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**Case report**                                                                         **Open Access**

# Obesity alone or with type 2 diabetes is associated with tissue specific alterations in DNA methylation and gene expression of *PPARGC1A* and *IGF2*

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## Abstract

**Background**: Epigenetic modifications of key genes have been linked to the development of aging related diseases, such as type 2 diabetes, with increased DNA methylation of the transcriptional co-activator, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A) in islets and skeletal muscle of patients with type 2 diabetes. Here, we examined DNA methylation and gene expression of PPARGC1A and insulin like growth factor-2 (IGF2) in adipose tissue and skeletal muscle of lean and morbidly obese individuals with or without type 2 diabetes.

**Methods**: Adipose tissue and skeletal muscle biopsies were collected from 24 lean, obese, and obese patients with type 2 diabetes (n=8/group). DNA methylation and gene expression of PPARGC1A and IGF2 were measured using pyrosequencing and quantitative real-time PCR respectively.

**Results**: DNA methylation and expression of both genes varied in a tissue specific manner (P<0.05). The highest levels of PPARGC1A methylation were observed in subcutaneous adipose tissue and lowest in muscle (P≤0.001), whereas IGF2 methylation was lowest in subcutaneous adipose tissue as compared with visceral adipose tissue and muscle (P≤0.04). Expression of PPARGC1A and IGF2 was highest in muscle and lowest in subcutaneous adipose tissue (P≤0.001) and PPARGC1A expression was conversely correlated with DNA methylation in skeletal muscle (r=-0.54, P=0.008). Obese patients with type 2 diabetes had higher PPARGC1A methylation in subcutaneous adipose tissue (P=0.01) and lower IGF2 DNA methylation in muscle (P=0.01) as compared with lean individuals. Obese patients with and without type 2 diabetes had reduced expression of both genes in subcutaneous adipose tissue (P≤0.04) as compared to lean individuals.

**Conclusions**: This study showed tissue specific DNA methylation and gene expression of PPARGC1A and IGF2, which may also be associated with obesity and type 2 diabetes. Further study of the effects of tissue specific DNA methylation on risk of obesity and type 2 diabetes in a larger cohort is now warranted.

**Keywords**: DNA methylation, Gene expression, *PPARGC1A*, *IGF2*, Type 2 diabetes, Obesity

## Background

An increasing number of studies suggest that epigenetic modifications in certain genes are associated with subsequent disease susceptibility later in life, both in humans and in animal models [1-4]. For instance, Godfrey *et al.,* reported that lower maternal carbohydrate intake in early pregnancy was associated with increased methylation of retinoid X receptor-α gene in umbilical cord tissue, and higher incidence of childhood adiposity at age 9 years [5]. Supporting this, Relton *et al.*, showed the association between altered DNA methylation patterns in cord blood at birth and later adiposity in childhood, with perturbed gene expression [6]. Individuals conceived during the "Dutch Hunger Winter" famine also had an elevated prevalence

of obesity, insulin resistance and cardiovascular disease [1], with reduced DNA methylation of imprinted gene, insulin-like growth factor 2 (*IGF2*), and increased DNA methylation of non-imprinted genes leptin and interleukin-10 as adults, as compared with their unexposed same-sex siblings [7,8]. Whether altered DNA methylation and expression of key genes occurs in peripheral insulin sensitive tissues is a source of current investigation.

Recent studies have demonstrated increased promoter DNA methylation and decreased gene expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PPARGC1A*) in pancreatic islets [9], in skeletal muscle from patients with type 2 diabetes [10], and in liver biopsies from individuals with non alcoholic fatty

liver disease [11]. Low birth weight, potentially reflecting *in utero* deprivation, was also associated with increased *PPARGC1A* DNA methylation in skeletal muscle in adults at increased risk of diabetes [12]. *PPARGC1A* is a master regulator of mitochondrial biogenesis and oxidative phosphorylation [13], and is linked to development of insulin resistance and type 2 diabetes [14]. Although reduced *PPARGC1A* expression in adipose tissue has been reported in insulin resistance [15-17], whether DNA methylation is also altered in this tissue is unknown. Furthermore, whether similar methylation changes in peripheral tissues exist in obese individuals without type 2 diabetes is also unclear.

*IGF2* is a key factor in human growth and development and is imprinted through the differentially methylated region (DMR), thus only the paternal allele is expressed. Aberrant imprinting of *IGF2* gene is associated with the overgrowth disorder Beckwith-Wiedemann syndrome [18] and the growth restriction disorder Silver-Russell syndrome [19]. Hypomethylation of *IGF2* DMR0 which is located 5'to the main *IGF2* promoters in humans [20] leads to bi-allelic expression of *IGF2* [21] and is associated with human colorectal cancer, Wilms tumour [21-23], and has also been linked to later development of obesity and insulin resistance in individuals exposed to famine in utero [1]. To date, no studies have examined *IGF2* DMR0 methylation and gene expression in adipose tissue and skeletal muscle from patients with obesity and type 2 diabetes.

The present study aimed to determine the tissue specificity of DNA methylation and expression of *PPARGC1A* and *IGF2* and their association with obesity, in the presence and absence of type 2 diabetes, compared with lean individuals, and also, to examine the relationships between DNA methylation patterns and gene expression. The data showed that DNA methylation and gene expression of *PPARGC1A* and *IGF2* occurs in a tissue specific fashion, and that altered DNA methylation and expression of both genes appears to be associated with obesity and type 2 diabetes.

## Methods
### Subjects
The characteristics of 24 subjects are presented in **Table 1**. Rectus abdominus muscle, subcutaneous adipose tissue and omental adipose tissue biopsies were obtained from eight obese individuals without type 2 diabetes and eight diet controlled obese patients with type 2 diabetes (four females and four males for each group) who were undergoing gastric bypass surgery for the treatment of morbid obesity. Rectus abdominus muscle and abdominal subcutaneous adipose tissue were obtained from four lean males who were undergoing hernia surgery. Vastus lateralis muscle and abdominal subcutaneous adipose tissue were collected from four lean female volunteers [24,25]. All tissues were snap frozen for later analysis. The study was approved by the human research ethics committees of the University of

Adelaide, Calvary Hospital, and Burnside Hospital. Informed consent was obtained from all participants.

### DNA and RNA extraction
Genomic DNA and total RNA were extracted from adipose tissue (120-150 mg) and muscle tissue (10-30mg) using a QIAamp DNA Mini kit (Qiagen, Valencia, CA) and Trizol (Invitrogen, USA) respectively following manufacturer's instructions. The concentration and purity of DNA and RNA were determined by Nanodrop (Thermo Fisher Scientific, California, USA).

### Bisulphite modification
0.4 - 1μg genomic DNA was bisulphite treated to convert unmethylated cytosines to uracil using the MethylEasy™ Xceed Rapid DNA Bisulphite Modification Kit (Human Genetic Signatures Pty Ltd, NSW, Australia) as per the manufacturer's instructions. All samples were bisulphite treated simultaneously to prevent possible batch effects. There was insufficient subcutaneous adipose tissue sample in one lean and one obese muscle sample for bisulphite conversion.

### Nested touchdown PCR
A nested touchdown PCR strategy was used to amplify bisulphite treated genomic DNA. PCR primers were designed to amplify regions containing specific CpG sites using Assay Design Software v1.0.6 (Biotage, Uppsala, Sweden).

5 CpG dinucleotides sites in the promoter of the *PPARGC1A* gene were examined (**Supplement figure 1**). The outer primers amplify the region on the minus strand of chromosome 4:23892115-23892603 (UCSC BLAT results from Assembly Feb 2009 (GRCh37/hg19)) to yield a 489 bp product, and the inner primers with biotinylated reverse primer and first-round product as template amplify the region minus strand chromosome 4:23892285-23892570 to yield a 286 bp product. The first 4 of these CpG sites were previously measured by Ling *et al.*, in human islets [9] and Brons *et al.,* in human muscle [12]. The 5th CpG site is identical to that measured by Gemma *et al.,* in umbilical cord blood [26].

**Table 1. Clinical characteristics of the subjects**

|  | Lean | Obese | Obese with diabetes | P value |
|---|---|---|---|---|
| Age (years) | 31 ± 6* | 49 ± 3 | 48 ± 2 | 0.005 |
| BMI | 22.3 ± 0.6* | 47.6 ± 1.8 | 46.3 ± 1.8 | <0.001 |
| Fasting Glucose (mmol/L) | 5.2 ± 0.6 | 4.9 ± 0.3 | 8.5 ± 1* | 0.007 |
| Total cholesterol (mmol/L) | 5.1 ± 0.2 | 5.2 ± 0.3 | 5.4 ± 0.4 | 0.76 |
| Triglycerides (mmol/L) | 1.5 ± 0.2 | 1.3 ± 0.1 | 2.6 ± 0.8 | 0.37 |

Data are presented as mean ± SEM. *denotes P<0.01 of the asterisked marked group as compared to both other groups.

5 CpG sites in the *IGF2* DMR0 (chr11:2,126,035-2,126,372 in NCBI build 36.1) [27] were also examined (**Supplement figure 1**). The outer primers amplify the region on Chromosome 11(+ strand): 2169429-2169753 (UCSC BLAT results from Assembly Feb 2009 (GRCh37/hg19)) to yield a 325 bp product. The inner primers with biotinylated reverse primer and first-round product as template amplify the region Chromosome 11:2169452-2169751 to yield a 300 bp product. These sites have been previously quantified by others: all 5 on the minus strand by Steegers-Theunissen *et al.* [27], and the first 3 on the plus strand by Murrell *et al.,* [23] both in peripheral blood.

All PCR reactions for one tissue were completed simultaneously with the ABI 7500 sequence detection system using two MicroAmp® fast 96-well reaction plates (Applied Biosystems, Foster City, CA). Each PCR reaction also included a no template negative control and unmodified DNA control (Qiagen, Valencia, CA). Only samples with single target size bands without primer dimers were used for pyrosequencing.

### Pyrosequencing
Quantification of cytosine methylation percentage was carried out on a PyroMark MD Pyrosequencing System (Biotage, Uppsala, Sweden) using PyroMark Gold Q96 CDT Reagents 972824 (Qiagen, Valencia, CA). Specific pyrosequencing primers (**Supplement table 1**) were designed using Biotage Assay Design Software v1.0.6 (Biotage, Uppsala, Sweden). For both genes, two different pyrosequencing assays utilized different forward sequencing primers, with the first to sequence the region containing the first 3 CpG's of interest, and the second to analyse the 4th and 5th CpG's of interest. Methylation was quantified using Pyro Q-CpG 1.0.9 Software (Biotage, Uppsala, Sweden) which calculated the percentage of methylated cytosines to total cytosines at each CpG site. The mean of five methylated sites was calculated for each sample.

### Quantitative real-time PCR
cDNA was synthesized from 1μg of each RNA sample in 20ul reactions using the QuantiTect reverse transcription kit (Qiagen, Valencia, CA) consistent with the manufacturer's protocol. Standard control samples (25ng/μl) pooled from each cDNA were diluted to create a standard curve. There was insufficient subcutaneous adipose tissue sample in two obese patients, and one visceral adipose tissue and one muscle sample in the obese group.

Quantitative real-time PCR was performed with the ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA) using TaqMan primers and probes for *IGF2* (Hs00171254_m1), *PPARGC1A* (Hs01016719_m1) and Beta-actin (Hs99999903-ml) (Applied Biosystems) according to manufacturer's instructions. To calculate the normalized relative expression levels, the individual expression levels of each sample were divided by the expression values of the beta-actin gene, which was not different by groups.

### Statistics
Quantitative data were presented as mean ± SEM unless indicated. Differences between groups were assessed by one-way ANOVA followed by a Fisher's LSD post hoc test. The effect of obesity on methylation was investigated by linear mixed models, with tissue, individual site methylation, group and sex as between factors and age as a covariate, Bonferroni post hoc test as appropriate. Correlations were calculated using Pearson correlation coefficients for normally distributed values and Spearman correlation coefficients when normality was rejected. Statistical analyses were performed with SPSS 18.0 (SPSS Inc., Chicago, IL, USA). All P values were 2-sided, and P≤0.05 was considered significant.

### Results
#### Clinical data
Lean patients were aged between 18-63 years, while obese and obese patients with type 2 diabetes were between 41-56 years of age (**Table 1**). Obese and obese patients with type 2 diabetes had higher body mass index (BMI) than those who were lean, whereas obese patients with type 2 diabetes had increased fasting plasma glucose compared to lean and obese patients (**Table 1**). Plasma lipids, including total cholesterol and triglycerides did not vary between groups.

#### DNA methylation and gene expression of *PPARGC1A*
DNA methylation of *PPARGC1A* varied significantly between tissues, with the highest DNA methylation in subcutaneous adipose tissue and lowest in muscle (**Figure 1A**). We also observed the highest expression of *PPARGC1A* in muscle and the lowest expression in subcutaneous adipose tissue, consistent with these tissue specific patterns of methylation (**Figure 1B**). Overall, there was also a significant negative relationship between average DNA methylation and gene expression of *PPARGC1A* gene in muscle (**Figure 1C**), but not in either visceral or subcutaneous adipose tissue (data not shown). DNA methylation of *PPARGC1A* in muscle was negatively correlated with age (**Figure 1D**).

In subcutaneous adipose tissue, obese patients with type 2 diabetes had increased DNA methylation of *PPARGC1A* compared with lean individuals (P=0.01, **Figure 1E**), and decreased *PPARGC1A* expression was also observed in obese patients with or without diabetes as compared to lean individuals (P=0.001, P=0.04, respectively) (**Figure 1F**). *PPARGC1A* DNA methylation or gene expression in visceral adipose tissue or muscle tissue did not vary between groups however (data not shown).

#### DNA methylation and gene expression of IGF2
As expected for an imprinted gene, DNA methylation of *IGF2* was higher than that of *PPARGC1A*. *IGF2* methylation was lowest in subcutaneous adipose tissue as compared to visceral adipose tissue or skeletal muscle (**Figure 2A**). The expression of *IGF2* was lower in visceral adipose tissue than in muscle (P≤0.001) (**Figure 2B**). *IGF2* methylation and
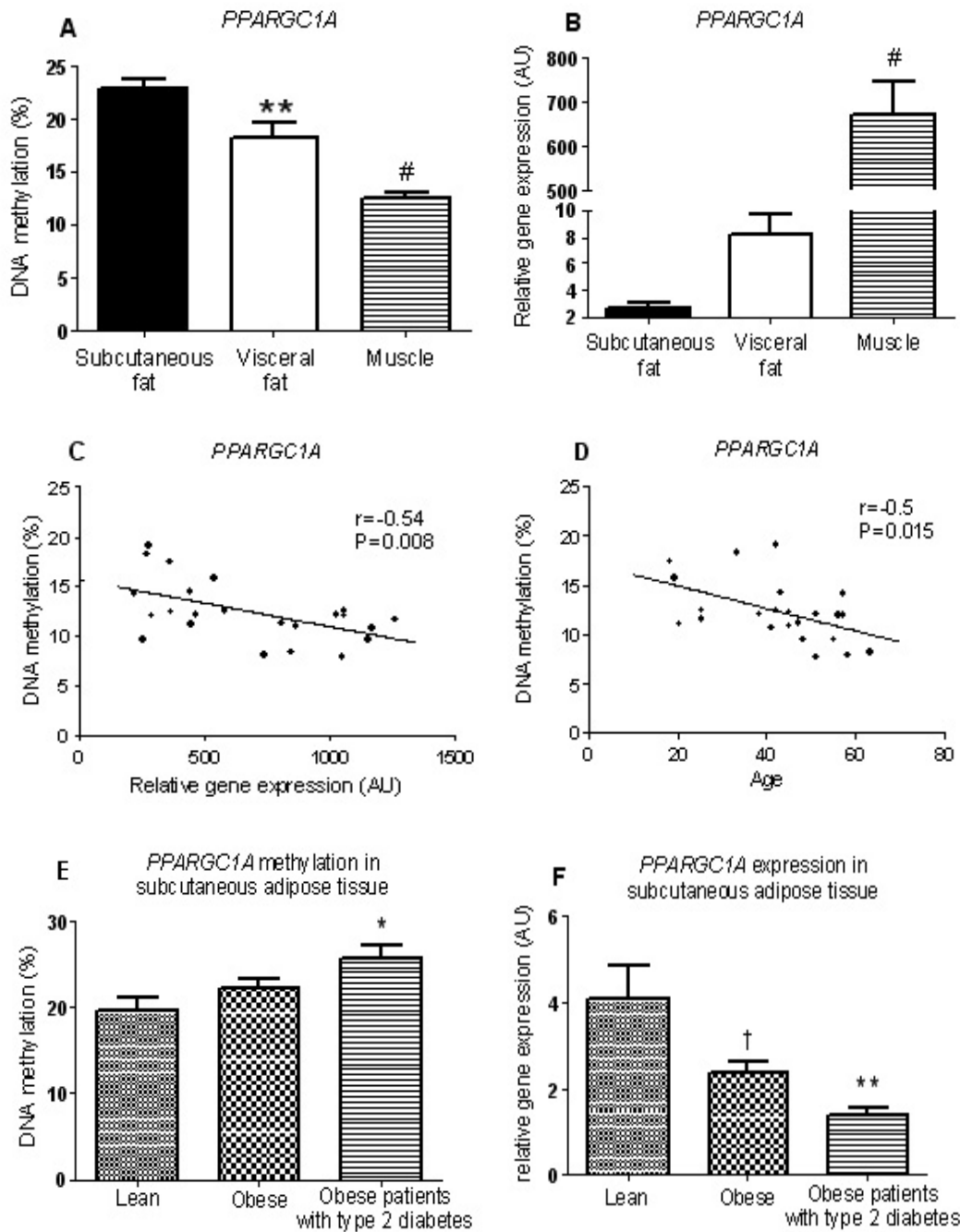
**Figure 1**. *PPARGC1A* DNA methylation and gene expression in muscle, subcutaneous and visceral adipose tissue. (1A) PPARGC1A DNA methylation levels, ** denotes significance between subcutaneous and visceral adipose tissue at P=0.001; # denotes significance between muscle and both subcutaneous and visceral adipose tissues at P<0.001; (1B) *PPARGC1A* expression in muscle is significantly higher than subcutaneous and visceral adipose tissue at P<0.001; *PPARGC1A* DNA methylation was negatively related to its expression (1C) and age (1D) in muscle; (1E) Obese patients with type 2 diabetes had higher DNA methylation of PPARGC1A in subcutaneous adipose tissue as compared to lean and (1F) obese patients with and without diabetes had lower *PPARGC1A* expression in subcutaneous adipose tissue as compared with lean individuals. *P=0.01; **P=0.001; # P<0.001; † P=0.04. AU: arbitrary units.

expression in muscle were also significantly correlated with age (**Figure 2C** and **2D**). In skeletal muscle, obese patients with type 2 diabetes had lower DNA methylation than lean individuals (59±2% vs 69±2%, P=0.01), but this was not significant between obese and lean individuals (P=0.1) (**Figure 2E**). *IGF2* methylation in adipose tissue was not different between groups (data not shown), but *IGF2* expression was lower in subcutaneous adipose tissue in obese patients with or without type 2 diabetes as compared with lean individuals (**Figure 2F**). There was no association between DNA methylation and expression of *IGF2* in any tissue examined (data not shown).

## Discussion

Epigenetic modifications in key genes may be important in the development of obesity and type 2 diabetes. In this study, we investigated the expression and DNA methylation of *PPARGC1A* and *IGF2* in subcutaneous and visceral adipose tissues and skeletal muscle from lean, obese and obese individuals with type 2 diabetes. Interestingly, we observed that *PPARGC1A* and *IGF2* gene had tissue specific differences in DNA methylation and expression, and that these may be influenced by obesity type 2 diabetes and aging.

DNA methylation plays a key role in regulating gene expression via the addition of a methyl group to the carbon-5 position of the Cytosine-phosphate-guanine (CpG) dinucleotide sequence in promoter regions, which interferes with the binding of particular transcription factors to DNA [**28**]. Our results indicate that higher DNA methylation was related to lower gene expression of *PPARGC1A* in a tissue specific manner. To our knowledge, this is the first study to demonstrate that both subcutaneous and visceral adipose tissue had significant higher DNA methylation of *PPARGC1A* compared with muscle in humans. Supporting this, our data also show a significant negative correlation between average DNA methylation and gene expression of *PPARGC1A* across the whole cohort in muscle, although this was not observed in adipose tissue. This is broadly consistent with Barres *et al.,* who showed methylation levels of *PPARGC1A* promoter were negatively correlated with mRNA expression and mitochondrial DNA in skeletal muscle that was collected from patients with or without type 2 diabetes [**10**]. Of interest, Ling *et al.,* observed a similar correlation between *PPARGC1A* promoter methylation and mRNA expression in islets from patients with type 2 diabetes [**9**]. It is not clear why this association is also not seen in adipose tissue in our study, and may be a reflection of tissue specific factors that separately influence expression.

Previous studies have shown increased promoter DNA methylation of *PPARGC1A* in skeletal muscle and islets in patients with type 2 diabetes [**9,10**]. Our data also showed this association, but only in subcutaneous adipose tissue. This may be related to different CpG sites being selected for study and the different DNA methylation detection methods employed. We did observe reduced *PPARGC1A*

expression in subcutaneous adipose tissue in obese patients with type 2 diabetes. Reduced *PPARGC1A* expression has been reported in both visceral and subcutaneous adipose tissues as well as muscle from patients with type 2 diabetes in many studies previously [**10,16,29,30**]. It is not clear why these associations were not observed in visceral adipose tissue, and again may be a reflection of size of cohort or other tissue specific factors that influence expression.

We next examined DNA methylation and expression patterns of *IGF2*. Firstly and as expected, we detected higher DNA methylation in paternally imprinted *IGF2* gene as compared to the non-imprinted *PPARGC1A gene* [**7,9,12,23,31**]. We also observed visceral adipose tissue and muscle had higher DNA methylation than subcutaneous adipose tissue. However the changes in methylation were not accompanied by inverse changes in expression of IGF2, in that muscle also had higher expression of *IGF2*. However, this is not necessarily contradictory, as shown by Murrell *et al.* [**23**], who reported that DNA methylation of the *IGF2* DMR0 examined here, may play a role in activation and not suppression of gene expression. This relationship is complex and still requires further investigation, as the expression of the human *IGF2* gene is controlled at least by 5 promoters [**20,32,33**].

To date, few studies have examined associations between DNA methylation and gene expression of *IGF2* in obesity and type 2 diabetes. Heijmans *et al.,* showed individuals who were prenatally exposed to the Dutch famine had lower methylation of *IGF2* in blood and increased prevalence of obesity, insulin resistance and type 2 diabetes in later life [**7,34**-**36**]. In our study, patients with type 2 diabetes also had lower *IGF2* DNA methylation in muscle as compared to lean individuals. We also observed lower expression of *IGF2* in subcutaneous adipose tissue in obese and obese patients with type 2 diabetes as compared to those who were lean. Together these results suggest that DNA methylation and gene expression of *IGF2* may be associated with obesity and type 2 diabetes, but this result needs to be confirmed in a larger, age-matched cohort.

Interestingly, our data showed that aging may be associated with decreased DNA methylation of both genes and increased *IGF2* expression in muscle. Supporting this, Heijmans *et al.,* demonstrated *IGF2* methylation in blood cells decreased 4% in 10 years within the age range of 43–70 years [**7**]. In contrast, Issa *et al.,* have reported increased methylation of the *IGF2* promoter which switched from monoallelic to biallelic methylation during aging in adult human colon, breast and lung tumors [**37**]. However, Cui *et al.,* reported leukocyte *IGF2* methylation was stable to middle age [**38,39**]. A recent longitudinal study also showed DNA methylation levels in five of eight imprinted loci were stable over 2–20 years in blood and buccal cell samples [**40**]. These results indicate tissue specific difference with aging in humans. Similarly, genome-wide analysis of DNA methylation in rats demonstrated significant
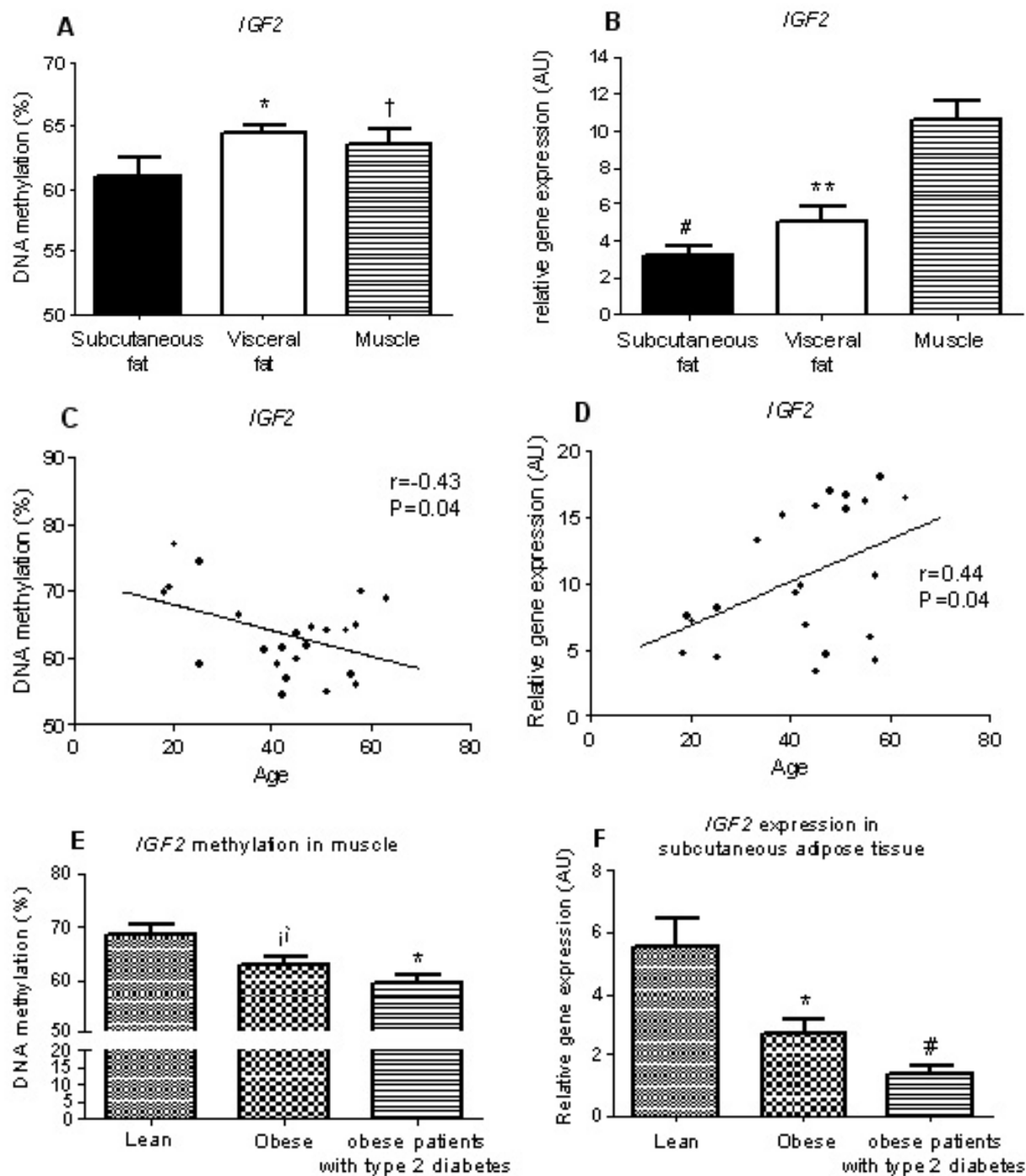
**Figure 2. IGF2 DNA methylation and gene expression in muscle, subcutaneous and visceral adipose tissue**.
(2A) Lower DNA methylation was observed in subcutaneous adipose tissue than visceral adipose tissue and muscle; (2B) Lower IGF2 expression was observed in subcutaneous adipose tissue and visceral adipose tissue versus muscle; (2C-2D) Age was negatively correlated with IGF2 DNA methylation (2C) and positively correlated with IGF2 expression (2D) in muscle; (2E) Patients with type 2 diabetes had significant lower IGF2 DNA methylation in muscle; (2F) IGF2 expression was decreased in obese and obese patients with type 2 diabetes in subcutaneous adipose tissue.
*P=0.01; **P=0.001; # P<0.001; † P=0.04; §P=0.1. AU: arbitrary units.

tissue differences and age-related tissue specific changes, including hypomethylation in intra-abdominal tissues and hypermethylation in liver [**41**]. However, care should be taken when comparing results of these various studies in humans, since different assays, tissues, and the examined loci varied by study [**42**].

## Conclusions

This study demonstrates tissue specific DNA methylation and gene expression of *PPARGC1A* and *IGF2*, which may be related to aging, obesity and type 2 diabetes. The strong negative correlation between DNA methylation and gene expression of *PPARGC1A* indicates epigenetic regulation may play a key role in expression of this gene, at least in muscle. These outcomes provide the rationale for further study of a larger cohort and emphasize the importance of assessing DNA methylation of these genes at least, in a tissue specific manner in humans.

## Additional files

**Supplement figure 1**
**Supplement table 1**

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

MC: carried out the epigenetic experiments; participated in the acquisition, analysis and interpretation of the data and drafted the manuscript.
AM: designed the pyrosequencing assays, participated in the acquisition of pyrosequencing data, and helped to draft the manuscript.
JO: participated in the design of the study and analysis and interpretation of the data and helped to draft the manuscript.
GW: participated in the conception and design of the study and helped to draft the manuscript.
LKH: participated in the conception and design of the study, revised the manuscript critically for important intellectual content and gave final approval of the version to be published. All authors read and approved the final manuscript.

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