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Circulating and tissue biomarkers as predictors of bromine gas inhalation

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Abstract

The threat from deliberate or accidental exposure to halogen gases is increasing as is their industrial application and use as chemical warfare agents. Biomarkers that can identify halogen exposure, diagnose victims of exposure or predict injury severity, and enable appropriate treatment are lacking. We conducted these studies to determine and validate biomarkers of bromine (Br₂) toxicity and correlate the symptoms and the extent of cardiopulmonary injuries. Unanesthetized rats were exposed to Br₂ and monitored noninvasively for clinical scores and pulse oximetry. Animals were euthanized and grouped at various time intervals to assess brominated fatty acid (BFA) content in the plasma, lung, and heart using mass spectrometry. Bronchoalveolar lavage fluid (BALF) protein content was used to assess pulmonary injury. Cardiac troponin I (cTnI) was assessed in the plasma to evaluate cardiac injury. The blood, lung, and cardiac tissue BFA content significantly correlated with the clinical scores, tissue oxygenation, heart rate, and cardiopulmonary injury parameters. Total (free + esterified) bromostearic acid levels correlated with lung injury as indicated by BALF protein content and free bromostearic acid levels correlated with plasma cTnI levels. Thus, BFAs and cardiac injury biomarkers can identify Br₂ exposure and predict the severity of organ damage.

Graphical abstract:

Our results make a case for the use of 2-bromopalmitic and 2-bromostearic acids as biomarkers for cardiopulmonary toxicity following exposure to Br₂. These findings are particularly significant

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The authors declare no competing interests.

because there are no known biomarkers for Br₂ exposure. We further provide evidence that the Br₂ inhalation biomarker content corresponds to the severity of the injury and that these biomarkers may serve as a tool for the first responders to identify, stratify, and prioritize emergency care of exposed individuals within hours of a mass casualty situation.

Keywords

bromine; halogens; brominated fatty acid; lung; heart; plasma; injury; biomarkers

Introduction

Stratification of patients following exposure to toxic chemicals can significantly improve their clinical outcome during a mass exposure situation and simplify the work of first responders. The abundance and use of many toxic chemicals, including halogens, in modern industries raise concerns about their accidental or intentional exposures. One such chemical, bromine (Br₂), is a highly volatile, dark reddish-brown liquid that rapidly evaporates at room temperature. Br₂, like other halogens, such as chlorine and iodine, is used as a disinfectant but mostly for small-scale water treatments where it is particularly effective against bacteria, viruses, and protozoan parasites ¹. Additionally, Br₂ is used commercially in the production of flame retardants, dyes, bleaches, and medicinal compounds, as well as gasoline additives. Br₂ toxicity is largely due to its inhalation and, therefore, some of the primary symptoms, such as coughing, difficulty in breathing, and irritation of oral or nasal mucous membrane, are related to its effects on the respiratory system. Exposure to Br₂ can also result in headaches, dizziness, nausea, vomiting, abdominal pain, and gastroenteritis. Our previous studies have also demonstrated extreme cardiotoxic effects of Br₂ exposure that are marked by cardiac ultrastructural damage and biventricular dysfunction potentially leading to heart failure ². Early and reliable disease diagnosis, particularly in the event of an exposure to potentially poisonous chemicals, can significantly affect the prognosis of patients. Moreover, identification of the exposure agent and extent of exposure is critical for providing care during a mass exposure scenario. Biomarkers provide us the confidence of accurate diagnosis, prognosis, and treatment. Among the biomarkers, lipids are increasingly being recognized as sensitive and accurate indicators of human diseases ³. Having established the presence of biomarkers, such as brominated palmitic (2-bromopalmitic) and brominated stearic (2-bromostearic) acids in the lungs and hearts of Br₂-exposed animals, our next task was to establish their correlation with established indicators of Br₂-induced clinical distress parameters and cardiopulmonary damage. Based on our observation that brominated fatty acids (BFAs), such as palmitic and stearic acids, are significantly increased at the onset of Br₂ exposure and remain elevated for significant durations of time after exposure, we designed this study to evaluate whether the elevated levels of BFAs can be exploited as biomarkers for quick and accurate diagnosis of cardiopulmonary toxicity ². We evaluated their levels in the lungs, heart, and blood, following exposure of rats to Br₂ and then correlated the contents with both pulmonary and cardiac damage, using parameters, such as BALF proteins, heart rate (HR), cardiac troponin I (cTnI) levels, oxygen saturation, and the clinical score. We demonstrate that the levels of some of these biomarkers can correlate with indices of cardiopulmonary injury severity after toxic halogen gas inhalation.

Materials and methods

In vivo exposures to Br₂ and clinical scoring

All animal procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Unanesthetized non-SPF male Sprague–Dawley rats (200–250 g, Envigo, Indianapolis, Indiana) were exposed (whole-body) to 600 ppm Br₂ for 60 min, as described previously^{4, 5}. Rats were then returned to room air and monitored continuously until the euthanasia criterion was met (Fig. 1). Clinical scoring is a composite score (0–9) of activity and respiratory quality as previously described⁶. Briefly, a score of 0 is given to an animal with normal respiratory quality and activity. Mild (score of 1), moderate (score of 2), and severe (score of 3) symptoms are assessed and added. For example, an animal demonstrating mild respiratory quality and stridor and moderate activity will have a clinical score of 4 (1 + 1 + 2). Oxygen saturation and HRs were monitored using the MouseOx® small animal oximeter (Starr Life Sciences, Oakmont PA) as previously described in our laboratory⁷. Plasma was analyzed for cardiac injury marker cTnI by ELISA⁷.

Extraction and quantification of brominated species

The brominated lipids were identified as previously described, in David Ford's laboratory⁸. The person performing the assay was blinded to the identity of the coded samples. Rats were euthanized at various time intervals post-Br₂ exposure as described above. Blood was collected from descending aorta and centrifuged at 2000 rpm for 10 min to obtain plasma fraction. The lung and left ventricular (LV) tissues were collected, flash-frozen, and stored at –80 °C before being shipped overnight to the Ford laboratory on dry ice. Free and total (free + esterified) BFAs and chlorinated fatty acids (CFAs) were measured by liquid chromatography/mass spectrometry after the Dole extraction was performed as described before⁹. The endogenous chlorinated lipids represent background levels of 2-CFAs in the tissues. Total fatty acids were measured after base hydrolysis and esterified BFAs calculated by subtracting free fatty acids from total fatty acids. Samples were also spiked with known amounts of [*d*₄]-2-chloropalmitic acid to use as internal standards.

Statistical analysis

Data are expressed as means ± standard error¹⁰. Data were analyzed by a one-way analysis of variance (ANOVA)¹¹¹¹ with Student's paired *t*-test. A *P*-value of < 0.05 was considered significant. Spearman's correlation coefficients were determined to assess the relationship between the biomarker expression and the clinical parameters. The analysis was conducted using GraphPad Prism® 8.0 software (GraphPad, La Jolla, CA).

Results

BFAs are detectable in the lungs, plasma, and hearts of Br₂-exposed rats

We have previously reported time kinetics of the generation of free and esterified BFAs in the plasma of Br₂-exposed rats². For the present study, we quantitated free and esterified BFAs in the plasma, lungs, and hearts of rats exposed to Br₂ and randomized into groups at time intervals to assess BFAs (Fig. 1) and compared them with clinical symptoms and injury

parameters. The levels of 2-bromopalmitic and 2-bromostearic acids were significantly increased in the lungs and hearts soon after Br₂ exposure was done (immediate or 0 min time point) (Fig. 2). However, no such change was observed for the endogenous CFAs. Of note, the brominated levels were not detectable in naïve (control) animals. We quantitated free and esterified 2-bromopalmitic and 2-bromostearic acid levels in the plasma as well and found the levels to be similar to what we have reported earlier with the 45-min exposure². The total (free + esterified) BFAs were detectable and at the levels significantly more than those in control animals in the 3-h postexposure group. These experiments also demonstrate that those that succumbed early or met the euthanasia criterion early (at 0–2 h) had the highest BFA content in the lungs and plasma. These studies are distinct from our previous studies (600 ppm for 45 min) as here we have utilized a higher Br₂ dose (600 ppm for 60 min) and that the animals were euthanized over different time intervals versus preplanned sac times of designated groups in our previous studies². We have additionally determined the BFA content in the lung, which was not reported in the previous study.

BFAs correlate with poor clinical scores and blood oxygen saturation

The clinical score is a measure of the severity of disease, and in our model system, we assigned a clinical score to every individual animal based on three parameters: respiratory quality, stridor, and activity. The scores for individual parameters range from 0 (normal) to 3 (severe) and, thus, a higher clinical score indicated severe symptoms. The cumulative clinical scores ranged from 5 to 8, suggesting moderate-to-severe clinical disease. Spearman's correlation coefficient suggested a strong correlation between BFAs (for both free and total (free + esterified) 2-bromopalmitic and 2-bromostearic acids) and poor clinical scores (Table 1 and Fig. S1, online only). However, respiratory quality (one of the determinants of clinical score) of all the subjects were equally affected and since it is a qualitative measure and limited by the score value (maximum of 3), the desired spread of values were not achieved and a threshold effect was observed. This qualitative data was, therefore, included in the online supplement for clarity.

Pulse oximetry is a routinely used noninvasive measure for a patient's blood oxygen saturation and respiratory health¹². Oxygen saturation can predict mortality in risk score models¹³. Therefore, we also measured oxygen saturations in animals exposed to Br₂ and correlated findings with the levels of BFAs, using Spearman's correlation. Oxygen saturation was observed to be in the normal range (>90%) in only a few exposed animals and was significantly reduced in most of the animals (Fig. 3 and Table 1), which again suggested pulmonary injury and respiratory distress. Spearman's correlation coefficient again suggested a strong inverse correlation, between BFA (for both free and total (free + esterified) 2-bromopalmitic and 2-bromostearic acids) contents of the lung, plasma, and heart, and oxygen saturation.

Total bromostearic acids correlate with biomarkers for lung injury

One of the hallmarks of acute lung injury is a compromised vasculature resulting in protein leakage, and increased protein content of BALF is an indicator of the severity of lung permeability¹⁴. Therefore, we measured BALF protein content and sought its correlation with the levels of BFAs in the lungs, plasma, and hearts. A majority of the total bromostearic

acid content of pulmonary, plasma, and cardiac samples correlated positively with BALF protein, with the leaked protein mostly ranging from 0.3 to 0.6 mg/mL (Fig. 4 and Table 1).

BFAs are biomarkers for cardiac injury

Next, we assessed the correlation of BFAs with cardiac damage. For this, we chose two independent parameters; HR and cTnI levels. We have demonstrated that Br₂ inhalation causes the HR to drop acutely (within 3 h) after exposure and it remains significantly low at 24 h after Br₂ inhalation when even the blood oxygen saturations are back to normal². By this time, significant cardiac dysfunction occurs. Thus decreased HR may also indicate cardiac injury or dysfunction. As shown in Figure 5 (see also Table 1), the HR decreased in many animals as they were exposed to Br₂. A Spearman's correlation, albeit negative, was observed between BFAs (for both free and total (free + esterified) 2-bromopalmitic and 2-bromostearic acids) and the HR. Free BFA (2-bromostearic acid) levels in all three samples (plasma, lung, and heart) also significantly correlated with the levels of cTnI in the plasma (Fig. 6 and Table 1). cTnI levels provide sensitive and specific identification of cardiac muscle damage¹⁵ and are good indicators of cardiac health. Taken together, the data from HR and cTnI levels clearly establish acute cardiotoxicity in animals exposed to Br₂ and a correlation between these cardiac markers and the BFAs content in the lung, plasma, and heart.

Discussion

The aim of this study was to identify predictive biomarkers of Br₂ inhalation and to associate them with cardiopulmonary injury severity. We examined these by exposing rats to Br₂ and evaluating BFA contents in the plasma, lung, and heart tissues and correlating them with clinical scores, oxygen saturations, BALF protein, HR, and cTnI in the plasma, parameters most of which can be easily assessed in clinic. The blood, lung and cardiac tissue BFA (2-bromopalmitic and 2-bromostearic acids) content significantly correlated with the clinical scores, tissue oxygenation, HR, and cardiopulmonary injury parameters. In particular, bromostearic acid levels were found to correlate with lung injury as indicated by BALF protein content and cardiac injury as indicated by plasma cTNI levels. Our studies also demonstrated that cardiac troponin remains elevated for a prolonged durations post Br₂ exposure. Thus, increased levels of circulating BFAs and cardiac injury biomarkers can be used to identify Br₂ exposure and predict the severity of organ damage.

There are several reports in the literature on the occupational/accidental lethal exposures to Br₂. Damage to skin, airway, respiratory, and circulatory systems are primary manifestations of Br₂ exposure, responsible for related mortality^{16–19}. According to a World Health Organization (WHO) report on human health effects after exposure to Br₂ vapors, 0.04 mg/m³ (0.006 ppm) Br₂ can cause eye irritation while 1.3–3.3 mg/m³ (0.02–0.5 ppm) Br₂ can additionally irritate the nose and throat with resulting cough and headache²⁰. The report recognized >3.33 mg/m³ (0.5 ppm) Br₂ exposure as intolerable with toxic pneumonitis and pulmonary edema at 261–392 mg/m³ (40–60 ppm) Br₂ exposure. Furthermore, 6536 mg/m³ (1000 ppm) Br₂ was determined to be fatal within a few minutes of exposure. In our previous study, Br₂ inhalation at 600 ppm for 45 min resulted in 50% mortality within 48 h

with severe morbidity². We used a 60-min exposure for the same concentration to obtain more severe symptoms and to model close proximity exposure. Computational modeling of the Graniteville, NC train chlorine spill disaster suggested exposure of more than 100,000 ppm at the site of release and about 670 ppm within 60 min at a distance of 2 miles from the accident²¹. Although no such modeling has been made for Br₂ accidents, we used a dose of 600 ppm for 60 min to mimic victims of such severe doses and compared them with unexposed controls.

Since Br₂ exposure occurs primarily through inhalation, pulmonary toxicity is expected and rather well characterized.²² However, acute Br₂ exposure can also result in cardiovascular morbidities resulting from hypoxemia, and cardiac arrhythmias, severe enough to progress to cardiac arrest²³. Our recently published work has characterized the cardiotoxicity of Br₂ inhalation². We demonstrated that inhaled Br₂ induces ultrastructural myocardial damage and acute cardiac dysfunction. The reaction of Br₂ with pulmonary plasmalogens produces highly reactive brominated fatty aldehydes that quickly oxidize to BFAs².

In the current study, we focused on lipids as biomarkers of Br₂-induced cardiopulmonary toxicity. Traditionally, nucleic acids or proteins have been evaluated for their utility as diagnostic and/or prognostic biomarkers. This is, in part, due to the availability of tools and techniques tailored for their quick detection. However, lipids are the other physiological macromolecules that can serve as indicators of various diseases. Besides being an integral part of the membrane structures, lipids also play an important role as steroids in cellular signaling³. Their role as biomarkers for cardiovascular^{24,25} and pulmonary diseases^{26,27} has been advocated. We specifically focused on palmitic and stearic acids as biomarkers for cardiopulmonary toxicity since their respective vinyl ether-linked aliphatic precursors are the predominant species in plasmalogens. Palmitic acid is the most ubiquitous saturated fatty acid in the human body and represents approximately a quarter of all fatty acids in membrane phospholipids²⁸. Its use as a biomarker has been suggested for multiple diseases, including diabetes^{29,30}, and lung³¹ and cardiovascular diseases³². Stearic acid is another common saturated fatty acid. Similar to palmitic acid the use of stearic acid as a biomarker for diabetes³³, and lung³⁴ and cardiovascular diseases³⁵ has also been suggested. Interestingly, the serum stearic acid/palmitic acid ratio has been evaluated as a prognostic marker for diabetes remission in obese patients that are a candidate for gastric bypass³⁶. A longitudinal study comprising of 38 obese type 2 diabetes patients, followed by validation in an independent cohort of 381 patients concluded that a higher baseline stearic acid/palmitic acid ratio correlates positively with remission of diabetes after gastric bypass in obese individuals³⁶.

The focus on brominated palmitic and stearic acids in this study, as putative biomarkers for cardiopulmonary damage, was based on our published results². With the background information that brominated compounds formed on the pulmonary epithelial surface are considerably stable³⁷ and that bioactive halogen intermediates produced in the pulmonary vasculature with direct drainage to the left atrium provide a “first-pass effect” in coronary arteries, resulting in severe cardiac dysfunction^{7,38}, we reported that generation of brominated palmitic and stearic acids was an early event that also persisted at least until 3 h postexposure to Br₂². We confirmed 2-bromopalmitic and 2-bromostearic acids in the

plasma of animals in the present study. Additionally, present work also confirmed BFAs in the lung and heart tissues of Br₂-exposed animals, where the levels were particularly high, up to 6–10-fold higher than plasma, arguably pointing to the relevance of brominated moieties as biomarkers for cardiopulmonary insult. Although, palmitic and stearic acids may play a differential role in pulmonary disease pathogenesis, increased circulating stearic acid has been shown to cause cardiac and pancreatic β cell lipotoxicity^{39–41}. We have demonstrated that brominated fatty aldehyde, when administered into the LV, can cause cardiac damage and dysfunction similar to Br₂ inhalation; however, the effects of elevated BFAs need to be investigated². Pulmonary damage as assessed by measuring BALF protein, which is indicative of alveolar–capillary disruption in the lung, correlated with total bromostearic acid contents in cardiopulmonary tissue and plasma. Changes in the HR associate with enhanced cardiovascular risk. Troponin is a biomarker of choice that indicates cardiac injury⁴². Whereas cTnI is an ideal marker for cardiac damage¹⁵. Similar to the indicator for pulmonary damage, cardiac damage was established in Br₂-exposed animals with a positive correlation with free bromostearic acid. These organ-specific differences in total versus free bromostearic acids may be related to either the esterification enzyme content or the content of stearic acid itself in the lungs and the heart.

For establishing correlation, we used Spearman's rank correlation coefficient. A coefficient of "1" denotes perfect correlation while "0" indicates no correlation⁴³. Moreover, a positive value suggests a positive correlation while a negative value is indicative of inverse relationship/negative correlation. Thus, the clinical score, BALF protein content, and cTnI with positive Spearman's coefficient mean that these parameters directly correlate with the levels of BFAs. Blood oxygen saturation and HR, with negative Spearman's coefficient, are indicative of an inverse correlation. We analyzed plasma in addition to the lungs and hearts of Br₂-exposed animals and the results on the correlation of different parameters for pulmonary and cardiac damage and clinical health with BFAs were similar. This suggests that BFAs in the plasma mirror cardiopulmonary damage after acute exposure to Br₂, and, therefore, a quick blood draw and assessment of 2-bromopalmitic and/or 2-bromostearic acids can be a quick test for Br₂-induced cardiopulmonary toxicity. We also report significantly elevated levels of BFAs acutely after Br₂ exposure. Thus, plasma levels are also indicative of the time since the exposure of an individual to Br₂. Early intervention can significantly abrogate the developing cardiopulmonary damage and thus aid a more efficient response in case of a mass exposure resulting from an accident.

Taken together, our results make a case for the use of 2-bromopalmitic and 2-bromostearic acids as biomarkers for cardiopulmonary toxicity following exposure to Br₂. These findings are particularly significant because there are no known biomarkers for Br₂ exposure. Further refining of the methods to detect these BFAs, particularly at the point-of-care facilities, can potentially reduce the mortality associated with exposure to such toxic chemicals. We further provide evidence that the Br₂ inhalation biomarker content corresponds to the severity of the injury and that these biomarkers may serve as a tool for the first responders to identify, stratify, and prioritize emergency care of exposed individuals within hours of a mass casualty situation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.

Schematic representation of the experimental design and sample collection after bromine (Br₂) inhalation. Sprague–Dawley rats were exposed to 600 ppm Br₂ for 60 minutes. Rats were euthanized according to the euthanasia criterion after monitoring their clinical symptoms and pooled into their corresponding time points and samples were collected for the analysis of BFAs. Unexposed controls were also utilized for comparisons.

Figure 2.

Formation of reactive brominated lipids in the lungs, blood, and heart after Br₂ inhalation. Sprague–Dawley rats were exposed to 600 ppm Br₂ for 60 minutes. Lung (A), plasma (B), and heart (C) tissues were analyzed for the production of BFAs by MS, demonstrating the formation of (free or esterified/total) 2-bromopalmitic (red bars) and 2-bromostearic (yellow bars) acids. The blue and green bars are the respective endogenous CFAs. Data are means ± SE; *n* = 5–6 for each group. **P* < 0.05 versus the naïve/0 ppm group.

Figure 3.

Reactive brominated lipid content in the lungs, blood, and heart correlates with blood oxygenation. Sprague–Dawley rats were exposed to 600 ppm Br₂ for 60 minutes. Lung (A), plasma (B), and heart (C) tissues were analyzed for the production of BFAs by MS, demonstrating the formation of (free or esterified/total) 2-bromopalmitic (blue circle) and 2-bromostearic (red square) acids. Pulse oximetry was performed as described in the methods to measure the oxygenation. The lipid biomarker values were correlated with oxygen saturation using Spearman's correlation. The correlation coefficient r and P values are shown on each figure for the corresponding biomarker.

Figure 4.

Reactive brominated lipid content in the lungs, blood, and heart correlates with lung injury. Sprague–Dawley rats were exposed to 600 ppm Br₂ for 60 minutes. Lung (A), plasma (B), and heart (C) tissues were analyzed for the production of BFAs by MS, demonstrating the formation of (free or esterified/total) 2-bromopalmitic (blue circle) and 2-bromostearic (red square) acids. Bronchoalveolar fluid (BALF) was collected after euthanasia and analyzed for total protein content. The lipid biomarker values were correlated with BALF protein using

Spearman's correlation. The correlation coefficient r and P values are shown on each figure for the corresponding biomarker.

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Figure 5.

Reactive brominated lipid content in the lungs, blood, and heart correlates with the HR. Sprague–Dawley rats were exposed to 600 ppm Br₂ for 60 minutes. Lung (A), plasma (B), and heart (C) tissues were analyzed for the production of BFAs by MS, demonstrating the formation of (free or esterified/total) 2-bromopalmitic (blue circle) and 2-bromostearic (red square) acids. The HR was measured by pulse oximetry. The lipid biomarker values were correlated with the HR using Spearman's correlation. The correlation coefficient r and P values are shown on each figure for the corresponding biomarker.

Figure 6.

Reactive brominated lipid content in the lungs, blood, and heart correlates with cTnI. Sprague–Dawley rats were exposed to 600 ppm Br₂ for 60 minutes. Lung (A), plasma (B), and heart (C) tissues were analyzed for the production of BFAs by MS, demonstrating the formation of (free or esterified/total) 2-bromopalmitic (blue circle) and 2-bromostearic (red square) acids. Blood was collected from descending aorta after euthanasia and plasma analyzed for cTnI by ELISA. The lipid biomarker values were correlated with cTnI using Spearman's correlation. The correlation coefficient r and P values are shown on each figure for the corresponding biomarker.

Table 1.

Correlation coefficients of biomarkers with physiological and injury parameters

Parameter/ symptom	Brominated palmitic acid			Brominated stearic acid		
	Pulmonary correlation (Spearman's <i>r</i> and <i>P</i> value)	Plasma correlation (Spearman's <i>r</i> and <i>P</i> value)	Cardiac correlation (Spearman's <i>r</i> and <i>P</i> value)	Pulmonary correlation (Spearman's <i>r</i> and <i>P</i> value)	Plasma correlation (Spearman's <i>r</i> and <i>P</i> value)	Cardiac correlation (Spearman's <i>r</i> and <i>P</i> value)
Clinical score						
Free	0.67, 0.002	0.47, 0.045	0.42, 0.08	0.67, 0.002	0.50, 0.03	0.68, 0.017
Total/ esterified	0.75, 0.000	0.60, 0.008	0.71, 0.001	0.68, 0.001	0.58, 0.01	0.63, 0.005
Oxygen saturation						
Free	-0.68, 0.004	-0.69, 0.002	-0.58, 0.01	-0.74, 0.02	-0.67, 0.003	-0.68, 0.002
Total/ Esterified	-0.53, 0.02	-0.60, 0.008	-0.88, 0.001	-0.67, 0.002	-0.71, 0.001	-0.69, 0.002
BALF protein						
Free	0.45, 0.10	0.43, 0.08	0.55, 0.02	0.40, 0.06	0.63, 0.007	0.58, 0.016
Total/ Esterified	0.41, 0.12	0.70, 0.002	0.79, 0.00	0.61, 0.017	0.73, 0.001	0.74, 0.001
Heart rate						
Free	-0.65, 0.003	-0.52, 0.03	-0.58, 0.01	-0.71, 0.000	-0.70, 0.001	-0.73, 0.000
Total/ Esterified	-0.72, 0.001	-0.71, 0.001	-0.720, 0.007	-0.68, 0.002	-0.66, 0.002	-0.68, 0.001
cTnI						
Free	0.58, 0.01	0.24, 0.33	0.09, 0.71	0.57, 0.012	0.33, 0.17	0.54, 0.019
Total/ Esterified	0.69, 0.001	0.53, 0.02	0.44, 0.06	0.58, 0.01	0.46, 0.05	0.44, 0.06

Note: Statistically significant *P* values are shown in bold.