Heartjnl-2011-300960 - Low-grade Inflammation and the Phenotypic Expression of Myocardial Fibrosis in Hypertrophic Cardiomyopathy

I Response to the comment by Editor

Editor Comment:

We have taken another look at your paper in the light of the rebuttal you sent us. We recognise the potential interest of your paper but will need to see a revised version addressing all the points of our reviewers before coming to a final decision. In particular we would like to see a more robust response to the issue of quantifying the inflammatory response.

Response: We appreciate the possibility, given by Editor-in-Chief, to address the points by Editor and Reviewers and send a revised version of our article to *Heart*. Regarding the issue of quantifying the inflammatory response, there are several aspects:

1. **The data of circulating proinflammatory cytokines, hS-CRP, as well as myocardial histological findings, immunohistochemical studies, and nuclear factor kappa B demonstrate a variable inflammatory response in patients with HCM, all pointing in the same direction, as stated by Reviewer 3 below.**
2. **To quantify the inflammatory response, the circulating proinflammatory cytokines and hS-CRP were measured in both patients and matched controls. In statistical analyses, the levels of 4 of 5 cytokines and hS-CRP were higher in patients with HCM compared to controls, with a statistically significant difference (three p values <0.01, one p value < 0.05, and one p value <0.001, Table 3), demonstrating a quantified inflammatory response in patients with HCM.**
3. **We recognize the inconsistency in inflammatory cell data in histological HE staining versus immunohistochemical analyses, which is caused by the fact that each staining was performed from a different EMB microscopic slide in the patients with HCM. Endomyocardial biopsy samples are necessarily small. The most representative EMB biopsy sample was always designated for the traditional histological staining, and the second best sample, which was not sufficient for all analyses in every patient, for immunohistochemical stainings. We have added a sentence on the fact in the Supplementary data in bold (page 1, first sentences in 1st and 2nd paragraph), and also a few words in the "Limitations of the study" in bold (page 16, 1st paragraph). Even taking these inconsistencies into account, the present study is the first human study to include both endomyocardial biopsy and CMRI information from genotyped subjects with HCM.**
4. **To quantify the histologically and immunohistochemically detected inflammatory response in the myocardium, we used a semiquantitave evaluation by an experienced pathologist. We acknowledge that counting the exact number of inflammatory cells would be a very good option. However, first, B lymphocytes were found in none of the myocardial samples, and just few macrophages were found in one patient sample only, preventing counting the number of B lymphocytes and CD68 positive macrophages per mm^2, as required by Reviewer 2. Second, in control myocardial samples, inflammatory cells (very few) were found only in one sample, making more sophisticated evaluation in controls insignificant. Variable inflammatory response in endomyocardial samples of HCM patients was demonstrated by HE histology showing eosinophilic granulocytes and mononuclear inflammatory cells in one third of patients with HCM. In immunohistochemistry, when using rabbit anti-human antibody, CD3 positive cells indicating T-lymphocytes were found in 7 of 11 patients. In addition, NF-kB activation, which is known to lead to proinflammatory phenotype, was detectable in half of the myocardial specimen of the patients but not in controls. The variable inflammatory response in patients with HCM is also clearly visible in figures 2A-2C. To clarify our findings, we have re-written the paragraph "Immunohistochemical findings in endomyocardial samples" (page 10, 3rd paragraph, text in bold) in a more comprehensible manner, and added a clarifying sentence to discussion (page 13, 3rd paragraph, text in bold).**

In our opinion, taking aforementioned aspects into account, our claim that there is a variable inflammatory response in the myocardium of the patients with HCM is adequately supported

by the semiquantitative scoring of histological and immunohistochemical characteristics by an experienced pathologist. Previously, we have used a similar semiquantitative evaluation of histological and immunohistochemical findings to show an active inflammatory process in stenosed aortic valves. Our findings were described in the article "Characterization of the early lesion of degenerative valvular aortic stenosis – histological and immunohistochemical studies" (*Circulation* 1994;90:844-853), which has been cited for 425 times (ISI Web of Knowledge, Nov 2011). Additionally, we have used a similar semiquantitative evaluation of histological findings in our article "Atherosclerosis-like lesions of the aortic valve are common in adults of all ages: a necropsy study", published in *Heart* (2005;91:576-82). We have now added the articles as references for our quantifying method in the Supplementary data (page 1, 1st paragraph, in bold).

II Response to the comments by Reviewers 1 and 3

Reviewer Comments:
Reviewer: 1
Comments to the Author
In this work Kuusisito et al. show that HCM is asscoicated with ECM remodeling and increased low grade inflammation.
This is an intersting paper and written clear.

1. Please discuss a possible interaction between fibrosis and inflammation. Is there a possible link?

Response: We have discussed the possible interaction between inflammation and fibrosis in Introduction (page 4, the second paragraph) and Discussion (page 12, the 3rd paragraph "Potential pathogenic mechanisms for myocardial fibrosis formation in HCM"). We have also edited the Discussion chapter "Myocardial NF-kB activity in HCM", and highlighted the role of NF-kB activation in myocardial fibrosis formation (page 14, 2nd paragraph, text in bold).

2. Would be nice to have immunhistological stainings revealing what types of monocytes invaded the myocardium? This would give interesting new informations ...

Response: Immunohistochemical stainings of mononuclear cells were performed as explained in the Supplementary data (pages 1-2). B lymphocytes were found in none of the myocardial samples, and few macrophages were found in one patient sample only. Mild to moderate mononuclear inflammatory cell infiltration showing CD 3 positivity with rabbit anti-human antibody indicating the presence of T lymphocytes was found in a major part of EMB patient samples but only in 0 to 2 controls (bold text "Immunohistochemical findings" page 10, 3rd paragraph, which we have re-written in a more comprehensible manner). T lymphocytes are shown also in Figure 2Cc. We have added a clarifying sentence to discussion (page 13, 3rd paragraph, text in bold).

3. Any data regarding MMP regulation available. Would be interesting in this setting.

Response: Regrettably, we do not have any data regarding MMP regulation. Instead, we have shown that in the patient population of the present study, heterogeneity of late enhancement in CMRI is associated with levels of circulating serum amino-terminal propeptide of type III collagen (published in *Heart* in 2006, see reference 16).

Reviewer: 3
Comments to the Author
This paper presents a interesting study which analyse the relation between inflammation and fibrosis in patients with hypertrophic cardiomyopathy. The authors analyse both circulating and histopathologic markers of inflammation and they also evaluate fibrosis by two different methods: magnetic resonance imaging with late gadolinium enhancement and histopathologic evaluation. One of the main strengths of the paper is that

all the included patients have the same mutation. These fact reduces the confusion usually subjacent to those studies including patients with different mutations which may have very variable consequences. Even thought this fact is also reported as a limitation (the results cannot be automatically extrapolated to other mutations) we think that it is mainly and advantage. The findings in patients are compared to the results in two different groups of controls: circulating biomarkers and MRI are obtained from healthy volunteers while the controls for the histopathologic studies are deceased individuals without cardiac disease (the only possible source for this controls).
The main findings of the study are that inflammatory circulating markers are higher in patients than in controls, that patients with HCM show inflammatory cell infiltration in histopathologic studies, and that there are significant and relevant correlations between histopathologic inflammation, fibrosis and LGE in MRI. The authors show that inflammation and fibrosis show variable degrees and characteristics in patients with the same mutation. Even thought some of the correlations are in the limit of significance (i.e inflammatory infiltration with LGE p value=0.046 with r=0.541) all the findings with the different techniques point in the same direction and we have to consider that the numbers of patients are relatively small (something difficult to avoid in this type of study including EMB and genotyped patients). Finally, the authors suggest that fibrosis could be caused or triggered by inflammation, opening the possibility to target inflammatory response to avoid or decrease fibrosis and arrhythmias in HCM patients.
We consider that the paper has an appropriate design, is well written and provides relevant and interesting information. The figures are very good and demonstrative.

Particular comments:
-Page 8, line 36: "histolopathological" should be "histopathological"
-Page 11, line 56: "There we no.." should be "There were no..."

Response

We thank Reviewer 3 for constructive comments. We have corrected line 36 on page 8 and line 56 on page 11 (now page 12, 1st paragraph) as suggested by Reviewer (corrections in bold).

III Response to the comments by Reviewer 2

Reviewer: 2
Comments to the Author

The current manuscript describes the presence of low grade inflammation in relation to fibrosis in patients with HCM
The strong point of the manuscript are the fact that inflammation is studied at different levels: blood and cardiac samples. However, the study population is small, and different methodologies used are difficult to accept.

Response: We appreciate that the study population is small, but human studies with genotype-verified diagnosis of HCM, especially with a single causative mutation, are generally small. E.g. in the study by Timmer SA et al. "Carriers of the hypertrophic cardiomyopathy MYBPC3 mutation are characterized by reduced myocardial efficiency in the absence of hypertrophy and microvascular dysfunction" . Eur J Heart Fail. 2011 Oct 21. [Epub ahead of print] included 15 genotyped patients. Another study by Oliva-Sandoval MJ et al. "Insights into genotype-phenotype correlation in hypertrophic cardiomyopathy. Findings from 18 Spanish families with a single mutation in MYBPC3 ." Heart. 2010 Dec;96(24):1980-4; included 65 genotyped subjects. Previous studies including endomyocardial biopsies and CMRI in genotyped HCM patient populations are so far nonexistent. We have discussed the matter in "Strengths and limitations of the study" (page 15, 2nd paragraph, and page 16, 1st paragraph, bold text).

Regarding the methodologies, CMRI methods of the study have been previously validated and published (Sipola et al. Heart 2006, see reference 16), as well as the semiquantitative method to evaluate histological and immunohistochemical findings (Otto et al. Circulation 1994;90:844-853). We agree that several methods (proinflammatory cytokines, histological and immunohistochemical detection of inflammatory cells and immunohistochemical verification of NF-kB) were used to

confirm the presence of inflammation in the myocardium of the patients with HCM, which we consider the strength of our study. In contrast, we wonder what Reviewer 3 means by a vague term "different methodologies are difficult to accept" and consequently, we cannot answer more specifically.

Methods:
CMRI Image analysis: performed short-axis images at the levels of tips of the mitralis valve leaflets and papillary muscles. In statistical analysis, max value of six segmental LGE heterogeneity values was used. Why? What is the scientific basis for this method?

Response: With this CMRI method we aimed to assess the severity and not the extent of late-enhancement. Consequently, it is natural to use the maximal LGE heterogeneity value of six measured segmental values in statistical analyses. In fact, the method has been validated and LGE heterogeneity has been shown to correlate with levels of serum amino-terminal propeptide of type III collagen in the patient population of the present study (Sipola et al. *Heart* in 2006, see reference 16).

Right ventricular biopsies of patients are compared to death controls, in the left ventricle. Data may be biased by these different locations.

Response: This is not a correct statement. The endomyocardial biopsies were obtained from the right side of the interventricular septum under fluoroscopic guidance. The cadaver myocardial samples were taken from several locations of the heart including interventricular septum (page 7, 3rd paragraph, section "Endomyocardial biopsy and autopsy myocardial samples"). Regarding cadaver controls, we are aware of the possibility of some bias. However, cadavers were the only possible source for control myocardial samples, as stated by Reviewer 3.

The methodology used to quantify inflammation is not acceptable. At least, the number of CD45- ,CD3 and CD68-staining inflammatory cells should be analysed by counting their number per mm². Just a H&E quantification by a subjective analysis (scoring) is not acceptable.

Response: We acknowledge that counting the exact number of inflammatory cells would be a very good option. However, first, B lymphocytes were found in none of the myocardial samples, and just few macrophages were found in one patient sample only, preventing counting the number of B lymphocytes and CD68 positive macrophages per mm^2, as required by Reviewer 2. Second, in control myocardial samples, inflammatory cells (very few) were found only in one sample, making more sophisticated evaluation in controls insignificant. Variable inflammatory response in endomyocardial samples of HCM patients was demonstrated by HE histology showing eosinophilic granulocytes and mononuclear inflammatory cells in one third of patients with HCM. In immunohistochemistry, when using rabbit anti-human antibody, CD3 positive cells indicating T-lymphocytes were found in 7 of 11 patients. In addition, NF-kB activation, which is known to lead to proinflammatory phenotype, was detectable in half of the myocardial specimen of the patients but not in controls. The variable inflammatory response in patients with HCM is also clearly visible in figures 2A-2C. To clarify our findings, we have re-written the paragraph "Immunohistochemical findings in endomyocardial samples" (page 10, 3rd paragraph, text in bold) in a more comprehensible manner, and added a clarifying sentence to discussion (page 13, 3rd paragraph, text in bold).

In our opinion, taking aforementioned aspects into account, our claim that there is a variable inflammatory response in the myocardium of the patients with HCM is adequately supported by the semiquantitative scoring of histological and immunohistochemical characteristics by an experienced pathologist. Previously, we have used a similar semiquantitative evaluation of histological and immunohistochemical findings to show an active inflammatory process in stenosed aortic valves. Our findings were described in the article "Characterization of the early lesion of degenerative valvular aortic stenosis – histological and immunohistochemical studies" (*Circulation* 1994;90:844-853), which has been cited for 425 times (ISI Web of Knowledge, Nov 2011). Additionally, we have used a similar semiquantitative evaluation of histological findings in our article "Atherosclerosis-like lesions of the aortic valve are common in adults of all ages: a necropsy study", published in *Heart*

(2005;91:576-82). We have now added the articles as references for our quantifying method in the Supplementary data (page 1, 1st paragraph, in bold).

Table 2: unclear what the control group is? P-values?

Response: The controls are the cadaver samples as indicated in the results section (the 2nd paragraph on page 10 under the heading "Histological findings in endomyocardial samples"). As the cadaver samples showed minimal inflammatory findings (mild inflammatory cell infiltration of mononuclear cells in one of 20 control cadaver samples) and other pathological characteristics (mild myocyte size heterogeneity and myofiber disarray in one sample, mild myocyte hypertrophy in 7 samples, mild interstitial fibrosis in 5 samples; hypertension in cadavers could not be excluded), we did not consider very informative to put the data in Table 2. Given the small numbers, the value of statistical analyses in this instance is also questionable. However, if regarded appropriate, statistical analyses can surely be performed and control data may be added to Table 2.

Laboratory determinations of cytokines and hs-CRP: which is the time point? When were the samples taken?

Response: The blood samples for laboratory determinations were all taken during one two-day visit at the Kuopio University Hospital, as indicated in Patients and Methods section (page 6, 1st paragraph). The laboratory determinations of cytokines and hs-CRP were performed all at the same time shortly after all patients had participated in the study.

Little is described on the patient characteristics: such as the presence or absence of heart failure at the time of biopsy taking. Confounders such as symptomatic heart failure (increase inflammation independent of HCM or whatever cardiac disease),medication, … should be mentioned. These confounders may also influence the cytokines profile measured in blood.

Response: The claim by Reviewer is incorrect. It is clearly stated in the manuscript that 90% of the patients had NYHA functional class I-II, and none had a history of decompensated heart failure, and all patients had a normal ejection fraction (Results/ clinical, echocadiographic and CMRI characteristics, pages 8-9). In contrast to the claim by the Reviewer, it is mentioned that one third of the patients used cardiac medication, mostly betablockers (Results section – clinical, echocardiographic and CMRI characteristics). We also have exercise test data on the study subjects, which we have previously published (reference 17). None of the patients was taking ACE inhibitors or AT1 receptor antagonists, or medication for heart failure. Taking into account the limited space available, we did no consider essential to include all the previously published patient data in the present manuscript, but we have put some additional patient data in the Results section (page 9, first paragraph, text in bold).

Numbers of patients are inconsistent through the manuscript: 16 out of 20 HE staining, CD3, IHC 7 out of 11 cases (CD3), 12 out of 15 for collagen staining.

Response: We recognize the inconsistency in inflammatory cell data in histological HE staining versus immunohistochemical analyses, which is caused by the fact that each staining was performed from a different EMB microscopic slide in the patients with HCM. Endomyocardial biopsy samples are necessarily small. The most representative EMB biopsy sample was always designated for the traditional histological staining, and the second best sample, which was not always sufficient for all analyses in every patient, for immunohistochemical stainings. We have added a sentence on the fact in the Supplementary data (page 1, first sentences in paragraphs 1 and 2, text in bold), and also a few words in the "Limitations of the study" (page 16, 1st paragraph, in bold). Even taking these inconsistencies into account, the present study is the first human study to include endomyocardial biopsy and CMRI information from genotyped subjects with HCM.

Inconsistence between 37 % of inflammatory influx studies by HE staining, but not reproducible with IHC.

Response: This is a flawed argument. Of 11 patient EMB samples available, 7 (63%) showed CD3 positivity with rabbit anti-human antibody in mononuclear inflammatory cells. See the 1st paragraph under the heading "Immunohistochemical findings…", which we have re-written in a more comprehensible manner (page 10, 3rd paragraph, text in bold). Regarding the small number of cases in this analysis see the previous response above.

Studies on different kind of inflammatory cells are completely lacking (cfr question above).

Response: We do not understand the statement by Reviewer and wonder the meaning of it. In our manuscript, we provide data of eosinophilic granulocytes, macrophages, and mononuclear inflammatory cells including T-lympohcytes and B-lymphocytes in the EMB samples of the patients and control cadaver samples (the Results section under headings "Histological findings…" and "Immunohistochemical findings…").