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# ASSOCIATION OF GLUTATHIONE TRANSFERASE A1, M1, P1 AND T1 GENE POLYMORPHISM WITH OXIDATIVE STRESS BYPRODUCTS AND CARDIOVASCULAR COMPLICATIONS IN PATIENTS WITH END-STAGE RENAL DISEASE

**Doctoral Dissertation**

**Belgrade 2016**

**UNIVERZITET U BEOGRADU**

**MEDICINSKI FAKULTET**

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# POVEZANOST POLIMORFIZAMA GLUTATION TRANSFERAZA A1, M1, P1 I T1 SA POKAZATELJIMA OKSIDATIVNOG STRESA I KARDIOVASKULARNIM KOMPLIKACIJAMA KOD BOLESNIKA SA TERMINALNOM BUBREŽNOM SLABOŠĆU

**Doktorska Disertacija**

**Beograd 2016**

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## Acknowledgments

I would like to thank people who made this Thesis possible. First of all I would like to express my sincere gratitude to my supervisor, Professor Tatjana Simić who provided unreserved and continuous support during my PhD education. I am thankful for her enthusisasm, inspiration and encouragement, but also for the hard question which incented me to widen my research from various perspectives

I would also like to thank to my co-supervisor dr Tatjana Damjanović for her assistance patients' recruitment and the help during the follow-up of the patients.

I am thankful to all board members; to Professor Nada Dimković for her patience and all advices she has given to me; to Professor Tatjana Pekmezović for her insightful comments and all useful conversations we have shared during last years; to Professor Marija Plješa-Ercegovac for being always there for me; and to Professor Zorana Jelic-Ivanovic and Professor Karmen Stankov for their kindness and constructive suggestions.

My sincere thanks also go to my colleagues Professor Ana Savic-Radojević and Professor Marija Matić, as well as to my fellow colleagues Tatjana Đukić, Vesna Ćorić, Tanja Radić, Đurđa Jovanović, and Sanja Sekulić. All their support and intriguing discussions meant a lot to me.

I am grateful to Professor Dragan Milenkovic and Irena Krga who provided me an opportunity to join their team and who gave access to the laboratory and research facilities.

I would like to thank to Professor Ivanka Marković, Professor Aleksandra Isaković and Anđelka Isaković, for their great assistance in flow cytometry learnig.

I am thankful to Professor Steva Plješa for his assistance in patients' recruitment.

Thanks to Aleksandra Stefanović and Jasmina Ivanišević for their assistance in laboratory.

I am grateful to the personnel of the Institute of medical and clinical biochemistry and my other coworkers at the Institute.

I take this opportunity to express my gratitude to my Family. Their love served as a secure anchor during the hard and easy times. I would also like to thank to my beloved husband

Milovan for supporting me in everything, and especially for encouraging me throughout this experience. To my beloved children, Pavle and Sara, who are my everything. I am also thankful to my father Radisav and sister Nataša for their support and love. Finally, I will be eternally grateful to my mother for always believing in me and encouraging me to follow my dreams. Thank you for your sacrifice and everything you have done for me.

*To my Family*

# ASSOCIATION OF GLUTATHIONE TRANSFERASE A1, M1, P1 AND T1 POLYMORPHISM WITH OXIDATIVE STRESS BYPRODUCTS AND CARDIOVASCULAR COMPLICATIONS IN PATIENTS WITH END-STAGE RENAL DISEASE


Summary

Chronic kidney disease is described as a progressive and irreversible deterioration in kidney function. When there is less than 10% of nephron function pertained, the patients face end-stage renal disease where renal replacement therapy is needed. Data show that hemodialysis is the most common method used to treat advanced and permanent kidney failure. Increased oxidative stress is a hallmark of end-stage renal disease (ESRD). Glutathione transferases (GST) are involved in the detoxification of xenobiotics and protection of oxidative damage. The role of polymorphic expression of GSTSs in end-stage renal disease development enhanced oxidative stress and prognosis of ESRD patients has emerged recently.

The aim of this study was to test if the genetic polymorphism in antioxidant enzymes *GSTA1, GSTM1, GSTP1* and *GSTT1* is more frequent in ESRD patients than in control group, and if it modulates the levels of oxidative stress byproducts and cellular adhesion molecules in these patients. The association between *GSTM1* and *GSTT1* deletion polymorphisms as well as SNPs in *GSTA*1/rs3957357, *GSTP1*/rs1695 and rs1138272 genes with overall and cause-specific cardiovascular mortality in patients with end-stage renal disease was also assessed. Furthermore, the predictive role of oxidative stress byproducts and adhesion molecules level was also tested.

Individuals with either *GSTM1-null* or *GSTP1*C/**T genotypes were at increased susceptibility towards ESRD development than individuals with *GSTM1-active* or *GSTP1*C/*C* genotypes (OR = 1.6, p = 0.024 and OR = 3.2, p = 0.001, respectively). This risk was even more increased when these genotypes were combined with other *GST null/low activity* genotypes. Polymorphic expression of *GST* gene influences the vulnerability to protein and lipid oxidation, as well as the levels of soluble cellular adhesion molecules in plasma of ESRD patients. The oxidative stress byproducts were even more increased in terms of combined *GST*

genotypes. Regarding predictive role of *GST* genotypes, only *GSTM1-null* genotype in ESRD patients was found to be independent predictor of overall and cardiovascular mortality (overall: HR = 1.8, p = 0.009; cardiovascular: HR = 2.3, p = 0.006 and myocardial infarction: HR = 2.3, p = 0.035). Possible predictive role for the byproducts of oxidative stress was found for higher concentrations (above the median for each product) of MDA, AOPP, PAB and VCAM-1 regarding overall and cardiovascular survival. Comparison of two endothelial cells with different *GSTM1* genotypes in terms of monocyte adhesion and reactive oxygen species production after incubation in uremic serum was not significantly different.

Taken together, the results presented in this study suggest a possibility for *GST* genotype-based stratification of ESRD patients which could improve the attempts towards individualization of antioxidant treatment. Besides, determination of oxidative stress byproducts may permit the targeting of preventive and early intervention in high-risk patients to reduce their cardiovascular risk.

**Keywords**: end-stage renal disease; glutathione S-transferases polymorphism; hemodialysis; oxidative stress; adhesion molecules

**Research area**: Medicine

**Research field**: Biochemistry

# POVEZANOST POLIMORFIZAMA GLUTATION TRANSFERAZA A1, M1, P1 I T1 SA POKAZATELJIMA OKSIDATIVNOG STRESA I KARDIOVASKULARNIM KOMPLIKACIJAMA KOD BOLESNIKA SA TERMINALNOM BUBREŽNOM SLABOŠĆU


Sažetak

Hronična bubrežna slabost se opisuje kao progresivno i ireverzibilno smanjenje bubrežne funkcije. Kada se funkcija bubrega smanji na ispod 10% od normalnih vrednosti, nastupa stanje koje se zove terminalna bubrežna slabost kada je neophodna primena terapije neke od meoda zamene bubrežne funkcije. Podaci pokazuju da je hemodijaliza najčešće primenjivan metod. Povećana produkcija slobodnih radikala i stanje oksidativnog stresa glavna su obeležja terminalne bubrežne slabosti. Glutation transferaze (GST) su enzimi koji su uključeni u procese eliminacije ksenobiotika i antioksidativne zaštite. Ipak do sada još uvek nije dovoljno ispitana njihova uloga u podložnosti za razvoj terminalne bubrežne slabosti, oksidativnog stresa kod ovih bolesnika kao i moguća prediktivna uloga.

Imajući ovo u vidu, cilj ove teze bio je da se ispita veza između polimorfizama gena za *GSTA1, GSTM1, GSTP1* i *GSTT1* u podložnosti za razvoj terminalne bubrežne slabosti, kao i da se utvrdi da li polimorfna ekpresija glutation transferaza utiče na vrednosti produkata oksidativnog stresa i adhezionih molekula u plazmi bolesnika sa terminalnom bubrežnom slabošću. Takođe, jedan od ciljeva bio je i da se ispita da li polimorfizam *GST* gena može da ima prognostički značaj u smislu opšteg ili kardiovaskularnog preživljavanja kod ovih bolesnika. Dodatno, ispitan je i prediktivni značaj biohemijskih pokazatelja oksidativnog stresa i adhezionih molekula.

Osobe koje su imale *GSTM1-nulti* i *GSTP1*C/*T* genotip imale su veću podložnost za razvoj terminalne bubrežne slabosti (OR = 1.6, p = 0.024 and OR = 3.2, p = 0.001, redom), koja je još više bila izražena kada su se ovi genotipovi kombinovali sa drugim *GST* nultim ili genotipovima smanjenih aktivnosti. Takođe, polimorfna ekspresija glutation transferaza kod bolesnika sa terminalnom bubrežnom slabošću utiče na nivo oksidativnog oštećenja proteina, lipida i adhezionih molekula. Produkti oksidativnog stresa su još više izraženi kod bolesnika

kod kojih postoji kombinacija *GST* nultog ili genotipova smanjenih aktivnosti. U ovoj tezi je pokazano i da *GSTM1-nulti* genotip ima značajnu ulogu kao prediktor opšteg i kardiovaskularnog uzroka smrti (opšti uzrok smrti: HR = 1.8, p = 0.009; kardiovaskularni uzrok: HR = 2.3, p = 0.006 i infarct miokarda: HR = 2.3, p = 0.035). Takođe, pokazano je i da više vrednosti (iznad nivoa medijane vrednosti određene za svaki produkt pojedinačno) MDA, AOPP, PAB i VCAM-1 imaju prognostički značaj kod opšteg i kardiovaskularnog preživljanja u bolesnika sa terminalnom bubrežnom slabošću. Produkcija slobodnih radikala i adhezija monocita za endotelne ćelije nije se razlikovala između HUVEC sa *GSTM1-aktivnim* i *GSTM1-nultim* genotipom.

Rezultati iz ove teze ukazuju da bolesnici sa terminalnom bubrežnom slabošću mogu da se stratifikuju prema *GST* genotipu sa ciljem unapređenja antioksidantne terapije. Takođe, merenje produkata oksidativnog stresa može da posluži da se odredi kod kojih bolesnika treba započeti rane preventivne mere da bi se smanjio rizik od kardiovaskularnih komplikacija.

**Ključne reči:** terminalna bubrežna slabost, glutation transferaze, polimorfizam, hemodijaliza, oksidativni stres, ćelijski adhezioni molekuli

**Naučna oblast**: Medicina

**Uža naučna oblast:** Medicinska biohemija

UDK:

# Table of Contents


1 **INTRODUCTION** ..... 1

1.1 CHRONIC KIDNEY DISEASE AND END-STAGE RENAL DISEASE ..... 1

1.1.1 Causes of CKD ..... 3

1.1.2 Treatment of end-stage renal disease ..... 3

1.2 END STAGE RENAL DISEASE AS A STATE OF REDOX IMBALANCE ..... 4

1.2.1 Oxidative stress ..... 4

1.2.2 Evolution of oxidative stress in the course of ESRD development ..... 5

1.2.3 Markers of oxidative stress in patients with end-stage renal disease ..... 9

1.3 CARDIOVASCULAR DISEASE IN END STAGE RENAL DISEASE PATIENTS ..... 12

1.3.1 Oxidative stress-endothelial dysfunction-cardiovascular disease triade in end-stage renal disease patients ..... 13

1.4 GLUTATHIONE TRANSFERASES ..... 16

1.4.1 Classification of GSTs ..... 17

1.4.2 Structure and substrate specificity ..... 18

1.4.3 Metabolism of exogenous and endogenous compounds by GST ..... 19

1.4.4 Polymorphism of glutathione transferases ..... 20

1.4.5 Glutathione Transferase M1 gene polymorphism ..... 22

1.4.6 Chronic renal failure and *GST* polymorphism ..... 26

2 **AIMS** ..... 29

3 **MATERIAL AND METHODS** ..... 30

3.1 STUDY SUBJECTS ..... 30

3.2 DETERMINATION OF GLUTATHIONE TRANSFERASE A1, M1, P1 AND T1 GENE POLYMORPHISM ..... 31

3.2.1 DNA extraction ..... 31

3.2.2 Determination of *GSTA1* gene polymorphism ..... 31

3.2.3 Determination of *GSTM1* gene polymorphism ..... 32
3.2.4 Determination of *GSTP1* genes polymorphisms ..... 33
3.2.5 Determination of *GSTT1* gene polymorphism ..... 35
3.3 MEASUREMENT OF OXIDATIVE STRESS BYPRODUCTS ..... 36
3.3.1 Plasma separation ..... 36
3.3.2 Measurement of malondialdehyde levels ..... 37
3.3.3 Determination of MDA protein adducts ..... 37
3.3.4 Determination of AOPP (Advanced Oxidation Protein Products) ..... 37
3.3.5 Determination of nitrotyrosine concentration ..... 37
3.3.6 Prooxidant-antioxidant balance (PAB) ..... 38
3.3.7 Total-oxidant status measurement ..... 38
3.3.8 Protein thiol groups ..... 39
3.3.9 Protein carbonyls ..... 39
3.4 DETERMINATION OF CIRCULATING ADHESION MOLECULES IN PLASMA ..... 39
3.4.1 Human soluble VCAM-1 measurement ..... 40
3.4.2 Human Soluble ICAM-1 Determination ..... 40
3.5 OVERALL AND CARDIOVASCULAR SURVIVAL ANALYSIS ..... 40
3.6 MOLECULAR CHANGES OF ENDOTHELIAL CELLS ..... 41
3.6.1 Cell cultures ..... 41
3.6.2 The treatment of the endothelial cells ..... 42
3.6.3 Cell counting ..... 42
3.6.4 The viability assay ..... 42
3.6.5 The monocytes adhesion assay ..... 43
3.6.6 The measurement of reactive oxygen species production ..... 43
3.7 STATISTICAL ANALYSIS ..... 44
4 **RESULTS** ..... 46

4.1 CLINICAL CHARACTERISTIC OF PATIENTS AND CONTROL GROUP ..... 46

4.2 DISTRIBUTION OF *GST* GENOTYPES ..... 46

4.3 Combined *GST* genotypes and ESRD risk ..... 48

4.4 ASSOCIATION BETWEEN *GST* GENOTYPE AND BYPRODUCTS OF OXIDATIVE DAMAGE ..... 51

4.4.1 Impact of *GST* genotype on protein oxidative damage ..... 51

4.4.2 Impact of *GST* genotype on lipid oxidative damage ..... 54

4.4.3 Impact of *GST* genotype on TOS and PAB ..... 57

4.4.4 Impact of *GST* genotype on circulating soluble adhesion molecules concentration ..... 59

4.4.5 Impact of combined *GST* genotype on markers of oxidative damage ..... 62

4.5 PREDICTIVE ROLE OF *GST* GENOTYPE ..... 66

4.5.1 Lack of association between *GSTA1* genotype and overall or cardiovascular mortality 66

4.5.2 A significant association of *GSTM1* deletion polymorphism with all cause and cardiovascular mortality ..... 68

4.5.3 Association of *GSTT1* genotype with all cause and cardiovascular mortality ..... 70

4.5.4 Association of *GSTP1* genotype with all cause and cardiovascular mortality ..... 71

4.6 PREDICTIVE ROLE OF OXIDATIVE STRESS BYRODUCTS ..... 74

4.6.1 Protein oxidative stress byproducts as possible predictors of patients' outcome ..... 75

4.6.2 The possible predictive role of malondialdehyde ..... 80

4.6.3 Prooxidant-antioxidant balance level as a predictor of overall and cardiovascular mortality 83

4.6.4 Soluble ICAM-1 and ICAM-1as potential predictors of all-cause and cardiovascular death 86

4.7 Effect of human umbilical vein endothelial cells incubation in uremic serum ..... 89

4.7.1 Viability of endothelial cells after incubation in uremic serum ..... 89

4.7.2 Effect of monocyte adhesion to endothelial cells after incubation in uremic serum ..... 89

4.7.3 Reactive oxygen species production in endothelial cells after incubation in uremic serum 91

5 **DISCUSSION** ..... 93

6 **CONCLUSIONS** ..... 106

7 **BIBLIOGRAPHY** ..... 108

# 1 INTRODUCTION

## 1.1 CHRONIC KIDNEY DISEASE AND END-STAGE RENAL DISEASE

Chronic kidney disease (CKD), is described as a progressive and most frequently irreversible deterioration in kidney function in which the body's ability to maintain metabolic, fluid and electrolyte balance fails, resulting in retention of urea and other nitrogenous wastes in the blood. Patients with chronic kidney disease have gradual loss of kidney function. Definition of CKD and international classification are introduced in National kidney foundation Disease Outcomes Quality Initiative criteria ("The National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI)," 2014) According to KDOQI criteria, the disease is divided into five stages based on increasing severity and glomerular filtration rate (Table 1.). Adverse outcomes of CKD can be prevented through early detection and treatment. Early stages can be detected by biochemical analysis including creatinine-based estimate of glomerular filtration rate (GFR) (Levey 1999). Criteria for CKD include GFR < 60 mL/min/1.73 $m^2$ for 3 months, irrespective of the presence or absence of kidney damage. As kidney function declines, the end products of protein metabolism accumulate in the blood resulting in uremia development. This adversely affects every system in the body. The greater the production of waste products, the more severe the symptoms are.

End-stage renal disease (ESRD) occurs when there is less than 10% of nephron pertained function remaining. End-stage renal disease represents severe kidney damage, measured by the reduction in the glomerular filtration rate to < 15 ml/min/1.73 m2. At this stage affected patients require dialysis or a kidney transplant. All of the normal regulatory, excretory and hormonal functions of the kidney are severely impaired.

Thus at this stage many exogenous and endogenous metabolites are accumulated, together with various uremic toxins in that way contributing to redox moiety and impaired antioxidant protection in patients on hemodialysis (Mimić-Oka et al., 1999).

**Table 1**. Stages of chronic kidney disease with glomerular filtration rate (GFR), short description and treatment options.

| Stage | GFR | Description | Treatment |
|---|---|---|---|
| 1 | > 90 | Normal kidney function but urine findings or structural abnormalities or genetic trait point to kidney disease | Observation, blood pressure control |
| 2 | 60 - 89 | Mildly reduced kidney function, and other findings (as for stage 1) point to kidney disease | Observation, control of blood pressure and risk factors. |
| 3 | 30 - 59 | Moderately reduced kidney function | Observation, control of blood pressure and risk factors |
| 4 | 15 - 29 | Severely reduced kidney function | Planning for endstage renal failure |
| 5 | < 15 | Very severe, or end-stage renal failure | Dialysis or transplantation |

Adapted from NKF-NDOQI CKD guidelines ("The National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI)," 2014)

Chronic kidney disease represents a major public health problem all over the world. In U.S. of America every 10$^{th}$ adult is affected with chronic kidney disease (Plantinga et al., 2008) while in other countries this problem is of similar magnitude. Worlwide, situation is very similar. Namely, around 11% of world population suffers from some stage of chronic kidney disease. There are disturbing data showing that incidence of end stage renal disease is progressively growing worldwide in the last two decades with over two million affected individuals in the world and approximately 650,000 in the Europe (Bello et al., 2005). According to the national dialysis register there are almost 6000 people in Serbia treated with some type of renal replacement therapy (RRT). Incidence of ESRD in Serbia was also growing in the last decade, rising from 107 to 189 patients per million individuals. It is important to note that Serbia is among the European countries with higher incidence of ESRD (Ing et al., 2012). There are at least two reasons for CKD to be considered as a serious condition: the first one is that these patients are at greater risk to develop cardiovascular complications, tumors and chronic infections; the second one is that majority of CKD patients will develop ESRD, the fifth stage of CKD, where renal replacement therapy is needed.

### 1.1.1 Causes of CKD

The main causes of CKD include: diabetes mellitus, hypertension and glomerulonephritis (ERA-EDTA annual reports, 2014). These three conditions are responsible for almost 70% of all CKD cases. Among other causes that may affect the renal function are:

- Hereditary diseases such as polycystic kidney disease, in which large cysts forming in the kidneys are damaging renal tissue.
- Malformations that occur during fetal development.
- Lupus and other diseases that affect the immune system.
- Obstructions (kidney stones, tumors or an enlarged prostate gland in men).
- Repeated urinary infections.

Apart from these well established causes, other factors may contribute to development and progression of renal failure including age, poverty, small gestational mass, and certain ethnic groups. Other factors that influence the progression of chronic kidney disease are smoking, diabetes, hypertension and obesity.

### 1.1.2 Treatment of end-stage renal disease

When glomerular filtration rate is below 15 ml/min/1.73 $m^2$ a renal replacement therapy should be considered. Data from Fresenius medical care network show that hemodialysis (HD) is the most common method used to treat advanced and permanent kidney failure (Grassman et al., 2005). Namely, from estimated 1.3 million patients in the world who received some form of renal replacement therapy, almost 89% received hemodialysis and 11% received peritoneal dialysis (Grassmann et al., 2005).

Hemodialysis is usually performed three times per week in 3-5 hours duration. Standard blood drawn rate is about 200-400 ml/min. During the treatment, the patient's entire blood volume (about 5 L) circulates through the machine every 15 minutes. Original cotton (also called "natural") dialyzer membranes were replaced with synthetic polysulfone "high flux" membranes that showed better biocompatibility. Dialysate acetate-based buffer is now replaced with the mixture of bicarbonates and acetic acid which showed many advantages regarding acid-base status of patients and impact on metabolism (Desai, 2015).

## 1.2 END STAGE RENAL DISEASE AS A STATE OF REDOX IMBALANCE

Patients affected with different stages of chronic kidney disease, especially patients with end-stage renal disease on chronic hemodialysis treatment, exhibit a huge burden of oxidative and inflammatory processes that lead to imbalance in redox homeostasis. This imbalance is shown to lead these patients to elevated risk of mortality and morbidity.

### 1.2.1 Oxidative stress

Oxidative stress is commonly defined as "a disturbance in the pro-oxidant–antioxidant balance in favor of the former." (Sies, 1991) (Figure 1). However, since data have shown that balance of pro-oxidants and antioxidants cannot be defined by a single entity, a new definition which defines oxidative stress as "a disruption of redox signaling and control." is introduced (Jones, 2006).

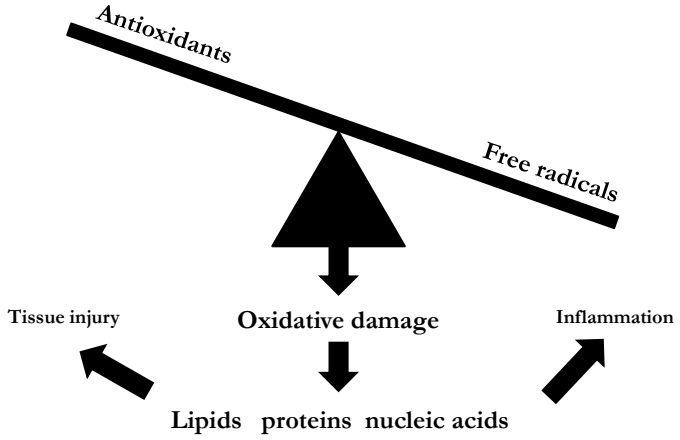


**Figure 1.** When free radicals production exceeds antioxidant defences, an oxidative stress developes. Free radicals attack and oxidise macromolecules such as lipids lipids, proteins, and nucleic acids consequently leading to tissue injury and in some cases, the influx of inflammatory cells to the sites of injury. Adapted from (Sies, 1991)

Reactive oxygen species (ROS) are a family of molecules that are produced in all aerobic cells. They possess unpaired electrons and are highly reactive. In physiological conditions ROS are continuously generated as products of cellular respiration and enzymatic reactions (xanthine oxidase, myeloperoxidase, NO synthase, cyclooxygenase, lypooxygenase).

Thus, ROS are physiologically produced in mitochondria, but could be synthesized in phagocytic cells, as well as, in vascular wall and many other tissues (Valko et al., 2007). ROS include radicals such as superoxide ion radical ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$), lipid hydroperoxides and non-radicals such as singlet oxygen ($^1O_2$), hydrogen peroxide ($H_2O_2$), hypochlorus acid (HOCl), chloramines (RNHCl) and ozone (Bedard and Krause, 2007). Besides ROS, reactive nitrogen species (RNS) also occur. They include nitric oxide radical (NO.), nitrogen dioxide radical ($NO_2$), peroxynitrite (ONOO-) and dinitrogen trioxide ($N_2O_3$). Reactive species, both oxygen and nitrogen ones, have many important roles including signal transduction, eliminating of invading pathogens, wound healing and tissue repair.

Although normally produced, amounts of free radical may surmount cellular antioxidant capacity resulting in oxidative stress. However, once the production of both ROS and RNS exceeds antioxidant defense system these free radicals start to react with macromolecules such as proteins, lipids, carbohydrates and nucleic acids resulting in their irreversible damage and loss of function. Exogenous factors leading to excessive free radical production are pollutants, radiation, cigarette smoking, drugs and xenobiotics. Many clinical conditions caused by inflammation are prone to oxidative stress development (Yamakura and Kawasaki, 2010) and a growing body of evidence suggests that oxidative stress is involved into pathogenesis of numerous diseases with cardiovascular one being the most prevalent (Dhalla et al., 2000).

### 1.2.2 Evolution of oxidative stress in the course of ESRD development

Oxidative stress represents a hallmark of end-stage renal disease. As renal disease is progressing and ESRD is developing, higher levels of free radical production have been reported (Mimic-Oka et al., 1992). An increased redox burden in uremic patients leads to oxidation of protein, lipids and carbohydrates. In hemodialysis patients many factors contribute to increased production of free radicals on one side and decreased antioxidant capacity on the other side, leading to development of well defined concept of oxidative stress. Furthermore, these patients have an electrolyte imbalance, high concentrations of cytokines and inflammatory mediators. Moreover, these patients are usually under treatment in order to correct anemia and different metabolic disorders. However, it is believed that main causes of

oxidative stress in hemodialysis patients represent accumulation of uremic toxins, bioincompatibility with dialyzer, and dialysate contaminants.

#### 1.2.2.1 Loss of Antioxidants

Both in chronic kidney disease and during hemodialysis sessions, a massive production of ROS and oxidatively modified lipids and proteins is described, contributing to huge oxidant burden in these patients (Locatelli et al., 2003). However, not only the excessive free radical production is responsible for systemic redox stress of this population. Patients undergoing hemodialysis deal with impaired antioxidant capacity, and the mechanisms leading to such a state have been relatively well described (Dursun et al., 2002). It was reported that erythrocyte antioxidant capacity increases with the progression of CKD while ESRD patients undergoing hemodialysis have defective antioxidant activity (Mimić-Oka et al., 1999). Namely, Karamouzis et al. have observed that only in patients with end-stage renal failure the levels of total antioxidant enzyme capacity (TAC) are decreased, which is not the case with other stages of CKD (Karamouzis et al., 2008). Reduced erythrocyte superoxide dismutase (SOD) and glutathione peroxidase (GPX) activity have been reported in patients with ESRD (Yilmaz et al., 2006). The GPX and SOD activity are shown to decrease in term of CKD progression with the lowest level observed in the fifth stage of CKD (Mimić-Oka et al., 1999). It seems that intravenous administration of heparin can displace extracellular SOD from endothelial cells (Faraci and Didion, 2004). Since extracellular SOD is an important determinant of nitric oxide bioavailability in endothelial cells, any loss of this enzyme would therefore affect normal endothelial function (Fukai et al., 2002). Mimic-Oka et al have also described significantly lower plasma catalase activity, associated with higher hydrogen peroxide production in ESRD patients (Mimić-Oka et al., 1999).

Even non-enzymatic defense systems are affected in ESRD patients. Namely, dialysis procedure itself contributes to the loss of vitamin C, major non-enzymatic antioxidant molecules (Deicher et al., 2005). There are evidence that low plasma vitamin C level may be useful in prediction of both fatal and non-fatal cardiovascular events among maintenance haemodialysis patients (Deicher et al., 2005). Additionally, Takahashi et al have shown that low

vitamin C levels can affect endothelium-dependent vasodilatation and contribute to endothelial dysfunction and atherosclerosis process (Taddei et al., 1998).

Regarding glutathione, a major low-molecular-mass thiol compound participating in cellular redox reactions and thio-ether formation, it should be emphasized that its content is also diminished in HD patients (Ceballos-Picot et al., 1996). Glutathione levels are decreased in both plasma (Ross et al., 1997) and erythrocytes in patients affected with ESRD (Francesco Galli et al., 1999).

#### 1.2.2.2 Uremic toxins

Patients with ESRD have an accumulation of more than hundred uremic solutes with potential pro-oxidant activity and toxicity. Knowledge of uraemic toxicity has grown very much during past years and the interest for them increased enormously. Based on their physicochemical characteristics, uremic retention solutes may be categorized into three groups: free water-soluble low molecular weight compounds, middle molecules and protein-bound uremic toxins (Duranton et al., 2012; Meert et al., 2007) (Table 2). The first one comprises the free water-soluble compounds such as urea, uric acid, myoinositol, oxalate, guanidino compounds and polyamines. On the other hand, indoxyl sulfate, indole-3-acetic acid, and p-cresol represent some of the compounds belonging to the group of protein-bounded uremic solutes which have been associated with endothelial dysfunction and cardiovascular disease development (Dou et al., 2015, 2004; Gondouin et al., 2013; Schulz et al., 2014).

Among uremic toxins identified in plasma of HD patients, indoxyl sulfate is mostly studied. Like all indolic uremic toxins, it is generated by gut bacteria metabolism of tryptophan (Schepers et al., 2010; Vanholder and Glorieux, 2015). Taki et al have shown that serum levels of indoxyl sulfate are increased in HD patients, contributing to endothelial dysfunction, and atherosclerosis progression (Taki et al., 2007). Furthermore, indoxyl sulfate has been shown to increase the expression of ICAM-1 and contributes to development of cardiovascular complications (Tumur et al., 2010). Uremic toxins present in the serum of patients with CKD alter endothelial cell properties in vitro by accelerating apoptotic processes in a dose dependent manner (Zafeiropoulou et al., 2012).

**Table 2**. Classification of uremic solutes/toxins

| Uremic toxins | | |
|---|---|---|
| **Small hydrophilic compounds** | **Middle molecules** | **Protein-bound uremic toxins** |
| • Urea | • IL-6, IL-1β | • p-cresyl sulfate |
| • Uric acid | • TNF-α | • Indoxyl-sulfate |
| • Guanidine | • Leptin | • Indole-3-acetic acid |
| • Oxalate | • Resistin | • Homocystein |
| • ADMA | • Endothelin | • Pentosidin |
| | • AOPP | • AGEs |

Indoxyl sulfate is also able to induce mitochondrial depolarization and cell death (Lee WC et al., 2014). A recent study conducted by Dou et al reported that indole-3 acetic acid (IAA), another uremic toxin found in serum, is associated with mortality and cardiovascular events in patients with CKD. (Dou et al., 2015). They have also demonstrated that IAA is able to induce oxidative stress and inflammatory response by activating AhR/p38MAPK/NF-κB pathway in vascular endothelial cells in vitro (Dou et al., 2015).

#### 1.2.2.3 Bioincompatibility of hemodialysis treatment

Although hemodialysis has many beneficial aspects, process itself is also able to provoke inflammatory reactions. An example of this is the inflammatory response due to bioincompatibility of dialysis membrane. Indeed, it is believed that bioincompatibility has a central role in oxidative stress development. From the first observation in the far 1968 that early phase of dialysis leads to significant drop of neutrophil leukocyte count (Kaplow and Goffinet, 1968) researchers focused on these phenomena and in years to come found an explanation. Craddock et al. suggested the complement activation as the main factor for neutrophil aggregation (Craddock et al., 1977) while it was later explained that alternate pathway leads to C3a and C5a production (Chenoweth et al., 1983) where these two anaphylatoxins stimulate cytokine synthesis and release from monocytes (Fischer et al., 1999). In this process a contact of the cells with dialysis membrane leads to activation of L-fucose dependent pathway (Betz et al., 1988). Fortunately, synthetic membranes are available in the

most dialysis centers around the world and the consequences are minimized nowdays. In addition, in some developed countries vitamin E-coated membranes are predominantly used place due to proven benefits on patients' survival and cardiovascular complications have been observed with the use of these membranes (Lin et al., 2009; Mune et al., 1999).

### 1.2.3 Markers of oxidative stress in patients with end-stage renal disease

Direct measurement of reactive oxygen and nitrogen species *in vivo* is very difficult due to the highly reactive nature of free radicals. Some techniques such as L-band electron spin resonance with nitroxyl probes and magnetic resonance imaging spin trapping are under development to measure free radicals directly in animal models (Han et al., 2001; Utsumi and Yamada, 2003). However, their application in human studies is still under investigations. Due to the fact of very short half-life of ROS, their concentration is changing in seconds, additionally compromising their determination. In attempt to overcome these difficulties, methods for determination of stable end-products of macromolecules' oxidation are introduced (Table 3). Oxidative modifications of biological macromolecules (proteins, lipids, DNA and carbohydrates) are excessively modified by ROS production *in vivo*. Their byproducts' concentrations are measured in different samples (plasma, serum, cell lysate, tissue homogenate) and are able to reflect degree of oxidative stress in different pathologies.

**Table 3**. Byproducts of oxidative damage measured in plasma or urine

| **Byproducts of oxidative damage** | | |
|---|---|---|
| **Proteins** | **Lipids** | **DNA** |
| • Thiol (-SH) groups | • MDA | • 8-OH-deoxyguanosine |
| • Carbonyl groups | • MDA-adducts | • 5-OH-methyl uracil |
| • AOPP | • F2-isoprostanes | |
| • Nitrotyrosine | • 4-HNE | |
| • AGEs | | |

AOPP-advanced oxidation protein products; AGEs-advanced glycation end products; MDA-malondialdehyde

Proteins are very prone to oxidative damage. Indeed, protein modifications by ROS include hydroxylation of aromatic and aliphatic amino acids residues, nitrosylayion of sulphhydryl

groups, chlorination of aromatic amino acids which result in formation of protein carbonyls, nitrotyrosine derivatives and many inter-moleculear cross-links (Galli 2007). Indeed, the most important protein oxidative damage byproducts are (a) protein thiol content (b) protein carbonyls tyrosine damage byproducts (c) advanced oxidation protein products (AOPP) (d) nitrotyrosine (e) advanced glycation end-products (AGEs). The significant byproduct of protein oxidative damage is the decrease in thiol (SH-) group content. The SH-groups of albumins are considered to be sacrificial targets for reaction with free radicals. The residues of amino acids Hcy and Cys are forming disulphides in oxidation reactions and are prone to further modifications. Loss of thiols has been described in terms of chronic kidney failure (Himmelfarb et al., 2002; Mimic-Oka et al., 1992).

Direct oxidation of Lys, Arg or Thr by ROS leads to formation of protein carbonyls (Stadtman and Levine, 2003), another byproduct of protein oxidative damage. Miyata et al have shown that protein carbonyls are related to uremic toxicity and introduced the term "carbonyl stress" (Miyata et al., 2001). Several studies confirmed that carbonyls content is significantly increased in plasma of hemodialysis patients (Galli et al., 2005; Michelis et al., 2003) and may cause a significant increase in intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 mRNA levels (Pavone et al., 2011). Massive accumulation of carbonyls, derived from polyunsaturated fatty acuds, sugars and amino acids, has an important role in the genesis of advanced glycation end-products (AGEs) (Himmelfarb and McMonagle, 2001). The AGEs content has been increased in patients with end-stage renal disease (Floridi et al., 2002; Galli et al., 2005; Noordzij et al., 2008) and it is known to contribute to the higher cardiovascular risk and mortality (Miyata et al., 1999; Raj et al., 2000).

Oxidative modifications of tyrosine include production of 3-chloro-tyrosine, 3-nitro-tyrosine or di-tyrosine (Figure 2). These are very sensitive byproduct of protein damage and are increased in patients on maintain hemodialysis (Himmelfarb and McMonagle, 2001). Di-tyrosine protein modifications and intraprotein cross-link creation have additional role in formation of yet another reliable marker of oxidative stress in ESRD patients-AOPP. In HD patients levels of AOPP are in correlation with markers of uremia and oxidative stress (Witko-Sarsat et al., 1998). This byproduct is also able to induce activation of human neutrophil and monocyte oxidative metabolism (Witko-Sarsat et al., 2003). Furthermore, increased AOPP

level is shown to increase macrophage infiltration in atherosclerotic plaques and in glomeruli in the remnant kidney model (Liu et al., 2006).

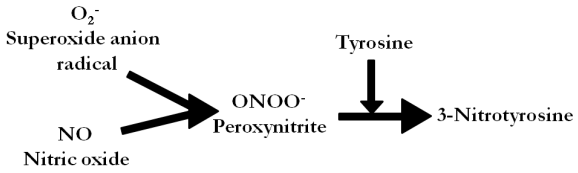


**Figure 2.** Nitrotyrosine formation during oxidative stress

Lipids are also affected by oxidative damage (Figure 3). Determination of the extent of lipid peroxidation includes measurement of (a) malondialdehyde (MDA) and other aldehydes that represent degradation products of lipid peroxidation and are able to react with thiobarbituric acid (b) conjugated dienes in LDL particles and (c) F-2 isoprostanes. MDA is a three-carbon, low-molecular weight aldehyde that can be produced by different mechanisms. Dahle et al. first described a mechanism of MDA formation based on the fact that only peroxides that possessed α or β unsaturated bonds capable of undergoing cyclization to finally form MDA (Dahle et al., 1962). There is a lack of MDA distribution in the general population and their referent concentrations. However, it has been reported that plasma MDA concentration is elevated in various diseases including hemodialysis (Nally, 1997) when compared to healthy volunteers. Furthermore, the increased level of MDA have been related to increased cardiovascular risk (Rumley et al., 2004; Smith et al., 1993).

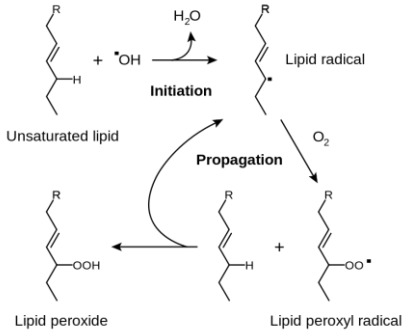


**Figure 3.** Mechanism of lipid peroxidation

Malondialdehyde is able to bind to proteins and form stable adducts, also termed advanced lipid peroxidation end products. These modifications may cause both structural and functional changes of oxidized proteins. In addition to the fact that byproducts of oxidative damage of macromolecules are elevated, it has been shown that their concentrations in ESRD patients are further increasing as disease is progressing and GFR is decreasing. For example, GFR shows negative correlation with levels of advanced oxidative protein products, AOPP (Witko-Sarsat et al., 1998)., MDA (Yilmaz et al., 2006) and F2-isoprostanes (Cottone et al., 2009). Furthermore, data suggest that elevated oxidative stress byproducts are associated to carotid intima media thickness, as a reflection of the atherosclerosis process (Drüeke et al., 2002).

## 1.3 CARDIOVASCULAR DISEASE IN END STAGE RENAL DISEASE PATIENTS

Although dialysis treatment is shown to improve the quality of life as well as the life span in patients with ESRD, attempts to decrease morbidity and mortality in this population have failed. Namely, these patients have shorter overall survival in comparison to general population (Choi et al., 2014). There are data indicating that within 5 years from the beginning of hemodialysis treatment, almost half patients will die (Foley et al., 1998). The main cause of death is of cardiovascular origin. Indeed, cardiovascular mortality is increased 20-30-fold in hemodialysis patients (Foley and Parfrey, 1998). Therefore patients on hemodialysis are prone to the development and progression of cardiovascular disease (Go, 2016). Interestingly, studies have shown that there is no statistically significant difference in terms of cardiovascular complications between patients treated with hemodialysis and peritoneal dialysis (Siamopoulos and Elisaf, 1997).

Cardiovascular diseases (CVD) can be divided into two groups: ischemic and nonischemic CVDs. The ischemic group of cardiovascular diseases comprises coronary artery disease, ischemic cardiomyopathy, cerebral vascular insult (CVI or stroke) and peripheral vascular disease. In the non-ischemic CVD are arrhythmia and valvular heart disease. It is noteworthy to mention that the risk of CVD is already increased even in early stages of CKD and increases rapidly in later stages where the renal function severely decreases (Vanholder et

al., 2005). It seems that with each decrease of 5 ml/min/1.73m$^3$ of GFR the risk of cardiovascular death rises for 22% (Hallan et al., 2007). The similar risk was accounted for albuminuria, where it was demonstrated that it has even a stronger association with atherosclerosis than GFR (Rodondi et al., 2007). Atherosclerosis represents the main pathological mechanism in the development of ischemic heart disease, cerebral vascular disease and periphery artery disease (Brevetti et al., 2010; Libby, 2002).

### 1.3.1 Oxidative stress-endothelial dysfunction-cardiovascular disease triade in end-stage renal disease patients

Well-known cardiovascular risk factors (hypertension, diabetes, smoking and metabolism disorders) cannot explain that high prevalence of cardiovascular diseases in this population, so other non-traditional risk factors, such as endothelial dysfunction and oxidative stress have increasingly been studied (Yao et al., 2004). A term of "accelerated atherosclerosis" has been introduced in order to explain high burden of CVD complications in patients with ESRD. Many questions arise as to which states contribute to such a bad prognosis and many studies conducted by different research groups tend to identify risk factors (Burmeister et al., 2014; Kotur-Stevuljevic et al., 2012). Indeed, specific metabolic, inflammatory and immune factors may contribute in multiplications of risk for cardiovascular diseases and mortality. Occlusion of the blood vessel by atheroma leads to myocardial infarction or stroke development. Atherosclerosis is now considered not only as a problem of lipid accumulation, but also as a result of complex inflammatory and oxidative processes. The pivotal role of oxidative stress in cardiovascular disease development and progression has been widely studied in the last few decades. Many studies showed that increased ROS production affects initiation, progression and clinical consequences of CVD (Csányi et al., 2012; Förstermann and Münzel, 2006; Sugiyama et al., 2004).

The endothelium has an important role in controlling vascular function. When intact, the endothelium produces regulatory molecules which provide anti-atherogenic environment. However, any abnormalities occurring may result in development of atherosclerosis. The highly reactive species, such as ROS, alter endogenous vasoactive mediators expression of endothelium, contributing to atherogenic processes. In further course, ROS oxidize low

density lipoproteins to form their oxidized forms (oxLDL), the process which is accepted as the most important one in atherogenesis. The oxLDL causes accumulation of cholesterol esters in macrophages (Bobryshev, 2006) (Figure 4). Additionally, oxLDL has cytotoxic effect and contributes to inhibition of macrophage motility (Collot-Teixeira et al., 2007; Yu et al., 2013). At this point endothelial cells are activated and will release many cytokines and express various receptors. Importantly, the free radicals are not only produced by endothelial cells, but also the smooth muscle cells and the adventitial cells (Madamanchi et al., 2005).

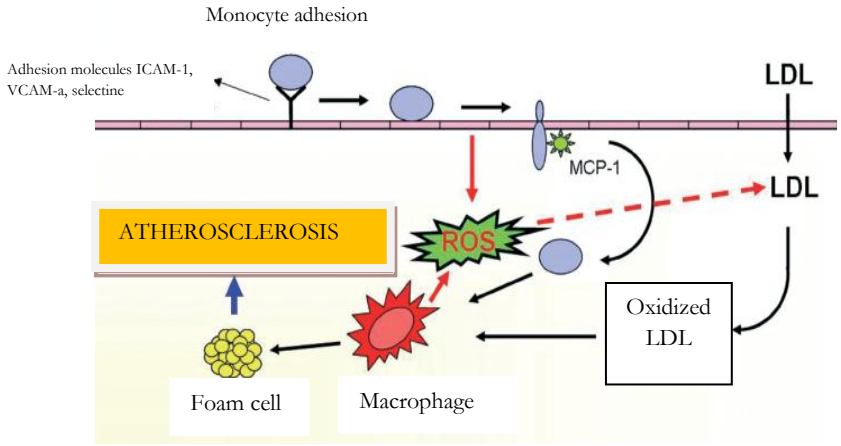


**Figure 4**. Oxidative stress in atherosclerosis. Free radicals provoke oxidation of LDL and monocyte adhesion and migration to subendothelial space. The oxLDL is taken by macrophages which become foam cells. The photo was adapted from (Bonomini et al., 2008).

Intact vascular endothelium plays an essential role in maintaining adequate vascular tone and vascular homeostasis. Furthermore, it prevents platelet aggregation and smooth muscle cell proliferation. Any alterations affecting endothelial physiology, also known as *endothelial dysfunction*, are shown to play an important role in early step in the development of atherosclerosis (Davignon and Ganz, 2004; Mudau et al., 2012; Sitia et al., 2010). Additionally, endothelial dysfunction contributes to plaque progression and the occurrence of atherosclerotic complications (Hadi et al., 2005). When ROS production exceeds antioxidant mechanisms, an excessive lipid peroxidation and protein oxidation occur. This induces cellular response reflected by increased vascular endothelium permeability, over-expression of redox genes, intracellular calcium overload and DNA fragmentation, resulting in damage to vascular smooth muscle cells (VSMCs) and endothelial cells (Dulak et al., 2000). Additionally, when

redox balance is disturbed and fatty acids are oxidized, MDA formation further contributes to LDL modifications (Holvoet et al., 1995).

When trombocytes encounter activated endothelial cells, they secrete monocyte chemoattractant protein-1 (MCP-1) which facilitates monocyte adhesion to endothelium and plays a crucial role in initiating atherosclerosis by recruiting macrophages and monocytes to the vessel wall (Boring et al., 1998). In further actions, expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and platelet–endothelial cell adhesion molecule-1 (PECAM-1) is also increased (Blankenberg et al., 2003; Nakashima et al., 1998).

Although the exact mechanism of atherosclerotic changes in uremic patients has not been well documented, it has been accepted that endothelium dysfunction is a prominent feature of end-stage renal disease. Chronic kidney disease leads to alteration of endothelium structure, its properties and function (Hayakawa and Raij, 1999; Kunz et al., 1999). An increase in oxidative stress favors endothelial dysfunction by reducing nitric oxide availability and its subsequent beneficial effects on vascular function (Lerman and Burnett, 1992). Even a single hemodialysis session may favor endothelial dysfunction due to sequestration of nitric oxide (NO) and increased levels of ROS (Errakonda et al., 2011).

Some authors suggested that in uremic patients, elevated values of AOPPs are able to provoke endothelial dysfunction and monocyte activation (Marsche et al., 2004; Witko-Sarsat et al., 2003). Indeed, AOPP is not only the oxidative stress byproduct but it also has the ability to induce both free radical production and vascular inflammation. Namely, Gou et al have shown that oxidized proteins are able to activate endothelial cells via RAGE (receptor for advanced glycation end products)-mediated signaling pathway (Guo et al., 2008).

It has been suggested that in inflammatory and pro-oxidant environment in patients with ESRD, endothelium is responding by expressing cellular adhesion molecules (ICAM-1 and VCAM-1) that facilitate the adhesion of leukocytes to the endothelial cells and their migration (Gou et al, 2008). It has been shown that TNF-α, an inflammatory marker, induces expression of ICAM-1 and VCAM-1 in cultured endothelial cells (Bevilacqua, 1993). Furthermore, increased levels of soluble CAMs are elevated in patients undergoing hemodialysis and show a positive correlation with von Wilebrand factor, another proposed marker of endothelial activation and thrombotic occurrence (Bolton et al., 2001).

## 1.4 GLUTATHIONE TRANSFERASES

Glutathione transferases (GSTs) (EC 2.5.1.18) are superfamily of enzymes belonging to phase II enzymes that play an important role in the detoxification reactions (Hayes et al., 2005). GSTs catalyze the conjugation of the tri-peptide glutathione (GSH, γ-glutamylcysteinyl-glycine) with a hydrophobic co-substrate possessing an electrophilic center, in order to produce an inactive hydrophilic conjugate which is easily excreted from the body (Figure 5). However, several GSTs do not use GSH as substrates but, as cofactors (in e.g. leukotriene biosynthesis).

Members of large GST enzyme family are able to detoxify numerous toxic compounds and possess strong antioxidant activity towards reactive oxygen species and peroxides (Habig et al., 1974; Hayes et al., 2005).

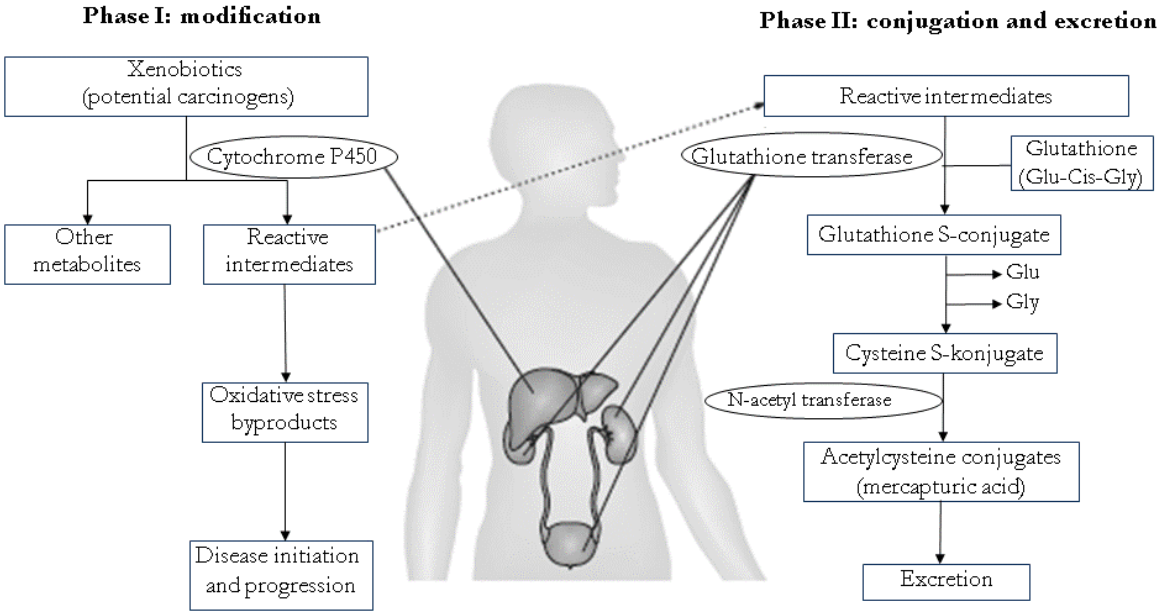


**Figure 5.** GST catalyze the GSH conjugation to a xenobiotic which generate GSH-conjugate. Photo adapted from (Simic et al., 2009)

Apart from conjugation with glutathione, GSTs catalyze hormone biosynthesis, peroxide breakdown, tyrosine degradation, dehydroascorbate reduction and many other reactions (Hayes et al. 2005). Furthermore, GSTs possess non-catalytic activity and the most

important one described so far are the role of GSTP1 and GSTM1 in the inhibition of c-Jun N terminal kinase (JNK) (Adler et al. 1999).

### 1.4.1 Classification of GSTs

Glutathione S-transferases are classified into three superfamilies: (i) cytoplasmic transferases, (the largest family consisting of dimeric and soluble enzymes), (ii) the mitochontrial and (iii) the microsomal GSTs. The microsomal GSTs are integral membrane proteins, now designated as membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEGs). Microsomal GST enzymes are trimeric and membrane-bound and they belong to MAPEG superfamily. A classification of GSTs is based upon their protein sequence and structure. Cytosolic GSTs consists of seven major classes designated by the names of Greek letters and abbreviated in Roman letters: alpha (GST A; five members), mu (GST M; five members), pi (GST P; one member), theta (GST T; two members), zeta (GST Z; one member), omega (GST O; two members) and sigma (GST S; one member) (Mannervik et al., 2005)(Table 4).

Each class has one or more protein sequences which are numbered using Arabic numerals. Amino acid sequence identity is greater than 40% for members of the same GST class while GSTs from different classes share less than 25% identity.

**Table 4.** Classification of human glutathione transferases based on amino acid sequence identity. The table has been adapted from (Hayes et al., 2005)

| GLUTATHIONE TRANSFERASES (GST) | | |
|---|---|---|
| **Cytosolic GST** | **Mitochondria GST** | **Microsomal GST** |
| Alpha class: A1-1,A2-2, A3-3, A4-4, A5-5 | Kappa class: GST K1-1 | MGST1 |
| Mu class: M1-1, M2-2, M3-3, M4-4, M5-5 | | MGST2 |
| Pi class: GST P1 | | MGST3 |
| Theta: GST T1, T2-2 | | |
| Zeta class: GST Z1-1 | | |
| Omega class: O1-1, O2-2 | | |
| Sigma class: GST S1-1 | | |

All GST isoenzymes play an important role in the biotransformation of toxic compounds and chemical environmental agents (Luo et al., 2011). However, individual GSTs have distinct substrate specificities and catalyze different chemical reactions.

### 1.4.2 Structure and substrate specificity

Soluble GSTs function as 50kD dimmers which are formed from subunits within a given GST isoenzyme class (Hayes et al., 2005). Dimeric structure of GSTs increases the stability and provides a proper structure of the active site that is required for the efficient catalysis (Abdalla et al., 2002; Armstrong, 1997). However, there are some evidence of GSTs monomer-dimer equilibrium important for protein : protein interactions, described in mitogen activated protein kinases (Adler et al., 1999; Cho et al., 2001).

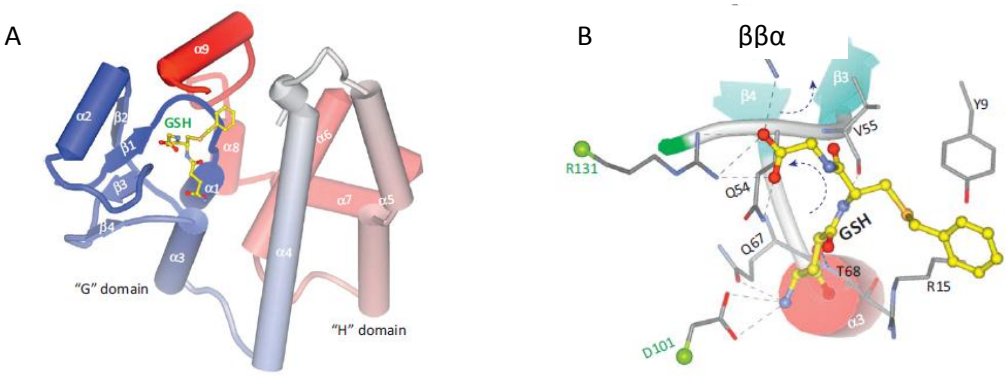


**Figure 6.** Three-dimensional structure of GST with G- and H-domain depicted (A). Interaction of glutathione with ββα motif residues of glutathione transferase (B) (adopted from (Wu and Dong, 2012))

A single GST unit consists of two important domains: (1) an N-terminal α/β-domain (G-domain) and (2) an all-α-helical domain (so called H-domain) (Wu and Dong, 2012). The G-domain represents a binding site for tripeptide cofactor glutathione (GSH, γ-glutamyl cysteinyl-glycine) and it is conserved throughout all classes (Figure 6). The sulfur from glutathione is hydrogen bonded to a catalytic residue in the N-terminal end of the protein (Atkinson and Babbitt, 2009). This hydrogen bond represents a crucial role in GST catalysis by stabilizing the activated GSH (thiolate anion, GS-) (Atkinson and Babbitt, 2009). Some amino-acid residues found in different GST classes contribute to their substrate heterogeneity: (a)

tyrosine (alpha, mu, pi and sigma class of GSTs) (b) serine (theta and zheta GST class) (c) cysteine (omega GST class) (Armstrong 2010).

The H-domain is, however, not conserved as G-domain and may vary in shape, size and biochemical characteristics, influencing enzyme hydrophobicity and electrophilic substrate binding (Oakley, 2011). Thus, alpha GST class is known for its ability to catalyze various structurally unrelated compounds, including steroids, benzo(a)pyrene dioal epoxides and products of lipid peroxidation (Wu and Dong, 2012). Based on its lack of selectivity towards substrate, GSTA1-1 is named "promiscuous" (Table 5).

**Table 5**. The list of substrates recognized by cytosolic GSTs (adapted from Hayes 2005)

| GST enzyme | Preferred substrates |
|---|---|
| GSTA1 | BCDE, BPDE, chlorambucil, busulfan, DBADE |
| GSTM1 | BPDE, CDE, DBADE, styrene-7.8-oxide |
| GSTT1 | Butadiene epoxides, ethylene oxide, EPNP, BCNU |
| GSTP1 | Acrolein, base propenals, BPDE, CDE, chlorambucil, EA, COMC-6 |

BCDE-benzo-chrysene diol epoxide; BCNU 1.3-bis(2-chloroethyl)-1-nitrosourea; BPDE benzo(a)pyrene diol epoxide; CDE chrysene-1,2-diol 3,4-epoxide; DBADE dibenzo(a,h)anthracene diol epoxide; DBPDE dibenzo(a,l)prene diol epoxide; EA ethacrynic acid; COMC-6 crotonyloxymethyl-2-cyclohexenone; EPNP 1,2-epoxy-3-(p-nitrophenoxy)propane

GST mu class possesses much larger active site that recognizes more electrophilic compounds such as aflatoxin B1-epoxides and benzopyrene diols (Wu and Dong, 2012). It is important to notify that different GSTs have overlapping substrate specificities, sometimes resulting in difficulty to identify isoenzymes based only on their catalytic properties.

### 1.4.3 Metabolism of exogenous and endogenous compounds by GST

The reaction catalyzed glutathione transferases consists of nuclephilic addition of the sulfur thiolate of glutathione to a wide range of electrophilic compounds (Dourado et al., 2008) (Dourado 2008). Once the GSH is activated, its nuclepohilic sulfur atom attacks the electrophilic compound present in H-site producing glutathione S-conjugate. This conjugate is

water-soluble, usually less toxic than the compound from which it originated. Namely, when methylen chloride is catalyzed by theta class of GST, more toxic compound formaldehyde, with potential cancerogenicity, is generated during this process (Meyer et al., 1991). Upon formation, glutathione conjugate is exported from the cells by ATP-dependent transmembrane pumps and metabolized via mercapturic acid metabolic pathway forming S-cysteine conjugates (Ballatori et al., 2009). Afterwards, all metabolites are excreted via urine or bile (Egner et al., 2008; Teichert et al., 2009).

Besides metabolizing various exogenous compounds, glutathione transferases are involved in intracellular binding, transport and catalysis of leukotriens, prostaglandins and steroid hormones (Hayes et al., 2005). Furthermore, many endogenously formed byproducts of oxidative stress are also inactivated by the action of GST. Especially the alpha class of GST takes major place in catalyzing phospholipids, fatty acid and cholesterol hydroperoxydes (Seeley et al., 2006). Other substrates for alpha class include degradation products of lipid peroxidaion, acrolein and 4-hydroxynonenal (Hubatsch et al., 1998). There is evidence that some GST exhibit selenium independent glutathione peroxidase activity that contributes to antioxidant protection of the cells along with other antioxidant enzymes (Hayes and McLellan, 1999). Another important role of glutathione transferases, especially omega class, is the catalysis of reaction of S-glutathionylation, implicated in protection against oxidative stress and protein structure and function (Tew and Townsend, 2011). In the reaction of S-glutathionylation the conjugation of GSH to low pKa cysteine sulfhydryl or sulfonic-acid occurs as a response to oxidative stress (Tew and Townsend, 2011).

### 1.4.4 Polymorphism of glutathione transferases

The term genetic polymorphism refers to the difference in the DNA sequence among individuals, groups, or populations. Genetic polymorphism presents the recurrence, within a population, of two or more discontinuous genetic variants (alleles) of a specific trait in such proportions that they cannot be maintained simply by mutation. Certain form of genetic variant is considered an allele if its frequency is more than 1% in general population. Sources of polymorphism include single nucleotide polymorphism (SNPs), sequence repeats, insertions, deletions and recombination. The most common polymorphisms are SNPs ("snip")

where a single base mutation occurs in the DNA. A result of a SNP can be (i) no alteration of amino acid in protein sequencing (so called "synonymous" substitution) or (ii) the substitution results in an alteration of the encoded amino acid ("non-synonymous" substitution). Some SNPs are found in regulatory regions of genes and therefore responsible of changing the amount of a protein's synthesis. However, deletion polymorphisms are not rare. By this type of polymorphism it is considered a complete lack of a functional gene which results in an absent protein transcript.

The *GST* genes located on different chromosomes are described as highly polymorphic (Krajka-Kuźniak et al., 2008; Oakley, 2005). Because of their well known xenobiotic-metabolizing activity, genetic variations in *GST* genes have gained much of the attention recently. Any change in the expression of GST protein level might possibly influence an individual's susceptibility to carcinogens and various diseases. From the clinical point of view, deletion and single nucleotide polymorphism of the classes *GSTA1*, *GSTM1*, *GSTP1*, and *GSTT1* have been widely studied.

#### 1.4.4.1 Glutathione transferase A1 Polymorphism

The human alpha class glutathione S-transferase consists of five genes (*GSTA1*, *GSTA2*, *GSTA3, GSTA4* and *GSTA5*) and seven pseudogenes, clustered on chromosome 6p12. The GSTA1 and GSTA2 have been known for years and probably the most extensively described. They catalyze the conjugation of glutathione with many electrophilic compounds and possess glutathione-dependent (selenium-independent) peroxidase activity (Figure 9). The alpha class has activity for polycyclic aromatic hydrocarbon, epoxides and alkenyl products of lipid peroxidation (4-hydroxynonenal) (Johansson and Mannervik, 2001). Their expression is present at high levels in liver, small intestine and kidney and at low levels in a wide range of tissues (Coles et al., 2001; Morel et al., 2002).

Genetic polymorphism has been described for several classes. GSTA1 expression is influenced by a genetic polymorphism in the proximal promoter, with three linked base substitutions at positions -567, -69 and -52 resulting in two alleles h*GSTA1*A* (-52G, -69C, -567T) and h*GSTA1*B* (-52A, -69T, -567G) (Morel et al 2002). Data suggest that promoter activity of *hGSTA1*A* and *hGSTA1*B* is mainly under influence of -52G>A base changing.

Experiments *in* vitro have showed high transcriptional activity of *GSTA1*A* gene (wild type) and very lower transcriptional activation with *GSTA1*B* allele (variant allelle) (Morel et al., 2002). It seems that alteration occurring at this position is a result of modified binding of the ubiquitous transcription factor Sp1 (Morel et al., 2002) (Figure 7).

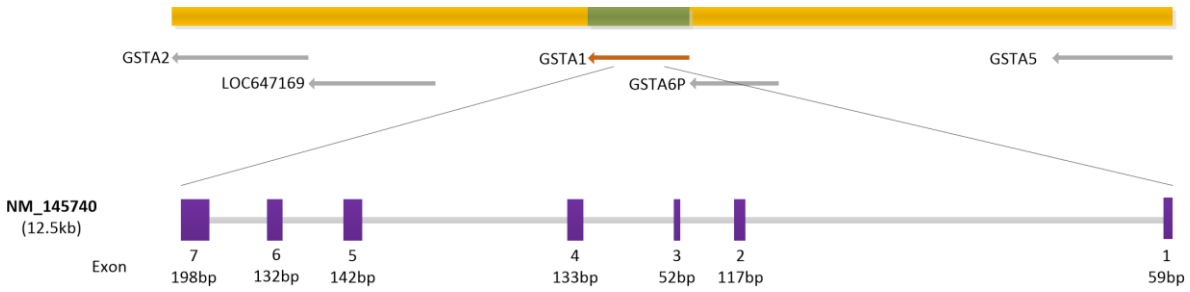


**Figure 7.** *GSTA1* gene (Savic-Radojevic and Radic, 2014)

Distribution of alleles varies within population. In Caucasians around 38% possess *GSTA1*A/*A* genotype, 48% *GSTA1*A/*B* genotype and 14% *GSTA1*B/*B* genotype (Magagnotti C at al., 2003) while in African and Asian population distribution is slightly different (61% *GSTA1*A/*A*, 26% *GSTA1*A/*B*, 13% *GSTA1*B/*B* and 78% *GSTA1*A/*A*, 19% *GSTA1*A/*B*, 1% *GSTA1*B/*B*, respectively) (Coles et al., 2001). Expression of *GSTA1*B* allele results in 4-fold lower enzyme activity, so far linked with many disease development such as colorectal carcinoma (Sweeney et al., 2002), prostate cancer (Komiya et al., 2005) and bladder cancer (Matic et al., 2013).

### 1.4.5 Glutathione transferase M1 gene polymorphism

Class mu genes are approximately 5 kb in length, and in humans all five genes are clustered together on chromosome 1p13.3 (Ross et al., 1993) arranged as 5'-*GSTM4-GSTM2-GSTM1-GSTM5*-3' (Xu et al., 1998). Three alleles of *GSTM1* class have been identified so far *GSTM1*A*, *GSTM1*B* and *GSTM1*0* with the latter resulting in deletion of the entire gene and therefore complete lack of enzyme activity. Since no evidence of functional difference between *GSTM1**A and *GSTM1*B* have been reported, these two alleles are considered as single functional phenotype (Widersten et al., 1991) (Figure 8). The other four genes belonging

to mu subfamily (*GSTM2*, *GSTM3*, *GSTM4*, and *GSTM5*) possess very similar sequence homology and substrate specificity with GSTM1 (Pearson et al., 1993).

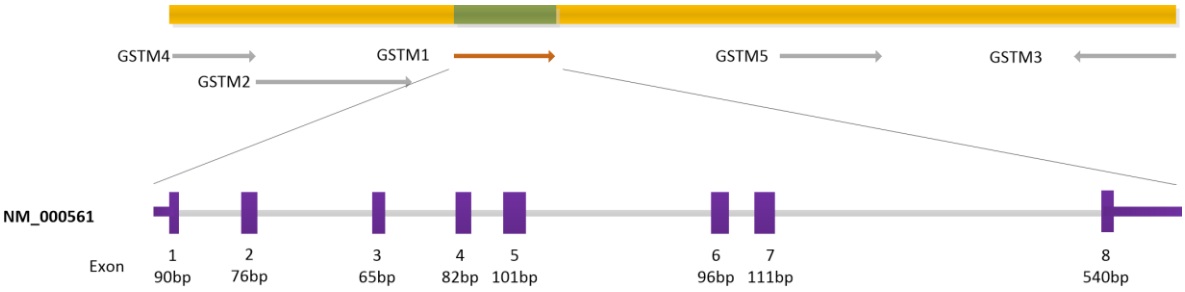


**Figure 8.** *GSTM1* gene (Pljesa-Ercegovac and Matic, 2014)

The GST mu class has been recognized to metabolize carcinogens from cigarette smoke such as polycyclic aromatic hydrocarbons (PAHs) and aromatic amines (Ketterer et al., 1992). About 50% of the Caucasians are homozygous for *GSTM1*0/GSTM1*0* (*GSTM1-null*) genotype and lack GSTM1 enzyme activity (Benhamou et al., 2002). Individuals with *GSTM1-null* genotype have no capability of detoxifying these carcinogens and therefore are possibly associated with an increased susceptibility to various types of cancers including lung cancer(Sharma et al., 2015; Yang et al., 2015; Zhang et al., 2014), urinary bladder carcinoma (Matic et al., 2013) and acute leukaemia (Arruda et al., 2001). Apart from enzymatic activity, GSTM1 acts as a modulator of protein kinase (MAPK) signal transduction pathway and mediates apoptosis via a mechanism involving protein-protein interactions thus forming complexes with apoptosis signal-regulating kinase 1 (ASK1), by inhibiting ASK1 activation during cellular stress (Cho et al., 2001; Townsend and Tew, 2003).

#### 1.4.5.1 Glutathione transferase P1 polymorphism

Gene for GSTP1 is located on chromosome 11q13. Pi class genes are about 3 kb long and contain seven exons. Within *GSTP1* gene two SNPs have been described so far: transition from Ile to Val at codon 105 and transition of Ala to Val at codon 114. This SNPs result in four polymorphic alleles *GSTP1*A*, *GSTP1*B*, *GSTP1**C, and *GSTP1*D*. Wild-type allele *GSTP1*A* has isoleucine (Ile) at codon 105 and alanine (Ala) at codon 114. *GSTP1*B* allele has one amino acid substitution (*Val105/Ala114*), *GSTP1*C* has two amino acids substitutions

(*Val105/Val114*), while *GSTP1*D* has Ile at position 105 and Val at 114 (*Ile105/Val114*) (Ali-Osman et al., 1997; Watson et al., 1998) (Figure 9). These SNPs are found in the active site of the GSTP1-1 protein consequently decreasing the enzyme's catalytic activity by 5-fold (Harries et al., 1997). However, these polymorphism do not change the GSH-binding site, but the formation of thioether conjugates between GSH and some small-molecule electrophiles (Ali-Osman et al., 1997; Zimniak et al., 1994).

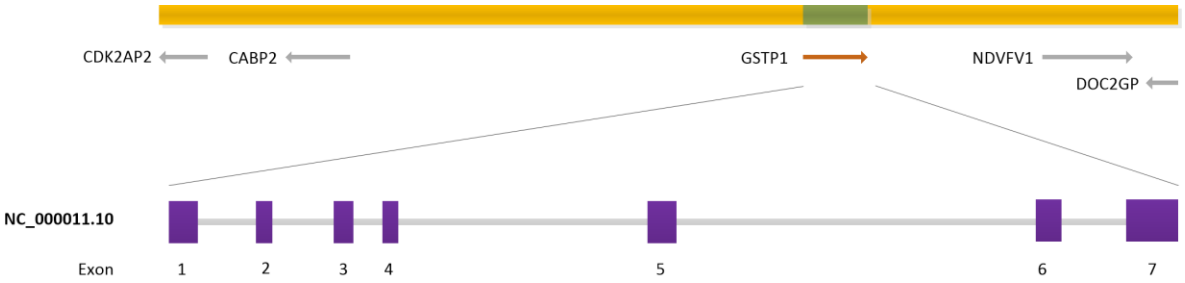


**Figure 9.** *GSTP1* gene polymorphism.

Most of the substrates for GSTP1 protein are different diol epoxides, polycyclic aromatic hydrocarbon (PAHs) found in a cigarette smoke. Therefore, polymorphic expression of *GSTP1* has been linked so far with various cancer developments including endometrial (Chan et al., 2005), testicular (Harries et al., 1997), hepatocellular carcinoma (Chen et al., 2010). As GSTP1 protein is highly expressed in lungs, patients with polymorphic expression of this gene were at greater risk for asthma (Kaymak et al., 2016; Lee et al., 2015; Su et al., 2013) and bronchial carcinoma (Feng et al., 2013; Ou et al., 2015; Xu et al., 2014). Furthermore, Harries et al (1997) suggested that patients homozygous to *GSTP1*B* allele had increased risk to develop testicular cancer (Harries et al., 1997).

In *GSTP1* gene several single nucleotide polymorphisms have been described with *GSTP1* rs 1695 and *GSTP1* rs1138271 being the most relevant one from the clinical point of view. In healthy, white populations, the frequencies of the genotype variants of *GSTP1* rs1695 polymorphism are **Ile/*Ile* (AA) 51.5%, **Ile/*Val* (AG) 39.4% and **Val/*Val (AG)* are 9.1%, (Garte et al., 2001). The distribution of *GSTP1* (rs1138272) genotype in European population **Val/*Val (CC)* and **Val/*Ala (CT)* are 92.1% and 7.9% respectively (Karaca et al., 2015). It is interesting that the distribution of variant allele is very low when general population is

examined (Karaca et al., 2015). It is common that individuals carrying both variant alleles are named with *GSTP1-low activity* genotype, while those who carry at least on referent allele are called *GSTP1-active* individuals.

### 1.4.5.2 Glutathione transferase T1 polymorphism

The theta class of glutathione transferases consists of two genes, *GSTT1* and *GSTT1*. These genes are found at 22q11.2 and the distance between them is about 50 kB (Landi, 2000). There are several substrates related to GSTT catalytic activity, including carcinogens PAHs found in tobacco smoke and in combustion products (Meyer et al., 1991). Another substrates include 1,3-butadiene and ethylene oxide, methyl-bromide, ethylene-dibromide and ethylene-oxide (Pemble et al., 1994; Wiencke et al., 1995).

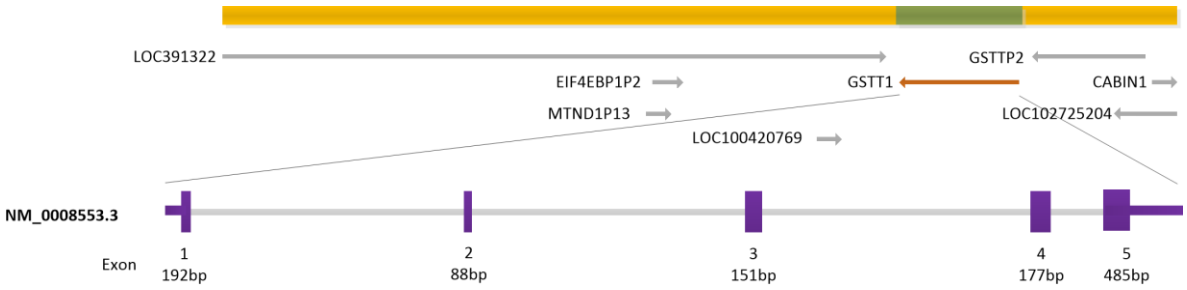


**Figure 10.** *GSTT1* gene polymorphism

The human *GSTT1* gene is 8.1 kb in length (Webb et al., 2016; Coogan et al., 1998) and like *GSTM1*, most commonly exhibits deletion polymorphisms (Figure 10). The complete deletion of the gene (homozygotes) represents an absence of both *GSTT1*0* alleles (*GSTT1-null* genotype), which results in a complete lack of enzyme activity. Heterozygotes with only one allele absent may possess lower enzyme activity and are called *GSTT1-active* genotype carriers. In the case of *GSTT1*, gene homozygous deletion, present in about 20% of Caucasians, leads to the lack of GSTT1 enzyme activity (Libetta et al., 2011). Besides deletion polymorphism of *GSTT1* gene, a single nucleotide polymorphism has also been described. This SNP results in forming two alleles *GSTT1*A* and *GSTT1*B* with different catalytic

properties (Alexandrie et al., 2002) (Figure 13). Subjects carrying *GSTT1*A/*A* genotype possess at least 2-fold higher enzyme activity in erythrocytes toward methyl-chloride compared to individuals who have at least one variant *GSTT1*B* allele (Alexandrie et al., 2002). This loss of enzyme activity is due to the conformational changes in enzyme structure which occurred as a consequence of amino acid substitution (threonine to proline) (Alexandrie et al., 2002).

Apart from detoxification of mono-halogen derivatives, GSTT1 is capable of bio-activation of compounds that have more than one halogen in their structure. During these reactions, highly reactive byproducts are formed, capable of reacting with DNA and proteins (Guengerich, 2005; Sherratt et al., 1997). In individuals with *GSTT1-null* genotype, the bladder cancer risk can be either decreased or increased, depending on to which substances the subjects were exposed (Cantor et al., 2010; Simic et al., 2009).

### 1.4.6 Chronic renal failure and *GST* polymorphism

Ever since glutathione transferases came into focus of research, a number of studies have confirmed or annuled an association between GST polymorphisms and the risk of disease developments (Moasser et al., 2014; Piacentini et al., 2012). These disparate findings may be due to insufficient power in some studies, differences between disease types, type of study populations and study design. In addition to well established mechanisms of oxidative stress related to the specific causes of renal failure, dialysis procedure and uremic state, recently the role of genetic predisposition to enhanced oxidative damage and consequent worse prognosis of MHD patients has started to emerge. In hemodialysis patients who encounter enhanced free radical production and accumulation of uremic toxins, up-regulated expression of enzyme glutathione transferase in peripheral blood cells is well described (Carmagnol et al., 1981; Mimic-Oka et al., 1992). However, as recently shown by Lin et al. (Lin et al., 2009), the capacity to evoke the GST response towards oxidative stress in MHD patients seems to be genetically determined. Namely, patients with *GSTM1-null* genotype had enhanced DNA damage based on higher lymphocyte level of 8- (OH) deoxy guanosine and higher mortality rate than HD patients with active GSTM1 enzyme (Lin et al., 2009). Homozygous deletion of *GSTM1* allele has attracted much attention in the epidemiologic studies as a result of risk linkage with lung and bladder cancer (Matic et al., 2013; Sharma et al., 2015) and increased

susceptibility to coronary heart disease among smokers (Li et al., 2000; Miller et al., 2003). Despite the fact that oxidative modifications of proteins and lipids have important role in the pathogenesis of cardiovascular complications in these patients, the question of whether *GSTM1* genotype influences the level of byproducts of protein and lipid oxidative damage in patients with ESRD has not been well established as yet.

In addition to *GSTM*1, polymorphisms of other GSTs (*GSTT1*, *GSTP1* and *GSTA1*) have also gained a lot of attention in genetic epidemiologic studies. It seems reasonable to assume that *GSTT1-null* or *GSTA1* or *GSTP1 low* activity genotypes might also influence the level of oxidative stress in HD patients and thus contribute to endogenous predisposition to oxidative damage in the setting of disrupted redox balance. Still, the role of polymorphic expression of *GSTT1*, *GSTP1* and *GSTA1* genes in increased oxidant-induced protein and lipid damage among HD patients has to be established. In this line, it seems reasonable to assess whether null or low activity *GSTM1*, *GSTT1*, *GSTP1* and *GSTA1* genotype alone or in combination correlate with eight byproductss of oxidative stress including protein thiol, carbonyl groups, nitrotyrosine, advanced oxidation protein products, malondialdehyde, malondialdehyde adducts, total oxidant status and prooxidant-antioxidant balance in patients with end-stage renal disease.

Many efforts have been made to highlight the pathogenic mechanisms leading to huge cardiovascular burden in hemodialysis patients. Thus the prevalence of cardiovascular diseases is up to 30 fold higher in the patients with ESRD than in general population. So far there are no data related to the link between *GST* polymorphism and fatal/non-fatal cardiovascular diseases in ESRD patients. In the non-ESRD population individuals with *GSTM1* and/or *GSTT1-null* genotypes seem to be at higher risk of cardiovascular disease (Nomani et al., 2011; Ramprasath et al., 2011). The observed link between *GST* polymorphism and CVD was further strengthened in smokers lacking *GSTM1* or *GSTT1* genes (Masetti et al., 2003; Wang et al., 2008). In addition, it seems that in patients with type 2 diabetes mellitus with *GSTT1-null* genotype and *GSTT1*0/GSTM1*0* genotype combination, the susceptibility to advanced atherosclerosis is higher than in controls (Taspinar et al., 2012). However, different studies have yielded conflicting results.

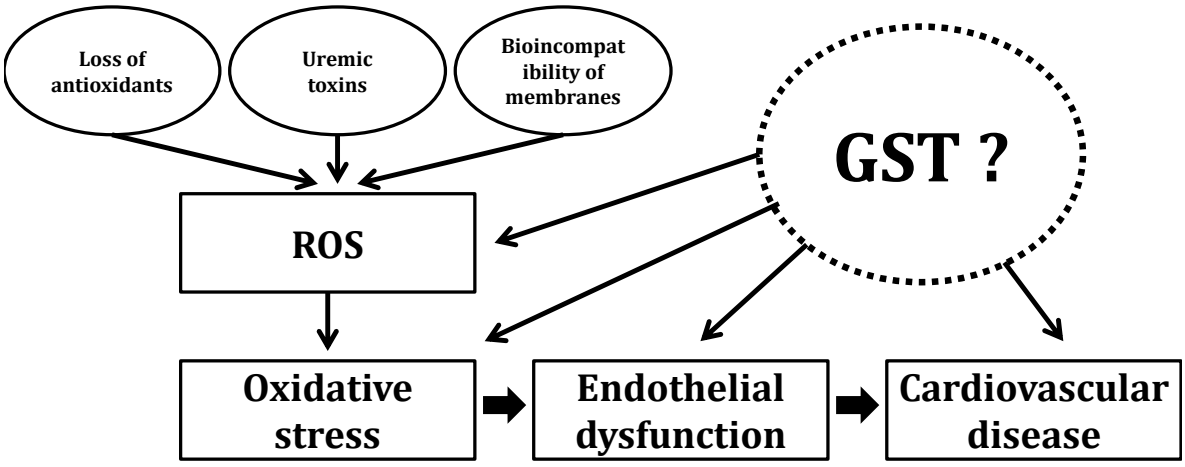


**Figure 11.** Possible association between glutathione transferase gene polymorphism and oxidative stress and cardiovascular disease

In ESRD patients, only polymorphic expression of *GSTM1* was studied with respect to prognostic significance. Although the presence of *GSTM1-null* genotype in ESRD patients was associated with lower overall survival compared to those with *GSTM1-active* gene variants (Lin et al., 2009), specific association of this and other common *GST* polymorphisms, which play a key role in antioxidant defense, with cause-specific cardiovascular mortality still has to be addressed.

# 2 AIMS

This thesis had several goals to asses:

1. To determine the distribution of glutathione transferase *A1*, *M1*, *P1* and *T1* gene polymorphism of in patients with end-stage renal disease and control group

2. To evaluate the association of *GSTA1*, *GSTM1*, *GSTP1* and *GSTT1* polymorphisms and byproducts of oxidative stress, as well as the level of adhesion molecules in the plasma of patients with end-stage renal disease

3. To assess the predictive value of *GST* polymorphism, oxidative stress byproducts and adhesion molecules level in 5-year overall and cardiovascular survival of ESRD patients

4. To test whether *GSTM1* genotype influences monocyte adhesion to the endothelial cells and reactive oxygen species production when exposed to uremic milieu

# 3 MATERIAL AND METHODS

## 3.1 STUDY SUBJECTS

This *case-control* study included a total of 199 patients (84 male and 115 female, mean age 60.0 ± 12.1 years) on maintained hemodialysis treatment three times a week in two dialysis centers in Belgrade (Center for Renal Diseases, Zvezdara University Medical Center and Department of Nephrology and Hemodialysis, University Teaching Hospital Zemun).

The inclusion criteria were that all recruited patients were stable, age over 21 and with hemodialysis treatment vintage over 3 months.. Furthermore, patients had to be HIV, HBV and HCV negative.

Exclusion criteria were previously registered malignancy or infectious co-morbidity based on C-reactive protein values and if patients, for any reason, did not want to participate in the study.

Patients did not receive any antioxidant therapy (vitamin C and/or E) at the beginning of the study.A total of 35.2% patients received angiotensin-converting inhibitors therapy and 15.1% received statins.

The causes of end-stage renal disease (ESRD) were hypertensive nephrosclerosis (n = 93), glomerulonephritis (n = 32), diabetic nephropathy (n = 25), polycystic renal disease (n = 19), pyelonephritis (n = 19), Balkan endemic nephropathy (n = 7) and obstructive nephropathy (n = 4). Patients were treated with single-use dialysers equipped with low- and high-flux polysulphone membranes, with a membrane surface area of 1.3–2.1. $m^2$.

A total of 199 consequtive controls (85 male and 114 female, mean age 59.3 ± 10.9 years) were recruited from individuals with nephrolithiasis and normal renal function who were admitted to the Clinic of Urology, Clinical Center Serbia during the same time period. All the participants provided written informed consent. This study protocol was approved by the Ethical Committee of Faculty of Medicine, University of Belgrade (No 28/XII-14, date: 28.12.2005), and the research was carried out in compliance with the Helsinki Declaration (as revised in 2013).

## 3.2 DETERMINATION OF GLUTATHIONE TRANSFERASE A1, M1, P1 AND T1 GENE POLYMORPHISM

So far, deletion polymorphisms of *GSTM1* and *GSTT1* genes, and single nucleotide polymorphisms (SNP) of *GSTA1* and *GSTP1* genes were described as most common type of polymorphisms. The *GST* polymorphisms were determined in extracted DNA.

### 3.2.1 DNA extraction

A total DNA was purified using QIAamp DNA Mini Kit kit (Qiagen, Inc., Chatsworth, CA) from the whole blood sample obtained from patients with end-stage renal disease and control group, according to manufacturer's instruction. This spin-column based method provides DNA purified of all contaminants and inhibitors. Briefly, in this method cell membranes of leukocytes are destroyed by incubation in lysis buffer. Hystones and other proteins are removed by enzyme digestion of proteinase K. In the following steps, DNA is transferred to the spin-columns, where it selectively binds silica-gel, and purified with adequate buffers provided by manufacturer. Purity of DNA sample was confirmed by analysis on Gene Quant Pro. If the 260/280 ratio was higher higher 1.8, the quality of isolated DNA was considered good.

Extracted DNA was stored in AE buffer (provided by QIAamp DNA Mini Kit) at -20°C until further usage.

### 3.2.2 Determination of *GSTA1* gene polymorphism

The analysis of the SNP of *GSTA1* C-69T (rs3957357) polymorphism was performed by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) by Coles et al. (Coles et al., 2001) with some modifications. The primers used were *GSTA1* C-69T forward: 5'-TGTTGATTGTTTGCCTGAAATT-3' and *GSTA1* C-69T reverse: 5'-GTTAAACGCTGTCACCCGTCCT-3'. The PCR reaction was performed in Mastercycler gradient thermal cycler (*Eppendorf, Hamburg, Germany*) with an initial denaturation step at 95 °C for 1 min and then 32 cycles as follows: 50 s at 94 °C, 56 s at 62 °C and 60s at 72°C, with final elongation step for 7 min at 72 °C. PCR products were electrophoretically separated on a 1%

agarose gel at 150V for 10 minutes and visualized with ethidium bromide in order to determine successful amplification.

In the next step, PCR products were digested with restriction enzyme EarI (*Thermo Fisher Scientific, Waltham, Massachusetts, USA*) overnight at 37°C. Digested products (*GSTA1 AA* 481 bp, *GSTA1 AB*: 385 bp and *GSTA1 BB:* 96 bp) were separated on 2% agarose gel stained with ethidium bromide and visualized on GelLogic2000 Camera (*Gel Logic Imaging System, Kodak, USA*). The presence of restriction site resulting in two fragments (385 bp and 96 bp) indicated variant genotype (*GSTA1*B/*B*) also named *GSTA1-low activity* genotype. If the result of restriction was the presence of three fragments (481 bp, 385 bp and 96 bp), it indicated heterozygous (*GSTA1*A/*B).* The presence of only one fragment of 481 bp was for referent genotype (*GSTA1*A/*A*).

Representative ethidium-bromide stained gel with *GSTA1* restriction products is presented in Figure 12.

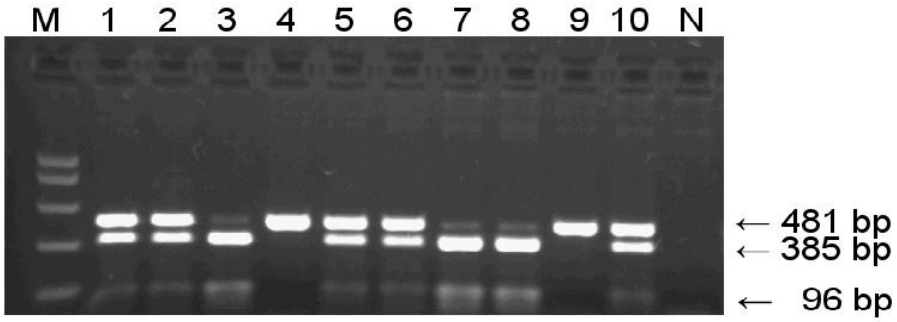


**Figure 12.** Agarose gel electrophoresis. PCR-RFLP products of the *GSTA1* gene. Lanes 4 and 9 represent *GSTA1 *A/*A* genotype (481 bp). Lanes 1, 2, 5, 6 and 10 represent *GSTA1 *A/*B* genotype (481bp, 385bp and 96bp) while lanes 3, 7 and 8 represent *GSTA1 *B/*B* genotype (385bp and 96bp). M-DNA marker. N-negative control.

### 3.2.3 Determination of *GSTM1* gene polymorphism

The analysis of GSTM1 gene deletion polymorphism was performed by multiplex PCR (Abdel-Rahman et al., 1996). Primers used were: *GSTM1* forward: 5'-GAACTCCCTGAAAAGCTAAAGC-3' and *GSTM1* reverse: 5'-GTTGGGCTCAAATATA CGGTGG-3'. Exon 7 of the *CYP1A1* gene was co-amplified and used as an internal control

using the following primers: *CYP1A1* forward: 5'-GAACTGCCACTT CAGCTGTCT-3' and *CYP1A1* reverse: 5'-CAGCTGCATTTGGAAGTGCTC-3'. PCR was carried out for 33 cycles in DNA thermal cycler (*Mastercycler PCR, Eppendorf, Hamburg, Germany*). PCR reaction mixture was incubated and initially denatured at 94°C for 4 minutes. After that, the following steps were performed: denaturation (94°C, 2 minutes), annealing (1 minute at 59°C) and primer extension (1 minute at 72°C). Final extension lasted for 10 minutes at 72°C. PCR products were separated on a 2.5% agarose gel at 150V for 10 minutes and stained with ethidium bromide. The *GSTM1* DNA fragments that were amplified were 215 bp in size. The absence of 215bp band was indicative for *GSTM1-null* genotype. The presence of the *GSTM1-active* genotype (referent genotype) was confirmed by the band at 215bp. The assay does not distinguish heterozygous from homozygous referent genotype (Figure 13).

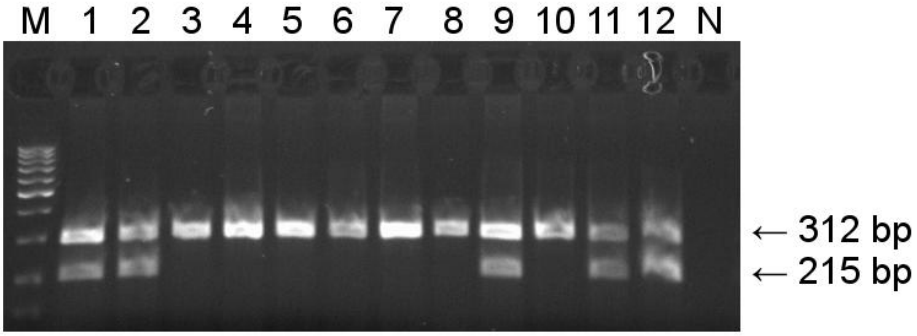


**Figure 13.** PCR products of the *GSTM1* gene deletion polymorphism. Lanes 1, 2, 9, 11 and 12 are patients with *GSTM1-active* genotype (both homozygous and heterozygous; 215bp) and lanes 3 through 8 with lane 10 represent *GSTM1-null* genotype. PCR products of *CYP1A1* gene (312 bp) are shown in upper line. M-DNA marker. N-negative control.

### 3.2.4 Determination of *GSTP1* genes polymorphisms

Single nucleotide polymorphism of *GSTP1 Ile105Val* (rs1695) was analyzed using the PCR–RFLP method by Harries et al. (Harries et al., 1997). Primers used were: *GSTP1 rs1695* forward: 5'-ACCCCAGGGCTCTATGGGAA-3' and *GSTP1 rs1696* reverse: 5'-TGAGGGCACAAGAAGCCCCT-3'. PCR was carried out in 35 cycles in PCR thermal cycler (*Eppendorf Mastercycler, Hamburg, Germany*). Reaction mixture for PCR was incubated and initially denatured at 94°C for 2 minutes. After that, following steps were performed: denaturation

(94°C, 30s), annealing (30s at 55°C) and primer extension (30s at 72°C). Final extension was performed for 5 minutes at 72°C. PCR products were separated on a 2.5% agarose gel at 150V for 10 minutes and stained with ethidium bromide. The presence of 176bp band indicated the presence of amplified *GSTP1* gene. The amplification products (20μL) were digested by 2U of restriction endonuclease Alw26I at 37°C for 16h. Restriction products were separated on a 3% agarose gel. The restriction site resulting in two fragments (91bp and 85 bp) indicated *GSTP1*Val/*Val* (*GSTP1-low activity*) genotype. The presence of three fragments (176bp, 91bp and 85bp) was indicative for *GSTP1*Ile/*Val* genotype, while the presence of one 176 bp band indicated *GSTP1*Ile/*Ile* genotype (referent genotype) (Figure 14).

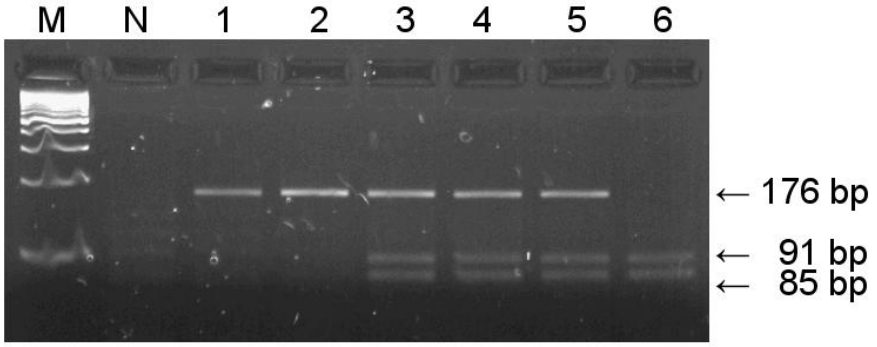


**Figure 14.** PCR-RFLP products of the *GSTP1* gene in 3% gel. Lanes 1 and 2 are wild type (*GSTP1*Ile/*Ile*; 176bp), lanes 3, 4 and 5 are heterozygotes (*GSTP1*Ile/*Val*; 176bp, 91bp and 85bp) and lane 6 is homozygote (*GSTP1*Val/*Val*; 91bp and 85bp). M-DNA marker. N-negative control.

The *GSTP1* rs1138272 polymorphism was determined by qPCR (*Applied Biosystem*) using Applied Biosystem Taqman Drug Metabolism Genotyping Assay (ID C_1049615_20). Briefly, DNA isolated from the blood of patients with ESRD was diluted to the concentration of 6ng/μL and a 5μL of sample was used. The samples were vaporized at 65°C for about 30 minutes. After that, 5 μl of solution consisting of Taqman probe and HotStart Master Mix was added to each well. According to the thermal protocol for qPCR, the initial denaturation step lasted for 4 minutes and was followed by 40 cycles (95°C for 15s and 60°C for 1 minute).

### 3.2.5 Determination of *GSTT1* gene polymorphism

*GSTT1* genotyping was performed by multiplex PCR according to Abdel-Rahman et al. protocol with some modifications (Abdel-Rahman et al., 1996). Primers used were *GSTT1*-forward: 5'-TTCCTTACTGGTCCTCACATCTC-3' and *GSTT1*-reverse: 5'-TCACGGGATCAT GGCCAGCA-3'. Exon 7 of the *CYP1A1* gene was co-amplified using the following primers: *CYP1A1*-forward 5'-GAACTGCCACTTCAGCTGTCT-3' and *CYP1A1*-reverse 5'-CAGCTGCATTTGGAAGTGCTC-3'. PCR reaction was performed with an initial denaturation step at 94 °C for 4 minutes. After initaial denaturation, 33 cycles were performed with following thermal protocol: 2 min at 94 °C, 1 min at 61 °C and 1 min at 72 °C. Final elongation was done for 6 min at 72 °C. PCR products were electrophoretically separated on a 1% agarose gel at 150V for 10 minutes and visualized with ethidium bromide to determine successful amplification.

The assay does not distinguish between heterozygous or homozygous wild-type genotypes; therefore, the presence of 480 bp bands was indicative for the *GSTT1-active* genotype. Lack of band was indicative for *GSTT1-null* genotype. Presence of 312 bp was indicative for the presence of *CYP1A1* gene, used as a positive control (Figure 15).

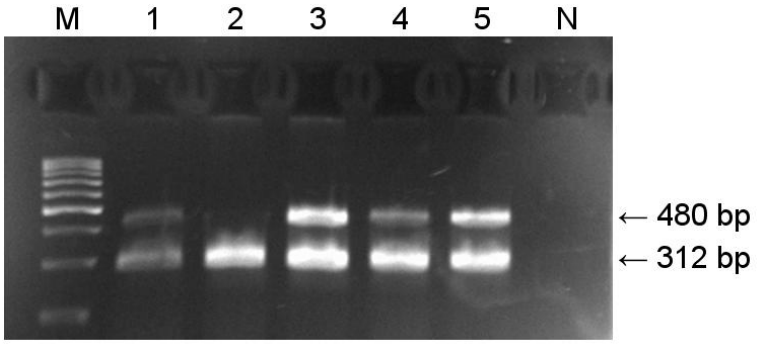


**Figure 15**. PCR products of the *GSTT1* gene. Lanes 1, 3, 4 and 5 are patients with *GSTT1 active* genotype and lane 2 represents *GSTT1-null* genotype. PCR products of *CYP1A*1 gene (312 bp) are showed in lower line. M-DNA marker. N-negative control.

All primers used for PCR reactions are synthesized and bought from *Metabion International AG* (*Planegg*, *Germany*) are presented in Table 6.

**Table 6**. A table summarizing all primer sequences and restriction enzymes used to determine *GST* genotype.

| Gene | Primer sequence (5'-3') | Polymorphism | Anilling T (°C) | PCR product | Restriction enzyme |
|---|---|---|---|---|---|
| *GSTA1* | F:TGTTGATTGTTTGCCTGAAATT<br>R:GTTAAACGCTGTCACCCGTCCT | rs3957357 | 62°C | 481bp | Eam1104I |
| *GSTM1* | F:CTGCCCTACTTGATTGATGGG<br>R: TGGATTGTAGCAGATCATGC | deletion | 59°C | 215bp | - |
| *GSTP1* | F: CCCCAGGGCTCTATGGGAA<br>R: TGAGGGCACAAGAAGCCCCT | rs1695 | 55°C | 176bp | Alw26I |
| *GSTT1* | F:TTCCTTACTGGTCCTCACATCTC<br>R: TCACGGGATCATGGCCAGCA | deletion | 61°C | 480bp | - |
| *CYP1A1* | F: ACTGCCACTTCAGCTGTCT<br>R: CAGCTGCATTTGGAAGTGCTC | positive control | 59-61°C | 312bp | - |

## 3.3 MEASUREMENT OF OXIDATIVE STRESS BYPRODUCTS

To estimate the redox state of patients with ESRD, byproducts of oxidative stress were measured in plasma samples. As markers of lipid peroxidation, malondialdehyde (MDA) and malondialdehyde-adducts (MDA adducts) were determined. In assessment of protein oxidative damage, protein thiol-groups content, nitrotyrosine, advanced oxidation protein products (AOPP) and protein carbonyls were determined. Additionally, prooxidant-antioxidant balance (PAB) and total oxidant status (TOS) were also evaluated.

### 3.3.1 Plasma separation

Venous blood samples (app 5 ml) from patients with ESRD were collected in standard sterile polystyrene vacuum tubes containing ethylene diamine tetra acetic acid (EDTA) at the beginning of the dialysis session prior to the administration of heparin. Blood samples were aliquoted for standard biochemical blood analysis, DNA and plasma isolation. For plasma separation, samples were centrifuged at 3600 rpm for 10 minutes. Plasma samples were aliquoted to avoid frequent thawing/freezing and stored in at -80°C until further usage.

### 3.3.2 Measurement of malondialdehyde levels

Concentration of malondialdehyde was measured using TBARS (thiobarbituric acid reactive substances) colorimetric method (Dousset et al., 1983). This method is based on the ability of MDA to conjugate with thiobarbituric acid forming an MDA-TBA compound. This red colored compound has a light absorption peak at 532nm and molar absorption coefficient of 1.56 x $10^5$ L/ (mol x cm). This method is not specific for MDA exclusively but can detect all TBARS. MDA concentrations in plasma of ESRD patients were read from standard curve, constructed based on MDA standards. All values for MDA are expressed as mmol/L.

### 3.3.3 Determination of MDA protein adducts

MDA protein adducts were measured by enzyme immunoassay (OxiSelectTM ELISA kits, Cell Biolabs). After a standard curve was constructed based on known MDA-BSA concentrations, a quantity of MDA adduct in protein samples was calculated. Detection sensitivity limit of the kit was 2 pmol/mg of MDA adduct.

### 3.3.4 Determination of AOPP (Advanced Oxidation Protein Products)

AOPP is considered as one of the possible markers of protein oxidative injury, especially found in hemodialysis patients. Method for its determination is developed by Witko-Sarsat et al (Witko-Sarsat et al., 1996) and slightly modified for this purposes. The assay is based on the spectroscopic analysis of modified proteins at 340 nm. Namely, when acetic acid and potassium-iodide are added into diluted sample, a specific absorbance at 340 nm is read providing a concentration of AOPP expressed in Chloramine T equivalents (CT equivalents). All measurements are done on STASAR , GILFORD III-Lighting spectrophotometer. EDTA plasma was diluted in phosphate buffer (1 : 5).

### 3.3.5 Determination of nitrotyrosine concentration

The nitrotyrosine is produced when oxidative modifications of tyrosine residues are introduced in proteins as a result of damage caused by peroxynitrite or other potential nitrating agent. Briefly, this assay is based on principle of competitive ELISA. Determination of protein

nitrotyrosine concentration in plasma sample (or nitrated BSA standards) is conducted in several steps. In the first one, sample is added to a nitrated BSA pre-absorbed EIA plate. After a brief incubation, an anti-nitrotyrosine antibody is added, followed by an HRP conjugated secondary antibody. The protein nitrotyrosine content in plasma sample was determined using a standard curve created from predetermined nitrated BSA standards.

### 3.3.6 Prooxidant-antioxidant balance (PAB)

In order to evaluate the prooxidant–antioxidant balance, the determination of both the oxidant and the antioxidant status is achieved. This method is a modification of previously described by Alamdari et al (Alamdari et al., 2007). In this assay a total amount of hydrogen-peroxide in antioxidant moiety is calculated using a chromogen 3,3´5,5´-tetramethylbenzidine (TMB) which reacts with both $H_2O_2$ and antioxidants (uric acid). These two compounds are chosen because they do not interact with each other and do not interfere with the course of reactions. Peroxidase catalyzes the reaction between TMB and $H_2O_2$ where TMB is oxidized to the cation which forms a blue colored compound. In the presence of antioxidants, TMB is reduced to form a colorless product. The PAB value is calculated using standard curve made on a basis of intensity of predetermined standard solution. The standard solutions were prepared by mixing varying proportions (0-100%) of hydrogen peroxide with uric acid. The capacity of antioxidants is determined as a value of uric acid concentration expressed in μmol x $L^{-1}$ of uric acid. The capacity of pro-oxidants is determined as a value of $H_2O_2$ concentration expressed in μmol x $L^{-1}$.

The PAB values were of plasma samples were calculated based on the standard curve and expressed in arbitrary HK units (the percentage of $H_2O_2$ in the standard solutions).

### 3.3.7 Total-oxidant status measurement

Hydrogen peroxide and lipid peroxides represent the main components of total oxidant status assay, a method described by Erel in 2005 (Erel, 2005). This assay is based on the ability of oxidants present in the sample to oxidize ferrous ion in o-diasinidine complex to ferric ion. Molecules of glycerol make this process easier. Ferric ion produced in the reaction will react with xylenol orange in an acidic medium to produce colored complex compound

which absorbance is measured spectrophotometrically at λ= 560 nm. Different concentrations of $H_2O_2$ (10-200 μmol/L) were used to plot standard curve where all TOS values of plasma samples were obtained.

### 3.3.8 Protein thiol groups

Total content of protein thiol groups in plasma samples from patients with ESRD was determined according to method previously described by Jocelyn (Jocelyn, 1987). This method is based on reactions of thiols with Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB. Protein thiol groups react with DTNB cleaving the disulfide bond to give 2-nitro-5-thiobenzoate ($TNB^-$), which will ionize to the $TNB^{2-}$ dianion in water at neutral and alkaline pH. This $TNB^{2-}$ ion has a yellow color. Molar extinction coefficient for TNB is 13.6 x $10^3$ Lmol/1cm at 412 nm wavelength. Since the sunlight can reduce DTNB, all reactions were performed in dark place or protected from sunlight.

### 3.3.9 Protein carbonyls

Protein carbonyls were determined by ELISA method (Oxiselect Protein Carbonyl Elisa Kit). This immunoassay is based on quantitation of protein carbonyls in protein samples by comparing with a standard curve that is prepared from predetermined BSA standards.

All the protein carbonyls are derivatized to dinitrophenyl (DNP) hydrazone and incubated with an anti-DNP antibody, as primary antibody, and after that with secondary HRP conjugated antibody. Absorbance was read at 450 nm as primary wavelength and fully reduced BSA standards were used as blank. Every measurement was performed in duplicate. Carbonyls concentration in plasma samples was expressed as nmol/mg.

## 3.4 DETERMINATION OF CIRCULATING ADHESION MOLECULES IN PLASMA

Having in mind that early atherosclerotic changes involve recruitment of inflammatory cells from the circulation and that this process is predominantly mediated by cellular adhesion molecules, predominantly expressed on the vascular endothelium and on circulating leukocytes

in response to several inflammatory stimuli, the concentration of soluble cell adhesion molecules have been determined in plasma isolated from the patients on hemodialysis.

### 3.4.1 Human soluble VCAM-1 measurement

The human soluble Vascular Cell Adhesion Molecule-1 (sVCAM-1) was determined in plasma of ESRD patients using commercial solid-phase sandwich ELISA kit (*Novex, Life Technologies*). In brief, standards or diluted samples were added to antibody coated 96-wells plate together with anti-sVCAM-1 Biotin Conjugate solution. After the incubation and plate washing, Streptaviridin-HRP was added to each well. This step was followed by addition of chromogen and stop solution after which absorbance was read at 450 nm plate reader. Standard curve was designed from values obtained from standards, and values for samples were read from standard curve. All values were expressed as ng/ml.

### 3.4.2 Human Soluble ICAM-1 Determination

The human soluble Intercellular Adhesion Molecule 1 (ICAM-1) was determined in plasma of ESRD patients using commercial enzyme linked immunosorbent assay (*Thermo Scientific*). This assay was performed using 96-well anti-human sICAM-1 precoated strip plate to which standards or diluted samples were added. After 4-times washing of the plate, a biotinylated antibody was added to each well, which was followed by another washing and addition of Straptaviridin-HRP solution. The reaction was ceased by adding TMB substrate to each well after which absorbance was measured at 450 nm and 550 nm. Values measured on 550 nm were substracted from those on 450 nm to correct for optical imperfections in the microplate. Standard curve was designed from values obtained using standards, and ICAM-1 concentrations were read from standard curve. All values were expressed as pg/ml.

## 3.5 OVERALL AND CARDIOVASCULAR SURVIVAL ANALYSIS

All patients with ESRD were followed for 5 years (from February 2010 to February 2015) Patients were followed until the death outcome or the end of follow-up period. Both overall (all-cause) and cardiovascular mortality was registered during this time.

Cardiovascular complications that were prospectively registered were myocardial infarction (MI) and cerebral vascular insult (CVI). Diagnosis of MI was performed by cardiologist, based on the presence of at least two out of three conditions (specific clinical presentation and/or specific changes in electrocardiogram and/or elevation of enzymes). Diagnosis of CVI was performed by neurologists, based on scanner findings and specific clinical presentation.

## 3.6 MOLECULAR CHANGES OF ENDOTHELIAL CELLS

In order to test if *GSTM1* deletion polymorphism has an impact on endothelial cells an experiment using primary Human Umbilical Vascular Endothelial Cells (HUVEC, Lonza) was performed.

### 3.6.1 Cell cultures

In this thesis two cell cultures were used. The first one was HUVEC and the second one were the cell line of human monocytes THP1.

Primary HUVECs were seeded on previously 0.2% gelatin coated Petri dishes in a phenol-red free endothelial growth medium (EGM) supplemented with fetal bovine serum (2%), fibroblast growth factor (0.4%), vascular endothelial growth factor (0.1%), heparin (0.1%), insulin-like growth factor (0.1%), ascorbic acid (0.1%), epidermal growth factor (0.1%) and hydrocortisone (0.04%). The cells were cultivated at 37°C in a humidified atmosphere with 5%$CO_2$ until they would reach 70-80% confluence when they were subcultured. For all experiments the cells at passage 4 were used.

The THP1 cell line was cultured in flasks in RPMI (Roswell Park Memorial Institute) medium enriched with L-glutamine, 10% FBS (fetal bovine serum) and phenol-red. Cells were subcultured on every 3 days, or when the color of medium was changed from red to yellow.

All the reagents used in the experiment (PBS, trypsin, medium were heated in water bath before added to cells). Plates were always moved in the number 8-like manner to spread evenly the reagents.

### 3.6.2 The treatment of the endothelial cells

The cells were incubated for 4 hours with 20% human serum obtained from healthy volunteers and patients on hemodialysis therapy. As a control, endothelial cells were incubated in 20% FBS in EGM (for monocyte adhesion assay only). All experiments were performed in 24-well plates in triplicates (determination of free radicals) or sextuplicates (monocyte adhesion test).

### 3.6.3 Cell counting

After the cells were collected from Petri or 24-well dish, they were transferred into separate tubes. The sieve was used to separate cells which formed agglomeration. Cells were counted with Beckman coulter Z1 particle counter. A total of 100 μl of sample was diluted in 10 ml of Z-pak balanced electrolyte solution isoton and measured in triplicates. Mean value was used and multiplied with 200 in order to get the number of cells per milliliter.

### 3.6.4 The viability assay

In this study crystal violet test was used to assess the cell viability analysis. This assay provides the relative number of living adherent cells. This test is based on ability of crystal violet to bind for proteins and DNA of cells. Cells that undergo cell death detach from the plates and are subsequently lost from the population of cells, reducing the amount of crystal violet staining in a culture. After the treatment, cells were washed with PBS solution and 200 μL of crystal violet/methanol solution was added. After 15 minutes, wells were washed with distilled $H_2O$ (500 μL/well, three times) and left upside down over night to dry. During following day 150 μL of 1% SDS was added to each well and absorbance was read at plate reader at 550 nm. The color intensity corresponded to the number of living adherent cells. The results of this assay were presented as % of living cells compared to the control which had arbitrary 100% viability.

### 3.6.5 The monocytes adhesion assay

The HUVECs in passage 3 that were frozen in liquid nitrogen were defreezed and seeded in the previously 0.2% gelatin-coated Petri dishes. Cells were diluted in 12 ml of previously heated EGM (37°C). The cells were left for three days to reach confluence after which they were subcultured to 24-well plate previously coated with gelatin. Approximately 65,000 cells were seeded per well. The incubation with control and uremic serum was obtained once the cells reached confluence. After the 4 hrs of incubation, around 250,000 monocytes/well were added. The plates were incubated for 15 minutes. After the end of the incubation period wells were aspirated and cells were washed 3 times with 400 μl of PBS. After this step 100 μl of trypsin/EDTA was added per well. Plates were left at the incubator for 4 minutes. Plates were tapped with hand or were banged on the table to help the detachment of cells. To stop the action of trypsin a total of 200 μl of previously prepared PBS with 10% FBS was added per well. Cells were collected and transferred into the corresponding tubes. A 200 μl of 4% paraformaldehyde (PFA) was added to each tube. PFA was previously prepared and stored at -20°C in 12 ml tubes. One tube was defreezed before used for experiment; PFA was kept at 4 after defreezing. Tubes were vortexed and left at 4°C until used for the flow cytometer reading the following day.

Preparation for the flow cytometry readings obtained following steps. Tubes were centrifuged for 10 minutes at 1800 rpm (300G) at 20°C and supernatant was aspirated. Then the FCR blocking was added (5 μl of the FCR Blocking/tube+35 μl of PBS/BSA/EDTA solution). Tubes were vortexed and centrifuged for few seconds before incubated in the dark (at room temperature) for 10 minutes (at least). After the incubation period, CD45 and $IgG_{2a}$ antibodies were added (1 μl of antibody+ 9 μl of PBS/BSA/EDTA solution).The THP1 positive staining was CD45 positive, while $IgG_{2a}$ antibodies were used for negative THP1 staining. For positive HUVEC staining CD45 antibodies were used, while for negative one $IgG_{2a}$ antibodies were used. All samples contained CD45 antibodies.

### 3.6.6 The measurement of reactive oxygen species production

To estimate the free radical production in endothelial cells incubated with uremic serum, both dihydroethidium (DHE) and dihydrorhodamine (DHR) staining was used. The

DHR is a non-fluorescent dye which detects all reactive oxygen species. This dye goes through the cell membrane and to react with free radicals found in the cells. Dihydrorhodamine is oxidized into rhodamine which has green fluorescence that is detected on flow cytometer in FL-1 channel. On the other hand, DHE detects superoxide ion radical. This dye is also cell permeable and in the interior of the cells it oxidizes to ethidium which has red fluorescence detected in flow cytometer in FL-2 channel.

Cells from passage 3 were transferred from Petri dish to 24-well plates. Around 50,000 cells/well were seeded. The treatment with standard medium, control serum and uremic serum was obtained once the cells have reached confluence. After the treatment was aspirated from the wells, a 300 μL of 10 μM DHE and 2.5 μM DHR were added into wells on corresponding plates. Plates were incubated for 30 minutes on 37 °C, after which wells were washed with PBS. Cells were detached using 100 μL trypsin/EDTA solution for 4 minutes. To stop the reaction of trypsin, 200 μL of 10%FBS in PBS was used. The content was transferred to the tubes and cells were centrifuged twice with 500 μl of PBS at 500g for 10 minutes. After this step, flow cytometry analysis was performed.

### 3.7 STATISTICAL ANALYSIS

In descriptive statistics, we summarized all continuous variables by means ± standard deviations (SD) or median with interquartile range (IQR), depending on normality if variables' distributio. Differences in investigated parameters were assessed by using analysis of variance (ANOVA) for continuous variables and $\chi 2$ for categorical variables. The associations between the genotypes and ESRD risk were calculated by using logistic regression to compute odds ratios (ORs) and corresponding 95% confidence intervals (CIs), adjusted according to age and gender as potential confounding factors.

In the first step of statistical evaluation of relationships between byproducts of oxidative damage (SH groups, carbonyls, AOPP, nitrotyrosine, MDA, MDA adducts, TOS, PAB), adhesion molecules (ICAM-1, VCAM-1) and combined *GSTA1*, *GSTM1*, *GSTP1* and *GSTT1* genotypes in ESRD patients, distribution was tested by using the Kolmogorov–Smirnov test. For normally distributed data, we performed ANOVA and, if necessary, the Bonferroni post hoc test for locating differences between multiple groups. For data with a

non-normal distribution, we used the Mann–Whitney rank-sum test (for between-two-group comparisons) and the Kruskal–Wallis non-parametric test that compared three unpaired groups.

Two-tailed P-values of $<0.05$ were considered significant. Data were analyzed using the Statistical Package for the Social Sciences (SPSS) (version 17.0, Chicago, IL).

Survival analysis was performed separately in the total cohort and according to the cardiovascular cause of death. The Kaplan-Meier method was used to estimate the cumulative survival probability. The long-rank test was performed for the assessment of differences in survival according to the different categories of variables.

The predictive value of different *GST* genotypes in overall and cardiovascular mortality was assessed by Cox proportional hazards regression models, adjusted by confounding factors in three models. The numbers of patients included in regression models were the same for the all tested GST polymorphisms. In Model 1 we adjusted for age and gender. Model 2 included the covariates in Model 1 plus an additional adjustment for the current smoking status. Model 3 included all covariates from Models 1 and 2, plus an additional adjustment for diabetes and cholesterol status. The associations are presented as hazard ratios (HR) with their corresponding 95% confidence intervals (95% CI).

# 4 RESULTS

## 4.1 CLINICAL CHARACTERISTIC OF PATIENTS AND CONTROL GROUP

Characteristics describing both demographic and clinical features of patients and control group are given in Table 7. Although the study previously included 202 patients, it turned out that 3 of them had previously registered malignant disease so they were removed from further analysis. For the 199 patients on regular hemodialysis and 199 healthy control there was no difference in terms of age (60.0 ± 12.1 years in patients vs. 59.3 ± 10.9 yrs in control group) or gender (male : female ratio in end-stage renal disease patients was 84 : 115 and 85 : 114 in control group) distribution. Concentrations of albumin, hemoglobin and serum iron were significantly decreased while urea, creatinine and ferritin concentrations were significantly increased in patients when compared to individuals from control group. These results were in respect with studied illness of these patients. Mean time spent on hemodialysis was 6.3 ± 4.4 years. Mean Kt/V value for patients was 1.3.

The causes of renal failure in our study group were: 26 had diabetes mellitus (13.1%), 93 had hypertension (46.7%), glomerulonephritis had 27 of them (13.6%), 19 with pyelonephritis (9.5%), 18 suffered from polycystic kidney disease (9.0%) and 7 had endemic nephropathy (3.5%). For 9 patients (4.5%) the cause of renal failure remained unknown. A total of 27 patients (13.6%) had registered previous cardiovascular disease. There were no significant correlations between age of the patients/control and any biochemical analysis (data not shown).

## 4.2 DISTRIBUTION OF *GST* GENOTYPES

The distributions of *GSTA1*, *GSTM*1, *GSTP1* and *GSTT1* genotypes in patients and controls are presented in Table 8. By the term active genotype we considered the presence of at least one allele in the case of *GSTM1* and *GSTT1*. In control group the frequency of *GSTA1* and *GSTP1* genotypes were in Hardy-Weinberg equilibrium ($p = 0.87$ and $p = 0.15$, respectively).

**Table 7**. Clinical characteristics of patients with end stage renal disease (ESRD) and control group

| Characteristic | ESRD patients | Controls |
|---|---|---|
| **Gender** | | |
| Male vs female (%) | 84 (42) vs 115 (58) | 85 (43) vs. 114 (57) |
| Age (year; mean ± SD) | 60.0 ±12.1 | 59.3 ± 10.9 |
| Time on hemodialysis | 6.3 ± 4.4 | - |
| Albumin (g/L; mean ± SD) | 39.6 ± 4.0 | 43.5 ± 2.6* |
| Urea | 24.4 ± 4.8 | 5.5 ± 1.2* |
| Creatinine | 880.2 ± 238.6 | 84.4 ± 10.3* |
| Cholesterol | 4.7 ± 1.0 | |
| HDL (mmol/L; mean ± SD) | 1.2 ± 0.3 | 1.3 ± 1.4 |
| LDL (mmol/L; mean ± SD) | 2.6 ± 0.8 | |
| Triacylglycerol (mmol/L; mean SD) | 2.1 ± 1.3 | 1.4 ±0.4* |
| Hemoglobin (g/L; mean ± SD) | 108.1 ± 15.4 | 144.0 ± 13.7* |
| Serum iron | 11.3 ± 5.6 | 21.1 ± 4.8* |
| Ferritin | 350.2 ± 278.0 | 48.0 ± 29.6* |
| Hematocrit (%; mean ± SD) | 32.1 ± 5.2 | 29.8 ± 4.9 |

The frequency of *GSTA1*, *GSTM1*, *GSTP1* and *GSTT1 null/low-activity* genotypes was higher in patients with end stage renal disease than in control group. In control group *GSTA1*A/A* genotype was found in 39.2% individuals, while *GSTA1*B/B* genotype was found in 13.6% individuals. In contrast, in patients group a total of 35.2% individuals possessed *GSTA1*A/A* genotype while 19.6% had *GSTA1*B/B* genotype. As far as *GSTP1* genotype is concerned, 23.6% of patients had the *GSTP1*Val/Val* genotype compared to 16.6% individuals in controls. Regarding *GSTM1* genotype, homozygous deletion was found in 59.8 % ESRD patients and in 48.7% individuals in control group. The frequency of *GSTT1-null* genotype was higher in patients (33.7%) than in controls (28.6%). Significant association between the *GST* genotype distribution and ESRD was found for the *GSTM1* genotype. Namely, individuals that were homozygous for *GSTM1-null* allele had 1.6-fold higher susceptibility towards ESRD development (OR = 1.6, 95% CI: 1.1 – 2.4, p = 0.024) than

individuals carrying at least one *GSTM1* referent allele. Very high significant association was also found for *GSTP1* rs1138272 polymorphism. Individuals who were carriers of at least one variant allele (*GSTP1*T*) had 3.2-fold increased risk for ESRD ($p = 0.001$) when compared to individuals who were homozygous for referent allele (*GSTP1*C*). In the case of another *GSTP1* rs1695 polymorphism, it was observed that individuals with *GSTP1-Val*Val* genotype were at increased susceptibility towards ESRD development. However, statistical significance lacked (OR = 1.6, 95% CI : 0.9 – 2.7, $p = 0.112$). Regarding *GSTA1* polymorphism, although OR was higher in individuals with low activity genotype (OR = 1.6), statistical significance was not observed. Grouping individuals who possesses at least one variant GSTA1*B allele did not increase susceptibility towards ESRD development. No statistical significance was observed for association with ESRD regarding *GSTT1* genotype.

### 4.3 Combined *GST* genotypes and ESRD risk

When combinations of various GST genotypes were analyzed, it was observed that the presence of the either variant *GSTP1* rs1138272 or *GSTM1-null* genotype influenced significantly the susceptibility towards ESRD development. Namely, the highest susceptibility of ESRD development was observed in individuals who carried both *GSTP1* (rs1138272)-*low activity* and *GSTT1-null* genotype (OR = 6.6, 95% CI : 2.1 – 10.5, $p < 0.05$). The risk was also significantly increased in individuals who had combination of *GSTP1-low activity* (rs1138272) genotype with *GSTA1-low activity* or *GSTM1-null* genotype (OR = 3.3, 95% CI : 1.7 – 6.6, $p < 0.05$ and OR = 5.9, 95% CI : 2.3 – 15.4, $p < 0.05$, respectively). Individuals who carried both *GSTP1-low activity* genotype combined had almost 3-fold increased risk for ESRD (OR = 2.9, 95% CI : 1.7 – 5.7, $p < 0.05$). Interestingly, when *GSTP1-low activity* genotype was combined with either *GSTA1-active* or *GSTP1-active* (rs1695) genotype, the ESRD risk development was also increased in the studied population (OR = 5.9, 95% CI : 2.3 – 15.4, $p < 0.05$ and OR = 3.8, 95% CI : 1.6 – 9.1, $p < 0.05$, respectively). The presence of *GSTM1-null* genotype in combination with other null or low activity genotypes (apart from *GSTP1* rs1138272 polymorphism) provided the certain risk for those who carried the combinations with this genotype. Namely, combination of *GSTM1-null* with the *GSTT1-null* or *GSTP1-low-activity*

genotype was also found to be significant (OR = 2.0, 95% CI : 1.1 – 3.7, p < 0.05 and OR = 1.8, 95% CI : 1.0–3.1, p < 0.05, respectively) (Table 9).

**Table 8.** *GSTA1*, *GSTM1*, *GSTP1* and *GSTT1* genotypes in relation to risk of ESRD

| GST genotype | Cases n (%) | Controls n (%) | OR (95%CI) | p |
|---|---|---|---|---|
| ***GSTA1*** | | | | |
| *AA* | 70 (35.2) | 78 (39.2) | 1.00 | |
| *AB* | 90 (45.2) | 94 (47.2) | 1.1 (0.7-1.7) | 0.110 |
| *BB* | 39 (19.6) | 27 (13.6) | 1.6 (0.9-2.9) | 0.746 |
| *AB+BB* | 129 | 121 | 1.2 (0.8-1.8) | 0.401 |
| ***GSTM1*** | | | | |
| **1 active* [a] | 80 (40.2) | 102 (51.3) | 1.00 | |
| **0 null* [b] | 119 (59.8) | 97 (48.7) | 1.6 (1.1-2.4) | 0.024 |
| ***GSTT1*** | | | | |
| **1 active* [a] | 132 (66.3) | 142 (71.4) | 1.00 | |
| **0 null* [b] | 67 (33.7) | 57 (28.6) | 1.2 (0.8-1.9) | 0.319 |
| ***GSTP1 rs1695*** | | | | |
| **Ile/*Ile* | 75 (37.7) | 82 (41.2) | 1.00 | |
| **Ile/*Val* | 77 (38.7) | 84 (42.2) | 1.0 (0.6-1.5) | 0.939 |
| **Val/*Val* | 47 (23.6) | 33 (16.6) | 1.6 (0.9-2.7) | 0.112 |
| *Ile/Val + Val/Val* | 124 | 117 | 1.1 (0.8-1.7) | 0.531 |
| ***GSTP1 rs1138272*** | | | | |
| **C/*C* | 111 (66.9) | 182 (86.3) | 1.00 | |
| **C/*T* | 55 (33.1) | 29 (13.7) | 3.2 (1.9-5.4) | 0.001 |

Adjusted for age and gender. [a]Active (present) if at least one active allele present. [b]Null (inactive) if no active alleles present. OR- odds ratio. CI- confidence interval.

When *GSTM1-null* and *GSTA1-low* activity genotypes were combined a certain risk on ESRD development was observed although statistically insignificant (OR = 1.8; 95% CI : 0.9 – 3.3, p = 0.058) (Table 9).

**Table 9.**Combined effects of *GSTA1*, *GSTM1*, *GSTP1* and *GSTT1* genotypes in terms of risk of end-stage renal disease.

| | GSTM1 | | GSTA1 | | GSTP1 | | GSTT1 | |
|---|---|---|---|---|---|---|---|---|
| | **Present**[a] | **Null**[b] | **Active**[a] | **Low activity**[c] | **Active**[a] | **Low activity**[c] | **Active**[a] | **Low activity**[c] |
| **GSTA1** | | | | | | | | |
| *Active*[a] | | | | | | | | |
| Ca/Co | 29/36 | 41/42 | | | - | - | - | - |
| OR (95%CI) | 1.0[d] | 1.2 (0.6-2.3) | | | | | | |
| *Low activity*[c] | | | | | | | | |
| Ca/Co | 48/64 | 78/55 | | | - | - | - | - |
| OR (95%CI) | 0.9 (0.5-1.8) | 1.8 (0.9-3.3) | | | | | | |
| **GSTP1 AB** | | | | | | | | |
| *Active*[a] | | | | | | | | |
| Ca/Co | 34/50 | 41/32 | 30/33 | 45/49 | | | - | - |
| OR (95%CI) | 1.0[d] | 2.1 (1.1-4.0)* | 1.0 [d] | 1.0 (0.5-2.0) | | | | |
| *Low activity*[c] | | | | | | | | |
| Ca/Co | 45/52 | 78/65 | 40/45 | 84/72 | | | - | - |
| OR (95%CI) | 1.3 (0.7-2.4) | 1.8 (1.0-3.1)* | 1.0 (0.5-1.9) | 1.2 (0.7-2.3) | | | | |
| **GSTP1 CD** | | | | | | | | |
| *Active* | | | | | | | | |
| Ca/Co | 47/88 | 20/14 | 35/71 | 76/101 | 45/73 | 66/99 | 74/119 | 38/23 |
| OR (95% CI) | 1.0 | 2.6 (1.2-5.7)* | 1.0 | 1.5 (0.9-2.5) | 1.0 | 1.1 (0.7-1.7) | 1.0 | 2.6 (1.4-4.7)* |
| *Low activity* | | | | | | | | |
| Ca/Co | 64/84 | 35/13 | 21/7 | 34/20 | 22/9 | 33/18 | 37/53 | 17/4 |
| OR (95% CI) | 1.4 (0.9-2.3) | 4.9 (2.4-10.02)* | 5.9 (2.3-15.4)* | 3.3 (1.7-6.6)* | 3.8 (1.6-9.1)* | 2.9 (1.7-5.7)* | 1.1 (0.7-1.8) | 6.6 (2.1-10.5)* |
| **GSTT1** | | | | | | | | |
| *Present*[a] | | | | | | | | |
| Ca/Co | 51/73 | 81/69 | 48/56 | 85/85 | 55/62 | 77/80 | | |
| OR (95%CI) | 1.0[d] | 1.7 (1.1-2.8)* | 1.0[d] | 1.2 (0.7-1.9) | 1.0[d] | 1.1 (0.7-1.7) | | |
| *Null*[b] | | | | | | | | |
| Ca/Co | 28/30 | 38/27 | 22/22 | 45/35 | 20/20 | 47/37 | | |
| OR (95%CI) | 1.3 (0.7-2.5) | 2.0 (1.1-3.7)* | 1.1 (0.5-2.3) | 1.5 (0.8-2.7) | 1.2 (0.6-2.5) | 1.4 (0.8-2.5) | | |

[a]Active (present) if at least one active allele present. [b]Null if no active alleles present. [c] Low activity if at least one lower activity allele present [d]Reference group. * Statistically significant difference when compared to reference group. *OR*- odds ratio. *CI*- confidence interval.

## 4.4 ASSOCIATION BETWEEN *GST* GENOTYPE AND BYPRODUCTS OF OXIDATIVE DAMAGE

The association between polymorphic *GST* expression and byproducts of oxidatively damaged macromolecules (proteins and lipids; prooxidant-antioxidant balance and total oxidant status) has been studied in the further course.

### 4.4.1 Impact of *GST* genotype on protein oxidative damage

In the Table 10 and Figures 16, 17, 18 and 19 are presented the results regarding protein oxidative damage measured in the plasma of ESRD patients. All values were stratified according to *GST* genotype.

Plasma protein thiol groups content was significantly lower in patients with *GSTM1-null*, *GSTT1-null* genotype as well as, *GSTP1 *Val/*Val* genotype in comparison to corresponding reference genotype (p = 0.001, 0.002 and 0.042, respectively) (Figure 16).

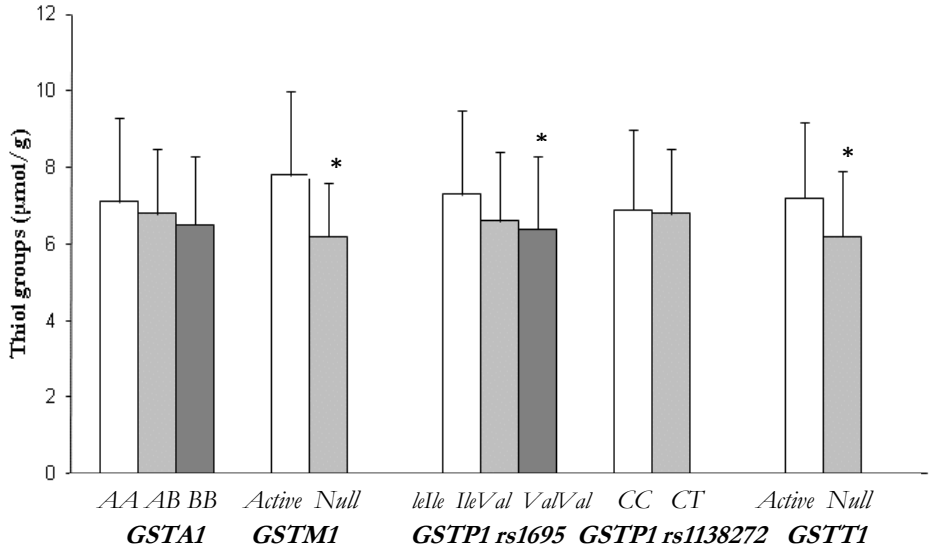


**Figure 16**. Plasma thiol groups in terms of *GST* genotype in ESRD patients. *p < 0.05, **p < 0.001

Plasma carbonyl group levels were found to be statistically increased in patients with *GSTA1 *B/*B* or *GSTM1-null* genotype (p = 0.007 and 0.005, respectively) (Figure 17). The *GSTP1* rs1695, *GSTP1* rs1138272 and *GSTT1* gene polymorphism did not significantly

influence the carbonyls level although modest increased related to the presence of variant allele was observed.

**Table 10**. Byproducts of protein oxidative damage stratified according to *GST* genotype in ESRD patients. All values are presented as mean ± SD or median with IQR

| Genotype | **SH-groups** μmol/g | **Carbonyls** nmol/g | **Nitrotyrosine** nmol/L | **AOPP** μmol/L |
|---|---|---|---|---|
| *GSTA1-AA* | 7.1 ± 2.2 *(100%)* | 2.2 ± 0.2 *(100%)* | 56.9 [43.3 – 85.1] *(100%)* | 65.4 [49.4 - 74.4] *(100%)* |
| *GSTA1-AB* | 6.8 ± 1.8 *(96%)* | 2.3 ± 0.3 *(105%)* | 62.4 [44.9 – 89.32] *(110%)* | 64.3 [45.0 – 75.1] *(98%)* |
| *GSTA1-BB* | 6.5 ± 1.9 *(90%)* | 2.6 ± 0.7* *(118%)* | 68.3 [55.0 – 101.2] *(120%)* | 61.4 [47.3 – 74.7] *(94%)* |
| *GSTM1-active*[a] | 7.8 ± 2.2 *(100%)* | 2.1 ± 0.2 *(100%)* | 64.5 [46.1-93.1] *(100%)* | 57.9 [32.8 – 69.4] *(100%)* |
| *GSTM1-null*[b] | 6.2 ± 1.4** *(79%)* | 2.4 ± 0.4* *(114%)* | 64.5 [46.1-93.1] *(100%)* | 66.3 [52.5 – 86.2]* *(115%)* |
| *GSTP1-Ile/Ile* | 7.3 ± 2.2 *(100%)* | 2.2 ± 0.2 *(100%)* | 54.5 [39.0 - 78.7] *(100%)* | 67.4 [50.3 – 84.5] *(100%)* |
| *GSTP1-Ile/Val* | 6.6 ± 1.7 *(90%)* | 2.3 ± 0.2 *(105%)* | 75.5 [50.1 – 101.2]* *(139%)* | 61.1 [43.9 – 70.5] *(91%)* |
| *GSTP1 Val/Val* | 6.4 ± 1.8* *(88%)* | 2.3 ± 0.3 *(105%)* | 72.3 [46.8 – 93.1]* *(133%)* | 64.6 [18.7 – 73.7] *(96%)* |
| *GSTP1 *C/*C* | 6.9 ± 2.1 *(100%)* | 2.3 ± 0.3 *(100%)* | 59.3 [47.1 – 91.2] *(100%)* | 64.1 [43.6 – 80.2] *(100%)* |
| *GSTP1 *C/*T* | 6.8 ± 1.8 *(98%)* | 2.3 ± 0.2 *(100%)* | 62.4 [42.4 – 93.1] *(105%)* | 65.6 [46.9 – 76.1] *(102%)* |
| *GSTT1 active*[a] | 7.2 ± 2.0 *(100%)* | 2.2 ± 0.3 *(100%)* | 61.1 [44.9 – 93.1] *(100%)* | 61.6 [45.1 – 73.7] *(100%)* |
| *GSTT1-null*[b] | 6.2 ± 1.7* *(86%)* | 2.4 ± 0.4 *(109%)* | 66.5 [49.5 – 101.1] *(109%)* | 66.4 [56.7 – 85.4]* *(108%)* |

[a]Active (present) if at least one active allele present. [b]Inactive (null) if no active alleles present; *if $p < 0.05$, **if $p < 0.01$ All values are presented as mean ± standard deviation or median with interquartile range, depending on normality of distribution

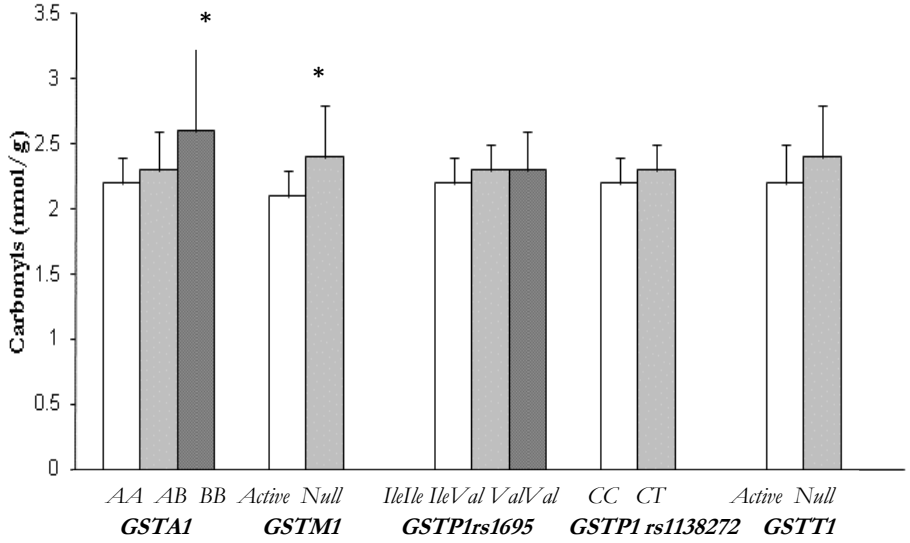


**Figure 17.** Protein carbonyls in plasma of ESRD patients in terms of *GST* genotype. *p < 0.05, **p < 0.01

The most pronounced effect regarding AOPP content was observed in the ESRD patients with *GSTM1-null* genotype when compared to the patients with *GSTM1-active* genotype (66.3 [52.5 – 86.2] and 57.9 [32.8 – 69.4], respectively). Another significant increase in AOPP concentrations has been shown in patients carrying *GSTT1-null* genotype (66.4 [56.7 – 85.4]) in comparison to the ESRD patients with *GSTT1-active* genotype (61.6 [45.1 – 73.7]).

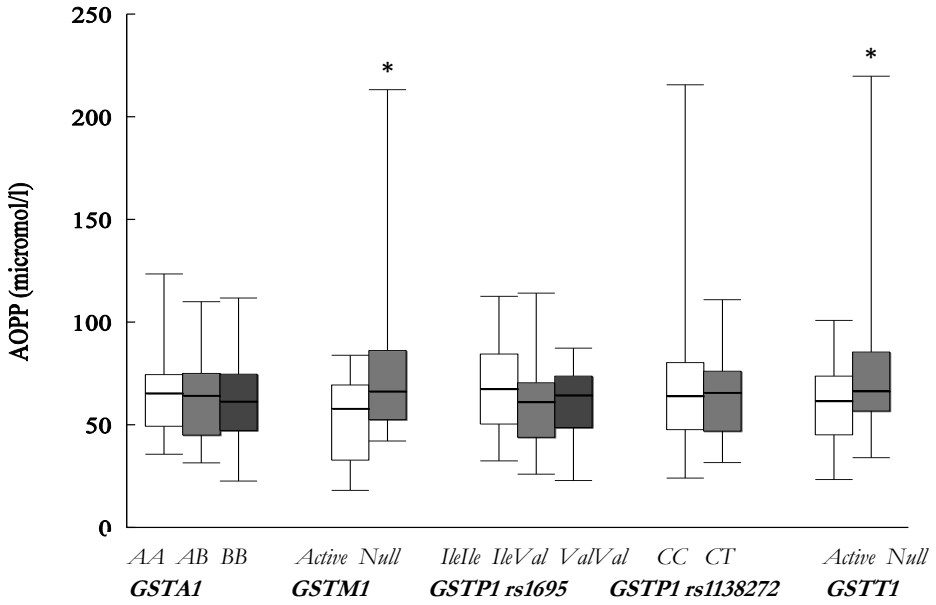


**Figure 18**. AOPP content in terms of *GST* genotype in plasma of ESRD patients. *p < 0.05, **p < 0.01

Furthermore, nitrotyrosine, a reliable marker of nitrosative damage of proteins, was found to be significantly higher in ESRD patients carrieng at least one variant allele *GSTP1*Val* (rs1695 polymorphism). Namely, the patients with *GSTP1*Ile/*Ile* genotype had 54.6 [39.0 – 78.7] nmol/L of nitrotyrosine measured in plasma, while the ESRD patients with present variant *GSTP1* gene had 75.5 [50.1 – 101.2] if they had *GSTP1*Ile/*Val* genotype and 72.3 [46.8 – 93.1] nmol/L of nitrotyrosine if they carried *GSTP1*Val/*Val* genotype. Other *GST* genotypes did not significantly influence the level of nitrosative oxidative damage in ESRD patients (Figure 19).

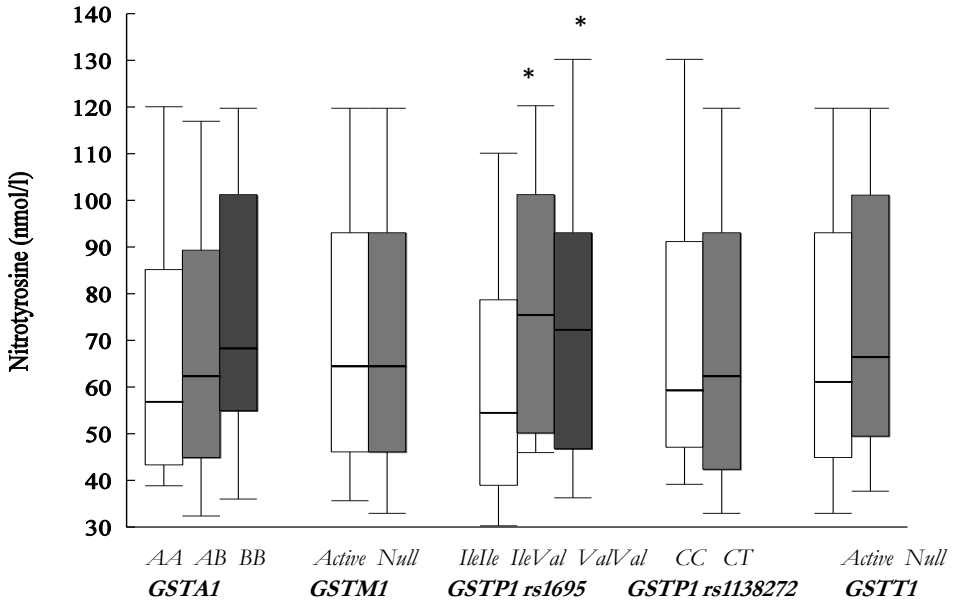


**Figure 19**. Nitrosative protein damage in terms of *GST* genotype in ESRD patients. *p < 0.05, **p < 0.01

### 4.4.2 Impact of *GST* genotype on lipid oxidative damage

Malondialdehyde (MDA), a commonly used byproductr of lipid oxidative damage, exists both as free and bound to proteins. When bound to proteins, it is designated as MDA adduct. Levels of both MDA and MDA adducts were changed in terms of *GST* genotype and these results are presented in Table 11 and Figures 20 and 21.

Malonidialdehyde levels were significantly changed in terms of *GSTP1* genotype (rs1695). Namely, patients with *GSTP1*Val/*Val* genotype had 2.6 [2.0 – 3.2] mmol/l of

MDA compared to 2.1 [1.7 – 2.8] mmol/l in patients with *GSTP1*Ile/*Ile* genotype (+24 %, $p < 0.05$). Although the increase of MDA concentrations has been observed in patients with *GSTT1-null* genotype when compared to the patients with *GSTT1-active* genotype, statistical significance was not observed. Polymorphisms of other *GST* genes did not influence significantly on the MDA values in patients with end-stage renal disease (Figure 20).

**Table 11**. Byproducts of lipid oxidative damage stratified according to *GST* genotype in ESRD patients. All values are presented as mean ± SD or median with interquartile range (IQR).

| Genotype | **MDA** mmol/L | **MDA adducts** pmol/mg |
|---|---|---|
| *GSTA1*A/*A* | 2.3 [2.0 – 3.0] *(100%)* | 39.8 ± 9.8 *(100%)* |
| *GSTA1*A/*B* | 2.4 [1.7 – 3.0] *(104%)* | 39.7 ± 9.1 *(99.7%)* |
| *GSTA1*B/*B* | 2.3 [1.7 – 3.1] *(100%)* | 40.2 ± 12.8 *(101%)* |
| *GSTM1-active*[a] | 2.3 [1.7 – 3.1] *(100%)* | 33.0 ± 7.9 *(100%)* |
| *GSTM1-null*[b] | 2.4 [1.8 – 3.0] *(104%)* | 44.8 ± 8.3** *(136%)* |
| *GSTP1*Ile/*Ile* | 2.1 [1.7 – 2.8] *(100%)* | 36.2 ± 7.7 *(100%)* |
| *GSTP1*Ile/*Val* | 2.4 [1.7 – 3.0] *(114%)* | 41.8 ± 10.2* *(115%)* |
| *GSTP1*Val/*Val* | 2.6 [2.0 – 3.2]* *(124%)* | 41.0 ± 7.8* *(113%)* |
| *GSTP1*C/*C* | 2.2 [1.7 – 2.5] *(100%)* | 38.2 ± 9.83 *(100%)* |
| *GSTP1*C/*T* | 2.4 [1.8 – 3.2] *(109%)* | 39.3 ± 10.1 *(103%)* |
| *GSTT1 active*[a] | 2.3 [1.7 – 2.8] *(100%)* | 36.7 ± 8.9 *(100%)* |
| *GSTT1-null*[b] | 2.6 [1.7 – 3.3] *(113%)* | 45.1 ± 9.7** *(123%)* |

[a]Active (present) if at least one active allele present. [b]Inactive (null) if no active alleles present; *if $p < 0.005$, **if $p < 0.001$

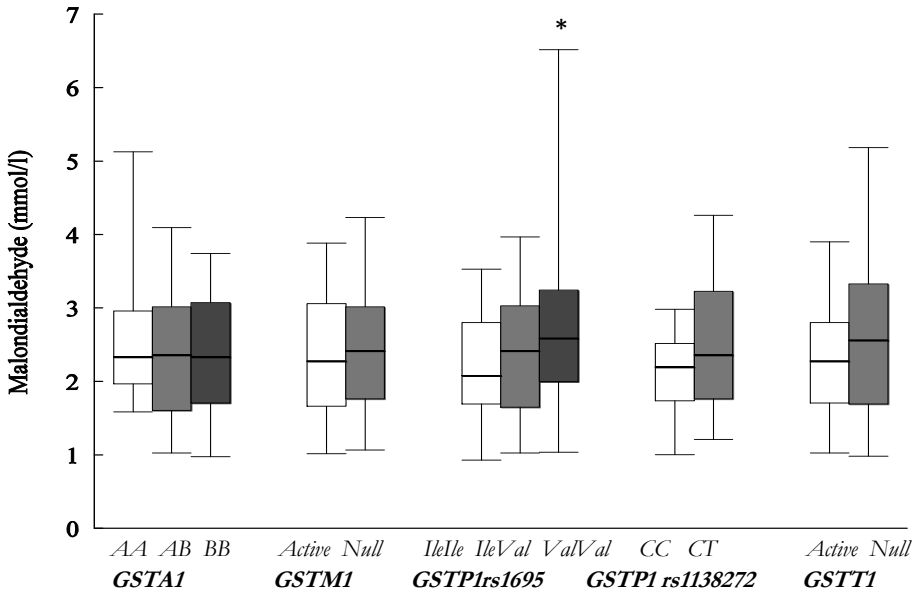


**Figure 20.** Malondialdehyde content in terms of *GST* genotypes. *p < 0.05, **p < 0.01

Patients with *GSTM1-null* genotype had 36% higher concentration of MDA adducts when compared to patients with *GSTM1-active* genotype (44.8 ± 8.3 vs 33.0 ± 7.9). The statistically significant increase of MDA adducts level was also observed in *GSTP1 rs1695* polymorphism where patients with heterozygous genotype had 41.8 ± 10.2 pmol/g of MDA adducts in comparison with 36.2 ± 7.7 pmol/g in ESRD patients with *GSTP1*Ile/*Ile* genotype. Similarly, the ESRD patients with *GSTT1-null* genotype had around 23% elevated MDA adducts when compared to the patients with *GSTT1-active* genotype (Figure 21).

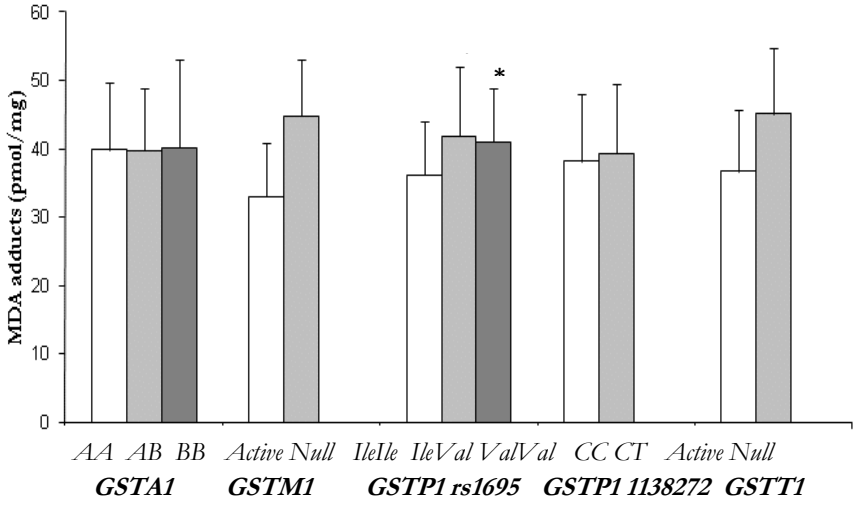


**Figure 21.** MDA adducts content in terms of *GST* genotypes. *p < 0.05, **p < 0.01

### 4.4.3 Impact of *GST* genotype on TOS and PAB

Since the effects of different oxidant molecules are additive, total oxidant status and prooxidant-antioxidant balance in plasma of hemodialysis patients were determined. Results are shown on Table 12 and Figure 22 and Figure 23.

.**Table 12**. Total oxidant status and prooxidant antioxidant balance in terms of *GST* genotype.

| Genotype | TOS<br>μmol of $H_2O_2$ equivalents/ml | PAB<br>HK units |
|---|---|---|
| *GSTA1*A/*A* | 16.2 [12.8 – 38.2]<br>*(100%)* | 119.0 [70.2 – 297.0]<br>*(100%)* |
| *GSTA1*A/*B* | 21.5 [13.9 – 51.5]<br>*(133%)* | 157.4 [75.8 – 232.4]<br>*(132%)* |
| *GSTA1*B/*B* | 17.8 [12.7 – 41.5]<br>*(110%)* | 147.2 [ 72.2 – 230.4]<br>*(124%)* |
| *GSTM1-active*[a] | 14.2 [11.5 - 21.2]<br>*(100%)* | 124.8 [69.8 – 202.5]<br>*(100%)* |
| *GSTM1-null*[b] | 29.5 [15.3 – 64.5]*<br>*(208%)* | 159.4 [75.0 -256.6]*<br>*(128%)* |
| *GSTP1*C/*C* | 17.6 [12.7 – 49.3]<br>*(100%)* | 152.4 [84.6 – 230.4]<br>*(100%)* |
| *GSTP1*C/*T* | 19.1 [13.1 – 42.2]<br>*(109%)* | 148.2 [63.4 - 237.0]<br>*(97%)* |
| *GSTP1*Ile/*Ile* | 16.9 [13.0 – 48.9]<br>*(100%)* | 148.9 [85.4 – 233.8]<br>*(100%)* |
| *GSTP1*Ile/*Val* | 19.5 [12.8 – 44.2]<br>*(115%)* | 154.4 [68.0 – 253.9]<br>*(104%)* |
| *GSTP1*Val/*Val* | 19.7 [14.8 – 48.3]<br>*(117%)* | 139.2 [70.2 - 227.8]<br>*(93%)* |
| *GSTT1 active* | 19.2 [12.8 – 41.5]<br>*(100%)* | 143.0 [66.6 – 232.2]<br>*(100%)* |
| *GSTT1-null* | 18.2 [13.4 – 64.5]<br>*(95%)* | 153.8 [94.6 – 259.4]<br>*(108%)* |

[a]Active (present) if at least one active allele present. [b]Inactive (null) if no active alleles present; *if $p < 0.005$, **if $p < 0.001$

Regarding the results on association of *GST* genotype and total oxidant status, the significant increase was observed in patients with *GSTM1-null* genotype when compared to the

patients with *GSTM1-active* genotype (29.5 [15.3 – 64.5] μmol of $H_2O_2$ equivalents/ml vs. 14.2 [11.5 – 21.2] μmol of $H_2O_2$ equivalents/ml, $p < 0.05$). The patients lacking GSTM1 antioxidant activity had doubled increased levels of TOS than end-stage renal disease patients with at least one present *GSTM1* gene. In other analyzed genotypes (*GSTA1*, *GSTP1* rs1138272, *GSTP1* rs1695, *GSTT1*) no significant increase of TOS level was observed. Interestingly, although the *GSTA1* heterozygous patients had an increase of TOS for 33% in comparison to *GSTA1*A/*A* genotype carriers, the ESRD patients with *GSTA1*B/*B* genotype had increase of 10% of total oxidant status when compared to the patients carrying referent genotype (*GSTA1*A/*A*: 16.2 [12.8 – 38.2]; *GSTA1*A/*B*: 21.5 [13.9 – 51.5]; *GSTA1*B/*B*: 17.8 [12.7 – 41.5]) (Figure 22).

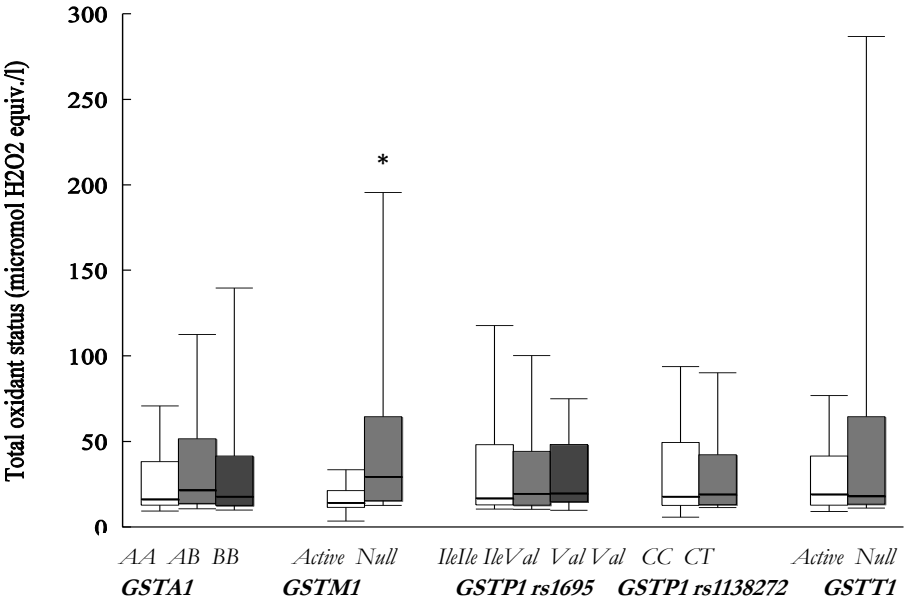


**Figure 22**. Total oxidant status in terms of *GST* genotype in ESRD patients. *p < 0.05, **p < 0.01

The levels of prooxidant-antioxidant balance assay were significantly increased only in ESRD patients with *GSTM1-null* genotype when compared to the patients with *GSTM1-active* genotype. Namely, the patients with homozygous deletion of *GSTM1* gene had 159.4 [75.0 - 256.6] HK units which is around 28% higher than concentration of PAB measured in patients with at least one *GSTM1* gene present (*GSTM1-active* genotype, 124.8 [69.8 – 202.5] HK units). The presence of variant *GSTA1*B* allele influced the levels of PAB in ESRD patients by

increasing them, but although that increase for geterozygous was more than 30%, the result remained statistically insignificant (*GSTA1*A/*A*: 119.0 [70.2 – 297.0]; *GSTA1*A/*B*: 157.4 [75.8 – 232.4]; *GSTA1*B/*B*: 147.2 [ 72.2 – 230.4], p > 0.05). The median concentrations of PAB were were not changed in terms of other *GST* genotypes (*GSTT1-null*, *GSTP1*Val/*Val* and *GSTP1*C/*T*) (Figure 23).

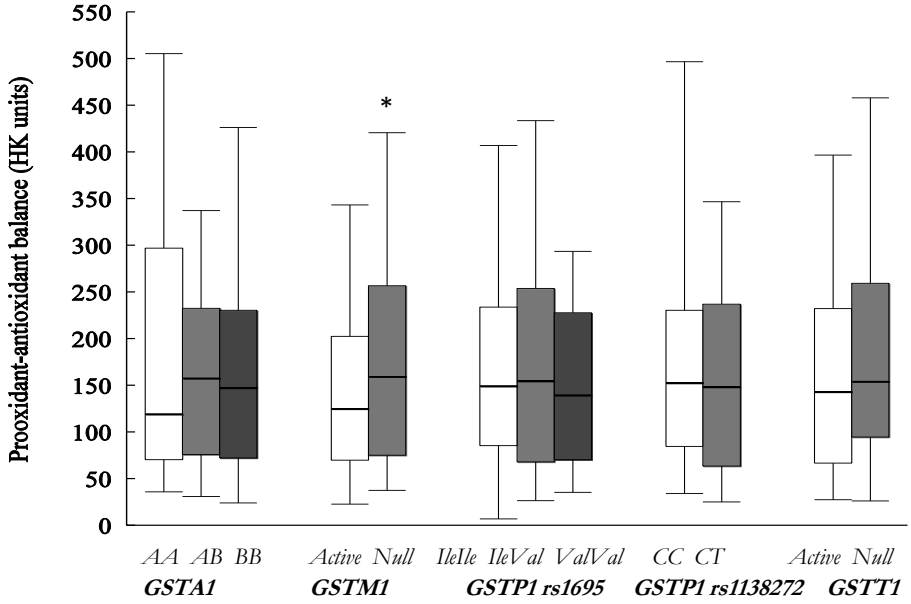


**Figure 23**. Prooxidant-antioxidant balance in terms of *GST* genotype in ESRD patients. *p < 0.05, **p < 0.01

### 4.4.4 Impact of *GST* genotype on circulating soluble adhesion molecules concentration

Giving a thought that oxidative stress contributes to initiation and progression of atherosclerosis through a process predominantly mediated by adhesion molecules, one of the aims in this thesis was to investigate the association between the concentrations of circulating soluble intercellular (sICAM-1) and vascular cellular (sVCAM-1) adhesion molecules, determined in plasma of ESRD patients, and *GST* genotype. These results are presented in Table 13 and Figures 24 and 25.

**Table 13.** Association of *GSTA1, GSTM1, GSTP1* rs 1138272, *GSTP1* rs1695 and *GSTT1* polymorphism with soluble adhesion molecule concentration in plasma of patients with end-stage renal disease. All results are presented as mean ± SD.

| Genotype | sICAM-1 (pg/mL) | sVCAM-1 (ng/mL) |
|---|---|---|
| *GSTA1*A/*A* | 87.1±27.6 *(100%)* | 607.5±131.4 *(100%)* |
| *GSTA1*A/*B* | 90.9±24.1 *(104%)* | 613.9±125.1 *(101%)* |
| *GSTA1*B/*B* | 106.8±15.0 *(123%)* | 637.4±115.2 *(105%)* |
| *GSTM1-active*[a] | 83.7±27.6 *(100%)* | 529.4±134.2 *(100%)* |
| *GSTM1-null*[b] | 96.0±25.4* *(115%)* | 669.7±82.6** *(127%)* |
| *GSTP1*Ile/*Ile* | 91.1±25.8 *(100%)* | 604.7±131.5 *(100%)* |
| *GSTP1*Ile/*Val* | 91.8±27.3 *(101%)* | 626.9±108.8 *(104%)* |
| *GSTP1*Val/*Val* | 89.2±21.6 *(98%)* | 616.6±139.3 *(102%)* |
| *GSTP1 *C/*C* | 88.5±21.3 *(100%)* | 599.0±132.5 *(100%)* |
| *GSTP1*C/*T* | 98.9±30.9 *(112%)* | 650.3±115.6* *(109%)* |
| *GSTT1-active* | 91.7±22.1 *(100%)* | 618.3±129.6 *(100%)* |
| *GSTT1-null* | 89.7±30.3 *(98%)* | 610.5±116.9 *(99%)* |

[a]Active (present) if at least one active allele present. [b]Inactive (null) if no active alleles present; *if $p < 0.005$, ** if $p < 0.001$

Concentration of soluble ICAM-1 was also increased in patients with double deletion of *GSTM*1 gene. Namely, *GSTM1-null* patients had 96.0 ± 25.4 pg/ml concentration of sICAM-1 and *GSTM-1-active* patients had 83.7 ± 27.6 pg/mL of sICAM-1 ($p < 0.05$) (Figure 24). Patients with *GSTM1-null* genotype had significantly increased values of soluble VCAM-1 concentration (669.7 ± 82.6 ng/mL) compared to patients with *GSTM1-active* genotype (529.4 ± 134.2 ng/ml, $p < 0.001$) (Figure 25). Although the presence of *GSTA1* gene polymorphism has shown the impact of sICAM-1 concentration, the statistical difference was not observed.

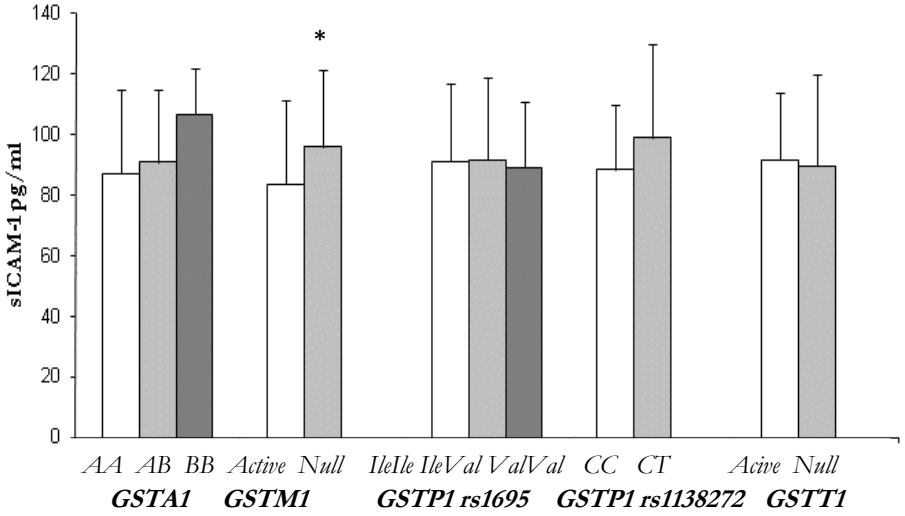


**Figure 24.** Soluble ICAM-1 concentrations in plasma of ESRD patients in terms of *GST* genotype. *p < 0.05, **p < 0.01

Regarding other *GST* genes, only *GSTP1* rs1138272 gene polymorphism influenced the soluble adhesion molecules concentration. Patients who were carriers of variant allele had significantly increased levels of sVCAM-1 in comparison with those with referent genotype. (*GSTP1*C/*T*: 650.3 ± 115.6 ng/ml, *GSTP1*C/*C*: 599.0 ± 132.5 ng/ml, p < 0.05).

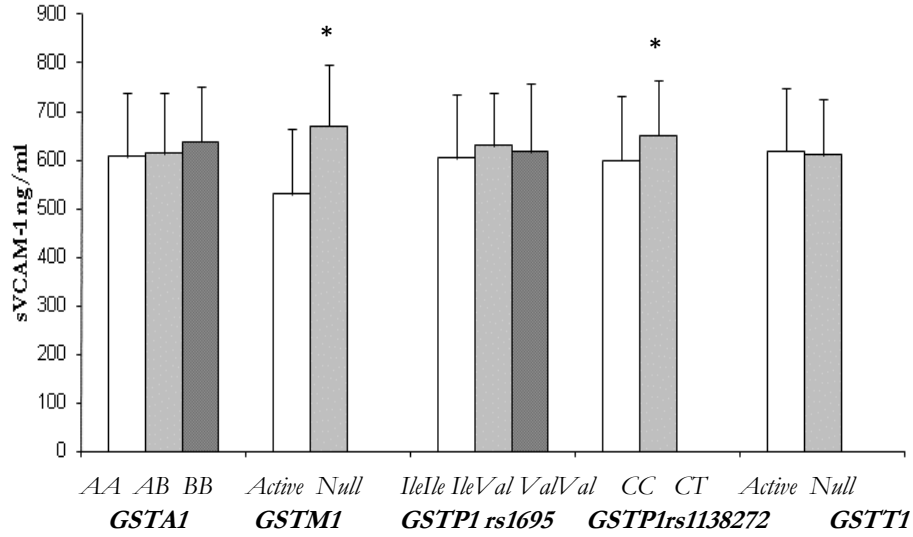


**Figure 25.** Soluble ICAM-1 concentrations in plasma of ESRD patients in terms of *GST* genotype. *p < 0.05, **p < 0.01

Other *GST*' gene polymorphism, such as *GSTT1* and *GSTP1* rs1695, did not show any significant influence on circulating levels of CAMs.

### 4.4.5 Impact of combined *GST* genotype on markers of oxidative damage

To assess whether the effects of *GST null/low-activity* genotypes are more pronounced when combined, we compared the level of eight oxidative damage byproducts and two cellular adhesion molecules with different combination of *GSTA1*, *GSTM1*, *GSTP1rs1695* and *GSTT1* genotype (Table 14a and 14b). The level of protein thiols was significantly lowered in terms of the presence of variant *GST* allele. Namely, patients with *GSTM1-active/GSTT1-active* genotype had thiols concentration of 8.2 ± 2.4 μmol/g of protein, while the presence of null or low activity genotype had diminishing effect on SH groups concentration. Thus, when *GSTM1* and *GSTT1* genotype were combined, it was observed that patients carrying *GSTM1-null* genotype had significantly lowered the amount of protein thiol groups. Patients with *GSTM1-null/GSTT1-active* genotype had 6.5 ± 1.2 μmol/g of protein SH group concentration while the presence of *GSTT1-null* genotype diminished thiols even more (5.5 ± 1.4 μmol/g of protein, p = 0.001). When *GSTA1-low activity* genotype was included in stratification, the effect was even more pronounced resulting in losing almost 30% of referent thiol concentration (*GSTM1/GSTT1/GSTA1 active genotype*). The lowest SH-groups concentration was observed in patients carrying *GSTM1-null/GSTT1-null/GSTA1-low activity/GSTP1-low activity* genotype where it was determined 5.3 ± 1.2 μmol/g of protein SH group concentration.

Protein carbonyls, another marker of protein oxidative damage, were significantly increased in patients with null or low activity *GST* genotype present. As presented in Table 14a, the protein carbonyls concentration gradually increased in terms of *GST* genotype. Thus, patients with both *GSTM1*- and *GSTT1-null* genotype had 123.8% increased concentration of carbonyls in comparison to ESRD patients with *GSTM1-active/GSTT1-active* genotype. The presence of *GSTA1-low activity* and *GSTP1-low activity* genotype further increased carbonyls in plasma of ESRD patients. It was determined that patients with all null or low activity *GST* genotype combination ha 126% increased protein carbonyls compared to the patients with all active *GST* genotypes (2.9 ± 0.6 vs. 2.3 ± 0.1, respectively).

Similar effect was observed in increased AOPP concentration in terms of *GST* genotype combinations. The ESRD patients who carried all null or low activity *GST* genotypes had up to 25% increased AOPP content when compared to appropriate referent group (72.3 [61.4 - 218.2] and 57.9 [28.3 - 70.0] mmol/L, respectively; p = 0.038).

The level of MDA was lowest in ESRD patients with both *GSTM1-active/GSTT1-active* genotype, and then gradually increased with the presence of *null* or *low activity* genotypes, reaching 2.6 [1.6 - 3.1] mmol/L of MDA concentration in ESRD patients with *GSTM1-null/GSTT1-null* genotype combination. However, when *GSTA1* and *GSTP1* genotypes were included in the model, it was observed that the presence of their low activity alleles further increases the level of MDA measured in plasma. Thus, the highest level of MDA was determined in patients carrying *GSTM1-null/GSTT1-nul/GSTP1-low* activity genotype although statistical significance was not observed.

As far as MDA adducts were concerned, similar effect was observed. Presence of *GST* null or low activity genotypes was influenced significantly the MDA adducts in ESRD patients. The highest concentration was measured in ESRD patients with *GSTM1-null/GSTT1-null/GSTA1-low activity* genotype who had almost 2-fold increase of MDA adducts when compared to the patients with referent (*GSTM1-active/GSTT1-active/GSTA1-active)* genotype (53.2 ± 8.9 pmol/mg and 31.1 ± 8.3 pmol/mg, respectively; p = 0.001).

Total oxidant status was further analyzed in terms of combined *GST* genotypes. When *GSTM1* and *GSTT1* genotypes were combined, it was observed that the presence *GSTM1-null* genotype influenced significantly the increase of TOS level in ESRD patients. Combination of *GSTA1-low activity* or *GSTP1-low activity* genotype with *GSTM1/GSTT1*, gave also higher TOS levels when compared to appropriate reference groups, although the statistical significance was present only in combination of *GSTM1-null/GSTT1-null/GSTA1-low activity* genotypes (p = 0.044). Values of PAB were also increased in favor of null or low activity *GST* genotype, although statistical significance was not present.

Combined *GST* genotypes did not influence the sICAM-1 concentration in plasma of ESRD patients. On the other hand, significant association of sVCAM-1 concentrations with different *GST* genotypes combinations was observed. Namely, the patients with both *GSTM1* and *GSTT1 null* genotypes present had significant increase of sVCAM-1 in plasma (49.6 ± 9.6 vs. 29.4 ± 6.8 in *GSTM1-active/GSTT1-active* patients, p = 0.001). This trend was continued further with the presence of *GSTP1-low activity* genotype in combination (35% increase in concentration, p = 0.001) (Table 14b).

**Table 14a.** Association of combined *GST* genotypes with byproducts of oxidative stress in plasma of ESRD patients

| Combined Genotypes | Thiols (µmol/g) | | Carbonyls (nmol/mg) | | AOPP (mmol/L) | | Nitrotyrosine (nmol/L) | | MDA adducts (pmol/mg) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | [C] | p | [C] | p | [C] | p | | p | [C] | p |
| *GSTM1 active/GSTT1 active* | 8.2±2.4<br>100% | | 2.1±0.3<br>100% | | 57.1[28.3-69.4]<br>100% | | 60.2[43.7-93.1]<br>100% | | 29.4±6.8<br>100% | |
| *GSTM1active/GSTT1 null* | 7.1±1.7<br>86.6% | 0.029 | 2.3±0.3<br>109.5% | 0.290 | 64.5[48.8-80.8]<br>113% | 0.450 | 62.4[46.1-93.1]<br>104% | 0.279 | 40.0±5.9<br>136.1% | 0.001 |
| *GSTM1 null/GSTT1 active* | 6.5±1.2<br>79.3% | 0.001 | 2.4±0.0.4<br>114.3% | 0.586 | 61.1[38.5-69.7]<br>107% | 0.021 | 72.3[50.1-101.2]<br>120% | 0.168 | 42.0±6.0<br>142.9% | 0.001 |
| *GSTM1 null-GSTT1 null* | 5.5±1.4<br>67.1% | 0.001 | 2.6±0.5<br>123.8% | 0.003 | 73.9[61.6-213.9]<br>129% | 0.001 | 64.5[48.8-78.7]<br>107% | 0.073 | 49.6±9.6<br>168.7% | 0.001 |
| *GSTM1/GSTT1/GSTA1 active* | 8.8±1.2<br>100% | 0.001 | 2.2±0.2<br>100% | 0.002 | 50.1 [26.7-66.9<br>100% | 0.001 | 62.8[46.1-93.1]<br>100% | 0.718 | 31.1±8.3<br>100% | 0.001 |
| *GSTM1/GSTT1/GSTA1 null or low activity* | 5.5±1.2<br>62.5% | | 2.9±0.5<br>131.8% | | 77.1[62.6-214.5]<br>154% | | 75.5[72.3-78.7]<br>120% | | 53.2±8.9<br>171.1% | |
| *GSTM1/GSTT1/ GSTP1 active* | 8.4±2.8<br>100% | 0.001 | 2.2±0.2<br>100% | 0.024 | 56.4[31.9-70.0]<br>100% | 0.022 | 62.8[46.1-89.3]<br>100% | 0.040 | 30.0±6.4<br>100% | 0.001 |
| *GSTM1/GSTT1/ GSTP1 null or low activity* | 5.2±1.2<br>61.9% | | 2.6±0.5<br>118.2% | | 72.9[31.9-184.9]<br>129% | | 59.3[46.8-78.7]<br>94% | | 49.4±9.9<br>164.7% | |
| *GSTM1/GSTT1/GSTA1/GSTP1 active* | 9.5±2.3<br>100% | 0.001 | 2.3±0.1<br>100% | 0.037 | 57.9[28.3-70.0]<br>100% | 0.038 | 55.4[39.5-72.6]<br>100% | 0.169 | 33.5±6.2<br>100% | 0.001 |
| *GSTM1/GSTT1/GSTA1/GSTP1 null or low activity* | 5.3±1.2<br>55.8% | | 2.9±0.6<br>126.1% | | 72.3[61.4-218.2]<br>125% | | 59.3[48.8-75.5]<br>107% | | 52.6±8.4<br>157.0% | |

**Table 14b.** Association of combined *GST* genotypes with MDA, PAB, TOS, sICAM-1 and sVCAM-1 from plasma of ESRD patients

| Combined Genotypes | MDA (mmol/L) | | PAB (HK units) | | TOS (μmol $H_2O_2$ equiv./L) | | sICAM-1 (ng/ml) | | sVCAM-1 (ng/ml) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | [C] | p | [C] | p | [C] | p | [C] | p | [C] | p |
| *GSTM1 active/GSTT1 active* | 2.2[1.7-2.8]<br>100% | | 113.4[67.4-192.2]<br>100% | | 14.2[12.2-21.2]<br>100% | | 85.5±24.4<br>100% | | 595.2±104.3<br>100% | |
| *GSTM1active/GSTT1 null* | 2.4[1.8-2.9]<br>109% | 0.269 | 161.2[64.0-264.6]<br>142% | 0.127 | 31.5[16.2-55.7]<br>222% | 0.315 | 95.9±19.7<br>112% | 0.115 | 653.7±66.6<br>110% | 0.001 |
| *GSTM1 null/GSTT1 active* | 2.6[1.8-3.3]<br>118% | 0.132 | 157.4[95.8-269.8]<br>139% | 0.08 | 16.2[12.7-23.9]<br>114% | 0.001 | 80.4±22.3<br>94% | 0.547 | 593.9±94.2<br>100% | 0.770 |
| *GSTM1 null-GSTT1 null* | 2.6[1.6-3.1]<br>118% | 0.098 | 151.4[95.0-244.0]<br>133% | 0.181 | 42.0[14.9-272.2]<br>296% | 0.002 | 96.1±34.1<br>112% | 0.290 | 658.6±59.1<br>111% | 0.001 |
| *GSTM1/GSTT1/GSTA1 active* | 2.2[1.5-2.8]<br>100% | 0.188 | 128.6[66.6-193.4]<br>100% | 0.402 | 14.2[12.1-19.2]<br>100% | 0.044 | 81.8±14.4<br>100% | 0.067 | 526.9±142.3<br>100% | 0.292 |
| *GSTM1/GSTT1/GSTA1null or low activity* | 2.5[1.1-2.9]<br>114% | | 163.4[151.4-175.4]<br>127% | | 11.5[9.6-13.4]<br>81% | | 116.0±23.6<br>142% | | 635.8±26.1<br>121% | |
| *GSTM1/GSTT1/ GSTP1 active* | 2.2[1.7-2.8]<br>100% | 0.026 | 107.8[68.0-186.8]<br>100% | 0.361 | 14.2[12.2-24.2]<br>100% | 0.068 | 88.8±23.5<br>100% | 0.473 | 522.6±129.2<br>100% | 0.001 |
| *GSTM1/GSTT1/ GSTP1 null or low activity* | 3.8[3.1-6.9]<br>173% | | 154.2[127.2-203.4]<br>143% | | 59.8[15.2-272.8]<br>421%% | | 101.3±2.8<br>114% | | 703.7±79.8<br>135% | |
| *GSTM1/GSTT1/GSTA1/GSTP1 active* | 2.3[1.5-2.7]<br>100% | 0.111 | 175.8[28.6-232.2]<br>100% | 0.791 | 14.2[11.5-14.8]<br>100% | 0.096 | 87.1±18.5<br>100% | 0.569 | 628.8±76.8<br>100% | 0.007 |
| *GSTM1/GSTT1/GSTA1/GSTP1 null or low activity* | 2.6[1.8-3.8]<br>113% | | 137.4[87.8-221.2]<br>78% | | 20.1[13.4-271.5]<br>141% | | 99.8±27.9<br>115% | | 671.7±75.4<br>107% | |

## 4.5 PREDICTIVE ROLE OF *GST* GENOTYPE

During a five year follow-up death outcomes and cardiovascular complications were prospectively registered in order to test predictive role of *GST* genotype on ESRD patients' outcome. A total of 186 patients were followed to the death or the end of 5 years follow up. Overall mortality rate was 51.6% (96 patients) with 58 cardiovascular deaths (cardiovascular mortality rate was 30.7%). Around 90 registered deaths were of other causes (cachexia, gastro-intestinal bleeding, infections, malignant disease). Within cardiovascular deaths, for 36 patients (19%) the myocardial infarction (MI) was the cause, while for the 25 (13.2%) the cerebral vascular insult (CVI) was the cause. Three patients were with both MI and CVI registered as the causes of death. The mean survival time for all patients was 44 ± 20 months. Multivariable Cox regression analysis was used to test the independent influence of *GST* gene polymorphisms on predicting all-cause (overall), cardiovascular mortality as well as the death of myocardial infarction and cerebral vascular insult (stroke). Three models were used. The first one calculated hazard ratio adjusted for age and gender. The second one included smoking status of patients, besides age and gender. The third one included everything as the second one with added adjustment for diabetes and cholesterol level. Kaplan-Meier survival curve analysis was used to test the influence of *GST* gene polymorphism on ESRD patients' survival and to estimate mean survival time.

### 4.5.1 Lack of association between *GSTA1* genotype and overall or cardiovascular mortality

Table 15 represents the association of *GSTA1* polymorphism with overall, cardiovascular mortality as well as the death of myocardial infarction and stroke. Patients were dichotomized on those homozygous for variant allele (*GSTA1 *B/*B* genotype) and those who carried at least one reference allele (*GSTA1 *A /*A* and *GSTA1 *A/*B* genotypes). The *GSTA1-low activity* genotype did not represent a predictor of higher risk for neither overall nor cardiovascular mortality among patients with end-stage renal disease. Slightly increase of hazard ratio was observed in Model 3 for cardiovascular mortality, although far from being statistically significant. Based on Kaplan-Meier survival curve analysis, ESRD patients with *GSTA1-low activity* genotype did not affect survival of patiens with ESRD (Figure 26).

**Table 15.** *GSTA1* polymorphism as a predictor for overall and cardiovascular mortality as well as death of myocardial infarction and cerebral vascular insult

| Model 1[a] | | Model 2[b] | | Model 3[c] | |
|---|---|---|---|---|---|
| HR (95% CI) | P value | HR (95% CI) | P value | HR (95% CI) | P value |
| **Risk for overall mortality comparing *GSTA1*B* homozygotes to *GSTA1*A* carriers** | | | | | |
| 0.87 (0.53-1.45) | 0.598 | 0.97 (0.56-1.68) | 0.92 | 0.98 (0.56-1.74) | 0.969 |
| **Risk for cardiovascular mortality comparing *GSTA1*B* homozygotes to *GSTA1*A* carriers** | | | | | |
| 1.09 (0.55-2.16) | 0.808 | 1.16 (0.56-2.39) | 0.697 | 1.23 (0.58-2.63) | 0.593 |
| **Risk for death from MI comparing *GSTA1*B* homozygotes to *GSTA1*A* carriers** | | | | | |
| 1.23 (0.42-3.59) | 0.709 | 0.81 (0.35-1.90) | 0.63 | 0.89 (0.36-2.2) | 0.795 |
| **Risk for death from CVI comparing *GSTA1*B* homozygotes to *GSTA1*A* carriers** | | | | | |
| 0.79 (0.36-1.74) | 0.56 | 1.24 (0.42-3.69) | 0.69 | 1.19 (0.40-3.53) | 0.75 |

Abbreviations: CI, Confidence Interval; HR, Hazard Ratio. [a]Adjusted for age and gender. [b]Adjusted for the covariates in Model 1 plus smoking status. [c]Adjusted for the covariates in Model 2 plus diabetes and cholesterol level.

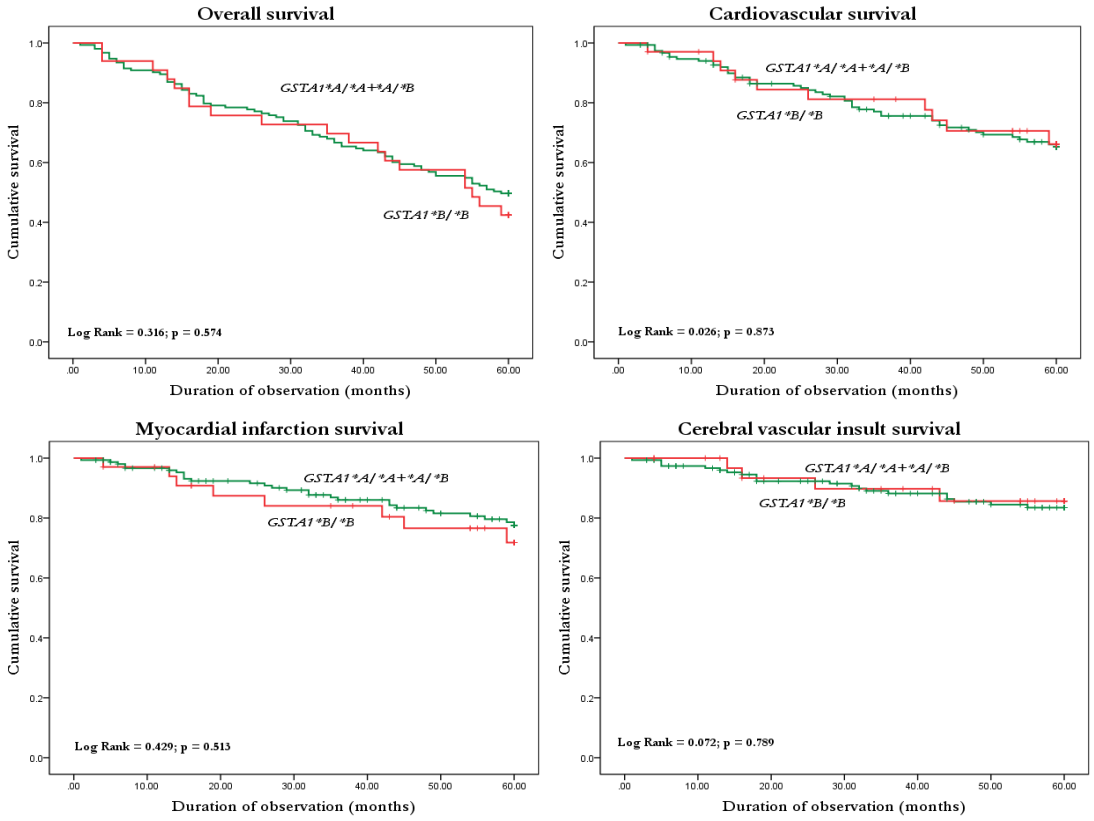


**Figure 26.** Kaplan-Meier survival analysis for overall, cardiovascular, cerebral vascular insult and myocardial infarction survival regarding *GSTA1* genotype.

### 4.5.2 A significant association of *GSTM1* deletion polymorphism with all cause and cardiovascular mortality

The presence of *GSTM1-null* genotype was independent predictor of overall and cardiovascular mortality risk among ESRD patients (Table 16). Patients with *GSTM1* homozygous deletion had a significantly increased risk for overall mortality (Model 1: HR = 1.8, 95% CI: 1.1 - 2.8, p = 0.009). Introducing additional parameters into the Cox regression model did not alter the risk which remained significantly increased (Model 2: HR = 1.8, 95% CI: 1.12 - 2.84, p = 0.015 and Model 3: HR = 1.7, 95%CI: 1.07-2.75, p = 0.025). Cardiovascular mortality risk was statistically significant increased in all three models considered (Model 1: HR = 2.3, 95% CI: 1.28 - 4.29, p = 0.006; Model 2: HR = 2.1, 95% CI: 1.14 - 3.93, p = 0.018, Model 3: HR = 2.02, 95% CI: 1.08 - 3.80, p = 0.028). Additionally, *GSTM1-null* genotype has shown significant effect on myocardial infarction mortality with HR=2.3 (95% CI: 1.06 - 4.83, p = 0.035). However, although HR for cerebral vascular insult mortality was high (HR = 2.3) this was shown statistically insignificant (95% CI: 0.91 -5.76 p = 0.078).

**Table 16** *GSTM1* gene deletion polymorphism as a predictor for overall and cardiovascular mortality as well as death of myocardial infarction and cerebral vascular insult

| **Model 1[a]** | | **Model 2[b]** | | **Model 3[c]** | |
|---|---|---|---|---|---|
| HR (95% CI) | P value | HR (95% CI) | P value | HR (95% CI) | P value |
| **Risk for overall mortality comparing *GSTM1-null* to *GSTM1-active* carriers** | | | | | |
| 1.8 (1.15-2.77) | 0.009 | 1.8 (1.12-2.84) | 0.015 | 1.7 (1.07-2.75) | 0.025 |
| **Risk for cardiovascular mortality comparing *GSTM1-null* to *GSTM- active* carriers** | | | | | |
| 2.3 (1.28-4.29) | 0.006 | 2.1 (1.14-3.93) | 0.018 | 2.0 (1.08-3.80) | 0.028 |
| **Risk for death from myocardial infarction comparing *GSTM1-null* to *GSTM1-active* carriers** | | | | | |
| 2.3 (1.06-4.83) | 0.035 | 2.0 (0.89-4.30) | 0.094 | 1.8 (0.80-3.99) | 0.155 |
| **Risk for death from CVI comparing *GSTM1-null* to *GSTM1-active* carriers** | | | | | |
| 2.3 (0.91-5.76) | 0.078 | 2.1 (0.82-5.36) | 0.122 | 2.1 (0.84-5.55) | 0.113 |

Abbreviations: CI, Confidence Interval; HR, Hazard Ratio. [a]Adjusted for age and gender. [b]Adjusted for the covariates in Model 1 plus an additional adjustment for smoking status. [c]Adjusted for the covariates in Model 2 plus an additional adjustment for diabetes and cholesterol level.

A Kaplan-Meier survival analysis demonstrated shorter overall (Log Rank = 5.718, p=0.017) and cardiovascular survival (Log rank = 6.275, p = 0.006) as well as shorter time to death of MI (Log Rank = 3.088, p = 0.038) (Figure 27). Mean overall survival time for *GSTM1-null* patients was 41.7 months (38.0 - 45.4), while for *GSTM1-active* patients it was 47.5 months (43.1 - 51.9). Patients with homozygous deletion of *GSTM1* gene had mean cardiovascular survival time of 46.9 months (43.4 - 50.5), while those with *GSTM1-active* genotype lived averagely 53.0 months (49.3 - 56.7). Average survival time for myocardial infarction was 52.0 months (48.9 - 55.1) for *GSTM1-null* genotype patients in comparison with *GSTM1-active* genotype patients who lived 55.0 months (51.6-58.1). Regarding cerebral vascular insult survival, Kaplan-Meier survival analysis curve has shown that *GSTM1-null* genotype did not affect it (Log Rank = 3.217, p=0.073) (Figure 27).

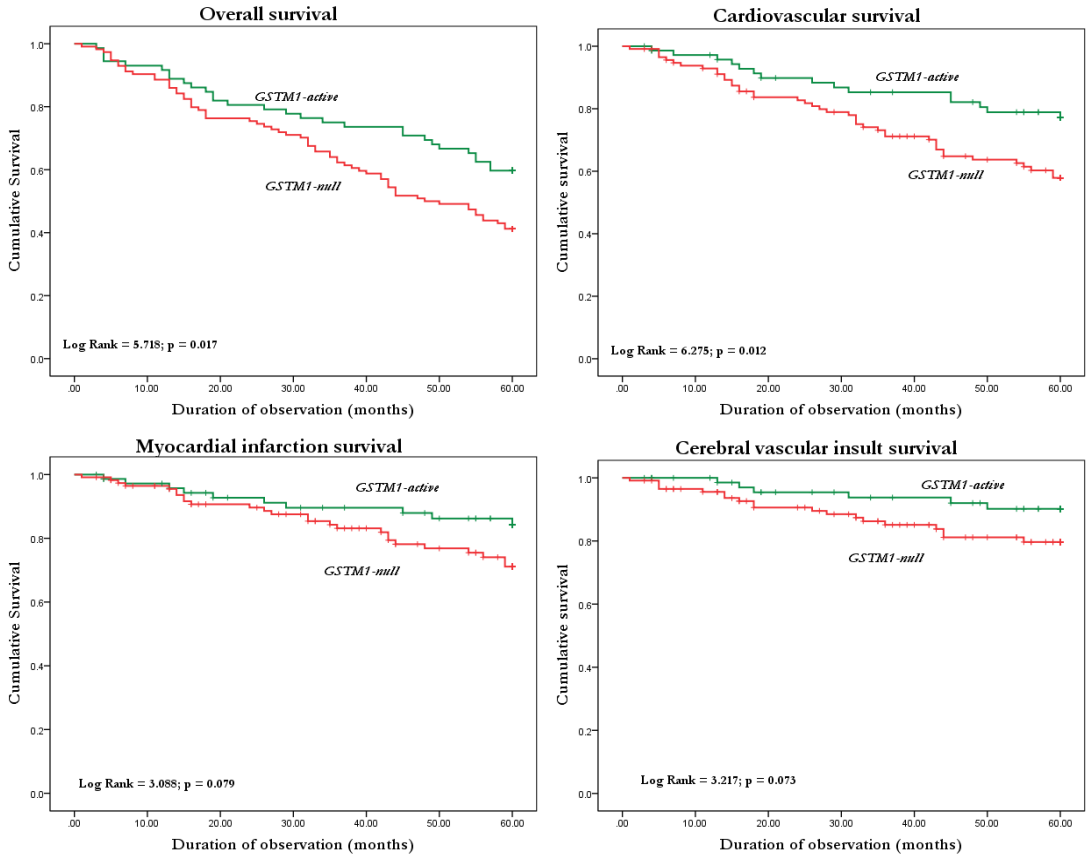


**Figure 27**. Kaplan-Meier survival analysis for overall, cardiovascular, cerebral vascular insult and myocardial infarction survival regarding *GSTM1* genotype

### 4.5.3 Association of *GSTT1* genotype with all cause and cardiovascular mortality

Table 17 summarizes the association of *GSTT1* gene deletion polymorphism with overall, cardiovascular mortality as well as the death of myocardial infarction and cerebral vascular insult (stroke). No association was found for any cause of the death regarding *GSTT1-null* genotype in patients with end-stage renal disease. Namely, patients lacking GSTT1 activity had no increased risk for either overall (HR = 1.1, 95% CI: 0.705 - 1.67, p = 0.712) or cardiovascular mortality (HR = 1.2, 95% CI: 0.68 - 2.2, p = 0.906). Homozygous deletion of this gene was not shown to be significant for special causes of death, including myocardial infarction (HR = 1.1, 95%CI: 0.56 - 2.28, p = 0.733) and cerebral vascular insult (HR = 1.1, 95% CI: 0.46 - 2.50, p = 0.879).

Kaplan-Meier survival analysis showed no impact of *GSTT1-null* genotype of ESRD patient's survival considering overall, cardiovascular, cerebral vascular insult and myocardial infarction cause of death (Figure 28).

**Table 17**. *GSTT1* gene deletion polymorphism as a predictor for overall and cardiovascular mortality as well as death of myocardial infarction and cerebral vascular insult among ESRD patients by Cox proportional hazards regression models

| **Model 1[a]** | | **Model 2[b]** | | **Model 3[c]** | |
|---|---|---|---|---|---|
| HR (95% CI) | P value | HR (95% CI) | P value | HR (95% CI) | P value |
| **Risk for overall mortality comparing *GSTT1-null* to *GSTT1-active* carriers** | | | | | |
| 1.1 (0.71-1.67) | 0.712 | 1.1 (0.59-1.70) | 0.712 | 1.1 (0.66-1.69) | 0.811 |
| **Risk for cardiovascular mortality comparing *GSTT1-null* to *GSTT1-active* carriers** | | | | | |
| 1.2 (0.68-2.03) | 0.569 | 1.4 (0.79-2.43) | 0.250 | 1.3 (0.74-2.41) | 0.332 |
| **Risk for death from MI comparing *GSTT1-null* to *GSTT- active* carriers** | | | | | |
| 1.1 (0.56-2.28) | 0.733 | 1.5 (0.74-3.14) | 0.250 | 1.5 (0.72-3.27) | 0.270 |
| **Risk for death from CVI comparing *GSTT- null* to *GSTT1-active* carriers** | | | | | |
| 1.1 (0.46-2.49) | 0.879 | 1.1 (0.45-2.53) | 0.880 | 1.0 (0.40-2.50) | 0.98 |

Abbreviations: CI, Confidence Interval; HR, Hazard Ratio. [a]Adjusted for age and gender. [b]Adjusted for the covariates in Model 1 plus an additional adjustment for smoking status. [c]Adjusted for the covariates in Model 2 plus an additional adjustment for diabetes and cholesterol level.

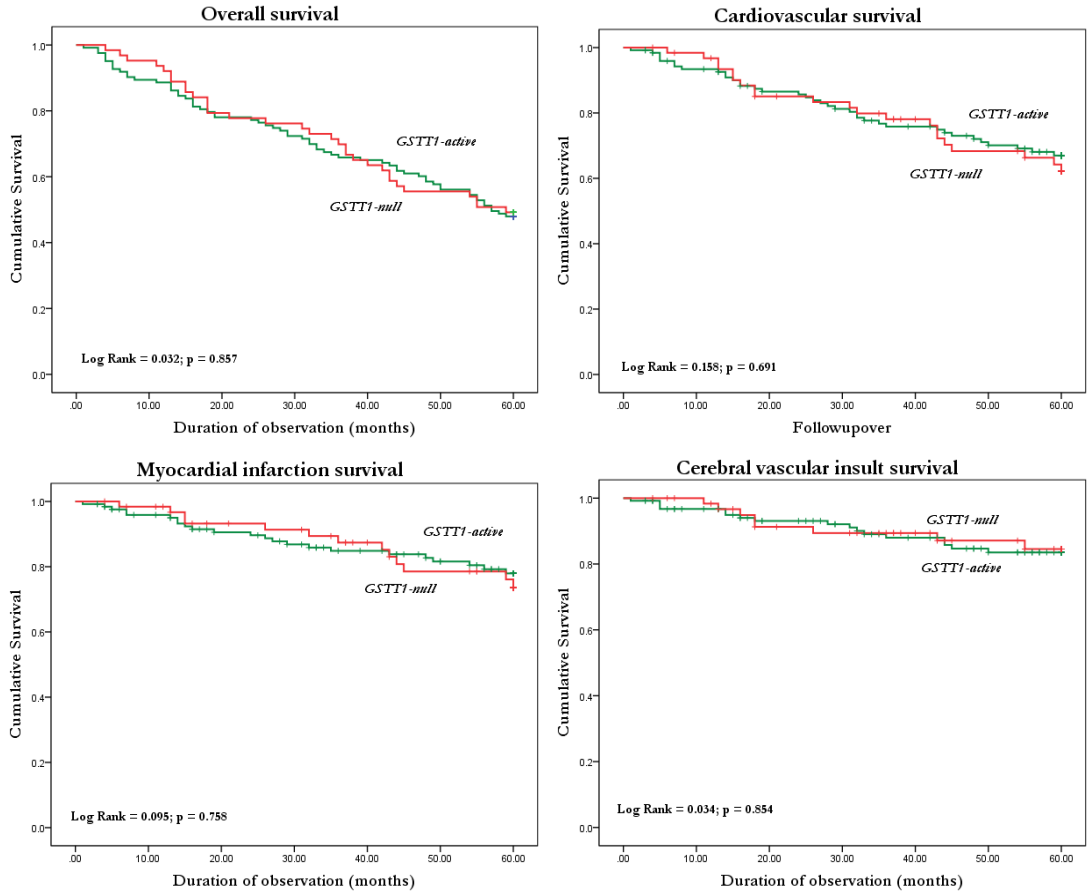


**Figure 28.** Kaplan-Meier survival analysis for overall, cardiovascular, cerebral vascular insult and myocardial infarction survival regarding *GSTT1* genotype

### 4.5.4 Association of *GSTP1* genotype with all cause and cardiovascular mortality

The Cox proportional hazard model was applied to assess whether polymorphism in both *GSTP1* gene examined in this thesis is associated with poor outcome of the patients on hemodialysis. These results are presented in Table 18.

As far as *GSTP1* rs1695 polymorphism is concerned, patients were dichotomized into those with active genotype (*GSTP1 *Ile/*Ile+*Ile/*Val*) and low activity genotype (*GSTP1 *Val/*Val* genotype). Although the risk was increased (HR = 1.4, 95% CI: 0.70-2.79), *GSTP1 low-activity* genotype was not significant predictor of overall death (p = 0.337). This genotype was not associated with neither cardiovascular cause of death (HR = 1.4, 95% CI: 0.79 - 2.47, p = 0.248) nor cerebral vascular cause of death (HR = 1.3, 95% CI: 0.53 - 3.04, p = 0.602). The *GSTP1 Val/Val* genotype was associated with doubled myocardial infarction risk of death (HR = 2.24, 95%CI: 1.06 - 4.76, p = 0.063) in tested Model 2 which was adjusted to age, gender and smoking status. Kaplan-Meier survival

analysis showed that patients *GSTP1*Val/*Val* genotype had significantly shorter myocardial infarction survival in comparison to those with *GSTP1-active* genotype (Log rank = 6.08, p = 0.014) (Figure 29). Mean survival time for these patients was 49.2 months (43.6 - 54.9), while the ESRD patients who carried at least one *GSTP1*Ile* allele lived averagely 54.5 monts (52.1 – 56.9).

**Table 18.** *GSTP1* rs1695 and *GSTP1* rs1138272 polymorphism as a predictor for overall and cardiovascular mortality as well as death of myocardial infarction and cerebral vascular insult among ESRD patients by Cox proportional hazards regression models

| **Model 1[a]** | | **Model 2[b]** | | **Model 3[c]** | |
|---|---|---|---|---|---|
| HR (95% CI) | P value | HR (95% CI) | P value | HR (95% CI) | P value |
| **Risk for overall mortality comparing *GSTP1*Val* homozygotes to *GSTP1 Ile* carriers** | | | | | |
| 1.2 (0.78-1.94) | 0.378 | 1.4 (0.84-2.29) | 0.203 | 1.4 (0.81-2.30) | 0.248 |
| **Risk for cardiovascular mortality comparing *GSTP1*Val* homozygotes to *GSTP1 Ile* carriers** | | | | | |
| 1.4 (0.79-2.47) | 0.248 | 1.7 (0.9-3.1) | 0.103 | 1.6 (0.84-3.09) | 0.151 |
| **Risk for death from myocardial infarction comparing *GSTP1*Val* homozygotes to *GSTP1 Ile* carriers** | | | | | |
| 1.9 (0.97-3.80) | 0.063 | 2.2 (1.06-4.76) | 0.035* | 2.0 (0.88-4.45) | 0.097 |
| **Risk for death from CVI comparing *GSTP1*Val* homozygotes to *GSTP1 Ile* carriers** | | | | | |
| 1.3 (0.53-3.04) | 0.602 | 1.7 (0.69-4.25) | 0.25 | 2.0 (0.78-5.00) | 0.15 |
| **Risk for overall mortality comparing *GSTP1*C* homozygotes to *GSTP1 *T carriers*** | | | | | |
| 0.8 (0.51-1.32) | 0.421 | 0.7 (0.44-1.31) | 0.313 | 0.8 (0.45-1.40) | 0.428 |
| **Risk for cardiovascular mortality comparing *GSTP1*C* homozygotes to *GSTP1 *T carriers*** | | | | | |
| 1.1 (0.60-1.92) | 0.811 | 1.0 (0.3-1.7) | 0.489 | 1.2 (0.58-2.24) | 0.694 |
| **Risk for death from MI comparing *GSTP1*C* homozygotes to *GSTP1 *T carriers*** | | | | | |
| 1.3 (0.61-2.62) | 0.522 | 1.3 (0.55-2.87) | 0.588* | 1.6 (0.65-3.71) | 0.326 |
| **Risk for death from CVI comparing *GSTP1*C* homozygotes to *GSTP1 *T carriers*** | | | | | |
| 0.9 (0.38-2.26) | 0.874 | 1.0 (0.38-2.52) | 0.428 | 1.0 (0.38-2.72) | 0.962 |

Abbreviations: CI, Confidence Interval; HR, Hazard Ratio. [a]Adjusted for age and gender. [b]Adjusted for the covariates in Model 1 plus an additional adjustment for smoking status. [c]Adjusted for the covariates in Model 2 plus an additional adjustment for diabetes and cholesterol level.

Regarding the impact of *GSTP1 rs1138272* polymorphism on patients' survival, results have shown that this SNP does not change the risk of death outcome in none of the

causes of death in any of three models considered. Patients with *GSTP1*C/*T* genotype had 1.6 increased risk of fatal outcome, but still statistically insignificant (p = 0.326). The risk was even slightly decreased when overall and cerebral vascular causes of death were considered but without reaching statistical significance in any of Models. Kaplan Meier analysis of survival is presented in Figures 29 (*GSTP1* rs1695 polymorphism) and Figure 30 (*GSTP1* rs1138272 polymorphism).

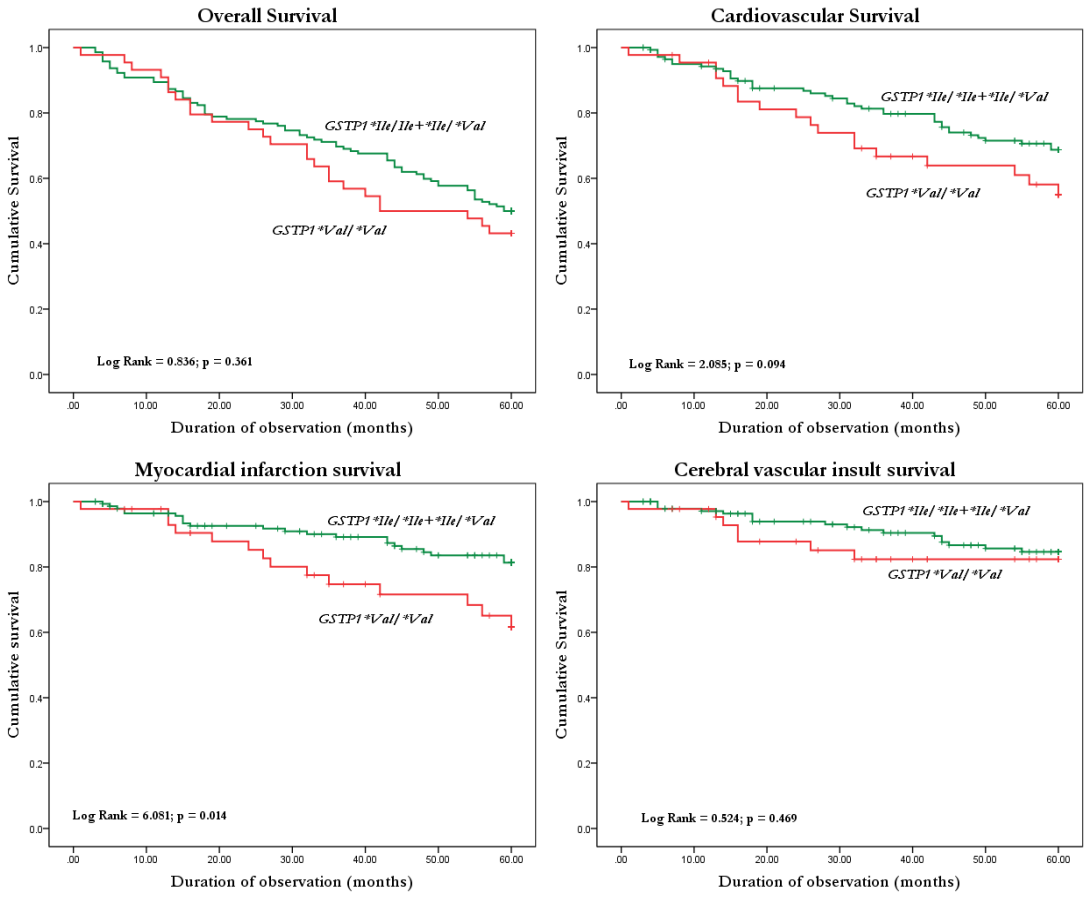


**Figure 29**. Kaplan-Meier survival analysis for overall, cardiovascular, cerebral vascular insult and myocardial infarction survival regarding *GSTP1* genotype (rs1695).

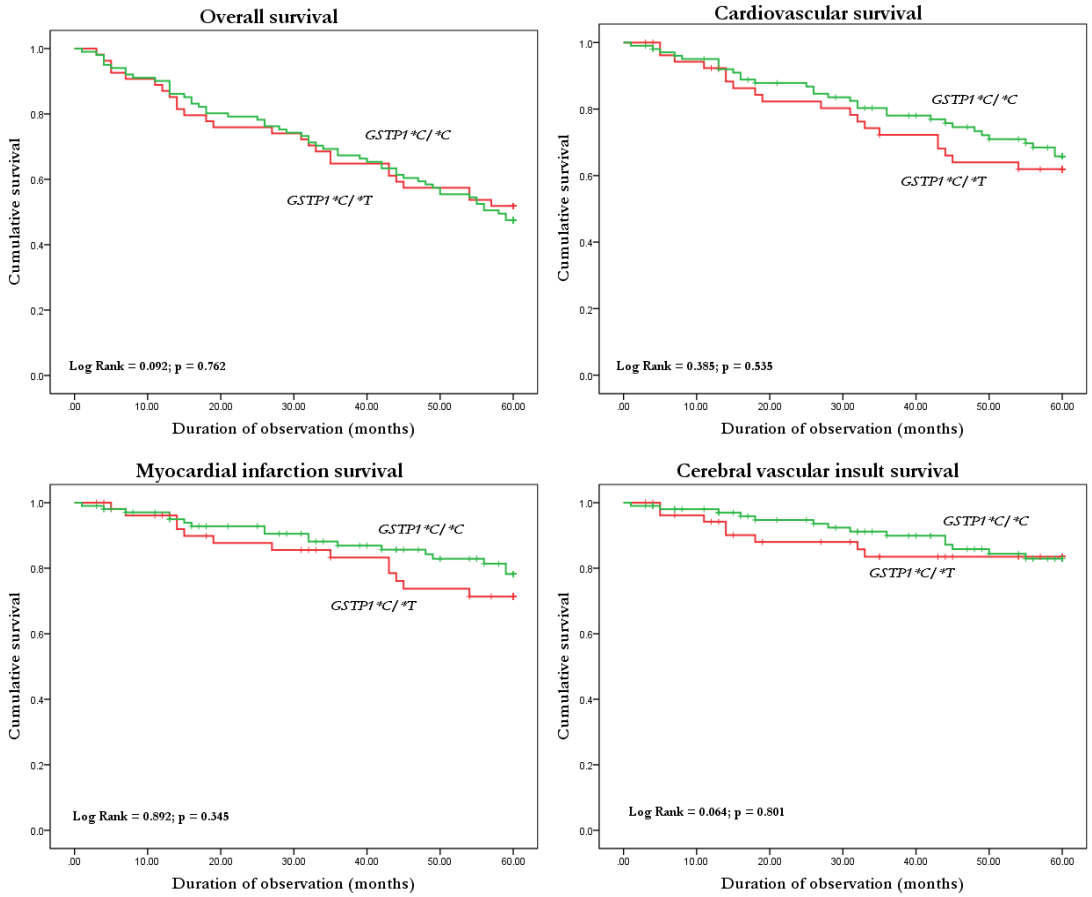


**Figure 30**. Kaplan-Meier survival analysis for overall, cardiovascular, cerebral vascular insult and myocardial infarction survival regarding *GSTP1* rs1138272 genotype

## 4.6 PREDICTIVE ROLE OF OXIDATIVE STRESS BYRODUCTS

In order to assess the potential predictive role of oxidative stress byproducts, "*case only*" study was performed. A total of 199 patients with ESRD was included in the study and followed during 5-years. To examine predictive role of oxidative stress byproducts and cellular adhesion molecules, a Cox hazard ration calculations and Kaplan-Meier survival analysis were performed. Patients were dichotomized on those with low (below the median (med)) and high values (above the median) of the parameter that was considered. The predictive role of each byproduct was tested in three Models, each of them being adjusted for specific parameters that have been linked to mortality in previous studies (Model 1 was adjusted for age and gender; Model 2 adjustment included age, gender and smoking status; Model 3 included age, gender, smoking status, cholesterol levels and presence of diabetes adjustment parameters).

### 4.6.1 Protein oxidative stress byproducts as possible predictors of patients' outcome

End-stage renal disease patients with low levels of thiols (above the median) had 1.7-fold greater risk for CV death in Model 2 (HR = 1.7; 95% CI: 0.96-3.04, p = 0.067). Patients with low thiols had almost doubled risk for MI death when compared to those with high levels (above the median)(Model 1: HR = 1.9; 95% CI: 0.96 - 3.99, p = 0.064). In Model 2 and Model 3 certain increase of MI risk was observed in ESRD patients with low thiol levels, but with no statistical significance (Model 2: HR = 1.9; 95% CI: 0.89 - 4.03, p = 0.098; Model 3: HR = 1.7; 95% CI: 0.80 - 3.71, p = 0.167). Furthermore, patients with thiol concentration below the median level had no shorter survival in comparison with those with higher values of thiol content. Kaplan-Meier survival analysis showed that ESRD patients with lower thiols concentration have shorter survival of fatal myocardial infarction survival (Log-Rank = 3.756, p = 0.053) (Figure 31A).

The dichotomization of patients based on the median levels of carbonyls showed no significant association with increased risk for fatal overall and cardiovascular outcome in this group of patients with ESRD (Table 19a). Concentration of carbonyls above the median level in plasma of patients with ESRD did not have prognostic level in terms of overall and cardiovascular survival, as it has been shown in Kaplan-Meier survival analysis (Figure 31B).

Advanced oxidation protein products (AOPP) level was a significant predictor of fatal cardiovascular and myocardial infarction outcome in all three models tested in the study. Increase in AOPP concentration above the median level was associated with an increased risk for cardiovascular death (Model 1: HR = 2.4; 95% CI: 1.31 - 4.50, p = 0.005; Model 2: HR = 2.2, 95%CI: 1.13 - 4.17, p = 0.020; Model 3: HR = 2.1, 95%CI: 1.10 - 4.12, p = 0.024). The risk was even more increased when only myocardial infarction death was considered. Namely, patients with values of AOPP higher than 64.29 μmol/ L had 3 times greater risk for MI compared to the ESRD patients with lower values of AOPP (Model 1: HR = 3.3; 95% CI: 1.41 - 7.93, p = 0.006; Model 2: HR = 2.5, 95%CI: 1.01 - 6.17, p = 0.047) (Table 19b). Although the patients with higher AOPP concentration were at greater risk for all-cause and fatal CVI outcome, this increase was not statistically significant. Kaplan-Meier survival analysis revealed that patients with high AOPP levels had poorer cardiovascular and MI survival than those with low AOPP levels (Log Rank =7.0, p =

0.003 and Log Rank = 7.1, p = 0.008, respectively) (Figure 32A). Dichotomization of patients based on the median levels of nitrotyrosine showed no significant association with any cause of death in these patients. Kaplan-Meier survival analysis did not show shorter survival of the patients with higher nitrotyrosine concentration (Table 19b and Figure 32B).

**Table 19a**. Protein oxidative byproducts (protein thiols and carbonyls) as predictors for overall and cardiovascular mortality as well as death of myocardial infarction and cerebral vascular insult among ESRD patients by Cox proportional hazards regression models.

| **Model 1[a]** | | **Model 2[b]** | | **Model 3[c]** | |
|---|---|---|---|---|---|
| HR (95% CI) | P value | HR (95% CI) | P value | HR (95% CI) | P value |
| **Protein thiol gropus (med = 6.7 µmol/ g)** | | | | | |
| **Risk for overall mortality** | | | | | |
| 1.3 (0.88-2.01) | 0.171 | 1.4 (0.90-2.15) | 0.137 | 1.3 (0.96-2.07) | 0.196 |
| **Risk for CV mortality** | | | | | |
| 1.6 (0.94-2.79) | 0.081 | 1.7 (0.96-3.04) | 0.067 | 1.6 (0.91-2.89) | 0.103 |
| **Risk for death from MI** | | | | | |
| 1.9 (0.96-3.99) | 0.064 | 1.9 (0.89-4.04) | 0.098 | 1.7 (0.80-3.71) | 0.167 |
| **Risk for death from CVI** | | | | | |
| 1.1 (0.48-2.33) | 0.889 | 1.1 (0.50-2.62) | 0.747 | 1.1 (0.49-2.58) | 0.782 |
| **Carbonyls (med = 2.2 nmol/ g)** | | | | | |
| **Risk for overall mortality** | | | | | |
| 0.9 (0.44-1.90) | 0.813 | 1.2 (0.56-2.57) | 0.635 | 1.1 (0.51-2.44) | 0.779 |
| **Risk for CV mortality** | | | | | |
| 1.1 (0.47-2.73) | 0.786 | 1.4 (0.57-3.58) | 0.453 | 1.4 (0.53-3.53) | 0.523 |
| **Risk for death from MI** | | | | | |
| 1.0 (0.32-3.17) | 0.988 | 1.3 (0.39-4.27) | 0.687 | 1.3 (0.39-4.54) | 0.655 |
| **Risk for death from CVI** | | | | | |
| 1.9 (0.59-6.11) | 0.282 | 2.2 (0.66-7.32) | 0.200 | 2.5 (0.67-9.09) | 0.173 |

Abbreviations: CI, Confidence Interval; HR, Hazard Ratio. [a]Adjusted for age and gender. [b]Adjusted for the covariates in Model 1 plus an additional adjustment for smoking status. [c]Adjusted for the covariates in Model 2 plus an additional adjustment for diabetes and cholesterol level.

**Table 19b**. Protein oxidative byproducts AOPP and nitrotyrosine as a predictor for overall and cardiovascular mortality as well as death of myocardial infarction and cerebral vascular insult among ESRD patients by Cox hazard regression model.

| Model 1[a] | | Model 2[b] | | Model 3[c] | |
|---|---|---|---|---|---|
| HR (95% CI) | P value | HR (95% CI) | P value | HR (95% CI) | P value |
| **AOPP (med = 64.3 μmol/L)** | | | | | |
| **Risk for overall mortality** | | | | | |
| 1.5 (0.97-2.36) | 0.075 | 1.5 (0.92-2.42) | 0.104 | 1.5 (0.91-2.41) | 0.119 |
| **Risk for CV mortality** | | | | | |
| 2.4 (1.3-4.5) | 0.005 | 2.2 (1.13-4.17) | 0.020 | 2.1 (1.10-4.12) | 0.024 |
| **Risk for death from MI** | | | | | |
| 3.3 (1.41-7.93) | 0.006 | 2.5 (1.01-6.17) | 0.047 | 2.4 (0.94-5.93) | 0.066 |
| **Risk for death from CVI** | | | | | |
| 1.5 (0.65-3.43) | 0.351 | 1.8 (0.73-4.39) | 0.203 | 1.8 (0.73-4.44) | 0.198 |
| **Nitrotyrosine (med = 64.5 nmol/L)** | | | | | |
| **Risk for overall mortality** | | | | | |
| 1.2 (0.77-1.86) | 0.432 | 1.1 (0.70-1.81) | 0.611 | 1.1 (0.70-1.82) | 0.618 |
| **Risk for cardiovascular mortality** | | | | | |
| 1.7 (0.90-3.05) | 0.104 | 1.6 (0.83-3.03) | 0.164 | 1.6 (0.83-3.13) | 0.523 |
| **Risk for death from MI** | | | | | |
| 1.8 (0.80-3.98) | 0.160 | 1.8 (0.73-4.38) | 0.200 | 2.0 (0.79-5.13) | 0.142 |
| **Risk for death from CVI** | | | | | |
| 1.6 (0.65-3.73) | 0.318 | 1.5 (0.60-3.50) | 0.404 | 1.4 (0.57-3.29) | 0.490 |

Abbreviations: CI, Confidence Interval; HR, Hazard Ratio.
[a]Adjusted for age and gender. [b]Adjusted for the covariates in Model 1 plus an additional adjustment for smoking status. [c]Adjusted for the covariates in Model 2 plus an additional adjustment for diabetes and cholesterol level

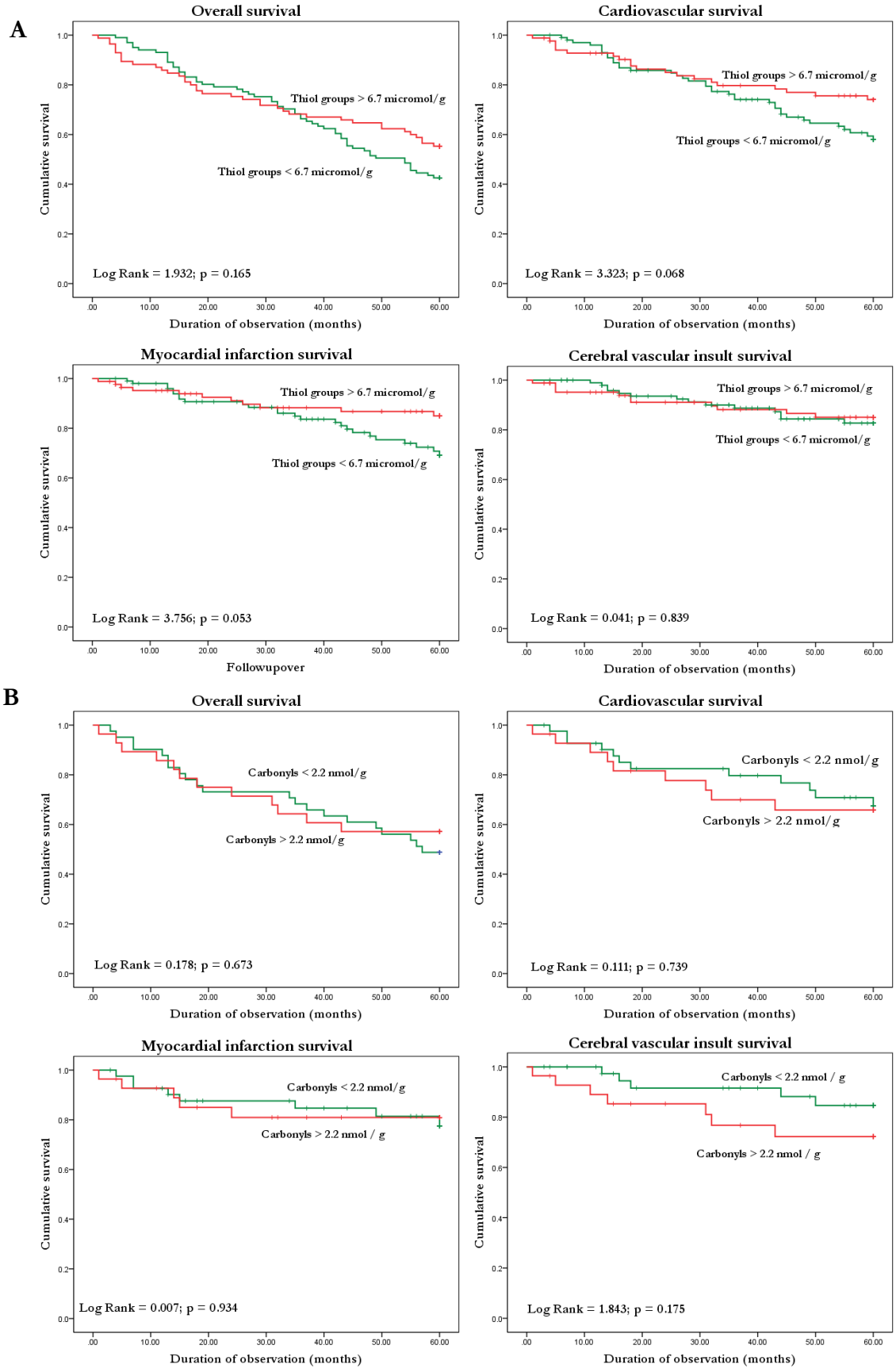


**Figure 31**. Kaplan-Meier survival curves for overall, cardiovascular, CVI and MI survival of ESRD patients dichotomized by median levels of thiol groups (A) and carbonyls (B).

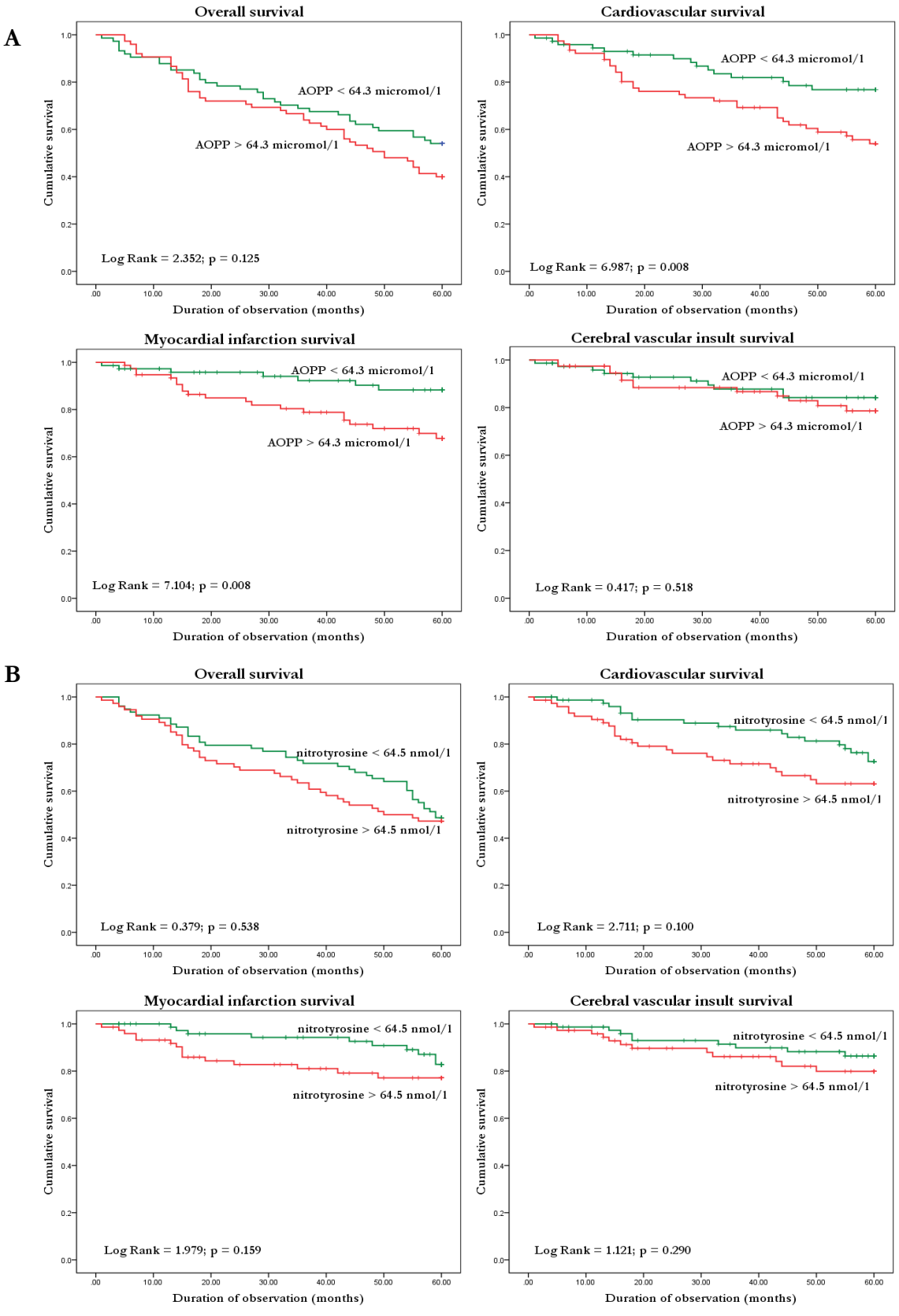


**Figure 32**. Kaplan-Meier survival curves for overall, cardiovascular, CVI and MI survival of ESRD patients dichotomized by median levels of AOPP (A) and nitrotyrosine (B)

### 4.6.2 The possible predictive role of malondialdehyde

Patients with ESRD were dichotomized based on median levels of malondialdehyde (median was 2.3 mmol/L) measured in plasma of these patients. Univariate Cox's proportional hazards regression analysis showed that high values of MDA (above median) are strong predictor of mortality in ESRD patients (Table 20). Namely, overall mortality of hemodialysis patients was significantly increased in patients with MDA concentration above median level (Model 1: HR = 1.5, 95%CI = 1.00 - 2.28, p = 0.049, Model 2: HR= 1.6, 95% CI = 1.05 - 2.52, p = 0.029). Patients with high MDA levels were at greater risk for cardiovascular mortality and this risk was even increased when data were adjusted to additional parameters considered in Model 2 and Model 3 (Model 1: HR = 1.9, 95%CI: 1.11 - 3.28, p = 0.019, Model 2: HR = 2.4, 95% CI: 1.33 - 4.20, p = 0.003; Model 3: HR = 2.2, 95% CI: 1.18 - 3.93, p = 0.012). Malondialdehyde has shown possible predictive role for cerebral vascular insult and myocardial infarction fatal outcome as well. Thus the risk for death of cerebral vascular insult was up to three times higher in patients with MDA levels above the median (HR = 3.0, 95%CI: 1.21-7.39, p = 0.018). Regarding fatal myocardial infarction outcome, the highest observed risk was seen in Model 2 (HR = 2.6, 95%CI: 1.21-5.44, p = 0.014).

Kaplan-Meier survival analysis has shown shorter cardiovascular and cerebral vascular insult survival for patients with high malondialdehyde levels (Log Rank = 4.41. p = 0.036 and Log Rank = 5.21, p = 0.022, respectively) (Figure 33A). Mean cardiovascular survival time for patients with MDA concentrations in plasma above the median levels was 46.7 months (42.5 – 50.9) while the patients with low MDA levels lived averagely 52.1 (49.0 – 55.2). Mean CVI survival time for patients with MDA concentrations in plasma above the median levels was 52.7 months (49.4 – 56.1) while the patients with low MDA levels lived averagely 56.8 (54.5 – 59.1).

Stratified malondialdehyde adducts (MDA adducts median was 39.1 pmol/g) level did not show any association with increased risk for overall, cardiovascular, myocardial infarction or cerebral vascular insult mortality of patients with end-stage renal disease in any of the three Models that were considered in the study.

Kaplan-Meier analysis has shown that high MDA adducts levels did not have any influence on ESRD patients' survival (Figure 33B).

**Table 20**. Lipid oxidative stress byproducts, malonidaldehyde and MDA adducts, as predictors for overall and cardiovascular mortality as well as death of myocardial infarction and cerebral vascular insult among ESRD patients by Cox proportional hazard regression models.

| Model 1[a] | | Model 2[b] | | Model 3[c] | |
|---|---|---|---|---|---|
| HR (95% CI) | P value | HR (95% CI) | P value | HR (95% CI) | P value |
| **MDA (med = 2.3 mmol/L)** | | | | | |
| **Risk for overall mortality** | | | | | |
| 1.5 (1.00-2.82) | 0.049 | 1.6 (1.05-2.52) | 0.029 | 1.5 (0.97-2.42) | 0.068 |
| **Risk for cardiovascular mortality** | | | | | |
| 1.9 (1.11-3.28) | 0.019 | 2.4 (1.33-4.20) | 0.003 | 2.2 (1.18-3.93) | 0.012 |
| **Risk for death from MI** | | | | | |
| 1.9 (0.96-3.74) | 0.064 | 2.6 (1.21-5.44) | 0.014 | 2.5 (1.11-5.40) | 0.026 |
| **Risk for death from CVI** | | | | | |
| 2.9 (1.20-7.14) | 0.018 | 3.0 (1.21-7.39) | 0.018 | 2.9 (1.18-7.39) | 0.021 |
| **MDA adducts (med = 39.1 pmol/ mg)** | | | | | |
| **Risk for overall mortality** | | | | | |
| 1.0 (0.63-1.73) | 0.871 | 1.0 (0.60-1.69) | 0.970 | 1.0 (0.60-1.73) | 0.931 |
| **Risk for CV mortality** | | | | | |
| 1.1 (0.58-2.34) | 0.631 | 1.1 (0.56-2.17) | 0.771 | 1.2 (0.60-2.41) | 0.609 |
| **Risk for death from MI** | | | | | |
| 1.4 (0.58-3.49) | 0.448 | 1.4 (0.56-3.49) | 0.474 | 1.5 (0.56-3.75) | 0.440 |
| **Risk for death from CVI** | | | | | |
| 1.1 (0.42-3.11) | 0.787 | 1.1 (0.42-3.14) | 0.788 | 1.3 (0.45-3.60) | 0.642 |

Abbreviations: CI, Confidence Interval; HR, Hazard Ratio.
[a]Adjusted for age and gender. [b]Adjusted for the covariates in Model 1 plus an additional adjustment for smoking status. [c]Adjusted for the covariates in Model 2 plus an additional adjustment for diabetes and cholesterol level

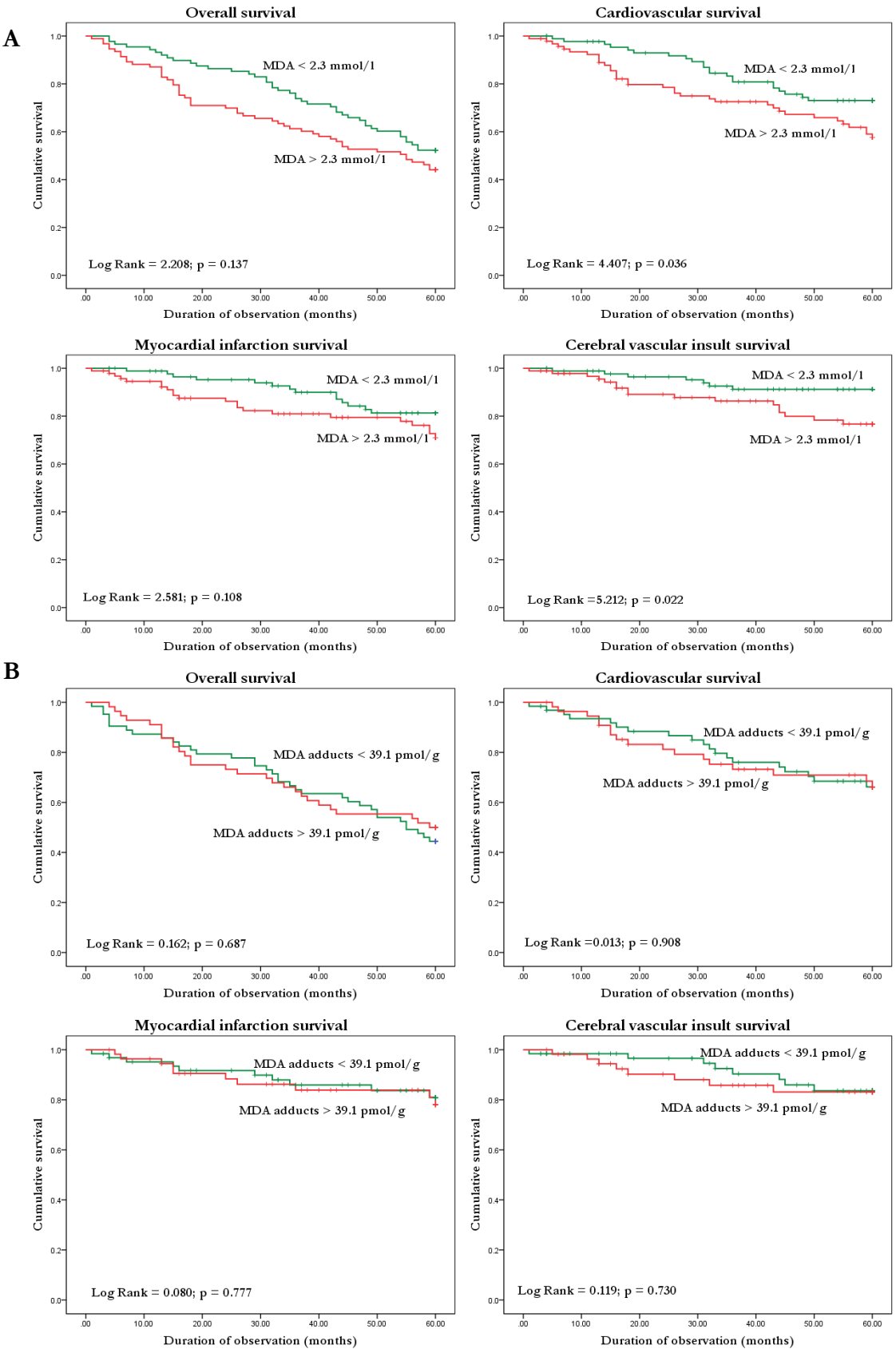


**Figure 33.** Kaplan-Meier survival curves for overall, cardiovascular, CVI and MI survival of ESRD patients dichotomized by the median levels of MDA (A) and MDA adducts (B).

### 4.6.3 Prooxidant-antioxidant balance level as a predictor of overall and cardiovascular mortality

The Table 21 summarizes the association between prooxidant-antioxidant balance and total oxidant status values with different models of overall (all-cause) and cardiovascular survival, as well as fatal myocardial infarction and cerebral vascular insult outcome.

Multivariate Cox regression analysis revealed that PAB is a strong predictor of hemodialysis patients' outcome. Namely, patients with PAB levels above the median value (148.6 HK units) were at increased risk for fatal all-cause and cardiovascular outcome, specifically cerebral vascular insult death. Thus the risk for overall mortality was up to 2.4 times higher in Model 2, adjusted for age, gender and smoking status (HR = 2.4, 95%CI: 1.45 - 3.87, p = 0.001). When cardiovascular cause of death was isolated, the mortality risk was even slightly higher than for all-cause moratlity (Model 1: HR = 2.5, 95%CI: 1.33 - 4.59, p = 0.004; Model 2: HR = 2.4, 95%CI: 1.27- 4.52, p = 0.007; Model 3: HR = 2.3, 95%CI: 1.22-4.41, p = 0.011). High values of PAB (above the median) were strong predictors of CVI death as well with the highest risk of 2.6 observed in Model 1 (HR = 2.6, 95%CI: 1.04 - 6.53, p = 0.04). Regarding MI cause of death, the risk was up to 1.9 times higher but without statistical significance (Model 1: HR = 1.9, 95%CI: 0.83 - 4.10, p = 0.131). Kaplan-Meier survival method based on median levels of PAB has showed that patients with high values have shorter cumulative overall, cardiovascular and cerebral vascular insult survival (Log Rank = 10.4, p = 0.001; Log Rank = 9.0, p = 0.003; Log Rank = 5.4, p = 0.020, respectively)(Figure 34). Mean overal survival time for ESRD patients with high PAB values was 37.0 months (32.0 – 42.0) wile for the patients with low PAB values it was 48.7 (44.8 – 52.7). Based on the Kaplan-Meier analysis results, ESRD patients with PAB above the median levels lived approximately 9 months shorter compared to the patient with low PAB values when cardiovascular survival was considered (44.1 (39.2 - 49.0) and 53.0 (49.6 – 56.4), respectively) and 5 months shorter when cerebral vascular insult was observed (51.4 (47.2 – 55.5) and 56.2 (53.4 – 59.0), respectively).

When patients were stratified based on median values of total oxidant status (20.0 μmol $H_2O_2$ equivalents) results have shown that patients with high TOS values (above the median) were not at greater risk for overall or cardiovascular death. The risk was at marginal statistical significance for CVI death when Model 1 was considered (HR = 2.3,

95%CI = 0.97 - 5.32, p = 0.058). Kaplan-Meier survival analysis has shown shorter CVI survival for patients with high levels of TOS but without reaching statistical significance (Log Rank = 3.2, p = 0.075) (Figure 34).

**Table 21**. The median levels of prooxidant-antioxidant balance (PAB) and total oxidant status (TOS) as a predictor for overall and cardiovascular mortality as well as death of myocardial infarction and cerebral vascular insult among ESRD patients by Cox proportional hazards regression models.

| **Model 1[a]** | | **Model 2[b]** | | **Model 3[c]** | |
|---|---|---|---|---|---|
| HR (95% CI) | P value | HR (95% CI) | P value | HR (95% CI) | P value |
| **PAB (med = 148.6 HK units)** | | | | | |
| **Risk for overall mortality** | | | | | |
| 2.2 (1.40-3.53) | 0.001 | 2.4 (1.45-3.87) | 0.001 | 2.3 (1.42-3.83) | 0.001 |
| **Risk for cardiovascular mortality** | | | | | |
| 2.5 (1.33-4.59) | 0.004 | 2.4 (1.27-4.52) | 0.007 | 2.3 (1.22-4.41) | 0.011 |
| **Risk for death from MI** | | | | | |
| 1.9 (0.83-4.11) | 0.131 | 1.7 (0.72-3.83) | 0.236 | 1.6 (0.67-3.74) | 0.297 |
| **Risk for death from CVI** | | | | | |
| 2.6 (1.04-6.53) | 0.040 | 2.5 (0.99-6.47) | 0.052 | 2.5 (0.97-6.36) | 0.058 |
| **TOS (med = 20.00 μmol $H_2O_2$ equivalents/L)** | | | | | |
| **Risk for overall mortality** | | | | | |
| 1.0 (0.67-1.59) | 0.884 | 1.1 (0.70-1.73) | 0.687 | 1.1 (0.66-1.68) | 0.819 |
| **Risk for cardiovascular mortality** | | | | | |
| 1.4 (0.82-2.50) | 0.211 | 1.5 (0.81-2.61) | 0.212 | 1.3 (0.73-2.45) | 0.343 |
| **Risk for death from MI** | | | | | |
| 1.2 (0.58-2.51) | 0.611 | 1.2 (0.54-2.60) | 0.676 | 1.1 (0.48-2.48) | 0.829 |
| **Risk for death from CVI** | | | | | |
| 2.3 (0.97-5.32) | 0.058 | 2.2 (0.93-5.34) | 0.070 | 2.2 (0.90-5.27) | 0.086 |

Abbreviations: CI, Confidence Interval; HR, Hazard Ratio.[a]Adjusted for age and gender. [b]Adjusted for the covariates in Model 1 plus an additional adjustment for smoking status. [c]Adjusted for the covariates in Model 2 plus an additional adjustment for diabetes and cholesterol level.

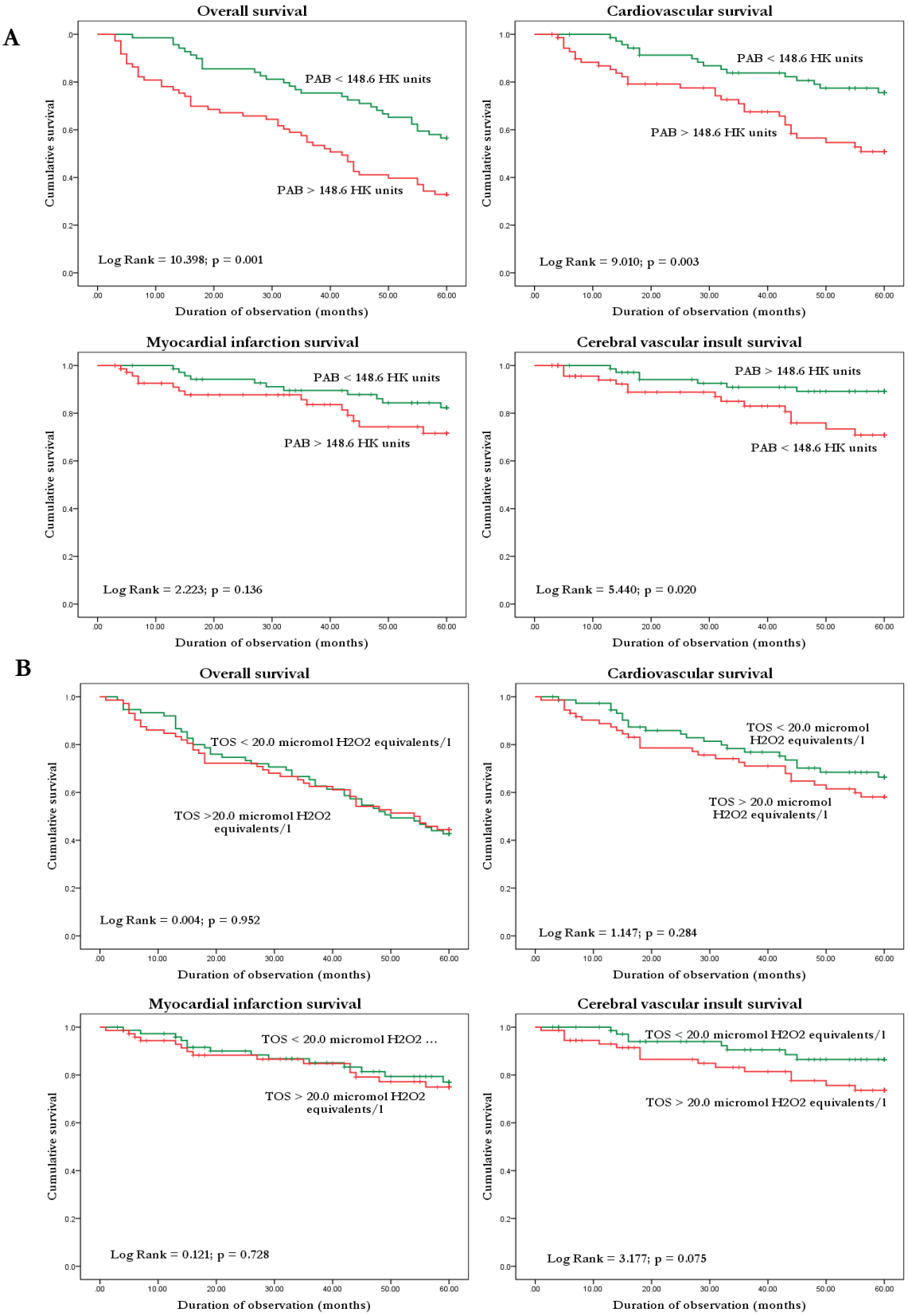


**Figure 34.** Kaplan-Meier survival curves for overall, cardiovascular, CVI and MI survival of ESRD patients dichotomized by the median levels of PAB (A) and TOS (B).

### 4.6.4 Soluble ICAM-1 and ICAM-1as potential predictors of all-cause and cardiovascular death

On the Table 22 are summarized results on the predictive role of soluble ICAM-1 and VCAM-1 concentrations measured in plasma of end-stage renal disease patients on overall and cardiovascular death outcome. Levels of cell adhesion molecules were dichotomized based on the median values and were 649.6 ng/ml for sVCAM-1 and 90.4 ng/ml for sICAM-1.

Multivariate Cox regression method has shown that end-stage renal disease patients with high sICAM-1 values had no statistically significant increase in risk for either overall or cardiovascular death, as well as for CVI or MI death.

Kaplan-Meier survival analysis revealed shorter overall survival for patients with sICAM-1 values above the median (Log Rank = 4.6, p = 0.032) (Figure 35 A). The ESRD patients with high sICAM-1 levels lived approximately seven months shorter in comparison with the patients with sICAM-1 concentrations bellow median levels when overall survival was considered (41.0 months (34.6 – 47.4) and 48.0 months (42.1 – 53.9), respectively). High sICAM-1 levels did not influence cardiovascular survival of ESRD patients (Log Rank = 2.821, p = 0.093).

Regarding the predictive role of sVCAM-1 values, Cox regression analysis has shown that the end-stage renal disease patients with sVCAM-1 values above the median have increased all-cause mortality risk. Thus sVCAM-1 was a strong predictor of overall mortality in the model adjusted for age and gender (Model 1: HR = 1.9, 95%CI: 1.2-3.1, p = 0.007). When additional parameters were introduced, such as smoking (for Model 2) and cholesterol and diabetes (Model 3), the risk was slightly higher (Model 2: HR = 2.0, 95%CI: 1.25 - 3.31, p = 0.004 and Model 3: HR = 2.0, 95%CI: 1.22 - 3.26, p = 0.006). Values of sVCAM-1 above the median increased the risk of cardiovascular and myocardial infarction death in ESRD patients, but without reaching statistical significance (Table 22). The sVCAM-1 concentration above the median level showed as a poor predictor of cerebral vascular insult death.

Kaplan-Meier survival analysis curve showed that patients with high sVCAM-1 level have significantly shorter overall survival (Log Rank = 6.2, p = 0.013) compared to the patients with low sVCAM-1 levels (Figure 35 B). Mean overall survival time for ESRD

patients with high sVCAM-1 values was 41.3 months (37.2 – 45.3) while for the patients with low sVCAM-1 concentration it was 47.5 (42.9 – 52.2).

**Table 22**. The median levels of sICAM-1 and sVCAM-1 as a predictor for overall and cardiovascular mortality as well as death of myocardial infarction and cerebral vascular insult among ESRD patients by Cox proportional hazards regression models.

| Model 1[a] | | Model 2[b] | | Model 3[c] | |
|---|---|---|---|---|---|
| HR (95% CI) | P value | HR (95% CI) | P value | HR (95% CI) | P value |
| **ICAM-1 (med = 90.4 ng/ml)** | | | | | |
| **Risk for overall mortality** | | | | | |
| 1.6 (0.78-3.19) | 0.209 | 1.5 (0.68-3.16) | 0.323 | 1.3 (0.53-2.95) | 0.601 |
| **Risk for cardiovascular mortality** | | | | | |
| 1.6 (0.67-3.76) | 0.288 | 1.4 (0.66-5.03) | 0.485 | 1.0 (0.29-2.58) | 0.790 |
| **Risk for death from MI** | | | | | |
| 1.6 (0.50-5.30) | 0.420 | 1.2 (0.61-11.67) | 0.786 | 1.4 (0.58-2.42) | 0.304 |
| **Risk for death from CVI** | | | | | |
| 0.9 (0.23-3.61) | 0.888 | 0.9 (0.20-3.71) | 0.835 | 0.57 (0.11-3.04) | 0.508 |
| **VCAM-1 (med = 649.6 ng/ml)** | | | | | |
| **Risk for overall mortality** | | | | | |
| 1.9 (1.20-3.05) | 0.007 | 2.0 (1.25-3.31) | 0.004 | 2.0 (1.22-3.26) | 0.006 |
| **Risk for cardiovascular mortality** | | | | | |
| 1.7 (0.92-3.10) | 0.089 | 1.7 (0.91-3.20) | 0.093 | 1.7 (0.91-3.21) | 0.097 |
| **Risk for death from MI** | | | | | |
| 1.9 (0.86-4.23) | 0.150 | 1.8 (0.79-4.19) | 0.157 | 1.8 (0.76-4.09) | 0.189 |
| **Risk for death from CVI** | | | | | |
| 1.3 (0.54-3.17) | 0.554 | 1.3 (0.51-3.18) | 0.608 | 1.3 (0.52-3.29) | 0.561 |

Abbreviations: CI, Confidence Interval; HR, Hazard Ratio. [a]Adjusted for age and gender. [b]Adjusted for the covariates in Model 1 plus an additional adjustment for smoking status. [c]Adjusted for the covariates in Model 2 plus an additional adjustment for diabetes and cholesterol level.

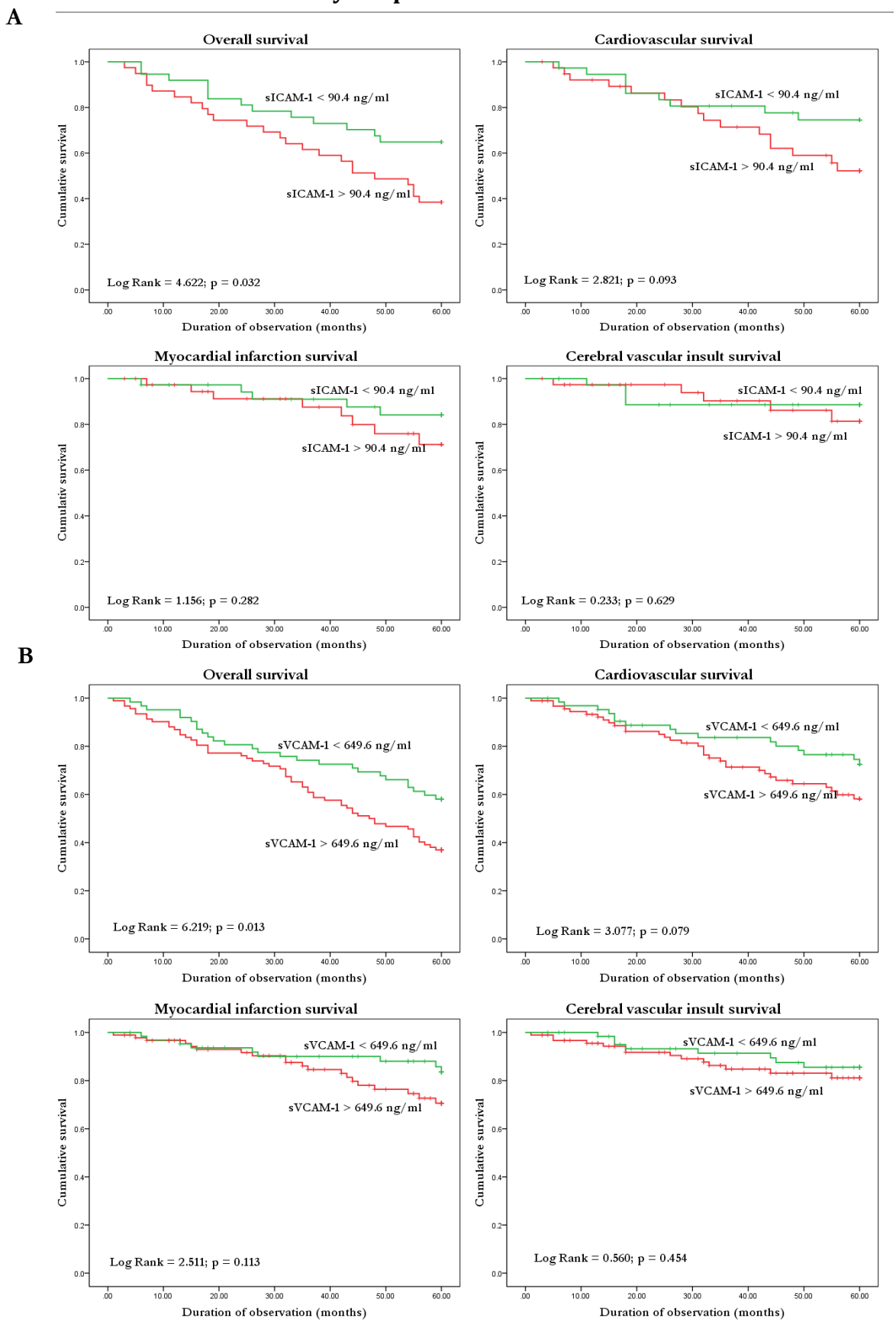


**Figure 35.** Kaplan-Meier survival curves for overall, cardiovascular, CVI and MI survival of ESRD patients dichotomized by the median levels of ICAM-1 (A) and VCAM-1 (B)

## 4.7 Effect of human umbilical vein endothelial cells incubation in uremic serum

In order to test whether polymorphic expression of *GSTM1* gene in *human umbilical vein endothelial cells (HUVEC)* influences their susceptibility to oxidative stress and atherogenic potential in terms of monocyte adhesion in uremic milieu, two primary cell cultures that differ with respect to *GSTM1* were incubated in uremic serum. The HUVECs were treated with control serum obtained from healthy volunteers and uremic serum from patients on hemodialysis. The 2% FBS and 20% FBS serums were used as a vehicle.

### 4.7.1 Viability of endothelial cells after incubation in uremic serum

Incubation of HUVECs in 20% uremic serum in EGM for 4 hrs did not change viability of endothelial cells (Figure 36). Namely, the cell viability was decreased less than 10% compared to the cells that were exposed to 2% FBS (standard EGM).

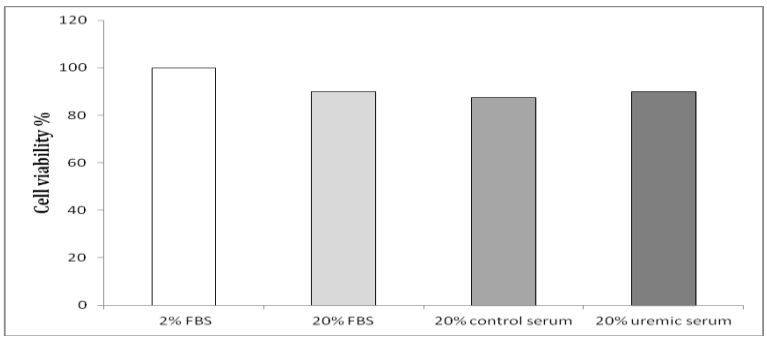


**Figure 36.** Endothelial cell viability assay when exposed to 2% FBS, 20% FBS, 20% control serum and 20% uremic serum.

### 4.7.2 Effect of monocyte adhesion to endothelial cells after incubation in uremic serum

In this study we have expected that endothelial cells with different *GSTM1* genotype will react differently with to monocyte adhesion, when exposed to uremic milieu. When endothelial cells are exposed to uremic serum, toxins with pro-oxidant activities activate endothelial cells, consequently provoking its dysfunction. These first cellular changes on endothelial cells can be detected by monocyte adhesion assay. A small number of monocytes adhered to untreated HUVECs, incubated with 2% FBS (standard medium) and 20% FBS (Figure 37).

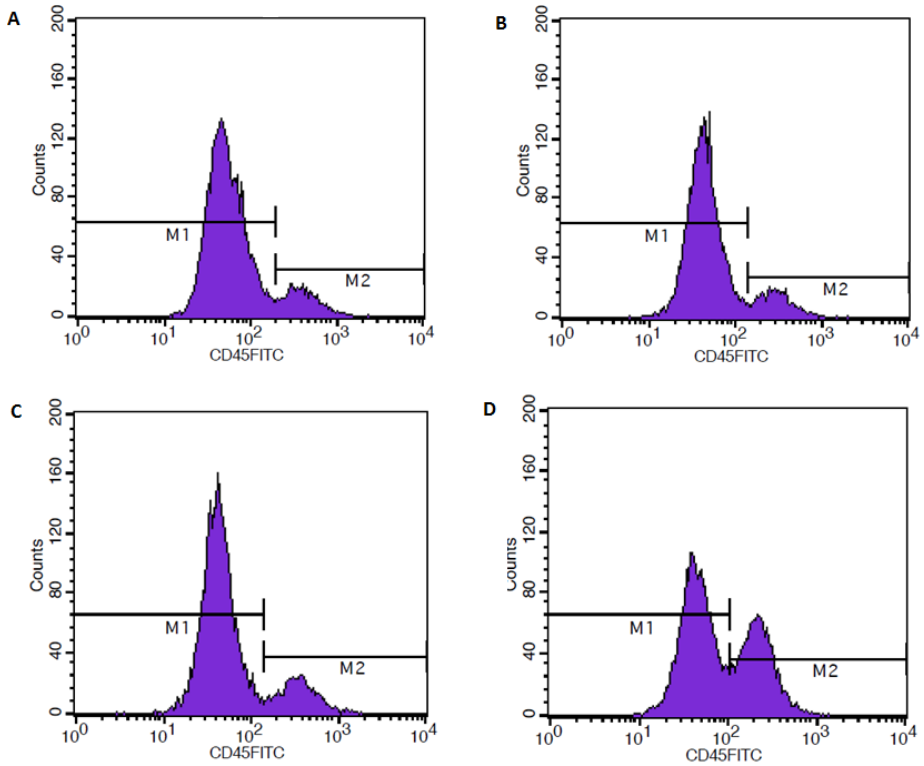


**Figure 37.** Representative Figures from flow cytometry analysis (A) Monocyte adhesion to HUVECs incubated in 2% FBS (B) Incubation in 20% FBS (C) Incubation in 20% control serum (D) Incubation in uremic serum. M1-marker for endothelial cells M2-marker for monocytes.

Two cell cultures with different *GSTM1* genotypes were tested for the difference between the relative monocyte adhesions. The HUVEC with *GSTM1-active* genotype had RMA of 21.05 ± 0.99 when incubated in control serum obtained from healthy volunteers. When these cells were exposed to 4-hour long incubation in uremic serum, the RMA increased up to 60.96 ± 13.50 (Δ 39.91, $p < 0.001$). On the other hand, *GSTM1-null* HUVECs had RMA of 38.29 ± 9.76 after exposure to control serum. After incubation with uremic serum, the relative monocyte adhesion increased up to 71.57 ± 14.20 (Δ 33.28, $p < 0.001$). Although the increase of RMA after exposure to uremic toxins was statistical significant in both cell cultures, the comparison solely of *GSTM1-active* HUVEC with *GSTM1-null* HUVEC after 4 hr treatment with 20% uremic serum was not statistically significant (Δ 10.61, $p = 0.248$) (Figure 38).

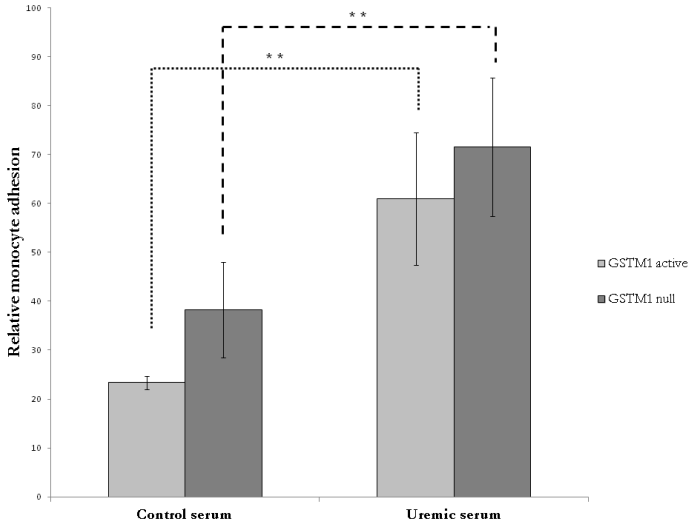


**Figure 38**. Comparison of monocyte adhesion test in two endothelial cells, one of them with *GSTM1-active* (light grey color) and the other one with *GSTM1-null* genotype (dark grey color).

### 4.7.3 Reactive oxygen species production in endothelial cells after incubation in uremic serum

In order to test whether *GSTM1* gene deletion may influence the reactive oxygen species production in uremic cells exposed to 4 hrs incubation in uremic serum, two endothelial cell cultures with different *GSTM1* genotype were cultivated to passage 4. After incubation period, fluorescence of DHE and DHR dyes was measured using flow cytometry. A total ROS production in HUVECs with *GSTM1-active* genotype was +15% after incubation in uremic serum in comparison to the endothelial cells incubated in control (healthy) serum. When endothelial cells with *GSTM1-null* genotype were incubated in uremic serum, an increase of 20% of total ROS production was observed after uremic treatment when compared to the incubation in control serum. Comparison of two endothelial cell culture total ROS production after uremic exposure did not differ significantly (Figure 39).

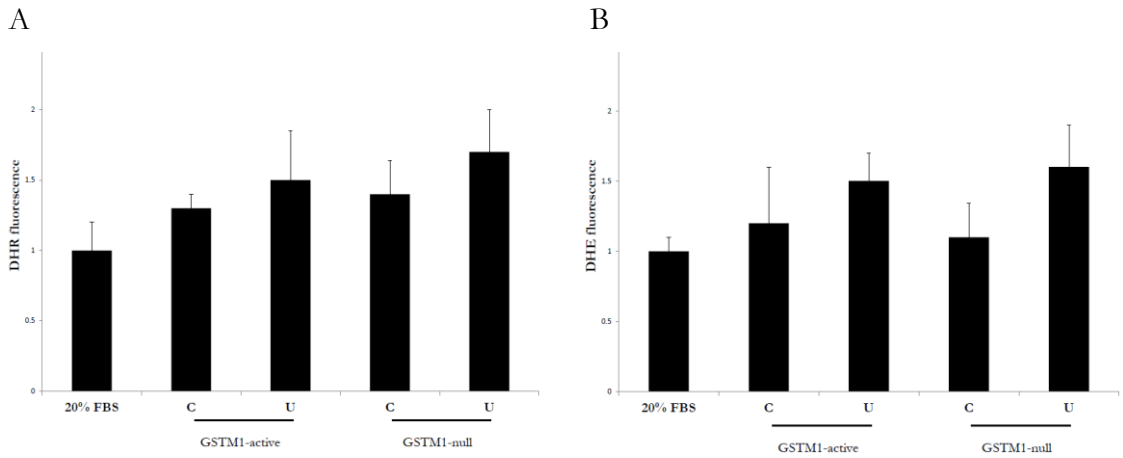


**Figure 39.** A total reactive oxygen species (A) and superoxide anion production (B) in endothelial cells in terms of *GSTM1* genotype after incubation in 20% uremic serum. The ROS production in endothelial cells incubated in 20% FBS was determined as 1.

In the next line, it was tested whether this increase of reactive oxygen species is a result of elevated superoxide anion production. The increase of superoxide anion production in *GSTM1-active* endothelial cells was 25% when compared to the control, while in *GSTM1-null* endothelial cell this increase was 45%. Although the endothelial cells with homozygous deletion of *GSTM1* gene produced more superoxide anion radical after uremic serum stimulation, statistical significance between two cell cultures in terms of *GSTM1* genotype was not observed.

# 5 DISCUSSION

In this doctoral thesis the association between *GSTA1*, *GSTM1*, *GSTP1* rs1695, *GSTP1* rs1138272 and *GSTT1* gene polymorphism and susceptibility for end-stage renal disease was analyzed. Additionally, influence of *GST* gene polymorphism, both individual and combined effect, on oxidative damage byproducts was also tested. A total of eight end-products of oxidative stress and two cellular adhesion molecules from ESRD patients plasma were analyzed in terms of *GST* genotype. Potential predictive role of *GST* gene polymorphisms, oxidative stress byproducts and soluble CAMs was also assessed. And finally, the influence of *GSTM1* gene polymorphism on monocyte adhesion to endothelial cells and their production of reactive oxygens species was tested in human umbilica ;vein endothelial cells.

In this thesis it was shown that *GSTM1-null* and *GSTP1*C/*T* genotype increased the susceptibility for end-stage renal disease development when compared to the control group of healthy individuals. This effect was even more pronounced if the *GST null/low activity* genotypes were combined. Furthermore, concentrations of protein and lipid byproducts of oxidative stress correlate with *GST null* or *low activity* genotypes. Additionally, *GSTM1-null* genotype and *GSTP1*C/*T* were associated with higher levels of soluble VCAM-1 in plasma of patients with ESRD, what is shown for the first time. Predictive role of *GSTM1-null* genotype in 5-year overall and cardiovascular death outcome was also shown to be significant.

There is mounting evidence for the presence of elevated oxidative stress in patients who undergo maintenance hemodialysis compared to healthy matched controls (Miyata et al., 1999; Tarng et al., 2000; Witko-Sarsat et al., 1996) that may contribute to poor cardiovascular and global outcome (Locatelli et al., 2003).

High levels of reactive oxygen species that occur in the course of CKD are associated with the disease pathogenesis, its progression and complications (Libetta et al., 2011; Kao et al., 2010). Based on this premise, it was reasonable to expect that variations in detoxifying and antioxidant activities of glutathione transferases modulate individual susceptibility towards ESRD development, regardless of its specific cause. Among five common GST polymorphisms analyzed in this thesis, only *GSTM1-null* and *GSTP1*C/*T* genotypes (rs1138272) have shown a significant association with increased ESRD susceptibility. The ESRD risk was even more pronounced if any of other three *null/ low*

*activity GST* genotypes (*GSTA1*, *GSTT1* or *GSTP1* rs1695) were combined. The results on association between *GSTM1-null* genotype and increased susceptibility of ESRD development are in accordance with several studies performed in Indian population (Agrawal et al., 2007; Datta et al., 2010a and 2010b). However, there are several studies which results are not in line with *GSTM1-null* genotype as a risk factor for ESRD development, such as the study among Taiwan Chinese (Yang et al., 2004), population of Taipei (Lin et al., 2009) and Asian Indians ESRD patients (Tiwari et al., 2009). Regarding *GSTT1-null* genotype, several studies suggested that *GSTT1-null* genotype increases the susceptibility for ESRD development (Datta et al., 2010a; Nomani et al., 2016; Yang et al., 2004). The role of low activity *GSTP1-*Val/*Val* polymorphism was addressed in only three studies (Agrawal et al., 2007; Nomani et al., 2016; Tiwari et al., 2009), in which it has shown conflicting results. In this thesis it was found that individuals with *GSTP1*Val/*Val* genotype are at increased risk (OR = 1.8) although statistical significance lacked. On the other hand, *GSTP1* rs1138272 polymorphism significantly altered the susceptibility to ESRD development. Thus, individuals carrying variant *GSTP1* allele were at 3-fold increased risk for ESRD when compared to subjects who were homozygous for referent allele (OR = 3.2, $p < 0.05$). So far this polymorphism has been assessed in several studies regarding malignant and non-malignant disease development, however with no results on its distribution and effects in population of chronic kidney disease patients (Ghatak et al., 2016; Qi et al., 2015). Taken together, these conflicting data may suggest the existence of ethnic-specific *GST* genetic susceptibility towards ESRD susceptibility. It is noteworthy of mentioning that *GST*s genes distribution is also ethnic specific (Garte et al., 2001; Karaca et al., 2015). To our knowledge, this is the first investigation that addressed the susceptibility to ESRD in European Caucasians in association with common *GST* polymorphisms.

In further course of the thesis it was explored whether combination of *GST* genes additionally influences the ESRD risk. The results suggest that there is an increase of ESRD susceptibility when *GST* genes are combined. These results are in accordance with previous studies of Datta et al. and Agrawal et al. who concluded that *GSTM1/GSTT1* and *GSTM1/T1/P1* genotype combinations are increasing ESRD risk in in Northern Indian patients although the magnitude of this association was higher when compared to our population (Agrawal et al., 2007; Datta et al., 2010a). Variances in susceptibility to end-stage renal disease development in different populations might not only be the

consequence of ethnicicity where significant variety of *GST*s distributions is observed, but also due to the different etiology of ESRD in various regions. Since ESRD among our patients had distinct etiology and could hardly be associated with environmental agents metabolized by GST enzymes, except for Balkan endemic nephropathy, weak association obtained for *GSTM1-null* and *GSTP1*C/*T* genotypes and its absence with other *GST* forms is not unexpected. It is reasonable to assume that *GST* polymorphic expression may be much more important in the course of chronic kidney disease progression, since accumulated end products of endogenous and exogenous origin and ROS in these patients act as GST substrates (Klemm et al., 2001). This is a situation in which gene interaction with disease specific mechanisms influences further course of disease.

Numerous studies showed significant redox imbalance in patients with end-stage renal disease. Various oxidative stress byproducts are measured in order to determine the level of oxidative damage in patients on hemodialysis, and eventually, to introduce a proper antioxidant therapy. Frequently are measured lipid hydroperoxides (Chao et al., 2002), sulfhydryl groups (Himmelfarb et al., 2002), carbonyl groups (Miyata et al., 2001), advanced oxidation protein products (Witko-Sarsat et al., 1996) mostly in plasma or serum, but there is a broad specter of new potential biomarkers of oxidative damage developing each day. Since the *GST* genes are very important in antioxidant reactions, it was reasonable to assume that lack or low activity *GST* gene variants will contribute to increased susceptibility to oxidative and carbonyl stress in ESRD patients. Additionally, taken that oxidative stress parameters correlate with cardiovascular complications and mortality, interaction between the uremic state and particular *GST* genotype would represent a potential mechanism explaining the inter-individual differences in terms of cardiovascular outcome in these patients.

This was conducted in the next phase of this thesis, in which the influence of polymorphic *GSTA1*, *GSTM1*, *GSTP1* and *GSTT1* genes was analyzed with respect to oxidative phenotype, as well as to the level of soluble adhesion molecules. A total of eight oxidative stress byproducts and two cellular adhesion molecule concentrations were measured in plasma of ESRD patients and correlated with *GST* genotypes. Among oxidative stress parameters, the measurement of total oxidant status and prooxidant-antioxidant balance was also conducted. We found that individual *GST* polymorphisms influence vulnerability to both protein and lipid oxidation, with *GSTM1-null* gene variant having the most pronounced effect. Moreover, ESRD patients with *GSTM1-null* genotype

had increased levels of both sICAM-1 and sVCAM-1 in plasma. Interestingly, *GSTP1* rs1138272 polymorphism correlated only with increased sVCAM-1 levels in plasma of these patients. Besides, our results have shown a strong combined effect of null or low activity *GSTM1*, *GSTT1*, *GSTA1* and *GSTP1* genotypes in terms of susceptibility towards oxidative and carbonyl stress in patients with ESRD. The data concerning genetic predisposition to worse oxidative phenotype in patients with ESRD are limited. Lin et al. showed that hemodialysis patients with *GSTM1-null* genotype are more vulnerable to oxidative DNA damage when compared to those who possess *GSTM1-active* genotype (Lin et al., 2009). Our results confirmed similar findings presented in patients with benign prostate hyperplasia in which combined *GSTM1-null*/*GSTT1-null* genotype was associated with increased plasma MDA level (Nomani et al., 2016). Results from this thesis are also consistent with the results of Tang et al.(Tang et al., 2010), who showed that coronary artery disease patients with *GSTM1-null*/*GSTT1-null* genotype have lower total antioxidant capacity than those with both active genotypes. Besides, several studies have suggested that this particular genotype combination was associated with enhanced DNA damage observed in cancer patients (Butkiewicz et al., 1999; Kato et al., 1995; McCarty et al., 2009; Rojas et al., 2000). In the light of the role that GST enzymes have in detoxification and as antioxidant enzymes, results of this and other studies on association between null/low activity *GSTM1*, *GSTT*1, *GSTP1* and *GSTA1* genotypes and increased oxidant damage of proteins and lipids are biologically plausible. Results regarding *GSTM1* gene deletion and CAMs association from this thesis are in accordance with the cross-sectional ARIC study performed in larger cohort of smokers where the evidence on modulating effect of *GSTM1* polymorphism in terms of inflammation was confirmed (Miller et al., 2003). According to the presence of various *GST* gene variants in combination, ESRD patients may be stratified in terms of level of oxidative and carbonyl stress. Since oxidative stress parameters correlate with cardiovascular complications and mortality (Bayes, 2005; Descamps-Latscha et al., 2005; Köse et al., 2011; Lin et al., 2009), it may be speculated that interaction between uremic state and combined *GST* genotype would represent at least one of potential mechanisms explaining inter-individual differences in terms of cardiovascular outcome in these patients.

Oxidative stress in ESRD patients is considered the cornerstone of atherosclerotic process. Carotid artery intima-media thickness in chronic hemodialysis patients correlates with lipid peroxidation byproducts (Dursun et al., 2008), while serum MDA is strong

predictor of prevalent CVD in these patients (Boaz et al., 1999). Besides, the content of AOPP independently predicts atherosclerotic cardiovascular (CV) events in non-diabetic pre-dialysis patients (Descamps-Latscha et al., 2005). It is important to note that GST family members play a dual role in defense mechanisms that counteract complex biochemical changes present in uremic state. Namely, all products of analyzed *GST* genes possess both glutathione conjugating and antioxidant enzymatic activity (Hayes and Strange, 1995; Simic et al., 2009). However, according to the results presented in this thesis, the level of protection against both oxidative and carbonyl stress in uremic syndrome is influenced by common *GST* polymorphisms which determine the quantity and/or activity of GST protein "echelons" that act upon organic hydroperoxides and accumulated harmful compounds.

In this study, we addressed the most relevant classes of oxidative protein and lipid damage byproducts encountered in ESRD, as well as, total oxidant status and pro-oxidant/antioxidant balance in relation to *GSTA1*, *GSTM1*, *GSTP1* and *GSTT1* genotypes.

The ESRD patients with homozygous deletion of *GSTT1* allele or carriers of *GSTP1-low activity (*Val/*Val)* genotype were significantly more susceptible to increased level of oxidative damage byproducts, but not as much as *GSTM1-null* patients. However, the effect modification with regard to oxidative phenotype in hemodialysis patients is most pronounced if *GSTM1-* and *GSTT1-null* as well as low activity *GSTA1* and *GSTP1* genotypes are present in combination. Similar result was obtained in few previous studies which have shown that the presence of both *GSTM1-* and *GSTT1-null* genotypes influenced significantly on the level of MDA in the plasma of chronic kidney disease patients (Datta et al., 2010b; Nomani et al., 2016). To our best knowledge this is the first study that encountered association between common *GST* polymorphisms and oxidative stress byproducts. Taken together these results suggest a possibility for *GST* genotype based stratification of ESRD patients which could improve the attempts towards individualization of antioxidant treatment. The suggested therapeutic interventions aimed at reducing oxidative stress in hemodialysis patients include biocompatible membranes, administration of antioxidants and substances indirectly affecting oxidative stress (Libetta et al., 2011). Recently, vitamin E-bonded membranes for hemodialysers have been developed, which have a wide spectrum of positive effects on antioxidant status in dialysis patients (F. Galli et al., 1999; Kalantar-Zadeh et al., 2006). The results obtained in this study suggest that the use of dialysers with vitamin E bonded membranes would be of

most benefit for patients with combined *GST null* or *low activity* genotypes. According to results of this and other investigations, the susceptibility to oxidative stress in hemodialysis patients depends at least partly on *GST* genotype.

During a five year follow-up death outcomes and cardiovascular complications were prospectively registered in order to test predictive role of *GST* genotype on ESRD patients' outcome. Data obtained in this study have shown that *GSTM1-null* genotype in ESRD patients is a significant independent predictor of overall and cardiovascular mortality. Besides, patients with *GSTP1-low activity* genotype (*GSTP1*Val/*Val*) exhibited an increased risk of cardiovascular mortality, specifically myocardial infarction where it reached statistical significance. For general cardiovascular mortality and cerebral vascular insult, although the increased hazard ratio was observed, it did not reach statistical significance in any of three models tested.

Several lines of evidence indicate an association between the *GSTM1-null* genotype and faster progression of kidney disease as well as worse outcome of dialysis patients. Very recently Chang et al. have shown that *GSTM1-null* allele influences the course of kidney disease progression in participants of the African American Study of Kidney Disease (AASK) trial (Chang et al., 2013). Patient groups with and without the common *GSTM1-null* allele, differed significantly in the time to a glomerular filtration rate event or dialysis, or death. Similarly, Lin at al. (Lin et al., 2009) showed that dialysis patients with the *GSTM1-null* genotype exhibit higher mortality rate when compared to those with the active enzyme. Our results confirmed these findings regarding the deleterious effect of *GSTM1-null* genotype on overall mortality in ESRD patients. It is interesting to mention that studies regarding the role of *GST* gene polymorphism in cancer risk and prognosis have suggested that individuals with referent, or so called *active* genotype, are at greater risk, but have poorer survival rate (Li et al., 2012; Liu et al., 2014).A possible explanation of this is that individuals with the *GSTs* variant genotypes are less able to detoxify the metabolites of drugs and carcinogens which make them prone to certain cancers development. It is reasonable to assume that these individuals (because of the lack of detoxifying enzymes) have therefore better respond to chemotherapeutic drugs, resulting in a survival advantage.

Furthermore, by the cause-specific analysis of the association between *GSTM1* and cardiovascular causes of death, such as MI and stroke, we provided a direct proof of the role of GSTM1 protein in prevention of oxidative stress related cardiovascular complications. Namely, patients lacking GSTM1 protein (*GSTM1-null* genotype)

demonstrated a significantly higher risk of cardiovascular death. When ESRD patients were further stratified according to the specific cause of death (MI or stroke), Kaplan Meier analysis demonstrated a significantly shorter time to death from both cardiovascular causes in patients with *GSTM1-null* genotype in comparison to those with the active enzyme. The independent significant association between *GSTM1-null* genotype remained in all three models tested for overall and cardiovascular survival as shown by Cox regression analysis. Risk for CVI death for ESRD patients with *GSTM1-null* genotype was increased, but statistical significance lacked out. To our knowledge this is the first study suggesting a significant association between *GSTM1-null* genotype and the fatal outcome from either overall or specific-cause cardiovascular mortality in end-stage renal disease patients. These results are biologically plausible given the role of GSTM1 in antioxidant protection and the progression of carotid atherosclerosis in pro-oxidant environment. Oxidized lipids are GST substrates while GSTM1 directly regulates intracellular levels of lipid peroxidation by product 4-hydroxynonenal (4-HNE) in vascular smooth muscle cells. A prospective study in Netherlands confirmed an increased progression of atherosclerosis among smokers lacking the enzyme GSTM1 (de Waart et al., 2001). Specifically, de Waart et al demonstrated that the male smokers with the *GSTM1 null* genotype had a higher mean 2-year progression of the common carotid artery intima-media thickness compared to those with *GSTM1-active* (de Waart et al., 2001). In contradiction to other studies is a report from Cora et al. who suggested that *GSTM1-null* genotype may protect against coronary artery disease development (Cora et al., 2013).

The question arises about the association between *GSTM1-null* genotype and cerebral vascular insult being stronger than death from myocardial infarction. Data from recent meta-analysis suggested that the individuals with homozygous deletion of *GSTM1* gene is associated with an increased risk of ischemic heart disease (OR=1.38; 95%CI=1.01-1.87). Although it has been shown that the presence of *GSTM1-null* genotype was associated with hypertriglyceridemia and low HDL-cholesterol levels in healthy humans (Danielski et al., 2003; Maciel et al., 2009), it has not been shown directly that the GST activity is actually changed during cardiac or coronary vascular disease. However, Noce et al have shown that the erythrocyte GST activity displays increased activity in uremic diabetic patients and represent potential predictor of disease outcome (Noce et al., 2014, 2012)

Taken together, our data suggest that patients with *GSTM1-null* genotype are at higher risk for all-cause and cardiovascular death in ESRD patients, while those with *GSTP1-low activity* were at increased risk for myocardial infarction. Results from this thessis implicate that *GSTM1-null* genotype could be considered as a potenitail biomarker of increased susceptibility towards ESRD development and violated redox balance in these patients. This deletional polymorphism may also be useful in targeting the high-risk patients for early prevention preventive and eventual intervention in order to reduce their cardiovascular risk. However, in order to accomplish that, prospective clinical trials with thousands subject included in different CKD stages are required. Further studies will provide reliably data of the antioxidant therapy effects in individuals with polymorphic *GST* expression. Data from this thesis suggest that the ESRD patients with *GSTM1-null* genotype could represent potential candidates for antioxidant therapy with the aim of MI or CVI prevention. Although there are no clear evidence that antioxidant therapy reduces the risk of major cardiovascular events in people with CKD (Coombes and Fassett, 2012; Jun et al., 1996) it may be speculated that some patients with particular genotypes will have benefits from such therapy.

In order to assess the potential predictive role of oxidative stress byproducts, we assessed to Cox hazard ration calculations and Kaplan-Meier survival analysis. Patients were dichotomized on those with low (below the median) and high values (above the median) of the parameter that was considered. The predictive role of each byproduct was tested in three Models, each of them being adjusted for specific parameters that have been linked to mortality in previous studies (Model 1 for age and gender; Model 2 for age, gender and smoking status; Model 3 for age, gender, smoking status, cholesterol levels and presence of diabetes). An increasing body of evidence supports a role for oxidative stress in the pathogenesis of cardiovascular diseases. As previously mentioned, oxidative stress in end-stage renal disease has multifactorial origin and it is considered to be the cornerstone of atherosclerotic process. However, its implication in prognosis of chronic renal failure is still lacking.

Due to the relatively lack of extracellular antioxidants, albumins are prone to oxidative damage and represent the main source of various protein byproducts (Capeillère-Blandin et al., 2004). In the plasma of hemodialysis patients the level of advanced oxidation protein products has been observed and described as one of the mediators of oxidative stress and monocyte respiratory burst (Witko-Sarsat et al., 1998, 1996). The AOPPs

represent dityrosine-containing, crosslinked protein products that are produced in the reaction of plasma proteins with chlorinated oxidants, originating from neutrophils that possess myeloperoxidase, the only enzyme that is able to generate a chlorinated oxidant (Witko-Sarsat et al., 1998). Its levels are increased in both acute and chronic renal failure, as well as in kidney injury (Lentini et al., 2010). Although the AOPP accumulation represents not only a result of redox imbalance in ESRD patients, but also a significant contributor to many cellular and molecular processes, (Guo et al., 2008; Wei et al., 2009; Zhou et al., 2009), the studies describing its prognostic relevance are rare. A study by Kaneda et al. has shown that accumulation of AOPP has been associated with ischemic cardiovascular disease development and that increased levels of AOPP may present independent predictors of cardiovascular disease in patients with chronic kidney disease (Kaneda et al., 2002). In this thesis AOPP levels above the median (64.29 μmol /L) were significant predictors of cardiovascular mortality in ESRD patients. Their prognostic significance remained even after additional adjustment for covariates relevant for cardiovascular disease development. Risk for myocardial infarction death was also increased in patients with high AOPP levels in Model 1 and 2, but lost on significance in Model 3. No association between AOPP levels and the risk of CV was observed in this cohort of ESRD patients. Based on our knowledge, this is the first study that describes potential prognostic relevance of AOPP. A relatively recent study by Zhou et al. suggested that accumulation of AOPP in hemodialysis patients was independently associated with ischemic heart disease development (Zhou et al., 2012), but the authors did not consider lethal outcome. It seems that AOPP levels are even increased in patients with normal renal function and may indicate early atherosclerosis even in relatively healthy population (Ashfaq et al., 2006).

During oxidative stress, polyunsaturated fatty acids (PUFA) are very prone to reactive oxygen species attack. When oxidatively modified, PUFA form lipid hydroperoxides which rapidly decompose to secondary products. Malondialdehyde is one of the products which serves as a reliable marker of lipid peroxidation and has been associated with broad specter of chronic and malignant diseases (Bakan et al., 2002; Dierckx et al., 2003; Kolanjiappan et al., 2002; Polidori et al., 2002). Its prognostic relevance, however, has not been widely studied, especially in patients on hemodialysis. So far it has been shown that MDA levels measured in red blood cells correlate with severity of hypertension and that its level is increased in acute myocardial infarction (REF). The levels of MDA in plasma are also increased in patients with coronary artery disease

(Sakuma et al., 1997). A PREVENT study that comprised a cohort of more than 600 patients revealed that serum levels of MDA were strongly predictive of cardiovascular events in patients with stable coronary artery disease, independently of traditional risk factors and inflammatory markers (Walter et al., 2004). In this thesis it was shown a possible predictive role of increased MDA concentration (above 2.3 μmol/L) in plasma of ESRD patients. Thus overall and cardiovascular death outcome risk was significantly increased in patients with extensive lipid peroxidation. Additionally, fatal cerebral vascular insult and myocardial infarction were also associated with high MDA levels, where the risk was almost tripled in comparison with the patients whose concentration of MDA was bellow median level. This result is not in accordance with Terrier-Lenglet et al who did not find any association between plasma levels of MDA and overall/cardiovascular mortality in patients with CKD (Terrier-Lenglet et al., 2011). However, in a study where oxidative modifications of lipids associated with LDL (MDA-LDL) were tested for possible cardiovascular predictive value, a significant correlation between increased MDA-LDL levels and cardiac events was found (Kotani et al., 2015). Besides binding to lipoproteins, MDA is able to generate cross-links with DNA molecule and proteins (Del Rio et al., 2005). When reacting with lysine on proteins, a stable lipid peroxidation protein adducts are formed, so called MDA adducts, which are found in atherogenic lesions (Fu et al., 1998; Heinecke, 1998; Holvoet et al., 1995; Stocker and Keaney, 2004). The MDA adducts are highly immunogenic and pro-inflammatory and represent a great threat to endothelial cells since they are a part of pathological processes involved in formation of atherosclerotic plaque (Matsuura and Lopez, 2008). We hypothesized that MDA-adducts measured in plasma of ESRD patients could have prognostic value. However, the results from this thesis have shown no association of increased MDA values with overall or cardiovascular mortality.

It is important to note that malondialdehyde in biological samples is measured as thiobarbituric acid reactive substances, a method that is very unspecific to MDA and compromises its exact determination (Knight et al., 1988). This problem is overcome with several modern techniques for MDA determination, with HPLC being accepted as the most common and relevant one. In this study, however, we have used spectrophotometrical method, which due to the lack of specificity and sensitivity, may overestimate actual MDA values and therefore this might be considered as limitation in the study.

Besides determining single oxidative stress byproducts, a prognostic value of prooxidant-oxidant status and total oxidant status was also tested. The PAB assay simultaneously measures prooxidants and antioxidants in the sample, determining the balance that exists between them. Previous studies have shown that PAB levels are increased in patients with coronary artery disease when compared to healthy controls, although the statistical significance was not observed (Rahsepar et al., 2011). Similarly, PAB values are increased in patients 12h after suffered myocardial infarction indicating its possible role in prognosis of cardiovascular disease, although data on this issue are very limited in the literature (Ghayour-Mobarhan et al., 2009). In this thesis it is reported that patients with PAB values above the median levels (148.6 HK units) were at increased risk of all-cause and cardiovascular mortality. It was not observed that increased PAB values may serve in the prognosis of acute myocardial infarction. In case of cerebral vascular insult, a possible predictive role of higher PAB levels is noted, although the statistical significance was seen only in Model 1, adjusted for age and gender only. Earlier study in stroke patients, however, was not able to confirm its 6-months predictive value (Parizadeh et al., 2011).

Soluble ICAM-1 and VCAM-1 measured in serum of patients, have been considered markers of endothelial activation (Nakai et al., 1995) and represent an early manifestation of atherosclerothic processes (Cybulsky and Gimbrone, 1991). They represent mediators of leucocyte adhesion and migration in the process of ischemic cardiovascular disease development (Blann et al., 1996). Although the prognostic value of inflammation in dialysis patients has been investigated extensively, their prognostic value has still been contradictory and various studies have yielded different conclusion. Dong et al have shown that sICAM-1, but not sVCAM-1, predicts mortality in peritoneal dialysis patients (Dong et al., 2014). Indeed, patients with cardiovascular diseases have significantly increased serum sICAM-1 levels compared to patients with no evidence of CVD (Papayianni et al., 2002) and may serve as predictors of CVD mortality in hemodialysis patients (Papagianni et al., 2003). Partially in contrast with the above mentioned results, in this thesis has been shown that the ESRD patients with the sICAM-1 levels above the median (90.4 ng / ml) were not at increased risk for neither all-cause nor cardiovascular mortality in any of the observed Models. On the other hand, the results of this thesis suggest that sVCAM-1 could be a potential predictor of overall mortality. Namely, the risk of overall death outcome was doubled in all of the three models used in this study with

high statistical significance. Although the risk of cardiovascular mortality was increased in ESRD patients with high levels of sVCAM-1, statistical significance lacked. No association between sVCAM-1 levels and myocardial or cerebral vascular insult cause of death was observed. A study performed by de Caterina et all has shown that VCAM-1 correlates with atherosclerothic processes and may serve as a potential *in vivo* biomarker of endothelial dysfunction (De Caterina et al., 1997). Thus the aortic expression of VCAM-1 correlates with cardiovascular risk factors (Mu et al., 2015). The result from this thesis is in accordance with the study performed by Papagianni et al who showed that that patients with higher sVCAM-1 levels had higher 5-year all-cause and CVD mortality (Papagianni et al., 2008). However, there are more studies indicating no association between this CAM and higher mortality in CKD patients (Dong et al., 2014; Tripepi et al., 2005; Wang et al., 2005). This apparent result's discrepancy between different studies is probably based on differences in study population (chronic renal failure versus hemodialysis patients or peritoneal dialysis) and on differences in covariates used in statistical modelling. Although the expression of both ICAM-1 and VCAM-1 is upregulated in atherosclerotic lesions, it seems that VCAM-1 has a more important role in the development of atherosclerosis (Cybulsky et al., 2001). Still, it remains unclear why sVCAM-1 values are better predictor than sICAM-1 of cardiovascular death outcome in hemodialysis patients and further studies should elucidate this problem.

This study was performed in a relatively large cohort of ESRD patients with a long-term follow-up period and was adjusted for multiple confounding factors that influence cardiovascular disease development. The consistency in hemodialysis treatment of our ESRD patients was also favored and represents an advantage in the study.

In order to test whether polymorphic expression of *GSTM1* gene in human umbilical vein endothelial cells *(HUVEC)* influences their susceptibility to oxidative stress and atherogenic potential in terms of monocyte adhesion in uremic milieu, two primary cell cultures that differ with respect to *GSTM1* were incubated in uremic serum.

One of the main steps in early steps of atherogenesis represents adhesion of monocyte to the endothelium of arterial vessels. Monocyte adhesion promotes the remodeling of endothelial cell membrane into specific features that serve as docking sites for leucocyte transmigration and are further implicated in endothelial dysfunction (Hordijk, 2006; Wittchen, 2009). The link between uremic toxins and endothelial dysfunction and cardiovascular disease development in end stage renal disease has been well established

during last several years. There is a growing body of studies trying to elucidate specific mechanism and particular factors contributing to the endothelial activation and dysfunction in uremic patients. In this thesis it was firstly shown that *GSTM1-null* genotype is associated with increased susceptibility towards ESRD and redox imbalance in these patients. In addition, this genotype was associated with increased plasma levels of soluble VCAM-1, protein which overexpression occurs within atherogenesis. In later course, it was revealed that patients with homozygous deletion of *GSTM1* gene are at increased risk of fatal overall and cardiovascular outcome. In the last stage of thesis it was reasonable to assume that we will be able to confirm this association on cellular level. Two human umbilical vein endothelial cell cultures, one with *GSTM1-active* and the other with *GSTM1-null* genotype, were incubated for 4 hours in uremic serum obtained from patients on regular hemodialysis. Both *GSTM1-active* and *GSTM1-null* genotype HUVECs have shown increased relative monocyte adhesion to endothelial cells. However, in the present experimental conditions we did not succeed to observe the *GSTM1* gene deletion effect on monocyte adhesion to endothelial cells ($p = 0.248$). It is interesting dough that HUVEC with *GSTM1-null* genotype when exposed to healthy serum incubation had higher monocyte adhesion when compared to endothelial cells with *GSTM1-active* genotype. In this study it was shown that *GSTM1-null* endothelial cells did not have increased reactive oxygen species production.

To our knowledge this is the first study where association between *GSTM1* gene deletion and endothelial dysfunction has been tested. In the view of the fact that oxidative stress byproducts correlate with the cardiovascular complications and mortality, it may be speculated that interaction between uremic state (with all its accompanying phenomena) and particular *GST* genotype would represent at least one of potential mechanisms that would explain inter-individual differences in terms of cardiovascular outcome in these patients.

# 6 CONCLUSIONS

Based on the results and discussion presented in this thesis it may be concluded:

- The distribution of glutathione transferases *A1*, *M1*, *P*1 and *T1* gene variants differs between patients with end-stage renal disease and control group.
    - Individuals with *GSTM1-null* confer 1.6 times higher risk of ESRD development in comparison to *GSTM1-active* subjects
    - Carriers of *GSTP1*C/*T* genotype have higher risk of ESRD development.
- *GSTA1, GSTM1, GSTP1* and *GSTT1* polymorphisms are associated with increased levels of oxidative stress byproducts and the level of cellular adhesion molecules levels.
    - Regarding individual polymorphisms, most pronounced association between genotype and byproducts of oxidative stress in ESRD was found for *GSTM1 null*. The presence of *GSTT1-null* or *GSTP1-low-activity* (*Val/Val* and *Ile/Val*) genotypes in ESRD patients also significantly influences the level of oxidative damage byproducts, but not as much as *GSTM1-null*.
    - Strong combined effect of *null/low activity GSTA1*, *GSTM1*, *GSTP1* and *GSTT1* genotypes in terms of susceptibility towards oxidative and carbonyl stress was found in ESRD paients.
    - *GSTM1-null* genotype in ESRD patients was associated with increased level of soluble ICAM-1 and VCAM-1, while the *GSTP1*C/*T* genotype influenced the concentration of soluble VCAM-1 in plasma of these patients
- *GST* polymorphisms, oxidative stress byproducts and cellular adhesion molecules levels influence 5-year overall and cardiovascular survival
    - *GSTM1-null* genotype has been confirmed as the risk factor for overall (HR = 1.8) and cardiovascular death (HR = 2.4), as well as myocardial infarction death (HR = 2.3) in ESRD patients

- Malondialdehyde concentration above median level in plasma of ESRD patients were prognostic in terms of overall (HR = 1.6) and cardiovascular mortality (HR = 2.4), as well as for fatal cerebral vascular insult outcome (HR = 3.0)
- Advanced oxidation protein products are also significant predictors of cardiovascular (HR = 2.4) and myocardial infarction mortality (HR = 3.3)
- Prooxidant-antioxidant balance above median level in plasma of ESRD patients were prognostic in terms of overall (HR = 2.4) and cardiovascular mortality (HR = 2.5), as well as for fatal cerebral vascular insult outcome (HR = 2.6)
- Soluble vascular cell adhesion molecule 1 in plasma of patients with ESRD are associated with overall mortality (HR = 2.0).
- These genetic markers may permit the targeting of preventive and early intervention on high-risk patients to reduce their cardiovascular risk.

➢ In vitro incubation of human umbilical vein endothelial cells in uremic serum resulted in increased monocyte adhesion to these cells and elevated production of free radicals. This response was not influenced by *GSTM1* genotype

➢ Taken together, the results presented in this study suggest a possibility for *GST* genotype-based stratification of ESRD patients which could improve the attempts towards individualization of antioxidant treatment. Besides, determination of oxidative stress byproducts may permit the targeting of preventive and early intervention in high-risk patients to reduce their cardiovascular risk.

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# List of abbreviations

| | |
|---|---|
| AGE | advanced glycation end-products |
| AMI | acute myocardial infarction |
| AOPP | advanced oxidation protein products |
| BSA | bovine serum albumine |
| CKD | chronic kidney disease |
| CVD | cardiovascular disease |
| CVI | serebral vascular insult |
| DNA | Deoxyribonucleic acid |
| DNP | dinitrophenyl |
| DTNB | 5,5'-dithiobis-(2-nitrobenzoic acid |
| EDTA | ethylene diamine tetra acetic acid |
| ELISA | enzyme-linked immunosorbent assay |
| ERA-EDTA | European Renal Association - European Dialysis and Transplant Association |
| ESRD | end-stage renal disease |
| GFR | glomerular filtration rate |
| GPX | glutathione peroxidase |
| GSH | glutathione |
| GST | glutathione transferase |
| HD | hemodialysis |
| HNE | 4-hydroxynonenal |

| | |
|---|---|
| HRP | horse radish peroxidase |
| IAA | indole-3-acetic acid |
| ICAM-1 | Intercellular Adhesion Molecule 1 |
| LDL | low density lipoprotein |
| MAPEG | membrane-associated proteins in eicosanoid and glutathione |
| MCP-1 | monocyte chemoattractant protein 1 |
| MDA | malondialdehyde |
| MDA-add | malondialdehyde adducts |
| mRNA | messenger rinonucleic acid |
| oxLDL | oxidized low density lipoprotein |
| PECAM-1 | platelet–endothelial cell adhesion molecule-1 |
| ROS | reactive oxygen species |
| RRT | renal replacement therapy |
| SH- | thiol |
| SOD | superoxide dismutase |
| TMB | 3,3´5,5´-tetramethylbenzidine |
| TNF-α | tumor necrosis factor α |
| VCAM-1 | Vascular cell adhesion molecule 1 |
| VSMC | Vascular smooth muscle cells |

# BIOGRAPHY

Dr Sonja Suvakov was born on September 22nd 1983. in Kladovo. She has finished Elementary and High School in Smederevo. She has graduated on the Faculty of Medicine, University of Belgrade with average mark of 9.11/10.0. During her studies, she was mentoring gifted High School and Graduate Students in Petnica Science Center (Valjevo, Serbia), was awarded with DAAD scholarship and had two internships in Austria via Lions Club. After graduation, dr Suvakov has enrolled PhD study program "Molecular Medicine" on the Faculty of Medicine, University of Belgrade in 2009 which she has also finished with the average mark of 9.80/10.0. From February to December 2010 she was employed as a research assistant on the project no 145009DJ "The Role of Glutathione S-transferases in urinary tract carcinoma" financed by the Ministry of Science and Technological Development, Republic of Serbia. From January 2011 she was a part of the project "The role of glutathione S-transferase in susceptibility to disease" no 175052 financed by the Ministry of Education, Science and Technological Development. In April 2011 dr Suvakov become teaching assistant associate. In January 2014 she became teaching associate at the Faculty of Medicine, on the subject Biochemistry.

During the May in 2010 dr Suvakov was at the Cangenin PhD training School "High-Throughput Screens in Genome Integrity and Cancer" held in Oxford, Great Britain. In 2011 dr Suvakov won the Young author best poster Presentation award on ERA-EDTA Congress, Prague in Chech Republic. In September 2015 dr Suvakov was awarded with Grant for the FEBS3+Conference "Molecules of life" in Portorož, Slovenia. During the June of 2016 she has spent a month on INRA institute in Clermont-Ferrand, France.

Dr Sonja Suvakov is the coauthor of 12 articles indexed in Current Contents-u (CC) or Science Citation Index (SCI). In two articles she is the first author.

She is married and mother of two kids.

**Prilog 1.**

# Izjava o autorstvu

Potpisana Sonja Šuvakov

broj upisa MM09/09

**Izjavljujem**

da je doktorska disertacija pod naslovom

**Povezanost polimorfizama glutation tansferaza A1, M1, P1 I T1 sa pokazateljima oksidativnog stresa i kardiovaskularnim komplikacijama kod bolesnika sa terminalnom bubrežnom slabošću**

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**Potpis doktoranda**

U Beogradu, 04.11.2016.

Sonja Šuvakov

**Prilog 2.**

# Izjava o istovetnosti štampane i elektronske verzije doktorskog rada

Ime i prezime autora: Sonja Šuvakov

Broj upisa MM09/09

Studijski program Molekularna medicina

Naslov rada **Povezanost polimorfizama glutation tansferaza A1, M1, P1 I T1 sa pokazateljima oksidativnog stresa i kardiovaskularnim komplikacijama kod bolesnika sa terminalnom bubrežnom slabošću**

Mentor prof. dr Tatjana Simić

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Potpisani Sonja Šuvakov

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**Potpis doktoranda**

U Beogradu, 04.11.2016.

Sonja Šuvakov

**Prilog 3.**

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