Author’s response to reviews

Title: Type 2 diabetes gene TCF7L2 polymorphism is not associated with fetal and postnatal growth in two birth cohort studies

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Author’s response to reviews: see over
Dear Sir/Madame,

Thank you very much for reviewing our manuscript: **Type 2 diabetes gene TCF7L2 polymorphism is not associated with fetal and postnatal growth in two birth cohort studies.** We are grateful for the detailed comments of your reviewers. Most importantly, we have changed the analysis regarding the difference in minor allele frequency between the SGA and non-SGA populations. Secondly, we have removed all the dominant and recessive models from the manuscript. Finally, the text we edited by a native speaker and several small grammatical and stylistic error were removed from the manuscript. We have made changes in our manuscript in response to the comments and hope you will find the revised manuscript acceptable for publication in *BMC Medical Genetics*.

Below you will find our specific responses to all questions or comments from your reviewers.

With kind regards,

Yours sincerely,

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Response to Reviewer: Rachel Freathy

Mook-Kanamori and colleagues have examined the evidence for association between the common rs7903146 variant in TCF7L2 and measures of fetal and infant growth using subjects from a prospective, population-based study (N=3419) and a cohort of SGA subjects (N=566). They compared allele frequency between the two cohorts and found no difference (P=0.47). No association was found between TCF7L2 genotype and any fetal or postnatal growth measure in either study, or with longitudinal growth rates from 0 to 2 years (P>=0.04). The authors conclude that "TCF7L2 would not appear to be involved in the previously demonstrated associations of low birth weight with T2D". Strengths of this paper include detailed fetal and infant growth phenotypes and reasonable sample power. The authors have tested a clear hypothesis using appropriate methods, and the manuscript is clear and well written. I have the following points of concern:

Minor essential revisions:
1. Introduction, paragraph 2: "The T-allele of rs7903146, which has an estimated [minor] allele frequency in Caucasians of about 25%..." This is a little vague, and should be clarified by, for example, giving the MAF range from cited publications or quoting the HapMap CEU minor allele frequency.

We agree that this is a bit too vague and have changed the text to the following (page 3): "The T-allele of rs7903146, which according to HapMap has an allele frequency amongst Caucasians (CEU) of 28%, has been shown to be associated with reduced insulin response and secretion in both diabetic and non-diabetic individuals [8-10], though results in non-diabetics are not consistent [11, 12]."

2. Introduction, final sentence: the SGA cohort is referred to as "replication" study. I do not think this is an accurate description because of the different nature of ascertainment and, crucially, the sample size. It would be more appropriate to speak of using two independent studies to investigate the same question, rather than describing them as primary and replication studies.

We agree that the word ‘replication’ is misplaced here. We have removed it and the sentence is now as follows (page 4): “Second, we assessed associations of this genotype with birth weight and postnatal growth in 566 small-for-gestational-age (SGA) children participating in an independent cohort study.”

3. Minor allele frequency was compared between the two studies. This analysis seems similar to a case-control study, comparing individuals who are small for gestational age with
those who are appropriate for gestational age. However, the authors did not remove individuals who were small for gestational age from the "control" (Generation R) study. I realise that the small number of these is unlikely to make much difference to the results, but the rationale for this analysis, and the decision not to exclude SGA subjects from the Generation R study for this analysis should be explained more clearly. We agree that assessing the difference between the general population and a SGA group can be misleading, since there are SGA subjects in the general population group. As the reviewer suggested, we have removed the SGA's from the Generation R study so that we can compare SGA’s with non-SGA’s. The p-value does not change much. We have also changed the text in the data analyses and results sections accordingly.

4. It is not clear why the authors tested dominant and recessive models in addition to the additive model. I am not aware of any study which reports association between TCF7L2 and type 2 diabetes as deviating significantly from an additive model. Testing these additional genetic models without clear rationale for doing so increases the number of tests carried out, without adding value to the study.

We agree with this point and have therefore removed all dominant and recessive models from the text and tables. Only the additive models are presented.

5. The power calculation in the methods is helpful to the reader. However, there were fewer individuals available for the postnatal analyses, so presumably the effect size detectable was not as small. It would be more helpful to give a little more detail than "able to detect differences in growth characteristics of about 0.05 SDS", perhaps giving the effect size detectable in (i) the fetal and (ii) the postnatal growth analyses. Additionally, in terms of study design, a P value threshold of 0.05 is too lenient, considering the number of tests that have been carried out.

We have added a power analysis regarding the postnatal analyses to the text. The first sentence of this paragraph now is as follows (page 12): “With sample sizes in the Generation R Study of 3,419 and 2,675 subjects for fetal and postnatal analyses respectively, and assuming a statistical power level (1 – β) of 0.80, a level of significance (α) of 0.05 and a variance of 1.0, we were able to detect differences in growth characteristics of 0.048 SDS and 0.054 SDS respectively.”

Furthermore, we absolutely agree that a P-value of 0.05 would be too lenient for this study, if we had found any association. However, we do not find any association and therefore we feel that it is not necessary to add this correction to the power analysis.
6. In Table 3, it would be helpful to include the numbers of subjects by genotype, as has been done in tables 4 and 5. Thank you for pointing this out. We have added the numbers to the table.

7. Was gestational age transformed for linear regression analysis? How was the distribution of this phenotype treated.
Gestational age was not normally distributed. However, in the linear regression analysis the residuals did not deviate much from normality, which makes it acceptable to use the linear regression. We also ran the analysis using an inverse normal and quadratic transformation. These had similar (null-)results and therefore we would prefer to show the data in an untransformed, easier to interpret, form.

8. The authors mention in the results section that one P value was < 0.05 (for weight at 2 years in the SGA cohort). I am curious as to why this result is picked out. Multiple testing considerations mean that this result could easily have occurred by chance. I do not think it is worth mentioning here.
We agree. This does not have any additional value to the paper and we have therefore removed this sentence from the manuscript.

9. While I agree with the authors’ conclusion (especially in the light of previous data) that fetal TCF7L2 genotype is not associated with fetal or infant growth (and therefore unlikely to influence metabolic phenotypes until after early childhood), I think it is important for the authors to qualify this with reference to the limits of detection in the current study. The confidence limits around their effect size estimates cannot rule out smaller effects of this variant on fetal growth. This is a minor point, but a statement in the discussion, acknowledging the power and detection limits of the study would be helpful.
We agree with the reviewer that a brief notice of this point in the discussion is necessary (page 8): “Nonetheless, our results also could be explained by a lack of power and we cannot rule out that we were not able to detect smaller effects of this variant on early growth.”

10. It should be mentioned that the results are not adjusted for maternal genotype. As the authors point out, associations between the maternal TCF7L2 risk allele and increased offspring birth weight have previously been documented. Fetal and maternal genotypes are 50% correlated. This raises the possibility that, in utero, where the risk allele is present in both mother and fetus, any small effects of fetal genotype reducing fetal growth could be masked by opposing effects of maternal genotype. Again, in the light of previous data, this is
unlikely, but the discussion should acknowledge that this possibility cannot be ruled out because maternal genotype was not available in the current study.

We agree and have changed this paragraph (page 7-8): “Freathy et al. found an increase of birth weight for each fetal and maternal risk allele [14]. They concluded that the most likely mechanism for this association was that maternal genotype was associated with a reduction of maternal insulin secretion, leading to increased fetal glucose and insulin levels and subsequently increased birth weight, rather than a direct effect of the fetal genotype on birth weight. Pulizzi et al. found no effect of the fetal genotype of this polymorphism on birth weight. Since fetal and maternal genotypes are 50% correlated, it cannot be excluded that, when the risk allele is present in both mother and child, small effects of fetal genotype that reduce fetal growth could be masked by opposing effects of maternal genotype. Since maternal genotype was not available in our study, we were not able to test this hypothesis. However, we did not find any effect of fetal genotype on birth weight in the general population nor in a specific population of children with insufficient fetal growth resulting in small size for gestational age at birth. Our findings are therefore in line with the conclusions of these previous studies. Furthermore, we found no effect of fetal genotype on estimated fetal weight or weight during infancy, indicating that there is no evidence for any association between this fetal genotype and weight or change in weight during early life either. The effect of this polymorphism on the metabolic phenotype found in adults would therefore appear to develop after early childhood. Nonetheless, our results also could be explained by a lack of power and we cannot rule out that we were unable to detect smaller effects of this variant on early growth.”

11. Discussion, page 7, paragraph 1: "Several studies investigated the effect of common genetic variants related to insulin action on early growth and found no or inconsistent associations [15,20,21], possibly because they investigated gene polymorphisms that appear to be less strongly associated with T2D than TCF7L2 rs7903146." There are a few important points to make about this statement: (a) I agree that power would be lower for variants that are less strongly associated with T2D (or more specifically, predispose to T2D with smaller effects) than TCF7L2, but it is important to make the distinction between the PPARG and KCNJ11 variants tested in reference 20 (which are confirmed diabetes susceptibility genes, verified genome-wide and widely replicated) and the IGF1 and INS-VNTR variants, which do not predispose to type 2 diabetes. (b) KCNJ11 is generally thought not to associate with insulin action, but insulin secretion. (c) A recently published study in Diabetes documents strong associations between the fetal CDKAL1 and HHEX T2D variants and birth weight (Freathy et al, 2009).
We agree that this statement and the references are a little outdated. Furthermore, more recent papers have shown a convincing association with birth weight. We have changed these sentences as follows (page 7). We also have changed the references (Freathy 2007 and 2009, Pulizzi 2009, and Bennett 2008): “Several studies have investigated the effect of common genetic variants related to insulin action and secretion on early growth [14, 15, 20, 21]. Of the initially identified T2D gene polymorphisms identified by the GWA, fetal CDKAL1 (rs7754840) and HHEX (rs1111875) genotype, and maternal TCF7L2 (rs7903146) genotype have been shown to affect birth weight. Pulizzi et al. demonstrated in the Helsinki Birth Cohort that fetal TCF7L2 genotype did not interact with birth weight to increase the risk of T2D in adulthood [15]. TCF7L2 rs7903146 has been shown to have the strongest genetic effect on T2D and this result has been replicated in several studies [3-5]. Therefore, TCF7L2 is a very important candidate gene for explaining the association between low birth weight and T2D risk.”

12. Figure 1 was unreadable from the pdf file, so I was unable to check it. This is a useful thing to include in the paper though.
I do not know what went wrong here. We will add this to the final manuscript.
Mook-Kanamori and colleagues investigated whether the most replicated type 2 diabetes gene, TCF7L2, is associated with growth pattern during fetal life and early infancy, a concept in the frame of the fetal insulin hypothesis. To do so, they used data from 2 cohort, (Generation R, representative of general population, and SGA cohort, including subjects small for gestational age). Of Generation R, fetal measurements during the second and third trimester were available.

The issue addressed by this manuscript is of interest, and there are some potentially relevant data on TCF7L2 genotype and fetal measurements. Though, I do not agree on the way some data were presented. The authors found:

1. The minor allele (T) frequency of the variant rs7903146 in TCF7L2 gene was similar between Generation R and SGA cohorts.
2. Birth size was similar among genotypes in both cohorts.
3. TCF7L2 was not associated with pre- and post birth growth characteristics in both cohorts.

The authors concluded that “TCF7L2 would therefore not appear to be involved in the previously demonstrated associations of low birth weight with T2D”. A key strength of the study is the availability of fetal measurements in Generation R cohort, which adds some exciting information in the puzzle of the fetal insulin hypothesis. As the authors clearly state, birth weight can be the result of very different growth pattern during fetal life, thus representing a rough proxy of fetal development. The manuscript is clear, even if throughout the text there is some redundancy (see Discretionary Revisions). However, there are some important areas of concern:

Major Compulsory Revisions:

1. Dominant, recessive and additive models are tested. This approach can be acceptable in a preliminary analysis of data, but according to the literature in this field, only the additive model earns credit. When testing different models, a Bonferroni correction for multiple testing multiple is usually required, and this often turns nominal significant findings into non significant results. Here the dominant and recessive models do not add any information, so I would suggest to skip them, showing only results drawn from the additive model.

We agree with this point a have therefore removed all dominant and recessive models from the text and tables. Only the additive models are presented.
2. Comparing the allele distribution between the general population (Generation R) and SGA cohort gives the reader some qualitative hint to think about, but, as it is, it sounds to me somehow misleading, whereas from that the authors seem to draw too strong conclusions. The ideal would have been to compare in the Generation R cohort the allelic distribution between SGA subjects and non SGA subjects, adjusting for available confounding factors and looking for eventual interactions. From table 1 it appears that the low number of SGA subjects in the Generation R cohort makes the suggested approach weak in terms of statistical power. If the authors want to compare the allelic frequency between the two cohorts, not merely to describe the data, but in a “case-control” approach, I suggest, at least in this specific analysis, to remove from the Generation R cohort subjects small for gestational age. Even after doing so, there will be left some newborn labeled as non SGA, but still with a birth weight lower than 2500 g, if I interpret correctly the data shown in table 1: in Generation R cohort 1.6% is SGA and 2.5% has a birth weight <2500 g. On the other hand in The SGA cohort there is 17.3% with a birth weight >2500 g. The authors should either to redesign this analysis or to better comment the results from the chi-square. Consistently, I would advocate more caution to state: “Furthermore, minor allele frequency was not different in SGA subjects than in non-SGA subjects from which we can conclude that there was no association between genotype and risk of being born SGA”.

We agree that assessing the difference between the general population and a SGA group can be misleading, since there are SGA subjects in the general population group. As the reviewer suggested, we have removed the SGA’s from the Generation R study so that we can compare SGA’s with non-SGA’s. The p-value does not change much. We have also changed the text in the data analyses and results sections accordingly. Furthermore, we agree that we should be careful with drawing a too strong conclusion and have rephrased the sentence as follows (discussion, final paragraph): “Furthermore, minor allele frequency was not different in SGA subjects than in non-SGA subjects indicating that it is unlikely that this polymorphism is associated with the risk of being born SGA.”

Discretionary Revisions:

1. The final statement in the abstract: “TCF7L2 would therefore not appear to be involved in the previously demonstrated associations of low birth weight with T2D” cannot be drawn from the data presented.

We agree that this cannot be drawn from the current data. We have removed this statement from the abstract and the final part is not as follows: “We found no evidence for an association between TCF7L2 genotype and fetal and early postnatal growth. Furthermore, this TCF7L2 polymorphism was not associated with an increased risk of SGA.”
2. The authors are redundant in the Introduction and in the Discussion sections, repeating some information from the literature, not strictly related to their own results.

The reviewer rightly points out that there were some redundancies. We have removed the review of the fetal insulin hypothesis from the discussion (first four sentences from 3rd paragraph of discussion) and also removed the review of the findings from Freathy et al (first two sentence from 5th paragraph), since these topics were already discussed in the introduction.

3. The authors cite previous studies from Cauchi and Freathy. To be more complete they might cite also the findings from the Helsinki Birth Cohort, which consistently did not show any association between this variant of TCF7L2 and birth size. We have added the Helsinki Birth Cohort to the references. Furthermore, we have made reference to their work (Pulizzi, 2009) in both the introduction and discussion of the manuscript.

4. “Furthermore, we demonstrated that this polymorphism is not related to size at birth...” : I would rather use “replicate”or “confirm”. On the other hand I would stress more throughout the text what seems the real novelty of this study. Since TCF7L2 is likely associated with impaired insulin secretion and insulin is a main anabolic factor during fetal life, it arises as a candidate gene of impaired fetal development, as well stated by the authors. Previous studies failed to demonstrate this hypothesis, but their findings were not definitive, being birth weight a proxy of development. In this unique study, the authors demonstrate that this variant of TCF7L2 does not influence the fetal development, by direct fetal measurements.

We agree that it would be better to highlight the novelty, namely the directly measured fetal growth. We have therefore changed this first paragraph of the discussion as follows (page 6):

“In the current study, we found that T2D gene polymorphism TCF7L2 rs7903146 is not associated with growth in fetal life in the general population or with growth in early postnatal life in both the general population and in a cohort of subjects born SGA. We also confirmed previous suggestions that this variant of TCF7L2 is not associated with birth weight and, more importantly, demonstrated that it does not influence the fetal development using direct fetal measurements. Finally, we showed that this polymorphism does not appear to be associated with the risk of being born SGA.”

Minor Essential Revisions

Introduction; 3rd line: These associations may be explained by common genetic variants; better to use “influenced”.
Agreed. We have changed this in the text.

**Introduction:** 4th line: “factor” is repeated twice in the same sentence.
Agreed, the sentence is now as follows: “Insulin is the most important fetal growth factor and insulin-mediated fetal growth might be affected by genetic polymorphisms that regulate fetal insulin secretion or insulin sensitivity.”

**Results:** 11th line: I would say nominal p value, to make clearer that it may a false positive
On recommendation of another reviewer, also because she wanted to highlight the fact that this most likely is a false positive, we have removed this sentence.

**Discussion:** 2nd line: “early life” I would specify “fetal life” for general population and early postnatal life for both cohorts.
We agree that we should highlight the “fetal life”. We have changed the sentence to the following: “In the current study, we found that T2D gene polymorphism TCF7L2 rs7903146 is not associated with growth in fetal life in the general population or with growth in early postnatal life in both the general population and in a cohort of subjects born SGA.”

**Discussion:** 24th line: These fetal genetic factors could also (partially) explain the association between low birth weight and T2D risk
Agreed. We have added the word ‘(partially)’ to the text.

**Materials and Methods/The Generation R study/Fetal growth...** 8th line: "using" is repeated twice in the same sentence.
Thank you. We have changed the sentence to the following: “Estimated fetal weight (EFW) was calculated by means of the formula from Hadlock using head circumference, abdominal circumference and femur length (log \(_{10}\) EFW = 1.5662 – 0.0108 (HC) + 0.0468 (AC) + 0.171 (FL) + 0.00034 (HC)\(^2\) – 0.003685 (AC * FL)).”
Response to Reviewer: Clive Osmond

Thank you for sending me this article for statistical review.

If I had been reading it for more general review I would have questioned the wisdom of searching for genetic causes of a condition that is showing epidemic behaviour in many populations.

We agree that obesity and type 2 diabetes are showing an epidemic behaviour. However, recent genome-wide association studies have identified genes that are related to both these traits. Nonetheless, we are still unable to explain the heritability of these metabolic disorders and therefore it is useful to look at the possible link with early growth (as stated in this manuscript). We feel that “questioning the wisdom of searching for genetic causes” of common complex diseases goes beyond the scope of this manuscript.

I would also say that the issue of postnatal growth as a predictor of type 2 diabetes could be described a bit more fully. As I understand it studies of infant growth (Hertfordshire, Helsinki, Delhi) suggest that infant weight gain (muscle rather than fat?) is protective, but later childhood weight gain is harmful. However, to the stats (which are nicely expressed)........

Indeed other studies indicated that the timing of fast growth is associated with the risk to develop type 2 diabetes in adulthood. Also body composition is associated with the risk of type 2 diabetes. We had changed the last sentence of the third paragraph of the introduction to the following (page 4): “Furthermore, rapid postnatal weight gain, especially in fat mass, has also been shown to be associated an increase risk of obesity and type 2 diabetes in later life, independent of birth weight [18, 19].”

The knee-jerk reaction of some statistical referees would be to ask for more detail about the robustness of the findings, given that many observations are missing. I don’t think that is actually too critical an issue here. The authors report some differential follow-up according to genotype. Maybe there is also differential follow-up according to size/growth. More critical is whether there is differential association between genotype and growth according to follow-up. But there is no way to assess that, nor does it seem very likely.

We agree with the reviewer that this is unlikely, and impossible to assess. Nonetheless, it should be a discussion point and therefore we have made the following change to that paragraph of the discussion (page 6): “Our effect estimates could be biased if the associations between genotypes and growth characteristics differed between those with and without postnatal growth data available. In the Generation R cohort, no differences were observed between children with and without postnatal growth measurements. In the SGA
cohort the T-allele was slightly more frequent in subjects with postnatal growth measurements than in subjects without these measurements ($p < 0.05$). Finally, it could be possible that there is differential effect of genotype on growth according to availability of follow-up data. This bias would affect our estimates, though such a bias seems unlikely."

I would suggest giving the n’s available in the tables, rather than the footnotes.

We have added these numbers

For some reason my Figure 1 came out as a page of empty boxes.

I do not know why this happened, but it will be added to the final manuscript.

I was a bit concerned about the growth units that are given as sds/week. The first value quoted in para 3 on page 5 gives a value of –0.04 sds/wk. That is an enormous difference between the genotypes. Over just one year it would imply a change of –2sds, which doesn’t seem plausible.

Thank you for pointing this out. We have corrected this and now show the data in SDS/year.

I missed the derivation of the SD scores. If they are internal to the study, which I doubt, it would be good to know how the authors cope with the variable ages at measurement.

We used growth analyser software from the Dutch Growth Research Foundation. We have added this text the data analyses section (page 12): “Standard deviation scores were obtained using Dutch reference growth curves (Growth Analyser 3.0, Dutch Growth Research Foundation).”