Author’s response to reviews

Title: Leptin receptor (LEPR) SNP polymorphisms in HELLP syndrome patients determined by quantitative real-time PCR and melting curve analysis.

Authors:

Tibor Varkonyi (Varkonyi.Tibor@noi1.sote.hu)
Levente Lázár (Lazar.levente@noi1.sote.hu)
Attila Molvarec (molvarec.attila@noi1.sote.hu)
Nandor G Than (Than@noi1.sote.hu)
János Rigó Jr (rigo@noi1.sote.hu)
Bálint Nagy (nagy.balint@noi1.sote.hu)

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Author’s response to reviews: see over
Dear Professor Edmunds,

Thank you for your e-mail and for the reviewer’s comments. We answered the questions point by point. We hope you will find the revised version suitable for publication in the BMC Medical Genetics.

Referee 1.

1. Describe and discuss the power of this study to confirm or exclude the association of the LEPR SNPs and HELLP syndrome.
Sample size estimation of the study was performed by using Quanto 1.2.4 (www.hydra.usc.edu) statistical program. Abate et al (Arch Med Res 40;306-310,2009) published their data on Lys109Arg LEPR polymorphism with a similar set up. Our sample size provided sufficient statistical power (>80% at Type I error rate of 0.05) to detect 21% difference in LEPR c.326A>G genotypes and 14% difference in LEPR (AG AG GG AG) haplotypes between cases and controls.
Based on our investigation there is no association with the LEPR SNP polymorphism and the development of HELLP syndrome. It is in agreement with previous study on preeclampsia using two LEPR SNP polymorphisms on same size of cases and controls (Rigo et al. Gynecol Endocrinol 2006).

2. The fetal genotype has not been investigated. Please, discuss the role of fetal genes in this context.
We did not investigate the fetal genotype, while it would be interesting, unfortunately the ethical regulation does not permit to carry out such kind of study in Hungary. It is not possible to collect blood or genetic material from newborns for research purposes. Our study was partly supported by the EU6th Framework, our ethical permission allowed only to study the maternal part, while it would be really interesting to collect data on fetal and maternal side.

3. Have the results been corrected for multiple testing of SNPs?
The multiple comparisons or multiple testing problem occurs when one considers a set, or family, of statistical inferences simultaneously. Errors in inference, including confidence intervals that fail to include their corresponding population parameters, or hypothesis tests that incorrectly reject the null hypothesis, are more likely to occur when one considers the family as a whole. We did not study families, parents and children in our study, so we did not perform multiple testing like ANOVA F-test or Bonferoni-Dunn test. I suppose this question is in connection with the Referee’s previous question.

SNPAnalyzer 2.0 software (Istech Corp., Korea) was used for testing the genotype distribution for the Hardy-Weinberg equilibrium with Multiple correction, results are shown in Table 5.
4. The description of sequence variations is somewhat confusing. Please, use the nomenclature of variations recommended by The Human Genome Variation Society.

Thank you for this comment, we were also confused when checked the literature and almost each publication used different name for same the SNP. We changed the nomenclature in the text according to the recommendation of The Human Genome Variation Society.

Minor comments:
1. Table 1: Please provide statistics of the clinical characteristics between the two groups.
   We calculated and provided the statistical calculations in the revised Table 1.

2. Table 3: Please correct: LEPR G- (the third SNP). The order of the allele 1 and 2 is not consistent given in the name of these SNPs. Please clarify these.
   We made the correction.

3. Table 5: Please correct: VEGF SNPs.
   It was corrected.

Referee 2.

Major compulsory revisions
1. Some results are redundantly presented in the abstract.
   We rechecked the results in the abstract and corrected accordingly, we removed the unnecessary duplications.

2. The introduction is insufficient to set the genetic background of the study. It could be expanded to introduce known or potential polymorphisms/haplotypes related with HELLP syndrome and/or associated conditions.
   There are only a few articles dealing with gene polymorphism and its relation with HELLP syndrome, we have publication on VEGF polymorphism, already. Most of the studies dealing only with the relation of different gene polymorphisms and preeclampsia. HELLP syndrome is a very rare disease so it is difficult and takes time to collect the samples.
   We inserted a new paragraph into the Introduction the give a rationale for the genetic background of this study and inserted 5 references (Ref. 18, 19, 20, 21 and 22). There are several articles on gene expressions in HELLP syndrome and all found higher expression of the leptin gene in HELLP syndrome. The SNP polymorphism could have effect on the gene expression (like in the case of VEGF). We decided to study four SNPs on leptin receptor gene to find out is there any difference in the allele and genotype distribution of these four leptinreceptor SNPs.

3. What is the rationale for choosing the LEPR SNP polymorphisms assessed in the study?
   It is discussed in the point 2. High levels of leptin were detected in RNA and protein determinations. It is known that couple of VEGF SNPs has effect on the VEGF protein expression, we determined the VEGF SNPs in HELLP syndrome previously and found different frequencies in HELLP syndrome (Clin Chim Acta 2008). We decided to make
similar study on LEPR as it has not been studied. There are only a few studies on LEPR SNPs in preeclampsia too. It seems logistic to study it in HELLP syndrome. The leptin somehow is involved in the development of PE and HELLP syndrome, but nobody knows the mechanism of the action. Do the LEPR SNPs have difference in the frequency of the alleles and genotypes? We decided to find it out.

4. Did study participants sign informed consent? If so, please write it clearly.
Yes all patients were informed and they signed consent. It was stated in the Methods section. We made it clear in the text now.

5. Did the authors carried out power studies to define the minimum sample size to avoid false negative overall results?
Yes, we made the statistical power calculation. It is discussed at answers for Referee no.1. at point 1.

6. Real time PCR and melting curves were used to assess the polymorphisms of interest. What’s the reliability of melting curves as compared with other PCR-based genotyping? Also, the authors claim to have introduced RT-PCR and melting curves to assess LEPR SNP polymorphisms. Yet, implications of this new approach has not been discussed in depth.

   Our real-time PCR and melting curve analysis method is replacing the PCR-RFLP method which is widely used for SNP determinations. We can reduce the number of pipetting meanwhile reducing the risk of contamination during the analysis of the samples. The melting curve analysis makes the reliable allele determination as there are 5-10 °C differences in the melting points of the different alleles. The reaction is faster than the conventional PCR and we can avoid the use of separate digesting, electrophoresis and detection step which is used during PCR-RFLP. We can reach shorter detection times, less labor and favorable price. We involved it in the Discussion section now.

Minor essential revisions:
1. Several acronyms are not defined in the text (eg. VEGF, IUGR, etc.).
   We defined all acronyms in the text.

2. English needs some revision.
   We made a second English revision.

Thank you for consideration for publishing our manuscript.

Sincerely yours,

Dr. Bálint Nagy Ph.D.