Elevated Plasma Marinobufagenin, An Endogenous Cardiotonic Steroid, Is Associated With Right Ventricular Dysfunction and Nitrative Stress in Heart Failure

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Background—Plasma levels of cardiotoxic steroids are elevated in volume-expanded states, such as chronic kidney disease, but the role of these natriuretic hormones in subjects with heart failure (HF) is unclear. We sought to determine the prognostic role of the cardiotoxic steroids marinobufagenin (MBG) in HF, particularly in relation to long-term outcomes.

Methods and Results—We first measured plasma MBG levels and performed comprehensive clinical, laboratory, and echocardiographic assessment in 245 patients with HF. All-cause mortality, cardiac transplantation, and HF hospitalization were tracked for 5 years. In our study cohort, median (interquartile range) MBG was 583 (383–812) pM. Higher MBG was associated with higher myeloperoxidase (r=0.42, P<0.001), B-type natriuretic peptide (r=0.25, P=0.001), and asymmetrical dimethylarginine (r=0.32, P<0.001). Elevated levels of MBG were associated with measures of worse right ventricular function (RV) and predicted increased risk of adverse clinical outcomes (MBG≥574 pmol/L: hazard ratio 1.58 [1.10–2.31], P=0.014) even after adjustment for age, sex, diabetes mellitus, and ischemic pathogenesis. In mice, a left anterior descending coronary artery ligation model of HF lead to increases in MBG, whereas infusion of MBG into mice for 4 weeks lead to significant increases in myeloperoxidase, asymmetrical dimethylarginine, and cardiac fibrosis.

Conclusions—In the setting of HF, elevated plasma levels of MBG are associated with right ventricular dysfunction and predict worse long-term clinical outcomes in multivariable models adjusting for established clinical and biochemical risk factors. Infusion of MBG seems to directly contribute to increased nitrative stress and cardiac fibrosis. (Circ Heart Fail. 2015;8:1068-1076. DOI: 10.1161/CIRCHEARTFAILURE.114.001976.)

Key Words: cardiac fibrosis • cardiotoxic steroids • heart failure • nitrative stress • outcome

Cardiotoxic steroids (CTS) are a class of endogenous volume-sensitive hormones that bind to the Na/K-ATPase and include cardenolides (such as digoxin and ouabain) and aglycone bufadienolides (such as telocinobufagin and marinobufagenin [MBG]). Increased circulating levels of CTS have been proposed as a compensatory mechanism for natriuresis and vascular tone in volume-expanded conditions, including salt-sensitive hypertension, chronic kidney disease, and preeclampsia. In these settings, CTS contribute to enhanced natriuresis by inducing endocytosis of proximal tubule cell Na+/K-ATPase. This serves to remove the Na+/K-ATPase from the basolateral membrane and thus reduces the transport of sodium from the tubular lumen to the blood compartment, thereby increasing sodium excretion. However, CTS may exert off-target signal transduction effects beyond their direct effects on the sodium pump. Hence, chronic stimulation of Na+/K-ATPase signaling by CTS has important implications not only for the natriuretic response to increased salt load but also for pathological adaptation to volume expansion, including hypertension, hypertrophy, and fibrosis. Accumulation of MBG and other CTS has been documented in a variety of cardiovascular disease states beyond conditions marked by plasma volume expansion and fluid retention. Furthermore, myocardial hypertrophy and growth effects of CTS have been proposed in the setting of hypertension and renal dysfunction and supported by both experimental...
and clinical data, demonstrating the association between an endogenous digoxin-like substance and the development and severity of heart failure (HF). Clinical and experimental evidence from our group and others has also demonstrated the pro-oxidant and profibrotic effects of these steroid hormones in both cardiac and renal tissue.20-23 Because the relationship between MBG and myocardial structure and performance in the contemporary HF population has not been examined, we hypothesized that an increase in circulating MBG levels may track with disease severity and provide important prognostic information in the setting of HF. Further, because MBG is increased in volume overloaded states and associated with diastolic dysfunction in animal models, we hypothesized that circulating levels would be increased in HF patients with preserved ejection fraction and associated with echocardiographic indices of diastolic dysfunction. Herein, we examined the relationship between circulating levels of MBG with echocardiographic parameters, as well as long-term adverse clinical outcomes, in patients with HF. We also used an animal infarction model to demonstrate the contribution of MBG to the clinical phenotype observed in our human study.

Methods

Study Design and Population
We prospectively enrolled 245 patients with HF (≥18 years) seen at the Cleveland Clinic with a clinical diagnosis of HF and New York Heart Association functional class I-IV symptoms, who were free of significant renal, hepatic, and valvular diseases. Study participants were excluded if they experienced any of the following: (1) major cardiovascular event (myocardial infarction [MI], unstable angina, stroke, transient ischemic attack, and pulmonary embolism) within the preceding 30 days; (2) significant lung diseases, including chronic obstructive pulmonary disease, pulmonary fibrosis, pulmonary arterial hypertension, and asthma; or (3) major surgery or use of inotropic agents within the past month. We prospectively followed a composite end point, including adverse clinical events (all-cause mortality, cardiovascular event [myocardial infarction, unstable angina, transient ischemic attack, or pulmonary embolism] within 6 months of blood draw. The study protocol was approved by the Cleveland Clinic Institutional Review Board, and written informed consent was obtained from each of the study participants before their participation in the study.

Biochemical Assays
Blood samples were collected in ethylenediaminetetraacetic acid plasma vacutainers at the time of clinical and echocardiographic and hemodynamic evaluation, were immediately aliquotted, and were stored at −80°C until analysis. Plasma samples were extracted for MBG measurements using C18 SepPak cartridges (Waters Inc., Cambridge, MA), and MBG levels were measured using a competitive fluoroimmunoassay (dissociation-enhanced lanthanide fluoroimmunoassay) as previously described.22,24 The MBG dissociation-enhanced lanthanide fluoroimmunoassay uses a murine monoclonal antibody (anti-MBG 4G4) and uses competition between immobilized antigen (MBG–glycoside–thryglobulin) and MBG, other cross-reactants, or endogenous CTS within the sample for a limited number of binding sites on the 4G4 anti-MBG monoclonal antibodies. Secondary (goat anti-mouse) europium-labeled antibody was obtained from PerkinElmer (Waltham, MA). Data on cross-reactivity of the MBG antibody have been reported previously.17,25 For analysis of MBG tissue levels, adrenal glands were homogenized (TissueLyser II, Quiagen; 5 mm stainless steel beads), and the homogenate was extracted with 10-fold excess of methyl tert-butyl ether before drying under nitrogen gas and resuspension in assay buffer (50 mmol/L Tris-hydrochloride, 154 mmol/L sodium chloride, 7.7 mmol/L sodium azide, pH 7.8). Plasma myeloperoxidase (MPO) levels were determined by an enzyme-linked immunosorbent assay (CardioMPO II test; Cleveland Heart Laboratories, Cleveland OH). Arginine metabolic profiles were quantified by liquid chromatography-electrospray ionization tandem mass spectrometry as previously described.25,26 All other assays, including B-type natriuretic peptide, basic metabolic profile, and lipid profile, were measured on the Architect platform (Abbott Laboratories, Abbott Park IL). The 4-variable Modification of Diet in Renal Disease equation was used to calculate estimated glomerular filtration rate.27

Transsthoracic Echocardiography
Comprehensive 2-dimensional echocardiography was performed in standard parasternal and apical views on all participants by an American Society of Echocardiography registered research sonographer using a Vivid 7 echocardiography machines (GE Healthcare, Waukesha WI) equipped with a phased-array transducer and following the American Society of Echocardiography recommendations.28,29 Further detail on echocardiographic analysis is described in the Data Supplement.

Animal Study
To assess MBG levels in a mouse model of post-MI HF, left anterior descending (LAD) artery ligation (LADx) was performed in C57BL/6J mice, and plasma MBG was assayed after 4 weeks. Briefly, mice were intubated and ventilated with 60% oxygen at 120 breaths per minute with an inspiratory pressure of 16 to 18 cm H2O using a rodent ventilator (Harvard Apparatus). After sternotomy was performed, the left atrium was retracted for visualization of the proximal LAD using a surgical microscope (Leica M500), and the LAD was ligated with 10-0 prolene suture. Blanching and dysfunction of the anterior wall verified LADx. To directly test for a potential contribution of MBG to promotion of cardiac dysfunction and nitrative stress, osmotic minipumps (Alzet model 1004) were placed intraperitoneally to deliver MBG (0.1 μg/g/d) or vehicle to mice for 4 weeks similar to what we have reported in the rat.22 Quantitative real-time PCR was used to measure gene expression with 18S rRNA used as the internal control (TaqMan, Life Technologies). These studies were approved by the Cleveland Clinic Institutional Animal Care and Use Committee, and the procedures followed were in accordance with the institutional guidelines.

Quantitative Histological Techniques
Mason’s trichome and picrosirius red staining was performed on deparaffinized 5 μm serial heart sections. The sections were mounted under a Leica DM 2500 microscope and digitized with a QImaging MicroPublisher 5.0 RTV camera. Further detail on quantitative morphometric analysis is described in the Data Supplement.

Statistical Analysis
Normally distributed continuous variables were summarized as mean±standard deviation if normally distributed or median (interquartile range) if non-normally distributed. If >2 groups were compared, 1-way analysis of variance was performed before comparison of individual groups with the unpaired Student’s t test with Bonferroni’s correction for multiple comparisons. If only 2 groups of normal data were compared, the Student’s t test was used without correction. P values for nonparametric comparisons or those based on small sample size were performed using the Mann-Whitney test. Associations between changes in MBG and clinical, biochemical, and echocardiographic measures were performed using the Spearman’s rank correlation method. Clinical risk and time to clinical adverse events associated with increased MBG levels were assessed by Cox
proportional hazard analysis, and the units for hazard ratios represent the dichotomized values of MBG ≥574 and <574 pmol/L. The optimal sensitivity and specificity cutoff of MBG (≥574 pmol/L) levels in our cohort was determined using receiver operator characteristic curve analyses in the context of the time to event, and the hazard ratios reported represent the risk associated with MBG levels ≥574 pmol/L. This value was chosen as the optimal receiver operator characteristic cut point that maximized sensitivity plus specificity in receiver operator characteristic curve analysis of the nominal logistic regression fit between MBG modeled as a continuous variable and adverse clinical outcomes modeled as a nominal dichotomous variable. Survival curves (all-cause mortality, cardiac transplantation, or HF hospitalization) were generated from Kaplan–Meier survival analysis. Continuous net reclassification improvement was used as a test of association, and integrated discrimination improvement was used to measure improvement in model performance. The $P$ values compare models with and without MBG. The model was adjusted for traditional risk factors, including age, sex, diabetes mellitus, and ischemic pathogenesis. Statistical analysis was performed using GraphPad Prism, JMP 10.0 (SAS Institute, Cary, NC), and R 3.1.2 (Vienna, Austria). $P$ values <0.05 were considered statistically significant.

## Results

### Subject Characteristics

Baseline characteristics of our study cohort are presented in Table. Median MBG level was 583 (interquartile range 383–812) pmol in patients with HF compared with 241 (interquartile range 212–281) pmol in non-HF controls (n=13, mean age 43±12 years, 64% male, body mass index 27±3, 15% black). Patients with HF have higher MBG levels when compared with the non-HF controls, regardless of whether they have reduced or preserved left ventricular ejection fraction (Figure 1). Furthermore, higher MBG was associated with higher indices of inflammation/oxidative stress (MPO: $r=0.42$, $P<0.0001$), myocardial stress (B-type natriuretic peptide: $r=0.25$, $P=0.001$), and nitrative stress (asymmetrical dimethylarginine [ADMA]: $r=0.32$, $P<0.001$; symmetrical dimethylarginine: $r=0.34$, $P<0.001$; and mono methyl arginine: $r=0.40$, $P<0.0001$). In our study cohort, there was no significant association between MBG and cystatin C ($P=0.485$) and estimated glomerular filtration rate ($P=0.345$).

### MBG Levels and Myocardial Structure and Performance

Table I in the Data Supplement presents the relationships between MBG levels and echocardiographic parameters of cardiac structure and performance. In univariate analysis, higher MBG was associated with indices of left ventricular diastolic function (mitral deceleration time: $r=−0.24$, $P=0.007$) and right ventricular (RV) diastolic function (tricuspid $E'/E$ ratio: $r=0.22$, $P=0.027$; tricuspid deceleration time: $r=−0.38$, $P=0.002$), and larger RV size (RV end-diastolic area: $r=−0.21$, $P=0.023$). Elevated levels of MBG were associated with measures of worse RV systolic function (RV $s'$: $r=−0.39$, $P<0.0001$), but not left-sided systolic function (Table I in the Data Supplement).

### MBG Levels and Prognosis

In our study cohort, 118 patients experienced an adverse event of death, cardiac transplantation, or HF hospitalization over the 5-year follow-up. When divided as dichotomous variable according to optimal cut point (574 pmol/L), elevated MBG was a predictor of increased risk of 5-year adverse outcomes, with higher MBG predicting increased risk of adverse clinical outcomes (Table II in the Data Supplement).
events (hazard ratio 1.58 [95% confidence interval 1.10–2.31], \( P = 0.014 \); Figure 2A). The predictive value of MBG remained statistically significant after adjustment for age, sex, diabetes mellitus, and ischemic pathogenesis, but not estimated glomerular filtration rate (Table II in the Data Supplement). Moreover, the addition of MBG to traditional risk factors, such as age, sex, ischemic pathogenesis, and diabetes mellitus, resulted in a 33.6% event-specific net reclassification (95% confidence interval 9.3%–57.8%, \( P = 0.007 \)) and a 3% integrated discrimination improvement (Table III in the Data Supplement). When defined by quartiles, this trend was confirmed using Cochran–Amitage test for the trend over quartiles (\( P = 0.0297 \)), although the trend was not monotonic (Table IV in the Data Supplement). MBG did not predict events when modeled as a continuous variable (hazard ratio 1.14 [0.95–1.37], \( P = 0.15 \), per 1 standard deviation increment 0.62 with MBG modeled as a natural log-transformed continuous variable).

We next selected a subgroup of the patients with HF who had serial blood draws available at the time of their initial presentation in the hospital and 48 to 72 hours after admission (n=115 and included 35 events) and measured MBG. Rising MGB levels over the course of admission predicted increased risk of adverse outcomes (%ΔMBG modeled as a continuous variable per standard deviation increments where 1 standard deviation =0.91%, hazard ratio 1.30 [95% confidence interval 1.04–1.56], \( P = 0.025 \), per 1 standard deviation increment; Figure 2B).

**MBG Promotes Cardiac Fibrosis and Nitrative Stress in Animal Models**

To assess MBG levels in a mouse model of post-MI HF, LADx was performed in C57BL6J mice. After 4 weeks of LADx, we observed a significant decrease in left ventricular ejection fraction and increases in left ventricular size (Figure 3A and 3B). We also observed changes in left ventricular homogenate of molecular markers of cardiac calcium handling (sarcoplasmic reticulum calcium ATPase 2a and sodium calcium exchanger) and cardiac hypertrophy (beta myosin heavy chain and atrial natriuretic peptide) consistent with a HF phenotype in this model (Figure 3C–3F). Further, we noted increases in MBG levels in the post-MI HF mice (Figure 3G), with MBG levels observed within the range of values detected among HF subjects studied (50th percentile 585 pmol/L). We also performed the LADx procedure on a separate group of mice (n=5) and, after 1 week, isolated and extracted the adrenal glands and performed MBG measurement. Here we found that adrenal MBG levels were elevated versus control mice, but this did not reach statistical significance (Mann–Whitney \( P \) value = 0.056; Figure 3H).

To directly test for a potential contribution of MBG to promotion of cardiac dysfunction and nitrative stress, mice were infused intraperitoneally with MBG (0.1 \( \mu g/g/d \)), which results in comparable levels to the post-MI HF mice (Figure 4A). MBG infusion did not result in decreased ejection fraction, but was accompanied by a significant increase in left ventricular size (Figure 3A and 3B). Importantly, mice infused with MBG demonstrated corresponding increases in MPO (Figure 4B), as well as increases in several methylated arginine markers of nitrative stress, including ADMA, symmetrical dimethylarginine, and mono methyl arginine (Figure 4C–4E). Furthermore, although MBG-infused mice experienced significant left ventricular cardiac fibrosis versus vehicle-treated controls, right ventricular cardiac fibrosis...
appeared to be increased versus vehicle-treated controls, but this did not reach statistical significance (Mann–Whitney \( P \) value =0.219; Figure 5).

**Discussion**

Although elevated CTS have been associated with cardiac hypertrophy and dysfunction in subjects with hypertension,30,31 hypertrophic cardiomyopathy,32 decompensated HF,33,34 and cardiomyopathy in chronic kidney disease,15,23 this is the first study to our knowledge that examines the relationship between plasma MBG and cardiac parameters, as well as adverse clinical events, in a broad and contemporary cohort of patients with HF. The relationships between elevated MBG and indices of inflammation/oxidative stress, myocardial stress, and nitrative stress and the predominantly diastolic dysfunction are consistent with the physiological effects of MBG on the myocardium in volume-expanded states. These findings support future investigations on the potential role of modulation of MBG levels or activity as a novel targeted therapy in the population of patients with HF burdened with significant cardiovascular disease and death.

**Contribution of CTS in HF**

Although previous human studies have demonstrated the detection of elevated CTS in the setting of acute MI13,35,36 and HF37, the novel findings in the current study was the relationship between elevated MBG and worsened right ventricular function (a condition often associated with venous congestion) as assessed by standard echocardiographic indices, as well as adverse clinical outcomes and nitrative stress. Further, the data from the subgroup of patients who had serial MBG measurements suggest that MBG levels are dynamic and

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**Figure 3.** Elevated marinobufagenin (MBG) levels contribute to nitrative stress. Echocardiographic measures of cardiac ejection fraction (A) and diastolic left ventricular internal dimension (LVIdd; B) after 4 weeks of either left anterior descending (LAD) ligation (LADx) or MBG infusion. Gene expression of calcium-handling proteins sarcoplasmic reticulum calcium ATPase (SERCA2a; C) and sodium calcium exchanger (NCX-1; D), and hypertrophic markers beta myosin heavy chain (\( \beta \)MHC; E) and atrial natriuretic peptide (ANP; F) after 4 weeks of LADx. Plasma MBG (G) levels are increased 4 weeks after LAD ligation in a post–myocardial infarction heart failure model. H. Adrenal tissue MBG levels 1 week after LAD ligation. \( P \) values were calculated using the Mann–Whitney U test.

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**Figure 4.** Elevated marinobufagenin (MBG) levels contribute to nitrative stress. After 4 week infusion of MBG, plasma levels of MBG (A) and myeloperoxidase (MPO; B), as well as methylated arginine metabolites asymmetrical dimethylarginine (ADMA, C), symmetrical dimethylarginine (SDMA, D), and monomethyl arginine (MMA, E), are increased vs vehicle-treated mice. \( P \) values were calculated using the Mann–Whitney U test.
Angiotensin II can be its secretagogue, we isolated and that MBG is synthesized by adrenocortical cells and that MBG production. Because we have previously demonstrated suggesting factors beyond renal insufficiency that influence associated with cystatin C or estimated glomerular filtration rate, Interestingly, in our study, MBG was not significantly associ-
ated HF, but also marked by an increase in the sensitivity of cardiac Na/K-ATPase to ouabain.33,34 Levels of circulating endogenous ouabain also predicted HF progression in idio-
pathic dilated cardiomyopathy patients and left ventricular hypertrophy in the setting of end-stage renal disease.39 Increased plasma MBG levels parallel the progression of HF and are associated with a uremic cardiomyopathy in chronic kidney disease.32,33,34 Experimentally, Dahl salt-sensitive rats fed a high-salt diet demonstrated compensated left ventricular hypertrophy progressing to dilated cardiomyopathy in parallel with increasing plasma MBG level as well as increased expression and sensitivity of the Na/K-ATPase α-1 to MBG.33 Interestingly, in our study, MBG was not significantly associ-
ated with cystatin C or estimated glomerular filtration rate, suggesting factors beyond renal insufficiency that influence MBG production. Because we have previously demonstrated that MBG is synthesized by adrenocortical cells and that Angiotensin II can be its secretagogue, we isolated and extracted the adrenal glands and performed MBG measure-
ment in a group of mice 1 week after LADx and noted that it was significantly elevated. This supports the adrenal tissue as a significant contributor to the pool of circulating MBG in a post-MI HF model. Taken together with our findings, these observations suggest that the elevated CTS levels that support the view that rising levels of MGB during admission predict worse long-term clinical outcomes.

The relationship between cardiac structure, hemodynamics, and CTS has also been observed in several other cohort studies. Endogenous plasma ouabain levels are elevated in patients with severely impaired left ventricular function (ejection fraction <30%) and demonstrated significant positive correlation with hemodynamics, such as blood pressure, and cardiac indices, such as left ventricular mass index, left ventricular end diastolic volume, and eccentric remodeling in hypertensive patients.30,31 Both circulating and myocardial tissue CTS immunoreactivity was found to be positively correlated with left ventricular end-diastolic pressures and inversely correlated with cardiac index in patients with hyper-
trophic cardiomyopathy.32 The shift from compensated left ventricular hypertrophy to HF is not only marked by a 3-fold increase in endogenous plasma ouabain levels in decompensated HF, but also marked by an increase in the sensitivity of cardiac Na/K-ATPase to ouabain.30,31 Levels of circulating endogenous ouabain also predicted HF progression in idio-
pathic dilated cardiomyopathy patients and left ventricular hypertrophy in the setting of end-stage renal disease.39 Increased plasma MBG levels parallel the progression of HF and are associated with a uremic cardiomyopathy in chronic kidney disease.32,33,34 Experimentally, Dahl salt-sensitive rats fed a high-salt diet demonstrated compensated left ventricular hypertrophy progressing to dilated cardiomyopathy in parallel with increasing plasma MBG level as well as increased expression and sensitivity of the Na/K-ATPase α-1 to MBG.33 Interestingly, in our study, MBG was not significantly associ-
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ment in a group of mice 1 week after LADx and noted that it was significantly elevated. This supports the adrenal tissue as a significant contributor to the pool of circulating MBG in a post-MI HF model. Taken together with our findings, these observations suggest that the elevated CTS levels that accompany edematous states like HF may promote down-
stream adverse cardiovascular consequences.

Mechanisms Linking CTS to Cardiovascular Pathology in HF

In addition to their well-known effects on the ion transporting functions of the Na/K-ATPase, CTS also bind to and initiate signaling through a nonpumping pool of the Na/K-ATPase, which reside in caveolae.6 CTS confer a conformational change to the Na/K-ATPase that releases the Src kinase domain, thus activating Src kinase and multiple downstream targets.2,8,11 This novel Na/K-ATPase-mediated signaling is responsible for a variety of key cellular roles involving cell growth/hypertrophy, reactive oxygen species production, and collagen synthesis.6

In the present study, we demonstrate for the first time that increased circulating levels of MBG in human HF associate with elevations in markers of inflammation and nitrative stress, including MPO and the methylated arginine metabo-
lites ADMA, symmetrical dimethylarginine, and mono methyl arginine. Using an animal model of post-MI HF, we also demon-
strate a significant increase in plasma MBG 4 weeks after ligation of the LAD artery in mice. Further, infusion of mice with MBG, which results in similar circulating levels as those seen in both human and experimental HF, recapitulated the increases in MPO, as well as ADMA, symmetrical dimethyl-
arginine, and mono methyl arginine seen in our human HF study.

The mechanism whereby MBG may increase methylated arginine metabolites is unclear. We and others have shown that CTS, such as MBG and ouabain, increase reactive oxygen species and inflammatory cytokines in cardiac and renal cell types and also decrease NO bioavailability without changes in endothelial nitric oxide synthase expression.44 Inflammation and increased oxidant stress can significantly impact methyltransferases necessary for arginine methyla-
tion, the proteases involved in release of free methylarginine metabolites, and the catabolic dimethylarginine dimethylami-
nohydrolases responsible for metabolism of ADMA.45 Thus, it is possible that some of the observed associations between methylated arginine metabolites and MBG may occur in part via reactive oxygen species-mediated perturbations in these enzymatic pathways.
The association of CTS with markers of inflammation and nitrative stress is not, however, without precedent. We have previously reported in the rat model that treatment of both cardiac myocytes and isolated perfused hearts with the CTS ouabain yielded increased nitrative modification and decreased activity of cardiac calcium handling proteins, as well as diastolic dysfunction.46 We have also shown that ouabain induces increases in inflammatory cytokine expression from both macrophage and renal proximal tubular cell types.43 Our findings are also in parallel with the finding from animal models, such as partial (5/6th) nephrectomy, showing elevations in circulating MBG levels that stimulate systemic oxidant stress, oxidative modification, and fibrosis of cardiac tissue and cardiac dysfunction in the rat.19,22,47 This cardiac phenotype can also be recapitulated by infusion of MBG. In contrast, both active and passive immunization against MBG, as well as lowering circulating MBG levels via adrenalectomy, significantly reduce the oxidant stress and cardiac dysfunction independent of changes in blood pressure.

In our study, mice infused with MBG also demonstrated significant increases in cardiac fibrosis. MBG and ouabain have both been shown to increase [H]proline incorporation in addition to collagen expression (both mRNA and protein) in cardiac and renal fibroblast cell types,20 and these effects were blocked by pharmacological antagonism of the transforming growth factor beta pathway.19 We have also noted that decreases in Fli-1 (a negative regulator of collagen synthesis) expression seem to be necessary for MBG to induce increases in collagen in several types of fibroblasts (cardiac, renal, and dermal). Additionally, MBG induces translocation of protein kinase C delta from the cytosol to the nucleus in a phospholipase C-dependent manner, and this translocation of protein kinase C delta causes the phosphorylation and subsequent degradation of Fli-1.20 In several fibroblast cell types, CTS also stimulate Na/K-ATPase and oxidant signaling, which induce collagen production.19,22,43 These signaling pathways are significantly attenuated not only by oxidant scavenging and inhibition of Src kinase, but also through competitive inhibitory mechanisms induced by spironolactone and canrenone binding to the Na/K-ATPase.19,22,48,49 Thus, the proinflammatory and profibrotic CTS-Na/K-ATPase signaling axis may provide a novel therapeutic target in settings, such as HF, where elevated CTS induce inflammation and cardiac fibrosis.

**Study Limitations**

Despite being the largest study to our knowledge reporting the relationship between clinical outcomes and MBG in HF, the current study is still limited in the relatively small number of patients, as well as the selection bias that potentially confounds interpretation of such cohort studies. We did not have sufficient data to analyze central venous pressure or other indices of right ventricular function; thus, the measurements indicating worsened RV function are based solely on echocardiographic indices. Our Cox proportional hazard analyses with multiple covariates were limited by lack of power because of missing data. Similarly, when defined by quartiles, although the trend between MBG and worse clinical outcomes was similar to that obtained by the optimal cut point analysis, it was not statistically significant. Nevertheless, our study examines for the first time the contribution of MBG to incident cardiovascular outcomes in a cohort of patients with HF and demonstrates a novel association between CTS and markers of inflammation and nitrative stress. We would emphasize that the purpose of this study was to demonstrate underlying physiology rather than to propose MBG as a biomarker because it tracks with worsening renal function. The increased inflammation, nitrative stress, and cardiac fibrosis seen with elevations in MBG may be an important mechanism of cardiovascular dysfunction in patients with HF and requires further investigation.

**Conclusions**

Elevated levels of MBG are associated with indices of worse cardiac dysfunction and increased risk for development of adverse clinical outcomes in patients with HF even after multivariable models adjustment for established clinical risk factors. Similarly, changes in MBG levels over time seem to be of prognostic benefit in patients with HF, and infusion of MBG in an animal model seems to directly contribute to increased nitrative stress, cardiac fibrosis, and dysfunction. Thus, MBG may serve as an important therapeutic target in patients with HF.

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**Disclosures**

None.

**References**


**CLINICAL PERSPECTIVE**

Previous studies have shown that cardiotonic steroids, such as marinobufagenin (MBG), contribute to pathological adaptation to volume expansion, including hypertension, hypertrophy, and fibrosis. We therefore examined the potential contribution of MBG to cardiac pathology and long-term adverse clinical outcomes in patients with heart failure and used an animal infusion model to demonstrate the contribution of MBG to the clinical phenotype observed in our human study. These studies indicate a pathological role and clinical relevance of MBG in the development and progression of cardiovascular pathology in heart failure. Our findings support a mechanistic link between MBG and adverse cardiovascular outcomes in this susceptible population and imply the need to focus efforts on therapeutic modulation of cardiotonic steroids, such as MBG, in the setting of heart failure to attenuate the development and progression of cardiac pathology and risk.
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SUPPLEMENTAL MATERIAL

Quantitative Histologic Techniques. Mason’s trichome and picosirius red staining was performed on deparaffinized 5 µm serial heart sections. The sections were mounted under a Leica DM 2500 microscope and digitized with a QImaging MicroPublisher 5.0 RTV camera. Quantitative morphometric analysis was performed on fields (at least 8 from each animal) and the collagen volume was determined using automated and customized algorithms/scripts for batch analysis (ImageIQ Inc., Cleveland, OH) written for Image Pro Plus 7.0. Briefly, a set of representative images were chosen that demonstrated a wide range of staining intensities and prevalence. In an automated script, these “training” images were loaded one after another prompting the user to delineate pixels representing positive collagen staining using an interactive color picking tool. An iterative color profile or classifier was generated and subsequently applied to all images in a given directory using a fully automated algorithm. Positive pixels, as defined by the color profile, were segmented and summed to provide positive staining area. Total tissue area was determined by extracting the “saturation” channel, applying a low-pass filter, and thresholding the result. Any area within the general tissue boundary that was empty (i.e. white) was removed by converting the original image to grayscale and applying a fixed threshold for non background pixels on adequately white-balanced images. Finally, total tissue area and total stained area were exported to Excel. For post-processing verification, segmented regions were superimposed onto the original image (green outlines) for each image analyzed.
**Transthoracic Echocardiography.** Comprehensive 2-dimensional echocardiography was performed in standard parasternal and apical views on all participants by an American Society of Echocardiography registered research sonographer using a Vivid 7 echocardiography machines (GE Healthcare, Waukesha WI) equipped with a phased-array transducer. Standard 2-dimensional and Doppler data, triggered to the QRS complex, was digitally stored in a cine-loop format. The Simpson biplane method was used to measure left ventricular ejection fraction (LVEF), left ventricular end-systolic volume index (LVESVI), and left atrial volume index (LAVI). Ventricular volume and mass measurements were indexed to body surface area. Right ventricular systolic pressure (RVSP) was calculated from the Doppler estimated tricuspid valve regurgitant jet velocity using the Bernoulli equation. Assessment and classification of diastolic parameters, mitral inflow patterns, and quantification of mitral regurgitation were made following the American Society of Echocardiography recommendations. Small animal echocardiography was performed using a Vivid 7 ultrasound machine (GE Medical) equipped an iL3L linear probe operated at 14 MHz in order to obtain 2-D (parasternal long- and short-axis views) and M-mode images as we have previously published. All echocardiographic measurements were averaged over three cardiac cycles.
### Supplemental Table 1. Univariate correlations between marinobufagenin (MBG) and clinical and echocardiographic characteristics

<table>
<thead>
<tr>
<th>MBG (pM)</th>
<th>Spearman’s r</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>0.06</td>
<td>0.392</td>
</tr>
<tr>
<td>Body mass index (kg/m$^2$)</td>
<td>0.02</td>
<td>0.768</td>
</tr>
</tbody>
</table>

#### Echocardiographic Indices

**LV Structure**

- LV mass index (g/m$^2$) | 0.15 | 0.073 |
- LVEDVi (mL/m$^2$) | -0.05 | 0.552 |

**LV Systolic function**

- LV ejection fraction (%) | -0.04 | 0.585 |

**LV Diastolic function**

- Mitral E/e’ ratio | 0.13 | 0.136 |
- Mitral E/A ratio | 0.12 | 0.202 |
- Mitral DT (ms) | -0.24 | 0.007 |
- LA volume index (mL/m$^2$) | -0.04 | 0.551 |

**RV Structure**

- RV end-diastolic area (cm$^2$) | 0.21 | 0.023 |

**RV Systolic function**

- RV s’ (cm/s) | -0.39 | <0.0001 |
- RV Fractional area change | -0.42 | <0.0001 |

**RV Diastolic Function**

- Tricuspid E/e’ ratio | 0.22 | 0.027 |
Tricuspid DT (ms)  
-0.38  0.002

RA volume index (mL/m$^2$)  
0.22  0.013

**Laboratory Data**

- Myeloperoxidase (pM)  
  0.42  <0.0001

- BNP (pg/mL)  
  0.25  0.001

- eGFR (mL/min/1.73m$^2$)  
  -0.06  0.345

- Cystatin C (mg/L)  
  0.06  0.485

- ADMA (μM)  
  0.32  <0.001

- SDMA (μM)  
  0.34  <0.001

- MMA (μM)  
  0.40  <0.0001

*Abbreviation: ADMA, Asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; MMA, monomethyl arginine*
**Supplemental Table 2.** Cox proportional hazards analyses of adverse long-term clinical outcomes.

<table>
<thead>
<tr>
<th></th>
<th>HR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBG</td>
<td>1.58 (1.10 – 2.31)</td>
<td>0.014</td>
</tr>
<tr>
<td>Adjusted for age, male gender</td>
<td>1.55 (1.07 – 2.26)</td>
<td>0.019</td>
</tr>
<tr>
<td>Adjusted for age, gender, LVEF</td>
<td>1.51 (1.04 – 2.21)</td>
<td>0.031</td>
</tr>
<tr>
<td>Adjusted for age, gender, LVEF, ischemic etiology</td>
<td>1.48 (1.01 – 2.17)</td>
<td>0.042</td>
</tr>
<tr>
<td>Adjusted for age, gender, ischemic etiology, diabetes mellitus</td>
<td>1.50 (1.04 – 2.19)</td>
<td>0.031</td>
</tr>
<tr>
<td>Adjusted for age, gender, smoking, diabetes mellitus</td>
<td>1.47 (1.02 – 2.14)</td>
<td>0.040</td>
</tr>
<tr>
<td>Adjusted for eGFR</td>
<td>1.43 (0.99 – 2.08)</td>
<td>0.060</td>
</tr>
<tr>
<td>Adjusted for ischemic etiology</td>
<td>1.53 (1.06 – 2.24)</td>
<td>0.023</td>
</tr>
<tr>
<td>Adjusted for mitral E/e' ratio</td>
<td>1.65 (0.97 – 2.87)</td>
<td>0.066</td>
</tr>
<tr>
<td>Adjusted for RV s'</td>
<td>1.36 (0.76 – 2.51)</td>
<td>0.305</td>
</tr>
<tr>
<td>Adjusted for tricuspid E/e'</td>
<td>1.36 (0.77 – 2.46)</td>
<td>0.290</td>
</tr>
<tr>
<td>Adjusted for BNP</td>
<td>1.40 (0.87 – 2.31)</td>
<td>0.169</td>
</tr>
</tbody>
</table>

*HRs represent the risk associated with MBG levels ≥ 574 pM*
**Supplemental Table 3. Net reclassification analysis**

<table>
<thead>
<tr>
<th>Discrimination Analysis</th>
<th>Percentage</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model with MBG</td>
<td>60.7%</td>
<td></td>
<td>0.33</td>
</tr>
<tr>
<td>Model without MBG</td>
<td>57.8%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDI</td>
<td>3%</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NRI</td>
<td>33.6%</td>
<td>9.3%-57.8%</td>
<td>0.007</td>
</tr>
<tr>
<td>Events correctly reclassified</td>
<td>41.9%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-events correctly reclassified</td>
<td>-8.3%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviation:** AUC, Area under the curve; IDI, integrated discrimination improvement; NRI, net reclassification index; CI, confidence interval.

Both models were adjusted for age, gender, ischemic etiology, and diabetes. The risk of mortality was estimated using the Cox model.
**Supplemental Table 4.** Cox proportional hazards analyses by quartiles for adverse long-term clinical outcomes.

<table>
<thead>
<tr>
<th></th>
<th>HR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBG Quartile 4 vs Quartile 1</td>
<td>1.57 (0.91 – 2.75)</td>
<td>0.102</td>
</tr>
<tr>
<td>MBG Quartile 3 vs Quartile 1</td>
<td>2.14 (1.27 – 3.68)</td>
<td>0.004</td>
</tr>
<tr>
<td>MBG Quartile 2 vs Quartile 1</td>
<td>1.43 (0.81 – 2.55)</td>
<td>0.222</td>
</tr>
</tbody>
</table>
Supplemental References


