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DNA methylation of ghrelin and leptin receptors in underweight and recovered patients with anorexia nervosa

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ABSTRACT

Epigenetic mechanisms, which modulate gene expression, are becoming increasingly important in the research on anorexia nervosa (AN). Patients with AN have difficulties with the perception of hunger even though hormones like high ghrelin and low leptin signal the need for energy intake. Given the prominent role of the growth hormone secretagogue receptor (GHS-R1a) and the leptin receptor (LEPR) in appetite regulation, a dysregulation of the receptors' expression levels, possibly resulting from altered DNA promoter methylation, may contribute to the pathophysiology of AN. Such alterations could be secondary effects of undernutrition (state markers) or biological processes that may play an antecedent, possibly causal, role in the pathophysiology (trait markers). Therefore, the objective of this study was to examine DNA promoter methylation of the GHS-R1a and LEPR gene promoter regions and investigate whether methylation levels are associated with AN symptoms. We studied medication-free underweight patients with acute AN as well as weight-recovered patients and normal-weight, healthy female control subjects. While DNA methylation of the LEPR gene was similar across groups, GHS-R1a promoter methylation was increased in underweight AN compared to healthy controls – a finding which can be interpreted within the framework of the "ghrelin-resistance" hypothesis in AN. The results of the current study suggest for the first time a potential epigenetic mechanism underlying altered GHS-R1a sensitivity or altered ghrelin signaling in acutely underweight AN. If a ghrelin-centered model of AN can be verified, a next step could be the search for a dietary or psychopharmacological modulation at the ghrelin receptor, potentially via epigenetic mechanisms.

1. Introduction

Anorexia nervosa (AN) is a serious eating disorder with one of the highest mortality rates among psychiatric disorders (standardized mortality ratio (SMR) = 5.86; Arcelus et al., 2011). Although the exact etiology of AN is still unknown, substantial evidence indicates that a complex pattern of inheritance of various traits, influenced by both genetic and environmental factors (Mazzeo and Bulik, 2009), may be a significant etiological factor. Therefore, epigenetic mechanisms, which modulate gene expression, are becoming increasingly important in the research on AN. The best-studied epigenetic alteration is encoded by DNA methylation, which is characterized by the attachment of methyl groups to cytosine in association with cytosine-guanine dinucleotides (5′-CpG-3′ sites). There is evidence that methylation may directly affect the affinity of transcription factors (TFs) for their binding sites (Tate and Bird, 1993; Clouaire and Stancheva, 2008).

Previous research on epigenetics in AN is still very sparse. To date, most studies about DNA methylation in AN have focused on the promoter-specific methylation of previously identified candidate genes. Overall, 16 candidate genes have been studied so far (Hübel et al., 2019), mainly focusing on reward-related pathways including central neurotransmission and homeostatic pathways. However, altered methylation patterns in AN have been identified only regarding the five following genes: SNCA (synuclein alpha), DAT (dopamine transporter), DRD2 (dopamine receptor D2), OXTR (oxytocin receptor) and LEP (Leptin) (Frieling et al., 2007, 2010; Kim et al., 2014; Neyazi et al.,

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2019). Except for LEP, all genes showed an increased methylation level in AN compared to healthy control participants. In addition, two pilot epigenome-wide studies in AN have been conducted recently (Booij et al., 2015; Steiger et al., 2019).

Patients with AN have difficulties with the perception of hunger and satiety which may maintain the disease (Klastrup et al., 2019). Considering the importance of appetite regulation in AN, it is surprising that only a few studies have addressed DNA methylation in genes involved in appetite regulation, which revealed a significant decrease of methylation in LEPR (leptin receptor) (Neyazi et al., 2019), heterogeneous findings regarding LEP (Pjetri et al., 2013; Neyazi et al., 2019), and no alterations of the POMC (Proopiomelanocortin) (Ehrlich et al., 2010) and CNR1 (Cannabinoid-Rezeptor 1) genes (Frieling et al., 2009).

Among the endocrine mediators regulating food intake, particularly the regulation of appetite and energy expenditure, ghrelin and its receptor (growth hormone secretagogue receptor, GHS-R1a) play a major role (Cummings et al., 2001). Activation of the GHS-R1a via ghrelin induces an orexigenic state, which facilitates food intake (Nakazato et al., 2001). However, the effects of ghrelin go beyond the control of appetite and food intake but also include the modulation of other reward-related behaviors via the mesocorticolimbic dopamine system (Müller et al., 2015). AN patients seem to have altered reward processing including abnormal brain responses to food (O'Hara et al., 2015). Moreover, it has been shown that changes in leptin and ghrelin occurring in the acute phase of AN may sustain aberrant rewarding behaviors (Monteleone et al., 2018). In particular, in patients with AN, several studies indicate higher serum ghrelin levels (Ogiso et al., 2011; Méquinion et al., 2013; Bernardoni et al., 2020). However, despite the elevated ghrelin levels, patients fail to increase their dietary intake. Therefore, several studies have consistently suggested that increased ghrelin levels in the acute phase of AN are an ineffective compensatory mechanism to chronic starvation (Schalla and Stengel, 2018). Although never formally demonstrated, some authors have speculated about a possible ghrelin resistance (Holsen et al., 2014; Berner et al., 2019). This inability of AN patients to respond appropriately to elevated levels of ghrelin may be due to decreased GHS-R1a expression, sensitivity or function (Denis et al., 2014; Schaeffer et al., 2013). However, to date, the cause of disrupted ghrelin signaling has not been clarified. Nonetheless, findings from a recent study in underweight AN patients suggest that certain ghrelin agonists, that help restore ghrelin sensitivity, can increase hunger and lead to weight gain after a short period of treatment (Fazeli et al., 2018).

The hormone leptin is a major opponent of ghrelin and acts through LEPR as a mediator of long-term regulation of energy balance, suppressing appetite and food intake (Schwartz et al., 2000; Mistrík et al., 2004). There is strong evidence that leptin levels are suppressed in acute AN and characterize the extent of malnutrition (Hebebrand et al., 2007). Despite low leptin levels, individuals with AN continue to restrict their food intake. With refeeding and weight gain, serum leptin levels increase (Hebebrand et al., 1997) to normal or possibly even higher-than-normal levels (Schorr and Miller, 2017; Holtkamp et al., 2004). Altered LEPR expression or sensitivity could be an explanation for these phenomena.

Given the prominent role of GHS-R1a and LEPR in appetite regulation, a dysregulation of the receptors' expression levels, possible resulting from a modified promoter methylation, may contribute to the pathophysiology of AN. Thus, the objective of this study was to examine DNA promoter methylation of the GHS-R1a and LEPR gene promoter regions and investigate whether methylation levels are associated with the psychopathology of AN. To differentiate between the secondary effects of malnutrition (state markers) and biological processes that may play an antecedent, possibly causal, role in the pathophysiology (trait markers), we studied medication-free (see SM 1.1 for details) underweight patients with acute AN as well as weight-recovered patients. In addition to examining the average level of promoter methylation, we also focused on specific CpG sites in particular loci that are related to the binding of TFs (Choy et al., 2010) while controlling for possible effects of age and smoking.

2. Methods

2.1. Study participants

This study included female patients with acute AN, weight-recovered AN and healthy controls. 46 patients with acutely underweight anorexia nervosa (acAN) according to DSM-IV were admitted to eating disorder programs of a University Child and Adolescent Psychiatry Department. We also included 26 female subjects, who were previously treated for AN and had successfully recovered from their illness (recAN). In order to prevent heterogeneity of subjects, we clearly separated between acutely ill and recovered patients in our sample. To be considered "recovered", subjects had to (1) maintain a BMI $>$ 18.5 (if older than 18 years) or a BMI $>$ 10th BMI percentile (if younger than 18 years, Kromeyer-Hauschild et al., 2001) for at least three months prior to the study and (2) have not binged, purged or engaged in significant restrictive eating patterns.

The control group consisted of 60 normal-weight, eumenorrheic, healthy female control subjects (HC), who were recruited through advertisement among middle school, high school and university students.

Information regarding exclusion criteria and possible confounding variables, including menstrual cycle and use of contraceptive medication, were obtained from all participants using the SIAB-EX (Fichter and Quadflieg, 2001), supplemented by our own semi-structured interview and medical records (see also SM 1.2).

HC were excluded if they had any history of psychiatric illness. Participants of the acAN or the recAN group were excluded if they had a lifetime history of any of the following clinical diagnoses: organic brain syndrome, schizophrenia, substance dependence, bipolar illness, bulimia nervosa or binge-eating disorder. Further exclusion criteria for all participants were: IQ less than 85, current inflammatory, neurological or metabolic illness, chronic bowel diseases, cancer, anaemia, pregnancy, breast feeding, treatment with cortisone and use of psychotropic medications within the past 6 weeks. Additionally, all subjects with indicated drug use were excluded from analyses, as well as subjects who reported to consume drugs (notably cannabis) from time to time.

This study was carried out in accordance with the latest version of the Declaration of Helsinki, the study design was approved by the Institutional Review Board of the Charité – Universitätsmedizin Berlin and all participants (and if underage their guardians) gave written informed consent after the nature of the procedures had been fully explained.

2.2. Clinical measures

The diagnosis of a current and/or past eating disorder was ascertained in all participants (i.e. including HC, see also SM 1.2) using the expert form of the Structured Interview for Anorexia and Bulimia Nervosa (SIAB-EX; Fichter and Quadflieg, 2001). The SIAB-EX is a semi-structured interview that assesses the prevalence and severity of specific eating-related psychopathology over the past three months according to DSM-IV diagnostic criteria. Additionally, eating disorder-specific psychopathology was assessed with the short version of the Eating Disorders Inventory (EDI-2), a self-report questionnaire comprising eight subscales (Rathner and Waldherr, 1997). Further, we addressed psychopathological symptoms in all participants through Symptom Check-List-90 item-Revised (SCL-90-R), a 90-item self-report questionnaire, which measures psychological symptoms and psychological distress in adults (Derogatis, 1994).

2.3. Blood collection, biochemical assessments and bisulfite sequencing

Venous blood was collected into vacutainer tubes containing EDTA between 7:30 and 9:30 a.m. after the subjects had fasted overnight. For the acAN group the blood collection took place within the first week after initiating intensive treatment. Plasma was stored at −80 °C until analyzed. DNA methylation of the GHS-R1a gene promoter and LEPR gene promoter was determined in genomic DNA of peripheral blood mononuclear cells by means of bisulfite conversion and sanger sequencing as described previously (Ehrlich et al., 2010) (for details please refer to SM 1.3).

2.4. Quality control (QC)

All sequences were checked in the Sequence Scanner. Sequences with low Quality Value (QV20) were repeated. Only samples that could be technically adequately sequenced were used. For analysis, only CpG positions with 95% valid values were included. Similarly, only subjects with 95% valid CpG-values were included. CpG with less than 0.001 of variance were excluded from analysis.

Following QC, 7 acAN, 4 recAN and 6 HC were excluded due to the above points. Finally, we included a total number of 115 subjects and a total of 64 CpG (24 GHS-R1a-CpG positions and 40 LEPR-CpG positions) in the subsequent analysis.

2.5. CpG islands and transcription factor binding

CpG islands and regions of transcription factor binding within the sequenced regions were identified with the Txn Factor ChIP Track Settings of the UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly, which is hosted by the University of California, Santa Cruz (UCSC).

Using ChIP-seq experiments, genomic regions corresponding to the binding regions of the TFs were identified (Encode project; Gerstein et al., 2012). Clusters of a total of 161 TFs in 91 cell types were created to display the occupancy regions for each TF (see Fig. 2; marked as grey boxes). Furthermore, DNA binding motifs with the highest score in the cluster were identified for a corresponding transcription factor (ENCODE Factorbook repository; Wang et al., 2013; marked green in the genome browser).

2.6. Statistical analyses

Residuals of analyzed variables were normally distributed according to Kolmogorov–Smirnov test and thus, parametric methods were applied. In our main analysis, mean methylation of GHS-R1a and LEPR was compared between groups with analysis of covariance (ANCOVA) and subsequent Least Significant Difference (LSD) post hoc test if appropriate. Given possible biasing effects of age (Hannum et al., 2013; Horvath, 2013) and smoking (Ehrlich et al., 2012; Lee and Pausova, 2013) on DNA methylation levels, we included age and the presence or absence of current cigarette smoking as covariates in all methylation analyses. False Discovery Rate (FDR) was applied at the 0.05 level for correction of multiple testing regarding the two target genes. Our sub-analyses for exploration of single methylated CpGs included two approaches: 1) ANCOVA of CpG methylation at specific transcription factor binding sites (TFBSs), 2) Exploratory analyses of DNA methylation levels at each CpG residue were conducted using multivariate analysis of covariance (MANCOVA). Finally, for associations of methylation and clinical variables, correlations were calculated using partial correlation (based on Pearson) to control for age and smoking. If not indicated otherwise, all values are presented as mean ± standard deviation (SD) (see SM 1.4 for flowchart illustrating our analyses). All tests were performed with SPSS statistical software version 25 (SPSS, Chicago, Illinois).

3. Results

3.1. Sample characteristics

Demographic and clinical characteristics of all participants are summarized in Table 1. As expected, patients with acAN had significantly lower BMI-standard deviation scores (BMI-SDS) and higher levels of psychopathology (EDI-2, SCL-90-R). While recAN patients had similar BMI-SDS as HC, they still had some residual psychopathology. RecAN patients were slightly older than HCs. In the acAN group, 28 patients belonged to the restrictive type (AN-R) and 11 patients to the binge/purging type (AN-B/P), with no difference regarding their clinical

Table 1
Demographic and clinical characteristics.

	acAN $N = 39$	recAN $N = 22$	HC $N = 54$	F/X^2	$p/FDR\ p_{corr}$	Post hoc (LSD)
Age (years)	18.0 ± 3.4	19.7 ± 3.8	17.5 ± 2.8	3.53	.033/-	recAN > HC**
BMI (kg/m^2)	15.0 ± 0.3	21.3 ± 0.4	21.4 ± 0.3	137.04	.000/-	acAN < HC*** acAN < recAN***
BMI-SDS	−2.1 ± 0.5	0.1 ± 0.8	0.2 ± 1.3	135.52	.000/-	acAN < HC*** acAN < recAN***
Age of onset	15.3 ± 2.0	14.3 ± 2.1	-	3.12	.083/-	-
EDI-2 (total score)	221.8 ± 50.2	166.3 ± 45.5	144.9 ± 30.8	40.13	.000/-	acAN > HC*** acAN > recAN*** recAN > HC*
SCL-90-R (GSI)	58.3 ± 12.0	46.1 ± 8.5	43.0 ± 9.4	26.42	.000/-	acAN > HC*** acAN > recAN***
Leptin (ng/mL)[a]	0.7 ± 1.5	14.1 ± 1.9	15.1 ± 1.2	31.72	.000/-	acAN < HC*** acAN < recAN***
Cigarette smoker (# of participants)	7	5	9	0.39 (X^2)	.823	-
Mean CpG methylation (%)						
GHS-R1a	17.07 ± 3.0	15.77 ± 2.5	15.58 ± 2.3	4.29	.016/.032	acAN > HC** acAN > recAN*
LEPR	5.55 ± 1.8	4.66 ± 1.9	5.46 ± 1.9	2.42	.094/-	-

Statistics of demographic and clinical variables according to QC. acAN = acute anorexia nervosa; recAN = recovered anorexia nervosa; HC = healthy control. BMI-SDS = Body mass index standard deviation score. EDI-2 = Eating disorder inventory 2; SCL-90-R (GSI) = Symptom Checklist-90-R (global severity index). Group differences were tested using X^2 (cigarette smoker), one-way ANOVA (age, BMI, BMI-SDS, EDI-2, SCL-90-R) and ANCOVA (Mean CpG Methylation), *$p < .05$, **$p < .01$, ***$p < .001$. Adjustment for multiple comparisons for two genes (ANCOVA): Least Significant Difference (LSD). Mean values ± standard deviation for each variable are shown separately for each sample. [a] N = 110; 35, 21, and 54 for acAN, recAN, and HC, respectively.

characteristics between subtypes (BMI, BMI-SDS, EDI-2, SCL-90-R).

3.2. Main analysis - mean methylation

ANCOVA indicated that there were significant differences in mean methylation of the GHS-R1a gene promoter across groups ($F = 4.29$, $p = .016$, FDR $p_{corr} = .032$, $\eta_p^2 = 0.072$). Post hoc test indicated a significantly higher promoter methylation in acAN patients when compared to HC ($p = .010$), and in acAN compared to recAN ($p = .022$). There was no difference in mean methylation between recAN and HC (see Fig. 1, Table 1). No significant differences in mean methylation of the LEPR gene promoter were observed between groups ($F = 2.42$, $p = .094$, see Fig. 1, Table 1). The ANCOVA models also showed a significant influence of age (covariate) on GHS-R1a ($F = 4.74$, $p = .032$, $\eta_p^2 = 0.041$) but not on LEPR methylation. Smoking had no effect on DNA methylation, neither for GHS-R1a nor LEPR. Supplementary analyses only covarying for age but not smoking confirmed our initial findings (see SM 2.1).

3.3. Sub-analyses - methylation at single CpG sites

A more detailed examination of DNA methylation levels at the level of the individual CpG sites across all groups showed high variability in percentage of GHS-R1a-methylation across the 24 sites, varying from 2.66 ± 4.18% (GHS-R1a _m1392) to 47.76 ± 11.13% (GHS-R1a _m1657) (see Fig. 2B). To explore whether single CpGs were driving the overall group difference in GHS-R1a-methylation we adopted two additional approaches.

First, we focused on transcription factor binding sites (using the UCSC Genome browser). Fig. 2 illustrate regions of transcription factor binding sites. We found 12 transcription factors to be associated with CpG positions of the GHS-R1a gene promoter. The binding sites stretch almost across the whole promoter region which we have focused on in this study – justifying our initial approach of averaging methylation levels across all CpGs.

Based on the data by ChIP-seq experiments (Factorbook; Wang et al., 2012) the highest-scoring canonical DNA motif in the cluster for a corresponding transcription factor was identified. Therefore, we focused on the individual CpGs in those areas (marked green, see Fig. 2). For GHS-R1a, the RE1-Silencing Transcription factor (REST) includes one CpG (m1430) in the region of the highest scoring binding site. In line with our analyses using mean promoter methylation, ANCOVA of this particular CpG showed a similar pattern, namely differences between groups ($F = 5.29$, $p = .006$, $\eta_p^2 = 0.088$) with overall higher methylation in acAN patients compared to healthy controls ($p = .002$), but no differences in acAN compared to recAN or between recAN and HC. For the CCCTC-Binding factor (CTCF), the two indicated CpGs (m1378, m1368) within the area of the highest TF score showed no significant group differences (see SM 2.2 for details).

Our second approach to test whether single CpGs are driving the overall group difference in DNA methylation included testing all individual CpG loci using MANCOVA. Even though CpG m1430 showed the highest significant effect size of all CpGs, the multivariate main effect of group (acAN, recAN, HC) on CpG methylation was not significant for GHS-R1a (see SM 2.3) which precluded the further exploration of effects of single CpG sites.

3.4. Relationship between DNA methylation levels and clinical variables

Partial correlation analyses between GHS-R1a mean promoter methylation levels and EDI-2 total score revealed a weak, positive partial correlation ($r(111) = 0.199$, $p = .035$). However, within-group analyses (acAN, recAN, HC) showed no significant associations. Methylation of CpG GHS-R1a _m1430 was also positively associated with EDI-2 total score across all groups ($r(111) = 0.190$, $p = .044$) and particularly in the HC group ($r(50) = 0.292$, $p = .036$). There were no significant associations between mean and GHS-R1a_m1430 methylation levels and general psychological symptoms (SCL-90-R GSI).

4. Discussion

The current study investigated associations of DNA promoter methylation of two genes, implicated in appetite regulation, in acutely underweight AN patients and patients recovered from AN. While DNA methylation of the LEPR gene was similar across groups, GHS-R1a promoter methylation was increased in acAN compared to HC – a finding which can be interpreted within the framework of the "ghrelin-resistance" hypothesis in AN. Given normal GHS-R1a promoter methylation levels in recAN, hypermethylation in acAN might be a state-related phenomenon secondary to acute undernutrition.

Several studies have shown that the extent of DNA methylation in promoter regions is often associated with the repression of transcription (Shen et al., 2007; Jones, 2001). A speculative interpretation of our findings would be that increased methylation in patients with AN may inhibit the expression of the GHS-R1a gene. GHS-R1a regulates the

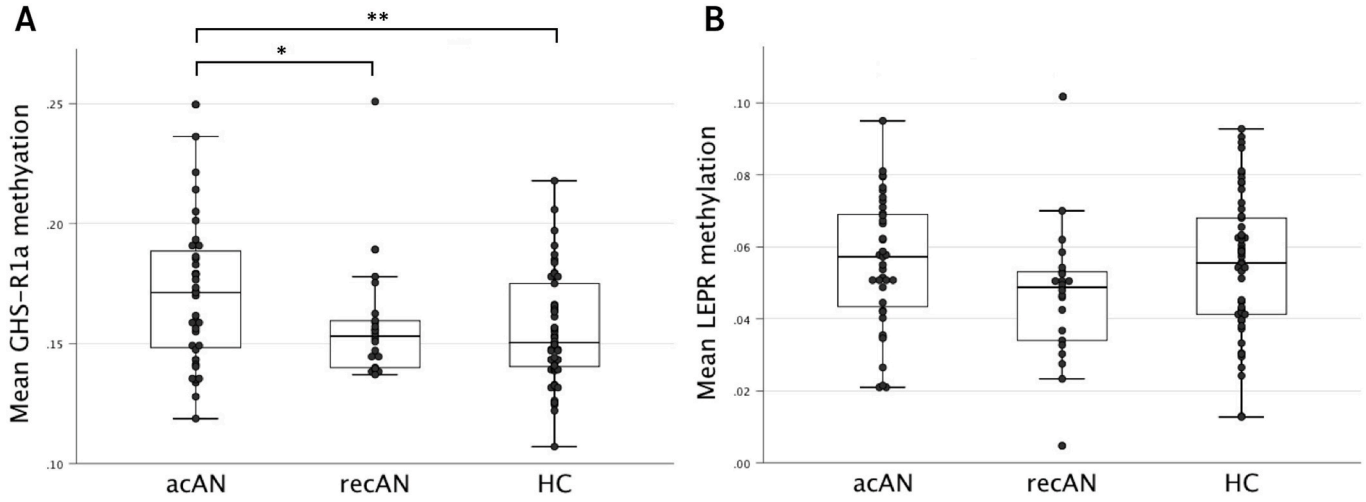

Fig. 1. Boxplots of methylation levels. Boxplot illustrating methylation levels in individuals with acute anorexia nervosa (acAN), recovered anorexia nervosa (recAN) and healthy controls (HC) in (A) GHS-R1a, (B) LEPR promoter region. The black dots represent each individual data point. The box includes methylation values between 25th and 75th quantile (median ± 1 interquartile range), the whiskers represent the range of estimates within 1.5-fold of the interquartile range. *$p < .05$, **$p < .01$.

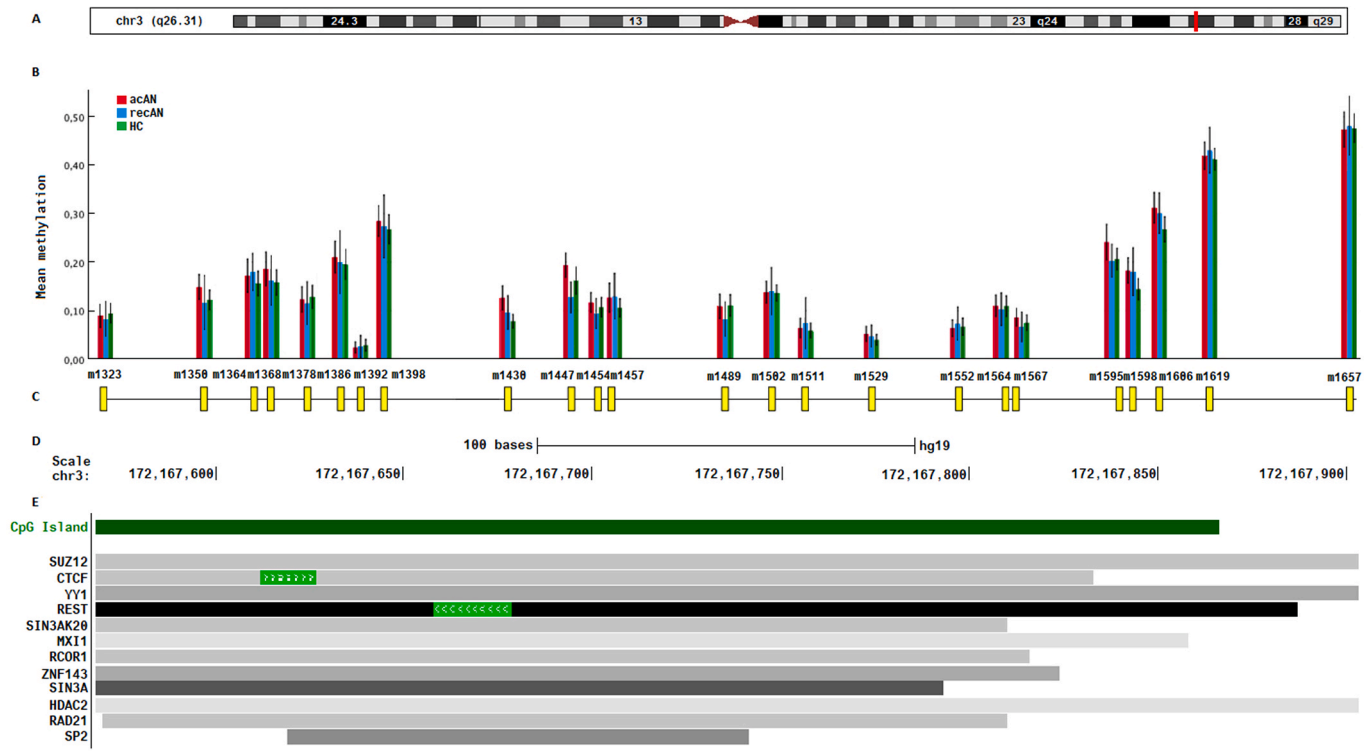

Fig. 2. Overview of target CpGs in the GHS-R1a gene and corresponding methylation and transcription factor binding sites. (A) Chromosome 3 and position of GHS-R1a gene. (B) Mean methylation level and standard deviations of acAN patients, recAN participants and HCs at each CpG. (C) CpG-positions in the GHS-R1a gene promoter (chr3: 172.167.569–172.167.903); minus (m) indicates the position of the respective CpG relative to exon1. (D) Scale of chromosome 3 in base pairs. (E) Transcription factor binding sites in the GHS-R1a gene promoter. The HGNC (HUGO Gene Nomenclature Committee) gene name for the transcription factor is shown to the left of each cluster. A grey box encloses each peak cluster of transcription factor occupancy, with the darkness of the box being proportional to the maximum signal strength observed in any cell line contributing to the cluster. Based on the data by ChIP-seq experiments (Factorbook), the highest-scoring canonical motif in the cluster for a corresponding transcription factor was identified (marked green). UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly (https://genome.ucsc.edu/) Web. Accessed 2019/09/10. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ability of cells to respond to ghrelin. Interestingly, our findings are consistent with several studies that proposed the hypothesis of a possible altered GHS-R1a sensitivity in AN, suggesting that AN patients may be "resistant" to the orexigenic effects of ghrelin. For instance, ghrelin administration does not cause normal appetite responses in patients with AN (Miljic et al., 2006), with patients reporting to feel less hungry than lean control participants. Animal models have shown that selectively attenuating GHS-R1a protein expression is associated with lower body weight, less adipose tissue as well as reduced daily food intake (Shuto et al., 2002). In contrast, obese patients were reported to be oversensitive to ghrelin, possibly due to an overexpression of the GHS-R1a (Klok et al., 2007). Ghrelin infusions in overweight individuals have been shown to result in a disproportionate increase of *ad libitum* energy intake and food intake in contrast to lean people (Druce et al., 2005). In a similar fashion, it may be possible that low expression of GHS-R1a leads to ghrelin hyposensitivity in AN patients.

Furthermore, a neuroimaging study supported the hypothesis of altered GHS-R1a sensitivity in AN. In contrast to controls, participants with active AN did not show positive associations between fasting ghrelin levels and neural activity in several limbic brain regions (which have been associated with reward and appetite regulation) in response to high-calorie foods (Holsen et al., 2014). Moreover, a recent study proposed a reverse effect of ghrelin on reward processing in AN which might also be related to altered GHS-R1a sensitivity (Bernardoni et al., 2020).

The origins of altered GHS-R1a promoter methylations are unclear. Speculatively, epigenetic changes could be related to early or even prenatal undernutrition, diets (many healthy young women experiment with diets, but future patients are often unable to get off the diet) or epigenetic imprinting (i.e. transmitted e.g. by mothers who themselves suffer from an eating disorder (Zhang and Kutateladze, 2018)) and early life stress (Steiger and Thaler, 2016).

Given that in our study the recAN group showed methylation levels similar to the HC group, it may be possible that the improvement of eating behavior leads to a normalization of GHS-R1a methylation and breaks the aforementioned vicious circle (see also Frieling and Buchholz, 2017). In line with our findings regarding the ghrelin receptor, many studies found that circulating ghrelin levels, which are increased in acute AN, also normalize with weight restoration (Schalla and Stengel, 2018). This interpretation would be in line with the fact that many mediators regulating appetite and food intake are altered in the acute phase of AN and normalize after weight recovery. Therefore, these changes could also be considered as adaptation mechanisms in response to malnutrition and food restriction.

In AN, it has not yet been sufficiently clarified which epigenetic alterations are due to acute undernutrition (state-related effects) and which belong to the underlying predisposing factors or vulnerabilities (trait effects). The findings presented here obtained from a sample with acute and weight recovered AN patients suggest that altered methylation of the GHS-R1a may rather be a state-related phenomenon. Another explanation of seemingly normalized GHS-R1a DNA methylation in recAN might be that patients with such an epigenetic pattern have a higher likelihood of recovery.

In many studies, the average promoter methylation is used, whereas recent results suggest that methylation of individual cytosines may also be important (Medvedeva et al., 2014). Therefore, apart from our main

approach considering mean DNA methylation across several CpGs, we were also interested whether a subset of CpGs would drive our findings. For GHS-R1a, we provided suggestive evidence for an important role of the CpG dinucleotide GHS-R1a_m1430 which is one of the main CpGs in the REST binding site.

Although leptin levels in peripheral blood differed significantly across groups, there were no group differences in leptin receptor promoter methylation. In contrast to our own findings, a recent study in adult AN patients found small differences in LEPR methylation between patients and controls (Neyazi et al., 2019). One possible explanation might be that these AN patients were on average 12 years older than our patients and often had a longer or chronic duration of illness. Another possible explanation for our diverging findings could be that leptin receptor methylation has also been associated with maternal smoking during pregnancy (Yousefi et al., 2013). Unfortunately, we have no data on smoking during pregnancy. However, smoking stated by the patients themselves did not seem to influence the current DNA methylation results. Last but not least, with the given methods we are unable to differentiate between membrane-bound and soluble leptin receptor. A previous study had shown that circulating levels of soluble leptin receptor were increased in underweight women with AN (Monteleone et al., 2002).

4.1. Limitations

Our findings have to be considered in light of the following limitations: First, since age had a significant effect on mean GHS-R1a DNA methylation, our findings may be specific to relatively young patients and may not be found in chronic patients with a longer duration of illness. Second, an alternative interpretation of seemingly normalized GHS-R1a DNA methylation in former patients is that patients with such an epigenetic pattern have a higher likelihood of recovery. Longitudinal studies are needed to clarify this question. Third, methylation studies in general are hampered by two challenges – tissue specificity (i.e. whether methylation patterns assessed in blood reflect methylation in the brain (Bediaga et al., 2017; Davies et al., 2012; Walton et al., 2016)) and the question of whether one should average methylation values across CpG in a certain genetic region, e.g. a specific gene promoter, since individual cytosines may also be important (Medvedeva et al., 2014). Our study cannot answer these questions, but we found suggestive evidence for an important role of the CpG dinucleotide GHS-R1a_m1430 which is one of the main CpGs in the REST transcription factor binding site. At the same time, a robust positive blood-brain methylation correlation (to brain tissue from the Brodmann areas 7,10 and 20) has been reported for the CpG1398 which is 32 bp away from CpG dinucleotide GHS-R1a_m1430 (see also SM 3, BECon: A tool for interpreting DNA methylation findings from blood in the context of brain; Edgar et al., 2017). Finally, we have to acknowledge that the current study follows a hypothesis-driven candidate gene study design and that the sample size is relatively small. Future (multi-center) studies with larger sample sizes and an epigenome-wide approach (EWAS) are warranted.

4.2. Conclusion

Taken together, the results of the current study suggest for the first time a potential epigenetic mechanism underlying altered GHS-R1a sensitivity or altered ghrelin signaling in acutely underweight AN. If a ghrelin-centered model of AN can be verified, a next step could be the search for a dietary (Zhang and Kutateladze, 2018) or psychopharmacological modulation at the ghrelin receptor, possibly via epigenetic mechanisms. For example, as previously demonstrated, the administration of ghrelin agonists seems to restore ghrelin sensitivity, thereby increasing hunger and weight gain (Fazeli et al., 2018).

Author statement

Victoria-Luise Batury: Conceptualization, Formal analysis, Writing - original draft, Esther Walton PhD: Supervision, Writing - review & editing, Friederike Tam MD: Writing - review & editing, Marie-Louis Wronski: Writing - review & editing, Vanessa Buchholz: Resources, Writing - review & editing. Helge Frieling MD: Resources, Writing - review & editing. Stefan Ehrlich: Supervision, Conceptualization, Writing - review & editing, Resources

Declaration of competing interest

All authors declare that there are no conflicts of interest in relation to the subject of this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpsychires.2020.08.026.

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