Differentiation of inflammatory cardiomyopathy from non-inflammatory cardiomyopathy by circulating High Mobility Group Box 1 protein

Short title: Selejan: HMGB1 in myocarditis

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Abstract

Background: HMGB1 (High-Mobility-Group-Box-1) exacerbates inflammation in myocarditis leading to autoimmune processes and development of dilated cardiomyopathy (DCM). This study aimed to evaluate HMGB1 levels in suspected myocarditis and/or DCM to explore whether it can separate inflammatory from non-inflammatory cardiomyopathy to guide differential therapies.

Methods: In this observational study, serum samples from 151 patients with suspected myocarditis (50 patients with biopsy-proven myocarditis, 101 with DCM (among those post-myocarditis DCM (n=62), non-inflammatory/myocarditis-unrelated DCM (n=39)), 30 patients with ischemic cardiomyopathy and 50 age-matched healthy subjects were analysed for HMGB1. Immunofluorescence staining and Western blot analysis for HMGB1 were performed in myocardial samples from patients with myocarditis, post-myocarditis and non-inflammatory DCM, ischemic cardiomyopathy and healthy controls. Follow-up measurements of serum HMGB1 were performed in 18 patients with myocarditis 2 years after the initial diagnosis.

Results: HMGB1 levels (expressed as median $+25^{th}/75^{th}$ percentiles) were significantly increased in myocarditis (5616 [4092, 8660] pg/ml, $p<0.001$) and in post-myocarditis DCM (4672 [3064, 7540] pg/ml, $p=0.03$) compared to healthy controls (3484 [3206, 4104] pg/ml). Higher HMGB1 levels were associated with an advanced New York Heart Association (NYHA) functional class (7504 [5084, 12560] pg/ml) in NYHA IV versus (4056 [2912, 4976] pg/ml) in NYHA I; $p=0.005$) and NYHA II (4204 [3072, 6620] pg/ml, $p=0.009$). Increases in HMGB1 levels during follow-up were associated with a further decrease in left ventricular ejection fraction (LVEF). HMGB1 levels were similar between controls (3484 [3208, 4104] pg/ml), ischemic cardiomyopathy (3136 [2784, 4000] pg/ml) and non-inflammatory DCM (3552 [2896, 4436] pg/ml). Myocardial expression of HMGB1 was increased in inflammatory versus ischemic and non-inflammatory cardiomyopathy, and also versus nonfailing myocardium. HMGB1 showed irregular cytosolic distribution in myocytes of inflammatory cardiomyopathy.

Conclusion: Serum levels of HMGB1 are increased in myocarditis and persist in post-myocarditis DCM suggesting a putative diagnostic and therapeutic role of HMGB1 in inflammatory heart disease.

Clinical Perspective

A) What is new?

>Serum HMGB1 is elevated in patients with inflammatory cardiomyopathy (myocarditis and post-myocarditis dilated cardiomyopathy), predominantly in men, distinguishing it from non-inflammatory dilated cardiomyopathy, ischemic cardiomyopathy and healthy controls.

>Serum HMGB1 levels in inflammatory cardiomyopathy are higher in patients with more advanced heart failure symptoms and further increase in patients with ongoing deterioration of cardiac function.

>Myocardial expression of HMGB1 is likewise increased in myocarditis and post-myocarditis dilated cardiomyopathy, but not in ischemic or non-inflammatory cardiomyopathy.

>While myocytes show regular nuclear HMGB1 in ischemic cardiomyopathy, in inflammatory cardiomyopathy, myocytic HMGB1 shows atypical cytosolic redistribution.

B) What are the clinical implications?

>Serum HMGB1 could serve as a non-invasive diagnostic criterion to differentiate between inflammatory and non-inflammatory cardiomyopathy in addition to the histopathological examination of endomyocardial biopsies as a gold standard. It could also serve as a target for the development of mechanism based new therapeutic options for the different cardiomyopathies, as HMGB1 antagonism has been shown to induce beneficial effects in animal models of autoimmune myocarditis.

Introduction

The clinical presentation of viral myocarditis ranges from asymptomatic, light to fulminant courses with cardiogenic shock. The course varies from spontaneous resolution to progression to dilated cardiomyopathy (**1-3**). Different viral agents **(4-5)** and the variability of the innate immune system, contribute to the broad spectrum of clinical presentations (**6-9**) and may modify ventricular remodelling involving myocardial fibrosis and dilatation (**3, 10**). Diagnosis is currently substantiated by endomyocardial biopsy and histopathological analysis using the Dallas criteria, immunohistochemical studies, and identification of the virus by molecular biology techniques (PCR and in situ

hybridization) (**10-12**). Immunohistological signs of inflammation, absence of ß-blocker therapy and advanced New York Heart Association (NYHA) functional class, low blood pressure, high heart rate and low left ventricular function are associated with poor outcome (**3, 13-14**). However, clearly defined biological markers predicting the clinical course of myocarditis with development of chronic dilated cardiomyopathy are not evaluated. HMGB1 (High mobility group box 1 protein) (**15**) is a ubiquitously expressed, non-histone nuclear protein, which can be secreted by cells and functions as a cytokine, activating pathogen and pattern recognizing receptors (PRRs) like Toll-like receptors (TLRs) and RAGE (Receptor for Advanced Glycation End products) boostering immune responses. HMGB1 has been shown to be an important mediator of inflammation and fibrosis in murine models of experimental autoimmune myocarditis (**16**), leading to autoimmune processes and development of dilated cardiomyopathy. In turn, HMGB1 blockade ameliorates myocardial pathophysiologic changes in experimental autoimmune myocarditis (**16-17**). The role of circulating serum HMGB1 in patients with myocarditis and patients with dilated cardiomyopathy (DCM) is still unknown. We investigated whether serum HMGB1 can be used to differentiate between histologically proven inflammatory, myocarditis-induced DCM and non-inflammatory DCM.

Materials and Methods

Data, analytic methods, and available study materials can be shared with other researchers for purposes of reproducing the results or replicating the procedure.

Study population

Patient recruitment and diagnostic procedures were performed in two cooperating heart centers (University Hospital of Saarland, University Hospital of Tübingen) and have been published in part (**1**, **13**). 151 consecutive patients underwent endomyocardial biopsy due to clinically suspected myocarditis (Figure 1) between 2000 and 2012. Myocarditis was suspected when patients experienced episodes of a febrile infection within the last 6 months and at least one of the following features not related to myocardial ischemia: impaired global or regional left ventricular systolic function, increase of myocardial necrosis markers, pericardial effusion, or sustained or nonsustained ventricular tachycardia or ventricular fibrillation of unknown origin. Coronary angiography was performed in all patients before biopsy, and patients with significant coronary artery disease were excluded from the study. Patients with overt cardiogenic shock requiring vasopressors at initial presentation, and also patients with relevant valvular

disease, were not included. Further exclusion criteria were sepsis, immunoincompetency or immunosuppressive therapy, autoimmune diseases, blood transfusion during the last 8 weeks, collagen diseases, advanced liver disease, malignancy, and operations during the last 6 months. All patients underwent a careful history and physical examination as well as selected laboratory studies. Left ventricular ejection fraction, left ventricular end-diastolic and end-systolic diameters were measured with 2-dimensionally guided M-mode echocardiography or cardiac MRI in case of poor echocardiographic conditions. A twelve-lead ECG was performed after 10 min of supine rest in all patients immediately after hospital admission. The heart rate was taken from that initial ECG.

Endomyocardial biopsies were investigated by histopathological analysis (Dallas criteria), immunohistochemistry, and molecular detection of viral genomes as described before (**1**). Viral genomes regularly investigated by PCR/RT-PCR were enteroviruses species (comprising coxsackieviruses and echoviruses), parvovirus B19, adenoviruses, Epstein-Barr virus, human herpesviruses type 6 and 7 and cytomegalovirus (CMV). Influenza viruses were investigated according to their seasonal patterns (80% of all patients with clinically suspected myocarditis assessed). The diagnosis of acute myocarditis requires the presence of myocyte necrosis in addition to inflammation (detection of T lymphocytes, macrophages and expression of HLA class II molecules), whereas chronic myocarditis was diagnosed, if inflammation was detected without ongoing myocyte necrosis (**1, 12**, **18**). If endomyocardial biopsy revealed no significant myocardial inflammation, post-myocarditis dilated cardiomyopathy (DCM after healed myocarditis) was diagnosed in patients with a clinical history of myocarditis and, according to the histopathological report, histopathological post-myocarditis signs of chronic myocardial damage with patchy interstitial fibrosis with or without detection of viral genomes. If biopsy revealed neither signs of ongoing myocarditis nor signs of previous myocarditis related inflammation with corresponding fibrosis pattern nor viral detection, and if medical history or further molecular-biological investigations delivered a high-probability myocarditis-unrelated reason for DCM development (e.g. mitochondriopathy), then non-inflammatory DCM was diagnosed (DCM of other origin, i.e. myocarditis unrelated).

In summary, if no exclusion criteria applied, diagnosis was based upon clinical scenario and pathology examination and the particular phenotypic classifications (diagnoses) were made on the following criteria:

142 >**Acute myocarditis**: Detection of myocyte necrosis plus inflammatory infiltrates (>14 leukocytes per 1 mm2 (CD3+
143 T lymphocytes and/or CD68+ macrophages) in the myocardium plus enhanced expression of HLA class II molecules)
144 with or without detection of viral genomes.

145 >**Chronic myocarditis**: No myocyte necrosis, but detection of inflammatory infiltrates (>14 leukocytes per 1 mm2
146 (CD3+ T lymphocytes and/or CD68+ macrophages) in the myocardium plus enhanced expression of HLA class II
147 molecules), with or without beginning fibrosis or detection of viral genomes.

148 >**DCM post-myocarditis**: No myocyte necrosis, no inflammatory infiltrates, variations in myocyte size, detection of
149 fibrosis patterns matching post-myocarditis chronic myocardial damage (i.e. focal/patchy and diffuse interstitial
150 fibrosis and/or perivascular myocardial fibrosis), with or without detection of viral genomes, plus clinical history of
151 myocarditis and lack of other reasons for DCM.

152 >**Non-inflammatory DCM**: No myocyte necrosis or inflammatory infiltrates, but variations in myocyte size and
153 diffuse rather than focal fibrosis, no detection of viral genomes (detection of viral genomes was a relative
154 counter-criterion for grouping into the non-inflammatory DCM group), plus high probability for myocarditis unrelated
155 cause (like chemotherapy induced, long-lasting therapy refractory arterial hypertension, alcohol abuse, Takotsubo
156 cardiomyopathy, peripartum cardiomyopathy, mitochondriopathy).

157

158 Serum samples were collected at the time of endomyocardial biopsy and were stored at -80°C until further analysis.
159 Follow-up blood samples were obtained only in one center in the heart failure outpatient clinic (University Hospital of
160 Saarland) after a mean average time of 2 years from 18 of the patients with biopsy proven active myocarditis (all
161 chronic myocarditis). All patients with signs or symptoms of heart failure received evidence-based medical treatment
162 (Table 1). Serum samples from 30 consecutive patients with established ischemic cardiomyopathy (ICM) recruited
163 between 2000 and 2012 and 50 age-matched healthy volunteers recruited 2012-2013 without a history of any disease
164 served as control groups.
165 Additionally, ventricular myocardium was obtained between 1991 and 2000 during heart transplantations from 5
166 patients with ischemic end-stage heart failure, NYHA IV and LV ejection fraction <35% (ICM n=5, all men, age 52-58
167 years). Cardiac medication included ACE inhibitors, ß-blockers, diuretics, digitalis and nitrates. Nonfailing
168 myocardium (no pathology on echocardiography) was obtained from 5 organ donors whose hearts could not be used

for transplantation after all (NF=5, all men, age 44-54 years, no cardiac medication). All explanted hearts were collected during the transplantations and quickly either snap frozen in liquid nitrogen for protein analysis or asserved in formaline.

Patients or substitute decision-makers gave written informed consent to include their data in the study. The study was approved by the local ethics committee (Nr. 122/09) and complies with the Declaration of Helsinki.

Materials

See online supplement.

ELISA

HMGB1 protein in serum samples was quantitatively determined by a human HMGB1 ELISA kit (Biomatik, CatNo EKF 57253) according to the manufacturer's instructions.

Western blot

The protocols are described in detail in the online-only data supplement.

Immunofluorescence analysis

To detect HMGB1 expression, HMGB1 co-immunostainings on 3 µm paraffin sections of the left ventricle were performed using heat-mediated antigen retrieval with 0,05% citraconic anhydride solution followed by overnight incubation at 4 °C with the 1:100 diluted primary antibodies anti-HMGB1 (Abcam; ab18256) and anti the myocyte marker α-sarcomeric actin (Sigma Aldrich, A2172, mouse monoclonal) and incubation with the appropriate secondary antibody at 37 °C for 1 h (dilution 1:30). TRITC-conjugated anti-rabbit IgG (Dianova, Germany) and FITC-conjugated anti-mouse IgM (Dianova, Germany) were used as secondary antibodies. 4xSSC buffer with 0,01% Tween was used for wash steps and for antibody dilution. Sections were counterstained with DAPI (Calbiochem, Germany) and mounted with fluorescent mounting medium (Vectashield, Vector Laboratories, USA) for fluorescence microscopic analysis. All sections were evaluated using a Nikon E600 epifluorescence microscope (Nikon, Germany) with appropriate filters at 100x and 400x magnification.

198 **Statistical analyses**

199 Statistical analyses were performed using Graph Pad Prism (version 5.0; GraphPad Software, San Diego California,
200 USA). Normal distribution of data was tested by Kolmogorov-Smirnov and Lilliefors test. Most data are not normally
201 distributed and are expressed as median and $25^{th}/75^{th}$ percentiles (continuous variables). Categorical variables are
202 presented as frequencies and percentages and were compared using the two-sided Fisher´s exact test. For comparisons
203 of continuous variables between patients with proven myocarditis, patients with dilated cardiomyopathy, ischemic
204 cardiomyopathy and healthy controls, Kruskal-Wallis-ANOVA with Dunn´s post-hoc analysis and correction for
205 multiple comparisons was applied. Non-parametric Mann-Whitney and t-test with Welch´s correction was used for
206 comparisons between two non-paired groups, if applicable. Analysis of follow-up HMGB1 measurements was
207 performed by Wilcoxon matched pairs test. Correlation analyses were performed by Spearman correlation. Statistical
208 significance was defined as a p-value of <0.05.
209

210 # Results

211

212 **Patient population**

213 Table 1 depicts the baseline characteristics of the patient population. Patients with non-inflammatory DCM had larger
214 ventricles than patients with myocarditis (LVEDDi median [25^{th}, 75^{th} percentiles]: 38 [34, 41] mm/m in
215 non-inflammatory DCM versus 32 [29, 37] mm/m in myocarditis, $p<0.001$), however left ventricular ejection fraction
216 (LVEF) was similarly low in the myocarditis and DCM groups (Table 1). Heart rate and body mass index were similar
217 between the groups (Table 1). Arterial hypertension was more prevalent in the ICM group than in the myocarditis
218 group. Also, the percentage of patients with diabetes mellitus was significantly higher in the ICM group than in the
219 other groups (Table 1). Leukocyte numbers in the blood, C-reactive protein levels and high-sensitivity Troponin T
220 levels, creatinine and liver enzymes did not differ significantly between the myocarditis and dilated cardiomyopathy
221 groups (Table 2).

222

223 **Endomyocardial biopsy results**

224 Immunohistologically, 50 patients (33%) revealed significant inflammatory cellular infiltrates, in addition to enhanced
225 expression of HLA class II molecules, indicating active myocarditis (Table 1, Figure 1). Six of those patients (12% of
226 all myocarditis patients) revealed additional myocyte necrosis corresponding to acute myocarditis, 44 patients were

diagnosed with chronic myocarditis. 62 patients (41%) showed the phenotype of post-myocarditis DCM (i.e. no signs of active myocarditis, but chronic myocardial damage with patchy and diffuse and/or perivascular myocardial fibrosis or wall thickened small arterioles after PVB19 myocarditis). 39 patients (26%) had dilated cardiomyopathy of other origin (not myocarditis related, non-inflammatory DCM including hypertension induced DCM, alcohol abuse related, chemotherapy induced cardiotoxicity, Takotsubo cardiomyopathy, peripartum cardiomyopathy, mitochondriopathy and DCM of unknown origin). Viral genome was detected in endomyocardial biopsies of 49 individuals (32 %). Viral genomes were detected in 24 subjects with post-myocarditis DCM (39 % viral persistence) and only in 1 patient with non-inflammatory DCM (1 patient with detection of PVB19 genome with low DNA copies, who was assigned to the "non-inflammatory DCM" group because of confirmed familial dilated cardiomyopathy as the most probable cause for DCM development in that case). In the myocarditis group, viral genome was detected in the heart of 24 patients (48% viral persistence).

Serum HMGB1 is increased in myocarditis and inflammatory dilated cardiomyopathy

HMGB1 serum levels were increased in patients with biopsy-proven myocarditis (Table 2; Figure 2; $p<0.001$ versus healthy controls, versus ICM controls and versus myocarditis unrelated, non-inflammatory DCM). Patients with post-myocarditis DCM also showed increased HMGB1 serum levels without significant difference to the myocarditis group (Table 2, Figure 2). Patients with acute myocarditis (n=6), were all males. Men with acute myocarditis showed significantly higher HMGB1 levels (8936 [8320, 10448] pg/ml) than men with chronic myocarditis (4692 [3956, 8624] pg/ml, $p=0.04$) (Supplemental Figure 1A). There were no female cases of acute myocarditis in our study group. While there were no significant differences in HMGB1 levels between the sexes in most individual subgroups as a whole and irrespective of age (control groups, the myocarditis group (chronic myocarditis), non-inflammatory DCM), women with post-myocarditis DCM displayed significantly lower HMGB1 levels than men (Supplemental Figure1 B-G).

Men with viral persistence in post-myocarditis DCM showed higher HMGB1 levels than those without viral persistence (Supplemental Figure 2E; $p=0.04$; 7432 [5368, 11544] in virus+ men versus 4280 [3032, 7384] in virus- men). Also, men with post-myocarditis DCM and viral persistence had significantly higher HMGB1 serum levels than women with documented viral persistence and post-myocarditis DCM (Supplemental Figure 3J; 7432 [5368, 11544] versus 3236 [2580, 4472], $p=0.001$). However, we observed no significant differences in HMGB1 levels between the detected viral entities (Supplemental Figure 4).

Serum HMGB1-levels correlated inversely with age in all men (Supplemental Figure 5A), in men with myocarditis (Supplemental Figure 5 B, n=34, r=-0.45, p=0.007) and post-myocarditis DCM (Supplemental Figure 5E; n=38, r=-0.36, p=0.03), but not in non-inflammatory DCM, ICM or healthy controls (Supplemental Figure 5G,I,K). In women, there was only in post-myocarditis DCM a significant correlation between age and HMGB1, and, contrary to male subjects, HMGB1 correlated positively with age in women (Supplemental Figure 5 B, D, F, H, J, L). When using the age of 50 years as a cut-off by unknown post-menopausal status (19), HMGB1 serum levels were significantly increased in men with chronic myocarditis as compared with women aged $\leq$ 50 years, so were HMGB1 concentrations in men with post-myocarditis DCM as compared to post-myocarditis women aged $\leq$ 50 years (Supplemental Figure 8).

In contrast to myocarditis and post-myocarditis DCM, serum HMGB1 was low in non-inflammatory DCM as compared to healthy controls. Also, patients with ischemic cardiomyopathy showed similar HMGB1 levels as seen in healthy controls (Table 2; Figure 2). However, in contrast to the male population (Supplemental Figure 6A), there were almost no significant differences between HMGB1 levels in the female population between the distinct groups (Supplemental Figure 6B).

Serum HMGB1 correlates with parameters of cardiac function

Patients in NHYA I and II showed significantly lower HMGB1 serum levels compared to patients in NYHA IV (Figure 3), the association being particularly strong in men (Supplemental Figure 7A), but without statistical significance in women (Supplemental Figure 7B). Additionally, in follow-up measurements, a serum HMGB1 decrease in patients with myocarditis was associated with improved left ventricular ejection fraction (Figure 4 A), whereas further increases in HMGB1 levels were associated with deteriorations of left ventricular ejection fraction (Figure 4 B). The changes in HMGB1 levels (Delta HMGB1) showed an inverse correlation with changes in ejection fraction (Delta EF) (Figure 4C).

Myocardial HMGB1 is increased in myocarditis and post-myocarditis dilated cardiomyopathy

Immunofluorescence staining for HMGB1 in endomyocardial biopsies revealed increased cytoplasmic staining for HMGB1 in myocytes in myocarditis and post-myocarditis DCM, while there was only regular nuclear staining for HMGB1 in ischemic cardiomyopathy (Figure 5A). Western blot analysis for HMGB1 (Figure 5B) in myocardial

biopsies confirmed an increased HMGB1 content in myocarditis and post-myocarditis DCM as compared with non-inflammatory DCM, ICM and non-failing myocardium (explanted heart samples for the latter two).

Discussion

We demonstrate increased serum HMGB1 levels only in patients with myocarditis and post-myocarditis DCM, thus distinguishing inflammatory cardiomyopathy from non-inflammatory and ischemic cardiomyopathy and from healthy controls.

In a mouse model of TnI-induced experimental autoimmune myocarditis, myocardial and systemic HMGB1 protein expressions were shown to be elevated **(16)**. In patients with myocarditis, myocardial and systemic HMGB1 expressions were increased **(16)**. Our results extend these findings, demonstrating that HMGB1 serum and myocardial levels remain increased in DCM after healed myocarditis, indicating ongoing myocardial injury.

HMGB1 inhibition with blocking antibodies (**17**) or the HMGB1 antagonist Glycyrrhizin (**16**) effectively reduced immune cell infiltration, myocyte necrosis and resulting fibrosis in experimental autoimmune myocarditis. Regulatory T-cells and a particular subset of T-helper cells (Th17-cells) are supposed to play a major role in myocarditis and its transition to dilated cardiomyopathy (**20-22**). HMGB1-blockade has been shown to suppress Th17-cell expansion and to attenuate experimental autoimmune myocarditis (**17**). Whether those positive effects of HMGB1 blockade can be extrapolated to myocarditis patients and whether HMGB1 blockade would be sufficient to stop myocardial injury, remains to be established.

Our results are in line with previous reports about men being diagnosed with myocarditis more often than women (**23**). Only ca. 30% of our patients with inflammatory cardiomyopathy were women. Men also seem to have a worse prognosis than women due to a more pronounced inflammatory pro-fibrotic response. Animal studies showed that females present a stronger M2-type activation pattern in heart infiltrating macrophages, which blunts inflammation and promotes cardiac healing, the opposite to the pro-inflammatory M1 activation pattern predominantly found in male mice (**23**). This is particularly interesting, as HMGB1 was described to facilitate macrophage reprogramming towards a pro-inflammatory M1-phenotype in experimental autoimmune myocarditis (**24**). We observed significantly higher

HMGB1 levels in young men with myocarditis versus women with myocarditis ≤50 years, and also in post-myocarditis DCM respectively. As menopause status was not obtained, we used an age of 50 years as a cut-off to assess the effect of aging on both men and women as applied in a recent study (**19**). Still, in most of our study subgroups (non-inflammatory DCM, ICM and healthy controls), we could not detect a significant difference in HMGB1 levels between men and women, not even when adjusting for age ≤50 years. We observed e.g. no difference in HMGB1 levels with regard to sexes in healthy individuals. Data between the sexes might have not reached significance because of the relatively low number of female subjects per group. It is also e.g. possible, that women who develop symptoms of myocarditis, and are thus diagnosed, are already preselected by still unknown molecular biological factors to be more sensitive to the development of myocarditis and its sequelae than the rest of the female population. Or maybe the different HMGB1 regulation only becomes apparent in infection. We observed no difference in HMGB1 levels in virus-negative patients with regard to sexes. Viral persistence seems to be associated with increased HMGB1 levels in men with post-myocarditis DCM as compared to men with virus-negative DCM, and also as compared to virus-positive women with post-myocarditis DCM. Also, the cross-over analysis of HMGB1-serum-levels between inflammatory and non-inflammatory cardiomyopathies rendered only in our male study population statistically significant results. This could indicate that HMGB1 plays a greater role in male myocarditis. Men have an increased incidence of myocarditis, dilated cardiomyopathy and heart failure and HMGB1 could play a hidden role in these pathological processes. HMGB1 serum levels also correlated inversely with age in our male subjects and it is known that young men are more susceptible to myocarditis than young women (**25**). Future investigations in animal models are warranted to further clarify a potential mechanistic role of HMGB1 in viral and autoimmune myocarditis and also in post-myocarditis DCM between the sexes.

In our study, increasing HMGB1 serum levels at a 2-year follow-up were associated with further deterioration of LV-EF in myocarditis. Of note, the number of followed-up female patients was far too low to enable any conclusions with regard to sex differences. Besides the well-characterized inflammatory and pro-fibrotic role of HMGB1 in experimental myocarditis **(15, 17)**, negative inotropic effects of HMGB1 were demonstrated in isolated contracting cardiac myocytes, which might contribute to the deterioration of cardiac performance (**21**). HMGB1 activates Pattern Recognizing Receptors (PRRs), and it has been shown that PRRs like RAGE and TLR4 levels in endomyocardial biopsies were higher in myocarditis and DCM patients than in controls (**16, 26**). Activation of PRRs by ligands like HMGB1 appears to be associated with enhanced production of IL-1ß and IL-18 and increased viral replication in the

heart **(27-28).** We observed higher HMGB1 levels in men with virus-positive post-myocarditis DCM than in virus-negative ones, but we saw no difference between individual viruses in this respect. Differences in the role of HMGB1 in certain viral entities, low numbers of subjects for the specific viral entities and different viral loads might account for this. Because HMGB1 can also bind other cytokines, such as IL-1ß, the inflammatory response can also be mediated through the receptors of partner molecules (**29-30**). Recently, myocardial TLR4 expression was suggested as a potential biomarker to predict the response to immunosuppressive therapy in patients with chronic inflammatory cardiomyopathy (**31**), which might improve prognosis (**32-33**). Here, we show for the first time that pro-inflammatory circulating TLR4 ligand HMGB1 is increased in patients with myocarditis and remains upregulated in post-myocarditis DCM, especially in men. Detection of increased HMGB1 serum levels in myocarditis as well as in post-myocarditis DCM might indicate persistent myocardial injury even at the level of so-called "post-myocarditis" DCM without specific histological signs of ongoing myocarditis. In agreement with this concept, follow-up cardiac magnetic resonance imaging in patients with myocarditis and LGE (late gadolinium enhancement) (**34**) indicated progressive remodelling in spite of normalization of cardiac enzymes and classical inflammatory parameters in the circulation (**34-35**). The time course of HMGB1 concentrations might be helpful in identifying patients at risk of developing dilated cardiomyopathy. Further investigations in larger patient cohorts and even preclinical models are needed to prove this hypothesis. In our study population, a decrease in HMGB1 serum concentration in follow-up measurements was associated with improved left ventricular ejection fraction and HMGB1 changes correlated significantly with EF changes.

The cellular origin of the measured serum HMGB1 is not clear and might even differ between the patient groups. Immune cells are widely known to secrete HMGB1 after activation. Interestingly, in this study, immunofluorescent staining for HMGB1 in myocardial samples revealed a strong cytoplasmic staining in myocytes from patients with both myocarditis and myocarditis related DCM, implying secretion of HMGB1 also from cardiomyocytes. In comparison to the difference in HMGB1 serum levels between myocarditis/post-myocarditis DCM and healthy controls, HMGB1 serum levels were not increased in ischemic cardiomyopathy and we observed only regular nuclear immunofluorescent staining for HMGB1 in myocardial control samples of ischemic cardiomyopathy. Western blot analysis of myocardial biopsies and myocardial samples of explanted hearts confirmed an increased expression of HMGB1 in myocarditis and myocarditis-related DCM as compared with non-inflammatory DCM, ICM and healthy myocardium.

This findings are particularly intriguing, because HMGB1 is also known to be an inducer of inflammasome formation promoting cell death (**36)**, and intracellular aggregates of inflammasome components indicative of inflammasome formation have also been described in cardiomyocytes in heart samples of acute myocarditis post-mortem cases (**37**). Intensity of inflammasome formation correlated with greater severity of heart failure at presentation and a higher NYHA level (**37**), which is consistent with our results. The role of HMGB1 and the inflammasome in the continuum of myocarditis and dilated cardiomyopathy warrants further pre-clinical investigations in the search for new potential therapeutic strategies in myocarditis, myocarditis-induced dilated cardiomyopathy and even long-term complications of present day established treatments for conditions like cardiac allograft rejection, where the HMGB1-inflammasome axis has also been shown to be involved in (**38-39**). HMGB1 serum levels might prove to be, at least in men, a useful non-invasive marker for diagnostic purposes helping to better distinguish between inflammatory and non-inflammatory cardiomyopathy.

Limitations

This study has some limitations. It is a two centers study from one country involving a limited number of subjects. This hampers the generalizability of our results and requires further large-scale studies, involving different regional areas and races. The number of women with myocarditis and DCM was low, which makes it difficult to make statements about the role and regulation of HMGB1 in female myocarditis. Also, we cannot determine the source of HMGB1 in any of the pathophysiologic settings.

In conclusion, persistently high HMGB1 serum levels are associated with cardiac dysfunction in human myocarditis and a higher risk for development of post-myocarditis DCM. It is concluded that, at least in men, HMGB1 could be a marker for myocarditis and myocarditis-induced DCM and might help design new diagnostic and therapeutic tools in those specific pathological settings.

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Figure legends

Figure 1

Consolidated scheme of reported clinical trial.

DCM: dilated cardiomyopathy; ICM: ischemic cardiomyopathy; CAD: coronary artery disease.

[a] Exclusion criteria: coronary artery disease, relevant valvular disease, systemic autoimmune disease, cardiogenic shock, sepsis, blood transfusion during the last 8 weeks, collagen diseases, advanced liver disease, malignancy, immunosuppressive therapy or immunoincompetency.

[b] Exclusion criteria: Acute or recent myocardial infarction or any operations within the last 6 months, relevant valvular disease, cardiogenic shock, sepsis, blood transfusion during the last 8 weeks, collagen diseases, advanced liver disease, malignancy, immunosuppressive therapy or immunoincompetency, systemic autoimmune disease.

* The diagnosis was based upon clinical scenario and pathology examination. The particular phenotypic classifications (diagnoses) were made on the following criteria:

>**Acute myocarditis**: Detection of myocyte necrosis plus inflammatory infiltrates (14 leukocytes per 1 mm2 (CD3+ T lymphocytes and/or CD68+ macrophages) in the myocardium, in addition to enhanced expression of HLA class II molecules) with or without detection of viral genomes

>**Chronic myocarditis**: No myocyte necrosis, but detection of inflammatory infiltrates (14 leukocytes per 1 mm2 (CD3+ T lymphocytes and/or CD68+ macrophages) in the myocardium, in addition to enhanced expression of HLA class II molecules) with or without detection of viral genomes or beginning fibrosis

>**DCM post-myocarditis**: No myocyte necrosis, no relevant inflammatory infiltrates, variations in myocyte size, detection of fibrosis with typical fibrosis pattern matching post-myocarditis signs of chronic myocardial damage (i.e.

focal, patchy and diffuse interstitial fibrosis and/or perivascular myocardial fibrosis), with or without detection of viral genomes, plus clinical history of myocarditis, plus individual lack of other plausible reasons for development of DCM.

>**Non-inflammatory DCM**: No myocyte necrosis, no inflammatory infiltrates, variations in myocyte size and diffuse rather than focal fibrosis, no detection of viral genomes (detection of viral genomes was a relative counter-criterion for grouping into the non-inflammatory DCM group), plus high probability for myocarditis unrelated origin according to medical history or previous diagnostics (f.e. chemotherapy induced, long-lasting therapy refractory arterial hypertension, alcohol abuse, Takotsubo cardiomyopathy, peripartum cardiomyopathy, mitochondriopathy).

Figure 2

Quantification of HMGB1 in serum samples from patients with myocarditis (n=50), patients with post-myocarditis DCM (n=62), patients with non-inflammatory DCM (n=39), ischemic cardiomyopathy controls (n=30) and healthy controls (n=50) by ELISA. HMGB1 in pg/ml serum. n.s.= not significant. Box plots indicate median, interquartile range and total range.

Figure 3

Association of serum HMGB1 content and NYHA-functional classes in patients with myocarditis and dilated cardiomyopathy (NYHA I n=17; NYHA II n=71; NYHA III n=48; NYHA IV n=15). All patients were summarized (myocarditis, post-myocarditis and non-inflammatory DCM). HMGB1 in pg/ml serum. n.s.= not significant. Box plots indicate median, interquartile range and total range.

The proportion of individual phenotypes in the NYHA groups the male/female ratio was as follows:

>**NYHA I**:
myocarditis 47% (n=8, 4 females), post-myocarditis DCM 32,4% (n=4, 2 females),
non-inflammatory DCM 29,5% (n=5, no females).
>**NYHA II**:
myocarditis 32,4% (n=23, 9 females), post-myocarditis DCM 38% (n=27, 11 females),
non-inflammatory DCM 29,6% (n=21, 8 females).
>**NYHA III:**
myocarditis 29,2% (n=14, 3 females), post-myocarditis DCM 45,8% (n=22, 8 females),
non-inflammatory DCM 25% (n=12, 4 females).
>**NYHA IV**:
myocarditis 33% (n=5, no females), post-myocarditis DCM 60% (n=9, 4 females),
non-inflammatory DCM 7% (n=1, no female).

Figure 4

2 years follow-up measurements in myocarditis (n=18, including 5 women): A) Serum HMGB1 with regard to the development of LV ejection fraction (LVEF). n=11 (including 3 women) for improvement of left ventricular ejection fraction (defined as a LVEF increase >10 percentage points); n=7 (including 2 women) for deterioration of left ventricular ejection fraction (defined as a LVEF decrease or increase <10 percentage points). p-value as determined by Wilcoxon matched pairs test. Box plots indicate median, interquartile range and total range. m/f (male/female ratio). B) Scatter plot between Delta EF and Delta HMGB1 from baseline to 2-years follow-up (Spearman correlation, p-value). EF deterioration: red squares; EF improvement: green circles.

Figure 5

A) Representative fluorescence microscopy for co-immunostaining for HMGB1 (red), the myocyte marker α-sarcomeric actin (green) and nuclei (stained blue by DAPI) in endomyocardial biopsies of patients with myocarditis (left panel), post-myocarditis DCM (middle panel), ischemic cardiomyopathy (right panel). Bars =10 µm.

B) Western blot analysis in myocardial biopsies (n=23: n=9 myocarditis, n=9 post-myocarditis DCM, n=5 non-inflammatory DCM) and explanted hearts (healthy controls n=5; ICM n=5).

Table 1: Baseline basic characteristics

	Healthy controls n=50	Myocar-ditis n=50	Post-myo-carditis DCM n=62	Non-Inflam-matory DCM n=39	Ischemic Cardio-myopathy ICM n=30	Healthy controls versus				Myocarditis versus			ICM versus		Post-Myo-carditis versus non-inflam-matory DCM
						Myo-car-ditis	post-myo-car-ditis DCM	non-inflam-matory DCM	ICM	post-myocar-ditis DCM	Non-inflam-matory DCM	ICM	post-myocar-ditis DCM	non-inflam-matory DCM	
Characteristic						**p-value**									
Age (years)	57 [39/ 70]	49 [34/60]	56.5 [45/68]	60 [51/70]	65.5 [52/71]	1.0	0.99	0.99	0.47	0.13	**0.019**	**<0.001**	0.35	0.99	0.99
Men (n,%)	32 (64%)	33 (66 %)	44 (71%)	26 (67%)	20(67%)	1.0	0.54	0.83	1.0	0.68	1.0	1.0	0.81	1.0	0.67
Arterial Hypertension (n%)	---	15(30%)	33(53%)	22(56%)	22(73%)	---	---	---	---	**0.02**	**0.02**	**<0.001**	0.07	0.21	0.84
Diabetes Mellitus (n%)	---	5(10%)	7(11%)	7(18%)	13(43%)	---	---	---	---	1.0	0.35	**<0.001**	**<0.001**	**0.03**	0.38
BMI	---	28(26/30)	27(25/30)	30(26/33)	28(25/32)	---	---	---	---	0.99	0.71	0.99	0.99	0.88	0.11
NYHA functional class (n,%)															
I	---	8 (16 %)	4 (6.5%)	5 (12%)	2 (6.7%)	---	---	---	---	0.13	0.77	0.31	1.0	0.69	0.48
II	---	23 (46 %)	27 (43%)	21 (54%)	8 (26.7%)	---	---	---	---	0.85	0.4	0.1	0.17	**0.02**	0.23
III	---	14 (28 %)	22 (35%)	12 (29%)	14 (46.7%)	---	---	---	---	0.42	1.0	0.1	0.36	0.15	0.53
IV	---	5 (10%)	9 (14.5%)	1 (2.4%)	6 (20%)	---	---	---	---	0.57	0.22	0.31	0.55	**0.037**	**0.048**
LV end-diastolic dimension index,mm/m	---	32 [29/37]	36 [32/39]	38 [34/41]	38 [35/41]	---	---	---	---	0.17	**<0.001**	**0.002**	0.36	0.99	0.27
LV ejection fraction [%]	---	41 [25/59]	30 [20/50]	39 [30/48]	28 [22/38]	---	---	---	---	0.35	0.99	**0.047**	0.99	0.12	0.75
Heart rate bpm	---	75[70/82]	77[72/82]	79[74/86]	73[69/79]	---	---	---	---	0.99	0.73	0.99	0.75	0.09	0.99
Endomyocardial biopsy results															
Immunohistology and histopathology															
Acute myocarditis (n,%)	---	6 (12 %)	---	---	---	---	---	---	---	---	---	---	---	---	---
Chronic myocarditis (n,%)	---	44 (88%)	---	---	---	---	---	---	---	---	---	---	---	---	---
Detection of viral genome (n,%)	---	24 (48%)	24 (39%)	1 (2,4%)	---	---	---	---	---	0.16	**<0.001**	---	---	---	**<0.001**
Medication															
ß-Blockers (n, %)	---	44 (88%)	55 (89%)	34 (87%)	26 (89%)	---	---	---	---	1.0	0.76	1.0	0.74	1.0	1.0
ACE inhibitors or angiotensin receptor antagonists (n,%)	---	43 (85%)	54 (87%)	35 (90%)	27(90%)	---	---	---	---	1.0	0.75	0.74	1.0	1.0	0.76
Aldosterone antagonists (n, %)	---	26 (52%)	44 (71%)	31 (79%)	24 (78%)	---	---	---	---	0.05	**0.015**	**0.017**	0.76	1.0	0.5

Continuous variables are expressed as median and 25th/75th percentiles. Categorical variables are presented as number (n) and percentages of patients.

Table 2: Baseline HMGB1 serum content and laboratory parameters

	Healthy controls n=50	**Myocarditis n=50**	**Post-myocarditis DCM n=62**	**Non-Inflammatory DCM n=39**	**Ischemic Cardio-myopathy ICM n=30**	**Healthy controls versus myocar-ditis**	**Healthy controls versus post-myocarditis DCM**	**Healthy controls versus non-inflam-matory DCM**	**Healthy controls versus ICM**	**Myocarditis versus post-myocar-ditis DCM**	**Myocarditis versus non-inflammatory DCM**	**Myocarditis versus ICM**	**Post-myocarditis versus non-inflammatory DCM**	**Post-myocarditis versus ICM**	**ICM versus non-inflam-matory DCM**
Parameters						**p-value**									
HMGB1 in serum (pg/ml)	3484 [3206/4104]	5616 [4092/8660]	4672 [3064/7540]	3552 [2895/4436]	3134 [2784/4000]	**<0.001**	**0.03**	0.99	0.99	0.99	**<0.001**	**<0.001**	**0.042**	**<0.001**	0.99
C-reactive protein (mg/l)	---	5.8 [2.1/23.3]	4.8 [3.0/16.4]	3.0 [1.0/11.0]	3.6 [1.4/7.3]	---	---	---	---	0.99	0.19	0.87	0.09	0.55	0.99
Leukocytes (10*6/l)	---	6150 [4363/10925]	7350 [5573/9800]	7200 [4180/7900]	8050 [7075/9250]	---	---	---	---	0.99	0.99	0.27	0.52	0.99	0.09
hsTroponin T (pg/ml)	---	1.0[1.0/11.3]	1.5[1.0/8.5]	2.0[1.0/6.5]	1.0[1.0/3.3]	---	---	---	---	0.99	0.99	0.99	0.99	0.99	0.99
Creatinine mg/dl	---	1.0[0.8/1.2]	1.0[0.8/1.2]	1.1[1.0/1.3]	1.1[0.9/1.3]	---	---	---	---	0.99	0.16	0.62	0.33	0.99	0.99
Blood Urea Nitrogen mg/dl	---	40[34/47]	42[31/46]	44[37/74]	52[36/68]	---	---	---	---	0.99	0.35	**0.043**	0.14	**0.014**	0.99
Aspartate aminotransferase U/l	---	32[25/44]	29[23/40]	27[23/35]	32 [23/38]	---	---	---	---	0.99	0.2	0.99	0.99	0.99	0.99
Alanine aminotransferase U/l	---	34[24/41]	29[19/37]	27[18/37]	25[22/39]	---	---	---	---	0.65	0.50	0.77	0.99	0.99	0.99
Gamma glutamyl transferase U/l	---	42[28/70]	34[27/54]	34[27/51]	41[28/71]	---	---	---	---	0.55	0.99	0.99	0.99	0.88	0.99

Continuous variables are expressed as median and 25th/75th percentiles. Categorical variables are presented as number (n) and percentages of patients.

Figure 1

Consolidated Scheme of Reported Clinical Trial

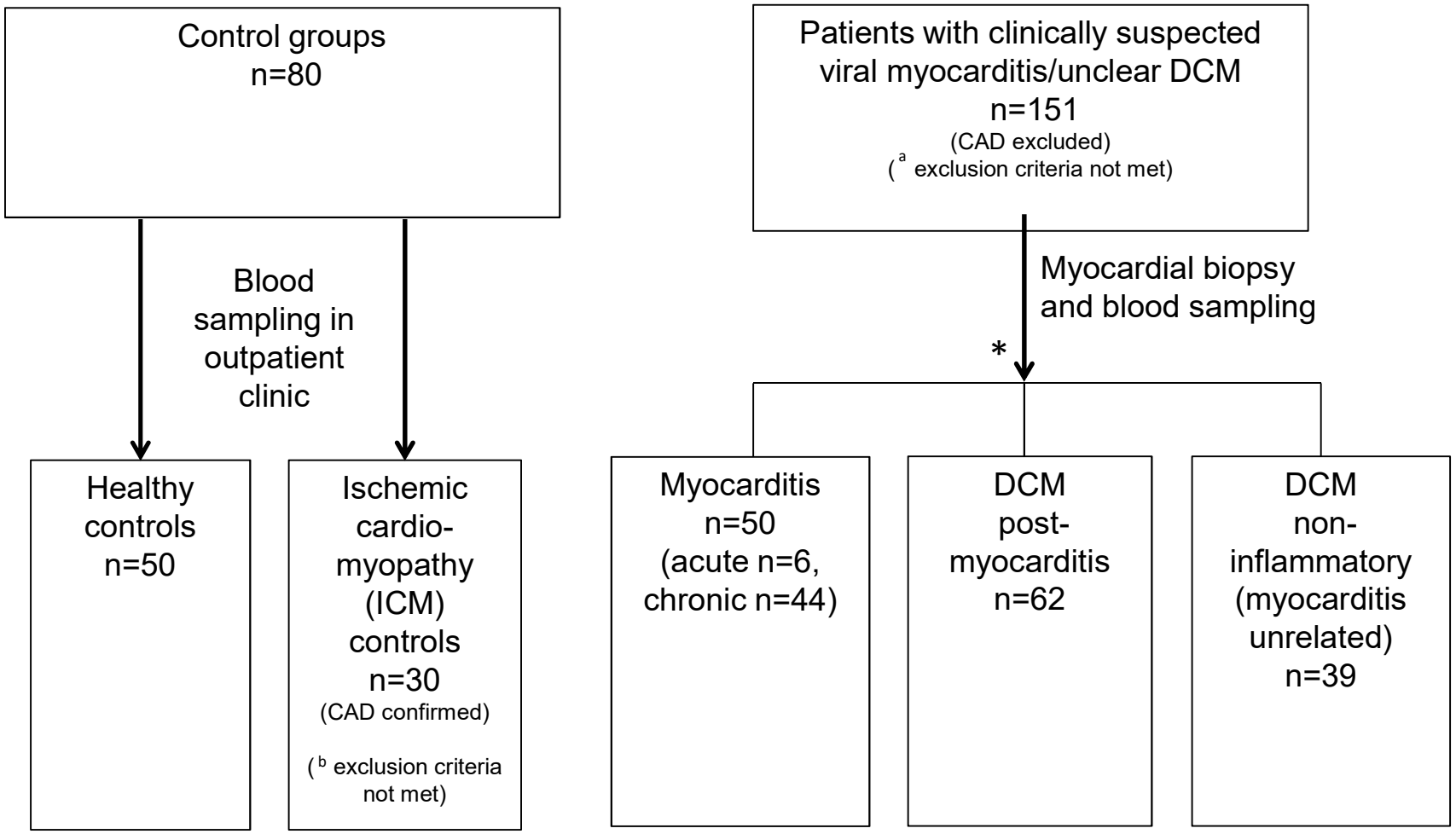
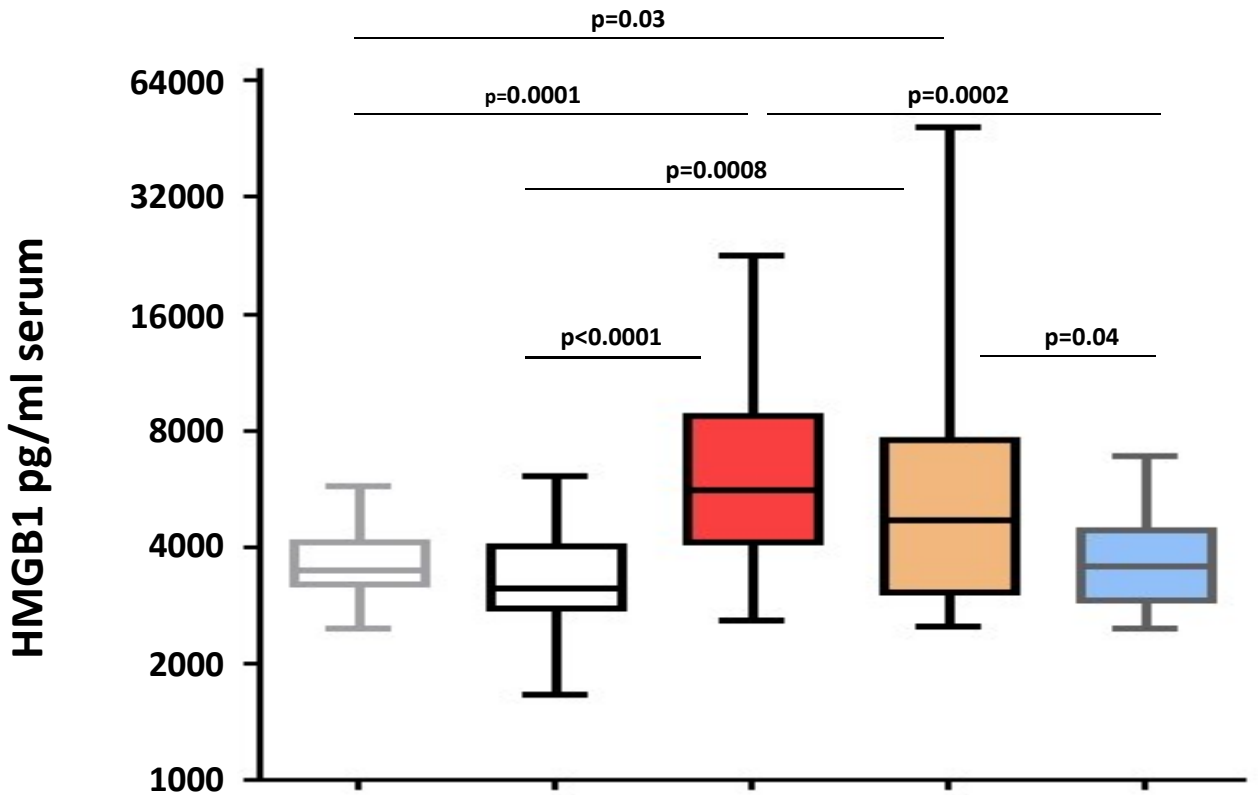
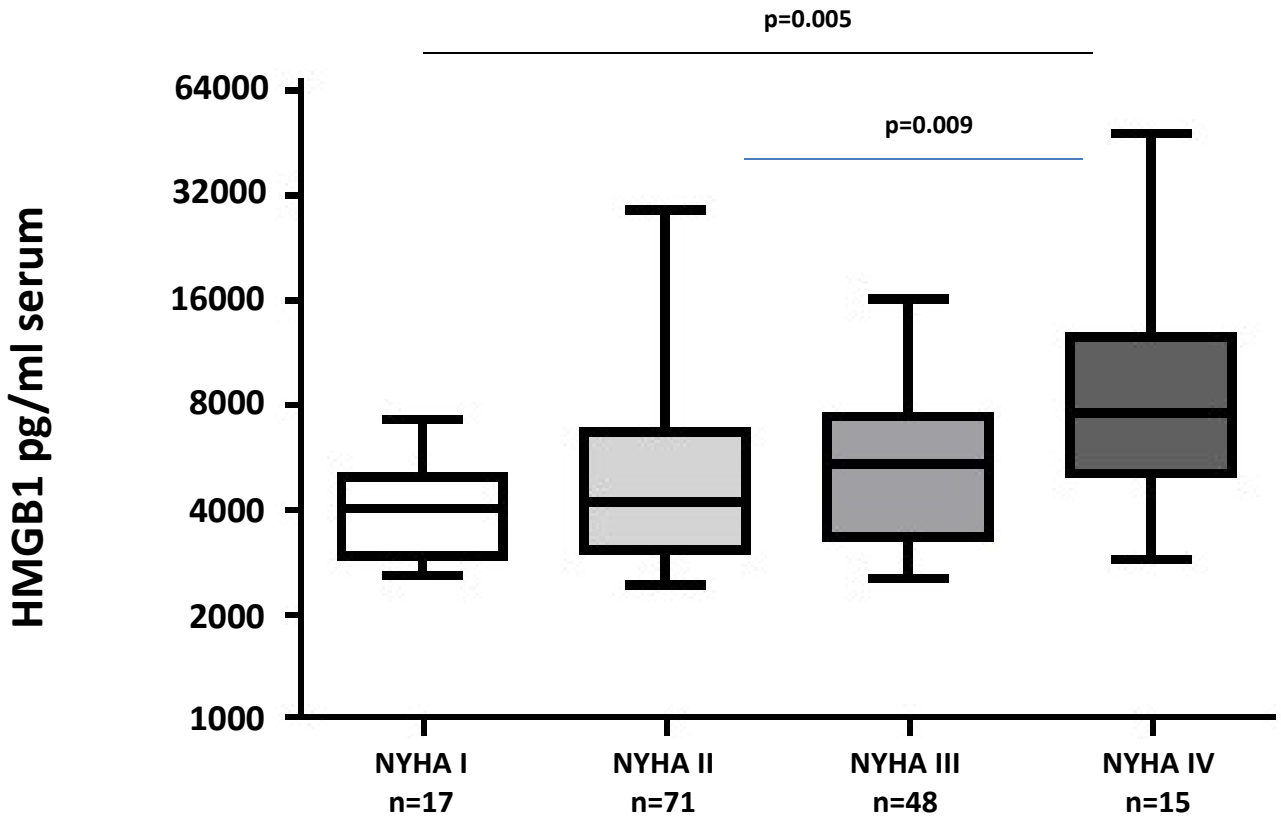


Figure 2



	Healthy n=50	ICM n=30	Myocarditis n=50	Post-myocarditis DCM n=62	Non-inflammatory DCM n=39
Median [Interquartile range]	3484 [3208,4104]	3136 [2784,4000]	5616 [4092,8660]	4672 [3064,7540]	3552 [2896,4436]

Figure 3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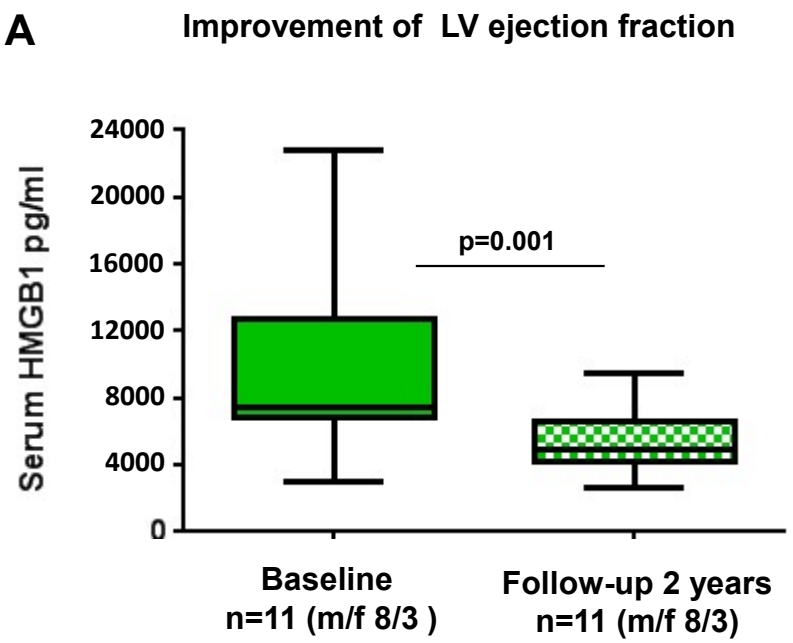
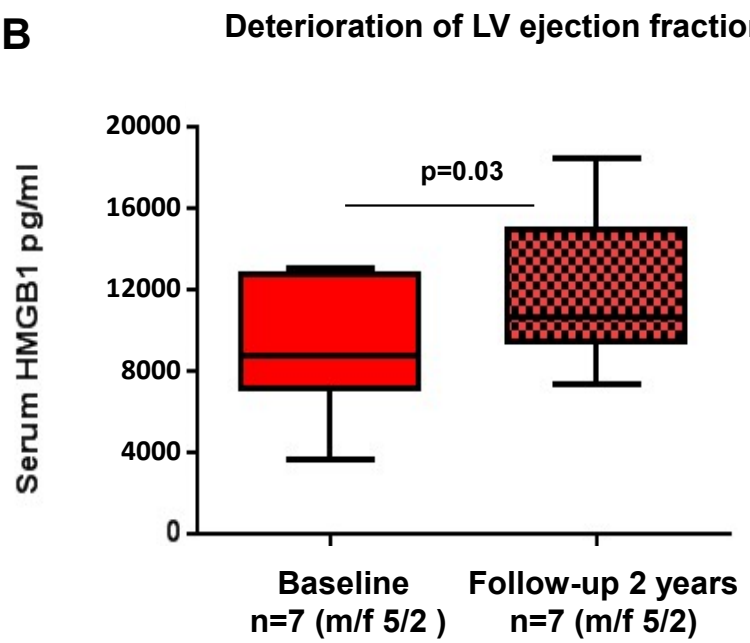
32000
16000
8000
4000
2000
1000
HMGB1 pg/ml serum
NYHA I
n=17
NYHA II
n=71
NYHA III
n=48
NYHA IV
n=15
Median
[Interquartile range]
4056
[2912,4976]
4204
[3072,6620]
5388
[3360,7356]
7504
[5084,12560]

Figure 4


A
Improvement of LV ejection fraction
Serum HMGB1 pg/ml
24000
20000
16000
12000
8000
4000
0
p=0.001
Baseline
n=11 (m/f 8/3)
Follow-up 2 years
n=11 (m/f 8/3)
Median
[Interquartile range]
7432
[6836,12672)
4924
[4184,6540]


B
Deterioration of LV ejection fraction
Serum HMGB1 pg/ml
20000
16000
12000
8000
4000
0
p=0.03
Baseline
n=7 (m/f 5/2)
Follow-up 2 years
n=7 (m/f 5/2)
Median
[Interquartile range]
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10696
[9432,14948]

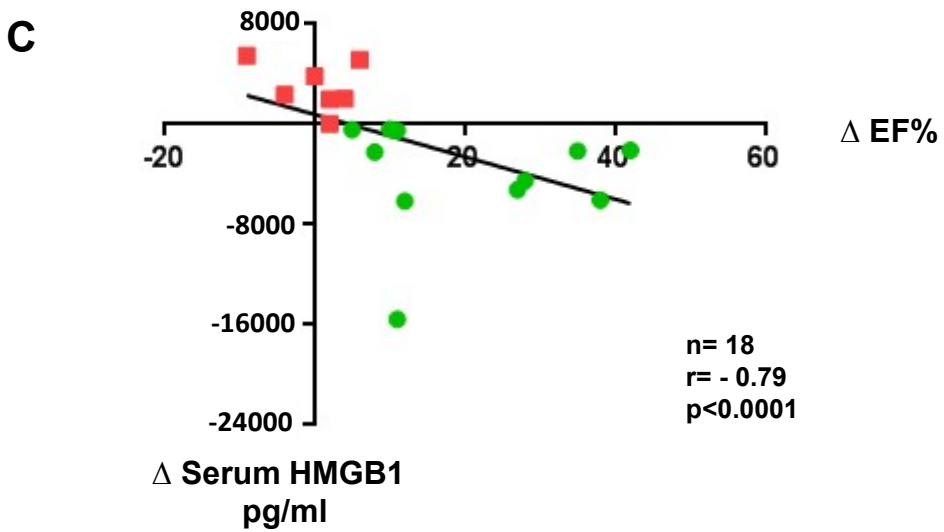
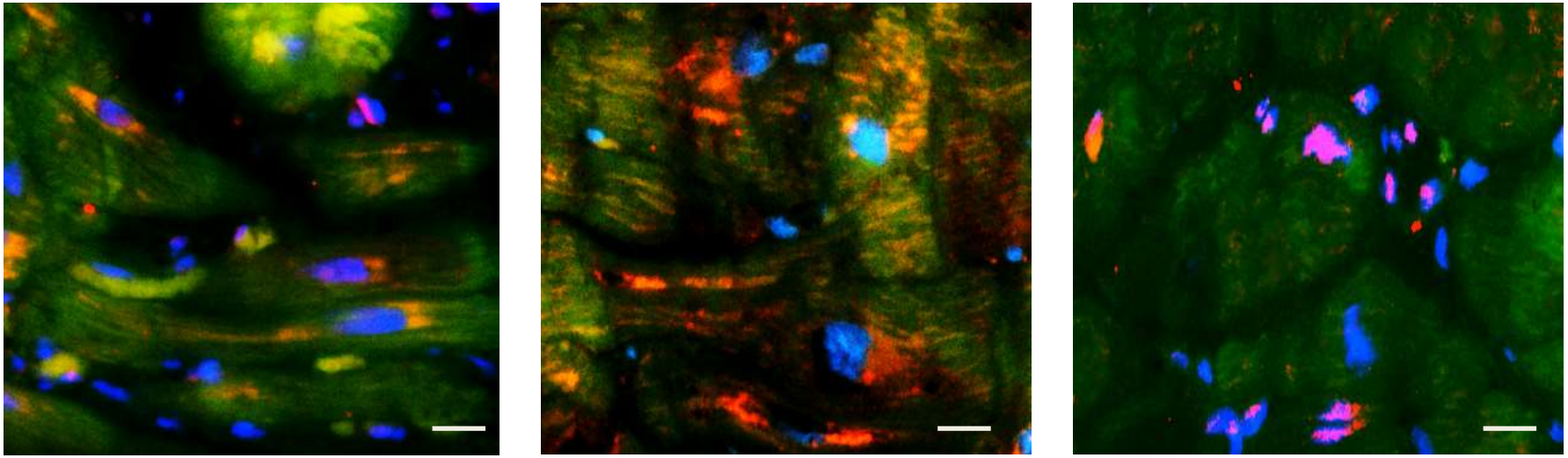

C
8000
-8000
-16000
-24000
-20
20
40
60
Δ EF%
n= 18
r= - 0.79
p<0.0001
Δ Serum HMGB1
pg/ml

Figure 5

A

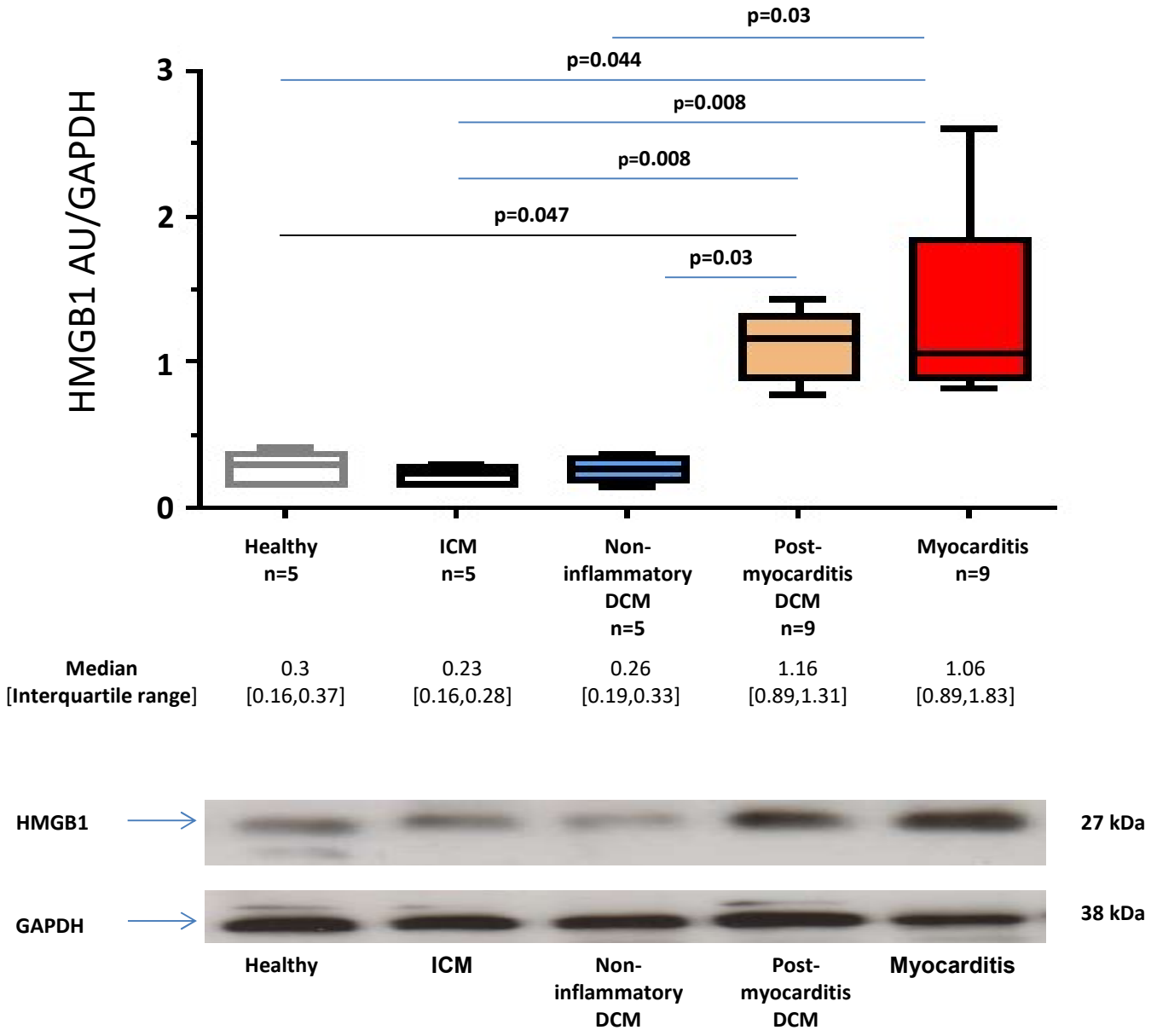


Acute myocarditis | **Post-myocarditis DCM** | **Ischemic cardiomyopathy (ICM)**

HMGB1; α-sarcomeric actin; Nuclei

Figure 5

B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p=0.044
p=0.008
p=0.008
p=0.047
p=0.03
HMGB1 AU/GAPDH
3
2
1
0
Healthy n=5
ICM n=5
Non-inflammatory DCM n=5
Post-myocarditis DCM n=9
Myocarditis n=9
Median [Interquartile range]
0.3 [0.16,0.37]
0.23 [0.16,0.28]
0.26 [0.19,0.33]
1.16 [0.89,1.31]
1.06 [0.89,1.83]
HMGB1
27 kDa
GAPDH
38 kDa
Healthy
ICM
Non-inflammatory DCM
Post-myocarditis DCM
Myocarditis

Supplementary Figure Legends

Supplementary Figure 1:
Analysis of serum HMGB1 in the individual groups according to sexes. Quantification of serum HMGB1 in A) acute versus chronic myocarditis in men, B) in healthy subjects, C) in ICM patients, D) in all myocarditis patients, E) in chronic myocarditis patients, F) in post-myocarditis DCM and G) in non-inflammatory DCM.
HMGB1 in pg/ml serum. Box plots indicate median, interquartile range and total range.

Supplementary Figure 2:
Analysis of serum HMGB1 according to viral persistence in A) all myocarditis patients, B) men with myocarditis, C) women with myocarditis, D) all post-myocarditis DCM patients, E) men with post-myocarditis DCM, F) women with post-myocarditis DCM.
HMGB1 in pg/ml serum. Box plots indicate median, interquartile range and total range.

Supplementary Figure 3:
Analysis of serum HMGB1 according to sexes and viral persistence in A) all virus-negative myocarditis versus post-myocarditis DCM patients, B) virus-negative men with myocarditis versus post-myocarditis DCM, C) virus-negative women with myocarditis versus post-myocarditis DCM, D) virus-negative chronic myocarditis men versus women, E) virus-negative post-myocarditis DCM men versus women, F) all virus-positive myocarditis versus post-myocarditis patients, G) virus-positive men with myocarditis versus post-myocarditis, H) virus-positive women with myocarditis versus post-myocarditis, I) virus-positive chronic myocarditis men versus women, J) virus-positive post-myocarditis DCM men versus women.
HMGB1 in pg/ml serum. Box plots indicate median, interquartile range and total range.

Supplementary Figure 4:
Quantification of serum HMGB1 according to viral entities. Double infections were included in both groups.
HMGB1 in pg/ml serum. Box plots indicate median, interquartile range and total range.

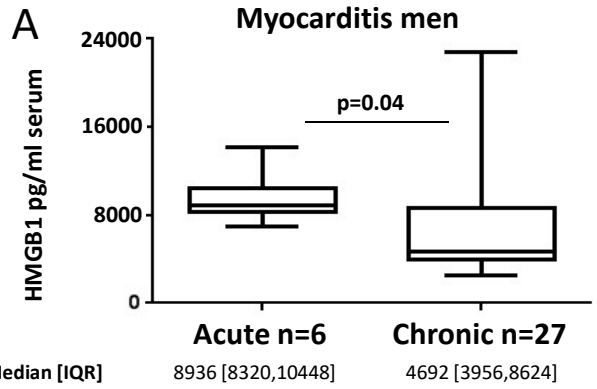
Supplementary Figure 5:
Quantification of HMGB1 in serum samples from A) healthy men, men with myocarditis, post-myocarditis DCM, non-inflammatory DCM and ICM, B) healthy women, women with myocarditis, post-myocarditis DCM, non-inflammatory DCM and ICM.
HMGB1 in pg/ml serum. Box plots indicate median, interquartile range and total range.

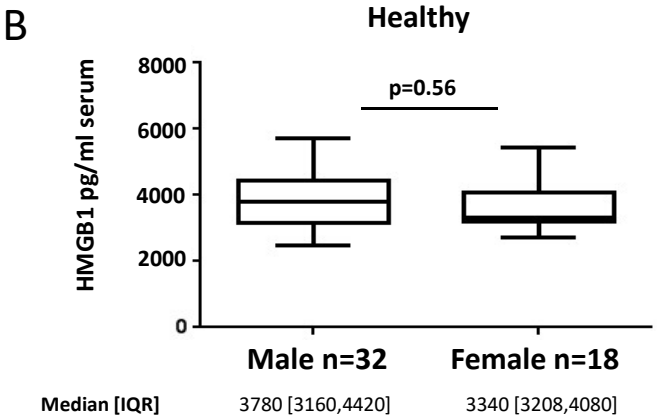
Supplementary Figure 6:
Analysis of serum HMGB1 according to NYHA stages in A) men and B) women.
HMGB1 in pg/ml serum. Box plots indicate median, interquartile range and total range.

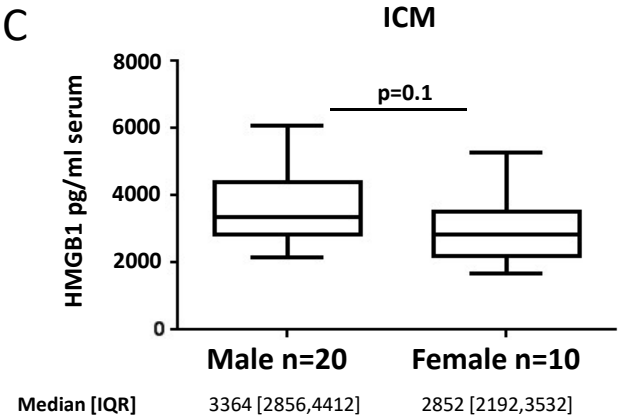
Supplementary Figure 7:
Correlations between serum HMGB1 and age in the individual groups according to sexes. Spearman correlation.
HMGB1 in pg/ml serum. Box plots indicate median, interquartile range and total range.

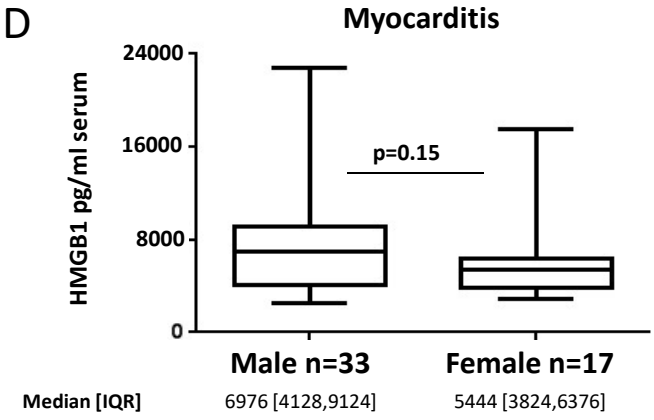
Supplementary Figure 8:
Analysis of serum HMGB1 in the individual groups according to sexes and age $\leq$50 years in A) healthy subjects, B) chronic myocarditis patients, C) post-myocarditis DCM, D) non-inflammatory DCM and E) ICM patients.
HMGB1 in pg/ml serum. Box plots indicate median, interquartile range and total range.

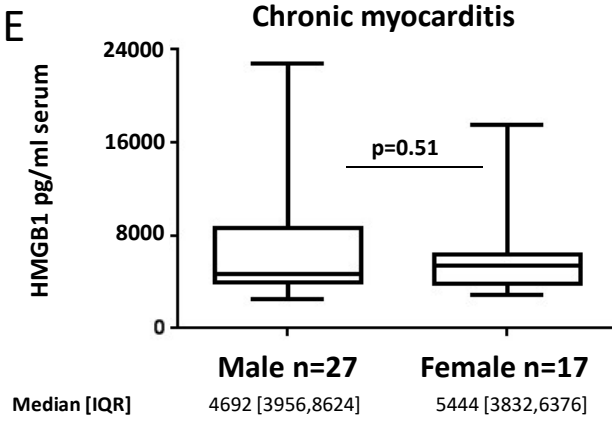
Supplementary Figure 1

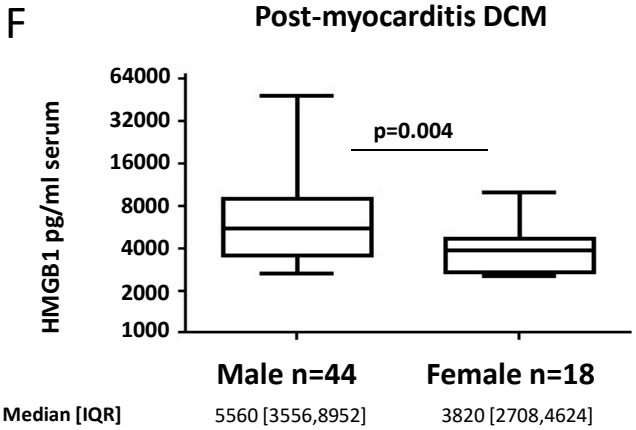


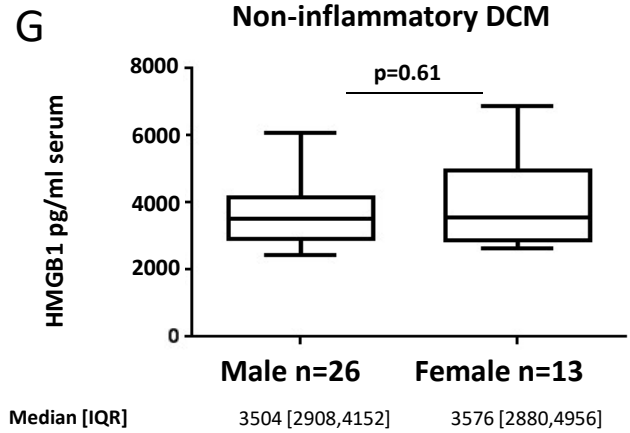




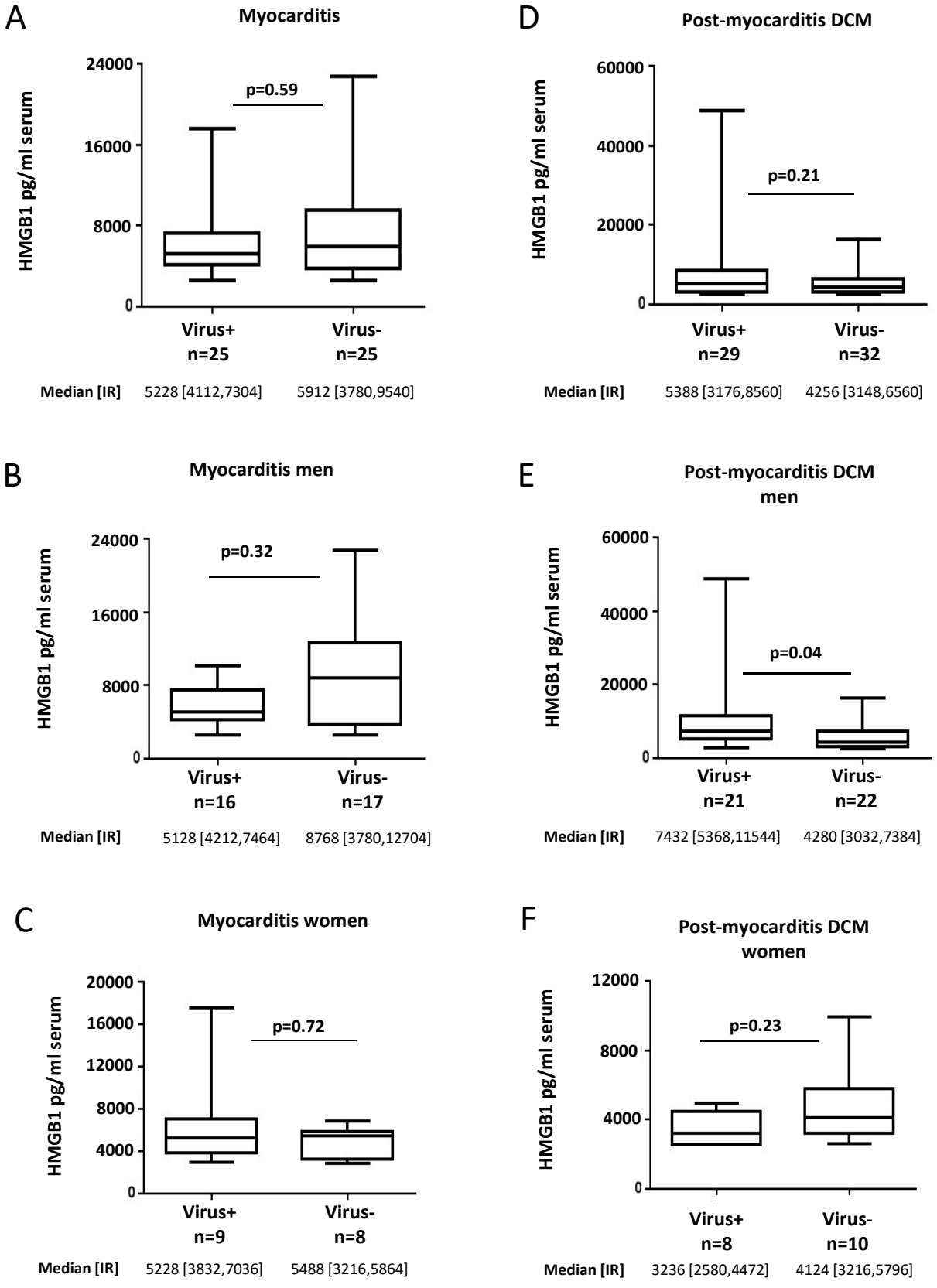




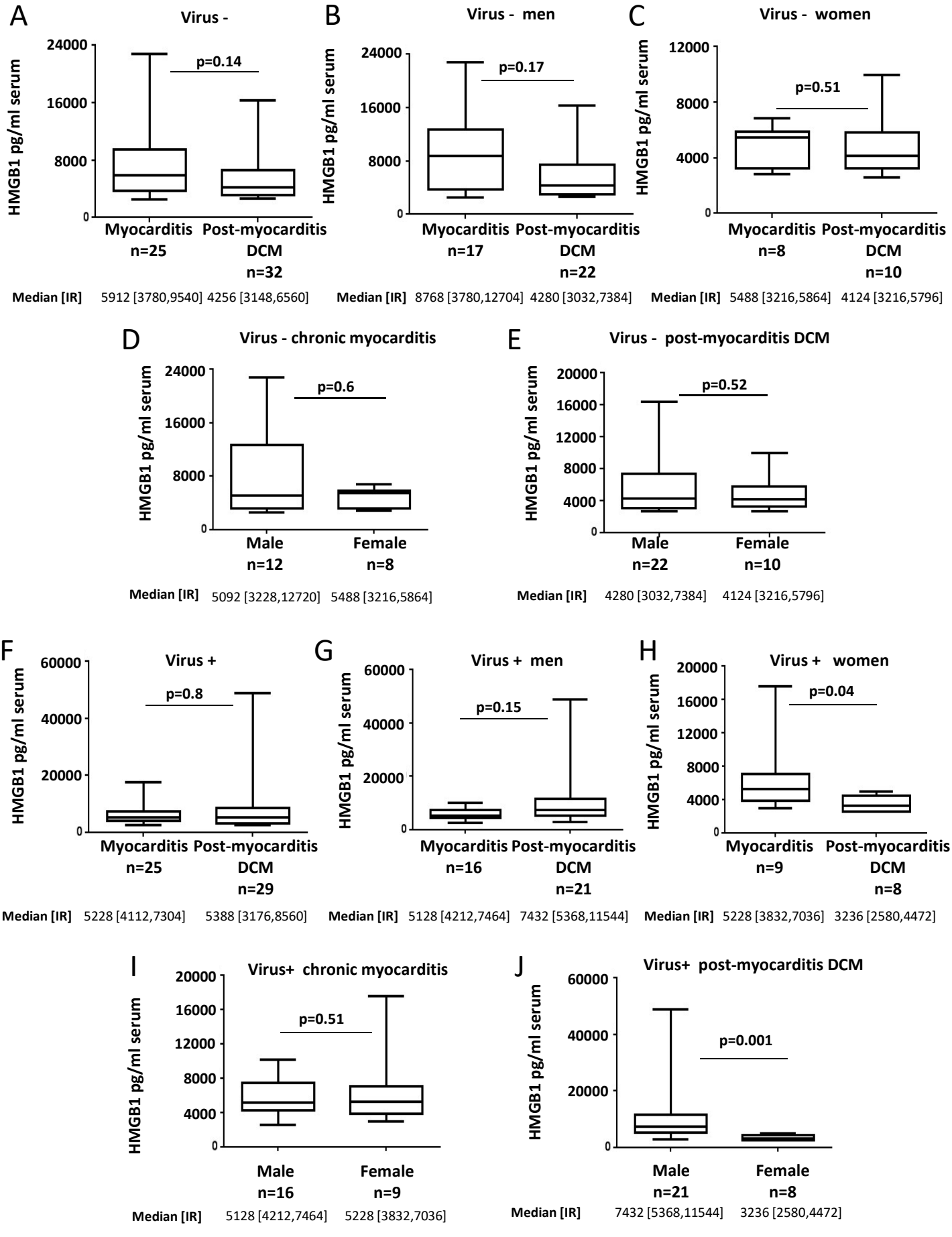




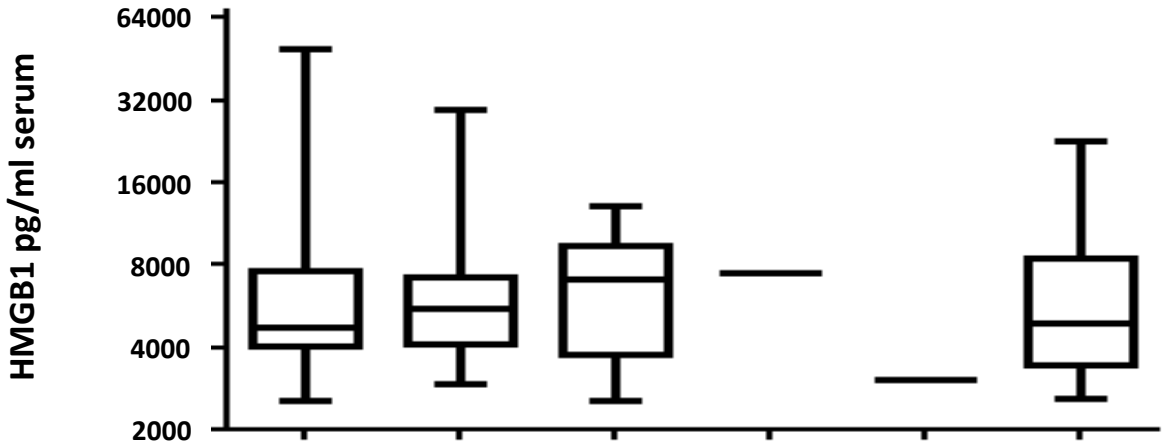
Supplementary Figure 2



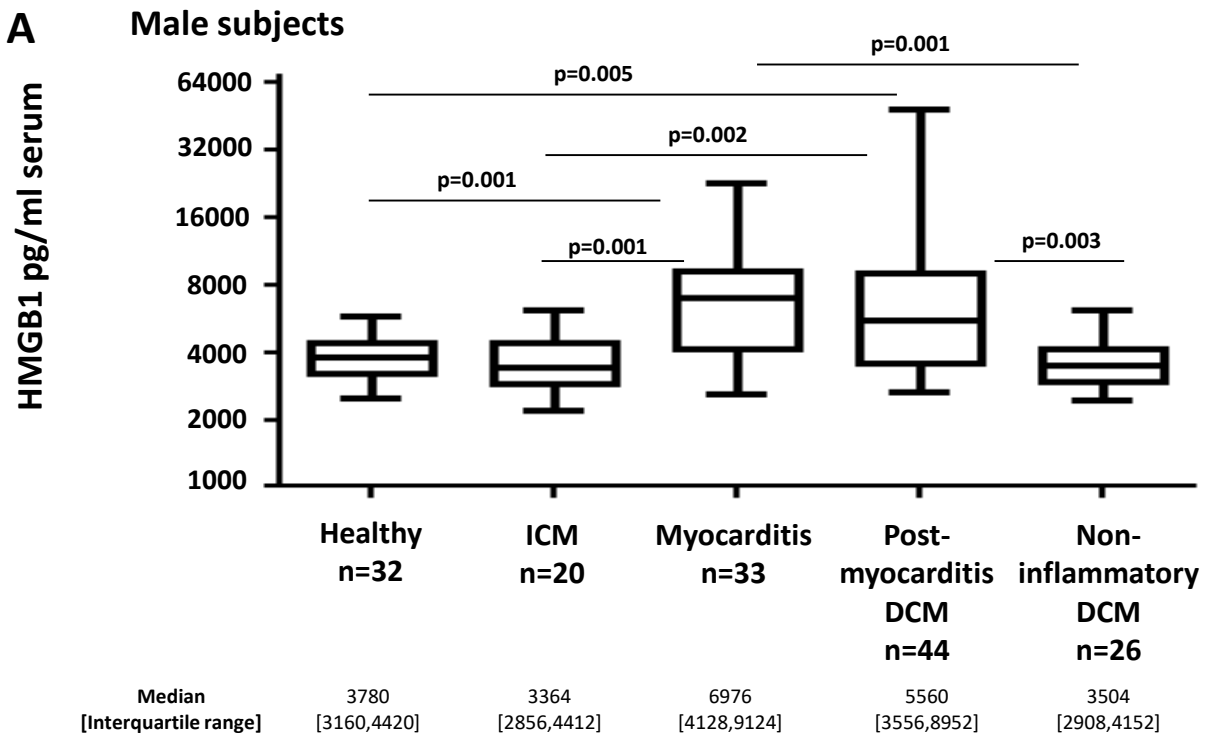
Supplementary Figure 3

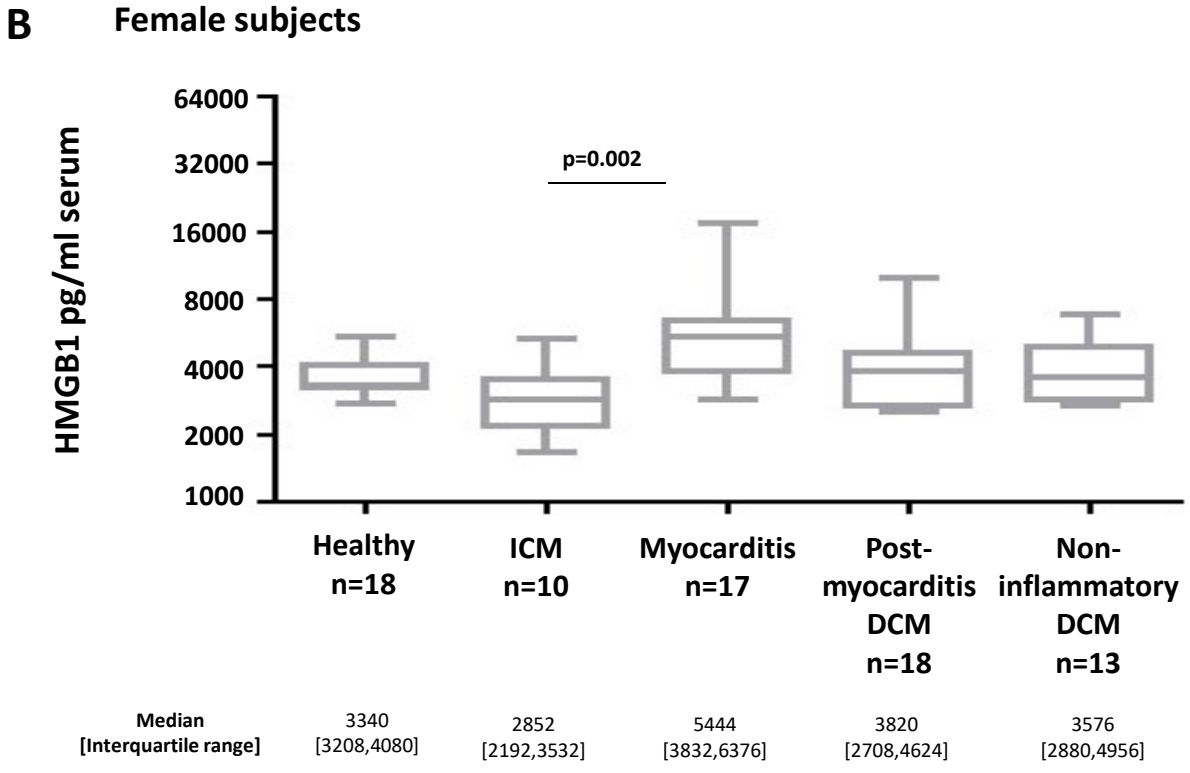


Supplementary Figure 4



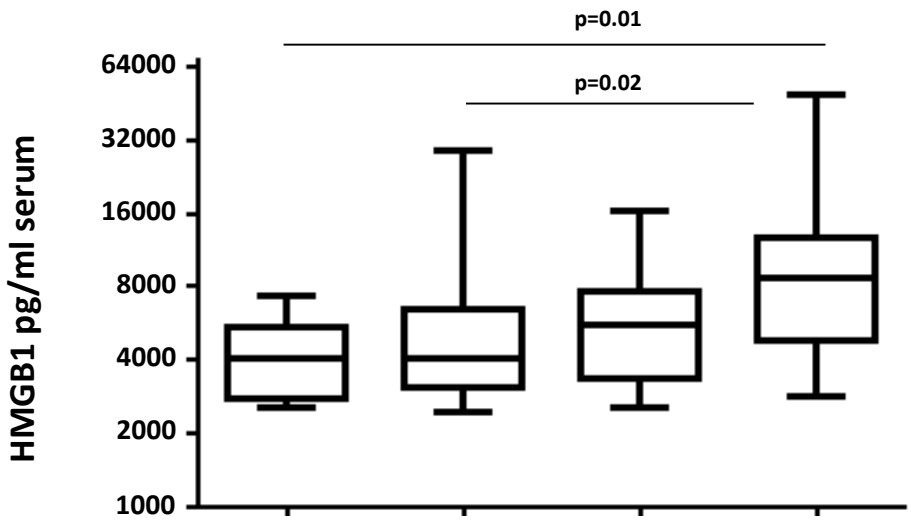
Supplementary Figure 5





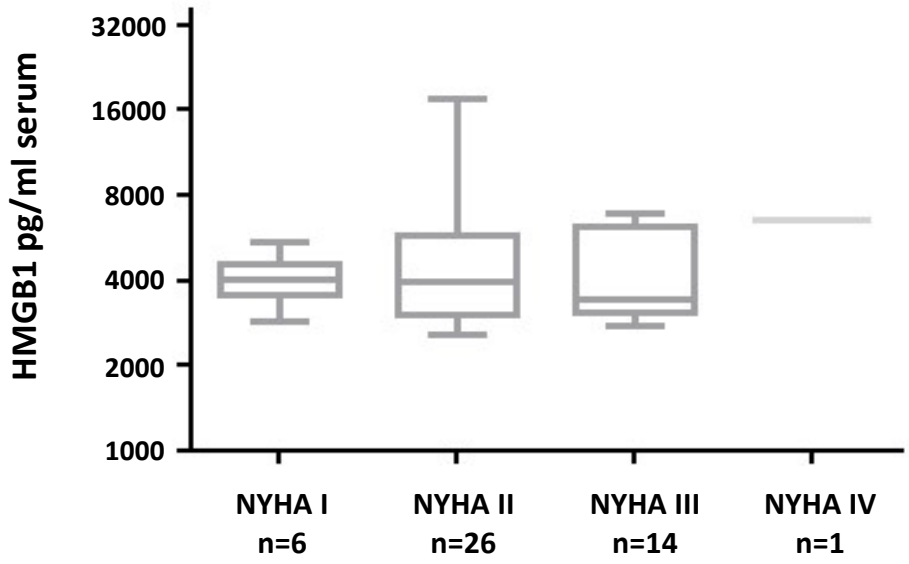
Supplementary Figure 6

A **Male subjects**



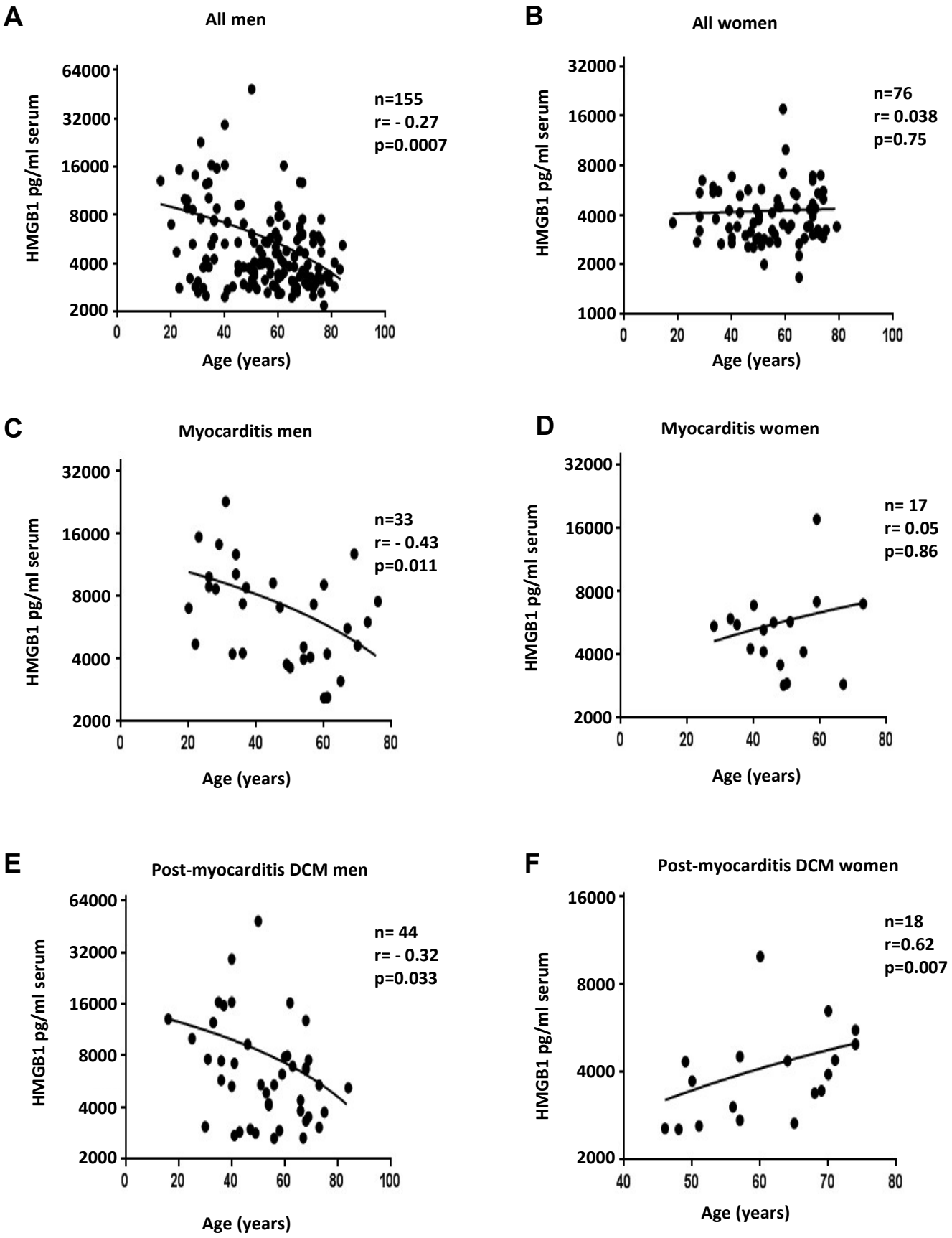
Median [Interquartile range]	4056 [2768,5480]	4100 [3112,6504]	5536 [3392,7612]	8688 [4840,12616]

B **Female subjects**

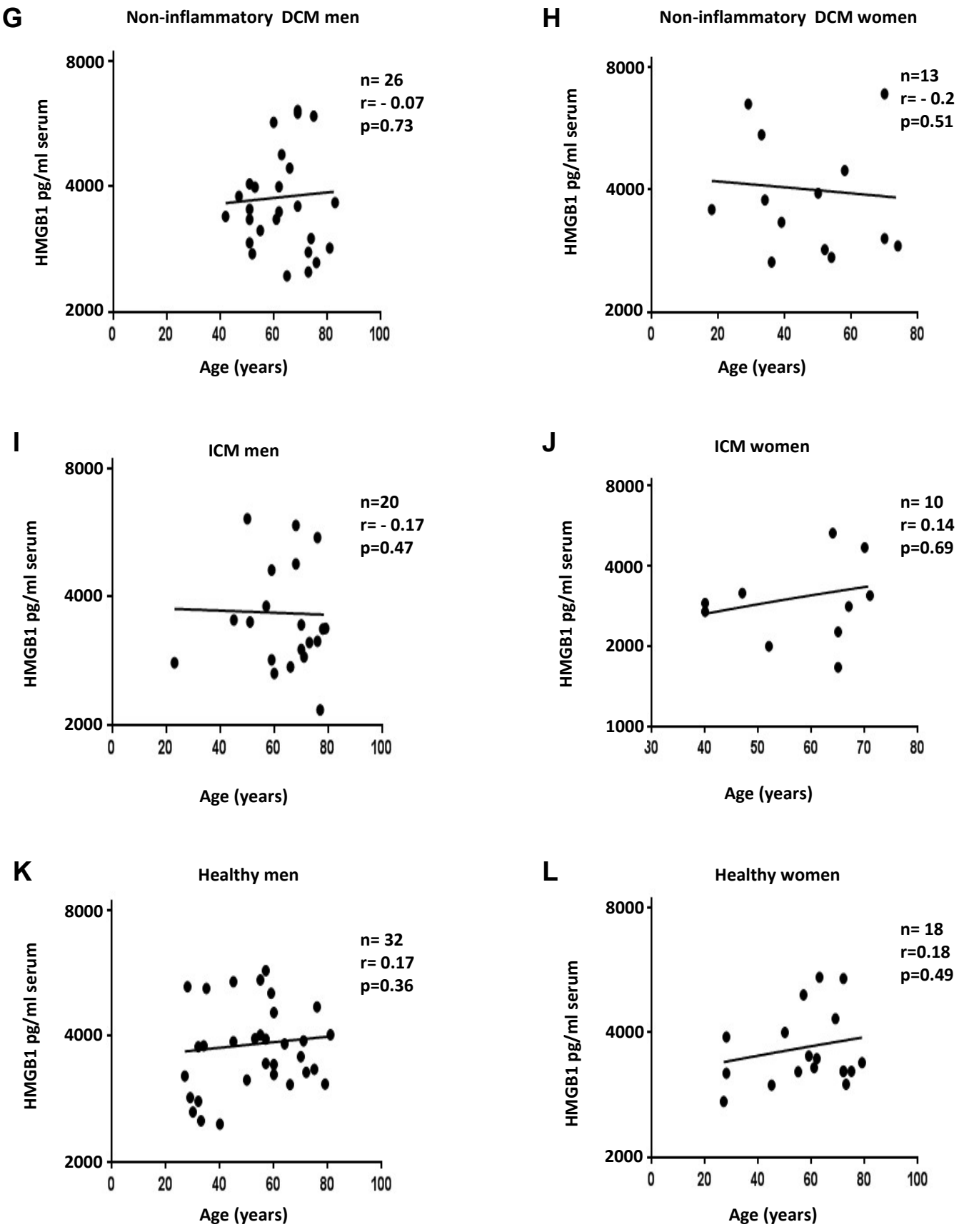


Median [Interquartile range]	4016 [3540,4548]	3952 [3000,5724]	3416 [2836,5808]	6472 [6472,6472]

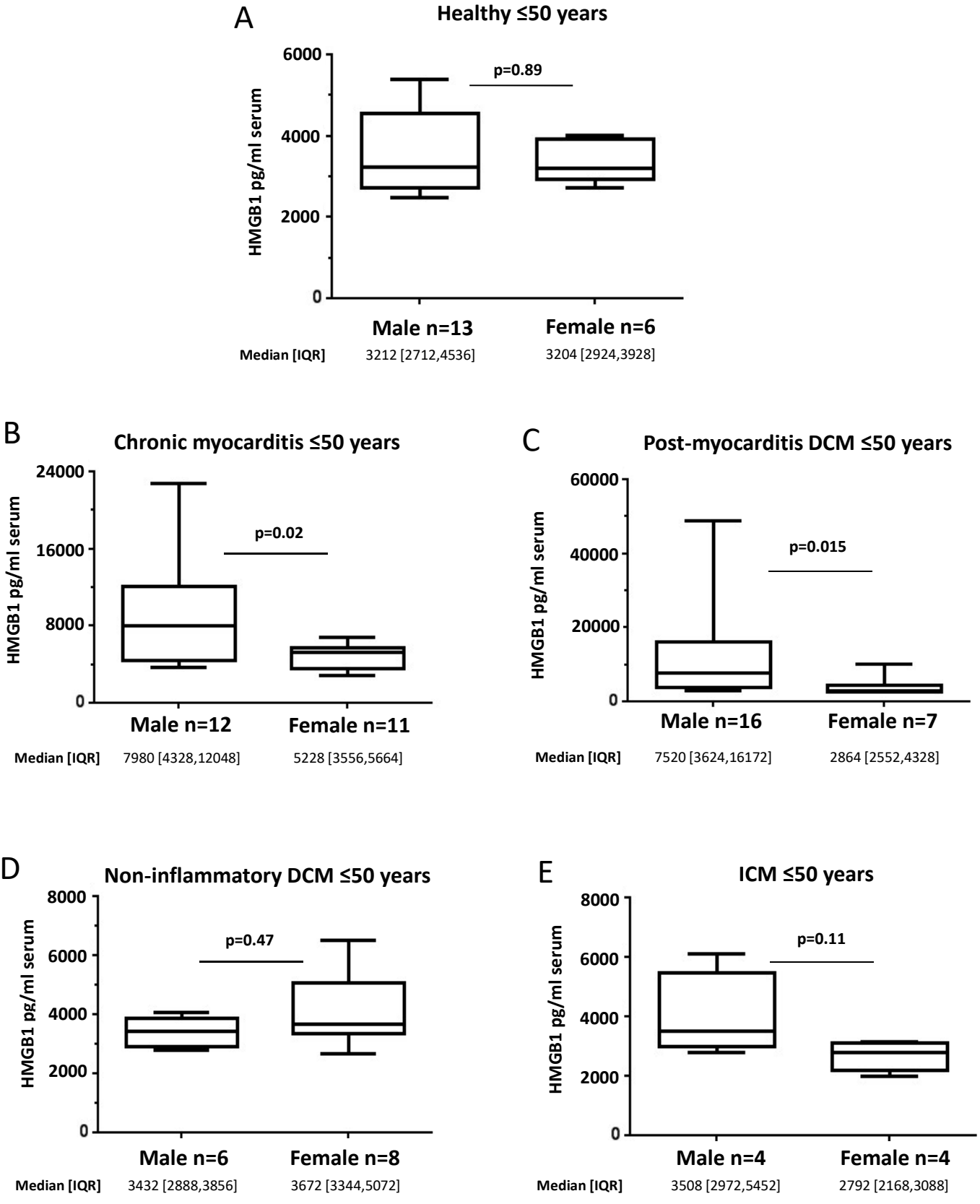
Supplementary Figure 7



Supplementary Figure 7 (continued)



Supplementary Figure 8



SUPPLEMENTARY METHODS

Materials

Rabbit polyclonal antibody against HMGB1 (Abcam; ab18256, Cambridge, UK), HMGB1 recombinant protein as positive control (Abcam; ab56525, Cambridge, UK) and mouse monoclonal anti-GAPDH (Millipore MAB374) were used for Western blot analysis. HRP (Horseradish peroxidase)-conjugated goat-anti-rabbit and goat-anti-mouse secondary antibodies were from Sigma-Aldrich (Deisenhofen, Germany). All other substances used were from Sigma-Aldrich, unless specified otherwise.

Western blot

Myocardial samples were pottered with extraction buffer containing 10 mmol/l cacodylic acid (pH 5.0) 0.15 mol/l NaCl, 1 µmol/l ZnCl2, 20 mmol/l CaCl2, 1.5 mmol/l NaN3, and 0.01% (v/v) Triton X-100 and mixed 2:1 v/v with 2xSDS-PAGE loading buffer. The denatured (95°C, 5 min) samples (25 ug /lane) were separated on 12% SDS polyacrylamide electrophoresis gels, transferred to nitrocellulose membranes (Protran®, Schleicher & Schuell GmbH, Dassel, Germany) by semi-dry electrophoretic blotting (0.8 mA/cm2) and subjected to Western blot analysis. Membranes were blocked with 0.1% Western Blocking Reagent (Roche, Mannheim, Germany) and probed with primary antibodies against HMGB1 1:1000 for 18h at 4°C. The goat anti-rabbit secondary antibody was diluted 1:10.000 and incubated for 60 min at RT. Proteins were visualized by enhanced chemiluminescence (ECL) according to the manufacturer´s guidelines (Amersham Pharmacia Biotech, Freiburg, Germany). Autoradiographs were quantified by imaging densitometry and analysed by the „LabWorks 4.6“ Software (LabWorks Image Acquisition and Analysis Software, UVP BioImaging Systems, Cambridge, UK). Data are presented as arbitrary units (AU) normalized to the same control sample/recombinant HMGB1.