Low-grade inflammation and the phenotypic expression of myocardial fibrosis in hypertrophic cardiomyopathy

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ABSTRACT
Objective To investigate the role of inflammation in the phenotypic expression of myocardial fibrosis in hypertrophic cardiomyopathy (HCM).
Design Clinical study.
Setting Kuopio University Hospital and University of Eastern Finland, Kuopio, Finland.
Subjects Twenty-four patients with a single HCM-causing mutation D175N in the α-tropomyosin gene and 17 control subjects.
Main outcome measures Endomyocardial biopsy samples taken from the patients with HCM were compared with matched myocardial autopsy specimens. Levels of high-sensitivity C-reactive protein (hsCRP) and proinflammatory cytokines were measured in patients and controls. Myocardial late gadolinium enhancement (LGE) in cardiac MRI (CMRI) was detected.
Results Endomyocardial samples in patients with HCM showed variable myocyte hypertrophy and size heterogeneity, myofibre disarray, fibrosis, inflammatory cell infiltration and nuclear factor kappa B (NF-κB) activation. Levels of hsCRP and interleukins (IL-1β, IL-1RA, IL-6, IL-10) were significantly higher in patients with HCM than in control subjects. In patients with HCM, there was a significant association between the degree of myocardial inflammatory cell infiltration, fibrosis in histopathological samples and myocardial LGE in CMRI. Levels of hsCRP were significantly associated with histopathological myocardial fibrosis. hsCRP, tumour necrosis factor α and IL-1RA levels had significant correlations with LGE in CMRI.
Conclusions A variable myocardial and systemic inflammatory response was demonstrated in patients with HCM attributable to an identified sarcomeric mutation. Inflammatory response was associated with myocardial fibrosis, suggesting that myocardial fibrosis in HCM is an active process modified by an inflammatory response.

INTRODUCTION
Hypertrophic cardiomyopathy (HCM), most commonly caused by mutations in sarcomeric genes, is a myocardial disease characterised by left ventricular hypertrophy, myocyte disarray and myocardial fibrosis.1 The cardiac phenotype in HCM varies between unrelated individuals and also between family members with an identical disease-causing sarcomeric mutation, suggesting that HCM is a complex inherited disease modified by other genetic and environmental factors.1–5 The molecular events triggered by the genotype or other factors that induce the cardiac phenotype, particularly fibrosis, remain to be determined.4–6

Previous studies have shown that chronic inflammation decreases myocardial contractility, induces hypertrophy and promotes apoptosis and fibrosis, thus contributing to myocardial remodelling.7–9 Inflammatory cytokines, tumour necrosis factor α (TNFα), interleukin (IL)-1 and IL-6, are not constitutively expressed in the normal heart, but are upregulated and produced as a stress response to myocardial injury or mechanical stress.3 TNFα and IL-6 can directly attenuate myocardial contractility and induce the development of myocyte hypertrophy, collagen deposition and fibrosis.6 IL-1β has been correlated with myocardial collagen deposition, cardiomyocyte apoptosis and inflammation.13 The expression of both inflammatory and anti-inflammatory cytokines is increased in heart failure.7 In addition, nuclear factor κ B (NF-κB), a transcription factor regulating inflammatory genes, has been shown to be activated in patients with heart failure of various causes.11

Circulating inflammatory cytokines are raised in HCM,12–14 but none of the previous studies has investigated the association of cytokine levels with cardiac phenotype determined by cardiac MRI (CMRI). Furthermore, there are no previous studies on NF-κB activation in HCM. Even more importantly, there are few data on myocardial histopathological findings or inflammatory response in patients with HCM caused by a specific sarcomeric gene mutation. Therefore, to investigate the role of inflammation in the phenotypic expression of myocardial fibrosis in HCM, we investigated cardiac histopathology, inflammatory cytokine and high-sensitivity C-reactive protein (hsCRP) levels and cardiac phenotype by CMRI in patients with HCM attributable to an identical HCM-causing mutation D175N in the α-tropomyosin gene (TPM1-D175N).

PATIENTS AND METHODS
The study protocol was approved by the ethics committee of the Kuopio University Hospital. All subjects gave written informed consent. Consent to the use of cadaver myocardial samples was.
Hypertrophic cardiomyopathy

obtained from the Finnish Center for Legal Protection in Health Care. Data of endomyocardial biopsy and cytokine findings have not been published in any form. Previously, we have published studies on CMRI-derived myocardial perfusion, myocardial late gadolinium enhancement (LGE), myocardial contractile impairment and inducibility of life-threatening arrhythmias in this same patient population.2 15−17

Patients with HCM
Twenty-four patients from five Finnish families (11 men, 13 women; mean age, 42±13 years; age range, 17−68 years) with HCM attributable to the mutation D175N in the α-tropomyosin gene (TPM1-D175N)18 19 were included in the study. In Finland, TPM1-D175N is one of the two founder mutations causing about 11% of all, and 25% of familial, HCM cases in eastern Finland.19 Of 24 subjects with TPM1-D175N, two did not meet the diagnostic criteria for clinical HCM and were regarded as healthy mutation carriers. All subjects with TPM1-D175N, however, are called ‘patients with HCM’ in the subsequent analysis. The study protocol described below was performed during one 2-day visit to the Kuopio University Hospital.

Control subjects
Seventeen healthy volunteers not related to patients with HCM and without a previous cardiac disease or drug treatment and with race, gender and age similar to the patients with HCM, were included into our study. Study protocol was identical to that for the patients with HCM, except for cardiac catheterisation and endomyocardial biopsy.

For histopathological analyses, control myocardial specimens were obtained from 20 cadavers matched for age, gender and race without known cardiac disease (no history of a cardiac disease, normal macroscopic myocardial findings and no clear evidence of a specific myocardial disease on microscopic examination).

Clinical and echocardiographic evaluation
All patients with HCM and control subjects underwent an interview, physical examination, 12-lead ECG recording and echocardiography, as previously described.2 15−17

CMRI protocol
CMRI cine, perfusion and LGE imaging were performed in patients with HCM and controls as previously described.2 15 16 CMRI cine imaging was performed in all patients, perfusion imaging in 17 patients with HCM and LGE imaging in 22 patients with HCM.

CMRI image analysis
Left ventricular (LV) characteristics and myocardial perfusion by CMRI were evaluated. LGE image analysis was performed in LV short-axis images at the levels of tips of the mitral valve leaflets and papillary muscles.16 In statistical analyses, the maximal value of the six segmental LGE heterogeneity values was used. Figure 1 shows LGE images of one control subject and three patients with HCM.

Coronary angiography
Coronary angiography was performed on 21 of 24 patients with HCM using standard angiographic techniques by the same cardiologist (JK).

Endomyocardial biopsy and autopsy myocardial samples
Myocardial specimens for histological analysis and immunohistochemistry were obtained from 20 patients with HCM.

Figure 1 Contrast-enhanced T1-weighted inversion recovery images in (A) a 52-year-old control subject; (B) a 45-year-old male patient with hypertrophic cardiomyopathy (HCM); (C) a 37-year-old female patient with HCM; (D) 19-year-old male patient with HCM. Patients with HCM (B−D) show intramyocardial focal high signal areas (arrows) with increased segmental late gadolinium enhancement.

Endomyocardial biopsy samples were obtained under fluoroscopic guidance from the right ventricle side of the interventricular septum with the standard endomyocardial biotome. Control cadaver samples were selected from the archives of the department of clinical pathology of Kuopio University Hospital. Representative myocardial routine autopsy specimens from the anterior, posterior or septal wall of the heart were taken from 20 matched cadavers.

Histological and immunohistochemical methods
Histological methods and immunohistochemistry analyses are described in detail in the online supplementary information.

Laboratory determinations of cytokines and hsCRP
Plasma concentrations of TNFα, IL-6, IL-10, IL-1β and IL-1RA were measured using assay kits from R&D Systems (Minneapolis, USA). hsCRP was measured using an Immulite analyser and a DPC hsCRP assay (DPC, Los Angeles, California, USA) in all 24 patients with HCM and in 17 controls.

Statistical analysis
Data are given as mean±SD. Statistical analyses were performed with a statistical software package (SPSS Win V11.5., SPSS Inc). Because of skewed distribution, LV mass and all cytokines were analysed after logarithmic transformation. The differences between the patients and controls were assessed by Student t test. Mann−Whitney test was used to investigate the association between endomyocardial findings and proinflammatory cytokines. Pearson’s correlation coefficients were calculated to investigate the association of cytokines with LGE in CMRI. Spearman’s correlation coefficient was used to investigate the association of different histopathological features, the association between histopathological inflammation and fibrosis and between histopathological inflammation/fibrosis and LGE in CMRI.
RESULTS

Clinical, echocardiographic and CMRI characteristics

Clinical, echocardiographic and CMRI characteristics of the patients with HCM and controls have been published previously and are summarised in Table 1. Patients with HCM had stable mild to moderate symptoms (90% of patients had New York Heart Association functional class I–II). None of the subjects with HCM had a history or clinical symptoms or signs of decompensated heart failure, myocarditis, systemic infection, chronic inflammatory disease or life-threatening arrhythmias. None of the patients had an intracardiac defibrillator. About one-third of the patients used cardiac medication, mostly β-blocking agents. None of the patients was taking ACE inhibitors or AT1 receptor antagonists or medication for heart failure.

None of the subjects with HCM had a significant LV outflow tract obstruction (>50 mm Hg) at rest. In CMRI, LV maximal wall thickness was increased in patients with HCM compared with controls. No difference was seen in global LV ejection fraction between patients with HCM and control subjects, but the number of hypokinetic segments was increased in patients with HCM compared with controls. The LV perfusion reserve was lower and the maximal LV LGE increased in patients with HCM compared with controls.

Coronary angiography in patients with HCM

Patients with HCM had normal coronary arteries except for one patient with HCM, who had <50% stenosis in the left anterior descending coronary artery and another patient who had <50% stenosis in the left anterior descending, intermediate and right descending coronary arteries.

Histological findings in endomyocardial samples

Table 2 shows the histopathological findings in haematoxylin–eosin stained endomyocardial samples in patients with HCM. Sufficient endomyocardial biopsy samples for histology were available for 16 of 20 patients with HCM. Histological samples showed variable amounts of heterogeneity of myocyte size, myocyte hypertrophy, myofibre disarray, myocardial fibrosis, inflammatory cell infiltration and intramyocardial small artery narrowing. Interstitial and perivascular fibrosis was found in about 90% of cases. Inflammatory cell infiltration, including mainly mononuclear inflammatory cells and eosinophilic granulocytes, was found in 57% of the patients. Narrowed intramyocardial small arteries were found in one-quarter of the patients. Figure 2A–C shows typical, mild and marked histopathological findings in patients with HCM, respectively.

In control cadaver myocardial samples, mild heterogeneity of myocyte size was found in one of 20 specimens, mild myocyte hypertrophy in seven samples, mild myofibre disarray in one sample, mild interstitial fibrosis in five samples, mild inflammatory cell infiltration of mononuclear cells in one sample and eosinophilic granulocytes and intramyocardial small artery narrowing in none of the samples.

Immunohistochemical findings in endomyocardial samples

Immunostaining with rabbit anti-human antibody showed that seven of 11 (64%) patient endomyocardial biopsy samples had CD3 positivity implicating T-lymphocytes (two with moderate and five with weak positive staining). We could, however, replicate the CD3 positivity with NCL-CD3-F51 in only one patient endomyocardial biopsy sample showing marked inflammatory response (figure 2C). In 20 control cadaver myocardial samples, weak CD3 positivity with rabbit anti-human antibody was found in two samples and CD3 positivity with NCL-CD3-F51 in none of the samples. B-lymphocytes with M755 staining were not found in patients or controls. Occasional macrophages with MO814 were found in one patient sample only.

Picrosirius collagen staining was positive in 12 of 15 HCM samples. Extensive fibrosis was found in two cases (figure 3), moderate fibrosis in four cases and mild fibrosis in six cases. In

Table 1 Clinical, echocardiographic and CMRI characteristics in control subjects and patients with hypertrophic cardiomyopathy

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control subjects (n = 17)</th>
<th>Patients with HCM (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men/women</td>
<td>8/9</td>
<td>11/13</td>
</tr>
<tr>
<td>Age, years</td>
<td>38±12</td>
<td>42±13</td>
</tr>
<tr>
<td>Vmax, m/s</td>
<td>1.3±0.2</td>
<td>1.4±0.5</td>
</tr>
<tr>
<td>LV MRI findings</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximal wall thickness, mm</td>
<td>9.7±1.7</td>
<td>19.5±4.9**</td>
</tr>
<tr>
<td>Mass, g</td>
<td>123±32</td>
<td>151±57</td>
</tr>
<tr>
<td>End-diastolic volume, ml</td>
<td>146±25</td>
<td>122±45**</td>
</tr>
<tr>
<td>End-systolic volume, ml</td>
<td>56±15</td>
<td>52±29</td>
</tr>
<tr>
<td>Hypokinetic segments, %</td>
<td>12±12</td>
<td>37±20**</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>61±7</td>
<td>58±7</td>
</tr>
<tr>
<td>Perfusion reserve</td>
<td>1.80±0.58</td>
<td>1.12±0.35*</td>
</tr>
<tr>
<td>The heterogeneity of late-enhancement, %</td>
<td>12±3</td>
<td>18±11*</td>
</tr>
</tbody>
</table>

*Vmax indicates maximum velocity in the Doppler signal from the jet in left ventricular outflow tract.

Data are means±SD.

**p<0.05, ***p<0.001.
CMRI, cardiac magnetic resonance imaging; HCM, hypertrophic cardiomyopathy; LV, left ventricular.

Table 2 Endomyocardial biopsy findings in 16/20 patients with hypertrophic cardiomyopathy (haematoxylin-eosin staining)

<table>
<thead>
<tr>
<th>Biopsy findings</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterogeneity in myocyte size</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>3 (19)</td>
</tr>
<tr>
<td>Moderate</td>
<td>6 (38)</td>
</tr>
<tr>
<td>Marked</td>
<td>7 (44)</td>
</tr>
<tr>
<td>Myocyte hypertrophy</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Mild</td>
<td>11 (69)</td>
</tr>
<tr>
<td>Moderate</td>
<td>4 (25)</td>
</tr>
<tr>
<td>Myofibre disarray</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>2 (13)</td>
</tr>
<tr>
<td>Mild</td>
<td>6 (38)</td>
</tr>
<tr>
<td>Moderate</td>
<td>6 (38)</td>
</tr>
<tr>
<td>Marked</td>
<td>2 (13)</td>
</tr>
<tr>
<td>Myocardial fibrosis</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>2 (13)</td>
</tr>
<tr>
<td>Mild</td>
<td>6 (38)</td>
</tr>
<tr>
<td>Moderate</td>
<td>6 (38)</td>
</tr>
<tr>
<td>Marked</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Inflammatory cell infiltration</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>10 (63)</td>
</tr>
<tr>
<td>Mild</td>
<td>4 (25)</td>
</tr>
<tr>
<td>Moderate</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Marked</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Intramyocardial small artery narrowing</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>12 (75)</td>
</tr>
<tr>
<td>Mild</td>
<td>2 (13)</td>
</tr>
<tr>
<td>Moderate</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Marked</td>
<td>1 (6)</td>
</tr>
</tbody>
</table>

*Eosinophilic granulocytes and mononuclear inflammatory cells.
20 control cadavers, mild fibrosis in Picrosirius staining was found in two cases.

NF-κB nuclear positivity was detected in eight of 15 patient endomyocardial biopsy samples. NF-κB nuclear activity was found in cardiomyocytes in four cases (three cases showed NF-κB positivity in 20–50% of nuclei, one case in 5% of nuclei) (figure 4). In three other cases, nuclear positivity of NF-κB was found in inflammatory cells. Endothelial NF-κB nuclear positivity was found in one case. In control cadaver myocardial specimens, no nuclear NF-κB positivity was detected.

Cytokines
Levels of hsCRP, IL-1β, IL-1RA, IL-6 and IL-10 were significantly higher in patients with HCM than in control subjects (table 3). There was a trend towards higher TNFα levels in patients with HCM compared with control subjects (p=NS).

Associations between histopathological myocardial fibrosis, LGE and inflammatory response
In patients with HCM, the degree of histopathological myocardial fibrosis significantly correlated with LGE in CMRI (r=0.568, p=0.034). The grade of myocardial inflammatory cell infiltration correlated with fibrosis in histopathological samples (r=0.614, p=0.011) and with LGE in CMRI (r=0.541, p=0.046).

Levels of hsCRP were significantly associated with histopathological myocardial fibrosis (p<0.05). All other cytokine levels tended to be higher in patients with moderate or marked histopathological findings compared with those with no or mild findings (p=NS, data not shown). Cytokine levels did not correlate significantly with NF-κB activation (data not shown).

Levels of hsCRP, TNFα and IL-1RA significantly correlated with maximal LGE in patients with HCM (table 4). There were no significant associations with IL-1β, IL-6, IL-10 and CMRI-derived maximal LGE. There were no significant associations of cytokine levels with CMRI-derived maximal LV thickness, LV mass, LV diastolic or systolic volumes, global ejection fraction, or myocardial perfusion (data not shown).

DISCUSSION
Our study demonstrates that a low-grade myocardial and general inflammatory response is present in HCM attributable to a single well-documented causative sarcomeric mutation (TPM1-D175N). Variable low-grade myocardial inflammation in the patients with HCM was indicated by the presence of myocardial inflammatory cell infiltration and enhanced nuclear NF-κB activity in the myocardium and by increased levels of hsCRP and circulating inflammatory cytokines. Myocardial inflammatory cell infiltration and levels of hsCRP significantly correlated with histopathological myocardial fibrosis and LGE in CMRI and TNFα and IL-1RA correlated with LGE, suggesting that myocardial fibrosis in HCM may be an active process modified by an inflammatory response.
ischaemia or neuroendocrinological activation, induces NF-κB upregulation in the myocardium. NF-κB invasion into the myocardium and activation of disorganised sarcomeric and cellular architecture, myocardial (arrows) in hypertrophic cardiomyopathy.

Potential pathogenic mechanism for myocardial fibrosis formation in HCM
Based on our findings we suggest a pathogenic mechanism for myocardial fibrosis formation in HCM. We propose that myocardial fibrosis in HCM is likely to be an active process, in which primary injury—for example, mechanical stress, due to disorganised sarcomeric and cellular architecture, myocardial ischaemia or neuroendocrinological activation, induces NF-κB upregulation in the myocardium. NF-κB, in turn, activates production of proinflammatory cytokines, inflammatory cell invasion into the myocardium and activation of fibroblasts, finally leading to myocardial fibrosis.

Histopathological myocardial phenotype and the causative mutation in HCM
Endomyocardial samples in our patients with HCM showed variable amounts of myocyte hypertrophy, myocyte size heterogeneity, myofibre disarray, myocardial fibrosis, low-grade inflammatory cell infiltration and intramyocardial small artery narrowing. Transgenic mice carrying a single missense mutation in codon 403 of the myosin heavy-chain gene exhibit variable amounts of histopathological hypertrophy, myocyte disarray, fibrosis and susceptibility to induced arrhythmias. In humans, only limited information on histopathological myocardial findings in patients with HCM attributable to an identified sarcomeric mutation has been published. As histopathology appears to vary to a great extent in humans and in transgenic animals with identical disease-causing mutations, other factors than the causative mutation necessarily contribute to histopathology in HCM.

Chronic myocardial inflammatory cell infiltration in HCM
Mild to marked interstitial and perivascular inflammatory cell infiltration of mononuclear inflammatory cells, showing CD5 positivity in immunostaining with rabbit anti-human antibody, and of eosinophilic granulocytes, was found in over one-third of histological endomyocardial specimens of the patients with HCM. In one patient with severe HCM, in histological endomyocardial specimens of the patients with HCM, but none of them has included chronic inflammatory cell infiltration was extensive (figure 2C). In contrast, practically no inflammatory cells were recognised in control myocardial samples. Three previous studies have reported that there is mild chronic inflammatory cell infiltration in the myocardium of patients with HCM, but none of them has included genotyped subjects.

Myocardial NF-κB activity in HCM
A new finding in our study was that immunohistochemical staining for nuclear NF-κB activity was positive in half of the endomyocardial samples of patients with HCM. NF-κB activation was detected particularly in cardiomyocytes, but also in inflammatory and endothelial cells. None of the control

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Control subjects (n = 17)</th>
<th>Patients with HCM (n = 24)</th>
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<tbody>
<tr>
<td></td>
<td>Mean±SD</td>
<td>Range</td>
</tr>
<tr>
<td>hsCRP, mg/l</td>
<td>1.29±1.50</td>
<td>0.09–5.69</td>
</tr>
<tr>
<td>TNFα, pg/ml</td>
<td>2.33±1.22</td>
<td>1.38–6.43</td>
</tr>
<tr>
<td>IL-1β, pg/ml</td>
<td>0.18±0.08</td>
<td>0.09–0.34</td>
</tr>
<tr>
<td>IL-1 RA, pg/ml</td>
<td>216±76</td>
<td>101–391</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>1.11±0.72</td>
<td>0.38–2.80</td>
</tr>
<tr>
<td>IL-10, pg/ml</td>
<td>0.86±0.55</td>
<td>0.49–2.04</td>
</tr>
</tbody>
</table>

*p<0.05; **p<0.01; ***p<0.001.
hsCRP, high-sensitivity C-reactive protein; IL-1β, interleukin 1β; IL-1 RA, interleukin 1 receptor antagonist; IL-6, interleukin 6; IL-10, interleukin 10; TNFα, tumour necrosis factor α.

Table 3 Circulating levels of cytokines in control subjects and in patients with hypertrophic cardiomyopathy

<table>
<thead>
<tr>
<th>LGE in CMRI</th>
<th>hsCRP, mg/l</th>
<th>TNFα, pg/ml</th>
<th>IL-1β, pg/ml</th>
<th>IL-1 RA, pg/ml</th>
<th>IL-6, pg/ml</th>
<th>IL-10, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.517*</td>
<td>0.486*</td>
<td>0.032</td>
<td>0.593**</td>
<td>0.267</td>
<td>0.038</td>
</tr>
</tbody>
</table>

*Analysis performed using log 10 transformed values.

Figure 3 Endomyocardial fibrosis in hypertrophic cardiomyopathy. Increased fibrosis is shown by Picrosirius Red staining (in red, asterisk). Cardiomyocytes are pale yellow stained. Original magnification ×400.

Figure 4 Nuclear NF-κB activation is detected in cardiomyocytes (arrows) in hypertrophic cardiomyopathy. ×400.
myocardial samples showed NF-κB activation. NF-κB is a pivotal intracellular mediator of inflammatory response, inducing the proinflammatory cytokine expression. NF-κB activation has been previously implicated in cardiac dysfunction and heart failure. NF-κB activation leads to proinflammatory phenotype including upregulation of TNFα, which activates inflammatory cell invasion and fibroblasts, resulting in perivascular fibrosis formation in the myocardium. Our finding of NF-κB activation in the myocardium of patients with HCM supports the concept that an inflammatory response may play an integral part in the phenotypic expression of myocardial fibrosis in HCM.

Proinflammatory and anti-inflammatory cytokines in HCM
In this study, both proinflammatory and anti-inflammatory cytokines were raised in patients with HCM. TNFα levels have been reported to be increased in HCM in some, but not in all, previous studies. IL-6 levels have been shown to be elevated in HCM in two previous studies. Decreased myocardial TNFα expression has been reported after non-surgical septal reduction in patients with obstructive HCM. There are, however, no previous studies evaluating systematically the levels of both proinflammatory and anti-inflammatory cytokines in patients with HCM and particularly, there are no studies correlating levels of cytokines with LGE and other LV characteristics in CMRI.

Clinical implications of the association of inflammatory response with myocardial fibrosis in HCM
CMR-derived LGE reflects collagenous scar formation in HCM. The notion that myocardial fibrosis is a potentially modifiable inflammatory process opens interesting clinical implications in preventing cardiovascular events in HCM, as myocardial fibrosis is a major determinant of malignant arrhythmias and end-stage systolic heart failure in HCM and consequently, increases the risk of cardiac death. In conclusion, we demonstrate that in patients with HCM attributable to an identical disease-causing mutation in the α-tropomyosin gene, myocardial inflammatory cells and enhanced NF-κB activity are present and circulating cytokine and hsCRP levels are increased. This inflammatory response is associated with myocardial fibrosis. Our findings suggest that a low-grade inflammatory response plays a significant role in the phenotypic expression of myocardial fibrosis in HCM.

Strengths and limitations of the study
We demonstrated for the first time, in a well-defined genotyped patient population with HCM, consistent signs of low-grade myocardial inflammation by serological, histopathological and immunohistochemical methods. We also showed that inflammatory response is associated with myocardial fibrosis, documented by histopathological methods and LGE in CMRI. Yet, there are some limitations to this study. First, the patient population with HCM is of limited size. Human studies including patients with genotype-verified diagnosis of HCM, especially those with a single causative mutation, are, however, few and generally small, as shown by recent studies. Second, it is possible that the findings of this study may not be applicable to all patients with HCM with different causative mutations in sarcomeric genes. However, myocardial fibrosis is a common manifestation of the HCM, LGE presenting in 80% of cases. Furthermore, according to current knowledge, no particular clinical HCM phenotype is mutation specific. Prob-ably, the findings of this study apply to HCM caused by other sarcomeric mutations as well, and confirmation of our findings in large genotyped patient populations is warranted. Third, in our CMRI method, severity and not the extent of LGE was measured. However, the method used has been regarded as scientifically valid and shown to be associated with serum amino-terminal propeptide of type III collagen. Fourth, cadaver myocardial specimens were used as controls and compared with endomyocardial biopsy findings of patients with HCM, since it is unethical to obtain endomyocardial samples from healthy controls. Fifth, there was a variable number of patient samples in different immunohistochemical analyses, because the second best endomyocardial biopsy sample designated for immunohistochemical stainings was not sufficient for all microscopic slides in every patient (see online supplementary information). Nevertheless, this study is, to our knowledge, the first human study to perform both endomyocardial biopsy and CMRI in genotyped subjects with HCM.

Key messages
- In patients with HCM attributable to an identical disease-causing mutation in the alpha-tropomyosin gene, myocardial inflammatory cells and enhanced NF-κB activity are commonly present, and circulating cytokine and hsCRP levels are increased.
- This inflammatory response is associated with histopathological and magnetic resonance derived myocardial fibrosis.
- Our findings suggest that a low grade inflammatory response plays a significant role in the phenotypic expression of myocardial fibrosis in hypertrophic cardiomyopathy.


Supplementary data: histological and immunohistochemical methods

Kuusisto J et al.: Low -grade inflammation and the phenotypic expression of myocardial fibrosis in hypertrophic cardiomyopathy

Histology

The most representative EMB biopsy sample was designated for the traditional histological staining. Endomyocardial biopsy and myocardial autopsy specimens were formalin fixed (pH 7.0), paraffin embedded, sectioned at 5µm and stained with haematoxylin and eosin, and Weigert van Gieson staining. The evaluation of histopathology was performed by an experienced pathologist (V.K.). The following histological characteristics of HCM were determined: heterogeneity of myocyte size, myocyte hypertrophy, myofiber disarray, myocardial fibrosis, inflammatory cell infiltration (mononuclear inflammatory cells, eosinophilic granulocytes and macrophages) and narrowing of intramyocardial small arteries. We used the semiquantitative evaluation of histological and immunohistochemical findings, which we have previously used to show an inflammatory response in aortic stenosis and aortic valves of necropsy subjects (1,2). The extent of these variables was graded as 0= none, 1= mild, 2= moderate, and 3=severe changes. All samples were blinded and examined twice by the same pathologist, and if a different grade was obtained, the samples were re-examined to obtain intraobserver consistency.

Immunohistochemistry

The second best EMB sample, which was not always sufficient for all analyses, was designated for immunohistochemical stainings. Each staining was performed from a different EMB microscopic slide in the patients with HCM.
To study CD3 positivity of mononuclear inflammatory cells, indicating presence of T-lymphocytes in endomyocardial samples and control cadaver samples, immunostaining was performed by an experienced pathologist (I.K.) with rabbit anti-human antibody (Sigma, 1:50 dilution) using trypsin pretreatment and avidin-biotin-HRP system (Vector Laboratories) with DAB as chromogen (Zymed).

The slides of EMB and control cadaver samples designated for antibody M755, MO814 and NCL-CD3-PS1 studies were pretreated in microwave oven in ChemMate Target Retrieval Solution (dilution 1:10, DAKO, Glostrup, Denmark) at 700W for two cycles of five minutes. Antibody M755 (DAKO, dilution 1:400) was used to label B-lymphocytes, and MO814 (DAKO, dilution 1:2000) to label macrophages. NCL-CD3-PS1 (Novocastra, Newcastle upon Tyne, UK, dilution 1:50) was used to label T-lymphocytes. The slides were stained in a Techmate 500 Plus automat (DAKO) using the labelled streptavidin biotin method. Peroxidase was the marker enzyme and it was visualized by hydrogen peroxide as a substrate and diaminobenzidine as a chromogen. Human tonsil was used as positive control in each staining batch and samples from the same series without primary antibody served as negative controls.

Myocardial fibrosis was verified in Masson’s Trichome and PicroSirius Red stained sections in endomyocardial biopsy and myocardial autopsy specimens (I.K).

To evaluate NF-κB activity, immunostaining was performed in endomyocardial samples of 15 patients with HCM and in 20 cadaver myocardial samples after pretreatment in citrate buffer in microwave oven and avidin-biotin-HRP system (Vector Laboratories) with DAB as chromogen (Zymed). Mouse anti-NF-κB, p65 subunit monoclonal antibody (Chemicon, 1:100 dilution), which
recognizes an epitope overlapping the nuclear location signal of the p65 subunit of the NF-κB heterodimer and thus selectively binds to activated form of NF-κB, was used.

References


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 semiquantitive 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 mm², 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 assoicated with ECM remodeling and increased low grade inflammation.
This is an interesting 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-κB activity in HCM”, and highlighted the role of NF-κB 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).

Ill 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 mm², 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, echocardiographic 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 beta-blockers (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-lymphocytes and B-lymphocytes in the EMB samples of the patients and control cadaver samples (the Results section under headings “Histological findings…” and “Immunohistochemical findings…”).
Low-grade Inflammation and the Phenotypic Expression of Myocardial Fibrosis in Hypertrophic Cardiomyopathy

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Short title: Hypertrophic cardiomyopathy, inflammation and fibrosis

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Words: 3011

Key words: hypertrophic cardiomyopathy, inflammation, fibrosis, genetics, magnetic resonance imaging, late gadolinium enhancement
Abstract

Objective - To investigate the role of inflammation in the phenotypic expression of myocardial fibrosis in hypertrophic cardiomyopathy (HCM).

Design - Clinical study.

Setting - Kuopio University Hospital and University of Eastern Finland, Kuopio, Finland.

Subjects: Twenty-four patients with a single HCM-causing mutation D175N in the α-tropomyosin gene and 17 control subjects.

Main outcome measures - Endomyocardial biopsy samples taken from the patients with HCM were compared to matched myocardial autopsy specimens. Levels of high-sensitivity C-reactive protein (hs-CRP) and proinflammatory cytokines were measured in patients and controls. Myocardial late gadolinium enhancement (LGE) in cardiac magnetic resonance imaging (CMRI) was detected.

Results - Endomyocardial samples in patients with HCM showed variable myocyte hypertrophy and size heterogeneity, myofiber disarray, fibrosis, inflammatory cell infiltration, and nuclear factor kappa B (NF-κB) activation. Levels of hs-CRP and interleukins (IL-1β, IL-1RA, IL-6, IL-10,) were significantly higher in patients with HCM compared to those in control subjects. In patients with HCM, there was a significant association between the degree of myocardial inflammatory cell infiltration, fibrosis in histopathological samples, and myocardial LGE in CMRI. Levels of hs-CRP were significantly associated with histopathological myocardial fibrosis. Hs-CRP, tumor necrosis factor α and IL-1RA levels had significant correlations with LGE in CMRI.

Conclusions - We demonstrated a variable myocardial and systemic inflammatory response in patients with HCM attributable to an identified sarcomeric mutation. Inflammatory response was associated with myocardial fibrosis, suggesting that myocardial fibrosis in HCM is an active process modified by inflammatory response.
Abbreviations and Acronyms

CMRI = cardiac magnetic resonance imaging

HCM = hypertrophic cardiomyopathy

hs-CRP = high-sensitivity C-reactive protein

IL-1 = interleukin-1

IL-6 = interleukin-6

IL-10 = interleukin-10

IL-1RA = interleukin-1 receptor antagonist

LGE = late gadolinium enhancement

LV = left ventricle, left ventricular

LVH = left ventricular hypertrophy

NF-κB = nuclear factor kappa B

SI = signal intensity

TPM1-D175N = mutation D175N in the α-tropomyosin gene

TNF-α = tumor necrosis factor α
Introduction

Hypertrophic cardiomyopathy (HCM), most commonly caused by mutations in sarcomeric genes, is a myocardial disease characterized by left ventricular hypertrophy (LVH), myocyte disarray and myocardial fibrosis (1). The cardiac phenotype in HCM varies not only between unrelated individuals but also between family members with an identical disease-causing sarcomeric mutation, suggesting that HCM is a complex inherited disease modified by other genetic and environmental factors (1-5). The molecular events triggered by the genotype or other factors that induce the cardiac phenotype, particularly fibrosis, remain to be determined (4-6).

Previous studies have shown that chronic inflammation decreases myocardial contractility, induces hypertrophy and promotes apoptosis and fibrosis, thus contributing to myocardial remodelling (7-9). Inflammatory cytokines, tumor necrosis factor α (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6), are not constitutively expressed in the normal heart, but are up-regulated and produced as a stress response to myocardial injury or mechanical stress (8). TNF-α and IL-6 can directly attenuate myocardial contractility and induce the development of myocyte hypertrophy, collagen deposition and fibrosis (8). IL-1β has been correlated with myocardial collagen deposition, cardiomyocyte apoptosis and inflammation (10). The expression of both inflammatory and anti-inflammatory cytokines is increased in heart failure (9). In addition, nuclear factor kappa B (NF-κB), a transcription factor regulating inflammatory genes, has been shown to be activated in patients with heart failure of various etiologies (11).

Circulating inflammatory cytokines are elevated in HCM (12-14), but none of the previous studies has investigated the association of cytokine levels with cardiac phenotype determined by cardiac
magnetic resonance imaging (CMRI). Furthermore, there are no previous studies on NF-κB activation in HCM. Even more importantly, there are little data on myocardial histopathological findings or inflammatory response in patients with HCM caused by a specific sarcomeric gene mutation. Therefore, to investigate the role of inflammation in the phenotypic expression of myocardial fibrosis in HCM, we investigated cardiac histopathology, inflammatory cytokine and hs-CRP levels, and cardiac phenotype by CMRI in patients with HCM attributable to an identical HCM-causing mutation D175N in the α-tropomyosin gene (TPM1-D175N).

Patients and Methods

The study protocol was approved by the Ethics committee of the Kuopio University Hospital. All subjects gave written informed consent. Consent to use cadaver myocardial samples was obtained from the Finnish Center for Legal Proctection in Health Care. Data of endomyocardial biopsy and cytokine findings has not been published in any form. Previously, we have published studies on CMRI derived myocardial perfusion, myocardial LGE, myocardial contractile impairment, and inducibility of life-threatening arrhythmias in this same patient population (15, 16,2,17).

Patients with HCM

Twenty-four patients from five Finnish families (11 men, 13 women; mean age, 42 ± 13 years; age range, 17–68 years) with HCM attributable to the mutation D175N in the α-tropomyosin gene (TPM1-D175N) (18,19) were included in the study. In Finland, TPM1-D175N is one of the two founder mutations causing about 11 % of all and 25 % of familial HCM cases in eastern Finland (19). Of 24 subjects with TPM1-D175N, two did not meet the diagnostic criteria for clinical HCM,
and were regarded as healthy mutation carriers. All subjects with \textit{TPM1-D175N}, however, are called HCM patients in the subsequent analysis. The study protocol described below was performed during one two-day visit at the Kuopio University Hospital.

\textbf{Control Subjects}

Seventeen healthy volunteers not related to patients with HCM and without a previous cardiac disease or medications, and with race, gender and age similar to the HCM patients, were included into our study. Study protocol was identical to that of the patients with HCM, except for cardiac catheterization and endomyocardial biopsy.

For histopathological analyses, control myocardial specimens were obtained from 20 age, gender and race matched cadavers without known cardiac disease (no history of a cardiac disease, normal macroscopic myocardial findings, and no clear evidence of a specific myocardial disease in microscopic examination).

\textbf{Clinical and Echocardiographic Evaluation}

All patients with HCM and control subjects underwent an interview, physical examination, 12-lead ECG recording, and echocardiography as previously described (2, 15-17).

\textbf{CMRI Protocol}

CMRI cine, perfusion and LGE imaging were performed in patients with HCM and controls as previously described (2, 15, 16). CMRI cine imaging was performed in all patients, perfusion imaging in 17 HCM patients, and LGE imaging in 22 HCM patients.
CMRI Image Analysis

Left ventricular (LV) characteristics and myocardial perfusion by CMRI were evaluated. LGE image analysis was performed in LV short-axis images at the levels of tips of the mitral valve leaflets and papillary muscles (16). In statistical analyses, the maximal value of the six segmental LGE heterogeneity values was used. Figure 1 shows LGE images of one control subject and three patients with HCM.

Coronary Angiography

Coronary angiography was performed on 21 of 24 HCM patients using standard angiographic techniques by the same cardiologist (J.K.).

Endomyocardial Biopsy and Autopsy Myocardial Samples

Myocardial specimen for histological analysis and immunohistochemistry were obtained from 20 patients with HCM. Endomyocardial biopsies were obtained under fluoroscopic guidance from the right ventricle side of the interventricular septum with the standard endomyocardial bioptome. Control cadaver samples were selected from the archives of the Department of Clinical Pathology of the Kuopio University Hospital. Representative myocardial routine autopsy specimens from the anterior, posterior or septal wall of the heart were taken from 20 matched cadavers.

Histological and Immunohistochemical Methods

Histological methods and immunochemistry analyses are described in detail in Supplementary information.

Laboratory Determinations of Cytokines and hs-CRP
Plasma concentrations of TNF-α, IL-6, IL-10, IL-1β and IL-1RA were measured using assay kits from R&D Systems (Minneapolis, USA). Hs-CRP was measured using an Immulite analyzer and a DPC High Sensitivity CRP assay (DPC, Los Angeles, CA) in all 24 patients with HCM and in 17 controls.

**Statistical Analysis**

Data are given as mean ± SD. Statistical analyses were performed with a statistical software package (SPSS Win 11.5., SPSS Inc., Chicago, IL, USA). Because of skewed distribution, LV mass and all cytokines were analyzed after logarithmic transformation. The differences between the patients and controls were assessed by Student’s t-test. Mann-Whitney test was used to investigate the association between endomyocardial findings and proinflammatory cytokines. Pearson’s correlation coefficients were calculated to investigate the association of cytokines with LGE in CMRI. Spearman’s correlation coefficient was used to investigate the association of different histopathological features, the association between histopathological inflammation and fibrosis, and between histopathological inflammation/fibrosis and LGE in CMRI.

**Results**

**Clinical, Echocardiographic and CMRI Characteristics**

Clinical, echocardiographic and CMRI characteristics of the patients with HCM and controls have been published before (2, 15-17) and are summarized in Table 1. Patients with HCM had stable mild to moderate symptoms (90 % of patients had NYHA functional class I-II). None of the subjects with HCM had a history or clinical symptoms or signs of decompensated heart failure, myocarditis, systemic infection, chronic inflammatory disease, or life-threatening arrhythmias.
None of the patients had intracardiac defibrillator. About one third of the patients used cardiac medication, mostly β-blocking agents. **None of the patients was taking ACE inhibitors or AT1 receptor antagonists, or medication for heart failure.**

None of the subjects with HCM had a significant LV outflow tract obstruction (>30 mmHg) at rest. In CMRI, LV maximal wall thickness was increased in HCM patients compared to controls. No difference was seen in global LV ejection fraction between patients with HCM and control subjects, but the number of hypokinetic segments was increased in patients with HCM compared to controls (2). The left ventricular perfusion reserve was lower and the maximal LV LGE increased in patients with HCM compared to controls (15, 16).

**Coronary Angiography in patients with HCM**

HCM patients had normal coronary arteries except for one patient with HCM, who had <50% stenosis in the left anterior descending coronary artery, and another patient who had <50% stenosis in the left anterior descending, intermediate and right coronary arteries.

**Histological Findings in Endomyocardial Samples**

Table 2 shows the histopathological findings in hematoxylin-eosin stained endomyocardial samples in patients with HCM. Sufficient endomyocardial biopsy samples for histology were available for 16 of 20 patients with HCM. Histological samples showed variable amounts of heterogeneity of myocyte size, myocyte hypertrophy, myofiber disarray, myocardial fibrosis, inflammatory cell infiltration and intramyocardial small artery narrowing. Interstitial and perivascular fibrosis was found in about 90% of cases. Inflammatory cell infiltration, including mainly mononuclear inflammatory cells and eosinophilic granulocytes, was found in 37 % of the
patients. Narrowed intramyocardial small arteries were found in one fourth of the cases. Figures 2 A-C show typical, mild and marked histopathological findings in patients with HCM, respectively.

In control cadaver myocardial samples, mild heterogeneity of myocyte size was found in one of 20 specimens, mild myocyte hypertrophy in 7 samples, mild myofiber disarray in 1 sample, mild interstitial fibrosis in 5 samples, mild inflammatory cell infiltration of mononuclear cells in one sample, and eosinophilic granulocytes and intramyocardial small artery narrowing in none of the samples.

**Immunohistochemical Findings in Endomyocardial Samples**

Using immunostaining with rabbit anti-human antibody, 7 of 11 (63%) patient endomyocardial biopsy samples studied showed CD3 positivity implicating T-lymphocytes (2 with moderate and 5 with weak positive staining). We could, however, replicate the CD3 positivity with NCL-CD3-PS1 in only one patient endomyocardial biopsy sample showing marked inflammatory response (Figure 2Cc). In 20 control cadaver myocardial samples, weak CD3 positivity with rabbit antihuman antibody was found in 2 samples, and CD3 positivity with NCL-CD3-PS1 in none of the samples. B-lymphocytes with M755 staining were not found in cases or controls. Occasional macrophages with MO814 were found in one patient sample only.

Picrosirius collagen staining was positive in 12 of 15 HCM samples. Extensive fibrosis was found in 2 cases (Figure 3), moderate fibrosis in 4 cases and mild fibrosis in 6 cases. In 20 control cadavers, mild fibrosis in Picrosirius staining was found in 2 cases.
NF-κB nuclear positivity was detected in 8 of 15 patient endomyocardial biopsy samples. NF-κB nuclear activity was found in cardiomyocytes in 4 cases (3 cases showed NF-κB positivity in 20-50% of nuclei, 1 case in 5% of nuclei) (Figure 4). In 3 other cases, nuclear positivity of NF-κB was found in inflammatory cells. Endothelial NF-κB nuclear positivity was found in 1 case. In control cadaver myocardial specimens, no nuclear NF-κB positivity was detected.

Cytokines

Levels of hs-CRP, IL-1β, IL-1RA, IL-6 and IL-10 were significantly higher in patients with HCM than in control subjects (Table 3). There was a trend towards higher TNF-α levels in patients with HCM compared to control subjects (P=NS).

Associations between Histopathological Myocardial Fibrosis, LGE and Inflammatory Response

In patients with HCM, the degree of histopathological myocardial fibrosis significantly correlated with LGE in CMRI (r=0.568, p=0.034). The grade of myocardial inflammatory cell infiltration correlated with fibrosis in histopathological samples (r=0.614, p=0.011), and with LGE in CMRI (r=0.541, p=0.046).

Levels of hs-CRP were significantly associated with histopathological myocardial fibrosis (p<0.05). All other cytokine levels tended to be higher in patients with moderate or marked histopathological findings compared to those with no or mild findings (P=NS, data not shown). Cytokine levels did not correlate significantly with NF-κB activation (data not shown).

Levels of hs-CRP, TNF -α and IL -1RA significantly correlated with maximal LGE in patients with HCM (Table 4). There were no significant associations with IL-1, IL-6, IL-10, and CMRI derived
maximal LGE. There were no significant associations of cytokine levels with CMRI derived maximal LV thickness, LV mass, LV diastolic or systolic volumes, global ejection fraction, or myocardial perfusion (data not shown).

Discussion

Our study demonstrates that a low grade myocardial and general inflammatory response is present in HCM attributable to a single well-documented causative sarcomeric mutation (TPM1-D175N). Variable low grade myocardial inflammation in the patients with HCM was indicated by the presence of myocardial inflammatory cell infiltration and enhanced nuclear NF-κB activity in the myocardium, and by increased levels of hs-CRP and circulating inflammatory cytokines. Myocardial inflammatory cell infiltration and levels of hs-CRP significantly correlated with histopathological myocardial fibrosis and LGE in CMRI, and TNF-α and IL-1RA correlated with LGE, suggesting that myocardial fibrosis in HCM may be an active process modified by inflammatory response.

Potential pathogenic mechanism for myocardial fibrosis formation in HCM

Based on our findings we suggest a pathogenic mechanism for myocardial fibrosis formation in HCM. We propose that myocardial fibrosis in HCM is likely to be an active process, in which primary injury, e.g. mechanical stress (20), due to disorganized sarcomeric and cellular architecture (21), myocardial ischemia (22), or neuroendocrinological activation (7) induces NF-κB up-regulation in the myocardium. NF-κB, in turn, activates production of proinflammatory cytokines, inflammatory cell invasion into the myocardium, and activation of fibroblasts finally leading to myocardial fibrosis (11, 7, 8).
Histopathological myocardial phenotype and the causative mutation in HCM

Endomyocardial samples in our patients with HCM showed variable amounts of myocyte hypertrophy, myocyte size heterogeneity, myofiber disarray, myocardial fibrosis, low grade inflammatory cell infiltration, and intramyocardial small artery narrowing. Transgenic mice carrying a single missense mutation in codon 403 of the myosin heavy chain gene exhibit variable amounts of histopathological hypertrophy, myocyte disarray, fibrosis, and susceptibility to induced arrhythmias (23). In humans, only limited information on histopathological myocardial findings in patients with HCM attributable to an identified sarcomeric mutation has been published (24). As histopathology appears to vary to a great extent as well in humans as in transgenic animals with identical disease-causing mutations, other factors than the causative mutation necessarily contribute to histopathology in HCM.

Chronic myocardial inflammatory cell infiltration in HCM

Mild to marked interstitial and perivascular inflammatory cell infiltration of mononuclear inflammatory cells, showing CD3 positivity in immunostaining with rabbit anti-human antibody, and of eosinophilic granulocytes, was found in over one third of histological endomyocardial specimens of the patients with HCM. In one patient with severe HCM inflammatory cell infiltration was extensive (Figure 2C). In contrast, practically no inflammatory cells were recognized in control myocardial samples. Three previous studies have reported that there is mild chronic inflammatory cell infiltration in the myocardium of HCM patients (3, 25, 26), but none of them has included genotyped subjects.

Myocardial NF-κB activity in HCM
A novel finding in our study was that immunohistochemical staining for nuclear NF-κB activity was positive in half of the endomyocardial samples of patients with HCM. NF-κB activation was detected particularly in cardiomyocytes, but also in inflammatory and endothelial cells. None of the control myocardial samples showed NF-κB activation. NF-κB is a pivotal intracellular mediator of inflammatory response, inducing the pro-inflammatory cytokine expression (11). NF-κB activation has been previously implicated in cardiac dysfunction and heart failure (11). NF-κB activation leads to proinflammatory phenotype including up-regulation of TNF-α, which activates inflammatory cell invasion and fibroblasts, resulting in perivascular fibrosis formation in the myocardium (27). Our finding of NF-κB activation in the myocardium of patients with HCM supports the concept that inflammatory response may play an integral part in the phenotypic expression of myocardial fibrosis in HCM.

Pro-inflammatory and anti-inflammatory cytokines in HCM

In the present study, both pro-inflammatory and anti-inflammatory cytokines were elevated in patients with HCM. TNF-α levels have been reported to be increased in HCM in some (12, 14) but not in all previous studies (13). IL-6 levels have been shown to be elevated in HCM in two previous studies (13, 14). Decreased myocardial TNF-α expression has been reported after nonsurgical septal reduction in patients with obstructive HCM (28). There are, however, no previous studies evaluating systematically the levels of both proinflammatory and anti-inflammatory cytokines in patients with HCM, and particularly, there are no studies correlating levels of cytokines with LGE and other LV characteristics in CMRI.

Clinical implications of the association of inflammatory response with myocardial fibrosis in HCM
CMR derived LGE reflects collagenous scar formation in HCM (29). The notion that myocardial fibrosis is a potentially modifiable inflammatory process opens interesting clinical implications in preventing cardiovascular events in HCM, as myocardial fibrosis is a major determinant of malignant arrhythmias and end-stage systolic heart failure in HCM, and consequently, increases the risk of cardiac death (30,5).

**Strengths and limitations of the study**

We demonstrated for the first time, in a well-defined genotyped patient population with HCM, consistent signs of low-grade myocardial inflammation by serological, histopathological and immunohistochemical methods. We also showed that inflammatory response is associated with myocardial fibrosis, documented by histopathological methods and LGE in CMRI. Yet, there are some limitations in the present study. First, the patient population with HCM is of limited size. Human studies including patients with genotype-verified diagnosis of HCM, especially those with a single causative mutation, are, however, few and generally small, as shown by recent studies (31, 32). Second, it is possible that the findings of the present study may not be applicable to all HCM patients with different causative mutations in sarcomeric genes. However, myocardial fibrosis is a common manifestation of the HCM, LGE presenting in 80% of cases (1). Furthermore, according to current knowledge, no particular clinical HCM phenotype is mutation specific (5). Probably, the findings of the present study apply to HCM caused by other sarcomeric mutations as well, but confirmation of the findings of the present study in large genotyped patient populations is warranted. Third, in our CMRI method, severity and not the extent of LGE was measured. However, the method used in the present study has been regarded as scientifically valid and shown to be associated with serum amino-terminal propeptide of type III collagen (16). Fourth, cadaver myocardial specimens were used as controls and compared to endomyocardial biopsy
findings of patients with HCM, since it is unethical to obtain endomyocardial samples from healthy controls. Fifth, there was a variable number of patient samples in different immunohistochemical analyses, because the second best endomyocardial biopsy sample designated for immunohistochemical stainings was not sufficient for all microscopic slides in every patient (see Supplementary information). Yet, the present study is, to our knowledge, the first human study to perform both endomyocardial biopsy and CMRI in genotyped subjects with HCM.

**Conclusions**

In conclusion, we demonstrate that in patients with HCM, attributable to an identical disease-causing mutation in the α-tropomyosin gene, myocardial inflammatory cells and enhanced NF-κB activity are present, and circulating cytokine and hs-CRP levels are increased. This inflammatory response is associated with myocardial fibrosis. Our findings suggest that a low grade inflammatory response plays a significant role in the phenotypic expression of myocardial fibrosis in hypertrophic cardiomyopathy.
References


Authors' contributions

All authors have contributed to the article by participating in the design (JK, VK, PS,KPe,KPu,AN,ML,IK,SY), performance of genetic studies (JK, PJ, ML), clinical and echocardiographic studies (JK,KPe), magnetic resonance studies (PS,JK), histological and immunohistochemical studies (JK,VK,AN,IK,SY), laboratory measurements (K Pu), drafting the manuscript and approving the final manuscript (all authors).

Funding

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Conflict of Interest: None declared

No additional data available.
Figure Legends

Figure 1. Contrast-enhanced T1-weighted inversion recovery images in (a) a 52-year-old control subject, (b) a 45-year-old male HCM patient, (c) 37-year-old female HCM patient, and (d) 19-year-old male HCM patient. Patients with HCM (b-d) show intramyocardial focal high signal areas (arrows) with increased segmental LGE.

Figure 2A. Typical histopathology in an endomyocardial biopsy of a patient with hypertrophic cardiomyopathy. (a) A general view, HE 200x; (b-c) moderate fiber disarray, interstitial fibrosis (stars), moderate myocyte size heterogeneity and hypertrophy, and scattered mononuclear inflammatory cells (arrows), HE 400x, 630x.  

2B. Mild histopathological findings in a patient with hypertrophic cardiomyopathy. (a) A general view, HE 200x; (b-c) mild fiber disarray, fibrosis (star), myocyte hypertrophy, and occasional mononuclear inflammatory cells (arrow), HE 400x, 630x.  

2C. Severe hypertrophic cardiomyopathy. (a) A general view: Weigert van Gieson staining highlights marked fibrosis (red, shown by star), 200x; (b) multiple mononuclear inflammatory cells (arrow); (c) showing CD 3 positivity in immunohistochemistry (staining in brown, arrow). Original magnification 400x. The patient had severe symptoms, marked LVH and inducible ventricular arrhythmia in ventricular stimulation. Intracardiac defibrillator was subsequently implanted.
Figure 3. Endomyocardial fibrosis in hypertrophic cardiomyopathy. Increased fibrosis is shown by Picrosirius Red staining (in red, star). Cardiomyocytes are pale yellow stained. Original magnification 400x.

Figure 4. Nuclear NF-κB activation is detected in cardiomyocytes (arrows) in hypertrophic cardiomyopathy. 400x.

Table 1. Clinical, echocardiographic and CMRI characteristics in control subjects and patients with hypertrophic cardiomyopathy

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control Subjects (n=17)</th>
<th>Patients with HCM (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men / Women</td>
<td>8 / 9</td>
<td>11 / 13</td>
</tr>
<tr>
<td>Age, y</td>
<td>38 ± 12</td>
<td>42 ± 13</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt;, m/sec</td>
<td>1.3 ± 0.2</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>LV magnetic resonance imaging findings</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximal wall thickness, mm</td>
<td>9.7 ± 1.7</td>
<td>19.5 ± 4.9*</td>
</tr>
<tr>
<td>Mass, g</td>
<td>123 ± 32</td>
<td>151 ± 57</td>
</tr>
<tr>
<td>End-diastolic volume, ml</td>
<td>146 ± 25</td>
<td>122 ± 45*</td>
</tr>
<tr>
<td>End-systolic volume, ml</td>
<td>56 ± 15</td>
<td>52 ± 29</td>
</tr>
<tr>
<td>Hypokinetic segments, %</td>
<td>12 ± 12</td>
<td>37 ± 20*</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>61 ± 7</td>
<td>58 ± 7</td>
</tr>
<tr>
<td>Perfusion reserve</td>
<td>1.80 ± 0.58</td>
<td>1.12 ± 0.35†</td>
</tr>
<tr>
<td>The heterogeneity of late-enhancement, %</td>
<td>12 ± 3</td>
<td>18 ± 11†</td>
</tr>
</tbody>
</table>

V<sub>max</sub> indicates maximum velocity in the Doppler signal from the jet in left ventricular outflow tract.

Data are means ± SD.

*P < 0.001; †P < 0.05.
Table 2. Endomyocardial biopsy findings in patients with hypertrophic cardiomyopathy (hematoxylin- eosin staining)

<table>
<thead>
<tr>
<th></th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heterogeneity in myocyte size</strong></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>3 (19%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>6 (38%)</td>
</tr>
<tr>
<td>Marked</td>
<td>7 (44%)</td>
</tr>
<tr>
<td><strong>Myocyte hypertrophy</strong></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1 (6%)</td>
</tr>
<tr>
<td>Mild</td>
<td>11 (69%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>4 (25%)</td>
</tr>
<tr>
<td><strong>Myofiber disarray</strong></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>2 (13%)</td>
</tr>
<tr>
<td>Mild</td>
<td>6 (38%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>6 (38%)</td>
</tr>
<tr>
<td>Marked</td>
<td>2 (13%)</td>
</tr>
<tr>
<td><strong>Myocardial fibrosis</strong></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>2 (13%)</td>
</tr>
<tr>
<td>Mild</td>
<td>6 (38%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>6 (38%)</td>
</tr>
<tr>
<td>Marked</td>
<td>2 (13%)</td>
</tr>
<tr>
<td><strong>Inflammatory cell infiltration</strong>*</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>10 (63%)</td>
</tr>
<tr>
<td>Mild</td>
<td>4 (25%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>1 (6%)</td>
</tr>
<tr>
<td>Marked</td>
<td>1 (6%)</td>
</tr>
<tr>
<td><strong>Intramyocardial small artery narrowing</strong></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>12 (75%)</td>
</tr>
<tr>
<td>Mild</td>
<td>2 (13%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>1 (6%)</td>
</tr>
<tr>
<td>Marked</td>
<td>1 (6%)</td>
</tr>
</tbody>
</table>

*Eosinophilic granulocytes and mononuclear inflammatory cells.
Table 3. Circulating levels of cytokines in control subjects and in patients with hypertrophic cardiomyopathy

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects (n=17)</th>
<th>Patients with HCM (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
</tr>
<tr>
<td>hs-CRP, mg/L</td>
<td>1.29 ± 1.50</td>
<td>0.09 - 5.69</td>
</tr>
<tr>
<td>TNF – α, pg/mL</td>
<td>2.33 ± 1.22</td>
<td>1.38 - 6.43</td>
</tr>
<tr>
<td>IL-1β, pg/mL</td>
<td>0.18 ± 0.08</td>
<td>0.09 - 0.34</td>
</tr>
<tr>
<td>IL-1RA, pg/mL</td>
<td>216 ± 76</td>
<td>101 – 391</td>
</tr>
<tr>
<td>IL – 6, pg/mL</td>
<td>1.11 ± 0.72</td>
<td>0.38 - 2.80</td>
</tr>
<tr>
<td>IL – 10, pg/mL</td>
<td>0.86 ± 0.55</td>
<td>0.49 - 2.04</td>
</tr>
</tbody>
</table>

hs-CRP, high sensitivity C-reactive protein; TNF-α, tumor necrosis factor-α; IL-1β, interleukin 1β; IL-1RA, Interleukin 1 receptor antagonist; IL-6, Interleukin 6; IL-10, Interleukin 10.

*P < 0.01; †P < 0.05; ‡P < 0.001.
TABLE 4. The association of circulating cytokine levels with myocardial maximal LGE heterogeneity at mid-ventricular level in patients with HCM (Pearson’s correlation coefficients).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>LGE in CMRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>hs-CRP, mg/L</td>
<td>0.517*</td>
</tr>
<tr>
<td>TNF – α, pg/mL</td>
<td>0.486*</td>
</tr>
<tr>
<td>IL-1β, pg/mL</td>
<td>0.032</td>
</tr>
<tr>
<td>IL-1RA, pg/mL</td>
<td>0.593 †</td>
</tr>
<tr>
<td>IL – 6, pg/mL</td>
<td>0.387</td>
</tr>
<tr>
<td>IL – 10, pg/mL</td>
<td>0.038</td>
</tr>
</tbody>
</table>

Abbreviations of cytokines as in Table 3. Analysis is performed using log 10 transformed values. *P<0.05; †P<0.01.
Supplementary data: histological and immunohistochemical methods

Kuusisto J et al.: Low-grade inflammation and the phenotypic expression of myocardial fibrosis in hypertrophic cardiomyopathy

Histology
The most representative EMB biopsy sample was designated for the traditional histological staining. Endomyocardial biopsy and myocardial autopsy specimens were formalin fixed (pH 7.0), paraffin embedded, sectioned at 5µm and stained with haematoxylin and eosin, and Weigert van Gieson staining. The evaluation of histopathology was performed by an experienced pathologist (V.K.). The following histological characteristics of HCM were determined: heterogeneity of myocyte size, myocyte hypertrophy, myofiber disarray, myocardial fibrosis, inflammatory cell infiltration (mononuclear inflammatory cells, eosinophilic granulocytes and macrophages) and narrowing of intramyocardial small arteries. We used the semiquantitative evaluation of histological and immunohistochemical findings, which we have previously used to show an inflammatory response in aortic stenosis and aortic valves of necropsy subjects (1,2). The extent of these variables was graded as 0= none, 1= mild, 2= moderate, and 3=severe changes. All samples were blinded and examined twice by the same pathologist, and if a different grade was obtained, the samples were re-examined to obtain intraobserver consistency.

Immunohistochemistry
The second best EMB sample, which was not always sufficient for all analyses, was designated for immunohistochemical stainings. Each staining was performed from a different EMB microscopic slide in the patients with HCM.
To study CD3 positivity of mononuclear inflammatory cells, indicating presence of T-lymphocytes in endomyocardial samples and control cadaver samples, immunostaining was performed by an experienced pathologist (I.K.) with rabbit anti-human antibody (Sigma, 1:50 dilution) using trypsin pretreatment and avidin-biotin-HRP system (Vector Laboratories) with DAB as chromogen (Zymed).

The slides of EMB and control cadaver samples designated for antibody M755, MO814 and NCL-CD3-PS1 studies were pretreated in microwave oven in ChemMate Target Retrieval Solution (dilution 1:10, DAKO, Glostrup, Denmark) at 700W for two cycles of five minutes. Antibody M755 (DAKO, dilution 1:400) was used to label B-lymphocytes, and MO814 (DAKO, dilution 1:2000) to label macrophages. NCL-CD3-PS1 (Novocastra, Newcastle upon Tyne, UK, dilution 1:50) was used to label T-lymphocytes. The slides were stained in a Techmate 500 Plus automat (DAKO) using the labelled streptavidin biotin method. Peroxidase was the marker enzyme and it was visualized by hydrogen peroxide as a substrate and diaminobenzidine as a chromogen. Human tonsil was used as positive control in each staining batch and samples from the same series without primary antibody served as negative controls.

Myocardial fibrosis was verified in Masson’s Trichome and PicroSirius Red stained sections in endomyocardial biopsy and myocardial autopsy specimens (I.K).

To evaluate NF-κB activity, immunostaining was performed in endomyocardial samples of 15 patients with HCM and in 20 cadaver myocardial samples after pretreatment in citrate buffer in microwave oven and avidin-biotin-HRP system (Vector Laboratories) with DAB as chromogen (Zymed). Mouse anti-NF-κB, p65 subunit monoclonal antibody (Chemicon, 1:100 dilution), which
recognizes an epitope overlapping the nuclear location signal of the p65 subunit of the NF-κB heterodimer and thus selectively binds to activated form of NF-κB, was used.

References
