PEER REVIEW HISTORY

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ARTICLE DETAILS

<table>
<thead>
<tr>
<th>TITLE (PROVISIONAL)</th>
<th>The role of Helicobacter pylori and interleukin 6 -174 gene polymorphism in dyslipidemia: a case-control study</th>
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<tbody>
<tr>
<td>AUTHORS</td>
<td>Pohjanen, Vesa-Matti; Koivurova, Olli-Pekka; Niemelä, Seppo; Karttunen, Riitta; Karttunen, Tuomo</td>
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VERSION 1 - REVIEW

<table>
<thead>
<tr>
<th>REVIEWER</th>
<th>Rinaldo Pellicano</th>
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<tr>
<td>Department of Gastroenterology, Molinette Hospital, Turin, Italy</td>
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<tr>
<td>REVIEW RETURNED</td>
<td>20-Sep-2015</td>
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GENERAL COMMENTS

This is an interesting original article assessing the role of Helicobacter pylori infection and interleukin 6 (IL6) polymorphism -174 (rs1800795) in dyslipidemia. Although limited by the small sample size this "pioneering" study is well conducted.

<table>
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<tr>
<th>REVIEWER</th>
<th>Natale Figura, Adjunct Professor</th>
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<tr>
<td>University of Siena, Italy</td>
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<td>REVIEW RETURNED</td>
<td>23-Sep-2015</td>
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GENERAL COMMENTS

These researchers compared the serum lipid levels of H. pylori infected dyspeptic patients with those measured in uninfected patients. They also investigated whether the aplotypes of interleukin-6 (IL-6) and the presence of peptic ulcer influenced the lipid levels. They observed that infected patients had a reduced mean concentration of high density lipoprotein (HDL) respect to those who were uninfected; HDL mean level was particularly decreased in infected patients with IL6 -174 CC aplotype and in infected patients with ulcer respect to infected patients with chronic gastritis only (without peptic ulceration). The levels of HDL in infected patients with other IL-6 aplotypes were not increased respect to uninfected patients. Peptic ulceration was not associated with a particular aplotype of such inflammatory cytokine.

The present study may contain interesting findings; however, I have some concerns that prevented me from accepting it in the present form.

1. The authors say that patient receiving ongoing antibiotic treatment were excluded. Also patients who took antibiotics potentially active against H. pylori in the past three month should be excluded.

2. The authors claimed that they performed a case-control study with H. pylori positive patients as cases and H. pylori negative patients as controls. This is hardly correct. Controls of dyspeptic patients should
be normal individuals, without dyspepsia. This problem could be avoid just saying that these researchers compared serum lipid levels of H. pylori infected with those of uninfected patients who underwent upper endoscopy for dyspepsia, once patients had been differentiated on the basis of their IL-6 aplotypes.

3. It is not reported whether patients suffered from dyslipidemia and were on statin. This is essential. The authors say that statin was hardly used in Finland; this is not enough; they should make an effort to review all medical records relating to patients examined in order to know who was on statin. The difference between HDL levels in infected and uninfected patients is not so wide; patients who took statin should be excluded from the study.

4. Also patients with deficient glucose regulation should be excluded for the relationships between glucose and triglycerides and lipids in general. In a study in which the authors examined unselected patients attending an emergency department, the ratio of undiagnosed and suboptimally controlled diabetes was 16 % (Menchine MD, et al. Prevalence of undiagnosed and suboptimally controlled diabetes by point-of-care HbA1C in unselected emergency department patients. Acad Emerg Med 2011;18:326–329).

5. Of the 114 H. pylori positive patients, 40 had an active duodenal ulcer and 16 an active gastric ulcer. Too much ulcers. Normally, out of 100 consecutive patients who undergo endoscopy, 10% circa have an ulcer; this proportion increases to 20% of H. pylori infected patients. This fact makes me suspect that the patients enrolled were not consecutive, as argued in the section Materials and Methods. Is it possible that the authors enrolled all consecutive patients with peptic ulceration together with a representative proportion of those who had simple chronic gastritis? In the end, patients examined endoscopically in three Finnish hospitals between years 1996 and 2000 ought to be more numerous than 216.

6. It is known that H. pylori infected individuals with serum antibodies to CagA have increased circulating levels of IL-6 (Figura N, et al. Cross-sectional study: CagA-positive Helicobacter pylori infection, acute coronary artery disease and systemic levels of B-type natriuretic peptide. J Clin Pathol. 2014 Mar;67(3):251-7). This could explain the finding that patients with peptic ulceration had reduced levels of HDL. Ulcer patients, in fact, are infected by strains expressing CagA significantly more often than patients with chronic gastritis only. If the authors do not want to examine the serological CagA status of their patients, at least, they could comment this point.

REVIEWER
Daniel P. Potaczek
Institut für Laboratoriumsmedizin und Pathobiochemie, Molekulare Diagnostik, Philipps-Universität Marburg, Germany

REVIEW RETURNED
08-Oct-2015

GENERAL COMMENTS
With interest I read the manuscript by Pohjanen et al. I find it a good manuscript on a well-conducted study. I enclose some suggestions how I believe it could be further improved.
1. The Authors state that the paper is on the role of HP and the -174 SNP in dyslipidemia. However, the SNP is used in their study mainly as an additional (“controlling”) factor, not as an independent determinant/predictor. It would be nice to analyze the direct effect of the SNP on lipid levels in the whole group, and in HP positive and negative subjects separately. In the next step, the analysis should include also adjustment for HP positivity and, in all (sub-)groups, for other potential confounders. Please, show the results of such analyses.

2. In case of a need of adjustment, in addition to logistic regression after binarization of HDL levels (or any other continuous variable) I would also (or even predominantly) suggest a multiple regression analysis in which the dependent parameter stays continuous (not dichotomized). Both types of calculations can be provided in the manuscript.

3. Was the logistic regression analysis on binarized HDL conducted also in the whole group, without sub-stratification for a genotype? In addition, what happens if the SNP is also included as an independent variable in the logistic regression analysis (the whole group) conducted by the Authors?


5. Please, describe your genotyping method in more detail. The paper you reference (http://www.ncbi.nlm.nih.gov/pubmed/9769329) describes a very complex methodology combining mutational screening (SSCP > sequencing), RFLP genotyping, etc. Which primers you actually used? Was the genotype determined by sequencing or RFLP? Which restriction enzyme was used then, Hsp92II or NlaIII, or any other isoschizomer?

Other comments:

1. Lack of BMI is kind of strange but can be accepted, especially since it is addressed in the study limitations. I would, however, tone down (or even delete) the sentence “Nevertheless, there are no signs or major doubt that obesity or diet would confound our results or influence our main conclusions.” as it seems to contain a much-too-strong speculation.
2. How was the normality of the distribution tested?

3. Lack of circulation IL-6 measurements should be included in the study limitations.

4. Page 8, Discussion. Since “trend” has its own meaning in statistics, I would write “non-significant tendency” not “non-significant trend”.

5. Figure legend is very ascetic. Medians with IQR are most probably shown but what are the whiskers? 10-90%? 5-95%? 2.5-97.5? How were extremes and outliers defined. Please, expand the figure legend. In addition, units, genotypes, and some other things may be not large enough to be clearly read after publication.

REVIEWER
Mitsushige Sugimoto
Shiga University of Medical Science Hospital
Division of Digestive Endoscopy
Seta Tsukinowa-cho, Otsu, Shiga, JAPAN

REVIEW RETURNED
13-Oct-2015

GENERAL COMMENTS
General:
Authors investigated the role of Helicobacter pylori and interleukin 6 -174 gene polymorphism for serum level of lipid. Authors demonstrated that the median HDL level was significantly lower in the H pylori positive group than in the negative group (p<0.001), but not other marker, such as TC and LDL. In addition, authors showed that a significant association between H pylori infection and HDL levels was seen within the IL6 -174 CC genotype group, but not GC or GG genotype groups. Authors concluded that the association between H pylori infection and serum HDL could be transmitted through IL6.

Although this study is well written, this version has any problems. Therefore, it will be required to revise.

Major comments:
1. Please show sample power in Methods section.
2. As authors suggested, IL-6 affects serum lipid level. Howe about differences of serum lipid level among IL-6-174 genotype groups? If not, authors are required to discuss this point.
3. Why did not other marker of lipid, such as TC and LDL, have significant different between H. pylori positive and negate groups, and among IL-6-174 genotype groups?
4. In this study, there was no significant different prevalence of IL-6-174 genotype among ulcer patients and non-ulcer patients. Why not? This observation may suggest loss of sample power.
5. To have peptic ulcer in patients with H. pylori affects serum lipid level. Therefore, it might be better to add presence of peptic ulcer as one of factor (logistic regression models).
6. Howe bout association with endoscopic severity of gastric mucosal atrophy and IL-6-174 genotype, HDL level and H. pylori?
7. Were there patients with previous H. pylori infection and endoscopic mucosal atrophy in this study?

Minor comments:
1. Table 1: Please add data of all patients.
2. Please make new Table summarized logistic regression models.
3. In this study, authors have no data about mucosal bacteria flora.
Therefore, authors should delete third paragraph of page 10 (Recent observation...?) and last sentence of conclusion of Abstract.

4. Please list limitations in before conclusion.

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**VERSION 1 – AUTHOR RESPONSE**

Reviewer 1:
We thank the reviewer for his feedback.

Reviewer 2:
We thank the reviewer for his feedback. Our replies to specific questions are below:

1. We do not have the data needed to exclude additional patients. Nevertheless we have tested H. pylori positivity with several methods (serology, culture, histology), which reduces the amount of both false positives and negatives.

2. BMJ Open requires that the authors state the study design clearly. According to the usual definition of case-control study, the “controls” need not to be in normal health. The requirement is that they need to be free of the outcome issue that the “cases” have (reference: http://www.ncbi.nlm.nih.gov/pubmed/15836892).

3. The issue of statin medication is relevant because of its possible confounding effect. Unfortunately we do not currently have the possibility to go through the medical records. Statins were not taken into account when the study was originally planned, so our ethics committee permission does not cover this data. In addition, the medical records are from three different hospitals from the years 1996-2000 and thus it would be also difficult to obtain the data. Despite the lacking data we are confident that any use of statins has not confounded our results significantly. We have come to this conclusion based on the following reasons: As Ruokoniemi et al. (http://www.ncbi.nlm.nih.gov/pubmed/18782143) show, the prevalence of statin medication in Finland in 1996-2000 was very low (1.3-4.2% respectively). Additionally, it’s probable that the statin users are distributed randomly among the study subjects, as we don’t see any logical (non-random) reason why the use of statins would be enriched in H. pylori negative patients (who had higher HDL levels). Lastly, some of the differences in the HDL concentrations are so big (eg. in the IL6 -174 GG group, the H. pylori negative patients had 47% higher HDL than the H. pylori positive patients), that the possible effects of statins would be of only small significance.

4. The issue with glucose levels is somewhat similar to the statin issue. We do not have the data. As it has been suggested, that H. pylori and IL6 -174 polymorphism might be risk factors to deficient glucose regulation, there might be relatively more diabetic patients among the H. pylori positive patients and IL6 GG genotype patients. In these cases the deviation in glucose regulation might be a mechanism for the observed lipid changes. But because the deviations in glucose regulation are secondary effects for the studied variables (H. pylori positivity and IL6 genotype), they should not be able to confound our results or cause false positive correlations.

5. The reviewer’s notion is correct. Our study patients were enrolled consecutively until a certain amount of ulcer and non-ulcer patients were achieved. This has been now corrected in the methods.

6. We have CagA data on majority (107/114) of H. pylori positive patients. Of 107 of the patients of which 97 (90.7%) were positive. CagA was not associated significantly to HDL, but considering rarity of CagA negative strains, the number of H. pylori positive patients is too low to exclude the role of cagA in the association between ulcers and HDL. These results are now added to the article and commented in the discussion accordingly.

Reviewer 3:
We thank the reviewer for his feedback. Our replies to specific questions are below:

1. We have done crude and adjusted (with H. pylori positivity, age and sex as cofactors) regression
analyses on the association between IL6 -174 genotypes and HDL, which both show that the IL6 -174 is not significantly associated with HDL in the whole group. In the HP negative groups there is an association with CC genotype and higher HDL. We left these results out the first draft because we wanted to keep the article compact and concentrated on the main results. But as suggested, we have included them in the revised version of manuscript.

2. We have considered the use multivariate analyses with continuous variables. The main problem in the use of continuous variables is the skewed distribution of HDL. Thus our data is not compatible with linear regression analyses without logarithmic normalization. Thus the additional analyses might be hard to present and interpret. And as the results of the dichotomized analyses are suitable and sensitive enough for the data and also clear and easily interpretable, we decided to refrain from using several types of analyses to keep the article compact and easily readable.

3. As suggested, the whole group of regression analyses has now been included (see answer 1). H. pylori positivity is significantly associated to low HDL in the whole group but IL6 -174 is not.

4. We have also tried to keep the introduction compact, especially in terms of coronary artery disease as it is not the subject of this article. But after the inclusion of the additional results, we agree that it is appropriate to expand the introduction concerning IL6 -174 and also including some of the suggested references.

5. We used RFLP method with NlaIII restriction enzyme using the published primers. We have now specified this in the article.

Other comments:

1. The intended meaning was that we do not see any non-random reason how BMI would confound our main results. We have reformulated this sentence as suggested.

2. The normality was assessed visually from the distribution curves and the results were verified numerically by performing a Kolmogorov-Smirnov-test on SPSS.

3. We agree.

4. We agree.

5. We have expanded the figure legend as suggested. Concerning the graphical issues, we’ll wait for possible editor feedback.

Reviewer 4:
We thank the reviewer for his feedback. Our replies to specific questions are below:

Major comments:

1. Power level calculations are included in the methods section.

2. We have now included additional analyses as we have described in the answers (1 and 3) to the previous reviewer.

3. This is an interesting question for which there is no clear explanation. In the previous studies concerning H. pylori and dyslipidemia, the association between H. pylori and HDL has been the most frequent and associations between H. pylori and LDL and triglycerides have been less frequent. As there is no good data or theories explaining this phenomenon, we have decided to not speculate on it in the article.

4. The IL6 -174 has not been previously associated with peptic ulcers and we did not expect an association here.

5. H. pylori positivity and the presence of ulcers cannot be used together as covariates in regression analyses as H. pylori is already strongly associated with the presence of ulcers and this association disturbs the regression analysis. A statistically significant difference in HDL between H. pylori positive and negative patients exists also after the exclusion of ulcer patients. We have now added this result in the article to clarify that the difference in HDL between H. pylori positive and negative patients is not (only) due to ulcers.

6. Atrophy was not assessed endoscopically. There were no strong correlations between histological scores of atrophy and H. pylori.

7. Previously treated H. pylori patients were excluded.

Minor comments:
1. All relevant data is included in the table.
2. We have added a figure in which all crude ORs are compiled.
3. We have reformulated this sentence.
4. The limitations are discussed in a separate chapter in the discussion as recommended by BMJ open.

**VERSION 2 – REVIEW**

<table>
<thead>
<tr>
<th>REVIEWER</th>
<th>Daniel P. Potaczek</th>
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<tr>
<td>Philmpp-Universität Marburg, Germany</td>
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| REVIEW RETURNED | 11-Nov-2015 |

| GENERAL COMMENTS | The Authors fully addressed my comments/suggestions in a very professional way. |

<table>
<thead>
<tr>
<th>REVIEWER</th>
<th>Mitsushige Sugimoto</th>
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<td>Japan</td>
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| REVIEW RETURNED | 16-Nov-2015 |

| GENERAL COMMENTS | The reviewer completed the checklist but made no further comments. |
Role of *Helicobacter pylori* and interleukin 6 -174 gene polymorphism in dyslipidemia: a case–control study

Vesa-Matti Pohjanen, Olli-Pekka Koivurova, Seppo E Niemelä, Riitta A Karttunen and Tuomo J Karttunen

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