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?

Dexamethasone, though synthetic, is extremely potent, already in its active form and highly specific for the GR; therefore, we do not believe that 11BHSD1 is required locally or otherwise to exert GC responsive effects. It has been shown that 11BHSD2 is able to metabolize dexamethasone into a non-active form (Best, Nelson, and Walker,1997-JoE). This being the case, we are seeing even less of an effect as this would indicate there being decreased activity of GC locally, though we did not measure 11BHSD2 transcripts. Also, the administration of dexamethasone does not mean that there is no endogenous GC signaling occurring, which would be acted on by 11BHSD1 locally and transcripts of this enzyme were highly upregulated in the adipose tissue of mice treated with dexamethasone for 12 weeks (see graph below). Additionally, aside from those with Cushing’s, many patients who are medically treated with GCs are receiving a synthetic form, making the data provided from the mouse studies even more applicable to the general population.

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.  
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 significiant. 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 [1]. 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.

**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), they 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 the following genes, most of which we have described in our analysis but have not reported a p-value. These data are all available in Supplementary Table 1:**

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* |

**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** [1]**. 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 useful as 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 conversion 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 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, 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:**

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 lipogenesis genes in human and mouse subcutaneous adipose tissue. In addition to a shift towards lipid storage, we also observed elevated expression of glycogen synthesis genes** , including *GYS2, UGP2* and *GBE1* **in the Cushing's disease patients.**

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.**