Obesity Facts

Research Article

Obes Facts
DOI: 10.1159/000547603

Received: April 8, 2025
Accepted: July 17, 2025
Published online: July 25, 2025

HOOK1 Gene Expression and DNA Methylation in Obesity and Related Cardiometabolic Traits

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Keywords
Obesity · Adipose tissue · Epigenetics · Gene expression · DNA methylation

Abstract
Introduction: Accumulation of fat in omental visceral adipose tissue (OVAT) is strongly linked to metabolic diseases. Our recent findings show a distinct and more accessible chromatin landscape of the visceral depot compared to its subcutaneous counterpart. Based on integrated analysis of chromatin accessibility and transcriptomics, we identified previously unrecognised genes linked with obesity. Here, we performed in-depth analyses of one of the candidates, *HOOK1*, and tested for depot-specific gene expression, correlation with clinical traits and regulatory mechanisms including DNA methylation. ***Methods:*** We utilised intra-individually paired adipose tissue samples of human OVAT and subcutaneous adipose tissue (SAT) from our in-house cohort (N = 78). Gene expression was measured using real-time quantitative PCR and pyrosequencing was used to determine DNA methylation levels. Data were analysed for differential gene expression and DNA methylation differences between SAT and OVAT, along with correlation analyses with clinical variables related to obesity. Results were validated in adipose tissue samples from 1,618 donors of the Leipzig Obesity Biobank. ***Results:*** We observed consistently higher *HOOK1* gene expression in OVAT compared to SAT and successfully confirmed this effect direction in several validation cohorts. We further

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identified that *HOOK1* gene expression correlated with body mass index and hip circumference. We discovered a relationship between DNA methylation of the *HOOK1* promoter with clinical variables important for liver function. ***Conclusion:*** Our data show that *HOOK1* gene expression is adipose tissue depot-specific. We observed that gene expression and DNA methylation are correlated to clinical variables of obesity, suggesting that *HOOK1* may play a role in obesity and its sequelae.

Introduction

Overweight and obesity rates are on a steady rise worldwide and are expected to affect 46% of all adults in 2025 (https://data.worldobesity.org/publications/?cat=22) threatening global health as obesity is linked to serious co-morbidities such as metabolic diseases [1]. Increased fat mass, especially in omental visceral adipose tissue (OVAT), is well known to more strongly correlate with metabolic disorders as compared to subcutaneous adipose tissue (SAT) [2, 3]. Strong evidence was shown for adipose tissue depot-specific gene expression and epigenetic markers such as DNA methylation in OVAT vs. SAT being differentially correlated with body mass index (BMI) and clinical variables related to obesity [4–7]. Recently, we were the first reporting (Saeed et al. [8]) adipose tissue depot-specific differential chromatin landscapes in intra-individually paired samples of human OVAT vs. SAT identifying a much more accessible visceral adipose tissue compartment than its subcutaneous counterpart. These data illustrated depot-specific regulatory chromatin patterns highlighting potential adipose tissue depot-specific epigenetic mechanisms. However, the implications of these differences on a molecular level are not fully understood. Here, we set out to perform in-depth analyses in a specific target gene identified from the integrated analysis of ATAC-seq and RNA-seq data described in Saeed et al. [8]. As OVAT is more strongly linked to metabolic complications linked to obesity [2, 3], here we focused on gene targets from OVAT compartment and with a higher chromatin accessibility and gene expression. Moreover, to identify novel targets we focused on candidate genes not being previously reported in the context of the pathophysiology of obesity. Finally, to identify targets potentially regulated by DNA methylation, we overlapped our data with genes observed in Keller et al. [9] reported to be differentially methylated between responders and non-responders in physical and dietary treatment of obesity. By applying these selection criteria, we selected *hook microtubule tethering protein 1* (*HOOK1*) as a possible obesity candidate gene, presenting an open chromatin state in its promoter region in OVAT while being less accessible in SAT along with higher gene expression in OVAT (Fig. 1).

HOOK1 is part of the hook family of coiled-coil proteins which through their N-terminal domain can indirectly bind to the cells microtubules [10]. *HOOK1* is further involved with clathrin-independent endocytosis [11] and its cargo regulation through an interaction with γ-taxilin as demonstrated in HeLa cells [12]. Moreover, *HOOK1* has previously been linked to Alzheimer disease [13] and multiple types of cancers, including ovarian cancer [14], renal cell carcinoma [15] or oesophageal squamous cell carcinoma [16], where it has shown conflicting results with both preventative and pathogenic involvement [12, 15, 16]. In humans, it is ubiquitously expressed with highest expression detected in testis while being important for spermatid differentiation (https://www.gtexportal.org/home/). *HOOK1* has not been previously linked to human obesity or related clinical variables. Of note, a recent study described *HOOK1* genotypes to be implicated in body weight in sheep with possible implications for sheep breeding supporting our selection of *HOOK1* as a possible novel target gene in human obesity [17]. No genome-wide association studies targeting human obesity revealed any genetic variants in or close to *HOOK1*. There is a need to extend the current understanding of the molecular regulatory differences between the two major adipose tissue depots and their link to metabolic disease. In this study, we set out (i) to confirm higher *HOOK1* expression in OVAT; (ii) to test whether *HOOK1* gene expression correlates with clinical variables of obesity; and (iii) to test the hypothesis whether DNA methylation regulates *HOOK1* gene expression and is correlated with clinical variables.

Materials and Methods

Study Design of the Discovery Cohort

We have used intra-individually paired samples of OVAT and SAT from our in-house adipose tissue biobank ($N = 78$ [23 male, 55 female]; age 43 ± 10 years; BMI 44.8 ± 6.4 kg/m^2). All samples were collected from individuals during laparoscopic bariatric surgery and were immediately snap frozen on dry ice and stored at −80°C. For all individuals, a wide range of anthropometric traits and metabolic variables are available. Characteristics of the study population are presented in Table 1. Moreover,

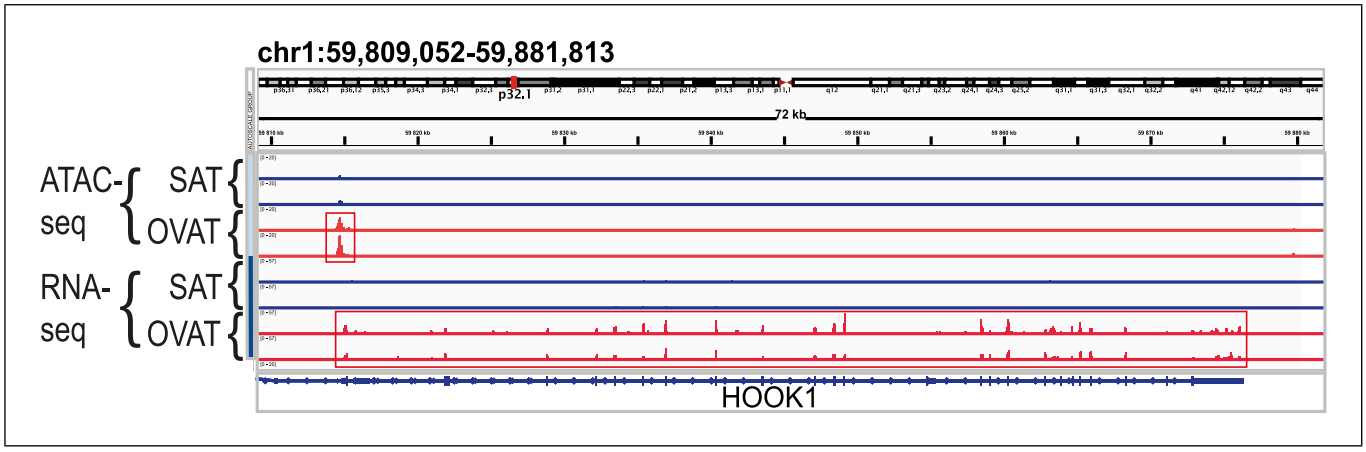


Fig. 1. Selection of *HOOK1* as a possible novel target gene for obesity. Illustration of ATAC-seq and RNA-seq results from in-house cohort. Blue lines represent SAT samples while red lines represent OVAT samples from patients. The promoter region of the *HOOK1* gene was targeted for the ATAC-seq and shows an open accessible region for OVAT with a high peak highlighted with a red box, compared to a small peak for SAT suggesting a closed or occupied chromatin state. This overlaps with the RNA-seq results which show a higher level of mRNA present in OVAT as highlighted in the large red box with peaks overlapping with the *HOOK1* exons visualised below. For SAT there are no peaks and, therefore, very low expression of the gene.

we extracted available data regarding *HOOK1* from bulk RNA-seq ($N = 5$) and ATAC-seq ($N = 8$) [8]. The study has been approved by the Regional Committee for Medical and Health Research Ethics, Southeast Norway (2013/2042; 2017/1528; 489516).

Study Design of the Validation Cohorts

The Leipzig Obesity Biobank (LOBB https://www.helmholtz-munich.de/en/hi-mag/cohort/leipzig-obesity-bio-bank-lobb) was used as a validation cohort comprising paired samples of abdominal SAT and OVAT as well as an extensive range of anthropometric traits and metabolic variables. Adipose tissue samples were collected during elective laparoscopic abdominal surgeries [18], while the anthropometric traits and metabolic variables were collected as described by Blüher [19] and Klöting et al. [20]. Exclusion criteria included participants under 18 years of age, those with chronic substance or alcohol misuse, smokers within the 12 months prior to surgery, individuals with acute inflammatory diseases, those taking glitazones as concomitant medication, patients with end-stage malignant diseases, individuals who experienced weight loss exceeding 3% in the 3 months leading up to surgery, and those with uncontrolled thyroid disorders or Cushing's disease. The cross-sectional cohort (CSC) includes 1,480 individuals (433 male, 1,047 female), 31 of the individuals were normal weight (age 56 ± 13 years; BMI 25.5 ± 2.6 kg/m^2) while the remaining 1,449 had obesity (age 46 ± 11 years; BMI 49.2 ± 8.3 kg/m^2). A LOBB 2-step bariatric surgery cohort (BSC) includes 65 individuals (22 male/43 female) with severe obesity and that have sampled SAT and OVAT during bariatric surgery for weight loss at two separate time points. The first step surgery was typically a sleeve gastrectomy called pre-surgery (age 45.3 ± 9.8 years; BMI 54.7 ± 9.3 kg/m^2), while the second step surgery was usually a gastric bypass, which is called post-surgery (age 47.3 ± 9.9 years; BMI 40.9 ± 7.3 kg/m^2). In addition, a LOBB metabolically healthy obesity versus unhealthy obesity sub-cohort includes 73 individuals (21 male/52 female), 42 insulin-resistant patients (age 47 ± 7.8 years; BMI 47.3 ± 8.2 kg/m^2), and 31 insulin-sensitive patients (age 38.8 ± 11.2 years; BMI 45.9 ± 6.9 kg/m^2). All patient characteristics of the LOBB cohorts are presented in online supplementary Table 1 (for all online suppl. material, see https://doi.org/10.1159/000547603). The study was approved by the Ethics Committee of the University of Leipzig (approval No. 159-12-21052012) and performed in accordance with the Declaration of Helsinki. All participants gave written informed consent before taking part in the study.

Gene Expression by Using Real-Time Quantitative PCR

For the in-house cohort, total RNA was extracted from adipose tissue using RNeasy Plus Mini Kit (Qiagen) with modifications as described in Saeed et al. [8]. Modifications to the protocol included lysis and homogenisation in QIAzol and removal of excess lipids before phase separation with chloroform. cDNA was

Table 1. Clinical characteristics of individuals from the in-house cohort

Variables	Total	Mean	SD
N	78		
Sex (m/f)	23/55		
Age, years	78	43	11
BMI, kg/m^2	78	44.87	6.47
Waist, cm	77	125.13	14.42
Hip, cm	77	132.86	11.95
Waist-to-hip ratio	77	0.98	0.36
AST, U/L	78	27.1	12.48
ALT, U/L	78	32.03	19.28
ALP, U/L	78	80.72	22.14
GGT, U/L	78	46.14	60.6
HbA1c, %	78	39.92	10.58
HDL cholesterol, mmol/L	77	1.36	1.28
LDL cholesterol, mmol/L	78	2.95	0.86
Triglycerides, mmol/L	77	1.77	0.69
Diabetes[a] (yes/no)	12/66		

Data are presented as mean ± SD (standard deviation). *N*, number of participants; m, male; f, female; BMI, body mass index; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; HbA1c, haemoglobin A1C; HDL, high-density lipoprotein; LDL, low-density lipoprotein. [a]Diabetes mellitus defined as either type I or type II.

generated from 500 ng total RNA with High-capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific) and utilised to perform qPCR in duplicates. The QuantStudio 7 Flex system (Thermo Fisher Scientific) was used with target specific assays for TaqMan Real-Time PCR Assays (Thermo Fisher Scientific). mRNA levels for gene expression were calculated by relative quantification with *PGK1* as a housekeeping gene ($2^{-[\mathrm{CT\ gene\ of\ interest}\ -\ \mathrm{CT}\ PGK1]}$). The commercially available assays (online suppl. Table 2) purchased, were designed to cross exon-intron borders.

DNA Methylation by Using Pyrosequencing in the In-House Cohort

DNA was extracted from intra-individually paired adipose tissues SAT and OVAT by utilising the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) with slight modifications of the protocol. Approximately 150 mg of tissue was used per sample, and all volumes in the protocol were adjusted accordingly for the increased starting material. The final elution step was performed with 100 µL Elution Solution. The EpiTect Bisulfite Kit (Qiagen) was used for bisulfite conversion of 500 ng of DNA and the EpiTect Whole Bisulfitome Kit (Qiagen) was used to amplify converted DNA. Pyrosequencing was performed on a PyroMark Q48 Autoprep (Qiagen). DNA methylation levels of 12 individual CpG site in the *HOOK1* promoter region were determined using two pyrosequencing assays (Hs_HOOK1_01_PM and Hs_HOOK1_2_PM, online suppl. Table 3; online suppl. Fig. 1). Each sample was analysed in duplicates, and the average methylation level of the replicates was used for further analysis. Positive (100% methylated) and negative controls (no template) were included in each run.

RNA-Seq Analysis of the Validation Cohorts

The library preparation and RNA sequencing (RNA-seq) data processing were performed as previously outlined [21]. In summary, RNA was extracted from adipose tissue samples using the SMARTseq protocol [22]. Single-end sequencing of the libraries was conducted on a NovaSeq 6000 instrument at the Functional Genomics Centre in Zurich. Adaptor and quality trimmed reads were aligned to the human reference genome (assembly GRCh38.p13, GENCODE release 32 [23]) and gene-level expression quantification was performed using Kallisto (v0.48) [24]. For samples with read counts exceeding 20 million, down sampling to 20 million was conducted using the R package ezRun (v3.14.1; https://github.com/uzh/ezRun, accessed on 23 March 2022). The data were normalised using a weighted trimmed mean of the log expression ratios and adjusted for age, transcript integrity number, and sex.

Statistical Analyses

SPSS statistics software 26 and GraphPad Prism 8 and R v4.2.3 were used for all statistical analyses. Within the in-house cohort, all variables underwent a Kolmogorov-Smirnov test and visual representation of histogram test for normal distribution. Non-normally distributed variables were logarithmically transformed to approximate normal distribution. A paired Student's *t* test was used to test for depot-specific gene expression of *HOOK1* in the adipose tissues and differential DNA methylation levels between the two adipose tissue depots. Bivariate Spearman's correlation was used to assess the

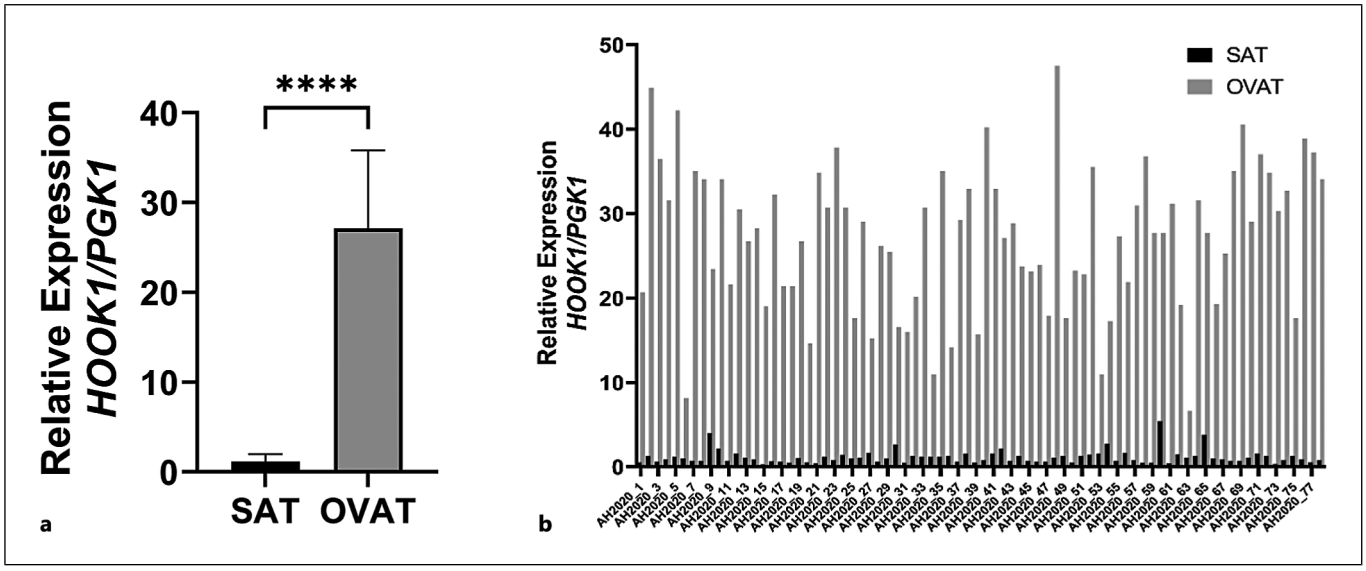


Fig. 2. Differential gene expression of *HOOK1* in intra-individual paired samples from SAT and OVAT in patients with obesity. **a** Average *HOOK1* expression in SAT and OVAT. **b** Individual *HOOK1* expression in SAT and OVAT. *p* value was calculated using two-sided paired *t* test (****$p = 6.03 \times 10^{-40}$) ($N = 78$). OVAT, omental visceral adipose tissue; SAT, subcutaneous adipose tissue.

relationships between gene expression with clinical variables and DNA methylation levels with gene expression and clinical variables. Linear regression analyses were employed to analyse for relationship between gene expression, DNA methylation and clinical variables while adjusting for age, gender, and BMI, where appropriate. Data are presented as mean ± standard deviation. To correct the statistical significance threshold for multiple testing in the discovery cohort, Bonferroni correction was applied (0.05/4 [highly correlated traits were summarised to three × 2 tissue depots = number of tests = 6]). We, therefore, lowered the significance threshold to $p = 0.0083$. All p values >0.0083 but <0.05 were considered to be of nominal statistical significance. All p values presented are uncorrected for multiple testing. For the validation cohorts, group comparisons were conducted using a non-parametric statistical approach. This involved performing a one-way Kruskal-Wallis ANOVA, followed by pairwise Mann-Whitney U tests, with corrections for multiple comparisons applied using the Hommel method. Univariate Spearman correlations were performed to assess the relationship between *HOOK1* gene expression and clinical phenotypes, with adjustments for multiple comparisons made through the application of the false discovery rate correction.

Results

HOOK1 *Is Differentially Expressed between SAT and OVAT*

First, we tested whether *HOOK1* gene expression is significantly higher in the visceral adipose tissue depot compared to SAT, in a larger sample set ($N = 78$) than reported in Saeed et al. [8]. The in-house cohort comprised intra-individually paired (OVAT and SAT) adipose tissue biopsies from patients with obesity and was used to determine *HOOK1* gene expression as visualised in Figure 2. We observed a consistently higher gene expression in OVAT compared to SAT in this larger sample set, confirming our previous RNA-seq data and being in line with higher chromatin accessibility in OVAT. Our results show a significant difference in gene expression between the two adipose tissue depots ($p = 6.03 \times 10^{-40}$) (Fig. 2a), an effect which was observed on the individual level for all included patients (Fig. 2b).

HOOK1 *Gene Expression Correlates with Clinical Variables Related to Obesity*

Considering its depot-specific gene expression and that *HOOK1* has not previously been linked to obesity in humans or its clinical variables, we next tested the hypothesis, whether *HOOK1* gene expression correlates with clinical traits of obesity (Table 1). A statistical

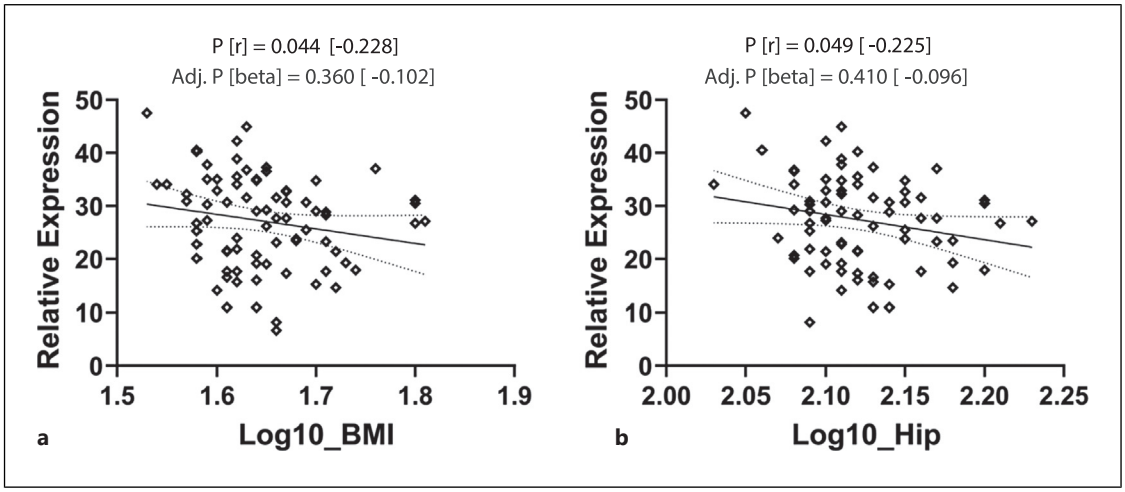


Fig. 3. *HOOK1* gene expression in OVAT negatively correlates with anthropometric traits. **a** BMI. **b** Hip circumference. *p* values were calculated by using bivariate Spearman correlation. Linear regression analysis was used to adjust for age and sex. BMI, body mass index; OVAT, omental visceral adipose tissue; P, *p* value; *r*, correlation coefficient from bivariate correlation; adj. P, *p* value from linear regression analysis adjusted for age and gender; beta, regression coefficient from linear regression.

analysis was conducted utilising Spearman's rank correlation coefficient, between *HOOK1* gene expression in SAT and OVAT and important clinical variables. We observed that *HOOK1* gene expression levels in OVAT negatively correlate with BMI ($p = 0.044$) and hip circumference ($p = 0.049$) (Fig. 3). Of note, these correlations do not withstand adjustment for age and gender in linear regression analyses. In SAT, none of the available clinical variables correlates with *HOOK1* gene expression levels (online suppl. Table 4a).

HOOK1 *DNA Methylation Correlates with Clinical Variables of Obesity*

Our data so far have shown that (i) *HOOK1* gene expression is adipose tissue depot-specific with a higher expression level in OVAT and (ii) that in unadjusted analyses *HOOK1* gene expression is correlated with the two anthropometric traits BMI and hip circumference, both important variables in obesity. Next, we tested our hypothesis that *HOOK1* gene expression might be regulated by DNA methylation, hence by an epigenetic mechanism possibly impacting on gene regulation. Therefore, in order to analyse DNA methylation, two locations in the promoter region of *HOOK1* were selected (Hs_HOOK1_01_PM and Hs_HOOK1_2_PM) and together included a total of 12 CpG sites (online suppl. Table 3). When employing two-sided paired *t* tests, we did not observe any significant depot-specific difference in DNA methylation between OVAT and SAT on an average level (Fig. 4a) or at the individual CpG level (Fig. 4b). To evaluate whether DNA methylation may regulate *HOOK1* gene expression differentially in the two adipose tissue depots, we tested whether the average DNA methylation levels at both assays in the promoter region correlate with *HOOK1* gene expression. Spearman's rank correlation analysis between the *HOOK1* DNA methylation levels and gene expression in OVAT did not show any significant correlation for both pyrosequencing assays (Hs_HOOK1_01_PM $p = 0.054$, Hs_HOOK1_02_PM $p = 0.717$) (Fig. 4c). Furthermore, the *HOOK1* DNA methylation and gene expression in SAT did not generate any significant correlation.

Next, we investigated the correlation between DNA methylation and clinical and anthropometric traits linked to obesity. DNA methylation of *HOOK1* in SAT was nominal significantly correlated with the anthropometric trait BMI ($p = 0.032$) and hip circumference ($p = 0.024$) using Spearman's rank correlation coefficient (Fig. 5a). Both bivariate correlations withstand adjustment for age and gender in general linear regression analyses. We further observed the liver function markers aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT) were to be correlated with DNA methylation of *HOOK1* in both SAT and OVAT (Fig. 5b). *HOOK1* DNA methylation in SAT at two CpG sites correlated with the liver marker AST ($p = 0.008$, $p = 0.048$), five CpG sites with ALT ($p = 0.008$, $p = 0.034$,

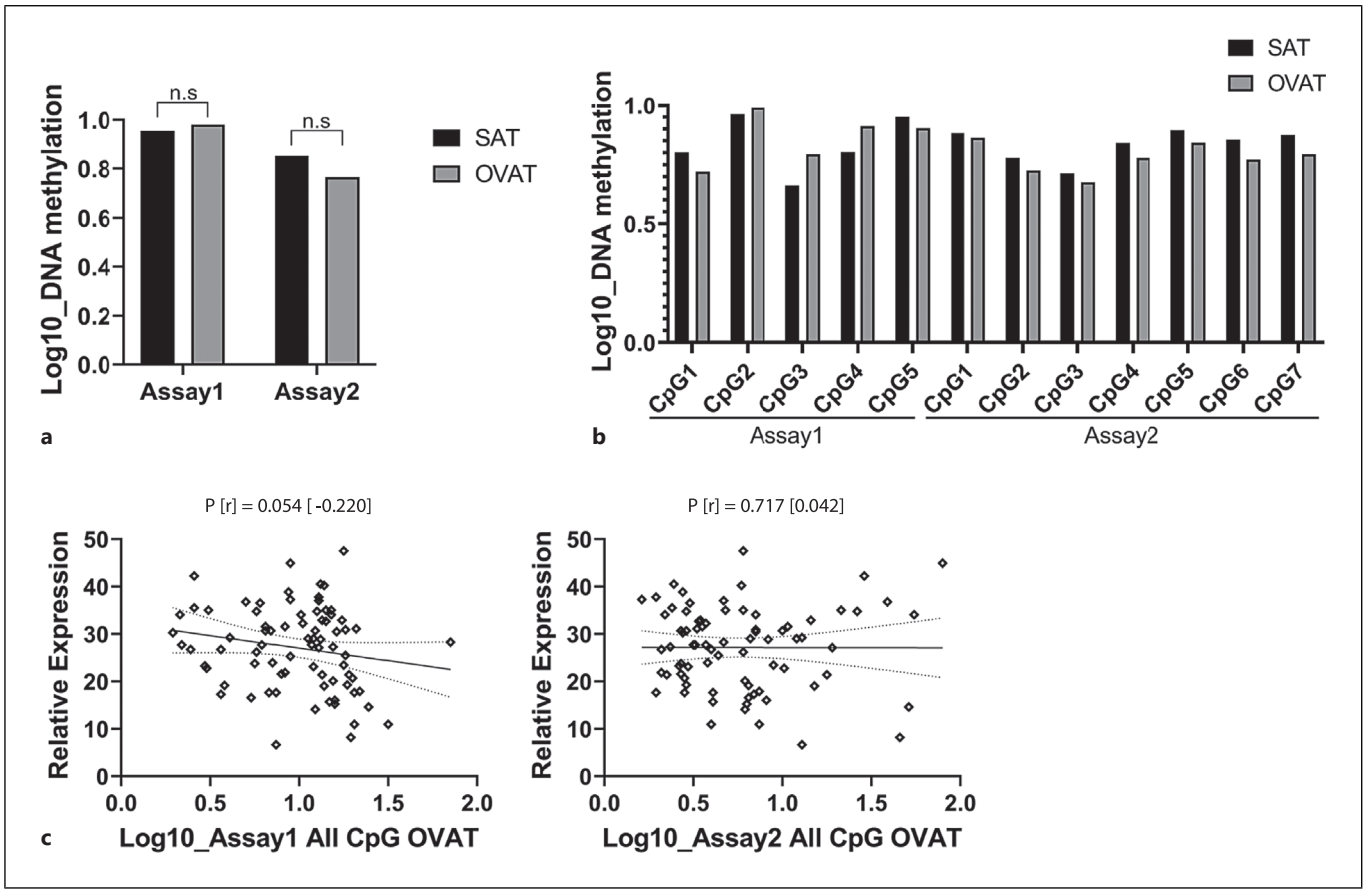
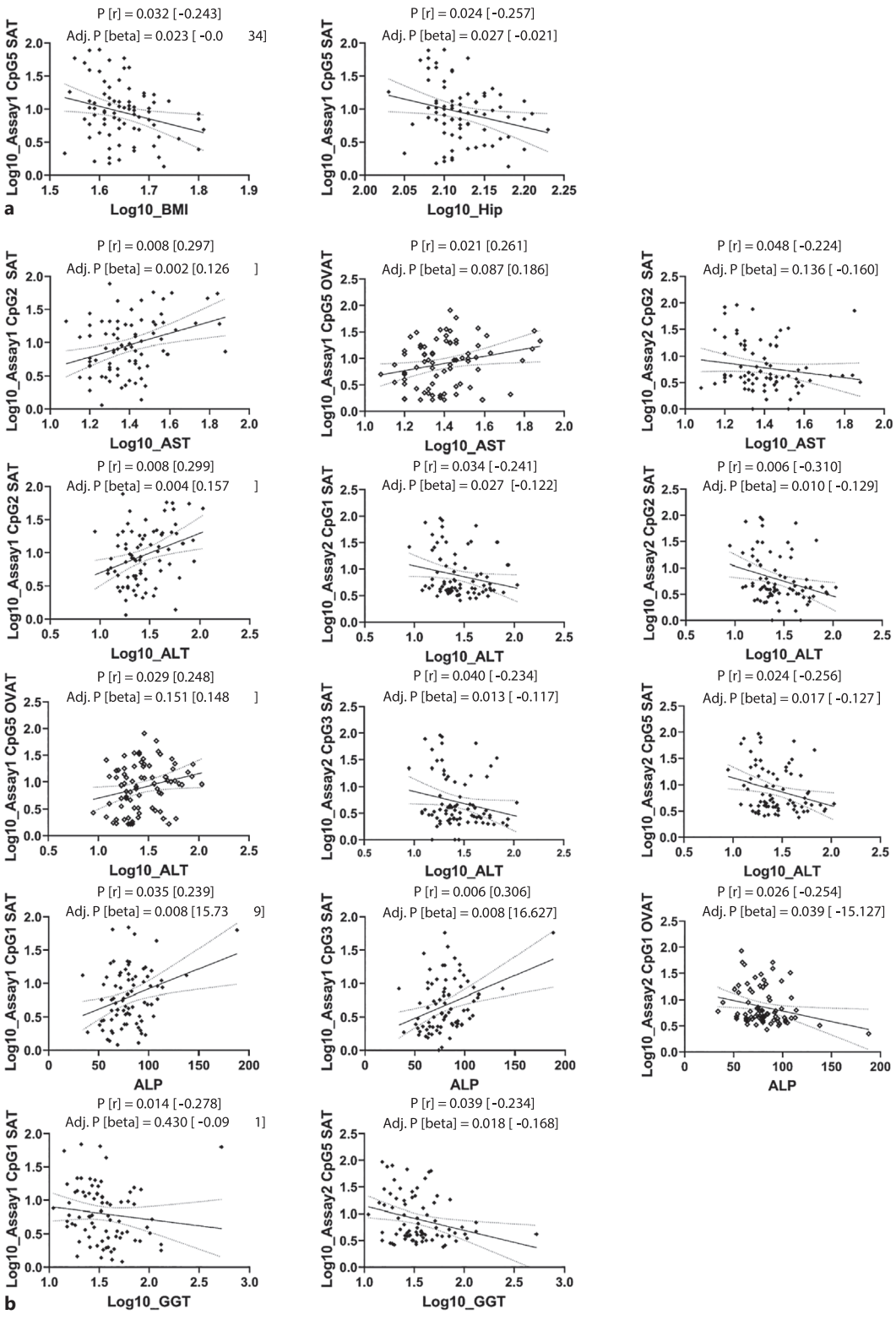


Fig. 4. DNA methylation at promoter of *HOOK1* is not the regulatory mechanism for *HOOK1* gene expression in adipose tissue. **a** Average DNA methylation of both assays in SAT vs. OVAT in the in-house cohort. **b** Each individual CpG site at promoter region of *HOOK1* and their DNA methylation level between SAT vs. OVAT. **c** DNA methylation vs. gene expression of *HOOK1* in OVAT. Two-sided paired sample *t* test was used to generate significance value, together with Spearman correlation analysis to generate *p* and *r* values. OVAT, omental visceral adipose tissue; P, *p* value; *r*, correlation coefficient from bivariate correlation; SAT, subcutaneous adipose tissue.

$p = 0.006$, $p = 0.04$, $p = 0.006$), two CpG sites with ALP ($p = 0.035$, $p = 0.006$), and two with GGT ($p = 0.014$, $p = 0.039$). *HOOK1* methylation in OVAT correlated at one CpG site with AST ($p = 0.021$), one CpG site with ALT ($p = 0.029$), and one site with ALP ($p = 0.026$). When applying adjustments for age and gender in a linear regression analysis, correlation at ten out of twelve CpG sites withstand this correction.

Validation of Depot-Specific HOOK1 *Gene Expression in Three Independent Cohorts*

In order to substantiate our findings of higher *HOOK1* gene expression in OVAT compared to SAT, we next extracted gene expression data from three independent validation cohorts comprising data originating from paired samples of human SAT and OVAT [25]. This includes a large CSC ($N = 1,449$ people with obesity, $N = 31$ individuals with normal weight), providing evidence for depot-specific *HOOK1* expression with higher levels in the OVAT compartment compared to SAT among individuals with obesity (adj. $p < 0.001$, Fig. 6a). Spearman's rank correlation analysis was performed on clinical variables (online suppl. Table 4b–d) and after adjustment for multiple testing adiponectin and cholesterol were significantly correlated with *HOOK1* expression in SAT (Table 2). In the 2-step bariatric surgery cohort (BSC) we also observed the same effect direction of higher gene expression in the visceral depot reaching significance among individuals having lost significant weight post-surgery (adj. $p = 0.0113$, Fig. 6b). Moreover, *HOOK1* expression in OVAT in the post-surgery group was negatively correlated with triglycerides after



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adjustments for multiple testing (p = 0.032) (Table 2). Finally, in a third validation cohort comprising metabolically healthy obesity vs. unhealthy individuals obesity, we discovered a consistently higher *HOOK1* gene expression in OVAT compared to SAT. Of note, these effects were strongest among subjects with healthy insulin sensitivity (metabolically healthy) (adj. p = 0.0013, Fig. 6c). Correlation analyses of *HOOK1* gene expression with clinical variables in this cohort did not pass adjustment for multiple testing (Table 2).

Discussion

In this work, we selected *HOOK1* as a possible novel target gene for obesity based on our data obtained in our recently published work on differential chromatin accessibility in human OVAT and SAT [8]. Here, we report that (i) *HOOK1* gene expression is consistently higher in OVAT compared to SAT in several cohorts, (ii) that the expression levels of *HOOK1* are related to clinical variables important for obesity such as anthropometric traits (unadjusted analyses), and (iii) that DNA methylation of the *HOOK1* promoter is correlated with anthropometric traits and liver function markers.

HOOK1 *Expression Is Adipose Tissue Depot-Specific*

The observed differences in expression levels between SAT and OVAT for *HOOK1*, obtained from Saeed et al. [8] were consistent across several large cohorts including the in-house adipose tissue cohort and various validation cohorts underlining the credibility of our data. *HOOK1* is ubiquitously expressed in multiple tissues with highest expression levels in testis (https://www.gtexportal.org/home/) where it plays an important role in the developmental pathway of spermatogenesis [26–29]. In line with this, genetic mutations within *HOOK1* were reported in patients with decapitated and decaudated spermatozoa syndrome [27]. In mice [29] and Drosophila [30], *HOOK1* is also predominantly expressed in testis and disruption leads to male infertility. In Drosophila, it is described to be involved in endocytosis of transmembrane ligands [11, 30, 31]. In humans, *HOOK1* was reported to function as an important player in endosomal sorting of clathrin-independent cargo [11, 31]. However, the role of *HOOK1* in human adipose tissue and its implication in obesity is not described. Our data show a higher expression level in OVAT compared to SAT and that *HOOK1* gene expression in OVAT negatively correlates with BMI and hip circumference. Considering that our gene expression data are based on bulk adipose tissue RNA-seq data, we cannot determine whether differences in *HOOK1* gene expression are contributed by adipocytes, immune cells or other cells present in the tissue. However, we show that primary adipocytes express *HOOK1* at very low levels when compared to bulk tissue from the same individuals (online suppl. Fig. 2), suggesting that other cells than adipocytes contribute to the depot-specific effects. Indeed, in a study deciphering cell type-specific expression in human SAT and OVAT, *HOOK1* was suggested to be contributed by cells of mesothelial origin in OVAT [32]. If *HOOK1* gene expression is mainly explained by mesothelial cells, the low expression level for SAT could potentially be explained by the lack of mesothelial cells in the subcutaneous depot [33]. Data from The Human Protein Atlas illustrate that the main *HOOK1* expression comes from mesothelial cells in visceral adipose tissue (single cell type – *HOOK1* – The Human Protein Atlas). Moreover, analysis of *HOOK1* expression using a single-nucleus RNA-seq dataset of subcutaneous and visceral adipose tissue from Reinisch et al. [34], via the Adiposetissue.org platform, revealed a clear enrichment of *HOOK1* in visceral mesothelial subclusters, particularly MesoCs3 and MesoCs5 (online suppl. Fig. 3) [35]. This underlines its depot- and cell-type specific expression.

HOOK1 *DNA Promoter Methylation Correlates with Liver Function Markers and Anthropometric Traits*

Our data show that the DNA methylation of *HOOK1* is negatively correlated with BMI and hip circumference in SAT. Further, we observed multiple correlations

Fig. 5. DNA methylation at promoter of *HOOK1* correlates with clinical variables. **a** Negative correlation of *HOOK1* methylation in SAT with anthropometric traits of BMI and hip circumference, both traits withstand correction for age and sex. **b** Correlation of *HOOK1* methylation in SAT and OVAT with liver function markers AST, ALT, ALP, and GGT. p values were calculated by using bivariate Spearman correlation. Linear regression analysis was used to adjust for age and sex. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma-glutamyl transferase; OVAT, omental visceral adipose tissue; SAT, subcutaneous adipose tissue; P, p value; r, correlation coefficient from bivariate correlation; adj. P, p value from linear regression analysis adjusted for age and gender; beta, regression coefficient from linear regression.

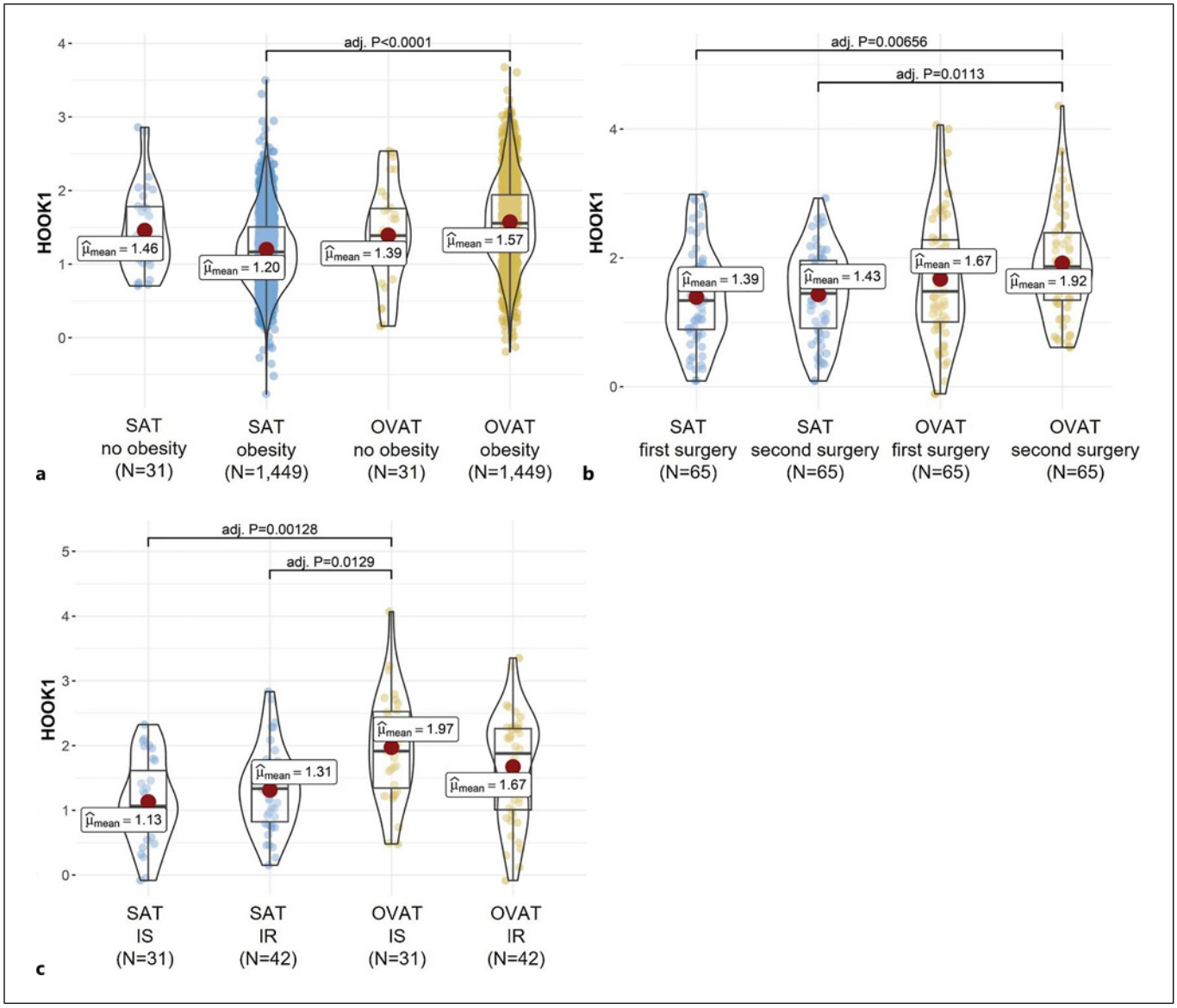

Fig. 6. *HOOK1* gene expression in SAT vs. OVAT from validation cohorts. **a** Cross-sectional cohort (CSC). **b** Bariatric surgery cohort (BSC). **c** Metabolically healthy versus unhealthy obesity (MHUO). IR, insulin resistance; IS, insulin sensitive; OVAT, omental visceral adipose tissue; post, post-surgery; pre, pre-surgery; SAT, subcutaneous adipose tissue.

between DNA methylation of the *HOOK1* promoter and liver function markers (AST, ALT, ALP, and GGT). DNA methylation of CpG sites covered by assay 1 (Hs_HOOK1_01_PM) positively correlate with these markers, while we found negative correlation of DNA methylation CpG sites in assay 2 (Hs_HOOK1_02_PM). Our results suggest that DNA methylation as an epigenetic mechanism might be related to liver function, potentially being linked to altered liver enzyme levels, that in turn may be related to liver fat deposition. Obesity has previously been correlated with serum levels of liver enzymes [36, 37]. AST, ALT, ALP, and GGT are commonly used as indicators of liver function and concentrations of these markers can indicate liver damage in the form of non-alcoholic fatty liver disease, whose development is not fully understood and underlies complex processes resulting in liver fat accumulation [38]. Katzke et al. [39] showed that higher serum levels of these liver markers correlate with chronic diseases and mortality. Our findings of DNA methylation in the

Table 2. Relationship of *HOOK1* gene expression with clinical variables in the validation cohorts

Clinical variables	*r*	*p* value	Significant *p* value	Adjusted *p* value	Significant adjusted *p* value	Gene	*N*	Cohort	Subgroup/tissue depot
WHR	0.1019	0.038772	*	0.52292		*HOOK1*	412	CSC	OVAT
Adiponectin	−0.08609	0.002336	**	0.039718	*	*HOOK1*	1,248	CSC	SAT
AST	−0.06326	0.042164	*	0.19299		*HOOK1*	1,032	CSC	SAT
Cholesterol	0.1011	0.00152	**	0.039718	*	*HOOK1*	982	CSC	SAT
FPG	0.06105	0.025826	*	0.19299		*HOOK1*	1,333	CSC	SAT
HbA1c	0.06959	0.008094	**	0.091734		*HOOK1*	1,447	CSC	SAT
LDL cholesterol	0.074	0.030106	*	0.19299		*HOOK1*	859	CSC	SAT
Mean SAT area	0.1307	0.035881	*	0.19299		*HOOK1*	258	CSC	SAT
Resistin	0.07698	0.04541	*	0.19299		*HOOK1*	676	CSC	SAT
BMI	−0.2914	0.018529	*	0.16712		*HOOK1*	65	BSC	OVAT pre-surgery
Body fat	−0.4973	0.003611	**	0.083063		*HOOK1*	33	BSC	OVAT pre-surgery
Creatinine	−0.2933	0.021798	*	0.16712		*HOOK1*	61	BSC	OVAT pre-surgery
Erythrocytes	0.2872	0.047817	*	0.21996		*HOOK1*	48	BSC	OVAT pre-surgery
LDL cholesterol	0.3412	0.038766	*	0.21996		*HOOK1*	37	BSC	OVAT pre-surgery
Leucocytes	−0.2683	0.03347	*	0.3849		*HOOK1*	63	BSC	OVAT post-surgery
Triglycerides	−0.5473	0.001411	**	0.032442	*	*HOOK1*	32	BSC	OVAT post-surgery
FPI	−0.4278	0.015302	*	0.35195		*HOOK1*	32	BSC	SAT pre-surgery
Triglycerides	−0.4029	0.023007	*	0.27362		*HOOK1*	32	BSC	SAT post-surgery
Waist	0.4799	0.023793	*	0.27362		*HOOK1*	22	BSC	SAT post-surgery
Fetuin A	−0.3621	0.046029	*	0.64418		*HOOK1*	31	MHUO	OVAT insulin sensitive
Triglycerides	−0.4041	0.024153	*	0.79704		*HOOK1*	31	MHUO	SAT insulin sensitive

Spearman correlation analyses were conducted to assess the relationship between gene expression values (log TMM, adjusted for age and sex), clinical parameters and blood sample analyses in the LOBB validation cohorts of OVAT and SAT. OVAT, omental visceral adipose tissue; SAT, subcutaneous adipose tissue; TMM, trimmed mean; MHUO, metabolically unhealthy obesity. *p value: <0.05. **p value: <0.01.

promoter region of *HOOK1* suggest a relationship with liver enzymes that may correspond with altered fat deposition in the liver, and potentially fatty liver disease or non-alcoholic fatty liver disease. However, no causative conclusion can be drawn, and further research including functional studies is warranted to explore a potential connection between DNA methylation of *HOOK1* in adipose tissue and liver function.

Furthermore, we found no significant differences in DNA methylation at the *HOOK1* promoter between SAT and OVAT. This held true for the average DNA methylation level of each assay, but also for each individual CpG site. These results suggest that DNA methylation of the two examined regions in the promoter region of *HOOK1* is not a major regulator of its gene expression. However, there is a possibility that other regions of the *HOOK1* promoter may have regulatory function driving gene expression through DNA methylation. In line with this, Keller et al. [9] reported significant differences for DNA methylated regions (downstream of our locations) of *HOOK1* between responders vs. non-responders for dietary and exercise

intervention methods. To fully establish whether DNA methylation is involved in regulating *HOOK1* gene expression further analyses are needed. Moreover, DNA methylation present at the selected promoter sites could potentially cause interference with important transcription factors overlapping this region [40]. The *HOOK1* promoter region includes binding sites for multiple transcription factors from the KLF family, known to be involved in glucose metabolism and adipose tissue development [41]. One may speculate that the *HOOK1* promoter could participate in these processes in a depot-specific manner. Additionally, other regulatory mechanisms might be involved in mediating depot-specific differences. The regulation of gene expression is complex, but other important players might be active or repressive histone marks and modifications, which could be a possible consequence for the difference generated in the transcriptome of the adipose tissues. Histone marks such as H3K4me3 and H3K27ac are associated with active transcription, while H3K27me3 is associated with transcriptional repression [42]. Histone marks are part of the complicated process of altering the higher-order chromatin structure, though, for example, the process of recruitment of nonhistone proteins, which carry enzymatic activities and can alter the chromatin landscape [43]. Acetylation is known to be a strong indicator of unfolding chromatin alterations as the modification will neutralise the basic charge of lysine, generating a change to the histone charge which will undoubtedly cause structural changes to the chromatin landscape [43]. Hence, differences in histone marks between the two depots could result in altered chromatin accessibility. The three modifications mentioned above could, therefore, potentially be involved in regulating *HOOK1* gene expression in SAT and OVAT, but further studies are needed in order to elucidate this.

HOOK1 *Expression and Correlation to Clinical Variables in Validation Cohorts*

When comparing the gene expression data from our in-house cohort with the three independent validation cohorts, we successfully validated that *HOOK1* gene expression is higher in OVAT. We validate differential expression between SAT and OVAT in a large cross-sectional study of individuals with obesity. We observed no clear differences in *HOOK1* gene expression between SAT and OVAT in lean controls, which may hint at depot-specific expression most prominent in patients with obesity. However, the number of lean individuals is low, and the effects observed might be false negative. Moreover, the same effect direction was verified in two additional cohorts of patients with obesity. In the 2-step bariatric surgery cohort, a higher *HOOK1* gene expression was observed in OVAT compared to SAT, while this reached only statistical significance in the post-surgery group. In the metabolically healthy vs. unhealthy obesity cohort, we observed stronger effects of depot-specific *HOOK1* gene expression among the insulin-sensitive individuals (metabolically healthy individuals). This may imply compromised gene expression mechanisms in individuals with impaired insulin sensitivity. Taken together, these data confirm our finding of higher *HOOK1* gene expression in OVAT compared to SAT.

Further, the results from the validation cohorts are in line with our findings regarding correlation of *HOOK1* gene expression with clinical variables related to obesity. Although we were not able to recapitulate the observed negative correlations with BMI and hip circumference in the CSC, the overall effect direction of the correlation between *HOOK1* gene expression in OVAT with BMI and hip circumference shows the same tendency as our in-house cohort. Additionally, several of the clinical correlations found in the validation cohorts support a possible active role of *HOOK1* in metabolic disease. These include a negative correlation with triglycerides in OVAT, a negative correlation with adiponectin in SAT and a positive correlation with total cholesterol in SAT. Although we observed somewhat different effect directions in SAT and OVAT in the validation compared to our in-house cohort, which may be due to differences in sample size, collectively, our data show a correlation with circulating blood lipids (total cholesterol and triglyceride levels) as well as with anthropometric markers (similar effect directions for BMI and hip circumference).

Limitations

This is, to the best of our knowledge, the first study showing a correlation of *HOOK1* gene expression with obesity and related clinical variables. The gene expression analysis is based on bulk human paired samples from two major adipose tissue depots. To support our initial findings, we included RNA-seq data from three large validation cohorts belonging to the LOBB. Despite these strengths, there are several limitations to consider. First, adipose tissue is heterogeneous consisting of multiple other cell types other than adipocytes. All datasets included in this study are based on whole adipose tissue; hence, the potential effects from differences in cell-type composition cannot be estimated or excluded.

However, the inclusion of purified adipocytes for each depot generated additional and more specific understanding of where *HOOK1* gene expression might originate from. Further insights into how specific cell types can affect the results would need a deeper analysis on a single cell expression basis and is beyond the scope of this study. Moreover, another limitation is that most of the individuals included in the cohort have obesity and stronger effects could be recognised more normal-weight individuals were included. Second, with respect to the DNA methylation analyses, and in addition to the above-mentioned issues regarding the investigated regions, the in-house cohort used has a limited sample size that could lead to false positive or negative results. Therefore, especially the results for DNA methylation and its correlation to clinical variables need to be interpreted with caution.

Conclusion

We show that *HOOK1* gene expression is adipose tissue depot-specific and that the gene expression and DNA methylation levels of the *HOOK1* promoter correlate with clinical variables relevant for obesity. Furthermore, our data suggest that DNA methylation in the *HOOK1* promoter may not be a major regulatory mechanism for *HOOK1* gene expression.

Statement of Ethics

The study was approved by the Regional Committee for Medical and Health Research Ethics, Southeast Norway (2013/2042; 2017/1528; 489516) and by the Ethics Committee of the University of Leipzig (approval No. 159-12-21052012) and performed in accordance with the Declaration of Helsinki. All participants gave written informed consent before taking part in the study.

Conflict of Interest Statement

Prof. Dr. med. Matthias Blüher received personal honoraria from Amgen, AstraZeneca, Bayer, Boehringer Ingelheim, Lilly, Novo Nordisk, Novartis, and Sanofi as well as payments from Boehringer Ingelheim to the institution and was a member of the journal's Editorial Board at the time of submission. The other authors on the manuscript declared no competing interests.

Funding Sources

S.S. was funded by an EU-Scientia postdoctoral Fellowship and project funding from the European Union's Horizon 2020 Research and Innovation Program under the Marie Skłodowska-Curie Grant (Agreement No. 801133). S.I.A.S. received a funding from the Robert og Ella Wenzins legat from the University of Oslo. M.B. received funding from grants from the DFG (German Research Foundation) – Project No. 209933838 – SFB 1052 (project B1) and by Deutsches Zentrum für Diabetesforschung (DZD, Grant No.: 82DZD00601).

Author Contributions

Conceptualisation: Y.B., S.S., and P.K.; data curation: S.I.A.S., A.C., Y.B., A.H., and A.G.; data interpretation and discussion: S.I.A.S., T.R., S.S., A.C., Y.B., and P.K.; funding acquisition: Y.B., S.S., S.I.A.S., and M.B.; methodology: S.I.A.S., M.B.D., B.B., and T.R.; contribution of biomaterial: T.M., J.A.K., T.G.V., M.B., and C.W.; writing – original draft: S.I.A.S. and Y.B.; writing – review and editing and final draft: all authors.

Data Availability Statement

The dataset supporting the final results and major findings of the study is included in the main article as well as online supplementary files. The human RNA-seq data from the LOBB are available from M.B. on reasonable request according to institutional guidelines. Further enquiries can be directed to the corresponding author.

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