

Editorial office  
World Journal of Gastroenterology  
Baishideng Publishing Group

**Oslo University Hospital**  
Rikshospitalet  
P.O. Box 4950 Nydalen  
N-0424 Oslo

Ph: +47 23 07 00 00

[www.ous-research.no/nopsc/](http://www.ous-research.no/nopsc/)

Oslo 10<sup>th</sup> of February, 2014

Dear Jin-Lei Wang, Dear Lian-Sheng Ma,

We thank you for the opportunity to submit a revised version of our manuscript “HLA variants related to primary sclerosing cholangitis influence rejection after liver transplantation” (ESPS Manuscript NO: 7829) to World Journal of Gastroenterology.

We have revised the manuscript according to all comments by the reviewers. Most importantly, all analyses have been re-done excluding the 5 patients who received a different immunosuppression protocol. The new analysis did not change any of the main findings or conclusions, yet we have, in accordance with this main concern of the reviewers, updated all results and tables with the numbers from this new analysis. Further details as to each specific comment from all reviewers are given in the below point-by-point responses to reviewers, in accordance with the World Journal of Gastroenterology instructions for responding to reviewer comments. We have also added a “Core tip” and “Comments” section to the revised manuscript.

We are grateful for the efforts made by the reviewers and believe the revised article has substantially improved and hope that you will find it publishable in World Journal of Gastroenterology in the present form.

Yours sincerely,



Tom H. Karlsen, MD, PhD, Professor of gastroenterology  
Leader, Norwegian PSC research center

**Title:** HLA VARIANTS RELATED TO PRIMARY SCLEROSING CHOLANGITIS INFLUENCE REJECTION AFTER LIVER TRANSPLANTATION

**Author:** Bjarte Fosby, Sigrid Næss, Johannes R. Hov, James Traherne, Kirsten M. Boberg, John Trowsdale, Aksel Foss, Pål-Dag Line, Andre Franke, Espen Melum, Helge Scott, Tom H. Karlsen

**Name of Journal:** *World Journal of Gastroenterology*

**ESPS Manuscript NO:** 7829

**1 Format has been updated**

**2 Revision has been made according to the suggestions of the reviewers**

**Reviewer 00053868**

(1)“Five of the patients received a different immunosuppression scheme. They should be excluded from the analysis”

**Response:** We are grateful for this comment by the reviewer. All analyses have been repeated after excluding the 5 patients with different immunosuppression. This did not significantly influence the results for any of the relevant alleles. However, we agree with the concern (which aligns with the main comment also from reviewers 02860955 and 02861055) and have thus changed all reported data and tables accordingly.

(2)“Previous studies have found that the risk of rejection is different depending on 1)the indication (higher in PSC, PBC and autoimmune, intermediate in hep C and lower in alcoholic and hep B? 2) age of the recipient(higher in younger patients) and 3)liver function (higher in patients with more preserved liver function. The potential influence of these variables in the risk of rejection should also be assessed”

**Response:** We agree with the reviewer that this is an interesting notion on which we already report on the differences between PSC patients and non-PSC patients in the first paragraph of the Results-section. We did investigate in further detail how these factors influence the risk of rejection in our study population and hereby provide detailed numbers for the convenience of the Editor. However, since these differences are well established and previously published on many instances we are reluctant to elaborate beyond the statement made as to the high frequency of acute rejection among patients with PSC. This is in line with the main logic of the manuscript, which serves to compare PSC with other indications for liver transplantation. The numbers of patients in the other

subgroups are also too small for robust statistical assessments to be made. Still, to somewhat expand on the topic of the importance of co-morbidity we have added a sentence describing the impact of inflammatory bowel disease among the PSC patients on risk of acute rejection. Also, the lack of association between acute rejection and age and gender in the present study population is now specifically noted in the results section.

PSC : n=44, acute rejections: 18 (40%)

PBC: n=15 , acute rejections: 5 (33%)

Autoimmune : n=7 , acute rejections: 2 (29%)

HCV : n=15, acute rejections: 4 (27%)

Alcoholic: n=17, acute rejections: 1 (5.6%)

HBV : n=5, acute rejections: 0 (0%)

(3)“ The association of PSC-associated HLA alleles and the risk of rejection is underlined in the title and in the title of the first section of the results. The association of certain HLA alleles should be mentioned in the results and the association of PSC and these alleles should be restricted to the discussion”

**Response:** We understand the request, but on this point we respectfully disagree with the reviewer, since it was an intrinsic part of the study design (and the a priori hypothesis) to stratify the analysis for the presence and absence of PSC on the basis of the particularly high risk of acute rejection observed among these patients. It is therefore important to make the emphasis on the PSC-associated alleles already at the point of the Results section, since assessments of the HLA data were made with the a priori knowledge on the importance of these alleles. The Scandinavian liver transplantation program, where PSC is the most common indication, provides an unique opportunity to make this specific assessment, which, in our opinion, should remain the focus of the presentation.

(4)“Some HLA alleles are very unfrequent. Thus, it is impossible to find a significant P value. This should be mentioned in the discussion.”

**Response:** We agree with the reviewer that a comment regarding type II statistical errors (false negative) should be mentioned. Indeed, the main impetus deriving from the present study is a motivation to collect larger study populations for the investigation of liver transplant recipient genetics and risk of acute rejection. The point is not only restricted to low-frequency HLA alleles, but also to risk alleles at other risk loci, where genome-wide association studies in immune mediated phenotypes have clearly demonstrated that the effect size for the majority of non-HLA loci are in the range of an odds ratio of 1.5 and below. We have thus emphasized the point on statistical power in the Discussion in accordance with the request from the reviewer.

(5)“The indications of transplantation should be revised (table1). It is likely that some of the patients had more than one indication (i.e. hepatitis C and HCC, or PSC and cholangiocarcinoma). These patients should be included in two categories. So, the sum of etiologies should be higher than 148.

**Response:** This is a very relevant point, and the table has been revised in accordance with the suggestion of the reviewer.

**Reviewer 02861055**

(6) "The time frame during which the acute rejections developed and patients were analysed could be better stated in the Material and Methods section"

**Response:** This is an important emphasis and we have thus added the following sentence to the Results section: "All episodes of AR occurred within the first 6 weeks after LTX, with a median of 10 days (range 5-40 days)."

(7) "In the first paragraph of the results, the authors clearly show an association between different HLA alleles and development of acute liver rejection. When available data, data on 1-year and 5-year graft survival in PSC and/or non-PSC patients could be added. Comparative analysis of the development of acute rejection and graft survival might provide useful information for possible treatment strategies in patients".

**Response:** This is an interesting point which we also considered when designing the study. The main concern, as from our own side, would be that the complexity of the "survival"-phenotype is likely to make any correlations hampered by uncertainty. I.e. known factors of importance to graft- and patient-survival are likely to be more important than the impact of acute rejection and genetics, surgical complications and disease recurrence (HCV and malignancy) in particular. The purpose of determining the genetics of acute rejection and the basis of the present study is to clarify biological factors involving in the pathophysiology of acute rejection. Long term, such understanding could provide mechanistic insights paving the way for novel means of immunosuppression, ultimately reducing morbidity and costs associated with acute rejections. Since the impact of acute rejections on graft loss and patient survival in liver transplantation (unlike e.g. kidney transplantation) is relatively small, a benefit of such means on these endpoints is more uncertain and in need of assessments in separate, and specifically designed studies. For all these reasons, we are reluctant to include survival data in the present manuscript, but are of course willing to do so at the discretion of the editor.

(8) "Data on HLA mismatch only show separate analysis for HLA-A, HLA-B and HLA-DR. Combined analysis of the three epitopes could be added to the analysis.

**Response:** This is a very good suggestion, and Table 5 has been updated accordingly.(9)"In the last paragraph of result, the authors present data about immunohistochemical staining of biopsies with acute rejection. However the rationale behind this evaluation is not clearly stated in the paper and should be introduced in the discussion. Markers used to specifically identify immune cell populations should be better discussed as well. Moreover, the very low amount of cell counts and the presence of clusters of immune cell infiltrates(with little discrimination between cells) hampers

the evaluation of the experiment.”

(9)“In the last paragraph of result, the authors present data about immunohistochemical staining of biopsies with acute rejection. However the rationale behind this evaluation is not clearly stated in the paper and should be introduced in the discussion. Markers used to specifically identify immune cell populations should be better discussed as well. Moreover, the very low amount of cell counts and the presence of clusters of immune cell infiltrates (with little discrimination between cells) hampers the evaluation of the experiment.”

**Response:** We agree with the reviewer that our motivation to perform the immunohistochemical analysis could be more clearly stated. The main impetus was derived from the conflicting results in previous genetic analyses of the impact of KIR genotypes and KIR-ligand genotypes on risk of acute rejection (references 19 through 23 in the article), and we therefore wanted to corroborate our negative genetic results with a characterization of the cellular infiltrate in different phenotypes. Similar data are extremely scarce in the literature and, despite the negative outcome (and low amounts of cell counts), we therefore wanted to include the data in conjunction with the genetic results. In accordance with the request of the reviewer, we have updated the Methods and Discussion sections with a specification of the background of this analysis. We are, however, willing to remove the section on immunohistochemical analysis at the discretion of the editor.

(10)“In the discussion, there is a clear discrepancy between the reasoning on the HLA-related results and the one in which KIR ligands and KIR genotypes are discussed. Since the latter represents a conspicuous part of the results, the author should better discuss and expand this section

**Response:** We agree on the notion by the reviewer. We have slightly expanded on the discussion of the NK cell/KIR aspects in accordance with the previous comment (immunohistochemistry), but are very reluctant to speculate further on the topic, given the negative results in our own analysis. Rather, we would prefer the current emphasis on the novelty aspects, which indeed relate to the HLA and the outcomes deriving from the comparison between PSC versus non-PSC indications for liver transplantation. As stated in the manuscript, the sum of evidence now seems to suggest that a genetically determined NK cell hyperreactivity is unlikely to play a major pathogenetic role in acute rejection in liver transplantation.

#### Reviewer 02861055

(11)“Title: Twelve words. The title is not objective. Can the authors please provide a title more centred on the objective of the article? Which HLA variants are more related to PSC and influence on acute LTX rejection?”

**Response:** We agree with the reviewer that a perfect title is difficult to establish. To include more specific descriptions on which HLA alleles (HLA-B\*08, HLA-B\*07, DRB1\*03, DRB1\*04) would clearly make the title excessively long (more than 12 words, which, at best of our knowledge, represents the length restriction given by the formatting guidelines of World Journal of Gastroenterology). Furthermore, a main emphasis of the paper, and also the emphasis which makes the paper original and provides the main novelty, derives from the specific analyses relating to primary sclerosing cholangitis. We therefore kindly ask that the title stays unchanged.

(12) "Abstract: 395 words. ? The description of the results is not written clearly"

**Response:** We respectfully disagree with the reviewer on this point. The results are written in accordance with the standard reporting of results in genetic association analysis. We have a long tradition in publishing genetic association results, including (as will be evident from PubMed) multiple publications in Nature Genetics and other high impact genetic journals, and feel confident that our present mode of reporting is up to normal standards.

(13) "Page 5, line 1 – the reference need to be described."

**Response:** We are grateful for the reviewer noting this point and have added the requested reference.

(14) "Material and methods: ? 1034 words? Although the authors have used "great care" to consider the diagnosis of acute rejection, five of these patients received a different immunosuppressive therapy. What is the influence of this conduct in the diagnosis and in the analysis of severity of AR?"

**Response:** We indeed agree with this main comment from the reviewer, which is also in line with the main notions from reviewers 00053868 and 02861055, and have repeated all analyses after excluding the 5 patients with different immunosuppression. This did not significantly influence the results for any of the relevant HLA alleles. However, in line with the request of the reviewer we have updated all results and tables in the manuscript according to the new analysis.

(15) "What were the criteria adopted by the authors in considering the diagnosis of PSC? Clarification should be provided for this matter?"

**Response:** We agree phenotype definitions are important and have added a sentence describing the diagnostic procedures for primary sclerosing cholangitis including a reference to one of our review articles describing these procedures. We are reluctant to elaborate further on the diagnosis of each of the underlying liver diseases, as this is all done according to standard international, clinical practice. Rather, the emphasis of our patient population descriptions is made on precisely making the phenotypic definition of acute rejection.

(16) "Clarification should be provided for the statistical strategies adopted regarding variance heterogeneity or non-normal distribution"

**Response:** Since no genome-wide genetic data (e.g. single nucleotide polymorphisms) are available for the study population in question, it is not possible to make adjustments for population heterogeneity according to standards of genome-wide association studies. As we have demonstrated by principle component analysis in our previous studies (e.g. Nature Genetics 2013;45(6):670-5 and Nature Genetics 2011;43(1):17-9), the Norwegian population is homogeneous to the extent that no such correction is required. Normality of predicted KIR copy number was assessed according to standard means (e.g. histogram, QQ-plots). We have added text to the statistical analysis section to comply with the questions of the reviewer.

(17)“Results ? 1139 words ? The text is not written clearly. ? Results should be reported in the Results Section and an analysis of the data should be presented in the Discussion Section. 5.1. Tables ? Table title and inferior legend should be adjusted to the table width. Legends should contain sufficient information to provide an adequate understanding of the table by the reader without reference to the text.”

**Response:** We agree with the reviewer and there is no discussion of data in the Results section. The reporting of data in the Results section is in accordance with standard practice in genetic association studies, with which we have extensive experience. The tables are given with full legends as well as abbreviations and can be interpreted without further references to the text. Formatting issues, including adjustment of legend to table with etc., will be managed at the typesetting stage by the World Journal of Gastroenterology’s publishing company.

(18)“Discussion ? 1102 words ? The authors do not address some important points as a limitation of their study: the retrospective nature of their study; the findings should be cautiously interpreted because of the small number of patients; other potential risks factors beyond the influence of genetic variants in the HLA complex on acute cellular rejection after liver transplantation should also be considered such as age, gender, coexistent of other comorbidities (some of the patients could have more than one potential factor related to liver damage)”

**Response:** We agree other risk factors could indeed be important, and therefore a major effort in the present project was put into the precision and homogeneity of the diagnosis of acute rejection, which we believe is critical for the study of genetic risk factors. In the present study, great care was taken to include only biopsy proven cases of acute rejection, and the resulting recipient group with acute rejection thus consisted of Banff RAI 3-6 cases with only two exceptions (who showed RAI > 6 in their biopsies). Cases with different etiologies with potentially similar immune manifestations (e.g. ischemia/reperfusion related injuries, biliary pathologies and infections, including early HCV recurrence) were systematically excluded. A multitude of immunological mechanisms are at play in the allograft during the first weeks after liver transplantation, and lack of precision in diagnosis of acute rejection will diminish power considerably in genetic association studies. For instance, lack of statistical power and phenotypic heterogeneity probably underlies the conflicting results of acute rejection associations for many non-HLA loci investigated in previous articles. All these aspects are specifically elaborated upon in the Discussion. In addition, and in accordance with the request of the reviewer, we have added to the discussions notions relating to the retrospective nature of the present analysis (“Ideally, data on confounders and rejection characteristics should be prospectively collected, not retrospectively as in the present analysis. ”). The main emphasis of the present study was to detect genetic associations for acute rejection, not risk factors for acute rejection in general, using a novel and extremely stringent approach; an aim we successfully accomplished. As for genetic association studies in general, the biological basis of the genetic findings, and their further elaboration in mechanistic studies, is more important than overall risk assessments. For that reason, and on the basis of the strict phenotype definitions adopted in the present analysis, we are convinced on the robustness, validity and importance of our findings. Notwithstanding, further

studies of liver transplant recipients, i.e. larger study populations, using the same stringent criteria, are highly warranted and are likely to shed risk on also non-HLA risk factors for acute rejection. Finally, in line with the suggestion of the reviewer, the lack of association between acute rejection and gender and age in the present study population is now specifically noted in the results section.

**Reviewer 02860955**

(19)“The 5 patients who received a calcineurin inhibitor (either cyclosporine or tacrolimus) in combination with prednisolone and azathioprine should be excluded.”

**Response:** See response to reviewers Reviewers 00053868 and 02861055. All analyses have been repeated after excluding the 5 patients with a different immunosuppression regimen. Although this did not influence the results regarding any of the relevant outcomes of the study, we have changed results and Tables in accordance with this concern of the reviewer.

(20)“It would be interesting to know if PSC patients with concurrent inflammatory bowel disease had an increased risk of AR?”

**Response:** We are most grateful to the reviewer for this interesting suggestion. We have collected the required phenotypic data and performed an analysis showing that among the 37 PSC patients (82%, in line with the general frequency inflammatory bowel disease [IBD] in PSC patients) there is a slightly higher occurrence of acute rejection in patients with IBD (43.2%) as compared with patients without IBD (25%). Although the difference did not achieve statistical significance ( $p=0.34$ ), we have included a sentence in the Results section reporting on these results.

(21)“No other risk factors concerning age, gender, ethnicity are mentioned. Could you comment on that?”

**Response:** There is minimal ethnic diversity with the Norwegian population and in the present study population (>96% of known Norwegian descent), as also demonstrated by principle component analysis in our previous studies (e.g. Nature Genetics 2013;45(6):670-5 and Nature Genetics 2011;43(1):17-9). Neither age nor gender had any significant influence on the occurrence of acute rejection in the present study population, and this information has now been added to the Results section (“We found no statistically significant influence from age or gender on risk of acute rejection (data not shown)”).

**Reviewer 02860875**

(22)“ Major: The strongest message from the paper is that an HLA association exists for ACR that is enriched



in, but not specific for, PSC. The authors should concentrate on this part with more supportive data. Do subjects (with or without PSC) with the AH8.1 haplotype have more rapid time to rejection, more episodes of rejection, worse graft/patient survival, higher immunosuppressive requirements at 1 year? Similarly, are the biopsy characteristics the same for ACR with or without the AH8.1 haplotype: more endothelialitis or more duct damage?"

**Response:** The point of the reviewer is intriguing and should indeed be the subject of further studies. In general, based on the general theme of experience from genetic association studies, extensive sub-phenotype analysis is hampered with the inevitable risk of type 1 statistical errors due to multiple testing. Furthermore, for most associations detected, sub-phenotype associations are scarce (e.g. only a few of the 163 inflammatory bowel disease risk loci associate with sub-phenotypic features, and are even not specific to ulcerative colitis and Crohn's disease). Furthermore, given the retrospective nature of the present analysis, we do not have available data to answer on all comments from the reviewer. We did not find a statistically significant influence of the AH8.1 on time to acute rejection (15 days with AH8.1 and 17 days without). The number of rejection episodes is likely biased by the occurrence of the first event of acute rejection, potentially providing an incentive for intensified immunosuppression. We did not find any statistically significant difference in number of rejection episodes between the different groups, but the numbers are too small to make conclusive statements. Similar statements apply to the proposed survival analysis. I.e. since the impact of acute rejections on graft loss and patient survival in liver transplantation (unlike e.g. kidney transplantation) is relatively small, an influence from detected genetic risk factors in acute rejection on these endpoints is uncertain and, in our opinion, in need of assessments in separate, and specifically designed studies. The proposal regarding immunosuppression is the most problematic proposal. At our center, PSC patients normally receive an intensified immunosuppression regimen including long-term prednisolone. Whether this practice could be stratified according to genetic risks, needs prospective evaluation in the context of a clinical trial and is clearly beyond scope of the present analysis. Finally, the biopsies were not assessed for the details requested by the reviewer, and we are extremely reluctant in performing. For all these reasons, we are reluctant to include proposed data in the present manuscript, for the simple reason to avoid adding speculative notions to what is at present a relatively robust reporting. Our data provide a strong impetus to further studies of involved mechanisms, and proposed aspects are better elaborated upon in such prospective contexts.

(23) "For the core cohort of the manuscript they have excluded over 50% of their original transplanted cohort. Most of the exclusions relate to lack of archival material. Perhaps for excluded subjects with only recipient DNA available they could look at the association between AH8.1 and ACR?"

**Response:** Indeed the present data provide a strong impetus to continue collecting DNA materials for larger studies of liver transplant recipient genetics. Indeed, this is one the main outcomes of the analysis, as also stated in the conclusion of the Discussion session; that for liver transplantation genetics, the host (recipient) genetics overshadow those of donor-recipient genetic matching. This provides important opportunities, given the difficulties that often exist in obtaining donor material

for research purposes, but we are afraid these opportunities need to be elaborated upon in future studies.

(24) "The data derived from immunohistochemistry is inconclusive and not supportive of the main conclusions. CD57 is not specific for NK or NKT cells, Ibegbu, J.Immunol, 2005, 174, 6088. I would suggest relegating to supplementals or dropping.

**Response:** We agree with the reviewer that the reporting on the immunohistochemistry preferably can be shortened. We have, in accordance with the suggestion of the reviewer, moved Table 6 into the Supplementary Material. We are reluctant to remove the immunohistochemistry altogether, since the outcome of the staining is in line with the second main outcome of our study (in addition to the positive findings for the HLA alleles), namely the negative findings regarding the KIR analysis. Given the conflicting results regarding the impact of KIR genotypes on risk of rejection it was felt necessary to include supportive data in the form of an assessment of CD56 and CD57 positive cells in archival material (which provided difficult, as stated in the manuscript, due to poor performance of antibodies specific to NK cells, necessitating the double staining approach employed). We have elaborated on these justifications in the revised version of the discussion. In the case of space restrictions, however, we will at the discretion of the editor remove also Figure 1 from the main manuscript

(25) "Minor 1. Introduction, Previous work has demonstrated that donor-recipient HLA-DR incompatibilities seem to impact graft survival in PSC patients to a greater extent than recipients for other diseases; further that HLA incompatibilities predicted the development of chronic rejection in PSC recipients only, Neumann, Transplantation 2003, 75, 132 2"

**Response:** This is an excellent suggestion and we have included a table showing the stratified results as Supplementary Table 7. The manuscript has been updated with a sentence referring to this stratified analysis, which unfortunately did not reproduce previous notions of a significant impact of HLA-DR incompatibility in PSC patients. Actually, our general impression, evaluating existing literature along with the present analysis is that for non-ABO genetics (blood type is also genetically determined), recipient genetics, rather than donor-recipient interactions, could be a major determinant and the substrate of further studies in other, and larger, study populations.

(26) "Methods, Excluding patients with suspected rejection without a biopsy is pragmatic and appropriate. It would be nice to know the relative proportions of subjects in this group transplanted for PSC and non-PSC indications. "

**Response:** As acknowledged by the reviewer, we took great care in maximizing the quality of the phenotypic assessments, and 17 patients were excluded due to lack of biopsy confirmation of acute rejection (8 PSC, 3 autoimmune hepatitis, 1 HBV cirrhosis, 2 HCV cirrhosis, 2 alcoholic cirrhosis, 1 PBC). In accordance with the request of the reviewer, these numbers have been added to the methods section of the manuscript.

(27)" Methods, It is not mentioned in the methods section, but I am making the assumption that treatment for rejection involved intravenous methyl-prednisolone. Did subjects receive only one round of ivMP before a diagnosis of steroid-resistant rejection? Was this confirmed on biopsy?"

**Response:** We agree this is of interest to the reader and have added a small notion to the results section on this point. Indeed, three to four rounds of i.v. methylprednisolone was administered (initial dose of 1g, followed by 0.5g over the following 2-3 days). A new biopsy was performed prior to ATG in 3 of the 10 patients in question, whereas the remaining 7 patients received ATG without any new biopsy based on persistence of the clinical and biochemical parameters..

**3 References and typesetting were corrected**