Author's response to reviews

Title: Quantifying circulating hypoxia-induced RNA transcripts in maternal blood to determine in utero fetal hypoxic status

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Author's response to reviews: see over
Dear Editors of BMC Medicine,

RE: MS 1337017938103039 – Quantifying circulating hypoxia-induced RNA transcripts in maternal blood to determine in utero fetal hypoxic status.

We thank the editors for the opportunity to respond to the reviewer’s comments. Here we are pleased to forward our rebuttal and a revised draft incorporating the suggested revisions. Additions to the revised manuscript are highlighted in yellow.

We look forward to your further correspondence,

Stephen Tong
Associate Professor Stephen Tong, corresponding author.

REVIEWER 1

This is an outstanding manuscript. I have several comments.

Major compulsory revisions:
1) Upload the array data to the GEO database.
Done.

Minor discretionary revisions: None

Discretionary revisions:
1) Please report the total blood sampling volume from the pregnant women.
This information was already provided in the methods section. To make the text clearer on this point, we have slightly rephrased the sentence. It now reads (line 134):

‘2.5 mls of either maternal peripheral whole blood and/or fetal umbilical cord blood samples were collected in PAXgene whole blood RNA tubes (PreAnalytix, Hombrechtikon, Switzerland)’

2) Please clarify that the quantitation and statistics in figure 3 and later are from the array intensity data.

The data for Figure 3A were calculated from a full quantitative PCR Taqman array, as opposed to a genomewide microarray which generates semi-quantitative data. While this was specifically stated in the results section (line 223-227), we are happy to also clarify this in the figure legend. It now reads (line 556, addition underlined):

‘(A) Expression of six hypoxia induced transcripts in paired maternal blood sampled before labour was commenced and moments before delivery. Data were generated from a Taqman PCR array.’

The data for Figure 3B also was not generated using a microarray, but by real time PCR with Taqman primers targeting these hypoxic genes. To clarify this, we have added the following to the figure legend for figure 3, line 564:

‘Data were generated using Taqman PCR’.

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.
This manuscript by Clare Whitehead and colleagues describes the circulating RNA pattern in maternal and fetal (cord) blood at term in either induced labor in uncompromised pregnancies or in pregnancies with a severely growth restricted fetuses (<10th percentile). In addition, they authors determined quantitative differences in key hypoxia related RNAs in both maternal and fetal blood in these both acute hypoxic (induced) and chronic hypoxic (IUGR/FGR) pregnancies. The overall concept that circulating RNAs (mRNA) would change in response to either acute or chronic hypoxia is novel and innovative.

Indeed, these RNA species have the potential to effect both maternal and fetal systems in an adaptive or maladaptive manner in response to the hypoxia, in addition to providing a means to identify potential fetuses that had been exposed to either chronic or acute hypoxic bouts in utero. Hypoxia is one of the most common of fetal perturbation. The methods- use of microarrays and qRT-PCR validation appear adequate and appropriate. The findings of changes in key hypoxic associated mRNAs is exciting and is of broad interest to the field.

We thank the reviewer for these positive comments.

Some concerns do however need addressing:

**MAJOR:**

1) The cutoff for fetal hypoxia (lactate <4 mmol/L) needs defining. Why this cutoff?

The reviewer is referring to line 115 in methods where it was erroneously written that the cut-off used to denote the presence of fetal hypoxia was an umbilical cord lactate concentration of <4 mmol/L. It was meant to read 6 mmol/L and we apologise for this typographical error. (Of note, the fact we used a cut-off of 6 mmol/L was clearly written in the results section).

This has now been amended (see line 115, of the resubmission, change highlighted in yellow). The cut-off of >6 mmol/L is now consistently written throughout the manuscript.

We suggest most clinicians would agree lactate concentrations >6 mmol/L in the umbilical cord signifies the likely presence of significant fetal hypoxia. It is a cut-off commonly used by tertiary institutions. Use of this cut-off is further supported by a recent paper by White et al (J Matern Fetal Neonatal Med 2012;25(9):1653-9). They found an umbilical cord lactate level of 5.7 mmol/L was strongly associated with an elevated risk of the newborn developing hypoxic ischaemic encephalopathy.

Given the complexity of the paper, we do not propose adding further text to justify the 6mmol/L cut off. Instead, we have referenced White et al for the interested reader (see line 194).

2) In the FGR group, preeclamptic pregnancies were included. While preeclampsia is known to lead to chronic fetal hypoxia, in itself, preeclampsia may alter placental miRNA/mRNA patterns independent of fetal hypoxia. This group should be evaluated separately.

We thank the reviewer for raising this important point. We have re-analysed our data, splitting the FGR group (ie the n=20) according to whether there was co-existent preeclampsia (n=8) or not (n=12) [there was no preeclampsia in the control group]. We examined the same four genes we have intensively examined throughout the manuscript: HIF1, HIF2, ADM and LDHA.

The results we obtained were very clear. In both FGR groups (ie FGR + preeclampsia and FGR without preeclampsia), expression of all four hypoxia-induced genes remained significantly
increased compared to gestationally matched controls (blood from normal pregnancies that progressed to term). Importantly, expression of all four hypoxia-induced genes were not significantly different between the FGR + preeclampsia cohort, and FGR without preeclampsia (see the new Supplementary Figure 3, shown below).

This data strongly supports the premise that hypoxia-induced mRNA genes increase with severe preterm FGR, irrespective of the presence of preeclampsia. We believe this sub-analysis substantially strengthens the findings in our manuscript. As such, we have included this analysis as an extra subsection in our manuscript and presented the data as supplementary figure 3. We thank the reviewer for the suggestion.

We have added the following in the body of the text (line 281):

‘Expression of hypoxia induced mRNA in maternal blood in the FGR cohort, split according to whether preeclampsia was also present.

To examine the possibility that co-existent preeclampsia may affect expression of hypoxia-induced mRNA in maternal blood (and thus, be a confounder), we split our FGR cohort according to whether there was concurrent preeclampsia (n=8) or not (n=12). mRNA expression of HIF1α, HIF2α, ADM and LDHA were all significantly elevated in both FGR cohorts (i.e. FGR with concurrent preeclampsia, and FGR without preeclampsia; see Figure S3) compared to gestationally matched controls (healthy pregnancies that progressed to delivery of an infant of normal birthweight at term). Importantly, mRNA expression levels of all four genes were no different between the FGR cohort with concurrent preeclampsia and FGR without preeclampsia. This data suggests mRNA coding hypoxia induced genes are increased in the presence of severe FGR, irrespective of the presence of preeclampsia.’

We have inserted the following as supplementary figure 3:
Figure S3: Expression of hypoxia-induced mRNA blood, with the FGR cohort split according to whether there was concurrent preeclampsia (FGR + PET, n=8) or not (FGR no PET n=12).

Comparisons between FGR - no PET vs FGR + PET groups were non-significant for all four genes (P≥0.41). Remaining comparisons, as shown in the graphs, were made between both FGR groups and gestationally matched controls (pregnancies that progressed to delivery of an infant of normal birthweight at term). *P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001

3) A table need(s) to be included with fetal pO2, lactate values for control, induced and FGR pregnancies. Or in graphical form with both hypoxic and normoxic fetuses depicted. It would seem appropriate to also separate preeclamptic and smoking from the uncomplicated FGR. As per smoking: these pregnancies (a small number) would seem to need to be excluded since smoking induces both FGR, fetal hypoxia but also nicotine effects independent of hypoxia.

Below we will consider these different parameters raised by the reviewer:

Fetal pO2 and lactate:
We did not obtain data on fetal pO2 and lactate for the entire FGR cohort. However, we wish to note we were focused on correlating hypoxia-induced mRNA in maternal blood with fetal blood pH. This is because fetal blood pH in the setting of FGR has the strongest evidence of an association with adverse neonatal outcomes [see manuscript]. Therefore, while we do not have the full set of data to present fetal pO2 and lactate in our cohort, we do not believe this significantly compromises this proof of principle manuscript.

Preeclampsia:
A separate analysis has been presented (see above)

Smoking:
We disagree smokers should be excluded. There were only 2 smokers in our FGR cohort and no differences in the number of smokers between the two groups (see table 1).

Most importantly, our intention is to develop a clinical test that will be broadly applicable. To be useful, our test should ideally be able to identify significant fetal hypoxia irrespective of whether mother smokes. We hypothesise even if smoking was an upstream contributing factor to the pathogenesis of placental hypoxia and FGR, our test should still be able to detect the presence of fetal hypoxia.

The possibility that a nicotine effect independent of hypoxia might explain an up-regulation of hypoxia induced mRNA in maternal blood is interesting. But we suggest at this stage, it is sufficiently speculative that it does not mandate removal of data obtained from the two smokers.
from our FGR cohort.

In our larger validation study, we will have sufficient power to examine whether there is an independent effect of smoking on hypoxia-induced mRNA levels in maternal blood.

4) In addition to pre-pregnancy diabetes, maternal obesity could also impact the pattern and level of mRNAs. Obesity is associated with an inflamed placenta and potential reduced placental function. The authors should consider obese pregnancies as an independent group to evaluate the contribution of obesity vs hypoxia per se, or the combination of obesity and hypoxia.

Unfortunately, we do not have reliable information on BMI given our hospital does not routinely measure height of patients at their first antenatal visit. Again, we would make the same arguments as above: like smoking, even if obesity (± the presence of diabetes) was an upstream cause of placental hypoxia, our test should be able to identify the presence of fetal hypoxia.

In recognition of this reviewer’s points regarding smoking and maternal obesity, we have inserted the following in the discussion (line 388, addition in bold):

‘To translate our potential test to the monitoring of fetuses with severe FGR, our test requires validation with a study of larger numbers. Such a validation study could also help define the possible influence of clinical factors, such as smoking and maternal obesity, on hypoxia induced mRNA levels. We are currently undertaking such a large prospective validation study.’

Quality of written English: Acceptable

Statistical review: Yes, and I have assessed the statistics in my report.

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REVIEWER 3

In this study, the authors quantified mRNAs from the tissues and maternal blood using microarray and RT-PCR. They found altered expression of hypoxia-induced genes in maternal blood correlates with degree of fetal hypoxia/acidaemia. It is an interesting and explorative research to measure mRNA in maternal blood for predicting degree of fetal problems in utero. It might provide a novel non-invasive approach potential helpful to clinical application.

However, the data in this study is insufficient to support the conclusion and the following issues should be addressed:

1. All the results of this study only demonstrate that hypoxia changed mRNA of hypoxia-induced gene in maternal blood and placenta tissue. There is very limited data that can support the altered expression of hypoxia-induced genes in maternal blood was caused by fetal hypoxia and correlated with degree of fetal hypoxia/acidaemia.

The reviewer is correct in that we have not decisively proven the altered expression of hypoxia-induced genes in maternal blood was caused by the fetal hypoxia. However, given we are examining humans, we are restricted in what we can do and limited in options regarding mechanistic studies.

However, we not only acknowledged the fact we have not decisively proven that the hypoxic-induced genes in the maternal blood are placentally derived, but we have already discussed it at length (line 376, discussion):
'A limitation of our study is that we have not decisively proven the hypoxia-induced mRNA we are measuring in the maternal blood originates from the fetoplacental unit. This may be possible with the use of next-generation sequencing technologies where sequence information could be used identify the origin of mRNA transcripts (maternal or fetal). However, we have presented strong circumstantial evidence to suggest the hypoxia induced mRNA are of fetoplacental origin: 1) they increase with situations of likely severe acute and chronic fetal hypoxia 2) they correlate with an increase of hypoxic mRNA transcripts in gestational tissues 3) their relative abundance displays a highly significant and tight correlation with fetal acidemic status at birth. Ultimately, if hypoxia-induced transcripts in maternal blood were validated to reflect fetal acidemic status, it would not be absolutely essential to establish their origin, although a fetoplacental source seems the most likely.'

We wish to note our focus is to develop a test for possible clinical translation. With this in mind, we believe the last point is particularly important. As long as we can validate a correlation between hypoxic mRNA transcripts in the maternal blood with fetal hypoxic status, we will have already developed an important clinical test. It would not be absolutely essential to prove a fetal origin, though this would seem to us extremely likely.

We are currently leading a large National Health and Medical Research Council of Australia funded international study to validate this potential test. Called the FOX study (Fetal Oxygenation Study), we aim to recruit 150 cases of preterm FGR from across 7 sites. The intention is to see whether we can develop a reference chart where hypoxic-induced mRNA transcript abundance can be measured to determine degree of fetal hypoxia in utero (ie Figure 7, but with greatly expanded numbers).

2. Is the hypoxia-induced genes expression change a common response to hypoxia? Was the similar study conducted to test non-pregnant woman under conditions of hypoxia? Did the authors check oxygen or lactate levels in maternal blood ? In addition, the authors should exclude other possible mRNA changes caused by maternal hypoxia itself.

AND

3. Is there a co-relationship between maternal oxygen levels and the fetal hypoxia condition? Checking oxygen levels is convenient and affordable.

We did not measure maternal hypoxia in this study.

Notably, we have acknowledged in the discussion that we have not absolutely established the hypoxic mRNA measured in the maternal blood were of fetal origin. However, for reasons listed in the discussion (kindly refer to our response to the previous question), we believe there is compelling circumstantial evidence to suggest these transcripts are of fetal origin.

4. In Fig7, please explain how to generate a gene hypoxia score?

The figure legend for figure 7, already contains a sentence explaining how the gene hypoxia score was generated (line 594):

‘Gene hypoxia score is the sum of relative HIF1α, HIF2α, ADM and LDHA expression in maternal blood on the day of delivery.’

The text in the results section also explains how the gene hypoxia score shown in figure 7 was generated (line 320):

‘Lastly we correlated the hypoxia gene expression score (sum of the relative expression of HIF1α,
HIF2α, ADM and LDHA) in maternal blood from samples taken on the day of delivery with fetal acidemic status at birth (pH of fetal blood drawn from the umbilical artery) in the FGR cohort. We observed a strong correlation (Figure 7; r=0.76, P=0.008) between…’

5. In qPCR analysis, the result should be representative of at least 3 individual experiments, as the data varies so much for each experiment.

We agree for conventional laboratory in vitro experiments it is mandatory to repeat experiments three times (biological replicates). However, this is not relevant to the current report since we are not describing functional or mechanistic studies. Instead, we are reporting observational data from a human cohort. Thus, there is only one read per gene, per patient. Importantly however, the qPCR was performed in triplicate wells (i.e. technical replicates were performed, as per best practice).

6. 30/20 women have selected for this study. Is the sample size big enough?

The reviewer is referring to our FGR study, where we had n=20 cases of preterm severe fetal growth restriction and 30 gestationally aged matched controls.

The sample size seems clearly adequate since examining this cohort was sufficient to demonstrate highly significant increases for all four hypoxia-induced mRNA of interest (HIF1α, HIF2α, Adrenomedullin and Lactate Dehydrogenase A) in the FGR cohort (P≤0.05 for all comparisons).

The concern of inadequate sample size is more of an issue if a study has not identified a difference in the parameter of interest. The question then arises whether it was due to inadequate power.

7. In line 166, “200ng of tRNA”? Does this mean total RNA?

We have amended this, spelling out ‘total RNA’. (see line 173):

‘Reverse transcription of 200ng of total RNA was performed…’

Quality of written English: Acceptable
Statistical review: Yes, and I have assessed the statistics in my report.