Diastolic Heart Failure
Evidence of Increased Myocardial Collagen Turnover Linked to Diastolic Dysfunction

Ramón Martos, MB; John Baugh, PhD; Mark Ledwidge, PhD; Christina O’Loughlin, DPhil; Carmel Conlon, BSc; Anil Patle, BTech; Seamas C. Donnelly, MD; Kenneth McDonald, MD

Background—The pathophysiology of diastolic heart failure (DHF) is poorly understood. One potential explanation is an active fibrotic process that produces increased ventricular stiffness, which compromises filling. The present study investigates collagen metabolism in hypertensive patients in different phases of diastolic function with and without proven DHF.

Methods and Results—We studied 86 hypertensive patients divided into groups according to the presence of DHF (32 with, 54 without) and phase of diastolic function (20 with normal function, 38 with impaired relaxation, 10 with pseudonormalization, and 16 with restrictive-like filling). Serum carboxy-terminal, amino-terminal, and carboxy-terminal telopeptide of procollagen type I, amino-terminal propeptide of procollagen type III, matrix metalloproteinases (MMPs; total MMP-1, active MMP-2, and MMP-9), and tissue inhibitor of MMPs levels were assayed by radioimmunoassay and ELISA. Doppler-echocardiographic assessment of diastolic filling was made with measurements of E/A ratio, E-wave deceleration time, and isovolumic relaxation time. Serum carboxy-terminal telopeptide of procollagen type I, carboxy-terminal telopeptide of procollagen type I, amino-terminal propeptide of procollagen type III, MMP-2, and MMP-9 levels ($P<0.001$ for all, controlled for age and gender) were greater in patients with DHF than in those without. When we controlled for age and gender, levels of serum carboxy-terminal telopeptide of procollagen type I, tissue inhibitor of MMP-1, amino-terminal propeptide of procollagen type III (all $P<0.001$), carboxy-terminal telopeptide of procollagen type I ($P=0.008$), and MMP-2 ($P=0.03$) were greater in more severe phases of diastolic dysfunction. Within phases of diastolic dysfunction, serum carboxy-terminal telopeptide of procollagen type I, amino-terminal propeptide of procollagen type III, MMP-2, and MMP-9 were elevated in those with DHF compared with those without DHF (all $P<0.001$).

Conclusions—These data demonstrate serological evidence of an active fibrotic process in DHF, which is more marked in more severe diastolic dysfunction. This observation may help explain the pathophysiology of DHF and may suggest new avenues for diagnostic and therapeutic intervention. (Circulation. 2007;115:888-895.)

Key Words: heart failure ■ diastole ■ hypertension ■ myocardium ■ metalloproteinases ■ collagen

Diastolic heart failure (DHF) is an important contributor to the heart failure syndrome, with reported prevalence rates ranging from 20% to 50% of the total heart failure population in clinical practice. The central disturbance in DHF involves abnormalities in myocardial relaxation and ventricular compliance. Although still poorly understood, a growing body of data has laid the foundation for a better understanding of the pathophysiological mechanisms that underlie DHF. A primary cause of DHF is hypertensive heart disease (HHD), a syndrome characterized by structural and functional abnormalities of the heart as a result of the hypertensive process. Experimental and clinical data have demonstrated serological and morphometric evidence of increased myocardial fibrosis in HHD. These observations have been directly linked to abnormalities in diastolic function and myocardial stiffness. Furthermore, therapies that reduce the fibrosis content of ventricles have been shown to improve diastolic function. These observations support the hypothesis that a change in the extracellular matrix of the myocardium, characterized by the formation of excess collagen tissue, is a cause of worsening diastolic dysfunction (DD), eventually leading to DHF. Although there has been considerable work done on collagen turnover in HHD, there are no data linking the extent of abnormalities of...
collagen turnover to the severity of DD and DHF. Recently, important work by Ahmed et al\textsuperscript{15} has demonstrated an association between elevated levels of tissue inhibitor of matrix metalloproteinase (TIMP)-1 with DD. Moreover, this work identified higher levels in patients with DHF than in those without.

Myocardial interstitial collagen content, historically measured by endomyocardial biopsy, can now be assessed with serum analysis of breakdown products of collagen I and collagen III, the major myocardial collagens.\textsuperscript{11,16} Enzymes that control collagen turnover, specifically, matrix metalloproteinase (MMP) and TIMP, can also be measured with serum analysis.\textsuperscript{15,16} Using similar methods, the present study aims to investigate the relationship between serum markers of collagen turnover, the extent of DD, and DHF.

**Methods**

**Subjects**

All subjects gave written informed consent to participate in the study. The Ethics Committee at St Vincent’s University Hospital approved the study protocol, which conformed to the principles of the Helsinki Declaration. The study population consisted of 86 white hypertensive patients, referred from the cardiology service at St Vincent’s University Hospital and subdivided by previously defined criteria into those with normal diastolic function and the following phases of DD: impaired relaxation, pseudonormalization, and restrictive-like filling.\textsuperscript{17} Furthermore, the study population was categorized according to the presence (DHF group, n=32) or absence (No-DHF group, n=54) of DHF. The diagnosis of DHF was based on the presence of all of the following criteria: 1 hospitalization for proven New York Heart Association class IV heart failure (confirmed by a consultant cardiologist) with continued signs or symptoms of heart failure (at least New York Heart Association class II level) as defined by European Society of Cardiology guidelines,\textsuperscript{18} left ventricular ejection fraction >45% with Doppler abnormalities of DD, and no significant evidence of valvular disease.

Patients were excluded if they had established pulmonary disease or anemia, which may make the diagnosis of DHF more difficult. We also excluded patients with renal insufficiency (serum creatinine >130 mmol) and conditions known to alter collagen turnover, including chronic liver disease, connective tissue disorders, metabolic bone diseases, and malignancy, and those who underwent recent trauma or surgery (<6 months).

A prerequisite of the present study dictated that all patients were clinically stable for 1 month (as defined by freedom from hospitalization or change in medication) before enrolment. All patients had appropriate clinical and laboratory evaluation to identify exclusion criteria and suitability for the study.

**Biochemical Measurements of Indices of Collagen Metabolism**

Peripheral venous blood samples were drawn during clinical assessment and immediately underwent serum isolation. Each sample was centrifuged for 10 minutes at 4°C. The serum was then separated into aliquots and stored at −80°C before simultaneous analysis of collagen turnover markers as described below.

Amino-terminal propeptide of procollagen type I (PINP) and type III (PIINP) and carboxy-terminal telopeptide of collagen type I (CTTP) were measured by radioimmunoassay with commercial antisem kits (Orion Diagnostica, Espoo, Finland). The intraassay variations for determining PINP, PIINP, and CTTP were 7%, <5%, and <8% respectively. The sensitivity (lower detection limit) of the assays was 13 μg/L for PINP, 1.9 μg/L for PIINP, and 0.5 μg/L for CTTP, respectively. Carboxy-terminal propeptide of procollagen type I (PICP) was measured with a specific ELISA according to the manufacturer’s method (Takara Biochemicals Co, Osaka, Japan). The sensitivity for PICP was 2 ng/mL.

All plasma MMP and TIMP levels were measured with 2-site sandwich ELISAs (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) per the manufacturer’s protocol. The MMP-1 assay (RPN2610; sensitivity 1.7 ng/mL) detects both free MMP-1 and that complexed with inhibitors such as TIMP-1. The MMP-2 assay (RPN 2617; sensitivity 0.37 ng/mL) detects the proform of MMP-2 and that complexed with TIMP-2; the MMP-9 assay (RPN 2614; sensitivity 0.6 ng/mL) detects the proform of MMP-9 and that complexed with TIMP-1; the TIMP-1 assay (RPN 2611; sensitivity 1.25 ng/mL) detects both free TIMP-1 and that complexed with MMPs. Duplicate measurements were performed, and the intra-assay coefficients of variation were <10% for all assays. Plasma B-type natriuretic peptide (BNP) levels were also measured with the Biosite Triage BNP test (Biosite, San Diego, Calif) in all patients.

**Echocardiography Study**

Two-dimensional echocardiography imaging, targeted M-mode echocardiography, and Doppler ultrasound measurements were obtained. M-mode measurements were taken according to the guidelines laid down by the American Society of Echocardiography. All echocardiography data represent the mean of 3 measurements on different cardiac cycles. Left ventricular ejection fraction was calculated by the Teichholz method. All measurements were made by blinded observers, with archive images recorded in a blinded fashion. The following pulsed Doppler measurements were obtained in the apical view with a cursor at the mitral valve inflow: maximal early (E) and late (A) transmitral velocities in diastole and E-wave deceleration time. Isovolumic relaxation time was measured in the apical 4-chamber view by continuous-wave Doppler placed between the mitral inflow area and the left ventricular outflow tract. Left ventricular DD was defined by the presence of alterations in E/A ratio, isovolumic relaxation time, and deceleration time. Left ventricular diastolic filling patterns were classified as previously described by Lubien et al.\textsuperscript{17} None of the patients studied exhibited left ventricular systolic dysfunction, as assessed by an ejection fraction ≤45%.

**Statistical Analysis**

Data are presented as the mean±SD for continuous variables, whereas frequencies and percentages (in parentheses) summarize categorical variables. Comparisons between No-DHF and DHF groups were conducted with independent sample t tests and ANCOVA for normally distributed continuous variables and Mann-Whitney U test and Kruskal-Wallis ANOVA for nonnormal distributions (α=0.05). We used χ\textsuperscript{2} analysis to compare categorical variables. Partial correlation coefficients, adjusted for age, were calculated to assess the relationship between echocardiographic Doppler parameters and biochemical markers. Variables with nonnormal distributions were log-transformed where appropriate. All statistical calculations were performed with SPSS software (version 11; Statistical Package for Social Sciences, Chicago, Ill, 2001).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Baseline Characteristics**

The demographics of the study population are presented in Table 1. The DHF group was older (72±11 versus 67±9 years), with a larger number of females (47% versus 24%), and higher body mass index (29.8±6.1 versus 27.3±4.5 kg/m\textsuperscript{2}) than the No-DHF group (P=0.03 for all). No differences were found in systolic or diastolic blood pressure between the groups. Pharmacotherapy was similar except for the anticipated higher use of loop diuretics (frusemide or
Markers of Collagen Turnover and DHF

PICP, CFTP, PIINP, MMP-2, and MMP-9 levels were greater in the DHF group than in the No-DHF group (P<0.001 for all; Figure 1), even when adjusted for the effects of age and gender. A trend toward higher levels of PINP in the DHF group (51.7±42.0 versus 42.0±22.1 μg/L, P=0.09) was also observed. There were no differences between the groups in measurements of MMP-1 (12.8±9.9 versus 10.4±6.7 ng/mL, P=0.42) or TIMP-1 levels (642.2±265.7 versus 562.4±157.2 ng/mL, P=0.30).

Relation Between Markers of Collagen Turnover and Phases of Diastolic Function

After adjustment for the effects of age and gender, patients in the restrictive-like filling group, most of whom had DHF, had shorter deceleration time and higher E/A ratios than those in the impaired relaxation and pseudonormal filling groups (P<0.001 for both; Table 2). No differences were found in isovolumic relaxation time between the groups (P=0.68).

The data demonstrate increases in PICP, CFTP, PIINP, MMP-2, and BNP across the phases of diastolic function. These differences remained significant when adjusted for age and gender effects. Although the numbers were small, it is also of interest that within similar phases of DD, several markers of collagen turnover (PICP, CFTP, PIINP, MMP-2, and MMP-9) were more elevated in the presence of heart failure (Table 3). There was a significant correlation (age-adjusted) observed between left atrial volume index and PICP (r=0.50, P<0.001; Figure 2), PINP (r=0.37, P=0.003), PIINP (r=0.35, P=0.006), CFTP (r=0.37, P=0.004), and MMP-2 (r=0.40, P=0.001; Figure 2).
Discussion

The results of the present study provide further information on collagen metabolism in patients with asymptomatic DD and DHF. Elevated levels of PICP and PIIINP and a trend toward an increase in PINP levels indicate increased collagen synthesis in DHF. In addition, elevated serum levels of CITP, MMP-2, and MMP-9 suggest increased degradation of myocardial collagen and other components of the extracellular matrix. Furthermore, greater collagen turnover is seen in more severe phases of DD, which suggests a direct relationship between DD and collagen excess. Finally, within a given phase of DD, several markers of collagen turnover are more elevated in the presence of heart failure.

It is generally accepted that the central problem of DHF is a stiff, noncompliant ventricle.3,19 The cause of this remains unknown, but earlier data from experimental and clinical studies in HHD,5,6,9,12,20–22 a frequent cause of DHF, provide some insight into this unresolved question. Studies using postmortem hearts20 or endomyocardial biopsy samples9,12,21,22 have demonstrated that myocardial interstitial fibrosis is one of the key pathological features of myocardial remodeling in HHD. These data have established the link between fibrillar collagen accumulation and tissue stiffness in HHD.

All of the above work was performed with tissue sampling, which presents an impediment to the clinical investigation of the fibrotic process in DD and heart failure. Recent work, however, has demonstrated that serum analysis of collagen-derived peptides and enzymes involved in their degradation provides a verified, noninvasive technique to measure this fibrotic process.9,11–16

With serum analysis, PICP has been one of the most widely studied procollagen markers.7,9,11–15 It is a breakdown product of fibrillar collagen type I, which accounts for 85% to 90% of myocardial collagen. A stoichiometric ratio of 1:1 exists between the numbers of collagen type I molecules produced and PICP released into blood stream and cleared by the liver.11,23 Type III collagen accounts for the majority of the remaining myocardial collagen. PIIINP is an extension peptide of procollagen type III, which is cleaved off during conversion from type III procollagen to type III collagen and then released into the blood stream. As with PICP, PIIINP is also eliminated from the blood by the liver. Although serum PIIINP has been proposed as a useful marker of fibrogenesis,1,24 this peptide is not completely removed from its procollagen precursor during the extracellular processing of collagen type III.24 In contrast, the removal of PICP is complete; hence, serum PICP may reflect fibrogenesis more accurately than serum PIIINP.
Other commonly assessed serum markers of myocardial collagen turnover include the metalloproteinase enzymes, their tissue inhibitors, and CITP, which is a breakdown product of type I collagen. Elevated levels of MMP-2, MMP-9, and TIMP-1 have been demonstrated in a population with HHD14,25,26 and in a population with hypertrophic obstructive cardiomyopathy.16

In a recent study examining a mixed heart failure population, Querejeta et al7 demonstrated a positive correlation between coronary sinus and peripheral serum levels of PICP in heart failure patients. Moreover, there was a significant increase in PICP levels when this heterogeneous heart failure population was compared with HHD patients. More recently, Ahmed et al15 have demonstrated elevated serum levels of TIMP-1 and MMP-9 in patients with left ventricular hypertrophy and DHF.

The specific pattern of activation of collagen markers noted in the present study consisted of a significant increase in PICP and PIIINP in patients with established DHF, supportive of the hypothesis that a predominant pathophysiological process in DHF is abnormal accumulation of fibrous tissue. This observation is consistent with the recent data of Lopez et al,27 who demonstrated a significant increase in total collagen volume fraction in patients with DHF. We also noted elevated levels of MMP-2 and MMP-9 in the DHF population, similar to previous studies on HHD.25,26 On one level,
this observation of elevated levels of enzymes known to be responsible for breakdown of interstitial proteins may seem counterintuitive in the setting of increased fibrosis. Elevated levels of metalloproteinase-2 and -9, however, have been associated with profibrotic remodeling.28,29 Furthermore, MMP-2 and MMP-9 have elastase activity and are associated with increased arterial stiffness in hypertensive patients.26 Therefore, increases in MMP-2 and MMP-9 in the present work may reflect loss of elastin and other components of the myocardial extracellular matrix, contributing to stiffness, more severe DD, and ultimately, DHF. The observation of increased MMP-2 is counter to the data of Ahmed et al,15 in which no change in this MMP was noted. The explanation for this difference is unclear, although the populations were not entirely similar and, in particular, the definition of DHF in the present study was more stringent and would have directed enrollment to older and more severely ill patients. Another notable result from the present study in the comparison of DHF and No-DHF groups was the lack of change in the MMP-1/TIMP-1 ratio. This observation is again consistent with the data of Lopez and colleagues,27 who noted no change in this ratio in DHF and an increase only in the presence of systolic heart failure.

The present study also demonstrated a close relationship between the extent of DD and increasing levels of many of the markers of collagen turnover, which supports the concept that this interstitial disease is responsible for diastolic impairment. This association was further supported by the close correlation between several of these markers and left atrial volume index, a continuous variable linked to diastolic function.30 Lubien et al17 demonstrated a relationship between BNP and phase of DD, a finding again confirmed in the present data. In fact, the increasing levels of BNP with worsening diastolic function may reflect a response to myocardial fibrosis, because several experimental data indicate that BNP has antifibrotic properties.31,32 Finally, we noted

### Table 3. Comparison of Markers of Collagen Turnover and BNP Across the Phases of DD in Patients With and Without DHF

<table>
<thead>
<tr>
<th></th>
<th>Impaired Relaxation</th>
<th>Pseudonormal</th>
<th>Restrictive-Like</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No-DHF (n=24)</td>
<td>DHF (n=14)</td>
<td>No-DHF (n=4)</td>
<td>DHF (n=6)</td>
</tr>
<tr>
<td>PICP, ng/mL</td>
<td>196.8±92.3</td>
<td>321.3±112.8</td>
<td>367.0±42.3</td>
<td>435.9±123.9</td>
</tr>
<tr>
<td>CTIP, μg/mL</td>
<td>4.7±2.0</td>
<td>5.6±1.9</td>
<td>4.9±0.9</td>
<td>7.9±2.2</td>
</tr>
<tr>
<td>PIIINP, μg/mL</td>
<td>4.0±1.2</td>
<td>4.2±0.6</td>
<td>4.0±0.3</td>
<td>5.8±2.2</td>
</tr>
<tr>
<td>MMP-2, ng/mL</td>
<td>1480.3±133.3</td>
<td>1781.5±265.0</td>
<td>1375.5±128.7</td>
<td>1834.2±168.8</td>
</tr>
<tr>
<td>MMP-9, ng/mL</td>
<td>87.5±51.8</td>
<td>197.2±119.2</td>
<td>79.5±35.9</td>
<td>240.0±181.1</td>
</tr>
<tr>
<td>MMP-1, ng/mL</td>
<td>11.2±6.3</td>
<td>13.6±12.2</td>
<td>8.3±4.8</td>
<td>11.7±6.1</td>
</tr>
<tr>
<td>TIMP-1, ng/mL</td>
<td>565.0±114.0</td>
<td>468.7±112.6</td>
<td>644.3±143.6</td>
<td>769.7±207.7</td>
</tr>
<tr>
<td>MMP-1/TIMP-1</td>
<td>0.019±0.011</td>
<td>0.035±0.035</td>
<td>0.013±0.006</td>
<td>0.016±0.009</td>
</tr>
<tr>
<td>PINP, μg/L</td>
<td>38.5±13.8</td>
<td>41.0±18.9</td>
<td>52.7±27.2</td>
<td>41.1±21.4</td>
</tr>
<tr>
<td>BNP, pg/mL</td>
<td>69.4±60.9</td>
<td>247.4±155.3</td>
<td>49.8±65.5</td>
<td>190.5±115.9</td>
</tr>
</tbody>
</table>

Values are mean±SD.

*The P value refers to the main effect of the absence or presence of DHF across the phases of DD for each marker of collagen turnover. Because the sample size in some groups is very small, caution must be taken when interpreting the results.

Figure 2. Scatterplots of the relationship between echocardiographic-Doppler parameter left atrial (LA) volume index with fibrosis markers PICP and MMP-2. All partial correlations are adjusted for age.
Finally, these data may support the development of new ventricular compliance characteristic of these syndromes. Between these 2 observations and may explain the reduced collagen turnover and degree of DD strengthens the link. Furthermore, the demonstrated association between extent of DHF, which may result in type II error.

Effective management of DHF has been impeded to date by a paucity of proven therapies, which possibly reflects a poor understanding of the pathogenesis of this syndrome. The growing evidence that points to a role for abnormal collagen accumulation provides a rationale for the examination of several potential therapeutic approaches in this syndrome. This includes therapies that alter the renin-angiotensin-aldosterone system, which have been shown in several clinical studies to modify the myocardial fibrotic process and improve diastolic function. Furthermore, it has been demonstrated that tonsensei, a loop diuretic, reduces PICP levels and reverses collagen volume fraction in patients with chronic heart failure.

In interpreting these data, certain limitations of the present study need to be taken into consideration. First, the present study relied on peripheral markers of collagen turnover without supportive endomyocardial biopsy data or coronary sinus sampling. Second, strict criteria were used to diagnose DHF, with all diagnoses confirmed at the time of presentation by a staff cardiologist. This was done to ensure that the heart failure population was truly representative of DHF syndrome, although in doing so, we clearly excluded those with less severe manifestations of this syndrome. Furthermore, it is also possible that some of the asymptomatic group had subtle symptoms of heart failure, especially those with more severe manifestations of DD. Third, although we included Doppler echocardiographic evidence of DD, we did not perform tissue Doppler, pulmonary venous flow measurements, or invasive confirmation of DD. Fourth, differences in medicines that can attenuate fibrosis across the groups may be a potential limitation to the interpretation of the results; however, because there was greater usage of these medicines in DHF and more severe DD, it may serve to emphasize the role of these markers. Finally, sample sizes were small, and groups were of unequal size when mean levels of collagen turnover markers were compared across the patterns of DD in those with and without DHF, which may result in type II error.

Conclusions

These original findings demonstrate the presence of increased collagen turnover in patients with established DHF and DD. Furthermore, the demonstrated association between extent of collagen turnover and degree of DD strengthens the link between these 2 observations and may explain the reduced ventricular compliance characteristic of these syndromes. Finally, these data may support the development of new diagnostic and therapeutic strategies in DHF and DD.

Disclosures

None.

References

The present study of 86 hypertensive patients provides original information on collagen metabolism in patients with asymptomatic diastolic dysfunction and diastolic heart failure. Elevated peripheral serum levels of carboxy-terminal propeptide of procollagen type I, amino-terminal propeptide of procollagen type III, and a trend toward an increase in amino-terminal propeptide of procollagen type I levels indicate increased collagen synthesis in diastolic heart failure. In addition, elevated serum levels of carboxy-terminal telopeptide of collagen type I, and matrix metalloproteinases 2 and 9 suggest increased degradation of myocardial collagen and other components of the extracellular matrix in diastolic heart failure. Levels of carboxy-terminal propeptide of procollagen type I, tissue inhibitor of matrix metalloproteinases-2 and 9, amino-terminal propeptide of procollagen type III, carboxy-terminal telopeptide of collagen type I, and matrix metalloproteinase-2 increased in more severe phases of diastolic dysfunction, which suggests a direct relationship between diastolic dysfunction and collagen excess. In addition, within phases of diastolic dysfunction and despite small numbers, several markers of collagen turnover were elevated in those with diastolic heart failure compared with those without. These findings demonstrate the presence of increased collagen turnover in patients with established diastolic heart failure and diastolic dysfunction. Furthermore, the demonstrated association between extent of collagen turnover and degree of diastolic dysfunction strengthens the link between these 2 observations and may explain the reduced ventricular compliance characteristic of these syndromes. Finally, these data may support the development of new diagnostic and therapeutic strategies in diastolic heart failure and diastolic dysfunction.
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