Cardiac Inflammation Contributes to Changes in the Extracellular Matrix in Patients with Heart Failure and Normal Ejection Fraction

Running Title: Westermann et al: Inflammation and Fibrosis in HFNEF

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Abstract

Background—The pathophysiology of heart failure with normal EF (HFNEF) is still under discussion. Here we study the influence of cardiac inflammation on extracellular matrix (ECM) remodeling in patients with HFNEF.

Methods and Results—We investigated the left ventricular systolic and diastolic function in 20 patients with HFNEF and 8 control patients by conductance catheter and echocardiography. Moreover, endomyocardial biopsies were obtained and ECM proteins as well as cardiac inflammatory cells were investigated. Furthermore, primary human cardiac fibroblasts were outgrown from the endomyocardial biopsies to investigate the gene expression of ECM proteins after stimulation with TGF-β. Diastolic dysfunction was present in the HFNEF patients compared to the control patients. In endomyocardial biopsies of HFNEF patients we found an accumulation of cardiac collagen, which was accompanied with a decrease in the major collagenases system (MMP-1) in the heart. Moreover, an increment of inflammatory cells, which expressed the pro-fibrotic growth factor TGF-β, could be documented in the HFNEF patients. Stimulation of primary human cardiac fibroblasts from HFNEF patients with TGF-β resulted in a transdifferentiation of fibroblasts to myofibroblasts, which produced more collagen and decreased the amount of MMP-1, the major collagenases in the human heart. A positive correlation of cardiac collagen as well as the amount of inflammatory cells to diastolic dysfunction was evident and suggests a direct influence of inflammation to fibrosis triggering diastolic dysfunction.

Conclusions—Cardiac inflammation contributes to diastolic dysfunction in HFNEF by triggering the accumulation of extracellular matrix.

Key Words: collagen; inflammation; remodeling; diastolic dysfunction;
Patients with heart failure with normal ejection fraction (HFNEF) have increased mortality\textsuperscript{1-4} and the morbidity was found to be higher compared to patients with HF and reduced EF.\textsuperscript{5} One of the underlying mechanisms leading to the clinical symptomatology in patients with HFNEF are diastolic function abnormalities with increased diastolic stiffness, but also non-diastolic function abnormalities with exercise induced changes in systolic velocity, chronotropic incompetence and ventricular-vascular uncoupling having been demonstrated to contribute to this disease.\textsuperscript{6-18} Despite the growing prevalence of HFNEF over the last 15 years, a disease affecting about half of the heart failure population, our knowledge about its molecular mechanisms is still limited, especially since the pathways leading to HFNEF are not confined to one pathology. Intracellular changes with elevated cardiomyocyte resting tension as well as a shift in titin isoforms are important in patients with severe HFNEF.\textsuperscript{19,20} Furthermore, accumulation of cardiac collagen was shown to be present in this disease and contribute to the aggravation of diastolic function.\textsuperscript{21-23} Enhanced endothelial migration of inflammatory cells into the myocardium might influence the development of these changes, especially in regard to changes of the extracellular matrix.\textsuperscript{24,25}

Therefore, we hypothesized that inflammation is one important trigger of cardiac fibrosis and therefore plays an important role in HFNEF. We show here, that increased inflammation triggers collagen accumulation in HFNEF patients, and that both, inflammation and fibrosis correlate to diastolic dysfunction. We show that the inflammatory cells express TGF-beta, which directly induces changes in cardiac fibroblasts, as increasing collagen accumulation and decreasing the degradation system (matrix metalloproteinases (MMP-1) and their tissue inhibitor (TIMP)). This was associated with a transdifferentiation of cardiac fibroblasts to myofibroblasts, knowing to be a potent contributor of pathological remodelling in diseases tissues. These findings suggest that cardiac inflammation by increased transendothelial migration is one potent trigger of diastolic dysfunction in the HFNEF population.
Methods

Study population

Patients presenting with NYHA status II and III, an EF>50% and diastolic dysfunction by echocardiography were evaluated for participation in this study. Persistent atrial fibrillation and pulmonary diseases were excluded. Significant coronary artery disease or heart-valve diseases were ruled out by angiography. Clinically relevant parvovirus B19 infection with a genome equivalent over 500 was ruled out in all patients included. 26 20 patients met the inclusion criteria and were enrolled in the study and are referred to as the HFNEF group in regard to recent guidelines27. In addition, 8 patients without signs of congestive heart failure, who underwent coronary angiography for evaluation of chest pain were also enrolled in this study and served as controls. In all patients endomyocardial biopsies were obtained the day after the hemodynamic function was analyzed. Cardiac conditions were stable prior to catheterization in all patients. All patients gave informed and written consent. The research protocol was approved by the local institutional review board.

Pressure-volume measurements and echocardiography

The conductance catheter was used to assess pressure volume measurements in all patients as described in more detail recently.6 Briefly, diastolic LV function was characterized by LV end-diastolic pressure (LVEDP) and the isovolumetric relaxation (Tau). Furthermore, we calculated the exponential curve fit to the diastolic LV pressure volume points during a transient preload reduction to determine the load independent diastolic stiffness constant (LV stiffness constant β). As parameter of systolic function, we analyzed the LV end-systolic pressure (LVP) and the ejection fraction (EF). Moreover, the slope of the end systolic pressure volume relationship (ESPVR) was calculated as a load independent parameter for
cardiac contractility. Mitral and pulmonary venous Doppler flow velocities were recorded in the apical 4-chamber view with a VingMed System FiVe (GE Healthcare, Chalfont St Giles, UK) as well as the LV filling index, by the ratio of transmitral flow velocity to annular velocity (E/E’ lateral) as previously described. Right ventricular systolic pressure (mmHg) was measured before the endomyocardial biopsies were taken.

**Endomyocardial biopsy**

Endomyocardial biopsies were obtained from the right side of the ventricular septum of each patient with use of a flexible bioptome (Westmed) via the femoral vein approach. Furthermore, RV systolic pressure was measured. The tissue pieces were frozen in liquid nitrogen and stored at -70 °C for subsequent analysis. From all patients, one endomyocardial biopsy was used for immunhistological stainings and one was used for measuring gene expression (all measurements performed once, n=20 in HFNEF and n=8 in controls).

**Cell culture**

Primary cardiac fibroblasts were obtained by outgrowth from biopsies from 5 HFNEF patients which were incubated in Iscove basal medium (Biochrom AG, Berlin, Germany) containing 10% human serum, 10% FCS, 100 U/ml penicillin (Biochrom AG, Berlin, Gemany) and 100 μg/ml streptomycin (Biochrom AG, Berlin, Germany). The cardiac fibroblasts were seeded out in 24-well plates and when the culture was about 80% confluence, they were serum-starved in Iscove basal medium containing 0.5% FCS (PAA, Cölbe, Germany) for 16 hours. They were then stimulated with 5 ng/ml TGF-β1 (PeproTech, Hamburg, Germany) for 6 hours or 24 hours compared to no stimulation for 6 hours or 24 hours. All experiments were
done with cells between the 2\textsuperscript{nd} and 4\textsuperscript{th} passage with n=6 wells per experiment for all time points and all patients. For each individual patients a mean was calculated from the 6 cell culture wells for each experiment (basal conditions and stimulated for all time points) and the means of all patients is presented in the figures.

Native fibroblasts were stained with antibodies against CD31, desmin, vimentin, P4HB and alpha-smooth muscle actin and compared to HL1 (experimental atrial cardiomyocytes) as well as to endothelial cells (HMEC).

The monocyte cell line THP-1 were cultured in RPMI 1640 medium (PAA, Cölte, Germany) containing 10% FCS (PAA), 100 units/ml penicillin, and 100 μg/ml streptomycin (PAA).

Type I carboxy-terminal telopeptide of collagen (ITCP) and propeptide of procollagen type I (PIP) in the serum

Serum was collected 1h before invasive measurements and ICTP as a break down product of collagen type 1 as well as PIP as a marker of type I collagen synthesis was measured as described previously.\textsuperscript{29}

\textit{RNA isolation}

Total RNA was extracted from the myocardial biopsies or cell culture wells by the Trizol method (GIBCO BRL, Carlsbad, California). Additional purification was performed using the ChargeSwitch® Total RNA Cell Kit (Invitrogen, Karlsruhe, Germany). The yield of purified total RNA was analysed by checking the UV absorbance at 260 nm on the NanoDrop® ND-1000 (Agilent Technologies, Boeblingen, Germany) spectrophotometer.
Real time RT-PCR

The ABI inventoried TaqMan® gene expression assays (each includes forward and reverse primers as well fluorescently marked probe) used for preamplification (from the biopsies) and for the real-time RT-PCR were obtained from Applied Biosystems. Prior real-time RT-PCR 1-250 ng cDNA samples of the biopsies were preamplified with the pooled gene expressions assays using TaqMan® PreAmp Master Mix (Early access) in a final volume of 25 μl. Quantification of housekeeping CDKN1B transcripts as an internal control for the amount and quality of cDNA was performed for all samples. Gene expression for collagen type I and III was analyzed from cardiac biopsies (primers from Applied Biosystems). Data for cell culture are as well normalized to human CDKN1b mRNA levels as an endogenous control (unaffected by TGF-β treatment) and are expressed relative to untreated control using the formula 2^(-ΔΔCT).

TGF-beta ELISA:

The THP-1 cells were starved in Iscove medium containing 0.5% FCS (PAA), 100 units/ml penicillin, and 100 μg/ml streptomycin (PAA) 16 hours prior to the experiment. 3 Mio/ml THP-1 cells were treated with 100 ng/ml PMA (Phorbol 12-myristate 13-acetate, Sigma-Aldrich) for 12, 24 and 48 hours. The cell culture supernatant was then used for measuring the total TGF-beta1 concentration using the TGFβ1 E\textsubscript{max} ImmunoAssay System (Promega). Therefore, the cell culture supernatant was first diluted 1:5 in Dulbecco’s PBS (PAA) and then acidified with HCl to pH 2.6 for 15 min followed by neutralization with NaOH before using the sampled for the TGFβ1 E\textsubscript{max} ImmunoAssay System.
**Histology**

Immunohistological stainings were performed using standard techniques. The antibodies were purchased from Chemicon (anti-MMP1, CD3, CD11a, CD45, VCAM-1, Collagen type I and III) and Calbiochem (anti-TIMP1 and anti-MMP2). Immunohistochemical stainings were quantified by digital image analysis (DIA). For double immunofluorescence we used Tissue Tec® embedded cryosections. The following primary antibodies were used: mouse anti α-sarcomer actin (Santa Cruz, USA, 1:50), mouse anti-TGF-b (Serotec, 1:50). The secondary antibodies were labelled with Fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) (Sigma, St. Louise, USA). Nuclei were visualized using DAPI (Sigma, St. Louise, USA). Slides were embedded in Fluorescent Mounting Medium (DakoCytomation, Glostrup, Denmark), cover slipped and analyzed using a fluorescence microscope (Zeiss, Jena, Germany). Moreover, sections were incubated with 10 μmol/L DHE to detect O$_2^-$ and visualized by fluorescent microscopy, where DHE positive nuclei were counted in regard to total nuclei number.

**Statistical analysis**

Data are shown in whisker blots (with min and max) in Figures 1-3, in Figure 4 and 5 mean ± S.E.M. For comparison HFNEF group with control group, after testing that the data were non-normally distributed, the non-parametric Mann-Whitney U test was used. Fisher’s exact test was used to analyze categorical variables. A probability value <0.05 was considered statistically significant. Data were analyzed with Graphpad 5.01 (PRISM, SanDiego, USA) and SPSS (version 15.0, SPSS Inc, Chicago, IL, USA).
Results

Patient demographics

Patient characteristics are summarized in Table 1. There were no significant differences between HFNEF patients and controls with respect to age, gender, or body surface area. Prevalence of hypertension and diabetes mellitus was not statistical different (Table 1).

Hemodynamic data

Systolic function was not changed in HFNEF compared to controls. In contrast, diastolic dysfunction was evident in the HFNEF group with increased LV stiffness constant β, increased LV end diastolic pressure, and prolonged isovolumetric. Echocardiography revealed, that cardiac dimensions of both groups were not statistically significant different. Diastolic dysfunction with increased E/E’ lateral was documented in the HFNEF group. Moreover, significant cardiac hypertrophy was documented in HFNEF compared to controls (Table 2). Right ventricular systolic pressure, as one parameter of pulmonary hypertension, was not statistically significant different in HFNF and controls (29.7 (24-33) vs. 25 (21–28.5), mmHg, p=0.4).

Extracellular matrix

Collagen type I and III expression was significantly higher in patients with HFNEF compared to controls in the cardiac biopsies. Moreover, mRNA abundance of both collagen subtypes were increased in HFNEF patients (Figure 1). The ratio of collagen type I / III was furthermore increased in the HFNEF patients. TGF-beta mRNA expression in the biopsies
was increased in HFNEF patients compared to controls (+59% compared to controls, p=0.004) Histological expression levels of cardiac MMP-1, the major human collagenases, were decreased in HFNEF, while TIMP-1 was increased in HFNEF compared to controls (Figure 2).

ICTP, as one serum marker for collagen degradation was increased in the serum of HFNEF groups compared to controls (mean ± S.E.M: 5.9 ± 2.1 vs 2.3 ± 1.4, μg/L, p=0.025). PIP, a serum marker for collagen production, was also increased in the HFNEF group compared to controls (mean ± S.E.M: 128.9 ± 26 vs. 65.1 ± 21, μg/L, p=0.018).

**Inflammation**

The inflammatory cells marked by CD3, CD11a and CD45 were all increased in the cardiac tissue of the HFNEF patients compared to controls (Figure 3). Moreover, the adhesion molecule VCAM-1 was increased in HFNEF compared to control patients (Figure 2). In double staining, inflammatory cells revealed a secretion of the profibrotic growth factor TGF-β (Figure 4). More cells (mostly cardiomyocytes and endothelial cells) in the HFNEF group were positive after DHE staining as a marker for radical oxygen production in comparison to controls (Figure 2). No change in systemic inflammation measured as CRP was found between the groups (1.3 ± 0.5 vs 1.1 ± 0.3, mg/dl n.s., p=0.45).

**Fibroblast cell culture**

After stimulation with TGF-β for 6 and 24 h CTGF and collagen type 1A1 mRNA was up regulated compared to their individual controls. The transdifferentiation to myofibroblasts, as suggested by α-SMA mRNA expression was observed after 24h of stimulation with TGF-β.
MMP-1 was not increased after 6h and 24h, while MMP-2 was up-regulated after 24h. With an increased TIMP-1 expression, the MMP-1/TIMP-1 ratio as one indicator of MMP-1 activity was down regulated after stimulation with TGF-β for 6h or 24h (Figure 5).

**THP-1 cell culture**

Activated THP-1 cells produced TGF-beta mRNA compared to their not activated controls. This was followed by a time dependent TGF-beta protein production, which showed significantly increased protein levels after 24h after stimulation of the THP-1 monocytes. (Figure 4b)
Discussion

The salient finding of the current study is that cardiac inflammatory cells documented in endomyocardial biopsies of patients with HFNEF induce extracellular remodelling with increased accumulation of collagen. TGF-β, excreted by inflammatory cells, is one potent stimulus for the regulatory changes of the extracellular matrix, as shown in cell culture experiments using primary cardiac fibroblasts derived from endomyocardial biopsies of patients with HFNEF. These changes contribute to diastolic dysfunction, which is one of the underlying pathologies of HFNEF, which was documented by a correlation of collagen as well as inflammatory cells to diastolic dysfunction.

The molecular changes leading to diastolic dysfunction are still debated and different pathways can affect the pathology of HFNEF. Altered isoform expression of the giant muscle protein titin can determine the elastic properties of the heart. Moreover, increased myocyte tension, which could be prevented by experimental phosphorylation of titin, contributes to diastolic dysfunction in patients with severe HFNEF. Additional modulatory effects on titin stiffness may arise from disulfide bonding under oxidant stress also affecting LV compliance. Nevertheless, the steep part of the diastolic pressure volume relationship is mainly modified by extracellular matrix, as one can see when comparing stretch lengths between whole muscle stripes compared to single myocytes, suggesting that an excess of collagen might furthermore aggravate diastolic dysfunction.

Cardiac collagen is a stable protein with a low turnover (80-120 days), but its balance can get lost in pathological conditions. Next to ischemia, which may lead to replacing fibrosis, e.g. increased wall stress, angiotensin II, and TGF-β may induce pro-fibrotic processes leading to pathological tissue fibrosis. The tensile strength of collagen type I approximates that of steel. Therefore, LV chamber stiffness in vivo will be affected by the amount of
cardiac extracellular matrix. We could show in this study, that total cardiac collagen content is increased in patients with HFNEF, which is in agreement with others showing increased fibrosis in this disease. Furthermore, not only collagen type I was found to be increased in this study population, but there was also a change in the collagen type I/III ratio in favour to the stiffer collagen type I, similar to patients with systolic heart failure. Next to increased protein levels of collagen type I and type III also their mRNA abundance was increased in endomyocardial biopsies of patients with HFNEF compared to controls. Another marker of excessive collagen production is ITCP, a degradation product of collagen showing increased collagen turnover together with PIP, a serum marker of collagen production, both seen increased in the HFNEF collective in this study. Oxidative stress was increased in HFNEF patients, as DHE staining revealed, which might be the results of increased LV stiffness.

Multiple studies show that one of the best known inductor of collagen production is the profibrotic growth factor TGF-beta. It has also profound effects on extracellular matrix homeostasis, in part via its ability to alter the balance between matrix proteinases (MMPs) and their inhibitors (TIMPs). The endogenous collagen degradation system is regulated by increased activity of MMPs overcoming their tissue inhibitors. Nevertheless, there exist different MMPs and the substrate affinity of those proteases is different. MMP-1 (interstitial collagenase) is known to degrade collagen fibers, and therefore it is suggested, that it favours collagen degradation. Via the transcription factor AP-1, TGF-β can repress MMP-1 gene expression and on the other hand increase TIMP-1 expression. Coherently to these ex vivo data, we show an up regulation of TIMP-1 protein and a down regulation of MMP-1 protein levels in the biopsies of the HFNEF patients, which leads to a significant decrease in the MMP-1/TIMP-1 ratio. This inhibition of the collagen degradation system could be one mechanism contributing to the accumulation of ECM in HFNEF patients and therefore trigger diastolic dysfunction over a longer time period. Interestingly, Lopez and colleagues showed
that this ratio was increased in patients with systolic heart failure, while it was unchanged in their hypertensive heart failure group.\textsuperscript{22} If this process is dynamic and may change with time or if this mechanism represents a distinct difference between both heart failure subtypes remains to be clarified.

Furthermore, and in contrast to the AP-1-mediated downregulation of MMP-1, we found increased levels of MMP-2 (controlled by AP-2), which is known as a gelatinase and has a substrate affinity for denatured fibrillar collagen as well as for the basement membrane.\textsuperscript{42} Studies exist, which describe the MMP-2 serum levels in patients with hypertensive and diastolic heart failure, but the results are not consistent, with some studies showing increment\textsuperscript{43}, others showing no difference or even decrement of MMP-2.\textsuperscript{33} Recently, increased levels of MMP-2 were shown to predict heart failure in patients with diastolic dysfunction and hypertension. In that study, MMP-2 was a better prognostic marker than the well known heart failure biomarker brain natriuretic peptide.\textsuperscript{29} There exist experimental studies utilizing MMP-2 knock out animals, which help to understand the molecular function of MMP-2. Matsumura and colleagues showed in MMP-2 knockout mice a decrease in invading inflammatory cells and a decrease in LV rupture after myocardial infarction.\textsuperscript{44} Those authors demonstrate that destruction of basement membrane proteins facilitate the transendothelial migration of immunocompetent cells, thereby triggering cardiac inflammation.

Therefore we investigated the amount of inflammatory cells in our patients and we show here that HFNEF is associated with increased cardiac inflammation with high numbers of CD3\textsuperscript{+}, CD11a\textsuperscript{+} and CD45\textsuperscript{+} cells. Moreover, the adhesion molecule VCAM-1, attracting immunocompetent cells to the endothelium to start the transendothelial migration, is also increased in the HFNEF group in our study. This is especially interesting, since VCAM-1 is up regulated by angiotensin II, which may be increased in regard to the known risk factors
like hypertension and diabetes mellitus in HFNEF. Recently, it was shown that
immunocompetent cells like T-cells (CD3+) can indeed alter tissue remodelling in vitro and
we could show that cardiac inflammation is associated with excessive collagen accumulation
in experimental diabetic cardiomyopathy, as one animal model of HFNEF. There is
experimental and clinical evidence that inflammatory cells might modulate cardiac function in
heart failure with reduced EF and normal EF. The direct effects of these cells are still
under debate, but it is suggested that increased inflammation is associated with the
development of systolic heart failure by distinct changes in the MMP/TIMP. In line with
these data, we show here that these inflammatory cells express TGF-beta in the cardiac tissue
shown by immunohistochemistry double staining. Moreover, THP-1 cells, when they become
activated, which is one hallmark to undergo transendothelial migration, produce TGF-beta on
mRNA and protein level, as we show here in vitro using PMA activated THP-1 cells.
Moreover, TGF-beta gene expression was increased in the HFNEF biopsies.

To test the direct effect of TGF-beta expressed by the inflammatory cells in this study, we
performed experiments using a cell culture system utilizing primary human cardiac fibroblasts
derived from endomyocardial biopsies of HFNEF patients. After stimulation with TGF-beta,
fibroblasts expressed increased amount of alpha-smooth muscle actin, one marker for their
transdifferentiation into the pathological activated myofibroblasts. Coherently, CTGF was
increased leading to higher collagen gene expression after 6h and 24h, which explains
collagen accumulation found in the endomyocardial biopsies. On the other hand, the
degradation system of collagen, especially MMP-1/TIMP-1 ratio, was down regulated after
stimulation with TGF-beta in the cell culture system, which is another explanation for the
extensive fibrosis seen in HFNEF hearts. MMP-2, in line with its known function in
degradating basement membrane to allow for easier transendothelial migration of inflammatory
cells into the cardiac tissue, was upregulated after TGF-beta stimulation. Interestingly, TGF-
beta gene expression was increased after stimulation, which suggests that already transdifferentiated myofibroblasts may induce an activation of further fibroblasts and therefore enhance the pro-fibrotic process in a paracrine fashion.

Viral agents are one possible inducer of cardiac inflammation. The parvovirus B19, which is associated with endothelial and diastolic dysfunction, is known to cause myocarditis. Nevertheless, recently it was shown in patients with dilated cardiomyopathy, that only a viral load above 500 genome equivalents is a clinically relevant threshold for the maintenance of myocardial inflammation. Since this was ruled out in all patients in this study, the exact mechanism of cardiac inflammation has still to be determined, although animal models of heart failure are often associated with cardiac inflammation. Recently, it was shown that activated myofibroblasts also produce chemokines in order to fuel the inflammatory process, which might induce a vicious cycle of inflammation triggering fibrosis and diastolic dysfunction. If hemodynamic changes in HFNEF induce pro inflammatory changes to start the cardiac inflammation has to be determined in detail in further studies.

We suggest that small numbers of invading inflammatory cells stimulate cardiac fibrosis by expressing TGF-beta and inducing a pathological transdifferentiation from fibroblasts to myofibroblasts. This will stimulate not only gene expression of collagen, but also induce a decrement of MMP-1 activity and an increment of MMP-2 mRNA abundance as well as a paracrine TGF-beta production. Increased stiffness due to extracellular matrix accumulation will increase oxidative stress, suggested to be associated with increased endothelial activation. Together with increased MMP-2, suggested to disrupt the basal membrane, this might induce a vicious circle fuelling inflammation leading to fibrosis and ultimately to progression of the disease.
Conclusion

In this study HFNEF was characterized by an increase in cardiac inflammation. This inflammatory process triggers cardiac collagen accumulation by inducing collagen gene expression and inhibiting the cardiac degradation system. Inhibiting the transendothelial migration of inflammatory cells into the cardiac tissue might be a future therapeutic concept in HFNEF.
Sources of Funding

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Disclosures

None.
References


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Table 1.

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<tr>
<th>Patient characteristics</th>
<th>Control (n=8)</th>
<th>HFNEF (n=20)</th>
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<td>573 (389-949)</td>
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Patients characteristics, data are shown as median (25% - 75% percentile). † Fischers exact test, # t-test
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<th>Table 2.</th>
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<tr>
<td><strong>Control</strong></td>
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<td><strong>Chamber Dimensions</strong></td>
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<tr>
<td>Septum (mm) 9.8 (8.3-10.2)</td>
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<td>Posterior wall (mm) 9.2 (8.6-9.5)</td>
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<td><strong>Tissue Doppler</strong></td>
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<td>LVP (mmHg) 131 (117-145)</td>
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<td>dP/dt max. (mmHg/sec) 1783 (1509-2106)</td>
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<td>ESPVR (mmHg/ml) 1.1 (0.8-1.3)</td>
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<td>LVEDP (mmHg) 6 (4-9)</td>
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<td>Tau (ms) 48 (40-49)</td>
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<td>Diastolic stiffness 0.01 (0.006-0.04)</td>
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Echocardiographic and hemodynamic results, data are shown as median (25% - 75% percentile). LVEDD: LV enddiastolic diameter, LVP: LV pressure, ESPVR: endystolic pressure volume relationship, LVEDP: LV enddiastic pressure, Tau: Isovolumetric relaxationtime. # t-test
Figure Legends

**Figure 1.** Increased protein levels of collagen type I and type III in endomyocardial biopsies of patients with HFNEF compared to controls. Increased collagen type 1/3 protein ratio as well as increased levels of CITP, measured in the serum. Moreover, mRNA abundance of collagen type I and type III were increased in HFNEF compared to controls. * p<0.05 compared to controls.

**Figure 2.** Decreased protein levels of matrix metalloproteinases-1 (MMP1) as well as increased levels of MMP2 and TIMP1 in endomyocardial biopsies of patients with HFNEF compared to controls. Increased oxidative stress by DHE staining was documented in HFNEF compared to controls. Moreover the adhesion molecule VCAM-1 was also increased in HFNEF compared to controls. * p<0.05 compared to controls.

**Figure 3.** Increased numbers of CD3⁺, Cd11⁺ as well as CD45⁺ cells in endomyocardial biopsies of patients with HFNEF compared to controls. * p<0.05 compared to controls.

**Figure 4.** (A) Representatives histological picture with double stainings of CD11⁺ cells with the profibrotic growth factor TGF-beta. (B) In vitro experiments with PMA activated monocytes (THP-1 cells) show increased production of TGF-beta mRNA as well as increased protein levels of TGF-beta in a time dependent manner. * p<0.05 compared to individual controls.
Figure 5. In vitro cell culture experiments utilizing primary cardiac fibroblasts stimulated with TGF-beta for 6 and 24 hours. Increased gene expression of alpha-smooth muscle actin (a-SMA), connective tissue growth factor (CTGF), collagen type I in stimulated cells compared to not stimulated cells. MMP-1 was decreased after stimulation with TGF-beta. MMP-2 was increased after stimulation.

* p<0.05 compared to the relative controls
CD45+ cells / mm²

Control HFNEF

P<0.001

CD11a+ cells / mm²

Control HFNEF

P=0.002

CD3+ cells / mm²

Control HFNEF

P<0.001

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