

Investigation of Biochemical Composition and Vasomotor Effect of Human Pericardial Fluid

Ph.D. Thesis

Zoltan Nemeth



Ph.D. Program leader
Istvan Szokodi, M.D., Ph.D., D.Sc.

Supervisor:
Prof. Akos Koller, M.D., Ph.D., D.Sc.

Co-Supervisor:
Prof. Attila Cziraki M.D., Ph.D.

Institute for Translational Medicine, and Szentagothai Research Centre
Doctoral School of Clinical Medical Sciences
Medical School, University of Pécs, Pécs, Hungary

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ABBREVIATIONS

ADMA	asymmetric dimethylarginine
AMI	acute myocardial infarction
Ang II	angiotensin II
ASE	American Society of Echocardiography
AVR	atrial valve replacement
BQ123	ET _A antagonist
CABG	coronary artery bypass graft surgery
CAD	coronary artery disease
CCS	Canadian Cardiovascular Society
CKD	chronic kidney disease
Dd	left ventricular end-diastolic diameter
DDAH	dimethylarginine dimethylaminohydrolase
Ds	left ventricular end-systolic diameter
eGFR	estimated glomerular filtration rate
eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
ET _A	ET-1 receptor
IVS	thickness of interventricular septum
LA	left atrial area
L-Arg	L-arginine
LVEF	left ventricular ejection fraction
LVM	left ventricular mass
miRNA	microRNA
MVR	mitral valve replacement
NAD(P)H-oxidase	nicotinamide adenine dinucleotide phosphate-oxidase
NCP	non-cardiac patients
NE	norepinephrine
NO	nitric oxide
PF	pericardial fluid
PF _{CABG}	pericardial fluid originated from CABG patient
PF _{VR}	pericardial fluid originated from VR patient
PRMT1	protein methyltransferase 1
PW	thickness of posterior wall
RA	right atrial area
RAS	renin-angiotensin system
ROS	reactive oxygen species
RV	right ventricular area
sCr	serum creatinine
VR	valve replacement surgery

INTRODUCTION

The pericardial fluid

The pericardial fluid (PF) is an approximately 15-50 ml viscous, pale yellow film layer placed between the layers of the pericardium¹. For a long time it was widely accepted by clinicians that the physiological role of the PF is that it reduces the friction between the pericardium and the surface of the heart. This idea has been disproved by researchers assessing the electrolyte and acid base composition of PF². This initiated intensive research focusing on the composition of PF and showed that it contains several cardiac biomarkers³.

Signaling molecule ADMA modulates nitric oxide

L-Arg/NO pathway and the ADMA

A methylated derivative of amino acid L-arginine (L-Arg) ADMA is known to reduce the bioavailability of nitric oxide (NO)⁴. NO is produced by nitric oxide synthases (NOSs) from the precursor amino acid L-Arg⁴. NO is a multirole molecule, among others modulating vasomotor tone, and attenuating tissue proliferation and growth⁴. Also, previous studies have established that ADMA, being a false substrate competitively inhibits the activity of endothelial NO synthase (eNOS) thus production of NO⁴. In addition, we have reported that ADMA activates the vascular renin-angiotensin system (RAS) and elicits the generation of reactive oxygen species (ROS)⁵. Elevated concentrations of ADMA in plasma have been reported in various cardiovascular diseases⁶. These results have been further confirmed by functional clinical findings investigating relationship between serum level of ADMA and cardiac functions⁷.

Potential role of ADMA in the cardiac remodeling

The process of cardiac remodeling is caused by pathophysiological/adaptive (injuries of the heart) processes, which are regulated by mechanical (wall stress) and molecular mechanisms⁸. Cardiac hypertrophy is a typical form of cardiac remodeling when – among others - the size of myocytes increases causing thickening of ventricular walls⁸. Cardiac hypertrophy is an adaptive or maladaptive process induced by physiological (exercise-induced hypertrophy) or pathological processes including pressure and/or volume overload, or occur after myocardial infarction⁹. Besides mechanical forces, locally acting factors, such as cytokines and growth factors are implicated in the development of cardiac hypertrophy¹⁰.

Based on the aforementioned, ADMA directly or indirectly could be involved in cardiac hypertrophy/remodeling.

Vasoactive substances in the pericardial fluid

Endothelin-1

Increasing data show that PF composes several vasoactive substances, such as endothelins¹¹, catecholamines¹², adenine nucleotides¹³, natriuretic peptides¹⁴, angiotensin II¹⁵, and prostaglandins¹⁶. It is well known that endothelins, such as endothelin-1 (ET-1) are potent vasoconstrictor peptides playing an important role in the regulation of vascular tone (through its receptors: ET_A and ET_B), and growth factors for many types of cells¹⁷. ET-1 receptor (ET_A) antagonist BQ123 has been widely used to reduce vasoconstriction induced by ET-1¹⁷.

ET-1 has been found involving in pathogenesis of hypertension and vascular diseases¹⁷. Moreover, it has been shown that concentration of ET-1 is more elevated in PF of patients with ischemic heart

disease as compared to non-ischemic patients¹⁵. Also it has been demonstrated that intrapericardial added ET-1 induces arrhythmias in ventricle of dog heart¹⁸. This may confirm that substances in PF may reach, moreover effect on cardiac interstitium, thus PF could behave as paracrine material.

In summary, as described above, PF has an important mechanical role, however, recently several studies have shown that PF has many other physiological roles as well, by which it can regulate coronary blood flow and cardiac remodeling¹⁹. In cardiac patients PF contains several vasoactive substances, growth factors and biomarkers, which levels are often higher than in plasma. Based on these data, it is very plausible that PF has many roles, other than mechanical, such as regulation of the function of the heart and coronary circulation.

HYPOTHESES AND AIMS

Based on the aforementioned, we have formulated two main hypotheses: **1)** The level of **ADMA** in PF of patients with valve disease - due to pressure and/or volume overload - could contribute to the morphological changes of the heart; **2)** In the PF of patients with cardiac disease - due to ischemia/hypoxia or ischemia/reperfusion - the level of vasoconstrictor factors, such as endothelins can reach levels that can elicit **vasomotor** responses in arterial vessels.

We aimed 1) to determine and investigate the level of L-Arg and ADMA of pericardial fluid of patients undergoing coronary artery bypass graft (CABG) or valve replacement (VR) surgery; and to investigate the correlations between PF ADMA and the morphology and function of the heart; **2)** to investigate the direct vasomotor effect of human PF on rat carotid arteries; and to elucidate the mechanisms by which PF elicits vasomotor responses of arteries.

MATERIALS AND METHODS

Study description

Patients

In the present study, 74 patients undergoing CABG or VR surgery (CABG: n=42; VR: n=32) were enrolled in the Heart Institute at the Medical School, University of Pecs, Hungary. Furthermore, we investigated peripheral blood plasma level of ADMA in 20 non-cardiac patients (NCP). The Local Ethical Committee of the Medical School of University of Pecs (RKEB-4123/2011) approved the study protocol. Full informed consent was obtained from all individuals before participation in the study. The investigation conforms to the principal outlined in the Declaration of Helsinki.

Animals

For the isolated vessels experiments 2 months old male Wistar rats (N=14) were used (vessels for ET-1 vasomotor responses: n=5; PF vasomotor responses: n=16; PF BQ123 responses: n=5). All experiments were approved in accordance with the general rules for animal protection in science work, a 2010 European Directive on ethical issues (European Communities Council) Directive 2010/63/ECC and Ethical Committee of the University for the Protection of Animals in Research and approved by the same committee. All procedures were approved by the Ethics Committee on

Animal Research of University of Pecs according to the Ethical Codex of Animal Experiments and license was given (No.: BA 02/2000-2/2012).

Harvesting of samples

Harvesting of human blood plasma and pericardial fluid

Plasma was harvested from NCP, and both plasma and PF were harvested from the cardiac patients after median sternotomy and collected into heparinized blood collecting tubes, and then stored at 5 °C for approximately 1 hour. After then they were centrifuged (1200 g, 15 min), and supernatant were stored at -75 °C until they used for the biochemical measurements and the experiments.

Isolation and preparation of rat common carotid arteries

The rats were anesthetized with an intraperitoneal injection of ketamine, and common carotid arteries were excised, and animals were then euthanized by an additional ketamine injection. With the use of microsurgery instruments and an operating microscope, the excised common carotid arteries (≈ 10 mm in length) were transferred into a cooled ($T=4^{\circ}\text{C}$) petri dish filled with oxygenized (95 % O_2 , 5 % CO_2) Krebs solution, and cut to pieces (≈ 2 mm in length).

Investigation of pericardial fluid ADMA

Echocardiography

All cardiac patients underwent complete two dimensional (2-D) transthoracic echocardiography before and after surgery. 2-D, M-mode and Doppler echocardiography were performed by Hewlett-Packard Sonos 5500 echocardiograph with a 2.5 MHz transducer (Hewlett-Packard, USA) according to the recent European guidelines²⁰. The following parameters were measured: left ventricular end-diastolic diameter (Dd), left ventricular end-systolic diameter (Ds), thickness of interventricular septum (IVS) and posterior wall (PW), right ventricular (RV), right atrial (RA), and left atrial (LA) area. Left ventricular mass (LVM) was calculated using the American Society of Echocardiography (ASE) convention: $\text{LV mass} = 0.8 (1.04 ([\text{LVIDD} + \text{PWTD} + \text{IVSTD}]^3 - [\text{LVIDD}]^3)) + 0.6 \text{ g}$. The left ventricular ejection fraction (LVEF) as the index of global systolic function was calculated according to the Simpson formula.

Measuring the concentration of L-Arg and ADMA

L-Arg and asymmetric dimethylarginine (ADMA) were determined using liquid chromatography – mass spectrometry. Quantification of ADMA and L-Arg derivatives was performed at the Department of Applied Chemistry, University of Debrecen.

Investigation of vasomotor effect of pericardial fluid

Measurements on isolated vessels

After preparation, vessels were placed in a 5 ml organ chambers of isometric myograph (DMT 610M, Danish Myo Technology, Aarhus, Denmark) between two stainless steel wires (diameter 0.04 mm), and their length tension curve were obtained (normalized to 2.0 g) then the vessels were incubated for stabilization in chamber solution (which continuously oxygenated with a gas mixture containing 95 % CO_2 , and 5 % O_2 , and kept at 36.5°C , pH 7.4). Isometric tension (mN) generated by the vessels was measured by using isometric myograph and acquisition of data was performed using Myodaq 2.01 M610+ program.

Adding of PF samples and vasoactive agents to the vessels

Following incubation, we tested the development of isometric force of isolated arteries using KCl, which was then washed out by Krebs solution. Before adding of PF samples into the organ chambers, they were thawed using warmed water ($T = 20^{\circ}\text{C}$). After that ET-1 (10^{-8} mol/L); PF (CABG and VR); BQ123 (10^{-6} mol/L) were added.

ET-1 induced vasomotor responses following BQ123 adding

We tested the vasoactive effect of ET-1 on isolated rat carotid arteries ($n=4$). Following wash out KCl (40 mM) (3 times, 20 min), we added ET-1 into the organ chambers. Following plateau phase of curves, ET-1 was washed out (6 times, 35 min), and then BQ123 (20 min) was added, and then adding of ET-1 was repeated.

PF_{CABG} and PF_{VR} -induced vasomotor responses

The vasoactive effect of PF of both CABG ($n=9$) and VR ($n=7$) were tested in isolated rat common carotid arteries ($N=8$, vessels: $n=16$). Following wash out KCl (60 mM) (3 times, 20 min), the PF samples were added into the organ chambers. Following plateau phase of curves PF was washed out (5 times, 20 min), and then the development of isometric force in isolated arteries was tested using KCl (60 mM).

PF_{CABG} induced vasomotor responses after adding of BQ123

The vasoactive effect of ET-1 in PF ($n=5$) was tested before and after adding of BQ123 (10^{-6} mol/L) on isolated rat common carotid arteries ($N=3$, vessels: $n=5$). Following wash out of KCl (60 mM) (3 times, 20 min), the PF samples were added into the organ chambers. After plateau phase of curves PF was washed out the PF (5 times, 20 min), then BQ123 was added into organ chambers for 20 minutes. Following that, the same PF samples were added into the organ chambers. Following plateau phase norepinephrine (10^{-6} mol/L) as another vasoconstrictor were added into the organ chambers to test the development of isometric force of the vessels.

Statistical methods

Results are expressed as mean \pm SEM. Statistical analyses were performed with Microsoft Excel and SPSS (Statistical Package for the Social Sciences) software. Statistically significant differences were determined using the Student's two-tailed unpaired t-test. $P<0.05$ was taken as a significant difference. The correlation studies were performed by linear regression analysis using SigmaPlot software.

RESULTS

Pericardial fluid ADMA

Clinical characteristics of patients

The mean ages and sex of both CABG and VR patients were similar. 35.7% of CABG and 80% of VR patients exhibited LV hypertrophy. In general, the CABG patients had hypertension and most of them had a history of earlier acute myocardial infarction (AMI). The serum creatinine (sCr), and eGFR were similar in both CABG and VR patients, albeit both patient groups mean eGFR indicate CKD stage 3. Pre-operative medications of the patients were similar, however patients of the CABG were treated with higher dose of aspirin and statin before surgery compared to patients of

the VR patients. In this study, 53 Caucasian patients underwent cardiothoracic surgery: 28 for CABG, and 25 for VR. CABG surgical interventions were as follows: x1 CABG-0; x2 CABG-3; x3 CABG-16; x4 CABG-8; x5 CABG-1. VR surgical interventions were as follows: AVR-17; MVR-7; AVR-MVR-1.

L-Arg and ADMA levels in NCP, CABG, and VR patients

We have found no significant differences in plasma levels of L-Arg and ADMA between the NCP, and the patients undergoing open-heart surgery (L-Arg_{NCP}: 70.8±6.0 µmol/L vs. L-Arg_{CABG}: 75.7±4.6 µmol/L, p = 0.513; L-Arg_{NCP}: 70.8±6.0 µmol/L vs L-Arg_{VR}: 58.1±4.9 µmol/L, p = 0.106; ADMA_{NCP}: 0.8±0.0 µmol/L vs. ADMA_{CABG}: 0.7±0.0 µmol/L, p = 0.144; ADMA_{NCP}: 0.8±0.0 µmol/L vs. ADMA_{VR}: 0.8±0.0 µmol/L, p = 1.707). In CABG patients, the plasma L-Arg levels were significantly higher compared to the VR patients (75.7±4.6 µmol/L vs. 58.1±4.9 µmol/L, p = 0.011), whereas there was no significant difference in pericardial fluid L-Arg levels between the CABG and the VR patients (76.9±4.4 µmol/L vs. 74.8±0.0 µmol/L, p = 0.748). VR patients exhibited significantly higher ADMA levels in PF than that of CABG group (0.9±0.0 µmol/L vs. 0.7±0.0 µmol/L, p = 0.009).

There was a significant difference in L-Arg/ADMA ratio in plasma between the NCP and CABG patients (94.2±9.5 vs. 125.4±10.7, p = 0.044), but not between NCP and VR patients (94.2±9.5 vs. 78.3±7.7, p = 0.197). Furthermore, the L-Arg/ADMA ratio both in plasma and PF was significantly higher in the CABG compared to the VR patients (in plasma: 125.4±10.7 vs. 76.1±6.6, p = 0.004, in PF: 110.4±7.2 vs. 81.7±4.8, p = 0.009).

Correlation between the levels of L-Arg and ADMA in plasma and PF

In NCP, there was no significant correlation between the levels of L-Arg and ADMA in plasma. However, we found positive significant correlation between levels of plasma L-Arg and ADMA in CABG patients, and between PF L-Arg and ADMA in both CABG and VR patients. Furthermore, we found correlation between L-Arg levels of plasma and PF in CABG patients, and ADMA levels of plasma and PF in VR patients. However, we did not find correlation neither between the pl L-Arg and PF ADMA, nor between the PF L-Arg and plasma ADMA in CABG and in VR group, respectively.

Echocardiographic parameters of CABG and VR patients

We found that the thickness of interventricular septum (IVS), posterior wall of left ventricle (PW) and also right ventricular (RV), and right atrial (RA) and left atrial (LA) areas were significantly greater in VR group than that of CABG group (**Fig 1A and B**). Also, statistically significantly greater LVM was found in VR group compared to CABG group (**Fig 1C**), whereas left ventricular ejection fraction (LVEF) did not show significant difference between the two groups.

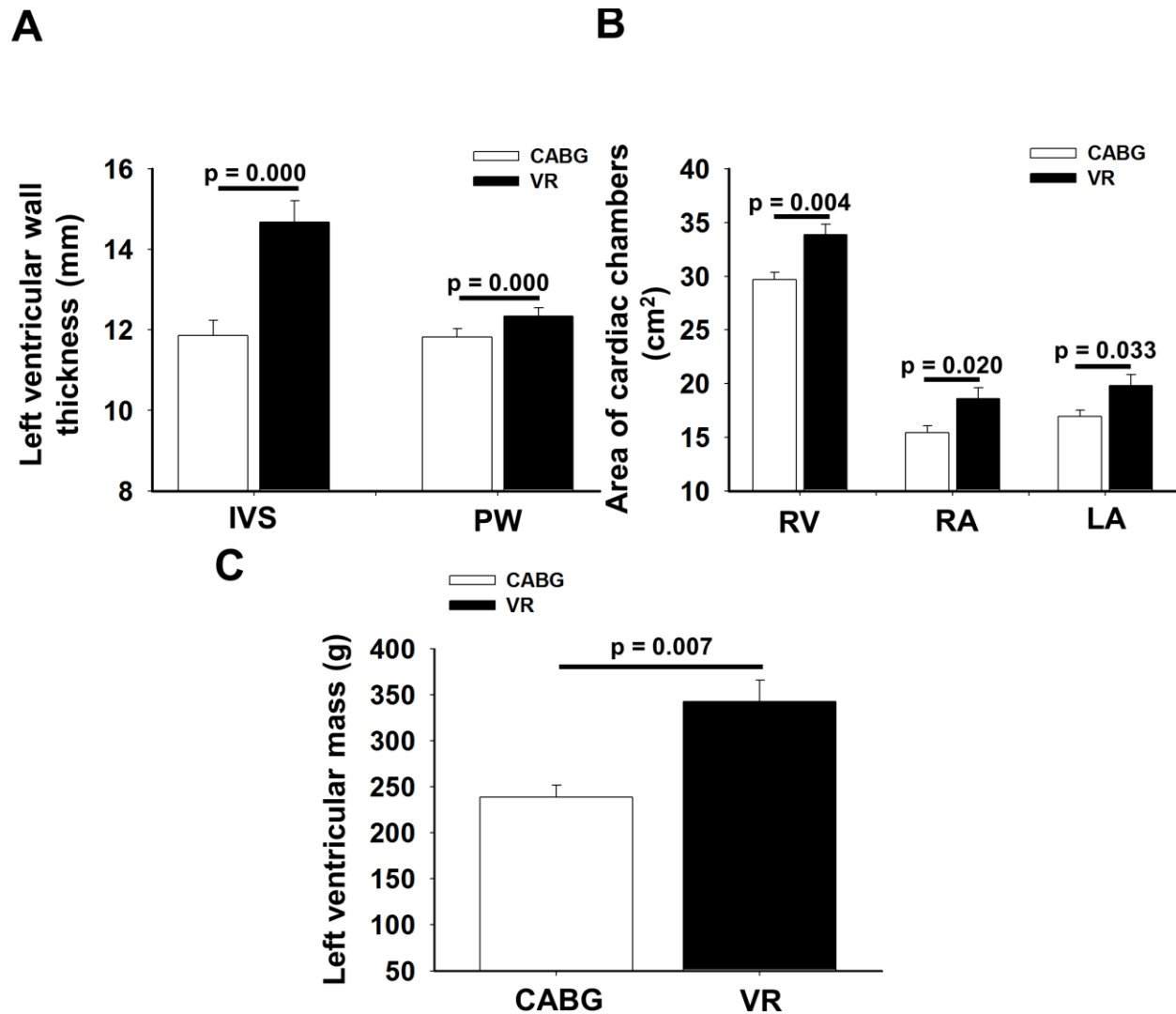


Figure 1. Morphological parameters of ventricles and atria of patients undergoing coronary artery bypass graft (CABG, n=28) or valve replacement (VR, n=25) surgery. (A) The thickness of interventricular septum (IVS) and posterior wall (PW), (B) the right ventricular (RV), the right atrial (RA) and the left atrial (LA) areas and (C) the left ventricular mass significantly higher in VR compared to CABG. Mean±SEM. $p < 0.05$.

Correlation between the levels of ADMA and echocardiographic parameters

We have found positive correlation between the ADMA levels of plasma and RV area ($r = 0.453$, $p = 0.011$; **Fig 2A**), PF ADMA and Ds of LV ($r = 0.487$, $p = 0.007$; **Fig 2B**), and Dd of LV ($r = 0.434$, $p = 0.015$; **Fig 2C**) in VR patients. Furthermore, we found negative correlation between ADMA levels of pericardial fluid and LVEF in VR patients ($r = -0.445$, $p = 0.013$; **Fig 2D**), but not in CABG patients. However, we did not find correlations between ADMA levels of plasma and pericardial fluid with other echocardiographic parameters, neither in CABG nor in VR patients.

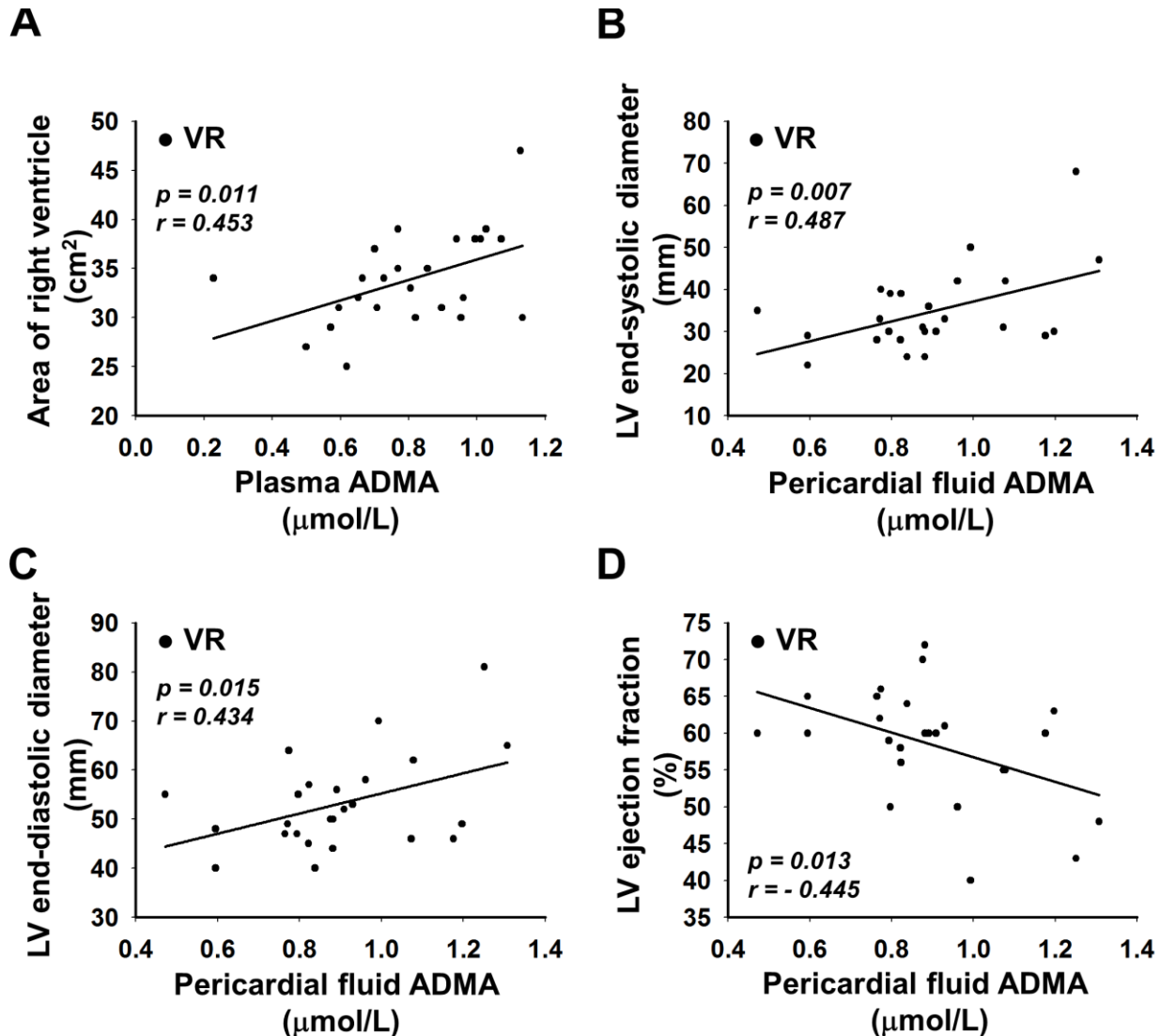


Figure 2. Correlations between the levels of asymmetric dimethylarginine (ADMA) and echocardiographic parameters of patients undergoing valve replacement (VR) surgery. (A) plasma ADMA vs. area of right ventricle ($y = 10.438x + 25.49$, $r = 0.453$, $p = 0.011$); (B) PF ADMA vs. left ventricular (LV) end-systolic diameter ($y = 23.689x + 13.53$, $r = 0.487$, $p = 0.007$); (C) PF ADMA vs. LV end-diastolic diameter ($y = 20.531x + 34.72$, $r = 0.434$, $p = 0.015$); (D) PF ADMA vs. LV ejection fraction ($y = -16.779x + 73.55$, $r = -0.445$, $p = 0.013$).

Vasomotor effect of the pericardial fluid

Clinical characteristics of patients

For these experiments pericardial fluid of 21 patients undergoing coronary artery bypass graft (CABG, $n=14$) or valve replacement (VR, $n=7$) were used. In VR group, 4 patients were undergoing mitral VR, and 3 patients were undergoing aortic VR surgery.

According to the classification of angina pectoris by the Canadian Cardiovascular Society (CCS) 1 patient exhibited mild or moderate angina (class 1 or 2), 5 patients exhibited moderate angina (class 2), 4 patients exhibited moderate or severe angina (class 2-3), and 4 patients had severe angina (class 3) before surgery.

ET-1 induced vasomotor responses before and after BQ123 adding

We have found that ET-1 elicited increases in isometric force of isolated arteries, which was significantly ($p < 0.05$) reduced following BQ123 (10^{-6} mol/L) adding (before BQ123: 5.5 ± 0.3 mN vs. after BQ123: 1.0 ± 0.4 mN).

Vasomotor responses induced by human PFs

Data in **Fig. 3A** show that PF elicited increases of up to 2.2 mN in the isometric force of isolated arteries. Summary data show that the PF of both the PF_{CABG} and PF_{VR} significantly increased the isometric force of isolated arteries (PF_{CABG}, 3.1 ± 0.7 mN; PF_{VR}, 3.0 ± 0.9 mN) (**Fig. 3B**). There was no significant difference between the isometric forces induced by PF_{CABG} and PF_{VR} ($p > 0.05$). The isometric forces produced by PF_{CABG} and PF_{VR} were significantly less ($p < 0.05$) than that of 60 mM/L KCl (PF_{CABG}, 3.1 ± 0.7 mN vs. before KCl, 6.1 ± 0.2 mN; PF_{VR}, 3.0 ± 0.9 mN vs. before KCl, 6.0 ± 0.1 mN).

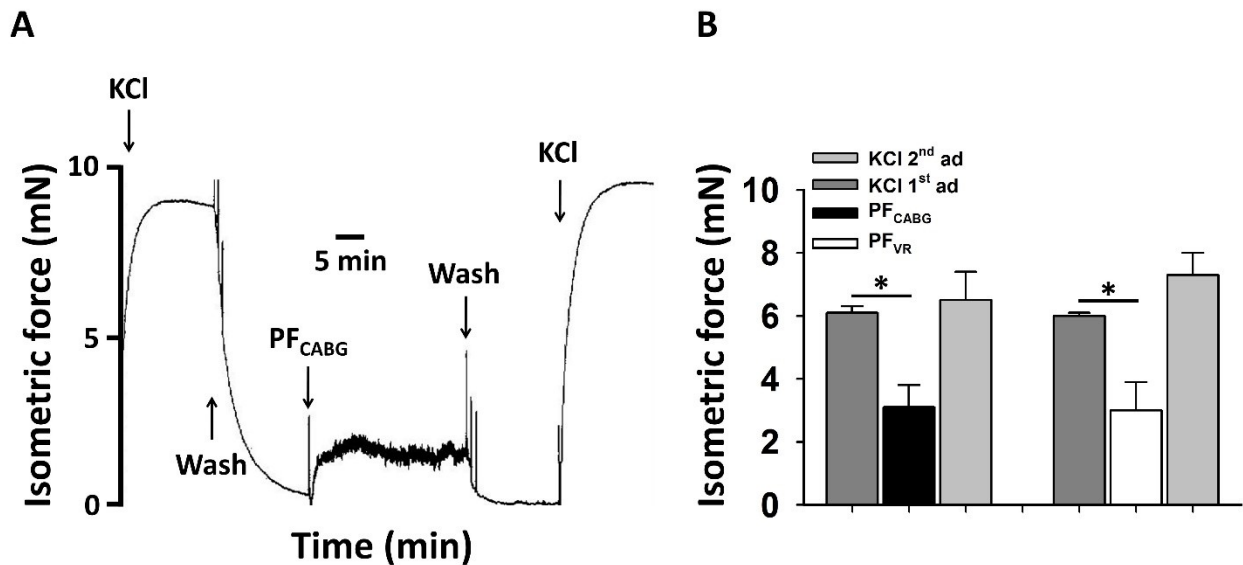


Figure 3. Vasomotor responses of rat carotid arteries to KCl (60 mM/L) and the pericardial fluid (PF) from patients undergoing coronary artery bypass graft (CABG; $n = 9$) or valve replacement (VR; $n = 7$) surgery. (A) Data showing that PF_{CABG} increases the isometric force of an isolated artery before and after the addition of KCl (60 mM/L). (B) The summary data show that PF_{CABG} and PF_{VR} increase the isometric forces of isolated arteries ($n = 16$) before and after the addition of KCl (40 mM/L). Values are the mean \pm SEM; *, $p < 0.05$ comparing the effects of KCl with PF_{CABG} and PF_{VR}.

PF_{CABG} induced vasomotor responses with BQ123

We have found that the addition of KCl also elicited increases in the isometric forces of isolated arteries (5.4 ± 0.5 mN), and following washout of the KCl, PF_{CABG} also significantly increased the isometric force (2.6 ± 0.5 mN) of isolated arteries. Following the addition of, and incubation with BQ123 (10^{-6} mol/L), PF_{CABG} elicited significant reductions in isometric force (0.8 ± 0.1 mN). The second addition of KCl also increased the isometric force (5.8 ± 0.6 mN). There was a significant ($p < 0.05$) difference between the isometric force induced by PF_{CABG} before and after the addition of BQ123 (before BQ123, 2.6 ± 0.5 mN vs. after BQ123, 0.8 ± 0.1 mN).

DISCUSSION

There were two salient findings of these investigations: **1)** Levels of methylated derivative of L-Arg, asymmetric dimethylarginine (ADMA) in the pericardial fluid of cardiac patients correlate with the magnitude of cardiac hypertrophy/remodeling; **2)** Pericardial fluids of cardiac patients elicit constrictions of isolated arteries, which is likely due to the presence of ET-1 in the PF.

Human pericardial fluid contains bioactive molecules and substances

Previous studies have demonstrated that human PF contains bioactive molecules and substances, such as ions, gases, and proteins, vasoactive substances and metabolites^{2, 11}. Also, it has been reported that the level of these substances varies in different cardiac diseases¹⁵. Furthermore, it has been revealed that in cardiac patients, certain bioactive substances, such as ET 1 present in higher concentration in PF compared to the plasma¹¹.

ADMA in the pericardial fluid

Recently, a signaling molecule, and false substrate for NOS, ADMA, which is a methylated derivative of L-Arg produced by PRMT1 and degraded by enzyme DDAH has gained attention. ADMA has been noted as a cardiovascular risk factor due to its increased plasma levels in several cardiovascular diseases⁶. Furthermore, it has been demonstrated that ADMA impairs NO-mediated arterial function partially by direct inhibition of endothelial NO synthase (eNOS) and by reducing bioavailability of NO by reactive oxygen species (ROS) due to activation of vascular renin-angiotensin system (RAS)⁵.

Human PF contains a high level of ADMA

In the present study, we have found that PF of patients undergoing CABG and VR contains L-Arg and ADMA. There are studies reporting values between 50 and 100 μ mol/L for L-Arg, and 0.3-0.8 μ mol/L for ADMA in humans²¹. The values of the plasma levels of L-Arg, and ADMA of NCP obtained in this study fell into this range. Because, PF of healthy people has not yet been investigated, therefore there are no exact reference values available for concentrations of L-Arg and ADMA in PF in healthy individuals.

Importantly, the level of ADMA has been found to be altered in various cardiovascular diseases⁶. We have found that both plasma and PF levels of L-Arg was about 100 fold higher than that of ADMA in both CABG and VR patients. In addition, we have found that in both CABG patients and VR patients the plasma level of ADMA was under or above the upper limit of the normal - healthy - range²¹. In VR patients, we found that the levels of PF ADMA were significantly higher as compared to the CABG patients.

ADMA in PF and left ventricular hypertrophy/remodeling

Left ventricular hypertrophy is resulted by interaction between a chronic hemodynamic overload and non-hemodynamic factors²². In the present study, the majority of VR patients suffered from aortic stenosis, which caused significant chronic pressure overload of the left ventricle²³. We found, that echocardiographic parameters, which are characteristics of left ventricular hypertrophy increased significantly in VR patients compared to CABG patients (**Fig 1A and C**). In the VR patients, areas of LA, RA and parameter of RV area exhibited significant increase in comparison of CABG patients (**Fig 1B**).

There are several lines of evidence presented in previous decades suggesting that presence of adequate level of NO limits the hypertrophic growth of the myocardium²⁴. One of the mechanisms that may explain the association between ADMA and cardiovascular disease is the ADMA-induced cardiac hypertrophy. Several alternative mechanisms have also been proposed to explain the association between ADMA and cardiac hypertrophy⁷. It has been demonstrated that ADMA can activate receptors for fibroblast growth factors in cardiomyocytes, thus leading to myocardial hypertrophy and fibrosis, or induce excessive local activation of the renin-angiotensin-aldosterone pathway²⁵. NO and normal NOS activity are essential for the prevention of heart remodeling, therefore decreased NO availability may lead to a loss of such protection.

In **Fig 4**, we have summarized the potential mechanism of action of ADMA in modulation cardiac morphology. We recently proposed a potential mechanism by which increased serum ADMA reduces the bioavailability of NO²⁶. We have also shown that elevated levels of ADMA activate the renin-angiotensin system in the arteriolar wall leading to increased production of Ang II, which then activates NAD(P)H oxidase leading to increased levels of reactive oxygen species, which interferes with the bioavailability of NO⁵. The activation of RAS increases the level of Ang II, which is known to be a growth hormone²⁷. These observations are in concordance with previous studies and suggest that reduced level of NO²⁸ and increase activation of RAS²⁹ together promote cardiac hypertrophy. Elevated level of ADMA in the pericardial fluid of patients in the VR patients correlates with left ventricular remodeling/hypertrophy and thus it can serve as a biomarker (**Fig 4**).

Role of ADMA in cardiac remodeling by attenuation of myocyte proliferation

Based on present and previous findings, we suggest that elevated levels of ADMA in the pericardial fluid of cardiac patients could indicate important pathophysiological mechanisms, such as absolute or relative cardiac ischemia and hypoxia leading to reduced bioavailability of NO, which – together with the locally released growth hormone Ang II - can contribute to the development of cardiac hypertrophy and remodeling (**Fig 4**). We propose that analyzing of pericardial fluid could be a valuable diagnostic tool, whereas interfering with the contents and effects of pericardial fluid open up new therapeutic options to beneficially modify cardiac function and structure.

Vasomotor effect of the pericardial fluid

In the present study we hypothesized that pericardial fluid of patients with cardiac diseases will increase the isometric tone of isolated rat arteries. For bioassay, we have used isolated carotid arteries of rats, because several papers demonstrated that carotid arteries mirror the events take place in coronary vessels of humans^{30-33 34-36}.

Pericardial fluid of humans elicits contraction of isolated arteries

We have found that PF of CABG and VR patients elicited substantial increase in isometric tone of isolated rat carotid arteries (**Fig 3**). The characteristics and potency of the responses of carotid

arteries to PF were different from those induced by KCl (**Fig 3**). Response curves induced by KCl were sigmoidal, whereas those induced by PF had an upswing slope and a maintained plateau phase (**Fig 3**).

The finding that the plateau phase of PF-induced response was maintained, suggest that the PF contains a constrictor agent(s) that effect is the long lasting. Earlier, Clarke et al found that ET-1 has a long lasting constrictor effect, which let us hypothesize that the main constrictor agent in human PF is endothelin³⁷. In this vascular preparation 60 mM KCl and 10^{-8} M NE elicit close to maximal contractions. In comparison, the maximum response produced by PF was less, but still substantial, suggesting major pathophysiological importance for PF-derived endothelin in modulation of diameter of surface coronary vessels. We have found no difference between arterial contractions induced by PF_{CABG} and PF_{VR}, suggesting the presence of common mechanism for the elevation of endothelin, in the PF of patients undergoing CABG or VR.

Increasing data suggest that vasoactive agents are implicated in both coronary artery disease and valve disease³⁸⁻⁴⁰ and the present data support potential underlying mechanisms contributing the vasoconstrictor effect.

Role of endothelin in the pericardial fluid induced isometric tone of isolated vessels

ET-1 is a potent vasoconstrictor peptide, which is involved in the development of endothelial dysfunction among others, via interacting with NO and eliciting cardiac dysfunction¹⁷. Several studies have reported that endothelin(s) play an important role in the development of cardiovascular diseases¹⁷. Moreover, ET-1 has been shown to be present in high concentrations in PF of patients undergoing cardiac surgery³, suggesting a potential regulatory role of ET-1 - not only in systemic blood circulation - but in coronary circulation and cardiac function, as well.

Previous and present data show that ET-1 elicits increases in isometric forces of isolated arteries, which is inhibited by the presence of a selective ET_A receptor antagonist BQ123¹⁷. In the present study we found that arterial contraction elicited by PF_{CABG} was significantly reduced by BQ123, indicating that the observed vasoconstrictor effect of PF_{CABG} is mainly due to the elevated concentration of ET-1, which acts particularly through ET_A receptor^{11, 32}.

Importantly, it is likely that these patients undergone several ischemic and ischemic/reperfusion period before CABG or VR surgery as shown in previous studies⁴¹. ET-1 has been suggested to be one of the mediators of ischemia/reperfusion injury⁴¹. Thus, it is logical to suggest that the vasomotor level of ET1 in PF of both groups of patients developed due to the frequent hypoxic periods. Based on the above findings and our present data we propose a novel pathway of regulation coronary circulation both in physiological and pathophysiological conditions via the vasomotor substances in the in the PF. Because vasoactive substances produced or secreted into the PF can freely diffuse in the pericardial fluid inside the pericardial sac, PF can serve as a medium for transporting molecules to various places of heart surface.

Thus - we suggest - pericardial fluid is involved in the regulation coronary blood flow and perhaps regulating other function of the heart providing a new therapeutic potential to treat cardiac diseases via the pericardial sac, a third circulatory pathway. Indeed, results of previous studies have shown that intrapericardial administration of substances are able to effect the function of the heart¹⁸ raising the possibility to modulate cardiac function and improve the coronary blood flow by intrapericardial administration of drugs. Furthermore, analysis of the pericardial fluid can provide biomarkers helping to diagnose cardiac diseases, such as pericarditis, cardiac hypertrophy, and ischemia.

SUMMARY AND NOVEL FINDINGS

ADMA in the pericardial fluid:

- 1) L-Arg and its methylated derivative ADMA are present in the PF_{CABG} and PF_{VR} patients,
- 2) In PF_{CABG} patients, plasma L-Arg concentration was higher compared to that of PF_{VR} patients, whereas in PF_{VR} patients, PF ADMA concentration was higher compared to that of PF_{CABG} patients,
- 3) We have found positive correlation between plasma L-Arg and ADMA levels in PF_{CABG} patients, between PF L-Arg and ADMA levels in both PF_{CABG} and PF_{VR} patients, between plasma L-Arg and PF L-Arg levels in PF_{CABG} patients, and between plasma and PF ADMA in PF_{VR} patients.
- 4) The L-Arg/ADMA ratio was lower in the PF and plasma of PF_{VR} than in PF_{CABG} patients.
- 5) We have found positive correlation between plasma ADMA levels and area of right ventricle, between PF ADMA levels and end-systolic, and end-diastolic diameter of the left ventricle, and negative correlation between PF ADMA levels and left ventricular ejection fraction in PF_{VR} patients.

Vasoconstrictor effect of pericardial fluid:

- 6) PF of patients with cardiac diseases increased the isometric tone of isolated rat common carotid arteries;
- 7) The magnitude of isometric tone induced by PF of PF_{CABG} and PF_{VR} patients were not different,
- 8) The arterial contraction elicited by PFs were significantly reduced by the selective ET_A receptor antagonist BQ123.

CONCLUSIONS

In this study we investigated the “non-mechanical” function of the pericardial fluid (PF). Accordingly we showed that - via signaling molecules (ADMA, endothelin) - the PF has important paracrine effects: influencing cardiac remodeling and vasomotor tone and thereby cardiac function and coronary circulation. Thus measuring of biologically active substances in PF, and investigation of vasomotor effect of PF may provide new approaches in the investigation of cardiac physiology, pharmacology, and therapy. **Because pericardial fluid can freely move around the surface of the heart it can reach both cardiac tissues and coronaries thus we suggest that pericardial fluid provides a “third pathway” for modulating cardiac homeostasis and coronary circulation.**

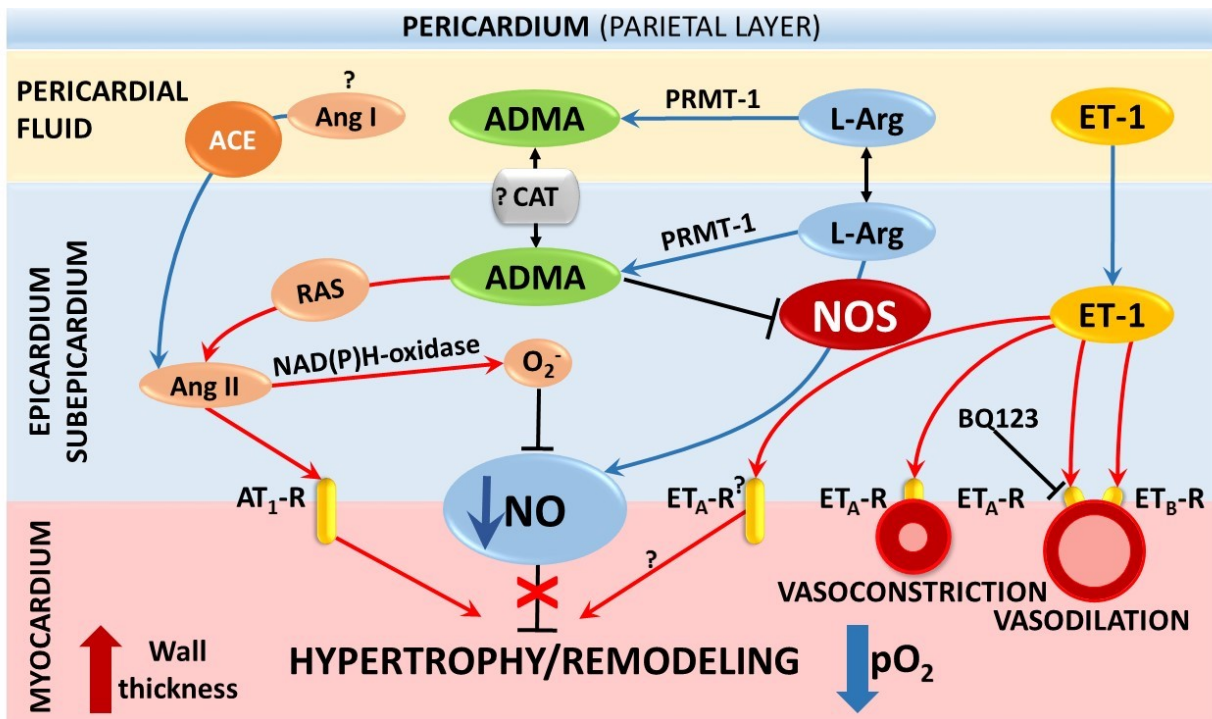


Figure 11. Proposed mechanisms by which bioactive substances of the pericardial fluid regulate cardiac tissues. Elevated level of ADMA of pericardial fluid elicits hypertrophy/remodeling of cardiac muscle: accordingly, reduced NO bioavailability and increased level of Ang II together leads to development of cardiac hypertrophy/remodeling. ET-1 of pericardial fluid elicits vasoconstriction of coronary vessels through ET_A-receptor and vasodilation through ET_B-receptor. Adding of ET_A-receptor blocker BQ123 causes vasodilation. ADMA – asymmetric dimethylarginine, ET-1 - endothelin-1, L-Arg - L-arginine, NO – nitric oxide, NOS – NO synthase, RAS – renin-angiotensin-system, Ang I - angiotensin I, ACE – angiotensin converting enzyme, Ang II – angiotensin II, ROS – reactive oxygen species, O_2^- - superoxide anion, PRMT-1 – protein methyltransferase-1, AT₁-R – Angiotensin II receptor, ET_A-R and ET_B-R – endothelin-1 receptors, CAT – cationic amino acid transporter, pO_2 – partial pressure of oxygen

PEER-REVIEWED PUBLICATIONS OF THE AUTHOR (IF: 5.0)

The thesis is based on the following publications:

1. **Nemeth Z**, Cziraki A, Szabados S, Biri B, Keki S, Koller A. Elevated Levels of Asymmetric Dimethylarginine (ADMA) in the Pericardial Fluid of Cardiac Patients correlate with Cardiac Hypertrophy. PLoS ONE. 10(8):e0135498. DOI: 10.1371/journal.pone.0135498. 2015 (**IF:3.23**)
2. **Nemeth Z**, Cziraki A, Szabados S, Horvath I, Koller A. Pericardial fluid of cardiac patients elicits arterial constriction: role of endothelin-1. Can J Physiol Pharmacol. 93(9):779-85. DOI: 10.1139/cjpp-2015-0030. 2015 (**IF: 1.770**)

Other publications:

3. Seffer I, **Nemeth Z**, Hoffmann Gy, Matics R, Seffer A. G, Koller A. Unexplored potentials of epigenetic mechanisms of plants and animals - Theoretical considerations. Genet Epigenet. 5:23-41. DOI: 10.4137/GEG.S11752. 2013.

Abstracts in peer-reviewed journals:

1. Koller A, **Nemeth Z**, Szabados S, Biri, Sandor Keki, Attila Cziraki. Asymmetric Dimethylarginine (ADMA) in the Pericardial Fluid may contribute to the Development of Cardiac Hypertrophy. Eur Heart J 36(Suppl 1):849-1187 DOI: <http://dx.doi.org/10.1093/eurheartj/ehv401>. 2015.
2. **Nemeth Z**, Cziraki A, Szabados S, Horvath I, Koller A. Potential role of endothelin-1 in pericardial fluid of cardiac patients in eliciting arterial constriction. J Vasc Res 52(Suppl 1):1-88 DOI:10.1159/000433498. 2015.
3. Nemeth A, Ajtay Z, Husznai R, **Nemeth Z**, Horvath I, Szokodi I, Szabados S, Koller A, Bode-Boger SM, Cziraki A. Investigation of asymmetric dimethylarginine in patients with coronary artery disease. Cardiologia Croatica 9:(5-6) p. 256. 2014.
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5. Cziraki A, Ajtay Z, Sulyok E, Horvath I, Nemeth A, Lenkey Z, **Nemeth Z**, Szabados S, Koller A, Bode-Boger SM. Investigation of asymmetric dimethylarginine in patients with coronary artery disease. Eur Heart J 34:(Suppl.) p. 563. 2013.
6. Koller A, Cziraki A, Szabados S, Biri B, Keki S, Seffer I, **Nemeth Z**. L-arginine and asymmetric dimethylarginine (ADMA) levels in pericardial fluid in patients undergoing open heart surgery. Eur Heart J DOI: <http://dx.doi.org/10.1093/eurheartj/eh309>. P3895. 2013.
7. **Nemeth Z**, Cziraki A, Keki S, Biri B, Szabados S, Horvath I, Parniczky A, Seffer I, Miseta A, Koller A. L-arginine and asymmetric dimethyl arginine (ADMA) levels in human pericardial fluid in patients with open cardiac surgery. Proceedings of The Physiological Society. Proc 37th IUPS, PCB072. 2013.

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