Author's response to reviews

Title: Adiponectin, chemerin, cytokines, and dipeptidyl peptidase 4 are released from human adipose tissue in a depot-dependent manner: An in vitro system including human serum albumin

Authors:

Henrik Svensson (henrik.svensson@gu.se)
Birgitta Odén (birgitta.oden@gu.se)
Staffan Edén (staffan.eden@sahlgrenska.gu.se)
Malin Lonn (malin.lonn@medic.gu.se)

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Author's response to reviews: see over
Dear Professor Shipley,

Thank you for the review of our manuscript “Adiponectin, chemerin, cytokines, and dipeptidyl peptidase 4 are released from human adipose tissue in a depot-dependent manner: An in vitro system including human serum albumin” (MS: 2127760056103508), and for your continued interest in our work. We have now revised the manuscript according to the thoughtful and constructive criticism raised by the reviewers. Please find below a list of all changes made in response to the suggestions.

We hope that the manuscript will now be acceptable for publication in BMC Endocrine Disorders.

Sincerely yours,

Malin Lönn, PhD
Associate professor
Reviewer comments:

Reviewer 1

We would like to thank Reviewer 1 for the valuable and constructive criticism of our manuscript. Please see below an itemized list of all changes made, or rebuttals, in response to the comments.

In this manuscript, Svensson et al. described that several adipokines and cytokines are released from human adipose tissue in a depot-dependent manner. These are not novel findings. They also recommended the supplementation of incubation medium with HSA rather than BSA to minimize induction of cytokine release.

It is true that several publications report that a number of adipokines/cytokines are released from human adipose tissue in a depot-dependent manner. However and importantly, many comparisons are based solely on gene expression analysis not always reflecting release. Other comparisons are based on incubation of adipose tissue/adipocytes and subsequent analysis of the medium. In a majority of these incubation studies, BSA is a component of the incubation medium. We believe this is a serious problem since previous (Schlesinger et al; Albumin inhibits adipogenesis and stimulates cytokine release from human adipocytes. Am J Physiol Cell Physiol 2006) and the present investigation demonstrate that the presence of BSA in the medium has considerable effects on adipose tissue/adipocyte function. Since the magnitude of the stimulating effect of BSA on cytokine release can never be predicted – depending on BSA preparation, intrinsic factors of the tissue depot, cytokine in focus etc - the present study was undertaken to evaluate an alternative to BSA, in order to minimize induction of cytokine release and allowing adequate depot comparisons. The present study shows that HSA is a better alternative. In this type of investigations, supplementation of the medium with HSA rather than BSA is strongly recommended to minimize induction of cytokine release.

In my opinion the main weakness of this study is the increased LPS levels showed in BSA. These experiments should be repeated in a new BSA batch, with lower LPS concentration.

We can not, and do not, rule out that there are commercially available BSA preparations, resulting in medium LPS concentrations corresponding to the ones detected in medium including HSA in the present study, and/or resulting in corresponding data on depot-depending adipokine release. However, one of the main results/messages in the present study is the recommendation to use HSA rather than BSA (please see the comment above). Both BSA preparations (fraction V and essentially fatty acid free) that were included in the present investigation are widely and commonly used in adipose tissue incubation experiments. We therefore believe it is important to share our information. Thus, in the present report we would like to pinpoint the demonstrated advantage of HSA in this context. Evaluation of other BSA preparations may be the focus of a future investigation.

Major comments
Adipose tissue necrosis or damage in all exploratory incubations should be tested, using for instance lactate dehydrogenase (LDH) test. This test is a useful assay to measure tissue damage, measuring LDH activity in media.

This is an important comment. We did not analyze LDH but have extensive documentation on the viability (lipoprotein lipase activity, lipolytic activity, receptor binding, adipokine release) of human adipose tissue incubated according to the protocol described in the present study (references from our own group including collaborators, my former surname was Ottosson):


It may also be noted that the tissue is handled under sterile conditions and cut in small fragments, 5-15 mg each (to avoid necrosis); incubations are performed at 37°C, 90% humidity, 4-5% CO₂, in an incubator; medium-to-adipose tissue ratio is high (250 mg/10 ml medium); pH is kept stable (7.3-7.5) throughout the incubation period - all conditions in favour of cell viability.

Since LPS concentration is around 10000 pg/ml in BSA compared with 6 pg/ml in HSA, three negative control treatments (with 10000, 10 and 1 pg/ml of LPS) should be added.

Please see our answers to the first and second comment above. The present report is focused on 1) the demonstrated advantage of HSA, in comparison with commonly used preparations of BSA, in minimizing cytokine induction, and 2) the findings on depot-dependent adipokine release using our incubation protocol including HSA. Evaluation of other BSA preparations, or effects of LPS in a dose-response-manner, may be the focus of a future investigation.
Mediators of NFkappaB pathway should be studied deeply, to investigate the effects of BSA-LPS and LPS in adipose tissue explant.

Please see above. The present report is focused on the demonstrated advantage of HSA, in comparison with commonly used preparations of BSA, in minimizing cytokine induction. Mechanisms behind effects of BSA or LPS may be the focus of a future investigation.

Cytokines gene expression should be measured to explore cytokine production, and not only cytokine release.

As mentioned in the Background and Discussion, a number of studies have revealed differences between subcutaneous and visceral depots regarding adipokine gene expression. However, gene expression levels are not always reflected at the protein level, or at the level of protein release. Further, the significance of different human adipose tissue depots in adipokine release is incompletely known. The aim of the present study was therefore to explicitly compare release of a number of adipokines/cytokines – all implicated in insulin resistance – from human subcutaneous and visceral adipose tissue in a short-term incubation system minimizing cytokine induction. However, in future investigations, measurements of adipokine gene expression will be added to our protocol to allow parallel assessments of mRNA and release levels, this is valuable advice.

How could the authors explain the increase LPS levels in BSA?? This should be discussed.

To our knowledge, standard grade fetal/bovine sera or BSA preparations (commonly used in incubation media) are high in endotoxin due to contamination during manufacturing. In contrast, HSA used in our medium is a pharmaceutical preparation intended for infusion during human intensive care.

It must be emphasized that we do not know for certain that endotoxin is the component in BSA causing induction of cytokine release in our system. Wheeler DS et al (The Immunomodulatory Effects of Albumin In Vitro and In Vivo. Advances in Pharmacological Sciences 2011) concluded that albumin induces TNF-α gene expression in peritoneal macrophages, and that this effect was not due to endotoxin contamination of the recombinant protein. This is now clarified in the Discussion section (page 11, second paragraph) and the Wheeler reference has been added (ref no 34).
Reviewer 2

We would like to thank Reviewer 2 for the valuable and constructive criticism of our manuscript. Please see below an itemized list of all changes made, or rebuttals, in response to the comments.

**Major Compulsory Revisions**

"We found that release of IL-6, unlike the other cytokines we studied, was similar in the subcutaneous and visceral depots, although it tended to be higher in visceral adipose tissue."

*IL-6 is increased in portal vein compared to systemic blood (see review article by Item and Konrad, 2012) and visceral fat released IL-6 is crucially involved in insulin resistance. The authors have to explain why IL-6 is similarly released from sc and vis fat in their studies.*

As stated in the Discussion section (page 12, last paragraph), most previous incubation studies, comparing human subcutaneous and visceral adipose tissue IL-6 release, included BSA or fetal calf serum in the medium which may have influenced the outcome. In one study (Fried SK et al; ref 13), incubations were performed without BSA in the medium and it was indeed reported that IL-6 release was higher in omental compared to subcutaneous adipose tissue. However, this comparison included only three observations (three severely obese subjects, mean BMI 52±2 kg/m²). Further, in the study by Fontana et al (Diabetes 56:1010-1013, 2007 - referred to in the review by Item and Konrad, 2012), the IL-6 concentrations in portal vein and radial artery blood were not possible to adjust for the amount of releasing adipose tissue. Thus, to our knowledge, with the exception of the Fried comparison including three observations, no investigation so far has presented convincing information on adipose tissue depot-dependent IL-6 release expressed per gram of whole adipose tissue. However, and also as stated in the Discussion section (page 12/13), previous clinical investigations show that in particular central/visceral fat accumulation is associated with increases in circulating levels of IL-6, in line also with the Fontana study, as well as with the trend in this direction in the present study. Further, in light of our finding that all the other eight investigated cytokines were more abundantly released from visceral than subcutaneous adipose tissue, we believe it is reasonable to assume that the non-significant difference between the depots, specifically regarding IL-6 release in the present study, is due to the relatively small number of observations which limited our power. We have clarified this in the Discussion section (page 12/13), and have also added the publication by Fontana et al (ref 54).

*Continuous release of adipokines from adipose tissues suggests a continuous increase in medium. This is not the case for adiponectin and omentin which increase until 2 h and stay constant up to 8 h. IL-6 is, however, continuously elevated. Please explain.*

It is reasonable to assume that the release pattern over time is dependent on the specific cytokine studied; its cellular origin/s, influence of vitro conditions on production/release mechanisms etc. It may also be noted that we incubated one
separate tube with tissue for each separate incubation period. Thus, local variation in for example cell composition in the different tissue pieces, influencing release, can not be excluded. Further, factors such as adipokine stability and feed back mechanisms may also influence the results.

*The authors measured protein in supernatants and not synthesis / release.*

Probably a misunderstanding. In the present study, adipose tissue pieces were incubated for 2, 4, 6, 8, and 24 h (one incubation tube for each incubation period). The medium was then removed and centrifuged for 5 min at 450 g to eliminate potential remaining tissue fragments. The concentration of a number of adipokines/cytokines in the medium was analyzed as a measure of adipokine/cytokine release.

*In principle there are no major differences in HAS and 0 albumin incubated cells. Please explain why incubation with HAS was chosen for subsequent experiments.*

It is correct that in principle, cytokine concentrations did not differ between albumin-free medium and medium containing BSA. This is also stated in the Discussion section (page 11, first paragraph) and we speculate that in a short-term incubation system like ours, with a high medium-to-adipose tissue ratio (dilute), supplementation of the medium with albumin may not be necessary. Still, to take every precaution, we chose to include albumin (HSA) for its stabilizing and fatty acid binding properties. Further investigations are required to clarify whether medium including HSA or albumin-free medium is the best choice in investigations of adipose tissue adipokine release.

*Are there differences in adipokine / cytokine levels related to gender of the donors. Is adiponectin higher in adipose tissues of females?*

This is an interesting question. However, the sample is too small to study gender differences (5/6 m/f; cytokines, 3/4 m/f; adiponectin). Further investigations are necessary.

*The authors have to show whether number of immune cells and / or macrophages is increased in visceral fat depots.*

We had the adipose tissue sections prepared and have now assessed macrophage density in the visceral and subcutaneous biopsies (1.74±0.61 vs 0.39±0.14 macrophages/mm$^2$, p<0.05, n=11). Corresponding information has been added to the Abstract, M & M (page 6), Results (page 9) and Discussion sections (page 13, first paragraph). We also added a figure (now Figure 2) showing representative sections, and a corresponding Figure legend. Professor Eva Jennische, providing expert advice regarding the macrophage analysis, has been included in the acknowledgments (page 14).