EDITORIAL REPORT  
  
Senior Editor  
Comments to the Author:  
The authors study gene expression in SC adipose tissue of Cushing's patients. The study is generally interesting, relevant and provides several novel findings. However, one concern is the age difference in the two cohorts, which should be accounted for statistically. Moreover, there are other limitations of the study, which should be appropriately discussed.  
  
Reviewers' Comments to Author:  
  
Reviewer: 1  
  
Comments to the Author  
Overall this is a study that identifies gene expression profiles in humans with Cushing's disease that are, more or less, endorsed in a mouse model of Cushing's. And provide a wealth of data that might prove to be useful when considering adipose tissue associated co-morbidities.  
The quality of the analysis seems to be of a good standard.  
The age of the human groups might add some underlying disparity but this I suspect this not a major issue. The mice were treated with dexamethasone, not the endogenous GC and so effects on genomic responses might be altered somewhat.

**We have now mentioned this point in our revised discussion (see the next point)**

A recent paper by Morgan et al, PNAS showed that the 11beta HSD1 enzyme is an important mediator of mouse Cushing's and Dex is not HSD1 metabolised. Might this alter the profiles in mouse compared to using corticosterone?

**In the Morgan *et al.* study, this group reported that treatment with corticosterone resulted in similar phenotypic results (increased fat mass, decreased lean mass and strength, and decreased insulin sensitivity). However they showed no change in mRNA levels of *Acaca1* and *Fasn* in adipose tissue though *Dgat2* transcripts were significantly elevated (Morgan et al., 2014). We observed slight elevations in all of these mRNA transcripts in our mice (Figure 4F), with elevations also seen in the human adipose tissue samples (Figures 4A and C). This group reported upregulation of the lipolytic genes *Lipe* and *Pnpla2,* which we did not observe in our human Cushings adipose samples (Figure 4D)*.* There are multiple differences between the Morgan *et al.* study and our study which could explain these differences. One is the use of corticosterone vs dexamethasone and another is the duration (5 weeks vs 12 weeks) and age of initial treatment (40 vs 70 days).**

**Another potential explanation is that dexamethasone is unable to be further activated by 11-HSD1. Dexamethasone is already more potent and specific to the glucocorticoid receptor than cortisol/corticosterone and therefore local activation may not be relevant. We have addressed this study in our revised discussion section as such:**

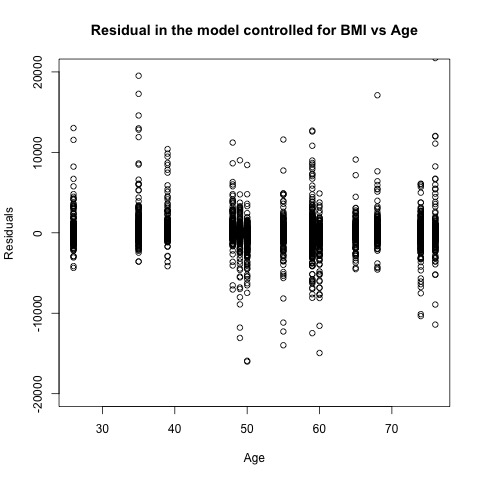
**Studies using a *Hsd11b1* knockout mouse showed similar findings to our data including increased fat mass, decreased lean mass and strength along with reduced insulin sensitivity (Morgan *et al.* 2014). Transcriptionally both of our studies report increases in *Dgat* mRNA, though we observed no effects of Cushing’s disease on lipolytic genes (Figure 4D) as that study did. In our study we did observe induction of fatty acid synthesis genes in both humans and mice (Figure 4A/F) which was not observed in the Morgan *et al.* study. Three differences could potentially explain these discrepancies. One is that in our case, dexamethasone is already active and cannot be further activated by 11-HSD1, wheras in their study corticosterone can be both inactivated by 11-HSD2 and reactivated by 11-HSD1. Another key difference is the duration of treatment, which for our study was three months and for the Morgan *et al.* study was just over one month. Finally they determined mRNA levels from gonadal adipose tissue, not subcutaneous adipose tissue, as we did in our work.**

At the end of my manuscript are a large number of pages of some sort of impenetrable table?? This needs either omitting or seriously re configuring.

**This was an error during the submission process, and will be rectified. Those pages are the supplementary data tables which will only appear in the online article**  
  
Figure 5a and 5b are a bit tricky to follow and might need making more visually clear to the reader- such as being re drawn more as a cartoon?

**This figure has now been modified for clarity**  
  
I found the discussion well balanced and did not over interpret the available data. It identified points of limitation (i.e. the age issue, the small numbers and potential confounders and overall I believe this is a good contribution to the literature in this field.  
  
Reviewer: 2  
  
Comments to the Author  
Major comments: This manuscript describes changes in adipose tissue resulting from chronic excess glucocorticoid exposure that may confirm suspected changes that occur in Cushings patients. While the mouse data strengthens the argument of the authors, the extremely small number of affected subjects has led to a number of conclusions that are not sufficiently supported by the data and statistical analysis.  
  
Suggested revisions:  
1.      The statistics section does not mention the use of a model that corrects for age in the Cushings and non Cushings groups. Has this been done? If so it should be addressed in this section and in the results. If not, a statistical model that corrects for age should be applied and reported.

**Due to our limited sample size, further stratification of our data by age/BMI reduced our statistical power dramatically. By subgrouping our subjects by age, we had only 2 Cushing’s patients between 40 and 60 and no control patients under 40. A power analysis showed that we would only achieve 27% power at a 0.05 significance level to detect gene expression changes of at least 2 fold when the sample size is 2. Nonetheless, a model controlled for BMI and age is reported as a Supplementary Table 6. This model does not provide more useful information than the model controlled for BMI alone. As shown in Figure 1 of this response, the residuals of BMI-controlled model are clustered around 0 across different ages, suggesting that there is no relationship between age and the residuals. As age is not a useful covariate, including age weakens the power of our analysis, especially when our sample size is limited, by needlessly consuming a degree of freedom. This will lead to many false negatives as reflected in just 14 significant differentially expressed genes (Supplementary Table 6).**



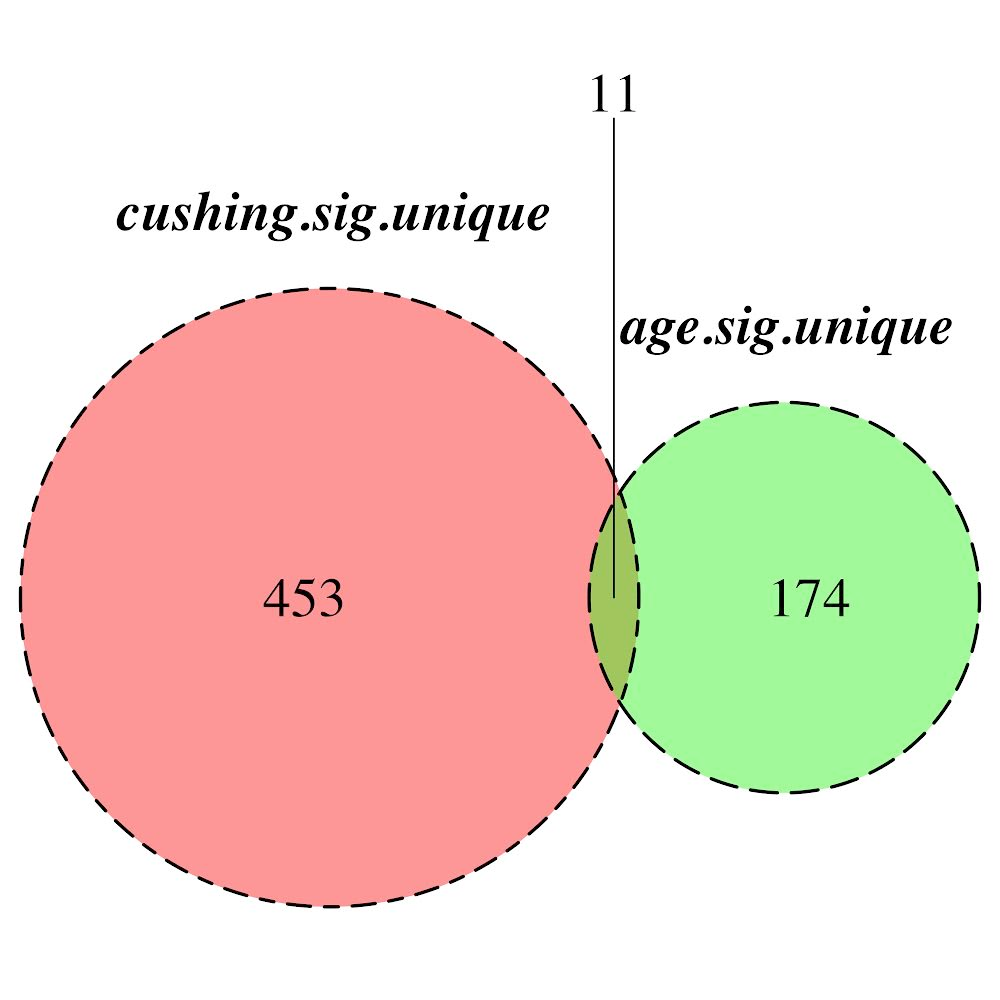
**Figure 1**. Age vs residuals of BMI-adjusted model.

**In spite of our limited experimental power, we have provided several alternate models in our revised manuscript. These are described in the revised methods section:**

**The analysis we focused on in this manuscript was without adjustment for BMI or age and is presented in Supplementary Table 1, with GSEA analyses in Supplementary Tables 2-3. A model adjusting for BMI as a linear covariate or stratified into obese or non-obese subjects is presented in Supplementary Tables 4-5. A model adjusting for both BMI and age were also constructed and is presented in Supplementary Table 6.**

**Cushing's patients age ranges from 26 to 50 years old. Age of control patients ranged from 48 to 74. We don't have any control patients that were younger than 40 years old nor any Cushing's patients that were older than 60. We have 6 control and 2 Cushing's patients that had the age greater than 40 but <=60.**

**To address the review concern, we compared our findings to the data from a study which analyzed gene expression changes with age in subcutaneous adipose tissue in 856 female twins in the age range of 39-85 years (Glass *et al.* 2013). The overlap of our gene list and the age-affected gene list (p adjusted < 0.01 for both analyses) contained only 11 genes (i.e. *ACACA, ACSS2, CLDN5, CLEC10A, ELOVL6, GCHFR, GPC6, HTATSF1, OLFM2, PNPLA3,* and *RET*). This limited overlap is described in Figure 2 of this response.**

Figure 2: Overlap between significantly differentially expressed genes in our dataset and those described to be altered with age from Glass *et al.* (2013).

**Finally, we validated many of the genes we identified as altered in the Cushing’s samples in a mouse model in which age was not a problematic covariate, as all mice had the same age. This suggests that for the majority of the genes that we have described as being different in Cushingoid adipose tissue, age was not the cause of these changes. This included several genes described in the above mentioned paper on age in subcutaneous adipose tissue including *Acaca1* and *Acss2*. Suggesting that these genes may be altered both by age and by excessive glucocorticoids. Overall we have described this potential limitation in the discussion section:**

**Another limitation in our study is the small sample size, especially the number of biological replicates in Cushing’s group (n=5). Adding a covariate such as BMI or age in the model further reduces the sample size to 2 or 3 replicates. Although this sample size is small, it is reasonable for a rare disease such as Cushing’s.**

2.      For the gene expression studies, results need to be reworded to describe the results that were and were not statistically significant. While it is possible that a larger sample size would have yielded statistically significant results, conclusions about classes of genes should not be based on results that are not statistically significant.

**We have clarified this point in the revised manuscript. GSEA analyses allow us to analyse groups of functionally related groups together. As described previously, this GSEA approach allows for identification of significantly altered pathways and networks, even if the underlying genes themselves are not quite significant. An example of this might be that if every ribosomal gene is downregulated 20%, but none quite reach our level of statistical significance (q<0.05), GSEA analysis would identify this pathway as significant. At times we are discussing the cluster of genes (for example genes involved in lipogenesis) and at other times we are discussing a specific gene. Each of the statistical tests (for the groups or for the genes) give separate p-values. We have specified throughout the revised manuscript precisely when we mean that the gene is significant and when we are referring to the group.**

a.      Be careful with the use of “trend” where there is a tendency toward a difference in the absence of statistical significance as trend means something else.

**We have removed this imprecise wording in several places in the revised manuscript.**

b.      Lines 267-69 and figure 4A, are these genes significantly increased? If so, please indicate on graph. If not, please reword the text accordingly.

**From Figure 4A, *FASN* (q= 0.0031), *ACACA* (q= 0.0039), *ACSL1* (q= 0.025), and *ELOVL5* (q= 0.016) reached statistical significance. *ACSL3* (q= 0.12), *ACSL4* (q= 0.10), *ELOVL1* (q=0.11) were increased, but did not reach statistical significance. *ELOVL6* was expressed below the detection limit cutoff and therefore no statistical tests were performed, as per the DESeq2 algorithm (Love *et al.* 2014). We have added asterisks to Figure 4A, and thank the reviewer for pointing out this error.**

c.      Lines 270-72 and figure 4B, as above.

Table 1: Genes which had high fold changes but did not have pairwise statistical tests performed by the DESeq2 algorithm.

|  |  |  |
| --- | --- | --- |
| Expression | Fold  Change | Gene |
| 413.57 | 4.45 | *FADS1* |
| 317.13 | 3.98 | *FADS2* |
| 93.02 | 3.81 | *ELOVL6* |
| 849.98 | 3.75 | *SPP1* |
| 66.31 | 3.18 | *BMP3* |
| 320.18 | 2.95 | *AACS* |

**For Figure 4B, *SCD* and *HSD17B12* were significantly increased in the Cushing’s adipose samples. Asterisks have been added to this figure. For *FADS1/2*, even though the fold change was quite high (4.0 and 4.5 fold respectively), these genes were excluded by the DESeq2 algorithm due to excessive variance. As described in the DESeq2 paper, this functionality was built in order to improve statistical power by ignoring genes with excessive variance that were unlikely to yield statistically significant results. This brings up an important limitation, in that we could potentially miss genes that have extremely high fold change but significant within-group variance. To ensure that we did not miss any high variance, but high fold change genes, we re-examined our dataset, looking for genes that had high fold change (>2 fold change), reasonable expression >50 counts and no calculated p-value. Other than ELOVL6, FADS1/2, we identified several genes, most of which we have described in our analysis but have not reported a p-value in Table 1 of this response. These data are all available in Supplementary Table 1:**

**We now describe this post-hoc analysis in the methods section as such:**

**The DESeq2 algorithm excludes genes with very high variance to improve statistical power (Love *et al.* 2014). To ensure that we did not miss any genes that had a high fold change, but that DESeq2 did not perform statistical tests for, we manually inspected genes that had expression at >50 reads a fold change >2.5 but no p-value calculated. These genes included *FADS1, FADS2, ELOVL6*, *SPP1*, *BMP3* and *AACS* (see Supplementary Table 1).**

d.      Line 286, and 291-94, same.

***LPL, LIPE* and *PNPLA2* did not reach statistical significance. Of the genes in Figure 4E, *DHCR24* was statistically significant, and an asterisk was added. To ensure that there were no other missing denotations of significance in the figures, we carefully re-examined all the bar graphs presented in this manuscript and checked their q-values. No other mistakes were found, we thank the reviewer for noting these.**

3.      Consider representing the gene expression data as a table that includes the p values. The graphs are cumbersome and don’t add to the manuscript.

**The gene expression data is provided in Supplementary Table 1.**

4.      Lines 337-39, 340 and figure 7A; this graph should be removed and text rewritten. There are no clear differences, statistically or otherwise.

**We do not claim any significant differences in these genes. We have added a direct statement to that effect in the revised manuscript. We feel that the lack of changes (especially the lack of downregulation) of these genes is important for our understanding of how insulin resistance occurs in Cushingoid adipose tissue, and therefore prefer to keep this important negative data in the manuscript.**

5.      Figure 7B should be provided as supplemental data; same for 7C, or include data as part of gene expression table that includes p values.

**For the reasons described above, though these are negative data, we would prefer it remain in the manuscript proper, as we feel that this provides important exclusionary data for the mechanism by which insulin resistance occurs in Cushingoid adipose tissue.**

6.      Likewise, figure 8A can be included in tabular form or expressed textually by describing the fold changes for obese and non-obese groups. Figure 8B should also be eliminated or included as supplemental data.

**We disagree with the reviewer on this point. We feel that this novel and unexpected data is more clear in the graphic representations we have provided in Figure 8, and would have less impact on the reader if provided in tabular form.**

7.      The discussion lacks depth in its explanation of gene expression profile changes (i.e. lines 386-89),

**In the revised manuscript we have provided more depth to this discussion point. This section now reads:**

**Broadly, these changes reflect a shift towards more rapid metabolism of glucose through glycolysis and the TCA cycle, and shifting of glucose and protein metabolites towards lipogenic pathways in adipose tissue. This is indicated by significant increases in glycolytic (*HK3, FBP1, ALDOC, ENO1, IDH1, ME1 and DLAT)*, proteolytic (PSMD1/12/14) and lipogenic (*ACACA, FASN, AACSL4/5, ACSL1/3/4, ELOVL1/5/6, GPAM*, *DGAT2*, *DGAT1*, *AGPAT2/3, GPD1,* and *LPIN1*) transcripts in human adipose tissue of Cushing’s patients, with similar transcript expression changes seen in mouse adipose and muscle tissue when treated with dexamethasone.**

a.      the statistical analysis and models used should be discussed here to explain how the limitation of small number of subjects was overcome, at least in part.

**We now describe this limitation in the discussion with the following addition:**

**Another limitation in our study is the small sample size, especially the number of biological replicates in Cushing’s group (n=5). Adding a covariate such as BMI or age in the model further reduces the sample size to 2 or 3 replicates. Although this sample size is small, it is reasonable for a rare disease such as Cushing’s. Realizing our limitation, we chose DESeq2 as the statistical method for our RNA-seq data. DESeq2 overcomes the small sample size problem by pooling information across genes. Maximum likelihood estimation is applied to estimate the dispersion or variance of a gene across all replicates in a group. Then, an empirical Bayes approach is used to get maximum a *posterior* as the final dispersion estimate. This method utilizes the available data to the maximum extent; therefore, help avoiding potential false positives (Love *et al.* 2014).**

b.      Lines 402-06 should be rewritten to reflect which differences were statistically significant and discussed in greater depth.

**We have re-written this section to provide more depth to this discussion point. It now reads:**

**These findings are consistent with our observed elevations of lipogenesic mRNA transcripts in human and mouse subcutaneous adipose tissue. Key transcripts in this category found to be significantly upregulated include Acetyl-CoA carboxylase alpha (*ACACA*), responsible for the first step of lipogenesis (the irreversible conversion of acetyl-CoA to malonyl-CoA) and Glycerol-3-phospahte acyltransferase (*GPAM*) is responsible for the first step in the synthesis of glycerolipids. In addition to a shift towards lipid storage, we also observed elevated expression of glycogen synthesis mRNA transcripts in the Cushing's disease patients. Most notably of these are significantly elevated mRNA transcripts Glycogen synthase 2 (*GYS2*) and UDP-glucose pyrophosphorylase 2 (*UGP2*), both of which are required for in glycogen synthesis.**

8.      In the methods section, a description of how subcutaneous fat mass was assessed should be included. It appears that inguinal fat pads were dissected and weighed but this should be clearly stated.

**This description has now added to the methods section;**

**Following cervical dislocation, a sagittal incision was made along the medioventral surface of each mouse and the skin carefully pulled back to expose the subcutaneous fat depots. The incision was extended along the anterior surface of each hindlimb to allow careful dissection of the inguinal fat pads. A small incision was then made into the rectus abdominus muscle to expose the abdominal cavity. The epididymal fats pads were identified and carefully dissected out. The right fat pads from each mouse were weighed and snap frozen in liquid nitrogen, whereas the left fat pads were each fixed in formalin.**

**References Cited in this Response**

Glass D, Viñuela A, Davies MN, Ramasamy A, Parts L, Knowles D, Brown A a, Hedman AK, Small KS, Buil A *et al.* 2013 Gene expression changes with age in skin, adipose tissue, blood and brain. *Genome Biology* **14** R75. (doi:10.1186/gb-2013-14-7-r75)

Love MI, Huber W & Anders S 2014 *Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2*. (doi:10.1101/002832)

Morgan SA, McCabe EL, Gathercole LL, Hassan-Smith ZK, Larner DP, Bujalska IJ, Stewart PM, Tomlinson JW & Lavery GG 2014 11β-HSD1 is the major regulator of the tissue-specific effects of circulating glucocorticoid excess. *Proceedings of the National Academy of Sciences of the United States of America* **111** E2482–E2491. (doi:10.1073/pnas.1323681111)