Author's response to reviews

Title: Leptin and adiponectin DNA methylation levels in adipose tissues and blood cells are associated with BMI, waist girth and LDL-cholesterol levels in severely obese men and women

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Author's response to reviews: see over
March 30, 2015

Dr. Matthias Blüher
Associate Editor
*BMC Medical Genetics*

Re: Manuscript 2663171631567161

Dear Dr. Blüher,

Thank you for the review of our manuscript entitled "*Leptin and adiponectin DNA methylation levels in adipose tissues and blood cells are associated with BMI, waist girth and LDL-cholesterol levels in severely obese men and women*” by Houde *et al.*

We would like to thank the three reviewers for their detailed review and constructive comments that helped us improve the quality of our manuscript. We hope that the revised version will now be found acceptable for publication in *BMC Medical Genetics*.

You will find attached our response to the reviewers’ comments and the revised version of our manuscript. Changes were made in red font for easier follow-up.

Sincerely yours,

_____________________
Luigi Bouchard, PhD MBA
ECOGENE-21 Laboratory
Assistant professor
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Reviewer's report:
The study by Houde et al. describes the potential relationship of DNA methylation levels at \textit{ADIPOQ} and \textit{LEP} with variables of obesity and obesity-related complications. The authors report that methylation levels at several CpG sites in the \textit{ADIPOQ} promoter in SAT are positively correlated to BMI, while \textit{LEP} CpG sites are negatively related to BMI in blood.

\textbf{Major Compulsory Revisions:}
1) Although the authors refer to Reference 30 the pyrosequencing strategy should be included describing the position and architecture of the analyzed CpG sites. Moreover, bisulfite conversion and target sequences should be provided.

As suggested, the pyrosequencing strategy used in the current study was included in the manuscript (page 17). Bisulfite conversion and target sequences were also provided in Additional Files 3 and 4.

2) What is the rational for using paired samples of adipose tissue? Did the authors detect adipose tissue specific variabilities that translate into clinical variables phenotypes?

Subcutaneous (SAT) and visceral (VAT) adipose tissues have distinctive biological functions, VAT being more strongly associated with diabetes and cardiovascular risk (Neeland et al., 2013). Previous studies have also showed that \textit{LEP} and \textit{ADIPOQ} expression profiles are different in SAT and VAT (van Harmelen et al., 2002; Lihn et al., 2004). DNA methylation levels were thus measured in both SAT and VAT because of their specific functions, and (related) gene expression profiles. These clarifications were added to the manuscript (page 6).

“SAT and VAT were both analysed because they show specific gene expression profiles (ex. \textit{LEP} and \textit{ADIPOQ}) [27, 28] and associations with cardiovascular risk factors [29].”

Yes, we found associations that were specific to SAT and VAT.
In SAT, \textit{ADIPOQ} DNA methylation levels were associated with anthropometric variables (BMI, waist and hip circumference). In addition, DNA methylations levels at \textit{LEP-CpG17} and \textit{ADIPOQ-CpGE1} gene loci in SAT were found to be correlated with LDL-C levels. \textit{ADIPOQ} DNA methylation levels were associated with total cholesterol levels in SAT only.
In VAT, we report tissue-specific correlations between LDL-C levels and DNA methylation levels of \textit{LEP-Mean} as well as between HDL-C levels and \textit{LEP-CpG7} DNA methylation levels.

3) Why were hyper or hypomethylated CpG sites excluded from the analysis? This need to be addressed.

Based on our extensive experience with pyrosequencing and HumanMethylation450 BeadChips results, we have observed that the CpG sites with DNA methylation levels
between 10% and 90% are those with the highest variability (unpublished results). Therefore, we excluded hypomethylated (≤10%) and hypermethylated (≥90%) CpG sites because they have low DNA methylation variability and are thus unlikely to explain phenotypic changes. Very similar selection criteria have been used in numerous epigenetic studies carried out by our group and others (Delgado-Calle et al., 2011; Guay et al., 2012; Bouchard et al., 2012; Domschke et al., 2013; Guay SP et al., 2013, Ruchat et al., 2013). We understand that the selection criteria were applied systematically, but we believe this remains a reasonable strategy.

The manuscript has been reviewed accordingly (page 18):

“Out of these 8 CpGs, we excluded the CpG sites that were found to be unmethylated (≤10.0%) or hypermethylated (≥90%) as they show low DNA methylation variability and are thus unlikely to explain the phenotypic variability.”

4) In the Methods 21 CpG sites (LEP) are mentioned and others on ADIPOQ. While repeatedly p-values for CpG7 and CpG11 are presented no data are shown for the other sites. The data should be provided.

The reviewer is right: up to 21 CpGs could have been analyzed. As suggested in Comment 7, we have included the associations between mean DNA methylation levels of LEP (21 CpG sites) and ADIPOQ and phenotypic variables in the revised version of the manuscript (see Comment 7 below). However, we (Houde et al., 2014) and other groups (Melzner et al., 2002; Stoger, 2006; Marchi et al., 2011) have previously showed that the selected CpG sites are of greater interest for LEP gene expression regulation through DNA methylation changes. Indeed, these CpG sites are found within a highly conserved region and near numerous transcription factor binding sites (Figure R1 below). We thus favour analysing CpG sites individually. The manuscript has been reviewed accordingly (page 18):

“The mean DNA methylation of the 21 CpGs analysed in the proximal promoter CpG island of LEP gene (Additional File 5) was first computed (LEP-Mean) and analysed. Moreover, since we [33] and other groups [48, 53, 54] have previously reported that CpGs located between CpG7 to CpG17 are of first interest for LEP gene expression regulation through DNA methylation changes, we analysed these CpGs individually to identify those more likely to be regulatory.”
Figure R1- Location of transcription factor binding sites and highly conserved regions within the CpG island of the LEP gene proximal promoter region in placental mammals, adapted from UCSC genome browser tracks. Track A- LEP proximal promoter sequence. Track B- Identification of epigenotyped CpG sites. Track C- Localisation of transcription factor binding sites. Track D- PhastCons conservation track for placental mammals. Track E- Single nucleotide polymorphisms (SNPs) previously identified in the LEP gene promoter. In blue, rs2167270 SNP analysed in the current study. UCSC genome browser was accessed on September 19 2013. (Adapted from Houde et al., 2014)

5) Page 8, last sentence: It is unclear why a correlation should be estimated between LEP and ADIPOQ. Please re-write the sentence.

This sentence was no longer required in the new version of the manuscript and was thus removed.

6) Did the authors adjust blood derived methylation levels for leukocyte counts?

Unfortunately, blood cell counts were not available in our study. However, because we have observed similar associations between LEP DNA methylation levels in SAT and blood with LDL-C levels (Table 2), it is unlikely that these associations are dependent on cellular count (page 14: “Moreover, as these associations are similar in SAT and blood, they are very likely independent of cellular count”). However, this does not apply to the associations with CRP levels and BMI. A sentence was added to the manuscript to highlight this potential limitation (page 12):

“Unfortunately, because blood cell counts were not available, we cannot exclude that LEP DNA methylation variability and the associations reported with CRP and BMI might be partially attributed to blood cell composition changes between samples. However, if cellular composition changes impact the results, this effect is likely modest as highlighted by Talens et al. [38]”
7) What is the mean methylation level of the CpG islands and is this correlated between the different tissues? Does this translate into clinically relevant parameters of obesity or related co-morbidities?

We want to thank the reviewer for this comment. As suggested, we assessed the correlations between mean DNA methylation levels of LEP and ADIPOQ CpG islands in SAT, VAT and blood and phenotypic variables. These data were added in the results section (pages 7, 8 and 9).

LEP and ADIPOQ mean DNA methylation levels were partially correlated between the three different tissues. These results are reported below for the benefit of this reviewer (see Table R1 and Table R2). A recently published paper by our group (Houde et al., 2014) reported comparisons of DNA methylation levels in SAT, VAT and blood.

Table R1- Pearson’s correlation coefficients between LEP mean DNA methylation levels in SAT, VAT and blood.

<table>
<thead>
<tr>
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<th>SAT and VAT</th>
<th>SAT and blood</th>
<th>VAT and blood</th>
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<tbody>
<tr>
<td>LEP-Mean</td>
<td>r=0.571; $p \leq 1 \times 10^{-6}$</td>
<td>r=0.321; $p=0.006$</td>
<td>r=0.354; $p=0.002$</td>
</tr>
</tbody>
</table>

Table R2- Spearman’s rank correlation coefficients between ADIPOQ mean DNA methylation levels in SAT, VAT and blood.

<table>
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<th>SAT and VAT</th>
<th>SAT and blood</th>
<th>VAT and blood</th>
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<tbody>
<tr>
<td>ADIPOQ-Mean</td>
<td>r=0.324; $p=0.005$</td>
<td>r=-0.174; $p=0.142$</td>
<td>r=-0.024; $p=0.838$</td>
</tr>
</tbody>
</table>

8) The corresponding m-RNA expression should be presented including the mentioned negative correlation with the respective methylation levels. Is the m-RNA level of ADIPOQ and LEP correlated with clinical phenotypes? Data on m-RNA expression should be included along with interpreting the data in the discussion.

We would like to thank the reviewer for this comment. As suggested the information on mRNA measurements for SAT and VAT were included in the methods (pages 16-17-18). RNA was not available for blood samples. In addition, we now report associations between mRNA levels, DNA methylation levels and clinical phenotypes in the results (pages 9 and 10; Tables 2 and 3; Additional Files 1 and 2) and the discussion (pages 12 and 13).
REFEREE 2: DAISUKE KOHNO
Reviewer's report:
This paper provides the evidence that the leptin DNA methylation level in blood cells and adiponectin DNA methylation level in SAT are associated with BMI. And also, this paper showed that higher LDL-C level is associated with higher DNA methylation levels of leptin and adiponectin genes. These data are important information for further studies. Data were carefully analyzed and manuscript is well written.

Minor Essential Revisions
1. If possible, authors should discuss why leptin DNA methylation levels in SAT and VAT are not associated with BMI and CRP. Is this related to lower mean DNA methylation levels in SAT and VAT compared to that in blood as shown in Additional file 1?

As this reviewer underlined correctly, LEP DNA methylation levels in SAT and VAT were lower than those in blood. This was somehow expected since a lower DNA methylation is associated with an active gene transcription and LEP is highly expressed in adipose tissue. Also, variability (SD) is lower in SAT and VAT as compared to blood suggesting that LEP DNA methylation is more tightly regulated in adipose tissue. This could also contribute to the missing associations with BMI and CRP.

In addition, DNA methylation is, at least partially, tissue-specific, to ensure tissue-specific functions (Song et al., 2005; Kitamura et al., 2007). DNA methylation tissue specificity appears to be regulated by different mechanisms including the transcription factors. Accordingly, the differences in DNA methylation and associations observed in adipose tissues and blood were expected and support that DNA methylation processes are independently regulated in different tissues. This could also contribute to explain the results we are reporting.

However, we have provided in the manuscript an explanation based on the role of inflammation in regulating gene expression in blood cells and its correlation with leptin levels (see pages 11-12). We believe that this hypothesis is so far the best one to explain the results we are reporting.

2. In line 12 of page 8, the file number corresponding to CRP data should be “Additional file 5” but not “Additional file 4”. In line 9 of page 7, “Figure S1” would be “Additional file 1”

We want to thank the reviewer for pointing this out. The numbering of Additional Files and Figures has been revised.
REFEREE 3: ASAF MARCO
Reviewer's report:
Major Compulsory Revisions
1. One of the major disadvantages of this paper is that methylation levels were not compared in any level with lean subjects. This comparison could have told us if this phenomenon is characterized only in a pathological state or also in the general population. In addition, this kind of information can indicate whether methylation levels in the adipocyte cells were re-programed (from "normal" state) in the obese subjects. If there are no available samples, the writers should address to this "gap" as a weak point in their paper.

We agree with the reviewer that DNA methylation levels of LEP and ADIPOQ genes may have been re-programmed in our metabolically-healthy obese participants. It would also be of great interest to compare the levels of DNA methylation in severely obese patients with the ones of lean subjects. Unfortunately, VAT and in a lesser extent SAT biopsies are not easy to obtain from lean subjects. We have highlighted this limitation in the discussion (page 15).

“This metabolically-healthy obese population has a unique obesity phenotype and metabolism, and LEP and ADIPOQ DNA methylation profiles might be distinct in these participants. Consequently, the results reported in the current study need to be validated in normal weight populations as well as in obese populations with cardiometabolic complications.”

2. The authors showed that LEP DNA methylations at specific sites were negatively correlated with BMI, but only in blood cells. These results weaken their statement that correlation between the methylation patterns of the LEP gene in blood cells and obesity related marks can serve as a biomarker for this pathology, since no proven connection was found between these marks and the adipocyte methylation profile.

As suggested in Comment 5, we toned down the discussion and removed this statement. Nevertheless, it remains that measuring DNA methylation in blood provides insights of interest for understanding the gene expression regulation of adipokines in obesity through DNA methylation changes.

3. In the results section (pg. 8) and throughout the paper the authors state that models were adjusted for age and sex. Please clarify what exactly was the amendment and how it was performed.

This basically means that age and sex were included as covariates in the Pearson’s correlation models. This test measures the strength of a relationship between two variables (DNA methylation levels and phenotypic variables), while controlling the effect of potential confounders (sex and age).

To clarify this statement in the paper, we have replaced “the models were adjusted for age and sex” for: “Pearson’s correlation coefficients were adjusted for age and sex”.
4. The discussion about the discrepancy between the LEP epigenetic signature in the blood and in the adipose tissues needs to be rewritten. The authors suggest that proinflammatory response contributed to the alteration of the LEP epigenetic profile in blood cells. In that case, why shouldn't it affect the profile of the adipose tissues as well? Moreover, the authors do not provide a reasonable explanation how an increase in CRP can affect epigenetic mechanisms.

We have re-written the second paragraph of the discussion to clarify the point mentioned by the reviewer (see pages 12 and 13).

The question “why shouldn't it affect the profile of the adipose tissues as well” has been answered above. Please see Comment 1, referee #2.

The molecular mechanism by which CRP could impact DNA methylation is still unknown. Other groups have previously reported associations between DNA methylation levels at multiple gene loci and CRP levels (Siedlinski et al., 201; Sun et al., 2013). However, functional studies are still needed to determine whether CRP can directly or indirectly (via other mechanisms) impact DNA methylation levels or whether DNA methylation regulates CRP levels.

5. Overall, authors should "tone down" the discussion part and stick to their results. For example, Fig. 3 is too descriptive with a lot of missing data and speculation that has not been tested.

As suggested by the reviewer, the discussion was toned down; we removed the figures or sentences that were too interpretative or speculative according to the data presented.

**Minor Essential Revisions**

1. Pg. 8 states that "...increased levels of CRP (r=-0.397, p=0.004, n=53) (Additional file 4)". Should be written "Additional file 5".

The numbering of Additional Files and Figures has been revised.

2. Regarding additional file 1, please clarify where is the CDC or the transcription start site in this sequence. In addition, it is not clear what the -10563 mark indicates (is it a mistake?)

The mark –10563 corresponds to the number of base pairs relative to the first leptin AUG codon. This is now specified in the Additional File 5 legend (page 30).

“The sequence (-11131 to -10563) was numbered relative to the first leptin AUG codon (UCSC Genome Browser (Human Feb. 2009)).”
Discretionary Revisions

1. I found myself going back and forth between the results and the additional files. If there is no space limit, I'd recommend merging the tables as much as possible.

We want to thank the reviewer for this suggestion. The results presented in Additional Files 4 and 5 have been merged and are now in Additional File 2.

2. There is a large body of literature showing extensive gender differences in the obesity profile. It would be nice to see if there are such differences in this study.

We agree with the reviewer that it would be of interest to compare the association between DNA methylation and cardiometabolic factors in both men and women separately. However, our sample size limits the possibility to perform such comparisons. We hope that these and other recent results in epigenetics will provide a strong foundation to obtain additional funding to build larger cohorts.
REFERENCES


Gene-specific DNA methylation association with serum levels of C-reactive protein in African Americans.


