Investigation of Biochemical Composition and Vasomotor Effect of Human Pericardial Fluid

Doctoral (Ph.D.) dissertation

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To the memory of my Father

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ABBREVIATIONS

ACE 2	angiotensin converting enzyme 2
ADMA	asymmetric dimethylarginine
AMI	acute myocardial infarction
Ang II	angiotensin II
ANP	atrial natriuretic peptide
ASE	American Society of Echocardiography
AT-1	Ang II type 1 receptor
AVR	aortic valve replacement
bFGF	basic fibroblast growth factor
BNP	brain natriuretic peptide
BQ123	selective ET_A endothelin receptor antagonist
CABG	coronary artery bypass graft
CAD	coronary artery disease
CSCs	cardiac stem cells
Dd	left ventricular end-diastolic diameter
DDAH	dimethylarginine dimethylaminohidrolase
Ds	left ventricular end-systolic diameter
eGFR	estimated glomerular filtration rate
eNOS	endothelial NO synthase
EP	epinephrine
ET-1	endothelin-1
ET_A	ET-1 receptor

HA	hyaluronic acid
IGF-1	insulin like growth factor-1
IL	interleukin
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
NCPs	non-cardiac patients
NE	norepinephrine
NO	nitric oxide
PCI	percutaneous coronary intervention
PF	pericardial fluid
PRMT-1	protein methyltransferase-1
PW	thickness of posterior wall
RA	right atrial area
RAS	renin-angiotensin system
RV	right ventricular area
TNF-α	tumor necrosis factor-α
VEGF	vascular endothelial growth factor
VR	valve replacement

INTRODUCTION

1. The pericardial fluid

The pericardial fluid (PF) is an approximately 15-50 ml viscous, pale yellow film layer placed between the layers of the pericardium [1]. The primary function of PF is to ensure a proper friction between the pericardium and the heart [1]. The pericardium and the pericardial fluid compose a functional unite, thus before introduction of pericardial fluid, it is important to describe the pericardium.

Pericardium surrounds the heart and the root of the large vessels. It is composed of the fibrous pericardium and serous pericardium. The fibrous pericardium is a dens fibrous layer blending with the adventitia of the roots of the large vessels and the central tendon of the diaphragm. The serous pericardium consists of two layers: the parietal layer, which lines the inner surface of the fibrous pericardium, and the visceral layer, which is the outer layer of the heart wall (epicardium) and the roots of the large vessels. The space between the epicardium and parietal layer of the serous pericardium is called pericardial cavity [2].

Pericardium is composed of dense connective tissue and the pericardial cavity is lined on either side by mesothelial cells. The mesothelial cells contain numerous microvilli playing a role in the distribution of the PF and gliding the two mesothelial surfaces during heartbeat, and facilitating PF and ion exchange [3, 4].

Most knowledge regarding physiological role of intact PF is derived from animal experimental data, because intact human PF from healthy individuals - obviously - cannot be harvested. Thus most of the data available derives from human pericardial effusions of patients undergoing open heart surgery.

2. Physiological role and composition of the pericardial fluid

Until the 1970's the widely accepted description of the physiological role of the pericardial fluid was that it reduces the friction between the pericardium and the surface of the heart. In the following decades, increasing number of data regarding its composition and subsequently supporting other ideas were published. It is important to note that many Hungarian researchers, amongst them Alexander Juhasz-Nagy A, Ferenc Horkay, Bela Merkely, Violetta Kekesi, and Istvan Szokodi worked and published on this field [5, 6]. Their findings opened new aspects for better understanding the function(s) of the pericardial fluid.

2.1. Mechanical role of the pericardial fluid

Mechanical functions of the PF relate to its viscous nature and that it is situated in a sub-atmospheric pressure condition. PF - due to its viscous characteristics - reduces the friction between the surface of the heart and the pericardium, thereby ensuring the smooth movement of the heart during every beat [1].

PF is placed in the pericardial cavity at subatmospheric pressure, which is approximately equal to the pleural pressure. Pericardial pressure affects the myocardial transmural pressure by modulating to the chamber distending (filling) pressure [4].

Cardiac tamponade also known as pericardial tamponade is an adverse mechanical effect of the PF. Cardiac tamponade is caused by the pathologic accumulation of pericardial fluid (pericardial effusion) in the pericardial cavity [7, 8]. Cardiac tamponade increases the intrapericardial pressure, which - due to the stiffness of the fibrous pericardium - compresses first the ventricles, and then increases the diastolic pressure and can limit ventricular filling, resulting increasing in atrial pressures. These hydro-hemodynamic changes can lead to increases in systemic and pulmonary venous pressures, consequent tachycardia, reduced ejection fraction and can even reduce coronary blood flow [4].

2.2. Composition of the pericardial fluid

In the ancient times the pericardium was believed to function solely as a sheath to protect the heart, and was observed that it contains a small amount of fluid, which resembles urine [9]. There are few data available regarding the origin and composition of the pericardial fluid, and even less is known regarding to the normal (healthy) composition because of the obvious limitation of harvesting of PF from non-diseased cardiac patients. Gibson et al have suggested that PF is produced both by active (pericardial cells) and passive (heart and blood tissue filtrate) mechanisms [10].

Honda et al found that PF of rabbits shows non-Newtonian flow behavior and has a lower viscosity (1.03-1.04 cP at 37 °C.) than that of blood plasma [11]. Also, it has been shown that viscosity of PF is the result of its high-molecular-weight hyaluronic acid (HA) content [11]. Furthermore, it has been revealed that PF contains IGF-1, which is an inductor of HA synthesis in pericardium or pericardial sac [12]. These molecular data reveal the viscous characteristic of PF.

Regarding the pH of PF, data are showing high variances, depending on the type of cardiac disease. Kindiq et al. in consecutive studies aimed at determining the pH of PF of patients with various pericardial diseases, found that it of ranged from 6.82 to 7.59. Also, he distinguished “inflamed” and “non-inflamed” PF (pH: 7.06 +/- 0.07 vs. 7.42 +/- 0.06) [13]. Furthermore, in dogs, it was found that values of pH of pericardial effusion were different in neoplastic vs. idiopathic pericarditis (7.85 vs. 6.40), however the median data were not different between the two groups [14].

2.2.1. Electrolyte and acid-base composition of the pericardial fluid

PF consists of several ions, gases, proteins which concentrations in most cases reflect the blood plasma [10, 15, 16]. First, Hutchin et al. (1971) investigated the electrolyte and acid-base composition of the human pericardial fluid enrolling 11 patients undergoing open-heart surgery [16]. They found no significant difference between the plasma and pericardial fluid ion concentration of Na^+, K^+, and Cl^-; however, Ca^{2+} and phosphorus were lower in PF as compared to plasma. The concentration of bicarbonate was higher in PF than in plasma. In addition, the pH of PF was found significantly higher as compared to plasma. They found that pCO_2, and buffer base, and total protein are significantly lower in PF than in plasma [16].

Gibson et al (1978) measured the composition of PF and simultaneously harvested plasma in rabbits and greyhounds [10]. They obtained similar findings as group of Hutchin regarding sodium and chloride. In addition, calcium and magnesium were similar in PF and plasma. The potassium concentration of PF was higher than the plasma concentration. The latter was evaluated by the lability of the cardiac intracellular potassium during systole. Other substances, such as proteins were found in a much smaller concentration in PF as compared to plasma; however, albumin was found in higher concentration as compared to other protein constituents. The osmolality of PF was found to be smaller than that of plasma [10]. These facts were elucidated with the hydrostatic pressure difference and osmotic concentration gradient between the plasma and pericardial fluid. Accordingly, these data suggest that PF is produced from the blood plasma by passive filtration forces, rather than by active transport systems.

Ben-Horin et al provided relatively detailed information regarding the composition of PF [15]. In this study 30 patients undergoing coronary artery bypass graft (CABG), or valve replacement (VR, or combined surgery were enrolled. They harvested both PF and blood plasma samples from the patients and found that the concentrations of small molecules, such

as urea, glucose, creatinine, and electrolytes were the same in both PF and plasma, confirming further the idea that the PF is an ultrafiltrate of blood plasma (**Table 1A and B**).

Table 1 Pericardial fluid composition compared to plasma composition of patients undergoing cardiac surgery

A: Electrolyte composition of the pericardial fluid compared to plasma

	PF mean value±SD	**Statistical differences from plasma**
Urea nitrogen (mg/100ml)	17±8	NS
Na (mEq/L)	138±4	NS
K (mEq/L)	5.0±1.0	NS
Cl (mEq/L)	109±5	NS
Ca (mg/100ml)	7.4±0.5	Sign. lower in PF
Phosphorus (mg/100ml)	3.7±1.0	Sign. lower in PF
Bicarbonate (mEq/L)	21.7±2.2	Sign. higher in PF

B: Metabolite and enzymatic composition of the pericardial fluid compared to plasma

	Pericardial fluid mean	Pericardial fluid: serum ratio mean
Total protein (g/dL)	3.3	0.6
Albumin (g/dL)	2.4	0.7
LDH (IU/L)	398	2.4
Glucose (mg/dL)	133	1.0
Urea (mg/dL)	33	1.0
Creatinine (mg/dL)	0.9	0.9
Calcium (mg/dL)	7.3	0.85
Total bilirubin (mg/dL)	0.6	1.4
AST (IU/L)	28	1.0
ALKP (IU/L)	13	0.2
Cholesterol (mg/dL)	43	0.3
Phosphorus (mg/dL)	2.6	0.7
Triglycerides (mg/dL)	34	0.3
Uric acid (mg/dL)	5.6	1.0
Amylase (IU/L)	56	0.4
White Blood Cells (K/µL)	1.4	0.2
Lymphocytes (%)	53	5.3
PMN (%)	31	0.4
Monocytes (%)	12	2.1
Eosinophils (%)	1.7	2.1
Basophils (%)	1.2	3.8

Based on the study of Hutchin and Ben-Horin [15, 16].

2.2.2. Bioactive molecules and metabolites in the pericardial fluid

Bioactive molecules and metabolites in PF are endothelins [5, 17], catecholamines [18], adenine nucleotides [19, 20], natriuretic peptides [21-23], angiotensin II [24], prostaglandins [25], cytokines [26] and growth factors [27]. It has been shown that level of certain substances increase in PF according to type of cardiac disease [17, 19, 20, 23, 28]. For instance, the pericardial level of ANP was higher of those who had left ventricle dysfunction, while BNP was higher of those who had left ventricular dilation [23, 29]. In addition, markedly elevated levels of adenine nucleotides and endothelin have been shown in ischemic heart disease [24, 30, 31]. Furthermore, an increase in intrapericardial endothelin-1 (ET-1) level has been reported in patients with ischemic heart disease [24]. Moreover, group of Alexander Juhasz-Nagy using dog heart found, that the intrapericardial administration of adenine nucleotides increased the pericardial level of endothelin, and vice versa [32, 33].

A methylated derivative of amino acid L-arginine (L-Arg) asymmetric dimethylarginine (ADMA) is known to reduce the bioavailability of nitric oxide (NO) [34] thereby modulating the regulation of vascular tone. In many studies, the level of ADMA has been shown to be elevated in plasma of patients with cardiovascular diseases [35-37].

3. Signaling molecule ADMA modulates nitric oxide

3.1. L-Arg/NO pathway and the ADMA

Nitric oxide is produced by nitric oxide synthases (NOSs) from the precursor amino acid L-Arg [38]. NO is a multirole molecule, among others modulating vasomotor tone, and attenuating tissue proliferation and growth [39-41] (**Fig 1**).

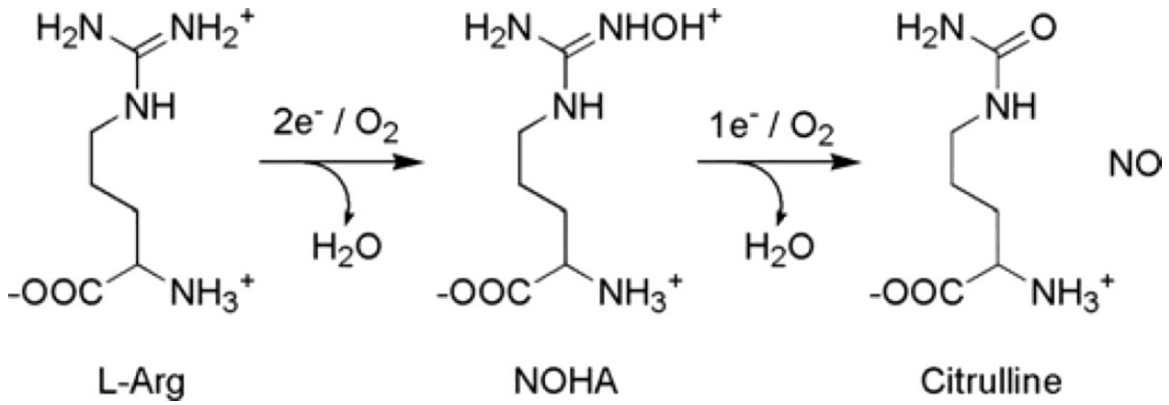

Figure 1. Simplified schema of NO synthesis. NOSs catalyze the conversion of L-Arg to N^{ω}-Hydroxy-L-Arginine (NOHA), and N^{ω}-Hydroxy-L-Arginine to L-Citrullin and NO. (Papale 2012)[42]

In addition, studies have shown that NO has anti-hypertrophic properties on cardiac muscle [43, 44]. Also, previous studies have established that ADMA, being a false substrate competitively inhibits the activity of endothelial NO synthase (eNOS) thus production of NO [45, 46].

L-Arg is substrate for also the protein methyltransferase-1 (PRMT-1) catalyzing of ADMA synthesis through methylation of L-Arg [47]. ADMA is degraded by the enzyme dimethylarginine dimethylaminohidrolase (DDAH) to citrullin and dimethylamine, and

excreted by the kidney [48, 49]. In addition, we have reported that ADMA activates the vascular renin-angiotensin system (RAS) and elicits the generation of reactive oxygen species [50, 51] (**Fig 2**).

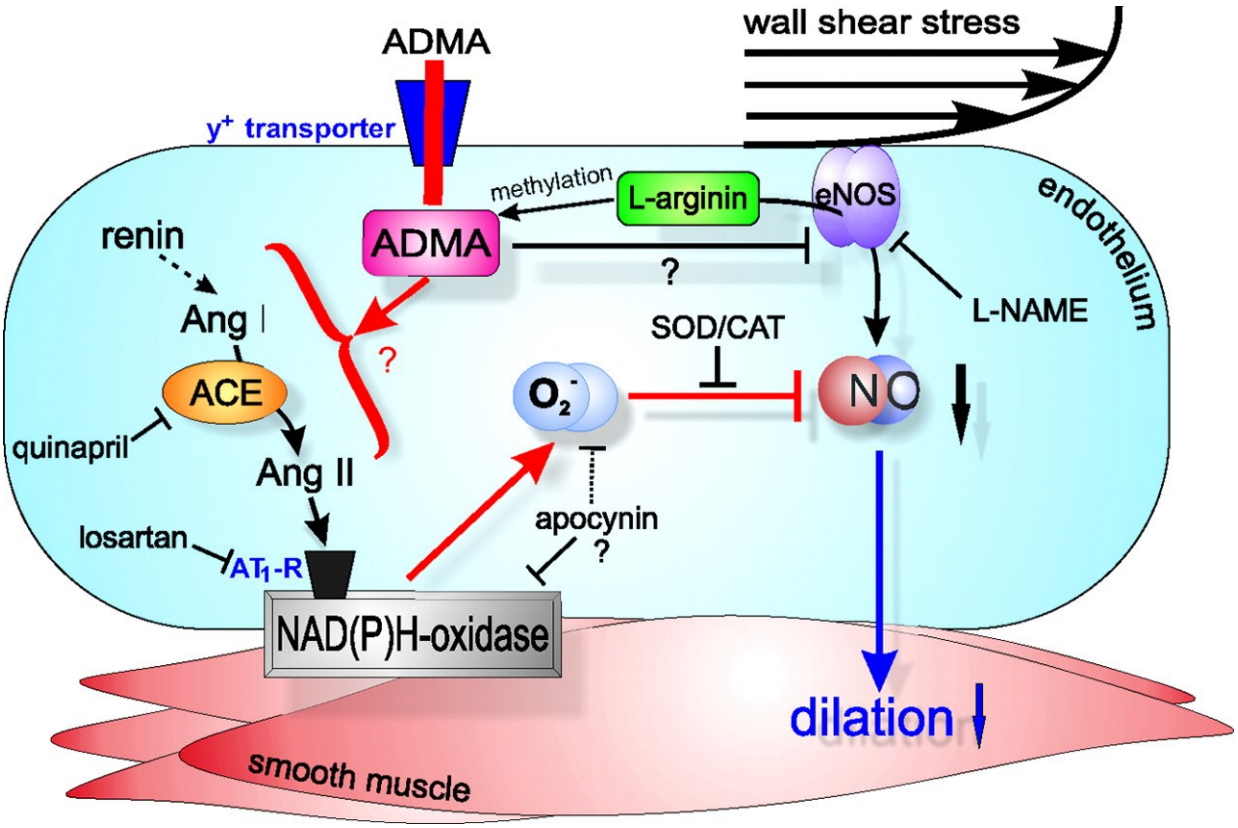

Figure 2. Proposed mechanisms by which ADMA induces enhanced oxidative stress and vasomotor dysfunction of arterioles. Elevated levels of ADMA activate the RAS in the arteriolar wall leading to increased production of angiotensin II, which then activates NAD(P)H oxidase. The consequent increased level of reactive oxygen species interferes with the bioavailability of NO released to increases in flow/shear stress, resulting in inhibition of flow-induced dilation and enhanced arteriolar tone, both of which favor the development of increased peripheral resistance. eNOS indicates endothelial NOS; O2−, superoxide; apocynin, proposed inhibitor of NAD(P)H oxidase; Ang I, angiotensin I; Ang II, angiotensin II; quinapril, ACE inhibitor; AT1-R, angiotensin type I receptor; losartan, AT1-R blocker (Veresh et al 2008) [52].

Also, elevated concentrations of ADMA in plasma have been reported in various cardiovascular diseases, such as hyperhomocysteinemia, type 2 diabetes mellitus, hypertension, hypercholesterolemia, and coronary artery disease [35, 37, 53-55]. Moreover, it has been previously established by us and others that L-Arg and ADMA are present in different concentrations in the plasma of patients with established coronary artery disease (CAD) as compared with non-CAD patients [56, 57].

These results have been further confirmed by functional clinical findings investigating relationship between serum level of ADMA and cardiac functions [58]. For example, it has been shown that adverse cardiovascular events in coronary artery disease (CAD) patients underwent percutaneous coronary intervention (PCI) were significantly associated with increased plasma ADMA levels [59].

3.2. Potential role of ADMA in the cardiac remodeling

Cardiac remodeling is initiated by altered mechanical stress, activating several cellular and molecular pathways in the cardiac tissues, including cardiac muscle, connective tissues and coronary vasculature, leading to changes in size, shape, and function of the heart [60, 61]. The process of cardiac remodeling is caused by pathophysiological/adaptive (injuries of the heart) processes, which are regulated by mechanical (wall stress) and molecular mechanisms [62-64].

Cardiac hypertrophy is a typical form of cardiac remodeling when – among others - the size of myocytes increases causing thickening of ventricular walls [64]. Although, during cardiac hypertrophy not only myocyte hypertrophy, but hyperplasia with increase in number of myocytes occur [65]. 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 infarction [66-70]. Besides mechanical forces,

locally acting factors, such as cytokines and growth factors are implicated in the development of cardiac hypertrophy [71, 72].

Several clinical studies have described that renin, angiotensin II (Ang II) and aldosterone are implicated in the development of cardiac remodeling [73]. Ang II effects on cardiac tissue through Ang II type 1 (AT1) and Ang II type 2 (AT2) receptors, and effect of Ang II related to cardiac hypertrophy can be inhibited using Ang II receptor blockers [74]. Also, it has been demonstrated that Ang-(1-7) prevented cardiac remodeling by overexpression of angiotensin converting enzyme 2 (ACE2) during chronic infusion of Ang II [75].

In summary, the processes of cardiac remodeling include restructuring and reshaping of the heart tissues [76, 77]. Recently, several signaling molecules have been found in PF that regulate cardiac remodeling, such as growth factors, microRNAs (miRNAs), and other regulatory molecules [64, 78, 79].

As mentioned above, NO is a multirole gas transmitter, which has been implicated in the regulation of cell proliferation [41]. Also, it has been mentioned that ADMA is an endogenous competitive inhibitor of NOS, and through activating of RAS, and likely NAD(P)H oxidase system involved in the generation ROS leading to reduced bioavailability of NO [50, 80]. Thus, ADMA directly or indirectly could be involved in cardiac remodeling.

4. Vasoactive substances in the pericardial fluid

As we described above, PF composes several vasoactive substances, such as endothelins [5, 17], catecholamines [18], adenine nucleotides (adenosine, inosine) [19, 20], natriuretic peptides (atrial and brain natriuretic peptides (ANP, BNP) [21-23], angiotensin II [24], and prostaglandins [25].

4.1. Endothelin-1

It is well known that endothelins are potent vasoconstrictor peptides playing an important role in regulation of vascular tone, and growth factors for many types of cells [81, 82]. Endothelins comprise three isoforms, and among them the ET-1 is the most biologically relevant [83]. ET-1 is a peptide building up by 21 amino acids, and released by vascular endothelial cells and cardiomyocytes [84, 85]. Receptors of ET-1, ET_A and ET_B are 7-transmembrane G-protein coupled receptors [86]. ET_A receptor antagonist BQ123 has been widely used to reduce vasoconstriction induced by ET-1 [87].

ET-1 has been found involving in pathogenesis of hypertension and vascular diseases [88]. Furthermore, it has been demonstrated that ET-1 causes cardiac dysfunction in animals [89]. ET-1 and its potential pathophysiologic role in PF of patients undergoing cardiac surgery were found in 1998 [90]. Also, others have shown the presence of – and characterized the function of ET-1 in PF [17, 91, 92]. 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 patients [24]. Szokodi et al. and Horkay et al. have demonstrated that intrapericardial added ET-1 induces arrhythmias with a prolonged QT time in ventricle of dog heart [92, 93]. This may confirm that substances in PF may reach, moreover effect on cardiac interstitium, thus PF could behave as paracrine material.

4.2. Catecholamines

Epinephrine (EP) and norepinephrine (NE) neurotransmitters and hormones have long been known as regulators of vascular tone [94]. Dopamine, precursor of NE exerts positive inotropic and chronotropic, and vasopressor effects in dose-dependent manner [95, 96]. However, there is little data regarding the level of pericardial catecholamine; it has been found that NE is present in rat PF, and its level approximately threefold higher in hypertensive rats than in

normotensive rat PF [18]. Also, EP, NE, and dopamine have been detected in post mortem human PF [97]. Moreover, it has been shown that intraperitoneal administered dopamine and NE elicited dose dependent increases in heart rate and significant elevation in left ventricular pressure and blood pressure in dogs [98].

4.3. Adenine nucleotides

Adenine nucleotides are importantly involved in the regulation of vascular tone and platelet function [99]. Adenosine is released by myocardial cells and elicits substantial dilations of coronary vessels mainly through A_2 adenosine receptor [100, 101]. It has been shown that level of adenosine was significantly higher in PF of patients with CAD as compared to VR patients [19]. Fazekas et al. found similar data in a comparative study using the same patients group [20].

4.4. Natriuretic peptides

Natriuretic peptides atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are cardiac hormones regulating natriuresis, diuresis and vasodilation, and inhibiting the renin-angiotensin-aldosterone system [102-104]. It has been well known for a long time that both ANP and BNP are released by atrial and ventricular myocytes into the blood during increases in cardiac wall stress [105, 106]. Also, it has been shown that they are present in pericardial fluid [23, 107] and their levels in the PF are significantly higher compared to plasma of patients with heart disease [108]. Several papers reported that elevated levels of natriuretic peptides are strongly associated with pathophysiological changes in the heart [23, 108, 109]. For example, the level of ANP in PF was higher in those who had left ventricle dysfunction, while BNP was higher in those who had left ventricular dilation [23, 29]. Also, ANP has been reported reflecting the peptide concentration of myocardium and may have a regulatory role in PF as a paracrine regulator [22].

Based on the aforementioned, natriuretic peptides seem to be novel candidates as biomarker in PF predicting pathophysiological changes in the cardiac interstitium.

4.5. Adrenomedullin

Adrenomedullin is a potent vasodilator peptide was first isolated from human phaeochromocytoma cells having natriuretic and diuretic functions [110]. Elevated levels of adrenomedullin have been found in patients with congestive heart failure [111], heart failure [112], congenital cyanotic heart disease [113]. Increasing levels of AM have been found in PF of patients with ischemic heart disease associating with left ventricular dysfunction and playing a role as a compensatory agent [114].

5. Signaling molecules in the pericardial fluid controlling cardiac remodeling

5.1. Inflammatory cytokines

Inflammatory cytokines are involved in the development of cardiovascular diseases, such as coronary atherosclerosis and myocardial infarction [115, 116]. Several inflammatory cytokines have been found in PF, such as interleukins (IL), tumor necrosis factor-α (TNF-α) in elevated level [116]. In a clinical study enrolling patients undergoing coronary artery surgery that due to inflammation events of the myocardium and pericardium IL-2 receptor, IL-6, IL-8 and TNF-alpha concentrations of PF were found significantly higher than blood plasma [117]. Also, significant correlations have been found between inflammatory cytokines and certain growth factors in patients with inflammatory pericardial disease [7]. These findings suggest that PF can be a marker of pathogenesis of inflammatory diseases.

5.2. Growth factors

The presence of these growth factors, such as basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) have been demonstrated in PF [27, 118]. Interestingly, bFGF was found in 20 fold higher concentration in PF as compared to serum [119]. Elevated levels of bFGF were reported in PF patients with angina pectoris [27] and its release from the myocardial tissue is associated with severe myocardial ischemia [120]. Also, increased levels of IGF1 were found in PF of cardiac patients, which was correlated with left ventricular dysfunction [121]. Furthermore, in an ex vivo study, pericardial fluid IGF1 induced HA synthesis in rabbit pericardium affecting the viscosity of PF [12]. In an in vitro study Corda et al. have found that PF induces growth of adult cardiomyocytes, which was assigned to the presence of bFGF in PF [119]. In addition, growth factors play important roles in the differentiation/fates of cardiac stem cells (CSCs) toward cardiomyocytes, endothelial cell, and smooth muscle cell thereby playing role in attenuating tissue regeneration and remodeling of the heart [118, 122].

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 remodeling [92, 123]. In cardiac patients PF contains several vasoactive substances, growth factors and biomarkers, which levels in PF 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.

To test these hypotheses we aimed the followings:

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

1. Study description

1.1. Patients

In the present study, 74 patients undergoing coronary 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.

1.2. 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).

2. Harvesting of samples

2.1. 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 stored at -75 °C until they used for experiments.

2.2. Isolation and preparation of rat common carotid arteries

The procedures were carried out as previously described [124, 125]. In brief, 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°C) petri dish filled with oxygenized (95 % O_2, 5 % CO_2) Krebs solution as described previously [124, 126]

3. Investigation of pericardial fluid ADMA

3.1. Echocardiography

All cardiac patients underwent complete 2-D transthoracic echocardiography before and after surgery. Two dimensional (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 guidelines [127]. 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: LV mass = 0.8 (1.04 ([LVIDD + PWTD + IVSTD]$_3$ - [LVIDD]$_3$)) + 0.6 g [128]. The left ventricular ejection fraction (LVEF) as the index of global systolic function was calculated according to the Simpson formula [129].

3.2. Measuring the concentration of L-Arg and ADMA

We followed the procedure as previously described in detail [130]. PF and blood samples were centrifuged (1200 g, 30 min) immediately after collection. Supernatants were maintained at -75 °C until biochemical analysis. L-Arg and asymmetric dimethylarginine (ADMA) were determined using liquid chromatography – mass spectrometry [131]. Quantification of ADMA and L-Arg derivatives was performed at the Department of Applied Chemistry, University of Debrecen.

4. Investigation of vasomotor effect of pericardial fluid

4.1. Measurements on isolated vessels

After isolation, vessels were placed in a 5 ml organ bath 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.

4.2. 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 °C). After that ET-1 (10^{-8} mol/L); PF (CABG and VR); BQ123 (10^{-6} mol/L) were added.

4.3. 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.

4.4. 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).

4.5. 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.

5. Data analysis, calculations and statistics

Results are expressed as mean±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

1. Pericardial fluid ADMA

1.1. Clinical characteristics of patients

Echocardiograph and electrocardiogram (ECG) indicated cardiac hypertrophy of the patients in VR group (**Fig 3**). Descriptive statistics of NCP and patients with CABG (n=28) or VR (n=25) surgery are summarized in **Table 2**, which shows the major demographic and clinical characteristics, as well as concomitant risk factors and medications of patients. Also, it shows that the vast majority of the patients in VR had cardiac hypertrophy (**Fig 3**). Types of surgery are summarized in **Table 3**.

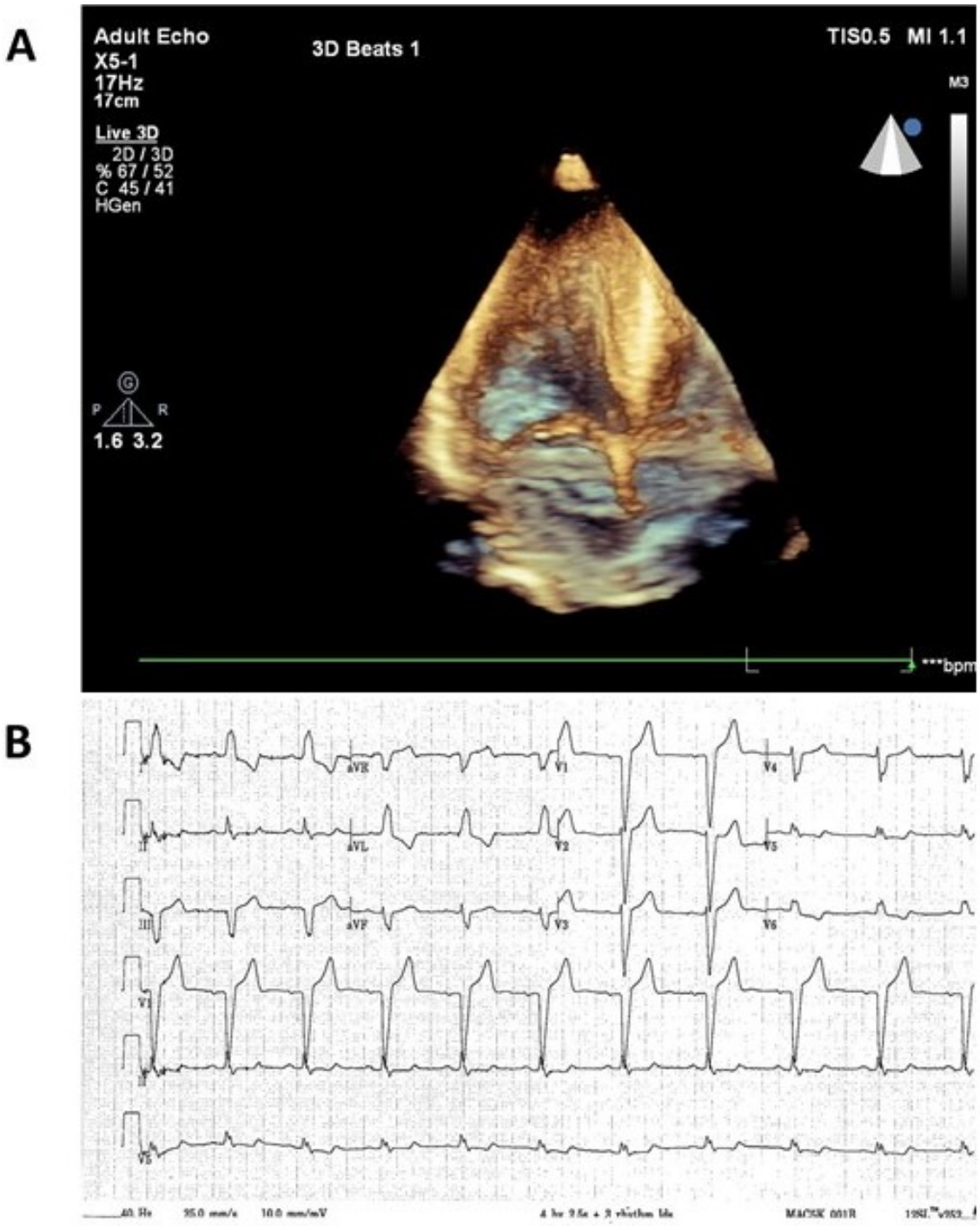

Figure 3. 3D echocardiograph and ECG signs of left ventricular hypertrophy (LVH).

A: Real time 3-D echo image of a patient with considerable concentric hypertrophy of left ventricle. B: The 12-leads echocardiographic pattern shows the left ventricular hypertrophy with a concomitant Left Bundle Branch block. The ECG paper speed is 25 mm/sec.

Table 2. Characteristics of patients and medications

Variable	NCP (n=20)	CABG (n=28)	VR (n=25)	p
Pre-operative data				
Age (year)	42.0±3.5	59.7±1.5	56.4±4.1	0.331
Sex (male/female)	11/9	17/11	15/10	0.870
Hypertension*	-	27	18	0.013
Cardiac hypertrophy	-	0	19	0.001
Diabetes mellitus	-	9	5	0.326
Previous AMI	-	12	0	0.000
sCr (μmol/L)	74.0±5.4	78.8±6.9	75.0±3.6	0.649
Estimated GFR (ml/min/1.73m^2)	-	58.54±1.3	58.64±0.8	0.947
Pre-operative medication		85% in combination/15% in monotherapy[#]	75% in combination/25% in monotherapy[#]	
Beta-blocker	-	23	18	0.809
Ca-channel blocker	-	8	5	0.671
ACE-inhibitor	-	14	16	0.129
AT-receptor blocker	-	4	1	0.208
Nitrate	-	0	0	0.000
Aspirin	-	21	4	0.000
Anti-diabetic	-	7	3	0.561
Statin	-	25	9	0.000
Diuretic	-	11	6	0.242

Data are mean ± SEM. *indicating blood pressure of 140/90 was considered normal in both cardiac groups [132]. [#]indicating medications in monotherapy for CABG (using beta-blocker or ACE inhibitor) and VR (using diuretic or ACE inhibitor). CABG: coronary artery bypass graft; VR: valve replacement; AMI: acute myocardial infarction, Estimated GFR: estimated GFR calculated by the Modification of Diet in Renal Disease (MDRD) GFR, sCr: serum creatitine, NCP – non-cardiac patients; CABG – coronary artery bypass graft; VR – valve replacement.

Table 3. Types of cardiac surgery

Operation	
CABGx1	0
CABGx2	3
CABGx3	16
CABGx4	8
CABGx5	1
AVR	17
MVR	7
AVR+MVR	1
Total	53

CABG: coronary artery bypass graft ("x" indicates the number of vessels involved); AVR: aortic valve replacement; MVR: mitral valve replacement.

1.2. 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 ($L\text{-}Arg_{NCP}$: 70.8±6.0 μmol/L vs. $L\text{-}Arg_{CABG}$: 75.7±4.6 μmol/L, p = 0.513; $L\text{-}Arg_{NCP}$: 70.8±6.0 μmol/L vs $L\text{-}Arg_{VR}$: 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) (**Fig 4A and B**). 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) (**Fig 4A**). 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; **Fig 4B**).

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) (**Fig 4C**). 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; **Fig 4C**). We found a significant inverse correlation between plasma L-Arg and eGFR in the CABG group (r = -0.367, p = 0.027). We found no significant correlation between the L-Arg/ADMA ratio and eGFR neither in plasma nor in PF of VR patients (pl L-Arg/ADMA ratio vs. eGFR: r = 0.200, p = 0.169; PF L-Arg/ADMA ratio vs. eGFR: r = 0.073, p = 0.128).

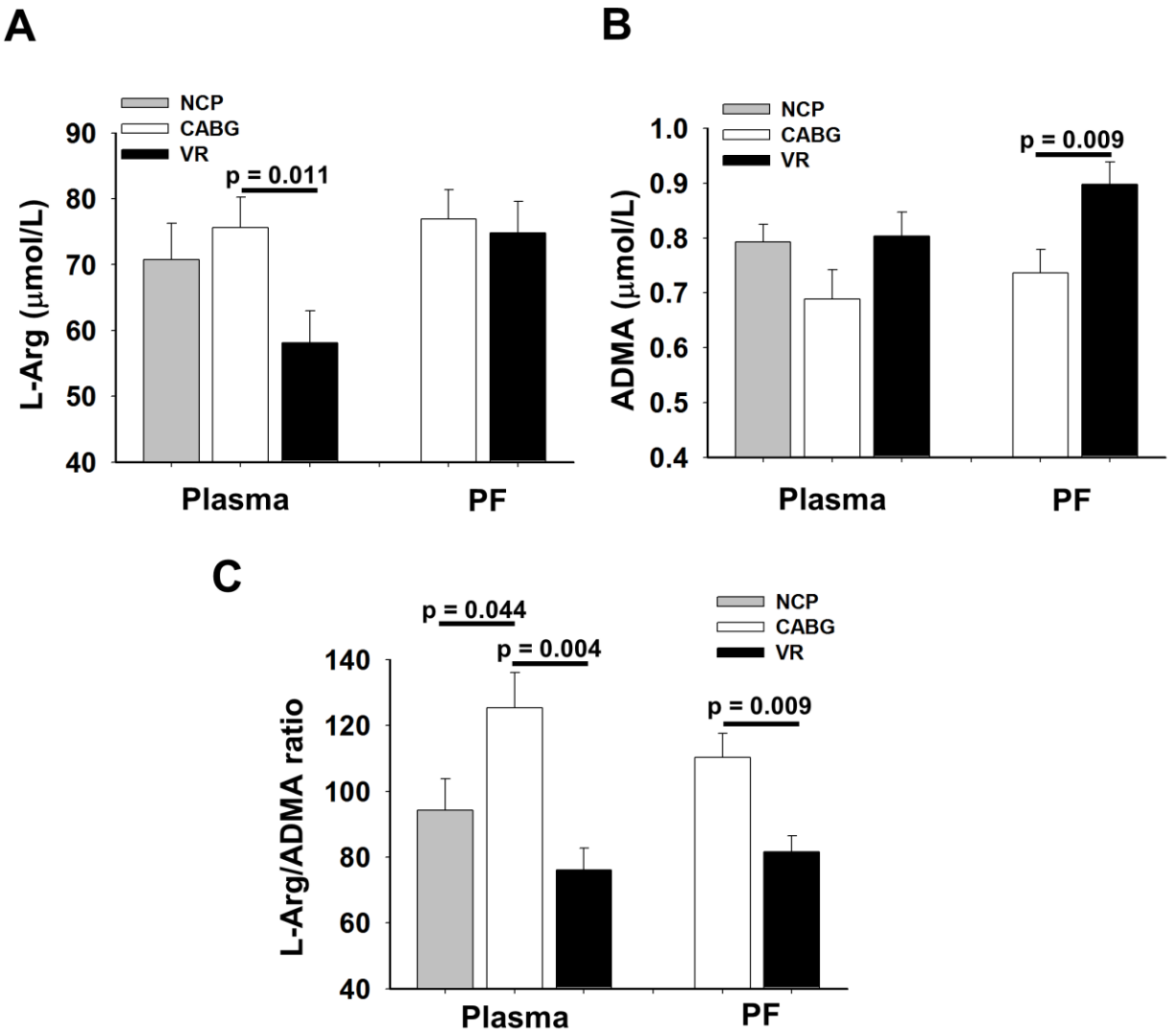

Figure 4. L-Arg and asymmetric dimethylarginine (ADMA) in plasma of non-cardiac patients (NCP) (n=20), and in plasma and pericardial fluid (PF) of patients undergoing coronary artery bypass graft (CABG, n=28) or valve replacement (VR, n=25) surgery. (**A**) Levels of L-Arg in plasma and PF. (**B**) Levels of ADMA in plasma and PF. (**C**) ratios of L-Arg and ADMA in plasma and PF. Mean ±SEM. * indicates significant ($p<0.05$) differences between CABG and VR patients. Substrate availability indicated by the ratio, which is significantly increased in CABG compared to NCP and VR.

1.3. 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 (**Fig 5A**), and between PF L-Arg and ADMA in both CABG and VR patients (**Fig 5B**). Furthermore, we found correlation between L-Arg levels of plasma and PF in CABG patients (**Fig 5C**), and ADMA levels of plasma and PF in VR patients (**Fig 5D**). 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.

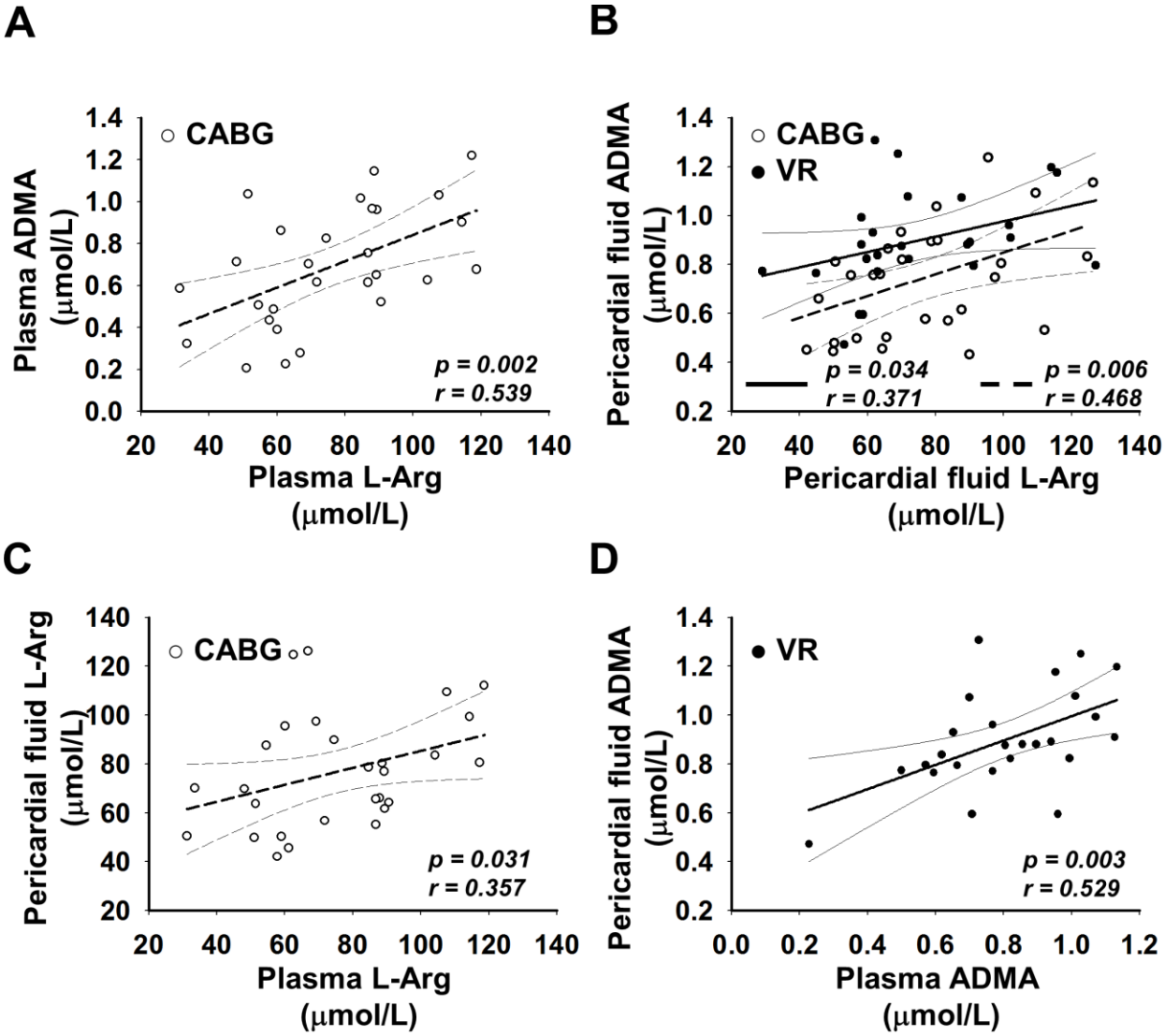

Figure 5. **Correlations between the levels of L-Arg and asymmetric dimethylarginine (ADMA) in plasma and pericardial fluid (PF) of patients undergoing coronary artery bypass graft or valve replacement surgery.** (**A**) plasma L-Arg vs. ADMA of CABG patients ($y = 0.006x + 0.21$, $r = 0.539$, $p = 0.002$); (**B**) PF L-Arg vs. ADMA of CABG and VR patients (CABG: $y = 0.005x + 0.39$, $r = 0.468$, $p = 0.006$; VR: $y = 0.003x + 0.67$, $r = 0.371$, $p = 0.034$); (**C**) plasma vs. PF L-Arg of CABG patients ($y = 0.347x + 50.69$, r = 0.357, p = 0.031); (**D**) plasma vs. PF ADMA of VR patients ($y = 0.498x + 0.50$, r = 0.529, p = 0.003).

1.4. Echocardiographic parameters of CABG and VR patients

Figure 6 shows, 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 6A and B**). Also, statistically significantly greater LVM was found in VR group compared to CABG group (**Fig 6C**), whereas left ventricular ejection fraction (LVEF) did not show significant difference between the two groups.

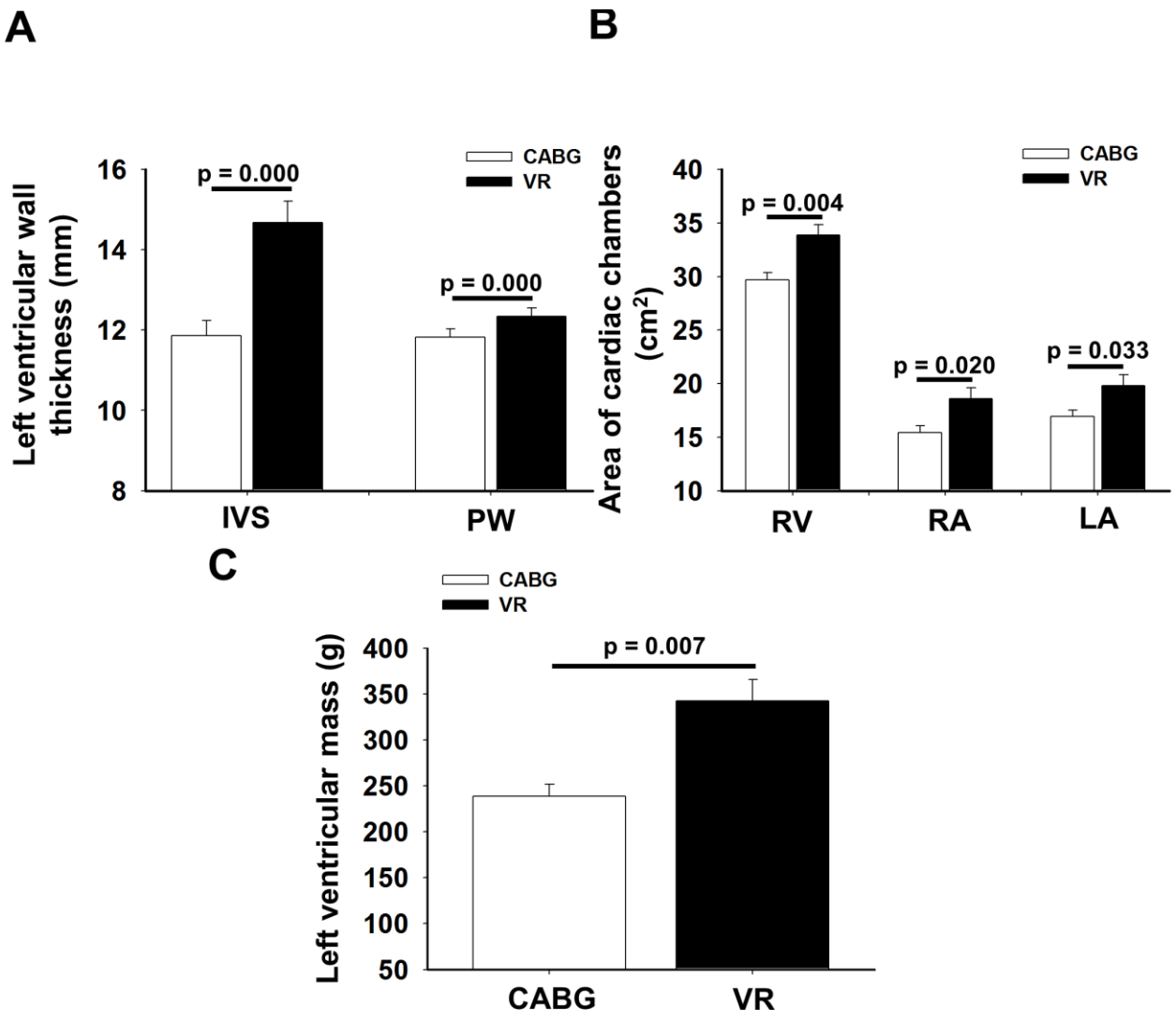

Figure 6. **Morphological parameters of ventricles and atria of patients undergoing coronary artery bypass graft (CABG, n=28) or valve replacement (VR, n=25) surgery.** (**A**) The thickness of interventricular septum (IVS) and posterior wall (PW), (**B**) the right ventricular (RV), the right atrial (RA) and the left atrial (LA) areas and (**C**) the left ventricular mass significantly higher in VR compared to CABG. Mean±SEM. $p<0.05$.

1.5. 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 7A**), PF ADMA and Ds of LV ($r = 0.487$, $p = 0.007$; **Fig 7B**), and Dd of LV ($r = 0.434$, $p = 0.015$; **Fig 7C**) 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 7D**), 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 (**Table 4**).

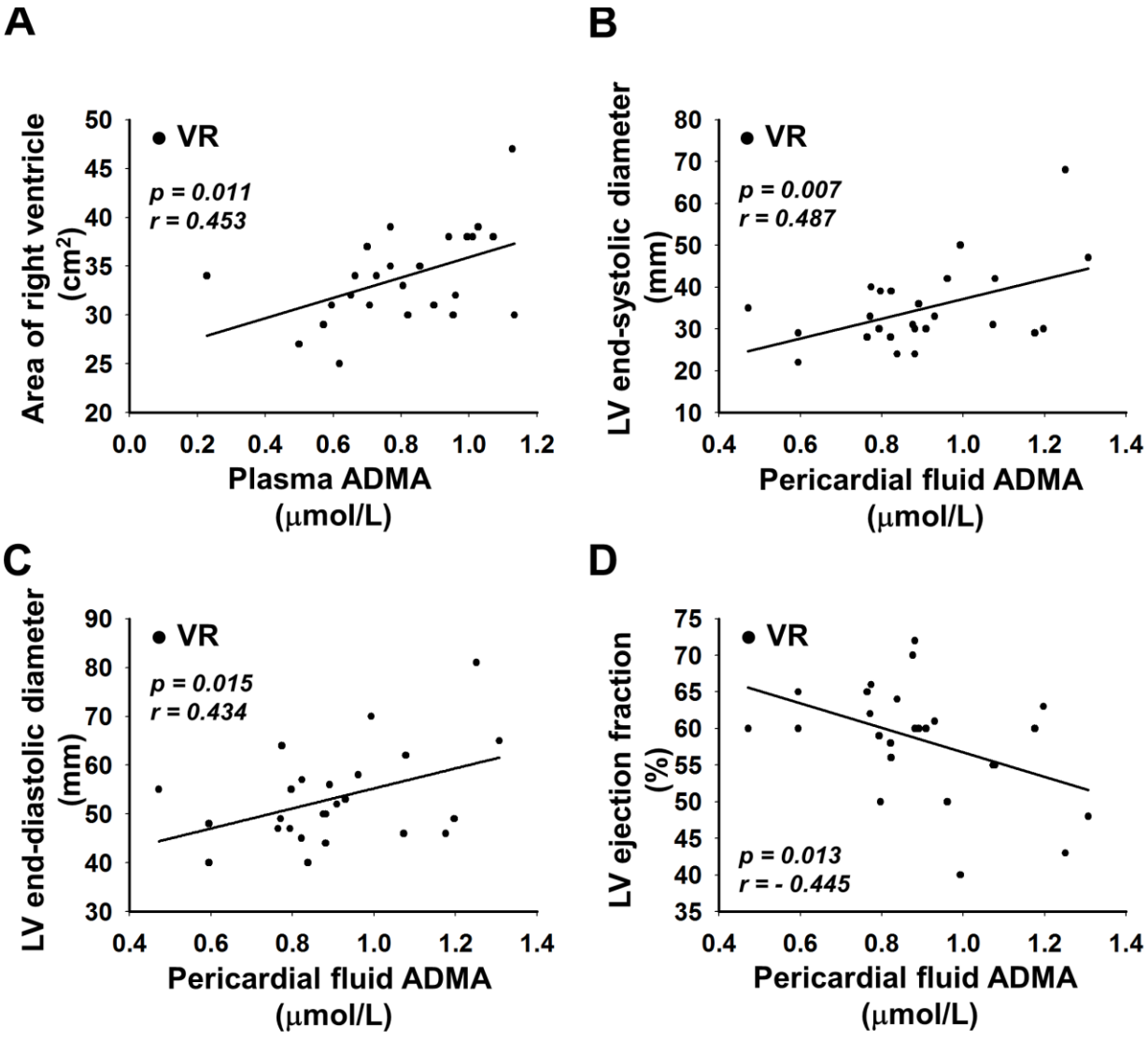

Figure 7. Correlations between the levels of asymmetric dimethylarginine (ADMA) and echocardiographic parameters of patients undergoing valve replacement (VR) surgery. (**A**) plasma ADMA vs. area of right ventricle ($y = 10.438x + 25.49$, $r = 0.453$, $p = 0.011$); (**B**) PF ADMA vs. left ventricular (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).

Table 4. Correlations between ADMA levels and echocardiographic parameters of patients undergoing VR surgery

ADMA levels vs. echocardiographic parameters	R	R^2	p
Plasma ADMA vs RV	0.453	0.206	0.011
PF ADMA vs RV	0.132	0.017	0.265
Plasma ADMA vs IVS	0.123	0.015	0.279
PF ADMA vs IVS	0.137	0.019	0.257
Plasma ADMA vs PW	-0.114	0.013	0.294
PF ADMA vs PW	0.176	0.031	0.200
Plasma ADMA vs Dd of LV	0.162	0.026	0.220
PF ADMA vs Dd of LV	0.434	0.189	0.015
Plasma ADMA vs Ds of LV	0.163	0.027	0.218
PF ADMA vs Ds of LV	0.487	0.237	0.007
Plasma ADMA vs RA	0.175	0.031	0.201
PF ADMA vs RA	0.050	0.003	0.406
Plasma ADMA vs LA	0.183	0.033	0.191
PF ADMA vs LA	0.104	0.011	0.310
Plasma ADMA vs LVM	-0.018	0.000	0.466
PF ADMA vs LVM	0.201	0.040	0.168
Plasma ADMA vs LVEF	-0.238	0.057	0.126
PF ADMA vs LVEF	-0.445	0.198	0.013

ADMA: asymmetric dimethylarginine; PF: pericardial fluid; VR: valve replacement; RV: area of right ventricle; IVS: thickness of interventricular septum; PW: thickness of posterior wall; Ds of LV: end-systolic diameter of left ventricle; Dd of LV: end-diastolic diameter of left ventricle; RA: area of right atria; LA: area of left atria; LVM: left ventricular mass; LVEF: left ventricular ejection fraction; R: Pearson's correlation coefficient; R^2: R-squared value.

2. Vasomotor effect of the pericardial fluid

2.1. 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 [133].

2.2. ET-1 induced vasomotor responses before and after BQ123 adding

Data show that ET-1 elicited increases in isometric force of isolated arteries, which was reduced following BQ123 (10^{-6} mol/L) adding (**Fig 8A**). Summary data show 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) (**Fig 8B**).

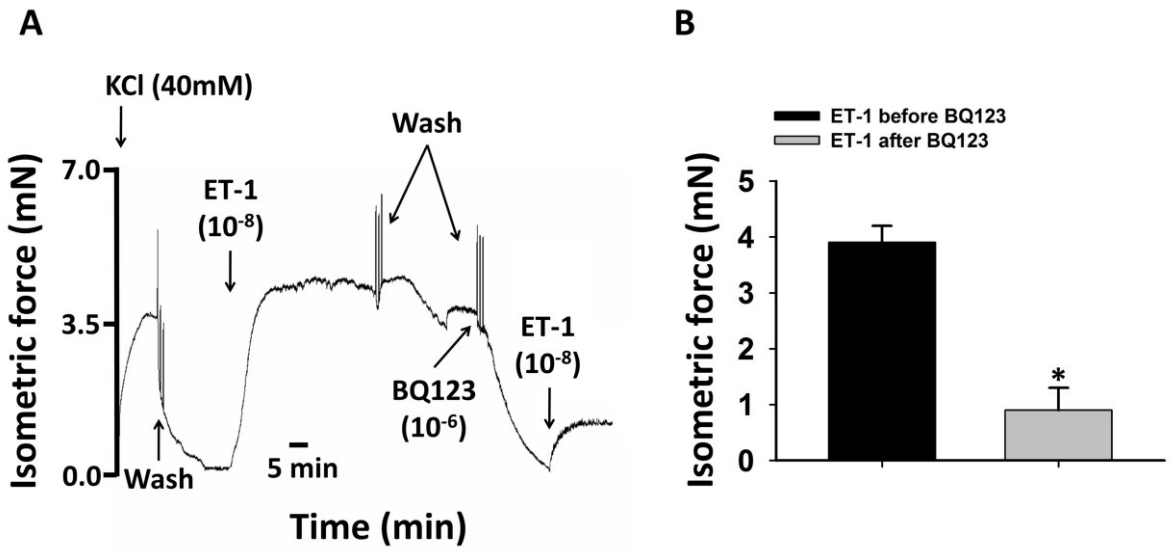

Figure 8. Endothelin-1 (ET-1)-induced vasomotor responses of isolated rat carotid arteries before and after addition of BQ123. (**A**) Data showing that KCl (40 mM/L) increases the isometric force of an isolated artery, and following washout of KCl, ET-1 (10−8 mol/L) also increases the isometric force of the isolated vessels; however, this is reduced following the addition of, and incubation with BQ123 (10−6 mol/L). (**B**) The summary data show that the isometric forces of isolated arteries (n = 5) induced by ET-1 (10−8 mol/L) are significantly reduced following the addition of, and incubation with BQ123 (10−6 mol/L). Values are the mean ± SEM; *, $p < 0.05$.

2.3. Vasomotor responses induced by human PFs

Data in **Fig. 9A** 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. 9B**). 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).

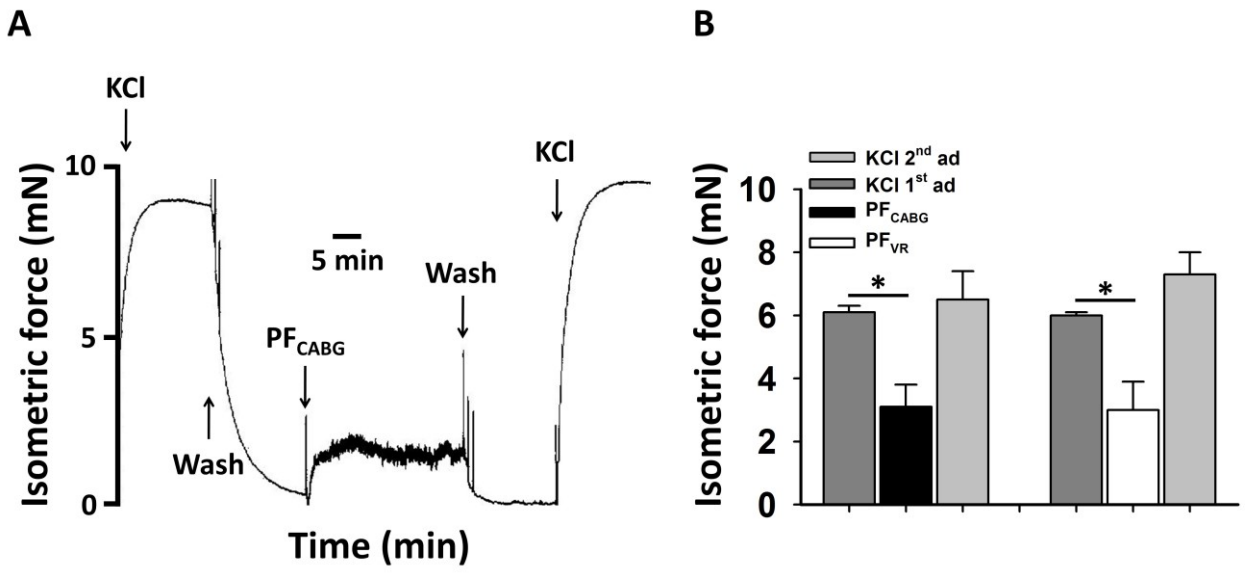

Figure 9. Vasomotor responses of rat carotid arteries to KCl (60 mM/L) and the pericardial fluid (PF) from patients undergoing coronary artery bypass graft (CABG; n = 9) or valve replacement (VR; n = 7) surgery. (**A**) Data showing that PF_{CABG} increases the isometric force of an isolated artery before 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 ± SEM; *, $p < 0.05$ comparing the effects of KCl with PF_{CABG} and PF_{VR}.

2.4. PF_{CABG} induced vasomotor responses with BQ123

Data in **Fig. 10A** show that PF_{CABG} elicited increases in the isometric force of isolated arteries, which were reduced following the addition of BQ123 (10^{-6} mol/L). Summary data show 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) (**Fig. 10B**). The second addition of KCl also increased the isometric force (5.8 ± 0.6 mN)

(**Fig. 10B**). 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) (**Fig. 10B**). Also, there was a significant difference between the isometric force induced by the first addition of KCl and the force induced by PF_{CABG} added before incubation with BQ123 ($p < 0.05$) (**Fig. 10B**).

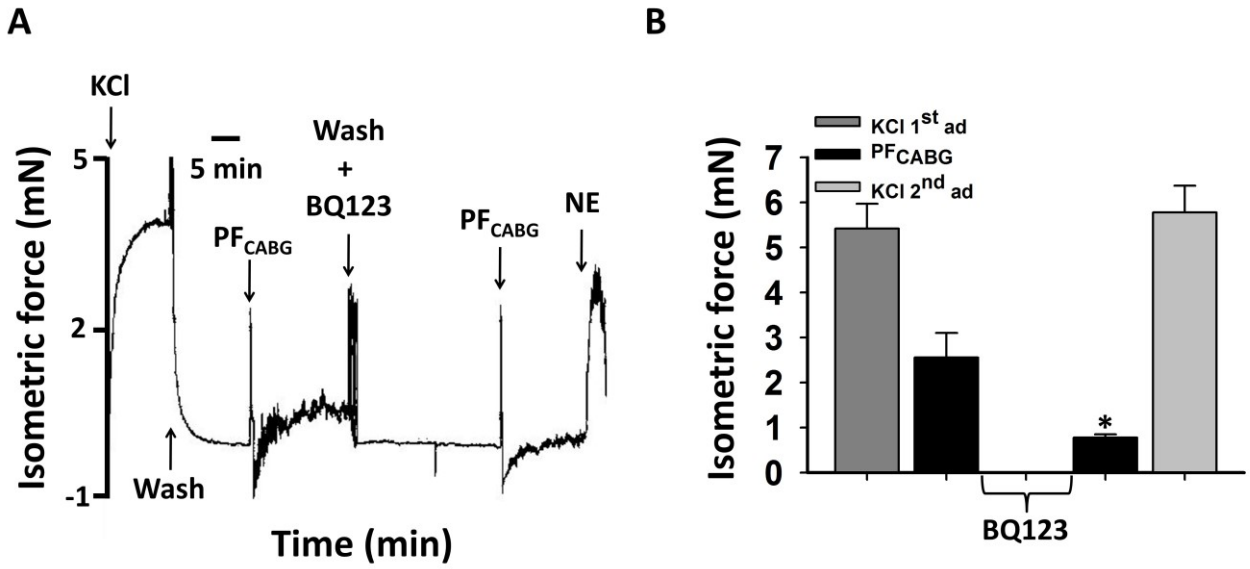

Figure 10. Vasomotor responses of rat carotid arteries to the pericardial fluid from patients undergoing coronary artery bypass graft surgery (PF_{CABG}) after the addition of BQ123. (**A**) Data showing that KCl (60 mM/L) increased the isometric force of an isolated artery, and after washout of KCl, PF_{CABG} also increased the isometric force; however, this increase was significantly reduced following the addition of BQ123 (10−6 mol/L). Following washout of PF_{CABG}, norepinephrine (NE; 10−6 mol/L) increased isometric force. (**B**) The summary data show that KCl (60 mM/L) increased the isometric force of isolated arteries ($N = 3$ arteries; $n = 5$ blood vessels). After washout of KCl, PF_{CABG} also increased isometric forces; however, this increase was significantly reduced following the addition of BQ123 (10−6 mol/L); following washout of PF_{CABG}, KCl (60 mM/L) also increased the isometric force generated by blood vessels. Values are the mean ± SEM. *, $p < 0.05$ comparing before and after the addition of BQ123 to the PF_{CABG}.

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.

In more details, the novel findings of the present studies are:

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.

1. Physiological role of the pericardial fluid

Pericardial fluid (PF) is a viscous film layer within the pericardial sac having mechanical function and containing biological active substances. Previously, mainly the mechanical role of PF has been recognized [4, 134]. Although reduction of mechanical friction is an important function of PF recently other physiological and pathological roles of PF have been suggested [23, 90, 91, 119]. Mounting evidence suggest that PF contains several bioactive substances [17-19, 27, 91] and that compositions of PF differ in various cardiovascular diseases [29-31].

In addition, previous studies, amongst them that of Alexander Juhasz-Nagy and Ferenc Horkay suggested that some of the substances in PF are potentially vasoactive [17, 20, 22] and that concentration of these substances may reach the level of vasomotor activity in certain cardiac diseases [17, 19, 20, 23, 28].

2. 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, inflammatory cytokines and growth factors [5, 7, 15]. Also, it has been reported that the level of these substances varies in different cardiac diseases [24, 135]. 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 plasma [136].

2.1. 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 diseases [137]. 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) [50].

2.1.1. 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 (**Fig 4**). There are studies reporting values between 50 and 100 μmol/L for L-Arg, and 0.3-0.8 μmol/L for ADMA in humans [138-140]. 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 diseases [141-144]. Also, it has been demonstrated, that plasma level of ADMA changed after stent placement in patients with coronary artery disease (CAD) [145]. Furthermore, Lu et al showed that plasma ADMA level significantly correlated with the severity of CAD [55]. 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 (**Fig 4A and B**). 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 - range (**Fig 4B**)[138]. In VR patients, we found that the levels of PF ADMA were significantly higher as compared to the CABG patients (**Fig 4B**).

2.1.2. L-Arg/ADMA ratio in plasma and PF as indicator of NO bioavailability

Previous studies have suggested that L-Arg/ADMA ratio indicates the availability of L-Arg for eNOS [146]. It has been shown that the L-Arg/ADMA ratio in plasma is about 100:1 in healthy individuals [131, 147]. Previously, it has been established that low L-Arg/ADMA ratio in acute myocardial infarction (AMI) can be linked to the severity of coronary insufficiency [148]. Also, it was previously reported that there is a correlation between coronary atherosclerotic score and plasma L-Arg/ADMA ratio indicating that changes in this ratio is linked to the severity of CAD [55].

In the present study, we found no significant difference in plasma L-Arg/ADMA ratio between the NCP, and the CABG and VR patients, whereas both the plasma and PF L-Arg/ADMA ratios showed a significant difference between the CABG and VR patients (**Fig 4C**).

Based on the aforementioned, it seems that production of ADMA is carried out by similar metabolic pathways in PF and plasma. Different levels of ADMA in CABG and VR patients

indicate that ADMA may have specific roles in the pathogenesis of cardiac events and reflects various pathological events of the heart. Taken together, these suggest that level of ADMA may reflect pathophysiological changes of the myocardium.

2.1.3. ADMA in PF and left ventricular hypertrophy/remodeling

Left ventricular hypertrophy is resulted by interaction between a chronic hemodynamic overload and non-hemodynamic factors [149]. The diastolic dysfunction of the heart is known to be the first predictor of left ventricular failure [150]. In the present study, the majority of VR patients suffered from aortic stenosis, which caused significant chronic pressure overload of the left ventricle [151]. We found, that echocardiographic parameters, which are characteristics of left ventricular hypertrophy, such as thickness of IVS, and parameter of LVM increased significantly in VR patients compared to CABG patients (**Fig 6A and C**). In the VR patients, areas of LA, RA and parameter of RV area exhibited significant increase in comparison of CABG patients (**Fig 6B**).

The role of NO in the development of hypertrophy and remodeling of the cardiac muscle in response to chronic changes in mechanical constraints (i.e., volume or pressure overload) is important, because altered morphology of the heart affects contractile performance [152]. There are several lines of evidence presented in previous decades suggesting that presence of adequate level of NO limits the hypertrophic growth of the myocardium [153]. 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 hypertrophy [58]. 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 pathway [154]. 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 11**, 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 NO [145]. 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 NO [52]. The activation of RAS increases the level of Ang II, which is known to be a growth hormone [155]. These observations are in concordance with previous studies and suggest that reduced level of NO [156] and increase activation of RAS [157] 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 11**).

2.1.4. Potential origins of ADMA in the pericardial fluid

PF has been considered as a passive ultrafiltrate of the blood plasma resulting by hydrostatic pressure difference between the plasma and PF, and osmotic concentration gradient [10]. It has also been shown that some substances in PF are derived from the cardiac interstitium, such as adenine nucleotides [20] and cardiac markers [158]. In the present study, we found that both plasma and pericardial fluid L-Arg levels in both CABG and VR patients were in the normal range (**Fig 4A**), whereas, ADMA levels reached the maximum of the normal range in these patients (**Fig 4B**).

The positive correlation between plasma L-Arg and ADMA in CABG patients (**Fig 5A**) suggests that ADMA originates mainly from the plasma. However, as the slope of the curve shows, ADMA may originate not only from the plasma, but also from cardiac tissues, which is then released into the pericardial fluid. In both CABG and VR patients, we observed significant positive correlations between L-Arg and ADMA in the pericardial fluid (**Fig 5**). These suggest that ADMA can be formed in PF from L-Arg, however as the slope of the curves suggest, that not only in the same compartment. Taken together, these suggest that part of ADMA in the PF origins from cardiac tissues and more ADMA is formed in VR than in CABG patients. Interestingly, in VR patients we did not find correlation between plasma L-Arg and plasma ADMA, and between plasma L-Arg and pericardial fluid ADMA. In our interpretation, these suggest that in VR patients ADMA may be generated in cardiac tissues from L-Arg, not just from plasma L-Arg. Although, the positive correlation between pericardial L-Arg and ADMA (**Fig 5B**), and between plasma and pericardial fluid ADMA (**Fig 5D**) suggest that in PF, ADMA is metabolized from the pericardial fluid L-Arg, however this correlation is not proportional. This latter findings and the positive correlation between plasma and pericardial fluid ADMA (**Fig 5D**) indicate that ADMA may diffuses between the two compartments.

2.1.5. 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 11**). 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.

2.2. 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 [159-162 163-165].

2.2.1. 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 (**Figs 9 and 10**). The characteristics and potency of the responses of carotid arteries to PF were different from those induced by KCl or other vasoactive substances (**Figs 9 and 10**). Response curves induced by KCl were sigmoidal, whereas those induced by PF had an upswing slope and a maintained plateau phase (**Figs 9 and 10**). However, the steepness of slope of response curves induced by PF was less than that of norepinephrine and Ang II, but similar to ET-1 (**Figs 8; 9 and 10**) [124].

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 endothelin [166].

In this vascular preparation 60 mM KCl, 10^{-8} M NE or 10^{-7} M Ang II [124] elicits close to maximal contractions. In comparison, the maximum response produced by PF was less (**Figs 8; 9 and 10**), 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 disease [167-169] and the present data support potential underlying mechanisms contributing the vasoconstrictor effect.

2.2.2. 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 dysfunction [81, 170, 171]. Several studies have reported that endothelin(s) play an important role in the development of cardiovascular diseases [172, 173]. For example, ET-1 has been found to play role in atherosclerosis, diabetes mellitus, and pulmonary artery hypertension [81, 174, 175]. Moreover, ET-1 has been shown to be present in high concentrations in PF of patients undergoing cardiac surgery [17], 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 BQ123 [176, 177] (**Fig 8A and B**). 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 [5, 161].

Importantly, it is likely that these patients undergone several ischemic and ischemic/reperfusion period before CABG or VR surgery as shown in previous studies [178]. ET-1 has been suggested to be one of the mediators of ischemia/reperfusion injury [178-180] and be present in high levels [181]. This conclusion was supported by findings in cultured human endothelial cells showing that hypoxia increases ET-1 release by increasing of expression of pre-pro-endothelin gene [182]. Furthermore, inhibiting ET receptors by BQ123 improved rat cardiac function after myocardial infarction [183]. 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 heart [93, 184, 185] 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.

Finally, it is of note that our study has limitations. Collecting human pericardial fluids of healthy persons (“control”) is unethical thus we could not obtain and investigate them in this

study. Also, measuring the ET-1 contents of PF and correlating them with the vasomotor responses would have provide further support for the conclusion of this study; however the limited amount of pericardial fluid available, precluded us performing simultaneous vascular and biochemical studies, thus we relied on literature data. Nevertheless, because our data derived from human pericardial fluids of patients they provide an important proof of concept regarding the role of ADMA in cardiac remodeling and vasomotor effect of ET in the human pericardial fluid.

CONCLUSIONS

In this study we investigated the "non-mechanical" function of the pericardial fluid (PF). According to our previous and present findings PF has paracrine effects: influencing cardiac remodeling and vasomotor tone.

In brief, elevated levels of asymmetric dimethyl-arginine (ADMA) in the pericardial fluid of patients with valve replacement could contribute and be a marker of cardiac hypertrophy. Furthermore, pericardial fluids of cardiac patients have substantial vasoconstrictor effect, which is mediated primarily by ET-1.

We suggest that measuring of biological 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 interstitium and coronary vasculature thus we suggest that pericardial fluid is providing a "third pathway" of cardiac homeostasis and coronary circulation**, which can have important physiological and pathophysiological ramifications and may help to develop new therapeutic strategies to improve injured myocardium and coronary circulation in case of impaired cardiac function.

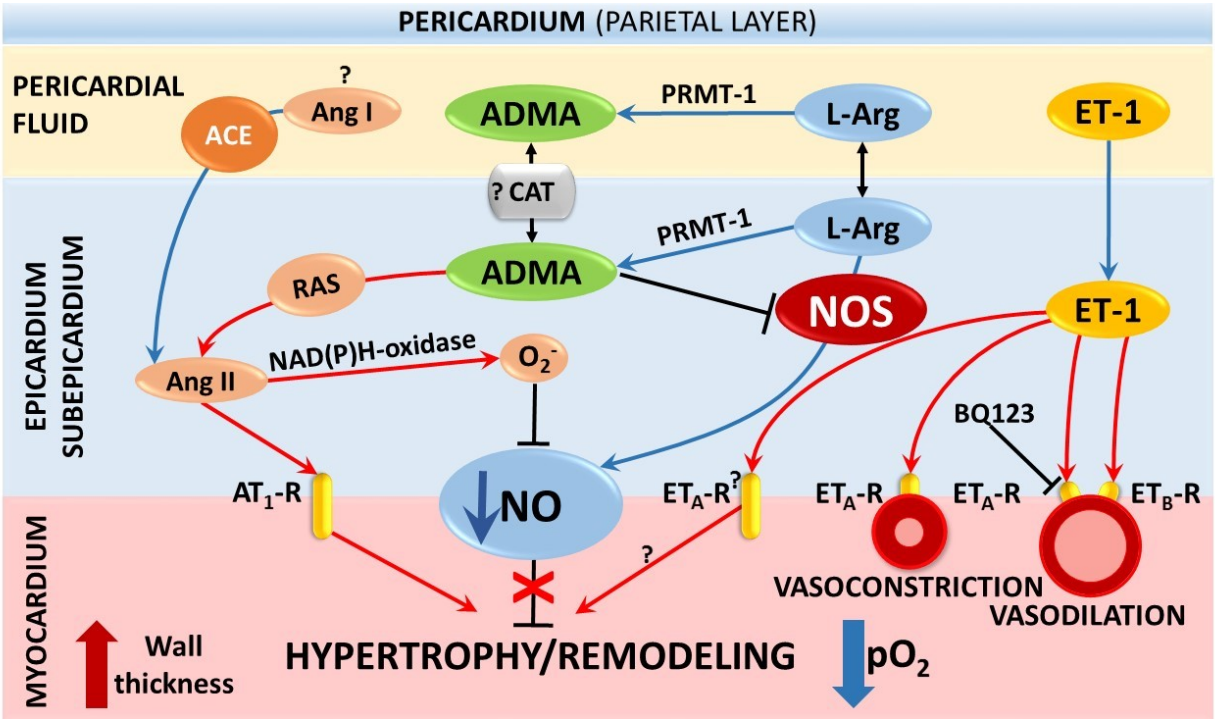

Figure 11. Proposed mechanisms by which bioactive substances of the pericardial fluid regulate cardiac tissues. Elevated level of ADMA of pericardial fluid elicits hypertrophy/remodeling of cardiac muscle: accordingly, reduced NO bioavailability and increased level of Ang II together leads to development 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, AT_1-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

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PEER-REVIEWED PUBLICATIONS OF THE AUTHOR

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:

4. 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.

5. **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.

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