Author's response to reviews

Title: Lack of association between polymorphisms of the IL18R1 and IL18RAP genes and cardiovascular risk: the MORGAM Project

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

We would like to thank you for giving us the opportunity to reconsider our manuscript "MS: 1247520950229495 - Lack of association between polymorphisms of the IL18R1 and IL18RAP genes and cardiovascular risk: the MORGAM Project". We did our best to account for reviewers' remarks as indicated in the point-by-point answers. In particular, we provided new information about SNP x environment interactions and power calculations.

We would also like to emphasize that we had not plagiarized any sentences drawn from published works by others. We had re-used some sentences we have already written in a manuscript previously published by the same authors (Grisoni et al. 2008) from the same studied populations as those described in the submitted document. However, to address your concern, all "self-plagiarised" sentences have been modified. The exact sentences we had re-used are listed at the end of this letter with the corresponding changes we made.

We hope this revised version that has been reformatted according to the recommendations of BMC Medical Genetics Journal would be suitable for publication.

Sincerely yours
Answers to reviewers

Reviewer 1: Andrew J Sandford

Major Compulsory Revisions

1. In the background section, the rationale provided for studying the IL18 receptor genes entirely relates to IL18. It would be informative for the authors to elaborate on their gene selection, for example, were the IL18R1 and IL18RAP genes picked up in genome-wide scans? Is there any other direct evidence of their plausibility as candidates for susceptibility to CVD?

Even if we do consider that the GWAS approach is a powerful and an attractive strategy to identify new susceptibility genes, it is not the panacea. Even if IL18R1 and IL18RAP genes have not been detected in recent GWAS, that does not preclude them from being candidates for susceptibility to CVD. We have completely modified the way the Introduction was presented in order to clarify the rationale of the current work.

2. Results section:

2.1. In the first paragraph of the results section, the abbreviation CHD is used for the first time without stating its meaning, whereas all other abbreviations in the paper were explained.

CHD abbreviation is now explained at its first occurrence in page 4 – line 19

2.2. In the IL18R1 gene results, the first sentence states there were “slight deviations from Hardy-Weinberg equilibrium” but contradicts this by stating “all genotypic distributions were compatible with HWE”. In the IL18RAP results, it is stated that all genotypic distributions were in HWE, but this is incorrect from the data presented in Table 2. For the IL18R1 data, out of the eleven SNPs genotyped, three different SNP genotyping data show departure from HWE, with p values below 0.05. The authors should present the actual p values so that the reader can judge whether the deviations are “slight”. Concerning the IL18RAP data, four departures from HWE were observed. Giving rise to more concern, is the fact that all of them are in the same cohort (PRIME/France). I believe that is in need of an explanation. It could be due to genotyping or plating errors or perhaps some contamination issues, or population stratification in this particular cohort. In any case, this should be discussed and details of quality control in the genotyping process should be clearly stated (see below).

We are sorry for the confusion. Our sentences were completely misleading. In each subcohort, no deviation from HWE was observed for any SNP. However, some SNPs failed HWE in subgroups of cases or in non-cases. This information has been omitted in the revised version, so as not to mislead readers.

However, we provided below the information about the seven SNPs that failed HWE at \( p < 0.05 \) so that the reviewer may decide by himself if this information is valuable or not and should be given in the manuscript. As correctly pointed out by the reviewer, four of these deviations from HWE were observed in PRIME/France samples. These were not observed in the whole PRIME/France sample but restricted only to the cases samples with only 114 individuals. Some of these deviations were in fact due to very small genotype counts and no genotyping error was detected after revalidation of the data.

Nevertheless, after correcting for the number of HW tests performed (44 = 11 x 4), none of the shown p-values passed the Bonferroni threshold of 0.0011 (=0.05/44) and then none was significant at corrected \( p < 0.05 \).

As a consequence, we don’t consider that the validity of the results presented in this work could be affected by these modest deviations from HWE.
List of SNPs that failed HWE at p < 0.05

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Cohort</th>
<th>Group.</th>
<th>uncorrected p-value</th>
</tr>
</thead>
<tbody>
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<td>IL18R1</td>
<td>rs1420096</td>
<td>PRIME/ France</td>
<td>Cases (n = 114)</td>
<td>0.032</td>
</tr>
<tr>
<td></td>
<td>rs11465656</td>
<td>Sweden</td>
<td>Non-cases (n = 128)</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>rs3732127</td>
<td>ATBC</td>
<td>Non-cases (n = 718)</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>rs11465660</td>
<td>PRIME/ France</td>
<td>Cases (n = 114)</td>
<td>0.009</td>
</tr>
<tr>
<td>IL18RAP</td>
<td>rs4851581</td>
<td>PRIME/ France</td>
<td>Cases (n = 114)</td>
<td>0.028</td>
</tr>
<tr>
<td></td>
<td>rs1420105</td>
<td>PRIME/ France</td>
<td>Cases (n = 114)</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>rs11465673</td>
<td>PRIME/ France</td>
<td>Cases (n = 114)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

2.3. In the results for both IL18R1 and IL18RAP, it is not mentioned if correction for multiple comparisons was performed in order to obtain the p values discussed in this section. In the case of IL18R1 +404SNP in the Swedish cohort, if the p value is corrected then it should not be considered borderline. If there was no correction performed then the authors’ conclusion that there is no association is reasonable. The same reasoning applies to IL18RAP +55/in7SNP in the FINRISK cohort.

As suggested, we now mentioned (Page 8 – Line 18 and Page 9– Line 12) that after correction for multiple testing, the borderline uncorrected p-value were no longer significant.

3. Methods section:

3.1. Using only the first stroke and/or CHD event for patients with multiple events does not fully utilize the data. The presence of multiple events samples could be used as an opportunity to stratify the cases into subgroups, which could then be compared by genotype. While there is no evidence of association when comparing cases with controls, it is possible that those SNPs or a portion of them will only be relevant in cases and would therefore be indicative of severity of CVD.

We do not consider that multiple events is a good indicator of the severity of CVD. If there is a severe event, the person dies, and therefore has no chance to get another event. If the person survives the event, the future depends very much on the medical and other interventions. In addition, in all MORGAM cohorts, only 68 subjects experienced multiple events, a number that is low for drawing reliable conclusions if we had investigated SNP association on this endpoint.

3.2. What are the sex specific markers used as part of the quality control?

The sex-specific markers include SRY marker in the Y-chromosome, HTR2C in the X-chromosome, 1 additional microsatellite in the X-chromosome, and 3 autosomal microsatellites. We don't think this information is relevant to the readers so we just wrote in the manuscript that "one marker in the Y-chromosome, 2 in X-chromosome and 3 autosomal markers" were used (Page 5 – Line 22).

3.3. In the quality control section, the part about the exclusion of some samples for contamination is not very clear. It. If contamination occurred in the process of plating or genotyping, the data from the samples in the same plate (or genotyping format used) would all need to be discarded and genotyping or plating repeated until evidence of contamination is no longer present. To that effect, the genotyping plates should contain appropriately placed negative controls. It is important to clarify the process of quality control.
We are sorry for the misleading of this paragraph as the reviewer may seem a bit confused to when the quality control genotyping was performed. It was not done in the same multiplexes as the real genotyping of the SNPs studied in this work but was independently performed before. We modified this section to indicate that the quality control was performed on random markers and independently of the SNPs genotyping. This quality control process was performed only once and independently any planned SNP-specific genotyping (Page 6 – Line 2).

3.4. It is mentioned that 0.85% of blinded duplicates was discrepant, but no such information is given for the known duplicates (~2% of genotyped samples).

We have modified this paragraph and mentioned that "we observed less than 0.25% genotyping errors based on the blinded and known duplicate comparison." (Page 6 – Line 1)

3.5. Which database was used to select the Tag SNPs and what were the selection criteria?

As indicated in page 6 (Lines 6-12), the Innate Immunity Programs for Genomic Applications database (http://innateimmunity.net/) was used to select SNPs. As the studied SNPs were exactly the same haplotype tagging SNPs studied in the AtheroGene study, we prefer to invite readers to refer to the Tiret et al. 2005 paper instead copy-pasting the description of the selection criteria. We however briefly explained that the studied SNPs were haplotype tagging SNPs selected to characterize the haplotypic variability of the corresponding genes and not single tag SNPs selected from pairwise r² only.

3.6. The justification to perform haplotype analyses is the linkage disequilibrium between SNPs of the same gene. But the SNPs used are Tag SNPs which by definition should be in very low LD. So was LD calculated in each cohort? It would be relevant to show these data.

As suspected by the reviewer, the pairwise LD between studied SNPs was moderate and is now reported in Figure 2. However, haplotypes defined by two or more SNPs of the same gene, even in very low LD, may differ functionally (for example if the function of a protein depends on a specific combination of 2 amino acids or if the expression of a gene is affected by SNPs that are close to each other in a promoter region (e.g. see Pearce et al. Circ Res 2005)) justifying the rationale of performing haplotype analysis.

3.7. In the statistical analysis section, it is mentioned that Mantel-Haenszel statistics were used to adjust for the cohorts' heterogeneity. It would be worthwhile to briefly explain this approach for the sake of the non-statistician reader.

A brief description of the Mantel-Haenszel statistics has been added in page 7 – line 12. "The Mantel-Haenszel method is a standard way to estimate averaged OR by weighting each log(OR) obtained in a given population by the inverse of their variance."

3.8. It is important to include power calculations in a negative study such as this. It would strengthen the article while helping other researchers make more informed decisions about pursuing the same line of work

We are grateful to the reviewer for this suggestion and we added a paragraph about the power of our study at the end of the Discussion (Page 11 – Lines 3-9)
Reviewer 2: Medea Imboden

1 General comment
As correctly pointed out by this reviewer, there was no evidence for any, even slight, association of the studied SNPs with CVD in our work, explaining why the presentation could be viewed as minimalistic. However, in the new proposed version, additional information have been given and we hope these could make the manuscript less minimalistic. As also noticed by the reviewer, the current work is closely related to the one published last year in *Eur J Hum Genet* by the same authors where the *IL18* gene was studied in the same populations as those investigated in this work. We do not think that copy-pasting some of our own sentences should be considered as plagiarism but, to address the reviewer's comment, we modified the text, in particular the 'MORGAM study populations' and 'statistical analysis' sections to limit the re-use of previously published phrases.

2 Major Compulsory Revisions

2.1 No data on the interactions tested is presented. The investigators are asked to present additional tables or figures showing their negative findings. Also the heading of this paragraph “multilocus analysis” should be termed more appropriately
As indicated now in the *Statistical Analysis* section (Page 7 – Lines 16-21), the DICE algorithm we used to search for interactions "explores in an automated way all combinations of covariates, that could be SNP or environmental factors, acting either additively or in an interactive way up to 3-orders interaction". As this program did not find any evidence of interaction, there are no significant findings to be illustrated and too much non significant interactions to be illustrated. However, to satisfy the reviewer's suggestion, we have added two figures illustrating the absence of haplotype x smoking and haplotype x BMI interaction.

The heading of the "multilocus analysis" paragraph has been changed into "Gene x Gene and Gene x environment interaction analyses"

2.2 Please state how the genetic variants were selected. Both genes, IL18R1 and IL18RAP lie in close chromosomal vicinity. Did the author look at LD or common haplotypes potentially covering both genes?
As now indicated in the *Genotyping* section (Page 6 – Lines 6-11), we have clarified that the studied SNPs were exactly the same as those studied in the AtheroGene cohort (Tiret et al Circulation 2005) and briefly explained " these SNPs were haplotype tagging SNPs selected to capture the haplotype diversity of the regulatory, coding and flanking intronic regions of the *IL18R1* and *IL18RAP* genes according to the information available in the Innate Immunity Programs for Genomic Applications database ([http://innateimmunity.net/](http://innateimmunity.net/))."

LD pattern between SNPs covering both genes has been given in Figure 2. Pairwise \( r^2 \) was moderate between SNPs within the same gene and also between SNPs of different genes.

2.3 A previous publication (Tiret et al.Circulation 2005; ref 9 in the manuscript) by partly the same authors contains a null finding on the association of the same genetic variants with cardiovascular disease. The author should cite this report and specify whether the present finding is an independent replication of the previous null finding. Is there overlap of the cases? If yes to what proportion?
In 2005, Tiret et al. have studied the same genetic variants in relation to cardiovascular mortality in the AtheroGene cohort that is composed of German individuals. In the
present study, individuals are from Finland, Northern Ireland, Sweden and France. Even if some of the authors from the Tiret et al. 2005 paper are also co-authors of the current manuscript, there is absolutely no overlap between the two studies. In addition, in the AtheroGene cohort, the clinical end point was cardiovascular death while CHD and stroke (including fatal and non fatal) events were the outcomes studied in this work. We have modified the introduction to clarify the rationale of the current work.

2.4 Could the lack of association also be due to a limitation of the study? This aspect and other potential limitations are not discussed and should be added in the discussion section.
We added at the end of the Discussion (Page 11 – Lines 3-9) a paragraph about power calculations to indicate whether the size of your study could be a limitation explaining the lack of association.

3. Minor Essential Revisions

3.1 Genetic variants of IL18 and of its receptor have been associated with a variety of diseases involving chronic inflammatory states. The authors are invited to mention the association with asthma and atopy more prominently in the introduction and to harmonize conclusion and introduction to this respect
The Introduction (Page 3 – Lines 17-19) has been modified accordingly to be harmonized with the Conclusion.

3.2 The numbering of the tables needs to be appropriately changed as there are no space constraints and all data can be presented online.
To satisfy all reviewers' suggestions, six new figures have been added to the main document. However, we decided to include some information in Supplementary Data to facilitate reading of the main document.
Data shown in these Supplements are not essential for the interpretation of our results but could be useful for readers interested in pursuing the same line of work.
If the reviewer still wishes to see all data in the main document, we would modify the manuscript accordingly.

3.3 Not all abbreviations were introduced in the text at their first appearance, e.g. CHD. We checked for correct abbreviations definition.

3.4 How was the recruitment of the majority of cases done (cases not belonging to the subcohort)? A short description would be greatly appreciated.
In a case-cohort design, the recruitment of cases is independent on being within or out the subcohort. It is a prospective design where all individuals are followed up in the same way. We clarified that point at the end of page 4 and beginning of page 5. "All individuals were followed up for clinical outcome that was obtained mostly from national death register, MONICA and hospital discharge registers, and regional health information system (Evans et al 2005). Genotyping was however restricted to all subcohort members and to all additional subjects who were not part of the subcohorts but who experienced cardiovascular outcomes during the follow-up".
3.1 Please indicate quantitatively the contribution of the current work in context of previous investigations of polymorphisms in these genes. Previous study of these genes in the AtheroGene project is mentioned in passing, but no detail is given. It's not clear at the moment from the manuscript exactly what this study has added to the existing literature.

The introduction of the manuscript has been modified to clarify the rationale of this work and results obtained in the AtheroGene project are now discussed (from Page 3 – Line 13 to Page 4 – Line 6).

3.2 Odds ratios and confidence intervals have not been provided for the SNP-by-SNP analyses. Please provide these to give some indication of the uncertainty remaining after this potentially important negative study.

We added a new figure (Figure 1) that displays Odds Ratio (and confidence intervals) associated with each SNP in the whole MORGAM cohorts. ORs associated with each SNP separately in each population can still be found in Supplementary data.

3.3 It is not clear what the strategy was for the testing of SNP X SNP X Covariate interactions. Please describe this in more detail. For instance, was each IL-18 SNP tested with each SNP in either of the receptor constituent genes? Or was each SNP in each gene tested in combination with every other SNP? Some comment on the power of the study to detect the interactions that were sought would be appropriate. It is not likely that there was much power to detect third order interactions.

The principle underlying the DICE program has been described in more details in the statistical analysis section. "DICE is an algorithm that explores in an automated way all combinations of one, two or three covariates, that could be SNP or environmental factors, acting either additively or in an interactive way. The selection for the most parsimonious model of interaction, if any, is based on a Information Criterion" Unfortunately, it is not really possible to exactly quantify the power of our study to detect third-order interactions because a large number of situations could be envisaged. We do agree with the Reviewer that our study may not be well powered to detected third-order interaction but that should not prevent us from looking for them. However, as mentioned in the last paragraph of the discussion (Page 11) our study is quite well powered to detect some second-order interactions.

3.4 What was the rationale for including both haemorrhagic and ischemic stroke in the case group?

As now detailed in page 5 – line9 , "both ischaemic and hemorrhagic stroke were included in the analysis because there is often insufficient data to separate between the subtypes in this population-based study. About 80% of the strokes are ischaemic in these populations"

4. Discretionary Revisions

4.1 The statement about Hardy-Weinberg equilibrium in the IL18R1 gene is confusing and seems contradictory. A supplementary table of those SNPs that failed HWE at p<0.01 might be more informative.

We are sorry for the confusion. Our sentences were completely misleading. In each subcohort, no deviation from HWE was observed for any SNP. However, some SNPs
failed HWE in subgroups of cases or in non-cases. This information has been omitted in the revised version, so as not to mislead readers. However, we provided below the information about the seven SNPs that failed HWE at p < 0.05 so that the reviewer may decide by himself if this information is valuable or not and should be given in the manuscript. As correctly pointed out by reviewer 1, four of these deviations from HWE were observed in PRIME/France samples. These were not observed in the whole PRIME/France sample but restricted only to the cases samples with only 114 individuals. Some of these deviations were in fact due to very small genotype counts. Nevertheless, after correcting for the number of HW tests performed (44 = 11 x 4), none of the shown p-values passed the Bonferroni threshold of 0.0011 and then none was significant at corrected p < 0.05. As a consequence, we don't consider that the validity of the results presented in this work could be affected by these modest deviations from HWE.

### List of SNPs that failed HWE at p< 0.05

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</table>

4.2 A statement on how the allele frequencies at typed SNPs compared with the HapMap frequencies would be helpful.

Six of the SNPs studied in this report (rs3732127, rs11465670, rs4851581, rs1420106, rs1420105 and rs11465673) have been genotyped in the Hapmap Phase III Caucasian data and their allele frequencies are very similar to those observed in our populations. For the 5 other SNPs, the allele frequencies observed in our study are also very similar to those available at the NCBI dbSNP European population database. A statement has been added to the discussion (Page 10 – Lines 14-16) "These allele frequencies were comparable to those observed in HapMap Phase III European samples populations ([www.hapmap.org](http://www.hapmap.org)) or in NCBI dbSNP European database ([http://www.ncbi.nlm.nih.gov/projects/SNP/](http://www.ncbi.nlm.nih.gov/projects/SNP))."

4.3 I found the description of the case-cohort design somewhat difficult to follow in the text. If I have understood it correctly, an age-stratified subcohort was randomly selected from each study, which as expected was mostly made up of controls. Additional cases were then selected from the remainder of the study population in each cohort. Were these all available cases or was there any additional selection (younger age, for example)? I also suggest simplifying Table 1 by removing the first two columns.

We have modified the description of the "MORGAM study populations" with the aim of clarifying the description of the case-cohort (First paragraph of page 4) : In each population cohort, a subset of individuals from the whole cohort was randomly selected independently of disease status to be part of a subcohort according to population-specific sampling probabilities depending on sex and age, older subjects having a higher selection probability to ensure that similar age distributions were obtained in cases and
the subcohort. All individuals were followed up for clinical outcome that was obtained mostly from national death register, MONICA and hospital discharge registers, and regional health information system (Evans et al 2005). Genotyping was however restricted to all subcohort members and to all additional subjects who were not part of the subcohorts but who experienced cardiovascular outcomes during the follow-up."

We prefer to keep the first two columns of Table 1 as we do consider that it provides valuable information about the proportion of "additional cases" relative to the total number of studied individuals. If the reviewer were to remain uncomfortable with that information, we would remove it.

4.4 Why are some numbers in Table 2 in italics?
It was indicated in footnotes of Table 2 that "Italics characters correspond to allele that showed slight deviation (p<0.05) from Hardy-Weinberg equilibrium." However, this information is no longer relevant (see point 4.1 above) and has been omitted.

4.5 I suggest the use of rs numbers throughout the manuscript to identify SNPs.
We followed this suggestion and rs numbers are now used in the document.
Interleukin-18 (IL-18) is a pro-inflammatory cytokine that is now the source of considerable research interest as it is implicated in the etiology of several diseases, including immune diseases, type I diabetes and cardiovascular diseases (CVD). In particular, increased IL-18 levels were shown to be strong predictors of cardiovascular mortality and associated with carotid intima-media thickness. Interleukin-18 (IL-18) is a pro-inflammatory molecule that has been shown to be involved in the susceptibility of several human complex diseases such as immune diseases, type I diabetes and cardiovascular diseases (CVD). The hypothesized mechanism by which IL-18 may be linked to CVD risk is related to atherosclerosis and its complication [2-9]. Consistent with this hypothesis, highest IL-18 levels were shown to be associated with increased carotid intima-media thickness [10] and with cardiovascular mortality in a cohort of patients with coronary artery disease [7, 8].

In the present work, five cohorts were studied, two from Finland (FINRISK, ATBC), one from France and one from Northern Ireland, (both issued from the PRIME Study), and one from Sweden, for a total number of 25,141 subjects with available DNA. The present report was based on the analysis of five cohorts, two from Finland (FINRISK, ATBC), one from France and one from Northern Ireland, (both issued from the PRIME Study), and one from Sweden. The FINRISK cohort comprised two surveys with baseline investigations five years apart (1992 and 1997). Both were pooled in this report after having checked for consistency across surveys and the analysis was adjusted for survey....

A case-cohort design has been adopted in MORGAM to enable the study of multiple endpoints, including CHD and stroke, while reducing the genotyping costs. To facilitate the study of multiple endpoints and to reduce genotyping costs, a case-cohort design has been adopted in MORGAM.

In each cohort, subjects were randomly selected to be part of a subcohort according to cohort-specific sampling probabilities depending on sex and age, older subjects having a higher selection probability to ensure that similar age distributions were obtained in cases and the subcohort.

In each population cohort, a subset of individuals from the whole cohort was randomly selected independently of disease status to be part of a subcohort. According to population-specific sampling probabilities. These probabilities were dependent on sex and age such that older subjects had a higher selection probability and that age distributions were similar in cases and in the subcohort.

In the presence of multiple events during the follow-up, only the first event was considered. Both fatal and non-fatal events were included.

Both fatal and non-fatal events were considered and, when multiple events occurred during the follow-up, only the first one was studied.
Analyses were first carried out in each population separately, adjusting for age at baseline, baseline status, gender and smoking status when appropriate. The Mantel-Haenszel statistics was used for testing the homogeneity of the association across cohorts and subsequently for providing combined odds ratio (OR) estimates in the whole study.

Analyses were performed separately in each population and were adjusted for age at baseline, baseline status, gender and smoking status when appropriate. Homogeneity of the associations across cohorts was assessed by use of the Mantel-Haenszel statistics that was subsequently used for estimating pool odds ratio (OR) in the whole study. The Mantel-Haenszel method is a standard way to estimate averaged OR by weighting each log(OR) obtained in a given population by the inverse of their variance.