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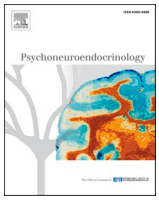

Alteration of serum leptin and LEP/LEPR promoter methylation in Prader-Willi syndrome

Jelte Wieting [a,*,1], Kirsten Jahn [a,1], Vanessa Buchholz [a], Ralf Lichtinghagen [b], Stephanie Deest-Gaubatz [a], Stefan Bleich [a], Christian K. Eberlein [a], Maximilian Deest [a,2], Helge Frieling [a,2]

[a] Hannover Medical School, Department for Psychiatry, Social Psychiatry and Psychotherapy, Carl-Neuberg-Str. 1, 30625 Hannover, Germany
[b] Hannover Medical School, Department for Clinical Chemistry, Carl-Neuberg-Str. 1, 30625 Hannover, Germany

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ABSTRACT

Prader-Willi syndrome (PWS) is a rare neurodevelopmental disorder based on a loss of paternally expressed but maternally imprinted genes in chromosome region 15q11–13. PWS individuals typically show insatiable appetite with subsequent obesity representing the major mortality factor unless food intake is inhibited. The neurobiological basis of PWS-typical hyperphagia has remained poorly understood. Many PWS-typical abnormalities are based on hypothalamic dysregulation, a region in which hunger and satiety are hormonally regulated, with the hormone leptin being a main long-term regulator of satiety. Previous studies in PWS have inconsistently shown leptin alterations solely in early childhood, without investigating the leptin system on an epigenetic level. The present study investigates serum leptin levels (S-leptin) and DNA methylation of the leptin (LEP) and leptin receptor gene (LEPR) promoter in 24 individuals with PWS compared to 13 healthy controls matched for sex, age, and body mass index (BMI) and relates the results to the extent of hyperphagia in PWS. S-Leptin levels were obtained by Enzyme-linked Immunosorbent Assay. LEP/LEPR-promoter DNA methylation was assessed by bisulfite-sequencing, hyperphagia by Hyperphagia Questionnaire for Clinical Trials (HQ-CT). PWS and control groups differed significantly in S-leptin levels with higher S-leptin in PWS. Methylation analysis showed significant differences in mean promoter methylation rate both for LEP and LEPR with a lower methylation rate in PWS. LEPR, but not LEP methylation correlated significantly with S-leptin levels. S-leptin and both LEP and LEPR methylation did not correlate with HQ-CT scores in PWS. The present study is the first to show significantly elevated S-leptin levels in an adult PWS cohort combined with an altered, downregulated LEP and LEPR promoter methylation status compared to sex-, age- and BMI-matched controls. Analogous to previous studies, no link to the behavioral dimension could be drawn. Overall, the results suggest an increased leptin dysregulation in PWS, whereby the findings partly mirror those seen in non-syndromic obesity.

1. Introduction

Prader-Willi syndrome (PWS) is a rare neuronal developmental disorder with an estimated prevalence of 1:15000 (Lionti et al., 2015). The syndrome is genetically determined by a loss of normally paternally expressed but maternally imprinted genes on chromosome 15q11–13. Essentially, three different genetic subtypes can lead to PWS. Most common, in about 70 % of cases, is a large deletion of the paternal allele of chr15q11–13 (delPWS). In about 25–30 % of cases a maternal uniparental disomy of chromosome 15 (UPD) and in rare cases, a defect of the imprinting center (IC) occurs (Angulo et al., 2015).

Besides typical morphological features, hormonal deficiencies mainly due to hypothalamic dysfunction and a certain behavioral phenotype, newborns with PWS initially present with a failure to thrive and muscular hypotension. During infancy, PWS individuals rapidly develop an insatiable appetite leading to massive weight gain unless food intake is regulated (Muscogiuri et al., 2019). Despite improved diagnostics and therapy, life expectancy is still assumed to be reduced in

* Corresponding author.
E-mail address: wieting.jelte@mh-hannover.de (J. Wieting).
[1] JW and KJ should be considered joint first author.
[2] MD and HF should be considered joint senior author.

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people with PWS. Obesity and its sequelae such as cardiovascular diseases, diabetes, as well as sleep apnea and other respiratory complications are the major driving factor for the increased mortality rate in people with PWS (Proffitt et al., 2019). The causes for obesity in PWS are currently understood in rudimentary terms at best. Misbalances in the hypothalamic satiety center and the various hormones influencing it have been suspected with the hyperphagia-behavior typical of PWS.

Satiety hormone leptin has an appetite suppressing, energy expenditure increasing and consequently body weight reducing effect (Kelesidis et al., 2010). In human, satiety is induced essentially via stimulation of melanocortin-4 receptors (MCR-4) by the proopiomelanocortin (POMC) derivative alpha- melanocyte stimulating hormone (alpha-MSH) in the paraventricular nucleus of the hypothalamus. Besides stimulation of POMC, satiety hormone leptin inhibits AgRP- and neuropeptide Y (NPY)-producing neurons in the arcuate nucleus of hypothalamus, hormones, which have an appetite-increasing effect via MCR-4 inhibition. In humans, the hormone leptin is encoded on the LEP gene located on chromosome 7q31.3. It is largely expressed in adipocytes, but also to a lesser extent centrally in the hypothalamus and pituitary gland, as well as other tissues (Morash et al., 1999). The physiological regulatory mechanisms behind leptin expression are complex. In healthy individuals, leptin blood levels correlate with body fat stores (Considine et al., 1996). In women, circulating leptin is significantly elevated compared to men. Fasting causes a decrease in leptin levels in humans, whereas levels re-increase after days of refeeding (Kolaczynski et al., 1996). It is important to note that leptin levels do not increase in response to individual meals (Korbonits et al., 1997). Leptin can be considered as a long-term indicator of the body's energy reserves.

Leptin exerts its effect via leptin receptors encoded by LEPR gene. LEPR is located on chromosome 1 (1p31). One of the splice variants of the LEPR gene, the longest isoform LEPRb is most widely expressed in the human brain, particularly in the hypothalamus. Only the long isoform LepRb contains the intracellular motif required for leptin-mediated POMC-activation in the hypothalamus, making this isoform the main mediator of the above-mentioned leptin-specific effects (Bjørbæk et al., 1997; Hill et al., 2008).

Based on prior studies, elevated levels of leptin can be expected in adults with PWS. Adult PWS showed elevated blood leptin levels compared to healthy controls (Hirsch et al., 2015), but there were no significant differences compared to weight-adapted or obese control subjects (Carlson et al., 1999; Kennedy et al., 2006; Pagano et al., 2005). In PWS, significant differences in leptin blood levels compared with obese controls have so far only been demonstrated for young children $<$ 6 years (Goldstone et al., 2012; Haqq et al., 2008; Orsso et al., 2019).

As no PWS-specific differences have been found in comparison, it could be assumed, that overweight-typical leptin results are reflected in adults with PWS. Leptin blood levels to be expected differ between previous studies in PWS and beyond. For example, Carlson et al. found leptin plasma levels of 35.5 ng/ml in obese (mean BMI 31) and 30.7 ng/ml in PWS (mean BMI 28), whereas larger scaled studies in aside overweight healthy people with comparably high BMI showed levels of 12.3 ng/ml (mean BMI 29.4 kg/m^2) (Gupta et al., 2016) and 8.2 ng/ml (mean BMI 29.2 kg/m^2) (Charles et al., 2015).

In non-syndromic obesity, elevated leptin levels continue to be seen in both blood and adipocytes, but no significant leptin effect can be discerned. Accordingly, leptin resistance in obesity is assumed. The cause of leptin resistance has not been conclusively clarified. Leptin enters the central nervous system (CNS) via an exhaustible transport system across the blood-brain barrier (Caro et al., 1996). Due to saturation effects the transport rate decreases with increasing leptin levels, so that no proportionally increased signaling in CNS can be expected with an increased peripheral blood level, as shown, for example, by Holtkamp et al. who found no further leptin increase in brain tissue or CSF above serum leptin levels of 25–30 ng/ml (Holtkamp et al., 2004). Hence, it is speculated that low leptin levels are more significant in leptin signaling. The extent to which this is also displayed in genetic or epigenetic changes is subject of research.

The present study focuses on the epigenetic regulation of leptin in PWS, on which there are no publications to date. In general, one important epigenetic mechanism is the methylation of cytosine bases in the DNA strand, being of regulative function in the context of cytosine-guanine dinucleotides (CpGs) in CpG-rich gene-promoters. Among other mechanisms, increased methylation impedes the binding of transcription factors so that protein expression is subsequently suppressed in most cases (Bird, 2002). Methylation of the gene promoter region around the first exon is of particular interest as it usually represents the transcription start site and thus an essential gene regulatory unit.

In the context of metabolic diseases, an association between decreased LEP-methylation status and obesity has been demonstrated in male children (Dunstan et al., 2017). In obese adolescents, a decreased methylation status of the LEP promoter region was detected in peripheral blood (García-Cardona et al., 2014). Overall, the data predominantly indicate hypomethylation of the LEP promoter region in metabolic disorders. In general, an inverse correlation between LEP promoter methylation and leptin expression is assumed (Melzner et al., 2002). Alterations of the LEPR gene in obesity have been less studied overall. A study on the influence of pre-pregnancy BMI on gestational weight suggested an inverse correlation between LEPR methylation and LepRb expression (Chavira-Suárez et al., 2019).

Based on the previous studies on the topic, we assumed that leptin misbalances seen in adult PWS might reflect findings seen in obesity in general. With our study, we aimed to investigate possible differences in leptin appetite regulation between PWS and obesity in general. To that end, we compared an adult PWS with a sex-, age- and BMI-matched control cohort in terms of serum leptin levels as well as DNA methylation of LEP and LEPR promoter region. Thereby, we wanted to explore possible functional relationships between epigenetic LEP and LEPR regulation and blood leptin levels in PWS. Based on previous studies on obesity, an inverse correlation between LEP promoter methylation status and S-leptin levels could be assumed. But, since no PWS-specific studies were available so far, our investigations regarding LEP/LEPR methylation in PWS were also exploratory in character. In a second step, we analyzed our laboratory findings for correlations with the behavioral phenotype of the PWS measured by a caregiver-report test instrument, since obesity in PWS is essentially based on hyperphagia-related behavior.

2. Methods

2.1. Study characteristics

The study included 24 subjects with PWS (14 male, 10 female) and 13 healthy control subjects (8 male, 5 female) matched for sex, age, and body mass index (BMI). Control subjects with a difference of $\pm$ 5 years and $\pm$ 1 kg/m^2 BMI compared to the study cohort were considered. The control subjects were recruited via public tenders analogously and online. The healthy control subjects confirmed during the interview that they had not been diagnosed with any mental illness at the time of the study. The participants of this study are part of a larger register study entitled PSY-PWS which takes place at Hannover Medical School and is described in more detail in former publications (Deest et al., 2021, 2020). The study followed the Declaration of Helsinki and was reviewed and approved by the local ethics committee of Hannover Medical School (No: 8129_BO_S_2020). All subjects or their legal representatives gave written informed consent to participation after all study procedures were explained to them.

The HQ-CT (Hyperphagia Questionnaire for Clinical Trials) in German language was used to assess the level of hyperphagia-related behavior in PWS. The HQ-CT is specifically designed and validated for PWS. It is a 9-item caregiver-report test instrument considered to be the current gold standard for hyperphagia assessment in PWS. The HQ-CT

total score results from the sum of the 9 item-level responses ranging from 0 to 4 with a maximum score of 36 (Fehnel et al., 2015). Cut-off values above which clinically significant symptoms can be assumed have not yet been established.

2.2. Molecular analysis

DNA was extracted from blood samples using chemagicStar DNA-Blood1k kit (PerkinElmer Chemagen Technology, Baesweiler, Germany). The genomic DNA was subsequently bisulfite converted using the EpiTect® 96 Bisulfite Kit (Qiagen, Hilden, Germany) to detect the methylation status of the cytosine bases. Our target sequences within the LEP and LEPR promoter region were amplified from the previously purified bisulfite-converted DNA by polymerase chain reaction. Primers were designed to cover a LEP fragment of 823 bp (Hg 38, Chr. 7: 128.240.657 to 128.241.478), including exon 1 (Hg 38, Chr.7: 128.241.278 to 128.241.306), with 65 CpG-sites covered, and a LEPR fragment of 461 bp length (Hg 38, Chr. 1: 65.420.416 to 65.420.876), including exon 1 (Hg 38, Chr. 1: 65.420.652 to 65.420.740). The number of covered CpG-sites was 46. Fig. 1 shows a schematic illustration of the explored gene segments within the entire genes.

Sequencing was performed using the Big-Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Individual PCR protocols and primer sequences can be found in Supplementary Material S1.

To determine the methylation status of the different CpG loci from the data obtained by DNA sequencing, Epigenetic Sequencing Methylation Analysis Software (ESME) was used (Lewin et al., 2004). Methylation for each CpG site was calculated per each subject by building the ratio between normalized peak values of cytosine and thymine. The methylation status of all CpG sites examined within the gene segment are summarized across all samples of each group as LEP or LEPR mean methylation rate.

S-leptin levels were determined by enzyme-linked immunosorbent assay (ELISA) from Serum blood samples using Quantikine® Human Leptin Immunoassay (R&D Systems, Minneapolis, MN, USA).

2.3. Statistics

Sequence Scanner v2.0 software (ABI Life Technologies, Grand Island, NY, USA) was used for sequence quality control. Only sequences showing a QV (quality value) > 20 were included for further analysis.

For statistical analysis, we used SPSS (IBM, Armonk, NY, USA). For graphical data processing, Graph Pad Prism (GraphPad, LaJolla, CA, USA) was used.

Data were further checked for missing values and variance. Single CpGs with more than 5 % missing values and inter-individual variability below 5 % were removed from the analysis. In addition, subjects with more than 5 % missing values in methylation rate were excluded (see Supplementary Material S2).

Mann-Whitney test was used to compare differences in S-leptin levels and BMI between PWS and control subjects. We calculated mixed linear models to detect fixed effects of the variable group (PWS or control), CpG-position, sex and BMI on methylation rate. BMI and age were set as parameters of covariance. Differences in methylation rates at individual CpG sites were assessed within the mixed linear model with data splitted for CpG site. Fixed effects of factor group were calculated for the individual CpG sites. Bonferroni correction was applied to correct p-values for multiple testing. p values of < 0.05 were considered significant.

To assess the correlation between HQ-CT and the LEP/LEPR methylation in total and for individual CpG sites Pearson's correlation coefficient was calculated.

2.4. Analysis of transcription factors

CpG sites statistically shown to differ significantly in mean methylation between the groups were further analyzed for transcription factor (TF) binding. Geneious 11 (Biomatters, Auckland, New Zealand) was used for software-assisted transcription factor prediction. Their functional relevance was assessed by PubMed and UniProt database research (Bateman et al., 2021). Mentioned below are those factors shown to be of functional relevance in central nervous system processes.

3. Results

3.1. Patient characteristics

The study included 24 subjects with Prader-Willi syndrome (male n = 14, 58.3 %; female n = 10, 41.7 %) and 13 healthy control subjects (male n = 8, 61.5 %; female n = 5, 38.5 %). In PWS subjects, the mean age was 27 years (minimum 12 y, maximum 55 y), whereas in controls, the mean age was 32 years (minimum 18 y, maximum 47 y).

As matched for this, BMI values differed only slightly between groups showing no significant difference according to Mann Whitney test, U = 166, Z = 0.318, p = 0.766. Mean BMI was 27.2 kg/m^2 in PWS (Minimum 19.1 kg/m^2, Maximum 39.1 kg/m^2) and 26.6 kg/m^2 in healthy controls (Minimum 19.4 kg/m^2, Maximum 40.0 kg/m^2). Genetic subtype was determined in all subjects with PWS. DelPWS (n = 11) and UPD (n = 7) were shown most frequently (UPD/IC n = 4 and IC n = 2). Mean HQ-RT score in the PWS group was 10.39 (median 8, minimum 1, maximum 33).

Table 1 presents the patient characteristics in detail.

3.2. S-Leptin

Mean S-Leptin in PWS subjects was 32.747 ng/ml (SD = 24.018) and 11.928 ng/ml in control group (SD = 12.787). Divided by sex, female subjects had higher serum leptin levels than males in both the PWS and control groups. Male PWS had a mean leptin level of 26.608 ng/ml (SD = 26.435), female of 41.316 ng/ml (SD = 18.002). Controls also showed a difference between males with an average of 5.324 ng/ml (SD = 4.740) to 22.493 ng/ml (SD = 14.986) in female controls.

According to Mann Whitney test for comparison of PWS and control groups in terms of S-leptin levels there was a significant difference between the groups, U = 245.00, Z = 2.831, p = 0.004 (see Table 1 and Fig. 2). Effect size was 0.465, displaying a moderate effect according to Cohen's classification of effect sizes.

Serum leptin levels correlate highly positively with BMI in both PWS subjects (linear R2 = 0.502) and healthy control subjects (linear R2 = 0.623). S-Leptin values do not correlate with the HQ results in the PWS subjects (linear R2 = 0.016).

3.3. LEP methylation

For LEP significant fixed effects on methylation rate of individual CpG position ($F_{(48,1660)} = 85.312$; $p < 0.001$) and group ($F_{(1,1660)} = 22.706$, $p < 0.001$) were detected. MLM revealed no significant CpG x group interaction for LEP gene ($F_{(48,1660)} = 1.247$; $p = 0.121$). We found independent effects of age ($F_{(1,1660)} = 34.185$; $p < 0.001$), but not of sex ($F_{(1,\ 1660)} = 2.657$, $p = 0.103$) nor BMI ($F_{(1,\ 1660)} = 1.52$, $p = 0.218$). Significance of group differences was not affected when age and gender were applied as additional factors.

Overall LEP mean methylation rate was shown to be lower in PWS (29.9 %) compared to healthy controls (32.5 %) (see Fig. 3). Analysis of fixed effects of factor group on single CpG sites methylation revealed significant effects for eleven individual CpG positions (m169, m199, m203, m329, m331, m338, m340, m349, m371, m373 and m378). All CpG sites named above showed a significantly lower mean methylation rate in the PWS group compared to controls. The CpG positions m329 and m331, m338 and m 340, and m349 to m373 sequentially follow each other in the base sequence. Fig. 4 shows a graphical representation of these results. Fig. 1A maps the statistically prominent CpG sites within

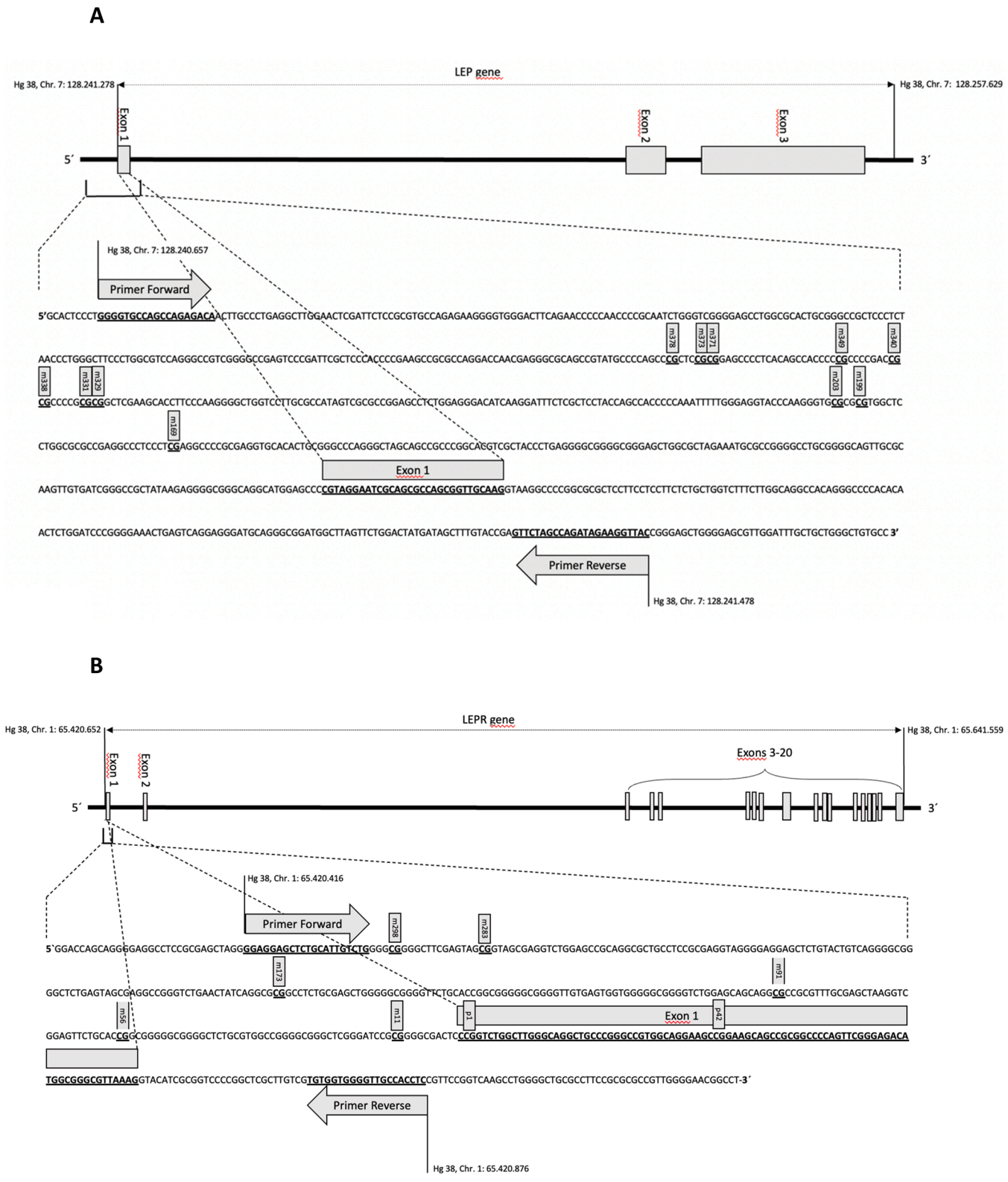

Fig. 1. A) Illustration of the explored segment within the entire LEP gene (Hg 38, Chr. 7: 128.241.278 to 128.257.629). The entire LEP gene is schematically shown as line at the top with grey boxes representing exons 1–3. The zoom below shows the genomic sequence we examined. The forward and reverse primer (gray arrows) frame the explored gene segment (Hg 38, Chr. 7: 128.240.657 to 128.241.478). We examined the region around exon 1 (horizontal box, Hg 38, Chr.7: 128.241.278 to 128.241.306), where the transcription start side is located. Additionally shown are the CpG sites that were significantly different in mean methylation in group comparison (upright boxes). The numbering is given in relation to the position of the first base of exon 1 (m = minus), B) analogously to 1 A illustrates the explored segment (Hg 38, Chr. 1: 65.420.416 to Hg 38, Chr. 1.: 65.420.876) within the entire LEPR gene (Hg 38, Chr. 1: 65.420.652 to 65.641.559), including exon 1 (Hg 38, Chr. 1: 65.420.652 to 65.420.740) and the CpG positions differing significantly in comparison between PWS and controls (upright boxes) with numbering again given in positional relation to the first base of exon 1 (m = minus, p = plus).

Table 1
showing basic demographics of PWS and control cohort in comparison. Statistical analysis revealed a significant difference in mean S-leptin but not in mean age and BMI.

	PWS	Control			
n=	24	13			
Sex	14 male (58.3 %), 10 female (41.7 %)	8 male (61.5 %), 5 female (38.5 %)			
Subtype	11 DelPWS, 7 UPD, 2 IC, 4 UPD/IC				
			Mann-Whitney-U-test		
			U	Z	p
Mean Age (in y)	27	32	107	1.561	0.124
Min/Max	12/55	18/47			
SD	12.044	8.140			
Mean BMI (kg/m^2)	27.20	26.60	166	0.318	0.766
Min/Max	19.100/39.111	19.370/40.009			
SD					
Mean S-leptin (ng/ml)	32.747	11.928	245	2.831	0.004 *
Min/Max	3.261/79.822	0.116/43.797			
SD	24.018	12.788			

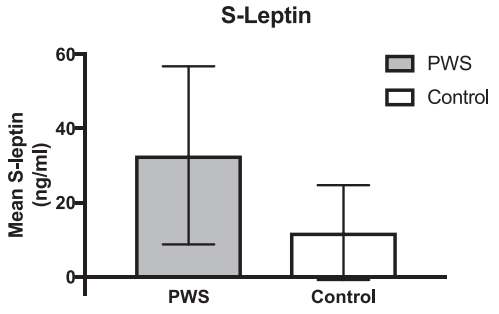

Fig. 2. Chart showing significant higher mean S-Leptin (with SD) in PWS compared to BMI-matched controls.

the covered LEP segment in relation to exon 1. No significant differences in mean methylation rate were found between the sexes with males showing an insignificantly higher methylation rate (31.6 %) compared to females (30.8 %).

For detailed statistical results see Supplementary Material S3.

Correlation analysis revealed no significant correlation between overall LEP promoter methylation rate and S-Leptin levels with Pearson's $r = -0.009$, $p = 0.720$. Also, there was no correlation between overall LEP methylation and HQ scores.

According to Pearson's r, there was a negative correlation between mean methylation at CpG sites m349 ($r = -0.473$, $p = 0.023$) and m371 ($r = -0.462$, $p = 0.026$) and HQ scores in subjects with PWS. No significant correlation was found for any other LEP CpG site shown to differ significantly between the groups in LEP methylation rate.

Software-based analysis of transcription factor binding to the significantly differing LEP CpG sites revealed binding of almost ubiquitously expressed factor Sp1 to m378, m338, m331, m329, m203 and m199. A rerun of the correlation analysis between LEP methylation and serum leptin levels only for the CpGs predicted to bind Sp1 was trending towards an inverse correlation, but nonsignificant at Pearsons $r = -0.121$, $p = 0.104$. Binding of NF-kappaB(-like) and E2F-1 was predicted for m349, whereby E2F-1 binding was also shown for m340. The TFs functional relevance is detailed in Supplementary Material S5.

3.4. LEPR methylation

MLM revealed significant fixed effects of factors group ($F_{(1813)} = 11.021$; $p = 0.001$) as well as CpG-position ($F_{(23,813)} = 8.984$; $p < 0.001$) on the LEPR methylation rate. Overall LEPR mean methylation rate was lower in PWS with 3.0 % compared to 4.4 % in controls. In contrast to LEP analysis, we showed a significant group x CpG site interaction for LEPR gene ($F_{(23,813)} = 2.414$; $p < 0.001$). No significant fixed effects were seen for factor sex on methylation rate ($p = 0.279$), nor for age ($p = 0.977$) or BMI ($p = 0.382$). Significance of group differences was not affected when age and gender were applied as additional factors.

Within the mixed linear model analysis split for single CpG sites, significant effects of factor group at eight individual CpG positions were seen (m298, m283, m173, m091, m56, m11, p1 and p42). No contiguous CpG positions could be identified. Fig. 5 shows a graphical representation of these results. For detailed statistical results see

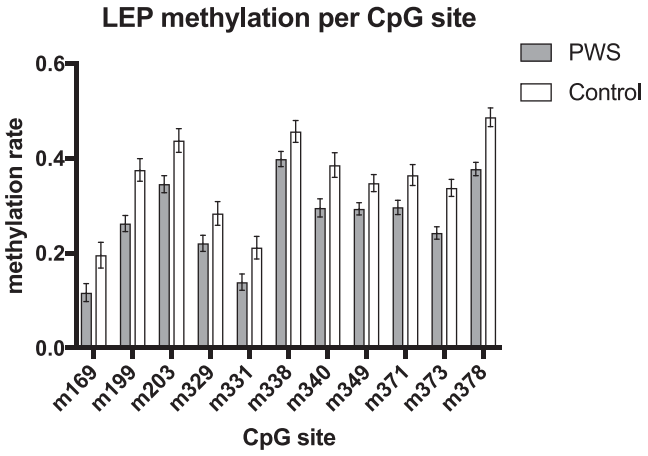

Fig. 4. Chart revealing significant lower mean methylation of LEP-promoter (with SD) in eleven of partially contiguous CpG regions in PWS compared to controls (only shown CpG sites with a significant difference).

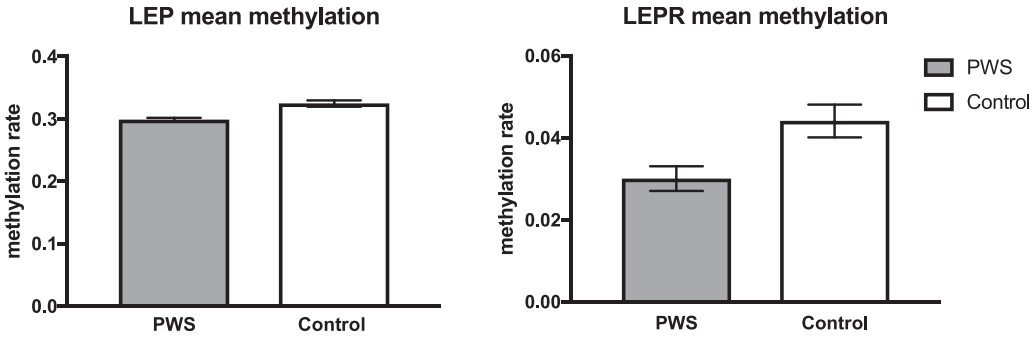

Fig. 3. Charts showing significant lower mean LEP and LEPR promoter methylation (with SD) in PWS compared to BMI-matched controls.

Supplementary Material S4. Fig. 1 B again maps the group-dependent differing CpG sites within the covered LEPR gene segment.

Correlation analyses showed a highly significant inverse correlation between overall LEPR methylation rate and S-Leptin levels with Pearsons $r = -0.120$, $p < 0.001$. No correlation was found between overall LEPR methylation rate and HQ scores. There were no correlations of individual CpG sites methylation rate with HQ scores.

For LEPR, analysis of transcription factor binding revealed binding of factors Sp1, octamer-binding factor and AP-2 to m298 (TFs functional relevance again detailed in Supplementary Material S5).

4. Discussion

The present study investigates serum leptin levels and methylation of the LEP and LEPR promoter in adult PWS compared to healthy BMI-matched controls. Our study reveals that PWS subjects have significantly higher S-leptin levels than controls while showing significantly lower LEP and LEPR promoter methylation. LEPR, but not LEP mean methylation correlated with S-leptin levels, while overall there was no correlation with the behavioral dimension in terms of the HQ-scores.

To our knowledge, the study is the first to demonstrate a significant difference in serum leptin levels between obese adults with PWS and weight-matched controls, a finding previously shown only for infants < 6 years of age that appeared to resolve with increasing age. In line with previous studies a correlation of leptin level with BMI was still observed in both PWS and control group in the present study, but as they were matched for BMI both the PWS and control group were only slightly overweight at 27.2 respective 26.6 kg/m^2. The mean BMI of our study seems to be considerably lower compared to other studies on the topic, for example, 37.2 kg/m^2 in Butler et al. or 41.2 kg/m^2 in Pagano et al. (Butler et al., 1998; Pagano et al., 2005). Since leptin levels in our healthy control subjects are in line with the expectations based on previous studies with comparable BMI profile, a BMI-independent effect of the factor PWS on S-leptin levels can be assumed. The comparatively high number of male subjects in our study might have a confounding effect, especially since sex-dependent differences in leptin regulation are known. An indication could be the study by Butler et al., which showed comparatively higher leptin blood levels only in male PWS subjects, who, unlike in our study, were non-obese (Butler et al., 1998). But this idea again conflicts with previous findings that women generally have higher leptin levels than men. In contrast, Kennedy et al. also showed no differences in leptin levels between PWS and overweight controls, although females significantly predominated in the PWS cohort (Kennedy et al., 2006). Our results displayed no significant differences between the sexes in terms of blood leptin levels.

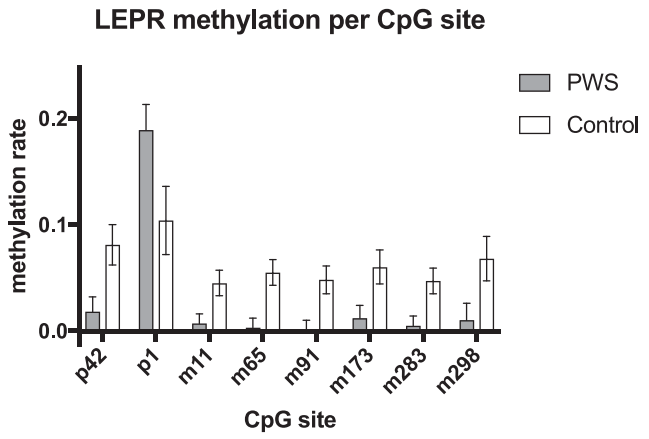

Fig. 5. Chart revealing significant difference in mean methylation of LEPR-promoter (with SD) in eight CpG regions in comparison of PWS and controls (only shown CpG sites with a significant difference).

Furthermore, the present study is the first to investigate the epigenetic regulation of the satiety hormone leptin in people with PWS. In terms of obesity in general the majority of previous human studies have demonstrated an association between decreased LEP methylation levels and elevated leptin serum levels in obese subjects, hence an inverse correlation between LEP promoter methylation and leptin expression was assumed. Our results appear partly conclusive on this point from a neurobiological perspective, although it should be mentioned that no significant correlation was shown between LEP promoter methylation and S-leptin levels. Also, Lindgren et al. found no difference in leptin mRNA levels between PWS and obese controls, although increased leptin protein biosynthesis might be expected with decreased LEP promoter methylation as found in the present cohort (Lindgren et al., 1997). On the other hand, recent data from our laboratory regarding LEP promoter methylation after bariatric surgery indicated decreased LEP promoter methylation with concomitant decreased serum leptin after surgically induced weight loss (Wilhelm et al., 2021). Overall, however, intervention-induced weight loss with the accompanying hormonal disruptions appears to bear little resemblance to the given conditions in PWS. Regarding the receptor, we found an inverse correlation between LEPR methylation and S-leptin levels in the present cohort, which is in line with the study by Wilhelm et al.

Taken together, the findings of hypomethylated LEP/LEPR promoter and elevated S-leptin made in our PWS cohort mirror those previously made in obesity in general and indicate an upregulation of the entire leptin system.

To reveal functional implications of altered LEP or LEPR promoter methylation, CpGs that differed significantly between groups were additionally analyzed for transcription factor binding.

While the analysis for LEPR offers little interpretive relevance, in LEP promoter region, half of the CpG sites showing group dependent differences were predicted to bind Sp1 with all sites showing hypomethylation in PWS compared to controls. Sp1, a Zinc finger TF known to bind to CG rich promotor regions of many genes and to be essential for cell growth, apoptosis and differentiation, has been shown to play a crucial role in LEP promoter activity with unbinding of Sp1 reducing promoter activity 2.5-fold (Mason et al., 1998). Conversely, one could assume that increased Sp1 binding might enhance promoter activation. Thus, PWS-associated hypomethylation at Sp1-binding sites of the LEP gene might represent a conclusive explanation for hyperleptinemia seen in the PWS mediated by an enhanced LEP promoter activity. Although we could only see a negative correlation of LEP methylation and serum levels in trend in the present study, which was enhanced when only those CpGs predicted to bind Sp1 were statistically considered, an inverse correlation of LEP methylation and leptin expression has recently been observed in female patients with multisomatoform disorder (Achenbach et al., 2022), suggesting a cross-disease functional relationship. In addition, NF-kappaB and E2F-1 binding was predicted for m349. NF-kappaB has been associated with leptin action and appetite regulation in various contexts. In a rodent model, inhibition of hypothalamic NF-kappaB was shown to block anorexigenic effects of leptin (Jang et al., 2010). Accordingly, reduced methylation of m349 could hypothetically result in facilitated NF-kappaB binding and thus increased leptin activity might serve as a counterregulatory mechanism in obesity. But however, it remains controversial whether NFkappaB exerts weight-reducing effects via indirect leptin-dependent activation of POMC or direct POMC promoter activation (Shi et al., 2013). E2F-1 is generally described to be relevant in neuronal processes, but no association with appetite regulation has been mentioned.

Our observation that LEPR promoter methylation correlated significantly with S-leptin levels, suggests a regulatory link between LEPR activity and leptin expression. It is known that leptin-mediated activation of anorexigenic POMC neurons in the arcuate nucleus of the hypothalamus is mediated via leptin receptors LepRb. Leptin-induced POMC-activation was absent in a Magel2 (Melanoma antigen gene family member L2) knockout mouse model (Hill et al., 2008), a gene

encoded in the PWS relevant chromosomal region 15q11.2. Magel2 and Necdin, another gene encoded in the PWS region, regulate the linkage of leptin receptors to an ubiquitination complex relevant for surface expression, internalization, and degradation processes. In contrast, while MAGEL2 is normally associated with cell surface abundance of LepR and its reduced degradation, the hypothalamus of Magel2-null mice showed less LepR and altered levels of proteins of the ubiquitin pathway that regulate LepR processing (Wijesuriya et al., 2017). Transferring these findings to our PWS collective, one might hypothesize that the above deduced PWS-associated downregulation of LepR might be counteracted by upregulated leptin hormone and leptin receptor expression mediated at the epigenetic level via decreased promoter methylation of LEP and LEPR as evidenced by our data. To that end, a functional inverse relationship between LEPR methylation and LEPRb expression has been previously suggested (Chavira-Suárez et al., 2019). However, it remains to be mentioned that Wijesuriya et al. also noted that Magel2 mutation rodent models showed an overall less pronounced hyperphagia phenotype than other models, for example compared to Snord116 gene deficiency.

Likewise, our study failed to provide a conclusive link between laboratory findings and the behavioral dimension. Overall, no correlation of serum leptin levels with HQ scores was demonstrated in the PWS subjects. Also, no correlation was found between overall methylation rate of LEP and LEPR with HQ-scores in PWS. Solely, LEP CpG sites m349 and m371 negatively correlated with HQ-scores.

But, from a neurobiological perspective, our finding that the methylation rates of the abovementioned CpG sites correlate negatively with the outcome of the HQ appears inconclusive. As stated above, it should be assumed that increased leptin expression via decreased LEP methylation should be accompanied by an increased feeling of satiety and, accordingly, by decreased food-seeking behavior, which would correspond to a lower score on the HQ. In this respect, the correlations found seem most likely to be incidental.

To this end, our findings appear to be in line with previous human studies on this topic like Goldstone et al. being unable to establish a connection between hormonal abnormalities in PWS and the behavioral dimension in the sense of an altered eating behavior (Goldstone et al., 2012). In synopsis of our and previous findings, no connection between leptin regulation and behavioral abnormalities in PWS can be assumed.

4.1. Limitations

Overall, the cohort size of our study is small, but in line with that of previous studies on the topic.

Especially limiting appears the use of peripheral blood material, although the regulation of satiety is essentially due to central processes. Therefore, only indirect conclusions can be drawn regarding the central processes behind leptin-dependent appetite regulation in PWS, especially, since tissue-specific differences in LEP promoter DNA methylation are known (Stöger, 2006). Moreover, it is important to keep in mind that central effects of leptin seem to unfold in a narrow concentration corridor. As shown by Holtkamp et al., peripheral serum leptin levels above 25–30 ng/ml cannot necessarily be expected to also result in higher levels in central tissue or CSF (Holtkamp et al., 2004). In this respect, the average leptin level of 32.747 in our PWS cohort should be evaluated with caution regarding its functional relevance for central processes. Unfortunately, we did not have central material such as CSF available for our study, as such sampling is hardly justifiable from an ethical point of view.

Furthermore, the HQ currently is described to be the gold standard for assessing food-seeking behavior in PWS. However, the overall data on the validity of the questionnaire appears limited. In general, it offers only an indirect assessment, since it is completed by relatives or caregivers, which could be subject to perceptual bias compared to the PWS individuals themselves. In addition, it should be noted that the HQ is not an ideal measurement tool for subjects who are accommodated in food-safe environments, such as professionalized residences. Also, the HQ was not performed in the control subjects, so comparability is not given.

Another crucial limitation of the study seems to be the use of BMI as determinant of obesity in the subjects. BMI values of the control group could likely be confounded by a comparatively increased muscle mass. Body fat percentage could be the much more accurate determinant for comparison at this point, especially since leptin is primarily produced in adipocytes. However, the comparability with previous studies still appears to be given insofar as these also mostly used body weight or BMI as assessment standard. Also, due to its pilot character, the lack of comparability to previous studies, especially for assessing the actual relevance of individual CpG positions, is a limiting factor in the interpretation of our results.

Furthermore, our study lacks a healthy, normal-weight control group for comparisons regarding leptin resistance in obese and non-obese subjects. Hence, the present study is not final able to disentangle PWS-related from general obesity-related effects.

4.2. Conclusion and future prospects

The observed differences in leptin expression and epigenetic regulation again pointed towards a dysregulation of the hormonal network regulating hunger and satiety in people with PWS without being able to provide a conclusive connection of leptin dysregulation and food-seeking behavior typical in PWS.

Our basic research could set a theoretical basis for future drug treatment options for hyperphagia in PWS. A promising drug, according to the signaling pathways underlying hyperphagia described above, could be the melanocortin 4 receptor agonist setmelanotide, which showed a good response in terms of an appetite suppressing effect at least in a MAGEL-2-null mouse model (Bischof et al., 2016). But to date, no data on the efficacy in PWS have been published.

Because the results of our and previous studies on leptin in PWS differ, future replication studies appear inevitable, preferably with larger study populations and a non-obese control group to delineate overweight effects. In addition, more specific measures such as body fat percentage should replace BMI measurement and hyperphagia assessment might be optimized. Last, since recent studies have pointed to a prominent role of the dopamine system in appetite regulation in PWS (Low et al., 2021), attempts might be made to focus on possible associations of dopamine system and hyperphagia-related behavior in PWS, as we were unable to draw a conclusive link between homeostatic appetite regulation and the behavioral dimension.

Conflicts of interest

All authors declare to have no conflicts of interest.

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Author contribution

Jelte Wieting contributed in acquisition of samples, drafted the manuscript and contributed to analysis and interpretation of data. Kirsten Jahn carried out part of the experiments, contributed to analysis and interpretation of data and revised the manuscript critically for important intellectual content. Vanessa Buchholz and Ralf Lichtinghagen carried out part of the experiments. Christian K. Eberlein contributed to acquisition of samples and contributed to interpretation of data and revised the manuscript critically for important intellectual content. Stephanie Deest-Gaubatz and Stefan Bleich contributed to interpretation of data and revised the manuscript critically for important

intellectual content. Maximilian Deest contributed to acquisition of samples, analysis, and interpretation of the data and was substantially involved in drafting the manuscript. Helge Frieling contributed to conception and design of the study, interpretation of data, and revised the manuscript critically for important intellectual content.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psyneuen.2022.105857.

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