Hypoxia-inducible factor 3A gene expression and methylation in adipose tissue is related to adipose tissue dysfunction

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Recently, a genome-wide analysis identified DNA methylation of the HIF3A (hypoxia-inducible factor 3A) as strongest correlate of BMI. Here we tested the hypothesis that HIF3A mRNA expression and CpG-sites methylation in adipose tissue (AT) and genetic variants in HIF3A are related to parameters of AT distribution and function. In paired samples of subcutaneous AT (SAT) and visceral AT (VAT) from 603 individuals, we measured HIF3A mRNA expression and analyzed its correlation with obesity and related traits. In subgroups of individuals, we investigated the effects on HIF3A genetic variants on its AT expression (N = 603) and methylation of CpG-sites (N = 87). HIF3A expression was significantly higher in SAT compared to VAT and correlated with obesity and parameters of AT dysfunction (including CRP and leucocytes count). HIF3A methylation at cg22891070 was significantly higher in VAT compared to SAT and correlated with BMI, abdominal SAT and VAT area. Rs8102595 showed a nominal significant association with AT HIF3A methylation levels as well as with obesity and fat distribution. HIF3A expression and methylation in AT are fat depot specific, related to obesity and AT dysfunction. Our data support the hypothesis that HIF pathways may play an important role in the development of AT dysfunction in obesity.

Obesity and its associated comorbidities constitute an evolving health burden worldwide1. Obesity is closely related to chronic inflammation in adipose tissue, liver and skeletal muscle2, which may contribute to chronic systemic inflammation, insulin resistance, and deterioration in glucose and lipid metabolism. Upon weight gain, adipocyte hypertrophy may lead to hypoxia in adipose tissue which is considered as a causative factor in adipose tissue dysfunction. It has been recently shown that adipose tissue expression of hypoxia inducible factor (HIF) 1A (HIF1A) increases in mice exposed to high fat diet3. In states of relative adipose tissue hypoxia, induction of HIF1α,β stimulates accumulation of macrophages in adipose tissue4 and the production of adipocyte-derived pro-inflammatory cytokines. HIFs are heterodimeric transcription factors that mediate hypoxia response in various tissues. They consist of an oxygen-labile α-subunit and a constitutively expressed β-subunit. Three existing isoforms of the α-subunit, HIF1α, HIF2α and HIF3α, allow the formation of transcription factors with different functions upon dimerizing with HIF3. Multiple isoforms of HIF3α exist6. HIF3α is capable of activating certain target genes independent or in collaboration with HIF1α, suggesting a role of HIF3α in glucose and amino acid metabolism, apoptosis, proteolysis, p53 signaling and PPAR signaling. In addition, HIF3α has been shown to play a role in adipocyte differentiation8,10.

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Recent genome-wide analysis of DNA methylation in whole blood and human adipose tissue revealed an association of methylation at three CpG sites in intron 1 of HIF3A with BMI\(^1\)–\(^3\). In addition, two single nucleotide polymorphisms (SNPs) rs8102595 and rs3826795, have been shown to be associated with methylation at these sites, yet to be independent of BMI\(^4\). The strong relationship of HIF3A methylation and obesity was also shown in nematodes\(^5\)–\(^7\). Furthermore, gene-diet interactions between the methylation-associated SNP rs3826795 and vitamin B intake were recently reported, providing a potential causal link between the epigenetic status and obesity\(^8\).

Further investigation of the relationship between HIFs and development of obesity-associated comorbidities might reveal important insights in pathophysiological processes concerning AT inflammation and/or insulin resistance in the etiology of obesity related metabolic diseases. We therefore tested the hypothesis that expression of HIF3A in human subcutaneous and visceral adipose tissue is related to obesity, parameters of fat distribution and adipose tissue function. We further assessed the relationship between the AT expression, genetic variation (rs8102595 and rs3826795) and methylation of CpG-sites in HIF3A.

**Material and Methods**

**Study participants.** A total of 288 Caucasian men and 577 women were included in the study (Table 1). According to the ADA criteria, 343 subject were diagnosed with type 2 diabetes (T2D) and 484 had normal glucose tolerance (NGT)\(^9\)–\(^14\). Paired samples of visceral adipose tissue and subcutaneous adipose tissue were obtained from 603 individuals following open abdominal surgery for gastric banding, cholecystectomy, weight reduction surgery, abdominal injuries or explorative laparotomy. Patients with end-stage malignant diseases were excluded from the study. All adipose tissue samples were frozen immediately in liquid nitrogen after explantation and stored at \(-80\) °C. Six-hundred and three subjects (mean age 50 ± 14 years, mean BMI 43.6 ± 13.0 kg/m\(^2\)) were included into adipose tissue expression analyses or dual-energy X-ray absorptiometry (DEXA) and metabolic parameters such as fasting plasma glucose and insulin, a 75-g oral glucose tolerance test (OGTT), HbA1c, lipoprotein-, triglyceride-, free fatty acid- and adipokine serum concentrations was performed as previously described\(^17\)–\(^18\). Measurement of abdominal visceral and subcutaneous fat areas (N = 245) was performed using computed tomography (CT) or MRI scans. All subjects had a stable weight, defined as the absence of fluctuations of >2% of body weight for at least 3 months before surgery. In addition, adipocytes and cells of the stromal vascular fraction were isolated from adipose tissue samples of 35 subjects (18 men, 17 women). Adipocytes were isolated by collagenase (1 mg/ml) digestion. To determine cell size distribution and adipocyte number, aliquots of adipocytes were fixed with osmic acid and counted in a Coulter counter as previously described\(^14\). The study was approved by the ethics committee of the University of Leipzig (approval number: 159-12-21052012) and all subjects gave written informed consent. All methods were carried out in accordance with the approved guidelines.

**Analysis of human HIF3A mRNA expression.** Briefly, human HIF3A mRNA expression was measured by qRT-PCR using TaqMan Gene Expression Assay (Applied Biosystems, Darmstadt, Germany). Total RNA was isolated from adipose tissue samples using the Qiacube System (Qiagen, Hilden, Germany), and 2 μg RNA were reverse transcribed with standard reagents (Life Technologies). Further details including PCR conditions are provided in the Supplemental material. The following Gene Expression Assay was used: Hs00541709_M1 (tagging the transcripts NM_022462.4, NM_152794.3, NM_152795.3, NM_152796.4). HIF3A mRNA expression was calculated relative to the mRNA expression of hypoxanthine guanine phosphoribosyltransferase 1 (HPRT1), determined by the assay Hs01003267_M1 (Applied Biosystems, Darmstadt, Germany). Expression of HIF3A and HPRT1 mRNA were quantified by using the second derivative maximum method of the TaqMan Software (Applied Biosystems).

For expression analysis of HIF3A in adipocytes and stromal vascular fraction, total RNA was isolated from adipocytes and stromal vascular fraction extracted from 35 paired samples of subcutaneous and visceral adipose tissue. 300 ng RNA were reverse transcribed with standard reagents and from each RT-PCR, 23.5 μl was amplified in a 40 μl PCR using the Taqman Gene Expression Assay and the TaqMan Fast Advanced Mastermix according to the manufacturer’s instruction. HIF3A mRNA expression was calculated relative to the mRNA expression of HPRT1 mRNA or 18S rRNA, determined by the assay Hs001003267_m1 (Applied Biosystems, Darmstadt, Germany).

**DNA extraction and bisulfite conversion.** Briefly, genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and bisulfite conversion was performed using the Epitect Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol.

**Determining CpG methylation levels.** PCR and sequencing primers were designed using the PyroMark Assay Design 2.0 software (Qiagen, Hilden, Germany). DNA fragments were amplified from bisulfite-converted DNA using forward primer 5′-TGGTTAAGGGTTATTAGGG-3′ and biotinylated reverse primer 5′-GACTCTATCCACCCCTTTT-3′. The PCR reaction mixture and cycling program are provided in the Supplemental material. Bisulfite pyrosequencing was performed on a PyroMark Q96MD pyrosequencing system (Qiagen) using the PyroMark Gold Q96 CDT reagent kit (Qiagen) and the Pyro Q-CpG software (Qiagen). Percentage of methylation at eleven individual CpG sites within intron 1 of HIF3A were determined using three different sequencing primers (Assay 1: 5′-TGTTAAGGGTTATTAGGG-3′; Assay 2: 5′-GACTCTATCCACCCCTTTT-3′; Assay 3: 5′-GTGAAGGAGGCTTTTATATT-3′). Assay 1 included seven CpGs, Assay 2 only one CpG and Assay 3 three CpGs. The sixth CpG site in Assay 1, the CpG site in Assay 2 and
Table 1. Anthropometric and metabolic characteristics of study participants. Data are means ± SD; *p < 0.05, **p < 0.01, ***p < 0.001. Body mass index, waist to hip ratio, systolic blood pressure, and diastolic blood pressure are not considered for group comparison. BMI – Body Mass Index, WHR – waist-to-hip ratio, ALAT – alanine aminotransferase, ASAT – aspartate aminotransferase, ALT – alanine transaminase, ALD – low density lipoprotein cholesterol, LDL-C – low density lipoprotein cholesterol, HbA1c – hemoglobin A1c, CRP – C-reactive protein, fT4 – free thyroxine. The difference between the lean and the overweight group was significant (p < 0.05) for all variables except for the body mass index and BMI.

The third CpG site in Assay 3 corresponded to the CpG sites on the 450 K array reported elsewhere. In our experience, the average methylation difference between technical replicates is approximately one percentage point.

Genotyping of HIF3A SNPs. Genomic DNA was extracted from blood using the Quick Gene DNA whole blood Kit (Kurabo, Japan). Genotyping of the two previously reported SNPs rs102595 (A/G) and rs3826795 (G/A) was performed using the TaqMan SNP Genotyping assay (Applied Biosystems, C_29247492_10; C_31640839_10). To assess genotyping reproducibility, a random ~5% selection of the sample were re-genotyped for all SNPs; all genotypes matched the initial designated genotypes. Potential functional significance of the studied genetic variants was checked using the Regulome Database, which includes public datasets from GEO, the ENCODE project, and published literature.
Statistical Analyses. All non-normally distributed parameters were logarithmically transformed to approximate a normal distribution. To analyze differences in HIF3A methylation/expression levels between visceral and subcutaneous adipose tissue, paired two-tailed t-tests were applied. To test for group differences (e.g. lean vs. obese, NGT vs. T2D) two tailed t-tests were used. Pearson’s correlation coefficients were used to assess bivariate correlation with phenotypes related to obesity, fat distribution and glucose and insulin homeostasis. Linear regression models were used to control for confounders such as age, gender and BMI. To test SNPs for genetic associations with mRNA expression, DNA methylation and metabolic traits, linear regression analysis adjusted for respective covariates was applied. Association studies on type 2 diabetes (T2D) and obesity (lean with BMI < 25 kg/m² vs. obese with BMI ≥ 30 kg/m²) were done using logistic regression analyses. P-values ≤ 0.05 were considered to provide nominal evidence for association. Two-sided p-values are reported without adjustments for multiple testing. The analysis of associations with quantitative traits was restricted to nondiabetic subjects to avoid diabetes status or treatment masking potential effects of the variants on these parameters. Statistical analyses were performed using SPSS statistics version 20.0.1 (SPSS, Inc., Chicago, IL, USA).

Results

HIF3A mRNA expression is fat depot related. Analysis of paired subcutaneous and visceral adipose tissue samples revealed significantly higher HIF3A mRNA expression in subcutaneous compared to visceral adipose tissue (Fig. 1A). The fat depot differences in HIF3A expression could be confirmed in both genders (Fig. 1B). There was no significant difference in both subcutaneous and visceral adipose tissue HIF3A mRNA expression between individuals with normal glucose tolerance (NGT) and with type 2 diabetes (Supplementary Figure).

We further analyzed the contribution of adipocytes and stromal vascular fraction cells on whole adipose tissue HIF3A mRNA expression. Analysis of visceral and subcutaneous stromal vascular fraction showed significantly higher HIF3A mRNA levels in subcutaneous compared to visceral stromal vascular fraction (p < 0.05) (subcutaneous 0.56 ± 0.84 and visceral 0.37 ± 0.57). In paired samples of adipocytes and stromal vascular fraction cells we found significantly higher HIF3A expression in adipocytes compared to stromal vascular fraction cells both in subcutaneous and visceral fat compartments (Fig. 1C). There was no significant fat depot-related difference in HIF3A mRNA expression of isolated adipocytes. We further sought to determine HIF3A mRNA expression...
in relation to adipocyte cell size. Comparison of HIF3A mRNA expression between individuals with the lowest versus highest mean adipocyte size decile reveals that HIF3A is more highly expressed in subjects with higher mean adipocyte volume (Fig. 1D).

HIF3A mRNA expression in adipose tissue correlates with parameters of obesity, systemic inflammation, glucose metabolism and mRNA expression of genes regulating adipogenesis (leptin, PPARG). HIF3A mRNA expression in visceral and subcutaneous adipose tissue correlated significantly with BMI, body weight, waist and hip circumferences, abdominal visceral and subcutaneous fat area, %body fat, free fatty acid, triglyceride, alanine aminotransferase (ALAT), leptin serum concentrations and with the mRNA expression of Leptin (Table 2 and Supplementary Table 1). Furthermore, there were significant inverse correlations between subcutaneous and visceral adipose tissue HIF3A expression and age, CT ratio, adiponectin and C-reactive protein (CRP) serum concentrations (Fig. 2A) and leucocyte count (Fig. 2B). Only in visceral adipose tissue, HIF3A expression correlated with fasting plasma insulin, thyroid-stimulating hormone (TSH),

Table 2. Correlation analyses of subcutaneous and visceral adipose tissue HIF3A mRNA expression with metabolic parameters, methylation levels and mRNA expression of leptin and PPARG. \( r \) - correlation coefficient (Pearson adj. – \( p \)-value adjusted to age, sex and BMI), \( * \)adjusted for sex and age; BMI – Body Mass Index, WHR – waist-to-hip ratio, sc - subcutaneous, CRP – C-reactive protein, Met Blood (%)/Met SAT (%)/ Met VAT (%) - Methylation of cg22891070 in HIF3A in blood/SAT/VAT.

| Parameter                  | Subcutaneous | Visceral | Subcutaneous | Visceral |
|---------------------------|--------------|----------|--------------|----------|
| Age (years)               | \(-0.23\)    | 0.032    | \(-0.237\)   | 0.076    |
| BMI (kg/m²)               | 0.239        | 0.017    | 0.283        | 8.84 \times 10^{-4} |
| Body weight (kg)          | 0.235        | 0.467    | 0.263        | 5.45 \times 10^{-4} |
| Height (m)                | 0.044        | 0.458    | 0.001        | 0.983    |
| Waist (cm)                | 0.472        | 0.100    | 0.515        | 1.89 \times 10^{-10} |
| Hip (cm)                  | 0.387        | 0.425    | 0.442        | 6.73 \times 10^{-7} |
| WHR                       | 0.172        | 0.139    | 0.135        | 0.033    |
| Visceral fat area (cm²)   | 0.391        | 0.636    | 0.442        | 1.71 \times 10^{-6} |
| SC fat area (cm²)         | 0.392        | 0.240    | 0.465        | 4.06 \times 10^{-7} |
| CT ratio (sc/vis)         | \(-0.259\)   | 0.165    | \(-0.319\)   | 0.325    |
| Body fat (%)              | 0.324        | 0.055    | 0.442        | 8.23 \times 10^{-4} |
| CRP (mg/dl)               | \(-0.138\)   | 0.021    | \(-0.153\)   | 3.19 \times 10^{-4} |
| Leucocytes/nl             | \(-0.127\)   | 0.032    | \(-0.133\)   | 1.13 \times 10^{-4} |
| Met Blood (%)             | 0.054        | 0.618    | 0.023        | 0.876    |
| Met SAT (%)               | \(-0.054\)   | 0.482    | \(-0.088\)   | 0.498    |
| Met VAT (%)               | 0.060        | 0.667    | \(-0.045\)   | 0.729    |
| Leptin mRNA sc            | 0.227        | 2.37 \times 10^{-4} | 0.216 | 5.28 \times 10^{-4} | 1.41 \times 10^{-4} |
| Leptin mRNA vis           | 0.017        | 0.063    | 0.195        | 2.0 \times 10^{-3} | 2.60 \times 10^{-2} |
| PPARG mRNA sc             | 0.001        | 0.939    | 0.002        | 0.967    |
| PPARG mRNA vis            | 0.050        | 0.253    | 0.439        | 0.011 |
| Table 2. Correlation analyses of subcutaneous and visceral adipose tissue HIF3A mRNA expression with metabolic parameters, methylation levels and mRNA expression of leptin and PPARG. \( r \) - correlation coefficient (Pearson adj. – \( p \)-value adjusted to age, sex and BMI), \( * \)adjusted for sex and age; BMI – Body Mass Index, WHR – waist-to-hip ratio, sc - subcutaneous, CRP – C-reactive protein, Met Blood (%)/Met SAT (%)/ Met VAT (%) - Methylation of cg22891070 in HIF3A in blood/SAT/VAT.



Figure 2. HIF3A mRNA expression in subcutaneous and visceral adipose tissue in relation to CRP serum concentration categories (\( n = 318 \)) and leucocyte counts (\( n = 326 \)). A significant inverse relationship between both CRP level (A) and leucocyte count (B) and expression of HIF3A in both compartments can be observed. Data are presented as means ± SEM. **p < 0.001, AU-arbitrary units.**

in relation to adipocyte cell size. Comparison of HIF3A mRNA expression between individuals with the lowest versus highest mean adipocyte size decile reveals that HIF3A is more highly expressed in subjects with higher mean adipocyte volume (Fig. 1D).
In the present study, we included 2 SNPs (rs8102595 and rs3826795) which have previously been shown to be associated with DNA methylation in a large cohort including >20001. Both studied polymorphisms were in Hardy-Weinberg Equilibrium (p > 0.05) with following minor allele frequencies: rs8102595-10.8%, rs3826795-21.6%. There was no significant association between the SNPs and HIF3A mRNA expression in any of the two adipose tissue depots (Table 3). However, rs8102595 was nominally associated with HIF3A DNA methylation in visceral and subcutaneous adipose tissue (p < 0.05 after adjusting for age, gender and BMI; Table 3). Subjects carrying the minor allele (G) had a higher HIF3A DNA methylation in visceral adipose tissue, which was in line with the lower HIF3A mRNA expression in visceral adipose tissue (albeit not significant). Association analyses with parameters of obesity and fat distribution revealed a nominal association between rs3826795 and total cholesterol and the mean fat cell size of visceral adipose tissue (Supplementary Table 2). Rs8102595 showed an association with HDL-cholesterol, glucose infiltration rate and maximum fat cell size of subcutaneous adipose tissue (Table 3 and Supplementary Table 2).
HIF3A DNA methylation in blood, subcutaneous and visceral adipose tissue. Methylation measured at the CpG site in Assay 2, corresponds to the published cg22891070, which has been reported to show the strongest correlation to BMI\(^{11}\). In our study, HIF3A DNA methylation at cg22891070 was significantly higher in blood (20.84 ± 7.74%) compared to subcutaneous (12.83 ± 6.82%; \(p < 0.001\)) and visceral adipose tissue (17.28 ± 5.61%; \(p < 0.001\)), whereas HIF3A DNA methylation in visceral adipose tissue was significantly higher than in subcutaneous adipose tissue (\(p < 0.001\); Fig. 3A). In addition, DNA methylation at cg22891070 in visceral adipose tissue correlated significantly with hip (\(p < 0.01\), \(r = 0.614\)), subcutaneous (\(p < 0.01\), \(r = 0.651\)) and visceral fat mass (\(p < 0.05\), \(r = 0.468\)) and inversely with the CT-ratio (\(p < 0.01\), \(r = -0.653\)). Correlations between methylation in visceral adipose tissue and subcutaneous fat mass (\(p < 0.01\), CT ratio (\(p < 0.01\)), hip (\(p < 0.01\)) and adiponectin (\(p < 0.05\), \(r = -0.187\)) remained significant even after adjusting for age, gender and BMI. Furthermore, methylation of cg22891070 in subcutaneous adipose tissue correlated with CT ratio (\(p < 0.05\), \(r = -0.571\)) and age (\(p < 0.05\), \(r = -0.268\)). After adjusting for gender and BMI the correlation remained significantly for age (\(p = 0.032\)). Albeit not significant, in all analyzed tissues, obese individuals displayed a higher methylation of cg22891070 compared to lean and overweight individuals (Fig. 3B). The analyses including other tested CpG sites did not reveal correlations beyond those observed for cg22891070 (data not shown).

Discussion

Recent studies revealed an association between BMI and methylation of HIF3A in whole blood and in adipose tissue\(^{11-13}\). It has been proposed that the HIF-system could play a role in mechanisms involved in the pathophysiology of adipose tissue-inflammation, obesity-induced insulin resistance and the etiology of obesity related diseases. We therefore sought to further elucidate the relationship between HIF3A mRNA expression in visceral and subcutaneous adipose tissue and obesity, but also methylation of CpG-sites in HIF3A. In summary, we show that HIF3A gene expression and methylation in adipose tissue are fat depot specific, and related to obesity and adipose tissue dysfunction.

We investigated the methylation and expression of HIF3A in two distinct fat depots, subcutaneous and visceral adipose tissue. We show that higher HIF3A mRNA expression in both subcutaneous and visceral adipose tissue is associated with higher BMI and obesity related traits. HIF3A has been shown to accelerate 3T3-L1 adipocyte differentiation and to induce the expression of adipocyte related genes\(^9\). Interestingly, we found higher adipose tissue HIF3A mRNA expression in individuals of the highest decile of mean adipocyte size (for both depots) compared to the lowest decile. This may suggest that HIF3A is involved in the determination of adipocyte size and may thereby contribute to adipose tissue expandability. Our results further support the hypothesis that expression of HIF3A might be induced in states of metabolic excess and mediate mechanisms involved in adipogenesis. Moreover, based on our data, the expression of HIF3A seems to be more pronounced in adipocytes compared to the stromal vascular fraction independent of the fat depot. To this end, adipocytes isolated from subcutaneous adipose tissue displayed higher expression levels of HIF3A than those isolated from visceral adipose tissue. Thus, the major proportion of HIF3A expression in adipose tissue might be attributed to primary adipocytes, which further supports the proposed regulatory role of HIF3A in adipogenesis. In further support of this, we found a positive correlation between the mRNA expression of HIF3A and leptin (in both visceral and subcutaneous adipose tissue) as well as PPARγ (in visceral adipose tissue), two genes involved in the regulation of adipogenesis.

It is noteworthy, that HIF3A expression inversely correlated with CRP level and leucocyte count, suggesting down-regulation of the HIF3A expression in inflammatory states. Chronic inflammation in adipose tissue, liver and skeletal muscle are commonly associated with obesity\(^4\), which results in secondary pathologies like insulin resistance, hyperinsulinemia and glucose intolerance\(^{12,21}\). Obesity promoted relative hypoxia in adipocytes stimulates HIF1A induction\(^{5,6}\), which then triggers the inflammation process by mediating the production of adipocyte-derived chemokines and adipose tissue macrophage accumulation\(^{47}\). HIF3A can inhibit HIF1A mediated signaling under certain circumstances\(^{22}\). The observed reduced expression of HIF3A in inflammatory states may facilitate increased HIF1A signaling, which in turn could activate an inflammatory cascade within adipose tissue.
HIF3A mRNA expression is regulated at different levels. Transcription of HIF3A can be induced by HIF1 via hypoxia response elements (HREs) in the promoter region and protein stability of HIF3α can be regulated in dependency of oxygen supply via the oxygen-dependent degradation domain (ODD)22–24. HIF3A expression has further been shown to be regulated by micro RNA (miRNA), thus be modified on a post-transcriptional level25. These different mechanisms can supplement one another in fine tuning of HIF3A expression. We hypothesize that the complex regulation of HIF3A expression can be influenced by DNA methylation in various ways by interfering with different mechanisms of regulation. An association between BMI and methylation at three CpG-sites in intron 1 of HIF3A in whole blood and in adipose tissue has recently been identified by employing genome-wide DNA-methylation analyses11–13. In contrast, we did not find a correlation between BMI and HIF3A methylation. This may be due to the smaller sample size and a different composition of our cohort, which is characterized by a relatively high BMI (32.9 kg/m2), and thus, strongly differing from the previously reported cohorts with average BMI ranging between 24.2 and 28.3 kg/m2. Rönn et al. were able to replicate the association between methylation of HIF3A and BMI in a female cohort only12 and Demerath et al. showed HIF3A methylation to be associated with BMI only in one of three cohorts investigated13. Considering multiple isoforms of HIF3Aα, it is plausible that methylation might be transcript-specific; yet, one would expect to observe consistent results upon expression analysis of the same transcript.

It is of note that the CpG sites at the HIF3A locus that were associated with BMI are situated within regions of open chromatin, suggesting that these sites lie in a regulatory region11–20. However, this regulation appears more complex than being dependent on methylation only. It is plausible that methylation of HIF3A results in altered expression profiles, networking with mechanisms in different stages of regulation. Yet, a linear effect between methylation and expression even of the same transcript cannot be confirmed.

Methylation analysis of HIF3A in our cohort revealed significant differences between methylation in blood, subcutaneous and visceral adipose tissue, being strongest in blood and weakest in subcutaneous adipose tissue. Since HIF3A mRNA expression in subcutaneous adipose tissue is higher than in visceral adipose tissue, it is possible that methylation could together with other regulatory mechanisms, cause a decrease in the expression of HIF3A. In line with this, rs8102595 was nominally associated with DNA methylation at cg22891070 in subcutaneous and visceral adipose tissue; thus supporting data by Dick et al.11 reporting associations of 2 SNPs (rs8102595 and rs3826795) with DNA methylation. Based on the Regulome Database20, rs3826795 might affect the binding of transcription factors POLR2A and SIN3A, and rs8102595 might influence DNA-protein binding. However, considering the lack of associations of the two SNPs with BMI, changes in HIF3A methylation seem to be mediated by obesity rather than promoting obesity itself11. It is also of note, that we did not observe an association between the SNPs and HIF3A mRNA expression in any of the two adipose tissue depots. We have to point out however, that the availability of the biomaterial (adipose tissue and blood samples) only allowed including 548 subjects for genotyping and for subsequent genotype-expression association analyses, which may have resulted in the lack of statistical power for correlation analyses.

In contrast to previous studies mostly investigating subcutaneous adipose tissue, the present study reveals mRNA expression and DNA methylation differences between subcutaneous and visceral adipose tissue. The two depots consist of different histological and biochemical compounds. The depot-specific expression of HIF3A may be important for the different functioning of the different depots. Whereas visceral adipose tissue is more vascular, innervated and contains a higher number of inflammatory and immune cells, subcutaneous adipose tissue has a higher preadipocyte differentiating capacity and a lower percentage of large adipocytes27. As such, it may be important for the different functioning of the different depots. Whereas visceral adipose tissue is more vascular, innervated and contains a higher number of inflammatory and immune cells, subcutaneous adipose tissue may be regulated by methylation, along with other factors. It is noteworthy that recently, we observed diminished hydroxymethylation levels in subcutaneous adipose tissue, as a measure of potential de-methylation mechanisms, which might be related to the higher number of pre-adipocytes in subcutaneous adipose tissue28.

We found methylation of HIF3A in both compartments to be correlated inversely with fat distribution, and methylation in VAT correlated significantly with subcutaneous fat mass. This suggests that methylation occurs rather in subjects with a preponderance of subcutaneous fat. We also detected an inverse association between age and methylation in subcutaneous adipose tissue, which leads to the assumption that the modification is dynamic and changes during lifetime.

Finally, it has to be acknowledged that the CpG site cg22891070 presented in our study is located between the 2 previously reported CpG islands11. Various HIF3A transcripts with different functions have been reported22 and it is also likely that they can be specifically affected by the methylation. Since the expression assay used in the present study tagged all potential HIF3A transcripts, we were not able to link cg22891070 to a specific transcript. However, in our own datasets based on genome-wide expression arrays (unpublished data) transcript variants 2 (NM_022462.4) and 3 (NM_152795.3) seem to be predominantly expressed in adipose tissue. Since Pasanen et al.23 suggested no functional relevance of the variant 3, it remains to be determined whether transcript variant 2 appears functionally relevant in adipose tissue.

In conclusion, our data suggest that HIF3A expression and methylation in adipose tissue is related to its dys-function, making HIF3A an important factor involved in the complex etiology of obesity and associated comorbidities. HIF3A might function as an accelerator of adipogenesis in situations of excess of energetic supply and might contribute to the etiology of secondary obesity-induced pathologies by allowing a stronger induction of HIF1α-mediated proinflammatory signaling.

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Author Contributions

S.P., J.K., A.M. and N.E.H. performed mRNA expression and DNA methylation experiments; Y.B., N.K., T.H. and P.K. designed the study; M.R.S., A.D., M.F., T.L., M.D. and M.B. collected and provided biomaterial and clinical phenotypes; S.P., J.K., D.S., M.S., M.B. and P.K. wrote the manuscript.

Additional Information

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