

# Cross-tissue comparisons of leptin and adiponectin DNA methylation profiles

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**Abbreviations:** *ADIPOQ*, adiponectin; BMI, body mass index; *C/EBP*, CCAAT/enhancer binding protein; *HLF*, hepatic leukemia factor; IGT, impaired glucose tolerance; *LEP*, leptin; MIF-1, macrophage migration inhibitory factor; RIN, RNA integrity number; SAT, subcutaneous adipose tissue; TFBS, transcription factor binding site; VAT, visceral adipose tissue

DNA methylation has been mostly studied in circulating blood cells. Although being readily accessible, metabolically active tissues such as adipose tissue would be more informative for the study of metabolic disorders. However, whether or not the blood DNA methylation profile correlates with that of adipose tissue remains unknown. In this study, DNA methylation patterns of variation at *LEP* and *ADIPOQ* gene loci were similar between individual CpGs across the different tissues. We also report that DNA methylation levels at biologically relevant CpGs are correlated between blood, subcutaneous, and visceral adipose tissue, and that these nearby CpGs are associated with *LEP* and *ADIPOQ* gene expression in adipose tissues. These results will be highly relevant for future epigenetic studies in metabolic disorders.

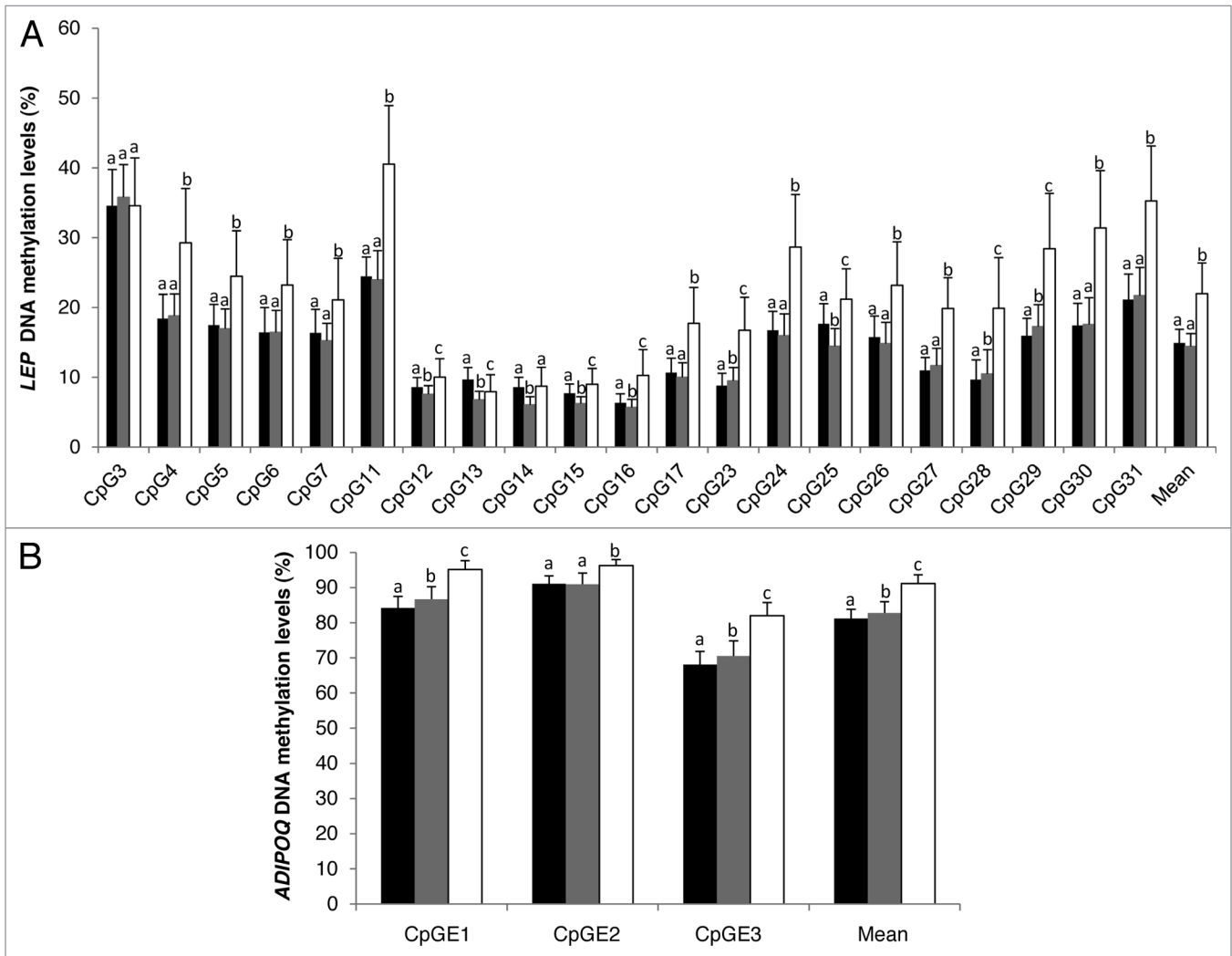
## Introduction

The *LEP* and *ADIPOQ* genes, encoding for leptin and adiponectin respectively, have drawn much attention in candidate gene studies on obesity and its related metabolic disorders. These adipokines are mainly produced and secreted by adipocytes and are important regulators of energy metabolism and insulin sensitivity.<sup>1,2</sup> Recent studies suggested that these two adipokines are involved in the fetal metabolic health programming of the newborn through epigenetic adaptations in response to a changing in utero environment. Indeed, we have recently reported correlations between placental DNA methylation levels at the *LEP* and *ADIPOQ* gene loci and maternal blood glucose concentrations (2 h post oral glucose tolerance test) at second trimester of pregnancy.<sup>3–5</sup> A high maternal glucose concentration has been previously associated with a long-term susceptibility to obesity and related metabolic disorders in the newborn.<sup>6–8</sup> Moreover, Tobi et al.<sup>9</sup> have observed higher *LEP* DNA methylation levels in leukocytes of men exposed prenatally to the ending World War 2 Dutch famine in comparison with unexposed siblings, suggesting that epigenetic marks imprinted during fetal life at the *LEP* locus could last over 60 y after birth.

DNA methylation marks consist in the addition of a methyl group at position 5' of the pyrimidine ring of the cytosines upstream of a guanine (dinucleotide CpG) catalyzed by DNA methyltransferases.<sup>10,11</sup> The establishment of DNA methylation marks during fetal life is crucial for cellular differentiation, X chromosome inactivation, genomic imprinting, and normal embryonic development.<sup>12,13</sup> Embryonic DNA methylation marks are mitotically stable thus they can have a long-term functional impact and can be partially transmitted to the next generation.<sup>14–18</sup> Although transcriptional regulation through DNA methylation is tissue-specific,<sup>19–21</sup> it has been suggested that embryonic epigenetic marks set early during cellular differentiation and division are similar in various tissues.<sup>22–24</sup> These similarities could be of importance when access to metabolically active tissues (e.g., adipose tissue) is limited, and especially when studying epigenetically affected genes in the context of in utero or fetal metabolic programming.

The objective of the current study was to compare *LEP* and *ADIPOQ* epigenetic profiles in blood, subcutaneous (SAT), and visceral (VAT) adipose tissue samples from the same obese class III<sup>22</sup> subjects in order to characterize tissue-specific DNA methylation at these two adipokine gene loci. The comparison of

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**Figure 1.** DNA methylation levels at *LEP* gene promoter (**A**) and *ADIPOQ* CpG island E (**B**). CpG sites in blood (white), subcutaneous (black), and visceral (gray) adipose tissues ( $n = 73$ ). Error bars represent the standard deviation (SD). Mean DNA methylation levels were compared with the paired sample Student *t* test. Bars with different letters indicate means that are significantly different from each other ( $P \leq 0.05$ ) after adjustment for multiple testing.

adipokine DNA methylation levels in blood and adipose tissues will tell us whether or not readily accessible blood cells can reflect the DNA methylation profile of metabolically active tissues and can eventually be used to predict a lifelong susceptibility to obesity and associated metabolic disorders.

## Results and Discussion

### DNA methylation and mRNA level analyses at the *LEP* gene locus

Blood, SAT, and VAT were obtained from 73 obese class III<sup>22</sup> subjects with body mass index (BMI) ranging from 40.0 to 60.0 kg/m<sup>2</sup> (mean: 49.6 ± 6.0 kg/m<sup>2</sup>) (sample characteristics in Table S1). DNA methylation levels were assessed in blood, SAT, and VAT at 21 CpGs out of the 31 that we<sup>3</sup> and Melzner et al.<sup>25</sup> have previously epigenotyped in the proximal promoter CpG island of the *LEP* gene (Fig. S1).

DNA methylation levels at these 21 CpG sites analyzed within the *LEP* gene promoter were found to be highly variable within the region and across the three tissues analyzed. On average, the correlation coefficients between DNA methylation levels at these 21 CpG sites were found to be modest to very high in blood ( $r = 0.12$  to  $0.92$ ), SAT ( $r = 0.01$  to  $0.82$ ), and VAT ( $r = 0.01$  to  $0.86$ ) (Fig. S3). For most of the CpG sites analyzed, methylation values were 1.5 to 2.0 times higher in blood than in SAT and VAT (Fig. 1A). These results confirm previous findings from Marchi et al.<sup>26</sup> and Stoger et al.<sup>27</sup> showing that *LEP* DNA methylation is higher in tissues known to express lower *LEP* mRNA levels. The highest DNA methylation differences (>10.0%) between blood and adipose tissues at the *LEP* gene promoter locus were observed at CpG4, CpG11, CpG24, CpG29, CpG30, and CpG31 with CpG11 being the most significant (Fig. S1). Interestingly, CpG4 is found on the macrophage migration inhibitory factor (*MIF-1*) transcription factor binding site (TFBS) and CpG11 on or nearby

**Table 1.** Pearson correlation coefficients between DNA methylation levels at *LEP* gene promoter CpG sites in blood, subcutaneous (SAT), and visceral adipose (VAT) tissues

CpG sites	Chromosomal location <sup>a</sup>	SAT vs. VAT	SAT vs. blood	VAT vs. blood
<i>LEP</i> -CpG3	chr7:127 881 368	0.295	-0.102	0.226
<i>LEP</i> -CpG4	chr7:127 881 349	0.589**	0.116	0.287
<i>LEP</i> -CpG5	chr7:127 881 343	0.414**	0.073	0.195
<i>LEP</i> -CpG6	chr7:127 881 338	0.435**	0.049	0.220
<i>LEP</i> -CpG7	chr7:127 881 329	0.367*	-0.049	0.189
<i>LEP</i> -CpG11	chr7:127 881 279	0.575**	0.433**	0.327
<i>LEP</i> -CpG12	chr7:127 881 268	0.142	0.160	0.156
<i>LEP</i> -CpG13	chr7:127 881 259	0.087	0.166	0.132
<i>LEP</i> -CpG14	chr7:127 881 256	0.134	0.447**	0.334
<i>LEP</i> -CpG15	chr7:127 881 245	0.079	0.213	0.232
<i>LEP</i> -CpG16	chr7:127 881 235	0.352*	0.488**	0.358*
<i>LEP</i> -CpG17	chr7:127 881 230	0.284	0.578**	0.268
<i>LEP</i> -CpG23	chr7:127 881 170	0.500**	0.339	0.203
<i>LEP</i> -CpG24	chr7:127 881 168	0.484**	0.408**	0.217
<i>LEP</i> -CpG25	chr7:127 881 160	0.202	0.186	0.315
<i>LEP</i> -CpG26	chr7:127 881 147	0.484**	0.311	0.289
<i>LEP</i> -CpG27	chr7:127 881 144	0.497**	0.240	0.232
<i>LEP</i> -CpG28	chr7:127 881 142	0.773**	0.611**	0.573**
<i>LEP</i> -CpG29	chr7:127 881 130	0.451**	0.212	0.205
<i>LEP</i> -CpG30	chr7:127 881 128	0.490**	0.277	0.310
<i>LEP</i> -CpG31	chr7:127 881 126	0.508**	0.200	0.204
<i>LEP</i> -MEAN	-	0.571**	0.321	0.354*

Statistically significant after corrections for multiple testing: \*\* $P \leq 0.01$  and \* $P \leq 0.05$ . <sup>a</sup>UCSC Genome Browser on Human Feb. 2009 (Feb. 2014).

the CCAAT/enhancer binding protein (*C/EBP*), hepatic leukemia factor (*HLF*), and *c-MYB* TFBS suggesting that these CpG sites may be important for tissue-specific *LEP* expression regulation (Fig. S2). The functional significance of CpG11 is also supported by its location within a highly conserved region across all placental mammals (Fig. S2). This CpG site was previously shown to be strongly differentially methylated between tissues with low (liver) and high (visceral adipocyte) mRNA levels of *LEP*.<sup>26</sup> Moreover, the regulation of *LEP* gene promoter activity by DNA methylation at *C/EBP* TFBS has previously been reported.<sup>25</sup> No transcriptional regulatory element was identified at CpG24, CpG29, CpG30, and CpG31 of the *LEP* gene promoter.

Next, we report that the correlation between DNA methylation levels at each individual CpG site was tissue- and site-specific. After multiple testing corrections, *LEP* DNA methylation levels at 14 individual CpG sites were found to be significantly correlated between SAT and VAT, whereas 6 and 2 CpGs were significantly correlated between blood and SAT and blood and VAT, respectively (Table 1). Interestingly, DNA methylation at CpG11, localized at *C/EBP* TFBS, was found to be among the most highly correlated between SAT and VAT and between SAT and blood (Table 1). For this site, a trend for association

was also observed between VAT and blood after multiple testing correction ( $r = 0.327$ ;  $P = 0.10$ ). CpG16 was also found to be highly correlated between the three tissues and CpG17 was correlated between SAT and blood (Table 1). These two CpG sites are localized within *SPI* TFBS and a highly conserved region of the *LEP* gene promoter locus (Fig. S2). *SPI* TFBS was shown to be involved in the regulation of *LEP* gene expression.<sup>25,28,29</sup> High correlation coefficients were observed between DNA methylation levels at *LEP* CpG28 in the three tissues (Table 1). Nevertheless, the cytosine at this CpG site was found to be polymorphic (single nucleotide polymorphism [SNP]; rs791620; C > A), and the genotype is likely driving the correlations observed. Among all the CpG sites analyzed in the current study, CpG11, CpG16, and CpG17 are the most promising as they are located in potentially functional regions and correlated between blood and adipose tissues. Nevertheless, further studies are needed to determine whether these CpGs are associated with the development of obesity in normal weight and obese populations. If such associations are observed, it could eventually raise the possibility of using blood *LEP* DNA methylation levels at these CpG sites as a surrogate for *LEP* DNA methylation levels in adipose tissues and as a marker of obesity susceptibility.

*LEP* gene expression analysis in SAT and VAT confirmed the functional importance of specific CpG sites in both adipose tissues. Considering that *LEP* expression is influenced by sex<sup>30,31</sup> and that associations between *LEP* DNA methylation and phenotypic variables have been previously reported to be sex-specific,<sup>9,32,33</sup> the relationships between *LEP* DNA methylation and mRNA levels were adjusted for sex. In addition, we observed significant interactions between sex and DNA methylation levels (CpG12 and CpG14) on VAT *LEP* mRNA levels. In VAT, DNA methylation levels at *LEP* CpG7, CpG11, and CpG27 to CpG30 were found to be positively associated with *LEP* mRNA levels (Table 2). These results are not supported by previous studies reporting negative associations between mean *LEP* promoter DNA methylation and expression in the visceral adipocyte fraction,<sup>26</sup> placenta,<sup>3</sup> and adipocyte cell cultures.<sup>25</sup> These contrasting results might be explained by the fact that *LEP* genetic variations were not taken into account in these studies. Indeed, an exploratory analysis revealed that *LEP* rs2167270, a variant previously associated with *LEP* expression,<sup>34</sup> tended to be associated with *LEP* gene expression in VAT ( $P = 0.06$ ) and was significantly associated with DNA methylation levels at CpG4 in SAT and at CpG3, CpG4, CpG6, and CpG7 in VAT (Table S2). In addition, rs2167270 was found to influence the relationship between *LEP* DNA methylation and mRNA levels in adipose tissues (Table 2). In VAT, positive significant correlation coefficients were found in GG genotypes for CpG11 and nearby CpG sites, while no correlation was observed in GA/AA genotypes (Table 2). Interestingly, in SAT a statistical interaction effect was observed between the rs2167270 genotype and *LEP* DNA methylation levels (CpG5 [ $p_i = 0.03$ ], CpG14 [ $p_i = 0.01$ ], CpG15 [ $p_i = 0.012$ ], and CpG25 [ $p_i = 0.009$ ]) on *LEP* mRNA levels. We observed that *LEP* DNA methylation levels (CpG14, CpG17, CpG23, CpG24, and CpG25) were negatively correlated with *LEP* mRNA levels in carriers of the A allele, and they tended to be positively correlated in the GG genotype at CpG14 and CpG25. These exploratory analysis results highlight, for the first time, the importance of both genetic and epigenetic variations in the regulation of *LEP* gene expression. Furthermore, they suggest that *LEP* gene expression regulation in SAT and VAT is independent and restricted to specific CpG sites. In VAT, CpG sites located close to CpG11; nearby the TATA box element and the *C/EBP* TFBS appear to be the most important regulator of *LEP* expression whereas in SAT, CpG sites near *SPI* TFBS (CpG16 and CpG17) may be central (Fig. S2). Further studies are needed to determine the contribution of the other SNPs (rs791620 and rs34104384) in the regulation of *LEP* gene expression in both types of adipose tissue. In addition, these preliminary genetic results will need to be confirmed in samples of larger size.

#### DNA methylation and mRNA level analyses at the *ADIPOQ* gene locus

At the *ADIPOQ* gene locus, DNA methylation levels were assessed in the 73 patients at three polymorphic CpG islands (A, C, and E) as previously described (Fig. S3).<sup>4</sup> DNA methylation levels at *ADIPOQ* CpG islands A and C were found to be hypomethylated (<10.0%) and hypermethylated (>90.0%), respectively in blood and both SAT and VAT (Fig. S3). The CpG sites

localized in these CpG islands were considered unmethylated and fully methylated and were thus not further analyzed.

As observed for the *LEP* gene locus, mean DNA methylation levels at *ADIPOQ*-CpGE1, CpGE2, and CpGE3 were also found to be higher in blood ( $91.2 \pm 2.4\%$ ) in comparison to SAT ( $81.2 \pm 2.7\%$ ) and VAT ( $82.8 \pm 3.3\%$ ) (Fig. 1B). At the *ADIPOQ* intron 1 locus, DNA methylation levels at CpGE1 and CpGE3 were highly correlated in SAT ( $r > 0.73$ ;  $P < 0.001$ ) and VAT ( $r > 0.73$ ;  $P < 0.001$ ), whereas in blood, the correlation was modest ( $r > 0.31$ ;  $P < 0.01$ ) (Fig. S5). DNA methylation levels at CpGE2 were the only ones significantly correlated between SAT and VAT after corrections for multiple testing (Table 3). CpGE2 DNA methylation was found to be negatively correlated with *ADIPOQ* mRNA levels in VAT, whereas in SAT, DNA methylation at CpGE3 was associated with *ADIPOQ* mRNA levels (Table 4). No transcriptional regulatory elements were identified at or near *ADIPOQ* CpG island E.

#### DNA methylation variations between CpG sites at the *LEP* and *ADIPOQ* gene loci

We also report that DNA methylation patterns of variation from one CpG site to the following were highly similar between the three tissues at both the *LEP* and *ADIPOQ* gene loci (Fig. 2). These similarities were previously observed by our group in cord blood and placenta samples<sup>5</sup> and by Marchi et al.<sup>26</sup> in the liver, visceral adipocyte, and stromal vascular fraction of white adipose tissue at the *LEP* gene locus. Similarities between inter-individual CpG site variations at specific gene loci have also been reported in several other tissues.<sup>24,35</sup> Following these observations, we previously suggested that DNA methylation levels vary concurrently between tissues around predefined (heritable) values, which seem independent from one CpG site to another.<sup>15</sup> Recently, Brons et al.<sup>36</sup> suggested that a detrimental fetal environment might decrease the acute regulation of the epigenome, which in turn might increase the risk to develop obesity and type 2 diabetes. This likely “predefined” pattern of DNA methylation that we and others have observed might be established early during embryonic development according to the maternal in utero environment and may determine the set point from which stochastic and environmental factors will induce a more or less acute regulation of the epigenome. Since this study was conducted with tissue samples from obese adults, longitudinal and dietary intervention studies will be needed to investigate whether *LEP* and *ADIPOQ* tissue-specific epipolymorphisms and associations are present in the newborn and normal weight populations, whether they predict the development of obesity or other metabolic disorders, and whether they respond to dietary and exercise interventions.

In summary, the current study compared, for the first time, *LEP* and *ADIPOQ* DNA methylation levels in blood and adipose tissues. We report that DNA methylation patterns of variation between one CpG to the next at the *LEP* and *ADIPOQ* gene loci is highly similar between blood, SAT, and VAT, suggesting that a setting point for DNA methylation at each CpG site may exist. In addition, we showed that DNA methylation levels at potentially functional CpG sites are correlated between blood and adipose tissues. DNA methylation levels at or near these CpG sites were

**Table 2.** Pearson correlation coefficients between DNA methylation and mRNA levels at *LEP* gene promoter CpG sites in subcutaneous (SAT) and visceral adipose (VAT) tissues according to rs2167270 genotype and adjusted for sex

	SAT			VAT		
	ALL <sup>a</sup>	GG <sup>a</sup>	GA/AA <sup>a</sup>	ALL <sup>a</sup>	GG <sup>a</sup>	GA/AA <sup>a</sup>
<i>n</i>	73	30	43	73	30	43
<i>LEP</i> -CpG3	-0.079	0.145	-0.215	0.070	0.286	-0.068
<i>LEP</i> -CpG4	-0.093	0.243	-0.294 <sup>†</sup>	0.201	0.266	0.194
<i>LEP</i> -CpG5	-0.060	0.318 <sup>†</sup>	-0.300 <sup>†</sup>	0.173	0.294	0.061
<i>LEP</i> -CpG6	-0.051	0.267	-0.239	0.211 <sup>†</sup>	0.350 <sup>†</sup>	0.093
<i>LEP</i> -CpG7	-0.076	0.228	-0.249	0.320*	0.575**	0.139
<i>LEP</i> -CpG11	-0.056	0.018	-0.070	0.290*	0.390*	0.238
<i>LEP</i> -CpG12	-0.124	0.004	-0.239	0.086	0.281	-0.039
<i>LEP</i> -CpG13	-0.057	0.192	-0.206	0.308*	0.627**	0.125
<i>LEP</i> -CpG14	-0.126	0.324 <sup>†</sup>	-0.497**	0.222 <sup>†</sup>	0.322 <sup>†</sup>	0.147
<i>LEP</i> -CpG15	-0.065	0.328 <sup>†</sup>	-0.285 <sup>†</sup>	0.180	0.281	0.097
<i>LEP</i> -CpG16	-0.093	0.031	-0.173	0.125	0.371*	0.003
<i>LEP</i> -CpG17	-0.172	0.078	-0.367*	0.103	0.212	0.014
<i>LEP</i> -CpG23	-0.168	0.083	-0.340*	0.225 <sup>†</sup>	0.200	0.256
<i>LEP</i> -CpG24	-0.134	0.202	-0.333*	0.114	0.117	0.116
<i>LEP</i> -CpG25	-0.093	0.329 <sup>†</sup>	-0.345*	0.193	0.152	0.207
<i>LEP</i> -CpG26	0.048	0.395*	-0.194	0.268*	0.282	0.264
<i>LEP</i> -CpG27	0.006	0.315	-0.238	0.301*	0.250	0.352
<i>LEP</i> -CpG28	-0.012	0.069	-0.090	0.265*	0.351 <sup>†</sup>	0.180
<i>LEP</i> -CpG29	-0.036	0.203	-0.217	0.252*	0.175	0.322
<i>LEP</i> -CpG30	-0.094	0.117	-0.233	0.215*	0.239	0.212
<i>LEP</i> -CpG31	-0.068	0.079	-0.188	0.172	0.317 <sup>†</sup>	0.102
<i>LEP</i> -MEAN	-0.060	0.268	-0.265 <sup>†</sup>	0.297*	0.372*	0.241

<sup>a</sup>Adjusted for sex. Statistically significant: \*\* $P \leq 0.05$  after corrections for multiple testing; \* $P \leq 0.05$  and <sup>†</sup> $P \leq 0.10$  without correction for multiple testing.

**Table 3.** Spearman rank correlation coefficients between DNA methylation levels at ADIPOQ CpG island E loci in blood, subcutaneous (SAT), and visceral adipose (VAT) tissues

CpG sites	Chromosomal location <sup>a</sup>	SAT vs. VAT	SAT vs. blood	VAT vs. blood
ADIPOQ-CpGE1	chr3:186 562 911	0.241	-0.252	-0.207
ADIPOQ-CpGE2	chr3:186 562 915	0.411**	-0.038	0.145
ADIPOQ-CpGE3	chr3:186 562 950	0.283	-0.019	0.043
ADIPOQ-MEAN	-	0.324	-0.174	-0.024

Statistically significant after corrections for multiple testing: \*\* $P \leq 0.01$  and \* $P \leq 0.05$ . <sup>a</sup>UCSC Genome Browser on Human Feb. 2009 (Feb. 2014).

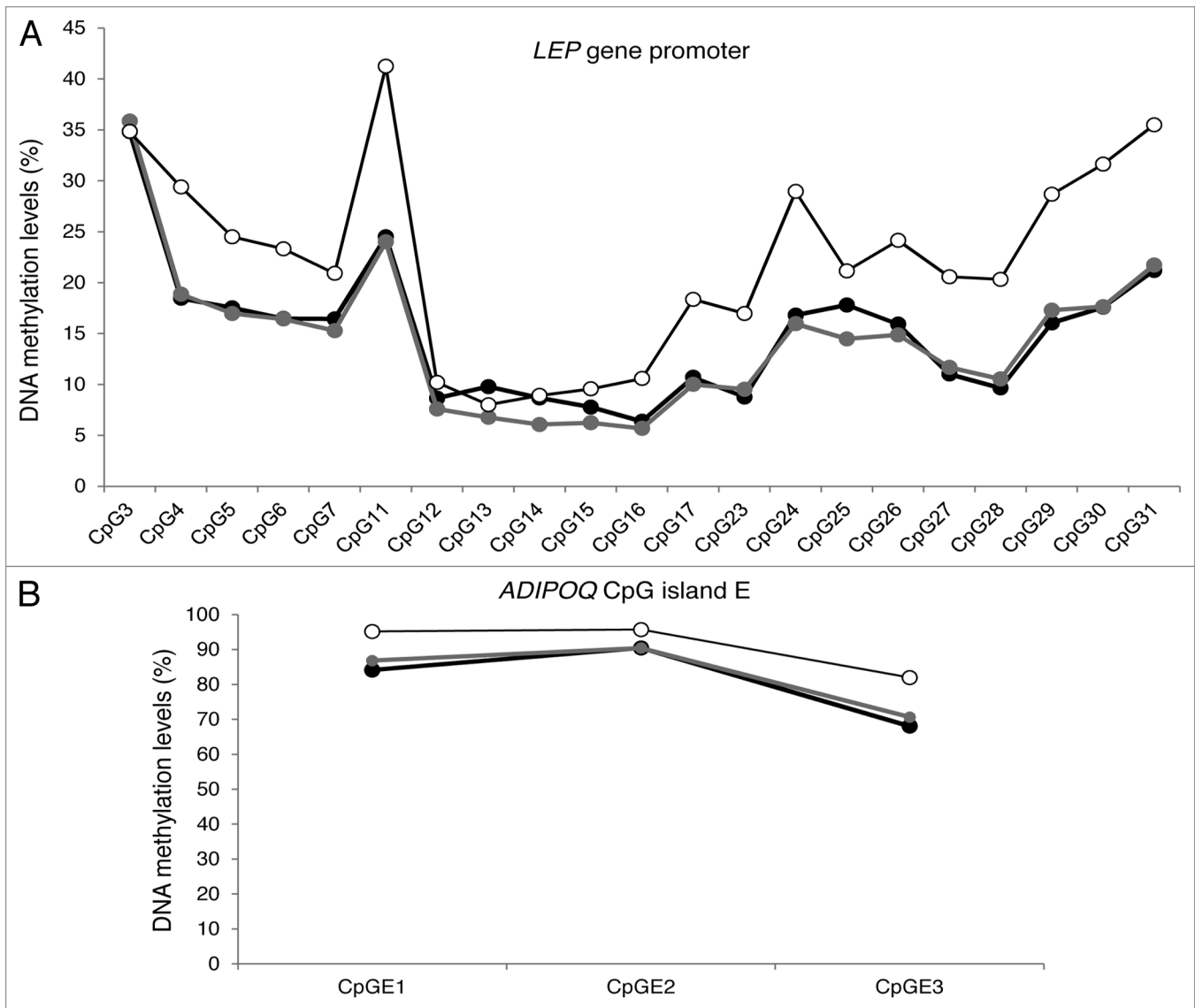
also found to be associated with adipokine gene expression levels. Hence, these specific CpGs might prove useful surrogates for DNA methylation measures, especially with SAT biopsies that require a much less invasive procedure than VAT sampling.

## Materials and Methods

### Patients

Blood, SAT, and VAT samples were obtained from 33 men and 40 pre-menopausal women (obese class III<sup>22</sup>: BMI  $\geq 40$  kg/m<sup>2</sup>) undergoing biliopancreatic derivation to treat obesity.

The 73 patients were free of treatment for dyslipidemia, diabetes, and hypertension. The surgical procedures have been previously described.<sup>37,38</sup> Briefly, fasting blood samples were taken on the morning or the day before the surgery. SAT and VAT samples were obtained at the beginning of the intervention. All samples were stored at  $-80$  °C until nucleic acid extraction. Anthropometric parameters (body weight, height, waist, and hip circumferences), resting blood pressure, and fasting plasma insulin, glucose and lipid levels were also measured before surgery according to standardized procedures.<sup>39</sup> All participants provided a written informed consent before their inclusion in the study, and



**Figure 2.** Average DNA methylation profile by CpG site for *LEP* gene promoter locus (A) and *ADIPOQ* CpG island E gene locus (B) in blood (white circles), subcutaneous (black circles), and visceral (gray circles) adipose tissues ( $n = 73$ ).

all clinical data were denormalized. This project was performed in collaboration with the Tissue bank for the study of obesity and its complications at the Institut Universitaire de Cardiologie et de Pneumologie de Québec. The project was approved by this institution's and the Université Laval's ethics committees and was conducted in accordance with the Declaration of Helsinki.

**Nucleic acid extraction**

DNA was purified from whole blood samples with the Gentra Puregene Blood Kit (Qiagen). DNA and RNA from SAT and VAT were extracted as previously described.<sup>40</sup> RNA quality was assessed with Agilent 2100 Bioanalyzer RNA Nano Chips (Agilent Technologies). Three RNA samples from SAT and VAT had low RNA integrity numbers (RIN < 6.0) and were excluded from the analysis. The other RNA samples in SAT and VAT showed a high quality with mean RIN values of  $8.0 \pm 0.8$  and  $8.3 \pm 0.6$  respectively.

**Table 4.** Pearson correlation coefficients between DNA methylation and mRNA levels at *ADIPOQ* CpG island E in subcutaneous (SAT) and visceral adipose (VAT) tissues

CpG sites	SAT <sup>a</sup>	VAT <sup>a</sup>
<i>ADIPOQ</i> -CpGE1	-0.164	-0.223 <sup>†</sup>
<i>ADIPOQ</i> -CpGE2	-0.078	-0.256*
<i>ADIPOQ</i> -CpGE3	-0.257*	-0.152

<sup>a</sup>Adjusted for sex. Statistically significant: \*\* $P \leq 0.05$  after corrections for multiple testing; \* $P \leq 0.05$  and <sup>†</sup> $P \leq 0.10$  without correction for multiple testing.

**DNA methylation analyses and genotyping**

DNA methylation levels at CpG sites were assessed using pyrosequencing (Pyromark Q24, QIAGEN-Biotage). Combined with the NaBis DNA treatment, pyrosequencing is a quantitative real-time sequencing technology that allows to measure DNA

methylation levels (%) at a single cytosine (CpG) of a given genomic region. NaBis treatment of DNA (EpiTect Bisulfite Kit, Qiagen) specifically converts unmethylated cytosines into uracil, while the methylated cytosines are protected from this transition creating a cytosine/thymine polymorphism. Once treated, NaBis-DNA is amplified (Pyromark PCR kit, Qiagen), and the cytosine and thymine alleles are quantified by pyrosequencing.<sup>41</sup> DNA methylation levels at the *LEP* gene CpG island proximal promoter region were assessed using the PCR and pyrosequencing primers described in Table S3. Specific PCR and pyrosequencing conditions and primer pairs for *ADIPOQ* DNA methylation analyses were the same as previously described.<sup>4</sup>

Pyrosequencing technology was also used for genotyping a single nucleotide polymorphism (SNP; rs2167270; G > A) within the *LEP* proximal promoter region (Fig. S1). The genotype of the 73 patients was determined with the same PCR and sequencing primers as for the methylation analysis of CpG3 to CpG7 (Table S3). The pyrogram signals obtained, representing the amount of G and A nucleotides added to the sequence, allowed the quantification of the two alleles and determination of the genotype.<sup>42</sup> The rs2167270 genotype was identical in all three tissues analyzed for the 73 patients. The genotype frequencies (GG: 30 [41.1%]; GA: 35 [47.9%] and AA: 8 [11.0%]) were found to be under Hardy–Weinberg equilibrium ( $P > 0.05$ ). Carriers of the minor A allele (GA/AA) were grouped together for the analysis since the number of homozygous AA was too low to provide adequate statistical power.

#### Expression analysis and identification of transcription factor binding sites and conserved regions in *LEP* and *ADIPOQ* genes

mRNA levels in SAT and VAT were quantified by quantitative real-time PCR (qRT-PCR). CDNA (cDNA) was generated from total RNA using a random primer hexamer provided with the High Capacity cDNA Archive Kit from Applied Biosystems. Equal amounts of cDNA were run in duplicate and amplified in a 20  $\mu$ L reaction containing 10  $\mu$ L of Universal PCR Master Mix (Applied Biosystems). Primers and Taqman probes were obtained from Applied Biosystems (*LEP*: Hs00174877\_m1 and *ADIPOQ*: Hs00605917\_m1). The *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*: Hs99999905\_m1; Applied Biosystems) housekeeping gene was amplified in parallel. *LEP*, *ADIPOQ*, and *GAPDH* amplifications were performed using the Applied Biosystems 7500 Real Time PCR System, as recommended by the manufacturer (Applied Biosystems). *LEP* and *ADIPOQ* mRNA (Ct) levels were quantified relative to change in *GAPDH* gene expression (Ct). *GAPDH/ADIPOQ* and *GAPDH/LEP* Ct ratios (1/ $x$ ) were used for the analysis.

UCSC genome browser<sup>43</sup> and a PubMed literature search were used to identify consensus sequences of TFBS sequence in the *LEP* gene promoter and *ADIPOQ* intron 1 DNA sequence. The relationship between *LEP* and *ADIPOQ* mRNA regulation and the identified transcription factors was further evaluated with a PubMed literature review.

#### Statistical analyses

The normal distribution of all variables was assessed using a Kolmogorov–Smirnov test. DNA methylation levels at the *ADIPOQ*-CpGE1, E2, and E3 loci in blood were not normally distributed. Hence, DNA methylation levels at the *ADIPOQ* gene locus were compared between blood, SAT, and VAT using the Wilcoxon signed-rank test, whereas paired sample  $t$  test was used for *LEP* gene loci cross-tissue comparisons. In addition, partial Pearson correlation and Spearman correlations were used to determine DNA methylation correlations across the three tissues for the *LEP* and *ADIPOQ* gene loci respectively. The associations between adipokines DNA methylation and mRNA levels were also assessed with partial Pearson correlations. Additionally, two-way ANOVA with interaction terms were used to determine the independent variables contributing to *LEP* mRNA level variance. The Student  $t$  test was used to compare DNA methylation and mRNA levels between *LEP* rs2167270GG genotypes (GG vs GA/AA). Anthropometric characteristics were also compared between men and women using the unpaired Student  $t$  test, while categorical variables were compared using the chi-square statistic. Because a large number of tests was performed, Bonferroni multiple testing corrections were applied. Results were therefore considered statistically significant when  $P$  values  $\leq 4.5 \times 10^{-4}$  ( $P \leq 0.01$ ) and  $P$  values  $\leq 2.3 \times 10^{-3}$  ( $P \leq 0.05$ ) (two-sided). All statistical analyses were performed with the IBM SPSS Statistics 20 software (release 20.0.0, SPSS).

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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#### Supplemental Materials

Supplemental materials may be found here:  
[www.landesbioscience.com/journals/adipocyte/article/28308](http://www.landesbioscience.com/journals/adipocyte/article/28308)

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