High-throughput DNA methylation analysis in anorexia nervosa confirms *TNXB* hypermethylation

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ABSTRACT

Objectives: Patients with anorexia nervosa (AN) are ideally suited to identify differentially methylated genes in response to starvation.

Methods: We examined high-throughput DNA methylation derived from whole blood of 47 females with AN, 47 lean females without AN and 100 population-based females to compare AN with both controls. To account for different cell type compositions, we applied two reference-free methods (FastLMM-EWASher, RefFreeEWAS) and searched for consensus CpG sites identified by both methods. We used a validation sample of five monozygotic AN-discordant twin pairs.

Results: Fifty-one consensus sites were identified in AN vs. lean and 81 in AN vs. population-based comparisons. These sites have not been reported in AN methylation analyses, but for the latter comparison 54/81 sites showed directionally consistent differential methylation effects in the AN-discordant twins. For a single nucleotide polymorphism rs923768 in *CSGALNACT1* a nearby site was nominally associated with AN. At the gene level, we confirmed hypermethylated sites at *TNXB*. We found support for a locus at *NR1H3* in the AN vs. lean control comparison, but the methylation direction was opposite to the one previously reported.

Conclusions: We confirm genes like *TNXB* previously described to comprise differentially methylated sites, and highlight further sites that might be specifically involved in AN starvation processes.

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Introduction

Anorexia nervosa (AN) is a pernicious condition characterized by restriction of energy intake and extremely low body weight. Epigenetic alterations are assumed to play a key role in moderating or mediating the impact of environmental/lifestyle exposures on gene function and can also be influenced by genetic variations (Haggarty 2015). Starvation is a key clinical feature of patients with AN and a strong environmental exposure can be expected to trigger epigenetic alterations (Heijmans et al. 2008). DNA methylation is one frequently investigated epigenetic mechanism, which is known to change over time (Fraga et al. 2005) and can also be influenced by the metabolome (Keating & El-Osta 2015). Previous investigations of DNA methylation in AN mainly focused either on the (promoter) methylation of specific candidate genes (Campbell et al. 2011; Pjetri et al. 2012) or on markers for a

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Table 1. Participant characteristics of the high-throughput DNA methylation analysis and the validation datasets.

				Replication data ($n = 5$ MZ pairs)	
	AN cases ($n = 47$)	LEAN controls ($n = 47$)	POP controls ($n = 100$)	AN co-twin	Healthy co-twin
Sex (females); n (%)	47 (100)	47 (100)	100 (100)	5 pairs (100)	
AN type; n (%):					
Restricting	36 (76.6)			4 (80)	
Binge-eating/purging	8 (17.0)			1 (20)	
Missing	3 (6.4)			0 (0)	
Age (years); median (Q1, Q3)	16 (14, 17)	22 (21, 23)	60 (54, 69)	22 (22, 23)	
Restricting type AN	15 (14, 17)			22 (22, 22)	
Binge-eating/purging type AN	16 (15, 17)			28 (–)	
BMI (kg/m^2); median (Q1, Q3)	13.7 (12.0, 14.6)	17.3 (16.9, 17.7)	26.6 (24.3, 32.1)	22.0 (21.5, 23.3)	20.7 (20.2, 24.5)
Restricting AN type	13.7 (13.0, 14.5)			21.8 (20.7, 22.3)	
Binge-eating/purging AN type	12.7 (12.1, 14.0)			29.8[a] (–)	

All twin individuals in the analysis have a life-time minimum BMI of <18.5 (all except one individual are currently weight restored but are still experiencing other significant AN symptoms).
AN, anorexia nervosa; POP, population-based females; BMI, body mass index; MZ, monozygotic.
[a]This individual has life-time AN diagnosis (minimum BMI of 18.2); is currently weight restored but still experiences binge-purge symptoms.

global methylation pattern (Saffrey et al. 2014). Recently, Booij et al. (2015) described a high-throughput DNA methylation analysis (450K Illumina bead arrays from lymphocytes) in 29 female patients with acute AN and 15 normal-weight female controls and reported 14 hypermethylated AN CpG sites at 11 genes (*PRDM16, HDAC4, TNXB, FTSJD2, PXDNL, DLGAP2, FAM83A, NR1H3, DDX10, ARHGAP1, PIWIL1*). Note that such high-throughput DNA methylation analyses rely on the pre-selected content of methylation sites (for details on technical properties of the 450K array: e.g., Dedeurwaerder et al. 2011).

Here, we analyse high-throughput DNA methylation data derived from whole blood for differences between AN patients and two control groups: lean (body mass index [BMI] ≤15th age- and sex-specific percentile) and population-based female controls. We included two control groups as they highlight different aspects of potential differential DNA methylation: the comparison of AN patients vs. lean females without AN will allow for assessing starvation as a key clinical feature of AN, while the comparison of AN patients vs. population-based female controls (without any weight restrictions) will allow for more broadly analysing the general aspects of AN. Lastly we compared methylation pattern in both control groups; these should not be different at the sites specific for AN.

To correct for known cell type distribution effects under starvation (Fukudo et al. 1993; Saito et al. 1999; Castro et al. 2004; Sabel et al. 2013; Bühren et al. 2014), we applied two recently proposed reference-free methods (Houseman et al. 2014; Zou et al. 2014) and investigated their overlapping results. We aimed to identify patterns of differential DNA methylation contrasting AN patients and controls. We discuss our findings on the background of a systematic literature search and data of the most recent and largest genome-wide association meta-analysis (GWAMA; Boraska et al. 2014) on 2,907 AN cases and 14,860 controls conducted by the Genetic Consortium for Anorexia Nervosa (GCAN) as part of the Wellcome Trust Case Control Consortium 3 (WTCCC3) to identify previously reported findings or potential overlapping epigenetic and genetic alterations associated with acute AN.

Materials and methods

Study subjects

We analysed 47 females with AN, 47 lean females (BMI ≤15th age- and sex-specific percentile) without AN, and 100 population-based females (POP; Table I). Females with AN prior to weight restoration were recruited and diagnosed (DSM-IV criteria fulfilled) at the Departments of Child and Adolescent Psychiatry of the Philipps-University of Marburg and of the University of Duisburg-Essen. All AN patients were interviewed with either the Composite International Diagnostic Interview (CIDI) or the Diagnostic Interview for Genetic Studies and the Eating Disorder Family History Interview (for details see Hinney et al. 1997). Lean females (BMI ≤15th percentile) without AN were recruited among the students of the Philipps-University of Marburg. They were reimbursed for their voluntary participation, had to have no somatic disorders and had to consume ≤10 cigarettes per day. Furthermore, the lean females were screened as follows: (1) life-time occurrence of AN and bulimia nervosa by the eating disorder section of the CIDI, (2) the German version of the Three-Factor Eating Questionnaire to ensure that only non-restrained eaters defined as individuals who scored five or less on the cognitive restraint factor were included, and (3) for their weight history by a semi-structured interview. For details we again refer to Hinney et al. (1997). Lean females that either fulfilled (1) or (2) or who reported that they had a higher

weight than same-aged individuals at ages 10, 15 and/or 18 were excluded. The 100 POP were a random sample from a dataset of 925 females of the population-based Cooperative Health Research in the Region of Augsburg (KORA) Survey F4 (Dick et al. 2014). Details on this part of the KORA cohort with a focus on high-throughput methylation profiling are provided in Dick et al. (2014). Control group participants were not specifically assessed for eating disorders. In the following sections, the group of females with AN is abbreviated by AN, the group of lean females without AN by LEAN and the population-based female controls selected from the KORA survey by POP. All studies were approved by the relevant institutional ethics committees, and all women provided written informed consent; the study was conducted in accordance to the *Declaration of Helsinki*.

High-throughput methylation data generation and pre-processing

High-throughput methylation profiling of whole blood of all samples was performed using Illumina HumanMethylation450 bead arrays at the German Center for Diabetes Research, Neuherberg (for details see Dick et al. 2014). The raw data were transformed into beta values using Illumina's Genome Studio methylation module according to the manufacturer's recommendation. We ran the pipeline of Touleimat and Tost (2012) for quality control (correction for batch effects, deletion of non-high-confidence probes), probe filtering (removing probes containing or extending on single nucleotide polymorphisms (SNPs) with minor allele frequencies of at least 5% in the Caucasian population to reduce the influence of genetic variations on methylation level variation), signal correction (colour-bias adjustment, background level correction based on negative control probes) and subset-based quantile normalisation (InfI/InfII shift correction, between-sample normalisation).

Cell type composition and high-throughput methylation association analyses

Methylation differences were investigated for two between-group comparisons: (1) AN vs. LEAN and (2) AN vs. POP. First, we used the high-throughput methylation data to estimate the individual cell type compositions applying the algorithm by Houseman et al. (2012) and the reference dataset provided by Reinius et al. (2012). Secondly and based on these results, the cell type admixture had to be taken into account for the high-throughput methylation association analyses. We applied two recently proposed reference-free methods to address the cell type admixture bias (FastLMM-EWASher, Zou et al. 2014; RefFreeEWAS, Houseman et al. 2014). We followed the recommendations by the authors of FastLMM-EWASher (Zou et al. 2014) and considered only those CpG sites with an average beta value across all samples between 0.1 and 0.9. We required that the genomic control factor λ had to be below 1.2 and a maximum of 10 principal components were allowed to be fitted. We decided to adjust for no further covariates even though the POP were for example considerably older than both the AN patients and the lean females. This decision was based on the observation that an inclusion of "age" would lead to an almost complete separation of the case–control groups resulting in unstable estimates. For both methods we applied an exploratory two-sided significance level α of 0.01 to detect CpG sites that are potentially differentially methylated between two groups. We then compared the results of FastLMM-EWASher and RefFreeEWAS for the two between-group comparisons at each CpG site. We call CpG sites identified by both reference-free methods "consensus CpG sites" and focused on them for robustness reasons. Supplementary Figure S1 (available online) is a flowchart of our procedure. As sensitivity analyses, these consensus CpG sites were assessed for quantitative age and BMI effects, potential menstruation effects and effects related to smoking (Supplementary Tables S8–S11 available online). Conversely, we screened the literature on these and related phenotypes and their overlap to our consensus CpG sites (Supplementary Table S12 available online). As another sensitivity analysis, we also performed a third between-group comparison (LEAN vs. POP) using the same approach as for the other two primary comparisons.

Comparison to the literature and to the AN GWAMA

We performed a systematic literature search and assessed the most recent and largest GWAMA for AN (Boraska et al. 2014).

We searched PubMed and the ISI Web of Knowledge at 2015/05/18 for articles dealing with AN or weight-loss intervention in relation to DNA methylation. The search term was "((anorexia nervosa) OR (weight-loss intervention) OR (underweight) OR (eating disorder)) AND (DNA methylation)". Articles were excluded if they: (1) exclusively dealt with animal models or (2) dealt with methylation globally (i.e., no genes were specified). Articles, which were automatically identified by the databases, were manually re-checked

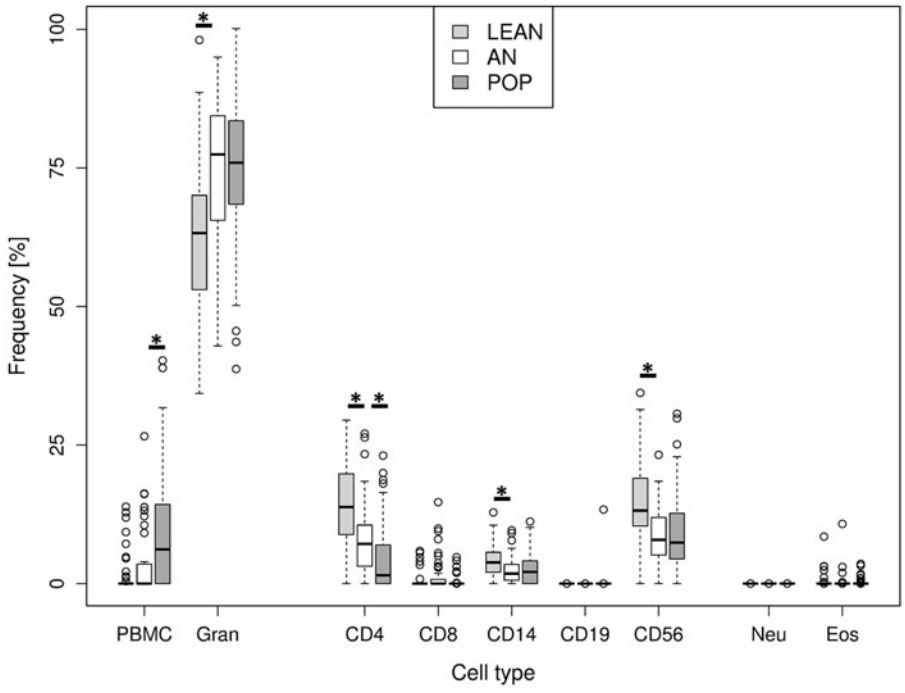

Figure 1. Boxplots of the estimated cell type distributions (between-group comparisons with *P* values $\leq$0.01 are indicated by asterisk; for details see Table S1).

for fulfilling the search criteria. Review articles were screened for further original research articles. From the included articles we extracted all candidate genes for which some evidence for DNA methylation was reported by the authors and reported the results for the corresponding CpG sites in our two between-group comparisons.

Finally, we compared the results of our consensus CpG sites with the genomic variation reported in the GCAN/WTCCC3 GWAMA by Boraska et al. (2014). More precisely, we worked with the effects of SNP associations expressed as estimated odds ratio, the corresponding nominal *P* value under a log-additive genetic model and the SNP-wise heterogeneity statistic (I^2 in %) from the GCAN/WTCCC3 GWAMA. To map these statistics to our high-throughput methylation association analyses, we normalised both datasets to the same genetic map (NCBI36, hg18) and reported the results for the best SNP (the SNP with the smallest *P* value within a maximal distance of 1 Mb to the consensus CpG site).

Validation in monozygotic twins

We used monozygotic (MZ) twin pairs discordant for AN as a validation set. These twin pairs were selected from two longitudinal population-based Finnish twin cohorts, FinnTwin12 and FinnTwin16, each consisting of five consecutive birth cohorts of Finnish twins (Kaprio 2013). Among 5,417 families we were able to identify 36 MZ twin pairs discordant for AN (cases fulfilling the DSM5 criteria for AN). However, only five pairs were AN-discordant at the time of blood draw for the methylation analysis (Table 1). Within-pair DNA methylation differences were computed for the consensus CpG sites using paired moderated t tests implemented in the R package limma.

Results

Cell type composition and high-throughput methylation association analyses

Figure 1 displays the cell type compositions by comparison group as derived from the DNA methylation data (for details see also Reinius et al. 2012). AN and LEAN females showed group differences in the global distribution of granulocytes (Gran; median frequency: 77 vs. 63%) and specifically in $CD4^+$ T cells (7 vs. 14%), $CD14^+$ monocytes (2 vs. 4%) and $CD56^+$ NK cells (8 vs. 13%). AN and POP groups differed globally in peripheral blood mononuclear cell (PBMC; 0 vs. 6%) and specifically $CD4^+$ T cell (7 vs. 2%) distributions (Figure 1; detailed results in Table S1).

Next, we addressed the cell type admixture effects using two reference-free methods for the high-throughput methylation association analyses. The QQ-plots indicated a residual inflation of small *P* values for RefFreeEWAS (Figure S2; Manhattan plots in Figure S3

available online). Consequently, we identified 26,769 differentially methylated CpG sites for AN vs. LEAN according to RefFreeEWAS ($P \leq 0.01$) but only 1,059 CpG sites for FastLMM-EWASher ($P \leq 0.01$). For AN vs. POP, these numbers were 11,395 and 2,607, respectively. Fifty-one differentially methylated CpG sites were identified as consensus CpG sites by both reference-free methods for AN vs. LEAN, and 81 consensus CpG sites for AN vs. POP (Tables 2 and 3). In both cases, the number of consensus CpG sites significantly exceeded that expected by chance ($P < 0.001$). There were no consensus CpG sites that were identified in both group comparisons (Tables S2 and S3, available online, contrast the consensus CpG sites from the first in the second comparison and vice versa). Moreover, the consensus CpG sites were not previously reported in the literature on age, BMI, menstruation or smoking effects (Supplementary Table S12 available online) and our sensitivity analyses also did not strongly support such associations (except for cg25361850 (*ZNF577*) all $P \geq 0.05$ after table-wise multiple testing adjustment (for 132 consensus CpG sites); Supplementary Tables S8–S11 available online). Furthermore, only one consensus CpG site from AN vs. POP (cg13826718 on chromosome 13 at 36919368 without an annotated gene; both $P \leq 0.01$) and none from AN vs. LEAN showed a consensus association signal for LEAN vs. POP (Supplementary Tables S13 and S14, Supplementary Figures S5 and S6 available online).

Comparison to the literature and to the AN GWAMA

In the systematic literature review, we identified 66 articles of which 24 met our inclusion criteria (see Figure S4, Tables S4 and S5 available online); 24 articles reported on 66 (DNA methylation) candidate genes that are summarized in Table S5 (available online). For *HDAC4, NR1H3, PRDM16, TNXB, WT1* and *ZNF783* we found CpG sites with suggestive evidence for a group comparison association (at $P \leq 0.01$) irrespective of the analysis method (bold genes in Table S5 available online). Interestingly, four of the six genes (*HDAC4, NR1H3, PRDM16, TNXB*) were derived from the study of Booij et al. (2015), which had a similar case–control study design also pertaining to patients with AN and controls. When comparing the unadjusted directions of effects, we observed that the *TNXB* CpG sites were hypermethylated in our AN patients compared to both control groups, which was similar to the results reported by Booij et al. (2015).

The comparison of our consensus CpG sites with the GCAN GWAMA by Boraska et al. (2014) (Tables 2 and 3) revealed one SNP (rs923768) in the chondroitin sulphate *N*-acetylgalactosaminyltransferase 1 gene (*CSGALNACT1)* with suggestive evidence for an association with AN susceptibility ($P = 1.31 \times 10^{-6}$). The corresponding CpG site was differentially methylated between the AN and POP samples. Apart from this signal, we detected no association signal meeting the threshold of suggestive evidence for an association ($P \leq 1 \times 10^{-5}$).

Validation in MZ twins

A total of 54/81 consensus CpG sites from the comparison of AN vs. POP showed mean methylation differences of the same direction in the AN-discordant MZ twin sample. This indicates greater than expected overlap in the direction of methylation differences between both of the samples (AN vs. POP, and AN-discordant MZ twin pairs; exact binomial test: $P_{\text{sign-test}} = 1.69 \times 10^{-3}$). Of the 51 consensus CpG sites from the comparison AN vs. LEAN, 26 showed a consistent direction of methylation differences in the discordant MZ twin sample (exact binomial test: $P_{\text{sign-test}} = 1.0$). At the individual CpG site level, none of the tested CpG sites were differentially methylated within the twin pairs ($P > 0.01$, Supplement Tables S6 and S7).

Discussion

Epigenetic marks can be altered by both genetic and environmental factors. For example, altered epigenetic profiles associated with BMI variability have already been described (e.g., Dick et al. 2014). We hypothesized that the epigenomic pattern in weight regulation can optimally be analysed by comparing starved individuals and lean or population-based controls. As starvation is a key feature of AN, we analysed high-throughput DNA methylation data derived from whole blood for differences between female AN patients prior to weight restoration and lean or population-based female controls. Initially, we estimated that the cell type distributions derived from DNA methylation data differed between the groups. However, there was also a large overlap of the group distributions and no clear group separation in accordance with data reported previously, which did not rely on DNA methylation (Fukudo et al. 1993; Saito et al. 1999; Castro et al. 2004; Sabel et al. 2013; Bühren et al. 2014). To address the resulting cell type admixture bias in our high-throughput DNA methylation analyses, we applied two recently published reference-free methods and focused on the CpG sites detected by both methods. There were 51 differentially methylated consensus CpG sites

Table 2. AN vs. LEAN: consensus CpG sites ($P \leq 0.01$) and the corresponding best GWAMA-SNPs (minimal *P* value) within a maximum distance of 1 Mb from the CpG site.

DNA methylation analyses							GWAMA				
CpG site	Gene[a]	Chromosome	Position	FastLMMEWASher *P* value	RefFreeEWAS *P* value	Unadj. mean difference	Best SNP	Position	OR (effect allele)	*P* value	I^2 (%)
cg13471521	*TNFRSF1B*	1	12167517	0.010	0.002	−0.029	rs11577773	11439108	0.91 (T)	0.003	0.0
cg20146909	*LRRC8D*	1	90062199	0.001	0.007	−0.004	rs2296883	89290783	0.89 (G)	0.003	0.0
cg03585329		1	102036107	0.007	0.001	−0.028	rs12022173	102874238	0.62 (C)	0.002	0.0
cg19377421	*ISG20L2; C1orf66*	1	154963665	0.008	0.008	0.026	rs7551218	154631344	1.09 (G)	0.008	45.4
cg27496793	*SOCS5*	2	46779937	0.002	0.008	−0.008	rs11899526	47085576	0.89 (T)	0.001	43.5
cg11577355	*AFF3*	2	100088906	0.002	0.004	-3.38×10^{-4}	rs17023084	99750195	0.70 (G)	1.60×10^{-4}	0.0
cg15474859	*KLF7*	2	207738549	0.003	0.007	0.002	rs13385352	207810750	1.11 (C)	0.002	0.0
cg16171858		3	58447774	0.010	0.006	0.002	rs9810192	58562367	1.19 (T)	0.008	27.8
cg08475266	*THOC7*	3	63820097	0.003	0.001	−0.007	rs1403700	63390235	0.91 (A)	0.005	0.0
cg15278957	*ACTL6A*	3	180762750	0.005	6.04×10^{-5}	0.011	rs5019580	180144300	1.11 (A)	4.99×10^{-4}	0.0
cg25105652	*DAB2*	5	39448795	0.006	0.009	−0.040	rs2962497	39618591	0.90 (A)	0.001	0.0
cg18891762	*PCDHGA4; PCDHGA2; PCDHGA5; PCDHGB2; PCDHGA1; PCDHGB1; PCDHGA3; PCDHGA5*	5	140726233	0.009	0.008	0.030	rs998794	140546893	1.10 (G)	0.014	0.0
cg05600864	*C1QTNF2*	5	159730492	0.005	0.003	0.004	rs2431579	159486555	1.12 (A)	3.94×10^{-4}	0.0
cg26316599	*ATP6V0E1*	5	172388661	0.007	0.001	0.006	rs1347155	171901953	1.17 (T)	0.003	8.8
cg09569760	*RNF130*	5	179408007	0.009	0.001	−0.012	rs4701136	178958156	1.14 (A)	2.34×10^{-4}	56.6
cg24068053	*C6orf41; GUSBL1*	6	27032529	0.002	0.008	0.008	rs1156457	27408559	1.12 (G)	0.005	0.0
cg25851176		6	29712185	2.20×10^{-4}	0.010	−0.013	rs1345228	29540369	0.87 (G)	0.001	0.0
cg27042983		6	44171125	0.006	0.002	0.004	rs10948152	44730276	1.11 (A)	0.002	26.5
cg10816283	*GPR116*	6	46979132	0.006	4.83×10^{-4}	−0.009	rs4236095	47476646	1.14 (G)	0.002	22.3
cg05041265	*PREP*	6	105914651	0.002	0.006	0.003	rs9486097	105996518	1.15 (C)	0.001	0.0
cg02913194	*PHACTR2*	6	143970207	0.004	0.001	0.006	rs6920034	143896036	1.14 (A)	0.005	0.0
cg09770410		6	155844810	0.005	0.008	−0.005	rs10499303	155656931	0.88 (A)	0.003	0.0
cg06885468		6	158049089	0.003	1.15×10^{-5}	−0.006	rs9384488	157051073	0.91 (G)	0.003	29.1
cg00564996		6	164310860	0.008	0.005	−0.011	rs6922725	163476188	1.15 (G)	0.001	20.2
cg19053223	*ATXN7L1*	7	105146306	0.002	6.44×10^{-5}	0.001	rs2704961	105755155	0.91 (T)	0.004	0.0
cg27506280		7	155281250	0.002	0.006	0.004	rs12534938	155458309	1.18 (T)	0.003	22.6
cg21597811		8	123756378	0.010	3.45×10^{-4}	−0.084	rs7008474	124236448	1.11 (T)	0.004	0.0
cg05623815		10	114592822	0.002	0.003	−0.011	rs17129837	114273106	1.18 (T)	0.007	0.0
cg21507528	*IGSF22*	11	18703891	0.005	0.004	−0.008	rs6483581	19165711	0.85 (C)	0.002	38.5
cg15668767	*CHRM4*	11	46363595	0.001	0.010	−0.014	rs4073513	46027829	0.92 (G)	0.015	0.0
cg09548275	*NR1H3*	11	47239575	0.004	0.007	−0.002	rs12286778	46278403	1.09 (C)	0.023	46.2
cg09494188	*SCGB1A1*	11	61943105	0.006	0.005	−0.017	rs11231409	62741444	1.08 (C)	0.016	30.2
cg01151584	*EHD1*	11	64384104	0.008	0.004	−0.008	rs555903	65223487	0.88 (A)	0.014	2.7
cg11067407		11	113105062	0.001	0.001	−0.024	rs948177	112709367	0.40 (A)	0.003	0.0
cg25849642		13	111675459	0.006	0.002	−0.023	rs7984371	110756667	0.89 (G)	4.76×10^{-4}	30.4
cg24453123		15	33161440	0.002	0.004	0.020	rs1510383	34045902	0.90 (C)	0.001	5.2
cg27119329	*ADAMTS7*	15	76842489	0.005	1.04×10^{-5}	0.013	rs16971252	77774780	1.22 (C)	0.001	0.0
cg16100530	*SH3GL3*	15	81937868	0.008	2.22×10^{-4}	0.006	rs7182482	82048355	0.93 (A)	0.014	0.0
cg21949830	*SLC43A2*	17	1456791	0.001	0.002	−0.011	rs8065878	1898829	0.89 (T)	0.001	0.0
cg12698834	*LLGL1*	17	18069460	0.008	0.005	−0.012	rs4646408	17351936	0.90 (T)	0.003	20.0
cg06519183	*UBE2O*	17	71898668	0.010	7.28×10^{-5}	−0.001	rs12450432	72204503	1.19 (A)	3.74×10^{-4}	6.9
cg05812008	*SNORD1B; SNORD1A*	17	72068216	5.00×10^{-4}	5.96×10^{-5}	0.038	rs12450432	72204503	1.19 (A)	3.74×10^{-4}	6.9
cg21204860	*SEPT9*	17	72958160	0.004	0.001	0.033	rs12450432	72204503	1.19 (A)	3.74×10^{-4}	6.9
cg03540494	*PIP5K1C*	19	3589778	0.005	0.003	0.006	rs4594	3160483	0.92 (T)	0.008	0.0
cg13727618	*INSR*	19	7096317	0.001	4.56×10^{-4}	−0.002	rs10409209	6096564	1.11 (G)	0.001	0.3

(*continued*)

Table 2. Continued.

		DNA methylation analyses					GWAMA				
CpG site	Gene[a]	Chromosome	Position	FastLMMEWASher *P* value	RefFreeEWAS *P* value	Unadj. mean difference	Best SNP	Position	OR (effect allele)	*P* value	I^2 (%)
cg02668248	*KLF2*	19	16298789	0.003	0.003	0.032	rs12608834	15381536	0.89 (A)	0.002	36.4
cg25361850	*ZNF577*	19	57083601	0.004	0.010	0.003	rs7251993	57043777	0.90 (C)	0.002	0.0
cg04229650	*C20orf12*	20	18341738	0.008	0.003	0.044	rs2618627	17728208	0.90 (C)	0.001	0.0
cg05343548		20	56395760	0.005	0.004	−0.033	rs6026369	56581846	0.91 (T)	0.003	50.8
cg03635685	*CABIN1*	22	22880642	0.004	0.004	−0.002	rs6003870	22422597	1.10 (A)	0.004	0.0
cg26549330		22	44850106	0.004	0.004	0.033	rs5767412	45692014	1.11 (T)	0.003	0.0

The table is sorted by chromosome and position of the CpG site on each chromosome. The mean difference reflects the unadjusted mean methylation difference between AN and LEAN females (derived from a *t*-test; a positive difference indicates hypermethylation in AN females).
[a]For some CpG sites no annotated gene was provided.

for the comparison of females with AN vs. lean controls and 81 consensus CpG sites for the contrast of AN patients vs. population-based controls. None of the consensus CpG sites were detected as differentially methylated in both group comparisons and none of the individual consensus CpG sites could be validated in the AN-discordant MZ twin pairs. However, we observed that 54 (out of 81) consensus CpG sites associated with AN in the AN vs. POP comparison showed the same direction of association with AN in the validation sample.

Interestingly, moving from CpG sites to genes, we confirmed one previously described AN candidate gene locus. Similar to Booij et al. (2015), we detected that multiple CpG sites at the *TNXB* gene were hypermethylated in our AN patients compared to both our control groups. The *TNXB* (Tenascin XB gene) encodes an extracellular matrix glycoprotein with anti-adhesive effects (Bristow et al. 1993). Absence of the protein in humans has been associated with the Ehlers-Danlos syndrome, a connective tissue disorder (Chen et al. 2009). Localization of the gene on chromosome 6 is within the major histocompatibility complex class III region (Weissensteiner & Lanchbury 1997). In a study based on a small sample size, gene expression levels of *TNXB* have been proposed as a potential diagnostic tumour marker to discriminate malignant mesothelioma from metastatic carcinoma in effusions (Yuan et al. 2009). There are hints for association of SNPs in the *TNXB* region with age-related macular degeneration; however, the result was not genome-wide significant (Cipriani et al 2012). It was recently shown that mutations in *TNXB* can cause hereditary primary vesicoureteral reflux, which is the most common congenital kidney and urinary tract anomaly (Gbadegesin et al. 2013). However, an effect on body weight regulation, starvation or AN has not yet been described.

Another interesting finding pertains to a differentially methylated CpG site that was recently described for AN (Booij et al. 2015). The methylation site is located at the *NR1H3* (Nuclear Receptor Subfamily 1, Group H, Member 3 gene) gene locus. Booij et al. (2015) detected two CpG sites at the chromosomal locus; one of which is identical to the CpG site identified here (cg09548275). However, the direction of the effect (hyper- vs. hypomethylation) is opposite to the previous study. *NR1H3* is a key regulator of macrophage function and controls transcriptional programs involved in lipid homeostasis and inflammation (Rébé et al. 2012). For $CD14^+$ cells (cellular marker for macrophages) our estimated cell type distributions (Figure 1) revealed a nominal difference between AN patients and lean controls (nominal $P < 0.01$), but not for AN vs.

Table 3. AN vs. POP: consensus CpG sites ($P \leq 0.01$) and the corresponding best GWAMA-SNPs (minimal *P* value) within a maximum distance of 1 Mb from the CpG site.

DNA methylation analyses							GWAMA				
CpG site	Gene[a]	Chromosome	Position	FastLMMEWAShe *P* value	RefFreeEWAS *P* value	Unadj. mean difference	Best SNP	Position	OR (effect allele)	*P* value	I^2 (%)
cg14270725	*MXRA8*	1	1279669	0.004	0.003	−0.009	rs260512	2172330	0.74 (G)	0.039	NA
cg09518245	*STMN1*	1	26106602	0.006	0.005	−0.017	rs12742115	26952518	1.16 (C)	0.006	4.7
cg18383660	*HCRTR1*	1	31854806	0.008	0.003	0.002	rs1149058	32773677	0.82 (A)	0.001	0.0
cg27300647	*L1TD1*	1	62432869	0.004	0.007	0.011	rs11207877	62199359	1.10 (C)	0.001	42.9
cg12796332	*ITGB3BP*	1	63679521	2.68×10^{-5}	0.002	−0.012	rs11208013	62983488	0.92 (G)	0.007	0.0
cg00893493	*MCOLN3*	1	85285779	0.002	0.005	−0.012	rs2068902	85888256	1.29 (G)	0.001	0.0
cg05598488		1	226221392	0.002	6.57×10^{-3}	−0.008	rs241315	226985825	0.59 (T)	0.002	NA
cg07769732		2	8732916	0.004	0.002	0.115	rs13399561	8667554	1.17 (A)	3.50×10^{-4}	0.0
cg06225840		2	19426930	0.008	0.005	−0.010	rs10186907	19661319	1.16 (T)	0.002	14.1
cg07317846		2	42501848	0.007	0.006	−0.037	rs10495895	42316384	1.17 (G)	0.003	0.0
cg08793877		2	70969502	0.003	0.004	0.046	rs2075221	70897719	0.91 (T)	0.003	14.5
cg11761200	*REG1A*	2	79203478	0.010	0.009	0.056	rs2043453	79943225	1.22 (A)	1.10×10^{-4}	0.0
cg05933789		2	96554135	0.010	4.81×10^{-5}	0.010	rs6576996	96987039	0.88 (T)	0.008	1.5
cg17321385		2	132146263	0.009	0.006	−0.014	rs13011826	131630364	0.91 (A)	0.002	1.2
cg05779559		2	193172214	0.002	0.002	0.011	rs4389279	192436569	0.92 (G)	0.013	0.0
cg15310568	*MAP2*	2	210151556	0.008	0.001	0.064	rs13420044	209179813	1.10 (A)	0.006	0.0
cg02901644	*DGKD*	2	234034526	0.009	0.001	0.054	rs2678484	233036874	0.88 (T)	0.006	0.0
cg03619761	*VHL*	3	10157854	0.001	0.002	0.012	rs350660	10772131	0.90 (A)	0.004	18.9
cg08081407	*ARF4*	3	57558733	0.007	0.006	−0.048	rs3772219	56746291	1.09 (A)	0.008	40.7
cg06640206	*MITF*	3	70068284	0.007	0.002	−0.017	rs9821048	69832777	0.89 (C)	1.24×10^{-4}	0.0
cg08177332		3	99826572	0.007	0.004	0.018	rs16841421	100810524	0.83 (A)	0.014	0.0
cg20074159		3	111729675	1.30×10^{-4}	0.006	−0.001	rs1881949	111038560	1.10 (G)	0.006	28.7
cg27260617	*AGTR1*	3	149929851	0.009	0.002	0.004	rs4681526	150767122	1.10 (C)	0.002	21.2
cg19402939	*CHRD*	3	185587175	6.96×10^{-3}	0.004	−0.041	rs10513793	184706797	0.87 (A)	0.002	0.0
cg21631156		4	148144725	0.007	0.001	0.018	rs4835297	147479926	1.09 (A)	0.019	0.0
cg20905984	*NCRNA00171*	6	30131091	3.89×10^{-4}	0.009	−0.030	rs1345228	29540369	0.87 (G)	0.001	0.0
cg24213189	*COL11A2*	6	33241513	0.003	0.001	0.054	rs12213869	33676665	1.14 (G)	1.92×10^{-4}	0.0
cg19773474		6	40435155	0.005	0.001	0.011	rs7759295	41243828	1.19 (T)	1.20×10^{-4}	35.4
cg26758810		6	148926237	0.004	0.001	0.038	rs17080218	149300228	1.55 (T)	0.004	NA
cg00467592		6	168359240	0.007	0.010	0.015	rs968333	167446188	0.90 (T)	0.001	0.0
cg02540477	*SMOC2*	6	168584884	0.007	0.009	−0.009	rs13219463	169220811	1.11 (C)	0.001	25.1
cg00562553	*HOXA4*	7	27136265	0.009	0.001	−0.081	rs916969	26480253	1.10 (A)	0.003	0.0
cg21934189		7	67890744	0.001	0.005	−0.009	rs11762852	67969225	1.22 (A)	0.001	5.0
cg12240237	*GTF2IRD1*	7	73584338	3.83×10^{-4}	0.005	0.015	rs17145361	73335263	1.13 (A)	0.002	0.0
cg13799941	*DLX6AS*	7	96470230	0.006	0.002	−0.007	rs2237590	95483258	1.09 (C)	0.007	16.6
cg20050761	*MEST; MESTIT1*	7	129919159	0.005	0.004	0.014	rs10265693	130371345	1.22 (G)	5.36×10^{-5}	0.0
cg00709423	*AGAP3*	7	150447767	0.004	0.005	0.053	rs4726070	150959151	0.92 (G)	0.012	25.9
cg25092989	*XKR6*	8	10969904	7.10×10^{-5}	0.007	0.002	rs10503406	10146382	1.11 (T)	0.001	0.0
cg00923880		8	19657458	0.007	0.007	0.008	rs923768	19575243	1.16 (C)	1.31×10^{-6}	0.0
cg23493018		8	37428981	4.24×10^{-3}	0.005	−0.034	rs6468413	37493810	1.14 (C)	0.001	0.0
cg04228202	*AP3M2*	8	42129496	0.006	0.000	−0.064	rs10504041	41584028	1.10 (A)	0.009	0.0
cg06045746	*RRM2B*	8	103316532	0.004	0.008	−0.033	rs4276657	103653827	0.92 (A)	0.005	0.0
cg04600798	*SLC39A4*	8	145611130	0.003	0.006	0.036	rs10100154	144863114	1.11 (A)	0.003	0.0
cg13576586	*TNFSF15*	9	116592306	0.006	0.003	−0.013	rs2989509	117041491	1.11 (T)	0.002	0.0
cg14351882	*GRIN1*	9	139181699	0.009	0.004	0.040	rs10870158	139005641	1.12 (C)	0.001	41.4
cg01066431	*LOC642826*	10	47805203	4.54×10^{-3}	0.008	−0.003	rs11593867	48063099	1.11 (A)	0.002	0.0
cg03463778	*LOC642826*	10	47805206	0.003	0.006	−0.024	rs11593867	48063099	1.11 (A)	0.002	0.0
cg07103124	*MAPK8*	10	49313092	0.002	0.005	−0.016	rs6537567	49419924	1.09 (A)	0.003	0.0

(*continued*)

Table 3. Continued.

				DNA methylation analyses			GWAMA				
CpG site	Gene[a]	Chromosome	Position	FastLMMEWAShe *P* value	RefFreeEWAS *P* value	Unadj. mean difference	Best SNP	Position	OR (effect allele)	*P* value	I^2 (%)
cg10415685	*ADRB1*	10	115795666	6.78×10^{-3}	0.010	−0.024	rs11196802	116357719	0.87 (T)	0.001	0.0
cg06155833	*MTG1*	10	135082926	0.009	0.004	0.027	rs11146587	134574228	2.12 (A)	0.005	NA
cg17566541	*LSP1*	11	1868863	2.71×10^{-4}	0.005	−0.030	rs2012618	2487832	1.10 (C)	0.004	0.0
cg07572949	*NLRP10*	11	7942139	0.001	4.44×10^{-4}	−0.006	rs11041822	8225663	1.28 (A)	0.001	16.6
cg15094908	*GALNTL4*	11	11499674	0.007	0.008	−0.039	rs10831605	11346019	1.14 (A)	4.49×10^{-4}	44.1
cg13963807	*PPP1CA; RAD9A*	11	66922283	0.003	0.004	0.024	rs7924398	67849769	1.15 (T)	0.017	0.0
cg15110243	*ODZ4*	11	78179392	2.48×10^{-4}	0.008	−0.018	rs2252969	79110524	0.89 (G)	0.001	23.7
cg18902148		11	134187256	0.010	0.003	−0.002	rs901035	133289709	1.11 (A)	0.005	0.0
cg15244786	*HOXC4*	12	52732546	0.008	0.004	−0.039	rs746423	52703526	1.13 (T)	0.003	0.0
cg14434755		12	113591923	0.003	0.006	−0.003	rs11066757	112724544	1.09 (C)	0.005	42.0
cg24622007	*GALNT9*	12	131253329	0.006	0.010	0.102	rs6486676	130469761	1.13 (A)	0.001	0.0
cg02659803		13	22307813	0.009	0.004	−0.022	rs7986997	22449919	1.13 (A)	0.001	0.0
cg21780859	*MTUS2*	13	28900907	0.001	0.008	−0.054	rs280942	29092288	1.10 (C)	0.003	0.0
cg13826718		13	36919368	0.002	0.005	0.006	rs4943552	37402142	1.12 (C)	3.12×10^{-4}	88.5
cg08377570	*LPAR6; RB1*	13	47917223	0.004	0.008	0.033	rs4942705	47317020	1.15 (G)	0.006	0.0
cg27317694	*RCBTB2*	13	48003051	1.02×10^{-5}	0.008	−0.069	rs7996852	48952347	0.91 (G)	0.005	0.0
cg27303211		13	108795300	0.005	0.008	0.009	rs9284251	108969732	1.10 (A)	0.005	0.0
cg09507215	*ATP11A*	13	112396392	0.002	0.001	0.062	rs2289152	111802399	1.10 (A)	0.002	47.3
cg11114503	*LTBP2*	14	74061771	0.003	0.002	0.007	rs11159135	74851854	1.10 (G)	0.003	0.0
cg13543254	*FMN1*	15	30918934	0.001	0.003	0.019	rs9672198	30301189	1.17 (T)	0.001	0.0
cg13161852		15	75978234	0.003	0.002	0.015	rs6495247	75856486	0.91 (T)	0.003	29.2
cg15029935	*CRTC3*	15	88927016	0.009	0.001	0.001	rs3803563	89332356	0.88 (C)	0.002	0.0
cg02528159	*PAQR4*	16	2961100	0.004	0.009	0.007	rs6500757	2946841	1.10 (A)	0.002	7.4
cg05650674	*CDH3*	16	67235498	0.009	0.007	0.002	rs4783689	67411172	0.91 (T)	0.004	0.0
cg07862889	*C17orf103*	17	21097508	0.007	0.003	−0.042	rs4381653	20805605	1.07 (A)	0.035	44.1
cg05652455	*MLLT6*	17	34135630	0.008	0.004	0.034	rs3744352	34515585	1.19 (T)	4.59×10^{-4}	0.0
cg21134182	*ZNF516*	18	72221903	0.002	0.003	−0.036	rs10514207	71984919	0.89 (G)	0.003	0.0
cg16774013	*NFATC1*	18	75261138	1.56×10^{-6}	0.008	0.131	rs507219	75450063	0.91 (G)	0.004	0.0
cg23188331	*ADAMTS10*	19	8567264	0.004	0.010	0.070	rs4442926	9311185	0.91 (A)	0.001	0.0
cg02493939	*LOC80054; CEBPA*	19	38486586	0.005	0.005	−0.008	rs494387	39256568	0.88 (C)	0.001	0.0
cg09310092	*SCN1B*	19	40221041	0.001	0.001	0.146	rs494387	39256568	0.88 (C)	0.001	0.0
cg07676859	*SSTR4*	20	22963932	0.005	0.003	−0.132	rs6047917	22052421	0.91 (G)	0.004	0.0
cg05291773		21	36422512	0.007	0.005	−0.115	rs11702555	36587841	0.87 (A)	0.001	11.5

The table is sorted by chromosome and position of the CpG site on each chromosome. The mean difference reflects the unadjusted mean methylation difference between AN and POP females (derived from a *t*-test; a positive difference indicates hypermethylation in AN females). NA, not available.

[a]For some CpG sites no annotated gene was provided.

population-based controls. However, as we corrected for cell type distribution, the association signal is likely not due to cell type distribution effects. The *NR1H3* protein is highly expressed in visceral organs (liver, kidney and intestine) (Zhao & Dahlman-Wright 2010; Basse et al. 2015). Studies in knock-out mice suggest an important role in the regulation of cholesterol homeostasis (Rippmann et al. 2009). Hence, this gene might well be relevant for starvation. Further epigenetic and functional analyses are warranted.

The comparison of the methylation patterns to the GCAN/WTCCC3 GWAMA (Boraska et al. 2014) yielded one SNP within 1 MB of a CpG site (rs923768) with suggestive evidence for an association ($P = 1.31 \times 10^{-6}$). The SNP is located in an intron of the *CSGALNACT1* gene. In our methylation analysis, differentially methylated CpG sites were detected for the comparison AN vs. POP. Studies in knock-out mice revealed a role of the gene product in cartilage formation. Thus, *CSGALNACT1* is relevant for: (1) normal cartilage development (Sakai et al. 2007; Watanabe et al. 2010), (2) normal endochondral ossification, an essential process during foetal development by which bone tissue is formed, and (3) aggrecan metabolism (Sato et al. 2011). As reduced bone strength is reported in former AN patients (e.g., Mueller et al. 2015), our result might implicate an epigenetic process induced by the semi-starvation that leads to a reduced bone mass.

Two studies in humans also depicted interesting findings with respect to *CSGALNACT1*: (1) a mutation screen in human *CSGALNACT1* in 114 patients with neuropathies (Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy, hereditary motor and sensory neuropathy and unknown aetiology) and 196 controls with other neurological diseases identified two novel non-synonymous mutations, not leading to *N*-acetylgalactosamineT-II activities in two patients with neuropathies (His234Arg; Met509Arg) (Saigoh et al. 2011). (2) Furthermore, a suggestive effect was reported for rs7816924 ($P = 2.11 \times 10^{-7}$), an intronic variant of *CSGALNACT1*, in a GWAS of major depressive disorder (Hunter et al. 2013). This finding is interesting as depression is a frequent comorbidity in AN (Abbate-Daga et al. 2015).

Strengths of our study include: (1) We performed a high-throughput methylation association analysis and did not focus on single candidate genes. (2) We combined our high-throughput methylation analysis with the results of a systematic literature search and the results of a AN GWAMA on genomic variation at our consensus CpG sites. (3) We assessed the largest GWAMA for AN susceptibility assuming that either marker genotypes might influence epigenetic factors (Schalkwyk et al. 2010; Yuen & Robinson 2011) or that DNA methylation might modify genetic effects resulting in heterogeneity when analysing the genetic effects in different populations (Abdolmaleky et al. 2004). (4) We followed up-to-date guidance to preprocess the raw data and accounted for cell type composition effects using two recently proposed reference-free methods. (5) The ignorance of the well-known cell type composition effects (Fukudo et al. 1993; Saito et al. 1999; Castro et al. 2004; Sabel et al. 2013; Bühren et al. 2014) resulting from semi-starvation is not an option as presented by our analysis using CpG sites to map cell type differences between groups. In contrast, our analyses underline the importance of an additional modelling level of the cell type differences (presumably of general importance for high-throughput DNA methylation analyses).

However, limitations should also be noted: (1) We did not address particular sub-phenotypes or more quantitative endophenotype comparisons, as we had insufficient statistical power for these analyses. (2) Unfortunately, there was only a small overlap of consensus CpG sites detected by both analysis methods, which nevertheless clearly exceeded the overlap expected by chance. This missing overlap might either be due to our datasets (e.g., residual confounding, sample size) or due to differences between the methods (e.g., considered CpG sites, cell type effect modelling) as underlined by the inflation of small *P* values for RefFreeEWAS. (3) We used a fairly liberal nominal significance level of 0.01 to screen for potentially differentially methylated CpG sites. Acknowledging that more stringent cut-offs would also lead to more false-negative findings, we focused on consensus CpG sites detected by both reference-free methods, to reduce false-positive findings. (4) The POP were considerably older than both the AN patients and the lean females. As a consequence, some of the potentially differentially methylated CpG sites between AN and POP might be confounded by age. We cannot refute this argument even though investigation results indicated that the majority of DNA methylation marks seem to be relatively stable after birth (Bocklandt et al. 2011; Gordon et al. 2012; Johansson et al. 2013) and even though both our sensitivity analyses and cross-checks of the literature did not reveal any major age-related CpG sites among the consensus sites. (5) Similarly, we cannot refute that other effects such as BMI effects, or effects of hormone levels (Lomniczi et al. 2015; Osborne et al. 2016) or smoking might have in part confounded findings. Again we refer to the literature and to our sensitivity analyses to attenuate such

arguments. (6) Clearly and probably the most limiting factors are our relatively small sample sizes for the high-throughput DNA methylation analyses and the choice of whole blood for the analyses. Furthermore, the sample size of the validation sample was again very small and very likely underpowered to make any firm conclusions. However, epigenetic studies on disease-discordant MZ twin pairs (completely matched for genetics, age, sex, cohort effects, maternal influences and common environment) are more powerful in detecting disease-associated epigenetic differences than unrelated cases and controls with different life-histories.

In sum, we confirmed hypermethylated CpG sites for AN near a gene with potential impact for AN, namely *TNXB*. Further studies should focus on this specific genetic region. In addition, we identified some evidence for differently methylated CpG sites with potential relevance to starvation.

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