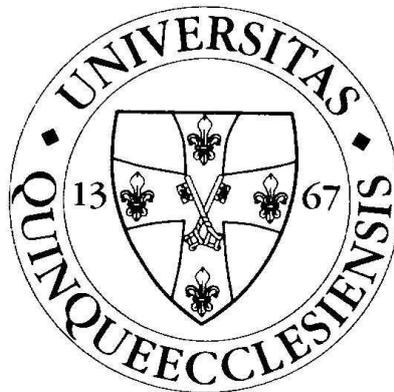


# **The role of glutathione S-transferase in myocardial ischaemic-reperfusion pathways and endogenous adaptation responses**

Summary of PhD Thesis

Viktória Kovács, MSc



University of Pécs, Faculty of Medicine  
Department of Surgical Research and Techniques

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## 1. INTRODUCTION

Myocardial ischemia-reperfusion injury is crucially involved in the pathogenesis of cardiovascular diseases. It has been well investigated that oxidative stress following ischaemic-reperfusion injury is a major apoptotic stimulus in many cardiac diseases. Under normal circumstances the endogenous antioxidant systems neutralise the harmful effects of free radicals. Pathophysiological conditions (such as hypoxia, ischemia, early reperfusion, etc) increase the amount of nascent oxygen free radicals and reactive oxygen intermediers (ROI) beyond the capacity of endogenous antioxidants and the oxidative stress develops.

Although recently surgical interventions more frequent in myocardial diseases, but to avoid the ischemic-reperfusion injury is not solved. The role of oxygen free radicals in reperfusion injury is well known, accordingly to decrease of these harmful agents is very important. Catalase, superoxide dismutase (SOD), glutathione peroxidase and repair enzymes are in the first line of antioxidant protection, but recently among other antioxidant enzymes researches are focus on glutathione S-transferase (GST).

Myocyte loss during ischemic-reperfusion injury involves both apoptotic and necrotic cell death. Therefore, it is reasonable to think that the balance of cell survival and death is critical during the pathological evolution of postischemic cardiac dysfunction. The intricate relationship between signal transduction and this balance of survival and death in oxidative stress and ischemic-reperfusion injury make the investigations necessary to be focused on MAP kinases and RISK pathways.

### 1.1. GLUTATHIONE AND GLUTATHIONE S-TRANSFERASE

Glutathione (GSH) is the predominant low-molecular-weight thiol (0.5–10 mmol/L) in cells. Most of the cellular GSH (85–90%) is present in the cytosol, with the remainder in many organelles (including the mitochondria, nuclear matrix, and peroxisomes).

Because of the cysteine residue, GSH is readily oxidized nonenzymatically to glutathione disulfide (GSSG) by electrophilic substances (e.g., free radicals and reactive oxygen/nitrogen species).

GSH/GSSG is the major redox couple that determines the antioxidative capacity of cells. It is important to note that shifting the GSH/GSSG redox toward the oxidizing state activates several signaling pathways (including protein kinase B, nuclear factor  $\kappa$ B, c-Jun N-terminal kinase, mitogen-activated protein kinase (MAPK) and Reperfusion Injury Salvage Kinase (RISK)), thereby reducing cell proliferation and increasing apoptosis.

A major factor that affects glutathione homeostasis is its utilization by conjugation, primarily via glutathione S-transferase (GST).

GSTs are members of a multigene family of isoenzymes ubiquitously expressed in most living organisms. It was subsequently shown that these enzymes catalyze the conjugation of GSH to a variety of electrophilic compounds, thus establishing the now widely accepted role of GSTs as cell housekeepers involved in the detoxification of endogenous as well as exogenous substances.

The GSTs encompass three major families of proteins: (1) cytosolic, (2) mitochondrial, and (3) microsomal, of which the cytosolic GSTs constitute the largest family. On the basis of amino acid sequence similarities, substrate specificity, and immunological crossreactivity, seven classes of cytosolic GSTs have been identified in mammals. These classes are designated by the names of the Greek letters  $\alpha$  (alpha),  $\mu$  (mu),  $\pi$  (pi),  $\sigma$  (sigma),  $\theta$  (theta),  $\omega$  (omega), and  $\zeta$  (zeta).

The ability of GST to alter levels of cellular glutathione in response to production of ROS has been implicated in protection of cells from ROS-inducing agents.

## **1.2. ISCHAEMIC/REPERFUSION INJURY**

Myocardial reperfusion is the restoration of blood flow to an ischemic heart. Early reperfusion minimizes the extent of damage of heart muscle and preserves the pumping function of the heart. However, after a prolonged period of ischemia reperfusion produces a marked damage in myocardium rather than restoration of normal cardiac function. This reoxygenation injury is mediated by a burst of ROS production. Thus, ischemia–reperfusion (I/R) injury could be defined as the damage to heart when blood supply is restored after a prolonged period of ischemia resulting in oxidative damage, inflammation and cardiac dysfunction. Reperfusion injury is an integrated response to the restoration of blood flow after ischaemia involving mechanical, extracellular and intracellular processes.

## **1.3. ISCHAEMIC POSTCONDITIONING**

Postconditioning (PC) is controlled reperfusion, defined as a series of brief interruptions of I/R applied at the very onset of reperfusion can protect the myocardium from I/R injury. The concept of PC was first revealed in 2002 by Vinten-Johansen and colleagues. The first studies published by Zhao et al., (2002).

## **1.4. CORONARY ARTERY BYPASS GRAFTING AND PERIOPERATIVE MYOCARDIAL INFARCTION**

Over the last decade, elective coronary artery bypass grafting (CABG) is the main revascularization modality in the treatment of the patients with multivessel coronary artery disease (CAD) and has progressed to a very standardized and safe surgical procedure with low mortality and low rates of myocardial, neurologic, renal and other adverse events. Perioperative myocardial infarction (PMI) is the most common cause of morbidity and mortality, it means a serious complication following CABG surgery.

## **2. AIMS AND HYPOTHESIS**

**In the first part** our aim was to identify the biological role of GST and assess the effect of GST inhibition (using its potent inhibitor, ethacrynic acid [EA]) by using an in vivo myocardial infarction model on rat.

- Firstly it was targeted to investigate the effect of GST inhibition on myocardial tissue damage and oxidative stress parameters.
- It was aimed to observe the alteration of proteins, MAP kinase and RISK pathways and finally cardiomyocyte apoptosis in case of occurrence of myocardial infarction.
- We targeted to investigate the effect of inhibited GST to the favorable effects of PC.

**In the second part** of our investigations we aimed to determine the existence of association between the genetic polymorphisms of metabolizing genes GSTP1, GSTM1, and GSTT1 and the presence of perioperative acute myocardial infarction in a cohort of patients undergoing cardiac surgery with cardiopulmonary bypass.

The variant GST alleles may increase susceptibility due to decreased detoxifying potential. Thus assumed that the differences of activity in the detoxification enzyme GST is associated with cardiovascular disease (PMI).

### **3. THE ROLE OF GLUTATHIONE S-TRANSFERASE IN AN IN VIVO ACUTE MYOCARDIAL INFARCTION RAT MODEL**

#### **3.1 INTRODUCTION**

A large collection of experimental data support the presence of apoptosis in a variety of cardiovascular diseases. It has also been well investigated that oxidative stress is a major apoptotic stimulus in many cardiac diseases. Among numerous defence mechanisms against oxidative injury, GST plays a crucial role. The GST family, which comprises a relatively high amount of total cytosolic protein, is responsible for the high capacity metabolic inactivation of electrophilic compounds and toxic substrates. Thus, GSH homeostasis is essentially regulated by GST activity, and the GSH redox status is critical for various biological events. Recently, novel roles for GSH homeostasis and GST in signal transduction, gene expression, apoptosis, protein glutathionylation, nitric oxide metabolism and inflammation have been discussed. It is important to note that alterations of cellular-reduced GSH metabolism and activity of GST can influence several signalling pathways. Certain types of GST play a key role in regulating MAP kinases and RISK pathways involved in the cellular response to stress, apoptosis and proliferation, influencing the decision regarding cell fate. At least five of the human GST genes display functional polymorphisms. These polymorphisms are likely to contribute to interindividual differences in response to xenobiotics and clearance of oxidative stress products and, therefore, may determine susceptibility to various inflammatory pathologies including cancer, and cardiovascular and respiratory diseases. In addition, some GST polymorphisms have also been associated with increased risk of lung adenocarcinoma. Recent studies highlight the potentially unique roles of GST enzymes as crucial determinants of the development of I/R. An association was found between different donor GST genotypes and primary graft dysfunction in patients following heart and lung transplantation. Other studies described the damaging effect of GST inhibition on peripheral and central motor neurons, cerebral astrocytes, isolated hepatocytes and vascular smooth muscle cells. Although the effect of GSH depletion in cardiomyocytes has been well described to be a result of different pathological states, the exact role of GST activity on cardiomyocyte apoptosis and alteration of signalling cascades of cardiomyocytes has not been determined.

#### **3.2. AIMS**

In present study we aimed to investigate the biological role of GST in heart under acute myocardial I/R. Because GST activity have adaptive response to oxidative stress in the heart, thus we hypothesized that GST inhibition via administration of EA might aggravate the severity and outcome of AMI. It was also aimed to determinate the effect of GST inhibition on postcondition. Furthermore it was hypotetised that alterations in MAPK (p38, GSK-3 $\beta$  and ERK) and Bcl-2 gene family (Bad), GST activity caused by inhibition of GST cause altered ischemic tolerance and postcondition in myocardium.

#### **3.3. MATERIALS AND METHODS**

##### **3.3.1 Animal model and surgical preparation**

70 male Wistar rats, weighing between 200-250 g were used in the present study from Charles River Breeding Laboratories (Hungary, Isaszeg).

The animals were anaesthetized with an intraperitoneal injection of ketamine hydrochloride and diazepam. The ratio was 1:1 (0,2 ml / 100 g) and the animals were placed

on a heating pad. The skin was disinfected and fine isolation a tracheotomy was performed. The lungs were ventilated at a frequency of 60-65 breaths/min and a tidal volume of 3-4 ml. ECG was placed and the carotid artery was catheterized for blood pressure measurement. The chest was opened by midline sternotomy. A 5-0 prolene ligature was passed around the left anterior descending (LAD) coronary artery and through a snare. In general the site of vessel encirclement was on the long axis of the left ventricle towards the apex approximately one-fourth of the distance from the atrioventricular groove to the left ventricular apex. Temperature was measured inside the pericardial cradle and maintained between 38,3°C and 38,7°C by adjusting a heating pad. The chest was then closed and the wound was covered with warm, wet compress to minimize heat and fluid losses. The ligature was after 40 minutes removed and the LAD was reperfused for 120 minutes. The vena cava preparation was performed for collecting blood samples.

### **3.3.2 Effect and administration of ethacrynic acid**

Present study used EA for pharmacological inhibition of GST. EA, a diuretic, inhibits GST in two ways. It has been shown to be a substrate of GST enzymes, on the other hand nonenzymatic GSH conjugation of EA also proved. Moreover this EA-GSH complex is an inhibitor of GSTs as well due to its stronger affinity for the enzymes. On the other hand EA itself inhibits GST through reversible covalent binding.

We recalculated the human diuretical dose of EA to rat. 86 mg of EA was suspended in 2 ml of 96% ethanol and 8 ml of saline solution and the concentration was 8,6 mg/ml. The dose of EA was 8,6 mg/kg and the solution was injected to the animals intraperitoneally 24 hours and 1 hour before the operation.

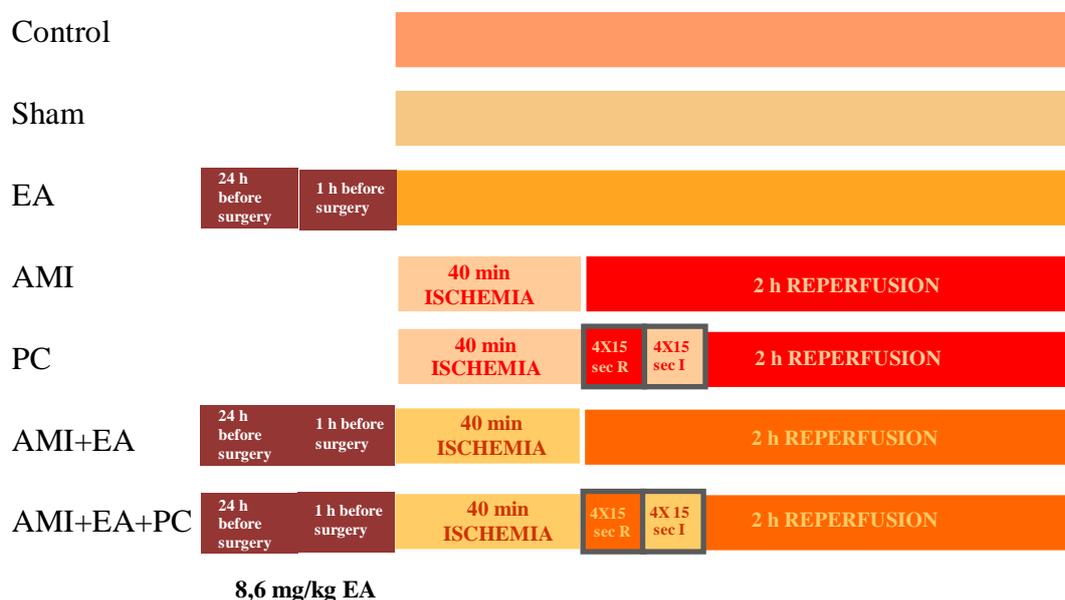
### **3.3.3 Protocol of ischaemic postconditioning**

In the ischaemic PC group and in the ethacrynic acid treated PC group, after the 40 minutes ischaemic phase 15 seconds reperfusion – 15 seconds ischaemic periods were used for 4 times. At the end of the 2-min postconditioned period of coronary artery, the suture was released and removed to provide the appropriate reperfusion.

### **3.3.4. Experimental protocol**

In our experiments the animals (70 rats) were divided into 7 groups (10 animals in each group). In control animals (group 1; control) the heart was excised right after thoracotomy. In the sham operated rats (group 2; sham) after the thoracotomy we opened the chest by midline sternotomy for two hours without intervention. Rats in the third group (group 3; EA) were treated with EA as described before. In the fourth group (group 4; AMI) after we closed the LAD for 40 minutes applied 2 hours reperfusion. Next group was the PC group (group 5, PC) as previously discussed. Rats in the sixth group (group 6; AMI+EA) underwent EA treatment combined with LAD occlusion and two hours reperfusion procedure. Finally, in the seventh group (group 7; AMI+EA+PC) the PC was applied following a pretreatment with EA. (Figure 1.)

**Figure 1.** Experimental protocol of rats



Peripheral blood samples were collected from the animals at the end of the reperfusion phase. The serum samples were harvested and stored at minus 80°C until biochemical assays. After the experimental period the heart was rapidly excised and rinsed in ice-cold physiological saline. The ischaemic zone was excised on the basis of the previously defined landmarks. The tissue was snap frozen in liquid N<sub>2</sub>, and stored for not more than 3 days at -82C before Western-blot analysis. Another part of the ischaemic zone was fixed with 10% neutral buffered formalin.

Measuring methods were as follows: ECG, heart rate and systemic blood pressures were monitored and recorded every 10 minutes during the whole procedure, tetrazolium staining which allows the early detection of myocardial infarction in a whole heart, quantification of plasma GST enzyme and oxidative stress parameters: analysis of malondialdehyde (MDA), reduced glutathione (GSH) and plasma thiol (SH) groups, superoxide dismutase (SOD) activity, serum myeloperoxidase, serum TNF-alpha and IL-1 quantification and western-blot analysis of pro/antiapoptotic (p38, ERK, Bad, GSK3β) signaling pathways.

### 3.3.5. Statistical analysis

All values are expressed as means ± standard error of the mean (S.E.M). Statistical comparisons include two samples Student t-test and one-way analysis of variance (ANOVA). p<0.05 was considered significant. Data presentation of Western blots are representative of series with similar results. Densitometric values are the mean±S.D. for the indicated number of independent experiments. Significance of differences was determined using ANOVA testing applying Bonferroni corrections for multiple samples. P values <0.05 were considered to be significant.

### **3.4. RESULTS**

#### **3.4.1. Haemodynamics**

Changes in heart rate did not show any significant fluctuations, even though most frequent rhythm disturbances were noted during early reperfusion. Similarly systolic and diastolic blood pressures only showed slight variations, however none of the changes were statistically significant.

#### **3.4.2. Measuring infarct size by the tetrazolium method**

The infarct size was significantly different in every groups (PC,AMI+EA,AMI+EA+PC) comparing to the AMI group. PC limited the infarction in groups PC and AMI+EA+PC compared to the groups AMI and AMI+EA. The infarction size was significantly higher in the presence of EA in the AMI+EA+PC group compared to the PC.

#### **3.4.3. Quantification of plasma GST enzyme**

The GST concentration in the plasma was significantly lower in AMI+EA and EA groups comparing to the control group. In the PC group there was a significantly raising of plasma GST compared to the AMI group. Our data showed significant differences between PC and AMI+EA+PC groups. GST level was significantly lower in the presence of EA.

#### **3.4.4. Malondialdehyde and plasma malondialdehyde levels**

In our experiment we measured the values of MDA level indicating membrane damage and lipidperoxidation. The MDA concentration was significantly higher in five groups (EA, AMI, PC, AMI+EA, AMI+EA+PC) comparing to the control group. Our data showed significant differences between PC and AMI+EA+PC groups. MDA level was significantly higher in the AMI+EA+PC group. We found significantly lower level of MDA in AMI+EA+PC group comparing to the AMI+EA group.

Compared the plasma malondialdehyde values we found the same significances. In the PC group there was a strongly significant decrease of plasma MDA compared to the AMI group. The membrane-damaging effect of AMI was significantly elevated in the presence of EA.

#### **3.4.5. Reduced glutathione levels (GSH)**

The values of reduced GSH levels were significantly lower in two groups (PC, AMI+EA) comparing to the control group. In postconditioned EA treated (AMI+EA+PC) group the values were significantly higher than in non-conditioned EA (AMI+EA) group, however this protecting factor of postconditioning between the similar groups in the presence of EA was not significant.

#### **3.4.6. Plasma thiol-groups (-SH)**

We detected in the AMI+EA group significantly lower levels of -SH comparing to control group or compared to the postconditioned AMI+EA group. We found in the AMI+EA and AMI+EA+PC groups lower values than in similar groups without administration of EA (AMI, PC).

#### **3.4.7. Enzyme activity of superoxide dismutase (SOD)**

The SOD concentration was significantly higher in two groups (sham, AMI+EA+PC) and besides significantly lower in two groups (EA, AMI+EA) comparing to the control group. Our data showed significant differences between AMI and AMI+EA groups. SOD level was significantly higher in the absence of EA. We found a strongly significant correlation between the AMI+EA and AMI+EA+PC groups. The amount of SOD were sharply increased when the animals were postconditioned.

#### **3.4.8. Serum myeloperoxidase results**

To characterize the neutrophil activation we measured the plasma myeloperoxidase (MPO) level and the induced ROS production of leukocytes. MPO level increased significantly in every group compared to the control group. In the postconditioned group there was significantly lower level of MPO, in the EA treated AMI group a strongly significantly higher level of MPO compared to the AMI group. To compare the values of PC and EA-treated postconditioned (AMI+EA+PC) groups we found a significant difference. When we were postconditioning the EA treated AMI group, it was seen a strongly significantly lower MPO level.

#### **3.4.9. Serum TNF-alpha levels**

In the study we measured the TNF-alpha levels in the groups. The values were significantly higher in five groups (EA, AMI, PC, AMI+EA, AMI+EA+PC) than in the control group. The EA treatment itself caused damage moreover the protecting effect of PC was not marked in the presence of EA.

#### **3.4.10. Serum interleukin-1 (IL-1)**

We investigated the serum IL-1 levels in our groups. The values were significantly higher in three groups (EA, AMI+EA, AMI+EA+PC) than in the control group. These groups are all EA treated groups. When this EA treatment was combined with AMI, the significance was even more apparent compared to the control. Postconditioning did not temper significantly that elevation of IL-1 level if the animals were co-treated with EA administration.

#### **3.4.11. The western-blot analysis of pro/antiapoptotic signaling pathways**

The activation of p38 MAPK phosphorylation was significantly higher in four groups (AMI, EA, AMI+EA, AMI+EA+PC) comparing to the control group. Postconditioning limited the phosphorylation of p38 in groups PC and AMI+EA+PC compared to the groups AMI and AMI+EA. The presence of EA increased the level of activated p38 in the AMI+EA and AMI+EA+PC groups compared to the AMI and PC groups. Significant decrease in p38 MAPK activation was found in the EA and PC treated group compared to the AMI group.

ERK phosphorylation strongly increased in every group compared with the control group, except the sham-operated group where weak increase can be observed. In the PC and AMI+EA group there was a significantly raising of activation of ERK compared to the AMI group. Our data showed significant differences AMI, PC and AMI+EA groups compared to the AMI+EA+PC group. The active phosphorylated form of ERK was significantly lower when AMI suffered animals were cotreated with EA and PC.

In this case we measured the values of phosphorylated Bad level. Proapoptotic activity of Bad is inhibited through its phosphorylation. The p-Bad concentration was significantly different in five groups (AMI, PC, AMI+EA, EA, AMI+EA+PC) comparing to the control group. Our data showed significant differences between AMI and AMI+EA and between AMI and AMI+EA+PC groups. In the presence of EA there was a decreased phosphorylation observed which has worsened the cell survival. On the contrary the postconditioning increased the p-Bad compared to the AMI group. We found significantly lower level of p-Bad in AMI+EA+PC group comparing to the PC group. The protective effect of PC seems to be weaker in the present of EA.

The GSK-3 $\beta$  concentration was significantly higher in five groups (EA, AMI, PC, AMI+EA, AMI+EA+PC) comparing to the control group. Our data showed significantly lower level of GSK-3 $\beta$  in AMI+EA+PC group comparing to the AMI+EA group.

### **3.5. DISCUSSION**

Measuring the plasma GST levels we were able to demonstrate that EA is working as an effective pharmacological inhibitor of GST and could markedly magnify oxidative stress-induced apoptosis in our in vivo model. It was reported double stress when animals simultaneously underwent a myocardial infarction. Our results were more confirmed by the measurement of total infarct size. The infarction size was significantly higher in the presence of EA while PC limited the extent of infarcted area. Using GST inhibition during AMI the protective effect of postconditioning is lost.

We have found that myocardial infarction caused I/R cause increased oxidative stress parameters which was further increased by administration of EA. The positive effect of ischaemic PC have been detected through the investigation of oxidative parameters and this positive effect has seen in the presence of EA (AMI+EA+PC). The increased level of ROS and a more disadvantageous GSH state may overact the intensity of insult and may explain the increased amount of apoptotic cells in GST-inhibited groups during myocardial I/R. Our results indicate that GST inhibition itself is able to increase the levels of the proinflammatory cytokines and the protective effect of PC can not prevail.

GSTs play an important role as antioxidant enzyme modulating MAPK and RISK pathways moreover GST inhibition was associated with increased activation of MAP and RISK kinases under stress conditions. Many studies have suggested that the MAPKs may be important regulators of apoptosis in response to myocardial I/R. In addition to this the present study showed that inhibition of GST by EA leads to increased phosphorylation of p38 proapoptotic and antiapoptotic ERK signaling proteins, increase in GSK-3 $\beta$  activation and decreased phospho Bad activation during myocardial I/R and what is more it was able to abolish the protective effect of PC. These findings represent the association between MAP kinases and GST during myocardial stress.

Our study has been conducted to examine the biological role of GST in vivo myocardial infarction rat model. We found that pharmacological inhibition of GST by EA augments the apoptosis as a result of oxidative stress and myocardial ischaemic injury. The study showed that GST inhibition was associated with increased activation of MAP and RISK kinases under stress condition.

## **4. POLYMORPHISMS IN GLUTATHIONE S-TRANSFERASE ARE RISK FACTORS FOR PERIOPERATIVE ACUTE MYOCARDIAL INFARCTION AFTER CARDIAC SURGERY**

### **4.1. INTRODUCTION**

For revascularisation of multiple coronary artery disease CABG remains the primary procedure of choice /treatment option. Despite the improvement in surgical, anaesthetic technique and treatment modalities CABG is frequently complicated by PMI. Multiple mechanisms play a role and interplay in pathomechanism of PMI, such as I/R during and following aortic cross clamping, furthermore cardiac manipulation, inadequate myocardial protection, and intraoperative defibrillation, and acute occlusion of bypass grafts. On the other hand primary mechanisms in the pathogenesis of perioperative myonecrosis is associated with further factors, like complex acute inflammatory response, global ischemia, manipulation. Recent studies have highlighted the significance of inter-personal variability of PMI suggesting the role of genetic, hereditary component in pathomechanism. Oxidative stress may occur as a consequence of multiple mechanisms during cardiac surgery resulting in myocardial injury. Among antioxidant enzymes eliminating the potential harm of reactive oxidants, the GSTs may play crucial part.

The GSTP1 isoform is one of the most investigated isoenzyme which is widely expressed in different human epithelial tissues and GSTP1 is predominantly expressed in myocardial cells compared with other isoenzymes. The gene of GSTP1 located on chromosome 11 has a wild type form named as GSTP1\*A (Ile105Ile/Ala113Ala) and two other single nucleotide polymorphic forms named as GSTP1\*B (Ile105Val/Ala113Ala) and GSTP1\*C (Ile105Val/Ala113Val). These are designated as A, B and C alleles that are expressed in various homozygous and heterozygous combinations modifying the activity of enzyme.

For both the GSTM1 and GSTT1 enzymes, the defect is due to a deletion of the entire genomic coding region, giving rise to the allele known as “null.” The deletion in a homozygous state determines the polymorphism called “null genotype,” with the consequent absence of total enzymatic activity.

Polymorphism altering or reducing these enzyme detoxification activities could increase a person’s susceptibility to pathologies mediated by oxidative processes.

In the present study it has been hypothesised that SNPs in GSTs are associated with the incidence of postoperative myocardial infarction in a cohort of patients undergoing cardiac surgery with cardiopulmonary bypass (CPB).

### **4.2. MATERIALS AND METHODS**

#### **4.2.1. Study design**

Between the period from January 2010 to July 2010 in Department of Cardiac Surgery, Zala County Hospital and from April 2010 to September 2010 in Heart Institute, University of Pécs patient subjected to open heart surgery were prospectively enrolled in the study. Exclusion criteria before enrolment were the following: perioperative MI, impaired left ventricular function, ejection fraction less than 40%, unstable angina, recent or occurring AMI (in 6 month), preoperative ischemia, acute heart failure, left ventricular end diastolic dimension (LVESD)>40mm, significant hypertrophy (septal, posterior wall diameter>15mm, left ventricular mass index>130g), and renal insufficiency. Total of 758 patients were enrolled for further assessment. Patients underwent first time elective CABG surgery on CPB. Eight and twenty four hours after surgery blood samples were taken for assessment of plasma

myocardial based creatinin kinase (CKMB) and troponin I (TI) levels. Patients with postoperative renal insufficiency, prolonged intubation, hypoxia, prolonged postoperative hypotension, excessive bleeding, ECG abnormalities referring regional ischemia, postoperative regional wall motion abnormality on echocardiography, graft occlusion and assessed by coronarography and those patients who undergone an operation with prolonged aortic cross clamp and extracorporeal circulation ( 90 and 120 min.), or receive more than 2 units of RBC transfusion, were excluded from study. Patients were divided into two groups: Group I: control patients (n=78) without any evidence of AMI in the postoperative period, those patients had less than 2.5 times elevation of CKMB or TI level of of upper limit of reference range as assessed by any of blood samples during the first postoperative 24 hours. Group II.: PMI patients (n=54) more than 5 times elevation of cardiac markers in 24 h following surgery, without any evidence of regional ischemia, or prolonged ischemia during surgery as described above in exclusion criteria. Blood samples were taken from both groups for genotype analysis. Ultimately 132 patients ( 89 male and 43 female, mean age 59,84 years) were selected for the trial.

#### **4.2.2. Genotype analysis**

The molecular analyses were performed using DNA extracted from peripheral EDTA-anticoagulated blood leukocytes with a routine salting out procedure. TaqMan SNP genotyping assay procedure were employed to analyze the GSTP1 gene region. Taqman Assays was used with standard PCR conditions.

TaqMan CNV genotyping assay procedure were performed to investigate the GSTM1 and GSTT1 regions. The analyzed chromosome location can be found at Chr.1:110231516 for GSTM1 and Chr.22:24376493 for GSTT1. The cytobands of GSTM1 and GSTT1 can be located at 1p13.3b and 22q11.23b positions. The amplication lengths were 100 and 82 nucleotids.

54 patients with AMI after open heart surgery and 78 patients without AMI after the open heart surgery were genotyped for polymorphism in the GST isoform P1, M1 and T1. In the case of GSTP1 there are 3 polymorphic GSTP1 alleles, GSTP1\*A, GSTP1\*B, and GSTP1\*C expressed in normal cells.

In contrast the GSTM1 and GSTT1 null phenotype appears to be caused by the deletion (two null alleles; -/-) of the GSTM1 or GSTT1 genes. Gene deletion in only one allele (+/-) also causes enzyme activity to decrease, so we could assume that this deletion also could be involved in PMI. We are talking about full enzyme activity in the case when both of the alleles are consummate (+/+).

The presence of genetic polymorphism that decrease or absence activity of the detoxification enzyme GST may contribute to heart disease and affect biomarkers of coronary health and oxidative stress.

#### **4.2.3. Statistical analysis**

All clinical data are presented as mean  $\pm$  standard error of the mean (SEM) where appropriate. Differences between the clinical parameters in patients with PMI and the control subjects were assessed with chi square ( $\chi^2$ ) test or the Mann-Whitney tests. Frequencies of different genotypes in PMI versus control groups were analyzed by  $\chi^2$  test. Odds ratios (ORs) originated from multiple logistic regression were used to determine whether the presence of the significant genotypes of the GSTP1-related variants were associated with the risk of the occurrence of PMI. The 95% confidence intervals (95% CI) for all ORs were calculated with

SPSS 20.0 package for Windows (SPSS Inc, Chicago, IL, USA) and were considered significant when P-value was less than 0.05.

### 4.3. RESULTS

There was no significant difference between groups regarding gender and age, preoperative risk factors and postoperative bleeding. Intraoperative factors such as aorta cross clamp time (ACCT), and CPB times, operation time were not significantly different between groups.

#### 4.3.1. Genotype analysis of GSTP1

There was no difference in the allele frequency of homozygous AA and the heterozygous AB and BC alleles between the groups, while it was almost three times elevated heterozygous AC allele combination observed in the patients who suffered PMI. There was 14% allele frequency of homozygous BB allele combination measured in the control group meanwhile in the PMI group BB allele combination was not present. (**Table 1.**)

It can be stated that in the presence of the A allele there is a significant difference between groups. This association is more markedly after adjusting the multiple regression analysis for age and gender (OR adjusted for differences in age and gender: 14.905 (1.859–119.495);  $p = 0.011$ ). The presence of A allele is presented as a risk factor. However we failed to evidence the statistically significant protective effect when B allele is present (OR adjusted for differences in age and gender: 0.610 (0.293–1.269);  $p = 0.186$ ). (**Table 2.**) Therefore it is possible that the A allele may have predisposing factor to PMI.

**Table 1** Genotype frequencies in PMIs and controls.

<b>PMI group (n=54)</b>			
	<b>A</b>	<b>B</b>	<b>C</b>
<b>A</b>	26 (48.1%)	17 (31.5%)	10 (18.5%)
<b>B</b>	-	-	1 (1.9%)
<b>C</b>	-	-	-
<b>control group (n=78)</b>			
	<b>A</b>	<b>B</b>	<b>C</b>
<b>A</b>	37 (47.4%)	20 (25.6%)	6 (7.7%)
<b>B</b>	-	11 (14.1%)	3 (3.8%)
<b>C</b>	-	-	1 (1.3%)

**Table 2** Multiple logistic regression analysis of disease risk of investigated variants from the perspective of haplotype carriers.

<b>Adjusted OR* frequency</b>		
<b>A</b>	<b>B</b>	<b>C</b>
<b>(AA+AB+AC)</b>	<b>(AB+BB+BC)</b>	<b>(AC+BC+CC)</b>
<b>14.905*</b>	0.610	1.755
<b>(1.859-119.495)</b>	(0.293-1.269)	(0.681-4.520)
<b>p= 0.011</b>	p=0.186	p=0.244

P<0.05 versus controls

OR\*- Adjusted for differences in age and gender.

The 95% confidence intervals (95% CI) for all ORs were calculated.

### 4.3.2. Genotype analysis of GSTM1

The analysis for GSTM1 gene showed that in the PMI group 8 of 54 samples (14,8%) analyzed showed the null genotype, whereas the remaining 85.2% (46 of 54) carry one or two copies of the allele. In the control group was the same, namely 7 of 78 samples (9,0%) carried the null genotype and 71 of 78 (91,0%) have one or two copies of the allele.

Using a statistical method, we found no significant correlation between the groups (P = 0.303).

### 4.3.3. Genotype analysis of GSTT1

Of 78 control patients, 21 (26.9%) were homozygous for the GSTT1 null allele. In contrast, 57 cases of the samples carrying the gene in hetero- or homozygosity (73,1 %) showed a stable GSTT1 genome (without detection of mutations or instability). In the PMI group this rate was 6 of the 54 patients (11.1%), while the hetero- or homozygous carrying was 46 patient (85,2%) and there was 2 (3,7%) missing data, where our measurement was unsuccessful. This opposite values of observed percentages indicate that the GSTT1 null genotype may be correlated with the absence of PMI occurrence after cardiac surgery. Evaluating this possibility with statistical methods, we observed that, effectively, there was a positive significant association (P=0,039) for the events mentioned above (**Table 3**). Moreover, the OR value obtained (OR = 0,354 [0,132–0,950]; 95% CI), confirms the correlation between the homozygous deletion of the gene as a protective factor for PMI incidence.

**Table 3** Multiple logistic regression analysis of disease risk of GSTT1.

<b>Not adjusted</b>	<b>Adjusted</b>
<b>OR frequency</b>	<b>OR* frequency</b>
<b>GSTT1</b>	<b>GSTT1</b>
0.354*	0.317*
(0.132-0.950)	(0.116-0.869)
p=0.039	p=0.026

P<0.05 versus controls

The 95% confidence intervals (95% CI) for all ORs were calculated.

#### **4.4. DISCUSSION**

PMI is one of the most frequent and important complication of cardiac surgery and its clinical risk factors are well described. The relative high inter-patient variance in occurrence of PMI independently of described risk factors may suggest the role of genetic predisposition. In our present study, for better differentiation between PMI and control groups, the patients with well known clinical risk factors for PMI were excluded thus conducting the further, genetic investigation in 132 patients among 750 consecutive, patients undergoing elective, first time cardiac surgery enrolled in our study.

PMI as a complication of cardiac surgery is associated with significant mortality and morbidity. Besides of classic, well-known risk factors of PMI, such as prolonged CPB or ACCT, longer surgery duration, preoperative, ongoing myocardial ischemia and inadequate revascularization, graft occlusion, unstable angina, emergent operation, other factors and genetic predisposition are expected to postoperative myocardial damage. In the present study we investigated the genetic contribution of GSTP1, GSTM1 and GSTT1 to occurrence of PMI. Our results suggest that the genetic polymorphism of GSTP1 is independently associated with the presence of PMI. The appearance of homozygous BB allele combination was significantly higher in the control group. In present paper it is suggested that the existence of the A allele carries complication for PMI. Although in the present report we mentioned that GSTM1 null genotype could be a risk factor for PMI presence, our results have shown that the GSTM1 null genotype (P = 0.303) have not significant association with the presence of PMI in CPB patients. A possible explanation is that, although expression of these genes was detected in the mammary tissue, the encoded enzymes only weakly expressed in cardiac muscle. This allows us to conclude that the action of metabolites is not mediated and/or regulated by this enzyme on a local level. So, the presence or absence of GSTM1 in PMI patients was independent from each other.

Our results have identified that GSTT1 null genotype play a protective role against PMI (OR:0.354, p=0.039).

The major limitations of this clinical part are the relatively small sample size which limits significant conclusions. The size of groups might be increased to improve the statistical power of the study. On the other hand it would be advantageous to follow up the patient in longer term for assessing the late complications associated with GST polymorphisms. Furthermore the enzymatic activity of GST was not measured in association of polymorphisms in groups.

In conclusion, genetic variants in GSTP1 are independently associated with an increased risk of PMI after surgical coronary revascularization. Our results highlight the potential genetic

role in etiology of PMI following cardiac surgery. To the best of our knowledge, this is the first study that has investigated the role of GST polymorphisms with PMI and we found GSTP1 A haplotype as a predisposing factor and identified BB homozygous allele combination as a possible protective factor with PMI and besides GSTT1 null genotype also may act a protective role with PMI.

Further large scale genetic association and molecular studies are required to delineate the clinical implications and mechanisms underlying these observations.

## 5. NOVEL FINDINGS

- 1) Firstly we demonstrated that the pharmacological inhibition of GST could markedly exaggerate oxidative stress parameters and induced apoptosis in vivo rat model. Measuring of total infarct size we were capable to detect that the infarction size was significantly higher in the presence of EA while PC limited the extent of infarcted area. We were able to confirm that presence of EA is capable to eliminate the protective effect of PC.
- 2) GST inhibition was associated with different activation of MAPK and RISK kinases regulating these pathways under myocardial stress conditions. We detected, that GST plays crucial role among pro- and antiapoptotic MAPK and RISK kinases in the process of oxidative stress-induced apoptosis in an in vivo rat model.
- 3) We were able to demonstrate that GST inhibition could markedly attenuate the protective effect of ischaemic PC and resulted in increasing apoptosis in in vivo rats during myocardial ischemia. It was clarified that in the process of PC GST inhibition is in the close association with activation of different MAP kinases and RISK protein kinases.
- 4) Our results propose that the genetic polymorphism of GSTP1 is independently associated with the presence of PMI. The appearance of homozygous BB allele combination was significantly higher in the group without complications (non-periop AMI group). In present paper it is suggested that the existence of the A allele carries complication for PMI.

As yet, there have been no previous analyses examining the association of GSTP1, GSTM1 and GSTT1 polymorphisms with the development of PMI. Our prior findings of genetic data from GSTM1 studies and PMI suggested that the null genotypes at GSTM1 had no effect on PMI.

Our results have identified that GSTT1 null genotype play a protective role against PMI. Our results highlight that polymorphisms could play predisposing role in progression of PMI following cardiac surgery.

New findings will enable us to have a better understanding about the mechanism of interrelationships which converge in the event of genomic variants, as well as for the real-time enzymatic processes themselves.

## 6. PUBLICATIONS

### 6.1 PUBLICATIONS RELATED TO THE THESIS

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**IF: 11,11**

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