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Use of Biomarkers of Collagen Types I and III Fibrosis Metabolism to Detect Cardiovascular and Renal Disease in Chimpanzees (*Pan troglodytes*)

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Cardiovascular disease is the leading cause of morbidity and mortality among captive chimpanzees. The most prevalent form of cardiovascular disease among chimpanzees is sudden cardiac death. Myocardial fibrosis was the only significant pathologic lesion observed in affected animals at necropsy. We previously showed an association between myocardial fibrosis and sudden cardiac death. The presumed pathogenesis was interstitial myocardial fibrosis that led to decreased myocardial contractility and interrupted signal propagation in the heart, leading to fibrillation and resulting in sudden cardiac death. In this pilot study, we assayed 5 biomarkers of collagen types I and III metabolism and fibrogenesis and studied their association with CVD in chimpanzees. The biomarker MMP1 did not crossreact in chimpanzee sera and could not be studied further. Two biomarkers (TIMP1 and PINP) and their difference showed no significant association with CVD in chimpanzees. The biomarkers ICTP and PIIINP were significantly increased in cases of CVD with concurrent renal disease. Furthermore, both biomarkers showed a significant trend to increase with disease severity. We conclude that ICTP and PIIINP warrant further study for antemortem detection of renal and myocardial fibrosis in chimpanzees.

Abbreviations: CVD, cardiovascular disease; ICTP, initial carboxyl-terminal telopeptide; LV, left ventricular; MF, myocardial fibrosis; MMP1, matrix metalloproteinase 1; PIIINP, procollagen III N-terminal protein; PINP, procollagen I N-terminal protein; SCD, sudden cardiac death; TIMP1, tissue inhibitor of metalloproteinase 1.

Cardiovascular disease (CVD), including cardiomyopathy, systemic hypertension, cardiac arrhythmias, congestive heart failure, premature ventricular complexes, and cardiac arrest, is the predominant cause of morbidity and mortality in captive chimpanzees.[9,14,28,29,51,57] Myocardial fibrosis (MF) has been observed widely in chimpanzees.[20,28,29,41] In many of these animals, cardiac amyloidosis and MF have been detected on necropsy.[20,28,29,41,57] In addition, sudden cardiac death (SCD) and MF have been reported in other apes, including western lowland gorillas *(Gorilla gorilla gorilla*), orangutans (*Pongo pygmaeus*), and white-handed gibbons (*Hylobates lar*).[10,41,43,49]

The most thorough study of cardiovascular disease and the potential pathogenic role of MF was provided by a recent retrospective study of clinical and necropsy findings in 36 chimpanzee deaths over a 6-y period at the Alamogordo Primate Facility.[28] SCD was the predominant cause of death in these animals (36%).[29] Postmortem histologic examination of the hearts revealed the presence of MF in 81% of the chimpanzees and was the only significant histopathologic finding. All animals that died of SCD had some degree of interstitial MF,[28] which was significantly associated with both arrhythmias and sudden cardiac death. The conjecture was that MF resulted in decreased contractility and potentially disrupted signal propagation, leading to arrhythmias (ventricular precontractions) and eventually SCD, as has been observed in humans.[37] Closely similar findings of diffuse or idiopathic cardiomyopathy have since been reported as the primary cause of death at 2 other chimpanzee colonies and in US zoological parks.[13,51,57]

Fibrogenesis was thought to play an important role leading to chimpanzee CVD because MF was the only histopathologic abnormality observed.[14,28,29] An accumulation of extracellular matrix in the cardiac interstitial space, MF consists primarily of fibrillar type I and III collagen and is a major determinant of ventricular dysfunction.[14,24] Because myocardial cells cannot regenerate, myocardial damage leads to replacement of the myocardium by fibrous, noncontractile collagenous tissue. In humans, MF occurs in numerous cardiovascular conditions including systemic hypertension, myocarditis, cardiomyopathy, and other conditions leading to myocardial damage.[46,58]All of these conditions have been observed in chimpanzees.[9,28,29,53]

The hypothesis underlying the present study was that chimpanzees with incipient CVD have increased collagenous tissue in the heart that can be detected clinically antemortem by use of fibrogenesis biomarkers developed for use in humans. To test this hypothesis, we investigated 5 fibrogenesis biomarkers that have been used extensively in humans. Matrix metalloproteinase 1 (MMP1) is involved in structural remodeling of the left ventricle (LV) during congestive heart failure by degrading extracellular matrix proteins.[2,23] Tissue inhibitor of metalloproteinase 1 (TIMP1)

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blocks MMP1. Serum concentrations of both MMP1 and TIMP1 are increased in human patients with heart failure.[2] Decreased synthesis of TIMP1 can be induced by mechanical stress of human fibroblasts in vitro, suggesting a mechanism for fibrotic processes in myocytes.[59] Procollagen carboxyl-terminal telopeptide (PINP) is a fragment cleaved from procollagen I and is a marker of collagen I synthesis.[38] Initial carboxyl-terminal telopeptide (ICTP) is the first degradation product of collagen type I by MMP1. The (PINP – ICTP) difference measures the balance between synthesis and degradation of collagen type I. Procollagen III amino-terminal propeptide is cleaved from procollagen III and is a marker of collagen III turnover.[22,44] We assayed these 5 fibrogenesis markers here in a pilot study of 10 chimpanzees with CVD and 10 control chimpanzees, to determine whether chimpanzees with CVD exhibited increased serum concentrations of fibrogenesis biomarkers when compared with heart-healthy controls.

Materials and Methods

Animals. The Alamogordo Primate Facility is a research-reserve colony of 222 chimpanzees maintained in accordance with the *Guide for the Care and Use of Animals*[21] and the Animal Welfare Act. The facility and its program are fully AAALAC-accredited. All chimpanzees at the facility were fed a commercial primate diet (Monkey Diet Jumbo 5LR2; Purina, St Louis, MO) and maintained in same-sex group housing, to comply with the NIH chimpanzee breeding moratorium. Animals are maintained in indoor dens (180 ft^2) with radiant heated floor, air conditioning, 24-h access to an outdoor den (242 ft^2, 12 ft high), and weekly access to an 802 ft^2 exercise yard. All animals take part in an enrichment plan offering novel foraging activities and devices designed to stimulate psychologic wellbeing. All protocols were approved by the Alamogordo Primate Facility Institutional Animal Care and Use Committee.

Clinical assessment. All chimpanzees were given a complete clinical evaluation annually under sedation (3.5 mg/kg Telazol, Wyeth, Ft Dodge, IA). Clinical assessments included complete blood count, serum chemistry, body weight, body temperature, pulse oxygen, heart rate, blood pressure assessment, electrocardiography, abdominal ultrasonography, dental prophylaxis, and tuberculosis testing. Blood samples were collected immediately prior to the examinations. Blood pressure measurements, electrocardiography, heart rate, SpO_2, and core body temperature were performed and recorded by using a handheld data-acquisition device (Datascope Passport 2, Soma Technology, Bloomfield, CT).

Cardiovascular disease status. Determination of CVD status was made on the basis of clinical evaluation. Animals that presented no clinical signs of cardiovascular abnormalities were evaluated by our clinicians as heart-healthy. Animals that did present clinical signs of cardiovascular abnormalities (arrhythmias, cardiomegaly, hypertension, and murmurs) were evaluated as having CVD and were scheduled for echocardiography for further characterization of structural heart disease. Echocardiography (Aloka Prosound 5000, Tokyo, Japan) was performed by a board-certified veterinary cardiologist (MSS) using a 2.5-mHz linear transducer. Complete (including Doppler) serial echocardiograms included diastolic LV wall thickness, diastolic and systolic LV internal diameters, aortic and left atrial diameters, and Doppler interrogation (color and spectral) of all valves. The LV shortening fraction was calculated by using the equation:

(LV diastolic internal diameter – LV systolic internal diameter) / LV diastolic internal diameter.

Blood pressure was measured (Passport 2, Datascope, Mahwah, NJ). Three chimpanzees with systemic hypertension (systolic blood pressure greater than 160 mm Hg or diastolic blood pressure greater than 90 mm Hg) were treated with amlodipine (Pfizer, New York, NY). CVD cases were placed into 1 of 4 categories: systemic hypertension without cardiac changes (n = 3 chimpanzees), cardiomyopathy (n = 4, with LV wall thickening, LV chamber enlargement or decreased shortening fraction, or ventricular ectopy), degenerative valve disease (n = 1, with moderate mitral regurgitation, LV chamber enlargement, and normal LV wall thickness), and equivocal (n = 3; 2 with LV wall thickening without other echocardiographic abnormalities and 1 with LV chamber enlargement with normal wall thickness). Mild renal disease was present in 2 CVD cases. Ten controls were selected from animals that failed to present any clinical signs of cardiovascular abnormalities and were assumed to be heart-healthy. Disease status was coded as a trichotomy: healthy, CVD, CVD plus mild renal disease.

Laboratory methods. Fresh blood samples were collected into serum separator tubes, centrifuged, stored on wet ice, and shipped by overnight courier to the lab (GI Lab, Texas A and M University, College Station, TX). Five serum biomarkers of fibrogenesis were studied: PINP, ICTP, MMP1, TIMP1, and PIIINP. Serum concentrations of TIMP1 and MMP1 were determined by using commercially available ELISA kits according to manufacturer protocols (Human Biotrak System, Amersham Biosciences, Piscataway, NJ). Reactions were stopped with 1 M sulfuric acid and then read with a spectrophotometer (Multiskan Ascent Photometric plate reader, Thermo Fisher Scientific, Waltham, MA) at 450 nm. Sample concentrations were estimated by interpolation from the standard curves. Serum concentrations of ICTP, PINP, and PIIINP were assayed by using commercially available competitive radioimmunoassays (Orion Diagnostica, Espoo, Finland). Tubes were counted for 1 min by using a gamma counter (1470 Wallace Wizard Automatic Gamma Counter, Perkin Elmer, Waltham, MA). Sample concentrations were estimated by interpolation from the standard curve for each assay. One biomarker (MMP1) did not crossreact with chimpanzee sera and was not studied further.

Statistical methods. A case-control study was designed to evaluate the information content for the 4 biomarkers that crossreacted in chimpanzees (ICTP, PINP TIMP1, and PIIINP). Ten male chimpanzees with CVD were compared with their age-matched, heart-healthy controls ($2n$ = 20). All animals tested negative for human infectious disease agents (HIV, hepatitis B virus, and hepatitis C virus). This experimental design excluded the potential confounding variables of age and sex and excluded hepatitis C infection known to induce fibrosis[35,7] and potentially interfere with biomarker clearance rates.[8] Power calculations used G*Power.[12] Effect sizes were estimated from published human literature.[5,11,16,23,25,26,30,31,36,44,47,50,56] This design could detect real statistical effects with 85% average power.[40] All statistical analyses were conducted with SYSTAT (version 11.0, SYSTAT Software, Richmond, CA). The Shapiro–Wilks test was used to assess normality.[6,15,18] The Box–Cox power transformation series, $x' = (x^{\lambda}-1)/\lambda$, was used to induce normality (Table 1).[4,15] Calculation of confidence intervals used methods developed for transformed variables.[3] ANOVA and the omnibus F-test, as well as focused

comparisons, were used to test for differences in biomarker serum concentrations.[54] Outliers that exceeded 1.5 interquartile ranges and had Studentized residuals with *P* values of less than 0.010 were excluded from analysis.[54] Fitted models included age as a quantitative covariate.[28,29] Logistic regression and nonparametric methods for a one-way layout were used also.[6,19] In this pilot study, the nominal level of statistical significance was set at $\alpha = 0.10$, to indicate when further study might be warranted.

Results

One (MMP1) of the five biomarkers initially selected for evaluation did not crossreact with chimpanzee sera and was not studied further.

The 4 remaining biomarkers (TIMP1, PINP, ICTP, PIIINP) and the PINP – ICTP difference showed minor between-group differences in serum concentrations. Cases had on average 13% higher biomarker concentrations than controls, with variation among biomarkers (Table 1). For TIMP1, concentrations for cases averaged 60% higher than for controls but with large standard deviation. Excluding TIMP1, cases averaged only 1.5% higher serum concentrations (range, –4% to +14%) for the other biomarkers (PINP, ICTP, PIIINP, and PINP – ICTP) relative to their controls.

For PINP, neither age ($F_{1,16} = 0.004$, $P = 0.950$) nor CVD status ($F_{2,16} = 0.717$, $P = 0.503$) were associated with biomarker concentration. For TIMP1, despite the large observed difference between cases and controls (+60%, Table 1), neither age ($F_{1,16} = 1.077$, $P = 0.315$) nor CVD status ($F_{2,16} = 1.151$, $P = 0.341$) were significant. The PINP – ICTP difference similarly lacked significance for both age ($F_{1,16} = 0.006$, $P = 0.939$) and CVD status ($F_{2,16} = 0.539$, $P = 0.593$).

For PIIINP, neither age ($F_{1,16} = 1.850$, $P = 0.193$) nor CVD status ($F_{1,16} = 2.383$, $P = 0.124$) were associated with serum concentrations. Exclusion of a single outlier (Studentized residual = –2.906, $P = 0.010$) resulted in significant effects of both age ($F_{1,15} = 4.115$, $P = 0.061$) and CVD status ($F_{1,15} = 3.214$, $P = 0.069$) on PIIINP concentrations. PIIINP showed a significant trend toward an increase from healthy controls (16.4 µg/mL) to cases of CVD alone (18.2 µg/mL) to cases of CVD with concurrent renal disease (25.6 µg/mL; $F_{1,15} = 6.404$, $P = 0.023$). This trend was significant even when the outlier was included ($F_{1,16} = 4.250$, $P = 0.056$). Pairwise comparisons indicated that the major difference was between healthy controls and CVD cases with concurrent renal disease ($P = 0.069$).

For ICTP, age was not a significant factor ($F_{1,16} = 0.444$, $P = 0.515$). However, CVD status was significantly related to serum concentration ($F_{1,17} = 5.203$, $P = 0.017$). The focused comparison indicated a significant trend for ICTP to increase across levels of disease severity ($F_{1,17} = 4.450$, $P = 0.050$). Pairwise comparisons indicated that this trend in ICTP levels resulted predominantly because CVD cases with concurrent mild renal disease had higher values (22.3 µg/L) than CVD cases alone (11.2 µg/L; $P = 0.018$). Interestingly, both cases of renal disease also had cardiomyopathy, whereas the single case of cardiomyopathy without renal disease did not differ from healthy controls.

ANOVA was useful to test for different biomarker concentrations between cases and controls. However, because the purpose of a biomarker is to predict disease status (CVD versus no CVD) on the basis of observed biomarker levels, ANOVA effectively conceptualized the causal chain in reverse. We therefore used logistic regression to assess whether these 4 biomarkers could be used in combination to predict disease, using age as a covariate.

Table 1. Descriptive statistics for 4 fibrosis biomarkers.

Biomarker	Lambda	Observed difference (%)	Expected difference (%)
PINP	+0.50	−4%	+22%
ICTP	−0.25	0%	+81%
TIMP1	−0.50	+60%	+41%
PINP – ICTP	0	−4%	−58%
PIIINP	−0.25	+14%	+47%

Lambda is the maximum likelihood estimate of the Box–Cox[4] λ parameter used to induce normality. Observed difference is the mean percentage difference observed between CVD cases and controls. Expected difference is the mean percentage difference expected based on a meta-analysis of human CVD patients and controls. Negative values for observed and expected differences indicate that controls had higher concentrations than CVD cases.

Results indicated that the tested biomarkers were not useful for prediction of whether a chimpanzee was a CVD case, a case of CVD with renal disease, or a healthy control (all $t < |1.00|$; all $P > 0.355$).

Discussion

Cardiovascular disease is the most common cause of chimpanzee morbidity and mortality and typically is accompanied by variable amounts of fibrosis observed postmortem in chimpanzee hearts.[9,14,20,28,29,41,51,57] PIIINP levels were significantly higher in CVD cases with renal disease and showed a significant trend to increase across disease categories. ICTP levels were also higher in CVD cases with renal disease than in CVD cases alone. The significant association between ICTP and PIIINP and mild renal disease with CVD was unexpected. However, these biomarkers have been used to detect fibrogenesis-related diseases in many different target tissues including liver,[45] lungs,[27,45,52] heart,[8,33,34] bone,[8,17,45] and synovial fluid.[45] The lack of tissue specificity of PIIINP and ICTP serendipitously enabled identification of ongoing fibrotic processes in the kidney. Interestingly, both cases of renal disease were mild at the time of the survey but since have progressed to severe renal disease. PIIINP appeared to detect fibrosis in both heart and kidney. Further studies on ICTP for kidney fibrosis and on PIIINP for cardiovascular fibrosis are warranted.

The lack of association between PINP and the PINP – ICTP difference and CVD was not surprising. Except for PINP, expected effect sizes were large (Table 1).[5,11,16,23,25,26,30,32,36,44,47,50,56] Significant group differences, if they existed, could be detected with a power of greater than 86%. Therefore, sample size did not compromise the results for TIMP1, PIIINP, or ICTP. But the expected PINP effect size was small[5,30,36] and even negative in one study,[56] indicating that inadequate power (41%) due to small sample size could explain the nonsignificant PINP findings. However, given the observed case – control difference of –4%, a sample of 1920 chimpanzees would be needed to detect a significant PINP difference at the recommended 85% power[40] but in the wrong direction (that is, with cases less than healthy controls). This result demonstrates that PINP and the PINP – ICTP difference remain poor biomarkers to study fibrogenic processes in chimpanzees.

PIIINP levels were higher in CVD cases with renal disease than in healthy controls, showed the expected effect of age, and showed a significant trend in the hypothesized direction. Assuming the interstitial MF–SCD conjecture is true, more complex pro-

cesses may be operative to make this association more difficult to detect in serum-based assays and in histopathology. In rats, pressure overload (systolic wall stress) increased interstitial collagen accumulation, whereas volume overload (diastolic wall stress) showed no change, suggesting differential regulation of collagen accumulation processes.[42] Collagen synthesis or degradation, as measured in serum biomarkers, does not necessarily equal collagen deposition and fibrogenesis, as measured in cardiac tissue histopathology.[45] The amount of collagen III actually deposited in the myocardium depends upon the balance of deposition and extracellular matrix degradation.[45] Because the collagen III amino-terminal propeptide is not always removed but may remain attached to the procollagen peptide, measurement of PIIINP probably underestimates actual collagen III synthesis.[8,45] Variation in the relative abundance of collagen I to collagen III could complicate histopathologic detection.[48] Differential serum detection of collagen type I versus collagen type III has also been reported.[39] Histopathology results were used to rank MF severity based on gross extent[28] but did not assess localization, distribution, or composition (collagen I:collagen III ratio) of MF, all of which factors could influence pathologic effects.[37,55] Future studies will examine the number, size, and distribution of fibrotic patches in the myocardium of deceased animals, compared with PIIINP serum levels collected prior to death, to compare CVD cases with heart-healthy controls. Such studies could advance our understanding of the role of MF in inducing chimpanzee CVD.

CVD heterogeneity may have obscured differences in PIIINP levels between CVD cases and healthy controls. In our current understanding, chimpanzee CVD appears to involve clinical progression from left ventricular hypertrophy to dilated cardiomyopathy, probably initiated by certain disease processes including systemic hypertension. This sequence effectively reduces CVD heterogeneity to stages in disease progression. The absence of dilated cardiomyopathy cases in the current study could mean that CVD had not progressed sufficiently to have detectable fibrosis.[28,29] An advantage to using a single heterogeneous disease category is that use of disease subtypes increases misclassification error and reduces sensitivity by increasing specificity.[1] Disease heterogeneity did not prevent us from finding statistically significant differences in serum concentrations in brain-type natriuretic protein and cardiac troponin I between these same CVD cases and controls. A larger sample size of CVD subtypes could yield more robust results by reducing within-group variability, particularly for TIMP1.

In summary, we have demonstrated that 2 commonly used biomarkers of fibrogenesis (ICTP, PIIINP) identified CVD cases with concurrent renal disease, whereas 1 (PIIINP) seems to detect trends in CVD severity. Both biomarkers warrant further study. Another 2 markers (PINP, TIMP1) were not informative. Due to its inherently weak effect size,[5,30,36,56] further study of PINP is not recommended. TIMP1 may benefit from larger samples of chimpanzee CVD subtypes. The use of ICTP to detect early development of renal fibrosis is recommended. Meanwhile, the conjecture that interstitial MF was the key pathologic element in inducing CVD-related mortality requires further study. We are planning to do such studies of PIIINP and ICTP using deceased animals to determine whether the distribution and amount of fibrosis in the myocardium is associated with biomarker concentrations in sera.

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