 'zomaar'-bezoekjes,… zodat ik toch even mijn gedachten kon verzetten van dit doctoraat. Maar uiteraard wil ik jullie voornamelijk bedanken voor de vele schouderklopjes en de *'kom op gaajz, you can do its'*. Ook **Fanny en Anouk**, jullie enorm bedankt voor alle steun en binnenkort zal het leuk worden als onze drie kapoenen met elkaar kunnen ravotten ☺.

De laatste paragraaf zou ik graag willen wijden aan de twee belangrijkste personen in mijn leven.

Jeroen, moppie/tijger, wat hebben wij in de periode van mijn doctoraat toch heel wat verwezenlijkt samen en daar ben ik oprecht trots op. Bedankt dat je er was voor mij in tijden dat ik het even echt niet meer zag zitten en om me duidelijk te maken dat ik toch maar moest geloven in mezelf. Dankjewel voor je grenzeloze steun en liefde, de nodige ontspanning en de zalige gibbermomenten met ons Elise. Je bent echt een fantastische papa voor onze dochter en een geweldige vriend! Ik ben enorm benieuwd naar wat de komende jaren ons nog gaan brengen! Love you!

Lieve **Elise**, jij eigenwijze meid, je was er verrassend vroeg bij waardoor bepaalde planningen van mijn doctoraat wat door elkaar werden gehaald, maar wat ben ik toch zo blij dat je in mama's en papa's leven bent gekomen! Ik ben ook ontzettend trots op jou, al van in het begin heb je laten merken dat je een enorm sterke meid bent en dat is tot op de dag van vandaag niet anders. Lieve schat, ik zie je enorm graag!