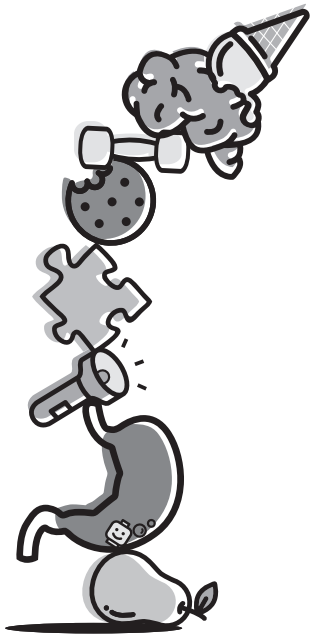
# NEW TREATMENT PERSPECTIVES IN PRADER–WILLI SYNDROME

Renske J. Kuppens

NEW
TREATMENT
PERSPECTIVES IN

# PWS



# NEW TREATMENT PERSPECTIVES IN **PRADER–WILLI SYNDROME**

Renske J. Kuppens

The studies described in this thesis were investigator-initiated studies, supported by independent research grants from Pfizer, Dutch Prader-Willi Fund and Dutch Growth Research Foundation.

Publication of this thesis was financially supported by the Dutch Growth Research Foundation, Pfizer and Nederlands Bijwerkingen Fonds.

Cover image and lay-out: Rosadinde Doornenbal
Printed by: Optima Grafische Communicatie
ISBN/EAN: 978-94-6169-950-3

Copyright © 2016 R.J. Kuppens, Rotterdam, The Netherlands
No part of this thesis may be reproduced, stored, in a retrieval system or transmitted in any form or by any means, without the written permission of the author or, when appropriate, of the publishers of the publications.

# New Treatment Perspectives in Prader-Willi Syndrome

# Nieuwe behandelinzichten in Prader-Willi syndroom

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
Rector Magnificus

Prof. dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

vrijdag 2 december 2016 om 11.30 uur

door

Renske Jozefien Kuppens
geboren te Zevenaar

**Erasmus University Rotterdam**

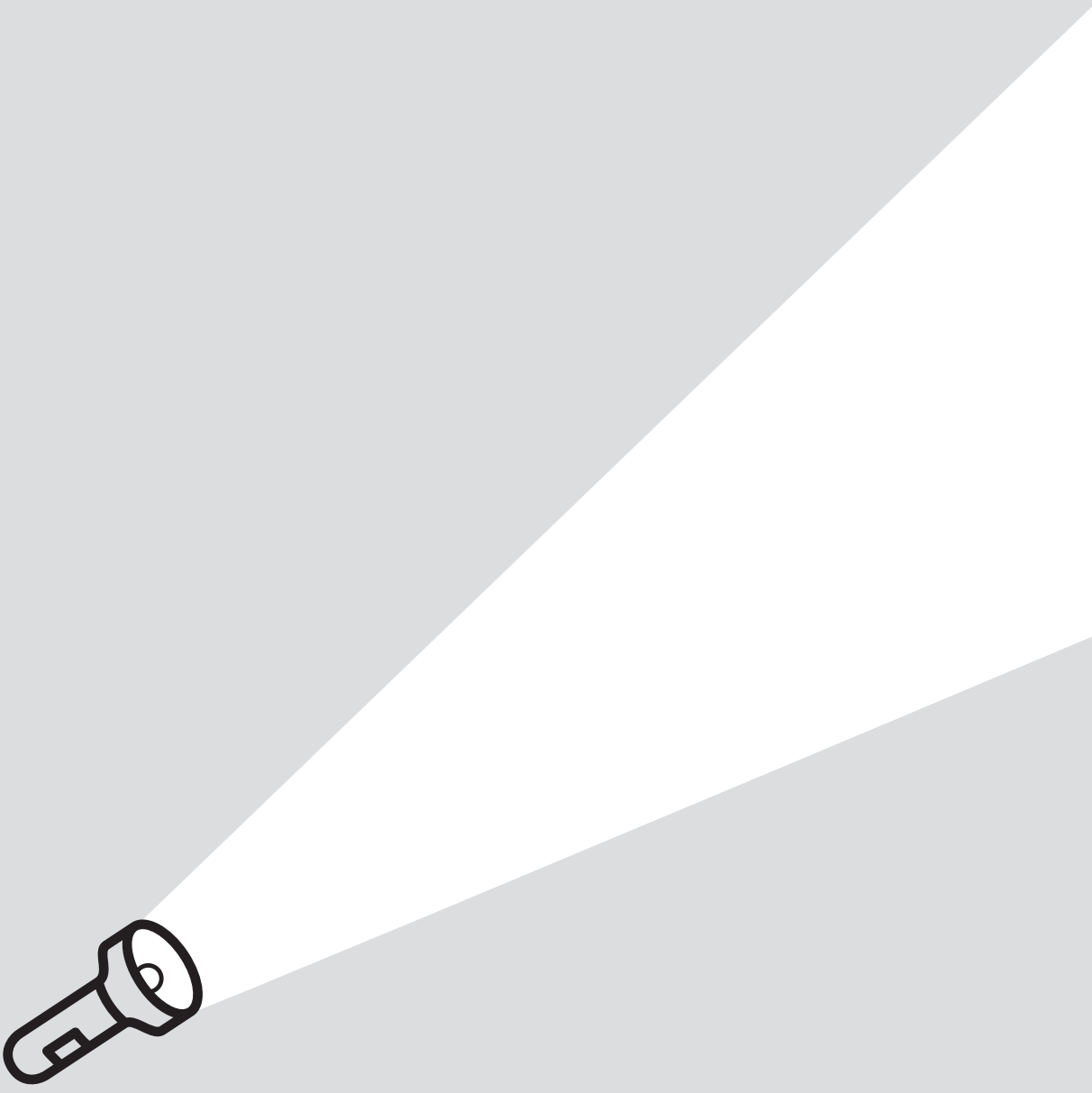
Erasmus

**PROMOTIECOMMISSIE**

| | |
|---|---|
| **Promotor** | Prof. dr. A.C.S. Hokken-Koelega |
| **Overige leden** | Prof. dr. A.J. van der Lelij |
| | Prof. dr. W. Kiess |
| | Prof. dr. A. Vogels |

*Voor alle kinderen en jongvolwassenen met het Prader-Willi syndroom en hun ouders*

## TABLE OF CONTENTS



# CHAPTER 1

## General introduction and aims of the thesis

## INTRODUCTION

In 2002, our research group started investigating various aspects of Prader-Willi syndrome (PWS) and the effects of growth hormone (GH) treatment in children with PWS. Since then the knowledge about PWS has markedly increased, which also improved the quality of life of the children and their families. This is the sixth thesis of our research group including new studies in children and young adults with PWS.

**Figure 1.** Young girl from the 17th century, believed to have had PWS.

The first patient with PWS was presented by Sir Langdon-Down in 1887[1]. He described an adolescent girl with short stature, obesity, hypogonadism and cognitive impairment (Figure 1). Seventy years later, in 1956, three Swiss endocrinologists Andrea Prader, Alexis Labhart and Heinrich Willi reported a series of patients with similar phenotypes[2]. The common characteristics included small hands and feet, abnormal growth and body composition with a very low lean body mass and early-onset obesity, hypotonia at birth, insatiable hunger and intellectual disability. Knowledge about different aspects of PWS markedly increased since these reports, but raised new questions as well. Studies are needed to unravel the challenges of PWS and to improve the quality of life of this patient group.

This chapter describes the genetic basis of PWS, the clinical characteristics of children and young adults with PWS in different stages of life, hypothalamic dysfunction and our studies regarding GH treatment in children with PWS. Subsequently, the clinical topics that needed to be further investigated are defined. Finally, the objectives of the studies described in the various chapters are presented.

### 1.1 PRADER-WILLI SYNDROME

Prader-Willi syndrome is a rare neurogenetic disorder resulting from the absence of expression of the paternally inherited genes located on chromosome 15 at the locus q11.2-13[3, 4]. PWS is considered the most common cause of genetic obesity and equally affects boys and girls, without association with race or social-economic status[5]. The incidence of PWS is estimated at 1 in every 12,000-15,000 live births[5-7]. Hypothalamic

dysfunction seems to be the underlying cause for most symptoms of PWS, although the phenotype of PWS is very variable[8].

## 1.2 GENETIC CAUSE

### History

For a long time, the diagnosis of PWS was exclusively based on a combination of clinical signs and symptoms, described as the Holm's criteria[9]. In 1976, it was for the first time reported that a clinically diagnosed patient with PWS had an abnormal karyotype with a 15/15 Robertsonian translocation[10]. In the 80s, it became clear that also deletions of the long arm of chromosome 15 at region q11-13 resulted in PWS[11], and that it specifically concerned the paternally inherited chromosome 15[12]. In 1991, a third subtype was reported, a maternal uniparental disomy (mUPD), leading to lack of a paternally inherited chromosome 15[13]. To date, it is known that PWS is caused by a lack of expression of the paternally inherited genes located on chromosome 15, on the locus 15q11-13, which is called the 'Prader-Willi region'. Lack of expression of the genes in the PWS region is lost due to either a deletion, an mUPD, an imprinting center defect (ICD) or a translocation[4, 14].

### Genomic imprinting

Genes are regions of DNA encoding information for functional proteins. Genes are located on chromosomes and vary in size from a few hundred DNA base pairs to more than 2 million (Figure 2). Humans have 23 pairs of chromosomes in the nucleus of each cell in the body. Children inherit 23 chromosomes from their father and 23 chromosomes from their mother, which result in 23 pairs in each cell. One of these 23 pairs is chromosome 15.

Genomic imprinting is an epigenetic phenomenon by which certain genes are expressed in a parent-of-origin specific manner without changing the DNA structure or base pair sequence[15]. During gametogenesis, genes are imprinted or silenced and only a small percentage of genes is active. In healthy subjects, the PWS region on the paternally inherited chromosome 15 is expressed, while this region on the maternally inherited chromosome 15 is silenced by imprinting. An abnormal or absent expression of the paternally inherited genes on the PWS region results in PWS.

The PWS region contains multiple imprinted genes and the loss of these genes contributes to the complete phenotype of PWS[16] (Figure 3). The exact function of each gene on the PWS region remains to be elucidated and in humans no single gene mutation has been found that will explain all the features of PWS.

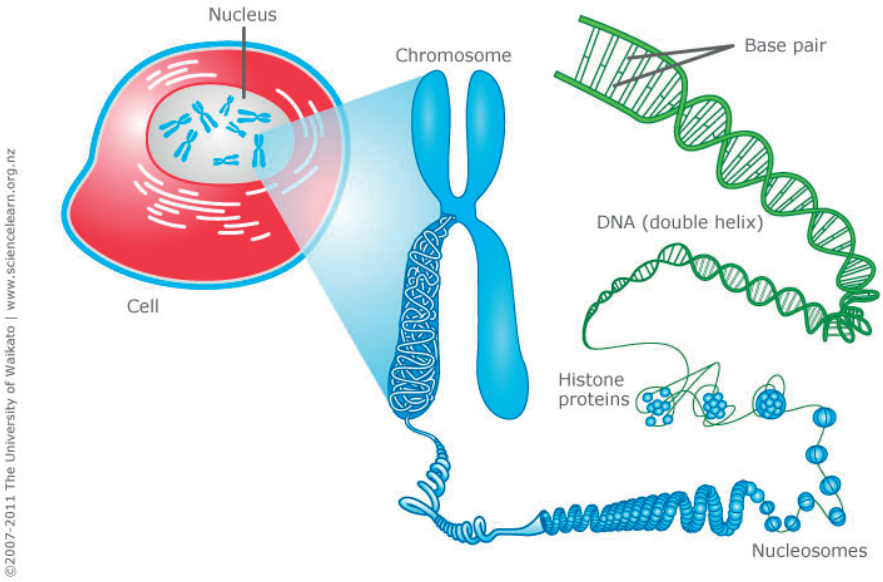



**Figure 2.** Cell with nucleus, chromosomes and DNA.

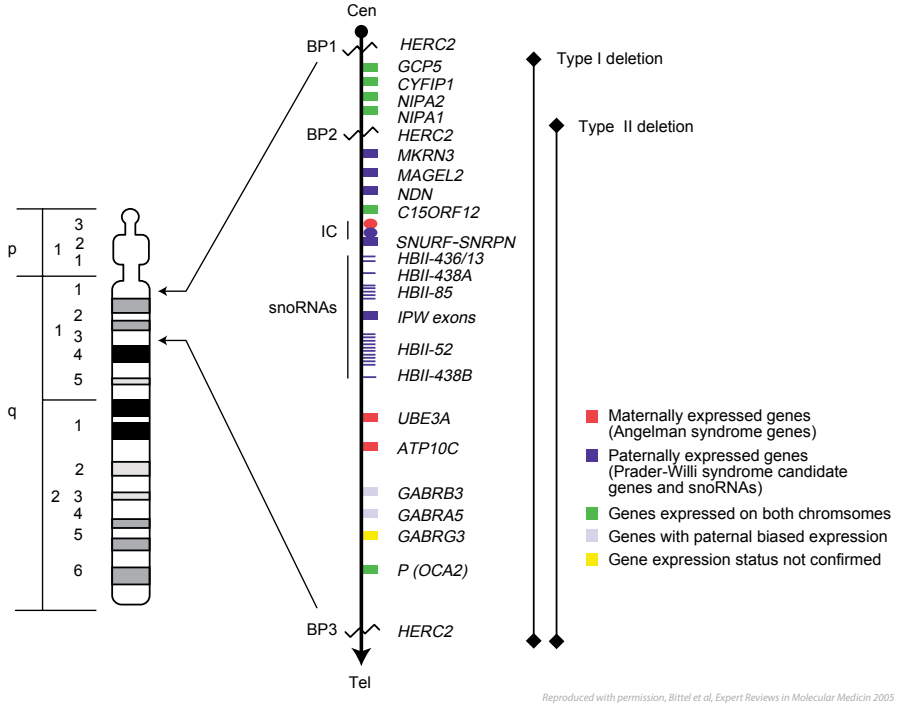



**Figure 3.** PWS region on chromosome 15. Order of genes on 15q11-q13 region, and patterns of expression.

## Genetic subtypes and phenotype

The lack of expression of the genes in the PWS region on the paternally derived chromosome 15q11-q13 can be caused by 4 different mechanisms, namely a deletion, an mUPD, an ICD or a translocation[4, 14, 16, 17] (Figure 4). The two proximal breakpoints (BP1 and BP2) and the distal breakpoint (BP3) appear to predispose to the typical deletion seen in PWS[16, 18]. The larger type I deletion involves BP1 to BP3, while type II deletion involves BP2 to BP3 (Figure 3). Besides the most common deletions, the second most frequent cause of PWS is an mUPD. In an mUPD, both chromosomes 15 are inherited from the mother, due to a gamete completion by the union of a nullisomic and a disomic gamete, or at trisomic conception followed by a trisomy rescue in early pregnancy and loss of the paternal chromosome 15[19].

An imprinting defect explains less than 5% of PWS cases[14]. Patients have apparently normal chromosomes 15 of biparental inheritance, but a mutation in the imprinting control region results in a maternal imprint, which leads to a complete loss of the paternally expressed genes of the PWS region. Less than 1% of the patients with PWS have a Robertsonian translocation, in which a part of the paternally inherited chromosome 15 has moved to another chromosome[4, 20].

Worldwide, a paternal deletion occurs in approximately 70% of people with PWS, an mUPD in 25%, and the rest is caused by an imprinting defect or translocation in the PWS region[16]. However, in the last decade, West-European studies demonstrate a shift towards equal rates of the genetic subtypes deletion and mUPD, most probably due to an increasing maternal age[21-23]. According to the literature, patients with a deletion have more typical facial characteristics and hypopigmentation, while those with an mUPD often have higher verbal IQ scores, but are also more at risk for psychiatric problems, like psychosis and autistic behaviour[18, 24-26].

Recurrence risk is typically very low in families with a person with a deletion or an mUPD, but if the father carries a translocation or if PWS is caused by an imprinting center mutation, the recurrence risk would be considerably higher, up to 50%[16]. Prenatal diagnosis for PWS is available[16].

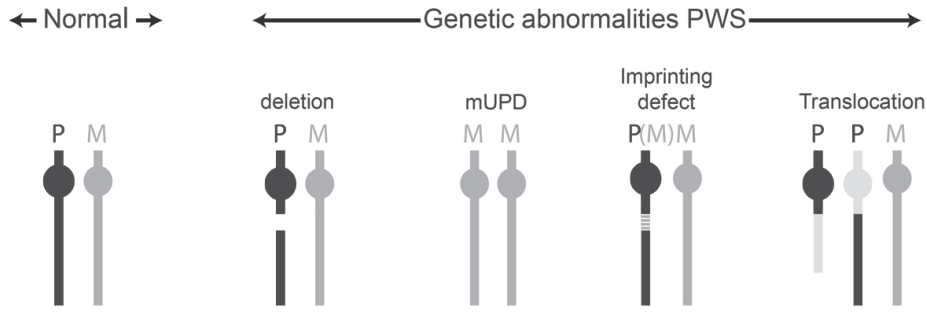



**Figure 4.** Schematic overview of the genetic abnormalities causing PWS. P=paternally inherited chromosome 15, M=maternally inherited chromosome 15, mUPD=maternal uniparental disomy.

## 1.3 PRADER-WILLI SYNDROME IN DIFFERENT PHASES OF LIFE

Various clinical features of PWS change during the different stages of life, but the most obvious change is the one in eating behaviour, from feeding problems to hyperphagia[9, 27]. It was traditionally described as 'the switch' in eating behaviour[28], although it is a gradual and complex process. Nowadays the clinical symptoms and signs are usually subdivided in nutritional phases[27] (Table 1). In the early phase after birth, severe hypotonia with poor feeding and failure to thrive are prominent, followed by a period of appropriate growth without feeding problems. Subsequently, generally between 18 and 36 months of age, body weight starts to increase without an increase in calorie intake or interest in food. Then in early childhood, interest in food and appetite increase abnormally and the child becomes overweight when leaving unattended. Thereafter, hyperphagia develops with impaired satiety leading to obesity and food seeking behaviour[9, 27]. In some adults, hyperphagia diminishes and appetite is no longer insatiable[13, 14].



### Prenatal period and birth

Around 85% of the mothers report decreased fetal movements during their pregnancy of a child with PWS[27]. The incidence of polyhydramnios and breech presentation are increased, and delivery by caesarian section is more common[16]. The latter two could be related to the hypotonia of the fetus, but it might also be due to hypothalamic dysfunction[8]. Babies with PWS are on average born at a gestational age of 38.2 weeks, but both preterm and post-term deliveries are more often seen[27]. Birth weight, length and BMI are generally about 15-20% lower in babies with PWS than in their siblings, even in full-term pregnancies[3, 27, 29].

**Table 1.** Nutritional phases

| Phases | Median ages | Clinical characteristics |
|---|---|---|
| 0 | Prenatal to birth | Decreased fetal movements and lower birth weight than sibs |
| 1a | Birth to 9 months | Hypotonia with difficulty feeding and decreased appetite |
| 1b | 9 to 25 months | Improved feeding and appetite and growing appropriately |
| 2a | 2.1 to 4.5 years | Weight increasing without appetite increase or excess calories |
| 2b | 4.5 to 8 years | Increased appetite and caloric intake, but can feel full |
| 3 | 8 years to adulthood | Hyperphagic, rarely feels full |
| 4 | Adulthood | Appetite is no longer insatiable (described in some adults) |

Modified from Am J Med Gen A[27]

### Newborn period

In the early phase after birth, severe hypotonia with poor feeding and failure to thrive are prominent. The marked central hypotonia causes decreased movements, a severe head leg, lethargy with decreased arousal, weak or absent cry and diminished reflexes, including a poor suck. Practically every newborn with PWS has feeding problems in the first period after birth and requires some kind of feeding assistance such as a nasogastric tube. The severe hypotonia is partly responsible for the feeding problems, but also the concomitant lack of appetite plays a role[27, 30]. Neonates with PWS show no evidence of being hungry and do not get excited at feeding time[9, 13]. If feeding would only occur when the neonate cries for milk, severe failure to thrive would develop. Most babies with PWS need a nasogastric tube until the age of 3 to 9 months (Figure 5).

The abnormal body composition is already present at birth. Even if the newborn has no failure to thrive and a normal body weight, an increased fat mass percentage and decreased lean body mass (LBM) can be found by skin fold measurements or dual energy-x-ray absorptiometry (DXA)[31, 32]. Other typical dysmorphic features include a narrow bifrontal diameter, almond-shaped eyes, a narrow nasal bridge and a thin upper vermillion with down-turned corners of the mouth. These may be present at birth, but become more pronounced during infancy and slowly evolve over time[16]. Other abnormalities include hypogonadism with genital hypoplasia, hip dysplasia and thermoregulation and breathing problems. Testing for PWS should be performed in all neonates with otherwise unexplained hypotonia with poor suck[33]. Nowadays, the diagnosis PWS can be genetically confirmed in the neonatal period[3].

**Figure 5.** Young infant with PWS. Photo is depicted with permission from parents.

### Infancy

After the newborn period with severe hypotonia and feeding problems, the eating behaviour gradually changes into approximately normal with appropriate growth[16]. Although the severe hypotonia considerably improves in the first year, there will always remain a decreased LBM and muscle strength which result in a delayed motor development[34, 35]. The mental development and speech and language development are delayed as well and milestones are on average reached at double the normal age[34-36]. Physical training and speech therapy are important to stimulate the development of the infant[36]. During the first years of life, children with PWS are friendly, easy going and affectionate[37] (Figure 6a-c).


**Figure 6a-c.** Young children with PWS. Photos are depicted with permission from parents.

### Toddlers age

Usually between 18 and 36 months of age, body weight starts to increase without an increase in calorie intake or interest in food[27]. The growth chart of weight for height will not parallel the reference charts anymore, but starts to increase and crosses the lines. The energy expenditure of a child with PWS decreases with age to 50% lower than normal, mainly due to the abnormal body composition and in particular the decreased LBM[32, 38]. A reduction of the energy intake with a well-balanced diet, in combination with enough physical exercise, is necessary to prevent the development of obesity [38, 39].

### Early childhood

In early childhood, rather soon after phase 2a, interest in food and appetite increases abnormally and the child becomes overweighed if there is no parental guiding. The preoccupation with food becomes more prominent and the child shows food-related behaviour, such as asking and talking about food, but also reading in cookbooks and acting like they work in a kitchen. The child is typically very concerned about the next meal and reminds others not to forget. If allowed, the child will eat more than peers and becomes obese. However, in this phase the child can be fairly easily redirected

about food and can feel full[27]. The median age of onset of phase 2b is around 4.5 years, although a wide variation is seen.

In the majority of children with PWS, the gradual change in eating behaviour coincide with a changing behavioural phenotype with more maladaptive behavioural and emotional disturbances. Most children with PWS have a psychomotor and cognitive impairment, resulting in a mild to moderate learning disability[40-42]. Mean total IQ scores are around 65 points, although there is a wide variation[34, 42]. They have a poor short-term memory and a deficit in sequential processing, but perform relatively well on visuospatial skills and exceptionally well on jigsaw puzzles[4].

Other signs and symptoms commonly seen during childhood are scoliosis[43], strabismus[44], sleep-related breathing disorders including apneas[45] and stress-induced central adrenal insufficiency[46].

### Childhood and puberty

Hyperphagia is the main characteristic of phase 3, which typically starts around the age of 8 years[27]. The profound preoccupation with food makes that the child thinks about food all day long. While eating they can already ask when the next meal will come and the child will continue eating if portions are not limited, as the child will rarely feel full. Common additional features are food-seeking behaviour, stealing food, lying about food, eating inedible things and temper tantrums related to food, and the impact on the quality of life of the patients and their families is huge[47]. As children with PWS could eat without stop when leaving unattended and their energy expenditure is very low, they could easily develop extreme obesity with concomitant problems. Besides, they are at risk for overeating, resulting in an acute life-threatening gastric rupture[48, 49]. Therefore, parental guidance and supervision are crucial and children must be supported 24 hours per day. Locks on kitchen doors and fridges will help children not to take food away and also give them rest that they do not have to seek for food.

Simultaneously with the gradual change in eating behaviour, the majority of children with PWS start to show more maladaptive behavioural and emotional characteristics, like temper tantrums, skin picking, stubbornness, mood lability, impulsivity, argumentativeness, and inappropriate social behaviour[50, 51]. Obsessive-compulsive symptoms are frequently seen in children with PWS and one third of children with PWS score positive for Autism Spectrum Disorder with mainly aberrations on maladaptive behaviour[52, 53]. A child with PWS is impaired in feeling empathy for others and has difficulties in social reciprocity (Figure 7a).

Pubertal development starts often spontaneously, but progresses delayed or incomplete[23, 54-56]. In most adolescents with PWS, sex steroid replacement therapy is necessary to attain a complete pubertal development and to achieve an adequate bone mineral density in adulthood[23].

**Figure 7a-b.** Child and young adult with PWS. Photos are depicted with permission from patients and parents.



## Adulthood

Some adults with PWS who were previously in phase 3 do no longer have an insatiable appetite and could feel full. This phase was described as nutritional phase 4 and has only been observed in adulthood[27]. It is unknown whether all adults with PWS eventually end up in this phase.

The behavioural phenotype of adults with PWS includes temper tantrums, skin picking, impulsiveness, lability of mood, inactivity and repetitive speech[57]. For most adults with PWS, it is not feasible to live independently (Figure 7b). Psychosis may occur, in particular in those with an mUPD[24].

The current generation of adults with PWS had a relatively late diagnosis and most did not receive growth hormone (GH) treatment. This resulted in a short adult height of 145-150 cm in women and 155-160 cm in men[29, 58]. Obesity was often already present at diagnosis and complications such as diabetes mellitus type 2 (T2DM), cardiovascular disease (CVD) and respiratory insufficiency impair life expectancy[59, 60]. However, when severe obesity can be avoided, life expectancy is reasonable[59].

## Nutritional phases in clinical practice

The nutritional phases according to Miller *et al.*[27] were used to score the feeding problems (Table 1), but in practice, the clinical presentation of children with PWS has significantly changed in today's population. Nowadays, some children have less signs

of classical hyperphagia without an increased weight or food-seeking behaviour. This might be due to an early diagnosis, improved health care, physiotherapy, diet regimen and the awareness of caregivers to limit the food intake. It could also be that GH treatment itself is able to suppress the hyperphagia. This controlled hyperphagia makes it difficult to categorize the child according to the nutritional phases. Development of a better hyperphagia scale for today's population is, therefore, warranted.

## 1.4 HYPOTHALAMUS

The hypothalamus is a part of the brain that contains a number of small nuclei with a variety of functions. One of the most important is the link between the nervous system and the endocrine system via the pituitary gland.

The hypothalamus synthesizes and secretes the neurohormones growth hormone-releasing hormone (GHRH), gonadotropin-releasing hormone (GnRH), corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH). These hypothalamic-releasing hormones are released to the blood stream and stimulate the anterior pituitary to secrete the following hormones: GH, gonadotropins (LH/FSH), corticotropin (ACTH), thyrotropin (TSH) and prolactin. Besides, the hypothalamus produces oxytocin, which is known to be involved in food intake, body weight and social skills.

Hypothalamic dysfunction seems to be the underlying cause for various signs and symptoms of PWS[8]. The hypothalamus acts as the control center for hunger and satiety, and seems to be involved in the regulation of appetite-regulating hormones, although there are still a lot of uncertainties. Other symptoms of PWS that have been related to dysregulation of the hypothalamus are temper tantrums, decreased activity level and energy expenditure, abnormal temperature control, excessive daytime sleepiness, abnormalities of sleep architecture, sleep-related breathing disorders, hypogonadotropic hypogonadism and hormonal insufficiencies such as a functional growth hormone deficiency and stress-related central adrenal insufficiency[8, 46, 61, 62].

## 1.5 GROWTH HORMONE TREATMENT

Children with PWS show similarities with those with GH deficiency. They have a decreasing growth velocity despite the onset of obesity, reduced lean body mass in the presence of adiposity, small hands and feet, and relatively low serum levels of insulin-like growth factor-I and low insulin[62]. However, most children do not fulfill the criteria for GH deficiency when they undergo a GH stimulation test[63]. This might argue for the presence of a functional GH deficiency, probably due to the hypothalamic dysfunction[62]. Without GH treatment, mean adult height in women is 145-150 cm and in men 155-160 cm[29, 58].

In the late 90s, the first positive effects of GH treatment on growth and body composition in children with PWS were reported[64-66]. In 2002, the Dutch national GH trial for children with PWS was started to investigate the effects on growth, body composition, activity level, psychological development and quality of life. It was started as a GH randomized controlled trial, lasting 1 year for infants and 2 years for prepubertal children, and children were subsequently followed during continuous GH treatment in the Dutch PWS Cohort Study until they attained adult height (for study designs see Appendix 1). Several long-term studies, including the Dutch PWS Studies, demonstrated that GH treatment is an effective and safe therapy for children with PWS[23, 66-70]. GH treatment improves body composition, bone mineral density, psychomotor development, cognition, adaptive functioning, linear growth and adult height and has substantially changed the phenotype of children with PWS[23, 34, 42, 70, 71]. GH treatment, in combination with an early diagnosis with early care and treatment, has resulted in a new generation of children with PWS[67, 70].

Nowadays, GH treatment is registered and reimbursed for children with PWS. Most Dutch infants start GH treatment soon after diagnosis and are followed in the Dutch PWS Cohort Study until adult height.

## 1.6 UNRESOLVED ISSUES

The hypothalamus seems to play an important role in various signs and symptoms in PWS. The underlying mechanism of hyperphagia is unknown, but appetite regulating hormones might be involved. There were only limited data about the appetite-regulating hormone ghrelin in PWS, and about the effects of glucose intake on this hormone. Therefore, these topics needed to be investigated. The effects of treatment with the hypothalamic neurohormone oxytocin on hyperphagia and social behaviour in children with PWS were unknown and were therefore investigated as well. Furthermore, an important clinical topic that needed to be investigated was the effect of GH treatment versus placebo in young adults with PWS who were GH-treated during childhood and had attained adult height, as there were no data about the effects of discontinuation or continuation of GH treatment on body composition, metabolic health profile and cognition.

### Appetite-regulating hormone ghrelin

As mentioned previously, the most striking symptom of PWS is hyperphagia, characterized by an excessive food intake and the lack of satiety. In the presence of a reduced metabolic rate and a decreased physical activity level, hyperphagia will often result in morbid obesity in children with PWS, when food intake is not restricted[72]. Remarkably, the complete opposite eating behaviour is present in the first period after birth with

feeding problems leading to severe failure to thrive when feeding assistance is not provided[27].

The mechanisms for the switch in eating behaviour from impaired suckling and failure to thrive to excessive weight gain and hyperphagia with impaired satiety are currently unknown, but abnormalities in appetite-regulating hormones might be involved. It is crucial to obtain knowledge about the pathophysiology of the switch, since this might help to find an appropriate treatment. One of the possibly involved hormones is ghrelin. Ghrelin is predominantly produced in the stomach and ghrelin cells are under the control of complicated mechanisms, which are still obscure[73-75]. It has an acylated (AG) and an unacylated (UAG) form in circulation[73-75] (Figure 8). The presence of the acyl group is necessary for the binding to the growth hormone secretagogue receptor (GHSR-1a)[74] and for that reason, unacylated ghrelin (UAG) was considered to be inactive. Nowadays there is increasing evidence that UAG has also several actions[78]. Both AG and UAG can be determined in blood samples, but AG is unstable and rapidly deacylates to UAG[76, 77]. UAG acts as a functional inhibitor of AG, suggesting an important role for the ratio between AG and UAG levels[78, 79]. Immediate addition of an esterase inhibitor at time of blood collection is therefore needed. In healthy individuals, AG and UAG levels are high in fasting state, while levels decline after food intake[80]. Furthermore, intravenous AG administration in healthy subjects increased food intake and appetite[81].

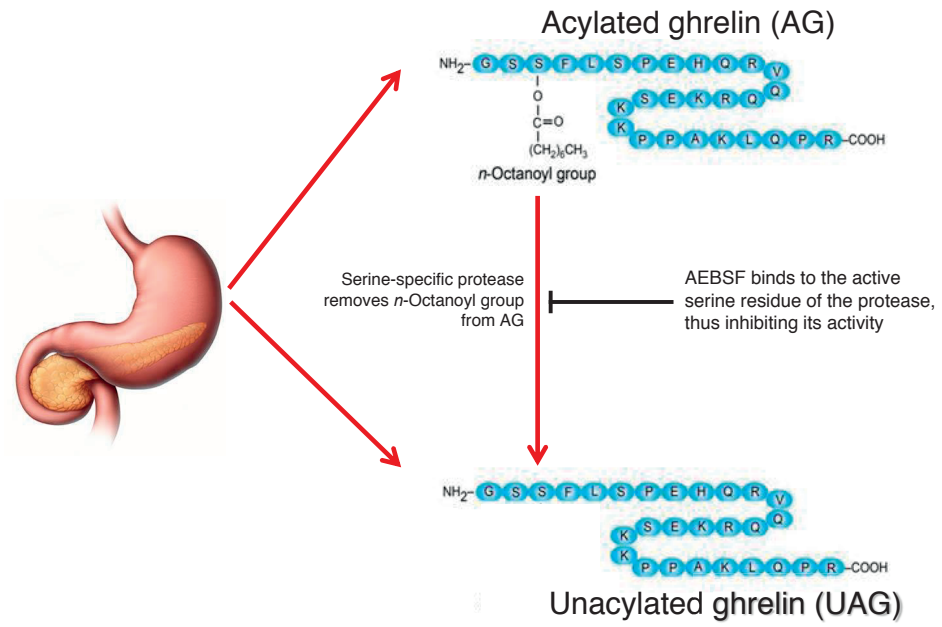



**Figure 8.** Schematic overview of acylated and unacylated ghrelin.

In patients with PWS, hyperghrelinemia has been reported[82-87]. As high AG levels are associated with increased food intake, they might explain part of hyperphagia in PWS, but these studies did not treat the samples with an esterase inhibitor to prevent deacylation[82-87]. Most studies described total ghrelin levels, thus did not distinguish between AG and UAG levels and did not report AG/UAG ratios. In addition, it was unknown whether there was a difference in ghrelin levels between patients in various nutritional phases. We therefore wanted to answer the question whether fasting ghrelin levels were abnormal in patients with PWS in comparison with obese and healthy controls.



**Ghrelin after glucose intake**

Hyperphagia, characterized by a combination of a preoccupation with food, an excessive food intake and the lack of satiety, has a major impact on the quality of life of patients with PWS and their surroundings. For a long time it was thought that patients experienced chronic hunger, but studies also demonstrated a decreased satiation in patients with PWS compared to obese and healthy controls[88]. It was unknown why patients with PWS do not feel satiated after a meal, but hypothalamic dysfunction in combination with abnormal peripheral endocrine responses was one of the possible causes.

In contrast to most other appetite-regulating hormones, ghrelin levels in healthy humans are high in a fasting state, while they decrease after food intake. In PWS, fasting ghrelin levels are abnormally high, but the response of the ghrelin system to food intake could be abnormal as well. The appropriate response would be a decrease in levels of AG and UAG, and AG/UAG ratio after food intake, possibly mediated by the hypothalamus. However, prior to our study, it was unknown whether this would occur in patients with PWS as well. We wanted to answer the question whether the lack of satiety in PWS was caused by an abnormal response of the orexigenic ghrelin to food intake.

**Oxytocin treatment**

Quality of life is hampered by temper tantrums and the preoccupation with food, which makes every day a challenge for many patients and their caregivers. Worldwide, there is ongoing research to find a solution for the main problems of patients with PWS. There is increasing interest in the hormone oxytocin, as oxytocin is involved in food intake, body weight and social skills, and these are seriously affected in patients with PWS.

Prader-Willi syndrome is caused by the lack of expression of the PWS region on chromosome 15 and one of the genes in this region is MAGEL2. This means that MAGEL2 is not expressed in PWS. The exact link between MAGEL2 and oxytocin is not clear, but animal studies demonstrated a major reduction of oxytocin secretion in the hypothalamus of MAGEL2-deficient mice. These mice had an altered onset of suckling activity resulting in impaired feeding and 50% mortality[89]. Human neonates with PWS show similar suckling

problems. Oxytocin treatment to MAGEL2-deficient mouse pups normalized suckling and feeding behaviour and rescued all of them[89].

In addition to the evidence in animal studies, there were clues that the oxytocin system is disturbed in humans with PWS. The number of oxytocin-expressing neurons in the hypothalamus of patients with PWS is significantly decreased by 42% and plasma levels of oxytocin are lower than in healthy controls[8, 90]. Thus, patients with PWS seem to have a deficiency of oxytocin and it was considered worth to investigate the effects of treating this deficiency with oxytocin. The oxytocin system is a promising target for therapeutic interventions, especially in obesity control and in aberrations in social function. A pilot study with intranasal oxytocin administration in adults with PWS showed positive effects on social behaviour[91], but prior to our study, the effects of oxytocin in children were unknown. We wanted to answer the question whether oxytocin treatment has positive effects on social behaviour and eating behaviour in children with PWS.

### Body composition in early adulthood

In children with PWS, the benefits of GH treatment are nowadays well established and GH treatment is approved by EMA and FDA. GH treatment in children with PWS improves body composition, bone mineral density, psychomotor development, cognition, adaptive functioning, linear growth and adult height[23, 34, 42, 70, 71]. The most important reason for treating these children is to optimize their abnormal body composition[67, 70]. Without GH treatment, body composition tends to deteriorate over time, leading to a BMI of 35 (+3.5 SDS) at the age of 18 years[29, 58]. A recent study showed that 8 years continuous GH treatment resulted in a healthy BMI around +1 SDS in adolescents with PWS, with a stable LBM and FM%[70]. This indicates that GH treatment is a potent force to counteract the clinical course of obesity in children with PWS[70]. GH treatment, in combination with an early diagnosis and care, has substantially changed the phenotype of these children and has resulted in a new generation of patients[67]. But it also brought new questions regarding those who reach adult height, as there is only a registration of GH treatment for PWS during linear growth. Although most adults with PWS are not GH deficient, it might be that they also need GH treatment for sustainment of their improved body composition like children with PWS do[92-94]. Should GH treatment thus be continued when a young adult with PWS has attained adult height? Prior to our study, there were no data on the effects of cessation or continuation of GH on body composition in young adults with PWS who were treated with GH during childhood and had attained adult height.

### Metabolic health profile in young adulthood

PWS patients have lower fasting insulin levels and higher insulin sensitivity than a weight-comparable group[95-97]. One of the suggested mechanisms for the higher insulin sensitivity in PWS is the reduced visceral adiposity compared to simple obesity[98]. Nev-

ertheless, adults with PWS are predisposed to develop diabetes mellitus type 2 (T2DM), probably due to their extreme obesity[99], and to cardiovascular disease (CVD) because risk factors for CVD such as hypertension and hyperlipidemia occur more often in PWS[100].

Without GH treatment, body composition tends to deteriorate over time[58], which is associated with an unfavourable metabolic health profile. On the other hand, GH treatment is a potent force to increase LBM and decrease FM%[70], which is associated with improved insulin sensitivity and metabolic health profile, but GH has also diabetogenic effects and treatment has been associated with decreased insulin sensitivity[101, 102]. Prior to our study, it was thus the question whether the benefits of GH treatment would outweigh the possibly unfavourable effects on glucose and insulin homeostasis in young adults. There were also no data on the effects of cessation or continuation of GH on metabolic health profile in these young adults.



### Cognition in young adulthood

In general, patients with PWS have a cognitive impairment with IQ scores between 50 and 85 points[42, 103]. We have demonstrated that GH-untreated children with PWS show a deterioration of cognitive functioning over time, while GH treatment prevents deterioration of certain cognitive skills on the short-term and significantly improves abstract reasoning and visuospatial skills during 4 years of GH treatment[34, 42]. The exact mechanism by which GH exerts its beneficial effects on cognitive functioning is not elucidated. MRI studies in PWS suggest that lower cortical complexity partially underlies cognitive impairment and developmental delay[104], and it might be that the brain tissue is affected by GH itself, or through the release of IGF-I. When young adults with PWS have attained adult height, they have to discontinue GH treatment. In untreated adults with PWS, lower IGF-I levels were correlated with poorer intellectual skills[105], which might suggest that discontinuation of GH treatment, which decreases IGF-I, could be disadvantageous.

Prior to our study, the effects of cessation or continuation of GH on cognitive functioning in this new generation of patients with PWS were unknown and required further research.

## 1.7 AIMS OF THE STUDIES AND OUTLINE OF THE THESIS

This thesis presents a detailed description of the studies, which were performed to increase the knowledge about PWS and to improve the care for patients with PWS. Serum levels of the appetite-regulating hormones acylated and unacylated ghrelin were measured in patients participating in the Dutch PWS Cohort Study, in collaboration with two French PWS centers. These hormone levels were also measured during oral glucose tolerance tests in participants of the Young Adult PWS Study. In the randomized, double-blinded, placebo-controlled PWS Oxytocin Study, the effects of 4 weeks of intranasally administered oxytocin versus 4 weeks of placebo on social behaviour and eating behaviour were investigated in children with PWS. In the randomized, double-blinded, placebo-controlled GH study (PWS Transition Study), the study population consisted of young adults with PWS who were GH-treated during childhood and had attained adult height, and thus had to stop GH treatment. In this specific group, we evaluated the effects of 1 year of GH treatment versus 1 year of placebo on body composition, metabolic health profile and cognition. Study designs are described in Appendix 1.

**Chapter 1** gives an introduction in PWS and the topics described in this thesis.
**Chapter 2** presents the results of a study evaluating the serum levels of the appetite-regulating hormones acylated and unacylated ghrelin in a large cohort of children and young adults with PWS, compared with age-matched obese subjects and healthy controls.
**Chapter 3** reports the response of the appetite-regulating hormones acylated and unacylated ghrelin to glucose intake during an oral glucose tolerance test in young adults with PWS.
**Chapter 4** describes the results of the randomized, double-blinded, placebo-controlled PWS Oxytocin Study, investigating the effects of 4 weeks of oxytocin versus 4 weeks of placebo on social behaviour and eating behaviour in children with PWS.
**Chapter 5** presents the PWS Transition Study, a 2-year randomized, double-blinded, placebo-controlled cross-over GH trial in young adults with PWS who were GH-treated during childhood and had attained adult height. This study investigated whether body composition deteriorated after cessation of GH treatment, and whether 1 year of GH treatment versus 1 year of placebo administration could maintain the improved body composition.
**Chapter 6** presents the effects on metabolic health profile in the PWS Transition Study, a 2-year randomized, double-blinded, placebo-controlled cross-over GH trial in young adults with PWS who were GH-treated during childhood and had attained adult height.
**Chapter 7** describes the effects on cognition in the PWS Transition Study, a 2-year randomized, double-blinded, placebo-controlled cross-over GH trial in young adults with PWS who were GH-treated during childhood and had attained adult height.

**Chapter 8** discusses the results and conclusions in the light of the current literature and presents clinical implications of our findings.
**Chapter 9** contains an English and Dutch summary of the results described in this thesis.
**Chapter 10** contains an overview of publications by PWS-team of Dutch Growth Research Foundation.
**Chapter 11** contains lists of abbreviations, co-authors and affiliations, and publications, and the PhD portfolio, CV and acknowledgements.

## APPENDIX 1: DUTCH PWS STUDIES

### Project Coordination

The Dutch Growth Research Foundation (Stichting Kind en Groei) is the initiator and coordinator of the Dutch PWS studies. The PWS team consists of MD-researchers, research nurses and a psychologist.

Both the Dutch randomized controlled GH trial (Dutch GH RCT) and the multicenter follow-up study (Dutch PWS Cohort Study) are multicenter studies. Three-monthly, 14 hospitals throughout The Netherlands are visited by the MD-researcher and the research nurse, where children are examined, in collaboration with the local pediatrician or pediatric endocrinologist (Figure 9). Standardized measurements take place at the Erasmus University Medical Center - Sophia Children's Hospital in Rotterdam, The Netherlands, at start, at 6 and 12 months and subsequently once a year. In the PWS Oxytocin Study, children visited the Sophia Children's Hospital at baseline, after 4 weeks and after 8 weeks. The PWS Transition Study was a single center study and young adults visited the Sophia Children's Hospital in Rotterdam twice a year for standardized measurements.

**Figure 9.** Participating centers Dutch GH trial and PWS Cohort Study.

| | |
|---|---|
| Rotterdam | R.J. Kuppens, S.H. Donze, L. Damen, E. Mahabier, P.M.C.C. van Eekelen, L. Schafthuizen, G.C.B. Bindels-de Heus, A.C.S. Hokken-Koelega<br>Previously: N.E. Bakker, S.T. Lo, E.P.C. Siemensma, R.F.A. Tummers-de Lind van Wijngaarden, D.A.M. Festen, A. Lukoshe, B. Kerkhof<br>*Dutch Growth Research Foundation and Erasmus University Medical Center Rotterdam-Sophia Children's Hospital (black dot)* |
| Nijmegen | A.A.E.M. van Alfen-van der Velden, *Radboud University Medical Center-Amalia Children's Hospital* |

From 2015: One accredited Dutch Prader-Willi Reference Center with 2 locations,
*Dutch Growth Research Foundation in collaboration with Erasmus University Medical Center-Sophia Children's Hospital, and Radboud University Medical Center-Amalia Children's Hospital*

| | |
|---|---|
| Amsterdam | J. Rotteveel, *VU University Medical Center* |
| Amsterdam | N. Zwaveling-Soonawala, *Academic Medical Center-Emma Children's Hospital* |
| Apeldoorn | L. Lunshof, *Gelre Hospitals* |
| Den Bosch | P.E. Jira, E.G.A.H. van Mil, *Jeroen Bosch Medical Center* |
| Den Haag | E.C.A.M. Houdijk, *Haga Hospitals-Juliana Children's Hospital* |
| Eindhoven | R.J.H. Odink, V. van Tellingen, *St. Catharina Hospital* |
| Enschede | M. Wegdam-Boer, *Medical Center Twente* |
| Groningen | G. Bocca, *University Medical Center Groningen-Beatrix Children's Hospital* |
| Harderwijk | M. van Leeuwen, *St. Jansdal Hospital* |
| Leeuwarden | E. van Pinxteren-Nagler, *Medical Center Leeuwarden(2002-2014)* |
| Leiden | D.A.J.P. Haring, *Alrijne Hospital* |
| Leiden | W. Oostdijk, *Leiden University Medical Center-Willem-Alexander Children's Hospital* |
| Lelystad | R. Lauwerijs, *MC Zuiderzee (2002-2013)* |
| Nijmegen | C. Westerlaken, *Canisius-Wilhelmina Hospital (2004-2011)* |
| Utrecht | J.J.G. Hoorweg-Nijman, H. van Wieringen, *St. Antonius Hospital* |
| Zwolle | E.J. Schroor, *Isala Hospital (2002-2014)* |

## Dutch GH RCT and PWS Cohort Study

*Patients*

Until July 2016, more than 185 Dutch children with PWS were included in the Dutch GH RCT and PWS Cohort Study, and nowadays, almost all Dutch infants are included in the PWS Cohort. For both studies, children had to meet the following criteria:

*Inclusion criteria*

- Genetically confirmed diagnosis of PWS;
- Age between 6 months and 16 years; (extended in 2016: between 0 months and 16 years)
- Maximal bone age of less than 14 years in girls, or 16 years in boys.

*Exclusion criteria*

- Non-cooperative behaviour;
- Extremely low dietary intake of less than minimal required intake according to guidelines set by the World Health Organization;
- Medication to reduce weight (fat);
- In children above 3 years of age: height above 0 SDS, unless weight-for-height is above 2 SDS;
- Previous treatment with GH (not applicable for the Dutch PWS Cohort Study).

*Design*

From 2002 to 2008, 61 infants, 47 prepubertal children and 7 pubertal children were included in the RCT (Figure 10). Infants and prepubertal children were randomized into either a GH-treated group or a control group for respectively 1 or 2 years, and subsequently all were treated with GH 1 mg/m$^2$ per day. All pubertal children were treated with GH, but were randomized to receive either 1 mg/m$^2$ per day or 1.5 mg/m$^2$ per day until adult height. All were prospectively followed and GH treated in the Dutch PWS Cohort Study in collaboration with pediatricians or pediatric endocrinologists throughout The Netherlands.

Since 2009, Dutch infants with PWS are directly included in the PWS Cohort Study for follow-up during long term GH treatment until attainment of adult height. All children are treated with GH 1 mg/m$^2$ per day and are prospectively followed in the Dutch PWS Cohort Study in collaboration with pediatricians or pediatric endocrinologists throughout The Netherlands (Figure 9).

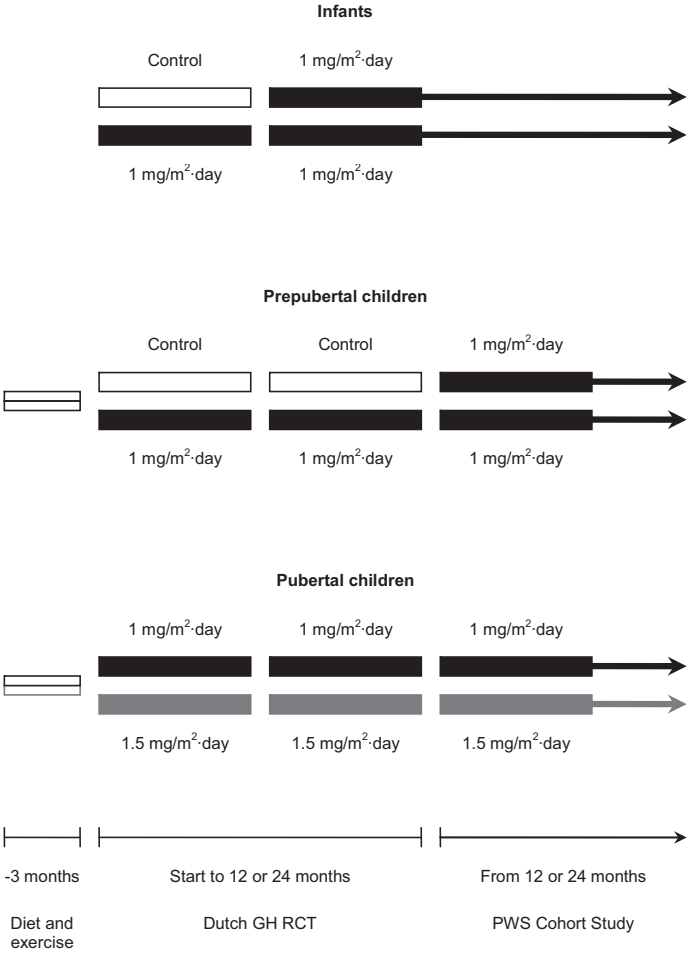



**Figure 10.** Design of the multicenter randomized controlled GH trial and the Dutch PWS Cohort study. GH dose 1.0 mg/m2·day≈0.035 mg/kg·day, GH dose 1.5 mg/m2·day≈0.052 mg/kg·day.

## PWS Oxytocin Study

*Patients*

Between January and September 2015, 25 children with PWS were included in the PWS Oxytocin Study. Children had to meet the following criteria:

*Inclusion criteria*

- Genetically confirmed diagnosis of PWS;
- Age between 6 and 15 years;
- Social behavioural problems and/or a preoccupation with food;
- Naïve for oxytocin treatment at time of enrollment;
- Used GH treatment for at least 1 year and were still receiving it.

*Exclusion criteria*

- Severe psychiatric problems, serious illness or cardiac abnormalities;
- Allergic reactions or hypersensitivity to oxytocin;
- Medication to reduce weight (fat) other than GH;
- Non-cooperative behaviour.

*Design*

A randomized, double-blind, placebo-controlled, cross-over study was conducted to investigate the effects of intranasal oxytocin administration on social behaviour, food intake and satiety (Figure 11). Children received either oxytocin or placebo for 4 weeks, after which they crossed-over to the alternative treatment for a further 4 weeks. No wash-out period was implemented. The children were stratified according to gender and age (6-10 or 11-14 years) and then randomly and blindly assigned to receive intranasal administration twice daily, before breakfast and dinner, of either oxytocin or identical appearing placebo. The dose was calculated according to body surface. Investigators were blinded for the allocation.

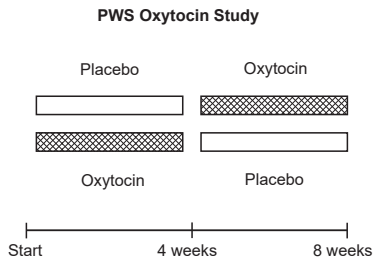



**Figure 11.** Design of the randomized, double-blinded, placebo-controlled cross-over PWS Oxytocin Study. Oxytocin dose calculated according to body surface

## PWS Transition Study

*Patients*

Until January 2014, 28 Dutch young adults with PWS who were treated with GH during childhood and attained adult height, were included in the PWS Transition Study. Young adults had to meet the following criteria:



*Inclusion criteria*

- Genetically confirmed diagnosis of PWS;
- GH treatment during childhood for at least 2 years and being on GH at time of inclusion;
- Adult height attainment, defined as a height velocity less than 0.5 cm per 6 months and a complete epiphyseal fusion.

*Exclusion criteria*

- Non-cooperative behaviour;
- Medication to reduce weight (fat).

*Design*

Two-year, randomized, double-blind, placebo-controlled, cross-over study investigating the effects of 1 year placebo versus 1 year GH on body composition, metabolic health profile and cognition (Figure 12). Young adults were stratified according to gender and BMI (below or above 25 kg/m$^2$) and then randomly and blindly assigned to receive 1 year of subcutaneous injections once daily at bedtime of either 0.67 mg/m$^2$/day GH or 1 year of identical appearing placebo, after which they crossed-over to the alternative treatment for another year. Investigators were blinded for the allocation.

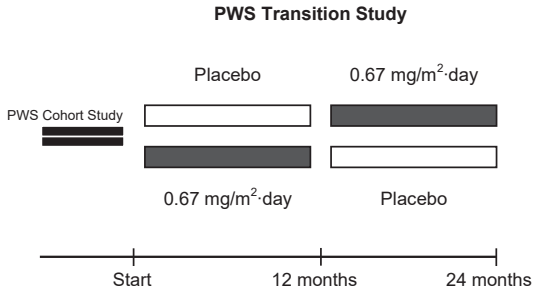



**Figure 12.** Design of the 2-year randomized, double-blinded, placebo-controlled cross-over GH trial. GH dose 0.67 mg/m$^2$·day≈0.023 mg/kg·day.

## REFERENCES


1. Down, J. L.: On polysarcia and its treatment (London Hospital Reports 1864). Mental affections of childhood and youth. London, 1990 (originally published in 1887)
2. Prader, A. L., A.; Willi, H.: Ein Syndrom von Adipositas, Kleinwuchs, Kryptorchismus and Oligophreniech myatonieartigen Zustand in Neugoborenanalter. Schweizerische medizinische wochenschrift, 86: 1260, 1956
3. Goldstone, A. P., Holland, A. J., Hauffa, B. P. et al.: Recommendations for the diagnosis and management of Prader-Willi syndrome. J Clin Endocrinol Metab, 93: 4183, 2008
4. Cassidy, S. B.: Prader-Willi syndrome. J Med Genet, 34: 917, 1997
5. Akefeldt, A., Gillberg, C., Larsson, C.: Prader-Willi syndrome in a Swedish rural county: epidemiological aspects. Dev Med Child Neurol, 33: 715, 1991
6. Whittington, J. E., Holland, A. J., Webb, T. et al.: Population prevalence and estimated birth incidence and mortality rate for people with Prader-Willi syndrome in one UK Health Region. J Med Genet, 38: 792, 2001
7. Burd, L., Vesely, B., Martsolf, J. et al.: Prevalence study of Prader-Willi syndrome in North Dakota. Am J Med Genet, 37: 97, 1990
8. Swaab, D. F.: Prader-Willi syndrome and the hypothalamus. Acta Paediatr Suppl, 423: 50, 1997
9. Holm, V. A., Cassidy, S. B., Butler, M. G. et al.: Prader-Willi syndrome: consensus diagnostic criteria. Pediatrics, 91: 398, 1993
10. Hawkey, C. J., Smithies, A.: The Prader-Willi syndrome with a 15/15 translocation. Case report and review of the literature. J Med Genet, 13: 152, 1976
11. Ledbetter, D. H., Riccardi, V. M., Airhart, S. D. et al.: Deletions of chromosome 15 as a cause of the Prader-Willi syndrome. N Engl J Med, 304: 325, 1981
12. Butler, M. G., Palmer, C. G.: Parental origin of chromosome 15 deletion in Prader-Willi syndrome. Lancet, 1: 1285, 1983
13. Chaillet, J. R., Knoll, J. H., Horsthemke, B. et al.: The syntenic relationship between the critical deletion region for the Prader-Willi/Angelman syndromes and proximal mouse chromosome 7. Genomics, 11: 773, 1991
14. Buiting, K., Saitoh, S., Gross, S. et al.: Inherited microdeletions in the Angelman and Prader-Willi syndromes define an imprinting centre on human chromosome 15. Nat Genet, 9: 395, 1995
15. Waterland, R. A., Jirtle, R. L.: Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. Nutrition, 20: 63, 2004
16. Cassidy, S. B., Schwartz, S., Miller, J. L. et al.: Prader-Willi syndrome. Genet Med, 14: 10, 2012
17. Webb, T., Whittington, J., Clarke, D. et al.: A study of the influence of different genotypes on the physical and behavioral phenotypes of children and adults ascertained clinically as having PWS. Clin Genet, 62: 273, 2002
18. Angulo, M. A., Butler, M. G., Cataletto, M. E.: Prader-Willi syndrome: a review of clinical, genetic, and endocrine findings. J Endocrinol Invest, 38: 1249, 2015
19. Mascari, M. J., Gottlieb, W., Rogan, P. K. et al.: The frequency of uniparental disomy in Prader-Willi syndrome. Implications for molecular diagnosis. N Engl J Med, 326: 1599, 1992
20. Bittel, D. C., Butler, M. G.: Prader-Willi syndrome: clinical genetics, cytogenetics and molecular biology. Expert Rev Mol Med, 7: 1, 2005
21. Sinnema, M., Boer, H., Collin, P. et al.: Psychiatric illness in a cohort of adults with Prader-Willi syndrome. Res Dev Disabil, 32: 1729, 2011

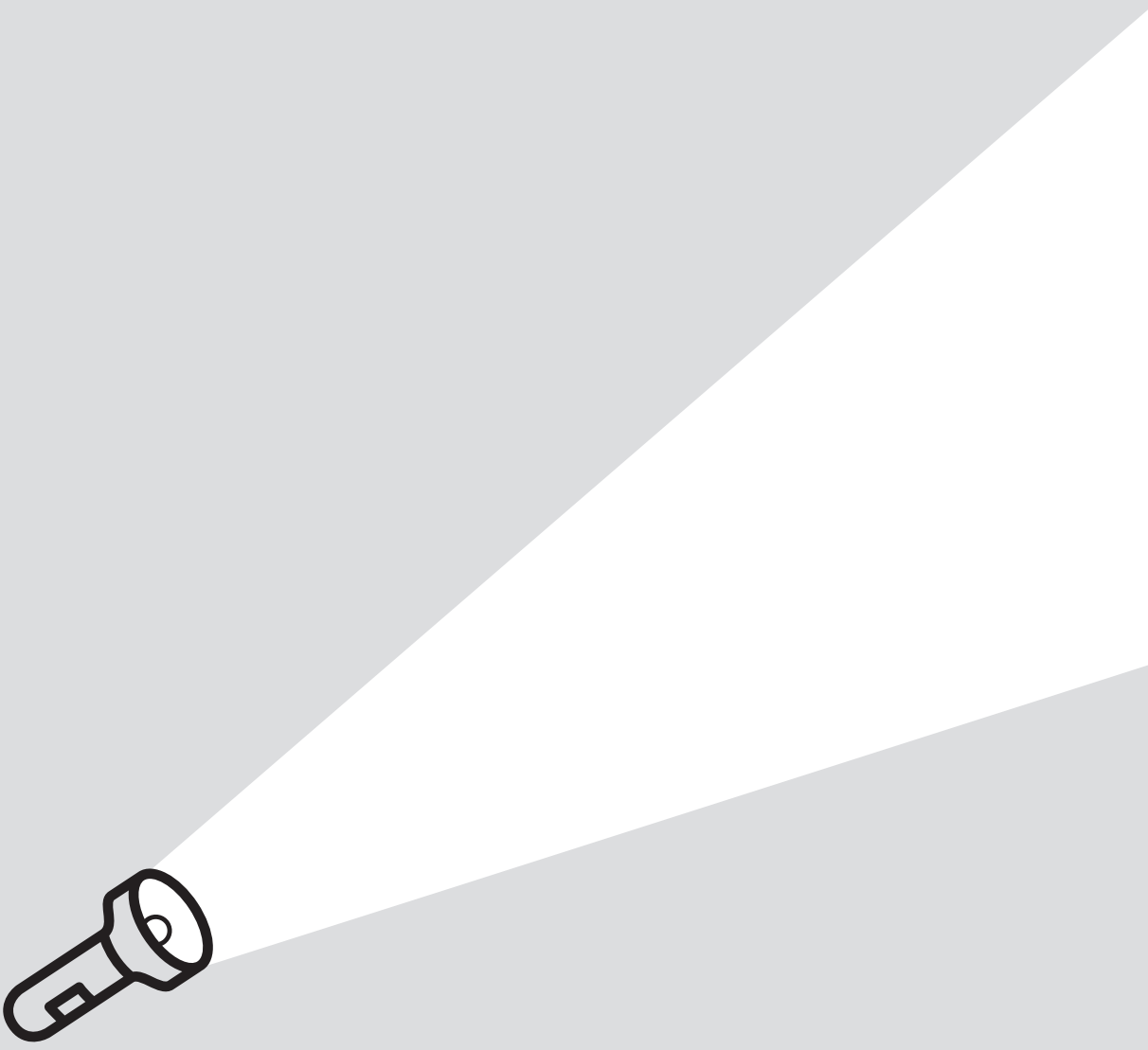
22. Whittington, J. E., Butler, J. V., Holland, A. J.: Changing rates of genetic subtypes of Prader-Willi syndrome in the UK. Eur J Hum Genet, 15: 127, 2007
23. Bakker, N. E., Kuppens, R. J., Siemensma, E. P. et al.: Bone mineral density in children and adolescents with Prader-Willi syndrome: a longitudinal study during puberty and 9 years of growth hormone treatment. J Clin Endocrinol Metab, 100: 1609, 2015
24. Boer, H., Holland, A., Whittington, J. et al.: Psychotic illness in people with Prader Willi syndrome due to chromosome 15 maternal uniparental disomy. Lancet, 359: 135, 2002
25. Spritz, R. A., Bailin, T., Nicholls, R. D. et al.: Hypopigmentation in the Prader-Willi syndrome correlates with P gene deletion but not with haplotype of the hemizygous P allele. Am J Med Genet, 71: 57, 1997
26. Butler, M. G., Bittel, D. C., Kibiryeva, N. et al.: Behavioral differences among subjects with Prader-Willi syndrome and type I or type II deletion and maternal disomy. Pediatrics, 113: 565, 2004
27. Miller, J. L., Lynn, C. H., Driscoll, D. C. et al.: Nutritional phases in Prader-Willi syndrome. Am J Med Genet A, 155A: 1040, 2011
28. Goldstone, A. P.: Prader-Willi syndrome: advances in genetics, pathophysiology and treatment. Trends Endocrinol Metab, 15: 12, 2004
29. Wollmann, H. A., Schultz, U., Grauer, M. L. et al.: Reference values for height and weight in Prader-Willi syndrome based on 315 patients. Eur J Pediatr, 157: 634, 1998
30. Beauloye, V., Diene, G., Kuppens, R. et al.: High unacylated ghrelin levels support the concept of anorexia in infants with prader-willi syndrome. Orphanet J Rare Dis, 11: 56, 2016
31. Eiholzer, U., Blum, W. F., Molinari, L.: Body fat determined by skinfold measurements is elevated despite underweight in infants with Prader-Labhart-Willi syndrome. J Pediatr, 134: 222, 1999
32. Bekx, M. T., Carrel, A. L., Shriver, T. C. et al.: Decreased energy expenditure is caused by abnormal body composition in infants with Prader-Willi Syndrome. J Pediatr, 143: 372, 2003
33. Gunay-Aygun, M., Schwartz, S., Heeger, S. et al.: The changing purpose of Prader-Willi syndrome clinical diagnostic criteria and proposed revised criteria. Pediatrics, 108: E92, 2001
34. Festen, D. A., Wevers, M., Lindgren, A. C. et al.: Mental and motor development before and during growth hormone treatment in infants and toddlers with Prader-Willi syndrome. Clin Endocrinol (Oxf), 68: 919, 2008
35. Festen, D. A., Wevers, M., de Weerd, A. W. et al.: Psychomotor development in infants with Prader-Willi syndrome and associations with sleep-related breathing disorders. Pediatr Res, 62: 221, 2007
36. Reus, L., Zwarts, M., van Vlimmeren, L. A. et al.: Motor problems in Prader-Willi syndrome: a systematic review on body composition and neuromuscular functioning. Neurosci Biobehav Rev, 35: 956, 2011
37. Descheemaeker, M. J., Vogels, A., Govers, V. et al.: Prader-Willi syndrome: new insights in the behavioural and psychiatric spectrum. J Intellect Disabil Res, 46: 41, 2002
38. Bakker, N. E., Siemensma, E. P., Koopman, C. et al.: Dietary Energy Intake, Body Composition and Resting Energy Expenditure in Prepubertal Children with Prader-Willi Syndrome before and during Growth Hormone Treatment: A Randomized Controlled Trial. Horm Res Paediatr, 83: 321, 2015
39. Miller, J. L., Lynn, C. H., Shuster, J. et al.: A reduced-energy intake, well-balanced diet improves weight control in children with Prader-Willi syndrome. J Hum Nutr Diet, 26: 2, 2013
40. Hoybye, C., Thoren, M., Bohm, B.: Cognitive, emotional, physical and social effects of growth hormone treatment in adults with Prader-Willi syndrome. J Intellect Disabil Res, 49: 245, 2005
41. Whittington, J., Holland, A., Webb, T. et al.: Cognitive abilities and genotype in a population-based sample of people with Prader-Willi syndrome. J Intellect Disabil Res, 48: 172, 2004

42. Siemensma, E. P., Tummers-de Lind van Wijngaarden, R. F., Festen, D. A. et al.: Beneficial effects of growth hormone treatment on cognition in children with Prader-Willi syndrome: a randomized controlled trial and longitudinal study. J Clin Endocrinol Metab, 97: 2307, 2012
43. de Lind van Wijngaarden, R. F., de Klerk, L. W., Festen, D. A. et al.: Scoliosis in Prader-Willi syndrome: prevalence, effects of age, gender, body mass index, lean body mass and genotype. Arch Dis Child, 93: 1012, 2008
44. Hered, R. W., Rogers, S., Zang, Y. F. et al.: Ophthalmologic features of Prader-Willi syndrome. J Pediatr Ophthalmol Strabismus, 25: 145, 1988
45. Festen, D. A., de Weerd, A. W., van den Bossche, R. A. et al.: Sleep-related breathing disorders in prepubertal children with Prader-Willi syndrome and effects of growth hormone treatment. J Clin Endocrinol Metab, 91: 4911, 2006
46. de Lind van Wijngaarden, R. F., Otten, B. J., Festen, D. A. et al.: High prevalence of central adrenal insufficiency in patients with Prader-Willi syndrome. J Clin Endocrinol Metab, 93: 1649, 2008
47. Dykens, E. M., Maxwell, M. A., Pantino, E. et al.: Assessment of hyperphagia in Prader-Willi syndrome. Obesity (Silver Spring), 15: 1816, 2007
48. Wharton, R. H., Wang, T., Graeme-Cook, F. et al.: Acute idiopathic gastric dilation with gastric necrosis in individuals with Prader-Willi syndrome. Am J Med Genet, 73: 437, 1997
49. Stevenson, D. A., Heinemann, J., Angulo, M. et al.: Gastric rupture and necrosis in Prader-Willi syndrome. J Pediatr Gastroenterol Nutr, 45: 272, 2007
50. Lo, S. T., Siemensma, E. P., Festen, D. A. et al.: Behavior in children with Prader-Willi syndrome before and during growth hormone treatment: a randomized controlled trial and 8-year longitudinal study. Eur Child Adolesc Psychiatry, 24: 1091, 2015
51. Dykens, E. M., Cassidy, S. B.: Correlates of maladaptive behavior in children and adults with Prader-Willi syndrome. Am J Med Genet, 60: 546, 1995
52. Akefeldt, A., Gillberg, C.: Behavior and personality characteristics of children and young adults with Prader-Willi syndrome: a controlled study. J Am Acad Child Adolesc Psychiatry, 38: 761, 1999
53. Lo, S. T., Siemensma, E., Collin, P. et al.: Impaired theory of mind and symptoms of Autism Spectrum Disorder in children with Prader-Willi syndrome. Res Dev Disabil, 34: 2764, 2013
54. Siemensma, E. P., de Lind van Wijngaarden, R. F., Otten, B. J. et al.: Testicular failure in boys with Prader-Willi syndrome: longitudinal studies of reproductive hormones. J Clin Endocrinol Metab, 97: E452, 2012
55. Siemensma, E. P., de Lind van Wijngaarden, R. F., Otten, B. J. et al.: Pubarche and serum dehydroepiandrosterone sulphate levels in children with Prader-Willi syndrome. Clin Endocrinol (Oxf), 75: 83, 2011
56. Siemensma, E. P., van Alfen-van der Velden, A. A., Otten, B. J. et al.: Ovarian function and reproductive hormone levels in girls with Prader-Willi syndrome: a longitudinal study. J Clin Endocrinol Metab, 97: E1766, 2012
57. Clarke, D. J., Boer, H., Chung, M. C. et al.: Maladaptive behaviour in Prader-Willi syndrome in adult life. J Intellect Disabil Res, 40 ( Pt 2): 159, 1996
58. Hauffa, B. P., Schlippe, G., Roos, M. et al.: Spontaneous growth in German children and adolescents with genetically confirmed Prader-Willi syndrome. Acta Paediatr, 89: 1302, 2000
59. Carpenter, P. K.: Prader-Willi syndrome in old age. J Intellect Disabil Res, 38 ( Pt 5): 529, 1994
60. Lionti, T., Reid, S. M., Rowell, M. M.: Prader-Willi syndrome in Victoria: mortality and causes of death. J Paediatr Child Health, 48: 506, 2012

61. de Lind van Wijngaarden, R. F., Joosten, K. F., van den Berg, S. et al.: The relationship between central adrenal insufficiency and sleep-related breathing disorders in children with Prader-Willi syndrome. J Clin Endocrinol Metab, 94: 2387, 2009
62. Eiholzer, U., Bachmann, S., l'Allemand, D.: Is there growth hormone deficiency in prader-willi Syndrome? Six arguments to support the presence of hypothalamic growth hormone deficiency in Prader-Willi syndrome. Horm Res, 53 Suppl 3: 44, 2000
63. Grugni, G., Crino, A., Pagani, S. et al.: Growth hormone secretory pattern in non-obese children and adolescents with Prader-Willi syndrome. J Pediatr Endocrinol Metab, 24: 477, 2011
64. Angulo, M., Castro-Magana, M., Mazur, B. et al.: Growth hormone secretion and effects of growth hormone therapy on growth velocity and weight gain in children with Prader-Willi syndrome. J Pediatr Endocrinol Metab, 9: 393, 1996
65. Carrel, A. L., Myers, S. E., Whitman, B. Y. et al.: Growth hormone improves body composition, fat utilization, physical strength and agility, and growth in Prader-Willi syndrome: A controlled study. J Pediatr, 134: 215, 1999
66. Lindgren, A. C., Ritzen, E. M.: Five years of growth hormone treatment in children with Prader-Willi syndrome. Swedish National Growth Hormone Advisory Group. Acta Paediatr Suppl, 88: 109, 1999
67. Carrel, A. L., Myers, S. E., Whitman, B. Y. et al.: Long-term growth hormone therapy changes the natural history of body composition and motor function in children with prader-willi syndrome. J Clin Endocrinol Metab, 95: 1131, 2010
68. Sipila, I., Sintonen, H., Hietanen, H. et al.: Long-term effects of growth hormone therapy on patients with Prader-Willi syndrome. Acta Paediatr, 99: 1712, 2010
69. Lindgren, A. C., Lindberg, A.: Growth hormone treatment completely normalizes adult height and improves body composition in Prader-Willi syndrome: experience from KIGS (Pfizer International Growth Database). Horm Res, 70: 182, 2008
70. Bakker, N. E., Kuppens, R. J., Siemensma, E. P. et al.: Eight years of growth hormone treatment in children with Prader-Willi syndrome: maintaining the positive effects. J Clin Endocrinol Metab, 98: 4013, 2013
71. Lo, S. T., Festen, D. A., Tummers-de Lind van Wijngaarden, R. F. et al.: Beneficial Effects of Long-Term Growth Hormone Treatment on Adaptive Functioning in Infants With Prader-Willi Syndrome. Am J Intellect Dev Disabil, 120: 315, 2015
72. Butler, J. V., Whittington, J. E., Holland, A. J. et al.: The transition between the phenotypes of Prader-Willi syndrome during infancy and early childhood. Dev Med Child Neurol, 52: e88, 2010
73. van der Lely, A. J., Tschop, M., Heiman, M. L. et al.: Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. Endocr Rev, 25: 426, 2004
74. Kojima, M., Hosoda, H., Date, Y. et al.: Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature, 402: 656, 1999
75. Yang, J., Brown, M. S., Liang, G. et al.: Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. Cell, 132: 387, 2008
76. Delhanty, P. J., Huisman, M., Julien, M. et al.: The acylated (AG) to unacylated (UAG) ghrelin ratio in esterase inhibitor treated blood is higher than previously described. Clin Endocrinol (Oxf), 2014
77. De Vriese, C., Gregoire, F., Lema-Kisoka, R. et al.: Ghrelin degradation by serum and tissue homogenates: identification of the cleavage sites. Endocrinology, 145: 4997, 2004
78. Delhanty, P. J., Neggers, S. J., van der Lely, A. J.: Mechanisms in endocrinology: Ghrelin: the differences between acyl- and des-acyl ghrelin. Eur J Endocrinol, 167: 601, 2012

79. Ozcan, B., Neggers, S. J., Miller, A. R. et al.: Does des-acyl ghrelin improve glycemic control in obese diabetic subjects by decreasing acylated ghrelin levels? Eur J Endocrinol, 170: 799, 2014
80. Liu, J., Prudom, C. E., Nass, R. et al.: Novel ghrelin assays provide evidence for independent regulation of ghrelin acylation and secretion in healthy young men. J Clin Endocrinol Metab, 93: 1980, 2008
81. Wren, A. M., Seal, L. J., Cohen, M. A. et al.: Ghrelin enhances appetite and increases food intake in humans. J Clin Endocrinol Metab, 86: 5992, 2001
82. Feigerlova, E., Diene, G., Conte-Auriol, F. et al.: Hyperghrelinemia precedes obesity in Prader-Willi syndrome. J Clin Endocrinol Metab, 93: 2800, 2008
83. Cummings, D. E., Clement, K., Purnell, J. Q. et al.: Elevated plasma ghrelin levels in Prader Willi syndrome. Nat Med, 8: 643, 2002
84. Purtell, L., Sze, L., Loughnan, G. et al.: In adults with Prader-Willi syndrome, elevated ghrelin levels are more consistent with hyperphagia than high PYY and GLP-1 levels. Neuropeptides, 45: 301, 2011
85. DelParigi, A., Tschop, M., Heiman, M. L. et al.: High circulating ghrelin: a potential cause for hyperphagia and obesity in prader-willi syndrome. J Clin Endocrinol Metab, 87: 5461, 2002
86. Haqq, A. M., Farooqi, I. S., O'Rahilly, S. et al.: Serum ghrelin levels are inversely correlated with body mass index, age, and insulin concentrations in normal children and are markedly increased in Prader-Willi syndrome. J Clin Endocrinol Metab, 88: 174, 2003
87. Kweh, F. A., Miller, J. L., Sulsona, C. R. et al.: Hyperghrelinemia in Prader-Willi syndrome begins in early infancy long before the onset of hyperphagia. Am J Med Genet A, 2014
88. Lindgren, A. C., Barkeling, B., Hagg, A. et al.: Eating behavior in Prader-Willi syndrome, normal weight, and obese control groups. J Pediatr, 137: 50, 2000
89. Schaller, F., Watrin, F., Sturny, R. et al.: A single postnatal injection of oxytocin rescues the lethal feeding behaviour in mouse newborns deficient for the imprinted Magel2 gene. Hum Mol Genet, 19: 4895, 2010
90. Hoybye, C., Barkeling, B., Espelund, U. et al.: Peptides associated with hyperphagia in adults with Prader-Willi syndrome before and during GH treatment. Growth Horm IGF Res, 13: 322, 2003
91. Tauber, M., Mantoulan, C., Copet, P. et al.: Oxytocin may be useful to increase trust in others and decrease disruptive behaviours in patients with Prader-Willi syndrome: a randomised placebo-controlled trial in 24 patients. Orphanet J Rare Dis, 6: 47, 2011
92. Hoybye, C., Hilding, A., Jacobsson, H. et al.: Growth hormone treatment improves body composition in adults with Prader-Willi syndrome. Clin Endocrinol (Oxf), 58: 653, 2003
93. Sode-Carlsen, R., Farholt, S., Rabben, K. F. et al.: One year of growth hormone treatment in adults with Prader-Willi syndrome improves body composition: results from a randomized, placebo-controlled study. J Clin Endocrinol Metab, 95: 4943, 2010
94. Grugni, G., Marzullo, P., Ragusa, L. et al.: Impairment of GH responsiveness to combined GH-releasing hormone and arginine administration in adult patients with Prader-Willi syndrome. Clin Endocrinol (Oxf), 65: 492, 2006
95. Talebizadeh, Z., Butler, M. G.: Insulin resistance and obesity-related factors in Prader-Willi syndrome: comparison with obese subjects. Clin Genet, 67: 230, 2005
96. Bray, G. A., Dahms, W. T., Swerdloff, R. S. et al.: The Prader-Willi syndrome: a study of 40 patients and a review of the literature. Medicine (Baltimore), 62: 59, 1983
97. Schuster, D. P., Osei, K., Zipf, W. B.: Characterization of alterations in glucose and insulin metabolism in Prader-Willi subjects. Metabolism, 45: 1514, 1996

98. Goldstone, A. P., Thomas, E. L., Brynes, A. E. et al.: Visceral adipose tissue and metabolic complications of obesity are reduced in Prader-Willi syndrome female adults: evidence for novel influences on body fat distribution. J Clin Endocrinol Metab, 86: 4330, 2001
99. Nagai, T., Mori, M.: Prader-Willi syndrome, diabetes mellitus and hypogonadism. Biomed Pharmacother, 53: 452, 1999
100. Laurier, V., Lapeyrade, A., Copet, P. et al.: Medical, psychological and social features in a large cohort of adults with Prader-Willi syndrome: experience from a dedicated centre in France. J Intellect Disabil Res, 59: 411, 2015
101. Moller, N., Jorgensen, J. O., Abildgard, N. et al.: Effects of growth hormone on glucose metabolism. Horm Res, 36 Suppl 1: 32, 1991
102. Saenger, P.: Metabolic consequences of growth hormone treatment in paediatric practice. Horm Res, 53 Suppl 1: 60, 2000
103. Curfs, L. M., Wiegers, A. M., Sommers, J. R. et al.: Strengths and weaknesses in the cognitive profile of youngsters with Prader-Willi syndrome. Clin Genet, 40: 430, 1991
104. Lukoshe, A., Hokken-Koelega, A. C., van der Lugt, A. et al.: Reduced cortical complexity in children with Prader-Willi Syndrome and its association with cognitive impairment and developmental delay. PLoS One, 9: e107320, 2014
105. van Nieuwpoort, I. C., Deijen, J. B., Curfs, L. M. et al.: The relationship between IGF-I concentration, cognitive function and quality of life in adults with Prader-Willi syndrome. Horm Behav, 59: 444, 2011



CHAPTER 2

# Elevated ratio of acylated to unacylated ghrelin in children and young adults with Prader-Willi syndrome

R.J. Kuppens, G. Diène, N.E. Bakker, C. Molinas, S. Faye, M. Nicolino, D. Bernoux, P.J.D. Delhanty, A.J. van der Lely, S. Allas, M. Julien, T. Delale, M. Tauber, A.C.S. Hokken-Koelega

*Endocrine 2015; 50, 633-642*

## ABSTRACT

**Context** Prader-Willi syndrome (PWS) is characterized by a switch from failure to thrive to excessive weight gain and hyperphagia in early childhood. Hyperghrelinemia may be involved in the underlying mechanisms of the switch.

**Purpose** To evaluate acylated ghrelin (AG) and unacylated ghrelin (UAG) levels in PWS and investigate their associations with hyperphagia.

**Design** Cross-sectional clinical study in three PWS expert centers in the Netherlands and France.

**Method** Levels of AG and UAG and the AG/UAG ratio were determined in 138 patients with PWS (0.2-29.4 years) and compared with 50 age-matched obese subjects (4.3-16.9 years) and 39 healthy controls (0.8-28.6 years). AEBSF was used to inhibit deacylation of AG.

**Results** As a group, PWS patients had higher AG but similar UAG levels as healthy controls (AG 129.1 vs 82.4 pg/ml, p=0.016; UAG 135.3 vs 157.3 pg/ml, resp.), resulting in a significantly higher AG/UAG ratio (1.00 vs 0.61, p=0.001, resp.). Obese subjects had significantly lower AG and UAG levels than PWS and controls (40.3 and 35.8 pg/ml, resp.), but also a high AG/UAG ratio (1.16). The reason for the higher AG/UAG ratio in PWS and obese was, however, completely different, as PWS had a high AG and obese a very low UAG.
PWS patients without weight gain or hyperphagia had a similar AG/UAG ratio as age-matched controls, in contrast to those with weight gain and/or hyperphagia who had an elevated AG/UAG ratio.

**Conclusion** The switch to excessive weight gain in PWS seems to coincide with an increase in the AG/UAG ratio, even prior to the start of hyperphagia.

## INTRODUCTION

Prader-Willi syndrome (PWS) is a neurogenetic disorder caused by the lack of expression of the paternally derived genes on chromosome 15 at locus q11-q13[1]. Clinical findings change when children become older: infancy is characterized by poor feeding, failure to thrive and muscular hypotonia, while hyperphagia with impaired satiety, obesity, short stature, psychomotor delay and behavioural problems are prominent during childhood and adulthood[2, 3]. The mechanism behind the switch from failure to thrive to excessive weight gain and hyperphagia in early childhood is not yet known, but hyperghrelinemia might be involved[4, 5].



Most children with PWS nowadays receive growth hormone (GH) treatment, which counteracts the clinical course of obesity and improves the metabolic profile, leading to better lipid levels, higher adiponectin levels and lower systolic blood pressure[6, 7]. However, GH treatment does not solve the problem of hyperphagia.

The appetite-stimulating hormone ghrelin has an acylated and an unacylated form in circulation[8-10]. Acylated ghrelin (AG) is known to be diabetogenic and has many actions such as stimulating appetite and inducing a positive energy balance, which can lead to weight gain[11-14]. Intravenous AG administration in healthy volunteers increased food intake and appetite[15], suggesting that the hyperphagia in PWS might be associated with increased ghrelin levels. For a long time, unacylated ghrelin (UAG) was considered to be an inactive degradation product of AG. Currently, there is increasing evidence that UAG has also distinct actions[16]. It is reported that UAG has protective effects on beta cells, endothelial progenitor cells and muscle cells, and UAG seems to improve glycemic control[14]. In addition, UAG acts as a functional inhibitor of AG and it was reported to suppress ghrelin levels in humans[16, 17]. This suggests a crucial role for the ratio of AG and UAG levels (AG/UAG ratio) in maintaining weight balance.

Ghrelin can be determined in blood samples, but AG is unstable and is rapidly deacylated to UAG through the action of esterases[18, 19]. Thus, reliable ghrelin determination needs the immediate addition of an esterase inhibitor at the time of blood collection. In patients with PWS, hyperghrelinemia has been reported, but in these studies samples were not treated in this way[4, 5, 20-23].

We formulated three hypotheses. Children and young adults with PWS have (1) higher AG levels and lower UAG levels than healthy and obese controls; (2) increasing AG and decreasing UAG levels with the rise of the nutritional phases, resulting in increased AG/UAG ratios in the higher nutritional phases; and (3) a higher AG/UAG ratio in the presence of weight gain and/or hyperphagia and a normal AG/UAG ratio in the absence of weight gain and/or hyperphagia, compared with age-matched healthy controls.

Therefore, a cross-sectional study was conducted in which we measured the plasma levels of AG and UAG in children and young adults with PWS and compared these levels

with those of obese and healthy controls. AEBSF, an inhibitor of deacylation of AG, was immediately added to all blood samples. In patients with PWS, we investigated the associations between AG and UAG ghrelin levels and the following factors: age, BMI, genotype, eating behaviour and food intake. In addition, we investigated whether the switch from failure to thrive to excessive weight gain and hyperphagia is associated with a change in the AG/UAG ratio.

## SUBJECTS AND METHODS

### Subjects

The study group consisted of 138 children and young adults with PWS, either participating in the Dutch PWS studies coordinated by the Dutch Growth Research Foundation, or followed at the PWS reference center in Toulouse or Children's Hospital in Lyon. PWS was genetically confirmed in all patients. One hundred and seven patients (77.5%) were treated with growth hormone (GH), the others had not yet started with GH or had reached final height without possibilities to continue GH treatment or the parents refused the GH treatment. Three patients with PWS had diabetes mellitus type 2 (T2DM) and all were treated with metformin. As their ghrelin levels were similar as in the total PWS group, we did not exclude them from analyses. None of the healthy or obese controls had T2DM.

Plasma ghrelin levels of subjects with PWS (PWS) were compared with 50 obese subjects (obese) and 39 healthy controls (controls). Obese and controls suffering from any systemic illness, growth disorder, syndrome or having dysmorphic features were excluded. Obese children had a BMI>+2 SDS and were regularly seen in the outpatient department of the pediatric endocrinology unit in Toulouse. Healthy controls were children and young adults with a normal BMI, who underwent a minor surgical procedure at Erasmus Medical Center in Rotterdam. Normal BMI was defined as a BMI between -2 SDS and +2 SDS[24].

Standing height was measured with a calibrated Harpenden stadiometer or, when appropriate, supine length with a Harpenden infantometer (Holtain Ltd). Weight was determined on a calibrated scale (Servo Balance KA-20-150S; Servo Berkel Prior) and BMI was calculated. Height, weight and BMI were expressed as SDS, adjusted for age and sex. The Dutch reference data were used for the Dutch children and young adults[24, 25] and the French reference data for height and weight and the Cole BMI reference data were used for the French patients[26, 27]. All SDS values were calculated with Growth Analyser (version 4.0; www.growthanalyser.org).

The Medical Ethics Committees of the 3 participating centers approved the study. Written informed consent was obtained from parents of PWS. For obese and controls,

written informed consent was obtained from themselves and, if they were younger than 18 years, also from their parents or custodians.

### Eating behaviour

The nutritional phases according to Miller were used to score the eating behaviour of the subjects with PWS[3]: 1a Hypotonia with difficulty feeding, 1b No difficulty feeding and growing appropriately on growth curve, 2a Weight increasing without an increase in appetite or excessive calories, 2b Weight increasing with an increase in appetite, 3 Hyperphagia, feels rarely full, 4 Appetite no longer insatiable. For each subject with PWS, the nutritional phase was assessed by the multidisciplinary teams or independently by two observers who knew them very well (physician and nurse)[3]. In case of disagreement, the case was discussed until consensus was reached. Subjects with PWS without weight gain or hyperphagia, defined as being in nutritional phase 1a or 1b, and subjects with weight gain and/or hyperphagia, defined as being in phases 2a, 2b and 3, were compared with age-matched controls.



### Collection of blood and plasma preparation

In children >2 years, blood samples were collected in the morning after a 12-h overnight fast. Infants <2 years were fasted for at least 5-h. To stabilize the plasma ghrelin levels, blood samples were collected in EDTA tubes and 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF, Sigma-Aldrich Chemicals) was added to a concentration of 2 mg/ml at the time of collection. Blood was centrifuged at 4 ºC to prepare plasma, which was quickly frozen on dry ice. Samples were stored at -80 ºC and assayed within 3 months following collection.

### Assays

Plasma AG and UAG levels were assessed in duplicate (10-50 µL per well) in one laboratory using two-step double antibody sandwich EIAs, obtained from SPIBio (Bertin Pharma, France; A05306 and A05319, resp.). Assays were performed according to manufacturer's instructions. In summary, standards, quality controls and samples were incubated in the plate for 2 hours at room temperature without tracer. After a 3x wash, tracer antibody was added and incubated for 2 hours at room temperature. Following a 5x wash, Ellman's reagent was added and incubated for approximately 45 minutes until satisfactory color development. Finally, the absorbance was measured at 405nm using a VictorX4 plate reader (PerkinElmer, Groningen, Netherlands).

Data were analyzed using Graphpad Prism 5 (La Jolla, California). A sigmoidal third order (cubic) polynomial fitting was used to determine concentrations from the calibration curves. This resulted in $r^2$ values >0.99 in the majority of the assays. Intra-assay coefficient of variations (CVs) for AG and UAG were 8.2 and 11.4% and interassay CV for

AG and UAG were 3.9 and 11.0%. CVs were determined over 10 and 9 assays for AG and UAG, resp. Samples had inter-duplicate CV of <20% for both AG and UAG. The AG/UAG ratio was computed as AG divided by UAG.

In Rotterdam, insulin levels were assessed using the Immulite 2000 assay (Siemens Healthcare Diagnostics). Interassay CV was 4.4%. Serum glucose levels were determined using the Hitachi 917 (Hitachi Device Development Center), detecting glucose levels between 0 and 42 mmol/l. Serum IGF-I levels were assessed using the IDS-iSYS (Immunodiagnostic Systems). The intra-assay CV was <6.0% and the interassay CV was <2.1%. In France, insulin and glucose were enzymatically assessed on the Beckman AU 2700 (Beckman Coulter Inc) and serum IGF-I levels were measured using IRMA assay from Immunotech. The intra-assay CV was <6.3% and the interassay CV was <8.8%. For Rotterdam and France, homeostasis model assessment of insulin resistance (HOMA-IR) was performed using the model HOMA-IR = (fasting insulin (mU/l) x fasting glucose (mmol/l))/22.5[28].

### Statistics

Statistical analysis was performed by the Statistical Package for Social Sciences (version 20.0; SPSS, Chicago, IL). Data are expressed as median [interquartile range (IQR)]. Differences between the groups were calculated using Kruskal Wallis tests when comparing three groups and Mann Whitney U tests when comparing two groups. Patients with PWS in each nutritional phase were compared with age-matched healthy controls. Obese PWS, defined as BMI>+2 SDS, were compared with obese controls. AG and UAG levels and AG/UAG ratios were log-transformed (natural logarithm), as they were not normally distributed. In PWS patients, we cross-sectionally assessed linear correlations between ghrelin levels and other parameters using Spearman's rho correlation coefficient (ρ). As levels of AG and UAG decreased with age, linear regression analysis was used to compare groups with adjustment for age. The AG/UAG ratio was not adjusted for age, as it remained stable across ages. Linear regression analysis was used to analyze correlations with adjustment for parameters such as age and gender. Regression coefficients are presented as percentages for better interpretation of the results. A positive value indicates that the dependent variable is increased by that % for every unit increase of the independent variable. Differences were considered significant if the p-value was <0.05.

## RESULTS

### Clinical characteristics

Median (interquartile range (IQR)) age of PWS was 9.9 (4.1-14.9) years (Table 1). There was no significant difference in age between the 138 PWS (61 boys), 39 controls (13 boys)

**Table 1.** Baseline characteristics of 138 PWS, 39 controls and 50 obese subjects.

| | PWS n=138 | | Controls n=39 | | Obese n=50 | | |
|---|---|---|---|---|---|---|---|
| | median | IQR | median | IQR | median | IQR | p* |
| Age (yrs) | 9.9 | (4.1 to 14.9) | 7.3 | (3.6 to 13.6) | 9.8 | (7.9 to 13.0) | 0.350 |
| Weight for age (SDS) | 0.8 | (-0.6 to 2.4) | -0.2 | (-0.8 to 0.5) | 5.9 | (4.5 to 7.0) | **<0.001** |
| Height for age (SDS) | -0.3 | (-1.3 to 0.6) | -0.1 | (-0.8 to 0.4) | 1.3 | (0.8 to 2.5) | **<0.001** |
| BMI for age (SDS) | 1.0 | (-0.2 to 2.1) | -0.2 | (-0.8 to 0.8) | 2.8 | (2.6 to 3.1) | **<0.001** |
| Fasting glycemia (mmol/l) | 4.5 | (4.0 to 5.0) | | | 4.3 | (3.9 to 4.7) | **0.043** |
| Fasting insulin (pmol/l) | 62.5 | (34.7 to 90.3) | | | 100.7 | (76.4 to 123.3) | **<0.001** |
| HOMA-IR | 1.8 | (1.0 to 2.8) | | | 2.6 | (1.9 to 3.6) | **0.001** |
| IGF-I (SDS) | 0.7 | (0.7 to 1.7) | | | -0.5 | (-1.2 to 0.3) | **<0.001** |

*p-value between the three groups; 138 PWS, 39 healthy controls and 50 obese controls. Bold values are statistically significant (p<0.05).



and 50 obese (16 boys). Height, weight and BMI were significantly different between the three groups and highest in obese (p<0.001). Fasting insulin and HOMA-IR were higher in obese (p<0.001 and p=0.001, resp.), while fasting glucose and IGF-I SDS were highest in PWS (p=0.043 and p<0.001, resp.).

PWS was genetically confirmed by an abnormal methylation test in all subjects with PWS. In 131 (94.9%) subjects, the genetic subtype was known; seventy (50.7%) had a deletion, 55 (39.9%) a uniparental maternal disomy (mUPD) and 6 (4.3%) an imprinting center mutation. One hundred and seven PWS used GH (77.5%) with a median dose of 0.85 mg/m$^2$/day (0.61-1.0) (≈0.028 mg/kg/day). Median age at start of GH treatment was 1.9 (1.1-4.3) years and median duration of GH treatment was 7.0 (3.2-9.1) years.

### Ghrelin levels in the three groups

Median (IQR) acylated ghrelin (AG) was significantly higher in the PWS group (129.1 (67.1-227.9) pg/ml) than in controls (82.4 (56.3-130.4) pg/ml, p=0.016). Unacylated ghrelin (UAG) was similar in PWS and controls. As a result, the AG/UAG ratio was significantly higher in PWS than in controls (p=0.001)(Table 2 and Figure 1).

In PWS, both AG and UAG levels were significantly higher than in obese (both p<0.001), as obese had the lowest AG and UAG levels of the three groups. Both PWS and obese had a significantly higher AG/UAG ratio than controls. The reason for the higher AG/UAG ratios in PWS and obese was, however, different. PWS had high AG levels, resulting in this higher AG/UAG ratio, while obese had low AG levels with even lower UAG levels.

In all three groups, both AG and UAG levels decreased with age, but the AG/UAG ratio remained stable.

**Table 2.** Ghrelin levels of the three groups

| | PWS n=138 | Controls n=39 | Obese n=50 | p between groups* | PWS vs Controls* | PWS vs Obese* | Obese vs Controls* |
|---|---|---|---|---|---|---|---|
| Parameter | median (IQR) | median (IQR) | median (IQR) | | | | |
| AG (pg/ml) | 129.1 (67.1-227.9) | 82.4 (56.3-130.4) | 40.3 (26.4-82.5) | **<0.001** | **0.016** | **<0.001** | **0.001** |
| UAG (pg/ml) | 135.3 (66.0-284.2) | 157.3 (79.3-261.0) | 35.8 (26.0-64.4) | **<0.001** | 0.868 | **<0.001** | **<0.001** |
| AG/UAG ratio | 1.00 (0.57-1.49) | 0.61 (0.37-0.81) | 1.16 (0.92-1.43) | **<0.001** | **0.001** | 0.069 | **<0.001** |

*p-value between the groups, bold values are statistically significant (p<0.05).

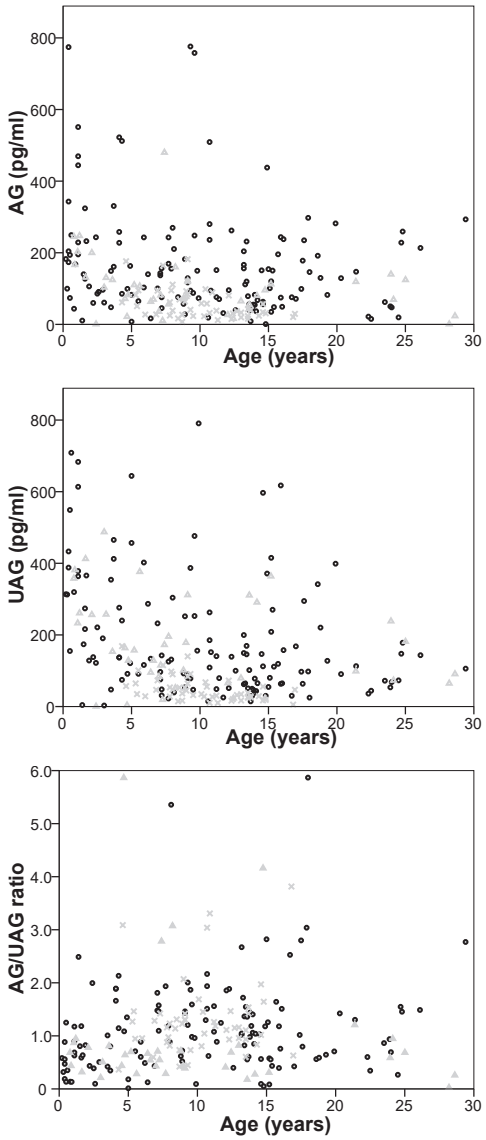



**Figure 1.** Ghrelin levels versus age for the three groups.
These figures show the AG and UAG levels and the AG/UAG ratio of children and young adults with PWS in black dots, of healthy controls in gray triangles and of obese controls in gray crosses.

### Ghrelin in nutritional phases in PWS

Of the 138 PWS who were classified according to the nutritional phases of Miller[3], 13 patients were in phase 1a, 37 in phase 1b, 12 in phase 2a, 44 in phase 2b, 31 in phase 3 and 1 in phase 4 (Table 3). The median age of the children with PWS in nutritional phase 1b was 8.9 years, while the median age of the children in nutritional phase 2a was 3.5 years younger, namely 5.3 years. It shows that the rise of the nutritional phases was not in line with an older age. A considerable number of older subjects with PWS were still in nutritional phase 1b. Parallel with the rise of the nutritional phases, children with PWS in the higher nutritional phases had a higher BMI, ranging from -2.0 SDS in phase 1a to +3.2 SDS in phase 4.



### Ghrelin levels and the switch in eating behaviour in PWS

Both AG and UAG levels of PWS patients decreased with the rise of the nutritional phases (Table 3, supplemental Figure). Between phase 1b and phase 2a, AG levels decreased from 161.0 to 117.2 pg/ml and UAG levels decreased from 252.0 to 125.5 pg/ml, but probably due to the low number in phase 2a this did not reach statistical significance. Subsequently, the AG and UAG levels of the patients in nutritional phase 2a, 2b and 3 remained consistently low. The AG/UAG ratio showed a marked increase between phase 1b and phase 2a and then remained consistently high in phases 2a, 2b and 3. This shows that the elevated AG/UAG ratio is already present in phase 2a, prior to phases 2b and 3 in which the hyperphagia occurs.

Differences in ghrelin levels between nutritional phase 1b and 2a did not reach statistical significance. As ghrelin levels were similar in phase 2a, 2b and 3, these nutritional phases were combined. Eighty-seven PWS had weight gain and/or hyperphagia (nutritional phase 2a, 2b and 3). These 87 PWS had significantly higher AG/UAG ratios than the 50 PWS who did not have weight gain or hyperphagia (nutritional phases 1a and 1b)(p=0.009)(Figure 2). This shows that the switch to weight gain and/or hyperphagia seems to occur simultaneously with the increase in the AG/UAG ratio.

### Ghrelin levels in PWS and age-matched controls

While the AG/UAG ratio was similar in PWS in phase 1a and 1b as in age-matched controls, the AG/UAG ratio was significantly higher in nutritional phase 2b and 3 than in age-matched controls. The AG/UAG ratio in PWS in phase 2a was similar as in phase 2b and 3, but was not significantly higher than age-matched controls, probably due to the low number in phase 2a (Table 3, Figure 2).

### Non-obese PWS vs healthy controls and obese PWS vs obese controls

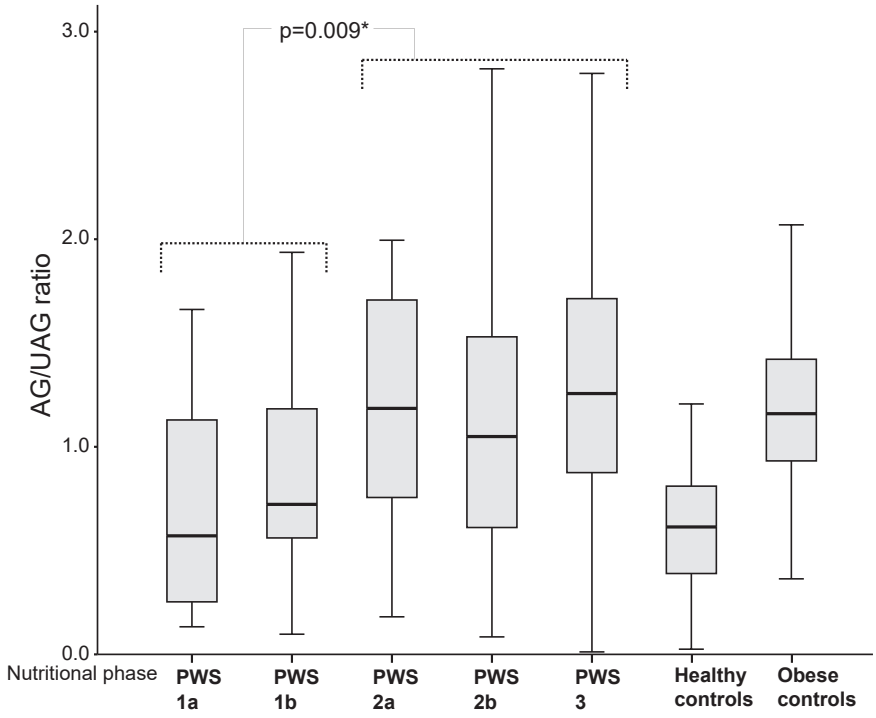
In an additional analysis, we compared 100 non-obese PWS with a median (IQR) BMI of +0.4 (-0.9-1.2) SDS with 39 healthy controls (BMI -0.2 SDS (-0.8-0.8)). The results were

**Table 3.** Age, BMI and ghrelin levels per nutritional phase

| | | Age (yrs) | | BMI (SDS) | | AG (pg/ml) | | UAG (pg/ml) | | AG/UAG ratio | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | median | range | median | p* | median | p* | median | p* | median | p* |
| PWS | 138 | 9.9 | 0.2-29.4 | 1.0 | **<0.001** | 129.1 | **0.016** | 135.3 | 0.868 | 1.00 | **0.001** |
| - Nutritional phase 1a | 13 | 0.5 | 0.2-4.1 | -2.0 | **0.001** | 182.6 | 1.000 | 350.8 | 0.521 | 0.57 | 0.837 |
| - Nutritional phase 1b | 37 | 8.9 | 0.6-22.3 | -0.6 | 0.167 | 161.0 | **0.022** | 252.0 | 0.180 | 0.72 | 0.225 |
| - Nutritional phase 2a | 12 | 5.3 | 1.5-9.3 | 0.9 | **<0.001** | 117.2 | 0.977 | 125.5 | 0.413 | 1.19 | 0.243 |
| - Nutritional phase 2b | 44 | 11.2 | 2.2-29.4 | 1.6 | **<0.001** | 114.4 | **0.020** | 120.1 | 0.848 | 1.05 | **0.002** |
| - Nutritional phase 3 | 31 | 14.5 | 4.7-26.1 | 2.7 | **<0.001** | 99.2 | 0.217 | 73.1 | 0.165 | 1.26 | **0.005** |
| - Nutritional phase 4 | 1 | 14.6 | | 3.2 | | 56.1 | | 596.8 | | 0.09 | |
| Healthy controls | 39 | 7.3 | 0.8-28.6 | -0.2 | | 82.4 | | 157.3 | | 0.61 | |
| Obese controls | 50 | 9.8 | 4.3-16.9 | 2.8 | **<0.001** | 40.3 | **0.001** | 35.8 | **<0.001** | 1.16 | **<0.001** |

*compared with age-matched healthy controls (HC). Phase: 1a PWS n=13 vs HC n=8 (median age 1.4 yrs, median BMI -0.1 SDS), 1b PWS n=37 vs HC n=26 (8.5 yrs, -0.1 SDS), 2a PWS n=12 vs HC n=12 (5.2 yrs, -0.9 SDS), 2b PWS n=44 vs HC n=29 (9.0 yrs, 0.0 SDS), 3 PWS n=31 vs HC n=18 (13.9 yrs, 0.0 SDS).
Bold values are statistically significant ($p<0.05$).

comparable with the data of the total group (Table 4). The AG levels were significantly higher in non-obese PWS than in healthy controls (median 140.1 vs 82.4 pg/ml, p=0.005), while the UAG levels were similar (median 150.7 vs 157.3 pg/ml, NS), resulting in a significantly higher AG/UAG ratio in non-obese PWS (0.89 vs 0.61, p=0.003).




| | PWS 1a | PWS 1b | PWS 2a | PWS 2b | PWS 3 | Healthy controls | Obese controls |
|---|---|---|---|---|---|---|---|
| N | 13 | 37 | 12 | 44 | 31 | 39 | 50 |
| Age (yrs)# | 0.5 (0.2-4.1) | 8.9 (0.6-22.3) | 5.3 (1.5-9.3) | 11.2 (2.2-29.4) | 14.5 (4.7-26.1) | 7.3 (0.8-28.6) | 9.8 (4.3-16.9) |
| BMI SDS§ | -2.0 | -0.6 | 0.9 | 1.6 | 2.7 | -0.2 | 2.8 |
| Deletion / mUPD^ | 7/6 | 14/18 | 4/6 | 27/14 | 17/11 | _ | _ |
| P-value of AG/UAG ratio vs healthy controls◦ | 0.837 | 0.225 | 0.243 | 0.002 | 0.005 | _ | <0.001 |

**Figure 2.** AG/UAG ratio of PWS per nutritional phase and of healthy and obese controls.
This boxplot shows the AG/UAG ratio of children and young adults with PWS in the 5 nutritional phases and of healthy controls and obese controls. The lower boundary is the 25th percentile and the upper boundary the 75th percentile. The line in the box represents the median. Lines are drawn from the smallest to the largest observed value that is not an outlier. #Median (range) age in years. §Median BMI SDS. ^Number of PWS patients with deletion and mUPD. ◦ P-value of AG/UAG ratio of the subjects in each nutritional phase compared with age-matched healthy controls. *All subjects in phase 1a and 1b combined and compared with all subjects in phase 2a, 2b and 3 combined.

**Table 4.** Comparison between non-obese PWS and healthy controls, and obese PWS and obese controls

| | | Gender | Age (yrs) | BMI (SDS) | AG (pg/ml) | UAG (pg/ml) | AG/UAG ratio |
|---|---|---|---|---|---|---|---|
| | n | m/f | median | median | median | median | median |
| Non-obese PWS (BMI<2SDS) | 100 | 43/57 | 8.9 | 0.4 | 140.1 | 150.7 | 0.89 |
| Healthy controls | 39 | 13/26 | 7.3 | -0.2 | 82.4 | 157.3 | 0.61 |
| *Non-obese PWS vs healthy controls* | *139* | *NS* | *NS* | *NS* | ***0.005*** | *NS* | ***0.003*** |
| Obese PWS (BMI>2SDS) | 38 | 18/20 | 13.5 | 2.7 | 100.1 | 94.9 | 1.08 |
| Obese controls | 50 | 16/34 | 9.8 | 2.8 | 40.3 | 35.8 | 1.16 |
| *Obese PWS vs Obese controls* | *88* | *NS* | ***0.013*** | *NS* | ***<0.001*** | ***<0.001*** | *NS* |

Bold values are statistically significant (p<0.05).

We also compared the ghrelin levels of 38 obese PWS with a median (IQR) BMI of +2.7 SDS (2.4-3.2) with 50 obese controls (BMI +2.8 SDS (2.6-3.1)). Both AG and UAG levels were significantly higher in obese PWS than in obese controls (median 100.1 and 94.9 pg/ml vs 40.3 and 35.8 pg/ml resp., both p<0.001, even after adjustment for age), but the AG/UAG ratio was similar in both groups (1.08 vs 1.16, p=0.730).

## Associations between ghrelin levels and clinical characteristics and HOMA-IR in PWS

There were no significant differences in AG and UAG levels and AG/UAG ratio between boys and girls, or between patients with a deletion and an mUPD. AG was inversely associated with age (p=0.012), weight SDS (p=0.004) and BMI SDS (p=0.001) and similar inverse associations were found between UAG and age, weight SDS and BMI SDS (all p<0.001). Since both AG and UAG decreased with age, the association analyses were adjusted for the variables age and gender. Higher BMI SDS was associated with lower AG and UAG levels, also after correction for age and gender (not shown). If the BMI SDS would increase with 1 SDS, the AG level would decrease by 22.8% and the UAG by 21.0%. The association between BMI SDS and AG/UAG ratio was not significant.

AG and UAG were both inversely associated with fasting insulin (p=0.023 and p<0.001, resp), and UAG with HOMA-IR (p=0.004). After adjustment for the variables age and gender, these associations remained significant (p=0.048 and p=0.031 for insulin, p=0.050 for HOMA-IR). The AG/UAG ratio was not associated with insulin or HOMA-IR.

## Associations between ghrelin and IGF-I levels in PWS

IGF-I levels were measured in 124 PWS of which 103 (83.1%) were treated with GH. IGF-I levels, adjusted for age and gender, were higher in the GH-treated versus untreated patients with PWS (p<0.001). The AG and UAG levels and AG/UAG ratio were not different between the GH-treated and untreated patients with PWS (p=0.423 for AG, p=0.374 for UAG and p=0.337 for AG/UAG ratio).

After adjustment for age and gender, IGF-I was inversely associated with AG and UAG (p=0.011 and p=0.008), but there was no significant association between IGF-I and the AG/UAG ratio (p=0.993).

### Obese and controls

In healthy and obese controls, no significant differences in ghrelin levels between boys and girls were found. Younger age was associated with higher levels of AG and UAG (p<0.001 and p=0.001, resp), but the AG/UAG ratio remained stable across age. BMI SDS was inversely correlated with AG and UAG levels (both p<0.001), but positively with the AG/UAG ratio (p<0.001).



## DISCUSSION

Our study shows that AG levels are significantly higher in PWS patients than in controls. In contrast to our expectations, UAG levels in PWS were similar to those in controls. This resulted in a significantly higher AG/UAG ratio in PWS than in controls. Remarkably, our study shows that PWS patients in nutritional phase 2a, 2b and 3, thus with weight gain and/or hyperphagia, had a higher AG/UAG ratio than those in nutritional phase 1a or 1b, without weight gain or hyperphagia, whose AG/UAG ratios were similar to age-matched controls.

In our large study group, we measured levels of acylated and unacylated ghrelin separately using double-antibody sandwich ELISAs specific for each isoform. This approach prevents detection of inactive peptide fragments in the samples. We also inhibited the deacylation of AG to UAG by adding AEBSF to the blood samples[18, 19]. Many previous studies used radioimmunoassays for total ghrelin which detect both full-length, as well as inactive fragments, of both ghrelin isoforms[4, 5, 20, 29-31], or samples were not stabilized with an esterase inhibitor to prevent deacylation of AG[32, 33]. As a result, our data cannot be compared with those in earlier studies.

Both PWS and obese had a significantly higher AG/UAG ratio than healthy controls. The reason for the higher AG/UAG ratio in both groups was, however, completely different. While PWS had higher AG levels with normal UAG levels, obese controls had low AG levels with even lower UAG levels. Based on the higher BMI in PWS patients, one would have expected lower AG and UAG levels like in obese subjects. This might indicate that the abnormalities of the ghrelin system are specific for PWS. Only two other studies reported AG or UAG levels in PWS and compared them with obese controls, but neither study added an inhibitor of AG degradation to the blood samples. Both demonstrated significantly higher AG levels in PWS than in obese controls[32, 33]. The ratio between AG and UAG was not presented. Paik *et al.* did not report a significant difference in UAG

levels between PWS and obese controls[33]. A possible explanation for these different observations might be the method that was used to collect the blood samples.

In contrast to our expectation, the median age of patients with PWS in nutritional phase 1b was 3.5 years higher than in phase 2a. Although hyperphagia is a constitutive marker of PWS, we found in our large group that several older patients were still in nutritional phase 1b, thus without weight gain or hyperphagia. An explanation might be that the patients in our group had an earlier diagnosis with earlier attention for diet, physical exercise and growth hormone treatment starting at a young age[34], although it is not proven that this approach can prevent hyperphagia. Parallel to the rise of the nutritional phases, we found an increasing BMI, supporting that the nutritional phases were correctly attributed.

Our results confirm our hypothesis that patients with PWS without hyperphagia have a similar AG/UAG ratio as age-matched controls, while patients with PWS with weight gain and/or hyperphagia have an AG/UAG ratio higher than that of age-matched controls. In nutritional phase 2a, children with PWS gain weight without a change in appetite or caloric intake. Phase 2b is associated with weight gain and an increased interest in food, and phase 3 is characterized by hyperphagia, typically accompanied by food-seeking and lack of satiety[3]. So, in nutritional phases 2a, 2b and 3 the switch to the typical weight and eating problems of PWS has already occurred. We found that the AG/UAG ratio of children with PWS is already increased in nutritional phase 2a, when there is only weight gain but no hyperphagia. There is a considerable change in the AG/UAG ratio between phase 1b and phase 2a and the AG/UAG ratio remained at a similar high level in phases 2b and 3. Compared to age-matched controls, the AG/UAG ratio in phase 2a, 2b and 3 was higher, although not significantly in phase 2a, probably due to the low number of patients in phase 2a. This considerable change in AG/UAG ratio between phase 1b and higher nutritional phases is in line with the switch to weight gain followed by hyperphagia, which happens in the same period and supports the hypothesis that ghrelin might be involved in this. Whether this modification is the cause or the consequence of the switch in the nutritional phases cannot be unraveled by our study. Delhanty *et al.* suggested that UAG is a functional inhibitor of AG which might suppress AG levels in humans[16, 17]. Elaborating on this idea, the ratio between AG and UAG levels (AG/UAG ratio) might be a more important parameter than individual AG and UAG levels. If this hypothesis is correct, it could be that in patients with PWS without weight gain and/or hyperphagia, in which the AG/UAG ratio is normal, UAG levels are sufficiently high to compensate for the elevated AG levels. However, the UAG levels in patients with PWS from phase 2a onwards are likely to be too low to modulate the effects of elevated AG levels. The resulting higher AG/UAG ratio might induce or contribute to the weight gain and hyperphagia. It is unknown which factors determine UAG levels in healthy subjects and PWS patients. Previous studies showed that somatostatin (agonist) administration

did not result in reduction of weight, food intake or appetite[35-37], but it might be that AG and UAG levels are equally affected by this treatment and that the AG/UAG ratio is not influenced. UAG might be secreted via different mechanisms than AG and rates of acylation and/or deacylation of AG might be differently modulated. Also differences in clearance of UAG relative to AG might play a role, but no reports are available. Our findings provide a rationale for a role of relatively decreased UAG levels in the abnormal eating behaviour in PWS. It would be interesting to investigate whether a more physiological AG/UAG ratio could be achieved by increasing the plasma UAG or decreasing the plasma AG levels and/or bioactivity, and whether this normalization of the AG/UAG ratio results in a reduction of the hyperphagia.



AG and UAG levels and the AG/UAG ratio at various ages in PWS show a wide variation. In the total group there was a distinct pattern with higher AG/UAG ratios in the higher nutritional phases than in age-matched controls. For individuals, this implies that a patient with PWS with a higher AG/UAG ratio has a higher chance to be in a higher nutritional phase.

As expected, PWS patients had a more favourable metabolic profile with lower insulin levels and a lower HOMA-IR than obese controls[7], while the IGF-I levels were higher in the PWS than in the obese group. In PWS, we found an inverse correlation between AG and UAG levels and BMI and in addition also an inverse association between UAG levels and HOMA-IR. This suggests that low UAG levels in PWS are associated with less favourable health aspects, such as a higher BMI and insulin resistance. Previous studies have reported similar results in non-PWS subjects, but no inhibitor was added to their blood samples[38-40].

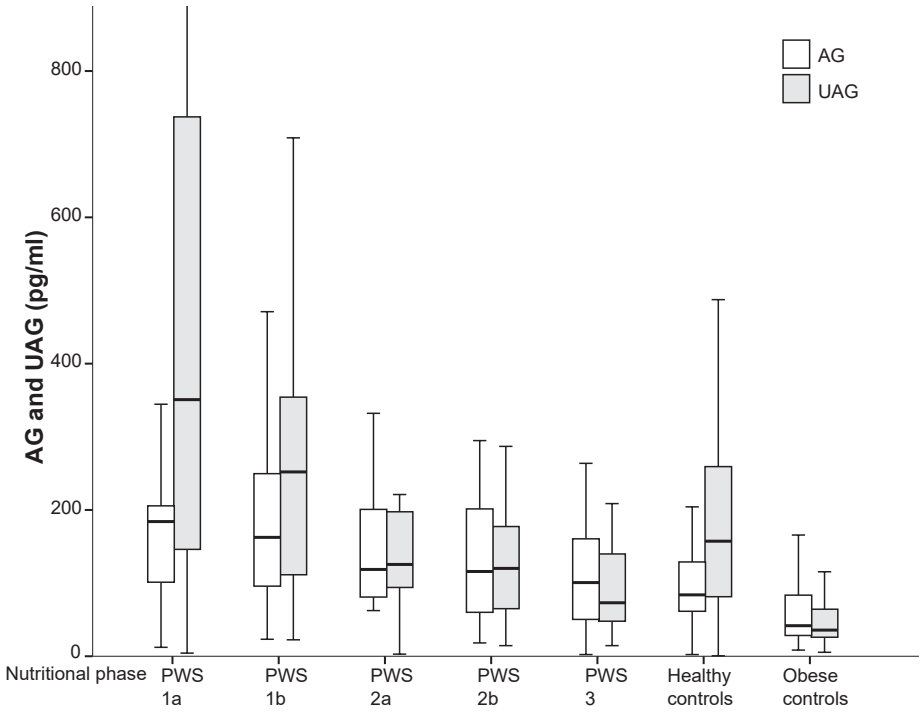
Our study showed no difference in AG/UAG ratios between GH-treated patients and untreated patients with PWS and there was also no significant association between IGF-I levels and AG/UAG ratio. Thus, in our study GH treatment seems to have no effect on the AG/UAG ratio, despite earlier notes of Hauffa and Petersenn in which GH treatment was assigned as confounding factor in the natural course of ghrelin concentrations[41]. We assume that this study was not designed to investigate the effects of GH treatment on ghrelin levels.

As blood sampling in children and young adults with PWS is difficult and quite invasive, we collected only one fasting sample. In our opinion, fasting samples are most appropriate and we present the AG and UAG levels of a large group of patients. It would be informative, however, to conduct a longitudinal study in children with PWS, to investigate whether the switch in eating behaviour is closely correlated with an increase in the AG/UAG ratio. In addition, it would be of interest to determine whether PWS patients show a postprandial decline in AG and UAG levels.

In conclusion, we report that PWS patients have higher AG levels but similar UAG levels compared to healthy controls, resulting in a significantly higher AG/UAG ratio in

PWS patients than in controls. Obese controls have significantly lower AG and UAG levels than PWS patients and healthy controls, but also a high AG/UAG ratio. The reason for the higher AG/UAG ratio in PWS and obese was, however, completely different, as PWS had a high AG and obese a very low UAG.

PWS patients without weight gain or hyperphagia had a similar AG/UAG ratio as age-matched controls, in contrast to those with weight gain and/or hyperphagia who had an elevated AG/UAG ratio. The switch to excessive weight gain in PWS seems to coincide with an increase in the AG/UAG ratio, even prior to the start of hyperphagia.




**Supplemental Figure 2.** AG and UAG levels of PWS per nutritional phase and of healthy and obese controls. This boxplot shows the AG (in white) and UAG (in gray) levels of children and young adults with PWS in the 5 nutritional phases and of healthy controls and obese controls. The lower boundary is the 25$^{th}$ percentile and the upper boundary the 75$^{th}$ percentile. The line in the box represents the median. Lines are drawn from the smallest to the largest observed value that is not an outlier.

## REFERENCES


1. Goldstone, A. P., Holland, A. J., Hauffa, B. P. et al.: Recommendations for the diagnosis and management of Prader-Willi syndrome. J Clin Endocrinol Metab, 93: 4183, 2008
2. Festen, D. A., Wevers, M., de Weerd, A. W. et al.: Cognition and behavior in pre-pubertal children with Prader-Willi syndrome and associations with sleep-related breathing disorders. Am J Med Genet A, 146A: 3018, 2008
3. Miller, J. L., Lynn, C. H., Driscoll, D. C. et al.: Nutritional phases in Prader-Willi syndrome. Am J Med Genet A, 155A: 1040, 2011
4. Feigerlova, E., Diene, G., Conte-Auriol, F. et al.: Hyperghrelinemia precedes obesity in Prader-Willi syndrome. J Clin Endocrinol Metab, 93: 2800, 2008
5. Cummings, D. E., Clement, K., Purnell, J. Q. et al.: Elevated plasma ghrelin levels in Prader Willi syndrome. Nat Med, 8: 643, 2002
6. Bakker, N. E., Kuppens, R. J., Siemensma, E. P. et al.: Eight years of growth hormone treatment in children with Prader-Willi syndrome: maintaining the positive effects. J Clin Endocrinol Metab, 98: 4013, 2013
7. Festen, D. A., van Toorenenbergen, A., Duivenvoorden, H. J. et al.: Adiponectin levels in prepubertal children with Prader-Willi syndrome before and during growth hormone therapy. J Clin Endocrinol Metab, 92: 1549, 2007
8. van der Lely, A. J., Tschop, M., Heiman, M. L. et al.: Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. Endocr Rev, 25: 426, 2004
9. Kojima, M., Hosoda, H., Date, Y. et al.: Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature, 402: 656, 1999
10. Yang, J., Brown, M. S., Liang, G. et al.: Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. Cell, 132: 387, 2008
11. Tschop, M., Smiley, D. L., Heiman, M. L.: Ghrelin induces adiposity in rodents. Nature, 407: 908, 2000
12. Theander-Carrillo, C., Wiedmer, P., Cettour-Rose, P. et al.: Ghrelin action in the brain controls adipocyte metabolism. J Clin Invest, 116: 1983, 2006
13. Asakawa, A., Inui, A., Fujimiya, M. et al.: Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. Gut, 54: 18, 2005
14. Allas, S., Abribat, T.: Clinical perspectives for ghrelin-derived therapeutic products. Endocr Dev, 25: 157, 2013
15. Wren, A. M., Seal, L. J., Cohen, M. A. et al.: Ghrelin enhances appetite and increases food intake in humans. J Clin Endocrinol Metab, 86: 5992, 2001
16. Delhanty, P. J., Neggers, S. J., van der Lely, A. J.: Mechanisms in endocrinology: Ghrelin: the differences between acyl- and des-acyl ghrelin. Eur J Endocrinol, 167: 601, 2012
17. Ozcan, B., Neggers, S. J., Miller, A. R. et al.: Does des-acyl ghrelin improve glycemic control in obese diabetic subjects by decreasing acylated ghrelin levels? Eur J Endocrinol, 170: 799, 2014
18. Delhanty, P. J., Huisman, M., Julien, M. et al.: The acylated (AG) to unacylated (UAG) ghrelin ratio in esterase inhibitor treated blood is higher than previously described. Clin Endocrinol (Oxf), 2014
19. De Vriese, C., Gregoire, F., Lema-Kisoka, R. et al.: Ghrelin degradation by serum and tissue homogenates: identification of the cleavage sites. Endocrinology, 145: 4997, 2004
20. Purtell, L., Sze, L., Loughnan, G. et al.: In adults with Prader-Willi syndrome, elevated ghrelin levels are more consistent with hyperphagia than high PYY and GLP-1 levels. Neuropeptides, 45: 301, 2011
21. DelParigi, A., Tschop, M., Heiman, M. L. et al.: High circulating ghrelin: a potential cause for hyperphagia and obesity in prader-willi syndrome. J Clin Endocrinol Metab, 87: 5461, 2002

22. Haqq, A. M., Farooqi, I. S., O'Rahilly, S. et al.: Serum ghrelin levels are inversely correlated with body mass index, age, and insulin concentrations in normal children and are markedly increased in Prader-Willi syndrome. J Clin Endocrinol Metab, 88: 174, 2003
23. Kweh, F. A., Miller, J. L., Sulsona, C. R. et al.: Hyperghrelinemia in Prader-Willi syndrome begins in early infancy long before the onset of hyperphagia. Am J Med Genet A, 2014
24. Fredriks, A. M., van Buuren, S., Wit, J. M. et al.: Body index measurements in 1996-7 compared with 1980. Arch Dis Child, 82: 107, 2000
25. Fredriks, A. M., van Buuren, S., Burgmeijer, R. J. et al.: Continuing positive secular growth change in The Netherlands 1955-1997. Pediatr Res, 47: 316, 2000
26. Sempé, M., Pédron, G., Roy-Pernot, M.: Auxologie: méthode et séquences. Paris: Théraplix. 1979
27. Cole, T. J., Freeman, J. V., Preece, M. A.: Body mass index reference curves for the UK, 1990. Arch Dis Child, 73: 25, 1995
28. Wallace, T. M., Levy, J. C., Matthews, D. R.: Use and abuse of HOMA modeling. Diabetes Care, 27: 1487, 2004
29. Goldstone, A. P., Holland, A. J., Butler, J. V. et al.: Appetite hormones and the transition to hyperphagia in children with Prader-Willi syndrome. Int J Obes (Lond), 36: 1564, 2012
30. Butler, M. G., Bittel, D. C.: Plasma obestatin and ghrelin levels in subjects with Prader-Willi syndrome. Am J Med Genet A, 143: 415, 2007
31. Haqq, A. M., Grambow, S. C., Muehlbauer, M. et al.: Ghrelin concentrations in Prader-Willi syndrome (PWS) infants and children: changes during development. Clin Endocrinol (Oxf), 69: 911, 2008
32. Park, W. H., Oh, Y. J., Kim, G. Y. et al.: Obestatin is not elevated or correlated with insulin in children with Prader-Willi syndrome. J Clin Endocrinol Metab, 92: 229, 2007
33. Paik, K. H., Choe, Y. H., Park, W. H. et al.: Suppression of acylated ghrelin during oral glucose tolerance test is correlated with whole-body insulin sensitivity in children with Prader-Willi syndrome. J Clin Endocrinol Metab, 91: 1876, 2006
34. Deal, C. L., Tony, M., Hoybye, C. et al.: GrowthHormone Research Society workshop summary: consensus guidelines for recombinant human growth hormone therapy in Prader-Willi syndrome. J Clin Endocrinol Metab, 98: E1072, 2013
35. Haqq, A. M., Stadler, D. D., Rosenfeld, R. G. et al.: Circulating ghrelin levels are suppressed by meals and octreotide therapy in children with Prader-Willi syndrome. J Clin Endocrinol Metab, 88: 3573, 2003
36. De Waele, K., Ishkanian, S. L., Bogarin, R. 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, 159: 381, 2008
37. Tan, T. M., Vanderpump, M., Khoo, B. et al.: Somatostatin infusion lowers plasma ghrelin without reducing appetite in adults with Prader-Willi syndrome. J Clin Endocrinol Metab, 89: 4162, 2004
38. Longo, K. A., Charoenthongtrakul, S., Giuliana, D. J. et al.: Improved insulin sensitivity and metabolic flexibility in ghrelin receptor knockout mice. Regul Pept, 150: 55, 2008
39. Nonogaki, K., Nozue, K., Oka, Y.: Hyperphagia alters expression of hypothalamic 5-HT2C and 5-HT1B receptor genes and plasma des-acyl ghrelin levels in Ay mice. Endocrinology, 147: 5893, 2006
40. Pacifico, L., Poggiogalle, E., Costantino, F. et al.: Acylated and nonacylated ghrelin levels and their associations with insulin resistance in obese and normal weight children with metabolic syndrome. Eur J Endocrinol, 161: 861, 2009
41. Hauffa, B. P., Petersenn, S.: GH treatment reduces total ghrelin in Prader-Willi syndrome (PWS) and may confound ghrelin studies in young PWS children. Clin Endocrinol (Oxf), 71: 155, 2009

CHAPTER 3

# Acylated and unacylated ghrelin during OGTT in Prader-Willi syndrome: Support for normal response to food intake

R.J. Kuppens, P.J.D. Delhanty, T.M. Huisman, A.J. van der Lely, A.C.S. Hokken-Koelega

*Clinical Endocrinology 2016; in press*

## ABSTRACT

**Background** Prader-Willi syndrome (PWS) is characterized by hyperphagia with impaired satiety. PWS patients have very high acylated ghrelin (AG) with normal unacylated ghrelin (UAG) levels, resulting in an elevated AG/UAG ratio, suggesting an intrinsic defect in the ghrelin regulation. Normally, food intake induces satiety and a drop in AG and UAG levels, but it is unknown if these levels also decline in PWS.

**Objective** To evaluate whether the high AG levels in PWS decline in response to glucose intake during an oral glucose tolerance test (OGTT), and to investigate the effects of growth hormone (GH) treatment on this response.

**Method** Serum levels of AG, UAG and AG/UAG ratio during an OGTT were determined in 24 GH-treated patients with PWS (median age 19.0, range 14.2-25.9 years) and in 10 GH-stop patients (of whom 5 were in GH-treated group; 18.5, 14.5-20.3 years).

**Results** In GH-treated and GH-stop young adults with PWS, there was a sharp decline of AG levels and a decrease of UAG levels in the first 30 minutes after the glucose load, which resulted in a lower AG/UAG ratio. GH-treated patients had significantly lower AG levels than GH-stop patients at baseline and during the OGTT. All UAG levels and AG/UAG ratios were lower in the GH-treated patients, although not significantly.

**Conclusions** In young adults with PWS, an oral glucose load significantly reduces AG and UAG levels, suggesting normal regulation of the ghrelin axis by food intake. GH treatment results in lower AG levels at baseline and during OGTT, suggesting a more favourable metabolic profile. Our findings might suggest that the impaired satiety is not the result of an abnormal response of the orexigenic ghrelin to food intake.

## INTRODUCTION

Prader-Willi syndrome (PWS) is a neurogenetic disorder caused by lack of expression of the paternally derived genes on chromosome 15 at locus q11-q13[1]. PWS is characterized by a number of signs and symptoms, including an abnormal body composition with a high body fat percentage and a low lean body mass (LBM), and hyperphagia resulting in morbid obesity when uncontrolled[2-4]. Nevertheless, euglycemic children and adults with PWS have lower fasting insulin and higher insulin sensitivity than weight-comparable subjects[5] and PWS children have higher adiponectin levels than healthy peers[6]. The mechanism behind the hyperphagia with impaired satiety is not yet known, but the appetite-stimulating hormone ghrelin seems to be involved[7-9].

To date, most studies measured total ghrelin levels, but did not differentiate between the two forms, acylated and unacylated ghrelin, in circulation[10-12]. Acylated ghrelin (AG) is known to be diabetogenic and has many actions such as stimulating appetite and inducing a positive energy balance, which can lead to weight gain[13-16]. The enzyme ghrelin O-acyl transferase (GOAT) mediates the acylation of the ghrelin peptide and the presence of the acyl group of AG is necessary for the binding to the growth hormone secretagogue receptor (GHSR-1a)[12]. For that reason, unacylated ghrelin (UAG) was considered to be inactive[11, 17], but nowadays there is increasing evidence that UAG has also several actions. UAG has protective effects on beta cells, endothelial progenitor cells and muscle cells, and it improves glycemic control[16, 17]. In addition, UAG acts as a functional inhibitor of AG and may suppress AG levels in humans[17, 18]. Thus, the ratio between AG and UAG levels (AG/UAG ratio) might be crucial in glucose homeostasis and maintenance of weight balance.

The fasting AG/UAG ratio is elevated in children and young adults with PWS, mainly due to very high levels of AG[7]. The effect of food intake on the AG/UAG ratio in PWS is unknown. During food intake, children with PWS continue to eat for a longer time than obese and normal weight controls[19]. This non-decelerating eating curve in PWS suggests impaired satiation rather than increased hunger. In healthy people, AG and UAG levels increase during the night, while feeding induces a strong decline of these levels[20]. After a standardized meal with 50% carbohydrates, AG declined by approximately 65% and UAG by 40%[20]. An OGTT proved to be as effective as a standardized meal in inhibiting ghrelin secretion[21].

It might be that the impaired satiation in PWS is related to a lack of decline of AG levels and particularly the AG/UAG ratio during food intake. Only one study has investigated both AG and UAG levels, but not the AG/UAG ratio, in PWS during an OGTT[22]. The inhibitor AEBSF was not added to the samples to prevent deacylation of AG to UAG[23], therefore these data must be interpreted with caution. Similar UAG levels, but higher AG levels with a larger AG decline after a glucose load were found in the 11 prepubertal obese

children with PWS who were not treated with GH, compared with 10 obese controls. But the change in AG/UAG ratio during an OGTT is unknown.

Considering the elevated AG/UAG ratio in fasting PWS patients and their impaired satiety during food intake, we hypothesized that ghrelin regulation is disturbed. We expected that glucose intake during an OGTT would neither influence the AG and UAG levels nor the AG/UAG ratio in PWS and that these levels would remain high, as high AG levels are a hallmark of patients with PWS. Given the fact that AG is diabetogenic and UAG improves glycemic control, we expected to find a positive correlation between the AG/UAG ratio and the insulin/glucose ratio. In addition, as GH treatment seems to have favourable influences on the natural course of PWS, we expected to find lower AG/UAG ratios in GH-treated than in GH-stop young adults. To further clarify the role of food intake and GH treatment in the regulation of AG and UAG levels and the AG/UAG ratio, we investigated the response of these levels to a standard oral glucose load in GH-treated compared to GH-stop young adults with PWS. AEBSF, an inhibitor of deacylation of AG, was immediately added to the blood samples to prevent deacylation of AG to UAG.

## METHODS

### Subjects

The study group consisted of 29 young adults with PWS, participating in the Dutch PWS studies coordinated by the Dutch Growth Research Foundation. PWS was genetically confirmed in all patients. Fourteen (48.3%) young adults received sex steroid replacement therapy. During childhood, all patients were treated with GH without oral glucose tolerance tests (OGTT) being performed. GH treatment was stopped due to attainment of adult height, as there is no registration of this treatment for adults with PWS. After stop an OGTT was performed (GH-stop group). A Young Adult study protocol was started in which patients were allowed to restart GH treatment. During the Young Adult study, patients had an OGTT (GH-treated group). After fasting blood collection, a standard OGTT according to the World Health Organization was performed with 75-g oral glucose. Blood samples for glucose and insulin determination were obtained after 30, 60, 90 and 120 minutes and for AG and UAG levels after 30 and 120 minutes after the oral glucose load.

Standing height was measured with a calibrated Harpenden stadiometer. Weight was determined on a calibrated scale (Servo Balance KA-20-150S; Servo Berkel Prior) and BMI was calculated. Final height and BMI were expressed as SDS, adjusted for age and sex[24, 25], and calculated with Growthanalyser (version 4.0; www.growthanalyser.org). The Medical Ethics Committee of Erasmus University Medical Center approved the study. Written informed consent was obtained from the young adults with PWS and their parents.

### Eating behaviour

The nutritional phases according to Miller were used to score the eating behaviour of the subjects with PWS[26]: 1a Hypotonia with difficulty feeding, 1b No difficulty feeding and growing appropriately on growth curve, 2a Weight increasing without an increase in appetite or excessive calories, 2b Weight increasing with an increase in appetite, 3 Hyperphagia, rarely feels full, 4 Appetite no longer insatiable. For each subject with PWS, the nutritional phase was assessed by the multidisciplinary teams or independently by two observers who knew them very well (physician and nurse)[26]. In the event of disagreement, the case was discussed until consensus was reached.

### Collection of blood and plasma preparation

Baseline blood samples were collected in the morning after an overnight fast for assessment of glucose, insulin, IGF-I, AG, UAG and AG/UAG ratio. Subsequently, a standard 75-g oral glucose tolerance test (OGTT) according to the World Health Organization was performed. Blood samples for glucose and insulin determination were obtained after 30, 60, 90 and 120 minutes after ingestion and immediately assayed as described elsewhere[7]. AG and UAG levels were determined after 30 and 120 minutes after oral glucose load. To prevent deacylation of AG to UAG, thus to stabilize the plasma AG and UAG levels, blood samples were collected in EDTA tubes and 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF, Sigma-Aldrich Chemicals) was added to a concentration of 2 mg/ml at the time of collection. Blood was centrifuged at 4 °C to prepare plasma, which was quickly frozen on dry ice. Samples were stored at -80 °C and assayed within 6 months following collection. The assay and its intra- and interassay coefficient of variation (CV) used in this study has been described[7].

To evaluate the overall responses to the oral glucose load, apart from the plasma levels at various time points, the insulin/glucose ratio at 0, 30 and 120 minutes was calculated as an index of relative insulin resistance.

### Statistics

Statistical analysis was performed by the Statistical Package for Social Sciences (version 20.0; SPSS, Chicago, IL). Data are expressed as median (interquartile range (IQR)) as they were not normally distributed. Differences between two groups were calculated using Mann Whitney U tests. As data were not normally distributed, Friedman's ANOVA was used for testing the ghrelin levels of the three different time points, and Wilcoxon Ranks tests were used to follow up this. Within the different groups, we cross-sectionally assessed linear correlations between ghrelin levels and other parameters using Spearman's rho correlation coefficient. Differences were considered significant if the p-value was $<0.05$.

## RESULTS

### Clinical characteristics

The 29 young adults underwent a total of 34 oral glucose tolerance tests (OGTT). Twenty-four tests were performed during GH treatment (GH-treated) and 10 tests after stop of GH treatment (GH-stop). Of the 29 young adults, five patients had two tests, one in the period after stop of GH treatment and one after restart of GH treatment.

The 24 young adults with PWS (6 male, 18 female) who underwent an OGTT during GH treatment, had a median (IQR) age of 19.0 (17.2-22.2) years and a BMI of 24.9 (22.2-29.1) kg/m$^2$, being +1.0 (0.5 to 2.0) SDS. Eleven (45.8%) had a paternal deletion, 9 (37.5%) a uniparental disomy (mUPD), 3 (12.5%) an imprinting center mutation and one (4.2%) a translocation (Table 1). Median (IQR) dose of GH was 0.45 (0.35-0.67) mg/m$^2$/day ≈ 0.015 (0.012-0.022) mg/kg/day.

Ten young adults with PWS (2 male, 8 female) underwent an OGTT in the period after stop of GH treatment (GH-stop group). Median (IQR) duration between the discontinuation of GH and the OGTT was 9 (6-12) months. Their age and BMI were similar to the GH-treated patients (Table 1).

### Ghrelin during OGTT in GH-treated young adults

Figure 1 shows the median fasting AG and UAG levels and AG/UAG ratio at baseline, and then 30 and 120 minutes after an oral glucose load in 24 GH-treated young adults. Levels of AG and UAG changed significantly during the OGTT (AG $p<0.001$ and UAG $p=0.001$, resp.). Median (IQR) baseline AG levels were 88.4 (37.0-143.1) pg/ml, but declined sharply after the glucose load to a nadir of 10.8 (2.0-60.2) pg/ml after 30 minutes ($p=0.001$) (Table 2). Median (IQR) decrease of AG was 90.8% (52.5-95.0%). In the second part of the OGTT, AG levels increased to 40.5 (2.0-83.7) pg/ml at 120 minutes ($p=0.020$). UAG levels showed a similar pattern with a significant decline ($p=0.001$) from 94.6 (67.4-295.2) pg/ml at baseline to 52.4 (36.2-151.0) pg/ml at 30 minutes after glucose load (median (IQR) decrease 48.9% (24.5-63.8%)), but it was followed by a more moderate increase to 84.9 (35.6-180.0) pg/ml after 120 minutes ($p=0.058$). As a result, the AG/UAG ratio declined significantly from 0.68 (0.48-1.81) to 0.29 (0.02-1.08) in the first 30 minutes ($p=0.045$) followed by a stable ratio of 0.44 (0.02-1.65) in the second part ($p=0.178$).

### Associations with AG and UAG levels and their ratio in GH-treated young adults

At baseline, there were no differences in fasting AG and UAG levels and AG/UAG ratio between boys and girls (p-values above 0.698), or between young adults with a deletion and an mUPD (p-values above 0.798). There were no associations with age, BMI, fat percentage, nutritional phase, sex steroid replacement therapy or IGF-I SDS.

**Table 1.** Baseline characteristics of 24 GH-treated and 10 GH-stop young adults with PWS.

| | GH-treated | GH-stop | p-value |
|---|---|---|---|
| **N** | **24** | **10** | |
| Age (yrs) | 19.0 (17.2 to 22.2) | 18.5 (16.6 to 20.0) | 0.406 |
| Age range | 14.2 to 25.9 | 14.5 to 20.3 | |
| Gender | | | |
| - Male | 6 (25.0%) | 2 (20.0%) | 0.758 |
| - Female | 18 (75.0%) | 8 (80.0%) | |
| Genetic subtype | | | |
| - Deletion | 11 (45.8%) | 4 (40.0%) | 0.967 |
| - mUPD | 9 (37.5%) | 5 (50.0%) | |
| - ICD | 3 (12.5%) | 1 (10.0%) | |
| - Translocation | 1 (4.2%) | - | |
| Adult height SDS | -1.7 (-2.1 to -1.1) | -1.9 (-3.1 to -0.4) | 0.664 |
| BMI (kg/m$^2$) | 24.9 (22.2 to 29.1) | 27.1 (23.6 to 34.0) | 0.257 |
| BMI SDS | 1.0 (0.5 to 2.0) | 1.9 (0.7 to 2.7) | 0.162 |
| Fat percentage | 43.8 (37.4 to 48.0) | 46.6 (39.7 to 55.5) | 0.174 |
| Waist (cm) | 77.3 (71.9 to 88.5) | 82.5 (73.7 to 91.9) | 0.347 |
| Waist-to-hip ratio | 0.76 (0.72 to 0.79) | 0.77 (0.72 to 0.81) | 0.611 |
| Duration of GH (years) | 10.7 (9.7 to 11.6) | 9.9 (8.4 to 11.5) | 0.210 |
| Pubertal stage Tanner | | | |
| - M or G 2 / 3 / 4 / 5 | 1 / 5 / 12 / 6 | 0 / 4 / 4 / 2 | 0.564 |
| - P 4 / 5 | 1 / 23 | 0 / 10 | 0.287 |
| Sex steroid therapy | 12 (50%) | 5 (50%) | 1.000 |
| Nutritional phase[a] | | | 0.275 |
| - Phase 1b | 10 (41.7%) | 2 (20.0%) | |
| - Phase 2b | 9 (37.5%) | 5 (50.0%) | |
| - Phase 3 | 5 (20.8%) | 3 (30.0%) | |

Characteristics at time of OGTT. Age, Adult height SDS, BMI, fat percentage, waist circumference, waist-to-hip ratio and duration of GH are expressed in median (IQR); gender, genetic subtype, sex steroid therapy and nutritional phase are expressed in n(%). Pubertal stage according to Tanner is expressed in n. P-value between GH-treated and GH-stop patients.
[a]Nutritional phase according to Miller *et al.*

In the 24 GH-treated young adults, a lower UAG at 30 and 120 minutes after glucose load was associated with a higher insulin/glucose ratio at 120 minutes (p=0.017 and p=0.006), while a lower baseline UAG tended to be associated with a higher insulin/glucose ratio at 120 minutes (p=0.082). Since there was no association between AG levels and the insulin/glucose ratio at the different time points, this resulted in a positive association with the AG/UAG ratio. A higher AG/UAG ratio at 0 and 120 minutes was associated with a higher insulin/glucose ratio at 120 minutes (both p=0.010), and a higher

AG/UAG ratio at 30 minutes tended to be associated with a higher insulin/glucose ratio at 120 minutes (p=0.071). Remarkably, AG and UAG levels and AG/UAG ratio at the different time points were not associated with the insulin/glucose ratio at 0 and 30 minutes.

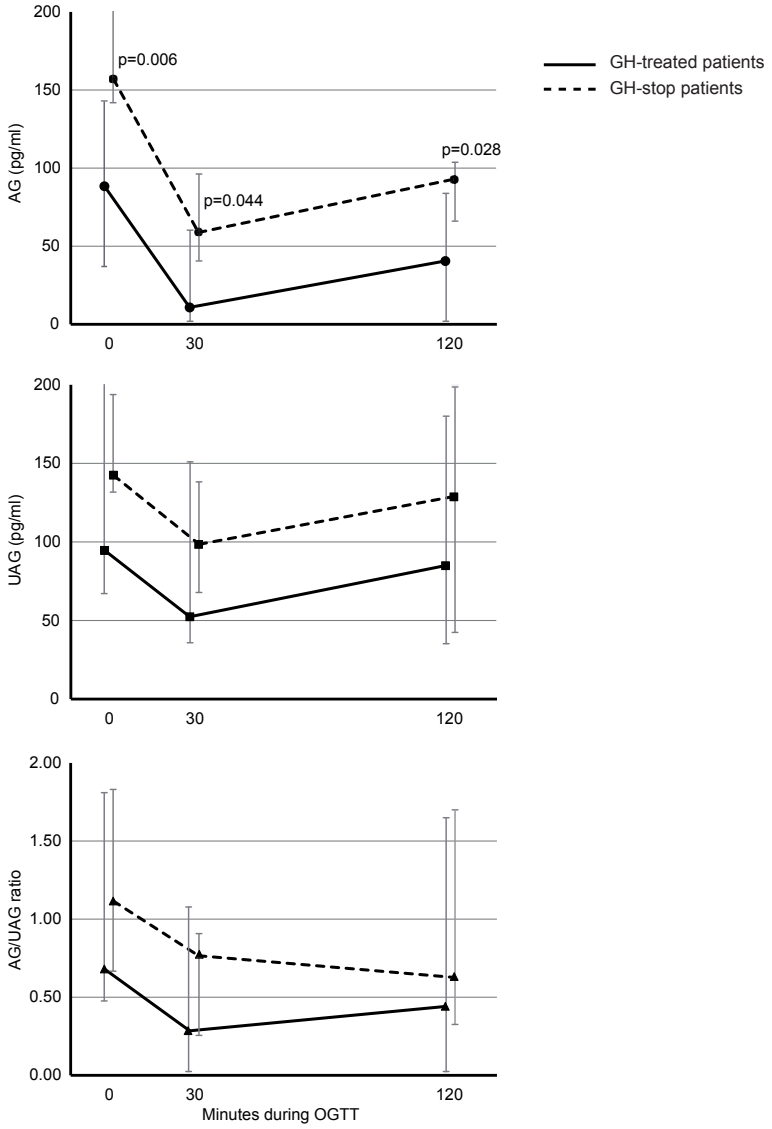



**Figure 1.** AG and UAG levels and AG/UAG ratio of 24 GH-treated and 10 GH-stop young adults with PWS. AG and UAG levels and AG/UAG ratio before and at 30 and 120 minutes after the glucose load of 24 GH-treated (solid line) and 10 GH-stop (dotted line) young adults with PWS. Lines are drawn from the 25$^{th}$ to the 75$^{th}$ percentile. P-value is given in case of a significant difference between GH-treated and GH-stop.

As these associations might be due to GH treatment, we also investigated the associations in the 10 GH-stop patients and found similar results; a higher AG/UAG ratio at 0, 30 and 120 minutes after glucose load was associated with a higher insulin/glucose ratio at 120 minutes (p=0.016, p=0.014 and p=0.004, resp.).

### Effects of GH treatment on ghrelin

To evaluate the effects of GH treatment, the AG and UAG levels of the 24 GH-treated young adults were compared with those of 10 GH-stop patients (Figure 1). At baseline, AG levels of the GH-treated young adults were significantly lower than those of the GH-stop patients (p=0.006), while baseline UAG levels were not significantly different. GH-treated and GH-stop young adults had a similar significant decline of AG and UAG levels after the oral glucose load to a nadir after 30 minutes (both p=0.001 in GH-group, both p=0.012 in GH-stop group, resp., relative to baseline). In the second part of the OGTT, from 30 to 120 minutes after the glucose load, AG and UAG increased in both the GH-treated and GH-stop young adults, although this increase was only significant for AG in the GH-treated group (p=0.020). As a result, the AG/UAG ratio declined in the first 30 minutes and did not change significantly in the second part (p=0.045 and p=0.178, resp.). At every time point of the OGTT, the AG/UAG ratio was lower in the GH-treated group, but not significantly.



**Table 2.** Biochemical characteristics during OGTT of GH-treated and GH-stop adolescents with PWS.

| Parameter | GH-treated n=24 | GH-stop n=10 | p-value° |
|---|---|---|---|
| Fasting glucose (mmol/l) | 4.7 (4.4 to 5.0) | 4.3 (4.0 to 4.6) | **0.034** |
| Fasting insulin (mU/l) | 69.5 (36.8 to 162.0) | 46.5 (25.3 to 74.5) | 0.145 |
| Fasting AG (pg/ml) | 88.4 (37.0 to 143.1) | 157.1 (141.7 to 251.5) | **0.006** |
| 30 min AG (pg/ml) | 10.8 (2.0 to 60.2) | 59.1 (40.5 to 96.1) | **0.044** |
| 120 min AG (pg/ml) | 40.5 (2.0 to 83.7) | 89.2 (66.0 to 103.6) | **0.028** |
| Fasting UAG (pg/ml) | 94.6 (67.4 to 295.2) | 142.4 (131.8 to 193.7) | 0.290 |
| 30 min UAG (pg/ml) | 52.4 (36.2 to 151.0) | 98.3 (68.1 to 138.3) | 0.498 |
| 120 min UAG (pg/ml) | 84.9 (35.6 to 180.0) | 128.4 (56.1 to 198.5) | 0.225 |
| Fasting AG/UAG ratio | 0.68 (0.48 to 1.81) | 1.11 (0.67 to 1.83) | 0.345 |
| 30 min AG/UAG ratio | 0.29 (0.02 to 1.08) | 0.76 (0.26 to 0.91) | 0.223 |
| 120 min AG/UAG ratio | 0.44 (0.02 to 1.65) | 0.64 (0.33 to 1.70) | 0.433 |
| Insulin/glucose ratio t=0* | 14.0 (7.1 to 33.6) | 13.3 (6.5 to 16.9) | 0.292 |
| Insulin/glucose ratio t=30* | 47.8 (30.8 to 88.6) | 41.4 (36.5 to 46.9) | 0.396 |
| Insulin/glucose ratio t=120* | 56.0 (30.0 to 83.0) | 35.2 (30.2 to 43.3) | **0.048** |

°P-value between GH-treated and GH-stop patients, bolded p-values are p-values below 0.05. *Insulin/glucose ratio in mU/mmol.

To further elucidate the effect of GH, we compared the ghrelin levels of 5 individuals who underwent two OGTTs: one in the period with and one without GH treatment. The median time difference between the two OGTTs was 1.0 year. The pattern of the AG levels and the AG/UAG ratio showed similar dynamics during OGTT in the period with and without GH treatment, with a sharp decline during the first 30 minutes after the glucose load in both periods. During GH treatment, AG levels declined from 99.0 to 60.2 pg/ml and the AG/UAG ratio from 0.80 to 0.40, while after stop of GH the AG levels declined from 150.2 to 95.0 pg/ml and the AG/UAG ratio from 1.18 to 0.89. The decline in the first 30 minutes was followed by an increase towards baseline levels in the second part. At every time point (0, 30 and 120 minutes) the AG levels and AG/UAG ratios were lower in the GH-treated than in the GH-stop period, although not significantly. In contrast, the baseline UAG levels were similar in both periods. In the GH-treated period, UAG decreased sharply in the first 30 minutes after the glucose load from 127.7 to 52.4 pg/ml, while it only slightly decreased in the GH-stop period from 133.5 to 115.0 pg/ml. This difference tended to be significant (p=0.068).

## DISCUSSION

As high AG levels are a hallmark of patients with PWS, we expected that glucose intake would neither influence the AG and UAG levels nor the AG/UAG ratio in PWS and that these levels would remain high. In contrast to our expectation, our study shows that glucose intake reduces AG and UAG levels in young adults with PWS. In the first 30 minutes after the glucose load, there was a sharp decline of AG levels and to a lesser extend of UAG levels, which resulted in a lower AG/UAG ratio. This demonstrates that even in the presence of very high fasting AG/UAG ratios[7], the ghrelin system of young adults with PWS shows a physiological response with a strong decline of AG and UAG levels after glucose intake.

Patients with PWS have hyperphagia and a non-decelerating eating curve, suggesting impaired satiety[19]. The exact mechanism of impaired satiety is not yet elucidated, but in the fasting state, the appetite-stimulating hormone AG is significantly elevated in PWS compared to healthy controls. AG's functional inhibitor UAG is similar in subjects with PWS and healthy controls, but in the presence of the high AG level in PWS, there is a state of relative UAG deficiency[7]. We expected that glucose intake would neither influence the AG and UAG levels nor the AG/UAG ratio in PWS and that these levels would remain high, as high AG levels are a hallmark of patients with PWS. In contrast to our expectation, we found that both AG and UAG levels strongly declined and that they reached a nadir at 30 minutes after an oral glucose load. The AG levels decreased markedly more than the UAG levels, which resulted in lower AG/UAG ratios at 30 minutes. The decline was similar

to the strong decline in AG of 65% and UAG of 40% after a standard meal in healthy controls[20], indicating that patients with PWS show a physiological response to the intake of glucose, in spite of the fact that they continue to eat. This suggests that there might be another explanation for the presence of impaired satiety in PWS. The hypothalamus is involved in many functions in the body such as regulating hormones, temperature control, circadian rhythm and respiratory control. In PWS, disturbances in these functions are common and it is most likely that also the hyperphagia and impaired satiety are related with a hypothalamic dysregulation. Our findings do suggest, however, that the impaired satiety is not the result of an abnormal response of the orexigenic ghrelin to food intake. The impaired satiety could e.g. be due to an insufficient response of anorexigenic hormones like GLP-1 after food intake. Oxytocin could also be involved, as recent studies demonstrated that this hormone controls food intake and weight balance, while dysfunction of oxytocin results in obesity[27].



Lower levels of UAG and AG/UAG ratio at 120 minutes, but also the UAG and the AG/UAG ratios at earlier time points, were associated with a higher insulin/glucose ratio at 120 minutes. This might suggest that a lower fasting UAG and higher fasting AG/UAG ratio are predictors for late insulin resistance. It would be interesting to further unravel this findings and investigate whether an elevated AG/UAG ratio can be used as a screening tool for insulin resistance. One may speculate that this association was induced by GH treatment, as GH increases insulin levels. For this reason, we also investigated the association between ghrelin levels and insulin/glucose ratio at 120 minutes in the GH-stop group, but found similar results. Our findings are in line with St-Pierre *et al.* who demonstrated that the AG/UAG ratio was higher in insulin-resistant than in insulin-sensitive non-diabetic obese individuals[28]. Our data support that AG is diabetogenic and UAG has protective effects on beta cells and improves glycemic control[16, 17]. Thus, it appears that the association between ghrelin and relative insulin resistance is not different for PWS.

At baseline, fasting AG levels were significantly lower in GH-treated patients than in GH-stop patients. During the OGTT, AG and UAG levels and the AG/UAG ratio followed the same pattern in the GH-treated and GH-stop patients, but during the entire test, the AG levels of the GH-treated patients remained lower. GH-treated patients had also lower fasting UAG levels and AG/UAG ratios although this did not reach significance. This lack of significance could be explained by the lower number of patients in the GH-stop group and the observed large variation in ghrelin levels. Similar results were found in the 5 patients in whom we performed an OGTT during both the GH-stop and GH-treated period. Apparently, GH treatment reduces AG levels, and to a lesser extent UAG levels, which results in a lower AG/UAG ratio. The acylated form of ghrelin stimulates appetite and induces a positive energy balance[13-16], while UAG seems to oppose these effects, acting as a functional inhibitor of AG[17, 18]. The lower AG/UAG ratio during GH treatment may, therefore, contribute to GH's more favourable metabolic effects. It is conceivable

that this contributes to the lower percentage of hyperphagia in our older GH-treated patients[7]. Ten out of 24 GH-treated young adults were still in nutritional phase 1b while their ages were between 14.2 and 23.1 years. This is in contrast to Miller *et al.* who reported a young median (IQR) age at onset of nutritional phase 2b of 4.5 (3.0-5.3) years and of phase 3 of 8.0 (5.0-13.0) years and showed that nearly all children and young adults with PWS progressed to the high nutritional phases[26]. It might be that GH reduces the severity of the hyperphagia. The 24 GH-treated young adults started GH treatment in infancy or childhood and used it for a median (IQR) duration of 10.7 (9.7-11.6) years. After attainment of adult height, young adults had to stop GH treatment for at least 6 weeks in order to perform a GH stimulation test. About half of the parents reported an aggravation of the hyperphagia with more food seeking behaviour (data not shown). Ghrelin is able to bind the GHSR, which stimulates GH release[11], but it has not been elucidated whether a reverse system exists in which GH influences ghrelin production or release. In our study it appears that GH affects especially AG and therefore the AG/UAG ratio. Hauffa *et al.* demonstrated that GH treatment was able to decrease both elevated basal and postcarbohydrate total ghrelin levels in PWS children and young adults, but AG levels were not changed by GH[29, 30]. However, they did not add an inhibitor to prevent deacylation of AG to UAG and they used other assays, which could explain the difference. Furthermore, UAG levels were not measured.

In our study, in contrast to our expectations, the ghrelin system in PWS works appropriately after an oral glucose load with a physiological decline of AG and UAG levels. In healthy controls, similar magnitudes of decline of AG (between 42% and 71%) and UAG (between 30% and 58%) after a glucose load[31, 32] and standardized breakfast meal[20] have been reported. The choice of a control population is difficult since obese patients have reduced levels of AG and UAG compared to healthy controls[7], whereas PWS patients have elevated AG levels, so that the comparison with obese patients would not be appropriate. Since the effect of a glucose load on AG and UAG levels is clearly defined in literature, the testing of new healthy controls was considered unethical, since it would add no new information, while causing unnecessary burden on the subjects involved. Our results show the decline of AG and UAG levels after an oral glucose load. An OGTT proved to be as effective as a standardized light breakfast in inhibiting ghrelin secretion[21]. Since the nadir of these levels was lowest after the ingestion of carbohydrates, compared to lipids and proteins[32, 33], our results might also be applicable for meals.

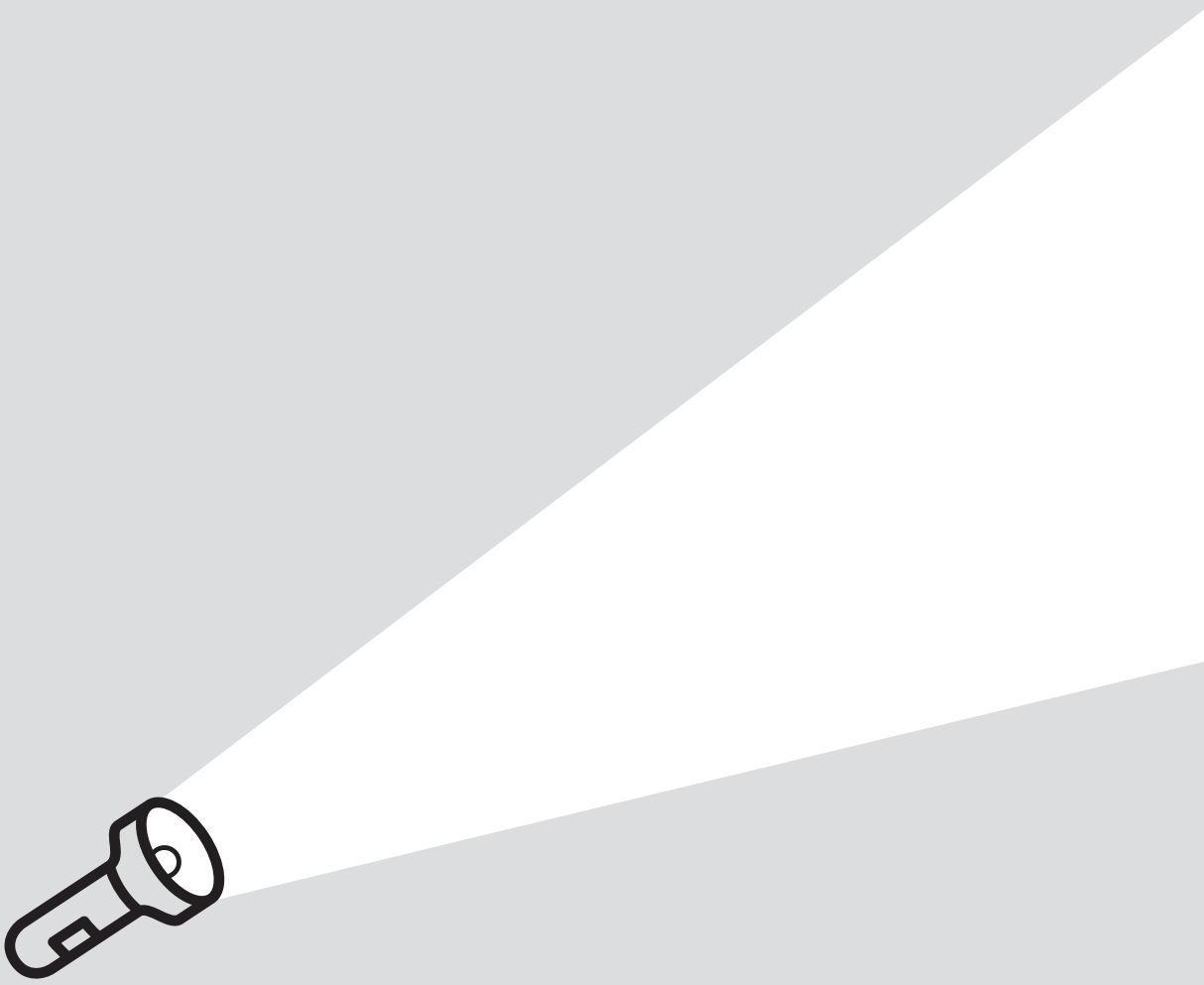
In conclusion, we found a sharp decline of AG levels and a decrease of UAG levels in the first 30 minutes after the glucose load in GH-treated and GH-stop young adults with PWS. This resulted in a lower AG/UAG ratio after glucose intake. Our observation that the ghrelin system in PWS responds similarly as in healthy controls suggests a normal regulation of the ghrelin axis by food intake. GH treatment results in lower AG levels at baseline and during OGTT, suggesting a more favourable metabolic profile. Our findings

might suggest that the impaired satiety is not the result of an abnormal response of the orexigenic ghrelin to food intake.

## REFERENCES


1. Goldstone, A. P., Holland, A. J., Hauffa, B. P. et al.: Recommendations for the diagnosis and management of Prader-Willi syndrome. J Clin Endocrinol Metab, 93: 4183, 2008
2. Festen, D. A., Wevers, M., de Weerd, A. W. et al.: Cognition and behavior in pre-pubertal children with Prader-Willi syndrome and associations with sleep-related breathing disorders. Am J Med Genet A, 146A: 3018, 2008
3. Holm, V. A., Cassidy, S. B., Butler, M. G. et al.: Prader-Willi syndrome: consensus diagnostic criteria. Pediatrics, 91: 398, 1993
4. Bakker, N. E., Kuppens, R. J., Siemensma, E. P. et al.: Eight years of growth hormone treatment in children with Prader-Willi syndrome: maintaining the positive effects. J Clin Endocrinol Metab, 98: 4013, 2013
5. Schuster, D. P., Osei, K., Zipf, W. B.: Characterization of alterations in glucose and insulin metabolism in Prader-Willi subjects. Metabolism, 45: 1514, 1996
6. Festen, D. A., van Toorenenbergen, A., Duivenvoorden, H. J. et al.: Adiponectin levels in prepubertal children with Prader-Willi syndrome before and during growth hormone therapy. J Clin Endocrinol Metab, 92: 1549, 2007
7. Kuppens, R. J., Diene, G., Bakker, N. E. et al.: Elevated ratio of acylated to unacylated ghrelin in children and young adults with Prader-Willi syndrome. Endocrine, 50: 633, 2015
8. Feigerlova, E., Diene, G., Conte-Auriol, F. et al.: Hyperghrelinemia precedes obesity in Prader-Willi syndrome. J Clin Endocrinol Metab, 93: 2800, 2008
9. Cummings, D. E., Clement, K., Purnell, J. Q. et al.: Elevated plasma ghrelin levels in Prader Willi syndrome. Nat Med, 8: 643, 2002
10. van der Lely, A. J., Tschop, M., Heiman, M. L. et al.: Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. Endocr Rev, 25: 426, 2004
11. Kojima, M., Hosoda, H., Date, Y. et al.: Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature, 402: 656, 1999
12. Yang, J., Brown, M. S., Liang, G. et al.: Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. Cell, 132: 387, 2008
13. Tschop, M., Smiley, D. L., Heiman, M. L.: Ghrelin induces adiposity in rodents. Nature, 407: 908, 2000
14. Theander-Carrillo, C., Wiedmer, P., Cettour-Rose, P. et al.: Ghrelin action in the brain controls adipocyte metabolism. J Clin Invest, 116: 1983, 2006
15. Asakawa, A., Inui, A., Fujimiya, M. et al.: Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. Gut, 54: 18, 2005
16. Allas, S., Abribat, T.: Clinical perspectives for ghrelin-derived therapeutic products. Endocr Dev, 25: 157, 2013
17. Delhanty, P. J., Neggers, S. J., van der Lely, A. J.: Mechanisms in endocrinology: Ghrelin: the differences between acyl- and des-acyl ghrelin. Eur J Endocrinol, 167: 601, 2012
18. Ozcan, B., Neggers, S. J., Miller, A. R. et al.: Does des-acyl ghrelin improve glycemic control in obese diabetic subjects by decreasing acylated ghrelin levels? Eur J Endocrinol, 170: 799, 2014
19. Lindgren, A. C., Barkeling, B., Hagg, A. et al.: Eating behavior in Prader-Willi syndrome, normal weight, and obese control groups. J Pediatr, 137: 50, 2000
20. Liu, J., Prudom, C. E., Nass, R. et al.: Novel ghrelin assays provide evidence for independent regulation of ghrelin acylation and secretion in healthy young men. J Clin Endocrinol Metab, 93: 1980, 2008

21. Gottero, C., Bellone, S., Rapa, A. et al.: Standard light breakfast inhibits circulating ghrelin level to the same extent of oral glucose load in humans, despite different impact on glucose and insulin levels. J Endocrinol Invest, 26: 1203, 2003
22. Paik, K. H., Choe, Y. H., Park, W. H. et al.: Suppression of acylated ghrelin during oral glucose tolerance test is correlated with whole-body insulin sensitivity in children with Prader-Willi syndrome. J Clin Endocrinol Metab, 91: 1876, 2006
23. Delhanty, P. J., Huisman, M., Julien, M. et al.: The acylated (AG) to unacylated (UAG) ghrelin ratio in esterase inhibitor treated blood is higher than previously described. Clin Endocrinol (Oxf), 2014
24. Fredriks, A. M., van Buuren, S., Burgmeijer, R. J. et al.: Continuing positive secular growth change in The Netherlands 1955-1997. Pediatr Res, 47: 316, 2000
25. Fredriks, A. M., van Buuren, S., Wit, J. M. et al.: Body index measurements in 1996-7 compared with 1980. Arch Dis Child, 82: 107, 2000
26. Miller, J. L., Lynn, C. H., Driscoll, D. C. et al.: Nutritional phases in Prader-Willi syndrome. Am J Med Genet A, 155A: 1040, 2011
27. Zhang, H., Wu, C., Chen, Q. et al.: Treatment of obesity and diabetes using oxytocin or analogs in patients and mouse models. PLoS One, 8: e61477, 2013
28. St-Pierre, D. H., Karelis, A. D., Coderre, L. et al.: Association of acylated and nonacylated ghrelin with insulin sensitivity in overweight and obese postmenopausal women. J Clin Endocrinol Metab, 92: 264, 2007
29. Hauffa, B. P., Petersenn, S.: GH treatment reduces total ghrelin in Prader-Willi syndrome (PWS) and may confound ghrelin studies in young PWS children. Clin Endocrinol (Oxf), 71: 155, 2009
30. Hauffa, B. P., Haase, K., Range, I. M. et al.: The effect of growth hormone on the response of total and acylated ghrelin to a standardized oral glucose load and insulin resistance in children with Prader-Willi syndrome. J Clin Endocrinol Metab, 92: 834, 2007
31. Harada, T., Nakahara, T., Yasuhara, D. et al.: Obestatin, acyl ghrelin, and des-acyl ghrelin responses to an oral glucose tolerance test in the restricting type of anorexia nervosa. Biol Psychiatry, 63: 245, 2008
32. Foster-Schubert, K. E., Overduin, J., Prudom, C. E. et al.: Acyl and total ghrelin are suppressed strongly by ingested proteins, weakly by lipids, and biphasically by carbohydrates. J Clin Endocrinol Metab, 93: 1971, 2008
33. Prodam, F., Me, E., Riganti, F. et al.: The nutritional control of ghrelin secretion in humans: the effects of enteral vs. parenteral nutrition. Eur J Nutr, 45: 399, 2006



CHAPTER 4

# Promising effects of oxytocin on social and food-related behaviour in young children with Prader-Willi syndrome: a randomized, double-blind, controlled cross-over trial

R.J. Kuppens, S.H. Donze, A.C.S. Hokken-Koelega

*Clinical Endocrinology 2016; in press*

## ABSTRACT

**Background** Prader-Willi syndrome (PWS) is known for hyperphagia with impaired satiety and a specific behavioural phenotype with stubbornness, temper tantrums, manipulative and controlling behaviour and obsessive-compulsive features. PWS is associated with hypothalamic and oxytocinergic dysfunction. In humans without PWS, intranasal oxytocin administration had positive effects on social and eating behaviour, and weight balance.

**Objective** To evaluate the effects of intranasal oxytocin compared to placebo administration on social behaviour and hyperphagia in children with PWS.

**Design** Randomized, double-blind, placebo-controlled, cross-over study in a PWS Reference Center in the Netherlands.

**Method** Cross-over intervention with twice daily intranasal oxytocin (dose range 24-48 IU/day) and placebo administration, both during 4 weeks, in 25 children with PWS (aged 6 to 14 years).

**Results** In the total group, no significant effects of oxytocin on social behaviour or hyperphagia were found, but in the 17 children younger than 11 years, parents reported significantly less anger (p=0.001), sadness (p=0.005), conflicts (p=0.010) and food-related behaviour (p=0.011), and improvement of social behaviour (p=0.018) during oxytocin treatment compared with placebo. In the 8 children older than 11 years, the items happiness (p=0.039), anger (p=0.042) and sadness (p=0.042) were negatively influenced by oxytocin treatment compared to placebo. There were no side effects or adverse events.

**Conclusion** This randomized, double-blind, placebo-controlled study suggests that intranasal oxytocin administration has beneficial effects on social behaviour and food-related behaviour in children with PWS younger than 11 years of age, but not in those older than 11 years of age.

## INTRODUCTION

Prader-Willi syndrome (PWS) is characterized by neonatal hypotonia with suckling problems, early onset of hyperphagia with impaired satiety, endocrine disturbances and a specific behavioural phenotype with stubbornness, temper tantrums, manipulative and controlling behaviour, obsessive-compulsive features and difficulties in changing routines[1-3]. This results from the absence of expression of the paternally derived genes located on chromosome 15 at the locus q11.2-13, caused by a paternal deletion, maternal uniparental disomy, imprinting center disorder or paternal chromosomal translocation[1]. One of the non-expressed genes in this region is MAGEL2.

MAGEL2-deficient mice have a major reduction of oxytocin in the hypothalamus and an altered onset of suckling activity resulting in impaired feeding and 50% mortality[4]. Injection of a specific oxytocin receptor antagonist in wild-type mouse pups resulted in a similar feeding deficiency as seen in MAGEL2 mutants[4]. Administration of oxytocin to MAGEL2-deficient mouse pups 3-5 hours after birth normalized suckling and feeding behaviour and rescued all of them[4]. Human newborns with PWS show similar suckling problems as found in the MAGEL2-deficient mice, which suggests that the lack of MAGEL2 gene might play a role in the suckling deficit seen in PWS newborns.

In adult patients with PWS, the number of oxytocin-expressing neurons in the hypothalamus was significantly decreased by 42% and plasma levels of oxytocin were relatively low in relation to their obesity[5, 6]. However in 23 children with PWS between 5 and 11 years of age, high plasma levels of oxytocin were reported[7]. Altogether, the oxytocin system in patients with PWS appears to be dysfunctional.

Oxytocin is known to be involved in food intake[8, 9], body weight[10, 11] and social skills[12], all of which are seriously affected in patients with PWS. The majority of patients with PWS have hyperphagia with impaired satiety and they are severely at risk to become obese. They show symptoms of autistic spectrum disorder (ASD) and 36% of them fulfill the criteria of ASD[13]. Social cognitive functioning is markedly reduced, which has major consequences for the family and surrounding and for the approach of patients with PWS[14, 15]. Currently, there are no treatment options for the hyperphagia and social behavioural problems of patients with PWS, but the oxytocin system is a promising target. Studies on intranasal oxytocin administration showed that oxytocin reduced body weight of obese non-PWS patients[10]. Also, a single oxytocin gift improved emotion recognition in healthy and autistic adults[16, 17] and reduced repetitive behaviours in those with ASD[18].

Only two studies have investigated the effects of oxytocin treatment in PWS. Tauber *et al.* administered a single gift of 24IU intranasal oxytocin to 24 adults with PWS. After two days, they showed increased trust in others and decreased sadness tendencies with less disruptive behaviour[19]. In the placebo-controlled cross-over study by Einfeld *et al.*,

22 individuals with PWS aged 12-30 years received 18-40IU intranasal oxytocin twice daily during 8 weeks, but they showed no benefit in the target behaviours or weight[20].

Given the possible dysfunction of the oxytocin system in PWS and the involvement of oxytocin in socials skills, food intake and body weight, we hypothesized that oxytocin supplementation in children with PWS would improve social behaviour and hyperphagia. We, therefore, investigated the effects of intranasal oxytocin administration on social behaviour, food intake and satiety in children with PWS in a randomized, double-blind, placebo-controlled, cross-over study.

## METHODS

### Subjects

In order to be eligible to participate in this study, subjects (1) had a genetically confirmed diagnosis of PWS; (2) were aged 6 to 14 years; (3) had social behavioural problems and/or a preoccupation with food; (4) were naïve for oxytocin treatment at time of enrollment; and (5) used growth hormone therapy for at least 1 year and were still receiving it. Exclusion criteria were (1) severe psychiatric problems such as psychosis, serious illness or cardiac abnormalities; (2) allergic reactions or hypersensitivity to oxytocin; (3) medication to reduce weight (fat) other than GH; and (4) non-cooperative behaviour resulting in inability to comply with intranasal administration and/or hospital visits.

Forty-two children with PWS were eligible. Parents of 17 children refused to participate; 10 due to too large burden, 5 due to practical issues and 2 because the children themselves did not want to participate. The study group consisted of 25 children (14 boys, 11 girls) with PWS, aged 6-14 years. GH therapy was prescribed at an initial dose of 1 mg/m$^2$/day and dose was lowered in case of high IGF-I levels. One child used levothyroxine and another used citalopram and aripiprazole.

### Design

A randomized, double-blind, placebo-controlled, cross-over study was conducted to investigate the effects of intranasal oxytocin administration on social behaviour, food intake and satiety. Children received either oxytocin or placebo for 4 weeks, after which they crossed-over to the alternative treatment for a further 4 weeks. No wash-out period was implemented, as the half life time of oxytocin is only 3-20 minutes. An independent statistician generated the random allocation sequence and only he and the independent pharmacist were unblinded. The children were stratified according to gender and age (6-10.99 or 11-14.99 years) and then randomly and blindly assigned to receive intranasal administration twice daily, before breakfast and dinner, of either oxytocin (Syntocinon®, 4IU/puff, Sigma Tau) or identical appearing placebo (placebo, 0IU/puff, Sigma Tau). The

dose was based on doses used in other trials[12, 20] and calculated according to body surface: a child of 0.8-1.15m$^2$ received 2dd3 puffs (12IU twice daily); 1.15-1.45m$^2$ had 2dd4 puffs (16IU twice daily); 1.45-1.75m$^2$ had 2dd5 puffs (20IU twice daily); and of >1.75m$^2$ had 2dd6 puffs (24IU twice daily).

### Measurements

Children were examined at the outpatient clinic, at baseline, after 4 weeks and after 8 weeks. Standing height was measured with a calibrated Harpenden stadiometer and weight was determined on a calibrated scale (Servo Balance). Height, weight and BMI were expressed as SDS according to Dutch reference data, adjusted for age and sex[21, 22]. Percentage fat was measured by DXA (Lunar Prodigy; GE Healthcare). All scans were made on the same machine and daily quality assurance was performed.

Blood samples were collected in the morning after 12-h overnight fast. Samples were quickly frozen on dry ice and stored at -80ºC until assayed. A breakfast meal consisting of 270 grams (560 kcal) industrially produced multigrain pancakes cut into pieces was used to examine the food intake and satiety. This amount was determined in collaboration with a dietitian; weight was 150%, and calorie-intake 185% of a normal healthy breakfast for a child with PWS. After blood collection, the fasted children were instructed to eat as much as they wished of the meal. The weight of the plate with pancakes was measured at baseline and after the child finished eating. Besides, the duration of eating was determined. The child stayed in the room together with the investigator, while their parents waited elsewhere.

Social and eating behaviour were carefully monitored by the parents at home and investigated by two parent questionnaires. Dykens Hyperphagia Questionnaire was used to determine (changes in) eating behaviour and hyperphagia[23]. Although the Dykens Hyperphagia Questionnaire is validated, we have experienced that not all items of this questionnaire are applicable nowadays. The new generation of children with PWS had an early diagnosis and received intensive support from (para)medics, and started with GH treatment at a young age, which has markedly changed the phenotype, but they are still preoccupied with food. Therefore, the Oxytocin Study Questionnaire was developed by three physicians and a psychologist, all very experienced in PWS. The questionnaire unravels (changes in) emotions, social and eating behaviour and possible side effects. Parents were asked to fill in the applicable change from -3 (much less frequently) to +3 (much more frequently), in which 0 was 'no difference'. An example of a question is '*In the last 4 weeks, my child was…sad*'. Comparable questions were asked regarding being angry, being happy, showing food-seeking behaviour, having conflicts with others, etc.

### Assays

All blood samples were determined in the same laboratory according to a standardized procedure. Levels of serum creatinine, hepatic enzymes and glucose were measured with COBAS 8000 systems of Roche and thyroid function was measured with Vitros ECIQ immunoanalyzer system of Ortho Clinical Diagnostics. Oxytocin levels in blood samples were measured in duplo with an oxytocin ELISA kit (Enzo Life Sciences). To assure the content of the vials, one puff per child per phase was measured by the same oxytocin ELISA kit. In summary, all children had one vial with and one vial without oxytocin, which confirmed perfect execution of the randomization.

### Statistics

Statistical analysis was performed by SPSS version 23.0. Calculation of the sample size was based on the Oxytocin Study Questionnaire. Parents answer questions about changes in eating behaviour and social behaviour, from -3 (much less frequently) to +3 (much more frequently), in which 0 was 'no difference'. A decrease of 4 points was considered clinically relevant. Based on an SD of 4, a power of 0.9 and significance level of 0.05, a total of 24 patients had to enter the two-treatment cross-over study. Data was not normally distributed, therefore non-parametric tests were used and data is expressed as median (interquartile range (IQR)) unless otherwise stated. Statistical analysis appropriate for cross-over trials were used, taking into account any carry-over or treatment-period effect, calculated by Wilcoxon Signed Rank test and Mann Whitney U tests, but these were not found. Depending on the data, results of the visit (ie questions about changes) or differences (Δ) between visit 1 and 2, and between visit 2 and 3 (ie Δweight) were used. The effect of oxytocin versus placebo was tested by Wilcoxon tests in case of continuous data and McNemar tests in case of binary data. Correlations between effect of oxytocin or oxytocin levels and other parameters were assessed using Spearman's rho. Differences were considered significant if p-value was $<0.05$.

### Study approval

Written informed consent was obtained from parents and from children older than 12 years; assent was obtained in children younger than 12 years. The study protocol was approved by the Medical Ethics Committee of Erasmus University Medical Center, The Netherlands, and registered at Nederlands Trial Register NTR4950 (www.trialregister.nl).

## RESULTS

### Baseline characteristics

Table 1 shows the baseline characteristics of the 25 children with PWS who were included between January and September 2015. Median age was 9.3 (range 6.0-13.7) years and BMI was 2.4 (0.7-4.3) SDS. Thirteen (52%) patients had a deletion and 12 (48%) an mUPD. All received GH treatment with a median dose of 0.8 (0.6-1.0) mg/m$^2$/day (≈0.024 mg/kg/day), started at a median age of 1.3 (1.0-2.2) years, with a median duration of GH treatment of 8.0 (5.7-9.2) years. The median dose of oxytocin was 16IU (range 12-24) twice daily. All 25 children completed the study.

### Effects on social behaviour, food intake and satiety

In the total group of 25 children with PWS between 6 and 14 years of age, no effects of oxytocin versus placebo treatment were found on social behaviour, food intake and satiety. In contrast to these nonsignificant effects of oxytocin in the total group, correlation analyses showed that a younger age was strongly associated with beneficial effects of oxytocin treatment on social and eating behaviour (ρ=-0.553, p=0.004 and ρ=-0.485, p=0.014, resp.) and therefore subanalyses were performed. In line with the stratification, we divided the total group in 17 patients younger than 11 years and 8 patients older than 11 years.

### Subanalysis in the younger children

*Effects on social behaviour*

Parents filled out questionnaires about their child. The items anger, sadness and conflicts improved significantly during oxytocin treatment compared to placebo (p=0.001, p=0.005 and p=0.010, resp.)(Table 2, Figure 1). The total Oxytocin Study Questionnaire score showed a significant improvement of -4 (-7.5 to -1) points during oxytocin treatment compared to placebo (p=0.001). Ten of 17 (58.8%) parents reported an improvement in social behaviour during oxytocin treatment, while 4 (23.5%) parents reported improvement during placebo (p=0.059).

*Effects on eating behaviour*

The 17 younger children showed a significant improvement in food-related behaviour during oxytocin treatment (p=0.011). During 4 weeks of oxytocin treatment, food-seeking behaviour and satiety remained similar (p=0.429 and p=0.713, resp.), however, at baseline, food-seeking behaviour was only reported by six (35.3%) of the 17 parents, and in three of them, food-seeking behaviour was seen a few times per year. In both phases, almost all children finished their standardized breakfast meal. Only three children left pancakes (median 95, range 70-140 gram) during oxytocin treatment and one

**Table 1.** Baseline characteristics of total group and per treatment schedule

| | **TOTAL GROUP OF PATIENTS OF 6 TO 14 YEARS** | | | |
|---|---|---|---|---|
| | PWS (n=25) | Oxytocin / Placebo (n=11) | Placebo / Oxytocin (n=14) | p* |
| Gender | 14 boys, 11 girls | 6 boys, 5 girls | 8 boys, 6 girls | |
| Genetic subtype (DEL/mUPD) | 13 / 12 | 6 / 5 | 7 / 7 | |
| Age (yrs) | 9.3 (6.9 to 11.9) | 9.0 (6.4 to 11.2) | 10.3 (7.0 to 12.5) | 0.403 |
| Height for age (SDS) | 0.8 (0.2 to 1.6) | 1.2 (0.4 to 1.7) | 0.5 (0.0 to 1.1) | 0.222 |
| Weight for height (SDS) | 2.0 (0.6 to 4.1) | 2.3 (0.3 to 6.1) | 1.9 (1.0 to 4.1) | 0.809 |
| BMI for age (SDS) | 2.4 (0.7 to 4.3) | 2.4 (-0.1 to 6.3) | 2.2 (1.2 to 3.9) | 1.000 |
| Age at start GH treatment (yrs) | 1.3 (1.0 to 2.2) | 1.3 (0.9 to 2.2) | 1.5 (1.0 to 2.6) | 0.609 |
| Duration of GH treatment (yrs) | 8.0 (5.7 to 9.2) | 7.3 (5.3 to 9.0) | 8.2 (5.9 to 9.3) | 0.687 |
| GH dosage (mg/m$^2$/day) | 0.8 (0.6 to 1.0) | 0.8 (0.5 to 1.0) | 0.8 (0.6 to 1.0) | 0.851 |
| Prepubertal/pubertal | 16 / 9 | 7 / 4 | 9 / 5 | |
| | **PATIENTS OF 6 TO 11 YEARS** | | | |
| | PWS (n=17) | Oxytocin / Placebo (n=8) | Placebo / Oxytocin (n=9) | p* |
| Gender | 9 boys, 8 girls | 4 boys, 4 girls | 5 boys, 4 girls | |
| Genetic subtype (DEL/mUPD) | 9 / 8 | 4 / 4 | 5 / 4 | |
| Age (yrs) | 7.3 (6.4 to 9.7) | 7.8 (6.1 to 9.3) | 7.3 (6.7 to 10.3) | 0.606 |
| Height for age (SDS) | 0.5 (0.0 to 1.3) | 1.2 (0.0 to 1.6) | 0.3 (-0.2 to 0.8) | 0.277 |
| Weight for height (SDS) | 1.7 (0.6 to 3.9) | 1.7 (0.4 to 5.3) | 1.7 (0.9 to 3.9) | 0.743 |
| BMI for age (SDS) | 1.9 (0.6 to 3.9) | 2.1 (0.0 to 5.7) | 1.8 (1.0 to 3.6) | 0.963 |
| Age at start GH treatment (yrs) | 1.3 (0.9 to 1.7) | 1.3 (0.8 to 2.0) | 1.2 (0.9 to 1.5) | 0.743 |
| Duration of GH treatment (yrs) | 6.3 (5.3 to 8.4) | 6.3 (5.2 to 8.2) | 6.3 (5.5 to 8.8) | 0.541 |
| GH dosage (mg/m$^2$/day) | 0.7 (0.5 to 1.0) | 0.7 (0.5 to 1.0) | 0.7 (0.5 to 1.0) | 0.963 |
| Prepubertal/pubertal | 14 / 3 | 6 / 2 | 8 / 1 | |
| | **PATIENTS OF 11 TO 14 YEARS** | | | |
| | PWS (n=8) | Oxytocin / Placebo (n=3) | Placebo / Oxytocin (n=5) | p* |
| Gender | 5 boys, 3 girls | 2 boys, 1 girl | 3 boys, 2 girls | |
| Genetic subtype (DEL/mUPD) | 4 / 4 | 2 / 1 | 2 / 3 | |
| Age (yrs) | 12.5 (11.6 to 13.7) | 12.3 (11.8 to 13.0) | 12.6 (11.9 to 13.7) | 0.393 |
| Height for age (SDS) | 1.2 (0.6 to 1.6) | 1.6 (1.1 to 1.8) | 1.0 (0.2 to 1.6) | 0.571 |
| Weight for height (SDS) | 2.2 (0.6 to 4.3) | 2.3 (0.4 to 4.6) | 2.0 (0.8 to 4.3) | 1.000 |
| BMI for age (SDS) | 2.8 (0.9 to 5.6) | 3.2 (1.3 to 5.5) | 2.5 (1.2 to 5.4) | 1.000 |
| Age at start GH treatment (yrs) | 2.7 (1.8 to 4.3) | 2.2 (1.6 to 2.9) | 2.9 (2.1 to 4.9) | 0.393 |
| Duration of GH treatment (yrs) | 9.5 (8.5 to 11.1) | 9.9 (9.5 to 10.6) | 8.5 (8.2 to 11.0) | 0.393 |
| GH dosage (mg/m$^2$/day) | 1.0 (0.7 to 1.0) | 0.9 (0.8 to 1.0) | 1.0 (0.7 to 1.0) | 0.786 |
| Prepubertal/pubertal | 2 / 6 | 1 / 2 | 1 / 4 | |

*p-value at baseline between the two treatment schedules. Data expressed as median with interquartile range.

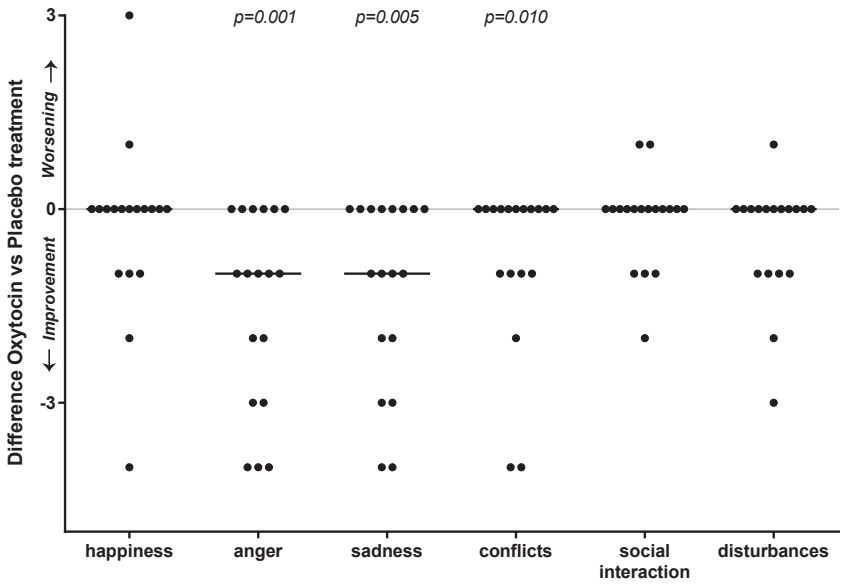



**Figure 1.** Effect of 4 weeks oxytocin versus placebo treatment on 6 items of social behaviour in children with PWS younger than 11 years of age. Individual differences between oxytocin and placebo treatment are presented as a black dot and the black horizontal lines display the median difference. In case of a significant difference between the oxytocin and placebo phase, p-values are given.

of them also left 88 grams during placebo. During the standardized breakfast meal, the rate and duration of eating was similar during oxytocin and placebo treatment (23.8 vs 22.9 gram/minute, p=0.887 and 10.5 vs 11.6 minutes, p=0.102)(Table 2).

The difference in weight, BMI and fat percentage between start and end (Δ) of the 4 weeks of oxytocin treatment were similar as during placebo (p=0.055, p=0.149 and p=0.136, resp.).

## Subanalyses in the older children

*Effects on social behaviour*

We did not find beneficial effects of oxytocin in the 8 children older than 11 years (Table 2). The items happiness, anger and sadness were negatively influenced by oxytocin treatment compared to placebo (p=0.039, p=0.042 and p=0.042, resp.). The Oxytocin Study Questionnaire showed an unfavourable score of +1.5 (0.3-5) points during oxytocin treatment compared to placebo (p=0.027). Three (37.5%) parents reported a deterioration of social behaviour during oxytocin treatment, while four (50%) parents reported an improvement during placebo treatment (p=0.038).

**Table 2.** Effects on social behaviour and hyperphagia of children younger and older than 11 years

| | PATIENTS OF 6 TO 11 YEARS | | | | | | | PATIENTS OF 11 TO 14 YEARS | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Items investigated | Oxytocin phase | | | Placebo phase | | | p* | Oxytocin phase | | | Placebo phase | | | p* |
| **Improvement social behaviour (n=)** | 10 of 17 | | | 4 of 17 | | | 0.059 | 0 of 8 | | | 4 of 8 | | | **0.038** |
| **Questionnaires about social behaviour** | *better* | *same* | *worse* | *better* | *same* | *worse* | | *better* | *same* | *worse* | *better* | *same* | *worse* | |
| Happiness | 6 | 10 | 0 | 2 | 14 | 1 | 0.344 | 0 | 7 | 1 | 5 | 3 | 0 | **0.039** |
| Anger | 11 | 6 | 0 | 0 | 10 | 7 | **0.001** | 0 | 4 | 4 | 5 | 3 | 0 | **0.042** |
| Sadness | 9 | 8 | 0 | 0 | 12 | 5 | **0.005** | 0 | 5 | 3 | 5 | 3 | 0 | **0.042** |
| Conflicts | 7 | 9 | 0 | 1 | 12 | 4 | **0.010** | 0 | 5 | 3 | 3 | 5 | 0 | 0.068 |
| Social interaction | 2 | 15 | 0 | 3 | 12 | 2 | 0.450 | 0 | 6 | 2 | 3 | 5 | 0 | 0.066 |
| Disruptive behaviour | 4 | 13 | 0 | 1 | 13 | 3 | 0.071 | 0 | 5 | 3 | 2 | 6 | 0 | 0.109 |
| **Improvement eating behaviour (n=)** | 6 of 17 | | | 2 of 17 | | | 0.096 | 1 of 8 | | | 3 of 8 | | | 0.157 |
| **Standardized breakfast meal** | | | | | | | | | | | | | | |
| Did not finished meal completely (n=) | 3 of 17 | | | 1 of 17 | | | 0.109 | 0 of 8 | | | 0 of 8 | | | 1.000 |
| Rate of eating (gram/minute) | 23.8 (15.8 to 29.5) | | | 22.9 (14.6 to 29.0) | | | 0.887 | 23.8 (14.0 to 59.6) | | | 24.5 (17.4 to 52.5) | | | 0.779 |
| Duration of eating (minutes) | 10.5 (8.8 to 14.5) | | | 11.6 (9.3 to 18.5) | | | 0.102 | 12.0 (4.4 to 21.1) | | | 11.6 (5.3 to 15.6) | | | 0.889 |
| **Measurements** | | | | | | | | | | | | | | |
| Δweight (kg) | 0.3 (-0.1 to 0.6) | | | -0.3 (-0.5 to 0.0) | | | 0.055 | 0.5 (-0.2 to 1.2) | | | 0.4 (0.0 to 0.7) | | | 0.833 |
| ΔBMI (kg/m$^2$) | -0.1 (-0.3 to 0.2) | | | -0.2 (-0.3 to 0.0) | | | 0.149 | 0.0 (-0.1 to 0.0) | | | 0.1 (-0.2 to 0.3) | | | 0.327 |
| Δfat percentage by DXA scan | -0.7 (-0.9 to -0.1) | | | -0.1 (-0.6 to 1.1) | | | 0.136 | 0.5 (-0.1 to 0.9) | | | -0.1 (-0.8 to 0.3) | | | 0.092 |
| **Questionnaires about food** | *better* | *same* | *worse* | *better* | *same* | *worse* | | *better* | *same* | *worse* | *better* | *same* | *worse* | |
| Food related behaviour | 7 | 10 | 0 | 1 | 9 | 7 | **0.011** | 0 | 7 | 1 | 3 | 5 | 0 | 0.066 |
| Food seeking behaviour | 1 | 15 | 1 | 1 | 13 | 3 | 0.713 | 0 | 7 | 1 | 0 | 8 | 0 | 0.317 |
| Satiety | 4 | 11 | 1 | 2 | 14 | 1 | 0.429 | 0 | 7 | 1 | 2 | 6 | 0 | 0.102 |
| Dykens hyperphagia | 8 | 8 | 1 | 3 | 11 | 3 | 0.285 | 0 | 7 | 1 | 3 | 5 | 0 | 0.068 |

Data expressed in number or median (IQR); *p-value between oxytocin and placebo phase, bolded p-values are p-values below 0.05.

*Effects on eating behaviour*

During oxytocin treatment, food-related behaviour, food-seeking behaviour and satiety remained similar (p=0.066, p=0.102 and p=0.317, resp.) in the children older than 11 years. In both treatment phases, all children finished their standardized breakfast meal, while the rate and duration of eating was similar during oxytocin and placebo treatment (23.8 vs 24.5 gram/minute, p=0.779 and 12.0 vs 11.6 minutes, p=0.889)(Table 2). Δweight, ΔBMI and Δfat percentage were similar in the two phases (all p>0.092).

## Associations

There were no significant differences in oxytocin versus placebo effects between boys and girls, between children with a deletion or mUPD, or between children with or without serious behavioural problems. The effects of oxytocin treatment were not associated with baseline BMI or BMI SDS. In the total group, the association between age and social and eating behaviour during oxytocin treatment was stronger than the association between pubertal stage and these outcomes (ρ=-0.553, p=0.004 and ρ=-0.485, p=0.014 versus ρ=-0.452, p=0.023 and ρ=-0.396, p=0.050, resp.).



## Oxytocin levels in blood

Serum oxytocin levels before and during study were determined to further unravel the different effects in younger and older children (Figure 2). At baseline, children younger than 11 years had a median fasting oxytocin level of 3156 (1864-5325) pg/ml, and 12-14 hours after the last oxytocin dose after 4 weeks of treatment it was 4685 (2809-9490) pg/ml (p=0.134). Children older than 11 years had a baseline fasting oxytocin level of 2692 (1737-3754) pg/ml, and 4750 (1976-7831) pg/ml after oxytocin treatment (p=0.327).

In the younger children, lower oxytocin levels after 4 weeks of oxytocin treatment were associated with positive effects on social behaviour (ρ=-0.540, p=0.027). This association was not found in older children. Baseline oxytocin levels or change in oxytocin levels during treatment were not associated with positive effects.

Only one patient older than 11 years had benefit from oxytocin treatment. Remarkably, this patient had the highest baseline oxytocin level of patients older than 11 years, which decreased considerably to the lowest level of 1126 pg/ml during oxytocin treatment (Figure 2). The other older patients without benefit had lower baseline oxytocin levels (Figure 2), which increased or only slightly decreased during oxytocin treatment.

## Dosing

The oxytocin/placebo dose was based on body surface (see Methods). In the total group, median dose was 16IU (12-20) twice daily, which was 12.3 (12.0-13.3) IU/$m^2$ and 0.39 (0.34-0.44) per kilogram body weight (IU/kg). Given the different effects of oxytocin in the younger and older group of patients, the correlation between dosage and age was

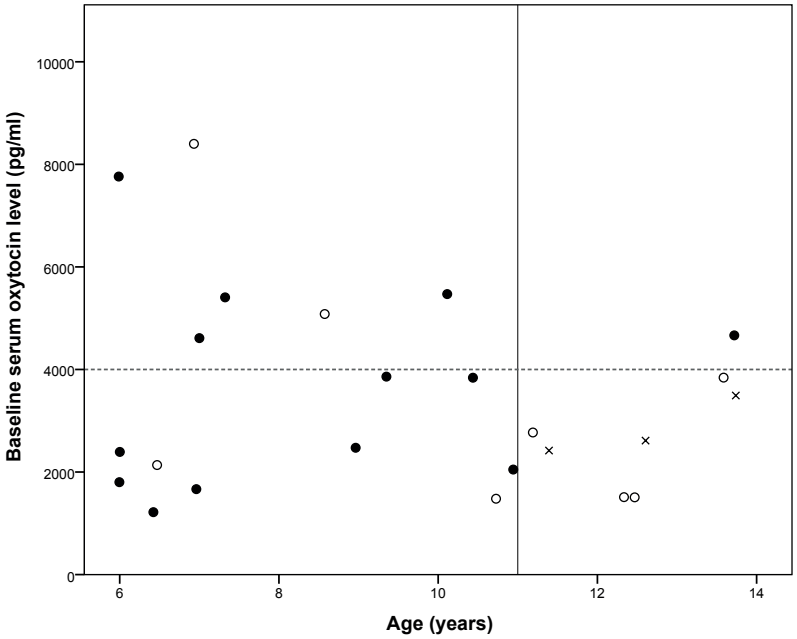



**Figure 2.** Fasting serum oxytocin levels at baseline in pg/ml per age. ● represents a patient with positive effect of oxytocin treatment. ○ represents a patient without effect of oxytocin treatment. x represents a patient with negative effect of oxytocin treatment. The grey line indicates the age of 11 years.

investigated. The dose in IU/m$^2$ did not correlate with age, but the given dose recalculated as IU/kg correlated inversely with age ($\rho$=-0.663, $p<0.001$), meaning that older patients received a lower dose when recalculated in IU/kg (Figure 3). The median dose was 0.42 (0.37-0.45) IU/kg in the children younger than 11 years and 0.34 (0.30-0.37) IU/kg in the older children (p=0.002). Remarkably, the only patient older than 11 years who had benefit from oxytocin treatment, had one of the lowest recalculated doses in IU/kg of the older patients and the lowest dose in IU/kg of all patients with beneficial effects of oxytocin.

## Safety parameters

The intranasal administration of oxytocin was very well tolerated and there were no side effects. Renal function, hepatic enzymes, thyroid function and glucose remained stable and normal for all patients, as did the systolic blood pressure. Diastolic blood pressure was lower during oxytocin treatment (median 64 vs 73 mmHg, p=0.008), but within normal limits. Similar results were found in patients younger and older than 11 years.

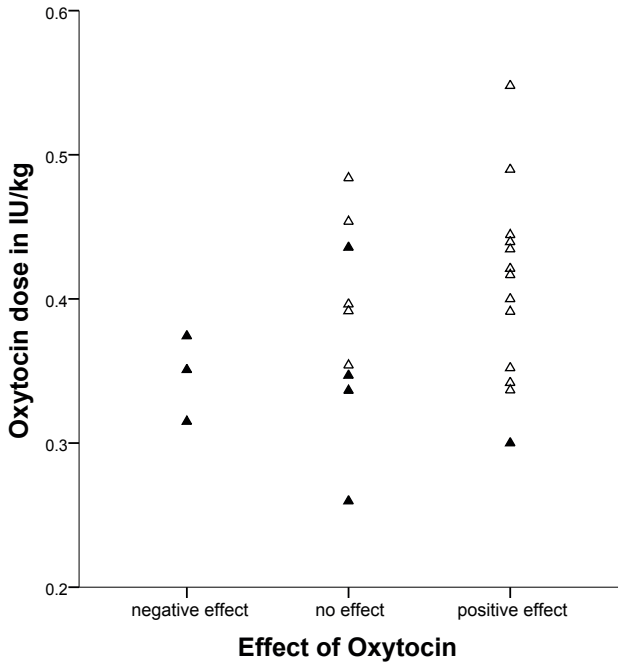



**Figure 3.** Dose of oxytocin recalculated as IU/kg versus effect of oxytocin on social and/or eating behaviour. Δ represents a patient younger than 11 years. ▲ represents a patient older than 11 years.



## DISCUSSION

Our randomized, double-blind, placebo-controlled, cross-over study is the first oxytocin study in children with PWS aged between 6 and 14 years. Although there were no effects in the group as a whole, subanalyses demonstrated that children with PWS between 6 and 11 years had beneficial effects of intranasal oxytocin administration on social behaviour and hyperphagia. Their parents reported significantly less anger, sadness, conflicts and food-related behaviour, and improvement of social behaviour during oxytocin treatment compared with placebo. In children with PWS older than 11 years the beneficial effects of oxytocin on social behaviour and hyperphagia were not found. We did not find side effects or adverse events.

Until now, there have been no effective treatment options for behaviour and food-related problems in PWS. Our study suggests that intranasal oxytocin administration is a novel and promising treatment for young children with PWS. Children with PWS have a specific behavioural phenotype with stubbornness, temper tantrums, manipulative and controlling behaviour, obsessive-compulsive features and difficulties in changing routines[1-3]. The social behavioural problems and hyperphagia seriously affect the quality of life of the children and their parents and care-takers.

Nowadays, most parents of children with PWS have made all kind of adjustments in everyday life to limit access to food, such as locks on the fridge[24]. This explains why, prior to the study, children had a low prevalence of food-seeking behaviour. It is, therefore, not surprising that we found no effects of oxytocin on food-seeking behaviour. However, the baseline food-related behaviour scores show that these children are still preoccupied with food, characterized by talking about food, asking for food, playing that they are cooking, etc., despite all the adjustments to control hyperphagia. Oxytocin treatment decreased this food-related behaviour, which argues that oxytocin has an inhibiting effect on the hyperphagia, despite the lack of effects on food-seeking behaviour and satiety. Studies on the long-term effects of 4 weeks treatment and also long-term oxytocin treatment trials are warranted to confirm our findings on efficacy and safety.

In contrast to the beneficial effects of oxytocin in the younger children, no positive effects were found in children with PWS older than 11 years. Some parents of the older subgroup even reported negative effects of oxytocin on social behaviour, especially regarding happiness, anger and sadness, in contrast to none of the parents of younger children. These findings are in line with the results of an 8-week oxytocin trial in 22 individuals with PWS between 12 and 29 years[20], in which no benefits in target behaviours or weight were found. The only significant difference found in that study was an increase in temper outbursts when the oxytocin dose was increased. Tauber *et al.* reported positive effects of oxytocin on social behaviour in adults with PWS, but comparison is difficult because they investigated a single dose of intranasal administration[19].

We measured plasma oxytocin levels prior and during the oxytocin trial. At baseline, oxytocin levels in children with PWS younger than 11 years showed high inter-individual variability, in line with findings in children with PWS of similar age by Johnson *et al.*[7], and there was no relation between baseline oxytocin levels and positive effects during treatment. In contrast, a lower oxytocin level after 4 weeks of oxytocin treatment was associated with stronger positive effects on social behaviour, suggesting that it is beneficial for young children to have a lower plasma oxytocin level during oxytocin treatment. Those older than 11 years had, however, lower and less widespread baseline oxytocin levels and their levels increased during oxytocin treatment. The only older boy with beneficial effects of oxytocin treatment, had a declining oxytocin level during treatment, like most of the younger children.

Why did the oxytocin treatment work in the younger, but not in the older children? One explanation could be that mistakes had been made in the preparation or delivery of the intranasal sprays in the older children. For that reason, an independent laboratory measured the content of the vials and was able to reject that explanation. Second, it could be that the sample size of the older subgroup was too small to show significant changes, but that argument is unlikely as several significant negative effects of oxytocin administration were found in the older subgroup. Third, there could have been a dosing

issue. We calculated the oxytocin doses according to body surface, a common way of hormone dosing in children, which resulted in a relatively lower dose in IU/kg in children older than 11 years. However, an inappropriately low dose is also an unlikely explanation, as the only older boy with positive effects of oxytocin had the second lowest dose of all children in IU/kg. Besides, Einfeld *et al.* reported adverse effects of higher oxytocin doses in older patients[20]. A fourth explanation could be that the behaviour and coping style of older patients with PWS are more embedded in their personality and are therefore not easy to change. A treatment period of 4 weeks is not short, but it could be that a longer period than 4 or 8 weeks of oxytocin treatment might be needed to induce beneficial effects in older children.

Another, more pathophysiological explanation might be that older children with PWS have developed an unresponsive oxytocin system over the years, with a lower number of oxytocin receptors and neurons in the hypothalamus. Adults with PWS have a 42% decrease in oxytocin neurons in the hypothalamus and relatively low plasma levels of oxytocin in relation to their obesity[5, 6]. One of the non-expressed genes in the PWS region on chromosome 15 is MAGEL2. This gene is known to be expressed in mouse hypothalamus during development and their knock-out alters the number and/or function of oxytocin neurons[25]. MAGEL2-knockout pups were not hypotonic, but had an altered onset of suckling activity resulting in impaired feeding and 50% mortality[4], while the survivors had deficits in social recognition and social interaction on the long-term[26]. These suckling problems in infancy and social problems later on are similar to children with PWS, suggesting that this gene might play a role in the suckling and behavioural problems in PWS. Adult MAGEL2-knockout mice expressed a significantly reduced number of oxytocin receptors in several regions of the brain[26]. Children with PWS, who are MAGEL2 deficient, could therefore be less or nonresponsive to oxytocin treatment when they become older, because their oxytocin system deteriorated over time. In contrast, it was shown that daily administration of oxytocin in the first postnatal week prevents the deficits in social behaviour in the adult mutant mice and partly restores a normal oxytocin system in the brain. This suggests that the postnatal period is a critical period for the oxytocin system in which social behaviour is programmed. Nevertheless, our study shows that oxytocin treatment has beneficial effects in children with PWS until the age of approximately 11 years, thus also beyond the postnatal period.

It is remarkable that the changeover in oxytocin effects occurred around the age of 11 years, the time of puberty onset. We previously demonstrated that GH-treated children with PWS have a normal age at onset of puberty, but that the majority shows a deterioration in pubertal development after Tanner stage 2-3 with a decline in gonadal function in boys[27-29]. It might be that the reactivation of the GnRH-axis just before the onset of puberty together with the lack of expression of MAGEL2 and other yet unknown genes, not only result in a rapid gonadal failure after the onset of puberty but also in an

enhanced deterioration of the oxytocin system in patients with PWS. We acknowledge that our supposition is very hypothetical, but we consider it noteworthy to mention that these processes seem to occur in the same timeframes. Studies are warranted to further unravel the pathophysiology and to determine whether others also find the 11-year cut-off for benefit of oxytocin treatment.

Present study was a placebo-controlled study in which we calculated the dose according to body surface, based on doses used in other trials[12, 20]. We did not perform a dose-finding study and no wash-out period was included to limit the number of hospital visits. We found no differences in food intake during the breakfast meal test between the oxytocin and placebo phase. This might be due to the lack of a satiety level in PWS or that their satiety level was much higher than the maximum amount of food that we offered[30], but we considered it unethical to present an unlimited amount of food as patients with PWS feel never satiated and have an increased risk of gastric rupture.

In conclusion, administration of intranasal oxytocin appears to have beneficial effects on social behaviour and food-related behaviour in children with PWS younger than 11 years of age without side effects or adverse events. Parents reported significantly less anger, sadness, conflicts and food-related behaviour, and improvement of social behaviour during oxytocin treatment compared with placebo. In contrast to the younger children with PWS, those older than 11 years of age did not benefit from oxytocin treatment.

## REFERENCES


1. Goldstone, A. P., Holland, A. J., Hauffa, B. P. et al.: Recommendations for the diagnosis and management of Prader-Willi syndrome. J Clin Endocrinol Metab, 93: 4183, 2008
2. Lo, S. T., Siemensma, E. P., Festen, D. A. et al.: Behavior in children with Prader-Willi syndrome before and during growth hormone treatment: a randomized controlled trial and 8-year longitudinal study. Eur Child Adolesc Psychiatry, 24: 1091, 2015
3. Holm, V. A., Cassidy, S. B., Butler, M. G. et al.: Prader-Willi syndrome: consensus diagnostic criteria. Pediatrics, 91: 398, 1993
4. Schaller, F., Watrin, F., Sturny, R. et al.: A single postnatal injection of oxytocin rescues the lethal feeding behaviour in mouse newborns deficient for the imprinted Magel2 gene. Hum Mol Genet, 19: 4895, 2010
5. Swaab, D. F.: Prader-Willi syndrome and the hypothalamus. Acta Paediatr Suppl, 423: 50, 1997
6. Hoybye, C., Barkeling, B., Espelund, U. et al.: Peptides associated with hyperphagia in adults with Prader-Willi syndrome before and during GH treatment. Growth Horm IGF Res, 13: 322, 2003
7. Johnson, L., Manzardo, A. M., Miller, J. L. et al.: Elevated plasma oxytocin levels in children with Prader-Willi syndrome compared with healthy unrelated siblings. Am J Med Genet A, 2015
8. Ott, V., Finlayson, G., Lehnert, H. et al.: Oxytocin reduces reward-driven food intake in humans. Diabetes, 62: 3418, 2013
9. Lawson, E. A., Marengi, D. A., DeSanti, R. L. et al.: Oxytocin reduces caloric intake in men. Obesity (Silver Spring), 23: 950, 2015
10. Zhang, H., Wu, C., Chen, Q. et al.: Treatment of obesity and diabetes using oxytocin or analogs in patients and mouse models. PLoS One, 8: e61477, 2013
11. Zhang, G., Cai, D.: Circadian intervention of obesity development via resting-stage feeding manipulation or oxytocin treatment. Am J Physiol Endocrinol Metab, 301: E1004, 2011
12. Macdonald, K., Macdonald, T. M.: The peptide that binds: a systematic review of oxytocin and its prosocial effects in humans. Harv Rev Psychiatry, 18: 1, 2010
13. Lo, S. T., Siemensma, E., Collin, P. et al.: Impaired theory of mind and symptoms of Autism Spectrum Disorder in children with Prader-Willi syndrome. Res Dev Disabil, 34: 2764, 2013
14. Festen, D. A., Wevers, M., de Weerd, A. W. et al.: Cognition and behavior in pre-pubertal children with Prader-Willi syndrome and associations with sleep-related breathing disorders. Am J Med Genet A, 146A: 3018, 2008
15. Siemensma, E. P., Tummers-de Lind van Wijngaarden, R. F., Festen, D. A. et al.: Beneficial effects of growth hormone treatment on cognition in children with Prader-Willi syndrome: a randomized controlled trial and longitudinal study. J Clin Endocrinol Metab, 97: 2307, 2012
16. Domes, G., Heinrichs, M., Michel, A. et al.: Oxytocin improves "mind-reading" in humans. Biol Psychiatry, 61: 731, 2007
17. Hollander, E., Bartz, J., Chaplin, W. et al.: Oxytocin increases retention of social cognition in autism. Biol Psychiatry, 61: 498, 2007
18. Hollander, E., Novotny, S., Hanratty, M. et al.: Oxytocin infusion reduces repetitive behaviors in adults with autistic and Asperger's disorders. Neuropsychopharmacology, 28: 193, 2003
19. Tauber, M., Mantoulan, C., Copet, P. et al.: Oxytocin may be useful to increase trust in others and decrease disruptive behaviours in patients with Prader-Willi syndrome: a randomised placebo-controlled trial in 24 patients. Orphanet J Rare Dis, 6: 47, 2011
20. Einfeld, S. L., Smith, E., McGregor, I. S. et al.: A double-blind randomized controlled trial of oxytocin nasal spray in Prader Willi syndrome. Am J Med Genet A, 164A: 2232, 2014

21. Schonbeck, Y., Talma, H., van Dommelen, P. et al.: The world's tallest nation has stopped growing taller: the height of Dutch children from 1955 to 2009. Pediatr Res, 73: 371, 2013
22. Schonbeck, Y., Talma, H., van Dommelen, P. et al.: Increase in prevalence of overweight in Dutch children and adolescents: a comparison of nationwide growth studies in 1980, 1997 and 2009. PLoS One, 6: e27608, 2011
23. Dykens, E. M., Maxwell, M. A., Pantino, E. et al.: Assessment of hyperphagia in Prader-Willi syndrome. Obesity (Silver Spring), 15: 1816, 2007
24. Ho, A. Y., Dimitropoulos, A.: Clinical management of behavioral characteristics of Prader-Willi syndrome. Neuropsychiatr Dis Treat, 6: 107, 2010
25. Grinevich, V., Desarmenien, M. G., Chini, B. et al.: Ontogenesis of oxytocin pathways in the mammalian brain: late maturation and psychosocial disorders. Front Neuroanat, 8: 164, 2014
26. Meziane, H., Schaller, F., Bauer, S. et al.: An Early Postnatal Oxytocin Treatment Prevents Social and Learning Deficits in Adult Mice Deficient for Magel2, a Gene Involved in Prader-Willi Syndrome and Autism. Biol Psychiatry, 78: 85, 2015
27. Siemensma, E. P., de Lind van Wijngaarden, R. F., Otten, B. J. et al.: Testicular failure in boys with Prader-Willi syndrome: longitudinal studies of reproductive hormones. J Clin Endocrinol Metab, 97: E452, 2012
28. Siemensma, E. P., de Lind van Wijngaarden, R. F., Otten, B. J. et al.: Pubarche and Serum Dehydroepiandrosterone Sulfate Levels in Children with Prader-Willi Syndrome. Clin Endocrinol (Oxf), 2011
29. Bakker, N. E., Wolffenbuttel, K. P., Looijenga, L. H. et al.: Testes in infants with Prader-Willi syndrome: human chorionic gonadotropin treatment, surgery and histology. J Urol, 193: 291, 2015
30. Lindgren, A. C., Barkeling, B., Hagg, A. et al.: Eating behavior in Prader-Willi syndrome, normal weight, and obese control groups. J Pediatr, 137: 50, 2000

CHAPTER 5

# Beneficial effects of growth hormone in young adults with Prader-Willi syndrome: a 2-year cross-over trial

R.J. Kuppens, N.E. Bakker, E.P.C. Siemensma,
R.F.A. Tummers-de Lind van Wijngaarden, S.H. Donze, D.A.M. Festen,
A.A.E.M. van Alfen-van der Velden, T. Stijnen, A.C.S. Hokken-Koelega

*Journal of Clinical Endocrinology and Metabolism 2016; in press*

## ABSTRACT

**Context** Patients with Prader-Willi syndrome (PWS) are severely at risk to develop morbid obesity, diabetes mellitus type 2 and cardiovascular disease, leading to high mortality. They have an increased fat mass (FM) and decreased lean body mass (LBM). During childhood, growth hormone (GH) treatment counteracts the natural course of increasing obesity. Discontinuation of GH treatment at attainment of adult height (AH) might deteriorate their improved clinical condition, whereas continuation might benefit them.

**Objective** To investigate the effects of GH versus placebo on body composition in young adults with PWS who were GH-treated for many years during childhood and had attained AH.

**Design** 2-year, randomized, double-blind, placebo-controlled cross-over study with stratification for gender and BMI in 27 young adults with PWS.

**Setting** PWS Reference Center in the Netherlands.

**Intervention** Cross-over intervention with GH (0.67 mg/$m^2$/day) and placebo, both during 1 year.

**Main outcome measures** Body composition, measured by DXA.

**Results** During placebo, FM increased (relative change +21.5%, $p<0.001$). Compared to placebo, GH treatment resulted in lower FM (-2.9 kg, $p=0.004$) and higher LBM (+1.5 kg, $p=0.005$), representing relative changes of -17.3% FM and +3.5% LBM. Both $FM\%_{limb}$ and $FM\%_{trunk}$ were lower during GH versus placebo (relative change +17.3% and +15.6%, $p<0.001$ and $p=0.007$, respectively). No GH-related adverse events occurred.

**Conclusion** GH-treated young adults with PWS who have attained AH benefit from continuation of GH treatment. FM increases during placebo, whereas GH versus placebo results in lower FM and higher LBM. Thus, GH treatment maintains the improved body composition without safety concerns.

## INTRODUCTION

Prader-Willi syndrome (PWS) is the most common genetic cause of life-threatening childhood obesity[1]. Hypothalamic dysfunction appears to be responsible for symptoms like severe hyperphagia, endocrine disorders, hypotonia, short stature, hypogonadism and an abnormal body composition[2]. Patients have a high body fat mass percentage (FM%) and a low lean body mass (LBM), even in the presence of a normal BMI[3-6].

The benefits of recombinant human growth hormone (GH) treatment in children with PWS are well established and GH treatment is approved by EMA and FDA. In children with PWS, BMI, FM% and LBM improve significantly during the first year of GH treatment and persist on the long-term[5, 7]. In addition, it is reported that GH has positive effects on BMD, psychomotor development, cognition, adaptive functioning, linear growth and adult height (AH)[5, 7-11], indicating that GH treatment is able to counteract the clinical course of PWS and has substantially changed the phenotype of children with PWS[5, 7].

When young adults with PWS without GH deficiency have attained AH, they have to cease GH treatment. We experienced that their body composition deteriorated after discontinuation of GH with an increase of FM% and a decrease in LBM. There are, however, no studies in PWS about the consequences of discontinuing GH treatment at AH. In addition, the effects of GH versus placebo on body composition in this new generation of PWS patients are also unknown. Studies in older PWS patients who were GH-untreated at inclusion, demonstrated that GH treatment decreased FM% and increased LBM[12, 13].

Given the well-established beneficial effects of GH on body composition during childhood, we hypothesized that placebo would deteriorate the body composition of young adults with PWS after attaining AH. Furthermore, we expected that GH versus placebo would maintain the improved body composition. We, therefore, investigated the effects of GH versus placebo on body composition, measured by DXA, in young adults with PWS who had attained AH, in a 2-year, randomized, double-blind, placebo-controlled cross-over study.

## METHODS

### Subjects

Inclusion criteria were (1) genetically confirmed diagnosis of PWS by a positive methylation test; (2) GH treatment during childhood for at least 2 years and being on GH at time of inclusion; and (3) AH attainment, defined as a height velocity less than 0.5 cm per 6 months and a complete epiphyseal fusion. Exclusion criteria were (1) medication to reduce weight (fat) or (2) non-cooperative behaviour.

From June 2008 to January 2014, 33 young adults with PWS fulfilled the inclusion criteria, but 2 did not want to continue daily injections and 3 refused due to too large burden of hospital visits. As low GH doses were not effective in improving body composition in children with PWS[14], patients were treated with the standard GH dose of 1.0 mg/m$^2$/day during childhood. GH dose was lowered in eight children, due to high serum IGF-I levels. In the present study, adolescent patients were in the transition phase and therefore GH dose was considerably lowered to 0.67 mg/m$^2$/day (≈0.023 mg/kg/day). Other medications were sex steroid replacement therapy in 12 (42.9%) young adults, thyroid hormone supplementation in eight (28.6%), modafinil in two (7.1%), and risperidone and citalopram in one (3.6%), and doses were not changed during the study. All patients were on a strict diet and exercise programs.

### Design

Two-year, randomized, double-blind, placebo-controlled, cross-over study investigating the effects of 1 year placebo versus 1 year GH on body composition. Young adults were stratified according to gender and BMI (below/above 25 kg/m$^2$) and then randomly and blindly assigned to receive 1 year of subcutaneous injections once daily at bedtime of either 0.67 mg/m$^2$/day GH (Genotropin®, 5 mg/ml, Pfizer) or 1 year of identical appearing placebo (Pfizer), after which they crossed-over to the alternative treatment for another year. An independent statistician generated the random allocation sequence. Investigators were blinded for the allocation. An independent physician monitored the safety during the study. During the entire study period, unblinding was not necessary.

### Measurements

Patients were assessed every 3 months by the PWS-team of the Dutch Growth Research Foundation in collaboration with pediatric endocrinologists and pediatricians. At each visit, the injection dose was adjusted to the calculated body surface area. In addition, patients visited the Sophia's Children Hospital at baseline, 6, 12, 18 and 24 months, to obtain: fat mass, FM%, LBM, height, weight, blood pressure, fasting blood levels of IGF-I, IGFBP-3, glucose and insulin, and (severe) adverse events ((S)AE).

FM and LBM were measured by DXA (Lunar Prodigy; GE Healthcare). All scans were made on same machine, with daily quality assurance. The intra-assay coefficients of variation were 0.41-0.88% for fat tissue and 1.57-4.49% for LBM[15]. LBM was calculated as fat-free mass minus bone mineral content. FM was also expressed as percentage of total body weight (FM%). FM% SDS and LBM SDS were calculated according to age- and sex-matched Dutch reference values[16].

Standing height was measured with a calibrated Harpenden stadiometer, weight was determined on a calibrated scale (ServoBalance KA-20-150S) and BMI was calculated.

Height, weight and BMI were expressed as SDS, adjusted for age and sex[17, 18]. SDS values were calculated with GrowthAnalyser 4.0.

After this 2-year study, GH treatment was discontinued for at least 6 weeks and fasting GH-stimulation test with bolus infusion of GHRH (1 µg/kg) and infusion of arginine (0.5 mg/kg) during 30 minutes was performed. The cut-off for GH deficiency[19] was 11.5 µg/l for BMI <25 kg/m$^2$, 8.0 µg/l for BMI 25-30 kg/m$^2$, and 4.2 µg/l for BMI >30 kg/m$^2$.

### Assays

Blood samples were collected after an overnight fast and measured in one laboratory. Glucose and insulin were immediately assayed[20]. IGF-I was measured using an immunometric technique on Immulite 1000 (LKGF1, Siemens Medical Solutions Diagnostics) with an interassay variation <7.3%, and IGFBP-3 by a specific RIA with an interassay variation <7.5%[21]. Levels of IGF-I and IGFBP-3 were expressed as SDS, adjusting for age and gender[21].

### Statistics

Statistical analysis was performed with SPSS 23.0. Calculation of sample size indicated that 20 subjects were required for a power of >90% with a significance level of 0.05. To account for attrition, 8 more patients were added. As data were normally distributed, parametric tests were used and data expressed as mean (standard deviation (SD)). Paired sample t-tests were used for differences over time during placebo. Effects of GH versus placebo were calculated using linear mixed model analysis with the outcomes measured at the end of the two treatment periods as dependent variable and with an unstructured covariance matrix. LBM was not adjusted for body size, as all had attained AH. Possible carry-over effects were analyzed by adding the interaction between period and treatment, but not found. Relative change during placebo was calculated as (delta during placebo)/(value at start of placebo). Relative change of GH versus placebo was calculated by (value after GH minus value after placebo)/(value after GH).

In addition to the 2-year cross-over data analysis, FM% SDS[16], LBM SDS[16], trunk and limb FM%, and GH dose during 4 years prior to attainment of AH were analyzed using mixed model analysis.

### Study approval

Written informed consent was obtained from patients and parents. The study protocol was approved by the Medical Ethics Committee of Erasmus University Medical Center, Rotterdam, and registered at Dutch Trial Register (www.trialregister.nl NTR1038).

## RESULTS

### Baseline characteristics

Twenty-eight subjects were included, but one 16.7-year old participant (BMI 25.0 kg/m$^2$) died due to gastric rupture 3 months after start while receiving placebo. Her data were not significantly different from the other patients and were excluded from analysis.

Mean age of the 27 young adults with PWS (8 boys, 19 girls) who completed all visits was 17.2 (1.8) years and BMI +0.9 (1.3) SDS (Table 1). At baseline (AH), FM% was significantly higher (1.9 SDS, p<0.001) and LBM significantly lower (-2.1 SDS, p<0.001) than average for age- and sexmatched controls, but similar in both treatment regimens (all p>0.487). During childhood, GH treatment was started at a mean age of 8.5 (3.5) years and continued for 8.7 (3.2) years until AH.

### Efficacy

*Placebo*

Figure 1 shows body composition during the 2-year cross-over study. During placebo, mean FM increased significantly (+4.1 kg, p<0.001), while LBM tended to decrease (-0.9 kg, p=0.069)(Table 2). This represents a relative change in FM of +21.5% and in LBM of -2.0%.

**Table 1.** Baseline characteristics of total group and per treatment schedule

| | PWS (n=27) | Placebo / GH (n=14)* | GH / Placebo (n=13)* |
|---|---|---|---|
| Boys / girls (n) | 8/19 | 4/10 | 4/9 |
| Genetic subtype | | | |
| Deletion | 9 | 2 | 7 |
| mUPD | 15 | 10 | 5 |
| ICD / translocation | 3 | 2 | 1 |
| Age (yrs) | 17.2 (1.8) | 17.2 (2.2) | 17.3 (1.2) |
| Height for age (SDS) | -1.3 (0.9) | -1.3 (0.9) | -1.2 (0.9) |
| Adult height (SDS) | -1.6 (1.0) | -1.7 (1.1) | -1.5 (0.9) |
| BMI for age (SDS) | 0.9 (1.3) | 1.0 (1.2) | 0.7 (1.3) |
| BMI for age PWS (SDS) | -1.4 (1.2) | -1.2 (1.2) | -1.5 (1.2) |
| Age at start GH treatment (yrs) | 8.5 (3.5) | 8.2 (3.8) | 8.9 (3.2) |
| Duration of GH treatment (yrs) | 8.7 (3.2) | 8.9 (3.8) | 8.4 (2.5) |
| IGF-I (SDS) | 2.2 (1.0) | 1.8 (1.2) | 2.5 (0.8) |
| FM% | 38.0 (10.9) | 39.4 (10.9) | 36.4 (11.0) |
| FM (kg) | 25.2 (10.0) | 26.3 (10.3) | 24.0 (9.9) |
| Lean (kg) | 37.5 (7.8) | 37.1 (8.3) | 37.9 (7.6) |

Data expressed as mean with (SD). *no significant differences between the two treatment schedules, all p-values above 0.128. GH: growth hormone, FM%: fat mass percentage, FM: fat mass.

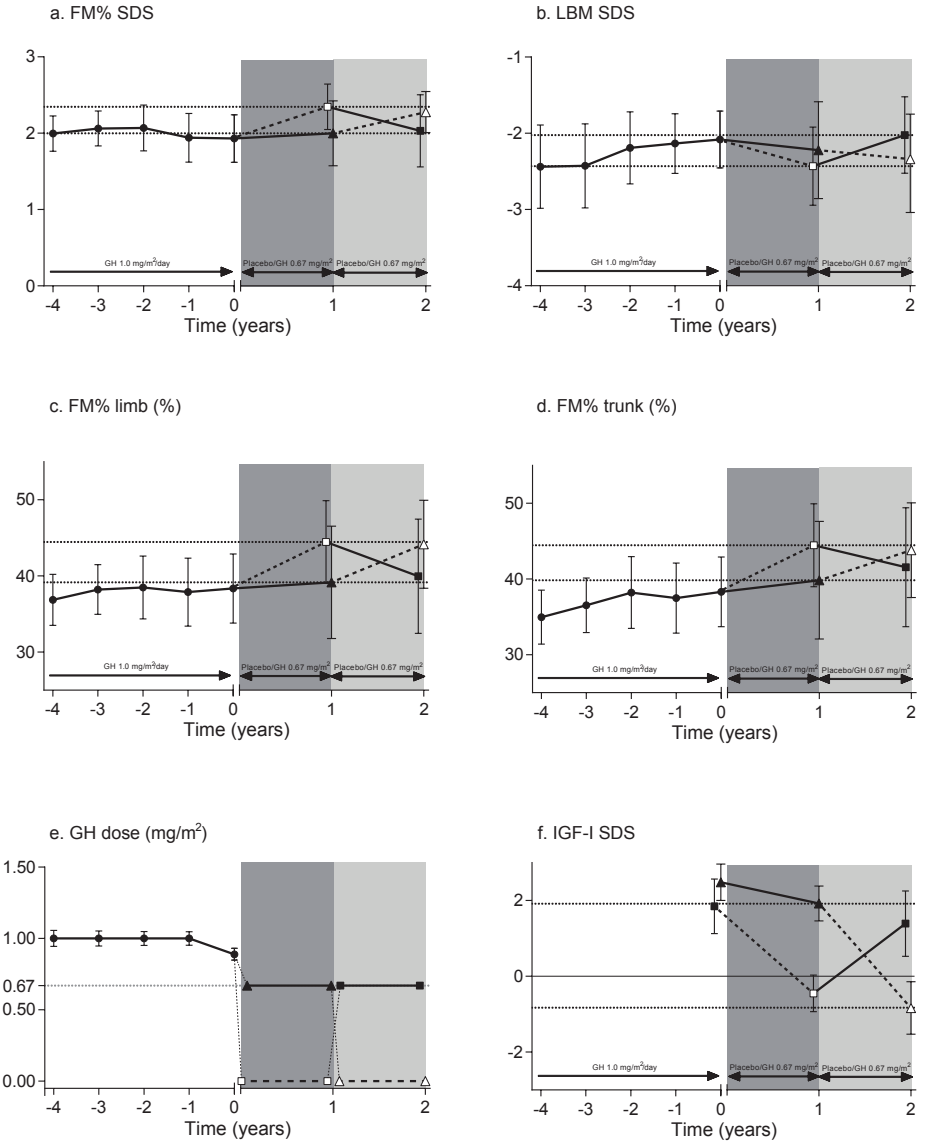



**Figure 1a-f.** Changes in body composition, GH dose and IGF-I SDS over time, presented as Means with 95%CI of 27 adolescents with PWS during the 2 years of this cross-over study (in dark and light grey). Longitudinal changes in Estimated Marginal Means with 95%CI in body composition and GH dose during the 4 years prior to attainment of AH (in white). FM% SDS, LBM SDS and IGF-I SDS were calculated according to age- and sex-matched Dutch references[16, 21]. Dotted lines to the white symbols represent the course during placebo.

**Table 2.** Body composition of 27 PWS adolescents at different stages in the study

| | Treatment schedule | | | | | | Relative change during placebo | Relative change GH vs placebo | Mean difference between GH and placebo | p-value* |
|---|---|---|---|---|---|---|---|---|---|---|
| | Placebo / GH (n=14) | | | GH / Placebo (n=13) | | | | | | |
| | Baseline | After 1 year placebo | After 1 year GH | Baseline | After 1 year GH | After 1 year placebo | | | | |
| FM (kg) | 26.3 (10.3) | 30.4 (9.9) | 29.1 (12.4) | 24.0 (9.9) | 27.4 (12.6) | 31.5 (11.7) | +21.5% | -17.3% | -2.9 | **0.004** |
| Total body FM% | 39.4 (10.9) | 43.7 (8.8) | 40.2 (12.0) | 36.4 (11.0) | 39.4 (11.8) | 43.7 (9.0) | +15.5% | -14.1% | -3.7 | **<0.001** |
| - Limb FM% | 39.8 (11.2) | 44.5 (9.4) | 40.0 (12.4) | 36.8 (12.0) | 39.2 (12.2) | 44.2 (9.6) | +17.3% | -17.1% | -4.7 | **<0.001** |
| - Trunk FM% | 40.1 (12.1) | 44.5 (9.5) | 41.6 (13.0) | 36.3 (11.3) | 39.8 (12.8) | 43.8 (10.3) | +15.6% | -13.1% | -3.3 | **0.007** |
| Total body LBM (kg) | 37.1 (8.3) | 36.1 (7.8) | 39.0 (8.7) | 37.9 (7.6) | 37.0 (6.9) | 36.3 (6.3) | -2.0% | +3.5% | 1.5 | **0.005** |
| - Limb LBM (kg) | 16.8 (4.4) | 16.2 (3.9) | 17.7 (4.1) | 16.7 (3.8) | 16.6 (3.4) | 16.0 (3.3) | -3.0% | +4.7% | 0.8 | **<0.001** |
| - Trunk LBM (kg) | 16.9 (3.7) | 16.6 (3.7) | 18.0 (4.3) | 17.9 (3.7) | 17.2 (3.3) | 17.1 (3.0) | -1.0% | +2.5% | 0.6 | 0.093 |
| BMI (kg/m$^2$) | 24.6 (4.1) | 26.1 (4.9) | 26.3 (5.2) | 23.6 (4.1) | 24.6 (5.0) | 25.9 (5.3) | +5.8% | -2.5% | -0.6 | 0.052 |
| - BMI SDS | 1.0 (1.2) | 1.2 (1.3) | 1.2 (1.4) | 0.7 (1.3) | 0.8 (1.4) | 1.0 (1.3) | x | x | -0.2 | **0.011** |
| - BMI PWS SDS | -1.2 (1.2) | -1.1 (1.2) | -1.3 (1.3) | -1.5 (1.2) | -1.6 (1.4) | -1.5 (1.3) | x | x | -0.1 | **0.028** |

Data expressed as mean with SD. FM: fat mass, FM%: fat mass percentage, LBM: lean body mass.

P-value of mean difference between GH and placebo, bolded p-values are p-values below 0.05.

Relative change during placebo: (delta during placebo)/(value at start of placebo). Relative change of GH versus placebo: (value after GH minus value after placebo)/(value after GH).

*GH versus placebo*

Table 2 shows the effects of GH versus placebo on body composition. Compared with placebo, GH treatment resulted in a lower mean FM (-2.9 kg, p=0.004) and higher LBM (+1.5 kg, p=0.005), representing a relative change of -17.3% FM and +3.5% LBM.

*Effects GH versus placebo on limb and trunk FM%*

During placebo, limb and trunk FM% increased (relative change +17.3% and +15.6%, both p<0.001), while limb LBM decreased (-3.0%, p=0.004). Compared to placebo, GH treatment decreased FM% of limbs and trunk with respectively -4.7% and -3.3% (p<0.001 and p=0.007, relative change -17.1% and -13.1%), and increased LBM of limbs with +0.8 kg (p<0.001, relative change +4.7%)(Table 2).

*GH-dose effect*

As GH effects might be dose-dependent[14], we investigated GH dose and serum IGF-I levels. The mean administered GH dose prior to present study was 0.88 (0.25) mg/m$^2$/day. In the 2-year cross-over study, GH dose was lower with 0.67 mg/m$^2$/day (p<0.001). Concurrent with the GH-dose reduction, IGF-I SDS decreased from 2.2 (1.0) SDS at baseline (AH) to 1.7 (1.2) during the GH year, although this was not significant (p=0.124). FM% increased in the group where the GH dose was lowered from 0.88 at baseline to 0.67 mg/m$^2$/day in the first year, while LBM remained stable (p=0.018 and p=0.122, resp.).

*GH deficiency*

After the 2-year study, twenty-four (88.9%) young adults underwent an arginine-GHRH test. Only 3 (12.5%) had a GH peak below the BMI-dependent cut-off[19]. There was no significant influence of the GH peak on the effects of GH versus placebo treatment on FM%, FM or LBM (p=0.649, p=0.170 and p=0.093).

*Other*

There was no difference in effect of GH versus placebo on FM, LBM or BMI between boys and girls, or between those whether or not receiving sex steroid replacement therapy.

## Safety

*Safety parameters*

GH treatment was very well tolerated. Compared to placebo, GH treatment resulted in a higher mean fasting glucose and insulin (+0.2 mmol/l, p=0.012, +18.4 pmol/l, p=0.037, resp.)(Table 3), but both remained below the upper limit in both phases. None of the patients developed T2DM. IGF-I and IGFBP-3 SDS were significantly lower during placebo than during GH (both p<0.001). Systolic and diastolic blood pressure were similar in both treatment phases.

**Table 3.** Safety parameters of 27 PWS adolescents at different stages in the study

| | Treatment schedule | | | | | | Mean difference between GH and placebo | p-value* |
|---|---|---|---|---|---|---|---|---|
| | Placebo / GH (n=14) | | | GH / Placebo (n=13) | | | | |
| | Baseline | After 1 year placebo | After 1 year GH | Baseline | After 1 year GH | After 1 year placebo | | |
| Glucose (mmol/l) | 5.0 (1.2) | 4.6 (0.5) | 4.6 (0.5) | 4.9 (0.3) | 4.6 (0.6) | 4.3 (0.3) | 0.2 | **0.012** |
| Insulin (pmol/l) | 87.6 (88.6) | 44.1 (43.5) | 56.4 (45.2) | 89.7 (60.7) | 72.3 (51.8) | 48.1 (30.2) | 18.4 | **0.037** |
| IGF-I SDS | 1.8 (1.2) | -0.5 (0.8) | 1.4 (1.5) | 2.5 (0.8) | 1.8 (1.1) | -0.8 (1.1) | 2.5 | **<0.001** |
| IGFBP-3 SDS | 0.4 (0.7) | -0.4 (0.6) | 0.3 (0.5) | 0.6 (0.5) | 0.4 (0.5) | -0.6 (0.8) | 1.0 | **<0.001** |
| Systolic BP (SDS) | 0.4 (1.0) | 0.8 (1.0) | 0.9 (1.1) | 0.6 (0.9) | 0.6 (0.8) | 0.5 (0.9) | 0.1 | 0.547 |
| Diastolic BP (SDS) | 0.6 (0.5) | 0.7 (0.8) | 0.8 (0.8) | 0.8 (0.6) | 0.8 (0.8) | 1.0 (0.7) | 0.0 | 0.779 |

Data expressed as mean with SD. P-value of mean difference between GH and placebo, bolded p-values are p-values below 0.05.
BP: blood pressure.

*Adverse events*

No (S)AEs considered to be GH-related were observed. During GH, no SAEs and 7 AEs occurred, all viral respiratory tract infections. During placebo, there were 2 SAEs and 12 AEs. First, a 16-year old female died 3 months after start of placebo due to gastric rupture with hemodynamic failure. Second, a 19-year old male with mUPD had a recurrence of psychosis after 6 months of placebo. He was treated with valproic acid in a psychiatric institution, whereupon he recovered. In both cases, unblinding was considered unnecessary, and after the study it appeared that both received placebo during the event. The 12 AEs during placebo were 1 patellar luxation, 1 knee rotation and 10 viral respiratory tract infections. In both phases, there were no cases of edema, arthralgia or carpal tunnel syndrome.

## DISCUSSION

This is the first randomized, double-blind, placebo-controlled cross-over GH study in young adults with PWS who were treated with GH during childhood and had attained AH. We demonstrate that the improved body composition which was attained during childhood deteriorates during placebo, with an increase in FM and BMI. Compared to placebo, GH treatment resulted in a lower FM and BMI, and higher LBM. We did not find GH-related adverse events. Thus, discontinuation of GH deteriorates body composition, while GH maintains the improved FM and LBM without adverse events.

Long-term GH treatment during childhood counteracts the clinical course of increasing obesity in PWS, with FM in the upper normal range and LBM in the lower normal range[5, 7]. Our study is the first placebo-controlled GH trial in young adults with PWS who had to cease GH because of attaining AH. In line with our expectations, we found a deterioration of FM during placebo. A study in 11 older GH-naïve adults with PWS showed that the improved body composition during 1-year GH treatment declined to pre-treatment levels after GH-withdrawal in the second year[22]. Another study in 14 adolescents with PWS who were GH-treated for 4 years demonstrated a significant increase of BMI SDS in the 2 years after cessation of GH[23]. These results concur with our findings that continuation of GH after attainment of AH benefits young adults with PWS, even in those without GH deficiency.

During placebo, mean FM rose impressively with a relative increase of 21.5% in 1 year. As FM references for the natural course of untreated PWS patients do not exist, BMI was used to compare. BMI of adolescent girls and boys with PWS aged 15-20 years increases with respectively +0.8 and +1.2 kg/m$^2$ per year[24]. Thus, the mean BMI-rise of +1.4 kg/m$^2$ during 1 year placebo aligns this natural development of increasing obesity in PWS.

Our trial shows that GH versus placebo results in a lower FM and BMI, and higher LBM. In the general population, less FM and lower BMI decrease the high risk of metabolic syndrome, T2DM and cardiovascular diseases[25, 26]. Not only total FM, but also the location of fat accumulation is important as more abdominal adipose tissue is associated with an unfavourable metabolic profile[27]. GH versus placebo reduced not only limb FM%, but also trunk FM%, emphasizing the importance of GH treatment for adults with PWS, as its continuation during adulthood might reduce the risk of future comorbidity.

It was demonstrated that children with PWS require high-normal to high serum IGF-I levels to maintain an improved body composition[5, 14]. However, we considered the approved GH dose for children with PWS of 1.0 mg/m2/day too high for young adults during the transition phase and we therefore treated all patients with 0.67 mg/m2/day. This GH-dose seems correct for the young adults during transition phase, as the majority had high-normal IGF-I levels without side effects. In the present study, we did neither titrate the GH dose on serum IGF-I levels nor on body composition. Further research is required to find the optimal GH dosing for young adults with PWS. Serum IGF-I levels seem inappropriate for titrating the GH dose in PWS, as they have a disrupted correlation between serum IGF-I levels and IGF-bioactivity, although this disruption is more pronounced in younger children than in the older ones[28].

Our other safety findings demonstrate that GH treatment induced only slightly higher fasting glucose and insulin levels compared to placebo, but both levels remained within normal ranges and no patients developed T2DM, in contrast to the high prevalence described[29]. GH versus placebo had no adverse effects on blood pressure. The two SAEs occurred during placebo, while none were reported during GH treatment. These findings indicate that GH during 1 year is not only an effective, but also a safe and well-tolerated treatment, although more research regarding the long-term effects, optimal dosing and clinical significance is required.

Our inclusion time was quite long, but PWS is a rare disorder and patients had to be GH-treated for at least 2 years. More female than male patients were included, probably due to the fact that the girls attained AH at a younger age. The ethical dilemma of 1 year placebo injections in mentally disabled young adults was extensively discussed with patients' caregivers. The high clinical relevance ensured them to vote for the strongest design, being a 2-year cross-over study allowing a smaller sample size.

Should GH treatment be continued when a young adult with PWS has attained adult height? The findings of our study suggest that the answer to the question is YES. The deterioration of body composition during placebo can be prevented by GH treatment.

In conclusion, this cross-over trial in young adults with PWS who were treated with GH during childhood shows that placebo treatment after AH deteriorates the improved body composition with an increase of FM. Compared to placebo, GH treatment results in a lower FM and higher LBM. This indicates that discontinuing GH treatment at AH

leads to deteriorated body composition, while GH maintains the improved FM and LBM without safety concerns.

## REFERENCES


1. Goldstone, A. P., Holland, A. J., Hauffa, B. P. et al.: Recommendations for the diagnosis and management of Prader-Willi syndrome. J Clin Endocrinol Metab, 93: 4183, 2008
2. Eiholzer, U., Bachmann, S., l'Allemand, D.: Is there growth hormone deficiency in prader-willi Syndrome? Six arguments to support the presence of hypothalamic growth hormone deficiency in Prader-Willi syndrome. Horm Res, 53 Suppl 3: 44, 2000
3. Holm, V. A., Cassidy, S. B., Butler, M. G. et al.: Prader-Willi syndrome: consensus diagnostic criteria. Pediatrics, 91: 398, 1993
4. Cassidy, S. B.: Prader-Willi syndrome. J Med Genet, 34: 917, 1997
5. Bakker, N. E., Kuppens, R. J., Siemensma, E. P. et al.: Eight years of growth hormone treatment in children with Prader-Willi syndrome: maintaining the positive effects. J Clin Endocrinol Metab, 98: 4013, 2013
6. Emerick, J. E., Vogt, K. S.: Endocrine manifestations and management of Prader-Willi syndrome. Int J Pediatr Endocrinol, 2013: 14, 2013
7. Carrel, A. L., Myers, S. E., Whitman, B. Y. et al.: Long-term growth hormone therapy changes the natural history of body composition and motor function in children with prader-willi syndrome. J Clin Endocrinol Metab, 95: 1131, 2010
8. Bakker, N. E., Kuppens, R. J., Siemensma, E. P. et al.: Bone mineral density in children and adolescents with Prader-Willi syndrome: a longitudinal study during puberty and 9 years of growth hormone treatment. J Clin Endocrinol Metab, 100: 1609, 2015
9. Festen, D. A., Wevers, M., Lindgren, A. C. et al.: Mental and motor development before and during growth hormone treatment in infants and toddlers with Prader-Willi syndrome. Clin Endocrinol (Oxf), 68: 919, 2008
10. Siemensma, E. P., Tummers-de Lind van Wijngaarden, R. F., Festen, D. A. et al.: Beneficial effects of growth hormone treatment on cognition in children with Prader-Willi syndrome: a randomized controlled trial and longitudinal study. J Clin Endocrinol Metab, 97: 2307, 2012
11. Lo, S. T., Festen, D. A., Tummers-de Lind van Wijngaarden, R. F. et al.: Beneficial Effects of Long-Term Growth Hormone Treatment on Adaptive Functioning in Infants With Prader-Willi Syndrome. Am J Intellect Dev Disabil, 120: 315, 2015
12. Hoybye, C., Hilding, A., Jacobsson, H. et al.: Growth hormone treatment improves body composition in adults with Prader-Willi syndrome. Clin Endocrinol (Oxf), 58: 653, 2003
13. Sode-Carlsen, R., Farholt, S., Rabben, K. F. et al.: One year of growth hormone treatment in adults with Prader-Willi syndrome improves body composition: results from a randomized, placebo-controlled study. J Clin Endocrinol Metab, 95: 4943, 2010
14. Carrel, A. L., Myers, S. E., Whitman, B. Y. et al.: Sustained benefits of growth hormone on body composition, fat utilization, physical strength and agility, and growth in Prader-Willi syndrome are dose-dependent. J Pediatr Endocrinol Metab, 14: 1097, 2001
15. Guo, Y., Franks, P. W., Brookshire, T. et al.: The intra- and inter-instrument reliability of DXA based on ex vivo soft tissue measurements. Obes Res, 12: 1925, 2004
16. Boot, A. M., Bouquet, J., de Ridder, M. A. et al.: Determinants of body composition measured by dual-energy X-ray absorptiometry in Dutch children and adolescents. Am J Clin Nutr, 66: 232, 1997
17. Fredriks, A. M., van Buuren, S., Wit, J. M. et al.: Body index measurements in 1996-7 compared with 1980. Arch Dis Child, 82: 107, 2000

18. Fredriks, A. M., van Buuren, S., Burgmeijer, R. J. et al.: Continuing positive secular growth change in The Netherlands 1955-1997. Pediatr Res, 47: 316, 2000
19. Corneli, G., Di Somma, C., Baldelli, R. et al.: The cut-off limits of the GH response to GH-releasing hormone-arginine test related to body mass index. Eur J Endocrinol, 153: 257, 2005
20. Kuppens, R. J., Diene, G., Bakker, N. E. et al.: Elevated ratio of acylated to unacylated ghrelin in children and young adults with Prader-Willi syndrome. Endocrine, 50: 633, 2015
21. Rikken, B., van Doorn, J., Ringeling, A. et al.: Plasma levels of insulin-like growth factor (IGF)-I, IGF-II and IGF-binding protein-3 in the evaluation of childhood growth hormone deficiency. Horm Res, 50: 166, 1998
22. Butler, M. G., Smith, B. K., Lee, J. et al.: Effects of growth hormone treatment in adults with Prader-Willi syndrome. Growth Horm IGF Res, 23: 81, 2013
23. Oto, Y., Tanaka, Y., Abe, Y. et al.: Exacerbation of BMI after cessation of growth hormone therapy in patients with Prader-Willi syndrome. Am J Med Genet A, 164A: 671, 2014
24. Hauffa, B. P., Schlippe, G., Roos, M. et al.: Spontaneous growth in German children and adolescents with genetically confirmed Prader-Willi syndrome. Acta Paediatr, 89: 1302, 2000
25. Mokdad, A. H., Ford, E. S., Bowman, B. A. et al.: Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. JAMA, 289: 76, 2003
26. Tirosh, A., Shai, I., Afek, A. et al.: Adolescent BMI trajectory and risk of diabetes versus coronary disease. N Engl J Med, 364: 1315, 2011
27. Despres, J. P., Lemieux, I.: Abdominal obesity and metabolic syndrome. Nature, 444: 881, 2006
28. Bakker, N. E., van Doorn, J., Renes, J. S. et al.: IGF-1 Levels, Complex Formation, and IGF Bioactivity in Growth Hormone-Treated Children With Prader-Willi Syndrome. J Clin Endocrinol Metab, 100: 3041, 2015
29. Sinnema, M., Maaskant, M. A., van Schrojenstein Lantman-de Valk, H. M. et al.: Physical health problems in adults with Prader-Willi syndrome. Am J Med Genet A, 155A: 2112, 2011

CHAPTER 6

# Metabolic health profile in young adults with PWS: Results of a 2-year randomized, placebo-controlled cross-over GH trial

R.J. Kuppens, N.E. Bakker, E.P.C. Siemensma, S.H. Donze, T. Stijnen, A.C.S. Hokken-Koelega

*Clinical Endocrinology 2016; in press*

## ABSTRACT

**Context** Patients with Prader-Willi syndrome (PWS) have an increased fat mass and decreased lean body mass. GH-treated young adults with PWS who have attained adult height benefit from continuation of growth hormone (GH) treatment, as GH maintained their improved body composition, whereas fat mass increased during the placebo period. Adults with PWS are predisposed to develop diabetes mellitus type 2 and cardiovascular disease. Whether GH affects metabolic health profile of this patient group is unknown.

**Objective** To investigate the effects of GH versus placebo on metabolic health, in young adults with PWS who were GH-treated for many years during childhood and had attained adult height (AH).

**Method** 2-year, randomized, double-blind, placebo-controlled cross-over study with stratification for gender and BMI in 27 young adults with PWS. Intervention with GH (0.67 mg/m$^2$/day) and placebo, both during 1 year.

**Results** Compared to placebo, GH treatment resulted in similar glucose and insulin levels during oral glucose tolerance test. Only fasting glucose and insulin were slightly higher during GH versus placebo (+0.2 mmol/l and +18.4 pmol/l), although both remained within normal ranges in both phases. Blood pressure and lipid profile were similar after GH versus placebo. At baseline (AH) and during GH, no patients had metabolic syndrome, while 1 developed it during placebo.

**Conclusion** GH treatment has no adverse effects on metabolic health profile. Thus, GH-treated young adults with PWS who have attained AH benefit from continuation of GH treatment without safety concerns regarding metabolic health.

## INTRODUCTION

Prader-Willi syndrome (PWS) is a neurogenetic disorder caused by the lack of expression of paternally expressed genes located on the PWS region of chromosome 15[1, 2], with clinical findings that change over age. Infancy is characterized by muscular hypotonia and failure to thrive, while obesity, hyperphagia, psychomotor delay and behavioural problems are prominent during childhood and adulthood[3, 4]. The body composition of patients with PWS is abnormal with increased fat mass (FM) and decreased lean body mass (LBM), even if there is no obesity[2, 4-8].

In children with PWS, the benefits of growth hormone (GH) treatment are well established without adverse effects on glucose parameters, lipid profile and blood pressure[5]. GH improves body composition, bone mineral density, psychomotor development, cognition, adaptive functioning, linear growth and adult height (AH)[5, 9-12] and has substantially changed the phenotype of children with PWS. Currently, when young adults with PWS without GH deficiency have attained AH, they have to cease GH treatment. We recently demonstrated an impressive increase in FM during the placebo period in GH-treated young adults with PWS who have attained AH, while continuation of GH maintains the improved body composition, indicating that they benefit from continuation of GH treatment[13].

Adults with PWS are predisposed to develop diabetes mellitus type 2 (T2DM) due to their abnormal body composition[14], and cardiovascular disease (CVD) as risk factors for CVD such as hypertension and hyperlipidemia occur more often in PWS[15]. GH has diabetogenic effects, but there are no studies about the metabolic effects of GH versus placebo in young adults with PWS who were treated with GH during childhood and had attained AH. Studies in older PWS-patients who were GH-untreated at inclusion, found no major side-effects of GH on metabolic health profile[16, 17].

As there were no negative effects of GH on the metabolic health profile in GH-treated children and GH-untreated adults with PWS, we hypothesized that GH versus placebo would result in a similar or even more favourable metabolic profile after attainment of AH. We, therefore, investigated the effects of GH versus placebo on the metabolic health profile in young adults with PWS after attainment of AH, in a 2-year, randomized, double-blind cross-over study.

## SUBJECTS AND METHODS

### Subjects

Inclusion criteria were (1) genetically confirmed diagnosis of PWS by a positive methylation test; (2) GH treatment during childhood for at least 2 years and being on GH at time

of inclusion; and (3) AH attainment, defined as a height velocity less than 0.5 cm per 6 months and a complete epiphyseal fusion. Exclusion criteria were (1) medication to reduce weight (fat) or (2) non-cooperative behaviour.

From June 2008 to January 2014, 33 young adults with PWS aged 14.1-20.2 years fulfilled the inclusion criteria, but 2 did not want to continue daily injections and 3 refused to participate due to too large burden of hospital visits. Twenty-eight subjects were included, but one 16.7-year old participant (BMI 25.0 kg/m$^2$) died due to gastric rupture 3 months after start while receiving placebo. Her baseline characteristics were not significantly different from the other patients and were excluded from analysis.

GH treatment was prescribed during childhood at an initial dose of 1 mg/m$^2$/day and dose was lowered in 8 children due to high serum IGF-I levels. In the present study, GH dose was 0.67 mg/m$^2$/day (≈0.023 mg/kg/day), which was considerably lower than during childhood. The most important other medications were sex steroid replacement therapy in 12 (44.4%) young adults, thyroid hormone supplementation in 8 (29.6%), modafinil in 2 (7.4%) and risperidone and citalopram in 1 (3.7%) adolescent, and doses were not changed during the study. All patients were on a strict diet and exercise programs.

### Design

Two-year, randomized, double-blind, placebo-controlled, cross-over study investigating the effects of 1 year placebo versus 1 year GH on metabolic health profile. Young adults were stratified according to gender and BMI (below/above 25 kg/m$^2$) and then randomly and blindly assigned to receive 1 year of subcutaneous injections once daily at bedtime of either 0.67 mg/m$^2$/day GH (Genotropin®, 5 mg/ml, Pfizer) or 1 year of identical appearing placebo (placebo, Pfizer), after which they crossed-over to the alternative treatment for another year. According to the FDA, a wash-out period should be at least 3 times the half life time of a drug. As the half life time of GH is only 2-3 hours and the study duration per phase was 1 year, no wash-out period was implemented. An independent statistician generated the random allocation sequence. Investigators were blinded for the allocation. An independent physician monitored the safety during the study. During the entire study period, unblinding was not necessary.

### Measurements

Patients were assessed every 3 months by the PWS team of the Dutch Growth Research Foundation in collaboration with pediatric endocrinologists and pediatricians. At each visit, the injection dose was adjusted to the calculated body surface area. In addition, patients visited the Sophia's Children Hospital at baseline, 6, 12, 18 and 24 months, to obtain: height, weight, waist circumference (WC), blood pressure, fasting blood levels of glucose and insulin, total cholesterol (TC), low-density lipoprotein cholesterol (LDLc)

and high-density lipoprotein cholesterol (HDLc), triglyceride (TG), IGF-I and IGFBP-3. In addition, a standard 75-g oral glucose tolerance test (OGTT) according to the World Health Organization was performed. To evaluate the overall responses to the oral glucose load, apart from the plasma levels at various time points, the 120-min area under the curve (AUC) for time-concentration for glucose and insulin was calculated using the trapezoidal rule. The insulin/glucose ratio at 0, 30 and 120 minutes was calculated as an index of relative insulin resistance. Impaired glucose tolerance (IGT) was defined as a fasting glucose level <6.1 mmol/l, and glucose between 7.8 and 11.1 mmol/l (140-200 mg/dl) 120 minutes after glucose load [18].

Standing height was measured with a calibrated Harpenden stadiometer, weight was determined on a calibrated scale (ServoBalance KA-20-150S) and BMI was calculated. Height, weight and BMI were expressed as SDS, adjusted for age and sex[19, 20]. SDS values were calculated with GrowthAnalyser 4.0 (www.growthanalyser.org). Systolic and diastolic blood pressure (BP) were measured using an appropriately sized cuff while patients were in sitting position. As height is an important determinant of BP, BP was expressed as SDS, adjusted for height and sex[21]. Fat mass percentage (FM) was measured by DXA[13].

### Assays

Blood samples were collected after an overnight fast and measured in one laboratory: glucose, insulin, IGF-I, IGFBP-3, TC, HDLc and TG. LDLc was calculated using the Friedewald formula: LDLc (mmol/l) = TC–HDLc–0.45*TG[22]. Blood samples during the OGTT for glucose and insulin determination were obtained after 30, 60, 90 and 120 minutes after ingestion and immediately assayed[23]. Levels of IGF-I and IGFBP-3 were measured and expressed as SDS, adjusting for age and gender[13, 24]. Lipids were determined as described[25].

### Metabolic syndrome

Revised criteria of the National Cholesterol Education Program (NCEP; Adult Treatment Panel III) were used to determine components of metabolic syndrome[26]. Metabolic syndrome was defined as having 3 or more of the following risk factors: 1) Abdominal obesity: waist circumference in men greater than 102 cm, and in women greater than 88 cm; 2) TG above 1.7 mmol/l; 3) HDLc in men below 1.03, in women below 1.3 mmol/l; 4) BP ≥130/≥85 mm Hg; 5) Fasting glucose above 5.6 mmol/l.

### Statistics

Statistical analysis was performed with SPSS version 23.0. Calculation of sample size indicated that 20 subjects would be sufficient for detection at a power of >90% with a significance level of 0.05. To account for attrition, 8 more patients were added. As data were normally distributed, parametric tests were used and data expressed as mean

(standard deviation (SD)). Effects of GH versus placebo were calculated using linear mixed model analysis with the outcomes measured at the end of the two treatment periods as dependent variable and with an unstructured covariance matrix. Possible carry-over effects were analyzed by adding the interaction between period and treatment, but not found. In addition to the 2-year results, we also presented systolic and diastolic BP SDS and lipid levels during the 4 years prior to attainment of adult height, as significant changes in the years prior to the study might influence the interpretation. Linear mixed model analysis was used.

### Study approval

Written informed consent was obtained from the patients and their caregivers. The study protocol was approved by the Medical Ethics Committee of the Erasmus University Medical Center, Rotterdam, and registered at Dutch Trial Register (www.trialregister.nl NTR1038).

## RESULTS

### Baseline characteristics

Table 1 shows the clinical characteristics of 27 young adults (8 boys, 19 girls) with PWS at adult height (AH). Their mean age was 17.2 (1.8) years and BMI +0.9 (1.3) SDS. Nine

**Table 1.** Baseline characteristics of total group and per treatment schedule

| | PWS (n=27) | GH / Placebo (n=13) | Placebo / GH (n=14) | p* |
|---|---|---|---|---|
| Boys / girls (n) | 8/19 | 4/9 | 4/10 | |
| Genetic subtype | | | | |
| Deletion | 9 | 7 | 2 | |
| mUPD | 15 | 5 | 10 | |
| ICD / translocation | 3 | 1 | 2 | |
| Age (yrs) | 17.2 (1.8) | 17.3 (1.2) | 17.2 (2.2) | 0.877 |
| Height for age (SDS) | -1.3 (0.9) | -1.2 (0.9) | -1.3 (0.9) | 0.695 |
| Adult height (SDS) | -1.6 (1.0) | -1.5 (0.9) | -1.7 (1.1) | 0.660 |
| Weight for height (SDS) | 0.6 (1.3) | 0.5 (1.4) | 0.8 (1.2) | 0.563 |
| BMI for age (SDS) | 0.9 (1.3) | 0.7 (1.3) | 1.0 (1.2) | 0.527 |
| BMI for age PWS (SDS) | -1.4 (1.2) | -1.5 (1.2) | -1.2 (1.2) | 0.506 |
| Fat mass percentage (%) | 38.0 (10.9) | 36.4 (11.0) | 39.4 (10.9) | 0.487 |
| Age at start GH treatment (yrs) | 8.5 (3.5) | 8.9 (3.2) | 8.2 (3.8) | 0.646 |
| Duration of GH treatment (yrs) | 8.7 (3.2) | 8.4 (2.5) | 8.9 (3.8) | 0.679 |

Data expressed as mean with (SD). *p-value at baseline between the two treatment schedules.

(33.3%) patients had a deletion, 15 (55.6%) an mUPD, 2 (7.4%) an ICD and 1 (3.7%) a translocation. During childhood, GH treatment was started at a mean age of 8.5 (3.5) years and continued for 8.7 (3.2) years until AH. Boys had a mean AH of 174.1 (3.0) cm, being -1.4 (0.4) SDS and girls reached 159.9 (7.3) cm, being -1.7 (1.1) SDS. Both treatment regimens had similar baseline characteristics.

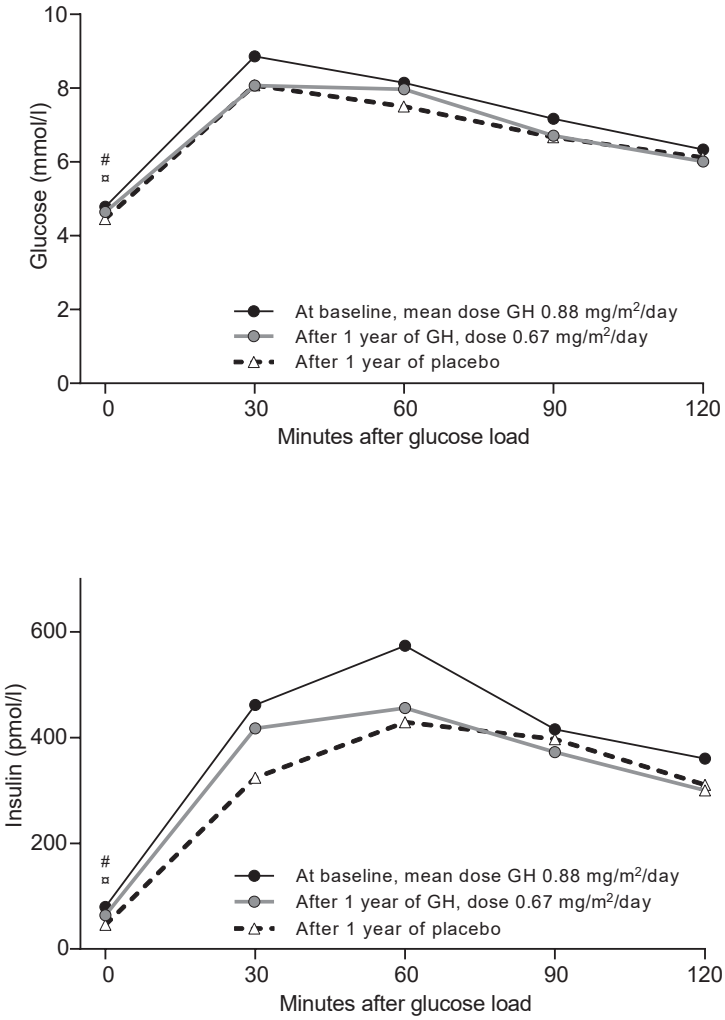



**Figure 1.** Mean glucose and insulin levels during OGTT after long-term GH treatment during childhood at baseline (AH) (black circles), after 1 year of GH treatment (grey circles), and after 1 year of placebo (white triangles) of 27 young adults with PWS. #GH vs placebo p-value <0.05, ¤Baseline vs placebo p-value <0.05, other not significantly different.

**Table 2.** Metabolic Health parameters of 27 PWS adolescents at different stages in the study

| | Stage of the study | | | Mean difference between GH and placebo | p-value* |
|---|---|---|---|---|---|
| | Baseline | After 1 year GH | After 1 year placebo | | |
| *Carbohydrate data* | | | | | |
| Fasting glucose (mmol/l) | 4.8 (4.6-4.9) | 4.7 (4.5-4.9) | 4.5 (4.3-4.7) | 0.2 | **0.012** |
| AUC glucose (mmol/l*120 min) | 897 (824-971) | 855 (782-929) | 808 (729-887) | 47 | 0.343 |
| Fasting insulin (pmol/l) | 79.4 (58.4-100.3) | 65.8 (49.1-82.5) | 47.4 (30.7-64.1) | 18.4 | **0.037** |
| AUC insulin (pmol/l*120 min*$10^3$) | 47.4 (36.6-58.2) | 43.5 (32.1-54.8) | 39.4 (27.7-51.0) | 4.1 | 0.457 |
| Ratio ins/gluc at 30 min | 52.2 (38.5-65.9) | 50.7 (38.1-63.3) | 41.2 (28.2-54.3) | 9.5 | 0.205 |
| Ratio ins/gluc at 120 min | 55.0 (39.4-70.7) | 50.7 (36.5-65.0) | 53.5 (38.1-68.9) | -2.8 | 0.752 |
| *Blood pressure* | | | | | |
| Systolic BP (SDS) | 0.5 (0.1-0.8) | 0.8 (0.4-1.1) | 0.6 (0.3-1.0) | 0.1 | 0.547 |
| Diastolic BP (SDS) | 0.7 (0.4-0.9) | 0.8 (0.5-1.1) | 0.9 (0.6-1.2) | 0.0 | 0.779 |
| *Serum lipids* | | | | | |
| TC (mmol/l) (*3.0-5.5) | 4.4 (4.1-4.7) | 4.5 (4.3-4.8) | 4.6 (4.4-4.8) | 0.0 | 0.851 |
| LDLc (mmol/l) (*1.7-3.8) | 2.7 (2.4-3.0) | 2.8 (2.6-3.0) | 2.8 (2.6-3.0) | 0.0 | 0.711 |
| HDLc (mmol/l) (*0.9-1.9) | 1.5 (1.3-1.6) | 1.5 (1.3-1.6) | 1.5 (1.3-1.6) | 0.0 | 0.974 |
| TG (mmol/l) (*0.4-1.6) | 0.9 (0.8-1.0) | 0.9 (0.7-1.0) | 0.8 (0.7-0.9) | 0.1 | 0.415 |
| *Growth factors* | | | | | |
| IGF-I SDS | 2.2 (1.7-2.6) | 1.8 (1.4-2.2) | -0.7 (-1.1--0.3) | 2.5 | **<0.001** |
| IGFBP-3 SDS | 0.5 (0.2-0.7) | 0.4 (0.2-0.6) | -0.6 (-0.8--0.3) | 1.0 | **<0.001** |

Data expressed as mean with 95%CI. *normal range for 13-18y. P-value of mean difference between GH and placebo, bolded p-values are p-values below 0.05.
GH: growth hormone, AUC: area under the curve, ins/gluc: ratio of insulin/glucose, BP: blood pressure, TC: total cholesterol, TG: triglyceride.

## Metabolic Health Profile

*Carbohydrate metabolism*

Figure 1 and Table 2 show glucose and insulin levels during OGTT after long-term GH treatment during childhood, at baseline of the present study at adult height (AH), after 1 year of GH and after 1 year of placebo. At baseline, fasting glucose was in none of the patients above 5.6 mmol/l, while IGT, defined as a glucose between 7.8 and 11.1 mmol/l at 120 minutes after glucose load, was present in 4 patients and none had T2DM.

Compared to placebo, GH treatment resulted in similar glucose and insulin levels at 30, 60, 90 and 120 minutes after glucose load (Figure 1). Only fasting glucose and insulin levels were higher after GH treatment versus placebo, although both remained within the normal ranges in both phases (glucose 4.7 versus 4.5 mmol/l, p=0.012, and insulin 65.8 versus 47.4 pmol/l, p=0.037, resp.)(Table 2). All other carbohydrate parameters were similar after GH versus placebo. Mean glucose at 120 minutes after glucose intake was similar (GH vs placebo 6.0 versus 6.1 mmol/l, p=0.998). The 120-min area under the curves (AUC) for glucose and insulin during OGTT were not significantly different after both treatment phases (p=0.343 and p=0.457, resp.), and the insulin/glucose ratios at 30 and 120 minutes were similar after GH and placebo. IGT was present in 2 patients after 1 year of GH and in 2 other patients after 1 year of placebo. None of the patients developed T2DM.

*Blood pressure*

Figure 2 shows systolic and diastolic BP during the 2-year cross-over study. At baseline, mean systolic and diastolic BP were significantly higher than height- and sexmatched controls (0.5 and 0.7 SDS, p=0.016 and p<0.001, resp.)(Table 2), but there were only 2 patients with a systolic BP above +2 SDS (both +2.2 SDS).

Compared to placebo, GH treatment resulted in a similar systolic and diastolic BP (p=0.547 and p=0.779). Four young adults had a systolic BP above +2 SDS after 1 year of GH, while 2 of them and 1 other had an elevated systolic BP after 1 year of placebo. Two young adults had a diastolic BP above +2 SDS after both GH and placebo, and 1 only after 1 year of placebo.

*Serum lipid levels*

Figure 3 shows fasting serum lipid levels during the 2-year cross-over study. At baseline, all mean lipid levels were within the normal range (Table 2). Levels above the normal range of TC and LDLc were found in respectively 2 and 1 patients, while 1 patient had a HDLc below the normal range.

Compared to placebo, GH treatment resulted in similar levels of TC, LDLc, HDLc and TG (p>0.415). One young adult had a TC above the upper limit of 5.5 mmol/l after 1 year of GH, while another had an elevated TC after 1 year of placebo. LDLc and TG were not

elevated after 1 year of GH treatment, while after 1 year of placebo, 1 patient had an LDLc higher than the upper limit of 3.8 mmol/l, and this and another patient had TG level higher than 1.6 mmol/l. HDLc was in none below the lower limit of 0.9 mmol/l.

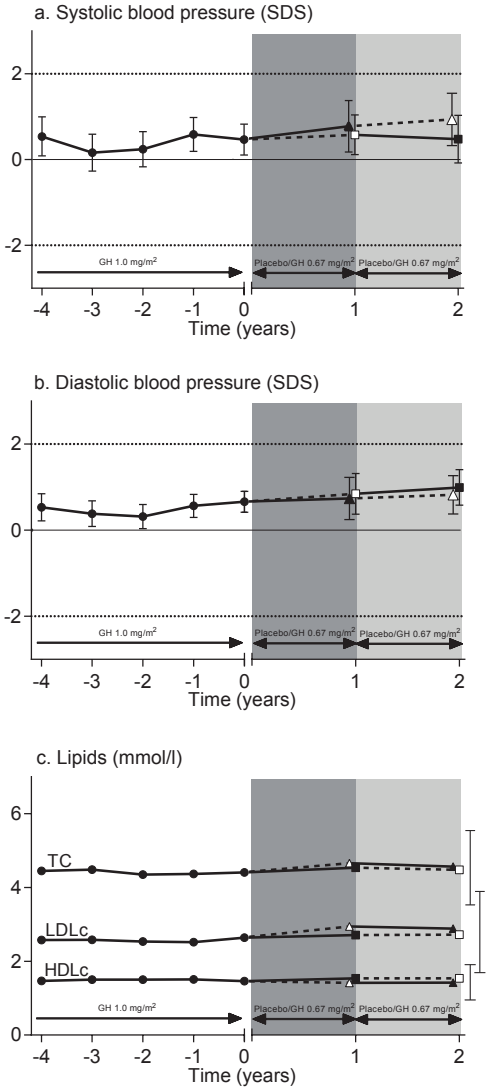



**Figure 2a-c.** Changes in systolic (fig 2a) and diastolic blood pressure (fig 2b), and total cholesterol, LDLc and HDLc (fig 2c) presented as Means with 95%CI of 27 adolescents with PWS during the 2 years of this cross-over study (in dark and light grey). Longitudinal changes in Estimated Marginal Means with 95%CI during the 4 years prior to attainment of AH (in white). BP SDS was calculated according to height- and sex-matched references [21]. Normal ranges for lipids are shown on the right side of figure 2c. Black lines with black symbols represent the course during GH treatment and dotted lines with white symbols represent the course during placebo.

**Table 3.** Metabolic syndrome components during the study according to ATP III criteria[26]

| | Stage of the study | | |
|---|---|---|---|
| Symptoms | Baseline | During 1 year GH | During 1 year placebo |
| Central Obesity | 2/27 (7.4%) | 4/27 (14.8%) | 5/27 (18.5%) |
| High TG levels | None | None | 1/27 (3.7%) |
| Low HDLc levels | 6/27 (22.2%) | 3/27 (11.1%) | 6/27 (22.2%) |
| High BP | 3/27 (11.1%) | 5/27 (18.5%) | 4/27 (14.8%) |
| High fasting glucose | None | None | 1/27 (3.7%) |
| More than 3 symptoms | None | None | 1/27 (3.7%) |

Number of patients (%) with MS-symptoms. No significant difference between GH and placebo year.
GH: growth hormone, TG: triglyceride, BP: blood pressure.

*Metabolic syndrome*

Table 3 shows the different components of the metabolic syndrome (MS). At baseline (AH), none of the patients had MS according to the revised NCEP criteria[26].

After 1 year of GH treatment, 12 components of MS were present; 4 patients had central obesity, 3 low HDLc levels and 5 a high BP, while none had high TG levels or high fasting glucose. After 1 year of placebo, there were 17 components of MS; 5 had central obesity, 1 high TG levels, 6 low HDLc levels, 4 high BP and 1 a high fasting glucose.

Compared to placebo, GH treatment did not result in MS. During the two years of study, one girl (3.7%) developed MS. She had no MS-symptoms after 1 year of GH, but after the subsequent year with placebo 3 symptoms were present.



## DISCUSSION

To our knowledge, this is the first two-year, randomized, double-blind, placebo-controlled study in young adults with PWS who were treated with GH during childhood until AH, which investigates the effects of GH versus placebo on metabolic health. Our findings demonstrate that the glucose-stimulated glucose and insulin levels during OGTT were similar during GH treatment versus placebo. Compared to placebo, GH treatment did not affect other carbohydrate parameters as AUC of glucose and insulin, and insulin/glucose ratios. Fasting glucose and insulin levels were slightly higher during GH versus placebo, but remained within the normal ranges during both phases and none developed T2DM. The prevalence of IGT and MS were similar after GH treatment and placebo, and BP and lipid profiles were not significantly different after GH versus placebo. This indicates that GH versus placebo does not affect carbohydrate parameters excepted a slightly higher fasting glucose and insulin, and that GH versus placebo does not disturb components of metabolic health profile.

Compared to placebo, GH treatment did not affect glucose-stimulated glucose and insulin levels, AUC for glucose and insulin and insulin/glucose ratios. These results of the OGTT are reassuring and demonstrate that glucose and insulin homeostasis reacts properly without developing glucose intolerance during GH versus placebo. The minor increase in fasting insulin with minimal influences on glucose metabolism is a well-known and physiological phenomenon during GH treatment[5, 27]. Our finding that GH treatment has only minimal effects without being pathologic concur with the conclusion of Höybye *et al.* that GH treatment did not elicit pronounced adverse effect on glucose and insulin homeostasis in older previously GH-untreated adults with PWS[16].

Lipid levels remained similar during GH versus placebo, while studies in other patient groups such as GHD or obesity found beneficial effects of GH treatment on lipid profiles[28, 29]. Our findings might be the result of the already quite favourable lipid profile of patients with PWS and the fact that hypercholesterolemia does not seem to be frequent, probably due to the smaller amount of visceral fat[30]. Findings in older adults with PWS who were GH-untreated at inclusion were in line with our results[27], although Sode-Carlsen *et al.* reported a small reduction in only LDLc during GH compared to placebo[17]. Their patients were, however, GH-untreated and heavier at baseline, while our patients received already 8 years of GH treatment before they participated in the present study, and this might have influenced the findings.

In our cohort with patients who were GH-treated during childhood for many years, none had MS at attainment of AH and none developed MS during GH after AH, while one developed MS during placebo. In obese adults with PWS a presence of MS of 41.4% was found, while it was with 4.7% much lower in the non-obese PWS subjects[31]. The authors concluded that obesity status plays a main role in metabolic health. Elaborating on this, it might be that the role of body composition, and subsequently GH treatment, is even larger. Continuation of GH prevents deterioration of body composition[13], and thus favorably affects metabolic health.

Patients with PWS have a relatively high insulin sensitivity with high adiponectin levels, which are thought to be protective with regard to T2DM and CVD[30, 32-34]. Besides the favourable effects of GH on adiponectin[33], it is even more important that continuation of GH treatment at AH prevents the deterioration of body composition in young adults with PWS. During placebo, FM rose dramatically with a relative increase of 21.5% in only 1 year, while GH treatment maintained the improved body composition with less FM and more LBM[13]. Thus, when GH treatment stops, the natural course of increasing obesity in PWS is not counteracted anymore. As LBM is the primary tissue of insulin-stimulated glucose uptake, disposal and storage[35], and obesity and FM are associated with MS, T2DM and CVD[36], it is likely that the deterioration of the body composition due to discontinuation of GH will impair the glucose and insulin homeostasis over time. This argues that discontinuation of GH at AH might result in a worse metabolic health profile

on the long-term in young adults with PWS, and that the benefits of GH far outweigh the minimal effects on glucose and insulin homeostasis.

The percentage of patients with an mUPD was higher than previously described37, but nowadays an mUPD is more often found, probably related to the increasing maternal age at conception in Western countries. Not only being diagnosed with PWS, but also other aspects such as obesity, advanced age, positive family history and low LBM are risk factors for metabolic health. By choosing a cross-over design, inter-individual risk factors were eliminated and this strong design allowed a smaller sample size as PWS is a rare disorder. Ongoing monitoring of young adults with PWS receiving GH treatment is highly recommended as they are at risk to develop T2DM and CVD, although our study demonstrates reassuring findings. Additional studies are needed to confirm this on the longer term, as patients are still young and received only 1 year of GH treatment.

In conclusion, this cross-over study demonstrates that 1 year of GH treatment has no adverse effects on glucose homeostasis, with similar glucose-stimulated glucose and insulin levels during OGTT, AUC of glucose and insulin, and insulin/glucose ratios during GH treatment and placebo. Fasting glucose and insulin levels remained within the normal ranges and were only slightly higher during GH treatment versus placebo. Blood pressure and lipid profile remained similar in both phases. None of the patients developed MS during GH treatment, while 1 developed MS during placebo. Thus, GH-treated young adults with PWS who have attained AH benefit from continuation of GH treatment without safety concerns regarding their metabolic health profile.

## REFERENCES


1. Goldstone, A. P., Holland, A. J., Hauffa, B. P. et al.: Recommendations for the diagnosis and management of Prader-Willi syndrome. J Clin Endocrinol Metab, 93: 4183, 2008
2. Cassidy, S. B.: Prader-Willi syndrome. J Med Genet, 34: 917, 1997
3. Eiholzer, U., Bachmann, S., l'Allemand, D.: Is there growth hormone deficiency in prader-willi Syndrome? Six arguments to support the presence of hypothalamic growth hormone deficiency in Prader-Willi syndrome. Horm Res, 53 Suppl 3: 44, 2000
4. Holm, V. A., Cassidy, S. B., Butler, M. G. et al.: Prader-Willi syndrome: consensus diagnostic criteria. Pediatrics, 91: 398, 1993
5. Bakker, N. E., Kuppens, R. J., Siemensma, E. P. et al.: Eight years of growth hormone treatment in children with Prader-Willi syndrome: maintaining the positive effects. J Clin Endocrinol Metab, 98: 4013, 2013
6. Emerick, J. E., Vogt, K. S.: Endocrine manifestations and management of Prader-Willi syndrome. Int J Pediatr Endocrinol, 2013: 14, 2013
7. Brambilla, P., Bosio, L., Manzoni, P. et al.: Peculiar body composition in patients with Prader-Labhart-Willi syndrome. Am J Clin Nutr, 65: 1369, 1997
8. Bekx, M. T., Carrel, A. L., Shriver, T. C. et al.: Decreased energy expenditure is caused by abnormal body composition in infants with Prader-Willi Syndrome. J Pediatr, 143: 372, 2003
9. Bakker, N. E., Kuppens, R. J., Siemensma, E. P. et al.: Bone mineral density in children and adolescents with Prader-Willi syndrome: a longitudinal study during puberty and 9 years of growth hormone treatment. J Clin Endocrinol Metab, 100: 1609, 2015
10. Festen, D. A., Wevers, M., Lindgren, A. C. et al.: Mental and motor development before and during growth hormone treatment in infants and toddlers with Prader-Willi syndrome. Clin Endocrinol (Oxf), 68: 919, 2008
11. Siemensma, E. P., Tummers-de Lind van Wijngaarden, R. F., Festen, D. A. et al.: Beneficial effects of growth hormone treatment on cognition in children with Prader-Willi syndrome: a randomized controlled trial and longitudinal study. J Clin Endocrinol Metab, 97: 2307, 2012
12. Lo, S. T., Festen, D. A., Tummers-de Lind van Wijngaarden, R. F. et al.: Beneficial Effects of Long-Term Growth Hormone Treatment on Adaptive Functioning in Infants With Prader-Willi Syndrome. Am J Intellect Dev Disabil, 120: 315, 2015
13. Kuppens, R. J., Bakker, N. E., Siemensma, E.P. C. et al.: Beneficial effects of growth hormone in young adults with Prader-Willi syndrome: Results of a 2-year randomized, double-blind, placebo-controlled cross-over trial. J Clin Endocrinol Metab, 2016
14. Nagai, T., Mori, M.: Prader-Willi syndrome, diabetes mellitus and hypogonadism. Biomed Pharmacother, 53: 452, 1999
15. Laurier, V., Lapeyrade, A., Copet, P. et al.: Medical, psychological and social features in a large cohort of adults with Prader-Willi syndrome: experience from a dedicated centre in France. J Intellect Disabil Res, 59: 411, 2015
16. Hoybye, C., Hilding, A., Jacobsson, H. et al.: Growth hormone treatment improves body composition in adults with Prader-Willi syndrome. Clin Endocrinol (Oxf), 58: 653, 2003
17. Sode-Carlsen, R., Farholt, S., Rabben, K. F. et al.: One year of growth hormone treatment in adults with Prader-Willi syndrome improves body composition: results from a randomized, placebo-controlled study. J Clin Endocrinol Metab, 95: 4943, 2010
18. World Health Organization: Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia: report of a WHO/IDF consultation, 2006

19. Fredriks, A. M., van Buuren, S., Wit, J. M. et al.: Body index measurements in 1996-7 compared with 1980. Arch Dis Child, 82: 107, 2000
20. Fredriks, A. M., van Buuren, S., Burgmeijer, R. J. et al.: Continuing positive secular growth change in The Netherlands 1955-1997. Pediatr Res, 47: 316, 2000
21. Rosner, B., Prineas, R. J., Loggie, J. M. et al.: Blood pressure nomograms for children and adolescents, by height, sex, and age, in the United States. J Pediatr, 123: 871, 1993
22. Friedewald, W. T., Levy, R. I., Fredrickson, D. S.: Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem, 18: 499, 1972
23. Kuppens, R. J., Diene, G., Bakker, N. E. et al.: Elevated ratio of acylated to unacylated ghrelin in children and young adults with Prader-Willi syndrome. Endocrine, 50: 633, 2015
24. Rikken, B., van Doorn, J., Ringeling, A. et al.: Plasma levels of insulin-like growth factor (IGF)-I, IGF-II and IGF-binding protein-3 in the evaluation of childhood growth hormone deficiency. Horm Res, 50: 166, 1998
25. van der Steen, M., Lem, A. J., van der Kaay, D. C. et al.: Metabolic Health in Short Children Born Small for Gestational Age Treated With Growth Hormone and Gonadotropin-Releasing Hormone Analog: Results of a Randomized, Dose-Response Trial. J Clin Endocrinol Metab, 100: 3725, 2015
26. Grundy, S. M., Cleeman, J. I., Daniels, S. R. et al.: Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. Circulation, 112: 2735, 2005
27. Sanchez-Ortiga, R., Klibanski, A., Tritos, N. A.: Effects of recombinant human growth hormone therapy in adults with Prader-Willi syndrome: a meta-analysis. Clin Endocrinol (Oxf), 77: 86, 2012
28. Maison, P., Griffin, S., Nicoue-Beglah, M. et al.: Impact of growth hormone (GH) treatment on cardiovascular risk factors in GH-deficient adults: a Metaanalysis of Blinded, Randomized, Placebo-Controlled Trials. J Clin Endocrinol Metab, 89: 2192, 2004
29. Mekala, K. C., Tritos, N. A.: Effects of recombinant human growth hormone therapy in obesity in adults: a meta analysis. J Clin Endocrinol Metab, 94: 130, 2009
30. Talebizadeh, Z., Butler, M. G.: Insulin resistance and obesity-related factors in Prader-Willi syndrome: comparison with obese subjects. Clin Genet, 67: 230, 2005
31. Grugni, G., Crino, A., Bedogni, G. et al.: Metabolic syndrome in adult patients with Prader-Willi syndrome. Nutr Metab Cardiovasc Dis, 23: 1134, 2013
32. Zipf, W. B.: Glucose homeostasis in Prader-Willi syndrome and potential implications of growth hormone therapy. Acta Paediatr Suppl, 88: 115, 1999
33. Festen, D. A., van Toorenenbergen, A., Duivenvoorden, H. J. et al.: Adiponectin levels in prepubertal children with Prader-Willi syndrome before and during growth hormone therapy. J Clin Endocrinol Metab, 92: 1549, 2007
34. Pagano, C., Marin, O., Calcagno, A. et al.: Increased serum resistin in adults with prader-willi syndrome is related to obesity and not to insulin resistance. J Clin Endocrinol Metab, 90: 4335, 2005
35. Smith, A. G., Muscat, G. E.: Skeletal muscle and nuclear hormone receptors: implications for cardiovascular and metabolic disease. Int J Biochem Cell Biol, 37: 2047, 2005
36. Mokdad, A. H., Ford, E. S., Bowman, B. A. et al.: Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. JAMA, 289: 76, 2003
37. Angulo, M.A., Butler, M.G., Cataletto, M.E.: Prader-Willi syndrome: a review of clinical, genetic, and endocrine findings. J Endocrinol Invest, 38: 1249, 2015

CHAPTER 7

# Effect of cessation of GH treatment on cognition in young adults with Prader-Willi syndrome: Results of a 2-year cross-over GH trial

R.J. Kuppens, E.F. Mahabier, N.E. Bakker, E.P.C. Siemensma, S.H. Donze, A.C.S. Hokken-Koelega

*Submitted*

## ABSTRACT

**Background** Patients with Prader-Willi syndrome (PWS) have a cognitive impairment. Growth hormone (GH) treatment during childhood improves cognitive functioning, while cognition deteriorates in GH-untreated children with PWS. Cessation of GH treatment at attainment of adult height (AH) might deteriorate their GH-induced improved cognition, while continuation might benefit them. We, therefore, investigated the effects of placebo versus GH administration on cognition in young adults with PWS who were GH-treated for many years during childhood and had attained AH.

**Method** Two-year, randomized, double-blind, placebo-controlled cross-over study in 25 young adults with PWS. Cross-over intervention with placebo and GH (0.67 mg/$m^2$/day), both during 1 year.

**Results** Total (TIQ), verbal (VIQ) and performance IQ (PIQ) did not deteriorate during 1 year of placebo, compared to GH treatment ($p>0.322$). Young adults with a lower TIQ had significantly more loss of TIQ points during placebo versus GH, in particular VIQ decreased more in those with a lower VIQ. The effect of placebo versus GH on TIQ, VIQ and PIQ was not different for gender or genotype.

**Conclusions** Compared to GH treatment, 1 year of placebo did not deteriorate cognitive functioning of GH-treated young adults with PWS who have attained AH. However, patients with a lower cognitive functioning had more loss in IQ points during placebo versus GH treatment. Our findings are reassuring, but do, however, not exclude that cessation of GH treatment for many years could result in a gradual deterioration of cognitive functioning on the long term.

## INTRODUCTION

Prader-Willi syndrome (PWS) is a neurogenetic disorder resulting from the lack of expression of the PWS region on the paternally derived chromosome 15, caused by paternal deletion, maternal uniparental disomy (mUPD), imprinting center defect (ICD) or balanced translocation[1]. PWS is characterized by a number of symptoms, such as muscular hypotonia, short stature, abnormal body composition with high fat mass and low lean body mass, severe hyperphagia, behavioural problems and cognitive impairment[1-4].

MRI studies in PWS suggested that lower cortical complexity partially underlies cognitive impairment and developmental delay[5], with structural brain abnormalities and different neurodevelopmental patterns between children with a deletion and an mUPD[6]. Certain cognitive skills improved significantly during GH treatment, while GH-untreated children with PWS showed a deterioration of cognitive functioning[7, 8]. Children with an mUPD started off with lower visuospatial skills, but showed a significant improvement during 4 years of growth hormone (GH), resulting into a similar cognitive functioning in all genotypes[7].

GH treatment has also positive effects on body composition, BMD, psychomotor development, adaptive functioning, linear growth and adult height (AH)[4, 7-11]. As a result, GH treatment has substantially changed the phenotype of children with PWS[4, 11], but when they attain AH, they have to stop GH treatment because most do not fulfill the criteria of adult GH deficiency. There are no studies about cognition after stop of GH treatment in this new generation of PWS patients. In untreated adults with PWS, lower IGF-I levels were correlated with poorer intellectual skills[12], which might suggest that discontinuation of GH treatment, which decreases IGF-I, could be disadvantageous.

Given the positive effects of GH and IGF-I levels on cognition in children with PWS, we hypothesized that the cognition in young adults with PWS would deteriorate after cessation of GH treatment compared to the continuation of GH administration. We, therefore, investigated the effects of placebo versus GH on cognition in young adults with PWS who had attained AH, in a 2-year, randomized, double-blind, placebo-controlled cross-over study.



## METHODS

### Subjects

Inclusion criteria of the present study were (1) genetically confirmed diagnosis of PWS; (2) GH treatment during childhood for at least 2 years and being on GH at time of inclusion; and (3) AH attainment, defined as a height velocity less than 0.5 cm per 6 months and complete epiphyseal fusion. Exclusion criteria were (1) use of medication to reduce

weight; (2) non-cooperative behaviour; or (3) inability to perform cognitive tests. Due to the last exclusion criterion, 2 patients could not participate; 1 due to poor cognitive skills and a severe hearing impairment, and with an IQ of 58 who refused to speak in the hospital. From June 2008 to January 2014, 33 young adults fulfilled the inclusion criteria. Two did not want to continue GH-injections and 3 parents refused participation due to too large burden of hospital visits. Twenty-eight young adults (8 boys, 20 girls) with PWS aged 14.1-20.2 years were included in the GH/placebo study. One participant died due to gastric rupture 3 months after start while receiving placebo. Twenty-five young adults completed the present study.

During childhood, the standard GH dose was 1 mg/m$^2$/day. In the present study during transition from childhood into adulthood, GH dose was set lower at 0.67 mg/m$^2$/day (≈0.023 mg/kg/day). Twelve (48%) young adults used sex steroid replacement therapy, 7 (28%) thyroid hormone supplementation, 2 (8%) modafinil and 1 (4%) risperidone and citalopram. All patients were on a strict diet and an exercise program.

### Design

Two-year, randomized, double-blind, placebo-controlled, cross-over study investigating the effects of 1 year placebo versus 1 year GH on cognitive functioning. The duration per phase was 1 year in order to prevent retesting phenomenon. A clinically relevant deterioration of cognitive functioning was defined as a decrease of 5 IQ points, taking into account the significant improvement of IQ during 4 years GH treatment in children with PWS[7]. Young adults were stratified according to gender and BMI (below/above 25 kg/m$^2$) and then randomly and blindly assigned to receive 1 year of subcutaneous injections once daily at bedtime of either 0.67 mg/m$^2$/day GH (Genotropin®, 5 mg/ml, Pfizer) or 1 year of identical appearing placebo (placebo, Pfizer), after which they crossed-over to the alternative treatment for another year. An independent statistician generated the random allocation sequence. Investigators were blinded for the allocation. An independent physician monitored the safety during the study. During the entire study period, unblinding was not necessary.

### Measurements

Patients were three-monthly seen by the PWS-team of the Dutch Growth Research Foundation in collaboration with pediatric endocrinologists and pediatricians. At each visit, the injection dose was adjusted to the calculated body surface area. In addition, patients visited the Sophia's Children Hospital every 6 months and at baseline, 12 and 24 months the following data were obtained: cognitive functioning, anthropometric measurements, fasting blood levels of IGF-I, and (S)AE.

All cognitive measurements were performed by a psychologist experienced in testing young adults with PWS. The 11 recommended subscales of Wechsler Adult Intelligence

Scale 3rd Edition (WAIS-III) were used to assess total IQ (TIQ) in patients over 16 years of age[13]. Verbal IQ (VIQ) subtests were Vocabulary, Similarities, Arithmetic, Digit Span, Information and Comprehension. Performance IQ (PIQ) subtests were Picture Completion, Coding, Block design, Matrix Reasoning and Picture Arrangement. The 10 recommended subscales of Wechsler Intelligence Scale for Children 3rd Edition (WISC-III) were used to assess TIQ in 4 patients younger than 16 years[14]. VIQ subtests were Information, Similarities, Arithmetic, Vocabulary and Comprehension. PIQ subtests were Picture Completion, Coding, Picture Arrangement, Block Design and Visual Puzzles. It was reported that WISC IQ and WAIS IQ are comparable in 16 year old young adults[15]. In both tests, scores on all subtests were expressed as standard deviation scores, based on Dutch population data for the same age[13, 14]. Standard subtest scores ranged from 1 (-3 SDS) to 19 (+3 SDS), with a mean of 10 (0 SDS).

Standing height was measured with a calibrated Harpenden stadiometer, weight was determined on a calibrated scale (ServoBalance KA-20-150S) and BMI was calculated. Height, weight and BMI were expressed as SDS, adjusted for age and sex[16, 17]. SDS values were calculated with GrowthAnalyser 4.0.

### Assays

Blood samples were collected after an overnight fast and measured in one laboratory. Serum levels of IGF-I were measured and expressed as SDS, adjusting for age and gender[18, 19].

### Statistics

Statistical analysis was performed with SPSS version 23.0. Calculation of sample size indicated that 22 subjects were required for a power of >80% with a significance level of 0.05. As data were not normally distributed, nonparametric tests were used and data expressed as median (interquartile range (IQR)), unless otherwise described. Statistical analysis appropriate for cross-over trials were used, taking into account any carry-over or treatment-period effect, calculated by Wilcoxon Signed Rank test and Mann Whitney U tests, but these were not found. Correlations between effect of GH treatment and other parameters were assessed using Spearman's rho. Differences were considered significant if p-value was <0.05.

### Study approval

Written informed consent was obtained from patients and parents. The study protocol was approved by the Medical Ethics Committee of Erasmus University Medical Center, Rotterdam, and registered at Dutch Trial Register (www.trialregister.nl NTR1038).

## RESULTS

### Baseline characteristics

Median age of the 25 young adults with PWS (7 boys, 18 girls) with cognitive functioning tests was 17.8 (15.7 to 18.5) years and BMI was +1.1 (-0.8 to +1.7) SDS (Table 1). Nine (36%) patients had a deletion, 13 (52%) an mUPD, 2 (8%) an ICD and 1 (4%) a translocation. At baseline (AH), both treatment arms had similar characteristics.

### Cognitive Functioning at AH

During childhood, GH treatment was started at a median (IQR) age of 8.8 (6.3 to 10.1) years and patients were treated for 8.6 (7.0 to 10.5) years until AH. Median total IQ (TIQ) at AH was 62 (56 to 73) points, with a non-significantly higher verbal IQ (VIQ) of 65 (57 to 72) points than performance IQ (PIQ) of 60 (54 to 72) points (p=0.157)(Table 1).

**Table 1.** Baseline characteristics of total group and per treatment schedule

| | PWS (n=25) | Placebo / GH (n=12) | GH / Placebo (n=13) | p* |
|---|---|---|---|---|
| Boys / girls (n) | 7/18 | 3/9 | 4/9 | |
| Genetic subtype | | | | |
| Deletion | 9 | 2 | 7 | |
| mUPD | 13 | 8 | 5 | |
| ICD / translocation | 3 | 2 | 1 | |
| Age (yrs) | 17.8 (15.7 to 18.5) | 17.2 (14.9 to 19.4) | 17.8 (16.9 to 18.0) | 0.852 |
| Adult height (SDS) | -1.7 (-2.2 to -1.0) | -1.7 (-2.4 to -1.1) | -1.8 (-2.0 to -0.9) | 0.852 |
| BMI for age (SDS) | 1.1 (-0.8 to 1.7) | 1.3 (-0.4 to 1.7) | 1.1 (-0.8 to 2.0) | 0.936 |
| BMI for age PWS (SDS) | -1.1 (-2.2 to -0.6) | -1.2 (-2.2 to -0.6) | -1.1 (-2.3 to -0.5) | 0.689 |
| Age at start GH treatment (yrs) | 8.8 (6.3 to 10.1) | 8.1 (5.8 to 9.8) | 8.8 (6.9 to 11.2) | 0.376 |
| Duration of GH treatment (yrs) | 8.6 (7.0 to 10.5) | 8.7 (7.2 to 11.4) | 8.3 (6.5 to 10.5) | 0.650 |
| IGF-I (SDS) | 2.1 (1.7 to 3.0) | 1.7 (1.1 to 3.0) | 2.2 (2.0 to 3.0) | 0.051 |
| FM% | 38.6 (32.3 to 44.9) | 39.0 (32.4 to 45.6) | 37.3 (30.5 to 44.9) | 0.810 |
| Lean body mass (kg) | 36.5 (30.6 to 41.5) | 35.0 (29.6 to 43.9) | 37.0 (33.2 to 40.2) | 0.611 |
| Total IQ | 62 (56 to 73) | 60 (54 to 63) | 67 (57 to 78) | 0.095 |
| Verbal IQ | 65 (57 to 72) | 66 (60 to 72) | 65 (55 to 74) | 0.713 |
| Performance IQ | 60 (54 to 72) | 59 (54 to 67) | 66 (52 to 74) | 0.635 |

Data expressed as median with (IQR). *p-value at baseline between the two treatment schedules.

### Placebo

TIQ, VIQ and PIQ had not changed after 1 year of placebo (p=0.422, p=0.220 and p=0.488) (Figure 1). The young adults had similar scores on the 11 subtests before and after 1 year of placebo (Table 2).

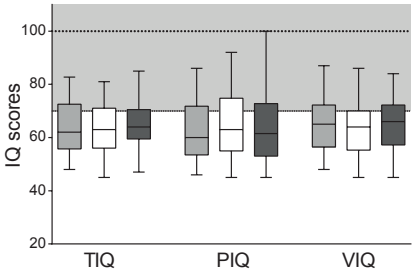



**Figure 1.** Cognitive functioning at baseline, after 1 year of GH treatment and after 1 year of placebo. Total, performance and verbal IQ at baseline (in light grey), after 1 year of placebo (in white) and after 1 year of GH treatment (in dark grey). Boxes represent 1st and 3rd quartile, with median in the middle. Whiskers indicate range. There are no significant differences.

### Associations during placebo

After 1 year of placebo, young adults scored lowest on subtest Coding (-2.7 SDS) and highest on the subtests Similarities, Information and Picture Arrangement (median score -1.7 SDS). After 1 year of placebo, there was no difference in TIQ, VIQ or PIQ between boys and girls (p>0.166) or between patients with a deletion and mUPD+ICD (p>0.138). The IGF-I SDS during placebo was not associated with TIQ, VIQ or PIQ (p>0.602).

### Placebo versus GH administration

Table 2 and Figure 1 show the effects of 1 year of placebo versus 1 year of GH administration on cognition. Compared with GH treatment, placebo did not deteriorate TIQ, VIQ or PIQ (p>0.322). The difference between placebo and GH administration was strongest in the subtest Block Design, as patients scored 0.3 SDS worse during placebo (median score placebo -2.0 SDS, GH administration -1.66 SDS, p=0.075), but the difference did not reach significance. The young adults had similar scores on the other 10 different subtests during placebo and GH administration (all p>0.123).

The effect of placebo versus GH administration on TIQ, VIQ or PIQ was neither different between boys and girls (p>0.418), nor between young adults with a deletion versus mUPD+ICD (p>0.138). Young adults with a lower TIQ had more loss in TIQ points during placebo versus GH (ρ=-0.407, p=0.043), in particular those with a lower VIQ had more decrease in VIQ points during placebo (ρ=-0.467, p=0.021).

Limited cognitive functioning is commonly defined as an IQ score below 70 points[20]. Seven (28%) young adults had a VIQ and 9 (36%) a PIQ higher than 70 points, during both placebo and GH treatment.

### Associations with GH peak during stimulation test

After the 2-year study, twenty-three young adults underwent an arginine-GHRH test. Only 3 (13%) had a GH peak below the BMI-dependent cut-off[21]. There was no significant influence of the GH peak on the effects of placebo versus GH administration on TIQ, VIQ or PIQ (p>0.604).

**Table 2.** Cognitive function of PWS adolescents at different stages in the study

| | Treatment schedule | | | | |
|---|---|---|---|---|---|
| | Placebo / GH (n=12) | | GH / Placebo (n=13) | | |
| | After 1 year placebo | After 1 year GH | After 1 year GH | After 1 year placebo | p-value |
| **Total IQ** | 61 (55 to 69) | 62 (58 to 69) | 69 (60 to 73) | 70 (57 to 80) | 0.832 |
| **Verbal IQ** | 62 (55 to 66) | 65 (57 to 70) | 66 (57 to 75) | 67 (55 to 76) | 0.486 |
| - Vocabulary | -2.3 (-2.7 to -2.0) | -2.2 (-2.9 to -2.0) | -2.3 (-3.0 to -1.3) | -2.0 (-2.7 to -1.5) | 0.650 |
| - Similarities | -1.8 (-2.2 to -1.7) | -1.7 (-2.3 to -1.1) | -1.3 (-2.3 to -1.0) | -1.3 (-2.3 to -1.0) | 0.943 |
| - Arithmetic | -2.0 (-2.2 to -2.0) | -2.0 (-2.0 to -1.7) | -2.0 (-2.0 to -1.7) | -2.0 (-2.0 to -1.8) | 0.320 |
| - Digit Span | -2.2 (-2.8 to -1.7) | -2.3 (-2.9 to -1.8) | -2.0 (-2.3 to -1.8) | -2.0 (-2.3 to -1.8) | 0.793 |
| - Information | -1.7 (-2.6 to -1.7) | -1.8 (-2.0 to -1.4) | -1.7 (-2.0 to -1.1) | -1.8 (-2.3 to -1.3) | 0.154 |
| - Comprehension | -2.3 (-2.7 to -2.3) | -2.2* (-2.6 to -2.0) | -2.0 (-2.6 to -1.4) | -2.0 (-2.6 to -1.4) | 0.123 |
| **Performance IQ** | 59 (53 to 72) | 57 (52 to 70) | 67 (57 to 75) | 69 (55 to 78) | 0.322 |
| - Picture Completion | -2.2 (-2.9 to -1.7) | -2.5 (-2.9 to -1.7) | -1.7 (-2.8 to -0.8) | -1.3 (-2.3 to -0.4) | 0.130 |
| - Coding | -3.0 (-3.0 to -2.2) | -2.7 (-3.0 to -2.3) | -2.5 (-3.0 to -1.5) | -2.3 (-3.0 to -1.5) | 0.903 |
| - Block design | -2.0 (-2.2 to -1.4) | -1.5* (-2.0 to -1.3) | -1.7 (-1.8 to -1.2) | -1.7 (-2.0 to -1.0) | 0.075 |
| - Matrix Reasoning | -1.7 (-2.1 to -1.3) | -2.3* (-2.3 to -1.7) | -2.2 (-2.3 to -1.2) | -2.2 (-2.3 to -1.3) | 0.376 |
| - Picture Arrangement | -2.3 (-2.6 to -1.2) | -2.0 (-2.3 to -1.4) | -1.7 (-1.7 to -1.0) | -1.3 (-2.0 to -1.0) | 0.611 |

Data expressed in SDS; median with IQR. P-value of mean difference between placebo and GH administration, tested by Wilcoxon tests.

*within Placebo/GH group; significantly different compared to placebo. No significant difference between placebo and GH within GH/placebo group.

## DISCUSSION

This is the first two-year, randomized, double-blind, placebo-controlled GH study in young adults with PWS who were treated with GH during childhood until AH, investigating the effects of cessation of GH (placebo) versus GH administration on cognitive functioning. Our data show that, compared to GH administration, one year of placebo did not deteriorate TIQ, VIQ or PIQ in the total group of young adults with PWS. However, patients with a lower cognitive functioning had more loss in IQ points during placebo versus GH treatment.

In this study, we investigated whether the GH-induced improvement in cognitive functioning during childhood would be lost during one year of placebo. Our results are reassuring, as there was no significant deterioration in TIQ, VIQ or PIQ after 1 year of placebo in young adults with PWS. If IQ had deteriorated after cessation of GH treatment, this would have suggested that sustained activation with GH was required to retain the improved cognitive functioning achieved during childhood. We found, however, that IQ remained similar after 1 year of placebo, which might indicate that GH treatment during childhood has long-lasting effects. To our knowledge, there are no studies investigating the effects of cessation of GH treatment on cognition after AH attainment.

The scores of the subtest Block Design, however, tended to deteriorate after 1 year of placebo compared to GH treatment, but the decrease of 0.3 SDS was not significant. An RCT in children with PWS showed that 2 years of GH treatment did not significantly improve Block Design scores compared to baseline. Only after 4 years of GH treatment, Block Design scores had increased approximately 0.3 SDS, which was significantly higher than at baseline[7]. Thus, our finding that TIQ did not change during 1 year of placebo compared to GH treatment, while Block Design scores tended to deteriorate, does not exclude that stop of GH treatment for many years could result in a deterioration of cognitive functioning on the long term. It might be that 1 year of placebo is too short to show a significant decrease in cognitive functioning.

There are no other studies on cognitive functioning in young adults with PWS who received long-term GH during childhood. Only one study investigated the effects of GH treatment versus placebo on cognitive functioning in adults with PWS, but these PWS adults were older and GH-untreated at inclusion. They demonstrated that the subtest Block Design and Coding improved during GH treatment and benefits were more pronounced in patients who were GH-treated for the longest time[22]. In untreated adults with PWS, lower IGF-I levels were correlated with poorer intellectual skills[12], which is in line with the beneficial effects of GH treatment, which increases IGF-I.

In contrast to our current findings of unaltered cognitive functioning after 1 year of placebo versus GH administration, we found an impressive deterioration of the body composition within this period[19]. This might suggest that the beneficial effects on cogni-

tion of GH treatment during childhood last into adulthood. The exact mechanism how GH exerts its beneficial effects on cognitive functioning is unknown. It has been suggested that GH may directly affect its GH receptors which are widespread throughout the brain, or through release of IGF-I. The neurotrophic effects of GH continue during adulthood, and it has been proposed that the age-related decline in GH secretion is involved in the decreased neurogenesis in healthy elderly[23]. Amongst them, those with higher IGF-I levels have better cognitive functioning and lower rates of cognitive decline[24, 25]. Besides, an RCT showed that GHRH administration in healthy elderly and in adults with a mild cognitive impairment had favourable effects on cognition[26]. Like in other syndromes, ageing in PWS might occur prematurely, and lower GH levels might be involved in this, but data are very limited[27, 28]. In other syndromes, like Down syndrome, age-related diseases as dementia with loss of function in multiple cognitive domains are more prevalent and occur earlier[29]. A deterioration in cognitive functioning over time is also seen in patients with Alzheimer's disease, and GH administration reduced learning and memory deficits in animals with this disease[23]. In adults with GH deficiency, GH administration improved long-term and working memory functions[30, 31]. Altogether, these results support the hypothesis that the GH-IGF-I axis is involved in cognitive functioning[32]. How GH treatment achieves its beneficial effects needs to be elucidated, but it suggests that GH might have neuroprotective effects. Thus, continuation of GH treatment might also benefit adults with PWS, while it cannot be excluded that cessation of GH impairs cognitive functioning on the long term.

Although there was no deterioration in TIQ, VIQ or PIQ during 1 year of placebo, we found that young adults with a lower cognitive functioning lost more IQ points during placebo versus GH treatment, indicating more benefit from GH treatment. This is in line with our previous finding that GH treatment was more beneficial for children with PWS with lower cognitive functioning[7]. In elderly and in patients with GH deficiency or Alzheimer, there was a positive correlation between IGF-I levels and cognitive functioning, while this was not found in a healthy adult group. The authors postulate that IGF-I levels are not involved in cognition in case of relatively good cognitive performance[33]. This is in line with our findings and suggests that patients with poor cognitive skills are more vulnerable for loss of function. The mechanism is, however, not clear.

Young adults with PWS in our study showed a moderate cognitive impairment, with median IQ scores being about 5 to 10 points higher than documented by other studies, who reported IQ scores between 50-60 or around 52 points in adults with PWS[22, 34]. These patients had not received GH treatment during childhood[34]. An explanation for the higher IQ scores in our groups might be that all our participants were treated with GH treatment for many years during their childhood, which is a crucial period for maturation of the brain. GH treatment during childhood prevents deterioration of cognitive skills and improves cognition on the longer term[7].

Besides the higher IQ scores than reported in the literature, we also found a very wide variation in IQ scores. One patient had a PIQ of 100 during GH and subsequently 92 during placebo, which is exceptionally high for an individual with PWS. Furthermore, the 3rd quartile of TIQ, PIQ and VIQ was higher than the cut-off for intellectual disability[20], meaning that more than 25% of the young adults had an IQ above 70 points. On the other hand, there was a patient who was not able to participate in this part of the study, as she had poor cognitive skills in addition to her hearing impairment. Previously reported percentages of poor cognitive skills in individuals with PWS were around 14%, while this was present in only 1 of 27 patients in our group[34, 35].

Cognition after stop of GH treatment and the effects of placebo versus GH administration on cognition were not different between patients with a deletion and those with an mUPD or ICD. It has been described that patients with an mUPD have better verbal skills than those with a deletion[7, 35]. These patients were, however, not treated with GH. During 4 years of GH treatment, children with an mUPD showed a larger improvement than children with a deletion, resulting in a similar Block Design score as those with a deletion after 4 years of GH treatment[7]. We now found no difference in cognition between those with a deletion versus mUPD+ICD, which suggests that long-term GH treatment during childhood improved cognitive functioning, particularly of those with mUPD+ICD.

The ethical dilemma of 1 year placebo injections in mentally disabled young adults was extensively discussed with patients' caregivers. The high clinical relevance ensured them to vote for the strongest design, being a 2-year cross-over study. It might be that 1 year placebo was too short to demonstrate alteration in cognitive functioning and that no GH treatment will lead to a deterioration on the longer term. It was, however, considered unethical to extend the placebo period beyond 1 year given the convincing positive effects of GH on body composition. It might be that the effect of GH versus placebo on cognitive skills would have reached significance if we could have studied a larger group. This was, however, not feasible as PWS is a rare disorder.

In conclusion, this cross-over trial in young adults with PWS who were treated for many years with GH during childhood shows that compared to GH treatment, 1 year of placebo did not deteriorate cognitive functioning. However, patients with a lower cognitive functioning had more loss in IQ points during placebo versus GH treatment. Our findings are reassuring, but do, however, not exclude that cessation of GH treatment for many years could result in a gradual deterioration of cognitive functioning on the long term.

## REFERENCES


1. Goldstone, A. P., Holland, A. J., Hauffa, B. P. et al.: Recommendations for the diagnosis and management of Prader-Willi syndrome. J Clin Endocrinol Metab, 93: 4183, 2008
2. Eiholzer, U., Bachmann, S., l'Allemand, D.: Is there growth hormone deficiency in prader-willi Syndrome? Six arguments to support the presence of hypothalamic growth hormone deficiency in Prader-Willi syndrome. Horm Res, 53 Suppl 3: 44, 2000
3. Holm, V. A., Cassidy, S. B., Butler, M. G. et al.: Prader-Willi syndrome: consensus diagnostic criteria. Pediatrics, 91: 398, 1993
4. Bakker, N. E., Kuppens, R. J., Siemensma, E. P. et al.: Eight years of growth hormone treatment in children with Prader-Willi syndrome: maintaining the positive effects. J Clin Endocrinol Metab, 98: 4013, 2013
5. Lukoshe, A., Hokken-Koelega, A. C., van der Lugt, A. et al.: Reduced cortical complexity in children with Prader-Willi Syndrome and its association with cognitive impairment and developmental delay. PLoS One, 9: e107320, 2014
6. Lukoshe, A., White, T., Schmidt, M. N. et al.: Divergent structural brain abnormalities between different genetic subtypes of children with Prader-Willi syndrome. J Neurodev Disord, 5: 31, 2013
7. Siemensma, E. P., Tummers-de Lind van Wijngaarden, R. F., Festen, D. A. et al.: Beneficial effects of growth hormone treatment on cognition in children with Prader-Willi syndrome: a randomized controlled trial and longitudinal study. J Clin Endocrinol Metab, 97: 2307, 2012
8. Festen, D. A., Wevers, M., Lindgren, A. C. et al.: Mental and motor development before and during growth hormone treatment in infants and toddlers with Prader-Willi syndrome. Clin Endocrinol (Oxf), 68: 919, 2008
9. Bakker, N. E., Kuppens, R. J., Siemensma, E. P. et al.: Bone mineral density in children and adolescents with Prader-Willi syndrome: a longitudinal study during puberty and 9 years of growth hormone treatment. J Clin Endocrinol Metab, 100: 1609, 2015
10. Lo, S. T., Festen, D. A., Tummers-de Lind van Wijngaarden, R. F. et al.: Beneficial Effects of Long-Term Growth Hormone Treatment on Adaptive Functioning in Infants With Prader-Willi Syndrome. Am J Intellect Dev Disabil, 120: 315, 2015
11. Carrel, A. L., Myers, S. E., Whitman, B. Y. et al.: Long-term growth hormone therapy changes the natural history of body composition and motor function in children with prader-willi syndrome. J Clin Endocrinol Metab, 95: 1131, 2010
12. van Nieuwpoort, I. C., Deijen, J. B., Curfs, L. M. et al.: The relationship between IGF-I concentration, cognitive function and quality of life in adults with Prader-Willi syndrome. Horm Behav, 59: 444, 2011
13. Wechsler, D.: Manual of Wechsler Adult Intelligence Scale (WAIS-III), 3rd ed. San Antonio, TX: Harcourt Assessment, 1997
14. Wechsler, D.: Manual of Wechsler Intelligence Scale for Children-Third Edition (WISC-III). New York: The Psychological Coorporation, 1991
15. Ross, R. T., Morledge, J.: Comparison of the Wisc and Wais at chronological age sixteen. J Consult Psychol, 31: 331, 1967
16. Fredriks, A. M., van Buuren, S., Wit, J. M. et al.: Body index measurements in 1996-7 compared with 1980. Arch Dis Child, 82: 107, 2000
17. Fredriks, A. M., van Buuren, S., Burgmeijer, R. J. et al.: Continuing positive secular growth change in The Netherlands 1955-1997. Pediatr Res, 47: 316, 2000

18. Rikken, B., van Doorn, J., Ringeling, A. et al.: Plasma levels of insulin-like growth factor (IGF)-I, IGF-II and IGF-binding protein-3 in the evaluation of childhood growth hormone deficiency. Horm Res, 50: 166, 1998
19. Kuppens, R. J., Bakker, N. E., Siemensma, E. P. C. et al.: Beneficial effects of growth hormone in young adults with Prader-Willi syndrome: Results of a 2-year randomized, double-blind, placebo-controlled cross-over trial. J Clin Endocrinol Metab, 2016
20. American Psychiatric Association., American Psychiatric Association. DSM-5 Task Force.: Diagnostic and statistical manual of mental disorders : DSM-5, 5th ed. Washington, D.C.: American Psychiatric Association, pp. xliv, 947 p., 2013
21. Corneli, G., Di Somma, C., Baldelli, R. et al.: The cut-off limits of the GH response to GH-releasing hormone-arginine test related to body mass index. Eur J Endocrinol, 153: 257, 2005
22. Hoybye, C., Thoren, M., Bohm, B.: Cognitive, emotional, physical and social effects of growth hormone treatment in adults with Prader-Willi syndrome. J Intellect Disabil Res, 49: 245, 2005
23. Arce, V. M., Devesa, P., Devesa, J.: Role of growth hormone (GH) in the treatment on neural diseases: from neuroprotection to neural repair. Neurosci Res, 76: 179, 2013
24. Aleman, A., Verhaar, H. J., De Haan, E. H. et al.: Insulin-like growth factor-I and cognitive function in healthy older men. J Clin Endocrinol Metab, 84: 471, 1999
25. Arwert, L. I., Deijen, J. B., Drent, M. L.: The relation between insulin-like growth factor I levels and cognition in healthy elderly: a meta-analysis. Growth Horm IGF Res, 15: 416, 2005
26. Baker, L. D., Barsness, S. M., Borson, S. et al.: Effects of growth hormone-releasing hormone on cognitive function in adults with mild cognitive impairment and healthy older adults: results of a controlled trial. Arch Neurol, 69: 1420, 2012
27. Sinnema, M., Schrander-Stumpel, C. T., Maaskant, M. A. et al.: Aging in Prader-Willi syndrome: twelve persons over the age of 50 years. Am J Med Genet A, 158A: 1326, 2012
28. Whittington, J. E., Holland, A. J., Webb, T.: Ageing in people with Prader-Willi syndrome: mortality in the UK population cohort and morbidity in an older sample of adults. Psychol Med, 45: 615, 2015
29. Tyrrell, J., Cosgrave, M., McCarron, M. et al.: Dementia in people with Down's syndrome. Int J Geriatr Psychiatry, 16: 1168, 2001
30. Arwert, L. I., Veltman, D. J., Deijen, J. B. et al.: Effects of growth hormone substitution therapy on cognitive functioning in growth hormone deficient patients: a functional MRI study. Neuroendocrinology, 83: 12, 2006
31. Arwert, L. I., Deijen, J. B., Muller, M. et al.: Long-term growth hormone treatment preserves GH-induced memory and mood improvements: a 10-year follow-up study in GH-deficient adult men. Horm Behav, 47: 343, 2005
32. Swaab, D. F.: Prader-Willi syndrome and the hypothalamus. Acta Paediatr Suppl, 423: 50, 1997
33. Licht, C. M., van Turenhout, L. C., Deijen, J. B. et al.: The Association between IGF-1 Polymorphisms, IGF-1 Serum Levels, and Cognitive Functions in Healthy Adults: The Amsterdam Growth and Health Longitudinal Study. Int J Endocrinol, 2014: 181327, 2014
34. Copet, P., Jauregi, J., Laurier, V. et al.: Cognitive profile in a large French cohort of adults with Prader-Willi syndrome: differences between genotypes. J Intellect Disabil Res, 54: 204, 2010
35. Whittington, J., Holland, A., Webb, T. et al.: Cognitive abilities and genotype in a population-based sample of people with Prader-Willi syndrome. J Intellect Disabil Res, 48: 172, 2004

# CHAPTER 8

## General discussion and conclusions, clinical implications, and recommendations for future research

## GENERAL DISCUSSION

Almost fifteen years ago, in April 2002, the Dutch GH RCT was initiated by the Dutch Growth Research Foundation to investigate the effects of GH treatment in children with PWS. Over time, it grew into the Dutch PWS Reference Center providing high-quality care, performing scientific research and sharing knowledge. Nowadays almost all Dutch infants with PWS are included in the PWS Cohort, receiving care by the PWS-team in close collaboration with pediatric-endocrinologists in The Netherlands.

GH treatment proved to have many beneficial effects in children with PWS and considerably changed their phenotype. The extreme classic hyperphagia appears to be less pronounced in this new generation of patients, but the persisting hyperphagia and clear preoccupation with food remains a large burden for these families. As the appetite-regulating hormone ghrelin might be involved, we investigated ghrelin in fasting state and after glucose intake. We also investigated the effects of oxytocin treatment on hyperphagia and social behaviour in children with PWS. In young adults with PWS who were GH-treated during childhood and had attained adult height, we studied the effects of (placebo) versus GH treatment on body composition, metabolic health profile and cognition. In this chapter, our results are discussed, also in view of literature data. Subsequently, clinical implications of our results are presented and recommendations for future research are provided.

### 8.1 GHRELIN



In PWS, clinical features change during the different life stages and one of the most striking symptoms is the abnormal eating behaviour. The first period after birth is characterized by feeding problems and failure to thrive, while excessive weight gain and hyperphagia with impaired satiety are prominent during childhood and adulthood[1]. Studies reported high serum levels of the appetite-regulating hormone ghrelin, but investigators did not distinguish between acylated (AG) and unacylated ghrelin (UAG), did not describe AG/UAG ratios and did not use an inhibitor to prevent deacylation of AG into UAG[2-7]. In the study described in Chapter 2, we evaluated the fasting AG and UAG levels, and AG/UAG ratios in patients with PWS in comparison with obese and healthy controls and investigated their associations with eating behaviour.

We showed that AG levels were significantly higher in PWS patients than in healthy controls. In contrast to our expectations, however, UAG levels in PWS were similar to those in healthy controls. This resulted in a significantly higher AG/UAG ratio in PWS than in healthy controls. Obese controls had significantly lower AG and UAG levels than PWS patients, but also a high AG/UAG ratio.Based on the higher BMI in PWS patients

than in healthy controls, one could expect similar levels in PWS as in obese subjects, but our data indicate that the abnormalities of the ghrelin system are specific for PWS.

Hypothalamic dysfunction is assumed to be the underlying cause of various signs and symptoms of PWS, such as abnormal temperature control, excessive daytime sleepiness, hypogonadotropic hypogonadism and hormonal insufficiencies[8-11]. The physiological regulation of some of these hormones is well-known[8-11]. Regarding appetite regulation, the mechanisms are not yet clear and there are still a lot of uncertainties. It seems obvious that the hypothalamic dysfunction in PWS is responsible for the disturbances in the appetite-regulating hormone ghrelin as well as the other hormonal insufficiencies in PWS, but as far as we know, this is not yet ascertained. On the other hand, some studies demonstrated that ghrelin might influence the hypothalamus[12, 13]. Intravenous administration of ghrelin in healthy males strongly stimulated GH secretion, but also ACTH, cortisol and prolactin were mildly elevated[12]. Combined administration of ghrelin and GHRH synergistically increased GH levels[13]. Besides, ghrelin is mainly produced in the stomach and this organ works differently in PWS who are known for their delayed gastric emptying, decreased ability to vomit and lack of satiety after a meal[14-16]. It might be that the production of ghrelin in PWS is different, resulting in elevated AG levels and AG/UAG ratios. It is unknown whether these high levels could induce desensitization resulting in a functional GH deficiency and other hormonal insufficiencies. More research on whether hypothalamic dysfunction is either the cause or consequence of abnormalities in the appetite regulating hormones can help to contrive a solution. An effective treatment would relief parents from the ongoing responsibility to retain food from their children, and would reduce the risk for obesity and comorbidities.

We have the clinical impression that GH treatment has an inhibiting effect on food intake. About half of young adults showed increasing hyperphagia after stop of GH treatment after attainment of adult height. We found, however, no difference in AG/UAG ratios between GH-treated patients and untreated patients with PWS and there was no significant association between IGF-I levels and AG/UAG ratio. The untreated patients were, however, very young (before start of GH) or older (after attainment of adult height) and this might have influenced these results.

We also subdivided the patients according to the nutritional phases and found that PWS patients in nutritional phase 2a, 2b and 3, thus with weight gain and/or hyperphagia, had a higher AG/UAG ratio than those in nutritional phase 1a or 1b, without weight gain or hyperphagia. The AG/UAG ratios of the latter were similar to age-matched controls[1]. Furthermore, the AG/UAG ratio changed considerably between phase 1b and higher nutritional phases, indicating that the switch in eating behaviour coincides with a change in the AG/UAG ratio, supporting the hypothesis that ghrelin might be involved in this. It would be valuable to determine longitudinally when the AG/UAG ratio is changing in parallel with the eating behaviour and which factors are involved. As there are

no other scoring systems for the switch in eating behaviour than the nutritional phases according to Miller *et al.*[1] we used this one. However, in practice the clinical presentation of children with PWS has significantly changed in today's population. We found a higher median age in patients with PWS in nutritional phase 1b than in those in phase 2a. Several older patients were still in nutritional phase 1b and have controlled hyperphagia without increased weight or food-seeking behaviour, which makes it difficult to categorize these children according to the nutritional phases. The phenotype has changed in the last decade, probably due to early diagnosis, improved health care, GH treatment, physiotherapy, diet regimen and the awareness of parents to limit the food intake.

*In conclusion, our study demonstrates that children and young adults with PWS have higher AG levels but similar UAG levels compared to healthy controls, resulting in a significantly higher AG/UAG ratio in PWS patients than in healthy controls. Obese controls have significantly lower AG and UAG levels than PWS patients, but also a high AG/UAG ratio.*

*PWS patients without weight gain or hyperphagia had a similar AG/UAG ratio as age-matched healthy controls, in contrast to those with weight gain and/or hyperphagia who had an elevated AG/UAG ratio. The switch to excessive weight gain in PWS seems to coincide with an increase in the AG/UAG ratio, even prior to the start of hyperphagia.*

## 8.2 GHRELIN AFTER GLUCOSE INTAKE

For a long time it was thought that patients with PWS are continuously hungry due to the higher ghrelin levels, but studies demonstrated a non-decelerating eating curve with impaired satiety in patients with PWS compared to obese and healthy controls[16]. In healthy individuals, AG and UAG levels are high in fasting state and food intake induces a strong decline of these levels[17]. Intravenous AG administration in healthy subjects increased food intake and appetite[18]. We demonstrated higher fasting AG and AG/UAG ratios in patients with PWS than in healthy controls[19], which might be involved in their hyperphagia. It was unknown why patients with PWS do not feel satiated after a meal, but findings suggested that the ghrelin system in PWS was not able to react physiologically with a decline of ghrelin levels after food intake. In the study described in Chapter 3, we investigated the response of AG and UAG levels and the AG/UAG ratio to a standard oral glucose load in young adults with PWS.

We showed that the ghrelin system in young adults with PWS responded physiologically after glucose intake, even in the presence of high fasting AG/UAG ratios. In the first 30 minutes after the glucose load, there was a sharp decline of AG levels and to a lesser extent of UAG levels, which resulted in a lower AG/UAG ratio. The magnitude of decline of these levels in PWS was similar as described in healthy controls[17, 20, 21]. Our

findings suggest that the impaired satiety is not the result of an abnormal response of ghrelin to food intake and that there must be other reasons. Hypothalamic dysfunction might play a role, but it could also be that abnormalities in other appetite-regulating hormones cause the impaired satiety in patients with PWS. Ghrelin is the only known appetite-regulating hormone which is high in the fasting state and decreases after food intake, while all other known appetite-regulating hormones are low when an individual is hungry and increase after food intake[22]. If patients with PWS have a lack of increase of anorexigenic hormones after food intake, this might result in a decreased satiation as well. Oxytocin is one of these hormones and studies demonstrated that it is involved in food intake, weight balance and obesity[23].

Our findings suggest that GH treatment reduces AG levels, and to a lesser extent UAG levels, leading to a lower AG/UAG ratio during GH treatment. One other study demonstrated that GH treatment in children with PWS seems to decrease high fasting total ghrelin levels, whereas they found no effects on AG levels[24]. These data of AG must be interpreted with caution, as no inhibitor was used to prevent the deacylation of AG into UAG. No other studies investigated the effects of GH treatment on UAG levels, and AG/UAG ratio in PWS.

AG has diabetogenic effects, stimulates appetite and induces a positive energy balance which might result in weight gain[25-28]. UAG on the other hand has protective effects, seems to improve glycemic control and acts as a functional inhibitor of AG[28-30]. Therefore, the ratio between AG and UAG is crucial and a lower AG/UAG ratio seems to be more favourable. We found that GH treatment had a decreasing effect on the AG/UAG ratio, which is in line with our impression that GH treatment influences eating behaviour in a positive manner (reduction of hyperphagia) and that young adults with PWS benefit from continuation of GH treatment after attainment of adult height.

*In conclusion, our study demonstrates that the ghrelin system of young adults with PWS shows a physiological response with a strong decline of AG and UAG levels after glucose intake, even in the presence of very high fasting AG/UAG ratios. This resulted in a lower AG/UAG ratio after glucose intake. Our findings suggest that the impaired satiety is not the result of an abnormal response of the orexigenic ghrelin to food intake.*

*Continuation of GH treatment after attainment of adult height results in lower AG levels during OGTT, suggesting a more favourable metabolic profile.*

## 8.3 OXYTOCIN TREATMENT IN CHILDREN WITH PWS

The quality of life of patients with PWS is hampered by temper tantrums and the preoccupation with food, which makes every day a challenge for patients and their caregivers.

Worldwide, there is ongoing research to find a solution for this major problem. There is increasing interest in the hormone oxytocin, as it is known to be involved in food intake, body weight and social skills[23, 31, 32]. In patients with PWS, the number of oxytocin-expressing neurons in the hypothalamus is significantly decreased by 42% and plasma levels of oxytocin are lower than in healthy controls[8, 33]. Thus, it seems that patients with PWS have a disturbance in the oxytocin system with an oxytocin deficiency.

Genetic information supports this hypothesis, as mice with a deficiency of one of the genes on the PWS region on chromosome 15 have a major reduction of oxytocin secretion in the hypothalamus[32]. These Magel2-deficient mice show an altered onset of suckling activity resulting in impaired feeding and 50% mortality after birth[32]. Human neonates with PWS show similar suckling problems. Oxytocin administration to Magel2-deficient mouse pups normalized suckling and feeding behaviour and rescued all of them[32].

Chapter 4 describes the results or our PWS Oxytocin Study. We conducted a randomized, double-blind, placebo-controlled, cross-over study to investigate the effects of 4 weeks of intranasal oxytocin versus 4 weeks of placebo administration in children with PWS aged between 6 and 14 years. Although there were no effects in the group as a whole, subanalyses demonstrated that children with PWS between 6 and 11 years had beneficial effects of intranasal oxytocin administration on social behaviour and hyperphagia. Significantly less anger, sadness, conflicts and food-related behaviour, and improvement of social behaviour were reported by the parents during oxytocin treatment compared with placebo.

In children with PWS older than 11 years, no beneficial effects of oxytocin on social behaviour and hyperphagia were found. Comparable results were reported by an Australian study in individuals with PWS between 12 and 29 years. For that reason, they doubled the oxytocin dose, but subsequently temper outbursts increased instead of the desired decrease[34]. It might be that a higher oxytocin dose not only simulates oxytocin receptors, but also the receptors of oxytocin's sister hormone arginine vasopressin (AVP), which is associated with stress-adaptation, anxiety and aggression[34]. If this hypothesis is true, a lower oxytocin dose might be better for older children. Other explanations like development of an unresponsive oxytocin system over the years with a lower number of oxytocin receptors and neurons in the hypothalamus, or an enhanced deterioration of the oxytocin system in patients with PWS around onset of puberty are also possible.

*In conclusion, administration of intranasal oxytocin appears to have beneficial effects on social behaviour and food-related behaviour in children with PWS younger than 11 years of age without side effects or adverse events. Parents reported significantly less anger, sadness, conflicts and food-related behaviour, and improvement of social behaviour during oxytocin treatment compared with placebo. In contrast to the younger children with PWS, those older than 11 years of age did not benefit from oxytocin treatment with the given dose.*

## 8.4 BODY COMPOSITION IN YOUNG ADULTHOOD

After several years of participation in the Dutch GH RCT, the oldest children attained adult height. Their phenotype had changed during GH treatment with a normalization of height, improvement of body composition, and a better psychomotor development. It was, however, disconcerting for parents to discontinue GH treatment when children had attained adult height. They had experienced the benefits for their child, but there was no information about the effects of GH in this new generation of young adults with PWS after adult height attainment. A few patients ceased GH treatment and parents and investigators saw a deterioration of the condition and behaviour of these young adults within 3 months after GH stop. We, therefore, started a new randomized, placebo-controlled GH trial investigating the effects of 1 year of continuation of GH treatment versus 1 year of placebo injections on body composition of young adults with PWS who were GH-treated during childhood and had attained adult height. The results are described in Chapter 5.

In children with PWS, the most important reason for treating them with GH is to optimize the body composition[35]. Without GH treatment, BMI and body composition deteriorate with age[36]. Our present results demonstrate that GH treatment remains required to maintain the body composition after attainment of AH. During 1 year of placebo, fat mass (FM) raised dramatically with a relative increase of 21.5%. The BMI increased by +1.4 kg/m$^2$, but it is debatable whether the change in BMI is representative in patients with PWS as BMI underestimates true body composition. Compared to placebo, GH treatment resulted in a lower FM and higher lean body mass (LBM). Thus, our data show that GH-treated young adults with PWS who have attained AH benefit from continuation of GH treatment.

The benefits of an improved body composition are clear. Less obesity and FM result in a more favourable metabolic profile and decrease the risk of cardiovascular diseases and T2DM[37]. Although BMI is not very representative in patients with PWS, it is known that a higher BMI is associated with higher mortality and morbidity, such as cardiovascular diseases, T2DM, kidney diseases, osteoarthritis and psychosocial problems[38-43]. Increase of LBM affects the metabolic profile positively, as LBM is the primary tissue for insulin-stimulated glucose uptake, disposal and storage[44]. In addition, a higherLBM has positive effects on energy expenditure, BMD and psychomotor development[45-51]. Thus, as we proved that discontinuation of GH treatment after AH attainment deteriorates body composition, cessation of GH treatment might result in more morbidity and mortality.

The majority of the parents reported a clear difference between the year with and the year without GH administration. Most important reported changes during the placebo period were more problem behaviour, worsening of weight and body composition, more preoccupation with food, less endurance and lower energy level. This clinical picture

during the placebo period resembles that of young adults with untreated GHD during the transition period, who also show altered body composition, low BMD, unfavourable lipid profile and decreased energy and quality of life[52, 53]. Although only 12.5% of the young adults with PWS had a GH peak below the BMI-dependent cut-off in a arginine-GHRH test[54], it seems that children and adults with PWS have a functional GHD[11]. This would explain why patients with PWS do not only benefit from GH treatment during childhood, but also in adulthood.

*In conclusion, this randomized cross-over trial in young adults with PWS who were treated with GH during childhood shows that placebo treatment after AH deteriorates the improved body composition with an increase of FM. Compared to placebo, GH treatment results in a lower FM and higher LBM. This indicates that discontinuing GH treatment at AH leads to deteriorated body composition, while GH maintains the improved FM and LBM. This is very important, because development towards morbid obesity is a major threat to these patients. Thus, it is likely that continued GH administration contributes to a better future health for these young adults.*

## 8.5 METABOLIC HEALTH PROFILE IN YOUNG ADULTHOOD

Adults with PWS are predisposed to develop T2DM due to their abnormal body composition[55], and risk factors for CVD such as hypertension and hyperlipidemia are more often present in PWS[56]. GH treatment is known for its diabetogenic effects, but there were no studies about the metabolic effects of GH versus placebo in young adults with PWS who were treated with GH during childhood and had attained AH. Studies in older PWS-patients who were GH-untreated at inclusion, found no major side-effects of GH on metabolic health profile[57, 58].

In Chapter 6, we report the results of the metabolic health profile of young adults with PWS during GH treatment versus placebo. Our study showed that the glucose-stimulated glucose and insulin levels during OGTT were similar during 1 year of GH treatment versus 1 year of placebo. Compared to placebo, GH treatment did not affect other carbohydrate parameters as AUC of glucose and insulin, and insulin/glucose ratios after glucose load. Fasting glucose and insulin levels were slightly higher during GH versus placebo, but remained within the normal ranges during both phases and none of the patients developed T2DM. The prevalence of IGT and MS were similar during GH treatment and placebo, and BP and lipid profiles were not significantly different during GH versus placebo. This indicates that GH versus placebo does not affect carbohydrate parameters except for a slightly higher fasting glucose and insulin, and that GH versus placebo does not negatively influence metabolic health profile.

Only few side effects of GH treatment in adults with PWS are reported, but studies are scarce and mainly uncontrolled or short term in a relatively small number of previously GH-untreated patients[35, 59, 60]. Our findings are in line with a study reporting a small increase in fasting glucose of +0.27 mmol/l and a trend towards an increase in fasting insulin of +20.2 pmol/l during GH treatment[59]. The results of our study indicate that GH treatment does not elicit pronounced adverse effect on glucose and insulin homeostasis.

The physiological phenomenon that GH treatment induces a minor increase in fasting insulin with minimal influences on glucose metabolism is well-known[59, 61]. Our findings show that the benefits of GH versus placebo outweigh the mildly unfavourable effects on glucose and insulin homeostasis. These effects of GH on carbohydrate metabolism are thus compensated, while the benefits on body composition remain. Furthermore, we found that GH versus placebo does not disturb blood pressure, lipid profile or components of metabolic syndrome.

*In conclusion, this cross-over study demonstrates no adverse effects of GH treatment on carbohydrate metabolism, blood pressure, lipid profile or the development of metabolic syndrome. Thus, GH-treated young adults with PWS who have attained AH benefit from continuation of GH treatment without safety concerns regarding their metabolic health profile.*

## 8.6 COGNITION IN YOUNG ADULTHOOD

We previously demonstrated that long-term GH treatment during childhood improves cognitive functioning[62]. We investigated in Chapter 7 whether this GH-induced improvement would not be lost after cessation of GH treatment at attainment of adult height. Our results are reassuring, as there was no significant deterioration in TIQ, VIQ or PIQ after 1 year of placebo in young adults with PWS. Furthermore, there was no significant difference in cognitive functioning between placebo and GH treatment. This might suggest that GH treatment during childhood, when the brain develops and matures, induces long-lasting effects, which remain present, at least for 1 year after cessation of GH treatment. Alternatively, it might be that 1 year of cessation is too short to show a significant decrease in cognitive functioning.

Although there was no deterioration in TIQ, VIQ or PIQ during 1 year of placebo, we found that patients with a lower TIQ had more loss in TIQ points during placebo than during GH treatment, in particular VIQ decreased more in those with a lower VIQ. These findings resemble those during childhood, where children with a greater deficit had more benefit from GH treatment[62].

Cognitive functioning influences daily functioning, but other aspects are relevant as well. In the old DSM-IV, IQ-score was an important diagnostic criterion, but this has

changed in the new DSM-V[63]. Now the diagnosis Intellectual Disability involves impairments of general mental abilities that impact adaptive functioning in 3 domains. The domains Conceptual, Social and Practical determine how well an individual copes with everyday tasks, and the IQ-score gives support for the diagnosis. This is relevant for individuals with PWS, as some have a relatively normal IQ-score, but are not able to live independently or to make their own decisions regarding money and food.

*In conclusion, our cross-over trial in young adults with PWS who were treated for many years with GH during childhood demonstrates that 1 year placebo versus 1 year of GH treatment after attainment of adult height did not deteriorate cognitive functioning. However, patients with a lower VIQ lost more VIQ points during placebo.*

## 8.7 GENERAL CONCLUSIONS AND CLINICAL IMPLICATIONS

Since 2002, our longitudinal PWS studies evaluated the short and long-term effects of GH treatment in a large group of children with PWS. Over the last decade, GH treatment has considerably changed the phenotype of children with PWS and we, therefore, also assessed the effects of cessation versus continued GH treatment after attainment of adult height. In addition, other aspects of PWS, like hyperphagia, appetite-regulating hormones and effects of intranasally administered oxytocin have been investigated and are described in this thesis.

We showed that the switch from failure to thrive to excessive weight gain in PWS coincides with an increase in the AG/UAG ratio, as PWS patients without weight gain or hyperphagia had a similar AG/UAG ratio as age-matched healthy controls, while those with weight gain and/or hyperphagia had an elevated AG/UAG ratio. In general, PWS patients have higher AG levels but similar UAG levels compared to healthy controls, resulting in a significantly higher AG/UAG ratio in PWS patients than in healthy controls. Despite these elevated fasting AG/UAG ratios, the ghrelin system of young adults with PWS showed a physiological response with a strong decline of AG and UAG levels, and AG/UAG ratio after glucose intake. This suggests that the impaired satiety in PWS is not the result of an abnormal response of the orexigenic ghrelin to food intake.

Our randomized, double-blind, placebo-controlled, cross-over study demonstrated beneficial effects of intranasal oxytocin on social behaviour and food-related behaviour in children with PWS between 6 and 11 years of age. In contrast to the younger children with PWS, those older than 11 years of age had no benefit from oxytocin treatment. This lack of benefit in the older children might be explained by dose issues or development of an unresponsive oxytocin system. Although more research is warranted, this first oxytocin trial in children with PWS demonstrated promising effects of a new treatment option.

The improved body composition of young adults with PWS who were treated with GH during childhood and had attained AH deteriorated after cessation of GH treatment, while GH treatment maintained the improved FM and LBM. Compared to placebo, GH treatment did not affect carbohydrate parameters except for a slightly higher fasting glucose and insulin which remained within the normal ranges. Other components of metabolic health profile did not change. Cognitive functioning did not deteriorate during one year of placebo in the total group, although patients with a lower VIQ lost more VIQ points during placebo than during GH administration. Taken together, this indicates that young adults with PWS who have attained adult height benefit from continuation of GH treatment without safety concerns. Additional studies are needed to confirm this on the longer term, as these patients are still young and received only 1 year of GH treatment during adulthood.

## 8.8 DIRECTIONS FOR FURTHER RESEARCH

In this thesis, we describe new perspectives of treatment, with promising treatments such as intranasal administration of oxytocin in younger children with PWS. We found encouraging effects on social and eating behaviour after 4 weeks of treatment, but further research is warranted to confirm our findings and to study the long-term effects. As both social and eating behaviour are quite subjective, more objective outcome measures such as brain imaging are preferable. Besides, the optimal dose and frequency of oxytocin administration should be investigated. Furthermore, it should be elucidated why children younger than 11 years of age showed promising effects, while the older ones did not benefit from oxytocin treatment.

Besides the elevated fasting AG/UAG ratios in PWS, we found that the switch to hyperphagia might coincide with an increase in AG/UAG ratio. This supports the hypothesis that ghrelin is involved in hyperphagia in PWS. This is the cause or the consequence of the switch in eating behaviour could not be elucidated. Further research should therefore focus on longitudinal observations identifying the phases of PWS in parallel with the changes in ghrelin levels.

The nutritional phases according to Miller *et al.*[1] were used to score the feeding problems, but in practice, the clinical presentation of children with PWS has significantly changed in today's population. The controlled hyperphagia makes it difficult to categorize the child according the nutritional phases. Development of a better hyperphagia scale for today's population is, therefore, warranted.

The crucial AG/UAG ratio is normal in the nutritional phases without weight gain and/or hyperphagia, suggesting that UAG is sufficiently high to compensate for the elevated AG level. From phase 2a onwards, when weight gain and hyperphagia develop, AG/UAG

ratio increases. It might be that UAG levels are then too low to modulate the effects of elevated AG levels. Elaborating on this idea, it might be worthwhile to investigate whether a more physiological AG/UAG ratio could be achieved by increasing the plasma UAG or decreasing the plasma AG levels and/or bioactivity, and whether this normalization of the AG/UAG ratio results in a reduction of the hyperphagia.

We are the first to demonstrate that the body composition of young adults with PWS who were treated during childhood and had attained adult height deteriorated dramatically during 1 year of placebo, while 1 year of GH treatment maintained the improved body composition achieved during childhood. Ongoing monitoring of young adults with PWS receiving GH treatment is highly recommended as they are at risk to develop T2DM and CVD, although our study demonstrates reassuring findings regarding metabolic health profile. Additional studies are needed to confirm this on the longer term, as these patients are still young and received only 1 year of GH treatment during adulthood. Also, studies are needed to individualize treatment with GH in adults with PWS.

Further research regarding the GH dose titration for young adults with PWS is recommended. We considered the approved GH dose for children with PWS too high and the adult GHD dose too low for young adults and therefore treated them with 0.67 mg/$m^2$/day, which resulted in high-normal IGF-I levels without side effects. It is, however, debatable whether IGF-I levels are appropriate for titrating the GH dose, as there is a disrupted correlation between serum IGF-I levels and IGF-bioactivity in PWS, although this disruption is more pronounced in younger children than in the older ones[64].

Current treatments focus on the main signs and symptoms of PWS, such as obesity, hyperphagia or behavioural problems, but further research should also give attention to the underlying cause of these signs and symptoms on different levels. Genomic imprinting induces activated genes in the PWS region of the paternal chromosome only, while the intact genes on the maternal chromosome 15 are silenced. Reactivation of the maternally silenced genes or other treatments specifically targeting the molecular defect might solve all or some aspects of PWS. Besides, a dozen of genes are situated on the PWS region, but the contribution of most of these individual genes is poorly understood. Animal models have attempted to address these questions, but have not been able to fully recapitulate the human phenotypes.

Finally, more national collaboration in the form of a National Reference Center and on European level as European Reference Network (ERN) can support the pooling of expertise and help to ensure that knowledge is shared across borders. This will contribute to improvements in clinical outcomes and the quality of life of patients living with a rare disease.

*Rare diseases are rare, but rare disease patients are numerous (Orpha.net)*

## PWS Oxytocin Study

*Randomized, double-blinded, placebo-controlled cross-over trial*
4 weeks of oxytocin versus 4 weeks of placebo
n=25 children with PWS

**6 to 11 years**
- Beneficial effects on social behavior and food-related behavior
- Less anger, sadness, conflicts and food-related behavior, and improvement of social behavior
- No side effects

**11 to 14 years**
- No beneficial effects on social behavior and eating behavior
- No side effects

**Oxytocin is a promising treatment for PWS, but more research regarding dose and longer term effects is warranted**

## PWS Transition Study

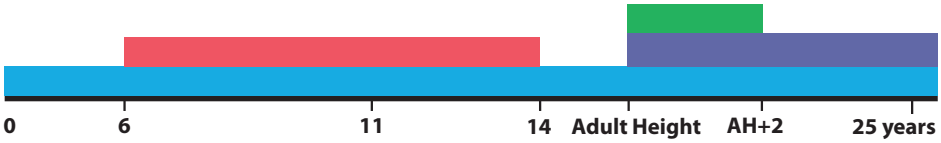
*Randomized, double-blinded, placebo-controlled cross-over trial*
1 year of GH versus 1 year of placebo
n=27 young adults with PWS

**Body composition:**
- Deteriorated during placebo with an increase in fat mass
- Maintained during GH versus placebo with a lower fat mass and higher lean body mass

**Metabolic Health profile:**
- GH versus placebo had no adverse effects on carbohydrate metabolism, blood pressure, lipid profile or development of metabolic syndrome

**Cognitive functioning:**
- Did not deteriorate during placebo
- Reassuring results, but do not exclude a deterioration on the long term

**GH-treated young adults with PWS benefit from continuation of GH treatment without safety concerns**




## PWS Ghrelin Study

*Observational study*
n=138 patients with PWS, aged 0 to 30 years, compared with 50 obese and 39 healthy controls

**Fasting AG/UAG ratio:**
- Was higher in PWS patients than in healthy controls, due to higher AG levels and similar UAG levels in PWS
- Was similar in PWS patients without weight gain or hyperphagia and age-matched healthy controls, but higher in PWS patients with weight gain and/or hyperphagia

**The switch to excessive weight gain in PWS seems to coincide with an increase in AG/UAG ratio, even prior to the start of hyperphagia**

## Ghrelin response in OGTT

n=25 GH-treated + 10 GH-stop young adults with PWS

- AG and UAG levels strongly declined after glucose intake, resulting in a lower AG/UAG ratio
- The ghrelin system responded physiologically
- GH treatment resulted in lower AG levels

**The impaired satiety does not seem to result from an abnormal reponse of the ghrelin system to food intake**

**Figure 1.** Overview of this thesis.

## REFERENCES


1. Miller, J. L., Lynn, C. H., Driscoll, D. C. et al.: Nutritional phases in Prader-Willi syndrome. Am J Med Genet A, 155A: 1040, 2011
2. Feigerlova, E., Diene, G., Conte-Auriol, F. et al.: Hyperghrelinemia precedes obesity in Prader-Willi syndrome. J Clin Endocrinol Metab, 93: 2800, 2008
3. Cummings, D. E., Clement, K., Purnell, J. Q. et al.: Elevated plasma ghrelin levels in Prader Willi syndrome. Nat Med, 8: 643, 2002
4. Purtell, L., Sze, L., Loughnan, G. et al.: In adults with Prader-Willi syndrome, elevated ghrelin levels are more consistent with hyperphagia than high PYY and GLP-1 levels. Neuropeptides, 45: 301, 2011
5. DelParigi, A., Tschop, M., Heiman, M. L. et al.: High circulating ghrelin: a potential cause for hyperphagia and obesity in prader-willi syndrome. J Clin Endocrinol Metab, 87: 5461, 2002
6. Haqq, A. M., Farooqi, I. S., O'Rahilly, S. et al.: Serum ghrelin levels are inversely correlated with body mass index, age, and insulin concentrations in normal children and are markedly increased in Prader-Willi syndrome. J Clin Endocrinol Metab, 88: 174, 2003
7. Kweh, F. A., Miller, J. L., Sulsona, C. R. et al.: Hyperghrelinemia in Prader-Willi syndrome begins in early infancy long before the onset of hyperphagia. Am J Med Genet A, 2014
8. Swaab, D. F.: Prader-Willi syndrome and the hypothalamus. Acta Paediatr Suppl, 423: 50, 1997
9. de Lind van Wijngaarden, R. F., Joosten, K. F., van den Berg, S. et al.: The relationship between central adrenal insufficiency and sleep-related breathing disorders in children with Prader-Willi syndrome. J Clin Endocrinol Metab, 94: 2387, 2009
10. de Lind van Wijngaarden, R. F., Otten, B. J., Festen, D. A. et al.: High prevalence of central adrenal insufficiency in patients with Prader-Willi syndrome. J Clin Endocrinol Metab, 93: 1649, 2008
11. Eiholzer, U., Bachmann, S., l'Allemand, D.: Is there growth hormone deficiency in prader-willi Syndrome? Six arguments to support the presence of hypothalamic growth hormone deficiency in Prader-Willi syndrome. Horm Res, 53 Suppl 3: 44, 2000
12. Takaya, K., Ariyasu, H., Kanamoto, N. et al.: Ghrelin strongly stimulates growth hormone release in humans. J Clin Endocrinol Metab, 85: 4908, 2000
13. Hataya, Y., Akamizu, T., Takaya, K. et al.: A low dose of ghrelin stimulates growth hormone (GH) release synergistically with GH-releasing hormone in humans. J Clin Endocrinol Metab, 86: 4552, 2001
14. Ariyasu, H., Takaya, K., Tagami, T. et al.: Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. J Clin Endocrinol Metab, 86: 4753, 2001
15. Arenz, T., Schwarzer, A., Pfluger, T. et al.: Delayed gastric emptying in patients with Prader Willi Syndrome. J Pediatr Endocrinol Metab, 23: 867, 2010
16. Lindgren, A. C., Barkeling, B., Hagg, A. et al.: Eating behavior in Prader-Willi syndrome, normal weight, and obese control groups. J Pediatr, 137: 50, 2000
17. Liu, J., Prudom, C. E., Nass, R. et al.: Novel ghrelin assays provide evidence for independent regulation of ghrelin acylation and secretion in healthy young men. J Clin Endocrinol Metab, 93: 1980, 2008
18. Wren, A. M., Seal, L. J., Cohen, M. A. et al.: Ghrelin enhances appetite and increases food intake in humans. J Clin Endocrinol Metab, 86: 5992, 2001
19. Kuppens, R. J., Diene, G., Bakker, N. E. et al.: Elevated ratio of acylated to unacylated ghrelin in children and young adults with Prader-Willi syndrome. Endocrine, 50: 633, 2015

20. Harada, T., Nakahara, T., Yasuhara, D. et al.: Obestatin, acyl ghrelin, and des-acyl ghrelin responses to an oral glucose tolerance test in the restricting type of anorexia nervosa. Biol Psychiatry, 63: 245, 2008
21. Foster-Schubert, K. E., Overduin, J., Prudom, C. E. et al.: Acyl and total ghrelin are suppressed strongly by ingested proteins, weakly by lipids, and biphasically by carbohydrates. J Clin Endocrinol Metab, 93: 1971, 2008
22. Perry, B., Wang, Y.: Appetite regulation and weight control: the role of gut hormones. Nutr Diabetes, 2: e26, 2012
23. Zhang, H., Wu, C., Chen, Q. et al.: Treatment of obesity and diabetes using oxytocin or analogs in patients and mouse models. PLoS One, 8: e61477, 2013
24. Hauffa, B. P., Haase, K., Range, I. M. et al.: The effect of growth hormone on the response of total and acylated ghrelin to a standardized oral glucose load and insulin resistance in children with Prader-Willi syndrome. J Clin Endocrinol Metab, 92: 834, 2007
25. Tschop, M., Smiley, D. L., Heiman, M. L.: Ghrelin induces adiposity in rodents. Nature, 407: 908, 2000
26. Theander-Carrillo, C., Wiedmer, P., Cettour-Rose, P. et al.: Ghrelin action in the brain controls adipocyte metabolism. J Clin Invest, 116: 1983, 2006
27. Asakawa, A., Inui, A., Fujimiya, M. et al.: Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. Gut, 54: 18, 2005
28. Allas, S., Abribat, T.: Clinical perspectives for ghrelin-derived therapeutic products. Endocr Dev, 25: 157, 2013
29. Delhanty, P. J., Neggers, S. J., van der Lely, A. J.: Mechanisms in endocrinology: Ghrelin: the differences between acyl- and des-acyl ghrelin. Eur J Endocrinol, 167: 601, 2012
30. Ozcan, B., Neggers, S. J., Miller, A. R. et al.: Does des-acyl ghrelin improve glycemic control in obese diabetic subjects by decreasing acylated ghrelin levels? Eur J Endocrinol, 170: 799, 2014
31. Zhang, G., Cai, D.: Circadian intervention of obesity development via resting-stage feeding manipulation or oxytocin treatment. Am J Physiol Endocrinol Metab, 301: E1004, 2011
32. Schaller, F., Watrin, F., Sturny, R. et al.: A single postnatal injection of oxytocin rescues the lethal feeding behaviour in mouse newborns deficient for the imprinted Magel2 gene. Hum Mol Genet, 19: 4895, 2010
33. Hoybye, C., Barkeling, B., Espelund, U. et al.: Peptides associated with hyperphagia in adults with Prader-Willi syndrome before and during GH treatment. Growth Horm IGF Res, 13: 322, 2003
34. Einfeld, S. L., Smith, E., McGregor, I. S. et al.: A double-blind randomized controlled trial of oxytocin nasal spray in Prader Willi syndrome. Am J Med Genet A, 164A: 2232, 2014
35. Deal, C. L., Tony, M., Hoybye, C. et al.: GrowthHormone Research Society workshop summary: consensus guidelines for recombinant human growth hormone therapy in Prader-Willi syndrome. J Clin Endocrinol Metab, 98: E1072, 2013
36. Hauffa, B. P., Schlippe, G., Roos, M. et al.: Spontaneous growth in German children and adolescents with genetically confirmed Prader-Willi syndrome. Acta Paediatr, 89: 1302, 2000
37. Mokdad, A. H., Ford, E. S., Bowman, B. A. et al.: Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. JAMA, 289: 76, 2003
38. Colditz, G. A., Willett, W. C., Rotnitzky, A. et al.: Weight gain as a risk factor for clinical diabetes mellitus in women. Ann Intern Med, 122: 481, 1995
39. Whitlock, G., Lewington, S., Sherliker, P. et al.: Body-mass index and cause-specific mortality in 900 000 adults: collaborative analyses of 57 prospective studies. Lancet, 373: 1083, 2009

40. Rosito, G. A., D'Agostino, R. B., Massaro, J. et al.: Association between obesity and a prothrombotic state: the Framingham Offspring Study. Thromb Haemost, 91: 683, 2004
41. Grotle, M., Hagen, K. B., Natvig, B. et al.: Obesity and osteoarthritis in knee, hip and/or hand: an epidemiological study in the general population with 10 years follow-up. BMC Musculoskelet Disord, 9: 132, 2008
42. Gortmaker, S. L., Must, A., Perrin, J. M. et al.: Social and economic consequences of overweight in adolescence and young adulthood. N Engl J Med, 329: 1008, 1993
43. Berrington de Gonzalez, A., Hartge, P., Cerhan, J. R. et al.: Body-mass index and mortality among 1.46 million white adults. N Engl J Med, 363: 2211, 2010
44. Smith, A. G., Muscat, G. E.: Skeletal muscle and nuclear hormone receptors: implications for cardiovascular and metabolic disease. Int J Biochem Cell Biol, 37: 2047, 2005
45. Halliday, D., Hesp, R., Stalley, S. F. et al.: Resting metabolic rate, weight, surface area and body composition in obese women. Int J Obes, 3: 1, 1979
46. Bosy-Westphal, A., Reinecke, U., Schlorke, T. et al.: Effect of organ and tissue masses on resting energy expenditure in underweight, normal weight and obese adults. Int J Obes Relat Metab Disord, 28: 72, 2004
47. Bakker, N. E., Siemensma, E. P., Koopman, C. et al.: Dietary Energy Intake, Body Composition and Resting Energy Expenditure in Prepubertal Children with Prader-Willi Syndrome before and during Growth Hormone Treatment: A Randomized Controlled Trial. Horm Res Paediatr, 83: 321, 2015
48. Bekx, M. T., Carrel, A. L., Shriver, T. C. et al.: Decreased energy expenditure is caused by abnormal body composition in infants with Prader-Willi Syndrome. J Pediatr, 143: 372, 2003
49. Bakker, N. E., Kuppens, R. J., Siemensma, E. P. et al.: Bone mineral density in children and adolescents with Prader-Willi syndrome: a longitudinal study during puberty and 9 years of growth hormone treatment. J Clin Endocrinol Metab, 100: 1609, 2015
50. Festen, D. A., Wevers, M., Lindgren, A. C. et al.: Mental and motor development before and during growth hormone treatment in infants and toddlers with Prader-Willi syndrome. Clin Endocrinol (Oxf), 68: 919, 2008
51. Reus, L., Pillen, S., Pelzer, B. J. et al.: Growth hormone therapy, muscle thickness, and motor development in Prader-Willi syndrome: an RCT. Pediatrics, 134: e1619, 2014
52. Clayton, P. E., Cuneo, R. C., Juul, A. et al.: Consensus statement on the management of the GH-treated adolescent in the transition to adult care. Eur J Endocrinol, 152: 165, 2005
53. Inzaghi, E., Cianfarani, S.: The Challenge of Growth Hormone Deficiency Diagnosis and Treatment during the Transition from Puberty into Adulthood. Front Endocrinol (Lausanne), 4: 34, 2013
54. Corneli, G., Di Somma, C., Baldelli, R. et al.: The cut-off limits of the GH response to GH-releasing hormone-arginine test related to body mass index. Eur J Endocrinol, 153: 257, 2005
55. Nagai, T., Mori, M.: Prader-Willi syndrome, diabetes mellitus and hypogonadism. Biomed Pharmacother, 53: 452, 1999
56. Laurier, V., Lapeyrade, A., Copet, P. et al.: Medical, psychological and social features in a large cohort of adults with Prader-Willi syndrome: experience from a dedicated centre in France. J Intellect Disabil Res, 59: 411, 2015
57. Hoybye, C., Hilding, A., Jacobsson, H. et al.: Growth hormone treatment improves body composition in adults with Prader-Willi syndrome. Clin Endocrinol (Oxf), 58: 653, 2003
58. Sode-Carlsen, R., Farholt, S., Rabben, K. F. et al.: One year of growth hormone treatment in adults with Prader-Willi syndrome improves body composition: results from a randomized, placebo-controlled study. J Clin Endocrinol Metab, 95: 4943, 2010

59. Sanchez-Ortiga, R., Klibanski, A., Tritos, N. A.: Effects of recombinant human growth hormone therapy in adults with Prader-Willi syndrome: a meta-analysis. Clin Endocrinol (Oxf), 77: 86, 2012
60. Grugni, G., Sartorio, A., Crino, A.: Growth hormone therapy for Prader-willi syndrome: challenges and solutions. Ther Clin Risk Manag, 12: 873, 2016
61. Bakker, N. E., Kuppens, R. J., Siemensma, E. P. et al.: Eight years of growth hormone treatment in children with Prader-Willi syndrome: maintaining the positive effects. J Clin Endocrinol Metab, 98: 4013, 2013
62. Siemensma, E. P., Tummers-de Lind van Wijngaarden, R. F., Festen, D. A. et al.: Beneficial effects of growth hormone treatment on cognition in children with Prader-Willi syndrome: a randomized controlled trial and longitudinal study. J Clin Endocrinol Metab, 97: 2307, 2012
63. American Psychiatric Association., American Psychiatric Association. DSM-5 Task Force.: Diagnostic and statistical manual of mental disorders : DSM-5, 5th ed. Washington, D.C.: American Psychiatric Association, pp. xliv, 947 p., 2013
64. Bakker, N. E., van Doorn, J., Renes, J. S. et al.: IGF-1 Levels, Complex Formation, and IGF Bioactivity in Growth Hormone-Treated Children With Prader-Willi Syndrome. J Clin Endocrinol Metab, 100: 3041, 2015

CHAPTER 9

# Summary
# Samenvatting

## SUMMARY

In this thesis we provide a detailed description of the studies performed to improve knowledge about Prader-Willi syndrome (PWS) and to optimize care for patients and parents. This chapter summarizes the studies and their most important outcomes.

### Chapter 1

This chapter provides an introduction in PWS by discussing the genetic background, symptoms in different phases of life, GH treatment, and unresolved issues, such as fasting and glucose-stimulated levels of the appetite-stimulating hormone ghrelin, effects of oxytocin treatment on social and eating behaviour, and effects of cessation versus continuation of GH treatment on body composition, metabolic health profile and cognition in young adults who were treated during childhood and had attained adult height. This chapter further describes the aims and outline of this thesis.

### Chapter 2

Prader-Willi syndrome is characterized by a switch from failure to thrive to excessive weight gain and hyperphagia in early childhood. The underlying mechanism of this switch is unknown, but the appetite-regulating hormone ghrelin might be involved. Acylated ghrelin (AG) is diabetogenic and stimulates appetite, while unacylated ghrelin (UAG) is known for its protective effects and is able to suppress AG. This suggests a crucial role for the ratio between AG and UAG. We, therefore, conducted an observational study in collaboration with PWS Centers in Toulouse and Lyon, France, and evaluated fasting AG and UAG serum levels and their associations with hyperphagia in a large group of 138 patients with PWS from 0.2 to 29.4 years. We compared them with levels of 50 age-matched obese subjects and 39 healthy controls. PWS patients had higher AG levels but similar UAG levels compared to healthy controls, resulting in a significantly higher AG/UAG ratio in PWS patients than in healthy controls. Obese controls had significantly lower AG and UAG levels than PWS patients, but also a high AG/UAG ratio. Remarkably, PWS patients without weight gain or hyperphagia had a similar AG/UAG ratio as age-matched healthy controls, in contrast to those with weight gain and/or hyperphagia who had an elevated AG/UAG ratio.

We conclude that the switch to excessive weight gain in PWS seems to coincide with an increase in the AG/UAG ratio, even prior to the start of hyperphagia.

### Chapter 3

Prader-Willi syndrome is characterized by hyperphagia with impaired satiety. Fasted PWS patients have very high AG with normal UAG levels, resulting in an elevated AG/UAG ratio, suggesting an intrinsic defect in the ghrelin regulation. Normally, food intake

induces satiety and a drop in AG and UAG levels, but it was unknown if these levels also decline in PWS. We, therefore, evaluated serum levels of AG, UAG and AG/UAG ratio before and after a glucose load during an oral glucose tolerance test (OGTT). These OGTTs were performed in 24 GH-treated and in 10 GH-stop young adults with PWS. In both groups, there was a sharp decline of AG levels and a decrease of UAG levels in the first 30 minutes after the glucose load, which resulted in a lower AG/UAG ratio. GH-treated patients had significantly lower AG levels than GH-stop patients at baseline and during the OGTT.

We conclude that, even in the presence of very high fasting AG/UAG ratios, the ghrelin system of young adults with PWS shows a physiological response with a strong decline of AG and UAG levels after glucose intake. Our findings might suggest that the impaired satiety is not the result of an abnormal response of the orexigenic ghrelin to food intake. Continuation of GH treatment after attainment of adult height results in lower AG levels at baseline and during OGTT, suggesting a more favourable metabolic profile.

**Chapter 4**

PWS is known for hyperphagia with impaired satiety and a specific behavioural phenotype with stubbornness, temper tantrums, manipulative and controlling behaviour and obsessive-compulsive features. PWS is associated with hypothalamic and oxytocinergic dysfunction. In humans without PWS, intranasal oxytocin administration had positive effects on social and eating behaviour. We, therefore, evaluated the effects of intranasal oxytocin compared to placebo administration on social behaviour and hyperphagia in a randomized, double-blind, placebo-controlled, cross-over study. Twenty-five children with PWS between 6 and 14 years of age received 4 weeks oxytocin and 4 weeks placebo twice daily. In the total group, no significant effects of oxytocin on social behaviour or hyperphagia were found, but in the 17 children younger than 11 years, parents reported significantly less anger, sadness, conflicts and food-related behaviour, and improvement of social behaviour during oxytocin treatment compared with placebo. In the 8 children older than 11 years, the items happiness, anger and sadness were negatively influenced by oxytocin treatment compared to placebo. There were no side effects or adverse events.

We conclude that intranasal oxytocin administration has promising effects on social and food-related behaviour in children with PWS younger than 11 years of age.

**Chapter 5**

Patients with PWS have an abnormal body composition with an increased FM and decreased LBM. During childhood, GH treatment counteracts the natural course of increasing obesity, but young adults have to stop GH treatment when they attain AH. The consequences of cessation of GH treatment were unknown, but it was conceivable that

it would deteriorate their improved clinical condition, while continuation might benefit them. We, therefore, investigated the effects of 1 year of GH versus 1 year of placebo on body composition measured by DXA in a 2-year, randomized, double-blind, placebo-controlled cross-over study with stratification for gender and BMI. Twenty-seven young adults with PWS who were GH-treated for many years during childhood and had attained AH participated and received GH treatment and placebo. During placebo, there was an increase of FM of +21.5%. Compared to placebo, GH treatment resulted in a lower FM and higher LBM, with changes of -17.3% and +3.5% respectively. Both FM% of the limbs and trunk were lower during GH versus placebo (change -17.3% and -15.6%). No GH-related adverse events occurred.

We conclude that GH-treated young adults with PWS who have attained AH, benefit from continuation of GH treatment. Cessation of GH treatment leads to a deterioration of body composition, while GH maintains the improved FM and LBM. This is very important, because development towards morbid obesity is a major threat to these patients. Thus, it is likely that continued GH administration contributes to a better future health for these young adults.

**Chapter 6**

Patients with PWS are severely at risk to develop morbid obesity, diabetes mellitus type 2 and cardiovascular disease, leading to high mortality. GH-treated young adults with PWS who have attained adult height benefit from continuation of GH treatment, because GH versus placebo maintains their improved body composition. It was, however, unknown whether GH affects metabolic health profile of this patient group. We, therefore, investigated the effects of 1 year of GH versus 1 year of placebo on metabolic health in a 2-year, randomized, double-blind, placebo-controlled cross-over study with stratification for gender and BMI. Twenty-seven young adults with PWS who were GH-treated for many years during childhood and had attained AH received GH treatment and placebo. Compared to placebo, GH treatment resulted in similar glucose and insulin levels during oral glucose tolerance test. Only fasting glucose and insulin were slightly higher during GH versus placebo, although both remained within the normal ranges. Blood pressure and lipid profile were similar during GH versus placebo. At baseline (AH) and during GH, no patients had metabolic syndrome, while 1 developed metabolic syndrome during placebo.

We conclude that GH treatment has no adverse effects on carbohydrate metabolism, blood pressure, lipid profile or the development of metabolic syndrome in young adults with PWS. Thus, GH-treated young adults with PWS who have attained AH benefit from continuation of GH treatment without safety concerns regarding to metabolic health.

### Chapter 7

Patients with PWS have a cognitive impairment. We have shown that GH treatment during childhood improves cognitive functioning, while GH-untreated children show a deterioration in IQ over time. The consequences of cessation of GH treatment on cognition at attainment of AH were unknown, but it could be that the GH-induced improved cognition would deteriorate. We, therefore, investigated the effects of 1 year of placebo versus 1 year of GH administration on cognition in a 2-year, randomized, double-blind, placebo-controlled cross-over study. Twenty-five young adults with PWS who were GH-treated for many years during childhood and had attained AH received GH treatment and placebo. One year of placebo did not deteriorate TIQ, VIQ or PIQ compared to GH treatment. However, young adults with a lower TIQ had significantly more loss of TIQ points during placebo than during GH treatment, in particular those with a lower VIQ lost more VIQ during placebo. The effect of placebo versus GH on TIQ, VIQ and PIQ was not different for gender or genotype.

We conclude that 1 year of placebo did not deteriorate cognitive functioning in young adults with PWS who were treated for many years with GH during childhood and had attained AH. However, patients with lower cognitive functioning had more loss of IQ points during placebo versus GH administration. These results are reassuring, but do not exclude that cessation of GH treatment for many years could result in a gradual deterioration of cognitive functioning on the long term.

### Chapter 8

In the general discussion, we discuss our results in view of the current literature and present the clinical implications of our findings. In addition, we report our general conclusions and give suggestions for further research.

## SAMENVATTING

In dit proefschrift geven we een gedetailleerd overzicht van de verschillende studies die zijn uitgevoerd om de kennis over Prader-Willi syndroom (PWS) te vergroten en de zorg voor patiënten en ouders te verbeteren. Hier wordt een samenvatting gegeven van deze studies en de belangrijkste resultaten worden besproken.

### Hoofdstuk 1

In dit hoofdstuk wordt achtergrondinformatie over PWS gegeven. De genetische achtergrond, de karakteristieke kenmerken per levensfase en groeihormoon (GH) behandeling worden besproken. Daarna volgt een korte inleiding van de onderwerpen die onderzocht zijn in dit proefschrift, namelijk de nuchtere en glucose-gestimuleerde spiegels van het eetluststimulerende hormoon ghreline, de effecten van oxytocinebehandeling op sociaal- en eetgedrag, en de effecten van stoppen versus voortzetten van GH behandeling op de lichaamssamenstelling, het metabole gezondheidsprofiel en cognitief functioneren van uitgegroeide jongvolwassenen met PWS die tijdens kindertijd behandeld zijn met GH. Daarnaast geeft dit hoofdstuk een overzicht van de vraagstellingen in dit proefschrift.

### Hoofdstuk 2

De verandering van 'failure to thrive' naar een overmatige gewichtstoename en hyperfagie is een typisch kenmerk van PWS. Het onderliggende mechanisme van deze verandering in de vroege kindertijd is onbekend, maar het eetlustregulerende hormoon ghreline zou betrokken kunnen zijn. Geacyleerd ghreline (AG) is diabetogeen en stimuleert de eetlust, terwijl ongeacyleerd ghreline (UAG) bekend staat om zijn beschermende effecten en de mogelijkheid om AG te onderdrukken. Dit suggereert een cruciale rol voor de verhouding tussen AG en UAG, oftewel de AG/UAG ratio. Samen met 2 andere PWS centra in Toulouse en Lyon (Frankrijk) hebben we nuchtere serum AG en UAG spiegels en hun associaties met hyperfagie onderzocht in een grote groep van 138 patiënten met PWS tussen 0.2 en 29.4 jaar. De spiegels werden vergeleken met die van 50 obese en 39 gezonde controles met dezelfde leeftijd. Kinderen en jongeren met PWS hadden hogere nuchtere AG spiegels, maar gelijke UAG spiegels in vergelijking met gezonde controles, resulterend in een significant hogere AG/UAG ratio in PWS patiënten dan in gezonde controles. Obese controles hadden significant lagere AG en UAG spiegels dan PWS patiënten, maar eveneens een hogere AG/UAG ratio. Het was opmerkelijk dat de AG/UAG ratio van PWS patiënten zonder gewichtstoename of hyperfagie gelijk was aan die van gezonde controles, terwijl PWS patiënten met gewichtstoename en/of hyperfagie een verhoogde AG/UAG ratio hadden.

Uit dit onderzoek concluderen we dat de verandering naar een overmatige gewichtstoename in PWS samen lijkt te gaan met een toename in de AG/UAG ratio, zelfs voorafgaand aan het ontstaan van de hyperfagie.

**Hoofdstuk 3**

Het Prader-Willi syndroom wordt gekenmerkt door hyperfagie in combinatie met een onverzadigbare eetlust. PWS patiënten hebben erg hoge nuchtere AG spiegels met normale UAG spiegels, leidend tot een verhoogde AG/UAG ratio. Dit zou kunnen wijzen op een intrinsiek defect in de ghreline regulatie. Voedselinname leidt normaal gesproken tot een verzadigd gevoel en een daling van de AG en UAG spiegels, maar het was onbekend of deze spiegels ook omlaag gaan bij mensen met PWS. Daarom hebben we de serum spiegels van AG, UAG en AG/UAG ratio onderzocht voorafgaand aan en na inname van een gestandaardiseerde hoeveelheid glucose tijdens een orale glucose tolerantie test (OGTT). Deze OGTTs werden verricht bij 24 GH-behandelde en 10 met GH gestopte jongvolwassenen met PWS. In beide groepen werd in de eerste 30 minuten na de glucose inname een sterke afname van AG spiegels gezien. De UAG spiegels daalden minder sterk, waardoor de AG/UAG ratio lager werd. GH-behandelde patiënten hadden significant lagere AG spiegels dan de met GH gestopte patiënten, zowel voorafgaand aan als tijdens de OGTT.

We concluderen dat het ghreline systeem van jongvolwassenen met PWS na inname van glucose een fysiologische reactie laat zien met een sterke daling van AG en UAG spiegels, zelfs in de aanwezigheid van erg hoge nuchtere AG/UAG ratio's. Onze bevindingen zouden erop kunnen wijzen dat de onverzadigbare eetlust niet wordt veroorzaakt door een afwijkende reactie van het eetluststimulerende ghreline op voedselinname. Het continueren van GH behandeling nadat een jongvolwassene is uitgegroeid leidt tot lagere AG spiegels voorafgaand aan en tijdens een OGTT, wat wijst op een gunstiger metabool profiel.

**Hoofdstuk 4**

Karakteristieke symptomen die bij PWS passen zijn hyperfagie met een onverzadigbare eetlust en een specifiek gedragsfenotype met koppigheid, driftbuien, manipulatief en controlerend gedrag en obsessief-compulsieve kenmerken. PWS is geassocieerd met hypothalame en oxytocinerge stoornissen. Bij mensen zonder PWS had intranasale oxytocinebehandeling een positief effect op sociaal- en eetgedrag. Daarom hebben we middels een gerandomiseerde, dubbelblinde, placebogecontroleerde cross-over studie de effecten van intranasale oxytocine op sociaal gedrag en hyperfagie onderzocht. Vijfentwintig kinderen met PWS tussen 6 en 14 jaar kregen tweemaal daags neusspray toegediend, eerst 4 weken oxytocine en vervolgens 4 weken placebo of in de omgekeerde volgorde. In de gehele groep werden geen significante effecten van oxytocine op

sociaal gedrag en hyperfagie gevonden, maar in de 17 kinderen die jonger waren dan 11 jaar, werd tijdens de oxytocinebehandeling significant minder boosheid, verdriet, conflicten en voedselgerelateerd gedrag, en een verbetering van sociaal gedrag gevonden dan tijdens placebo. In de 8 kinderen ouder dan 11 jaar had oxytocinebehandeling een negatief effect op de items geluk, boosheid en verdriet. Er waren geen bijwerkingen of ongewenste gebeurtenissen.

We concluderen dat intranasale oxytocinebehandeling veelbelovende effecten heeft op sociaal- en voedselgerelateerd gedrag bij kinderen met PWS jonger dan 11 jaar. Toekomstig onderzoek moet uitwijzen of oudere kinderen gebaat zijn bij een lagere dosering oxytocine.

**Hoofdstuk 5**

Patiënten met PWS hebben een afwijkende lichaamssamenstelling met een toegenomen vetmassa en weinig spiermassa. Tijdens de kinderleeftijd gaat GH-behandeling het natuurlijk beloop van toenemende obesitas en een afwijkende lichaamssamenstelling tegen, maar jongvolwassenen moeten stoppen met deze behandeling op het moment dat ze uitgegroeid zijn. De consequenties van het stoppen van de GH-behandeling waren onbekend, maar het was voorstelbaar dat de verbeterde klinische conditie zou verslechteren, terwijl jongvolwassenen met PWS ze baat zouden kunnen hebben bij voortzetting van GH-behandeling. We hebben daarom de effecten van GH versus placebo op lichaamssamenstelling via DXA-scans onderzocht in een 2-jarige, gerandomiseerde, dubbelblinde, placebogecontroleerde cross-over studie met stratificatie voor geslacht en BMI. Zevenentwintig uitgegroeide jongvolwassenen met PWS die tijdens de kinderleeftijd behandeld zijn met GH participeerden in de studie en werden behandeld met GH-behandeling en placebo, beiden gedurende 1 jaar. Tijdens placebo nam de vetmassa toe met +21.5%. In vergelijking met placebo resulteerde GH-behandeling in een lagere vetmassa en hogere spiermassa, -17.3% en +3.5% respectievelijk. Zowel het vetpercentage van de ledematen als van de romp waren lager tijdens GH-behandeling dan tijdens placebo (-17.3% en -15.6%, resp.). Er waren geen GH-gerelateerde bijwerkingen.

Uit dit onderzoek concluderen we dat uitgegroeide GH-behandelde jongvolwassenen met PWS baat hebben bij het voortzetten van GH-behandeling. Stoppen van GH-behandeling zorgt voor een verslechtering van de lichaamssamenstelling, terwijl voortzetten van GH de verbeterde vetmassa en spiermassa behoudt. Dit is van groot belang, aangezien de ontwikkeling van morbide obesitas een ernstige bedreiging is voor deze patiënten. Voortzetting van GH-behandeling zou bij kunnen dragen aan een betere en gezondere toekomst voor jongvolwassenen met PWS.

**Hoofdstuk 6**

Patiënten met PWS lopen een ernstig risico op het ontwikkelen van morbide obesitas, diabetes mellitus type 2 en cardiovasculaire ziekten, wat zorgt voor een hoog risico op sterfte. Uitgegroeide GH-behandelde jongvolwassenen met PWS zouden baat kunnen hebben bij het voortzetten van GH-behandeling, aangezien placebo zorgt voor een toename van vetmassa, terwijl GH versus placebo de verbeterde lichaamssamenstelling kan behouden. Het was echter onbekend of GH-behandeling het metabole gezondheidsprofiel van deze patiëntengroep beïnvloedt. We hebben daarom de effecten van GH versus placebo op het metabole gezondheidsprofiel onderzocht in een 2-jarige, gerandomiseerde, dubbelblinde, placebogecontroleerde cross-over studie met stratificatie voor geslacht en BMI. Zevenentwintig uitgegroeide jongvolwassenen met PWS die tijdens de kinderleeftijd behandeld waren met GH participeerden in de studie en werden behandeld met GH-behandeling en placebo, beiden gedurende 1 jaar. In vergelijking met placebo resulteerde GH-behandeling in vergelijkbare glucose en insuline spiegels tijdens een OGTT. Enkel het nuchtere glucose en insuline waren iets hoger tijdens GH dan tijdens placebo, hoewel beide in beide periodes binnen de normale grenzen bleven. Bloeddruk en lipiden waren gelijk tijdens GH-behandeling en placebo. Voorafgaand aan de studie en gedurende de GH-behandeling had geen van de patiënten metabool syndroom, terwijl 1 patiënt dit ontwikkelde tijdens placebo.

We concluderen dat GH-behandeling geen negatieve effecten heeft op het koolhydraatmetabolisme, de bloeddruk, het lipidenprofiel of op de ontwikkeling van metabool syndroom. Al met al hebben uitgegroeide GH-behandelde jongvolwassenen met PWS baat bij voorzetting van GH-behandeling zonder dat er ongerustheid hoeft te bestaan over de veiligheidsaspecten aangaande het metabole gezondheidsprofiel.

**Hoofdstuk 7**

Patiënten met PWS hebben een cognitieve beperking. Onze onderzoeksgroep heeft voorheen laten zien dat GH-behandeling tijdens de kinderleeftijd het cognitief functioneren verbetert, terwijl GH-onbehandelde kinderen achteruitgaan in IQ. De gevolgen van het stoppen met GH-behandeling op het moment dat jongeren uitgegroeid zijn waren onbekend, maar het was voorstelbaar dat de GH-geïnduceerde verbeterde cognitie zou verslechteren, terwijl jongvolwassenen met PWS baat zouden kunnen hebben bij voortzetting van GH-behandeling. We hebben daarom de effecten van placebo versus GH op het cognitief functioneren onderzocht in een 2-jarige, gerandomiseerde, dubbelblinde, placebogecontroleerde cross-over studie. Vijfentwintig uitgegroeide jongvolwassenen met PWS die tijdens de kinderleeftijd behandeld waren met GH participeerden in de studie en werden behandeld met GH-behandeling en placebo, beiden gedurende 1 jaar. Eén jaar placebo gaf geen verslechtering van het totaal IQ (TIQ), verbaal IQ (VIQ) of performaal IQ (PIQ), en TIQ, VIQ of PIQ waren niet verschillend tijdens GH of placebo.

Jongvolwassenen met een lager TIQ verloren echter significant meer TIQ punten tijdens placebo versus GH-behandeling, met name jongvolwassenen met PWS met een lager VIQ verloren meer VIQ punten tijdens placebo. Het effect van placebo versus GH op TIQ, VIQ en PIQ was niet verschillend voor geslacht of genotype.

We concluderen dat 1 jaar placebo geen achteruitgang gaf van het cognitief functioneren van uitgegroeide jongvolwassenen met PWS die tijdens de kindertijd langdurig behandeld zijn met GH. Echter, patiënten met een lager VIQ verloren meer VIQ punten tijdens placebo versus GH behandeling.

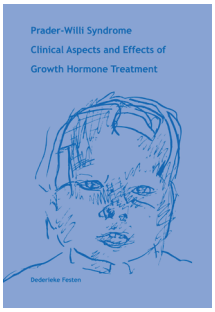
**Hoofdstuk 8**

In de algemene discussie bespreken we onze resultaten in het kader van de huidige literatuur en presenteren de klinische consequenties van onze bevindingen. We geven onze algemene conclusies en suggesties voor verder onderzoek.

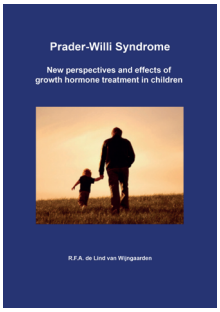
# CHAPTER 10

## Overview publications by PWS-team of Dutch Growth Research Foundation

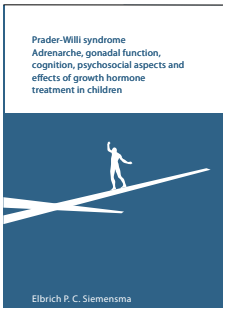
# OVERVIEW PUBLICATIONS BY PWS-TEAM OF DUTCH GROWTH RESEARCH FOUNDATION



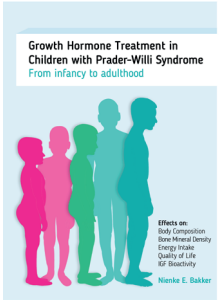

***2007*** **Prader-Willi syndrome**
**Clinical aspects and effects of growth hormone treatment**
*Dederieke A.M. Festen*



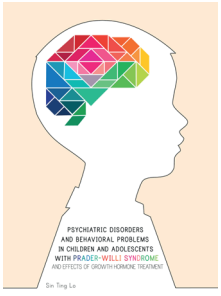

***2009*** **Prader-Willi syndrome**
**New perspectives and effects of growth hormone treatment in children**
*Roderick F.A. de Lind van Wijngaarden*



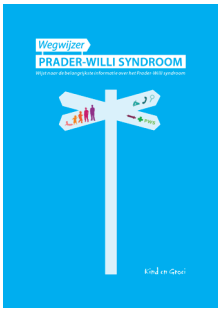

***2012*** **Prader-Willi syndrome**
**Adrenarche, gonadal function, cognition, psychosocial aspects From infancy to adulthood**
*Elbrich P.C. Siemensma*




***2015*** **Growth hormone treatment in children with Prader-Willi syndrome. From infancy to adulthood Effects on body composition, bone mineral density, energy intake, quality of life and IGF-bioactivity**
*Nienke E. Bakker*




***2015*** **Psychiatric disorders and behavioral problems in children and adolescents with Prader-Willi syndrome and effects of growth hormone treatment**
*Sin Ting Lo*




***2014*** **Wegwijzer Prader-Willi syndroom (in Dutch)**
**Binder for parents with information about PWS**
*Published by Dutch Growth Research Foundation*

## PUBLICATION LIST PWS-TEAM DUTCH GROWTH RESEARCH FOUNDATION

Festen DA, de Weerd AW, van den Bossche RA, Joosten K, Hoeve H, Hokken-Koelega AC 2006 Sleep-related breathing disorders in prepubertal children with Prader-Willi syndrome and effects of growth hormone treatment. The Journal of clinical endocrinology and metabolism 91:4911-4915

Festen DA, Visser TJ, Otten BJ, Wit JM, Duivenvoorden HJ, Hokken-Koelega AC 2007 Thyroid hormone levels in children with Prader-Willi syndrome before and during growth hormone treatment. Clinical endocrinology 67:449-456

Festen DA, Wevers M, de Weerd AW, van den Bossche RA, Duivenvoorden HJ, Otten BJ, Wit JM, Hokken-Koelega AC 2007 Psychomotor development in infants with Prader-Willi syndrome and associations with sleep-related breathing disorders. Pediatric research 62:221-224

Festen DA, van Toorenenbergen A, Duivenvoorden HJ, Hokken-Koelega AC 2007 Adiponectin levels in prepubertal children with Prader-Willi syndrome before and during growth hormone therapy. The Journal of clinical endocrinology and metabolism 92:1549-1554

Festen DA, Wevers M, de Weerd AW, van den Bossche RA, Duivenvoorden HJ, Hokken-Koelega AC 2008 Cognition and behavior in pre-pubertal children with Prader-Willi syndrome and associations with sleep-related breathing disorders. American journal of medical genetics. Part A 146A:3018-3025

Festen DA, de Lind van Wijngaarden R, van Eekelen M, Otten BJ, Wit JM, Duivenvoorden HJ, Hokken-Koelega AC 2008 Randomized controlled GH trial: effects on anthropometry, body composition and body proportions in a large group of children with Prader-Willi syndrome. Clinical endocrinology 69:443-451

de Lind van Wijngaarden RF, Otten BJ, Festen DA, Joosten KF, de Jong FH, Sweep FC, Hokken-Koelega AC 2008 High prevalence of central adrenal insufficiency in patients with Prader-Willi syndrome. The Journal of clinical endocrinology and metabolism 93:1649-1654

de Lind van Wijngaarden RF, de Klerk LW, Festen DA, Hokken-Koelega AC 2008 Scoliosis in Prader-Willi syndrome: prevalence, effects of age, gender, body mass index, lean body mass and genotype. Archives of disease in childhood 93:1012-1016

Festen DA, Wevers M, Lindgren AC, Bohm B, Otten BJ, Wit JM, Duivenvoorden HJ, Hokken-Koelega AC 2008 Mental and motor development before and during growth hormone treatment in infants and toddlers with Prader-Willi syndrome. Clinical endocrinology 68:919-925

de Lind van Wijngaarden RF, Siemensma EP, Festen DA, Otten BJ, van Mil EG, Rotteveel J, Odink RJ, Bindels-de Heus GC, van Leeuwen M, Haring DA, Bocca G, Houdijk EC, Hoorweg-Nijman JJ, Vreuls RC, Jira PE, van Trotsenburg AS, Bakker B, Schroor EJ, Pilon JW, Wit JM, Drop SL, Hokken-Koelega AC 2009 Efficacy and safety of long-term continuous growth hormone treatment in children with Prader-Willi syndrome. The Journal of clinical endocrinology and metabolism 94:4205-4215

de Lind van Wijngaarden RF, Festen DA, Otten BJ, van Mil EG, Rotteveel J, Odink RJ, van Leeuwen M, Haring DA, Bocca G, Mieke Houdijk EC, Hokken-Koelega AC 2009 Bone mineral density and effects of growth hormone treatment in prepubertal children with Prader-Willi syndrome: a randomized controlled trial. The Journal of clinical endocrinology and metabolism 94:3763-3771

de Lind van Wijngaarden RF, Joosten KF, van den Berg S, Otten BJ, de Jong FH, Sweep CG, de Weerd AW, Hokken-Koelega AC 2009 The relationship between central adrenal insufficiency and sleep-related breathing disorders in children with Prader-Willi syndrome. The Journal of clinical endocrinology and metabolism 94:2387-2393

de Lind van Wijngaarden RF, de Klerk LW, Festen DA, Duivenvoorden HJ, Otten BJ, Hokken-Koelega AC 2009 Randomized controlled trial to investigate the effects of growth hormone treatment on scoliosis in children with Prader-Willi syndrome. The Journal of clinical endocrinology and metabolism 94:1274-1280

de Lind van Wijngaarden RF, Cianflone K, Gao Y, Leunissen RW, Hokken-Koelega AC 2010 Cardiovascular and metabolic risk profile and acylation-stimulating protein levels in children with Prader-Willi syndrome and effects of growth hormone treatment. The Journal of clinical endocrinology and metabolism 95:1758-1766

Siemensma EP, de Lind van Wijngaarden RF, Otten BJ, de Jong FH, Hokken-Koelega AC 2011 Pubarche and serum dehydroepiandrosterone sulphate levels in children with Prader-Willi syndrome. Clinical endocrinology 75:83-89

Siemensma EP, van Alfen-van der Velden AA, Otten BJ, Laven JS, Hokken-Koelega AC 2012 Ovarian function and reproductive hormone levels in girls with Prader-Willi

syndrome: a longitudinal study. The Journal of clinical endocrinology and metabolism 97:E1766-1773

Siemensma EP, Tummers-de Lind van Wijngaarden RF, Festen DA, Troeman ZC, van Alfen-van der Velden AA, Otten BJ, Rotteveel J, Odink RJ, Bindels-de Heus GC, van Leeuwen M, Haring DA, Oostdijk W, Bocca G, Mieke Houdijk EC, van Trotsenburg AS, Hoorweg-Nijman JJ, van Wieringen H, Vreuls RC, Jira PE, Schroor EJ, van Pinxteren-Nagler E, Willem Pilon J, Lunshof LB, Hokken-Koelega AC 2012 Beneficial effects of growth hormone treatment on cognition in children with Prader-Willi syndrome: a randomized controlled trial and longitudinal study. The Journal of clinical endocrinology and metabolism 97:2307-2314

Siemensma EP, de Lind van Wijngaarden RF, Otten BJ, de Jong FH, Hokken-Koelega AC 2012 Testicular failure in boys with Prader-Willi syndrome: longitudinal studies of reproductive hormones. The Journal of clinical endocrinology and metabolism 97:E452-459

Lukoshe A, White T, Schmidt MN, van der Lugt A, Hokken-Koelega AC 2013 Divergent structural brain abnormalities between different genetic subtypes of children with Prader-Willi syndrome. Journal of neurodevelopmental disorders 5:31

Bakker NE, Kuppens RJ, Siemensma EP, Tummers-de Lind van Wijngaarden RF, Festen DA, Bindels-de Heus GC, Bocca G, Haring DA, Hoorweg-Nijman JJ, Houdijk EC, Jira PE, Lunshof L, Odink RJ, Oostdijk W, Rotteveel J, Schroor EJ, Van Alfen AA, Van Leeuwen M, Van Pinxteren-Nagler E, Van Wieringen H, Vreuls RC, Zwaveling-Soonawala N, de Ridder MA, Hokken-Koelega AC 2013 Eight years of growth hormone treatment in children with Prader-Willi syndrome: maintaining the positive effects. The Journal of clinical endocrinology and metabolism 98:4013-4022

Lo ST, Siemensma E, Collin P, Hokken-Koelega A 2013 Impaired theory of mind and symptoms of Autism Spectrum Disorder in children with Prader-Willi syndrome. Research in developmental disabilities 34:2764-2773

Lukoshe A, Hokken-Koelega AC, van der Lugt A, White T 2014 Reduced cortical complexity in children with Prader-Willi Syndrome and its association with cognitive impairment and developmental delay. PloS one 9:e107320

Bakker NE, Siemensma EP, van Rijn M, Festen DA, Hokken-Koelega AC 2015 Beneficial Effect of Growth Hormone Treatment on Health-Related Quality of Life in Children with Prader-Willi Syndrome: A Randomized Controlled Trial and Longitudinal Study. Hormone research in paediatrics 84:231-239

Lo ST, Festen DA, Tummers-de Lind van Wijngaarden RF, Collin PJ, Hokken-Koelega AC 2015 Beneficial Effects of Long-Term Growth Hormone Treatment on Adaptive Functioning in Infants With Prader-Willi Syndrome. American journal on intellectual and developmental disabilities 120:315-327

Bakker NE, van Doorn J, Renes JS, Donker GH, Hokken-Koelega AC 2015 IGF-1 Levels, Complex Formation, and IGF Bioactivity in Growth Hormone-Treated Children With Prader-Willi Syndrome. The Journal of clinical endocrinology and metabolism 100:3041-3049

Kuppens RJ, Diene G, Bakker NE, Molinas C, Faye S, Nicolino M, Bernoux D, Delhanty PJ, van der Lely AJ, Allas S, Julien M, Delale T, Tauber M, Hokken-Koelega AC 2015 Elevated ratio of acylated to unacylated ghrelin in children and young adults with Prader-Willi syndrome. Endocrine 50:633-642

Lo ST, Collin PJ, Hokken-Koelega AC 2015 Visual-motor integration in children with Prader-Willi syndrome. Journal of intellectual disability research : JIDR 59:827-834

Bakker NE, Siemensma EP, Koopman C, Hokken-Koelega AC 2015 Dietary Energy Intake, Body Composition and Resting Energy Expenditure in Prepubertal Children with Prader-Willi Syndrome before and during Growth Hormone Treatment: A Randomized Controlled Trial. Hormone research in paediatrics 83:321-331

Lo ST, Collin PJ, Hokken-Koelega AC 2015 Psychiatric disorders in children with Prader-Willi syndrome-Results of a 2-year longitudinal study. American journal of medical genetics. Part A 167A:983-991

Bakker NE, Kuppens RJ, Siemensma EP, Tummers-de Lind van Wijngaarden RF, Festen DA, Bindels-de Heus GC, Bocca G, Haring DA, Hoorweg-Nijman JJ, Houdijk EC, Jira PE, Lunshof L, Odink RJ, Oostdijk W, Rotteveel J, Van Alfen AA, Van Leeuwen M, Van Wieringen H, Wegdam-den Boer ME, Zwaveling-Soonawala N, Hokken-Koelega AC 2015 Bone mineral density in children and adolescents with Prader-Willi syndrome: a longitudinal study during puberty and 9 years of growth hormone treatment. The Journal of clinical endocrinology and metabolism 100:1609-1618

Lo ST, Siemensma EP, Festen DA, Collin PJ, Hokken-Koelega AC 2015 Behavior in children with Prader-Willi syndrome before and during growth hormone treatment: a randomized controlled trial and 8-year longitudinal study. European child & adolescent psychiatry 24:1091-1101

Bakker NE, Wolffenbuttel KP, Looijenga LH, Hokken-Koelega AC 2015 Testes in infants with Prader-Willi syndrome: human chorionic gonadotropin treatment, surgery and histology. The Journal of urology 193:291-298

Kuppens RJ, Delhanty PJ, Huisman TM, van der Lely AJ, Hokken-Koelega AC 2016 Acylated and unacylated ghrelin during OGTT in Prader-Willi syndrome: Support for normal response to food intake. Clinical Endocrinology 2016, in press

Kuppens RJ, Delhanty PJ, Huisman TM, van der Lely AJ, Hokken-Koelega AC 2016 The Authors' Reply: Acylated and unacylated ghrelin during OGTT in Prader-Willi syndrome, a rebuttal to the conclusions by Kuppens *et al.* Clinical Endocrinology 2016, in press

Kuppens RJ, Donze SH, Hokken-Koelega AC 2016 Promising effects of oxytocin on social and food-related behavior in young children with Prader-Willi Syndrome: a randomized, double-blind, controlled cross-over trial. Clinical Endocrinology 2016, in press

Kuppens RJ, Bakker NE, Siemensma EPC, Tummers-de Lind van Wijngaarden RFA, Donze SH, Festen DAM, van Alfen-van der Velden AAEM, Stijnen T, Hokken-Koelega ACS 2016 Beneficial effects of growth hormone in young adults with Prader-Willi syndrome: Results of a 2-year randomized, double-blind, placebo-controlled cross-over trial. The Journal of clinical endocrinology and metabolism 2016, in press

Kuppens RJ, Bakker NE, Siemensma ECP, Donze SH, Stijnen T, Hokken-Koelega ACS, Metabolic health profile in young adults with PWS: Results of a 2-year randomized, placebo-controlled cross-over GH trial. Clinical Endocrinology 2016, in press

## PUBLICATION LIST COLLABORATIONS

Deal CL, Tony M, Hoybye C, Allen DB, Tauber M, Christiansen JS 2013 GrowthHormone Research Society workshop summary: consensus guidelines for recombinant human growth hormone therapy in Prader-Willi syndrome (collaborator Hokken-Koelega AC). The Journal of clinical endocrinology and metabolism 98:E1072-1087

van den Berg-Emons R, Festen D, Hokken-Koelega A, Bussmann J, Stam H 2008 Everyday physical activity and adiposity in Prader-Willi syndrome. Journal of pediatric endocrinology & metabolism : JPEM 21:1041-1048

Goldstone AP, Holland AJ, Hauffa BP, Hokken-Koelega AC, Tauber M 2008 Recommendations for the diagnosis and management of Prader-Willi syndrome. The Journal of clinical endocrinology and metabolism 93:4183-4197

Tauber M, Hokken-Koelega AC, Hauffa BP, Goldstone AP 2009 About the benefits of growth hormone treatment in children with Prader-Willi syndrome. The Journal of pediatrics 154:778; author reply 779

Reus L, Pelzer BJ, Otten BJ, Siemensma EP, van Alfen-van der Velden JA, Festen DA, Hokken-Koelega AC, Nijhuis-van der Sanden MW 2013 Growth hormone combined with child-specific motor training improves motor development in infants with Prader-Willi syndrome: a randomized controlled trial. Research in developmental disabilities 34:3092-3103

Bakker NE, Hokken-Koelega ACS 2013 Growth hormone treatment in children with PWS. Prader-Willi syndrome by Höybye C. Nova Science Publishers, Inc New York. ISBN: 978-1-62618-993-5

Reus L, Pillen S, Pelzer BJ, van Alfen-van der Velden JA, Hokken-Koelega AC, Zwarts M, Otten BJ, Nijhuis-van der Sanden MW 2014 Growth hormone therapy, muscle thickness, and motor development in Prader-Willi syndrome: an RCT. Pediatrics 134:e1619-1627

Beauloye V, Diene G, Kuppens R, Zech F, Winandy C, Molinas C, Faye S, Kieffer I, Beckers D, Nergardh R, Hauffa B, Derycke C, Delhanty P, Hokken-Koelega A, Tauber M 2016 High unacylated ghrelin levels support the concept of anorexia in infants with prader-willi syndrome. Orphanet journal of rare diseases 11:56

# CHAPTER 11

**List of abbreviations**
**List of co-authors and affiliations**
**List of publications**
**PhD portfolio**
**Dankwoord**
**Curriculum Vitae**

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ACTH | corticotropin |
| AE | adverse event |
| AEBSF | 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride |
| AG | acylated ghrelin |
| AH | adult height |
| ASD | autism spectrum disorder |
| AUC | area under the curve |
| BMD | bone mineral density |
| BMI | body mass index |
| BP | breakpoint |
| BP | blood pressure |
| CRH | corticotropin-releasing hormone |
| CVD | cardiovascular disease |
| CV | coefficients of variation |
| CV | curriculum vitae |
| DNA | deoxyribonucleic acid |
| DXA | dual-energy X-ray absorptiometry |
| EMA | European Medicines Agency |
| FDA | Food and Drug Administration |
| FM | fat mass |
| FM% | fat mass percentage |
| FSH | follicle stimulating hormone |
| GH | growth hormone |
| GHRH | growth hormone-releasing hormone |
| GHSR | growth hormone secretagogue receptor |
| GLP-1 | glucagon like peptide type 1 |
| GnRH | gonadotropin-releasing hormone |
| GOAT | ghrelin O-acyltransferase |
| HDLc | high-density lipoprotein cholesterol |
| HOMA-IR | homeostatic model assessment of insulin resistance |
| ICD | imprinting center defect |
| IGF-I | insulin-like growth factor type I |
| IGFBP-3 | insulin-like growth factor binding protein 3 |
| IGT | impaired glucose tolerance |
| IQ | intelligence quotient |
| IQR | interquartile range |
| LBM | lean body mass |

| | |
|---|---|
| LDLc | low-density lipoprotein cholesterol |
| LH | luteinizing hormone |
| MD | medical doctor |
| MRI | magnetic resonance imaging |
| MS | metabolic syndrome |
| mUPD | maternal uniparental disomy |
| OGTT | oral glucose tolerance test |
| PIQ | performance IQ |
| PWS | Prader-Willi syndrome |
| RCT | randomized controlled trial |
| SAE | severe adverse event |
| SPSS | Statistical Package for Social Sciences |
| SDS | standard deviation score |
| T2DM | diabetes mellitus type 2 |
| TC | total cholesterol |
| TG | triglyceride |
| TIQ | total IQ |
| TRH | thyrotropin-releasing hormone |
| TSH | thyrotropin |
| UAG | unacylated ghrelin |
| VIQ | verbal IQ |
| WC | waist circumference |

## LIST OF CO-AUTHORS AND AFFILIATIONS

A.A.E.M. van Alfen-van der Velden, MD, PhD
Department of Pediatric Endocrinology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands

S. Allas, MD, PhD
Alizé Pharma, Ecully, France

N.E. Bakker, MD, PhD
Dutch Growth Research Foundation, Rotterdam, The Netherlands. Department of Pedatric Endocrinology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands

D. Bernoux, MD
Hôpital Femme-Mère-Enfant, Division of Pediatric Endocrinology, University of Lyon, Bron/Lyon, France

T. Delale, PhD
Alizé Pharma, Ecully, France

P. Delhanty, PhD
Department of Internal Medicine, Erasmus MC, Rotterdam, The Netherlands

G. Diène, MD
Centre de Référence du syndrome de Prader-Willi, Hôpital des Enfants, CHU de Toulouse, Toulouse, France

S.H. Donze, MD
Dutch Growth Research Foundation, Rotterdam, The Netherlands. Department of Pedatric Endocrinology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands

S. Faye
Centre de Référence du syndrome de Prader-Willi, Hôpital des Enfants, CHU de Toulouse, Toulouse, France

D.A.M. Festen, MD, PhD
Dutch Growth Research Foundation, Rotterdam, The Netherlands. Erasmus MC-Sophia, Rotterdam, The Netherlands. Ipse de Bruggen, Nootdorp, The Netherlands

A.C.S. Hokken-Koelega, MD, PhD
Dutch Growth Research Foundation, Rotterdam, The Netherlands. Department of Pedatric Endocrinology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands

T.M. Huisman
Department of Internal Medicine, Erasmus MC, Rotterdam, The Netherlands

M. Julien, PhD
Alizé Pharma, Ecully, France

A.J. van der Lelij, MD, PhD
Department of Internal Medicine, Erasmus MC, Rotterdam, The Netherlands

E.F. Mahabier, MSc
Dutch Growth Research Foundation, Rotterdam, The Netherlands. Department of Pedatric Endocrinology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands

C. Molinas, MD
Centre de Référence du syndrome de Prader-Willi, Hôpital des Enfants, CHU de Toulouse, Toulouse, France

M. Nicolino, MD, PhD
Hôpital Femme-Mère-Enfant, Division of Pediatric Endocrinology, University of Lyon, Bron/Lyon, France

E.P.C. Siemensma, MD, PhD
Dutch Growth Research Foundation, Rotterdam, The Netherlands. Department of Child Psychiatry, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands

T. Stijnen, PhD
Department Medical Statistics and Bioinformatics, Leiden University Medical Center, Leiden, The Netherlands

M. Tauber, MD
Centre de Référence du syndrome de Prader-Willi, Hôpital des Enfants, CHU de Toulouse, Toulouse, France

R.F.A. Tummers-de Lind van Wijngaarden, MD, PhD
Dutch Growth Research Foundation, Rotterdam, The Netherlands. Department of Internal Medicine, University Hospital Maastricht, Maastricht, The Netherlands

## LIST OF PUBLICATIONS

**RJ Kuppens**, G Dièn e, NE Bakker, C Molinas, S Faye, M Nicolino, D Bernoux, PJ Delhanty, AJ van der Lely, S Allas, M Julien, T Delale, M Tauber and ACS Hokken-Koelega, Elevated ratio of acylated to unacylated ghrelin in children and young adults with Prader-Willi syndrome, *Endocrine,* 50 (2015), 633-42

**RJ Kuppens**, PJ Delhanty, TM Huisman, AJ van der Lely and ACS Hokken-Koelega, Acylated and unacylated ghrelin during OGTT in Prader-Willi syndrome: support for normal response to food intake, *Clinical Endocrinology* (2016), in press

**RJ Kuppens**, SH Donze and ACS Hokken-Koelega, Promising effects of oxytocin on social and food-related behavior in young children with Prader-Willi syndrome: a randomized, double-blind, controlled cross-over trial, *Clinical Endocrinology* (2016), in press

**RJ Kuppens**, NE Bakker, EPC Siemensma, RFA Tummers-de Lind van Wijngaarden, SH Donze, DAM Festen, AAEM van Alfen-van der Velden, T Stijnen and ACS Hokken-Koelega, Beneficial effects of growth hormone in young adults with Prader-Willi syndrome: Results of a 2-year randomized, double-blind, placebo-controlled cross-over trial, *Journal of Clinical Endocrinology and Metabolism* (2016), in press

**RJ Kuppens**, NE Bakker, EPC Siemensma, SH Donze, T Stijnen, ACS Hokken-Koelega, Metabolic health profile in young adults with PWS: Results of a 2-year randomized, placebo-controlled cross-over GH trial, *Clinical Endocrinology* (2016), in press

**RJ Kuppens**, EF Mahabier, NE Bakker, EPC Siemensma, SH Donze, ACS Hokken-Koelega, Effect of cessation of GH treatment on cognition in young adults with Prader-Willi syndrome: Results of a 2-year cross-over GH trial (2016), submitted

**RJ Kuppens**, PJ Delhanty, TM Huisman, AJ van der Lely and ACS Hokken-Koelega, The Authors' Reply: Acylated and unacylated ghrelin during OGTT in Prader-Willi syndrome, a rebuttal to the conclusions by Kuppens *et al.*, *Clinical Endocrinology* (2016), in press

NE Bakker, **RJ Kuppens**, EP Siemensma, RF Tummers-de Lind van Wijngaarden, DA Festen, GC Bindels-de Heus, G Bocca, DA Haring, JJ Hoorweg-Nijman, EC Houdijk, PE Jira, L Lunshof, R J Odink, W Oostdijk, J Rotteveel, EJ Schroor, AA van Alfen, M van Leeuwen, E van Pinxteren-Nagler, H van Wieringen, RC Vreuls, N Zwaveling-Soonawala, MA de Ridder and ACS Hokken-Koelega, Eight years of growth hormone treatment in children with

Prader-Willi syndrome: maintaining the positive effects, *Journal of Clinical Endocrinology and Metabolism,* 98 (2013), 4013-22

NE Bakker, **RJ Kuppens**, EP Siemensma, RF Tummers-de Lind van Wijngaarden, DA Festen, GC Bindels-de Heus, G Bocca, DA Haring, JJ Hoorweg-Nijman, EC Houdijk, PE Jira, L Lunshof, RJ Odink, W Oostdijk, J Rotteveel, AA van Alfen, M van Leeuwen, H van Wieringen, ME Wegdam-den Boer, N Zwaveling-Soonawala and ACS Hokken-Koelega, Bone mineral density in children and adolescents with Prader-Willi syndrome: a longitudinal study during puberty and 9 years of growth hormone treatment, *Journal of Clinical Endocrinology and Metabolism,* 100 (2015), 1609-18

V Beauloye, G Diène, **RJ Kuppens**, F Zech, C Winandy, C Molinas, S Faye, I Kieffer, D Beckers, R Nergardh, B Hauffa, C Derycke, P Delhanty, ACS Hokken-Koelega and M Tauber, High Unacylated Ghrelin levels support the concept of anorexia in infants with Prader-Willi syndrome, *Orphanet Journal of Rare Diseases,* 11 (2016), 56

**RJ Kuppens**, PLA Fraaij, RJ Houmes and CMP Buysse, H1N1 in children requiring intensive treatment; the Rotterdam experience, *Pediatric Research,* 68 (2010), 680

**RJ Kuppens** and RIF van der Waal, Dermatologie in beeld: de novo ontstane, snel groeiende tumor in het gelaat, *Nederlands Tijdschrift voor Dermatologie en Venereologie,* 20 (2010), 28

## PHD PORTFOLIO

| | |
|---|---|
| Name | Renske Jozefien Kuppens |
| Promotor | Prof. dr. A.C.S. Hokken-Koelega |
| PhD period | April 2012 – December 2016 |
| Affiliations | Dutch Growth Research Foundation, Rotterdam<br>Department of Pediatric Endocrinology, Erasmus MC-Sophia Children's Hospital, Rotterdam |

| **Summary of PhD training** | Year | Workload (ECTS) |
|---|---|---|
| **General courses** | | |
| Biostatistical methods: Basic principles, NIHES, Erasmus MC | 2012 | 5.7 |
| Good clinical practice (BROK), Erasmus MC | 2013 | 1.0 |
| Research Integrity, Erasmus MC | 2013 | 2.0 |
| Biomedical English Writing and Communication, MolMed, Erasmus MC | 2014 | 4.0 |
| **Specific courses** | | |
| PubMed and EndNote, Medical Library, Erasmus MC | 2012 | 0.3 |
| Photoshop & Illustrator CS5, MolMed, Erasmus MC | 2012 | 0.3 |
| Basic and Translational Endocrinology, MolMed, Erasmus MC | 2013 | 1.6 |
| Radiation protection 5A, Zorgacademie, Erasmus MC | 2013 | 1.0 |
| MRI safety training, Erasmus MC | 2014 | 0.3 |
| **Seminars and workshops** | | |
| Weekly research meeting, department of pediatric endocrinology | 2012-2016 | 4.0 |
| Weekly patient presentation | 2012-2013 | 2.0 |
| Annual PhD day | 2012 | 0.3 |
| PWS Patient Care Day, Diegem/Brussel, Belgium | 2013 | 0.2 |
| Research Day, Sophia Children's Hospital | 2013 | 0.2 |
| Minisymposium 'social media in health care', Medical Ethics, Erasmus MC | 2013 | 0.2 |
| Methodology for patient-related research, CPO, Erasmus MC | 2013 | 0.3 |
| **International and national conferences** | | |
| 51st Annual Meeting of the European Society of Pediatric Endocrinology (ESPE), Leipzig, Germany (2 poster presentations) | 2012 | 1.0 |
| IPWSO 8th International PWS conference, Cambridge, UK | 2013 | 0.6 |
| 9th Joint Meeting of the European Society of Pediatric Endocrinology (ESPE), Milan, Italy (poster presentation) | 2013 | 1.0 |
| PWS Expert Meeting, Toulouse, France (oral presentation) | 2014 | 1.0 |

| | | |
|---|---|---|
| 53rd Annual Meeting of the European Society of Pediatric Endocrinology (ESPE), Dublin, Ireland (poster presentation) | 2014 | 1.0 |
| 54th Annual Meeting of the European Society of Pediatric Endocrinology (ESPE), Barcelona, Spain (poster presentation) | 2015 | 1.0 |
| Meeting section Pediatric Endocrinology, Utrecht (oral presentation) | 2015 | 1.0 |
| IPWSO 9th International PWS conference, Toronto, Canada | 2016 | 0.6 |
| 55th Annual Meeting of the European Society of Pediatric Endocrinology (ESPE), Paris, France (oral presentation) | 2016 | 1.0 |

**Lecturing**

| | | |
|---|---|---|
| PWS Parents information day, Capelle aan den IJssel (oral presentation) | 2014 | 1.0 |
| PWS Parents information day, Antwerp, Belgium (oral presentation) | 2014 | 1.0 |
| Annual IMC Weekendschool 'Growth and Development', Rotterdam | 2013-2014 | 1.0 |
| Education lecture study day of the Dutch Society for Physicians for people with intellectual disability (NVAVG), Vianen | 2014 | 1.0 |
| PWS Parents information day, Rotterdam (oral presentation) | 2015 | 1.0 |
| PWS Parents information day, Nijmegen (oral presentation) | 2016 | 1.0 |

**Research proposals**

| | | |
|---|---|---|
| Intranasal administration of oxytocin in children with Prader-Willi syndrome.<br>A randomized, double-blind, placebo-controlled cross-over trial<br>Effects on satiety and food intake, and social behaviour | 2014 | 5.0 |

**Miscellaneous**

| | | |
|---|---|---|
| Co-author National Guideline 'Diagnostiek en behandeling van kinderen met het Prader-Willi syndroom' | 2013 | 4.0 |
| Co-author 'Wegwijzer PWS | 2014 | 3.0 |
| 55th Annual Meeting of the European Society of Pediatric Endocrinology (ESPE), Paris, France: Travel Award | 2016 | |

## DANKWOORD

Het is zover, mijn boekje is af! De afgelopen jaren heb ik met ontzettend veel plezier en enthousiasme gewerkt, maar promoveren doe je nooit alleen. De inspirerende betrokkenheid van velen heeft veel voor mij betekend en zonder hen was het niet gelukt. Ik wil iedereen daarvoor hartelijk bedanken. Een aantal mensen wil ik in het bijzonder noemen.

Allereerst wil ik alle kinderen en jongeren met PWS en hun ouders enorm bedanken voor hun inspanningen en enthousiasme voor onze studies. We zagen elkaar soms wel 4 keer per jaar en ik heb velen in de afgelopen jaren zien groeien. Jullie zorgden voor afwisseling en brachten emotie, lach en verbazing. Wat waardeer ik jullie inzet, geduld en openheid. Jullie maken onderzoek naar het verbeteren van de kwaliteit van leven van kinderen en jongeren met PWS mogelijk.

Mijn promotor, prof. dr. A.C.S. Hokken-Koelega. Beste Anita, wat ben ik blij dat ik jouw promovenda mocht zijn! Je hebt me ongelooflijk veel geleerd en je grenzeloze vertrouwen heeft me laten groeien. Ik bewonder je passie voor het onderzoek, je gedrevenheid en ambitie, en je altijd vlotte, kritische en waardevolle commentaar. Ik ben onder de indruk van je warme hart voor mij, je promovendi en kinderen en jongeren met PWS. Mede hierdoor kijk ik met veel plezier terug naar de afgelopen jaren. Enorm bedankt!

Prof. dr. A.J. van der Lelij. Beste Aart Jan, bedankt dat u altijd laagdrempelig beschikbaar was voor overleg en voor de snelle, opbouwende commentaren op de manuscripten. Hartelijk dank voor uw bereidheid om plaats te nemen als secretaris in de kleine commissie.

Prof. dr. A. Vogels. Beste Annick, wat fijn dat u beschikbaar was voor overleg en advies. Hartelijk dank voor uw bereidheid om plaats te nemen in de kleine commissie.

Prof. dr. W. Kiess. I am very grateful and honored by your willingness to participate in my inner committee. Thank you for your interest and for travelling to Rotterdam.

Prof. dr. T. Stijnen. Beste Theo, hartelijk dank voor de laagdrempelige statistische hulp bij onze belangrijke studies en de prettige samenwerking. Bedankt voor de bereidheid plaats te nemen in mijn promotiecommissie.

Dr. A.A.E.M. van Alfen-van der Velden. Beste Janiëlle, ik waardeer je betrokkenheid bij de studies en je inzet voor kinderen met PWS. Samen zijn we nu Expertisecentrum PWS!

Bedankt voor de prettige samenwerking en dat je plaats wilt nemen in mijn promotiecommissie.

Dr. L.C.G. de Graaff. Beste Laura, wat fijn dat we de jongvolwassenen met PWS nu met een gerust hart kunnen overdragen naar de volwassengeneeskunde. Mede dankzij jouw inzet is de zorg voor volwassenen met PWS in Nederland flink verbeterd en kunnen ook zij terecht in het Expertisecentrum PWS. Bedankt voor het plaatsnemen in mijn promotiecommissie.

Alle kinderartsen van de samenwerkende centra. G.C.B. Bindels-de Heus, G. Bocca, D.A.J.P. Haring, J.J.G. Hoorweg-Nijman, E.C.A.M. Houdijk, P.E. Jira, L. Lunshof, R.J. Odink, W. Oostdijk, J. Rotteveel, E.J. Schroor, M. van Leeuwen, E. van Pinxteren-Nagler, V. van Tellingen, H. van Wieringen, M.E.J. Wegdam-den Boer, N. Zwaveling-Soonawala en alle poliassistenten. Bedankt voor jullie gastvrijheid, betrokkenheid en inzet voor onze studies en voor kinderen met PWS. Ook dank aan andere betrokken artsen; K.P. Wolffenbuttel, K.F.M. Joosten, H. Bruggenwirth, M. Smits, T.C.J. Sas, B. Otten, K. Haasnoot, B. Bakker, R. Moonen, N. Stikkelbroeck en vele anderen.

Erica van den Akker, Annelies Brandsma, Sandra de Kort en Sabine Hannema, bedankt voor alle leerzame momenten tijdens de endobesprekingen.

Philippe Collin, Sylvia Rasenberg, Jacqueline Heeren en José Veen. Bedankt dat jullie altijd bereid waren om mee te denken en om advies te geven. Wat fijn dat jullie de ouders zo goed kunnen ondersteunen.

Martin en Patric, bedankt voor alles rondom de ghrelinbepalingen en de fijne samenwerking. En Martin, ook voor de gezellige koffietjes.

Co-authors G. Diène, C. Molinas, S. Faye, M. Nicolino, D. Bernoux, S. Allas, M. Julien, T. Delale and M. Tauber, I would like to thank you for our collaboration on the ghrelin project. International Infant PWS group, M. Tauber, R. Nergardh, V. Beauloye, B. Hauffa and all their colleagues involved in the project, thank you for the kind collaboration.

Pfizer, en in het bijzonder Joli van der Lans en Joost van Middendorp, bedankt voor de prettige samenwerking en de onafhankelijke research grants voor de PWS-studies.

Prader-Willi Fonds, Ingeborg en Gertjan, jullie inzet voor kinderen en volwassenen met PWS is buitengewoon. Veel dank voor de financiële ondersteuning voor de oxytocinestudie. Stichting PWS, bedankt voor de fijne samenwerking.

Het belang van fijne collega's om je heen mag niet worden onderschat. Ineke, Gladys, Connie, Sandra, Sunita, Trudie, José, Manouk, Elise, Sander, Akvile, Berber, Momoko en Rosadinde. Wat kon het tussendoor gezellig zijn met koffie en theetjes in koude en warme tijden, en lunches in het kopieerhok of liever nog in de zon op het balkon. Sunita, wat fijn dat je altijd bereid bent om te helpen. Ineke, we kunnen op de stichting niet zonder jou! Sander, onwijs fijn dat je altijd wilt meedenken en een oplossing hebt voor bijna elk computerprobleem. Manouk, wat bijzonder dat we de afgelopen tijd allebei zo hard gewerkt hebben om ons boekje af te maken. Roos, wat is mijn boekje mooi hè? Bedankt voor alles! Ik wens jullie allemaal een hele mooie toekomst en veel geluk.

Mijn voorgangers binnen het PWS-team, Dederieke, Roderick, Elbrich, Nienke en Sinddie. Ik voel me vereerd dat ik jullie plekje heb mogen opvolgen. Bedankt voor jullie snelle en enthousiaste reacties bij vragen, de gezellige momenten in de PWS-wereld en het meedenken met manuscripten. Jullie hebben een stevige basis gelegd voor de studies en dat merken we nu nog. Ik waardeer jullie betrokkenheid en inzet, en wens jullie een prachtige carrière en veel geluk.

Het huidige PWS-team met Mariëlle, Stephany, Eva, Layla en Laura. Lieve Mariëlle, je bent voor zowel het hele PWS-team als voor vele kinderen en ouders onmisbaar. Mede door jou zijn de studies zo'n succes. Bedankt voor je toewijding, zorg, steun en onvoorwaardelijk inzet. Stephany, wat fijn dat jij Nienke's plek in nam. Bedankt dat je me op het juiste moment werk uit handen nam, zodat ik mijn proefschrift kon schrijven, en bovenal voor de fijne samenwerking. Eva, bedankt voor je interesse en alle gezelligheid. Layla en Laura, jullie zijn nu al zo betrokken, dat komt helemaal goed. Samen een prachtig team, wat fijn om te weten dat jullie het werk met zoveel toewijding voortzetten. Ik heb er alle vertrouwen in dat het nog veel moois gaat brengen.

Collega's van de Endo, Annemieke, Elbrich, Florentien, Gerthe, Judith, Kirsten, Laura, Layla, Lidewij, Lin, Manouk, Nienke, Sinddie, Stephany en Yvonne. Lieve meiden, naast de vele inspirerende endobesprekingen en waardevolle adviezen, vooral veel gezelligheid zoals op borrels en congressen. Wat is het fijn om in zo'n mooie groep te mogen promoveren. Bedankt voor alles. Ik wens jullie veel geluk en succes in de toekomst.

Collega's van het Sophia, jullie hebben gezorgd voor vele mooie herinneringen aan ski-reizen, borrels, barbecues en diners. Bedankt voor de gezelligheid. Laura, wat een eer dat ik naast je mocht staan, ik ben er van overtuigd dat je een prachtige toekomst tegemoet gaat. Suzanne, als je terug bent doen we snel weer een koffietje.

Mijn geweldig lieve vrienden en vriendinnen, die ik enorm waardeer om wie ze zijn. Ieder zijn eigen uitdagingen, maar altijd betrokken en geïnteresseerd in mij. Jullie staan altijd voor mij klaar. Bedankt voor de vele gezellige drankjes, etentjes en uitjes. Wat fijn dat ik mezelf mag zijn bij jullie en dat we samen zo kunnen ontspannen en genieten.

Lieve Marieke, Martha, Sabine en Susanne, een vijf-eenheid sinds dag één van de geneeskundestudie en al meer dan 12 jaar delen we lief en leed. Hopelijk blijft dat nog heel lang zo. Lieve Anne en Sawadi, wat voor ons drieën begon als onze eerste echte baan in Dordt, is uitgegroeid tot een bijzonder dierbare en hechte vriendschap, die ik enorm waardeer.

Lieve schoonfamilie, bedankt dat jullie mij zo snel in jullie familie hebben opgenomen. Ab en Richtje, dank voor alle interesse in mijn onderzoek, en voor de gezelligheid en betrokkenheid voor ons beiden.

Mijn paranimfen Nienke en Nine, wat ben ik blij dat jullie vandaag zo dicht bij mij staan. Lieve Nienke, jaren werkten we nauw en intensief samen, aan een half woord hadden we genoeg. Je enthousiasme en liefde voor de patiënten werkte aanstekelijk. Door de jaren heen ben ik je als veel meer dan zomaar een collega gaan beschouwen. En dan onze mannen die samen bier of whisky gaan proeven in Barcelona of Dublin terwijl wij bij een congres zitten. Bedankt voor al je support! Lieve Nine, als kind hebben we samen heel wat gekibbeld, maar ik kan me nu geen fijnere zus wensen. Jij en Simon zijn meestal druk in de weer, maar als het nodig is dan ben je er altijd. Nog even en dan ben je huisarts. Ik wens jullie samen alle geluk toe, ik ben zo trots op jou! Simon, wat fijn om jou als schone broer te hebben. Wat hebben we samen een heerlijk gezin.

Lieve papa en mama, ik hou zoveel van jullie, dat dat nooit in één dankwoord past. Jullie hebben Nine en mij een liefdevolle opvoeding gegeven vol met kansen. Jullie zullen me nooit tegenhouden en geven ons altijd onvoorwaardelijke steun en vertrouwen. Ik ben trots dat ik jullie dochter ben!

Lieve Reyer, bedankt voor je onvoorwaardelijke liefde, grenzeloze relativeringsvermogen, en gewoon dat je er altijd bent. Ondanks dat het Denemarkenavontuur ons gemakkelijk af ging, ben ik toch blij dat je letterlijk weer dichter bij me bent, want samen met jou is alles leuker. Op naar een nieuw avontuur, ik hou van ons!!

## CURRICULUM VITAE

**Renske Jozefien Kuppens** was born on June 24th 1985 in Zevenaar, the Netherlands. She passed her secondary school exam (Atheneum) at the Liemers College in 2003. She discovered she wanted to study medicine and at the age of 19 years, she packed her belongings and moved to live in Utrecht, to start a life as a medical student at the Utrecht University Medical Center.

During her study, Renske got acquainted with multiple medical disciplines, among which she educated local clinical officers throughout Malawi, Africa. During this period, Renske's love for travelling and seeing the world developed simultaneously with her enthusiasm for medicine. In 2010, Renske conducted a pediatric internship at the Erasmus MC-Sophia Children's Hospital, which gave rise to her specific interest in pediatrics.

Renske obtained her medical degree in October 2010, after which she soon started working as a pediatric resident at the Albert Schweitzer hospital in Dordrecht. During her study, Renske left Utrecht to live together with her boyfriend Reyer in Rotterdam, from which Dordrecht had become an apparent choice. After a joyful and instructive period in Dordrecht, Renske decided that she wanted to increase her experience in scientific research before moving on to a medical specialization. In April 2012, Renske happily accepted a clinical research project at the Dutch Growth Research Foundation and the department of Pediatric Endocrinology of the Erasmus MC-Sophia Children's Hospital, Rotterdam.

As a PhD fellow, Renske committed her research to Prader-Willi syndrome under supervision of Prof. A.C.S. Hokken-Koelega. During her four years of performing scientific research, Renske has had the opportunity to concentrate on both the scientific and the humane aspects of medicine. This enlarged Renske's commitment to the care and consideration for the children as well as their parents. The result of four years of extensive research on several facets of Prader-Willi syndrome has resulted in this thesis bundled before you.

Although pediatrics in general, and children and young adults with Prader-Willi syndrome in particular, have obtained a special place in Renske's heart, Renske decided that it was time to broaden her perspective. She will surely continue her medical career with the same care and consideration as she applied to this research.

# NEW TREATMENT PERSPECTIVES IN **PRADER–WILLI SYNDROME**