Author's response to reviews

Title: Association of adipocyte genes with ASP expression: a microarray analysis of subcutaneous and omental adipose tissue in morbidly obese subjects

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Author's response to reviews: see over
Dear Dr Edmunds,

We would like to resubmit our paper entitled: Association of adipocyte genes with ASP expression: a microarray analysis of subcutaneous and omental adipose tissue in morbidly obese subjects (MS: 1211627805229324) by authors Robin MacLaren, Wei Cui, HuiLing Lu, Serge Simard and Katherine Cianflone.

We have revised the manuscript in detail in order to respond to all of the helpful comments by the reviewers. In particular, we have revised the statistics, as well as the presentation of graphs, and modified the introduction, results and discussion to clearly state the specific aims of the study, clarify the results, and expand on the discussion with respect to the issues raised. Finally, we have also added the details and additional statements as requested by the editor.

We appreciate the opportunity to submit to BMC Genomics, and look forward to the response.
Sincerely,
Katherine Cianflone PhD
Response to Reviewer #1

“This is a very interesting study focused on the comparison of adipocyte gene expression assessed by microarray in morbidly obese subjects divided into two groups: subjects with low ASP and low triglyceride levels (LAT) and high ASP and high triglyceride levels (HAT).”

We appreciate the positive comments by the reviewer on the interest in this study.

“Overall, the study is well conducted and the results are interesting although rather surprising in some respects such as no differences in omental adipose tissue between the groups”

This is a particularly interesting point. While differences in omental adipose tissue in relationship to insulin resistance have been noted (in microarray studies and others), our present study specifically identifies differences in subcutaneous adipose tissue with respect to ASP related metabolism. While differences in subcutaneous adipose tissue have been identified using other biochemical parameters (such as differences in triglyceride synthesis in subcutaneous adipose tissue in non-obese vs obese subjects) this is the first to identify this based on ASP metabolism. We speculate (as a hypothesis to be tested) that this may indicate a compensatory mechanism, however this remains to be tested in future studies.

“The authors should clearly state how many diabetic subjects were in each group (it would be nice to see individual fasting glucose data for each subject). They should also rephrase their statement on page 4, line 5 “subjects with “very high” fasting glucose levels, but none of the subjects have been diagnosed as diabetic”

We have added details as to this point. While none of the subjects had been pre-operatively identified as diabetic, at the time of sampling (preoperative fasting), three were identified with plasma glucose levels above 7. This has now been clarified and added to the text (Results paragraph 1 and Methods paragraph 1).

“Also, I would strongly question the statement of authors that none of the 11 obese subjects had metabolic syndrome unless they show triglyceride levels, waist circumference and blood pressure for all subjects were normal. I believe that clarifying these important points is crucial for the correct interpretation of the study results.”
The reviewer’s point is well taken. In the absence of blood pressure, metabolic syndrome cannot be evaluated. Accordingly, we have revised the statement to clarify the subject’s status as clearly as possible (Methods paragraph 1).
Response to Reviewer #2

“The work presented is original and interesting, contributing to the better understanding the relation between gene expression in adipose tissue and circulating ASP concentrations and, vice versa, the potential effect of high/low levels of ASP/TG on the genomic of adipose tissue.”

We appreciate very much the positive comments of the reviewer.

“1. The microarray study provides evidence of the major (almost exclusive) contribution of subcutaneous adipose tissue in comparison to the omental one on the differences between LAT and HAT. One would expect the opposite, with major alterations in the omental adipose tissue contributing to the higher “lipemic index” in the context of morbid obesity. However, this finding is only commented in one line in the last paragraph of page 8 and in a merely descriptive way. In my opinion, this is one of the most important findings of the paper and the potential pathophysiological implications of this difference should be better discussed.”

This is a particularly interesting point. While differences in omental adipose tissue in relationship to insulin resistance have been noted (in microarray studies and others), our present study specifically identifies differences in subcutaneous adipose tissue with respect to ASP related metabolism. While differences in subcutaneous adipose tissue have been identified using other biochemical parameters (such as differences in triglyceride synthesis in subcutaneous adipose tissue in non-obese vs obese subjects) this is the first to identify this based on ASP metabolism. We speculate (as a hypothesis to be tested) that this may indicate a compensatory mechanism, however this remains to be tested in future studies. Comments with respect to this interpretation have been added to the Discussion (paragraph 4).

“2. Expression of the receptor for ASP C5L2 is higher in HAT in comparison to LAT in both the subcutaneous and the omental adipose tissue depots. However, as the authors previously reported, the binding of ASP is significantly higher to the subcutaneous depot (AJP 1999; 26:E815.). How can these findings be reconciled?”

As the reviewer points out, in a previous publication, we demonstrated that radiolabelled ASP binding was on average greater in subcutaneous (SC) vs omental (OM) tissue, and greater in obese vs non-obese. Further, we also demonstrated that binding affinity was also greater in subcutaneous (ie lower Kd). This suggests that
regulation is not only at the level of mRNA expression, but also related to membrane structural components on the plasma membrane (as suggested due to differences in binding affinity, and not only total binding). Further, we also demonstrated in the same paper that there was a correlation between ASP specific binding in SC vs OM tissue, such that subjects with SC adipose tissue with high ASP binding also had high ASP binding in OM tissue. In the present study, although overall there does not appear to be differences in mRNA expression between SC and OM tissue, nonetheless, there is a correlation between expression in SC and OM tissues such that those with high expression in SC also have high expression in OM tissues. This regression has now been added to the Results section (paragraph 9) and further information added to the Discussion (paragraph 6).

“3. It must be specified in the conclusion of the abstract and the discussion that the differences are mainly related to the subcutaneous adipose tissue depot.”

This has been added to both the abstract and the discussion (paragraph 4).

“4. Adipose tissue samples from lean volunteers were obtained during hysterectomy surgery or valve replacement. Do these procedures have an inflammatory component that may be affecting gene expression in adipose tissue? In addition, does the pathophysiology of these diseases may play a role on the gene expression levels? This should be briefly mentioned in the manuscript.”

The hysterectomy cases were elective hysterectomy (rather than emergency hysterectomy) there is no indication of any current inflammatory response. Of course this issue cannot be completely ruled out. However, all of the adipose tissue samples were obtained during surgery under the same sampling conditions. Accordingly, this comment has been added to the information on subject recruitment (Methods, paragraph 2).

“5. Data regarding the expression of IL-4, IL-13 and IL-10 seem not to correspond with the numerical data detailed on sup Table 1 unless the histogram starts on 50%.”

In graphs where absolute data are used (in correlation graphs), all axes begin at 0. In graphs where the ratio to LAT is used (% LAT), no change would be indicated as 100%, with changes in HAT either increasing or decreasing relative to LAT. Thus we have tried to clearly present the changes relative to % LAT. However, in order to present the axes clearly, the value at the origin is now clearly indicated.
“6. The ASP triad (FD, C3, and FB) increases in HAT vs LAT, while IL13 tends to decrease. However, they exhibit a significant positive correlation (sup Table 1 and sup fig 3). How is this possible?”

In fact, average IL13 LAT vs HAT does not change significantly (supplementary figure 3). The lack of correlation, in spite of significant correlation with C3, B and adipsin, is likely a consequence of the relatively narrow range in IL13 values, and the wider range in C3, B and adipsin values. Accordingly, these graphs have been removed.

Minor Essential Revisions
1. In the second paragraph of “Lipolysis Genes” in the Results’s section it seems that perilipin is referred to as “… other lipases..” However, perilipin limits the access of cytosolic lipases to lipid droplets thus promoting TG storage. This information may be misleading.

We have clarified by indicated “lipolysis related genes”, as well as added clarification regarding the role of perilipin.

2. In the same paragraph, carboxylesterase1 is also named adipose TG lipase. While carboxylesterase1 may be also called triacylglycerol hydrolase, to my knowledge, the name “adipose TG lipase” is more frequently used for PNPLA2 (aka ATGL).

The phrasing has been corrected.

3. Some explanation (although hypothetical) for the decreased expression of FABP2 and 7 (sup Fig 2) should be provided.

This has been added to the results, in the appropriate FABP section.

4. The sentence (second paragraph of “Differentiation Genes…..” in the Results’ section) “…In accordance with decreased expression…” should be amended.

This has been corrected.

5. In the third paragraph of “Inflammatory Genes” in the Results’ section it should be specified that the information detailed is referred to the subcutaneous adipose tissue depot.

This has been specified.
6. Genes regarding panels A and B are interchanged in the legend of figure 4.

   This has been corrected.

Discretionary Revisions

1. ** and *** corresponding to p<0.01 and p<0.001, respectively, is not used in the figure and therefore may be removed from figure 1.

   This has been corrected.
Response to Reviewer #3

1. As the authors acknowledge in the introduction the role of the ASP in the storage of TG and its local synthesis by the AT are already well known, as are the relations of this protein with various metabolic or “inflammatory” pathways that compose the functional profile of the AT. The authors must state clearly the rationale of the study, their objectives, the means employed in following them and what exactly is original in their findings.

   Cellular studies have demonstrated that acylation stimulating protein (ASP) is a main anabolic stimulator of TG storage in adipose tissue and is produced by adipocytes. Cell signalling has been evaluated using various cell models. On the other hand, a number of human studies have evaluated plasma ASP and associations with obesity and other metabolic factors. However, direct association between plasma ASP on one hand, and the metabolic function of the subcutaneous and omental adipose tissue on the other hand are unknown, and this is the first study to examine this. The purpose of this study was to characterize the expression profile of subcutaneous and omental adipose tissue from morbidly obese subjects separated based on the plasma levels of ASP and TG, using a microarray approach. This has now been clearly indicated in the introduction, in particular the last paragraph.

2. The microarray data has not been made available on a public repository previous to the submission of the manuscript.

   The data has now been deposited to the GEO repository under accession number GSE 15524. This has now been clearly indicated in the results section and in the methods section.

3. The functional analysis of the genes showing significant differential expression in between the LAT and HAT patients appears to be inappropriate and biased by strong a priori assumptions. Besides this no statistical analysis of any kind seems to have been performed to test the overrepresentation of the manually selected pathways.

   Analysis was carried out using all of the genes in the array, using SAM analysis. This is described in detail in the results section as well as in the methods section. This analysis provided an overview of the comparison in SC tissue and in OM tissue of LAT vs HAT subjects.

   Following this, the specific aim of this study was to evaluate whether there were differences in identified pathways related to metabolism and inflammation in LAT vs
HAT subjects. As the reviewer notes, there were specific a priori aims in targeting those pathways. We have now clarified this. Accordingly we identified every gene possible within the pathways that were expressed on the microarray, and presented the data for all the genes that we were able to identify.

We have clarified the specific aims of the study, and the approach used in the introduction (last paragraph). We apologize that the specific aims of the study, and the reasons for the strategy used were not clearly stated.

4. The analysis of transcriptional co-expression interactions (i.e. expression profiles’ similarities in between selected genes of interest) is equally inappropriate for at least two reasons: Pearson correlation coefficients are not robust to non normality of the distributions, Spearman or Kendall coefficients being more appropriate in these cases... see Zhang, B. and Horvath, S. (2005) A general framework for weighted gene co-expression network analysis. Stat Appl Genet Mol Biol, 4, Article17

We appreciate the comments related to statistical analysis. In particular the reference that was indicated was helpful. As suggested, all of the correlations in the Table 1 have now been presented using Spearman coefficients.

5. The results presented in this study suggest a different ASP profile in subcutaneous and omental AT. It is well known that the morphology and the function of these tissues are profoundly altered in morbidly obese subjects, although with a number of particularities depending on the type of AT depot...This study would have greatly benefited from a morphological analysis conducted in parallel with the microarray expression profiling.

We agree with the reviewer that morphological analysis would have been advantageous. However, there is a limited amount of subcutaneous and omental adipose tissue that the surgeons can remove at the time of operation (for ethical concerns), and we required usage of all the tissue in order to obtain sufficient amounts of mRNA of high enough quality to be used for microarray evaluation. Due to the large amount of lipid in adipose tissue (98%), isolation of RNA is problematic.

Further, due to the widely recognized sensitivity of adipose tissue to RNA degradation, samples were immediately frozen in liquid N2 in the operating room to prevent degradation, preventing any morphological analysis following this. Based on the present results, however, future studies targeting functional assays will be possible.