Author's response to reviews

**Title:** Sleep quality, BDNF genotype and gene expression in individuals with chronic abdominal pain

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**Author's response to reviews:** see over
Answers-to-Reviewer Comments

Reviewer: #1
Reviewer’s report:

This is a well-written manuscript that addresses sleep and chronic abdominal pain using
a whole genome expression approach as well as a single candidate gene/single SNP
association approach. The manuscript has several strengths including use of validated
sleep measures, use of appropriate genomic data collection approaches, and report of
interesting findings. The manuscript does have some weaknesses that if addressed
would further strengthen the manuscript.

Major Compulsory Revisions

1) The manuscript is written with the cohort of 59 subjects in mind; however microarray
gene expression data (a major data element for the project) was conducted in less than
half of this cohort (n=26). We are never told what this subset looks like? Are they
different in any manner from the larger cohort that is defined? How many controls were
included in that n=26? How was this subset selected and why?

Response: The microarray cohort was limited due to cost and control of sample. The
sample included only the Caucasian participants information for 26 samples used for
microarray data is- Pain: CAP (11) vs healthy control (15), Sleep quality: poor sleep
quality (7) vs good sleep quality (19) and BDNF: homozygous (17) vs heterozygous (9).
The information is included in the revised manuscript, Methods, Page 14, 3rd
paragraph, Line 3. Per quality measures of microarray processing only samples from
26 Caucasian participants were analyzed.

2) The controls for this study are important since most of the evaluations are relative
comparisons with controls; however we are given no information about the controls in
the manuscript? How was a control defined? Were they recruited in the same manner as the cases? Were they just as rigorously evaluated for sleep and CAP phenotypes as the cases?

**Response:** The procedure followed to determine categories of Pain and Sleep quality groups are mentioned prior in ‘Methods’ section of the manuscript (Pages 9–10). All study participants (cases and controls) were recruited per protocol now specified in more detail as requested and included in a supplemental file. The participants were grouped based in the pain group if they had a history of abdominal pain for greater than 6 months. Controls had no history of abdominal pain and no other organic disorders or GI diseases (e.g., inflammatory bowel disease, celiac disease, biliary disorders, bowel resection) and had no cardiac, pulmonary, neurologic, renal, endocrine, or gynecological pathology. This added information is as now specified in the added supplemental material. Sleep groups were based on PSQI scores as noted in the manuscript. All participants had the PSQI administered regardless of pain group. All participants had the same assessments.

3) Information related to recruitment of subjects in general is thin. It is mentioned that they were recruited through the NIH clinical center during an outpatient visit? But why were they visiting the center in the first place? Were they recruited from a specialty clinic? What was the inclusion and exclusion criteria for the study?

**Response:** Information related to study participant’s recruitment, inclusion and exclusion criteria are given in ‘Methods’ of revised manuscript (Page 9, 2nd paragraph) and details in Supplemental Information. Participants visited NIH Clinical Center exclusively to participate in the study and are directly recruited from clinicaltrials.gov,
mass media (newspaper ads and flyers). Participants in the study are not recruited from a specialty clinic.

4) CAP is a heterogeneous phenotype and how it was defined for the analyses of this project could be better explicated? How many bouts of pain and what level of severity for over 6 months would constitute CAP? Any attempts to focus on organic vs functional etiologies (see inclusion and exclusion comment above)?

Response: Agreed CAP is a heterogeneous phenotype. We have clarified in the ‘Methods’ section of the manuscript that ‘CAP’ is defined as greater than 6 months of abdominal pain group and was determined by self-report and confirmed by medical record review (Page 9, 3rd paragraph, Line 2). The study focus is to compare the features of CAP to Healthy controls. The abdominal pain severity during the 6 months prior to inclusion in the study is not known or a data point that is gathered as part of the protocol. Participants with known organic causes for their abdominal pain are not included in the study.

5) The manuscript does discuss why BDNF was chosen as a candidate for evaluation, but why only BDNF and not other equally plausible candidates? Why the chosen SNP in particular? Why not variation in the genes that look interesting from the gene expression data?

Response: Due to evidence in literature that BDNF (rs6265) modulates sleep intensity and correlates with higher abdominal pain scores, the focus was to study association of such SNP on Pain and Sleep quality groups, besides exploring effects of BDNF on gene expression. In future, would certainly plan studies to investigate SNPs of other genes of biological and medical relevance.
6) Evaluation of the BDNF polymorphism data was conducted by comparing homozygotes vs heterozygotes and it is not clear why this was done. Given the small sample size it is understandable that they would dichotomize genotypes particularly if the number of homozygote variants were small, however most studies would have dichotomized into presence/absence of the variant allele and not combined the two homozygote groups for comparison. The authors should clearly state why they did the groupings that they did for this analysis.

**Response:** Based on TaqMan assay of BDNF (rs6265) SNP genotyping results and ‘Quality value’ threshold, samples were classified for BDNF: homozygous (samples having only allele X or allele Y) and heterozygous (samples having both allele X and allele Y). Mutants were batched for heterozygous group categorizing BDNF into two levels for data analysis. Information of the categories of the BDNF group is given in Table 3, and in revised manuscript (Page 5, 2\textsuperscript{nd} Paragraph and Page 14, 2\textsuperscript{nd} Paragraph).

**Minor Essential Revisions**

1) Background, 2nd paragraph, sentence? Brain derived neurotropic factor (BDNF) is a neuropeptide located on the gene rs6265?? is awkward and should be reframed.

**Response:** Thank you for your recommendation we have modified the sentence as suggested in revised manuscript (Page 4, 2\textsuperscript{nd} Paragraph, Line 2).

2) There are a few things that are presented in the results/discussion that are not fully explained in the methods. The methods for the qRT-PCR should mention what genes were evaluated in this manner and why they were chosen.
Response: Most differentially expressed genes comparing CAP to healthy controls, IGF-1, IGHG1, and SPATS2L were evaluated by qRT-PCR (Page 13, 3rd Paragraph, Lines 2-3). Also, added the gene names in ‘Methods’, Page 15, 3rd Paragraph, Line 1.

2) The methods do not mention if/how pathway based analyses were conducted although the results/discussion mention it.

Response: We have added a paragraph in ‘Methods’ for pathway analyses (Page 15, 2nd Paragraph) as suggested.

3) Table 1 should list p-values for gender and race.

Answer:

Discretionary Revisions

1) Given the focus on BDNF and information presented in the background about BDNF expression and abdominal pain scores, it would be interesting, since the data is there, to say what BDNF gene expression looked like in this study.

Response: As recommended, in the revised manuscript we have now included a paragraph in the Results and Discussion section related to differential gene expression of BDNF group (comparing heterozygous to homozygous), Page 6, 3rd Paragraph.

2) Given that BDNF genotypes factored into the analysis, it would be interesting for the authors to note in the discussion if BDNF in any way is involved with the significant genes identified from the gene expression data.

Response: We have now included a table related to differential gene expression list of BDNF is given in Supplemental Information, Table S2, in which ‘BDNF’ gene is not present. Also, the genes have no overlap with gene lists obtained for Pain or Sleep
quality group. This information is now included information in the revised manuscript (Page 7, 1st Paragraph).

Level of interest: An article whose findings are important to those with closely related research interests Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests: 'I declare that I have no competing interests'

Reviewer: #2

Reviewer's report:
The interaction between sleep disorder and chronic abdominal pain (CAP) remains unclear. Here Reddy et al. try to investigate the association among sleep quality, CAP and gene expression. The authors found some differentially expressed genes which may be related to poor sleep quality and CAP. Though the manuscript provided us some information about gene expression and sleep quality and CAP, there are several major concerns about the manuscript.

Specific points #
1. I do not think genotyping of 59 participants is enough to compare the difference no matter in the two groups of sleep quality or in the two groups of CAP. What's more, the participants are different races; confounders may be hard to control.

Response: Thank you for your review. We have revised the paper extensively as recommended and hope you find it more suitable. We plan in the future to extend our genotyping study to a larger cohort.

2. The author showed that CAP is associated with poor sleep quality. The author also showed that the IGF1 expression was significantly reduced in poor sleep and was
increased in CAP. I don’t understand the causal relationship among IGF1 expression, poor sleep and CAP.

**Response:** Differential gene expression between CAP and healthy control indicate genes such as IGF1, IGHG1 and SPATS2L are up-regulated. Interestingly, the same genes are down-regulated comparing poor sleep quality to good sleep quality. The intensity of each gene across Pain and Sleep quality were studied using X-Y plots (Figure 2). As observed in Figure 2a, expression in IGF-1 associated with CAP is decreased in poor sleep quality sleep compared to good sleep quality (Page 6, 2nd Paragraph).

3. How to get the subset of 26 participants (page 13, line 7) should be discussed.

**Response:** As noted above in the Review #1 recommendation we have now included cohort information related to microarray data of 26 participants in ‘Methods’ (Page 14, 3rd Paragraph) as suggested.

4. I think the BDNF SNP is not a major point related to the manuscript.

**Response:** We agree that there is no significant main effect of BDNF. The interested focus and findings of this manuscript are the interaction effects in co-morbid symptoms of chronic abdominal pain and altered sleep quality. The focus is to study the associations of pain, sleep quality and BDNF and also explore their effects on gene expression. The effects of BDNF are subtle but additive with the current sample data, though gene expression profiles indicate BDNF interactions either with pain group or sleep quality (Figure 3). These finding indeed warrant further investigation.

**Minor points#**
1. Page 4, line 12: on the gene rs6265 is not appropriate. rs6265 is just a SNP but not a gene.

Response: Thank you for your recommendation. We have modified the sentence in the revised manuscript (Page 4, 2nd Paragraph, Line 2).

2. Page 4, line 14: Since the 16th reference has proved the phenomenon that BDNF Val66Met modulates sleep intensity, then details should be introduced.

Response: We have now included additional information related to the reference (Page 4, 2nd Paragraph, Line 4) as suggested.

3. Page 5, line 18: Data of genotyping studies should be given.

Response: More details related to genotyping are provided in 'Results and Discussion' of the manuscript (Page 5, 2nd Paragraph). The genotyping data of the samples is now given in supplemental information, Table S1. Also, the categories of BDNF SNP used for data analysis are given in Table 3.

4. Page 10, line 5: DNA extraction should be changed into DNA extraction and Genotyping. The primers and probes of genotyping and the experimental conditions should be provided.

Response: In the revised manuscript title of the sub-section, ‘DNA extraction’ is changed to ‘DNA extraction and Genotyping’. Information related to primers and probes of genotyping are now given in ‘Methods’ of revised manuscript (Page 11, 2nd Paragraph).

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests: I declare that I have no competing interests