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MULTIPLEXED IMMUNOASSAYS FOR SIMULTANEOUS QUANTIFICATION OF CARDIOVASCULAR BIOMARKERS IN THE MODEL OF H^{G}-NITRO-L-ARGININE METHYLESTER (L-NAME) HYPERTENSIVE RAT

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The multimarker approach using Luminex technology represents a new tool for studying the pathogenesis of cardiovascular disease. Although many cardiac biomarkers in heart failure have been well established, the role and significance of their measurement in hypertensive patients is still questionable. The aim of our study was to evaluate the relationship of selected biomarkers in L-NAME-induced hypertension to the left ventricular remodeling in the two different periods of hypertension development. Four groups of 3-month-old male Wistar rats were investigated: (1) control 4 (placebo for 4 weeks), (2) control 7 (placebo for 7 weeks), (3) L-NAME 4 (40 mg/kg/day for 4 weeks), and (4) L-NAME 7 (40 mg/kg/day for 7 weeks). BNP, cTnI, TNF-α, and VEGF were measured using Rat CVD Panel 1 Kit (Milliplex® MAP). Cardiac troponin T was determined using Elecsys® Troponin T high sensitive immunoassay (Roche, Switzerland). Although the systolic blood pressure increases about 50% in L-NAME-induced hypertension in rat, both hypertrophy and fibrosis were expressed only slightly in this experiment. The levels of BNP, TNF-α, or VEGF did not differ significantly among groups. However, cardiac troponin T measured by high sensitive ELISA was significantly ($P<0.05$) increased in L-NAME 4 (0.229 μg/l versus 0.034 μg/l) and L-NAME-7 groups (0.366 μg/l versus 0.06 μg/l) in comparison with the controls. We conclude that the slightly increased cTnT levels could indicate ischemic damage of L-NAME-hypertensive heart. Importantly, to our best knowledge, this is the first study indicating that CVD rat panel may be a useful methodological tool in experimental cardiology.

Key words: *cardiac biomarkers, multiplex assay, cardiac troponin, B-natriuretic peptide, vascular endothelial growth factor, tumor necrosis factor-alpha, hypertension, heart remodeling, myocardial hypertrophy*

INTRODUCTION

Cardiac biomarkers provide important information regarding the pathogenesis of cardiac diseases and appear to be useful in risk stratification, in the diagnosis of cardiac diseases, or in monitoring of therapy. Furthermore, many biomarkers may represent risk factors themselves and, therefore, may be potential targets of therapy. Multimarker approaches represent a new paradigm in recent biomarker research (1). Proteins in serum and plasma have traditionally been quantified with ELISA. "Multiple studies" have shown that the combination of several risk factors was a more powerful tool in cardiovascular risk determination than a single-marker approach. The current approach in the research of biomarkers should not be the identification of additional players but rather the understanding of the interplay between the well-established markers (1). The fact that a combination of several biomarkers reflects different aspects of interrelated pathophysiological processes of the particular disease may account for the beneficial nature of this multimarker approach, thus contributing to additional and sometimes completely new information. However, although single biomarkers have been studied extensively, the interplay between the various biomarkers remains a rather misty issue (1).

The basic principle of multiplex assay is based on the Luminex® xMAP® technology, which is a unique combination of flow cytometry and sandwich immunoassays. This technique involves 100 distinctly coloured bead sets created by the use of two fluorescent dyes at distinct ratios. Each bead set can be coated with a reagent specific to a particular bioassay, allowing the capture and detection of specific analytes from a sample. The use of different coloured beads enables the simultaneous detection of many analytes (up to 100) in the same sample. Imaging or laser excitation is used to determine the different assays by bead colours, and determine analyte concentration by measuring the reporter dye fluorescence. The main advantage of multiplex assay in comparison with ELISA is the low sample volume (20 μl), the same conditions of analysis, the savings on cost and time, and the complex information about the pathological process.

However, investigations of large animal and patient cohorts are needed for a more thorough understanding of available panels (2, 3). Rats are the most common laboratory animals being used for the study of the pathogenesis of cardiovascular diseases, new drug screening, and preclinical trials. Hence, Millipore released the rat CVD 1 Panel, providing an important tool for the study of pathogenesis of cardiovascular disease, which can simultaneously detect 10 biomarkers: B-natriuretic peptide (BNP), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor 1 (PAI-1), tissue inhibitor of matrix metalloproteinases type I (TIMP-1), tumor necrosis factor alpha (TNF-α), cardiac troponin I (cTnI), cardiac troponin T (cTnT), vascular endothelial growth factor (VEGF), and von Willebrand factor (vWF). In our study, only four of them (BNP, cTnI, TNF-α and VEGF) have been analyzed in the model of L-NAME (N^G-nitro-L-arginine-methyl ester) hypertension. In this pilot study, our aim was to involve the principle categories of cardiac markers according to Braunwald´s classification (4). Hence we have choosen four markers: the first one indicating left ventricular dysfunction and failure, another one reflecting myocardial injury, the third one as a member of the cytokine family and the forth marker referring to angiogenesis. As a marker of extracelullar-matrix alteration in the left ventricle hydroxyproline concentration was determined.

The aim of our study was to evaluate the significance of selected biomarkers in hypertension and state of end-organ damage, where their contribution to the understanding of pathophysiological disorders is still questionable. Importantly, the study using the multiplex approach in experimental cardiology, according to our best knowledge, has not been published yet. We wanted to show whether there is some relation between the particular multiassay-determined biomarkers to the left ventricular remodeling in the two different periods of L-NAME-induced hypertension.

MATERIALS AND METHODS

Animals

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 8532, revised 1985).

Four groups (n=8–10) of 3-month-old male Wistar rats were investigated: (1) control 4 (placebo for 4 weeks), (2) control 7 (placebo for 7 weeks), (3) L-NAME 4 (40 mg/kg/day dissolved in water, for 4 weeks), and (4) L-NAME 7 (40 mg/kg/day dissolved in water, for 7 weeks). Systolic blood pressure (SBP) was measured using the tail-cuff method twice before the start of experiment and then every week. The weight of the left ventricle (LVW) was determined at the end of the experiment.

Procedures

After finishing the experiment, the plasma for cardiac markers was collected using EDTA and the plasma samples were stored at –80°C. Cardiac troponin T was determined using Elecsys® Troponin T high sensitive immunoassay (Roche, Switzerland).

The selected parameters (cTnI, BNP, TNFα, VEGF) were measured using Rat CVD Panel 1 Kit (Milliplex® MAP) precisely according the enclosed guideline. Briefly, 25 µl of standard or assay buffer, 25 µl of matrix solution or sample, and 25 µl of beads was added to the appropriate well and incubated overnight at 4°C with shaking. After vacuuming and washing twice with wash solution, 25 µl of detection antibodies was added and incubated 2 hours at room temperature. Then, 25 µl of streptavidin-phycoerythrin was pipetted and incubated for 30 min before vacuuming and washing. Then 100 µl of sheath fluid was added into each well and the plate was analyzed on Luminex 200™ (Biorad, Hercules, USA).

Collagenous proteins were isolated from left ventricle samples according to Pelouch *et al*. (5). Briefly, the trimmed tissues (about 50 mg of wet weight) were treated stepwise with different buffers with increasing strength in order to obtained noncollagenous (*i.e*. metabolic and contractile proteins) and then collagenous proteins. Soluble collagenous proteins constituted mainly by collagen I and III was extracted into 0.5 mol/l acetic acid containing 1 mg of pepsin/ml, pH=1.45 for 24 hours at 4°C. After centrifugation the pellet was further suspended in 1.1 mol/l NaOH and heated two times for 10 min. This fraction contained insoluble collagenous proteins including collagen aggregates, elastins and other proteins of the extracellular matrix. Hydroxyproline (estimate of fibrosis) in both fractions was measured spectrophotometrically at 550 nm.

Statistical analysis

Values of individual cardiovascular parameters were declared as mean ±S.E.M. and were evaluated by ANOVA. Values of cardiac markers were expressed as median and were evaluated by Kruskal-Wallis nonparametric analysis of variance followed by multiple comparison by Dunn´s test with Bonferroni´s correction. Concomitantly, Spearman correlation coefficient was calculated. Statistical evaluation was performed using the SigmaStat software program (revision STAT 32 2.0, Jandel GmbH, Erkrath, Germany) and the NCSS program 2007 (version 07.1.21, LLC., Kaysville, Utah, USA).

RESULTS

Cardiovascular parameters

Blood pressure and ventricular weights

The administration of L-NAME caused hypertension. After 4 weeks SBP was 122.5±1.58 mm Hg in control rats. In the L-NAME 4 group, SBP was significantly higher than in controls by 51.8% ($P<0.05$; 185.0±2.98 mm Hg). After 7 weeks, SBP was 124.0±0.56 mm Hg in control rats. In the L-NAME 7 group, SBP was higher even by 58% than in the control 7 group ($P<0.05$; 195.8±0.85 mm Hg) (*Fig. 1*).

The absolute LVW was 430.6±17.6 mg and 454.0±14.9 mg in the control 4 and L-NAME 4 group in 4 weeks, respectively. After 7 weeks of treatment, LVW was increased significantly in L-NAME 7 rats in comparison to control 7 (434.3±16.1 mg versus 501.3±24.8 mg, $P<0.05$) (*Fig. 2*).

Hydroxyproline concentration

In the L-NAME 4 group, the total hydroxyproline concentration was 15% higher in comparison with the control 4 group. The same non-significant increase was also found in the L-NAME 7 group (0.19±0.016 mg/g versus 0.22±0.013 mg/g). The hydroxyproline content was 21% in the L-NAME 4 group and 35% higher in the L-NAME 7 group, respectively. The only statistically significant increase was found in the fraction of soluble hydroxyproline in L-NAME-4 (*Fig. 3*).

Cardiac biomarkers

Our results observed using Multiplex assay in controls are summarized in *Table 1*. The difference in the median values of

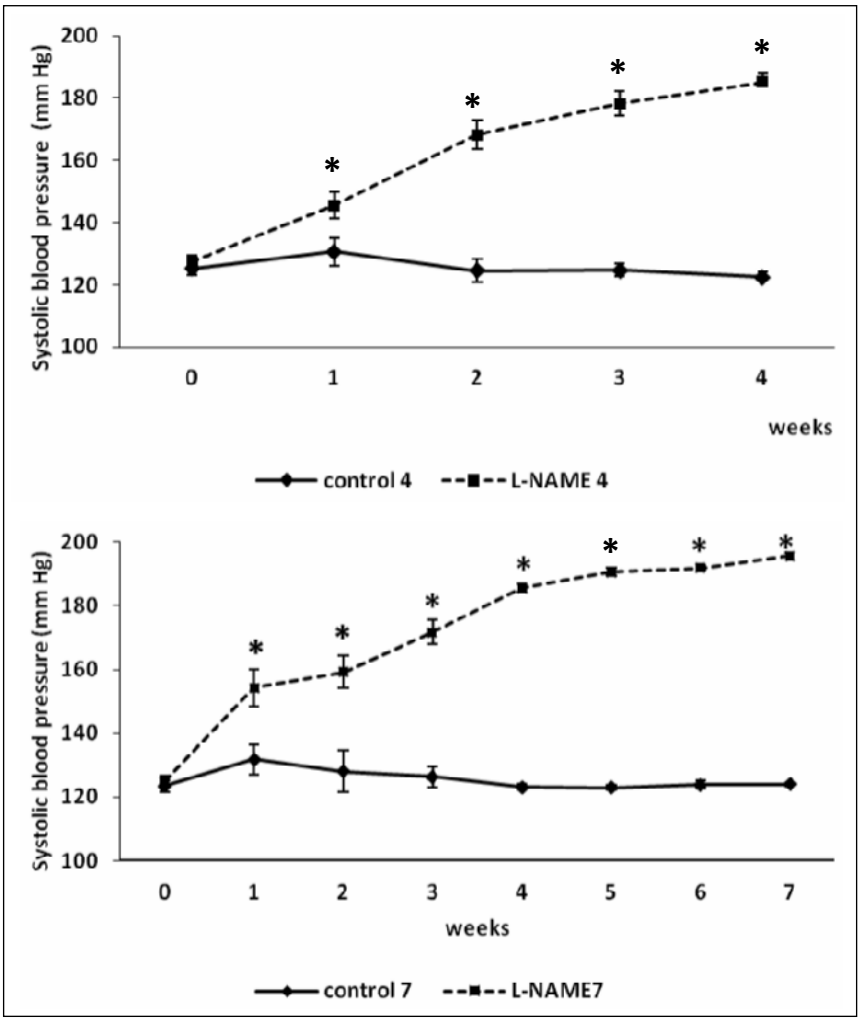

Fig. 1. Effect of 4 weeks and 7 weeks of N^G-nitro-L-arginine-methyl ester (L-NAME) treatment on systolic blood pressure. *P<0.05 versus control.

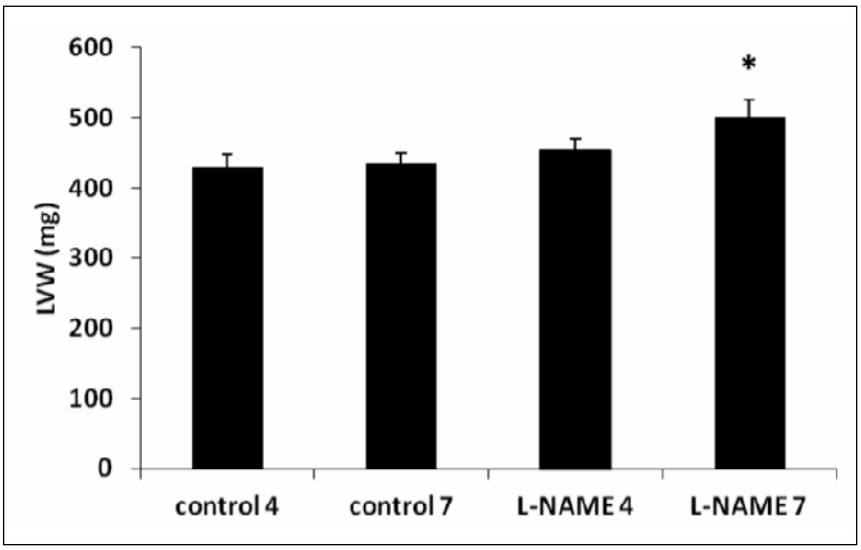

Fig. 2. Effect of 4 weeks and 7 weeks of N^G-nitro-L-arginine-methyl ester (L-NAME) treatment on the absolute weight of the left ventricle. *P<0.05 versus control 7.

brain natriuretic peptide (BNP) among the treatment groups was not statistically significant. After 7 weeks of treatment, the BNP was 21.14 pg/ml and 30.33 pg/ml in the control 7 and L-NAME 7 group, respectively (*Fig. 4*). The maximal measured value in the L-NAME 7 was 527.62 pg/ml. The median of cardiac troponin I was 0.04 µg/l in both control groups and 0.114 µg/l in L-NAME 7. The values out of range were considered to correspond to minimal detectable concentration. The percentage of detectable values of both TNF-α and VEGF was comparable with the controls.

Cardiac troponin T measured by high sensitive ELISA was 0.034 and 0.06 µg/l, in the control 4 and control 7 groups, respectively. The cTnT plasma levels were significantly (P<0.05) increased in L-NAME 4 and L-NAME-7 groups (0.229 and 0.366 µg/l, respectively) (*Fig. 5*). The detectable levels of cTnI tightly correlated with cTnT levels (R=0.83, P<0.001).

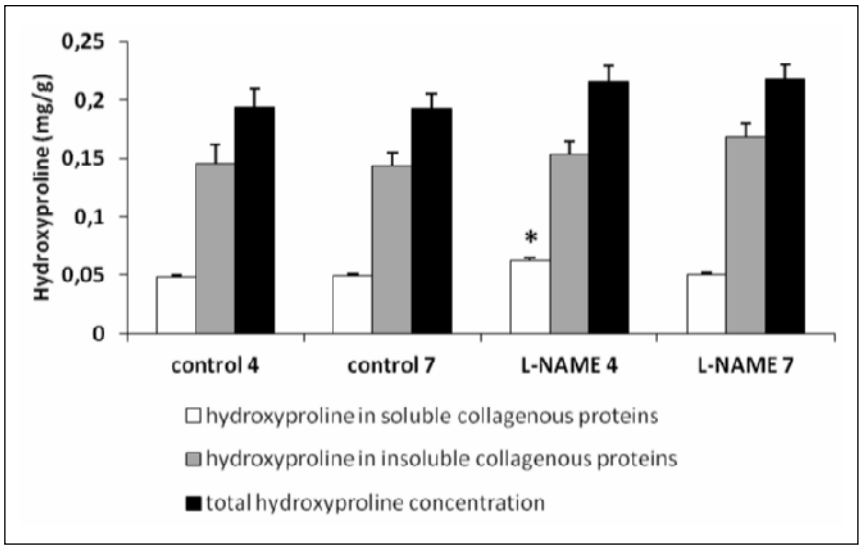

Fig. 3. Effect of 4 weeks and 7 weeks of N^G-nitro-L-arginine-methyl ester (L-NAME) treatment on hydroxyproline concentration of left ventricle; measured in the fraction of soluble and insoluble collagenous proteins. *P<0.05 versus control 4.

Table 1. Data on cardiac biomarkers from Rat CVD Panel 1 from Multiplex (Millipore), involving 20 controls.

	BNP (pg/ml)	cTnI (pg/ml)	TNF alfa (pg/ml)	VEGF (pg/ml)
OOR	1.43	40.02	5.07	2.51
Min	5.57	60.32	18.95	5.04
Max	36.45	179.15	22.87	17.44
Mean	20.54	55.92	6.83	5.33
Median	20.72	40.020	5.07	2.51
STDEV	9.64	10.15	15.17	5.26
% Detectable values	100	20	10	20

B-type natriuretic peptide (BNP), cardiac troponin I (cTnI), tumor necrosis factor alpha (TNF-α), vascular endothelial growth factor (VEGF); OOR (out of the range) = below the detection limit. For the statistical analysis the non-detectable values are considered to be equal to OOR.

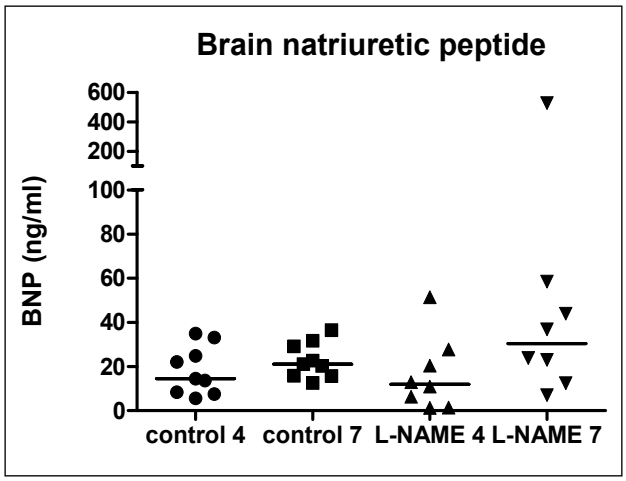

Fig. 4. Effect of 4 weeks and 7 weeks of N^G-nitro-L-arginine-methyl ester (L-NAME) treatment on the plasma levels of brain natriuretic peptide.

DISCUSSION

The systolic blood pressure was enhanced by about 50% in L-NAME-induced hypertension in this experiment. However only the weight of the LV and hydroxyproline concentration in soluble fraction of L-NAME7 were enhanced significantly, although the tendency to fibrosis was shown referring to all collagenous parameters in both investigated groups of rats. One should be aware that the remodeling of the targeted organs in L-NAME-induced hypertension was shown to be unusually complex issue. Along with the blood pressure rise representing the pressure overload, the disbalance among several humoral systems participate. Beside the attenuated production of NO with antiproliferative effect, the activation of several systems with proproliferative nature take part on the rebuilding of the LV, including the increase of the local-tissue angiotensin converting enzyme activity (6) or elevation of the plasma aldosterone level (7). As a result of this multivariant pathogenesis, the peripheral tissue rebuilding is reported to be variably expressed in different laboratories. Although most authors reported that L-NAME administration resulted in hypertrophy of the LV (8-11) with the enlargement of cross sectional area of myocytes corresponding to the concentric hypertrophy (12), several researchers did not observe LVH development in this model (13, 14). Analogically,

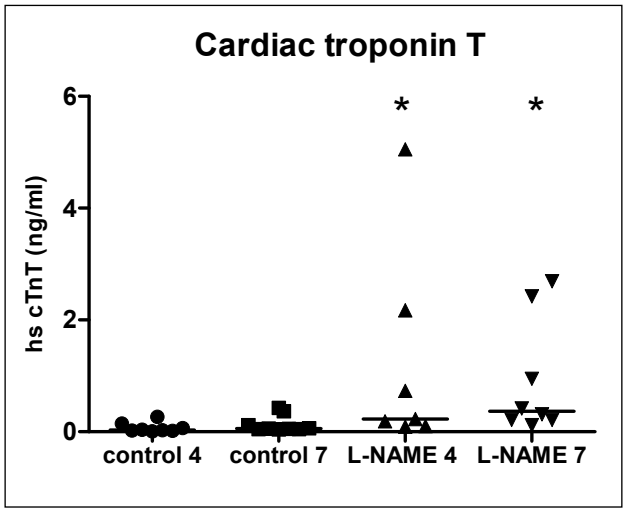

Fig. 5. Effect of 4 weeks and 7 weeks of N^G-nitro-L-arginine-methyl ester (L-NAME) treatment on the plasma levels of cardiac troponin T. *P<0.05 versus control 4 and control 7, respectively.

although fibrosis of the LV (as a result of myocardial ischemia) was mostly observed (15-18), while especially cross-linked collagen was augmented (17, 18), in some experiments the proportion of collagen I and III was not altered (9). The underlying mechanism of these discrepancies might be associated with the variable time and dosage of L-NAME administration and perhaps with other (hitherto unknown) modulating factors such as age and strain of the animals or mode of their handling (19). A multiplexing approach could deepen the insight into the complex pathogenesis of LVH in L-NAME administration and other models of hypertension including spontaneously hypertensive rats (20) or SHR combined with inhibition of neural NOS (21).

Hypertension seems to be associated with the structural and functional alterations of the microvascular network growth resulting partly from the abnormal regulation of the vascular endothelial growth factor (VEGF), one of the most potent known angiogenic factors. Elevated VEGF levels in hypertensive patients were shown to correlate with cardiovascular risk. Accordingly, treatment of hypertension significantly reduced VEGF levels. More recently, it has been suggested that VEGF could be a marker for early microvascular damage in hypertension, which are currently regarded as a sum of platelet activation and endothelial dysfunction in hypertensive patients (22).

In our study, the plasma VEGF in most healthy animals was below detection limits and chronic administration of L-NAME did not cause any significant change. Kazim *et al*. (23) even showed that chronic NOS inhibition significantly decreased cardiac VEGF mRNA and protein expressions, indicating the down-regulation of VEGF expression in the heart through depressed NOS expressions and NO levels. One explanation could be the reciprocal regulation between NO level and VEGF expression (24).

In coronary circulation, the activated angiogenesis mediated by VEGF may significantly contribute to the preservation of the physiologic structure and function in the overloaded heart, especially that of the sport heart. However, pathologic cardiac hypertrophy in hypertensive heart disease is not associated with microvascular growth proportional to cardiac hypertrophy, which thereby renders the myocardium vulnerable to ischemia. The possible utility of angiogenic gene programming in preserving the coronary microvasculature, cardiac function and altering disease course is under study (25). The findings of Xu *et al*. (26) showed that VEGF attenuates the transition from cardiac hypertrophy to heart failure through mitochondrial-mediated apoptosis and cardiomyocyte proliferation.

Natriuretic peptides are considered to be a marker of cardiac dysfunction (4). Atrial natriuretic peptide (ANP) is synthesized mainly by atrial myocytes. B-type natriuretic peptide (BNP) is produced by both atria and ventricles, but, the majority of BNP secretion is derived from the ventricles because of their greater mass. The molecule is initially translated as a 134 amino acid protein (pre-proBNP). The first 26 amino acids are a signal peptide which is removed in the first phase of the post-translational modification. Then prohormone proBNP is splited into N-terminal pro-BNP (NT-proBNP, 76 amino acids) and finally to biologically active BNP (32 amino acids). While the half-life of BNP is 21 minutes, the NT-proBNP half-life is 70 minutes, hence the NT-proBNP measurement is more suitable for clinical practice (27).

The BNP and/or NT-proBNP values are helpful for the detection of congestive heart failure (28). Regarding BNP, values below 100 ng/l make heart failure unlikely, with a negative predictive value of 90%. If the value is above 500 ng/l, heart failure is highly probable with a positive predictive value of 90% (29).

Recently, the potential of these markers in hypertension has been questioned (30). Experimental data indicate that natriuretic peptide appears to play an important role in preventing the development of arterial and pulmonary hypertension. Overexpression of the genes encoding ANP and BNP results in raised circulation of these peptides and a substantially decreased arterial BP (31). Furthermore, it has been shown that plasma ANP and BNP levels were significantly reduced in normotensive subjects with a family history of hypertension (32). In the study of Tsioufis *et al*. (33), on the other hand, BNP levels were higher in hypertensive (with increased LV mass index) compared with normotensive subjects but the changes were not statistically significant (20.4 versus 17.1 pg/ml). However, there have been suggestions that the extent of natriuretic peptides alteration is more closely related to the BP elevation itself than to left ventricular hypertrophy (30, 32).

Zhang *et al*. (34) described that left ventricular ANP mRNA, but not left ventricular BNP mRNA, correlated significantly with LVW/BW only in five out of twelve 8-week L-NAME-treated animals with expressed LV hypertrophy. No changes were observed in atrial gene expression or plasma concentration of ANP or BNP. Similarly, our data display well-known biological variability of BNP levels without any statistically significant difference between groups. Most values were below 100 ng/l; only one value in the L-NAME 7 treated group was in the zone indicating the risk of heart failure but one should take into account that 2 out of 10 animals died prematurely without the possibility of measuring BNP. The correlation between weight of the left ventricle and BNP was not significant.

Cardiac troponins are considered to be BNP-independent prognostic markers in acute or chronic heart failure (35, 36). Furthermore, recent studies suggested that cardiac troponins can be a novel predictive marker of adverse outcome in patients with hypertension (37, 38). Higher serum values probably indicate a more important cardiac involvement in the setting of a hypertensive disease (39).

Nowadays, six potential major mechanisms for troponin elevation have been proposed: (1) necrosis, (2) apoptosis, (3) normal myocyte cell turnover, (4) release of proteolytic troponin degradation products, (5) increased cellular membrane permeability due to stretch-related mechanisms mediated by integrins, and (6) formation and release of membrane blebs (40). In our study, the cTnI measured by multiplex-assay did not show significant changes between groups but there was a statistically significant increase of cTnT in L-NAME groups measured by the high sensitivity assay. The values of cTnT tends to increase in accordance with the enhancement of hydroxyproline concentration, however, the changes were not significantly altered. Similarly, we have found increased cTnT levels in the SHR rat in comparison with control (unpublished data). Although similar, or even better, detection limits have been demonstrated for Luminex analyses compared with ELISA, cardiac troponins represent the exception. Luminex minimum detectable concentration is theoretically 322.8 and 24.2 ng/l for cTnI and cTnT, respectively. For example, the measuring range of the 4th generation of ELISA for cTnT (Roche) is 0.01–25 μg/l and for the 5th generation is even 0.001–10 μg/l. These data clearly show that the use of high sensitivity cardiac troponin assays is more helpful for the detection of discrete injury of cardiac disease in laboratory animals than the multiplex-assay.

Tumor necrosis factor alpha is thought to be part of an integral network that orchestrates inflammatory, immunological, and neurohormonal signalling. TNF-α acts primarily as a paracrine or/and autocrine regulator of leukocytes and endothelial cells; only when TNF-α production far exceeds the number of TNF receptors in a given tissue does TNF-α occur in plasma (41, 42). In the non-failing heart, neither TNF

mRNA nor TNF protein appears to be constitutively expressed. However, several studies have shown enhanced levels of inflammatory cytokines including TNF-α in HF patients in plasma, as well as in the failing myocardium itself. There is enough evidence that these mediators are associated with processes being involved in cardiac remodeling such as hypertrophy, fibrosis, and apoptosis (43).

Recently, it was suggested that inflammation may also play a role in the development of hypertension, occurring either as a primary or secondary event, but the results from the studies are not convincing. Gullestad *et al*. (43) described that plasma levels of inflammatory cytokines, such as TNF-α, directly correlate with blood pressure in essential hypertension. On the other hand, in the study of Peeters *et al*. (44), circulating concentrations of TNF-α did not differ between patients with essential hypertension. The precise mechanism by which TNF-α interferes with hypertension is unclear, but the TNF-α-induced decrease of eNOS mRNA levels by increasing the rate of mRNA degradation and by the reduction in NO bioavailability seems to play an important role (45).

To our knowledge, only Miguel-Carrasco *et al*. (46) described increased plasma levels and heart expression of TNF-α in L-NAME-treated animals. However, our results did not reveal any significant changes between L-NAME and controls. Furthermore, although there is great cross-talk between TNF-α and VEGF-mediated signalling pathways (47), the evident association between increased TNF-α and VEGF levels has not been found.

The literature data on cardiac biomarkers in the model of L-NAME-induced hypertension are sparse. Furthermore, the data on cardiac markers in hypertension are conflicting and their role in elucidating the pathogenesis and prognosis of hypertension is under unremitting discussion. We conclude that the level of TNF-α or VEGF in L-NAME hypertension was not increased. The slightly increased cardiac troponin levels (measured by recently released high sensitive assays) could indicate ischemic damage of L-NAME-hypertensive heart. A multiplexing approach may contribute to a deeper insight on the role of biomarkers in hypertensive heart diseases. Importantly, to our best knowledge, this is the first study indicating that CVD panel may be a useful methodological tool in experimental cardiology.

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