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Plasma Profiling Determinants of Matrix Homeostasis in Pediatric Dilated Cardiomyopathy

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Abstract

Objective—Dilated cardiomyopathy is an important cause of heart failure in both children and adults, but is more progressive in children. In adult dilated cardiomyopathy, left ventricular remodeling is associated with changes in plasma levels of matrix metalloproteinases, and tissue inhibitor of metalloproteinases. Plasma matrix metalloproteinases, and tissue inhibitors of metalloproteinase changes in pediatric dilated cardiomyopathy has not been examined. The present study developed a low blood volume, high sensitivity assay to test the hypothesis that unique and differential plasma matrix metalloproteinases and tissue inhibitors of metalloproteinase profile exists in pediatric dilated cardiomyopathy patients.

Methods/Results—A systemic blood sample (1 milliliter) was obtained from 7 children with dilated cardiomyopathy (age 8 plus or minus 7 years), and 26 age-matched normal volunteers. Using a high throughput multiplex suspension immunoassay, plasma levels were quantified for: collagenases (matrix metalloproteinase-8), gelatinases (matrix metalloproteinase-2, -9), lysins (matrix metalloproteinase-3, -7), and tissue inhibitor of metalloproteinases-1, -2, and -4. Matrix metalloproteinase to tissue inhibitor of metalloproteinases ratios were also calculated. Plasma matrix metalloproteinase-2, -7, -8 and -9 levels were increased by greater than 2-fold in dilated cardiomyopathy patients than normals (with p less than 0.05). Dilated cardiomyopathy patients also had significantly higher tissue inhibitor of metalloproteinases-1 and -4 (298 percent and 230 percent; with p less than 0.05).

Conclusions—These unique findings demonstrated that a specific plasma matrix metalloproteinase/tissue inhibitors of metalloproteinase profile occurs in pediatric dilated cardiomyopathy when compared to normal children. These distinct differences in the determinants of myocardial matrix structure and function may contribute to the natural history of dilated cardiomyopathy in children, and may provide a novel biomarker platform in pediatric dilated cardiomyopathy.

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DISCLOSURES

None.

INTRODUCTION

Pediatric dilated cardiomyopathy is the most common form of cardiomyopathy in children, but also the most aggressive, where death or transplantation within one year of the initial diagnosis is a common outcome.¹ Despite a more ominous prognosis, pediatric dilated cardiomyopathy shares a similar phenotype of left ventricular dilatation and systolic dysfunction as adult dilated cardiomyopathy. While the primary etiologies of dilated cardiomyopathy in adults are due to acquired diseases, the large majority of pediatric dilated cardiomyopathies are idiopathic. Nonetheless, the clinical features of pediatric dilated cardiomyopathy are not unlike that seen in adults: a chronic and progressive alteration in myocardial structure that leads to myocardial systolic dysfunction, arrhythmias, congestive heart failure, and sudden death.

Maladaptive myocardial remodeling is the hallmark of dilated cardiomyopathy, and significant changes in the myocardial extracellular matrix are known to facilitate the structural and functional changes in adult dilated cardiomyopathy.² Maintenance of extracellular matrix homeostasis is coordinated through the proteolytic degradation and synthesis of extracellular matrix components by the matrix metalloproteinases and their endogenous tissue inhibitors of metalloproteinase. Thus, changes in matrix metalloproteinase levels as well as the relative balance to tissue inhibitor of metalloproteinases, can result in a conditions of enhanced matrix remodeling. Indeed, increased myocardial levels of matrix metalloproteinase, and abnormal balances between matrix metalloproteinases and tissue inhibitor of metalloproteinase levels have been identified in adult forms of dilated cardiomyopathy.³⁻⁵ While myocardial sampling can provide a direct assessment of matrix metalloproteinase and tissue inhibitor of metalloproteinase levels, this approach can be problematic for serial studies. Indeed, a number of past studies have identified that it is possible to perform matrix metalloproteinase and tissue inhibitor of metalloproteinase measurements from a plasma sample in adult patients with chronic heart failure as well as following myocardial infarction.⁶⁻⁹ These past studies in adult patients suggest that plasma matrix metalloproteinase and tissue inhibitor of metalloproteinase levels have been reported to have prognostic, and predictive value. However, whether, and to what degree, plasma matrix metalloproteinase and tissue inhibitor of metalloproteinase levels in children with dilated cardiomyopathy differ from age-matched normal subjects remains unknown. In addition, the past adult studies primarily focused upon a limited number of matrix metalloproteinases and tissue inhibitor of metalloproteinases, and thus a more robust examination of matrix metalloproteinases, tissue inhibitor of metalloproteinase, and the stoichiometric relationship between matrix metalloproteinase/tissue inhibitor of metalloproteinases has not been fully explored in any dilated cardiomyopathy state. Accordingly, we developed a high-sensitivity multiplex suspension assay to allow measurement of plasma matrix metalloproteinase and tissue inhibitor of metalloproteinase levels in children, using small (~1 milliliter) blood volume. The central purpose of this study was to establish a plasma matrix metalloproteinase /tissue inhibitor of metalloproteinase profile in children diagnosed with idiopathic dilated cardiomyopathy, and compare these results to age matched children with no history of cardiovascular disease.

METHODS

Patients

Children presenting with dilated cardiomyopathy were initially screened for inclusion into this study. All patients underwent standard cardiomyopathy evaluation, including echocardiography, left and right heart catheterization, coronary angiography, and myocardial biopsy with both light and electron microscopic examinations. A comprehensive viral workup including parvoviral, enteroviral, and Epstein-Barr viral polymerase chain

reaction was performed. In all patients, the diagnosis of idiopathic dilated cardiomyopathy was established based on normal coronary arteries on echocardiogram/angiography, and the absence of valvular or pericardial diseases. Patients with confirmed myocarditis, hypertrophic or restrictive cardiomyopathy, or structural heart defects were excluded. This study also did not include patients with a history of hematologic, neoplastic, or primary renal/liver diseases, or those with acute or chronic infections, autoimmune diseases, and patients receiving anti-inflammatory drugs. From this initial screening, 7 children with idiopathic dilated cardiomyopathy were identified for inclusion. The mean age of this sample cohort was 8 plus or minus 7 yrs (range 3 months to 14 years) with a time interval of 2.7 plus or minus 1.4 years from the initial diagnosis to sample collection. The left ventricular fractional shortening computed from the echocardiogram most coincident with the sample collection was 18 plus or minus 2 percent, revealing significant systolic dysfunction. For the purposes of providing referent normal values for these dilated cardiomyopathy patients, 26 age-matched normal volunteers (mean age: 6 plus or minus 6 years, range 3 months to 17 years) were enrolled. These patients were carefully screened by history and physical, which included blood chemistries and an electrocardiogram to exclude any history of cardiovascular disease. All of the referent control subjects were in American Society of Anesthesiologists functional status class 1, and in New York Heart Association function class I. The study protocol was reviewed and approved by the Medical University of South Carolina Institutional Review Board (HR#17161) and parental/patient consents were obtained for all subjects enrolled in this study. Following informed consent, 1 milliliter of blood obtained from a peripheral venous site was collected into a chilled EDTA tube, centrifuged, and the plasma decanted and stored at minus 70 degrees Celsius.

Analytical Measurements

The plasma samples were thawed on ice and subjected to multiplex suspension array for matrix metalloproteinases (Human Fluorokine MAP matrix metalloproteinase Kit, R&D Systems, LMP000) and TIMPs (Human Fluorokine MAP TIMP Kit, R&D Systems, LKT003) in which all samples could be measured in simultaneous fashion, thereby minimizing inter-assay coefficient of variation. For the matrix metalloproteinases, the matrix was assembled in order to measure representative subtypes from each class of matrix metalloproteinases, which included the collagenases (matrix metalloproteinase-8, matrix metalloproteinase-13), the gelatinases (matrix metalloproteinase-2 and matrix metalloproteinase-9), and the stromelysins/matrilysins (matrix metalloproteinase-3, matrix metalloproteinase-7). The four known tissue inhibitors of metalloproteinases (tissue inhibitor of metalloproteinase-1, -2, -3, -4) were also measured. The relative fluorescence obtained for each distinct matrix metalloproteinase/tissue inhibitor of metalloproteinase (Bio-Plex 200, BioRad Laboratories) was converted to an absolute concentration using standards that were included in each assay and the specifications for each reagent and sensitivity are shown in Table 1. The coefficient of variation for these assays was 16 percent or less. Matrix metalloproteinase-13 and tissue inhibitor of metalloproteinase-3 levels were undetectable for both normal and dilated cardiomyopathy patients, and were excluded from further analysis. For all other matrix metalloproteinase and tissue inhibitor of metalloproteinase multiplex assays, the sample readings fell within the targeted dynamic range for the analyte of interest. Representative standard curves and the pediatric sample results for all of the measured matrix metalloproteinases and tissue inhibitor of metalloproteinases are shown in Figure 1.

Data Analysis

The fluorescence emissions for each set of matrix metalloproteinase/tissue inhibitors of metalloproteinase standards were first fit to a 5-parameter logistic equation (Figure 1), following a conventional format for non-linear fluorescence profiles.¹⁰ Using this 5-

parameter logistic curve fitting algorithm, the fluorescence emissions for the samples were converted to actual matrix metalloproteinase and tissue inhibitors of metalloproteinase values. The comparisons of matrix metalloproteinase and tissue inhibitors of metalloproteinase profiles between the dilated cardiomyopathy and referent normal values were performed using a stepwise, confidence interval based t-statistic (PRCOMP, STATA Statistical Software, Intercooled V 8.0. College Station, TX). In order to examine stoichiometric relationships between matrix metalloproteinase and tissue inhibitors of metalloproteinase profiles, matrix metalloproteinase/tissue inhibitors of metalloproteinase ratios were computed. For this analysis, a normal distribution was not achieved for a number of the matrix metalloproteinase/tissue inhibitors of metalloproteinase ratio computations, and therefore pair-wise comparisons were performed using the nonparametric Mann-Whitney test. Results are presented as mean plus or minus standard error of the mean. Values of p less than 0.05 were considered to be statistically significant, and actual p-values obtained from the individual comparisons have been tabulated.

RESULTS

Patient Demographics

Of the seven dilated cardiomyopathy patients, 5 were in New York Heart Association class II. The other 2 patients presented at initial diagnosis in New York Heart Association class IV: one patient (age 9 years old) underwent successful heart transplantation, and the other (age 10 months old) Berlin Heart (Berlin Heart GmbH, Berlin, Germany) left ventricular assist device implantation. Histopathologic characteristics of the ventricular biopsies for all patients showed nonspecific features of dilated cardiomyopathy including variable degrees of myocyte hypertrophy and atrophy, interstitial and replacement fibrosis, and myofibrillar loss (myocytolysis) with no evidence of active myocarditis. In these dilated cardiomyopathy patients, the plasma brain natriuretic peptide at the time of sample collection was 1400 plus or minus 828 picogram/milliliter which was approximately 14 times higher than that for referent normal values (less than 100 picogram/milliliter). Despite significant systolic dysfunction, renal function as determined by plasma creatinine values, was preserved in these dilated cardiomyopathy patients when compared to the referent control subjects (0.6_{plus or minus} 0.2 versus 0.6_{plus or minus} 0.9, milligrams/deciliter, p equals 0.99).

Plasma Matrix Metalloproteinase and Tissue Inhibitors of Matrix metalloproteinase Levels

Quantitative plasma levels of all matrix metalloproteinases and tissue inhibitors of metalloproteinase examined in this study are summarized in Table 2. Plasma levels of the collagenase matrix metalloproteinase-8, the gelatinases matrix metalloproteinase-2 and -9, and the matrilysin matrix metalloproteinase-7 were increased by over 2-fold in pediatric dilated cardiomyopathy when compared to referent normal values. With respect to the tissue inhibitors of metalloproteinases, tissue inhibitors of metalloproteinase-1 and tissue inhibitors of metalloproteinase-4 were increased by over 2-fold when compared to referent normal values. The relative increase in tissue inhibitors of metalloproteinase-4 values observed in this study hold relevance, as this tissue inhibitors of metalloproteinase is highly expressed within the cardiovascular system, particularly the myocardium.^{11,12}

Matrix Metalloproteinase to Tissue Inhibitor of Metalloproteinase Ratios

One of the critical post-translational control points which determines overall net matrix metalloproteinase proteolytic activity is through the interactive binding between the matrix metalloproteinases and tissue inhibitors of metalloproteinase. In general terms, the relative affinity for of tissue inhibitors of metalloproteinases to bind to active matrix metalloproteinases is equivalent and bind in a 1:1 stoichiometric relation. Accordingly, in order to more carefully examine the relative balance between matrix metalloproteinases and

tissue inhibitors of metalloproteinase plasma profiles in pediatric dilated cardiomyopathy, the matrix metalloproteinase/tissue inhibitors of metalloproteinase ratios were computed and are summarized in Table 3. In this analysis, a higher matrix metalloproteinase/tissue inhibitors of metalloproteinase ratio would imply a greater proteolytic potential, whereas a lower matrix metalloproteinase/tissue inhibitors of metalloproteinase ratio would indicate reduced proteolytic potential. In this analysis, the matrix metalloproteinase-2, matrix metalloproteinase-9, and matrix metalloproteinase-7 to tissue inhibitor of metalloproteinase-2 ratios were increased by over 3-fold when compared to referent normal values. The matrix metalloproteinase-9/ tissue inhibitor of metalloproteinase-4 ratio was also significantly increased from referent normal values, but did not reach statistical significance.

DISCUSSION

There have been a large number of studies in adult cardiac disease states that have identified abnormalities in matrix metalloproteinase and tissue inhibitors of matrix metalloproteinase within the myocardium.^{4-5,13,14} These studies have identified that changes in the three major classes of matrix metalloproteinases, the collagenases, gelatinases, and stromelysin/lysins, can occur within the myocardium of adults with severe left ventricular dysfunction. These past studies have also identified that changes in tissue inhibitors of matrix metalloproteinase levels occur within the myocardium, and alterations in the balance between myocardial matrix metalloproteinases and tissue inhibitors of metalloproteinases occur which would favor extracellular matrix remodeling. While myocardial sampling can provide a direct measure of matrix metalloproteinase and tissue inhibitors of metalloproteinase levels in the context of left ventricular remodeling and developing failure, repeat sampling can be problematic. Since a majority of matrix metalloproteinases and tissue inhibitors of metalloproteinases are released into the interstitial space, then egress into the vasculature will occur, resulting in the ability to measure these analytes within a systemic blood sample. Indeed, a number of past studies in adults with left ventricular remodeling and dysfunction have been performed where plasma matrix metalloproteinases and tissue inhibitors of metalloproteinases have been utilized as a surrogate index, or biomarker for structural remodeling which may be occurring at the myocardial level.^{6-9,15-23} These studies have demonstrated a strong relationship between changes in matrix metalloproteinase/tissue inhibitors of metalloproteinase profiles to left ventricular remodeling, progression of heart failure, and also to hold prognostic significance. However, little is known about changes in matrix metalloproteinases and tissue inhibitors of metalloproteinases in children with left ventricular failure, and more importantly whether and to what degree plasma matrix metalloproteinase and tissue inhibitors of metalloproteinase profiles are altered in the context of pediatric dilated cardiomyopathy remained completely unexplored. Accordingly, the present study utilized a validated high-sensitivity multiplex suspension array in order to directly quantify a large number of soluble matrix metalloproteinases and tissue inhibitors of metalloproteinases in a small plasma sample, and thereby provided a means to explore potential differences in age matched normal children and in children with idiopathic dilated cardiomyopathy. The novel findings from this study were 2-fold. First, a large number of matrix metalloproteinases and tissue inhibitors of metalloproteinases could be reproducibly measured from a small volume peripheral blood sample in both normal and dilated cardiomyopathy patients. Second, a significant increase in a subset of matrix metalloproteinases and tissue inhibitors of metalloproteinases was observed in pediatric dilated cardiomyopathy patients. These results underscore that fundamental alterations in the extracellular remodeling pathways exists in pediatric dilated cardiomyopathy. These results also set the stage for future studies in which plasma matrix metalloproteinase and tissue inhibitors of metalloproteinase profiling may serve as a potential novel biomarker platform for the development of prognostic algorithms

as well as evaluating treatment strategies in this aggressive form of left ventricular failure in children.

The matrix metalloproteinases comprise a large family of proteolytic enzymes which process structural proteins as well as signaling molecules within the interstitial space.^{2,4,5,24} The alterations in matrix metalloproteinase profiles within the adult myocardium, has been the focus of a large number of past studies.^{2,3-5,13,14,25} Specifically, in adult forms of dilated cardiomyopathy, increased overall matrix metalloproteinase activity and increased induction of certain matrix metalloproteinase types has been clearly established. Using large animal models of dilated cardiomyopathy, a clear time dependent association in matrix metalloproteinase induction and left ventricular dilation has been demonstrated, which was abrogated by selective matrix metalloproteinase inhibition.^{2,26} Based upon these past studies, the current operational hypothesis in adult forms of left ventricular remodeling and failure is that increased myocardial matrix metalloproteinase expression results in increased matrix turnover, a loss of structural support to myocytes and myocardial fascicles, and progressive left ventricular remodeling. The potential utility of measuring matrix metalloproteinase levels from a systemic blood sample in adults with established left ventricular dysfunction, or in adult subjects with increased risk of developing left ventricular dysfunction, has been examined previously.^{6-9,15-23} These past studies have uniformly demonstrated a prognostic relationship between changes in plasma matrix metalloproteinase levels to that of progressive left ventricular dysfunction and clinical outcomes. Correlating plasma matrix metalloproteinase-2 and 9 levels with left ventricular systolic function, investigators in the RESOLVD trial reported that in patients with symptomatic heart failure and low ejection fractions, matrix metalloproteinase-2 levels increased with worsening disease, and matrix metalloproteinase-9 levels correlated with lower ejection fraction and left ventricular dilatation.¹⁹ In a cohort of subjects in the Framingham Heart Study, higher plasma matrix metalloproteinase-9 levels were associated with more than twofold risk of adverse left ventricular remodeling.²⁰ Moreover, increased plasma matrix metalloproteinase 2 levels have been correlated with worsening symptoms, and identified as an independent predictor of death in adults with congestive heart failure.^{8,17,18,21} The present study builds upon these past reports by performing plasma profiling of a number of matrix metalloproteinase types in the context of pediatric heart failure. The results from the present study demonstrated that robust increase in certain matrix metalloproteinase types, such as matrix metalloproteinase-2 and matrix metalloproteinase-9 occurred in the plasma of children with established dilated cardiomyopathy.

In the present study, increased plasma levels in matrix metalloproteinase subtypes from the 3 major matrix metalloproteinase classes was observed in a cohort of pediatric dilated cardiomyopathy patients when compared to referent age matched normal subjects. The interstitial collagenase matrix metalloproteinase-8 was increased in pediatric dilated cardiomyopathy, which has also been previously termed “neutrophil collagenase”.^{2,27} In addition, plasma levels for the gelatinases matrix metalloproteinase-9 and matrix metalloproteinase-2 were increased in pediatric dilated cardiomyopathy. A predominant source of matrix metalloproteinase-9 is through inflammatory pathways and activation of inflammatory cells such as neutrophils and macrophages.²⁷⁻²⁹ Finally, the present study demonstrated a robust increase in the matrilysin, matrix metalloproteinase-7 with pediatric dilated cardiomyopathy which has been co-localized to macrophages and resident interstitial cells such as fibroblasts. Taken together, the changes in these matrix metalloproteinase types likely reflect activation of upstream inflammatory-bioactive signaling cascades. Specifically, matrix metalloproteinases are tightly regulated at the transcriptional level,²⁸ in which both ligand and adhesion mediated receptor interactions alter intracellular formation of transcription factors which specifically bind to regulatory elements within the matrix metalloproteinase promoter region. Thus, the absolute changes in matrix metalloproteinase

profiles with pediatric dilated cardiomyopathy are likely the result of altered transcription pathways that are reflective of the underlying disease process. Past studies have demonstrated an association between increased levels of plasma matrix metalloproteinase-8 and matrix metalloproteinase-2 with changes in soluble tumor necrosis factor receptor levels in adult dilated cardiomyopathy patients.⁷ Moreover, matrix metalloproteinase-7 can proteolytically process membrane-bound tumor necrosis factor to soluble form.^{2,24} Taken together, the emergence of these matrix metalloproteinase types in the plasma of pediatric dilated cardiomyopathy patients suggests that local inflammation may be a contributory factor. However, this study did not include any patients with an acute inflammatory response (i.e. increased white blood cell count) and rigorously excluded any dilated cardiomyopathy patients with suspected myocarditis. Thus, it remains unclear what upstream signaling pathways may be evoking the specific plasma proteolytic profile in pediatric dilated cardiomyopathy. More importantly, the relationship between the biological function of certain matrix metalloproteinase types and the quantitative measurements performed in the present study remain associative at best with respect to any cause-effect relation to the progression of the pediatric dilated cardiomyopathy process. For example, due to the limited sample size and single time point measurements performed in the present study, any direct relationships between plasma matrix metalloproteinase profiles and the left ventricular remodeling process could not be determined. Nevertheless, the present study has clearly established that plasma profiling of matrix metalloproteinases in pediatric dilated cardiomyopathy results in a differential pattern from normal children, and that the small sample volumes required for assay would be amenable for future serial studies.

A critical control point for net matrix metalloproteinase activity is through forming non-covalent complexes with tissue inhibitors of metalloproteinases. In general terms, tissue inhibitors of metalloproteinases bind to the catalytic domain of active matrix metalloproteinases and thereby prevent access to substrates. The present study examined this important determinant of matrix metalloproteinase proteolytic activity through quantifying all 4 of the known tissue inhibitors of metalloproteinases. The results from this analysis revealed that plasma tissue inhibitors of metalloproteinase-3 levels were below detection limits in both referent normal and pediatric dilated cardiomyopathy samples. In contrast, plasma tissue inhibitors of metalloproteinase-1, -2 and -4 levels were readily quantified and were within the dynamic range of the analytical assay system. In past adult studies of left ventricular failure, measurements of plasma tissue inhibitors of metalloproteinase-1 have been a predominant focus.^{6-9,17,21,22} In a Framingham Heart substudy with over 1000 patients, plasma tissue inhibitors of metalloproteinase-1 levels was associated with major cardiovascular risk factors, and to indices of left ventricular hypertrophy and systolic dysfunction.³⁰ Elevated plasma tissue inhibitors of metalloproteinase-1 levels have also been shown to adversely impact prognosis, and are associated with increased mortality, in patients with congestive heart failure.^{9,17,21} Interestingly, the fact that increased plasma tissue inhibitors of metalloproteinase-1 levels also occur in pediatric dilated cardiomyopathy suggests that plasma tissue inhibitors of metalloproteinase-1 levels may reflect a common biological event in adverse myocardial matrix turnover, which in turn would influence myocardial structure and function. Tissue inhibitors of metalloproteinases-4 is the most recently discovered member of the tissue inhibitors of metalloproteinase family, and is noted for its high cardiovascular specificity.^{2,11,12} As with other tissue inhibitors of metalloproteinases, it complexes with matrix metalloproteinases in a 1:1 stoichiometric kinetics. The present study identified a 2-fold increase in plasma tissue inhibitors of metalloproteinase-4 levels in pediatric dilated cardiomyopathy which would further imply that the predominant source for changes in the tissue inhibitors of metalloproteinase profiles observed in the present study was the myocardium. In light of the fact that the net level of matrix metalloproteinase proteolytic activity is dependent on the relative concentrations of matrix metalloproteinases and tissue inhibitors of metalloproteinases, then one approach is

to examine the relative stoichiometry between specific matrix metalloproteinase and tissue inhibitors of metalloproteinases. For example, Wilson reported increased matrix metalloproteinase-9/tissue inhibitors of metalloproteinase-1 ratios in adult patients with congestive heart failure.⁷ Others have also reported adult patients with systolic heart failure or idiopathic dilated cardiomyopathy have elevated matrix metalloproteinase/ tissue inhibitors of metalloproteinase ratios, including matrix metalloproteinase-2/tissue inhibitors of metalloproteinase-2.^{16,17,23} In the present study, an increase in the relative matrix metalloproteinase/tissue inhibitors of metalloproteinase-2 ratio occurred which would imply a net loss of endogenous matrix metalloproteinase inhibitory control, and increased matrix metalloproteinase activity within the myocardial interstitium in pediatric dilated cardiomyopathy. However, the present study performed a quantitative analysis of matrix metalloproteinase and tissue inhibitors of metalloproteinase levels in the plasma, and therefore how these plasma levels may be reflective of local myocardial proteolytic activity can only be inferred. Future studies will be required to directly examine changes in plasma matrix metalloproteinase/ tissue inhibitors of metalloproteinase levels to that of local matrix metalloproteinase activation states.

Limitations and Summary

Although different techniques have been employed to quantify systemic circulating matrix metalloproteinase and tissue inhibitors of metalloproteinase levels, the most commonly reported has been enzyme lined immuno-sorbent assay. However, enzyme lined immuno-sorbent assay is limited by the need for large amount of blood sample volume, when multiple analytes are to be assessed. This presents a particular problem in infants and small children, who have less tolerance for large volume blood sampling. Therefore, a novel technique that employs multiplex suspension array was developed to simultaneously measure multiple analytes with greater sensitivity, but requiring small volume of plasma. Multiplex suspension array has been validated by tradition enzyme lined immuno-sorbent assay, and this study demonstrated the feasibility of using 1 milliliter of blood in children to quantify the various matrix metalloproteinase and TIMP levels using multiplex suspension array. However, several limitations in adopting plasma matrix metalloproteinase and tissue inhibitors of metalloproteinase levels as surrogate markers of myocardial proteolytic process must be acknowledged. First, plasma matrix metalloproteinase and tissue inhibitors of metalloproteinase levels do not necessarily be reflective of myocardial tissue concentrations. For example, matrix metalloproteinase-1 and -13 have very low or nondetectable plasma levels, but abundant levels of these matrix metalloproteinase types can be measured from myocardial samples.^{2,5,25} Second, the multiplex assay measures the absolute total amounts of matrix metalloproteinases and tissue inhibitors of metalloproteinases that are synthesized locally, and reflects a spillover from local sources. Accordingly, increased plasma levels of matrix metalloproteinases or tissue inhibitors of metalloproteinases would reflect a change in synthesis kinetics at the local level, and in this particular instance most likely the myocardium. However, these measurements may also be influenced by differences in clearance of these low molecular weight proteins, which is predominantly through renal clearance.^{30,3} In order to address this potential confounding issue, plasma creatinine levels were assessed as an index of renal function where no evidence for renal function impairment was found in the pediatric dilated cardiomyopathy group. While remaining speculative these findings would imply that the increased plasma matrix metalloproteinase and tissue inhibitors of metalloproteinase levels were due to increased synthesis and spillover rather than impaired clearance kinetics. Finally, in the present study, matrix metalloproteinase and tissue inhibitors of metalloproteinase plasma levels were only measured at one point in time, and how the temporal relation to the natural history of the myocardial remodeling process and progression to heart failure remains to be established in pediatric dilated cardiomyopathy. Nevertheless, the matrix metalloproteinase/ tissue inhibitors of

metalloproteinase measurements obtained in the present study can be utilized to provide some differential comparisons to past findings in adults with dilated cardiomyopathy (Table 4). The robust increase in plasma matrix metalloproteinase-7 appears to be a unique observation in pediatric dilated cardiomyopathy as this has not been reported previously in adult studies. Moreover, the relative increase in plasma matrix metalloproteinase-9 and the matrix metalloproteinase/tissue inhibitors of metalloproteinase-2 ratios are significantly higher in pediatric dilated cardiomyopathy when compared to adult dilated cardiomyopathy. These differential plasma matrix metalloproteinase and tissue inhibitors of metalloproteinase profile suggests a more aggressive proteolytic milieu exists in pediatric dilated cardiomyopathy which in turn would promote a more rapidly progressive left ventricular remodeling. While additional studies are necessary, these distinct differences in determinants of myocardial matrix structure and function may contribute to the ominous nature of dilated cardiomyopathy in children, and may be potentially useful as biomarkers for diagnosis and prognosis in pediatric dilated cardiomyopathy.

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Figure 1.

The fluorescence emissions for each set of MMP and TIMP standards were fit to a 5-parameter logistic (5-PL) equation, following a conventional format for non-linear fluorescence profiles. (13) Using this 5-PL curve fit, the fluorescence emissions for the samples were converted to actual MMP/TIMP values and finally corrected for dilution.