REVIEWER 1 Manuscript ID ijms-1763216

	Yes	Can be improved	Must be improved	Not applicable
Does the introduction provide sufficient background and include all relevant references?	( )	( )	(x)	( )
Are all the cited references relevant to the research?	(x)	( )	( )	( )
Is the research design appropriate?	(x)	( )	( )	( )
Are the methods adequately described?	(x)	( )	( )	( )
Are the results clearly presented?	( )	( )	(x)	( )
Are the conclusions supported by the results?	( )	( )	(x)	( )

Comments and Suggestions for Authors

Manuscript ID. ijms-1763216

Uptake of phosphate, calcium, and vitamin D by the pregnant uterus of sheep in late gestation: Regulation by chorionic somatomammotropin hormone.

This work deals with an interesting topic and the authors present new findings on the relative abundance and utilization of calcium, phosphate and vitamin D in late gestation. Important evidence for the role of CSH in mineral regulation is provided.

However, there are serious problems in the presentation of the results and also in the discussion that make it difficult to understand the work.

The authors need to rewrite the results and the discussion sections according to the results presented and not based on trends.

References to results which were tendencies have been removed from the discussion. We have made edits to the results and discussion sections based on your specific comments below however we still feel that it is important to highlight the tendencies towards significance in the data set in the results section, especially given the small number of pregnancies in the CSH RNAi IUGR group. We are willing to consider removing these references in the results section at the request of the editor but would prefer to leave the tendencies in the results section.

Major comments

· Introduction

The authors mention two studies on fetal phenotypes with different consequences, however, it would be interesting to know what determines the condition, whether non-IUGR or IUGR, when does one or the other condition occur?

Thank you for this interesting question. This is a question that studies such as this are trying to address to identify what is different in the response between the IUGR and non-IUGR pregnancies as this could have significant translational importance. Using the sheep lentiviral knockdown, the non-IUGR pregnancies in response to CSH RNAi have reduced uterine but not umbilical blood flow, greater uteroplacental glucose uptake (µmol/min/kg placenta) but lower umbilical glucose uptake (µmol/min/kg fetus). In contrast, the IUGR phenotype in response to CSH RNAi is characterized by decreased uterine and umbilical blood flows, reduced transport of many essential nutrients for development, and altered fetal IGF1 concentrations. The previous studies utilizing this model have demonstrated that CSH RNAi has significant physiological ramifications, even in the absence of IUGR, and comparing CSH RNAi pregnancies exhibiting both IUGR and non-IUGR phenotypes may help determine the direct effects of CSH and its potential impact on fetal development. Interestingly, in human pregnancies, it has also been demonstrated that CSH deficiency does not always result in IUGR (Daikoku, N.H.; Tyson, J.E.; Graf, C.; Scott, R.; Smith, B.; Johnson, J.W.C.; King, T.M. The relative significance of human placental lactogen in the diagnosis of retarded fetal growth. Am. J. Obst. Gynecol. 1979, 135, 516–521).

· In the hypothesis, the authors postulate that CSH RNAi could decrease the utilization of phosphate, calcium and vitamin D utero-placenta. If this occurs, which of the phenotypes are they referring to? Or would both conditions be affected?

Given the previous data published using these simples, we had initially hypothesized that the IUGR pregnancies would have more severely impacted phosphate, calcium, and vitamin D uptake and utilization than the non-IUGR pregnancies but that both would be affected compared to the controls.

· In general, tables 1, 2 and S1 are very confusing, since results are explained that are not found in the table or results that are significant are not explained.

For example, in Table 1 the authors begin by comparing non-IUGR CSH RNAi and IUGR results, which are not statistically significant and are referred to as a trend. Then the authors make a comparison between IUGR CSH RNAi and control RNAi. However, it is very confusing that the results that are significant are not mentioned while results that have a "trend" are outlined. Moreover, it is completely unclear why different comparisons are made between the control and others between the phenotypes. Thus, the results part should be completely rewritten in a scientific manner that is understandable.

The results sections have been updated in light of some of your suggestions. As described in the statistics section of the methods, ANOVA was performed to assess the

overall effect of treatment where there were 3 groups, and in instances where there were 2 groups t-tests were used – which was presented as overall P value column. Tukey post-hoc analysis or t-tests were performed to identify significant differences between the treatment groups, and these were identified by different superscripts. As we know that the IUGR pregnancies often have differences in nutrient utilization compared to the Non-IUGR, it is important to compare Control vs Non-IUGR, Control vs IUGR, and Non-IUGR vs IUGR treatment groups.

· Results of section 2.2.2 Phosphate: Although it was mentioned in the methodology section that the results and samples for non-IUGR are not available, it should be stated again in the results section when the results are described and the groups are compared. It was stated that there are no differences between CSH RNAi concentrations. However, since it was not measured in non-IUGR, it should be underlined that there were no differences in the IUGR phenotype, because the values for the non-IUGR group were missing. This is important because otherwise the interpretation is quite misleading.

The results sections for both the phosphate and vitamin D sections have been updated according to your suggestion.

· Line 270, in the discussion, the authors claim that phosphate signaling in cotyledon and canuncules was affected, but it should be specified that it was only in cotyledon, since in canuncules it was not significant.

The wording has been changed however, as described in this paragraph, there was a significant alteration in the expression of the phosphate transporter SLC20A2 in the caruncles.

This way if dealing with the data is found in the description or interpretation of many results presented in this manuscript. The authors claim that there were changes, but indeed there were no statistically significant differences. Why doing statistics if differences are proposed where no significant changes were found?? This is quite unscientific.

· In the discussion the authors mention that there are no differences in phosphate in utero and umbilical vessels or uptake etc. However, this cannot be seen in the table, indeed it even seems that phosphate concentrations might be decreased. It is (once again!) not clear which statistical analyses were made: between groups or between umbilical or uterine vessels?

The authors apologize if this was not clear but we felt that the way the data are presented in the supplementary table with the phosphate data shows that we are comparing the three treatment groups for each vessel/gradient/uptake/utilization parameter listed in the first line of the table and the p value in the last column is the overall p value for the ANOVA comparing the groups. There were no statistically significant differences detected for each parameter when comparing the three treatment groups. We have changed the wording of this sentence in the discussion to hopefully make this clearer.

Minor comments

· In result 2.1 the authors describe that RNAi control plasma samples were used. However, in the caption they mention CSH RNAi non-IUGR and CSH RNAi IUGR. If they included the others, where are those results?

We apologise for this mistake and have corrected this in the resubmission. The data presented in section 2.1 is only comparing these things in the control samples as one of the objectives of the study was to examine the differences in concentration of these molecules in normal pregnant animals across the vessels of interest.

· In Table 1, N/A appears in the legend, however, there is no N/A in the table. Please do not copy and paste the legend of the figures of the tables in all the others.

Apologies for this oversight, this has been removed.

In table 1 and 2 the values <0.05 are in bold, for what reason? This was to highlight the significant results in the table for the ease of the reader, but this has been removed.

· 2.2.3 fix punctuation between 25(. OH)D. This has been corrected.

· The figures are too small, it is not possible to read the axis. We apologise for any inconvenience caused with this. We have attempted to increase the size of fonts within the figures and the size of the figures themselves where possible but if this is still an issue we can resolve this during the proofing process.

· In line 156, it should say calcium and phosphate in caruncule is **not** altered, since it is not significant.

· In the discussion on line 265 specify that lower expression of mRNA is found in cotyledon. This has been added.

REVIEWER 2

Review Report Form
Open Review
English language and style
( ) Extensive editing of English language and style required
( ) Moderate English changes required
(x) English language and style are fine/minor spell check required
( ) I don't feel qualified to judge about the English language and style

	Yes	Can be improved	Must be improved	Not applicable
Does the introduction provide sufficient background and include all relevant references?	(x)	( )	( )	( )
Are all the cited references relevant to the research?	(x)	( )	( )	( )
Is the research design appropriate?	(x)	( )	( )	( )
Are the methods adequately described?	( )	(x)	( )	( )
Are the results clearly presented?	(x)	( )	( )	( )
Are the conclusions supported by the results?	(x)	( )	( )	( )

Comments and Suggestions for Authors

In the present study, the Authors investigated phosphate, calcium and vitamin D metabolite 25(OH)D concentrations in uterine and umbilical blood vessels. Additionally, they determined the impact of CSH RNAi as well as the expression of candidate mRNAs on the concentration and regulation of phosphate, calcium and 25(OH)D in uterine vessels, caruncles and placental cotyledons in both Non-IUGR and IUGR pregnancies.

The Authors demonstrated that CSH RNAi Non-IUGR pregnancies had a lower umbilical vein – umbilical artery calcium gradient and less cotyledonary and phosphate compared to Control RNAi pregnancies. Moreover, they showed that CSH RNAi IUGR pregnancies had less umbilical calcium uptake, lower uterine arterial and venous concentrations of 25(OH)D, and trends for lower umbilical 25(OH)D uptake compared to Control RNAi pregnancies. Finally they showed that CSH RNAi IUGR pregnancies had decreased umbilical uptake of calcium, less uterine venous 25(OH)D, lower caruncular expression of SLC20A2 mRNA and lower cotyledonary expression of FGFR1 and FGFR2 mRNAs compared to CSH RNAi Non-IUGR pregnancies. The Authors concluded that CSH is actively involved in the regulation of mineral transport and utilization at the ovine maternal-conceptus interface, thus providing further mechanistic understanding of the hormonal regulation of mineral transport during pregnancy in mammalian species.

This is a very interesting study addressing an important issue as hormonal regulation of mineral transport during pregnancy thorough the placenta, uterine and umbilical blood vessels. Thus, it is of great interest to the readers of the International Journal of Molecular Sciences.
However, there are some points that the Authors must address before publication

1. Abstract. Please, clarify that caruncles and cotyledons are the maternal and fetal parts of placentome (e.g. feto-maternal interface). Moroever, add that 25(OH)D

represent vitamin D metabolite and the meaning SLC20A2, FGFR1 and 2 (first time you mentioned them). Thank you for this suggestion, this information has now been added.

2. It is clear that the Authors previously published the experimental design but it could be very useful to add a brief description in the methods section (e.g. why you choose day 132 to perform your analyses).
Additional information has been added to the materials and methods. As described in the introduction, peak concentrations of CSH in peripheral serum are between Days 120 to 140 of gestation, before rapidly declining approximately 5 days prior to parturition so Day 132 was selected to correspond with the peak in CSH production by the cotyledon.

3. The Authors stated that they included in the study 10 Control RNAi; 6 CSH RNAi Non-IUGR, and 4 CSH RNAi IUGR. However, when you analyzed the effects of CSH RNAi on serum calcium (table 1) and 25(OH)D (table 2) abundance you included only 4-10 Control RNAi. Please, clarify. Due to some issues with catheters being lost there were not always all samples from the Control RNAi group from all vessel types available for phosphate and vitamin D analyses so 4-10 reflects the range in n of samples available from the Control RNAi group. Data for calcium was available from all of the 10 control RNAi animals.

4. Please, check title legend of table 2. Probably there is a mistake since you reported the effect of CSH RNAi on serum 25(OH)D abundance but you titled it “Effect of CSH RNAi on serum calcium abundance” Apologies for this error. This has been corrected in the resubmission.

5. It is very interesting that when that Authors evaluated the effect of CSH RNAi on calcium and phosphate abundance and on candidate mRNAs with roles in the regulation of calcium, phosphate and vitamin D signaling in cotyledons and caruncles, they showed main effects on fetal part of the placentome rather that maternal part. Can you, please, comment these results? Thank you for this interesting point, this has been added to the discussion.

6. The Authors should add in the Methods section the list of genes that they analyzed explaining the rationale (e.g. sodium dependent phosphate transporter). Thank you for this suggestion, this has been added to the methods section.

7. In Figure 2B and 2D, the Authors reported a significant differences in calcium and phosphate levels between controls and non IUGR in cotyledons. However, it is very strange that you did not find significant differences between non-IUGR and IUGR (graphically the difference is greater relative to control-non IUGR). Please, clarify. We have checked the statistics on this and confirm that these comparisons do not provide statistically significant differences. We would speculate that these differences may be significant with more animals.

8. Can the Authors, please check the statistic of Figure 3A, 3I, 3K, 3M and 3N? The bars are really different between groups but you did not report significant differences.

We have checked these statistics and have updated KL, TRPV6, and CYP24. However, there are no differences in S100G or STC1 expression.

9. It could be very interesting if the Authors could correlate their data with maternal and fetal biometry data maybe adding two tables (Data on maternal and on fetal biometrics). Thank you for this interesting suggestion. We have correlated the data generated with maternal weight, uterine weight, fetal weight, and placental weight and added these data to the manuscript.