Author's response to reviews

Title: Paternal obesity is associated with IGF2 hypomethylation in newborns: results from a Newborn Epigenetics Study (NEST) cohort.

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Author's response to reviews: see over
Dear Editor,

Please find our revised manuscript “9009423171612976”, titled: “Paternal obesity is associated with IGF2 hypomethylation in newborns: results from a Newborn Epigenetics Study (NEST) cohort”, authored by Soubry et al. We appreciate the reviewer’s thoughtful consideration of our manuscript and have addressed their comments and concerns as described below. Changes are highlighted in the manuscript, and line numbers are added below.

Reviewer: GM

1. **Expression analysis of IGF2 and H19 levels in the umbilical cord. This should then be correlated with the methylation changes seen.**

   We have addressed this concern in our two latest papers:
   
   
   “Gender-specific methylation differences in relation to prenatal exposure to cigarette smoke” by S. Murphy et al.; Gene 494; 2012 (supplementary data).

   In brief, expression analyses in cord blood were performed and showed an inverse relationship between methylation at the IGF2 DMR and IGF2 protein levels (Cancer, Causes and Control publication) as well as IGF2 transcript levels (Gene publication). The references have been added and are discussed in lines 295-298.

   **H19** expresses a non-coding RNA that has been associated with embryonic development and cancer. Its exact function remains to be elucidated. Hence, we added that **H19** is a non-coding gene (line 68), and briefly described the upstream ICR (imprint control region) region (line 69).

2. **The sample number should be increased and the analysis should be done separately for different ethnicities.**

   We agree that our sample size of 98 families is small (with paternal data from 79 newborns). We address this as one of the limiting factors of the study (line 362). Recruitment dates from 2005-06, which makes it difficult to increase the sample size because of the potential of recall bias.

   Other related epidemiological studies of similar size have previously been published and have revealed new scientific findings. We included these studies in our report: C. Gemma et al., 2009 (n=88 pregnant women), in line 325; A. Figueroa-Colon et al., 2000 (n=47 offspring), in line 262; and B. Heijmans et al., 2008 (n=120 offspring), in lines 79 and 275.
We calculated the significance level and the power of our results. The power was 0.7 and the p-value was 0.003 for our main finding at the IGF2 DMR in offspring from obese fathers (beta-coefficient was -5.3). It is therefore unlikely that these data have insufficient power.

Besides the dichotomized analysis of obesity, we have now extended our regression analysis by use of parental BMI as an independent variable in our models. We found a significant decrease in DNA methylation with increasing BMI of the father (p=0.009), these new findings have now been incorporated into the manuscript (see lines 243-249, and Figure 2).

We stratified our data by race/ethnicity and found the same trend in results at both DMRs for African Americans and Caucasians. However, stratification decreased statistical power resulting in unstable estimates.

**Reviewer: MF**

1. The questions posed by the authors are new, well defined and clearly responded. The methods are appropriate but there is a problem. The results are based on data (parental obesity) that have not been directly measured by the authors, but reported by the mothers. The data are not well controlled because they do not come from direct measurements, which is not "scientific". They could vary if the fathers would have measured by the authors. Therefore, there is a lack of reliability in the parental anthropometric data.

We are pleased that this reviewer found our questions well defined, clear and new. We agree that self-report (responses to questions by the subject), or by proxy (when reporting is done by others) may result in misclassification. However, this technique of data collection has been widely and successfully used in clinical studies regarding obesity, cigarette smoking, etc. We do not believe that such misclassification of obesity differs by DNA methylation status. Hence, the results in this study may represent an attenuation of the true results, e.g. the methylation differences between obese and non-obese parents may be larger than what we found. We address the issue regarding potential misclassification in our study limitations (lines 343-346).

2. The manuscript adheres to the relevant standards for reporting. The discussion and conclusions are supported by the data and respond to the objectives. The literature is properly cited and up-to-date. Abstract and title are correct. The article is well written. However, some minor errors can be found: “a continues variable”, “this results was”, “regression analyzes shows that”.

We thank the reviewer for noticing this. We corrected the typos (lines 139, 237 and 328).

3. Two questions: Is 3% enough to establish "biologically significant differences" in DNA methylation?

We have added a number of references regarding the biological significance of small methylation changes at the IGF2 and/or H19 DMRs.
In lines 296-299, we included that very small changes in DNA methylation may lead to a significant increase in circulating IGF2 protein and mRNA levels (C. Hoyo et al., 2012) (S. Murphy et al., 2012).

We describe associations found between small changes in DNA methylation at the IGF2 or H19 DMR and exposures to malnutrition in lines 272-274 (B. Heijmans et al., 2008); exposures to smoking (S. Murphy et al, 2012), assisted reproductive technologies (S. Katari et al., 2006), and the use of antidepressants during pregnancy (A. Soubry et al., 2011), in lines 300-301. All these changes were of the same magnitude as our findings.

Furthermore, very small yet significant differences (of only 1%) in DNA methylation at other genes, such as ARG, have been suggested to play a role in modulating the production of the airway inflammatory biomarker FeNO in children with asthma (C. Breton et al., Am J Respir Crit Care Med, 2011).

4. Child weight is related to parental obesity?

We have verified this potential association, but found no relationship at birth. This was added in line 264. We aim to further follow the weight trajectories of these children over time, and hope to answer this question in more detail in the future.

Reviewer: JG

The manuscript by Soubry and colleagues describes a study of the effect of paternal and maternal obesity on neonatal methylation in blood leucocytes using bisulfite pyrosequencing. The analysis is well described and the conclusions generally well thought out and convincing. However, there are a small number of details and discussion points missing that should be addressed.

1. The two IGF2/H19 DMRs used in this study should be better described. The IGF2 DMR they use is a somatic DMR whose methylation is set after conception. This has profound implications for the interpretation of the authors’ findings and must be discussed. The H19 DMR used in this study is presumably part of the imprinting control region (ICR), which is a germline DNA whose methylation levels are set during spermatogenesis. Can the authors please confirm that this is indeed the case? Has anyone else measured DNA methylation within their H19 region?

We thank the reviewer for this comment. In the introduction we have more thoroughly described the IGF2 and H19 DMRs (lines 67-75). The chromosomal regions analyzed are specified in the methods, and we refer now to our most recent paper (S. Murphy et al. PloS One, 2012), where the structural characteristics of the DMRs and genomic coordinates of the assays have been described in detail (lines 130-131).

The methylation marks associated with the IGF2 and H19 DMRs are established in the male germ line. While this has been known for the H19 DMR for some time (Kerjean, et al., 2000), this has only recently been shown for the IGF2 DMR (Boissonnas et al., 2010). Thus, methylation at the IGF2 DMR
is not somatically established and furthermore, is not maternally methylated as was widely believed. Regarding analyses in human germ cells, Boissonnas et al. studied alterations at the IGF2/H19 loci in human spermatozoa, and Kerjean et al. analyzed the human H19 DMR during spermatogenesis. We included these references in line 72. In addition, our own unpublished analyses of mature human spermatozoa from 16 individuals also support that both the IGF2 and H19 DMRs are fully methylated in male gametes and therefore constitute germline imprints. We have clarified the origins of methylation at these two DMRs in the manuscript (lines 71-75).

Furthermore, numerous other studies have measured DNA methylation at the IGF2 and/or H19 DMR, e.g. H. Cui et al., 2003, M. Cruz-Correa et al., 2004, S. Murphy et al., 2006, A. Feinberg et al., 2006, B. Heijmans et al., 2008, R. Steegers-Theunissen et al., 2009, M. Ollikainen et al., 2010, S. Murphy et al., 2012; all included in this manuscript (lines 79, 272, and 298-299).

2. The authors should cite human epidemiological studies and animal epigenetic studies implicating paternal obesity as a transgenerational risk factor for childhood obesity/diabetes etc.


We thank the reviewer for this valuable remark. We have rewritten parts of the discussion, and added these and other references (lines 260-271).

3. The authors should discuss the weakness of analyzing whole blood e.g. cell heterogeneity.

We have added our latest results regarding this potential issue (S. Murphy et al, 2012). In this publication, we performed an extensive analysis of DNA methylation in multiple fetal and newborn’s tissues, among which the major cell fractions in cord blood were also analyzed. The different umbilical cord blood fractions did not differ significantly at the DMRs studied here. We further discuss this in our limitations (lines 338-342).

We thank the reviewers for their helpful critique and considerations. We thank the editor for the interest in our manuscript, and are looking forward to hear about your decision.

Sincerely,

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