Author's response to reviews

Title: Gene sequence variations of the platelet P2Y12 receptor are associated with coronary artery disease.

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Version: 3 Date: 26 July 2007

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

we wish to submit the revised version of the manuscript entitled “Gene sequence variations of the platelet P2Y₁₂ receptor are associated with coronary artery disease” by U. Cavallari et al. for publication on the BMC Medical Genetics as a Research article.

We thank the editorial board and the Reviewer for the evaluation of our manuscript.

We have carefully considered the points raised by the three reviewers and have revised the manuscript accordingly.

We enclose a point by point reply to the objections of the reviewers. To facilitate the identification of the changes made we indicated “Section title” at each point.

We hope that this manuscript is now suited for publication in BMC Medical Genetics and look forward to your reply.

Kind regards, Yours sincerely

Elisabetta Trabetti

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Referee 1

Reviewer's report
Gene sequence variations of the platelet P2Y12 receptor are associated with coronary disease.

Version: 1  Date: 9 May 2007
Reviewer: Robert F Storey

Reviewer's report:

General

Cavallari et al have genotyped samples from a previous study of subjects who have undergone coronary angiography, assessing the relationship between a P2RY12 SNP that has previously been reported to define H1 and H2 haplotypes in this gene and presence or absence of angiographic evidence of coronary artery disease. They have concluded that the H2 haplotype is associated with an increased risk of coronary artery disease.

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

The authors should state whether the determination of p values for interaction, which identified non-smoking H2 carriers as being at higher risk of CAD, incorporated an adjustment for multiple analyses and whether this interaction remained significant after such an adjustment.

The main goal of the present study was to detect an association between P2RY12 gene polymorphism and CAD. As secondary end-point we aimed to identify subgroups of individuals showing a possible interactive effect between the gene variant and conventional risk factors. This was performed searching for putative interactions between the gene variant and each of the 9 (age, sex, BMI, triglycerides, HDL, LDL, hypertension, smoking and diabetes) covariates, setting a relative loose threshold (p<0.1) to target the interacting factors. Moreover, in the case of interacting binary factor, the plan of the analysis required that the association should have been tested in the subgroups of individuals having or not the interacting factor (accounting for two association tests). Since we identified only smoke as interacting factor, the non adjusted significance of the association between non-smokers and P2RY12 gene polymorphism (p=0.007) becomes p=0.007 X 2=0.014 after adjustment for multiple analyses. Therefore this association remained significant after such an adjustment. We added a sentence reporting p-value after adjustment for multiple tests at the end of Results section.

This is particularly important since the OR for the association between H1/H2 haplotype and CAD is less than the OR which the study was powered to assess for.

The power is related to the probability of success in finding a true association (generally accepted to be 80%). However true associations can be detected even if the power is low (<<80%). The power cannot help in determining the strength (i.e. OR) or the goodness of the association to be tested. Therefore it is possible to find an association with OR less than the one used to estimate the power of the study, even if the chance to detect true associations is low (<80%).

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)
Discretionary Revisions (which the author can choose to ignore)
The small study by Fontana et al suggesting an association between P2RY12 genotype and PAD raises an interesting hypothesis but is not conclusive. Some investigators have failed to confirm the findings of Fontana et al that the H1/H2 haplotype influences platelet reactivity (e.g. Hetherington SL et al), which undermines the rationale for this haplotype to influence risk of atherosclerotic disease. In the 14th line of the Discussion, I suggest insert 'might' before 'lead to an increased atherothrombotic risk...' and refer to data that questions the relevance of the H1/H2 haplotype to provide a balanced discussion.

As suggested by the Reviewer, we have mitigated our conclusions: we have inserted “might” and we added a sentence related to data from Hetherington et al in the Discussion (Page 9 lane 17) to emphasize that the genotype-phenotype correlation at this time could be at best only mild. Furthermore, a new paragraph about study limitations has been added at the end of Discussion section.

What next?: Accept after minor essential revisions
Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: Yes, and I have assessed the statistics in my report.
Declaration of competing interests:
I declare that I have no competing interests
Reviewer's report

Title: Gene sequence variations of the platelet P2Y12 receptor are associated with coronary artery disease.

Version: 1 Date: 7 July 2007
Reviewer: Jean-Luc Reny

Reviewer's report:

General
Gene sequence variations of the platelet P2Y12 receptor are associated with Coronary artery disease by Ugo Cavallari et al.
The authors present data from a case-control study on a genetic association between the P2Y12 receptor gene H1/H2 polymorphism and coronary artery disease. The study is well powered, the results are clearly presented and the strength of the association is significant though it certainly needs to be confirmed by an independent study. Some concerns need to be addressed.

1. Major Concerns

1.1. It is unclear whether cases with significant CA stenosis were actually free of any known atherothrombotic disease prior to the qualifying coronary angiogram of the present study.

CAD patients were candidate to coronary artery bypass grafting (CABG) and underwent coronography after the first manifestation of symptoms of ischemic heart disease, therefore no patient had a long previous history of CAD. We added this description in “Methods – Study population”.

1.2. If part of the CAD patients had prior atherothrombosis and/or specific treatment, data on antiplatelet treatment, particularly clopidogrel, should be provided and included in the multivariate analysis. Unbalanced clopidogrel treatment in cases and controls may cause a bias in the selection of subjects.

The selection process of the patients was essentially based on an angiographically-derived criteria. Most of the patients have not been treated with antiplatelet drugs before the coronography (recruitment time, that corresponds with the onset of CAD symptoms). After CABG, 90% of patients have been treated with aspirin, about 9% with ticlopidina and 1% with indobuphen. No patients had therapy with clopidogrel. Furthermore, the design of the study is a case-control setting and no longitudinal so that effects from the therapy do not influence the results. Therefore, we did not include therapy in the multivariate analysis. However, as suggested by the Reviewer, we have stated more clearly these points and have added some sentences in “Methods – Study population” section.

2. Minor Essential Revisions

2.1. P5, line 11: inclusion criteria should be detailed a bit further in the present article (see major concern).

As suggested by the Reviewer, a more complete and detailed description of the enrollment criteria was provided in “Methods – Study population” section.
2.2. The limitations of the study should be discussed

As suggested by the Reviewer, a new paragraph about study limitations has been added at the end of Discussion section.

2.2.1. Case-control design; risk of a false-positive finding; importance of confirmatory data

The main goal of the present study was to detect an association between P2RY12 gene polymorphism and CAD (as now reported in the last paragraph of Discussion section). The association was tested only for CAD phenotype, so that only one statistical test was performed, showing a p-value=0.03. Therefore the probability to observe this significant association by chance (false-positive rate) is 3%.

As secondary end-point we aimed to identify subgroups of individuals showing a possible interactive effect between the gene variant and conventional risk factors. This was performed searching for putative interactions between the gene variant and each of the 9 (age, sex, BMI, triglycerides, HDL, LDL, hypertension, smoking and diabetes) covariates, setting a relative loose threshold (p<0.1) to target the interacting factors. Moreover, in the case of interacting binary factor, the plan of the analysis required that the association should have been tested in the subgroups of individuals having or not the interacting factor (accounting for two association tests). Since we identified only smoke as interacting factor, the non adjusted significance of the association between non-smokers and P2RY12 gene polymorphism (p=0.007) becomes p=0.007 X 2=0.014 after adjustment for multiple analyses. Therefore this association remained significant after such an adjustment. We added a sentence reporting p-value after adjustment for multiple tests at the end of Results section.

The limitations of the study have been discussed in the added paragraph at the end of Discussion section, as well as the importance of our clear-cut selection of CAD phenotype on the basis of coronary angiography, so that no CAD patient had a not significant coronary artery disease (at least 50% stenosis) and no control had a subclinical coronary artery disease. Furthermore, as correctly noted also by Referee one, our conclusions about genotype-phenotype association have been mitigated and a sentence about the need of confirmatory data has been added in the Conclusion section.

2.2.2. H2/H2 subjects display an increased platelet reactivity that is significant and biologically important. Why is H2/H2 a more frequent genotype in control (CAD free) subjects: chance finding? comment on the lack of a “dose-response” effect.

Considering the low number of H2/H2 subjects (n=22), which impairs the statistical power and the significance of the analysis and does not allow to consider adequately a gene dose effect, we join together H1/H2 and H2/H2 subjects and compare subjects homozygous for the wild-type allele with subjects carrying the minor allele, according to a dominant model. With regard to this point a sentence has been added in the last paragraph of Methods – Genotyping section.
The frequency of each genotype could be given in the smokers and non-smokers CAD and CAD free subjects.

We added T/C and C/C genotype frequencies in smokers and non-smokers CAD and CAD-free subjects in Table 4, as requested.

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3. Discretionary Revisions
3.1. P8 line 9: “variations” in place of “mutations” may be more appropriate with respect to the frequency of the polymorphic variations (H1/H2 for instance)
   We wrote “variations” in place of “mutations”

3.2. P4 line 11: “its” instead of “it’s”
   We wrote “its” instead of “it’s”

3.3. P4 line 16: “types” in place of “type”
   We have not found the wrong word at Page4, we thought to be right in changing “types” in place of “type” at Page 8 line 16

What next?: Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions
Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I hold a patent related to the initial description of the H1/H2 polymorphism and its relation with symptomatic atherothrombosis. Aside from this patent I declare that I have no competing interests.
Referee 3

Reviewer's report
Title: Gene sequence variations of the platelet P2Y12 receptor are associated with coronary artery disease.
Version: 1 Date: 9 July 2007
Reviewer: Dirk Sibbing

Reviewer's report:
General
Genetic polymorphisms in platelet receptor genes may in part explain part of the individual risk for atherosclerotic disease. The H2 haplotype of the P2Y12 receptor has been shown to be associated with peripheral arterial disease and an increased ADP-induced platelet aggregation ex vivo in healthy volunteers. Cavallari and co-workers sought to assess in a large population of 1378 unrelated patients the impact of the H1/H2 haplotype on coronary artery disease (CAD). The study population comprised 991 patients with angiographically determined CAD and 387 patients without CAD. The major results of the study were that (a) H2 haplotype carriers were more frequent in the CAD group and that (b) this association was strongest in the non smoking population. Based on their results the authors conclude that gene sequence variations of the P2Y12 receptor are associated with the presence of CAD.

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Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)
None

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Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)
1. The authors state that consecutive patients were included into the study. However, gender distribution and the different mean age of both patient groups (CAD and CAD-free) might be the result of inhomogeneity between the two groups. The authors should comment on this and should furthermore add a flow chart to the Methods section of their manuscript describing the generation of their study population.

The study population was selected within the frame of angiography cardiovascular survey in an Italian population, on the basis of availability of P2RY12 genotype data. Instead of a flow chart, a more complete and detailed description of the enrollment criteria was provided in “Methods – Study population” section.

As expected, we found age and male gender associated with CAD phenotype; nevertheless the association of CAD with carriers of the P2RY12 minor haplotype remained close to significance also after adjustment for classical risk factors, including age and sex. Moreover, as requested in the next point by the Reviewer, we analysed genotype distribution in males and females, both in CAD and CAD-free subjects.
2. Gender distribution differed significantly in both groups (79.8% vs. 65%; P<0.001) and gender specific differences for associations of genotypes with a clinical phenotype have been observed in other studies (e.g. McDermott et al. Circulation. 2005;112:1113-1120; Wu et al. Ann Hum Genet. 2007 Jul;71:519-25.). The authors should comment on this and should add a separate gender specific calculation (male and female) for haplotype distribution und CAD association.

As suggested by theReviewer, we performed the analysis separately in each sex group. In male group the association between H2 haplotype carriers and CAD was close to significance, whereas no significant association was found in females, as given in the Table below. On the other hand, we should emphasize that the low number of female subjects may impair the statistical power of the analysis and that also in females the H2 haplotype carriers tended to be more frequent among CAD patients. This suggests that the effect of the gene is mild and it requires a large sample of subjects to be detected. We added these important information in some sentences in Results and Discussion sections.

Table. Genotype frequency of the i-T744C SNP of the P2RY12 gene in male and female subgroups with and without significant coronary artery disease.

<table>
<thead>
<tr>
<th></th>
<th>male</th>
<th>female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CAD (p-value*)</td>
<td>CAD-free (p-value*)</td>
</tr>
<tr>
<td></td>
<td>(CI 95%)</td>
<td>(CI 95%)</td>
</tr>
<tr>
<td>T/T</td>
<td>584 (73.83%) 201 (80.08%)</td>
<td>153 (76.5%) 108 (80.0%)</td>
</tr>
<tr>
<td>T/C</td>
<td>197 (24.9%) 45 (17.93%)</td>
<td>44 (22%) 23 (17.04%)</td>
</tr>
<tr>
<td></td>
<td>1.42 (0.99-2.05)</td>
<td>1.28 (0.70-2.17)</td>
</tr>
<tr>
<td></td>
<td>p= 0.05</td>
<td>p=0.53</td>
</tr>
<tr>
<td>C/C</td>
<td>10 (1.27%) 5 (1.99%)</td>
<td>3 (1.5%) 4 (2.96%)</td>
</tr>
<tr>
<td></td>
<td>p= 0.05</td>
<td>p=0.53</td>
</tr>
<tr>
<td>TC+CC</td>
<td>207 (26.17%) 50 (19.92%)</td>
<td>47 (23.5%) 27 (20.03%)</td>
</tr>
</tbody>
</table>

(*)T/C and C/C genotypes (H1/H2 and H2/H2 haplotypes, respectively) are pooled in the CAD and in the CAD-free group for p-value and OR calculation.
3. In addition to genotype distribution allele frequencies should be presented in the Results section.

We added allele frequency distribution in the Results section, as requested.

4. The heterozygous genotype (T/C) of the minor allele C was found more often in CAD group, whereas the homozygous genotype (C/C) of the minor allele was found more often in the CAD-free group. The authors should comment on this (lack of gene dose effect etc.).

As already answered to Reviewer 2, considering the low number of H2/H2 subjects (n=22), which impairs the statistical power and the significance of the analysis and does not allow to consider adequately a gene dose effect, we join together H1/H2 and H2/H2 subjects and compare subjects homozygous for the wild-type allele with subjects carrying the minor allele, according to a dominant model. With regard to this point a sentence has been added in the last paragraph of Methods – Genotyping section.

5. Background section (line 1): “atherosclerotic” should read “atherosclerotic”
We have corrected the word.

6. Background section (line 5): the words “high risk” can be deleted.
We have deleted the words.

7. Discussion section (line 15): the sentence “…and smokers exhibit higher platelet P2Y12” should read “… and smokers exhibit higher P2Y12 expression”.
We have corrected the sentence as requested.

8. Table 1: “Oligonucleotyde” should read “Oligonucleotide”
We have corrected the word.

Discretionary Revisions (which the author can choose to ignore)
None

What next?: Accept after minor essential revisions
Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that I have no competing interests.