






BRIEF COMMUNICATION

Circulating S-Glutathionylated cMyBP-C as a Biomarker for Cardiac Diastolic Dysfunction

Xiaoxu Zhou, MD, PhD; Euy-Myoung Jeong, PhD; Hong Liu, MD, PhD; Bahaa Kaseer, MD; Man Liu , PhD; Suvash Shrestha, MD; Hafiz Imran , MD; Kylie Kavanagh , DVM, MS, MPH; Ning Jiang, MD; Lori-Ann Desimone, BSN; Feng Feng, BS; Guangbin Shi, MD; Go Eun Jeong, BA; Anyu Zhou, MD, PhD; Philip Stockwell , MD; Samuel C. Dudley Jr, , MD, PhD

BACKGROUND: cMyBP-C (Cardiac myosin binding protein-C) regulates cardiac contraction and relaxation. Previously, we demonstrated that elevated myocardial S-glutathionylation of cMyBP-C correlates with diastolic dysfunction (DD) in animal models. In this study, we tested whether circulating S-glutathionylated cMyBP-C would be a biomarker for DD.

METHODS AND RESULTS: Humans, African Green monkeys, and mice had DD determined by echocardiography. Blood samples were acquired and analyzed for S-glutathionylated cMyBP-C by immunoprecipitation. Circulating S-glutathionylated cMyBP-C in human participants with DD (n=24) was elevated (1.46±0.13-fold, $P=0.014$) when compared with the non-DD controls (n=13). Similarly, circulating S-glutathionylated cMyBP-C was upregulated by 2.13±0.47-fold ($P=0.047$) in DD monkeys (n=6), and by 1.49 (1.22–2.06)-fold ($P=0.031$) in DD mice (n=5) compared with the respective non-DD controls. Circulating S-glutathionylated cMyBP-C was positively correlated with DD in humans.

CONCLUSIONS: Circulating S-glutathionylated cMyBP-C was elevated in humans, monkeys, and mice with DD. S-glutathionylated cMyBP-C may represent a novel biomarker for the presence of DD.

Key Words: cMyBP-C ■ diastolic dysfunction ■ S-glutathionylation

Heat failure with preserved ejection fraction (HFpEF) accounts for >50% of heart failure in patients¹ and cardiac diastolic dysfunction is the pathological condition underlying most HFpEF.² HFpEF is increasing in prevalence, causing increased morbidity and mortality.³ Few pharmacological approaches are effective in treating HFpEF.^{4,5}

cMyBP-C (Cardiac myosin binding protein C) plays a principally structural role in the assembly and stabilization of the sarcomere.⁶ Evidence shows that cMyBP-C is a key determinant of the speed and force of cardiac contraction.⁷ cMyBP-C modification can result in cardiac diastolic dysfunction (DD).⁸ Recently, we have shown that altered myofilament properties and S-glutathionylated myocardial cMyBP-C are associated with DD.^{9,10}

Circulating cMyBP-C has been suggested as a novel biomarker for myocardial infarction.¹¹ In this study, we tested whether circulating S-glutathionylated cMyBP-C is correlated with the presence of DD.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Patient Enrollment

This was a prospective, observational, cross-sectional clinical study and was conducted at the Lifespan

Correspondence to: Samuel C. Dudley Jr, MD, PhD, Division of Cardiology, University of Minnesota, VCRC 286 - MMC 508, 420 Delaware St., SE, Minneapolis, MN 55455. Email: sdudley@umn.edu

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Health System (the Miriam Hospital and Rhode Island Hospital) in Providence, Rhode Island. The study was approved by Lifespan Institutional Review Board. Participants were adults (age ≥ 18 years) and were identified at the time of echocardiography. Only participants with normal regional wall motion, wall thickness, cardiac dimensions, and left ventricular ejection fraction were enrolled. Participants were divided into DD and non-DD groups based on E/E' (the ratio of transmitral Doppler early filling velocity E to tissue Doppler early diastolic mitral annular velocity E') value, an echocardiographic index of cardiac diastolic function. All study participants signed written informed consent before enrollment. All study participants were subjected to phlebotomy at the time of enrollment.

Animal Experiments

Monkey blood samples were kindly provided by the Non-Human Primate Program of Wake Forest Baptist Medical Center in Winston-Salem, North Carolina. African green monkeys with either diabetes (by hemoglobin A1c level) and/or hypertension ($\geq 135/85$ mm Hg) were screened by echocardiography for DD as in the previous study.¹² Age-matched healthy monkeys were used as non-DD controls. All monkeys experienced the same housing conditions and diet.

High-fat diet (60 kcal% fat, Research Diet, New Brunswick, NJ) induced diabetic mice and age-matched normal diet mice (C57BL/6J) were obtained from Jackson Laboratory (Bar Harbor, ME) at 26 weeks old and were screened by fasting glucose level for diabetes and by echocardiography for DD. Animal care and interventions were provided in accordance with the National Institutes of Health *Guide for the Care and Use of Experimental Animals*, and all animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Measurement of S-Glutathionylated cMyBP-C

Circulating S-glutathionylated cMyBP-C was determined quantitatively using multimeric immunoprecipitation method (Pierce Classic Magnetic IP/Co-IP Kit, ThermoFisher Scientific, Waltham, MA).

For human blood samples, fresh drawn blood samples (1 mL) were mixed with 100 μ L of non-reducing preservative reagents including 25 mmol/L N-Methylmaleimide, neocupporine, and diethylenetriaminepentaacetic acid to inhibit further thiol oxidative degradation. Non-reducing plasma samples (200 μ L) were bound with 2 μ g of mouse anti-glutathione primary antibody (Virogen, Watertown, MA) at 4°C overnight. For monkey or mouse blood samples, blood was prepared without reducing reagents and ≈ 500 μ L serum was bound with 20 μ g of anti-cMyBPC mouse

primary antibody (Santa Cruz Biotechnology, Dallas, TX) at 4°C overnight. Antigen-antibody complexes were precipitated using protein A-conjugated magnetic beads and eluted with 100 μ L of nonreducing sample buffer. Immunoprecipitated samples were separated on a 4% to 20% prestained SDS-PAGE gel and transferred onto a nitrocellulose or a polyvinylidene difluoride membrane. The membrane was blocked by 5% non-fat dry milk for 1 hour. Rabbit anti-cMyBP-C primary antibody (H120, Santa Cruz Biotechnology, Dallas, TX) for human samples or mouse anti-glutathione primary antibody (VroGen, Watertown, MA) for animal samples were applied to detect glutathionylated cMyBP-C. The ChemiDoc MP System (Bio-Rad, Hercules, CA) was used to measure the optical density of the bands, and then the bands were analyzed by ImageJ imaging analysis software.

Statistical Analysis

Continuous variables were represented as mean \pm SEM when normally distributed. Categorical variables were expressed in percentages. Two-tailed Student *t*-test and Fisher exact test were used for comparisons of continuous and categorical variables, respectively, between DD and non-DD groups. Data that were not normally distributed were represented as median and interquartile range and compared with the Mann-Whitney test between groups. The Pearson correlation coefficient was used to determine the correlation between E/E' and circulating S-glutathionylated cMyBP-C level in human participants. All statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). A $P < 0.05$ was considered statistically significant.

RESULTS

Overall, 24 participants with DD and 13 with non-DD met eligibility criteria and were enrolled in the study. As shown in Table 1, there was no difference in sex, race, body mass index, or tobacco use between the participants with or without DD. The mitral E/E' was significantly higher in participants with DD (12.7 ± 1.4) than participants with non-DD (8.0 ± 0.84 , $P = 0.010$), while the left ventricular ejection fraction was comparable. The age of the participants with DD (76 ± 2) was ≈ 20 years older than the participants with non-DD (55 ± 5 , $P < 0.0001$). The hypertension incidence was higher in the participants with DD (83.3%) than the non-DD controls (46.2%, $P = 0.028$). The incidence of other coexisting diseases, including diabetes and chronic kidney diseases and the medications were similar between groups. Because of the small sample size, adjustment of confounding variables such as patient age differences between groups was not possible.

Table. Participant's Demographic and Clinical Characteristics

	Control	n	DD	n	P value
Age, y	55±5	13	76±2	24	<0.0001*
Sex (men, %)	51.5	13	50	24	0.731
Race (White, %)	84.6	13	91.7	24	0.602
BMI	27.3 (24.1–31.9)	12	28.2 (24.2–34.8)	24	0.580
Past tobacco, %	50	12	73.9	23	0.261
Current tobacco, %	0	12	13.0	23	0.536
LVEF, %	60.3±2.1	12	63.1±1.5	23	0.279
Mitral E/E'	8.0±2.8	11	12.7±4.8	12	0.010*
Diabetes, %	15.4	13	37.5	24	0.262
Hypertension, %	46.2	13	83.3	24	0.028*
CKD, %	0	13	12.5	24	0.538
ACEI, %	30.8	13	50.0	24	0.315
β-blocker, %	61.5	13	66.7	24	1.000
ARB, %	15.4	13	8.3	24	0.602

Data are expressed as mean±SEM or median and interquartile range. ACEI indicates angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; CKD, chronic kidney disease; DD, diastolic dysfunction; and LVEF, left ventricular ejection fraction.

* $P<0.05$; P value from *t*-tests or Mann-Whitney tests for continuous variables and Fisher exact tests for categorical variables.

As shown in Figure 1, circulating S-glutathionylated cMyBP-C in participants with DD was elevated (1.46 ± 0.13 -fold, $P=0.014$) compared with the non-DD controls.

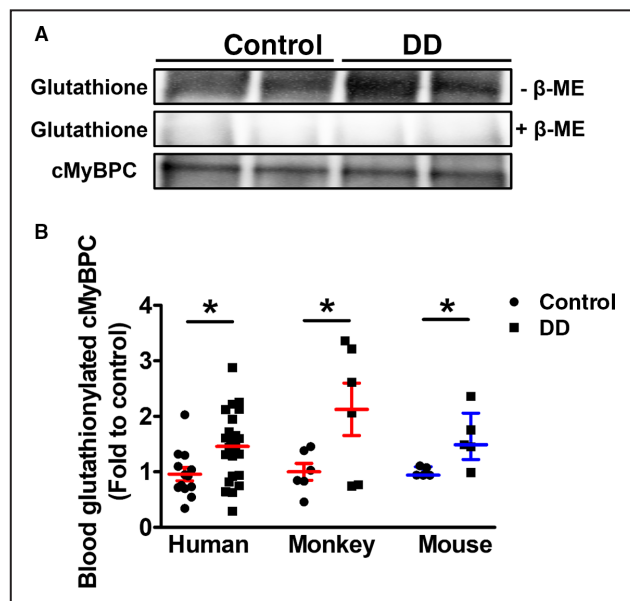


Figure 1. Circulating S-glutathionylated cMyBP-C in diastolic dysfunction.

A, Representative Western blot images of glutathione and cMyBP-C bands under reducing and nonreducing conditions. **B**, S-glutathionylated cMyBP-C in blood was elevated in humans, monkeys, and mice with diastolic dysfunction compared with the non-diastolic dysfunction controls ($n=13$, 24 in human, 6 in monkey, 5 in mouse). Bars are mean±SEM (in red), or median and interquartile range (in blue). Unpaired *t*-tests or Mann-Whitney tests were used. * $P<0.05$, vs controls. cMyBP-C indicates cardiac myosin binding protein C; DD, diastolic dysfunction; and β-ME, beta-mercaptoethanol.

Elevation in circulating S-glutathionylated cMyBP-C was also observed in primates and mice with DD (Figure 1). The circulating S-glutathionylated cMyBP-C was upregulated by 2.13 ± 0.47 -fold ($P=0.047$) in DD primates and by 1.49 (1.22–2.06)-fold ($P=0.032$) in DD mice when compared with the respective non-DD controls.

A Pearson correlation analysis was performed between circulating S-glutathionylated cMyBP-C level and mitral E/E' value in human participants, showing a significant positive correlation between the 2 variables (Figure 2, $r=0.496$, $P=0.016$).

DISCUSSION

Currently, there are no specific biomarkers for DD. Our previous studies have implicated myocardial S-glutathionylated cMyBP-C as contributing to the pathogenesis of DD.^{9,10} In this study, we found that circulating S-glutathionylated cMyBP-C was elevated in humans and animals with cardiac DD and were significantly correlated with diastolic function.

cMyBP-C can be released from heart tissue into the blood, especially in patients with acute myocardial infarction. Our data shows that modified cMyBP-C is elevated in the plasma when DD is present. In mouse studies, we have not seen significant cardiomyocyte apoptosis, suggesting that this increased release is secondary to some other stress response.

S-glutathionylation is the formation of a mixed disulfide between a cysteine moiety of GSH and a protein cysteine moiety. Increased S-glutathionylation can occur because of disulfide thiol exchange in the presence of excess glutathione disulfide, oxidative

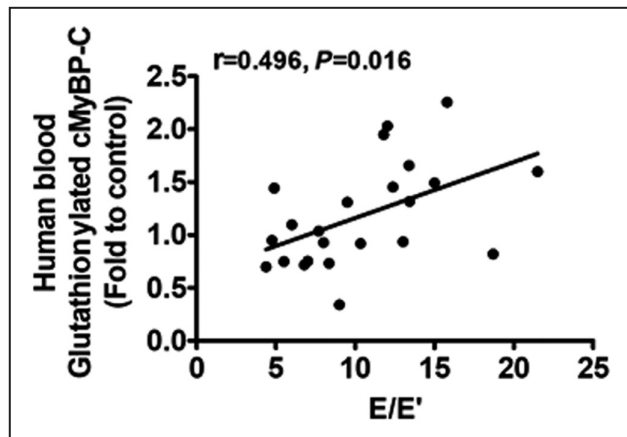


Figure 2. Correlation between circulating S-glutathionylated cMyBP-C level and cardiac diastolic function.

S-Glutathionylated cMyBP-C in blood was positively correlated with the ratio of transmitral Doppler early filling velocity E to tissue Doppler early diastolic mitral annular velocity E', an echocardiographic indicator of diastolic dysfunction, by Pearson correlation test (11 participants with non-diastolic dysfunction and 12 participants with diastolic dysfunction, $r=0.496$, $P=0.016$). cMyBP-C indicates cardiac myosin binding protein C.

activation of the protein sulfhydryl group, or formation of an S-nitroso adduct. Previously, we have shown glutathionylation of cMyBP-C leads to alteration in calcium affinity explaining DD in the hypertensive mouse model.⁹ In that study, no other posttranslational modification of any contractile protein was correlated with changes in DD. Therefore, we hypothesized that this modification of cMyBP-C was likely causative of DD.

Hypertension, diabetes, and aging are known risk factors for DD. As expected, humans with DD were older and had higher incidence of hypertension than non-DD subjects. Likewise, primates with diabetes and/or hypertension displayed impaired diastolic function. Finally, DD was induced in mice by high-fat diet, which is known to cause diabetes.¹³ Elevation of circulating S-glutathionylated cMyBP-C was observed in all those subjects, suggesting that DD under various risk factors share a common pathophysiology.

The standard diagnosis of HFpEF usually depends on the echocardiographic evaluation, which generally happens only after the onset of heart failure symptoms.¹⁴ Useful roles for a biomarker of DD might include screening of asymptomatic populations at risk for HFpEF, assisting in rapid diagnosis of acute heart failure occurrences, and directing future therapies.

CONCLUSIONS

Our results support the hypothesis that circulating S-glutathionylated cMyBP-C can serve as a diagnostic biomarker for DD and HFpEF.

ARTICLE INFORMATION

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Affiliations

Division of Cardiology, Department of Medicine, The Lillehei Heart Institute, University of Minnesota, Minneapolis, MN (X.Z., H.L., M.L., F.F., S.C.D.); Lifespan Cardiovascular Research Center, Brown University, Providence, RI (E.J., B.K., S.S., H.I., N.J., L.D., G.S., A.Z., P.S.); Department of Pathology, Wake Forest Baptist Medical Center, Winston-Salem, NC (K.K.); and Department of Biology and Medicine, Brown University, Providence, RI (G.E.J.).

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Disclosures

Dudley is the inventor on patent applications: (1) 11/895,883 Methods and Compositions for Treating Diastolic Dysfunction, (2) 13/503,812 Methods of Diagnosing Diastolic Dysfunction, (3) 13/397,622 Methods for Treating Diastolic Dysfunction and Related Conditions, (4) 13/658,943 Method of Improving Diastolic Dysfunction, (5) 13/841,843 Myosin Binding Protein-C for Use in Methods Relating to Diastolic Heart Failure, and (6) 61/728,302 Mitochondrial Antioxidants and Diabetes. The remaining authors have no disclosures to report.

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