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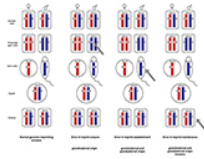
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RESEARCH PAPER



Methylation of imprinted *IGF2* regions is associated with total, visceral, and hepatic adiposity in postmenopausal women

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ABSTRACT

Excess body fat, especially intra-abdominal fat, is a leading risk factor for metabolic diseases. Differentially methylated regions (DMRs) of two imprinted genes, *insulin-like growth factor 2 (IGF2)* and *H19*, have been associated with obesity due to their important roles in regulating body composition, but have not been examined in relation to intra-abdominal fat depots. Total body fat from whole-body dual energy X-ray absorptiometry and visceral and liver fat contents from abdominal magnetic resonance imaging in 48 healthy women aged 60–65 years (of White or Japanese ancestry) were each regressed on circulating leukocyte DNA methylation levels of *IGF2* (at DMR0, DMR2a, and DMR2b) and *H19* (at CTCF3) as assessed by pyrosequencing, while adjusting for age and race/ethnicity. Total fat mass was inversely associated with methylation levels of *IGF2* DMR2b ($P = 0.016$). Total fat-adjusted visceral fat area ($P = 0.062$) and percent visceral fat measured at L4-L5 ($P = 0.045$) were associated with higher methylation levels of *IGF2* DMR2b. Both total fat-adjusted percent liver fat ($P = 0.039$) and the presence of fatty liver ($P = 0.015$) were positively associated with *IGF2* DMR2a methylation. Methylation levels of *H19* CTCF3 were not associated with overall or intra/abdominal adiposity. The findings indicate that methylation levels of *IGF2* DMR regions in leukocytes are associated with total body fat and with fat distribution in the viscera and liver independently of total adiposity.

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Introduction

Obesity is currently one of the most serious epidemics in the US and globally [1,2]. It is well established that obesity increases the risk of diabetes, cardiovascular disease [3,4], and various cancers [5–8]. While body mass index (BMI) [weight (kg)/height (meter)²] is often used as a surrogate measure of body fatness in obesity studies [9], it does not differentiate body composition [10]. Further, body fat amounts in the abdomen and intra-abdominal cavity, compared to fat in the extremities and subcutaneous layers, has been associated with a greater risk of metabolic disturbances [11–15]. Also, the proportion of the total fat stored as high-risk fat depots, such as viscera fat or liver fat, has been observed to vary among individuals, most notably by sex and race/ethnicity [16]. Such variations in body fat distribution have been only partially explained by genetic susceptibility: for example, genotypes for the known risk variant in the

PNPLA3 gene explained ~ 5% of the variation in non-alcoholic fatty liver disease (NAFLD) [17].

Epigenetic factors may also be involved in the regulation of total and regional adiposity [18]. One of the hypothesized molecular mechanisms involved in the life-long effects of early-life obesity and heritability of parental obesity is through epigenetic alterations in imprinted regions [19–22]. Imprinted genes are particularly vulnerable to epigenetic deregulation because only one allele is functional. The DNA methylation status of two imprinted genes on chromosome 11p15.5, namely *insulin-like growth factor 2 (IGF2)* and *H19*, the most-studied epigenetically imprinted genes, has been associated with greater body weight and metabolic diseases [19–22]. Specifically, methylation levels in the differentially methylated regions (DMRs) of these imprinted genes have been implicated in birth weight and intrauterine hyperglycemia [20], skinfold thickness [21], insulin resistance [22],

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insulinomas [23], and high-fat diet-induced obesity [24] in both animal and human epidemiological studies. Past studies of *IGF2/H19* methylation and adiposity have been mostly based on body weight or BMI. Accurate measurements of total adiposity and high-risk fat depots would provide a more valid assessment of the potential associations. As current gold standards, dual energy X-ray absorptiometry (DXA) is used to estimate total and regional fat mass in the trunk and extremities [25], and computed tomography (CT) and magnetic resonance imaging (MRI) are performed to determine the amounts of visceral fat in the intra-abdominal cavity and ectopic fat in organs such as the liver [26,27].

The goal of this study was to investigate blood DNA methylation levels of *IGF2* and *H19* DMR regions in relation to DXA and MRI-based overall and relative intra-abdominal adiposity in 48 healthy post-menopausal women with a wide range of adiposity.

Materials and methods

Study participants

Sixty women aged 60–65 years (30 White and 30 Japanese American) were recruited in 2009 from Oahu residents among the ongoing Multiethnic Cohort Study participants. Detailed recruitment and data collection methods are described elsewhere [28–30]. Briefly, participants were recruited stratified by 5 BMI categories (18.5–21.9, 22–24.9, 25–26.9, 27–29.9, and 30–35 kg/m²) based on self-reported weight and height within each racial/ethnic group to optimize the comparison of body fat distribution between the two ethnic groups with different adiposity profiles. Technician-measured BMI ranged between 18.8 and 39.6 kg/m² and included Class I and II obesity [31]. Exclusion criteria included: current or recent smoking (<2 years); use of medications that may interfere with metabolism and body composition (chemotherapy, insulin or weight-loss prescriptions); substantial weight change (loss or gain of 20 pounds or more) in the past 6 months; pre- or peri-menopausal status; and any soft or metal implants/objects in the body or claustrophobia that could interfere with the MRI protocol. Participants underwent anthropometric measurements, a DXA scan, and a fasting venous blood collection at the University of Hawaii Clinical Research Center. Forty-eight of the 60 women (28

White and 20 Japanese American) also participated in an MRI scan at the University of Hawaii and Queen's Medical Center MR Research Center. The study was approved by the Institutional Review Boards of the University of Hawaii and the Queen's Medical Center, Honolulu, and all participants signed an informed consent.

Body composition assessment

A whole-body DXA scan (GE Lunar Prodigy, Madison, WI) was performed to estimate total fat mass and regional fat mass in the trunk, arms, and legs, as well as lean mass and bone mineral content. An abdominal MRI scan was obtained on a 3 Tesla TIM Trio scanner (Siemens Medical Systems, Erlangen, Germany; software version VB13) as detailed previously [19,20]. Visceral fat area (cm²) at the L4-L5 intervertebral position was estimated from an axial gradient-echo sequence with breath holds by measuring the area of the intra-abdominal adipose tissue, including intra-peritoneal and retro-peritoneal fat. Liver fat content, as percent of volume, was estimated in the lateral segment of the right lobe of the liver using a series of axial triple gradient-echo Dixon-type scans [27,32].

DNA methylation analysis

Genomic DNA (gDNA) was isolated from fasting blood buffy coat stored at –80°C using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) and 500 ng of the gDNA was treated with sodium bisulfite using the EZ DNA Methylation-Direct Kit (Zymo Research, Orange, CA) according to the manufacturer's instructions. Bisulfite treated gDNA (20 ng) was amplified with a primer set for each DMR [23] using PyroGold reagents (Qiagen). The list of target DMRs in *IGF2* and *H19* and the individual CpGs in each DMR is presented in Supplementary Table 1. Shortly, 3 CpGs in *IGF2* DMR0 (NCBI GeneBank: Y13633.1, Nt 626–799; converted genome coordinates to UCSC Genome Browser (hg19): chr11:2,169,467–2,169,640), 18 CpGs (DMR2a, NCBI GeneBank: AC130303.8, Nt 155440–155238; converted genome coordinates to UCSC Genome Browser (hg19): chr11:2,154,754–2,154,956) and 9 CpGs (DMR2b, NCBI GeneBank: AC130303.8, Nt 155,737–155,911; converted genome coordinates to UCSC Genome Browser (hg19):

chr11:2,154,283-2,154,457) in two amplification products covering the DMR2, and 11 CpGs in the third CTCF binding region of the *H19* DMR were analyzed [22,23]. The quantification of methylation levels was carried out on a PyroMark Q24 system (Qiagen). A fully methylated and fully unmethylated DNA set was included in each batch as positive and negative controls (Zymo Research). Blind duplicate quality control samples were included on each plate to estimate inter-assay variation: the coefficient of variation (CV) was 0.7–2% for all assays.

Statistical analyses

All statistical analyses were performed using JMP 10 software (SAS Institute Inc., Cary, NC). The main adiposity variables of interest were total body fat mass (kg) for overall adiposity, trunk fat mass (kg) for abdominal adiposity, percent visceral fat area over total abdominal area at L4-L5 cross-section for visceral adiposity, and percent liver fat for hepatic adiposity, all measured on a continuous scale. Also, presence of fatty liver was defined based on liver fat content above 5.5% [33]. Methylation status (range 0–100%) at each DMR locus, as well as the average for each DMR region CpG (at *IGF2* DMR0, DMR2a, DMR2b; *H19* CTCF3), was determined. The association between DMR methylation and adiposity was assessed with multiple linear regression models (for continuous adiposity variables) or logistic regression models (for binary fatty liver), with each adiposity variable as the outcome and each DMR methylation measurement as the predictor. Models were adjusted for age and race/ethnicity since they are known to be important determinants of total body fat and distribution [34]: adjusting for height was considered, but not retained in the models since it was not associated with adiposity and did not confound the methylation-adiposity associations. Models for regional fat in the trunk, viscera, and liver were additionally adjusted for DXA total fat mass for associations with methylation independently of overall adiposity.

Results

Characteristics of the study participants are shown in Table 1. The women had a broad range of measured

Table 1. Characteristics of study participant women (n = 48).

Characteristics	Mean (SD; range) or n (%)
Age, years	63.3 (1.4; 61.9–64.7)
Ethnicity	
Japanese American	20 (42%)
White	28 (58%)
Smoking history	
Never	28 (58%)
Former	20 (42%)
Height, cm	157.8 (5.5; 143.1–166.8)
Body mass Index, kg/m ²	26.9 (5.1; 18.8–39.6)
Total fat mass, kg	27.4 (9.4; 11.1–53.5)
Percent total fat, %	40.4 (7.1; 22.2–53.6)
Trunk fat, kg	14.6 (5.8; 4.3–29.2)
Visceral fat ¹ , %	20.8 (8.0; 4.6–39.9)
Visceral fat area at L4L5, cm ²	138 (94; 16–502)
Liver fat, %	6.2 (5.6; 1.5–20.9)
Fatty liver (>5.5% liver fat)	17 (35%)
DNA methylation	
<i>IGF2</i> DMR0, %	42.1 (3.3; 34.3–50.0)
<i>IGF2</i> DMR2a, %	54.8 (7.3; 36.2–78.1)
<i>IGF2</i> DMR2b, %	51.9 (5.7; 41.0–65.1)
<i>H19</i> CTCF3, %	46.2 (2.6; 42.1–59.2)

¹ fat as % of total abdominal cross sectional area at L4/L5.

BMI (18.8–39.6 kg/m²) and overall adiposity (total fat mass, 11.1–53.5 kg), as well as regional adiposity in the trunk, viscera, and liver (Table 1). As reported previously [29], the women of White and Japanese ancestry had comparable mean overall adiposity as a result of the study design and, yet, Japanese American women had significantly greater amounts of abdominal, visceral, and liver fat compared to Whites. The methylation levels of the analyzed DMRs in *IGF2* and *H19* showed limited variation for the 41 analyzed individual CpGs (Supplementary Table 1) and for the DMR regions on average (Table 1): 42.1% ($\pm 3.3\%$) for DMR0, 54.8% ($\pm 7.3\%$) for DMR2a, and 51.9% ($\pm 5.7\%$) for DMR2b in *IGF2*, and 46.2% ($\pm 2.6\%$) for *H19* CTCF3. Methylation levels in none of the gene regions studied was associated with race/ethnicity (Supplementary Table 1), age or BMI (data not shown).

Higher total body fat mass was significantly associated with lower methylation of the *IGF2* DMR2b ($P = 0.016$) (Table 2). Trunk fat was not associated with methylation levels, and neither was abdominal subcutaneous fat at L4-L5. A greater percentage of visceral fat at the lower abdomen (L4-L5), independent of total fat mass, was associated with higher methylation levels of *IGF2* DMR2b ($P = 0.045$) (Table 2). Both liver fat ($P = 0.039$) and presence of fatty liver ($P = 0.015$) were positively associated with *IGF2* DMR2a methylation status: a 1% increase in *IGF2*

Table 2. Association* between DMR methylation in *IGF2* and *H19* and adiposity measures.

Adiposity Measures	<i>IGF2</i> DMR0		<i>IGF2</i> DMR2a		<i>IGF2</i> DMR2b		<i>H19</i> CTCF3	
(1) Multiple linear regression [beta (standard error)] for each adiposity measure associated with 1% increase in methylation	Beta (SE)	<i>P</i>	Beta (SE)	<i>P</i>	Beta (SE)	<i>P</i>	Beta (SE)	<i>P</i>
Total fat mass, g	496 (416)	0.240	120 (190)	0.530	-591 (236)	0.016	-615 (539)	0.260
Trunk fat mass, g	-17 (62)	0.779	14 (28)	0.621	35 (39)	0.374	-28 (80)	0.724
Visceral fat, % of total abdominal area at L4L5	1.1 (31)	0.971	20.9 (13.3)	0.124	38.0 (18.4)	0.045	-18.0 (39.6)	0.652
Visceral fat area at L4L5,mm ²	1.00 (1.02)	0.880	1.02 (1.01)	0.115	1.03 (1.01)	0.062	-1.03 (1.29)	0.256
Liver fat**, % in volume	-1.0 (1.0)	0.751	1.03 (1.01)	0.039	1.02 (1.02)	0.318	1.05 (1.04)	0.280
(2) Logistic regression [odds ratio (OR) and 95% confidence intervals (CI)] for fatty liver associated with 1% increase in methylation	OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>
Fatty liver (>5.5% liver fat)	0.93 (0.76, 1.14)	0.505	1.18 (1.03, 1.34)	0.015	1.12 (0.97, 1.29)	0.132	0.90 (0.65, 1.24)	0.504

* Both multiple linear regression and logistic regression models were adjusted for age and ethnicity. Regional adiposity measures in the trunk, viscera, and liver (and fatty liver) were further adjusted for total fat mass.

** Natural log-transformed.

DMR2a methylation levels was associated with a 18% greater likelihood of having fatty liver. Associations for individual CpGs are presented in Supplementary Table 2. For the significant intra-abdominal fat associations (presented above) with *IGF2* DMRs (visceral fat-DMR2b, liver fat-DMR2a), a consistent pattern of associations was observed at the individual CpG level. The CpG associations, however, reached only a borderline statistical significance level for fatty liver after adjustment for multiple comparisons (false discovery rate-adjusted $P < 0.10$) (Supplementary Table 2). A concurrent assessment of alcohol intake was not available to definitively classify fatty liver cases into alcoholic and non-alcoholic etiology groups; however, the results remained the same when we excluded five women who reported high alcohol intake (>20 g/day) [35] at cohort baseline (1993–1996).

Discussion

DNA methylation at the *IGF2* and *H19* loci has been shown to be associated with adiposity (e.g., body weight or BMI) and metabolic diseases [19–24]. There is little information about their associations with different body fat distributions, including with the metabolically more active forms of fat located in the intra-abdominal cavity and ectopic regions. Here, we provide evidence that methylation of *IGF2* DMR regions in peripheral blood leukocytes is associated with total body fat mass and also with visceral and liver fat independently of total adiposity, in post-

menopausal women. Specifically, higher methylation of the *IGF2* DMR2b was significantly associated with a lower total fat mass, whereas an opposite direction of associations was found with total fat-adjusted percent visceral fat (and visceral fat area, at a borderline significance level). We also found a significant positive association of *IGF2* DMR 2a methylation with both liver fat and percent liver fat, after adjusting for total fat.

Since obesity is highly heritable [36,37], many studies have focused on identifying genetic susceptibility regions in relation to adiposity traits [38,39]. Adding to genomic variations, epigenetic modifications have also been shown to influence risk of obesity and metabolic diseases. Studying DNA methylation of imprinted genes is important because epigenetic traits in these genes may be heritable [40] through a mechanism that is still unclear, resulting in parent-specific monoallelic gene expression [41]. Specifically, lower methylation of *IGF2* DMR has been associated with higher birth weight [20] and lower methylation at *IGF2* DMR in newborns was found to be associated with paternal obesity [42]. In humans, only the paternal *IGF2* allele is expressed, and its imprinting is known to be regulated by at least three DMRs including *IGF2* DMR0 (in exons 2 and 3), the DMR2 (in exons 8 and 9), and the *H19* DMR (at 3rd CTCF binding site) [22,23,43,44] and these DMRs have been shown to be linked to birth weight and risk of metabolic diseases including obesity [19–24]. *H19*, a long noncoding RNA, lies about 100 kb upstream of *IGF2*, and is only expressed from the maternal allele [38]. Importantly, although DNA methylation is well

known to be cell- and tissue-specific [45], methylation changes at the *IGF2* and *H19* DMRs are some of the most consistently observed epigenetic associations in relation to adiposity and metabolic diseases in blood cells [19–24,46]. This is likely because methylation marks regulating genomically imprinted genes are established prior to gastrulation and tissue differentiation, thus allowing blood leukocyte DNA to be a viable source for systemic imprinted gene methylation status [47]. Thus, although our study did not prove it, altered DNA methylation may lead to loss of imprinting and altered gene expression and may, consequently, contribute to a higher risk for obesity and metabolic diseases.

In the present study, we found a significant association between lower *IGF2* DMR2b methylation with higher total body fat measured by a whole-body DXA scan. Body composition measured by DXA provides more accurate measurements of body adiposity compared to BMI [48–50]. Given that *IGF2* is preferentially expressed in early embryonic and fetal development and plays a critical role in metabolic disorders, many studies have reported association of *IGF2* methylation with weight at birth and during childhood, as well as parental obesity and obesity-related diseases [20–23,42,51,52]. Thus, our study revealed a novel association of *IGF2* DMRs with total fat mass in obese but otherwise healthy older adult women.

Another interesting finding is the significant inverse association of *IGF2* DMR2b methylation with total fat-adjusted percent visceral fat area and *IGF2* DMR2a with liver fat. The direction of the association of DMR2b methylation with visceral fat was opposite to that with total body fat, suggesting that the associations with DMR2b may differ by body fat location and total body fat may preferentially deposit in the subcutaneous vs. visceral areas depending on the methylation of DMR2b. The mechanism by which the methylation status of a given DMR would affect body fat distribution remains to be determined, especially as the present results were obtained in circulating leukocytes. Also, we observed that locus-specific DNA methylation was associated with visceral vs. liver fat, implicating the involvement of potentially different regulatory machineries or enzymatic activity [53]. Although *IGF2* DMR2a and DMR2b are located within the same DMR2 region and under the same promoters [23], we analyzed them separately because

they are only moderately correlated (Spearman $\rho = 0.4898$), and they are located in different exons (*IGF2* DMR2a in exon 8 and DMR2b in exon 9) and are shown to have different regulatory features based on the UCSC Genome Browser (<http://genome.ucsc.edu/>). Specifically, *IGF2* DMR2b, located in a GC-rich genomic sequence, partially overlaps with a CpG island (CpG Island at position: chr11:2,154,034–2,154,387), while *IGF2* DMR2a is not linked to any CpG island. Also, the two regions are shown to bind to different transcription factors (*NF1C* and *CEBPD* for *IGF2* DMR2a and *USF1* for *IGF2* DMR2b) according to the ENCODE data in the UCSC Genome Browser.

We also observed a significant direct positive association of methylation levels in the *IGF2* DMR2a with percent liver fat and liver fat. Other studies have shown that *IGF2* may play an important role in abnormal liver conditions [54–56]. For example, *IGF2* was shown to be involved in lipid metabolism in liver diseases [54], and to be highly expressed in non-alcoholic steatohepatitis [55] and hepatocellular carcinoma [56]. Thus, higher methylation of *IGF2* DMR2a may result in an increase in liver fat in the healthy population, but additional studies are needed to investigate whether this link is associated with chronic liver disease.

A key strength of the present study is that we confirmed and further extended the reported association between *IGF2* and *H19* DMR methylation levels and overall adiposity found in simple anthropometric studies by accurately measuring total, intra-abdominal, and ectopic adiposity through high resolution abdominal imaging. We analyzed 41 individual CpGs and observed consistent associations across the CpGs within each DMR in non-diseased individuals. Study limitations include a relatively small sample size and the cross-sectional study design, which precludes causal inferences. Additional larger studies are warranted, especially to explore the associations of *IGF2* DMRs with body fat distribution in men and women with a greater age range, to compare multiple ethnicities, and to examine the mediating effects of dietary and physical activity factors, ideally in prospective cohorts with multigenerational data. Also, further investigation is needed to confirm methylation status of other *H19* CTCF sites [57,58] in association with adiposity.

In summary, we found significant associations of methylation levels at *IGF2* DMRs in circulating

leukocytes with total body fat and visceral and liver fat, among healthy postmenopausal women. Furthermore, this study revealed that body fat distribution may be associated with methylation of specific regions of *IGF2* DMRs. These findings in healthy women may provide further biological insights regarding epigenetic predisposition, particularly at the *IGF2*, which may contribute to obesity and related metabolic diseases in adulthood. Confirmation of these findings is needed in larger studies to better understand the interplay of the environment, parental obesity, and diet with DNA methylation of the *IGF2* DMRs.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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