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Original Paper

Interleukin-6 CpG Methylation and Body Weight Correlate Differently in Type 2 Diabetes Patients Compared to Obese and Lean Controls

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Key Words
Epigenetics · Inflammation · Diabetes mellitus type 2 · *Interleukin-6* · Metabolic syndrome · Adiposity · CpG methylation

Abstract
Background/Aims: Diabetes mellitus type 2 (DMT2) is accompanied by systemic low-grade inflammation with elevated levels of interleukin-6 (IL-6), which is encoded by a gene *(IL-6)* previously shown to be regulated by DNA methylation. We investigated seven CpG sites in *IL-6* in individuals with DMT2, obese individuals and lean controls. Further, the DMT2 group received the glucagon-like peptide 1 agonist liraglutide. ***Methods:*** Blood samples were taken at the beginning of the study and after 4 months. The DNA methylation was assessed using pyrosequencing. ***Results:*** Methylation levels at the CpG sites –664, –628 and +13 at the first sampling time point (T1) and at –666 and –664 at the second sampling time point (T2) correlated negatively with initial body weight in the DMT2 group. We found positive correlations for the obese and the lean control group. In the obese group, CpG +27 methylation at T1 correlated with initial body weight ($r = 0.685$; $p = 0.014$). In the lean group, CpG –664 at T1 ($r = 0.874$; $p = 0.005$) and CpG –628 at T2 ($r = 0.632$; $p = 0.050$) correlated with initial body weight. ***Conclusion:*** These findings are an informative basis for further studies to elucidate epigenetic mechanisms underlying DMT2. Additionally, our results might provide starting points for the development of biomarkers for prevention and therapy strategies.

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Background

The International Diabetes Federation estimated that 382 million patients suffered from diabetes in 2013, 95% of them having diabetes mellitus type 2 (DMT2) [1]. In addition to the individual suffering, DMT2 is an enormous burden for the national health care systems. DMT2 is often a consequence of adiposity [body mass index (BMI) ≥30 kg/m^2] associated with a so-called unhealthy lifestyle. However, not all individuals with adiposity fall ill, and the underlying mechanisms of the association between extended body fat and DMT2 are not yet fully explained. Besides lifestyle and environmental factors, genetic and epigenetic factors have been discussed in the literature, as has the role of the gastrointestinal microbiota [2, 3], exposure to chemical agents and other factors.

The familial aggregation of DMT2 cannot be explained only by the shared family environment, as was shown in twin studies [4, 5]. However, the identified genetic risk loci can only explain 10–20% of the overall heritability [6]. Further, adverse events in early life (e.g. intrauterine conditions) were linked to an increased risk of DMT2 in later life [7]. Epigenetic processes are likely to be the underlying mechanisms according to this fetal origin hypothesis and, thus, may explain the missing heritability. A growing number of studies have already identified altered DNA methylation associated with the DMT2 phenotype on CpGs located in or near different genes [8–10]. Interestingly, altered DNA methylation in DMT2 is often reported on the genes that also carry genetic DMT2 risk variants, for example the *fat mass and obesity-associated protein (FTO)* gene. *FTO* has a known BMI-associated SNP, and the methylation levels of CpG sites in the first intron correlate with the genotype [11].

Of special interest in research on the mechanisms underlying DMT2 is the association of obesity and DMT2 with systemic low-grade inflammation in the affected person. Patients show a high white blood cell count and elevated plasma levels of coagulation factors, acute-phase proteins and pro-inflammatory cytokines and chemokines [12]. These inflammatory markers are positively correlated with insulin resistance and are known to decrease with weight loss [13, 14]. Obesity induces the infiltration of macrophages into adipose tissue, where they produce pro-inflammatory cytokines [15, 16], which are also synthesized by the enlarged adipocytes themselves [17]. Additionally, the adaptive immune system is involved in these processes through the infiltration of adipose tissue by lymphocytes, which modify the number and activity of the macrophages [18].

One of the major inflammatory mediators is interleukin-6 (IL-6), which possesses pro- as well as anti-inflammatory effects [19]. IL-6 serum levels were shown to be elevated in obese individuals by a factor of 2–3 compared to non-obese individuals [20]. Additionally, circulating IL-6 is directly correlated with adiposity and insulin resistance [21]. Several mechanisms by which IL-6 might induce insulin resistance are discussed in the literature. IL-6 might be responsible for the phosphorylation of the insulin receptor substrate 1, which has an inhibitory effect on the insulin signaling cascade [22]. Further, IL-6 stimulates lipolysis and inhibits lipoprotein lipase, which can trigger insulin resistance indirectly [23]. Another mechanism might be the IL-6-induced expression of cellular proteins, for example members of the suppressor-of-cytokine-signaling family, which then inhibit insulin signaling pathways [24].

As with many inflammatory mediators, IL-6 is also known to be regulated through epigenetic mechanisms. A correlation between the methylation of different CpG sites in the promoter region of *IL-6* and its mRNA expression has been reported in several studies. In our study, we focused on seven CpG sites which are not located in transcription factor-binding motifs but flank such a region (fig. 1). However, these CpG sites were described to be involved in the remodeling of chromatin and, consequently, to alter the accessibility for transcription factors [25, 26].

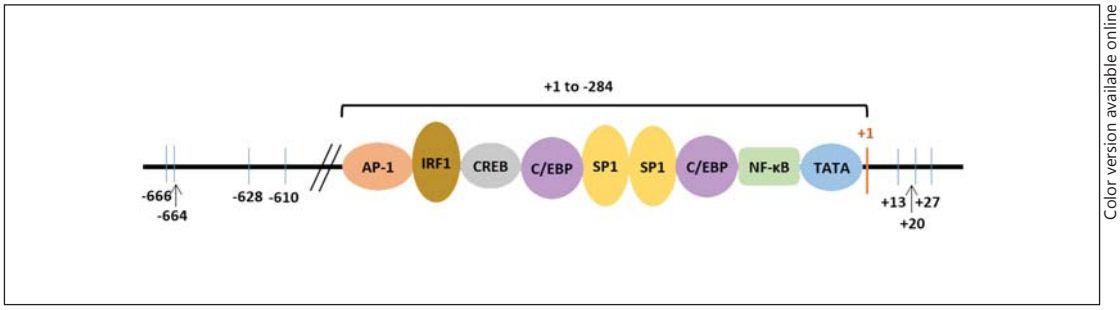

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Fig. 1. Schematic overview of the IL-6 promoter region. The investigated CpG sites (vertical lines) and their locations (numbers) counted from the transcription start site (+1) as well as the transcription factor motifs are shown. AP-1 = Activator protein 1; C/EBP = CCAAT-enhancer-binding protein; CREB = cAMP response element-binding protein; IRF1 = interferon regulatory factor 1; NF-κB = nuclear factor kappa B; SP1 = specificity protein 1; TATA = TATA-Box.

Further, we wanted to investigate the influence of the glucagon-like peptide 1 receptor (GLP-1R) agonist Victoza®, which contains the active component liraglutide, on the methylation levels. Thus, the participants of the DMT2 group started an intervention after the first sampling time point. Native GLP-1 is secreted within minutes during nutrition intake and induces insulin secretion, suppresses glucagon secretion and decelerates gastric emptying [27]. The half-life of naturally occurring GLP-1 is approximately 2 min [28]; the synthetic GLP-1R agonists are modified by different means to prolong the half-life, which is 11–15 h for liraglutide. The most important effects of GLP-1R agonists are the stimulation of insulin secretion [29], the suppression of glucagon secretion [30] and the decrease in gastrointestinal motility [31].

The aim of this study was to identify CpG sites which can act as possible biomarkers for the development of DMT2 in obese individuals and for the monitoring of prevention and therapy strategies. For this purpose, we investigated the DNA methylation levels of seven CpG sites located in the promoter region and the first exon of the *IL-6* gene in obese and lean study participants or DMT2 patients. Furthermore, we looked for differences in responses to an intervention with liraglutide over 4 months in the DMT2 group.

Methods

Study Design and Study Participants

For this study, 61 participants were enrolled: 25 diagnosed patients suffering from DMT2 were recruited at the Diabetes Outpatient Clinic at the Health Care Centre South, Vienna, and 18 non-diabetic but obese (BMI >30 kg/m^2) and 18 lean (BMI ≤25 kg/m^2) individuals were recruited through the Department of Nutritional Sciences at the University of Vienna (table 1). All study participants signed a written consent form. The present study was approved by the Vienna Human Ethics Committee.

Blood samples for the analysis of DNA methylation were collected by venous puncture at the beginning of the study and again after 4 months. After the first sampling time point, the DMT2 group started an intervention with the GLP-1 agonist liraglutide (Victoza®) in addition to their previous treatment regime. Victoza® was administered via subcutaneous injection at a dose of 0.6 mg daily for the first month and 1.2 mg thereafter.

Additionally, all study participants were given a questionnaire assessing their dietary habits including fortified foods or nutrient supplementation over the previous 3 months as well as lifestyle habits such as smoking and physical activity. Further, weight and height were measured.

Table 1. Characterization of the study population

Group	Participants	Gender		Age, years	BMI	Weight, kg
		female	male			
DMT2	25	10	15	58.4±9.4	38.4±5.1	113.1±18.7
Obese controls	18	12	6	37.8±13.8	38.4±7.2	110.0±15.8
Lean controls	18	15	3	25.7±3.1	21.2±1.9	58.6±8.0

Values are numbers or means ± SD.

Table 2. Primers for the analysis of IL-6 DNA methylation

CpG sites		Sequence
–610; –628; –664; –666	forward reverse sequencing	AGTAAAGTTTTTATTGGGAGGA Biotin-AAAACTACCTAACCATCCTCA GGTGAAGAAAGTGGT
+13; +20; +27	forward reverse sequencing	AAATGTGGGATTTTTTTATGA Biotin-AATTCCAAAACTAAAAATTTCCT ATGTTTGAGGTTTATTTTGTT

Analyses of IL-6 DNA Methylation

Blood samples were collected into PAXgene Blood DNA Tubes (PreAnalytiX, Hombrechtikon, Switzerland) and stored at –20°C until use. DNA was isolated using the PAXgene Blood DNA Kit (PreAnalytiX) and bisulfite-converted with the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). The primers for the analysis of seven CpGs in the promoter region and first exon of *IL-6* were designed with the PyroMark Assay Design Software 2.0 (Qiagen) using the reference sequence with the gene accession number NM_000600.2 (NCBI). The reverse primers were biotinylated to allow sequencing on the forward strand. Subsequently, PCR to amplify the target regions was performed under the following conditions: the 25 µl total volume for each reaction contained 12.5 µl PyroMark 2× PCR Master Mix (Qiagen), 2.5 µl CoralLoad (Qiagen) 10×, 5 pmol of each primer (table 2) and 10 ng of template DNA. The cycling program was 10 min at 95°C followed by 45 cycles of each 95°C for 30 s, 50°C for 60 s and 72°C for 60 s and a final elongation for 10 min at 72°C. Then, sequencing was performed on a PyroMark Q24 MDx work station (Qiagen) using specific sequencing primers (table 2).

Statistical Analyses

The different groups were compared using univariate ANOVA and univariate ANOVA with repeated measures to compare the sampling time points. The correlations between data sets were calculated using the Spearman rank correlation. The statistical analyses were performed with SPSS Statistics 20 (IBM, Armonk, N.Y., USA). A p value ≤0.05 was considered significant.

Results

The analysis of the questionnaire showed no significant differences in the consumption of grain products, fruits, meat, dairy products and fish between the three groups (DMT2, obese and lean). There were differences in the consumption of sweets: the category for sweet consumption chosen most often in the DMT2 group was ‘less than once per week’ (40%), and for the obese and lean control group it was ‘three to five times a week’ (50 and 28%, respec-

tively). Furthermore, the level of physical activity differed, with the lean group being the most active group and the diabetes group the least active (table 3).

The methylation states of the seven investigated CpG sites in the promoter region and first exon of the *IL-6* gene showed no significant differences between the three groups at the beginning of the study (T1) or after 4 months (T2; mean overall CpGs: DMT2 group at T1: 3.62 ± 0.82%; obese controls at T1: 3.93 ± 1.75%; lean controls at T1: 4.29 ± 1.17%; DMT2 group at T2: 3.67 ± 0.56%; obese controls at T2: 3.32 ± 0.69%; lean controls at T2: 3.52 ± 0.83%; detailed results are given in online suppl. table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000381714).

The liraglutide treatment over 4 months lowered the methylation at CpG +27 slightly but significantly (T1: 3.92 ± 0.90%; T2: 3.70 ± 0.92%; p = 0.026). The other investigated CpG sites did not show significant alterations (detailed results are given in online suppl. table 2).

However, we observed correlations between initial body weight and methylation on distinct CpG sites within the *IL-6* promoter region in all three study groups (fig. 2). In the diabetic group, the CpG sites –664 (r = –0.574; p = 0.008), –628 (r = –0.450; p = 0.036) and +13 (r = –0.518; p = 0.033) showed a negative correlation with body weight at T1, and the CpG sites –666 (r = –0.701; p = 0.001) and –664 (r = –0.602; p = 0.004) showed a negative correlation with body weight at T2. Notably, we found only positive correlations between body weight and methylation levels in the obese (CpG +27/T1: r = 0.685; p = 0.014) and lean (CpG –664/T1: r = 0.874; p = 0.005 and CpG –628/T2: r = 0.632; p = 0.050) control groups.

In short, the pattern of the correlations between initial body weight and CpG methylations differs between the three investigated groups. The diabetic group shows more correlations than the control groups. A correlation with CpG +27 was seen only in the obese control group but not in the diabetic or in the lean group. Interestingly, the correlations in the diabetic group were negative, whereas the obese as well as the lean control group showed only positive correlations.

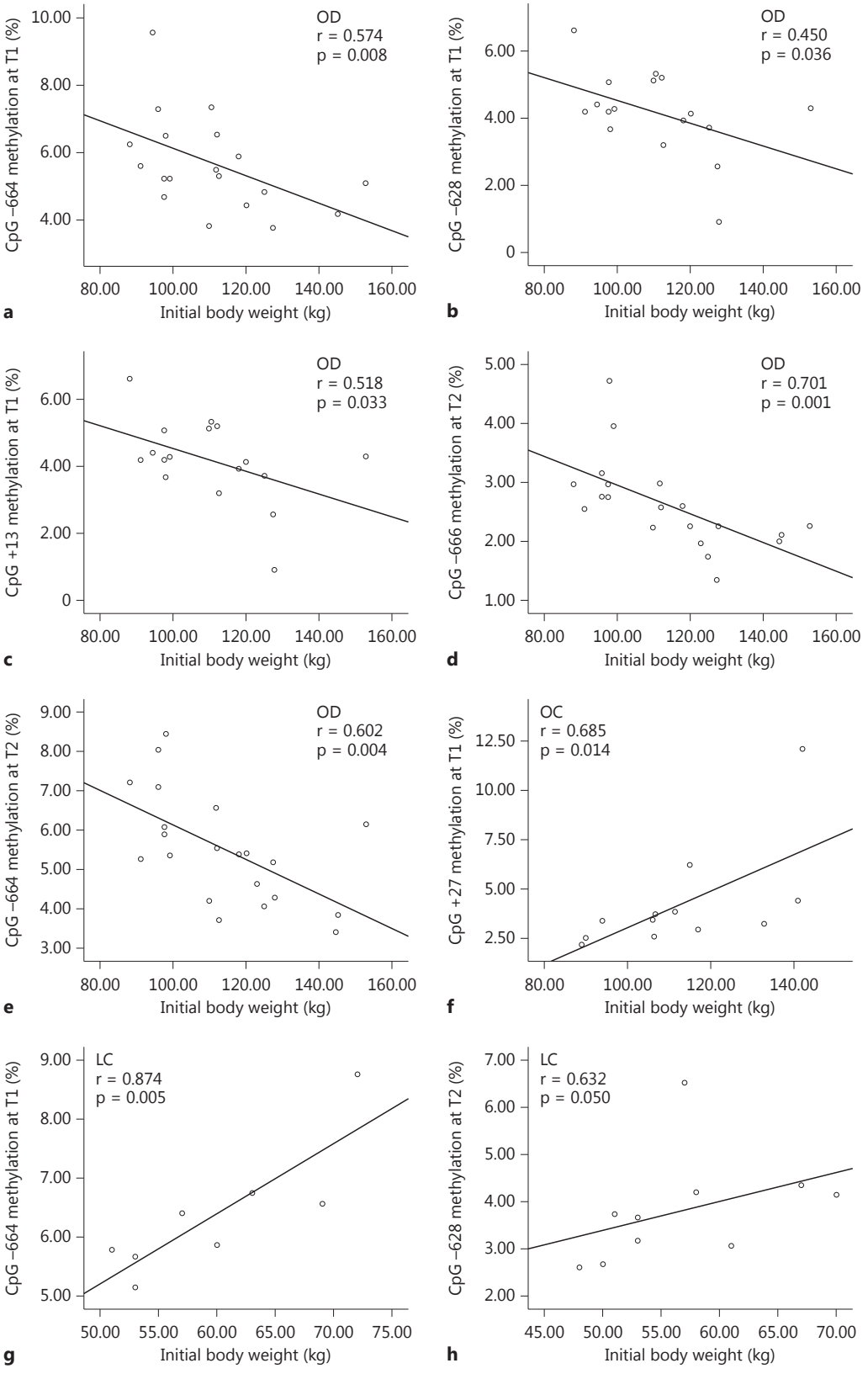
Table 3. Results of the food frequency questionnaire

Group	Grain products	Fruits and vegetables	Meat	Dairy products	Fish	Sweets	Physical activity	Smokers
DMT2	5–10 p/w (64%)	5–10 p/w (52%)	3–5 p/w (44%)	5–10 p/w (61%)	1–3 p/w (48%)	<1 p/w (40%)	<1/w (64%)	3 (12%)
Obese controls	5–10 p/w (44%)	5–10 p/w (56%)	3–5 p/w (50%)	5–10 p/w (33%)	1–3 p/w (50%)	3–5 p/w (50%)	1–3/w (50%)	5 (28%)
Lean controls	5–10 p/w (50%)	5–10 p/w (65%)	3–5 p/w (83%)	5–10 p/w (67%)	1–3 p/w (50%)	3–5 p/w (28%)	3–5/w (44%)	10 (56%)

The categories which were chosen most often by the study participants are indicated. The values in parentheses indicate the percentage of study participants who chose this category. p/w = Portions per week; number/w = times per week.

Fig. 2. Correlations between CpG methylation and initial body weight. **a–e** Correlations of the diabetic group. **f** Correlation of the obese control group. **g**, **h** Correlations of the lean control group. Correlations were calculated using the Spearman rank correlation. OD = DMT2 group; OC = obese control group; LC = lean control group.

(For figure see next page.)



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Discussion

The objective of this study was to detect differences in the methylation of seven CpG sites in the promoter region and first exon of the *IL-6* gene between patients with DMT2 and obese and lean individuals and to assess the impact of liraglutide on these methylation sites.

To our knowledge, the influence of liraglutide on the methylation of *IL-6* has not been investigated before, nor have any other published studies dealt with the influence of liraglutide on DNA methylation. CpG site +27 showed a slight alteration after 4 months of treatment. This finding hints at an epigenetic activity of liraglutide and provides a basis for further studies investigating the underlying mechanisms.

Moreover, we did not observe any significant differences in the methylation levels of each CpG or in the mean overall CpGs between the different groups. However, the methylation levels at the investigated CpG sites are rather low (1–10%); thus, it is possible that the variation within a group is higher than the differences between the groups. This would explain why we could not reveal differences between the means of the study groups but saw different patterns of correlations with anthropometric data. CpG site –664 might be of special interest since it shows a negative correlation with initial body weight in the DMT2 group and a positive correlation with initial body weight in the lean control group. Further studies should focus on this CpG site.

It is of interest which mechanisms lead to the different patterns of correlation between DNA methylation and body weight in individuals with and without DMT2. Several studies have observed differing methylation of CpG sites depending on the diet and intake of particular food components. Our group reported different methylation levels in the promoter region of the *manganese-dependent superoxide dismutase (MnSOD)* gene in buccal cells of vegetarians compared to omnivores [32]. For *IL-6*, similar results were reported by Zhang et al. [33], who observed a correlation between a so-called prudent diet and methylation of a CpG island located in the promoter region of *IL-6* in white blood cells. Our food frequency questionnaire showed hardly any difference in the nutritional behavior of the DMT2 and the control group, but it covered only the last 3 months of the participants' diets. The altered methylation pattern we found in the DMT2 patients might thus be a consequence of their nutrition earlier in their lives. Subsequently, the shifted DNA methylation can lead to changes in the expression of genes involved in disease pathogenesis.

On the other hand, there is also the possibility that an altered interindividual *IL-6* promoter methylation exists independently of the diet and influences the vulnerability to distinct diseases. This would explain why not all obese individuals develop DMT2 during their life course and would be in agreement with the results of our food frequency questionnaire.

Besides nutrition, other lifestyle factors can also influence DNA methylation. Several studies report a shift in DNA methylation with age, often a loss of methylation [34, 35]. In our study, we did not age match the study groups to reflect the classic course of the development of DMT2 [young and lean healthy individuals (lean control group) gaining weight with increasing age (obese control group) and contracting DMT2 with enduring obesity (DMT2 group)]. We saw neither significant differences in the DNA methylation between the three groups nor a correlation between age and methylation. These results were also reported in former studies, so that we assume that the differing age of the study participants does not influence our observations [33]. Further, the DMT2 group had already received a standard DMT2 treatment regime before the beginning of this study; the majority of DMT2 patients is diagnosed and treated in industrialized countries. Furthermore, Zhang et al. [33] reported no correlations between *IL-6* methylation and gender, ethnicity, physical activity and alcohol consumption.

Consequently, the question arises as to what the consequences of this different methylation pattern between participants with and without diabetes are. Several studies showed that methylation of distinct CpG sites within the *IL-6* promoter region correlates with mRNA expression [25, 26, 36]. This may imply a mechanistic link between promoter methylation and regulation of mRNA expression.

The CpG sites investigated in this study have been described to play important roles in the regulation of *IL-6* expression. From base pair –284 upstream of the transcription start site of the *IL-6* gene, the sequence does not contain binding motifs for transcription factors (fig. 1). However, studies have shown that the CpG methylation of this region influences the remodeling of chromatin and, consequently, the accessibility of chromatin for transcription factors [25, 26]. Dandrea et al. [26] reported that the repression of *IL-6* is mediated by the binding of methyl-CpG-binding protein 2 (MeCP2) and histone 3 methylated on lysine 9 to the methylated CpG sites in pancreatic adenocarcinoma cell lines. Similar results were observed by Poplutz et al. [25].

The present study focused on the differences in the DNA methylation of the *IL-6* promoter region in diabetes patients undergoing an intervention with liraglutide compared to an obese and a lean control group. Thus, it can be regarded as a basis for future studies investigating underlying mechanisms and consequences of the observed effects. In any case, this study also suggests the use of DNA methylation as a biomarker for the development and prognosis of DMT2, as was done by Campion et al. [37] for the methylation of the promoter region of the *tumor necrosis factor alpha (TNF-α)* as a biomarker for the prediction of weight loss response.

DNA methylation might have some advantages which make it a more robust biomarker than mRNA or protein expression. The expression of a gene occurs in several stages, and each one is regulated through a multitude of fine-tuning processes. Thus, the expression can be influenced by many effects, making it complicated to determine an association with the target parameter. In particular, the blood level of IL-6 is a problematic biomarker for the development of diabetes, even though it is involved in its pathogenesis. As one of the major inflammatory mediators, IL-6 is easily influenced by sudden inflammatory events. Moreover, the sampling method was shown to impact the results of the determination of circulating IL-6 [38], as did the time point at which the sample was taken, because IL-6 levels undergo a circadian rhythm [20].

In conclusion, we could not find any differences in the means of the methylation levels of the investigated CpGs, but we saw different correlation patterns between specific CpG methylations and anthropometric data in type 2 diabetics and obese and lean individuals. To our knowledge, this is the first study which compares the methylation levels of distinct CpG sites on the *IL-6* gene between DMT2 patients and obese and lean controls. Thus, these results are an important basis for further studies to elucidate epigenetic mechanisms underlying DMT2. Furthermore, these specific correlations should be the focus of further studies to develop robust biomarkers for individual prevention, prediction and monitoring of DMT2. In addition, our findings might serve as starting points for epigenetic active therapy strategies, which have already been established for other diseases.

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Disclosure Statement

The authors declare that they have no competing interests.

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