Multi-Analyte Profiling Reveals Matrix Metalloproteinase-9 and Monocyte Chemotactic Protein-1 as Plasma Biomarkers of Cardiac Aging

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Background—We have previously shown that cardiac sarcopenia occurs with age in C57/BL6J mice. However, underlying mechanisms and plasma biomarkers of cardiac aging have not been identified. Accordingly, the objective of this study was to identify and evaluate plasma biomarkers that reflect cardiac aging phenotypes.

Methods and Results—Plasma from adult (7.5±0.5 months old, n=27) and senescent (31.7±0.5 months old, n=25) C57/BL6J mice was collected, and levels of 69 markers were measured by multi-analyte profiling. Of these, 26 analytes were significantly increased and 3 were significantly decreased in the senescent group compared with the adult group. The majority of analytes that increased in the senescent group were inflammatory markers associated with macrophage functions, including matrix metalloproteinase-9 (MMP-9) and monocyte chemotactic protein-1 (MCP-1/CCL-2). Immunoblotting (n=12/group) showed higher MMP-9 and MCP-1 levels in the left ventricle (LV) of senescent mice (P<0.05), and their expression levels in the LV correlated with plasma levels (ρ=0.50 for MMP-9 and ρ =0.62 for MCP1, P<0.05). Further, increased plasma MCP-1 and MMP-9 levels correlated with the increase in end-diastolic dimensions that occurs with senescence. Immunohistochemistry (n=3/group) for Mac-3, a macrophage marker, showed increased macrophage densities in the senescent LV, and dual-labeling immunohistochemistry of Mac-3 and MMP-9 revealed robust colocalization of MMP-9 to the macrophages in the senescent LV sections, indicating that the macrophage is a major contributor of MMP-9 in the senescent LV.

Conclusions—Our results suggest that MCP-1 and MMP-9 are potential plasma markers for cardiac aging and that augmented MCP-1 and MMP-9 levels and macrophage content in the LV could provide an underlying inflammatory mechanism of cardiac aging. (Circ Cardiovasc Genet. 2011;4:455-462.)

Key Words: aging ■ biomarker ■ macrophage ■ metalloproteinase ■ myocardium

The prevalence of cardiovascular disease increases with age, and aging is a major risk factor for cardiac morbidity and mortality.1,2 Studies in human patients and in animal models report higher mortality rates in aged groups with cardiovascular disease. Specifically, the incidence of death positively associated with post–myocardial infarction (MI) left ventricular (LV) rupture increases with age.3-5 The contributing mechanisms of cardiac aging, however, are not well understood. We have previously shown that cardiac sarcopenia occurs with age in C57/BL6J mice, in the absence of hypertension.6 Senescent C57/BL6J mice exhibit increased LV collagen deposition, increased end-diastolic and end-systolic dimensions, and decreased fractional shortening and ejection fraction.6 In the present study, we identified potential biomarkers for cardiac aging by evaluating plasma markers that reflect the cardiac aging phenotype previously described.

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Matrix metalloproteinase-9 (MMP-9) is a 92-kDa gelatinase that proteolytically processes extracellular matrix (ECM) substrates, including native and denatured collagens, fibronectin, and laminin.7,8 In addition, MMP-9 processes non-ECM substrates, including cytokines and growth factors such as endothelin-1, interleukin (IL)-1β, IL-6, pro-tumor necrotic factor-α, and latent transforming growth factor-β.9,10 Ducharme et al11 studied the role of MMP-9 in post-MI LV remodeling and reported that MMP-9 deletion attenuates LV dilation and collagen accumulation after MI. Plasma MMP-9
levels correlate with the extent of post-MI remodeling, and MMP-9 is a plasma biomarker for acute MI patients. Blankenberg et al. showed that baseline plasma MMP-9 levels predict cardiovascular mortality in patients with cardiovascular diseases. In a previous study, we have shown that total MMP-9 levels in the LV and LV end-diastolic dimension increase from middle-aged to old CB6F1 mice, but the relationship between MMP-9 and cardiac aging is yet to be determined.

In addition to MMP-9, monocyte chemotactic protein-1 (MCP-1/CCL-2) is also a plasma biomarker for acute MI, due to its critical role in monocyte recruitment. In 2270 patients with acute MI, baseline plasma MCP-1 levels above the 75th percentile were associated with an increased risk of death or reoccurring MI during the 10-month follow-up period. The same group also demonstrated that high plasma MCP-1 levels (>238 pg/mL) at 4 months after MI was an independent predictor of mortality from 4 months to 2 years after MI. Plasma MCP-1 levels have been shown to increase in humans with age, but an association of plasma MCP-1 levels and cardiac aging has not been demonstrated. To identify plasma biomarkers of cardiac aging, we measured the levels of 69 analytes in the plasma of adult and senescent C57BL/6J mice by multi-analyte profiling. We demonstrated that MMP-9 and MCP-1 plasma levels were elevated in senescent mice and were positively linked to increased LV end-diastolic dimensions and increased levels of both MMP-9 and MCP-1 in the LV. These results suggest that MMP-9 and MCP-1 are potential plasma biomarkers of cardiac aging.

**Methods**

**Animals**

All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington, DC, 1996) and were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio. Male and female C57BL/6J wild-type adult (age, 7.5 ± 0.5 months; n = 27; 13 male and 14 female) and senescent (age, 31.7 ± 0.5 months; n = 25; 13 male and 12 female) mice were used.

**Echocardiography**

The echocardiography procedures and results have been previously published. Transthoracic echocardiography was performed under light anesthesia (0.5% isoflurane) with spontaneous respiration. Heart rate was determined from a surface ECG and maintained at a minimum of 400 beats per minute. Two-dimensional targeted M-mode echocardiographic measurements were obtained with an optimized 15.7-MHz transducer (ATL, HDI 5000). LV dimensions and wall thickness were measured from the short-axis view.

**Plasma Collection**

The mice were euthanized under a continuous flow of 2% isoflurane in oxygen mix from a ventilator. Heparin (100 μL of 1000 USP U/mL) was injected intraperitoneally, and 5 minutes after heparin injection, blood was collected from the carotid artery of the mice. Arterial blood was centrifuged for 5 minutes, the plasma was separated into 100 μL aliquots, and the tubes were snap frozen in liquid nitrogen and stored at −80°C.

**Multi-Analyte Profiling of Plasma**

Plasma samples (100 μL) were analyzed by the Rodent Multi-Analyte Profiling (MAP) version 1.9 (Rules-Based Medicine), and concentrations of 69 analytes were measured by a Clinical Laboratory Improvement Amendments (CLIA)-certified biomarker testing laboratory using reproducible, quantitative, multiplexed immunoassays.

**LV Protein Extraction**

Hearts were arrested in diastole by intraventricular injection of cardioplegia. The LV and right ventricle were separated. The LV was homogenized in soluble protein extraction buffer (250 mmol/L sucrose, 1 mmol/L EDTA, and 10 mmol/L Tris-HCl, pH 7.4) supplemented with 1× Complete Protease Inhibitor Cocktail (Roche). The tissue homogenate was centrifuged at 14 000 rpm, and the supernatant (soluble protein fraction) was transferred to a clean tube. The pellets were homogenized in insoluble protein extraction buffer (Sigma; Protein Extraction Reagent Type 4; 7 mol/L urea, 2 mol/L thiourea, 40 mmol/L Trizma base, and the detergent 1% CHBzO) with 1× Complete Protease Inhibitor Cocktail (Roche) to obtain the insoluble protein fraction.

**Immunoblotting**

To determine if changes in MMP-9 and MCP-1 levels in plasma could be attributed to their expression levels in the LV, MMP-9 and MCP-1 immunoblotting of myocardial tissue was performed. Immunoblotting of cleaved caspase-3 was also performed to study the extent of apoptosis in the LV. LV extracts from 12 adult mice (age, 7.3 ± 0.8 months; n = 12; 6 female and 6 male) and 12 senescent mice (age, 30.8 ± 0.5 months; n = 12; 6 female and 6 male) were used for immunoblotting. The insoluble protein fraction (10 μg) was used for MMP-9 immunoblotting and the soluble protein fraction (10 μg) was used for MCP-1 and cleaved caspase-3 immunoblotting.

The samples were resolved on a 4% to 12% Criterion Bis-Tris gel (Bio-Rad) in XT MES buffer (Bio-Rad). Protein samples in the gel were transferred to a nitrocellulose membrane (Bio-Rad). The membrane was incubated with 5% blocking grade nonfat dry milk (Bio-Rad) in X1 PBS (Sigma) for 1 hour at room temperature, followed by incubation with MMP-9 antibody (Abcam ab88989 at 1:5000 dilution), MCP-1 antibody (Cell signaling No. 2029 at 1:1000 dilution), or cleaved caspase-3 antibody (Cell signaling No. 9661 at 1:1000 dilution) for overnight at 4°C. The membrane was washed three times with 1X PBS with 1% Tween-20 (PBS-T) for 15 minutes each and incubated in anti-rabbit IgG (Vector PI-1000 at 1:5000 dilution) for 1 hour at room temperature. After washing 3 times with PBS-T for 15 minutes each, the membrane was incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) for 3 minutes and exposed to BioMAX MR film (Kodak). The films were scanned and Image J Software (NIH) was used for image analysis to quantify the densitometry. To confirm equal loading and sample transfer between the adult and senescent groups, the nitrocellulose membrane was stained with MemCode Reversible Protein Stain (Thermo Scientific) for total protein. The membrane was scanned by Kodak Image Station, and densitometry of each lane was quantified by Molecular Imaging Software (Kodak). The average densitometry value of the lanes for adult was 3185 ± 28 U and for senescent was 3202 ± 23 U (P = 0.64).

**Immunohistochemistry**

To determine age-related change in macrophage density in the LV, a second cohort of adult (n = 3) and senescent (n = 3) mice were euthanized and mid sections of the LV were fixed in formalin and then paraffin-embedded. Paraffin-embedded LV sections were deparaffinized by incubating 2 times in Citric-Solv (Fisherbrand) for 10 minutes each. The sections were rehydrated by incubating 2 times in 100%, 95%, and 70% ethanol for 5 minutes each and 5 minutes in distilled water. Rehydrated sections were then boiled for 10 minutes in Antigen Retrieval Solution (Dako). After the sections were cooled down for 20 minutes, the sections were washed 3 times with distilled water for 5 minutes each. The sections were incubated in 3% hydrogen peroxide in water for 20 minutes to quench endogenous peroxidase activity. The sections were blocked with rabbit normal serum (Vector Labs) for 20 minutes and incubated in Mac-3 antibody.
(Cedarlane CL8943AP at 1:100 dilution) overnight at 4°C. After washing 5 minutes in PBS, the sections were incubated in rabbit anti-rat IgG (Vector Labs) for 30 minutes at room temperature and washed with PBS for 5 minutes. After 30 minutes of incubation in ABC reagent (Vector Labs) and 5 minutes of PBS wash, the HistoMark black Peroxidase Substrate Kit (KPL) was used for color development. The sections were counterstained with eosin for 20 seconds, dehydrated, and mounted.

To determine the contribution of macrophages to MMP-9 expression in the LV, dual-label immunohistochemical staining of MMP-9 and Mac-3 were performed. LV sections were deparaffinized and rehydrated, and antigen retrieval and hydrogen peroxide treatment were performed as described above. The sections were blocked with 2.5% normal horse serum from the ImmPRESS kit (Vector Labs) for 20 minutes and incubated in MMP-9 (Abcam ab38893) for 2 hours at room temperature. After washing with PBS, the sections were incubated in ImmPRESS anti-rabbit IgG (Vector Labs) for 30 minutes at room temperature and washed with PBS for 5 minutes. After color development with the NovaRED substrate kit, Mac-3 immunohistochemistry was performed on the same sections as described above except that a 2-hour incubation of Mac-3 antibody was performed at room temperature, and the Vector Blue Alkaline Phosphatase Substrate Kit (Vector Labs) was used for color detection. To confirm staining specificity, no primary antibody negative controls and single antigen labeling controls were also performed.

Statistical Analyses
Data are expressed as mean ± SEM. The Shapiro-Wilk normality test was performed for each plasma analyte to determine the normality of the concentrations. Because the concentrations of most analytes were not normally distributed, nonparametric tests were used for statistical analysis. Specifically, we used the Mann-Whitney U test to compare values between the 2 age groups. Spearman rank correlation was performed to determine correlation coefficients between plasma analyte concentrations and end-diastolic dimensions or LV analyte levels. Data from both adult and senescent groups were combined for the correlation analyses. Densitometry values for MMP-9 and MCP-1 immunoblotting were normally distributed and were analyzed by Student t test and linear regression. A value of P < 0.05 was considered statistically significant. Stata version 11 was used for the statistical analyses.

Regression Modeling
Regression modeling was performed to study the relationship between plasma analyte concentrations and echocardiographic parameters (end-diastolic dimension, end-systolic dimension, and posterior wall thickness in diastole). End-diastolic dimension, end-systolic dimension, and posterior wall thickness in diastole were set as response $Y$ variables, and the 29 differentially expressed plasma analytes were set as independent $X$ variables. Every independent variable was fitted with each response variable individually, and each response variable was fitted with $k$ independent variables ($k = 1, 2, \ldots, 29$) by multiple regression. Considering that each $Y$ variable may depend on 1 or more $X$ variables, there will be at least $(2^{31} - 1)$, or more than 2 million possible models. The total number of models to analyze would be extremely large if we also include cross-product terms in the models. Therefore, we used a stepwise regression, that is, adding $X$ variables one by one. After adding each new $X$ variable, fitting of the regression model was evaluated by the closeness of the coefficient of multiple determination $R^2$ to 1. The coefficient of multiple determination is the percentage of the total variation in $Y$ that is due to the relationship between $Y$ and the predictors. We calculated the $(1-R^2)$, the percentage of the total variation in $Y$ that is due to experimental error, and set an upper bound for $(1-R^2)$ as 0.10, or equivalently, a lower bound 0.90 for $R^2$. We chose $R^2 > 0.9$ and statistically significant probability values < 0.05 for the model as well as for each $X$ variable in the model as the selection criteria for a good-fit model in this study. Statistical tests were applied to check the adequacy of the model. For each model that met this criterion, we also determined if any other $X$ variables could be included with the above criteria. The assumptions underlying the regression analysis were verified to confirm that all assumptions were met, namely that errors were random variables with means of 0, no errors correlated, the variance of error was constant for the parameters, and the predictors were independent. The fitted models were validated by residual analysis. We used the Durbin-Watson test to check if residuals were uncorrelated, the plot of residuals versus fits to check the constancy of the variance, and the variance inflation factors to detect the presence of multicollinearity or intercorrelation among predictors. For each good-fit model selected, the $X$ variables were selected as predictors of the response variable in response to age. Similarly, the predictors of plasma MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1 concentrations were also evaluated by regression modeling. Minitab and an algorithm by Polansky, Chou, and Mason were used for regression modeling.20

Results
Plasma MAP Revealed Increased MMP-9 and MCP-1 Levels With Senescence
To identify plasma biomarkers that reflect the cardiac aging phenotype, plasma from adult and senescent C57BL6J WT mice were collected and levels of 69 analytes in the plasma were measured by multi-analyte profiling. Of the 69 analytes measured, 26 analytes were at significantly higher levels in the senescent plasma, and 3 analytes were at significantly lower levels (Table 1). The majority of analytes presenting at higher levels in the plasma of senescent mice were inflammatory markers associated with macrophage functions, including MMP-9 and MCP-1. No sex-related differences were observed in MMP-9 and MCP-1 levels in both adult and senescent groups. Some inflammatory markers, including CD40 ligand, IL-1β, and IL-18 levels, did not change with senescence, suggesting that changes in inflammatory marker levels were specific.

Plasma MMP-9 and MCP-1 Levels Correlated With End-Diastolic Dimensions
Cardiac aging is associated with LV dilation, and we have previously demonstrated an increase in LV end-diastolic dimension in the senescent mice compared with adult mice.6 To determine if increased plasma MMP-9 and MCP-1 levels in the senescent mice reflect the cardiac aging phenotypes observed, Spearman rank correlation was performed to determine the correlation of plasma MMP-9 and MCP-1 levels with end-diastolic dimensions. Spearman rank correlation showed that the plasma MMP-9 and MCP-1 levels positively correlated to the end-diastolic dimensions of the LV (Table 2).

MMP-9 and MCP-1 Levels Increase With Age in the LV
To determine if increases in plasma analyte levels could be attributed to direct changes at the LV level, LV tissue was examined by immunoblotting. Immunoblotting for MMP-9 confirmed higher MMP-9 levels in the LV of senescent mice compared with adult mice (Figure 1A). Immunoblotting also demonstrated higher MCP-1 levels in the senescent LV compared with the adult LV (Figure 1B). Spearman rank correlation analysis revealed that plasma MMP-9 levels correlated with LV MMP-9 levels ($\rho = 0.50$, $P < 0.05$), and plasma MCP-1 levels correlated with LV MCP-1 levels ($\rho = 0.62$, $P < 0.01$) (Figure 2A and 2B). At the same time, LV MMP-9 levels correlated with LV MCP-1 levels ($r = 0.52$, $P < 0.01$).
Table 1. Plasma Analytes Differentially Expressed With Age

<table>
<thead>
<tr>
<th>Increased</th>
<th>Adult (n=27)</th>
<th>Senescent (n=25)</th>
<th>A/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2-Microglobulin, µg/mL</td>
<td>0.59±0.14</td>
<td>3.11±0.68</td>
<td>A/M</td>
</tr>
<tr>
<td>Calbindin, ng/mL</td>
<td>0.09±0.01</td>
<td>0.27±0.03</td>
<td>A</td>
</tr>
<tr>
<td>Cystatin C, ng/mL</td>
<td>444±12</td>
<td>546±39</td>
<td>A/M</td>
</tr>
<tr>
<td>Granulocyte chemotactic protein-2, ng/mL</td>
<td>4.0±0.3</td>
<td>7.0±0.9</td>
<td>A/M</td>
</tr>
<tr>
<td>Growth hormone, ng/mL</td>
<td>34.4±19.5</td>
<td>188.2±43.9</td>
<td>A/M</td>
</tr>
<tr>
<td>Haptoglobin, µg/mL</td>
<td>62.8±10.6</td>
<td>96.4±7.2</td>
<td>A/M</td>
</tr>
<tr>
<td>Immunoglobulin A, µg/mL</td>
<td>61.4±9.3</td>
<td>186.9±45.1</td>
<td>A/M</td>
</tr>
<tr>
<td>Interleukin-1α, pg/mL</td>
<td>13.6±1.3</td>
<td>33.1±5.7</td>
<td>A/M</td>
</tr>
<tr>
<td>Interleukin-7, ng/mL</td>
<td>0.06±0.01</td>
<td>0.11±0.01</td>
<td>A/M</td>
</tr>
<tr>
<td>Interleukin-11, pg/mL</td>
<td>30.4±3.1</td>
<td>66.2±10.5</td>
<td>A/M</td>
</tr>
<tr>
<td>Interferon-inducible protein-10, pg/mL</td>
<td>67.2±6.2</td>
<td>127.5±16.8</td>
<td>A/M</td>
</tr>
<tr>
<td>Lymphotactin, pg/mL</td>
<td>64.8±3.6</td>
<td>106.6±12.7</td>
<td>A/M</td>
</tr>
<tr>
<td>Monocyte chemotactic protein-1, pg/mL</td>
<td>147±17</td>
<td>510±148</td>
<td>A/M</td>
</tr>
<tr>
<td>Monocyte chemotactic protein-5, pg/mL</td>
<td>59.1±4.8</td>
<td>363.2±146.2</td>
<td>A/M</td>
</tr>
<tr>
<td>Macrophage inflammatory protein-1α, pg/mL</td>
<td>56.2±6.3</td>
<td>144.9±30.1</td>
<td>A/M</td>
</tr>
<tr>
<td>Macrophage inflammatory protein-2, pg/mL</td>
<td>14.6±0.7</td>
<td>25.6±2.6</td>
<td>A/M</td>
</tr>
<tr>
<td>Macrophage inflammatory protein-3β, pg/mL</td>
<td>0.65±0.02</td>
<td>1.13±0.21</td>
<td>A/M</td>
</tr>
<tr>
<td>Matrix metalloproteinase-9, ng/mL</td>
<td>50.6±2.9</td>
<td>95.1±9.2</td>
<td>A/M</td>
</tr>
<tr>
<td>Myeloperoxidase, ng/mL</td>
<td>69.1±6.6</td>
<td>120.3±10.9</td>
<td>A/M</td>
</tr>
<tr>
<td>Oncostatin M, ng/mL</td>
<td>0.04±0.01</td>
<td>0.08±0.02</td>
<td>A/M</td>
</tr>
<tr>
<td>Osteopontin, ng/mL</td>
<td>243±23</td>
<td>446±40</td>
<td>A/M</td>
</tr>
<tr>
<td>RANTES, pg/mL</td>
<td>6.25±0.46</td>
<td>15.15±2.61</td>
<td>A/M</td>
</tr>
<tr>
<td>Stem cell factor, pg/mL</td>
<td>154±5</td>
<td>198±16</td>
<td>A/M</td>
</tr>
<tr>
<td>Tissue inhibitor of metalloproteinase I, ng/mL</td>
<td>1.06±0.24</td>
<td>1.88±0.33</td>
<td>A/M</td>
</tr>
<tr>
<td>Tumor necrosis factor-α, ng/mL</td>
<td>0.03±0.01</td>
<td>0.05±0.01</td>
<td>A/M</td>
</tr>
<tr>
<td>Vascular cell adhesion molecule I, ng/mL</td>
<td>1612±30</td>
<td>2573±296</td>
<td>A/M</td>
</tr>
</tbody>
</table>

A/M indicates previously associated with aging (A) or macrophages (M); RANTES, regulated on activation, normal T-cell expressed and secreted. Values are mean±SEM. All analytes were significantly different between the 2 groups, P<0.05.

P<0.01) by linear regression (Figure 2C). Levels of cleaved caspase-3 were very low to undetectable in the LVs from both age groups, indicating that the extent of apoptosis in the myocardium was very low even into senescence. There was, however, a significant increase in myocardial fibrosis in the LVs from the senescent group.6
MMP-9 levels were higher in the senescent LV, we determined whether the LV of senescent mice had a higher macrophage content compared with adult mice. Immunohistochemical staining revealed higher numbers of Mac-3–positive cells in the senescent LV compared with adult LV (Figure 3A and 3B). Dual-label immunohistochemical staining of Mac-3 and MMP-9 in the LV of senescent mice showed robust MMP-9 staining localized to the macrophage (Figure 4), indicating the macrophage is a major source of MMP-9 in the senescent LV.

Spearman Rank Correlation Identified Other Potential Plasma Biomarkers

From MAP, levels of 29 analytes were significantly different between adult and senescent groups. For these 29 analytes, 19 analytes, including MMP-9 and MCP-1, had detectable levels in every sample. We performed Spearman rank correlation to study the association of these 19 analytes to LV end-diastolic dimensions. In addition to MMP-9 and MCP-1, levels of 10 other analytes correlated with LV end diastolic dimensions (Table 2). These analytes include osteopontin and TIMP-1, which have been demonstrated as plasma biomarkers of cardiovascular disease.21–23

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Regression Modeling Identified Good-Fit Models Predictive of Echocardiographic Parameters

In the present study, we were interested in exploring relationships between echocardiographic parameters (end-diastolic dimension, end-systolic dimension, and posterior wall thickness in diastole) and the 29 proteins altered in senescent mice. We used regression analysis to determine if there was a relationship between response $Y$ variables (end-diastolic dimension, end-systolic dimension, posterior wall thickness in diastole, plasma MMP-9 levels, and plasma TIMP-1 levels) and any of the independent $X$ variables (levels of the 29 plasma analytes altered with age). For the $Y$ variable end-diastolic dimension, there were 12 good-fit models that matched the selection criteria. For the $Y$ variable end-systolic dimension, there were 6 good-fit models; for posterior wall thickness in diastole, there were 3 good-fit models; and for MMP-9 and TIMP-1, there was 1 good-fit model for each. The predictors that were in common with the models are shown in Table 3.

Discussion

The goal of this study was to identify plasma biomarkers of cardiac aging. The major findings were (1) MMP-9 and MCP-1 levels increase in plasma with senescence; (2) increased MMP-9 and MCP-1 levels in plasma mirror increased
MMP-9 and MCP-1 levels in the senescent LV, which are concurrent with increased macrophage density; and (3) regression modeling indicates that the levels of macrophage markers link with the measurements of echocardiographic parameters, suggesting an underlying inflammatory mechanism of cardiac aging. Overall, we conclude that MMP-9 and MCP-1 are plasma biomarkers of cardiac aging and that the macrophage is a prominent component of the cardiac aging phenotype.

In this study, we showed that plasma MMP-9 levels were significantly higher in senescent mice compared with adult mice. We also demonstrated by Spearman rank correlation that plasma MMP-9 levels positively correlated with end-diastolic dimension of the LV. MMP-9 plays an important role in cardiac ECM remodeling after MI, and plasma MMP-9 levels have been shown to predict cardiovascular mortality in patients with cardiovascular diseases. Plasma MCP-1 levels were also significantly higher in senescent mice compared with adult mice and were positively correlated with end-diastolic dimension of the LV. The increased plasma MCP-1 levels in the senescent group are consistent with a previous report in humans showing higher circulating MCP-1 levels with age. MCP-1 has also been shown as a plasma biomarker for acute coronary syndromes. Our results showed that both plasma MMP-9 and MCP-1 levels increased with age and correlated with end-diastolic dimension, which suggests that MMP-9 and MCP-1 are potential markers of cardiac aging.

Immunoblotting of MMP-9 confirmed that MMP-9 levels were higher in the LV of senescent mice compared with adult mice. This result agrees with a previous study from our laboratory that MMP-9 levels increase with age in old CB6F1 mice. MCP-1 levels were also higher in the senescent LV compared with the adult LV. At the same time, plasma MMP-9 levels correlated with LV MMP-9 levels, and plasma MCP-1 levels correlated with LV MCP-1 levels. These results indicate the LV is a major tissue source contributing to the increased plasma MMP-9 and MCP-1 levels. LV MMP-9 levels positively correlated with LV MCP-1 levels, indicating a relationship between these 2 markers.

Aging is accompanied with an increased chronic inflammatory status. The effect of aging on macrophage numbers in the LV, however, has not previously been well characterized. Indeed, we were not able to locate any references to document that macrophages increase in the LV with age. In the present study, concurrent with an increase in macrophage chemokine MCP-1 levels, we demonstrated an increase in macrophage number in the senescent LV compared with the adult LV by immunohistochemistry, using the macrophage marker Mac-3. A previous study from our group has shown that the cardiac fibroblast is not the source of increased MMP-9 levels in the aged LV because MMP-9 levels decline in old cardiac fibroblasts. Our current study revealed increased macrophage numbers and robust colocalization of MMP-9 staining with macrophages, indicating that increased number of macrophages contributes to the increased MMP-9 levels in the senescent LV. The switch in MMP-9 being derived primarily from a fibroblast source to a macrophage source could change the function of this MMP with age. Net MMP-9 function is determined by the substrates that colocalize around the enzyme. Fibroblast-derived MMP-9 substrates include ECM proteins such as collagens, fibronectin, and laminin. This suggests that MMP-9 released in younger animals primarily serves a homeostatic role to maintain the cardiac ECM. In contrast, macrophage-derived MMP-9 substrates include cytokines, chemokines, and growth factors that regulate immune responses. MMP-9 in the aged LV therefore may primarily serve a role in modifying the immune response.

In the present study, we observed an age-related increase in MMP-9 levels in the LV; however, it is unclear if MMP-9 directly mediates the cardiac aging phenotype. Interestingly, previous studies have shown that aging in skeletal muscles and endothelial cells is associated with reduced levels of...
MMP-2, another gelatinase in the MMP family. This age-related reduction in MMP-2 results in impairment of neovascularization in ischemic hind limb muscle, a phenotype that can be rescued with exercise training. Aging, therefore, differentially regulates expression of different MMP family members and results in different aging phenotypes that can vary among tissue types. As discussed above, net MMP function depends on the substrates that colocalize around the enzyme; thus, it is likely that the effects of age-related changes in MMP expression levels are tissue-specific, due to changes in substrate composition. We have previously shown that aging does not alter MMP-2 levels in LV extracts, but whether aging reduces MMP-2 in the endothelial cells in coronary arteries and impairs neovascularization after MI in old patients remains to be explored.

We identify MMP-9 and MCP-1 as plasma biomarkers reflecting the age-related LV dilation, but the predictive power of a single biomarker is limited. With multivariate regression modeling, we identified several good-fit models that correlate with LV end-diastolic dimension, LV end-systolic dimension, or posterior wall thickness at diastole, with $R^2 > 0.9$ and $P < 0.05$. These models comprise predictors including MCP-1 and other macrophage markers and have high predictive power for the echocardiographic parameters. MMP-9 is not a predictor in these models, but MCP-1 and other predictors of these models can predict plasma MMP-9 levels. This suggests that MMP-9 increases after changes in macrophage markers, which agrees with our immunohistochemistry experiment that the macrophage is the source of MMP-9 in the senescent LV. The fact that LV MMP-9 levels correlated with LV MCP-1 levels supports the hypothesis that increased MCP-1 levels in the senescent LV increase macrophage recruitment into the LV, which, in turn, generate increased levels of MMP-9 in the senescent LV.

Besides MCP-1, 2 other C-C chemokines, macrophage inflammatory protein 1β (MIP-1β/CCL-4) and regulated on activation, normal T-cell expressed and secreted (RANTES/CCL-5), were also predictors of all 3 echocardiographic parameters and MMP-9 levels. Increased MIP-1β and RANTES levels have been associated with cardiovascular diseases. MIP-1β expression increases in infarcted myocardium after ischemia reperfusion. Increased circulating levels of RANTES are demonstrated in acute MI patients and in patients with congestive heart failure. At the same time, previous studies have shown that MIP-1β and RANTES levels increase in T-cells, in the brain, and in the thymus with age. The potential of MIP-1β and RANTES as biomarkers and their roles in cardiac aging remain to be investigated.

A strength of this study is that we were able to evaluate effects of cardiac aging in isolation from the effects of vascular aging or cardiac disease superimposed on aging. As we have previously reported, mice do not develop hypertension with age. The senescent mice used in this study were 32 months old, and we have previously reported that their systolic blood pressure was $111 \pm 3$ mm Hg, which was not altered from adult values of $114 \pm 3$ mm Hg ($P = 0.48$). The ejection fraction in the senescent group was reduced compared with the adult mice group; however, ejection fraction values were still within the normal physiological range.

Percentages of water composition in the lung were also comparable to adult controls. These results indicate the senescent mice did not show signs and symptoms of heart failure. Therefore, MMP-9 and MCP-1 are markers of normal cardiac aging without concomitant cardiovascular disease.

One limitation of the present study was that we did not rule out the possibility that other tissue sources also contributed to the changes in analyte levels in the plasma. Although we confirmed that MMP-9 and MCP-1 levels did increase in the LV and that plasma levels mirrored LV levels, it is possible that increased levels of MMP-9, MCP-1, and macrophages occur in other tissues as a common mechanism of aging. For example, increased MMP-9 expression has been shown in the lungs of 20-month-old DBA/2J mice compared with 2-month-old mice. A second limitation is that only 2 time points were evaluated, which limits our ability to determine the kinetic time course of the changes we observed. Current studies are underway to evaluate cardiac aging over multiple age groups. This will tell us whether MMP-9 and MCP-1 are markers that only increase at later time points of cardiac aging or if the increased levels of these analytes contribute to the cardiac aging phenotype. This will further validate the predictive capacities of these analytes.

In conclusion, we showed that circulating MMP-9 and MCP-1 levels are higher in senescent mice, and both levels positively correlated with the cardiac sarcopenia phenotype. This reveals MMP-9 and MCP-1 as novel biomarkers of cardiac aging. The augmented MMP-9, MCP-1, and macrophase levels in aging LV provide an underlying mechanism in the cardiac aging response.

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Disclosures
None.

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