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

Kennedy et al: Marinobufagenin in Heart Failure

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Abstract

Background—Plasma levels of cardiotonic steroids (CTS) 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 CTS 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 HF patients. 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 (MPO, r=0.42, p<0.0001), BNP (r=0.25, p=0.001), and asymmetric dimethylarginine (ADMA, r=0.32, p<0.001). Elevated levels of MBG were associated with measures of worse right ventricular function (RV s': r= -0.39, p<0.0001) and predicted increased risk of adverse clinical outcomes (MBG ≥574 pM: HR 1.58 [1.10-2.31], p=0.014) even after adjustment for age, gender, diabetes mellitus, and ischemic etiology. In mice, a left anterior descending coronary artery ligation model of heart failure lead to increases in MBG, while infusion of MBG into mice for 4 weeks lead to significant increases in MPO, ADMA, and cardiac fibrosis.

Conclusions—In the setting of heart failure, 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 appears to directly contribute to increased nitrative stress and cardiac fibrosis.

Key Words: heart failure, cardiotonic steroids, outcome, nitrative stress, cardiac fibrosis
Cardiotonic 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) as well as aglycone bufadienolides (such as telocinobufagin and marinobufagenin [MBG])\(^1\).\(^2\). 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\(^3\)\(^-\)\(^5\). 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\(^6\),\(^7\). However, CTS may exert “off-target” signal transduction effects beyond their direct effects on the sodium pump\(^2\),\(^8\). Hence, chronic stimulation of Na/K-ATPase signaling by CTS has important implications for not only for the natriuretic response to increased salt load\(^9\) but also has been implicated in pathological adaptation to volume expansion including hypertension, hypertrophy, and fibrosis\(^10\),\(^11\). 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\(^4\),\(^12\)\(^-\)\(^15\). Furthermore, myocardial hypertrophy and growth effects of CTS have been proposed in the setting of hypertension and renal dysfunction\(^16\), 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)\(^17\),\(^18\). Clinical and experimental evidence from our group and others has also demonstrated the pro-oxidant and pro-fibrotic effects of these steroid hormones in both cardiac and renal tissue\(^19\)\(^-\)\(^23\). As the relationship among 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, as MBG in 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 associate with echocardiographic indices of diastolic dysfunction. Herein, we examined the relationship among circulating levels of MBG with echocardiographic parameters, as well as long-term adverse clinical outcomes in patients with HF. We also used an animal infusion 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 HF patients (≥18 years) seen at the Cleveland Clinic with a clinical diagnosis of HF and New York Heart Association (NYHA) 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, unstable angina, stroke, transient ischemic attack, 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 endpoint including adverse clinical events (all-cause mortality, cardiac transplantation and HF hospitalization) and all-cause mortality through a process confirmed by chart review and the Social Security Death Index. Thirteen sex-matched apparently healthy volunteer participants without a history of heart failure served as non-heart failure controls. They were prospectively recruited outside of any healthcare institution setting, and did not report
any active medical conditions at the time 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 prior to their participation in the study.

**Biochemical Assays.** Blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-plasma vacutainers at the time of clinical and echocardiographic and hemodynamic evaluation, were immediately aliquotted and 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 fluoroimmunoassay (DELFIA)] as previously described\(^\text{22, 24}\). The MBG DELFIA uses a murine monoclonal antibody (anti-MBG 4G4) and employs competition between immobilized antigen (MBG-glycoside-thyroglobulin) and MBG, other cross-reactants, or endogenous cardiotoxic steroids 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 Perkin-Elmer (Waltham, MA). Data on cross reactivity of the MBG antibody have been reported previously\(^\text{3, 23}\). For analysis of MBG tissue levels, adrenal glands were homogenized (TissueLyserII, Quiagen, 5 mm stainless steel beads) and homogenate was extracted with 10 fold excess of methyl tert-butyl ether before drying under nitrogen gas and resuspension in assay buffer (50 mM Tris-hydrochloride, 154 mM sodium chloride, 7.7 mM sodium azide, pH 7.8). Plasma myeloperoxidase (MPO) levels were determined by an enzyme-linked immunosorbent assay (CardioMPO II test, Cleveland Heart Labs, Cleveland OH). Arginine metabolomic profiles were quantified by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) as previously described\(^\text{25, 26}\). All other assays including BNP, 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 (MDRD) equation was used to calculate estimated glomerular filtration rate (eGFR)27.

**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 and following the American Society of Echocardiography recommendations28, 29. Further detail on echocardiographic analysis is described in the Supplemental Material.

**Animal study.** In order to assess MBG levels in a mouse model of post-myocardial infarction heart failure, left anterior descending (LAD) artery ligation was performed in C57BL6J 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 LAD ligation. 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 in order to deliver MBG (0.1 ug/g/day) or vehicle to mice for 4 weeks similar to what we have reported in the rat22. 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 institutional guidelines.

**Quantitative Histologic 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 Supplemental Material.

**Statistical Analysis.** Normally distributed continuous variables were summarized as mean ± standard deviation if normally distributed, or median (interquartile range [IQR]) if non-normally distributed. If more than two groups were compared, one-way analysis of variance (ANOVA) was performed prior to comparison of individual groups with the unpaired Student’s t-test with Bonferroni’s correction for multiple comparisons. If only two groups of normal data were compared, the Student’s t-test was used without correction. P values for non-parametric 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 pM and <574 pM. The optimal sensitivity and specificity cutoff of MBG (≥ 574 pM) levels in our cohort was determined using receiver operator characteristic (ROC) curve analyses in the context of the time to event and the Hazard Ratios reported represent the risk associated with MBG levels ≥ 574 pM. This value was chosen as the optimal ROC cut-point that
maximized sensitivity plus specificity in ROC 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 (NRI) was used as test of association and integrated discrimination improvement (IDI) was 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 etiology. 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 the Table. Median MBG level was 583 [IQR 383-812] pM in patients with HF compared to 241 [IQR 212, 281] pM in non-HF controls (n=13, mean age 43±12 years, 64% male, body mass index 27±3, 15% African American). Patients with HF have higher MBG levels when compared to that of non-HF controls, regardless of whether they have reduced or preserved LVEF (Figure 1). Furthermore, higher MBG was associated with higher indices of inflammation/oxidative stress (MPO: r=0.42, p<0.0001), myocardial stress (BNP: r=0.25, p=0.001), and nitrative stress [asymmetric dimethylarginine (ADMA): r=0.32, p<0.001; symmetric dimethylarginine (SDMA): r=0.34, p<0.001; and mono methyl arginine (MMA): 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 GFR (p=0.345).
**MBG Levels and Myocardial Structure and Performance.** Supplemental Table 1 presents the relationships between MBG levels and echocardiographic parameters of cardiac structure and performance. In univariate analysis, higher MBG was associated with indices of LV diastolic function (Mitral deceleration time: \( r = -0.24, p=0.007 \)) and RV diastolic function (Tricuspid E/e’: \( r = 0.22, p=0.027 \); Tricuspid deceleration time: \( r = -0.38, p=0.002 \)), as well as 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 (Supplemental Table 1).

**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 a dichotomous variable according to optimal cut-point (574 pM), elevated MBG was a predictor of increased risk of 5-year adverse outcomes, with higher MBG predicting increased risk of adverse clinical 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, gender, diabetes mellitus, and ischemic etiology, but not eGFR (Supplemental Table 2). Moreover, the addition of MBG to traditional risk factors such as age, gender, ischemic etiology, and diabetes resulted in an 33.6% event-specific net reclassification (95% confidence interval 9.3%-57.8%, \( p=0.007 \)) and a 3% integrated discrimination improvement (Supplemental Table 3). 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 (Supplemental Table 4). 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 HF patients that had serial blood draws available at the time of their initial presentation in the hospital and 48-72 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 [%ΔMGB 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.** In order to assess MBG levels in a mouse model of post-myocardial infarction heart failure, left anterior descending artery ligation (LADx) was performed in C57BL6J mice. After 4 weeks of LADx we observed a significant decrease in left ventricular ejection fraction and increases left ventricular size (Figure 3A and B). We also observed changes in left ventricular homogenate of molecular markers of cardiac calcium handling [sarcoplasmic reticulum calcium ATPase 2a (SERCA2a) and sodium calcium exchanger (NCX-1)] and cardiac hypertrophy [beta myosin heavy chain (βMHC) and atrial natriuretic peptide (ANP)] consistent with a heart failure phenotype in this model (Figure 3C-F). Further, we noted increases in MBG levels in the post-MI heart failure mice (Figure 3G), with MBG levels observed within the range of values detected among HF subjects studied (50th percentile 585 pM). We also performed the LAD ligation 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 vs 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 ug/g/day) which results in comparable levels to the post-MI heart failure 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 B). 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, SDMA, and MMA (Figure 4 C-E). Furthermore, while MBG infused mice experienced significant left ventricular cardiac fibrosis vs vehicle treated controls, right ventricular cardiac fibrosis appeared to be increased vs vehicle treated controls but this did not reach statistical significance (Mann-Whitney p value = 0.219, Figure 5).

Discussion

While elevated CTS have been associated with cardiac hypertrophy and dysfunction in subjects with hypertension30, 31, hypertrophic cardiomyopathy32, decompensated HF33, 34, and cardiomyopathy in chronic kidney disease15, 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 is consistent with the physiologic 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 HF patients burdened with significant cardiovascular disease and death.

**Contribution of CTS in Heart Failure.** While previous human studies have demonstrated the detection of elevated CTS in the setting of acute myocardial infarction\(^{13, 35, 36}\) and HF\(^{37}\), 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 suggests that MBG levels are dynamic and supports 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 (EF< 30%)\(^{34}\) and demonstrated significant positive correlation with hemodynamics such as blood pressure as well as cardiac indices such as left ventricular mass index, left ventricular end diastolic volume, as well as eccentric remodeling in hypertensive patients\(^{30, 31}\). Both circulating and myocardial tissue CTS immunoreactivity was positively correlated with left ventricular end-diastolic pressures and inversely correlated with cardiac index in patients with hypertrophic cardiomyopathy\(^{32}\). The shift from compensated left ventricular hypertrophy to CHF is not only marked by a three-fold increase in endogenous plasma ouabain levels in decompensated CHF, but also an increase in the sensitivity of cardiac Na/K-ATPase to ouabain\(^ {33, 34}\). Levels of circulating endogenous ouabain also predicted HF progression in idiopathic dilated cardiomyopathy patients\(^ {38}\) and left ventricular
hypertrophy in the setting of end-stage renal disease. Increased plasma MBG levels parallel the progression of HF and are associated with a “uremic cardiomyopathy” in chronic kidney disease. 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. Interestingly, in our study, MBG was not significantly associated with cystatin C or estimated GFR, suggesting factors beyond renal insufficiency that influence MBG production. As we have previously demonstrated that MBG is synthetized by adrenocortical cells and that Angiotensin II can be its secretagogue, we isolated and extracted the adrenal glands and performed MBG measurement in a group of mice one week after LADx and noted it was significantly elevated. This supports the adrenal tissue as a significant contributor to the pool of circulating MBG in a post-MI heart failure model. Taken together with our findings, these observations suggest that the elevated CTS levels which accompany edematous states like HF may promote downstream adverse cardiovascular consequences.

Mechanisms Linking CTS to Cardiovascular Pathology in Heart Failure. In addition to their well known effects on the ion transporting functions of the Na/K-ATPase, CTS also bind to and initiated signaling through a non-pumping pool of the Na/K-ATPase which reside in caveolae. CTS confer a conformational change to the Na/K-ATPase that releases the Src kinase domain, thus activating Src kinase and multiple downstream targets. 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.

In the present study we demonstrate for the first time that increased circulating levels of MBG in human heart failure associate with elevations in markers of inflammation and nitrative
stress including MPO and the methylated arginine metabolites ADMA, SDMA, and MMA. Using an animal model of post-MI heart failure, we also demonstrate a significant increase in plasma MBG 4 weeks after ligation of the left anterior descending artery in mice. Further, infusion of mice with MBG, which results in similar circulating levels as those seen in both human and experimental heart failure, recapitulated the increases in MPO as well as ADMA, SDMA, and MMA seen in our human heart failure study.

The mechanism whereby MBG may increase methylated arginine metabolites is unclear. We and others have shown that cardiotonic steroids such as MBG and ouabain increase ROS and inflammatory cytokines in cardiac and renal cell types, and also decrease NO bioavailability without changes in eNOS expression. Inflammation and increased oxidant stress can significantly impact methyltransferases necessary for arginine methylation, the proteases involved in release of free methylarginine metabolites, and the catabolic dimethylarginine dimethylaminohydrolases responsible for metabolism of ADMA. Thus, it is possible that some of the observed associations between methylated arginine metabolites and MBG may occur in part via ROS 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 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. We have also shown that ouabain induces increases in inflammatory cytokine expression from both macrophage and renal proximal tubular cell types. Our findings are also in parallel with animal models such as partial (5/6\textsuperscript{th}) 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. 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 [³H]proline incorporation in addition to collagen expression (both mRNA and protein) in cardiac and renal fibroblast cell types and these effects were blocked by pharmacological antagonism of the TGF beta pathway. We have also noted that decreases in Fli-1 (a negative regulator of collagen synthesis) expression appear to be necessary for MBG to induce increases in collagen in several types of fibroblasts (cardiac, renal, and dermal). Additionally, MBG induces translocation of PKCdelta from the cytosol to the nucleus in a PLC dependent manner, and this translocation of PKCdelta causes the phosphorylation and subsequent degradation of Fli-1. In several fibroblast cell types, CTS also stimulate Na/K-ATPase and oxidant signaling which induce collagen production. These signaling pathways are not only significantly attenuated 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. Thus, the pro-inflammatory and pro-fibrotic 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 of 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 covariables was limited by lack of power due to missing data. Similarly, when defined by quartiles, while the trend between MBG and worse clinical outcomes was similar to that obtained by the optimal cutpoint 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 as 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 heart failure and requires further investigation.

Conclusion

Elevated levels of MBG are associated with indices of worse cardiac dysfunction as well as 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 appear to be of prognostic benefit in HF patients, and infusion of MBG in an animal model appears to directly contribute to increased nitrative stress, cardiac fibrosis and dysfunction. Thus, MBG may serve as an important therapeutic target in patients with heart failure.
Sources of Funding

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Disclosures

None.

References


Table. Baseline Characteristics (n=245).

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<td>Ischemic etiology, n (%)</td>
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**Medications:**

- Angiotensin converting enzyme inhibitors or angiotensin receptor blockers, n (%) 143 (61%)
- Beta-blockers, n (%) 171 (73%)
- Spironolactone, n (%) 87 (37%)
- Loop diuretics, n (%) 151 (64%)
- Digoxin, n (%) 70 (30%)

**Laboratory data:**

- MBG (pM) 583 [383, 812]
- Myeloperoxidase (pM) 124 [77, 253]
- BNP (pg/mL) 431 [97, 1417]
- eGFR (mL/min/1.73m²) 71 ± 32
- Cystatin C (mg/L) 1.85 [1.31 – 2.61]
- ADMA (µM) 1.05 [0.82 – 1.33]
- SDMA (µM) 1.70 [1.05 – 2.96]
- MMA (nM) 50 [34 – 92]

**Abbreviation:** NYHA, New York Heart Association; LV, left ventricular; RV, right ventricular; MBG, marinobufagenin (MBG); eGFR, estimated glomerular filtration rate; BNP, B-type natriuretic peptide, ADMA, Asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; MMA, mono methyl arginine.
Figure Legends

**Figure 1.** Marinobufagenin levels in heart failure. Comparison of plasma marinobufagenin levels between non-heart failure (non-HF) participants and HF participants with reduced (≤ 40) or preserved (>40) left ventricular ejection fraction (EF). **p <0.001 vs control, by Student’s t-test with Bonferroni’s correction.

**Figure 2.** Kaplan-Meier analysis of major adverse cardiac events (MACE) in participants with heart failure. (A) Heart failure patients (n=245) stratified according to optimal cutoff for plasma marinobufagenin (MBG) as follows: “Low” MBG (<574 pM) or “High” MBG (≥ 574 pM). (B) Subgroup analysis of heart failure patients (n=115) with serial blood draws from admission to pre-discharge at 48-72 hours stratified according to optimal cutoff for change in plasma MBG as follows: “decreasing” MBG (<5%) or “increasing” MBG (≥ 5%).

**Figure 3.** Elevated 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 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 (βMHC) (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.
Figure 4. Elevated MBG levels contribute to nitrative stress. After 4 week infusion of MBG, plasma levels of MBG (A) and MPO (B), as well as methylated arginine metabolites asymmetric dimethylarginine (ADMA, C), symmetric dimethylarginine (SDMA, D), and mono methyl arginine (MMA, E) are increased vs vehicle treated mice. P values were calculated using the Mann-Whitney U test.

Figure 5. Elevated MBG levels contribute to fibrosis. Representative picosirius red histology (A) and quantitative morphometry (B) from mouse hearts after 4 week MBG infusion. Scale bar represents 100 um. P values were calculated using the Mann-Whitney U test.
Elevated Plasma Marinobufagenin, An Endogenous Cardiotonic Steroid, Is Associated with Right Ventricular Dysfunction and Nitrative Stress in Heart Failure

David J. Kennedy, Kevin Shrestha, Brendan Sheehy, Xinmin S. Li, Anuradha Guggilam, Yuping Wu, Michael Finucan, Alaa Gabi, Charles M. Medert, Kristen Westfall, Allen Borowski, Olga Fedorova, Alexei Y. Bagrov and W.H. Wilson Tang

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SUPPLEMENTAL MATERIAL

Quantitative Histologic Techniques. Mason’s trichome and picrosirius red staining was performed on deparafinized 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\textsuperscript{1,2}. 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\textsuperscript{3,4}. 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/m²)</td>
<td>0.02</td>
<td>0.768</td>
</tr>
</tbody>
</table>

**Echocardiographic Indices**

**LV Structure**

- LV mass index (g/m²) | 0.15 | 0.073 |
- LVEDVi (mL/m²) | -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/m²) | -0.04 | 0.551 |

**RV Structure**

- RV end-diastolic area (cm²) | 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 |
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value 1</th>
<th>Value 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tricuspid DT (ms)</td>
<td>-0.38</td>
<td>0.002</td>
</tr>
<tr>
<td>RA volume index (mL/m²)</td>
<td>0.22</td>
<td>0.013</td>
</tr>
</tbody>
</table>

**Laboratory Data**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value 1</th>
<th>Value 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloperoxidase (pM)</td>
<td>0.42</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BNP (pg/mL)</td>
<td>0.25</td>
<td>0.001</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73m²)</td>
<td>-0.06</td>
<td>0.345</td>
</tr>
<tr>
<td>Cystatin C (mg/L)</td>
<td>0.06</td>
<td>0.485</td>
</tr>
<tr>
<td>ADMA (µM)</td>
<td>0.32</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SDMA (µM)</td>
<td>0.34</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MMA (µM)</td>
<td>0.40</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*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><strong>AUC</strong></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><strong>IDI</strong></td>
<td>3%</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>NRI</strong></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


