CARDIOVASCULAR RISK AND STRESS ADAPTATION IN METABOLIC DISEASES

Ph.D. Thesis

(Summary)

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1 INTRODUCTION

Cardiovascular diseases (CVD) are the major cause of morbidity and mortality in the industrialized world. There are different forms of CVD conditions, among which ischemic heart disease (IHD) is the most predominant form of all cases and deaths. IHD develops due to atherosclerosis leading to different clinical phenotypes from a less severe angina to more severe forms including acute myocardial infarction, chronic ischemia or even sudden cardiac death. IHD is a multifactorial pathological condition developing as a consequence of both genetic and environmental factors as well as interaction between them. It is well known that IHD generally coexists with other metabolic risk factors and comorbidities including hyperlipidemia, metabolic syndrome (defined as the coexistence of visceral obesity, dyslipidemia, hyperglycemia, and systemic arterial hypertension), atherosclerosis, insulin resistance as well as diabetes mellitus, hypertension-related left ventricular hypertrophy, heart failure, and aging. Moreover, increasing prevalence of hyperlipidemia and diabetes mellitus in the aging population results in a dramatic rise in the prevalence of chronic kidney disease characterized by severe metabolic changes generally termed as uremia. In addition, uremia and especially end-stage renal failure have been shown to increase the risk of cardiovascular morbidity and mortality.

Myocardial ischemia develops when coronary flow to the myocardium is relatively or absolutely reduced which results in myocardial ischemic injury. Under experimental circumstances and in clinical situations, ischemia may be followed by reperfusion. Although, reperfusion is essential to salvage ischemic tissue, it has the potential to cause further irreversible cell damage termed as reperfusion injury. In summary, myocardial ischemia and reperfusion leads to "ischemia/reperfusion (I/R) injury" which might result in the development of myocardial infarction, contractile dysfunction and arrhythmias.

Coronary interventions and thrombolytic therapies are the most widely used clinical interventions to reduce infarct size by reopening the occluded arteries. Pharmacologic agents (e.g., nitrates and beta blockers) reducing energy demand and/or increasing coronary flow are also common strategies to ameliorate infarct size. An alternative approach to minimize the development of myocardial infarction is the triggering of endogenous adaptive mechanisms of the heart. Experimentally, the most powerful endogenous adaptive cardioprotective mechanisms are the ischemic precondonditioning (IPre) and ischemic postconditioning (IPost). IPre is a well described endogenous adaptive mechanism in which brief exposure to I/R markedly enhances the ability of

the heart to withstand a subsequent, potentially lethal I/R injury. Brief cycles of I/R applied after a longer period of ischemia also confer cardioprotection against myocardial I/R injury. This phenomenon is termed as IPost. The cardioprotective effect of IPre and IPost results in attenuation of I/R injury characterized by improvement of post-ischemic contractile function, a decrease in the occurrence and severity of arrhythmias, and a reduction in infarct size. These endogenous cardioprotective mechanisms, IPre and IPost have been demonstrated to be abolished by several metabolic risk factors and comorbidities such as e.g. hypercholesterolemia, metabolic syndrome, and diabetes mellitus.

Although, the molecular mechanisms of IPre and IPost are extensively studied, the exact signal transduction pathways of IPre and IPost are still unknown due to the complexity of these mechanisms. Beside the traditional biochemical and pharmacological approaches, genomics may provide new potentials to investigate key molecular events in the mechanisms of IPre and IPost. We and others have previously shown that IPre significantly influenced myocardial gene expression pattern as assessed by miRNA chip as well as DNA microarray and qRT-PCR. We have previously demonstrated that hypercholesterolemia resulted in marked changes in preconditioning-induced gene expression which might lead to significant alterations of oxidative/nitrative stress signaling and to the loss of the cardioprotective effect of IPre. Nevertheless, certain cardiovascular risk factors and comorbid conditions including type 2 diabetes mellitus (T2DM) and CKD have been shown to influence cardiac gene expression profile. Therefore, in the present thesis we investigated the effect of another cardiovascular risk factor, the metabolic syndrome on cardiac gene expression pattern in order to examine the basic differences in cardiac metabolic changes which might provide a new potential to understand the mechanisms of different susceptibility to myocardial I/R injury in metabolic syndrome.

We and others have previously shown that cardiovascular risk factors and comorbid states including hypercholesterolemia as well as T2DM attenuate the cardioprotective effect of IPre and IPost. However, it has been demonstrated previously that the cardioprotective effect of both IPre and IPost are preserved in experimental subacute renal failure. Therefore, in the present thesis, we examined whether the state of prolonged uremia, a relevant cardiovascular rsik factor affects the I/R injury and cardioprotection by IPre.

2 AIMS

The aims of the present thesis were to

1. investigate the effect of metabolic syndrome on cardiac gene expression pattern in male ZDF rats which is a well-known model of metabolic syndrome.

2. examine the influence of another severe metabolic state, prolonged uremia (30 weeks), on the severity of

    a. myocardial I/R injury and

    b. the infarct size limiting effect of IPre.
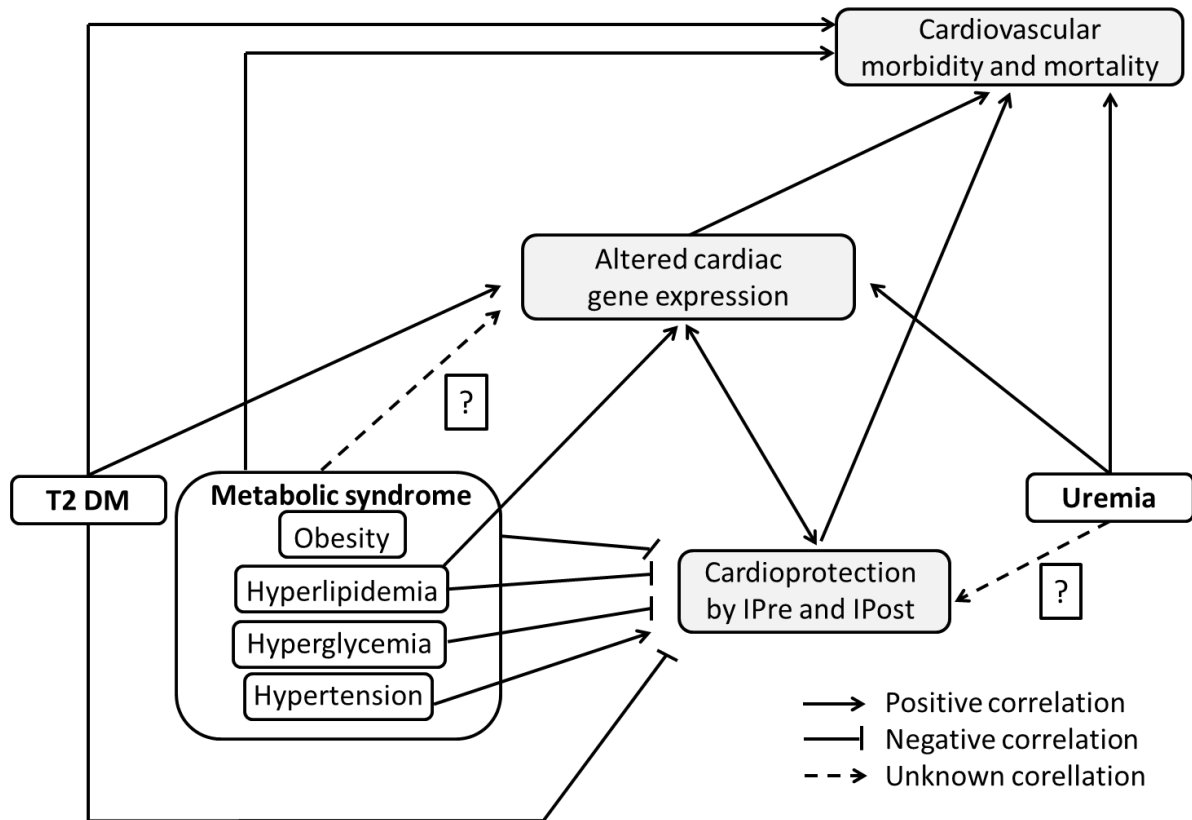
1. Figure Effects of comorbidities on cardiovascular morbidity and mortality, cardiac gene expression and cardioprotection conferred by IPre and IPost.

3 MATERIALS AND METHODS

These investigations conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996) and was approved by the Animal Research Ethics Committee of the University of Szeged.

3.1 Investigation of cardiac gene expression pattern in metabolic syndrome

Male Zucker Diabetic Fatty (ZDF/Gmi-fa/fa) rats and their lean controls were obtained from Charles River Laboratories at the age of 5 weeks and were housed at $22\pm2°C$ with a 12:12-h light-dark cycle. The rats received Purina 5008 chow and water ad libitum for 20 weeks after their arrival. The Zucker diabetic fatty rat with a point mutation in the leptin receptor is a recognized model of obesity, hyperlipidemia, hyperglycemia and hypertension. In the present study, only male rats were used, since female ZDF rats are less prone to the development of metabolic syndrome. Male ZDF rats develop an age-dependent obese and hyperglycemic phenotype at 10-12 weeks of age accompanied by a metabolic state of obesity, dyslipidemia, hyperinsulinemia and insulin resistance which develops to a hyperglycemic insulin-deficient state. The metabolic features manifested in this animal model are in many ways similar to the pathogenesis of metabolic syndrome in humans. Therefore, the ZDF rat is an ideal model for investigation of cardiac gene expression pattern changes related to human metabolic syndrome.

Body weight, serum glucose (AccuCheck, Roche Diagnostics Corporation, USA, Indianapolis, insulin (Mercodia, Ultrasensitive Rat Insulin ELISA), cholesterol and triglyceride levels (Diagnosticum Zrt., Budapest, Hungary) and homeostasis model assessment-estimated insulin resistance (HOMA-IR) were determined at 6, 16 and 25 weeks of age in order to monitor the basic parameters of glucose and lipid metabolism and insulin resistance in ZDF and lean rats. Oral glucose tolerance test (OGTT) was performed at week 16 and 25 in order to further characterize glucose homeostasis in ZDF and lean rats. At 25 weeks of age, rats were anaesthetized using diethyl ether. Hearts and pancreata were isolated, and then hearts were perfused according to Langendorff. After 10 min perfusion, ventricular tissue was frozen and stored at -80°C until DNA microarray investigation and gene expression analysis. RNA preparation, DNA microarray analysis and qRT-PCR were performed in the Department of Functional Genomics, Biological Research Center, Szeged, Hungary as described in the article related to the subject of the thesis. To validate the well-known nitrative stress-inducing effect of metabolic syndrome on the heart, frozen ventricular tissue was used for determination of cardiac free 3-nitrotyrosine level (ELISA, Cayman Chemical).

3.2 Investigation of the influence of prolonged uremia on IPre

Adult male Wistar rats were used in the study. Animals were housed in pairs in individually ventilated cages (Sealsafe IVC system, Italy) and were maintained in a temperature controlled room with a 12:12 h light: dark cycles throughout the study. Standard rat chow and tap water were supplied ad libitum. Drinking water contained 1mg/100g body weight iron (II) sulfate to attenuate the development of severe anemia.

Experimental prolonged uremia was induced by partial (5/6) nephrectomy. Animals underwent sham operation or partial nephrectomy in two phases. There was no difference in mortality between sham operated and partially nephrectomized groups. At week 29, cardiac function was assessed by transthoracic echocardiographic examination (Vivid 7 Dimension ultrasound system and EchoPac Dimension software, General Electric Medical Systems, USA). At week 29, a group of animals were placed for 24 hours in metabolic cages (Tecniplast, Italy) in order to estimate creatinine clearance and to measure urine creatinine (colorimetric assay kit, Diagnosticum Ltd., Budapest, Hungary) and protein levels. At week 30, rats were anesthetized; hearts were isolated and perfused ex vivo by oxygenated Krebs-Henseleit solution according to Langendorff as described previously. Immediately after excision of the heart, blood was collected from the thoracic cavity to measure plasma uric acid, carbamide and creatinine levels (colorimetric assay kits, Diagnosticum Ltd., Budapest, Hungary) in order to verify the development of uremia. Hematocrit and hemoglobin were measured from whole blood by means of a blood gas analyzer (Radiometer ABL 77, Radiometer Medical, Bronshoj, Denmark) in order to verify the development of renal anemia. Some plasma was used for determination of angiotensin II (ELISA, Phoenix Pharmaceuticals) as an indirect marker of hypertension and hypertrophy and nitrotyrosine as a marker of systemic nitrative stress (ELISA, Cayman Chemical). To assess the cardioprotective effect of IPre in the hearts of uremic and sham operated animals, the perfused hearts were subjected to I/R with or without preconditioning protocol. At the end of the appropriate perfusion protocol, the coronary artery was reoccluded and the area at risk and the infarcted area were delineated using planimetry following an Evans blue/triphenyltetrazolium chloride double staining method.

4 Results

4.1 Characterization of metabolic syndrome in ZDF rats

In order to verify the development of metabolic syndrome in male ZDF rats, concentrations of several plasma metabolites and body weight were measured at week 6, 16 and 25. ZDF rats showed a significant rise in serum fasting glucose level starting from week 16 as compared to lean controls. Parallel with hyperglycemia, serum insulin levels were significantly increased in ZDF rats compared to lean ones during the 25 weeks showing the presence of hyperinsulinemia in ZDF animals. However, serum insulin concentration in ZDF rats was significantly lower at week 25 as compared to serum insulin level measured at week 16 indicating beta-cell damage. HOMA-IR was significantly higher at week 6, 16 and 25 in ZDF rats when compared to lean controls showing insulin resistance in ZDF animals. Body weight increased throughout the study and was significantly higher in ZDF animals compared to lean ones showing obesity. Both serum cholesterol and triglyceride levels were significantly increased in ZDF rats as compared to lean ones throughout the study duration representing hyperlipidemia.

Oral glucose tolerance test (OGTT) was performed at week 16 and 25 in order to verify the development of impaired glucose tolerance in ZDF rats. Glucose levels during OGTTs were markedly increased in ZDF rats in every time point of blood glucose measurements both at weeks 16 and 25. Area under the curve (AUC) of blood glucose concentration during OGTTs was significantly elevated in ZDF rats at both weeks 16 and 25 representing impaired glucose tolerance.

Pancreas weight and pancreatic insulin content were measured at the end of the experiment in order to investigate the severity of diabetes mellitus in ZDF rats. Pancreas weight and pancreatic insulin concentration were significantly decreased in ZDF rats at week 25 showing impaired pancreatic function.

In order to verify the increased oxidative/nitrative stress in ZDF animals, myocardial 3-nitrotyrosine levels were determined in both groups at week 25. A marker molecule of peroxynitrite, 3-nitrotyrosine level was significantly elevated in the heart of ZDF animals.

4.2 Cardiac gene expression profile in metabolic syndrome measured using cDNA microarrays and by qRT-PCR

In order to determine alterations in cardiac gene expression profile, a DNA microarray was carried out. Among the 14921 genes surveyed, 10244 genes were expressed on the cDNA microarray, and 85 genes whose expression was > ~1.7-fold up- or down-regulated (log2 ratio <-0.75 or log2 ratio >0.75) in hearts of ZDF rats relative to levels of lean control rats showed significant change in expression.

According to our results, 49 genes showed down-regulation and 36 genes showed up-regulation in hearts of ZDF rats. Many of these differentially expressed genes in the heart due to metabolic syndrome includes functional clusters of metabolism (e.g. 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2; argininosuccinate synthetase; 2-amino-3-ketobutyrate-coenzyme A ligase), structural proteins (e.g. myosin IXA; aggrecan1), signal transduction (e.g. activating transcription factor 3; phospholipase A2; insulin responsive sequence DNA binding protein-1) stress response (e.g. heat shock 70kD protein 1A; heat shock protein 60; glutathione S-transferase Yc2 subunit), ion channels and receptors (e.g. ATPase, Na^+/K^+ transporting, beta 4 polypeptide; ATPase, H^+/K^+ transporting, nongastric, alpha polypeptide). Moreover some other genes with no definite functional clusters were also changed such as e.g. S100 calcium binding protein A3; neuronatin; ubiquitin carboxy-terminal hydrolase L1 etc.

The expression change 18 genes out of 23 selected genes have been confirmed by qRT PCR. Most of these genes have not been shown to be involved in the development of cardiovascular complications of metabolic syndrome yet.

4.3 Characterization of prolonged uremia

In order to verify the development of long term uremia induced by partial nephrectomy, body weight was monitored during the experiment and concentrations of several plasma and urine metabolites were measured at week 29. Partially nephrectomized rats showed significantly lower body weight starting from week 7. Plasma carbamide and creatinine levels were markedly increased in partially nephrectomized rats representing the uremic state of these animals. Plasma glucose levels were similar in both the sham-operated and the partially nephrectomized groups. Urine protein concentration was significantly increased in the partially nephrectomized rats as compared to sham-operated controls showing an impaired renal function. Moreover, urine creatinine level and creatinine clearance showed a marked but statistically not significant decrease at the level of $p < 0.05$ in partially nephrectomized rats. Hematocrit and hemoglobin levels were significantly decreased in uremic animals when compared to sham rats showing renal anemia. There was no difference in plasma sodium, potassium, calcium, chloride, standard bicarbonate, pH and anion gap between the groups.

4.4 Effect of prolonged uremia on myocardial morphology and function

Transthoracic echocardiography was performed at week 29 in order to investigate whether the development of prolonged uremia leads to alteration of myocardial morphology and function. Left ventricular systolic anterior and septal wall thicknesses were increased in uremic rats as compared to sham-operated controls. However, there was no difference in diastolic anterior and septal wall thickness between the uremic and sham operated group. In addition, left ventricular lateral and posterior wall thickness both in systole and diastole were similar in the uremic and the sham operated groups. Uremic animals demonstrated a mild reduction both in left ventricular systolic and diastolic function at week 29. Ejection fraction was significantly reduced in the uremic group as compared to the sham-operated control group. Stroke volume showed a tendency of decrease in uremic animals as compared to sham-operated controls. Heart rate and left ventricular end-systolic and end-diastolic volumes were similar in both uremic and sham-operated groups. Isovolumic relaxation time was decreased in uremic rats as compared to sham-operated controls. Other diastolic functional parameters including early and late ventricular filling velocity, E/A ratio, deceleration time, maximal and mean left ventricular gradient were not changed in the uremic group as compared to sham-operated controls.

4.5 Effect of prolonged uremia on ex vivo morphological and functional parameters

Coronary flow and morphological parameters including heart weight, left kidney weight and tibia length were measured at week 30 to investigate whether advanced uremia influences ex vivo functional and morphological parameters of the heart. Coronary flow was significantly reduced in uremic groups when compared to corresponding sham-operated control groups as assessed by two-way ANOVA.

The ratio of heart weight to body weight showed a tendency of increase in uremic animals, as a result of a significantly lower body weight in the uremic group. However, there was no difference in heart weight and heart weight to tibia length ratio between the uremic and the sham-operated control group. In addition, the weight of the whole left kidney in the sham-operated group and the remaining one third of the left kidney in the uremic group were similar suggesting a marked renal hypertrophy in the uremic animals.

4.6 Effect of ischemic preconditioning on infarct size

Infarct size was measured at week 30 to investigate the severity of ischemia/reperfusion injury and the cardioprotective effect of IPre in prolonged uremia. Preconditioning significantly decreased infarct size, however, the presence of prolonged uremia did not significantly influence the size of infarction as assessed by two-way ANOVA. Additional analysis with unpaired t-tests showed a significant infarct size limiting effect of preconditioning in hearts of both uremic and sham-operated rats. The area at risk zone was not affected significantly in any of the groups.

4.7 Effect of prolonged uremia on plasma angiotensin II, 3-nitrotyrosine and uric acid levels

Partially nephrectomized rats showed significantly higher plasma angiotensin II level which is a well-known marker of hypertension and left ventricular hypertrophy. Plasma 3-nitrotyrosine level was determined as a marker of peroxynitrite and systemic nitrative stress. Plasma 3-nitrotyrosine level was markedly increased in uremic rats as compared to sham operated controls representing increased systemic nitrative stress in uremic animals. Plasma level of uric acid, a well-known anti-oxidant, was significantly increased in the partially nephrectomized rats as compared to sham-operated controls showing increased anti-oxidant capacity in uremic rats.

5 CONCLUSIONS

In the present thesis, we investigated the effect of a cardiovascular risk factor, the metabolic syndrome on cardiac gene expression pattern, and we examined whether another cardiovascular risk factor and comorbid state, prolonged uremia, affects the I/R injury and cardioprotection by IPre.

1. In the present thesis we have found that 25 weeks old ZDF rats develop severe metabolic syndrome (dyslipidemia, obesity, hyperglycemia, hyperinsulinemia, impaired glucose tolerance and insulin resistance). We have demonstrated for the first time that metabolic syndrome is associated with profound modifications of the cardiac transcriptom. Several of the genes showing altered expression in the hearts of ZDF rats have not been implicated in metabolic syndrome previously. We conclude that metabolic syndrome alters the gene expression pattern of the myocardium which may be involved in the development of cardiac pathologies in the presence of metabolic syndrome (Figure 15). The precise role of these genes in the cardiac consequences of metabolic syndrome needs to be further investigated in future studies.

2. We have also found here that another metabolic disease, chronic renal failure although leads to severe metabolic changes and the development of myocardial dysfunction as well as minimal cardiac hypertrophy, the cardioprotective effect of IPre is still observed. In conclusion, our present study suggests that patients suffering from long-term uremia may also benefit from cardioprotection by IPre. This is particularly important as acute myocardial infarction frequently occurs in patients with late stages of renal failure. Since uremic patients are regularly excluded from clinical trials, there is a need for clinical studies to investigate the cardioprotective effect of conditioning techniques in patients with chronic renal failure suffering from acute myocardial infarction (Figure 2).
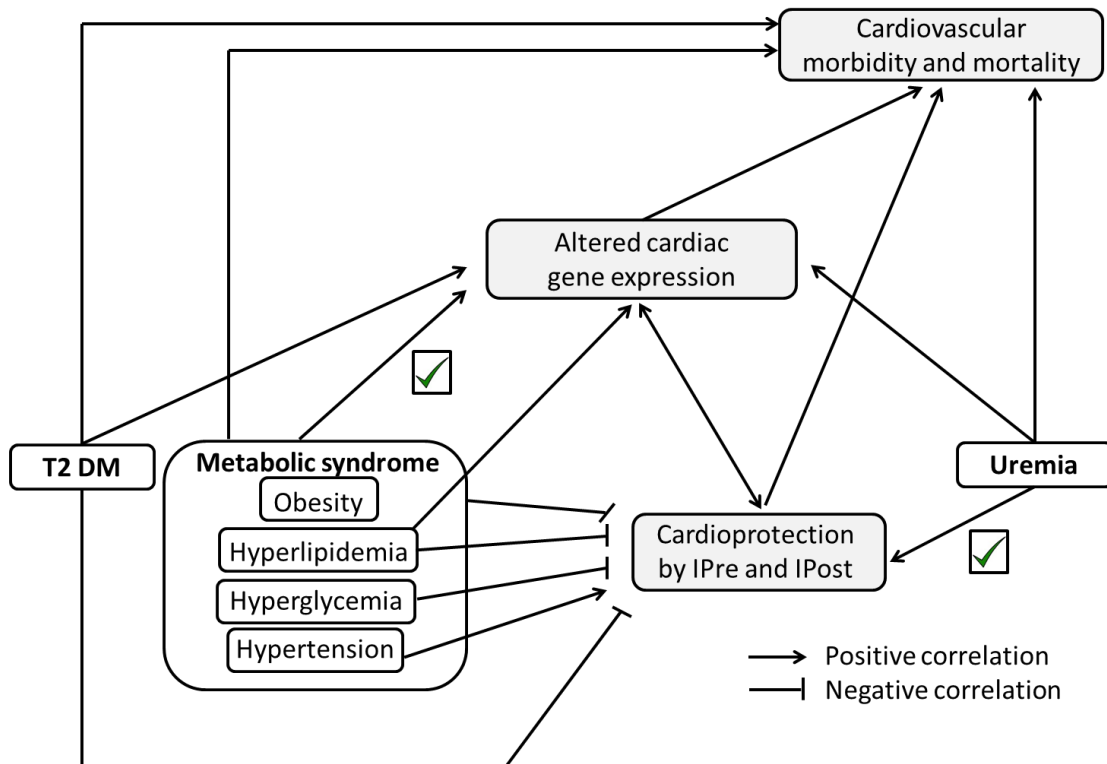
2. Figure Conclusion of the present thesis: Metabolic syndrome alters cardiac gene expression pattern and cardioprotection is preserved by ischemic preconditioning (IPre) in prolonged uremia. (Ischemic postconditioning (IPost), T2DM type 2 diabetes mellitus).

6   LIST OF ABBREVIATIONS

ANOVA: analysis of variance , **CKD**: chronic kidney disease, **CVD**: cardiovascular disease, **DNA**: deoxyribonucleic acid, **ELISA**: enzyme linked immunosorbent assay, **HOMA-IR**: homeostasis model assessment-estimated insulin resistance, **IHD**: ischemic heart disease, **IPre**: ischemic precondonditioning, **IPost**: ischemic postconditioning, **I/R**: ischemia/reperfusion, **OGTT**: oral glucose tolerance test, **qRT-PCR**: quantitative real time polymerase chain reaction, , **T2DM**: type 2 diabetes mellitus, **ZDF**: Zucker Diabetic Fatty

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8 LIST OF PUBLICATIONS

1. The subject of the thesis is based on the following full papers:

  I.   Kocsis GF, <u>Sárközy M</u>, Bencsik P, Pipicz M, Varga ZV, Pálóczi J, Csonka C, Ferdinandy P, Csont T. *Preconditioning protects the heart in a prolonged uremic condition.* **Am J Physiol Heart Circ Physiol.** 2012, **303**:H1229-1236, **IF: 3.629**

  II.   <u>Sárközy M</u>, Zvara Á, Gyémánt N, Fekete V, Kocsis GF, Pipis J, Szűcs G, Csonka C, Puskás LG, Ferdinandy P, Csont T. *Metabolic syndrome influences cardiac gene expression pattern at the transcript level in male ZDF rats.* **Cardiovasc Diabetol.** 2013, **12**:16, **IF: 4.21**

  Cumulative impact factor of papers the thesis based on: 7.839

2. Other full papers published during the Ph.D. fellowship:

  I.   Monostori P, Kocsis GF, Okrös Z, Bencsik P, Czétényi O, Kiss Z, Gellén B, Bereczki C, Ocsovszki I, Pipis J, Pálóczi J, <u>Sárközy M</u>, Török S, Varga IS, Kiss I, Fodor E, Csont T, Ferdinandy P, Túri S. *Different administration schedules of darbepoetin alfa affect oxidized and reduced glutathione levels to a similar extent in 5/6 nephrectomized rats.* **Clin Exp Nephrol.** 2013, 17:569-574, **IF: 1.246**

  II.   Fekete V, Murlasits Z, Aypar E, Bencsik P, <u>Sárközy M</u>, Szénási G, Ferdinandy P, Csont T. *Myocardial postconditioning is lost in vascular nitrate tolerance.* **J Cardiovasc Pharmacol.** 2013, **62**:298-303, **IF: 2.290**

  III. Csont T, <u>Sárközy M</u>, Szűcs G, Szűcs C, Bárkányi J, Bencsik P, Gáspár R, Csonka C, Kónya C, Ferdinandy P. *Effect of a multivitamin preparation supplemented with phytosterol on serum lipids and infarct size in rats fed with normal and high cholesterol diet.* **Lipids Health Dis.** 2013, **12**:138, **IF: 2.19**

  Cumulative impact factor of papers published during the Ph.D. fellowship: 5.726

  Total impact factor: 13.565