**UNIVERSITY OF BELGRADE**
**FACULTY OF MEDICINE**

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# ASSOCIATION OF *NRF2, SOD2* AND *GPX1* GENE POLYMORPHISMS WITH MARKERS OF OXIDATIVE STRESS AND PROGNOSIS IN PATIENTS WITH END STAGE RENAL DISEASE

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# POVEZANOST POLIMORFIZAMA *NRF2*, *SOD2* I *GPX1* GENA SA POKAZATELJIMA OKSIDATIVNOG DISTRESA I PROGNOZOM KOD BOLESNIKA SA TERMINALNOM BUBREŽNOM SLABOŠĆU

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## ABSTRACT

## ASSOCIATION OF *NRF2, SOD2* AND *GPX1* GENE POLYMORPHISMS WITH MARKERS OF OXIDATIVE STRESS AND PROGNOSIS IN PATIENTS WITH END STAGE RENAL DISEASE

**Đurđa D. Jerotić**

**Background**: Impaired redox homeostasis is a hallmark of end-stage renal disease (ESRD). Both excessive production of reactive oxygen species (ROS) and impaired antioxidant function play role in systemic oxidative stress in these patients. Polymorphisms of antioxidant genes may influence individual susceptibility towards ESRD, oxidative stress, cardiovascular complications, as well as prognosis in ESRD patients.

**Aims:** The aim of this study was to assess the association of antioxidant nuclear factor E2-related factor 2 (*Nrf2)* rs6721961, superoxide dismutase 2 (*SOD2)* rs4880 and glutathione peroxidase 1 *(GPX1)* rs1050450 gene polymorphisms with the risk of ESRD development and their functional significance in terms of the level of oxidative stress byproducts and soluble cellular adhesion molecules in ESRD patients. Furthermore, the predictive power of two biomarker panels in terms of the 8-year overall and cardiovascular survival in ESRD patients was evaluated. The first biomarker panel was comprised of a specific combination of *Nrf2, SOD2*, *GPX1* and glutathione S-transferase M1 (*GSTM1)* genotypes. The second biomarker panel was consisted of a combination of byproducts of oxidative stress, circulating adhesion molecules and *GSTM1* deletion polymorphism. A functional role of GSTM1 deletion on endothelial dysfunction in uremic milieu was explored *in vitro*, using human umbilical vein endothelial cells (HUVECs).

**Methods**: Polymorphisms of *Nrf2* rs6721961, *SOD2* rs4880, *GPX1* rs1050450 and *GSTM1* genes were determined by PCR in 256 ESRD patients and 374 controls. Byproducts of oxidative stress (thiol and carbonyl groups, advanced oxidative protein products (AOPP), nitrotyrosine, malondialdehyde (MDA), malondialdehyde adducts (MDAadd), total oxidant status (TOS) and prooxidant-antioxidant balance (PAB)), were analyzed in plasma of ESRD patients spectrophotometricaly or by ELISA. Concentration of soluble cell adhesion molecules (soluble vascular cell adhesion molecule-1 (sVCAM-1) and soluble intercellular adhesion molecule-1 (sICAM-1)) was determined by ELISA. *In vitro* part of this study was conducted on $GSTM1^{+/+}$ HUVECs and HUVECs silenced for the GSTM1 ($GSTM1^{+/-}$) which were treated with 30% control or uremic serum for 6 h. Oxidative stress parameters in HUVECs were analyzed as follows: total ROS by flow cytometer, MDA by ELISA and SOD and GPX activity spectrophotometrically. Expression of 105 cytokines in HUVECs was determined by Proteome Array. Expression of ICAM-1 and VCAM-1 proteins in HUVECs was assessed by Western blot.

**Results**: *SOD2 Val/Val* genotype increased the risk of ESRD development (OR=2.01, p=0.002), which was even higher in carriers of combined *SOD2 Val/Val* / *GPX1 Leu/Leu* genotypes (OR=3.27, p=0.019). *SOD2* polymorphism also showed an effect on oxidative phenotype. Overall survival in ESRD patients was dependent on the combination of the *Nrf2 C/C* and *GPX1 Leu/Leu* genotypes in addition to patients' age and *GSTM1* polymorphism. Similarily, *GPX1 Leu/Leu* genotype contributed to longer cardiovascular survival. Oxidative stress byproducts (AOPP, PAB, MDA) and cell adhesion molecules (sVCAM-1 and sICAM-1) demonstrated a significant predictive role in terms of overall and cardiovascular survival as well. When 6 biomarkers (*GSTM1* genotype, high AOPP/PAB/MDA/sVCAM-1/sICAM-1) were combined into a scoring model, a significantly shorter overall and cardiovascular survival was observed for patients with the highest score ($p < 0.001$). HUVECs treated with uremic serum exhibited impaired redox balance characterized by decreased antioxidant enzyme activities and enhanced lipid peroxidation, independently of the GSTM1 knockdown. In response to uremic injury, HUVECs exhibited alteration in the expression of a series of inflammatory cytokines including retinol-binding protein 4 (RB4), regulated on activation, normal T cell expressed and secreted (RANTES), C-reactive protein (CRP), angiogenin, dickkopf-1 (Dkk-1) and platelet factor 4 (PF4). GSTM1 knockdown in HUVECs led to upregulation of monocyte chemoattractant protein-1 (MCP-1) and ICAM-1.

**Conclusion**: New developments in the field of antioxidant polymorphisms in ESRD patients could lead to better stratification of ESRD patients based on a prognostic panel of antioxidant genes and biomarkers of oxidative stress, and provide a more personalised medicine approach for the need of targeted antioxidant therapy in these patients. The association of GSTM1 downregulation with the altered expression of adhesion molecules might be at least partly responsible for the increased susceptibility of ESRD patients with *GSTM1-null* genotype to cardiovascular diseases.

**Key words**: end stage renal disease, Nrf2, SOD2, GPX1, GSTM1, gene polymorphism, oxidative stress, HUVECs, cytokines

**Field**: Medicine

**Scientific Discipline**: Tumor biology and oxidative diseases

**UDC:**

## SAŽETAK

## POVEZANOST POLIMORFIZAMA *NRF2, SOD2* I *GPX1* GENA SA POKAZATELJIMA OKSIDATIVNOG DISTRESA I PROGNOZOM KOD BOLESNIKA SA TERMINALNOM BUBREŽNOM SLABOŠĆU

**Đurđa D. Jerotić**

**Uvod:** Poremećaj redoks homeostaze predstavlja obeležje terminalne bubrežne slabosti (TBS). Prekomerno stvaranje slobodnih radikala i smanjena antioksidativna zaštita imaju značajnu ulogu u pojavi sistemskog oksidativnog stresa kod bolesnika sa TBS. Polimorfizmi antioksidativnih gena mogu uticati na individualnu podložnost za TBS, oksidativni stres, kardiovaskularne komplikacije, kao i prognozu bolesnika sa TBS.

**Ciljevi:** Cilj ove studije bio je da se ispita povezanost polimorfizama gena koji kodiraju regulatorne i katalitičke antioksidativne proteine, *Nrf2* rs6721961, superoksid dismutazu 2 (*SOD2*) rs4880 i glutation peroksidazu 1 (*GPX1*) rs1050450 sa podložnošću za nastanak TBS, kao i njihov funcionalni značaj u pogledu nivoa pokazatelja oksidativnog oštećenja lipida i proteina i nivoa adhezionih molekula u plazmi bolesnika sa TBS. Pored toga, cilj ove studije bio je da se ispita prognostički značaj dva panela biomarkera u odnosu na opšte i kardiovaskularno preživljavanje bolesnika sa TBS nakon 8 godina praćenja. Prvi panel biomarkera sastojao se od specifične kombinacije *Nrf2, SOD2, GPX1* i glutation S-transferaze M1 (*GSTM1)* genotipova. Drugi panel biomarkera se sastojao od kombinacije pokazatelja oksidativnog stresa, adhezionih molekula i polimorfizma *GSTM1* gena. Funkcionalna uloga *GSTM1* delecionog polimorfizma u nastanku endotelne disfunkcije u uremijskim uslovima ispitana je *in vitro*, korišćenjem kulture endotelnih ćelija (*engl. human umilical vein endothelial cells - HUVECs*).

**Materijal i metode:** Polimorfizmi *Nrf2* rs6721961, *SOD2* rs4880, *GPX1* rs1050450 i *GSTM1* gena su određeni PCR metodom kod 256 TBS bolesnika i 374 pripadnika kontrolne grupe. Pokazatelji oksidativnog stresa (tiol i karbonilne grupe, napredni produkti oksidacije proteina (AOPP), nitrotirozin, malondialdehid (MDA), malondialdehid adukti (MDAadd), ukupni oksidativni status (TOS) i prooksidativni-antioksidativni balans (PAB)) analizirani su u plazmi TBS bolesnika spektrofotometrijski ili ELISA metodom. Koncentracije humanog solubilnog vaskularnog adhezivnog molekula-1 (sVCAM-1) i humanog solubilnog intracelularnog adhezivnog molekula-1 (sICAM-1) analizirani su ELISA metodom. *In vitro* deo ove studije sproveden je na $GSTM1^{+/+}$ HUVEC ćelijama i HUVEC ćelijama utišanim za GSTM1 gen ($GSTM1^{+/-}$) koje su tretirane 30% kontrolnim ili uremijskim serumom tokom 6 sati. Pokazatelji oksidativnog stresa u HUVEC ćelijama su analizirani na sledeći način: protočnim citometrom – ukupne reaktivne vrste kiseonika, ELISA metodom – MDA, spektrofotmetrijski – aktivnost SOD i GPX enzima. Metodom

proteoereja je određena ekspresija 105 citokina u HUVEC ćelijama. Ekspresija ICAM-1 i VCAM-1 proteina analizirana je metodom imunoblota.

**Rezultati:** Osobe nosioci *SOD2 Val*/*Val* genotipa su imale veću podložnost za razvoj TBS (OR=2,01, p=0,002), koja je bila još više izražena kod nosioca kombinovanog *SOD2 Val*/*Val* / *GPX1 Leu*/*Leu* genotipa (OR=3,27, p=0,019). Polimorfizam *SOD2* gena je takođe imao uticaja na oksidativni fenotip. Ukupno preživljavanje bolesnika sa TBS zavisilo je od kombinacije *Nrf2 C*/*C* i *GPX1 Leu*/*Leu* genotipova, pored starosti pacijenata i *GSTM1* polimorfizma. Takođe, *GPX1 Leu*/*Leu* genotip je doprineo dužem kardiovaskularnom preživljavanju. Pokazatelji oksidativnog stresa (AOPP, PAB, MDA) i ćelijski adhezioni molekuli (sVCAM-1 i sICAM-1) imali su prognostički značaj u pogledu ukupnog i kardiovaskularnog preživljavanja. Značajno smanjeno opšte i kardiovaskularno preživaljvanje je uočeno kod bolesnika sa visokim skorovima panela koji se sastojao od kombinacije šest biomarkera (*GSTM1* genotip, visok AOPP/PAB/MDA/sVCAM-1/sICAM-1). HUVEC ćelije tretirane uremijskim serumom imale su smanjenu aktivnost antioksidativnih enzima praćenu povećanom lipidnom peroksidacijom, nezavisno od utišavanja GSTM1 gena. Tretman HUVEC ćelija doveo je do promena u ekspresiji niza inflamatornih citokina, uključujući retinol-vezujući protein 4 (RB4), RANTES, C-reaktivnog protein (CRP), angiogenin, Dkk-1 i trombocitni faktor 4 (PF4). Utišavanje GSTM1 gena u HUVEC ćelijama je dovelo do ushodne regulacije monocitnog hemoatraktantnog proteina-1 (MCP-1) i ICAM-1.

**Zaključak:** Razvoj istraživanja u oblasti polimorfizama antioksidativnih gena kod bolesnika sa terminalnom bubrežnom slabošću može doprineti boljoj stratifikaciji ovih bolesnika na osnovu panela antioksidativnih gena i biomarkera oksidativnog stresa. Navedeni paneli bi mogli da budu dalje korišćeni u kontekstu prognoze preživljavanja bolesnika sa terminalnom bubrežnom slabošću, što bi takođe doprinelo razvijanju personalizovanog pristupa u lečenju ovih bolesnika, uključujući primenu ciljane antioksidativne terapije. Povezanost nishodne regulacije GSTM1 sa promenama u ekspresiji adhezionih molekula u endotelnim ćelijama bi mogla biti odgovorna za veću podložnost ka kardiovaskularnim komplikacijama bolesnika sa terminalnom bubrežnom slabošću koji su nosioci *GSTM1-nultog* genotipa.

**Ključne reči:** terminalna bubrežna slabost, Nrf2, SOD2, GPX1, GSTM1, polimorfizam gena, oksidativni stres, HUVEC, citokini

**Naučna oblast**: Medicina

**Uža naučna oblast:** Biologija tumora i oksidativna oboljenja

**UDK:**

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# 1. INTRODUCTION

## 1.1 Chronic kidney disease and end-stage renal disease

Chronic kidney disease (CKD) is a syndrome characterised as persistent alterations in kidney structure, function or both with implications for the health of the individual [1]. Approaches in defining and classifying CKD have evolved over the past years [2]. The Kidney Disease Improving Global Outcomes (KDIGO) initiative defines CKD as "structural or functional kidney abnormalities which persist for ≥ 3 months or decreased glomerular filtration rate (GFR) of less than 60 mL/min/1.73 m², irrespective of the underlying cause" [3]. CKD can be further graded and divided according to severity, treatment and prognosis. With respect to GFR, KDIGO classifies CKD into the five stages (G1-G5, Table 1) [4]. CKD may progress to the end stage renal disease (ESRD), when GFR falls below 15 mL/min/1.73 m² (category G5) [3]. At this point kidney function is severely compromised and long-term strategies for replacing this loss of function are necessary. These renal replacement therapies (RRT) include peritoneal dialysis (PD), haemodialysis (HD) or kidney transplantation.

**Table 1.** The KDIGO classification of CKD

| Prognosis of CKD by GFR and Albuminuria Categories: KDIGO 2012 | | | | Persistent albuminuria categories Description and range | | |
|---|---|---|---|---|---|---|
| | | | | A1 | A2 | A3 |
| | | | | Normal to mildly increased | Moderately increased | Severely increased |
| | | | | <30 mg/g <3 mg/mmol | 30–300 mg/g 3–30 mg/mmol | >300 mg/g >30 mg/mmol |
| GFR categories (ml/min/ 1.73 m²) Description and range | G1 | Normal or high | ≥90 | | | |
| | G2 | Mildly decreased | 60–89 | | | |
| | G3a | Mildly to moderately decreased | 45–59 | | | |
| | G3b | Moderately to severely decreased | 30–44 | | | |
| | G4 | Severely decreased | 15–29 | | | |
| | G5 | Kidney failure | <15 | | | |

This Kidney Disease Improving Global Outcomes (KDIGO) 2D matrix incorporates the level of albuminuria (given as a ratio to creatinine (in mg per g) and divided into three categories) and the glomerular filtration rate (GFR) to describe the risk of patients with chronic kidney disease (CKD) progressing to adverse outcomes (such as progression to end-stage renal disease (ESRD), cardiovascular disease, hospitalization, acute kidney injury or death). Adopted from *the Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group*

CKD is a common disorder, and has been recognized as a global public health problem, with rising incidence and prevalence, a poor quality of life, adverse outcomes, and significant economic burden. The prevalence of CKD ranges between 7–12% in different regions of the world [1]. The Global Burden of Disease (GBD) study indicates an increasing burden of CKD during the past three decades. According to this report, in 2016 there were more than 21 million incident cases of CKD per year, 276 million prevalent cases and nearly 1.2 million deaths due to CKD [5]. Along with the increasing prevalence of CKD leading to ESRD, the world is facing a rising need for RRT. According to the national dialysis register there are almost 6000 people in Serbia treated with some type of RRT [6]. The incidence and prevalence of RRT patients in Serbia was also growing in the last decade, rising from 108 to 179 and 435 to 699 patients per million population (pmp) respectively [6].

CKD has a significant mortality rate. Indicators of outcome show that, in these patients, death is five to ten times more probable than the progression into ESRD. This increased risk of death rises exponentially with eGFR decline and increasing albuminuria, and is highest in patients on RRT; almost one half of patients receiving either haemodialysis or peritoneal dialysis will die during the course of 5-years [1,7]. Patients receiving a kidney transplant have a better prognosis. These patients have a 5-year survival rate of 86% after receiving a cadaveric kidney and 93% after receiving a kidney from a living donor [1,7]. The most common causes of death in these patients are cardiovascular diseases. Interestingly, the development of cancer is also frequent in these patients, with the consequent rise of mortality due to this cause [2]. A report from the World Health Organization concluded that during 2012. more than 800 000 deaths were due to CKD [2]. In the span of approximately 25 years, the number of deaths caused by CKD has nearly doubled, from around 600 000 in 1990, to more than 1 million deaths in 2016 [5]. Subsequently, CKD moved from the 18$^{th}$ place in the leading causes of deaths to the 11$^{th}$ cause of death in 2016. Even though, to our knowledge, there are no more recent studies examining global death rates, and considering the previously mentioned rise in the incidence of CKD, it is plausible to assume that even more people die because of this illness today. Approximately 1650 people die of this disease in Serbia every year primarily due to associated cardiovascular diseases (52%) and infections (24%) [6]. Causes of death among dialyzed patients in Serbia are presented in Figure 1.

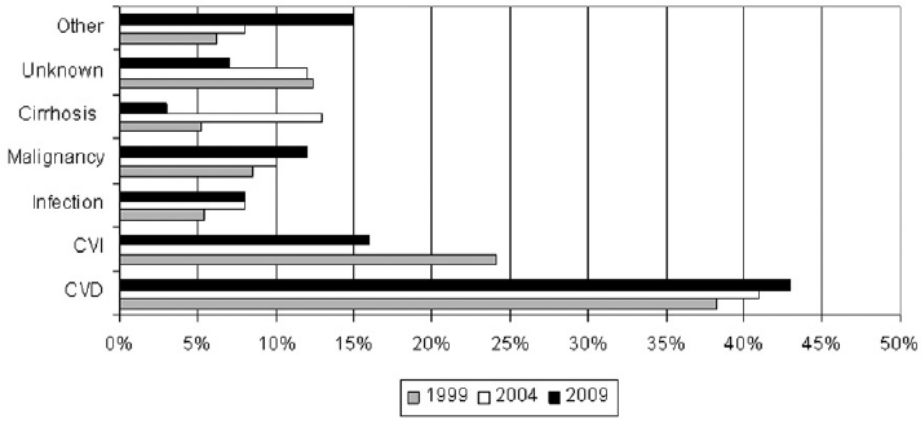



**Figure 1.** Causes of death of hemodialysis patients in Serbia in 1999, 2004, and 2009. CVD, cardiovascular disease; CVI, cerebrovascular insult. Adopted from *Đukanović et al., 2012*

### 1.1.2 Risk factors for the CKD onset and progression

Etiologic factors that lead to the development of CKD vary across different world regions. In high and middle-income countries main etiological factors include conditions such as diabetes mellitus (DM), hypertension and glomerulonephritis [2]. Notably, diabetes accounts for almost a half of CKD cases, affecting around 285 million adults worldwide [2]. However, in low-income countries, CKD is rather associated with infectious diseases, glomerulonephritis, and inappropriate use of medications (such as traditional remedies with potential nephrotoxins, NSAIDs and nephrotoxic antibiotics) [2]. Moreover, additional factors which may contribute to the CKD pathogenesis include low birth weight, congenital abnormalities, obstructive uropathy, pregnancy, obesity, and ageing [1]. The most common causes of ESRD in Serbia are presented in Figure 2.

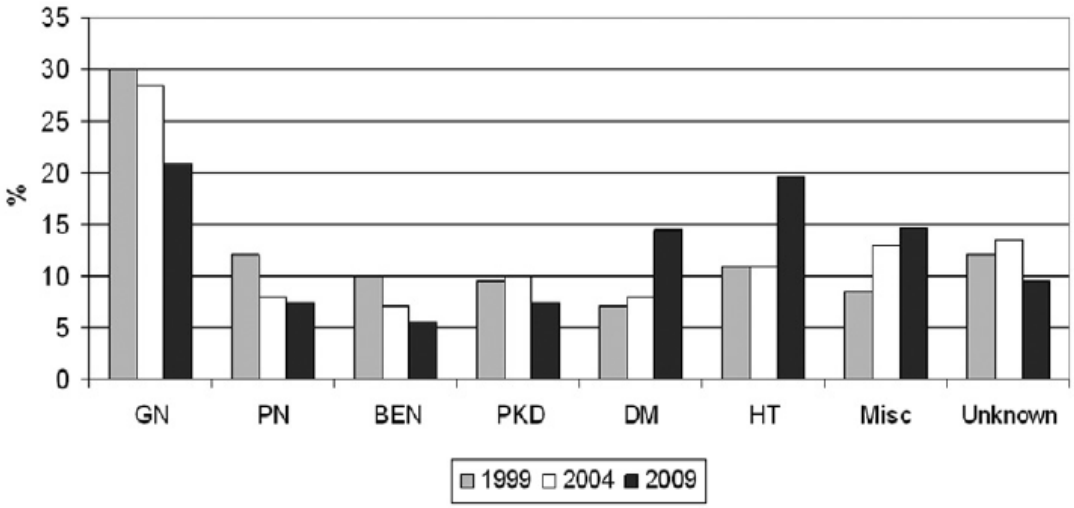



**Figure 2**. Frequency of causes of end-stage renal disease in patients on renal replacement therapy in Serbia at the end of 1999, 2004, and 2009. GN, glomerulonephritis; PN, pyelonephritis; BEN, Balkan endemic nephropathy; PKD, polycystic kidney disease; DM, diabetes mellitus; HT, hypertension; Misc, miscellaneous. Adopted from *Đukanović et al., 2012*

### 1.1.3 Signs, symptoms and complications

CKD might be asymptomatic, and diagnosed through a routine medical check-up. In contrast, the clinician may arrive at a diagnosis after a patient presents with multiple complications which indicate advanced CKD. As nephron number declines, patients experience complications including metabolic acidosis, anaemia, mineral bone disorder, arterial hypertension, hyperuricaemia and expansion of effective circulating fluid volume [1]. Dyslipidaemia, endocrine abnormalities and growth impairment in children can also occur [1]. Of these complications, cardiovascular diseases (CVDs) are the leading cause of death in patients with CKD worldwide and is associated with dyslipidaemia, hyperuricaemia and hypertension [1]. Moreover, CKD patients are at an increased risk of cancer development [8,9]. Nonspecific symptoms, commonly present in CKD patients, also include fatigue, anorexia, nausea, vomiting, weight loss, pruritus, oedema, muscle cramping and shortness of breath [1].

### 1.1.4 Diagnosis

CKD can be detected during a routine medical check-up, during examination of individuals at risk of CKD, as a consequence of the incidental finding of abnormal laboratory values in connection with another acute or chronic illness or during an investigation of symptoms and/or signs relating to the kidneys or urinary tract (such as haematuria) [1]. Importantly, the two biochemical parameters — GFR and albuminuria are used in the KDIGO matrix (Table 1). Biomarkers often used for the estimation of GFR are creatinine and cystatin C [1]. Creatinine can vary because of individual differences in the body muscle mass. Important factors for the assessment of creatinine variability include determinants such as age, sex, ethnicity and body surface area. In contrast, cystatin C is relatively independent of muscle mass and diet variations. By combining these two factors in the GFR estimation (creatinine and cystatin C), accuracy of GFR is substantially increased. Abnormal urinary excretion of albumin or total protein is also essential to detect CKD when GFR is normal and contributes to the assessment of prognosis [2].

Detection and determination of the cause of CKD also rely on renal imaging (ultrasonography, CT and MRI), examination of the urinary sediment and specialized biochemical and serological tests suitable to detect specific disorders that cause CKD [1]. Genetic testing is also emerging as an important tool for determining the cause of CKD, particularly in children and young adults [1].

**Table 2**. Criteria of CKD, according to international guidelines

| Either one, or both, of the following two criteria for at least 3 months: |
|---|
| 1 GFR <60 mL/min per 1·73m² (categories G3a–5,) |
| 2 Markers of kidney damage (1 or more) |
| • Albuminuria (albumin : creatinine ratio [ACR] ≥30mg/g) |
| • Urinary sediment abnormality |
| • Electrolyte or other abnormality due to tubular disorder |
| • Abnormalities on histology |
| • Structural abnormalities detected by imaging |
| • History of kidney transplantation |

### 1.1.5 Kidney replacement therapy

Most people reaching ESRD are treated with either haemodialysis or peritoneal dialysis, with a global prevalence of 280 per million people, compared with 65 per million people who have a functioning kidney transplant [2]. Globally, haemodialysis is the most commonly used. KDIGO recommends the initiation of dialysis when symptoms or signs of kidney failure are evident (typically when GFR is 15 ml/min/1.73 $m^2$). In many European countries, >50% of patients on HD or PD receive transplants. Comparing outcomes in people treated with dialysis to kidney transplant recipients, a systematic review of 110 cohort studies found reduced mortality, cardiovascular events, and better reported quality of life among kidney recipients [2].

Unfortunately, there is no single treatment to improve kidney function in CKD nowadays. Current therapeutic approaches are restricted mostly to the normalization of blood pressure and hyperglycaemia, since the hypertension and DM are the main contributors to CKD. However, as will be shown in the following text, oxidative stress, along with inflammation, is currently believed to be an underlying driver of pathological changes of CKD progression and its cardiovascular and other complications. Noteworthy, it is likely that individual susceptibility to oxidative stress might be determined by functional variations of the genes involved in antioxidant defence. In the following section of this thesis, based on findings from the literature, the interplay between oxidative stress, antioxidant defence, as well as gene polymorphisms of key antioxidant enzymes in CKD will be explored.

## 1.2 Oxidative stress in ESRD

The original concept of oxidative stress was defined in 1985 as "a disturbance in the prooxidant-antioxidant balance in favour of the former"[10]. Over the past several decades discoveries in the understanding of redox regulation and redox signalling led to a new concept of oxidative stress which may be defined as "an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signalling and control and/or molecular damage" [11]. Terms of oxidative eustress and oxidative distress arose from this definition and implicate that oxidative stress can be classified according to the intensity [11]. Intensity may range from physiological oxidative stress (eustress) to toxic oxidative burden (distress) which damages biomolecules. Therefore, low exposure to reactive oxygen (ROS) and nitrogen species (RNS) is utilized for redox signalling activating stress responses that may be beneficial, whereas high exposure results in disruption of redox signalling, oxidative damage and tissue dysfunction [11].

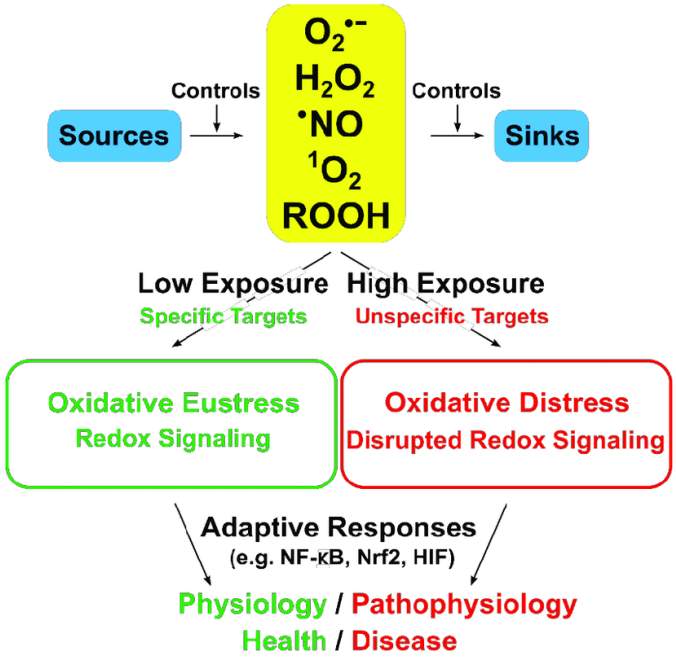



**Figure 3**. Oxidative stress and its relationship to redox signalling. Adopted from *Sies, 2019*

### 1.2.1 Mechanisms of excessive production of ROS in ESRD

Both excessive generation of ROS and impaired antioxidant function are frequently reported in ESRD patients. Several factors increase ROS production in ESRD including: mitochondrial dysfunction, increase in oxidative enzymes activity (nicotinamide adenine dinucleotide phosphate (NADPH)-oxidases (NOX), myeloperoxidase (MPO), xanthine oxidase (XO)), cytokines (interleukin (IL) 1$\beta$, IL-8 and tumor necrosis factor $\alpha$ (TNF-$\alpha$)) release from activated monocytes, uremic toxins, hyperhomocysteinemia, endoplasmic reticulum stress, the accumulation of secondary radicals and transition metals, endothelial nitric oxide synthase (eNOS) uncoupling etc. [12–18]. Moreover, the increased production of ROS in ESRD patients on haemodialysis is highly mediated by this treatment itself, mainly through membrane bio-incompatibility and endotoxin (LPS) release [19,20].

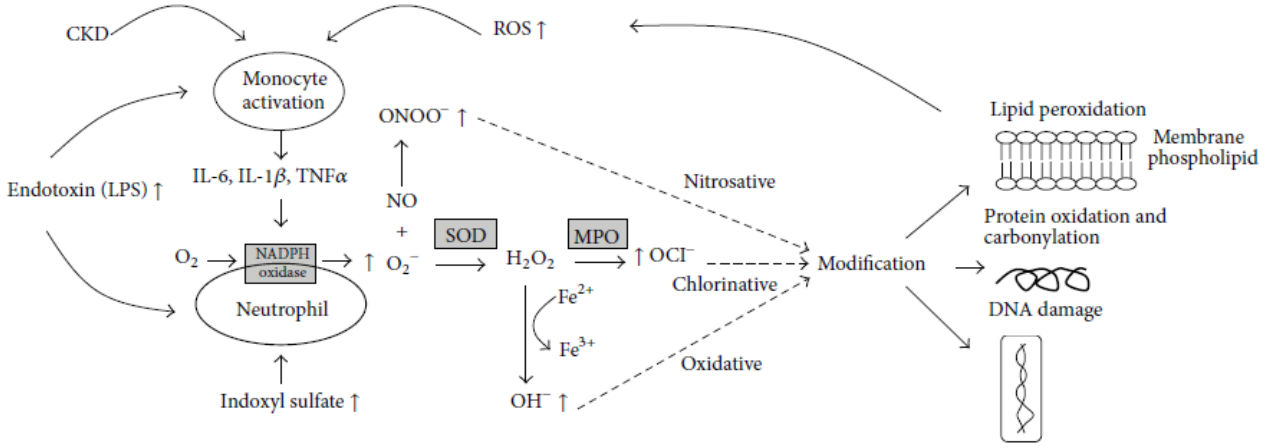



**Figure 4.** Synthesis of reactive oxygen species (ROS) in patients with CKD. Excessive reactive ROS including $ONOO^-$, $OH^-$, and $OCl^-$ are generated from oxygen through several main enzymes (NADPH oxidase, superoxide dismutase (SOD), and myeloperoxidase (MPO)). Several factors can also increase ROS generation, including cytokines (IL-8, IL-1$\beta$, and TNF-$\alpha$) released from activated monocytes, uremic toxin (indoxyl sulfate), and bacterial-derived material such as endotoxin (LPS) from the HD procedure. The resulting excessive ROS can lead to nitrosative (ONOO−), chlorinative (OCl−), and oxidative (OH−) modifications to lipids, proteins, and DNA. Adopted from *Sung et al. 2013*

ROS are generated in several cellular systems localized on the plasma membrane, membranes of mitochondria and endoplasmic reticulum, in the cytosol and in the peroxisomes [21]. Among these, mitochondria were identified as a major source of ROS [17]. During cellular respiration a large majority of oxygen is converted into water via 4-electron reduction by hydrogen. Nevertheless, one electron reduction of $O_2$ produces a "primary" ROS, superoxide ($O_2^-$) which is converted by mitochondrial superoxide dismutase (SOD) into $H_2O_2$. Both $O_2^-$ and $H_2O_2$ are precursors of more potent oxidants. Namely, $O_2^-$ has a high affinity for reacting with the free radical nitric oxide (NO), whereby rapidly producing the RNS peroxynitrite ($ONOO^-$), whereas $H_2O_2$ reacts with intracellular iron to form the hydroxyl radical ($OH^-$) via the Fenton reaction [21]. The resulting $ONOO^-$ and $OH^-$ can lead to extensive nitrosative and oxidative modifications of proteins, lipids, and nucleic acids [21]. Mitochondrial dysfunction which leads to an enhanced mitochondrial ROS production has been reported as one of the key factors in the development of kidney damage, as well as in the progression of a variety of kidney

diseases leading to CKD [17]. Impaired mitochondrial respiration system and higher levels of oxidative stress markers were observed in HD patients as well [22].

Apart from the alterations in mitochondrial function in CKD, the increased activity of cytosolic ROS-generating enzymes such as NADPH oxidase and xanthine oxidase participate in the aggravation of oxidative stress in the damaged kidney. NADPH oxidase is a membrane-associated enzyme that catalyses the production of $O_2^-$. Upregulated NADPH oxidase activity, which leads to enhanced superoxide generation has been reported in both CKD experimental animal models as well as in patients with CKD [23,24]. Moreover, single nucleotide polymorphism (SNP) in the coding region of p22phox gen, a key component of NADPH oxidase, was associated with elevated levels of oxidative stress and requirement of dialysis treatment among patients with acute renal failure [25]. Regarding xanthine oxidoreductase, this enzyme acts both as a xanthine dehydrogenase (XDH) and XO. Under physiological conditions, XDH uses hypoxanthine or xanthine as a substrate and $NAD^+$ as a cofactor to produce uric acid and NADH [26]. Nevertheless, under inflammatory conditions, posttranslational modification of the cysteine residues converts XDH to XO, which has an increased affinity for oxygen as a cofactor to finally produce uric acid and $O_2^-$ or $H_2O_2$ [26]. Xanthine oxidase activity is elevated in CKD patients and could be an independent predictor of cardiovascular events in CKD and HD patients [27]. On the other hand, a number of experimental evidence indicated that inhibitors of XO may exhibit nephroprotective effects through reduction of circulating uric acid levels (indirect benefit), but also through reduction of inflammation and oxidative stress in kidneys (direct benefits) [28–30].

Inflammatory cells have also been confirmed as an important source of free radicals in CKD patients, especially in those on HD [31]. HD leads to activation of polymorphonuclear leucocytes (PMNs), and consequent secretion of myeloperoxidase [21]. MPO catalyses the production of the very reactive hypochlorous acid ($HOCl^-$) from the reaction between $H_2O_2$ and $Cl^-$ [21]. MPO-derived HOCl mediates the oxidative modifications of proteins leading to AOPP and 3-chlorotyrosine production, and therefore has been recognized as a primary cause of protein damage in uraemia [32]. Higher MPO plasma levels were found in HD patients when compared to healthy controls [33]. Several studies have provided valuable data implicating the role of MPO-catalysed oxidation in atherosclerosis in individuals with uraemia [34,35]. Notably, high MPO levels has been reported to correlate with increased mortality risk in HD patients as well [35].

In uremic conditions, eNOS can be a source of excessive production of ROS, through the process called “eNOS uncoupling” [36]. Under conditions of increased oxidative stress the expression of the eNOS has been shown to be paradoxically increased rather than decreased [37]. However, regardless of the increased expression of eNOS, its capacity to produce NO is limited and eNOS itself becomes a superoxide source. In the uncoupled state (e.g., in the absence of the eNOS substrate L-arginine or the cofactor tetrahydrobiopterin (BH4)), electrons normally flowing from the reductase domain of one subunit to the oxygenase domain of the other subunit are diverted to molecular oxygen rather than to L-arginine [38,39], resulting in production of $O_2^-$ rather than NO. $O_2^-$ then combines rapidly with NO to generate very reactive $ONOO^-$.

Oxidative stress in ESRD patients can also be potentiated by iron therapy, which is frequently used to treat anaemia [40]. The administration of intravenous iron is a source of the free iron which has pro-oxidants properties.

### 1.2.2 Uremic toxins as a source of excessive production of ROS

Uremic toxins have been shown to play a crucial role in the oxidative stress onset. Uremic toxins represent compounds that are excreted by the healthy kidneys under physiological conditions. To classify a substance as uremic toxin it should meet the following criteria: 1) it must be a chemical or biological agent capable of producing a response; 2) it must interact with biological systems and produce a biological response; 3) the response should be considered deleterious to the biological system [41]. According to their physicochemical characteristics uremic toxins are divided to: small water-soluble compounds, larger (middle) molecules, and protein-bound compounds (Table 3) [42]. Small water-soluble compounds are easily removed by HD. However, removal of middle size molecules requires dialyzers which contain larger pore size membranes. Finally, membranes with larger pore size have nearly no effect on the removal of protein-bound toxins [42]. In a study of Dou et al. among 11 uremic solutes that were significantly increased in HD patients, particularly indoxyl sulphate (IS) and p-cresyl sulphate (PCS) could not be removed efficiently by HD due to their high protein-binding ratios [43]. In that line, numerous studies were focused especially on the biological response to these uremic toxins.

**Table 3.** Uremic toxins: characteristics and dialysis removal

| Type | Hydrophobic | Protein bound | Dialytic removal parallel with urea |
|---|---|---|---|
| Small water soluble molecules | | | |
| Guanidines | - | - | - |
| Purines | - | - | ± |
| Oxalate | - | - | + |
| Phosphorus | - | - | - |
| Urea | - | - | |
| Middle molecules | | | |
| Cystatin C, leptin | - | - | - |
| AGEs | ± | ± | - |
| Oxidation products (oxLDL) | ± | ± | - |
| Peptides (β-endorphin, methionine-enkephalin, β-lipotropin, etc.) | - | - | - |
| $\beta_2$-microglobulin | - | - | - |
| Parathyroid hormone | - | - | - |
| Protein bound compaunds | | | |
| Indoles (indoxyl sulfate, indol-3-acetic acid) | + | + | - |
| CMPF | + | + | - |
| Hipuric acid | ± | + | - |
| P-cresol | + | + | - |
| Polyamines | + | + | - |

Abbreviations: Advanced glycosylation end products (AGEs); 3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF). Adopted from *Dhondt et al., 2000*

The harmful effects of uremic toxins are highly mediated by oxidative stress and inflammation. Several mechanisms by which uremic toxins may trigger excessive ROS production have been described until now. Dou et al. showed that uremic toxin IS induces oxidative stress in endothelial cells by the activation of NADPH oxidase and decrease of total glutathione (GSH) levels [43]. A few more studies confirmed that IS, as well as PCS related ROS production primarily resulted from the NADPH oxidase activation [44–47]. It has been shown that other uremic toxins, asymmetric dimethylarginine (ADMA), advanced glycation end products (AGEs) and homocysteine also increase ROS production via increased NADPH oxidase expression [18,48,49]. On the other hand, treatment with N-acetyl cysteine (NAC), which increases intracellular levels of GSH, and apocynin, NADPH oxidase inhibitor, reversed the effect of uremic toxins on ROS production *in vitro* [43–46,50].

The synthesis of uric acid can aggravate oxidative stress via the activity of XO, which generates reactive oxygen species [51], although some other reports suggested that uric acid itself may have reducing and antioxidant features [52]. Nevertheless, under conditions of oxidative stress, such present in uremic milieu, elevated uric acid levels have pro-oxidant properties, especially when antioxidant systems are impaired. Indeed, *in vitro* studies reported a marked decrease in antioxidant enzymes SOD, glutathione peroxidase (GPX) and catalase (CAT)) expression and/or activities in a response IS and PCS treatments [45,53]. Importantly, uremic serum treatment led to the decrease of total nuclear factor erythroid 2–related factor 2 (Nrf2), and increase of Kelch-like ECH-associated protein 1 (Keap1) expression at both protein and mRNA level [45]. Since the Nrf2 represents a cytoprotective transcription factor responsible for regulating induction of numerous antioxidant genes (SOD, CAT, GPX, Glutathione S-transferase M1 (GSTM1) etc.), the uremic serum-evoked oxidative stress may be at least partially dependent on altered Keap1/Nrf2 signalling pathway.

Accumulation of uremic toxins leads to uremic syndrome, denoting multiple organ dysfunctions. According to the published data, uremic toxins affect especially cardiovascular system exerting a detrimental effect on cells involved in the functioning of myocardium and vessels, including smooth muscle cells, endothelial cells (ECs), as well as platelets and leukocytes [54]. The particular influence of uremic toxins on endothelial dysfunction is presented in details in a separate section (Page 29).

### 1.2.3 Haemodialysis as a source of excessive production of ROS

Given that the redox imbalance is a hallmark of uremic state, it is straightforward to assume that RRT would consequently improve this imbalance [55]. Indeed, some studies suggest that byproducts of oxidatives stress can be efficiently filtered during the HD process [56]. However, having in mind that HD can influence the activation of the complement system, as well as the activation of leukocytes, and cause the loss of antioxidants, this procedure might also be a source of oxidative stress and inflammation in ESRD patients [55]. These events are widely triggered by the dialyzer membrane bio-incompatibility and LPS release [57].

Concisely, during HD blood contact with foreign surface - dialyzer membrane, which promotes an activation of complement factors, platelets and PMNs. Moreover, bacterial-derived material such as LPS is returned from the dialysate back to the blood. Within minutes upon initiation of HD session, aforementioned events stimulate the release of an array of inflammatory mediators, including cytokines and ROS into the extracellular environment [16, 18–24]. Thus, HD leads to the imbalance of a number of homeostasis systems and correspondingly to wide variety of side effects denoted as "bio-incompatibility phenomena" [58].

As already underlined, PMNs activation is one of the major sources of free radicals in patients on haemodialysis [31]. The direct relationship between HD and pro-oxidant state was substantiated by the findings of higher $O_2^-$, ROS, malondialdehyde (MDA) and F2-isoprostanes and decreased plasma levels of antioxidants (selenium, vitamin C, GPX) in HD patients when compared to non-dialysed ESRD patients [33,59,60]. Moreover, the levels of $O_2^-$, ROS and oxidized low-density lipoprotein (oxLDL) were found to be elevated after HD session [33].

In order to prevent oxidative stress, several intracellular and extracellular antioxidant systems, including enzymatic (SOD, CAT, and GPX) and nonenzymatic systems (thiol, Vitamin E, and Vitamin C), are employed. Therefore, the well documented loss of antioxidants during the HD procedure may also explain the high oxidative burden in dialysed patients [61–63].

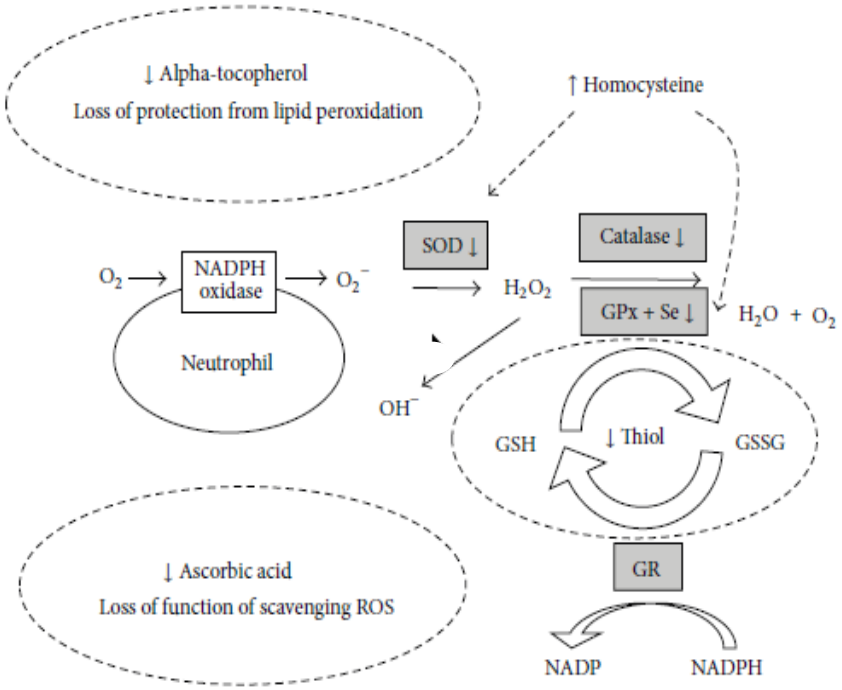



**Figure 5.** Impairment of antioxidant system in patients with CKD. Antioxidant systems, including nonenzymatic systems (thiol, alpha-tocopherol, and ascorbic acid) and enzymatic systems (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX)), are impaired or deficient in patients with CKD. Hyperhomocysteinemia can lead to inhibition of the activity of the antioxidant enzymes SOD and GPX. GR: glutathione reductase; GSH: glutathione; GSSG: glutathione disulfide; Se: selenium. Adopted from *Sung et al. 2013*

### 1.2.4 Key antioxidant enzymes - Superoxide dismutase

SOD and GPX are antioxidant enzymes that play primary role in decreasing oxidative stress, abundantly present in ESRD. SOD converts $O_2^-$ to $H_2O_2$ which is further reduced to $H_2O$ by GPX. Altered SOD expression and/or activity has been well documented under uremic conditions *in vitro* [45,53], on experimental CKD animal models [64,65], as well as in patients with different stages of CKD [66–68]. Notably, decreased SOD activity was reported in patients on haemodialysis, when compared to both healthy controls and CKD patients [27].

Until now, three SOD isoforms have been identified [69]. SOD1 contains copper (Cu) and zinc (Zn) within the active site (CuZnSOD), and it is present in red blood cells [20]. SOD2 has an active site that contains manganese (MnSOD), and is located in mitochondria. SOD3 also has Cu and Zn within the active site, and presents the extracellular form of SOD [20]. SOD1 have two identical subunits of about 32 kDa, each containing a metal cluster, the active site, constituted by a Cu and a Zn atom bridged by a common histidine ligand [45]. SOD1 also acts, but less efficiently, as a nonspecific peroxidase [70]. SOD3, also called extracellular superoxide dismutase, is a secretory, tetrameric, Cu and Zn - containing glycoprotein found in the interstitial spaces of tissues and extracellular fluids. Therefore, antioxidant activity in plasma, lymph, and synovial fluid, as well as, blood vessel wall and interstitium rely on SOD3 [71]. Human MnSOD functions as a homotetramer of 96 kDa, with each subunit containing an active site surrounding manganese ion (Figure 6) [70].

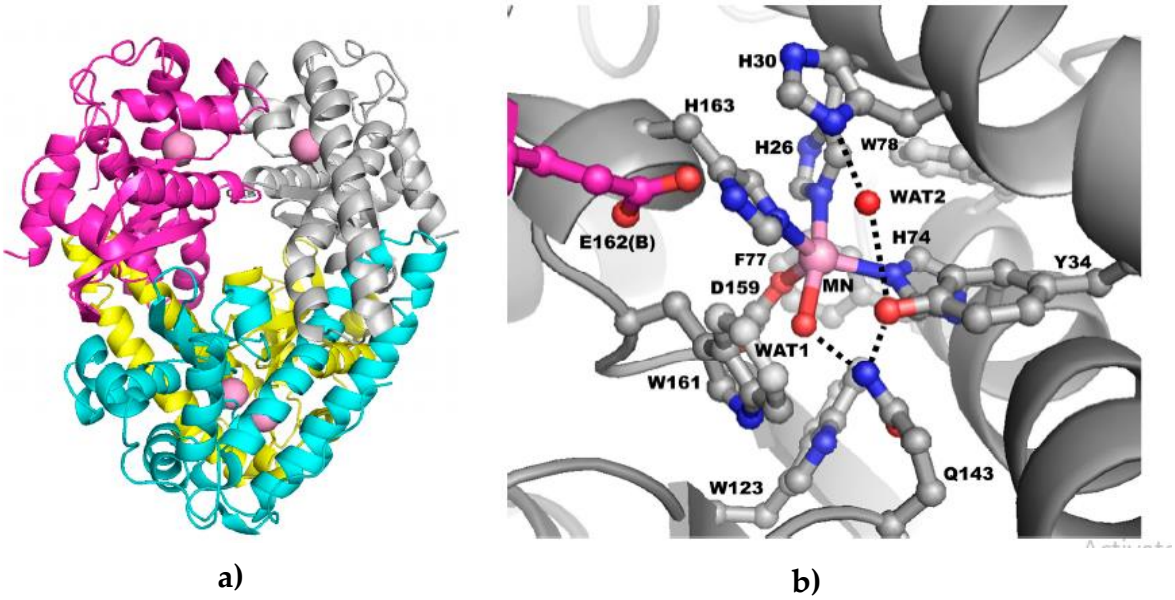



**Figure 6.** Human MnSOD. a) Each subunit contains a manganese ion at the catalytic centre, indicated by pink spheres. (b) The active site. Red spheres denote oxygen atoms, blue denotes nitrogen atoms, grey denotes carbon atoms from one subunit of the tetramer, and magenta denotes carbon atoms from the adjacent subunit. The dashed lines represent the hydrogen bond network hypothesized to be the proton relay to the manganese ion used for catalysis. WAT1: single oxygen-containing molecule; WAT2: single oxygen-containing molecule. Adopted from *Azadmanesh et al., 2018*

Despite the fact that all three SOD isoforms have substantial role in scavenging $O_2^-$ and thus implications in oxidative stress-related diseases, mitochondrial - SOD2 isoform is the only one found to be essential for life [72]. Studies started from the early 1970s provided the evidence on the importance of SOD2 for the cells' survival in aerobic environment *in vitro* [73], while the SOD2 gene knockout proved to be lethal in murine models [74,75]. A broad range of studies have shown that cytokine treatment, ultraviolet light, irradiation, certain tumours, amyotrophic lateral sclerosis, etc. can induce SOD2 activity [76]. In addition, overexpression of SOD2 has been shown to prevent apoptosis [77]. On the other hand, there are studies reporting decrease in SOD2 activity in aging, asthma, cancer, progeria, and transplant rejection [76].

In a comprehensive review of Macmillan-Crow et al., the authors emphasized the importance of SOD2 activity in transplantation, ischemia/reperfusion (I/R), and cancer [76]. Notably, two of them, transplantation and I/R are firmly related to kidney pathology. In a study of MacMillan-Crow et al., despite an elevation in overall SOD2 protein levels in rejecting renal extracts, its activity was dramatically reduced at the same time, compared to non-rejecting renal extracts [78]. The inactivation of SOD2 laid in nitration of SOD2 tyrosine residues. The authors suggested that the impaired SOD2 activity leads to increased $O_2^-$ levels and simultaneous increase in $ONOO^-$ within the mitochondria [78]. This cascade of events could further result in tyrosine nitration/oxidation of key mitochondrial proteins leading to mitochondrial dysfunction and cell death [79]. The I/R injury is a major cause of acute kidney injury (AKI) also called acute renal failure. Noteworthy, it is important risk factor for progression to CKD. Several studies showed a tyrosine nitration and decline in SOD2 activity during I/R in kidneys which was accompanied with increased ROS [78,79]. The loss of SOD2 activity during I/R contributed to the renal tissue injury. Therefore, therapy using mitochondria-targeted antioxidants and SOD mimics might be some of the future strategies in preventing kidney I/R injury [80].

### 1.2.5. Key antioxidant enzymes - Glutathione peroxidase

Glutathione peroxidase is one of the key enzymes of the antioxidant system, which catalyse the reduction of organic hydroperoxides and $H_2O_2$ by glutathione, to water. GPX activity was found to be significantly altered in all stages of CKD.

Several isozymes of GPX family that vary in cellular location and substrate specificity have been identified so far, out of which the best characterised are five human GPXs: cytosolic (GPX1), gastrointestinal (GPX2), plasma-extracellular (GPX3), phospholipid (GPX4) and sperm nuclei (GPX5). GPX1 is the most abundant isozyme in the family of glutathione peroxidases. It is produced in all cells and tissues, although mainly in erythrocytes, kidney and liver [81]. Apart from the cytosol, it was found in mitochondrial, and, in some cells, peroxisomal compartments [82]. Active purified mammalian GPX1 is a homotetramer with molecular mass ranging between 83 and 95 kDa [83]. It is a selenoprotein containing the rare amino acid selenocysteine (Sec) at its active site (Figure 7) [84].

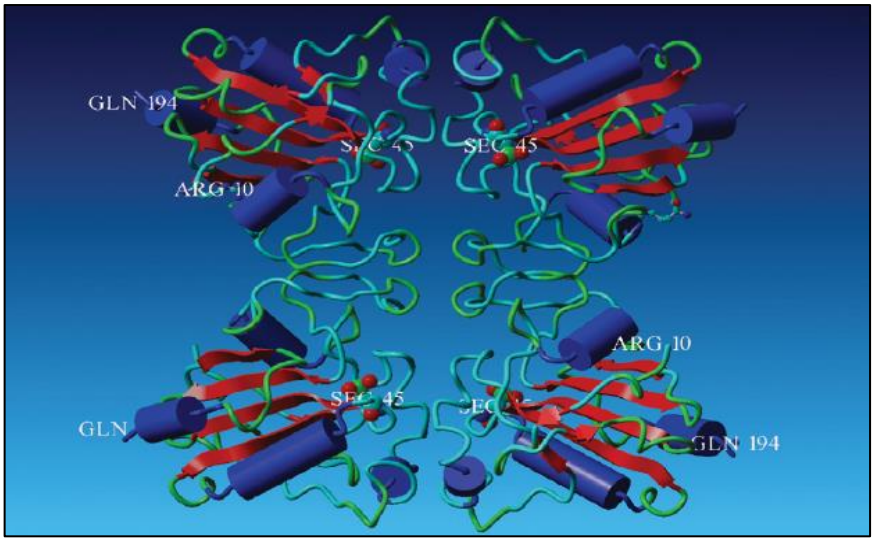



**Figure 7.** Structure of the tetrameric form of red blood cell GPX. In each subunit, Sec is located in the active site at position 45. Adopted from *Zachara et al., 2015*

Enzymatic reduction of peroxides catalysed by GPX1 includes the creation of intermediate modifications of the Sec within the active site [85]. Following the reaction with peroxide, selenol (Se-H) active site is transformed into a selenenic acid (Se-OH) (Figure 8) [85]. One GSH molecule reduces the Se-OH into a glutathiolated selenol (Se-SG) intermediate. A second GSH reduces the Se-SG bond which consequently restores the active site and forms the oxidized glutathione (GSSG) [85]. The following resolution of GSSG is mediated by NADPH-dependent glutathione reductase. Hence, GPX1 eliminates hydroperoxides thereby decreasing oxidative stress, but at the same time it consumes GSH which is the major low-molecular-weight thiol within cells [82]. By modulating the extent of cellular $H_2O_2$, GPX1 can influence either pro-survival or pro-apoptotic pathways [82]. The lack of GPX1 has been found to enhance cell injury, apoptosis and cell death in many *in vitro* and *in vivo* models of disease and toxicity [82,86–88].

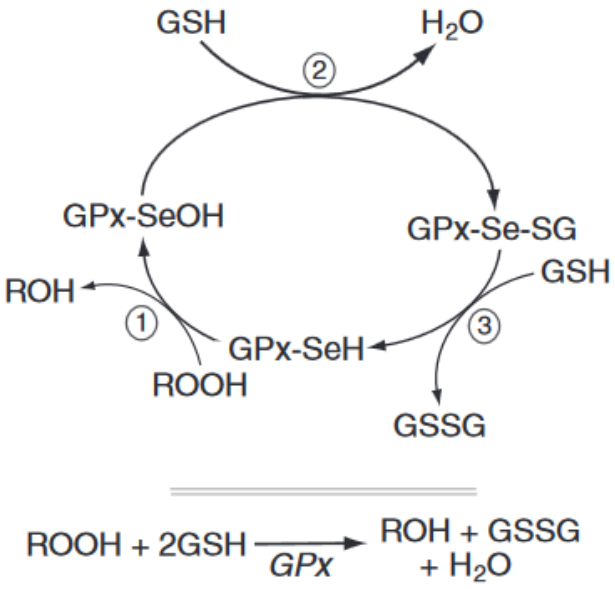



**Figure 8.** Reduction of hydrogen peroxide by GPX1. Adopted from *Lubos et al., 2011*

The specific contribution of GPX1 to pathophysiology of various diseases has been extensively studied, with the emphasis on cancer, diabetes, endothelial dysfunction, atherogenesis, and cardiac dysfunction [82]. GPX1 exerted either protective or harmful effects in these conditions as reviewed by Lubos et al. (Figure 9) [82].

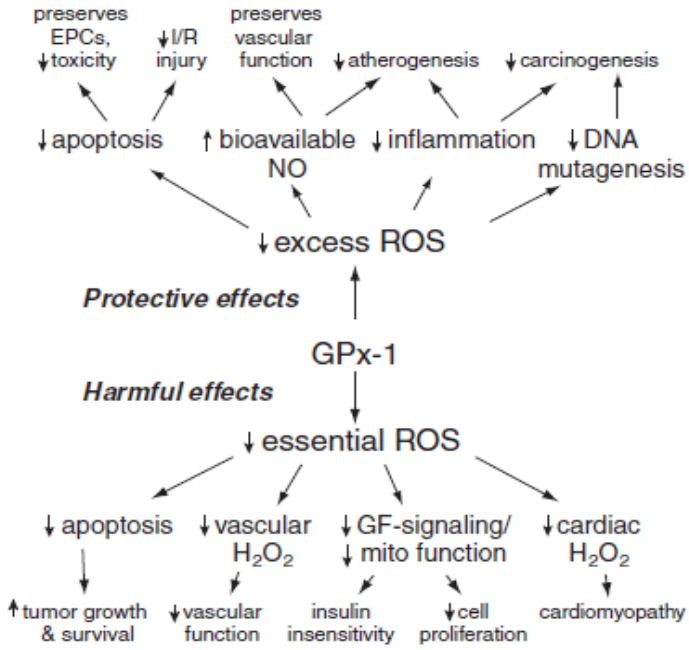



**Figure 9.** Protective and harmful effects of GPX1. Adopted from *Lubos et al., 2011*

Considering the fact that the erythrocyte - GPX1 and plasma - GPX3 represent two GPX isoforms present in human blood, studies investigating the relationship between these GPXs and CKD have emerged over the past years. Studies examining the plasma GPX3 activity in the kidney diseases consistently reported that the activity of this enzyme is significantly lower in CKD patients comparing to the healthy controls, decreases with the progression of uraemia, and decreases markedly in ESRD patients [67,89,90]. As the kidney is the main source of this GPX isoform, the decrease in plasma GPX3 activity may represent an early consequence of active nephron mass reduction. On the other hand, results regarding red blood cell (RBC) GPX1 activity in CKD patients are rather conflicting. Apart from a study of Zachara et al. and a few early reports on small number of participants that showed reduced RBC GPX1 activity in uremic patients [83,84,91], other studies reported unchanged, or significantly higher activity of this enzyme [67,68,89,92]. One of the possible explanations for the higher RBC GPX1 activity in uremic patients given by the authors lye in reduced RBCs life and more frequent replacement of RBCs in subjects with declining renal function [93]. Namely, mature erythrocytes are anucleate and GPX1 synthesis occurs during erythropoiesis. Since the bone marrow synthesis of GPX1 is unaffected in CKD, and a greater percentage of RBC are younger in CKD patients, the amount of GPX1 in RBC may be consequently relatively high [67,84]. Importantly, inconsistent results regarding GPX1 activity in uremic patients may also be explained by functional variations of GPX1 gene, which will be discussed in the separate section.

### 1.2.6 Biomarkers of oxidative stress in ESRD

Free radicals are highly reactive compounds with a very short half-life, measured in seconds. Therefore, measuring ROS *in vivo* is generally not achievable. On the other hand, lipids, proteins, carbohydrates and nucleic acids, after undergoing oxidative modifications, have lifetimes ranging from hours to weeks, making them ideal markers of oxidative stress [57]. The well established shift towards oxidative stress in ESRD patients leads to the extensive damage of biological macromolecules. Therefore, a broad range of studies attempted to assess markers that reflect oxidant-mediated molecular damage and their implications in ESRD pathophysiology, complications and prognosis. Some of the most explored oxidative stress biomarkers in uremic patients will be discussed in the following paragraphs.

Oxidatively modified amino acids and plasma proteins can serve as valuable biomarkers of oxidative stress in CKD patients [55]. Oxidant-mediated injury of proteins may lead to cross-linking, production of aggregation products that may be resistant to proteolysis, fragmentation, as well as loss of enzymatic or other functional properties [94]. Due to its biochemical properties and relative abundance, serum albumins are the main target of oxidation and glycation reactions in the circulation [94–96]. Nevertheless, since they contain a very reactive thiol group (Cys-34) rather present in the reduced state, albumins, together with low molecular weight amino thiols (cysteine, homocysteine and glutathione), behave as the important antioxidants at the same time [94]. Given that the extracellular fluids, such as plasma, contain low or no CAT activity and low levels of SOD and GPX enzymes, several reports suggested that protein-associated thiols actually present the major extracellular antioxidant defence system. An impaired homeostasis of blood thiols has been described in CKD. Plasma protein thiols are extensively oxidised in CKD patients in comparison to the healthy subjects [12,97]. Mimic-Oka et al. described a marked fall in plasma thiol group levels, independent of the degree of renal failure [90]. Similarly, Oberg et al. demonstrated that decreased content of reduced plasma protein thiols is a nearly universal finding in patients with stage 3–5 CKD, without being closely related to GFR [12]. Moreover, the redox status of low molecular weight amino thiols, which are accumulated in uremia, is shifted towards oxidized form in HD patients [89,98]. Taken together, an increase in thiol-oxidation and a concomitant decrease in both protein-associated, as well as low molecular weight reduced plasma thiols, are present in the plasma of uremic patients.

Along with high levels of thiol groups' oxidation, protein carbonylation can be a consequence or even contributor to progressive renal dysfunction in CKD patients [99]. Direct oxidation of Thr, Lys or Arg residues by ROS results in the formation of a protein derivates that contain highly reactive carbonyl groups [100]. Marked increase of reactive plasma carbonyl compounds in ESRD patients was confirmed by several studies [101,102]. The significance of elevated concentrations of reactive carbonyl compounds in uraemia is most clearly demonstrated by their role in the formation of AGEs. AGEs are formed non-enzymatically by irreversible reaction of reducing sugars or other reactive carbonyl compounds with numerous amino groups in proteins [57]. Prominent increase in serum levels of AGEs in ESRD patients might be explained by their increased generation under

conditions of enhanced oxidative stress, as well as by the impaired removal by kidneys due to progressive loss of renal function [57]. The most important adverse impact of AGEs lays in their contribution to endothelial dysfunction and consequent rapidly progressive atherosclerosis. This may be due to the interactions between AGEs with their receptors (RAGE), causing increased expression of adhesion molecules and enhanced attraction of circulating monocytes to the vessel wall [103,104]. Of note, interaction of AGEs with its RAGE receptor also leads to the increased production of interleukin-6 (IL-6) by monocytes and indirectly to the excess formation by C-reactive protein (CRP) in the liver, thus participating in the inflammation [105,106].

Furthermore, detection of tyrosine modifications currently represents one of the most sensitive and specific tools to detect the extent of oxidative protein modifications. Namely, oxidation of tyrosine residues results in the formation of 3-nitrotyrosine, 3-chlorotyrosine or dityrosine depending on the oxidizing species: RNS, HOCl or ROS [55]. It has been shown that plasma proteins in patients on HD have elevated levels of 3-nitrotyrosine [107] and 3-chlorotyrosine [108]. Moreover, Witko-Sarsat et al. provided biochemical and immunological characterization of dityrosine-containing proteins, called advanced oxidation protein products (AOPPs), in the plasma of dialyzed patients [109]. AOPPs are derived from oxidation-modified albumin, fibrinogen and lipoproteins [109,110]. Patients with advanced CKD, especially those on haemodialysis have elevated plasma levels of AOPP [109]. It has been found that AOPP plasma levels negatively correlate with creatinine clearance, thus indicating their potential role as a biomarker of CKD progression [111]. Moreover, increased AOPP levels have been associated with increased plasma concentration of neopterin, a marker of monocyte activation, hence reflecting the impact of AOPPs on inflammation in chronic uraemia [109]. Elevated AOPP levels have also been also recognized as risk factors for atherosclerotic cardiovascular events in CKD patients [112]. Interestingly, apart from AGEs, AOPPs may also bind to the RAGE in endothelial cells, which subsequently induces NADPH oxidase and ROS production, as well as intracellular and vascular cell adhesion molecules (ICAM-1 and VCAM-1) expression [47]. These events may explain the mechanisms by which AOPPs contribute to endothelial dysfunction, which is the underlying mechanism of accelerated atherosclerosis in ESRD patients.

Polyunsaturated fatty acids (PUFAs) are also very prone to oxidative modifications. Lipid peroxidation triggers the production of many reactive compounds such as MDA, thiobarbituric acid reactive substances (TBARS), acrolein and 4-hydroxynonenal (HNE). MDA is generated by both lipid peroxidation and as a by-product of prostaglandin and thromboxane synthesis [113]. It behaves as a reactive nucleophilic agent that can attack macromolecules, including amino acids or sulfhydryl moiety of proteins leading to alterations in their functions [113]. 4-HNE is a major toxic aldehyde produced by ROS attack to $\omega$-6 PUFAs. This aldehyde can react with proteins forming advanced lipid oxidation end-products (ALEs) [55]. Increased levels of ALEs may also serve as valuable marker of enhanced oxidative stress in HD patients [114]. Moreover, F2-isoprostanes, products of arachidonic acid oxidation, are indicators of free-radical attack of the cell membrane phospholipids [115]. Increased plasma levels of the aforementioned markers of lipid peroxidation were found in CKD patients, and have been positively correlated with

the CKD progression [12,116–118]. On the other hand, MDA levels were found to decline after using vitamin E-coated HD membranes [119]. Similarly, MDA and F2-isoprostane levels significantly decreased following kidney transplantation [120–122]. Noteworthy, besides its potential as a marker of CKD progression, MDA as a marker of CVD complications and prognosis have emerged. According to several studies, elevated MDA levels positively correlated with cardiovascular risk [123] and negatively correlated with cardiovascular survival in dialysed patients [124].

Finally, free radicals may react with nucleic acids thus contributing to mutagenesis and oncogenesis [55]. Oxidative damage of DNA results in a formation of the 8-hydroxy-2′-deoxyguanosine (8-OHdG). Furthermore, RNA molecules undergo significant oxidative damage, leading to ribosomal dysfunction and consequent alterations in protein function. Elevated 8-OHdG were found in patients undergoing dialysis implicating that these patients suffer from enhanced oxidative DNA damage [125].

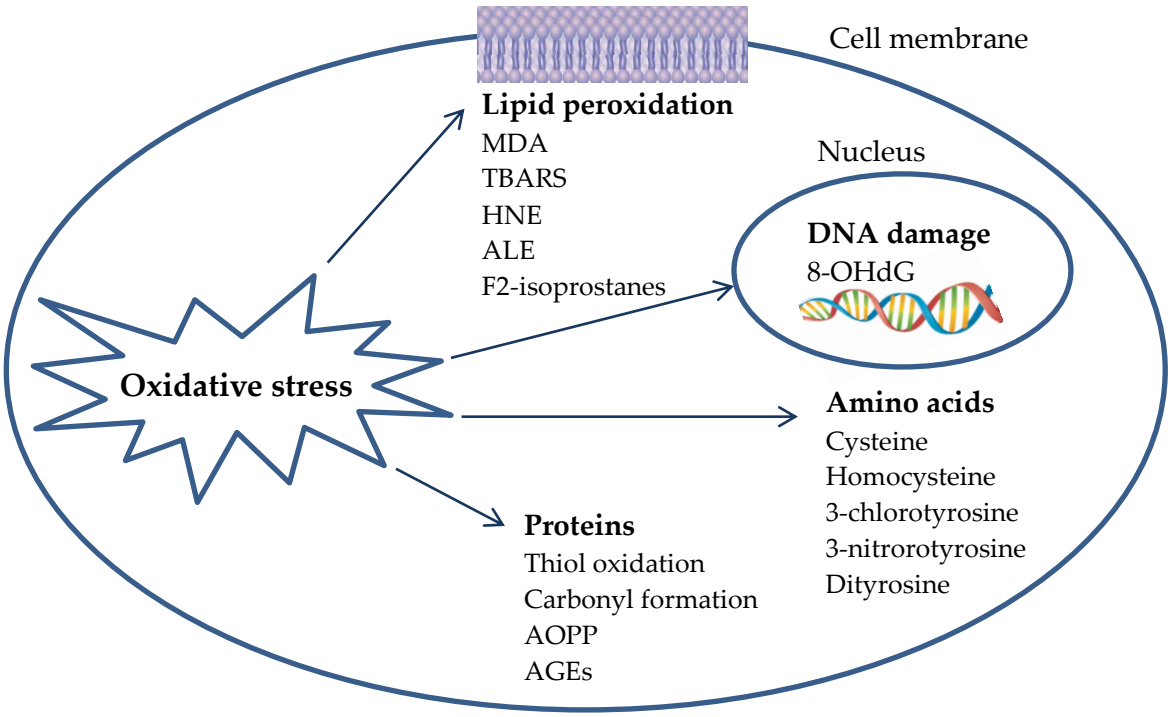



**Figure 10.** Oxidative stress biomarkers

In summary, high levels of oxidative stress byproducts have been indubitably shown in uremic patients. Measurement of these byproducts can be a valuable tool in prevention, early diagnosis, and individualization of therapy in CKD. Moreover, it has been suggested that the value of the assessment of aforementioned biomarkers may lie in their prediction of ESRD patients' survival. Interestingly, not all patients with this disease present with the same extent of oxidatively modified macromolecules. Suvakov et al. has recently shown that functional variations of genes encoding GSTs influence the severity of oxidative stress in ESRD patients [126]. Therefore, it can be hypothesized that in a similar fashion, polymorphisms of antioxidant regulatory and catalytic proteins, such as Nrf2, SOD2 and GPX1, may also have functional relevance in terms of oxidative phenotype in these patients.

### 1.2.7 Nrf2 as modulator of anti-oxidant and anti-inflammatory response

Oxidative stress and inflammation are integrated and inseparable hallmarks of CKD pathophysiology. Indeed, the interplay between them has been shown in cell lines exposed to uremic toxins and animal CKD models. Moreover, the link between markers of oxidative stress and inflammation has been reported in HD patients as well [55]. For instance, interaction between AOPPs, biomarkers of protein oxidative damage which are elevated in CKD patients, and RAGE might activate NF-kB inflammatory signalling pathway and consequently induce ICAM-1 and VCAM-1 cytokines expression *in vitro* [47]. The association between AOPPs and inflammation *in vivo*, in uremic patients, through elevated neopterin levels was already underlined. On the other hand, uremic toxin IS-induced monocyte adhesion, and ICAM-1, VCAM-1 and E-selectin expression *in vitro* were found to be supressed by the antioxidants such as N-acetyl cysteine [44]. In HD patients, high levels of TNF$\alpha$, IL-1$\beta$, CRP and IL-6 were found [55]. Noteworthy, the positive correlation between lipid oxidation biomarkers, F2-isoprostanes, and CRP levels has been reported in these patients [12]. Finally, constant activation of inflammatory cells, PMNs, by haemodialysis treatment, is one of the dominant causes of ROS overproduction and its consequences such as LDL oxidation and MPO-catalysed oxidative protein damage [31,110,127]. It has been suggested that Nrf2 signalling has a crucial role in renal protection against oxidative damage, but also in modulation of inflammatory response [128].

Nrf2 is cytoprotective transcription factor responsible for regulating basal activity, as well as coordinating induction of genes encoding numerous antioxidant and phase II detoxifying enzymes (SOD, CAT, GPX, NADPH:quinone oxidoreductase-1, heme oxygenase-1, glutamate cysteine ligase, GST, thiroredoxin, etc.) [129]. Nrf2 belongs to the cap "n" collar (CNC) subfamily of basic-region leucine zipper (bZIP) transcription factors [130]. Nrf2 protein comprises seven Nrf2-ECH homology domains (Neh1–7), each possessing distinct functions (Figure 11) [131].

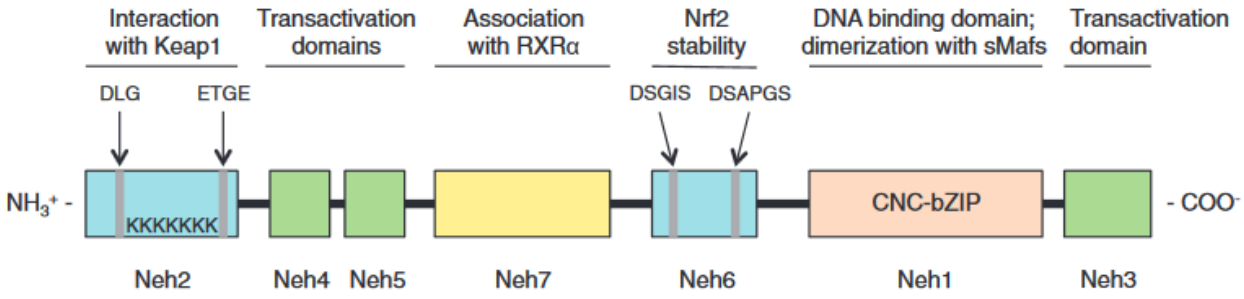



**Figure 11.** Structure of the human Nrf2 protein. The Neh1 CNC-bZIP domain is responsible for DNA binding and dimerization with the small Maf proteins; the Neh2 domain mediates the interaction with Keap1 through the DLG and ETGE motifs and contains seven lysine residues that are targets of ubiquitylation; the Neh3, Neh4 and Neh5 domains are transactivation domains; the Neh6 domain is a serine-rich region that regulates Nrf2 stability; and the Neh7 domain is involved in RXR$\alpha$ binding. bZIP, basic-region leucine zipper; CNC, cap ''n'' collar; Neh, Nrf2-ECH homology; RXRa, retinoid X receptor. Adopted from *Tonelli et al., 2018*

Nrf2 is located in the cytoplasm within an inactive complex bound to a Keap1 [132]. Under homeostatic conditions, two molecules of Keap1 are bound to the Neh2 domain of Nrf2. Keap1 functions as an adaptor protein for the Cul3 E3 ubiquitin ligase, which is responsible for the continuous ubiquitylation and degradation of Nrf2 [132]. Keap1 contains several reactive cysteine residues that serve as sensors of intracellular redox state. Electrophilic and oxidative insults modify thiol residues on Keap1 resulting in dissociation of Nrf2 from Keap1 and its translocation to the nucleus. Besides the cysteine modification of Keap1, nuclear translocation of Nrf2 may also occur by phosphorylation of specific serin and threonine residues in Nrf2 by upstream kinases such as protein kinase C, mitogen-activated protein kinases (MAPK) and phosphatydiinositol-3 kinase/Akt [133]. In the nucleus, Nrf2 heterodimerises with other transcription factors, such as small Maf protein and binds to the regulatory sequences, termed antioxidant response elements (AREs), located in the promoter region of antioxidant and phase II detoxifying target genes [134].

There is compelling evidence showing that Nrf2 exerts antagonistic effect on the NF-kB signalling pathway, which suggests that Nrf2 might also be involved in modulation of inflammatory response [133]**.** The Nrf2/Keap1 pathway controls NF-kB through reduction of IkB$\alpha$ (NF-kB inhibitory protein) phosphorylation, resulting in NF-kB degradation. This is substantiated by the fact that *Nrf2* knockout mice showed elevated NF-κB activity and TNF$\alpha$ expression, while the Nrf2 overexpression inhibited the activation of NF-κB [135]. Moreover, Nrf2 inducers, such as curcumin or bardoxolone methyl led to inhibition of NF-κB through the down-regulation of IκB kinase. Thus, the Nrf2 mediates regulation of both cellular antioxidant and anti-inflammatory machinery in order to protect against oxidant- and xenobiotic-induced cellular injury [136].

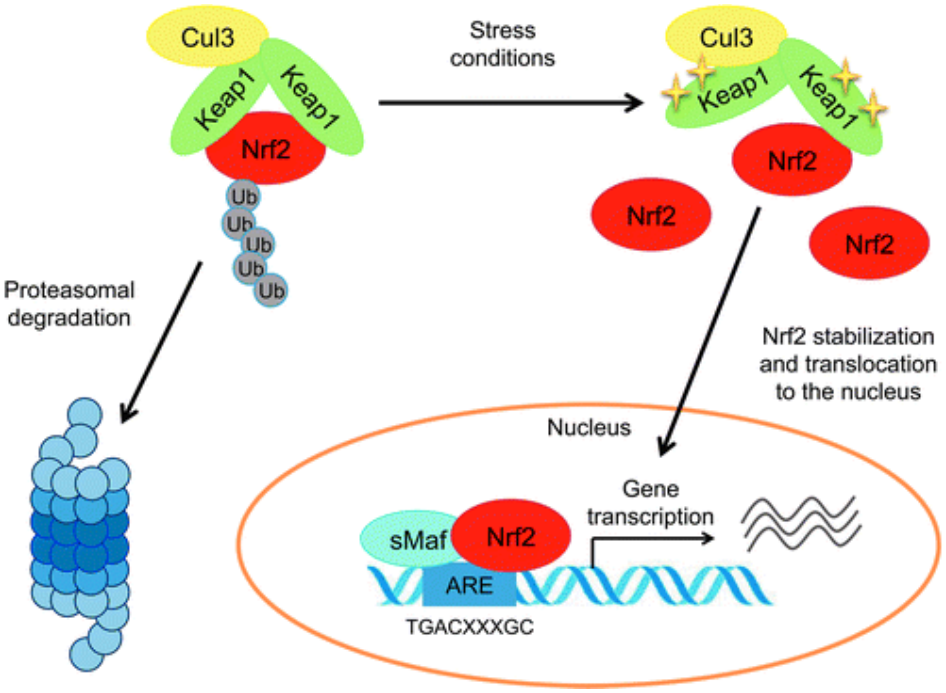



**Figure 12.** Nrf2 activation and response. Adopted from *Tonelli et al., 2018*

Increasing evidence from animal models supports the critical role of Nrf2 in renoprotection and kidney disease pathogenesis. Studies conducted in animals with 5/6 nephrectomy–induced CKD have revealed a marked decline in nuclear Nrf2, in contrary to the expected Nrf2 activation and upregulation, pointing to the impaired remnant kidney ability to deal with the oxidative stress and inflammation [137]. One of the uremic toxins, IS, was shown to repress renal expression of Nrf2. Namely, IS administration in rats reduced the level of Nrf2 and its target gene expression in the kidney, thereby increasing the renal level of 8-OHdG [138]. Moreover, *Nrf2* gene ablation has been shown to cause a lupus-like autoimmune nephritis and exacerbate diabetes-induced inflammation, oxidative stress, and renal injury in the experimental animals [139,140]. Experimental evidence showing the involvement of Nrf2 in diabetic nephropathy was also provided by other groups [141,142]. In addition, in a model of I/R injury, renal function and survival of *Nrf2*-knockout mice were significantly worse than wild-type mice [143]. Histological analysis of kidney tissue in *Nrf2*-knockout mice showed increased oxidative damage, including enlarged glomeruli, mesengial cell proliferation, thickening of the glomerular basal membrane and glomerulosclerosis, which was accompanied with decreased creatinine clearance and reduced life-span [143]. On the other hand, the administration of Nrf2 activators, such as bardoxolone methyl, resveratrol, curcumin and sulforaphane can ameliorate kidney dysfunction in CKD [144]. Therefore, studies using animal models with targeted deletion of *Nrf2* have provided insights into the role of Nrf2 transcription factor in kidney disease pathogenesis, indicating that dysregulation of Nrf2 signalling is involved in human CKD pathology as well. Indeed, subsequent studies investigating the genetic and molecular function of human Nrf2, including the relationship of Nrf2 polymorphisms with the CKD and its final stage - ESRD, have emerged recently [145,146].

## 1.4 Polymorphisms of regulatory and catalytic antioxidant proteins

Excessive oxidative stress in CKD patients could be associated with specific genetic patterns. Genome-wide association study (GWAS) identified several genetic loci with highly significant associations with CKD [147]. A variation in the DNA sequence that occurs in a population with a frequency of at least 1 % is defined as polymorphism [148]. Polymorphisms include SNPs, sequence repeats, insertions, deletions and recombination. The SNPs, where a single base mutation occurs in the DNA, are the most common since they arise every 1,000 base pairs in the human genome [149]. SNPs are used as genetic signatures in populations to study the predisposition to certain diseases [150]. Gene polymorphisms of regulatory and catalytic antioxidant proteins, such as Nrf2, SOD2, GPX1, as well as GSTM1, result in alteration in their proteins' activity profile hence affecting individual's antioxidant capacity. Therefore, polymorphisms of these genes may have functional relevance in terms of the severity of oxidative stress and consequent worse prognosis of ESRD patients.

### 1.4.1 *Nrf2* polymorphism

In order to investigate *Nrf2* as a candidate susceptibility gene for the risk of development of acute lung injury (ALI) in humans, Marzec et al. identified several *Nrf2* SNPs [151]. However, one of them, *Nrf2* rs6721961 *(-617C/A)* polymorphism, affected basal *Nrf2* expression and function at the greatest extent and consequently had the highest influence on the susceptibility to ALI in this study [151]. Since than, multiple studies assessed and found significant association between this polymorphism and other diseases' onset and progression. These include cancer, diabetes, CVD, as well as ESRD [145,152–156]. The frequencies of *Nrf2* rs6721961 genotypes in Polish healthy individuals, which can reflect their distribution in European population, were as follows: 78% *C/C*, *20% C/A* and *2% A/A* [157].

Human *Nrf2* is located on the cytogenetic band 2q31.2 of chromosome 2 [152]. *Nrf2* rs6721961 *(-617C/A)* SNP, characterised by a *C>A* substitution, is located in the ARE-like motif in the promoter region of the *Nrf2* gene [151]. Given its position in the promoter region, this SNP affects basal expression of Nrf2. This is confirmed by the study of Suzuki et al., showing that minor *Nrf2 A/A* homozygotes exhibit significantly decreased *Nrf2* gene expression [153]. Moreover, this *Nrf2* SNP affects ARE-like promoter binding sites attenuating the efficient binding of Nrf2 to AREs [158]. Indeed, Marzec et al. reported that formation of Nrf2 protein-DNA complex was significantly diminished in *Nrf2 C/A* and *Nrf2 A/A* variants in the ARE-like sequence [151]. In addition, since this SNP is located in the middle of the ARE motif and weakens the affinity of Nrf2 binding to the ARE, this SNP appears to disrupt the positive-feedback regulation of Nrf2 expression by Nrf2 itself [153]. In summary, reduced basal expression of Nrf2, together with altered ability of Nrf2 to bind efficiently to AREs, results in attenuation of ARE-mediated transcription of antioxidant and detoxifying genes.

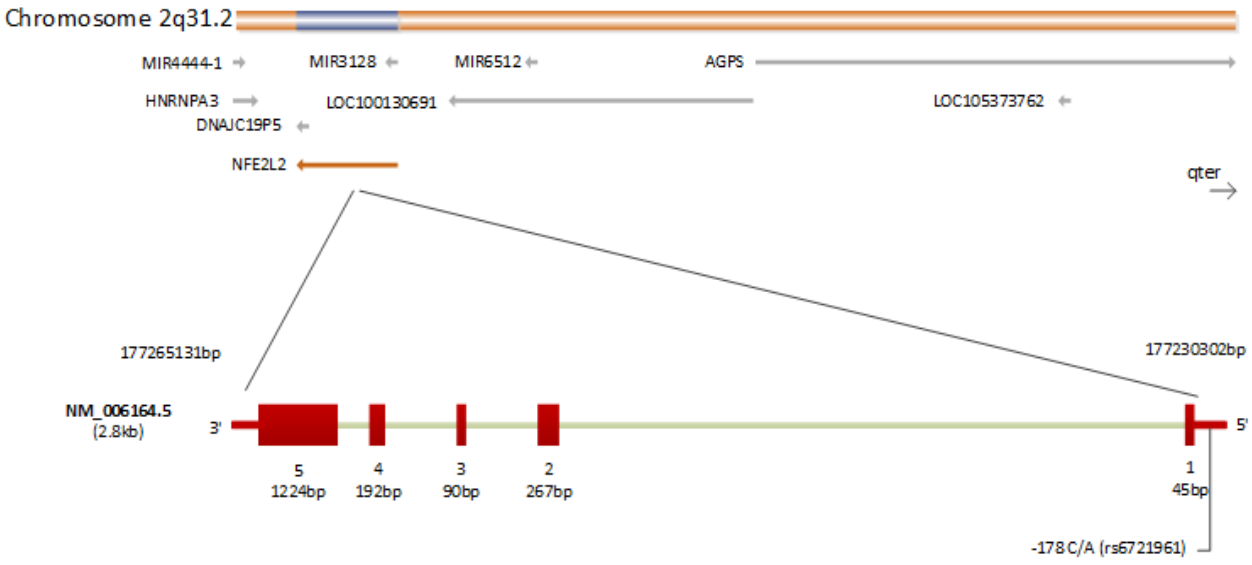



**Figure 13.** *Nrf2* gene Adopted from *Jerotic et al., 2021*

Despite recent progress in elucidation of *Nrf2* polymorphisms in the course of susceptibility and progression of several aforementioned diseases, only one study, conducted on Japanese cohort, aimed to investigate the association of *Nrf2* rs6721961 polymorphism with laboratory data, risk and mortality in ESRD patients [145]. In that study, *Nrf2* rs6721961 polymorphism did not influence either the risk of ESRD development or overall/cardiovascular survival. On the other hand, female patients, carriers of *Nrf2 A/A* variant genotype were reported to have significantly higher levels of both systolic and diastolic blood pressure than women having at least one wild type allele (*C/C* or *C/A* genotype) [145]. Since this has been the only data reported so far regarding the *Nrf2* rs6721961 polymorphism's association with ESRD, this field remains unexplored. Nevertheless, having in mind convincing data on functional relevance of this polymorphism on other oxidative stress-associated diseases, as well as results from animal models that support the critical role of Nrf2 signalling in renal protection against oxidative damage and in modulation of inflammatory response, it may be postulated that *Nrf2* rs6721961 polymorphism plays an important role in kidney deterioration in ESRD patients as well.

As already discussed, variant *Nrf2 A* allele diminishes Nrf2 expression and function, leading to disrupted expression of a broad range of protective, antioxidant and detoxifying molecules. Therefore, it is reasonable to assume that the ESRD patients carrying this allele would be the first to benefit from novel therapeutic agents capable of inducing Nrf2, such as bardoxolone methyl. Bardoxolone methyl is described as a synthetic triterpenoid, derived from the natural product oleanolic acid [128]. Bardoxolone methyl is preferentially a potent Nrf2 activator; however, it can also inhibit NF-κB pathway and therefore suppresses pro-inflammatory cytokine production [159]. Its effects in patients with CKD have been observed in Phase 2 (BEAM) and Phase 3 (BEACON) clinical trials [160,161]. Indeed this treatment had beneficial effects such as a significant increase in GFR. Nevertheless, Phase 3 trial has been terminated prematurely due to potential severe adverse events. Therefore, other Nrf2 activators, such as naturally occurring plant-derived substances – curcumin, resveratrol and sulphoraphane have emerged as promising therapeutic strategy to improve the renal function in CKD patients [144].

### 1.4.2 *SOD2* polymorphism

SOD2 is the antioxidant enzyme present within mitochondria. Still, it is a nuclear encoded protein which has to be first transported to mitochondria in order to catalyse dismutation of $O_2^-$. Considering that mitochondria are membrane-enclosed organelles with two membranes (an outer and an inner membrane), proteins destined to be imported into mitochondria face the challenge of being transported across two membranes and routed to their correct submitochondrial compartments [162]. Mitochondria-targeted proteins can be classified into two main classes; proteins with pre-sequences and proteins with internal targeting sequences [162]. Pre-sequences are N-terminal cleavable sequences which form positively charged extensions that interact with the mitochondrial import receptors;

whereas internal sequences are not cleavable, do not necessarily contain charged amino-acid residues, and are incorporated into the mature protein. The pre-sequences show a high tendency to form an amphipathic $\alpha$-helix [163]. SOD2 belongs to proteins transported into the mitochondria via an N-terminal targeting sequence [163]. This sequence can be affected by the SOD2 rs4880 gene polymorphism resulting in compromised SOD2 ability to neutralize superoxide radical. This polymorphism is therefore extensively studied, and suggested to be crucial in individual susceptibility to the development of diseases such as cancer, neurodegenerative and cardiovascular diseases.

SOD2, encoded by nuclear gene located on chromosome 6q25 DNA, is synthesized with a 24 amino acid-long mitochondrial targeting sequence (MTS) [164]. The mitochondrial import of the SOD2 precursor protein occurs both co-translationally and post-translationally [164], and is driven by the MTS. Following the transport into mitochondrial matrix, the MTS is subsequently cleaved forming the mature homotetrameric protein [165]. The *SOD2* rs4880 SNP is present in exon 2 and substitutes a *C>T* at the position 2734, which changes the amino acid from alanine (Ala) to valine (Val) at the position 16 of the SOD2 MTS (*SOD2 Ala16Val* genotype) [166]. The distribution of SOD2 rs4880 genotypes in a Caucasian Southeastern European general population is *22.4% Ala/Ala (C/C), 53.3% Ala/Val (C/T)* and *24.3% Val/Val (T/T)* [167].

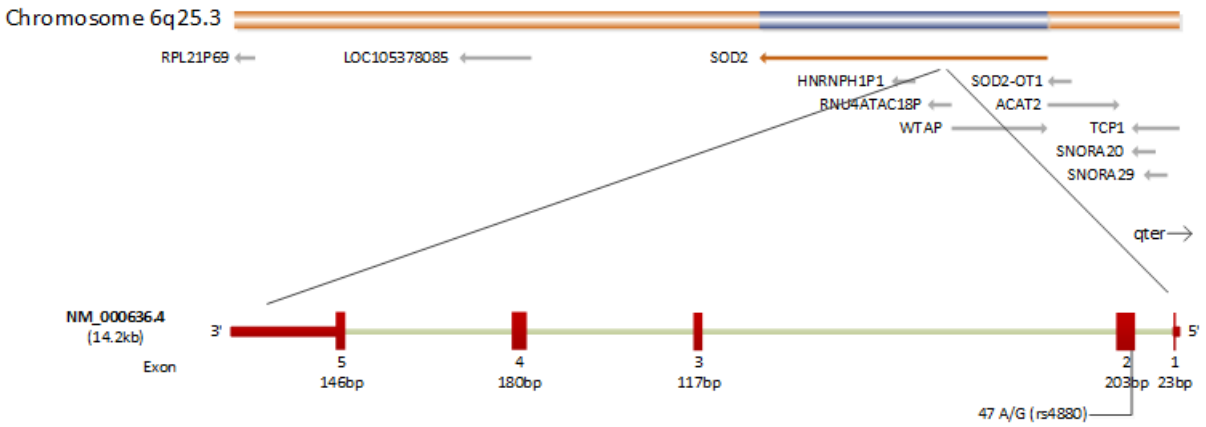



**Figure 14.** *SOD2* polymorphism. Adopted from *Jerotic et al., 2021*

Importantly, the *Val* allele of this SNP results in markedly reduced activity of the SOD2 within mitochondria which may be explained by several mechanisms [168]. First, this SNP affects mRNA stability and causes a more rapid degradation of the SOD2 *Val* mRNA, while the SOD2 *Ala*-variant has a higher synthesis rate in cells [168]. Second, the MTS of SOD2 *Ala* precursor achieve $\alpha$-helix structure resulting in rapid and full import into mitochondria, whereas the MTS of SOD2 *Val* precursor have $\beta$-sheet structure and is less efficiently transported to the mitochondrial matrix [165]. Therefore, the SOD2 activity was found to be approximately 40% higher after mitochondrial import of the SOD2 *Ala* precursor than after import of the SOD2 *Val* precursor [165]. Moreover, in cells transfected with SOD2 *Ala* vector, SOD2 activity 48 h post-transfection was 4-fold higher than in cells transfected with SOD2 *Val* vector [165]. Biological consequences of the *Ala>Val* substitution lie in compromised ability of SOD2 to neutralize superoxide radicals within the cell [165]. In this line, previous studies have suggested that the *Ala* allele, associated with higher

SOD mitochondrial activity, may be more protective against progression of diseases that have been linked to oxidative stress, such as CKD.

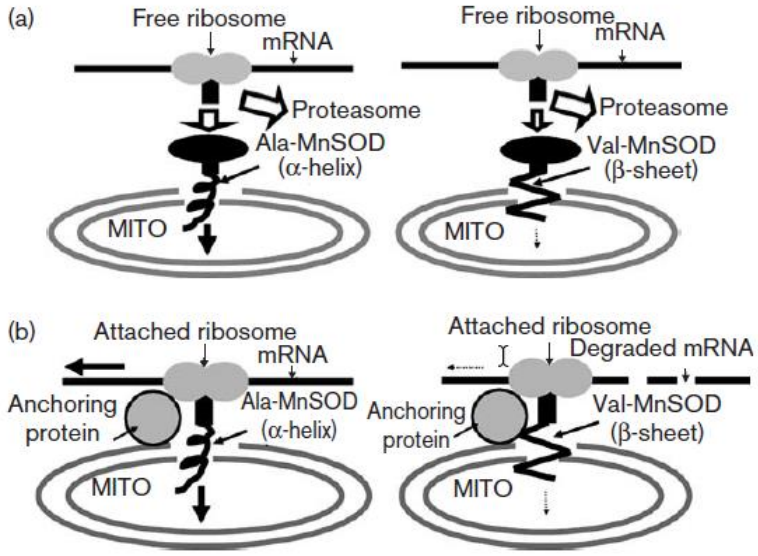



**Figure 15.** Schematic representation of post- and co-translational import of MnSOD, and suggested effects of the *Ala16Val* dimorphism on mitochondrial import and mRNA stability. (a) MnSOD can be synthesized by cytosolic polysomes and then imported post-translationally into the mitochondria. The SOD2 *Ala* MTS may achieve an α-helix structure, insuring some mitochondrial import, despite extensive proteasomal degradation of the precursor synthesized in the cytosol. By contrast, the SOD2 *Val* MTS may achieve a β-sheet structure, impairing mitochondrial import. (b) MnSOD mitochondrial import may also occur co-translationally. The decreased mitochondrial import of SOD2 *Val* is associated with decreased levels of the corresponding mRNAs. Hypothetically, impaired co-translational mitochondrial import could cause stalling of the translated mRNA and increased degradation of the stalled mRNA. Adopted from *Sutton et al., 2005*

Noteworthy, several original investigations have so far tested an association between *SOD2 Ala16Val* SNP and either CKD or ESRD, with contradictory results (Table 4). In the study of Crawford et al., amongst 185 CKD patients included, those carrying *SOD2 Val* allele had a significantly greater decline in eGFR compared with patients carrying the *Ala*/*Ala* genotype [169]. Moreover, the *SOD2 Val* allele has been shown to influence the risk of the CKD and ESRD development, as well. In an Iranian cohort of 280 T2DM patients with CKD and 280 T2DM controls, patients with the *Val*/*Val* genotype exhibited higher CKD risk than those with the *Ala*/*Ala*+*Ala*/*Val* genotypes [170]. The most widely studied impact of *SOD2* rs4880 polymorphism in terms of kidney pathology was the one associated with the onset and progression of diabetic nephropathy. These studies consistently showed that the low activity, *Val* allele increases the risk and the progression of diabetic nephropathy with a faster decline in eGFR [171–174]. On the other hand, in the study conducted on 671 ESRD patients and 780 healthy controls from China, the *Val* allele appeared to be protective in this cohort given that the *Ala*/*Ala* genotype was associated with increased risk of ESRD [175]. This could be explained by the differences in the genotype distribution between Asian and Caucasian population [146,175]. On the contrary to the aforementioned reports, two studies conducted in Australia (230 CKD patients and 224 healthy controls) and Spain (722 CKD patients and 172 healthy controls), reported no association between *SOD2* rs4880 polymorphism and CKD risk [169,176].

Although it is reasonable to assume that the increased risk of CKD in *SOD2 Val* carriers is due to higher oxidative burden, the exact association of *SOD2* polymorphism with an antioxidant status, levels of oxidative stress biomarkers and their implications to CKD triggered by either diabetes or other factors, still need to be clarified in future studies. The value of such studies may lie in benefits from targeted antioxidant therapy in CKD patients with SOD2 *Val* variant using SOD2 drugs or mimetics. Until now, the most well explored benefits of recombinant SOD2 (rSOD2) treatment are its oncotoxic and radio-protective effects *in vitro.* Importantly, when injected *in vivo*, the rSOD2 can easily enter inside the cells and organs and exert its antioxidant activity. In rats with liver cirrhosis, treatment with rSOD2 reduced the portal hypertension by 90%, resulted in the disappearance of ascites and in considerable reduction of liver fibrosis [177]. The authors explained that removing the free radicals, present in high concentration in liver vessels, allows the endothelial cells of these vessels to reuse NO and resume their responses to vasodilatory and vasoconstrictor stimuli [177]. Interestingly, rSOD2 treatment has been described as beneficial in a model of Cyclosporin-A (CsA) induced renal impairment [178]. Namely, Damiano et al. reported that rSOD2 treatment normalized CsA induced ROS levels in aorta and renal tissue, CsA -induced reduction of the GFR and improved the renal morphology at certain extent [178]. The authors suggested that rSOD2 may represent a novel therapeutic option in the treatment of CsA nephrotoxicity. Given the well-known, potent ischemic effect of the CsA, rSOD2 could be useful in the treatment or prevention of the kidney ischemic damages. Moreover, administration of SOD2 mimetics such as tempol, led to a correction of oxidative imbalance, improved oxidative-stress induced renal injury and decreased albuminuria and fibrosis in experimental animals with AKI, diabetes and hypertension [179,180].

### 1.4.3 *GPX1* polymorphism

The GPX1 gene is located on the 3p21.3 chromosome and has two exons [181]. Out of around forty GPX1 gene polymorphisms found so far, *GPX1* rs1050450 SNP is the most commonly studied due to its frequency and resulting functional alterations in the amino acid sequence. This SNP induces *C>T* substitution changing the amino acid proline (Pro) with leucine (Leu) at position 198 (*GPX1 Pro198Leu* genotype) [182]. The distribution of GPX1 rs1050450 genotypes in a Caucasian Southeastern European general population is *43.9% Pro/Pro (C/C), 47.3% Pro/Leu (C/T)* and *8.8% % Leu/Leu (T/T)* [167].

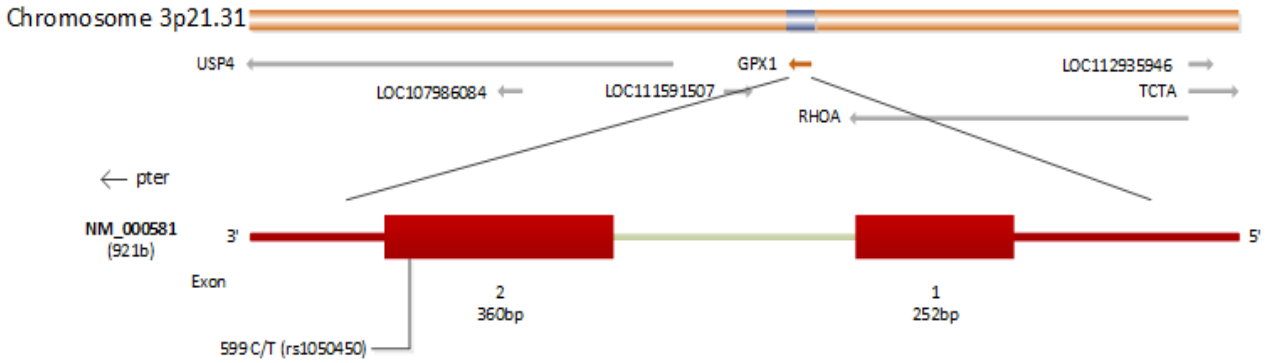



**Figure 16.** *GPX1* polymorphism. Adopted from *Jerotic et al., 2021*

The *Leu* allele of *GPX1* gene alters the catalytic enzyme activity, its affinity to the substrate, and structure stability [183]. Namely, purified GPX1 *Leu* variant has been shown to possess lower enzymatic activity compared to the GPX1 *Pro* enzyme [184]. Moreover, reduced activity of the GPX1 *Leu* variant compared to the GPX1 *Pro* variant was reported in transfected bovine aortic endothelial cells [185]. The association of *GPX1* rs1050450 polymorphisms with GPX1 activity has been also assessed in RBC in human cohorts. In a study conducted on young and healthy individuals in the United States, males with the *Leu*/*Leu* genotype were reported to have the lowest GPX1 activity, whereas this genotype had no effect on GPX1 activity in females [186]. Importantly, the association between *GPX1* rs1050450 polymorphisms and RBC GPX1 activity has been performed in CKD patients as well [67]. However, the observed RBC GPX1 activity was independent of the *GPX1* genotypes [67]. Nevertheless, the association between GPX1 gene polymorphisms with GPX1 activity is not straightforward, as nutritional, environmental, and other factors can also influence the expression and activity of this antioxidant enzyme [82]. The effect of a genotype on GPX1 activity may depend on selenium levels, and CKD patients, especially those on haemodialysis, have significantly lower levels of selenium. It has been found that GPX1 activity derived from the *Leu*-containing allele was less responsive to increasing selenium supplementation as compared with the *Pro*-containing allele [187]. This was also confirmed by the comprehensive study on 400 participants showing that the correlation of GPX1 activity with plasma selenium concentrations was higher for the *Pro*/*Pro* genotype than the *Leu*/*Leu* genotype [184].

*GPX1* rs1050450 polymorphism has been extensively studied in human diseases. The *Leu* allele and *Leu*/*Leu* genotype were associated with increased risk of breast, lung and bladder cancer [182]. *GPX1 Leu* allele also affected an increased risk of vascular calcifications and atherosclerosis [82]. To date, several studies have reported on its association with CKD (Table 4). In addition to *SOD2* rs4880 polymorphism, *GPX1* rs1050450 polymorphism has also been associated with an increased risk and progression of CKD [67]. According to these results, significantly more CKD patients had the *GPX1 Leu*/*Leu* genotype compared to controls. Moreover, *Leu*/*Leu* genotype was associated with lower eGFR in CKD patients. On the other hand, there are several studies reporting no individual association between this polymorphism and the risk of CKD, although its influence appeared significant when combined with the other gene polymorphisms including *SOD2* and PPAR-γ [146,175]. Despite the recent progress in examining the role of *GPX1* rs1050450 polymorphism in CKD, the extent of its influence on GPX1 activity, oxidative stress and prognosis in uremic patients is still unclear.

Based on a presumption that this polymorphism abolishes GPX1 enzyme activity and therefore results in oxidative stress mediated tissue injury, CKD patients would probably benefit from GPX1 targeted therapy. With this regard, selenium therapy was firstly considered. It has been shown that supplementation with selenium can augment GPX1 expression [188]. Along with selenium, antioxidant N-acetyl-cysteine may possibly preserve or enhance GPX1 function through regeneration of liver GSH stores. However, since several studies reported that *GPX1 Pro>Leu* polymorphism makes GPX1 enzyme being less responsive to selenium supplementation, it may be speculated that patients caring low activity, *GPX1 Leu* allele may rather benefit from more targeted GPX1 therapy,

such as of small molecule, GPX1 mimics - ebselen. Noteworthy, ebselen is thought to detoxify hydrogen peroxide, lipid and phospholipid hydroperoxides, by utilizing GSH as a cofactor [189]. To date, ebselen and its analogues have been shown to be protective in an animal models of CsA-induced nephrotoxicity, diabetes-associated renal injury and I/R renal injury [190–192].

**Table 4.** The association of *SOD2, GPX1* and *Nrf2* polymorphisms with CKD risk, progression and mortality

| **Reference** | **Location** | **Study group** | ***SOD2* rs4880** | ***GPX1* rs1050450** | ***Nrf2* rs6721961** |
|---|---|---|---|---|---|
| Crawford et al. 2011. | Australia | 185 CKD patients | *Ala/Val* and *Val/Val* genotypes had a significantly greater eGFR decline compared to those with the *Ala/Ala* genotype | No significant association with the progression of CKD | NR |
| Crawford et al. 2012. | Australia | 230 CKD patients / 224 controls | No significant association with CKD risk | *Leu/Leu* genotype was associated with increased risk of CKD and lower eGFR | NR |
| Shimoyama et al. 2014. | Japan | 216 ESRD patients / 464 controls | NR | NR | No significant association with ESRD risk, overall and cardiovascular survival |
| Chao et al. 2016. | China | 671 ESRD patients / 780 controls | *Ala/Ala* genotype was associated with increased risk of ESRD | No individual significant association with ESRD risk. GPX1 *Leu/Leu* genotype was associated with increased risk of ESRD when combined with PPAR-*γ G/G* genotype. | NR |
| Abbasi et al. 2018. | Iran | 280 CKD patients/ 280 T2D controls | *Val/Val* genotype was associated with increased risk of CKD | NR | NR |
| Corredor et al. 2020. | Spain | 722 CKD patients / 172 controls | No significant association with CKD risk | No significant association with CKD risk | NR |

Abbreviations: N.R. not reported. Adapted from *Jerotic et al. 2021*

## 1.5 Molecular mechanisms of cardiovascular complications in ESRD

Cardiovascular diseases are one of the main comorbidities and causes of death among patients with ESRD [193,194]. Notably, more than half of patients undergoing dialysis suffer from some kind of CVD, and the cardiovascular mortality is 10- to 20-fold higher in these patients than in individuals with normal renal function [195,196]. Moreover, approximately 50% of deaths in ESRD patients can be attributed to atherosclerotic cardiovascular disease [197]. Previously, it was assumed that the increased risk of CVD in these patients resulted from the primary underlying causes of CKD, such as diabetes mellitus and hypertension. However, in recent years, opposing data has shown that CKD *per se* may be a potent risk factor for the development of CVD [198]. Therefore, a magnitude of research has been performed in order to elucidate the contributing factors and underlying molecular mechanisms for such high burden of CVD morbidity and mortality in ESRD.

Both arteriosclerosis and atherosclerosis are present in ESRD patients' vasculature [199]. Disturbance of calcium and phosphate homeostasis associated with impaired kidney function, leads to accelerated calcifications of arteries in these patients playing a key role in the process of arteriosclerosis. The extent of vascular calcifications and arterial stiffness has been shown to be strong predictors of cardiovascular and overall mortality in dialyzed patients [200]. Pathophysiology of atherosclerosis in CKD patients can be explained by the same molecular mechanisms as in the general population; however, in CKD this process appears to be more rapid. Therefore, the term "accelerated atherosclerosis" was introduced in 1974. by Lindner et al. [201]. Factors that are thought to contribute to such acceleration of the atherogenic process in patients with CKD may be divided into traditional (age, male gender, smoking, diabetes mellitus, hypertension, dyslipidaemia) and non-traditional, disease specific factors, such as the accumulation of uremic toxins, hyperhomocysteinaemia, anaemia, CKD–mineral bone disorder (CKD-MBD), enhanced vascular calcifications, coagulation disorders, malnutrition, as well as chronic inflammation and oxidative stress [200].

Given the prominent role that oxidative stress and inflammation have in CKD and CVD pathophysiology, numerous studies aimed to investigate the predictive role of biomarkers of oxidative stress and inflammation in terms of CVD development and mortality in uremic patients. So far, elevation of several inflammatory biomarkers (CRP, TNF-$\alpha$, IL-6, ST2, matrix metalloproteinases (MMPs), etc.) [202–205], and oxidative stress byproducts (AGEs, AOPPs, MDA) showed a strong positive correlation with the severity of CVD and/or cardiovascular mortality in ESRD, and thus a potential for clinical use [123,206,207]. Besides, circulating levels of biomarkers of endothelial dysfunction (ED) can reflect the extent of endothelial injury in uremic patients, and hence indicate the severity of CVD. For instance, ADMA, endogenous amino acid able to inhibit eNOS, is potentially a promising marker of ED, and has been considered as a predictor of cardiovascular events and death in ESRD patients [208]. Moreover, a reduction in endothelial-derived NO production or bioavailability represents a measurable parameter of ED in patients [209]. In addition, in inflammatory and pro-oxidant environment in patients with ESRD, endothelium responds by expressing ICAM-1 and VCAM-1 that facilitate migration and

adhesion of leukocytes to the endothelial cells [47]. Given that these markers are upregulated in patients with CKD and represent an important predictors of mortality in patients with acute myocardial infarction and coronary artery disease (CAD), these molecules can be also considered as predictors of cardiovascular development and mortality in ESRD patients as well [210,211].

In order to elucidate exact molecular mechanisms that underlie the excessive cardiovascular morbidity in ESRD patients, the course of the investigations was translated *in vitro*, using endothelial cell lines. These studies proposed that endothelial dysfunction may be the underlying mechanism of thrombosis, hypertension and accelerated atherosclerosis, present in dialysed patients [201,212–214].

### 1.5.1 Human umbilical vein endothelial cells as a model to investigate endothelial dysfunction in uremic conditions

The vascular endothelium is the primary place where uremic toxins exert their pathophysiological influence, due to the fact that the endothelium is permanently exposed to the uremic toxins. The endothelial dysfunction is characterised by altered basal membrane synthesis, increased vascular tone and permeability, decreased bioavailability of NO, loss of antithrombotic and profibrinolytic properties, increased adhesion of platelets and leukocytes and inflammatory activation [212,215–217]. A great body of evidence suggests that uremic toxins can contribute to such impairment through the process of oxidative stress and inflammation, as reviewed very recently [218]. In fact, oxidative stress and inflammation appear to be common underlying mechanisms of endothelial dysfunction in majority of aforementioned traditional risk factors.

In 1973, Jaffe et al. described the method for isolation of human umbilical vein endothelial cells (HUVECs) [219]. Since that time, HUVECs have been used as a suitable model to investigate the biological response of endothelial cells to various pathological stimuli, such as the one found in uremic milieu. The overview of the main methods and results of studies investigating the effects of uremic serum or particular uremic toxins on HUVECs published so far is given in the Table 1S, Supplement. These studies confirmed detrimental effects of uraemia on endothelium on multiple levels, as will be discussed in the following text.

Uremic solutes inhibit endothelial cell proliferation and migration and increase apoptosis, thus diminishing the physical integrity of the endothelium [218]. Moreover, pre-dialysis uremic sera increases MMPs HUVECs, which are enzymes involved in focal destruction of the vascular extracellular matrix and weakening of the atherosclerotic plaques [212]. As previously discussed, the position of oxidative stress in endothelial injury is indubitable. Uremic toxins increase ROS levels in HUVECs, mostly by increasing NADPH oxidase activity, inducing mitochondrial dysfunction and decreasing cellular antioxidant defence characterized by depleted glutathione levels and inhibited SOD, GPX and CAT activity [43,45,53,220]. Increased nitrotyrosine, protein carbonyls and MDA levels found in HUVECs exposed to uremic milieu confirm enhanced oxidant injury of endothelial macromolecules [45,53]. Excessive ROS production, seen in HUVECs under uremic conditions, leads to activation of NF-$\kappa$B signalling pathway. The upregulation of this pathway is responsible for increased endothelial cell adhesion molecules expression in

HUVECs, such as VCAM-1, ICAM-1, E-selectin, as well as monocyte chemoattractant protein-1 (MCP-1) [44,50,221]. Another signalling pathway shown to be involved in VCAM-1 and ICAM-1 expression in HUVECs is MAPK signalling pathway [222]. VCAM-1, ICAM-1 and E-selectin facilitate leukocyte adhesion to the endothelium, whereas MCP-1 enables transmigration of monocytes into the intima, where they transform into macrophages, and after lipids uptake, into foam cells. These events have pivotal role in the pathogenesis of atherosclerosis. Besides, uremic toxins increase expression of pro-inflammatory cytokines such as IL-6, IL-8, IL-1β, vascular endothelial growth factor (VEGF), stromal cell-derived factor-1 (SDF-1), and TNF-$\alpha$ in HUVECs [50,53,223,224]. It has been shown that IL-1β and TNF-$\alpha$ may also induce expression of ICAM-1 and VCAM-1 in these cells [46]. Not only that uremic serum treatment leads to the increased expression of pro-inflammatory molecules, but it also downregulates the protective molecules in endothelial cells, such as KLF2 [225]. Noteworthy, uremic serum treatment inhibits eNOS activity and NO release in HUVECs as well [45]. Given that NO represents a major vasodilatator released by the endothelium, decreased NO production is accompanied with impaired vasodilation and may be one of the earliest signs of atherosclerosis [226]. Finally, the decreased Nrf2 and increased Keap1 expression were found in uremic serum treated HUVECs [45]. The diminished Nrf2 activity results, not only in decreased expression of a magnitude of cytoprotective antioxidant and detoxifying genes, but also in the upregulation of pro-inflammatory, NF-$\kappa$B signalling pathway.

In summary, these studies provided valuable mechanistic clues about endothelial dysfunction development in uraemia. Besides, several therapeutic agents, shown to ameliorate oxidative stress and inflammation, arose from the comprehensive research conducted so far with a promising potential for clinical use.

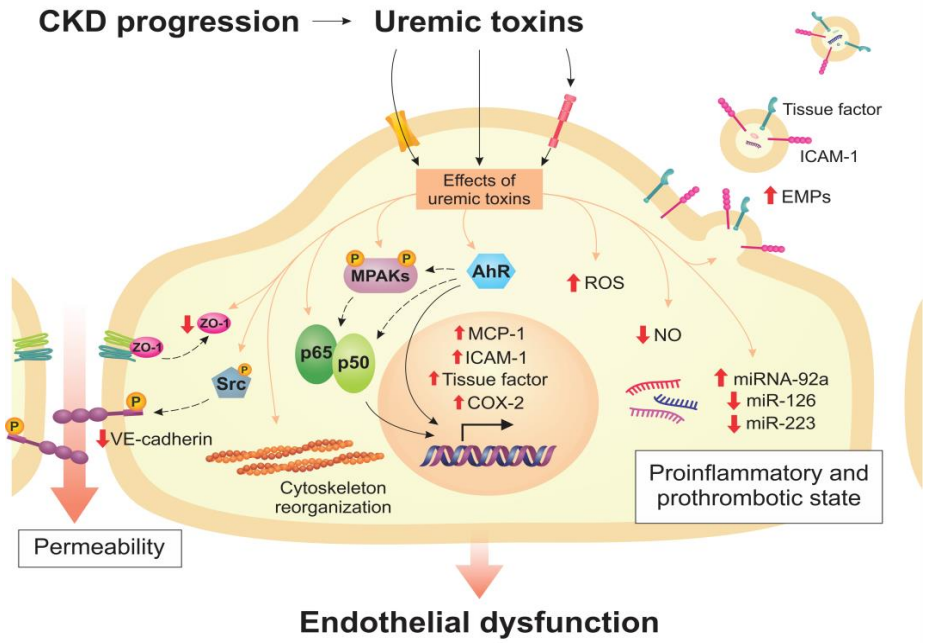



**Figure 17.** Endothelial dysfunction in uraemia. Transporters and receptors mediate the interaction between endothelial cells and uremic toxins, with subsequent activation of signalling pathways, expression of proinflammatory and prothrombotic molecules, increase in ROS, decrease in NO, modulation of miRNAs, cytoskeleton remodelling, formation of endothelial microparticles (EMPs), and loss of cell–cell junctions. Adopted from *Cunha et al., 2020*

### 1.5.2 The role of *GSTM1* polymorphism in cardiovascular complications of uremic patients

GSTM1 belongs to a superfamily of glutathione *S*-transferases, enzymes involved in cell detoxification by catalysing the conjugation of reduced glutathione to a wide variety of xenobiotics that have electrophilic centres [227,228]. Lately its antioxidant role has been investigated as well. GSTM1 is a part of the cellular antioxidant network, since it belongs to a group of enzymes whose expression is regulated by the redox-sensitive Nrf2 transcription factor [229]. The antioxidant function of GSTM1 probably lies in its ability to reduce organic hydroperoxides using glutathione as a substrate. Thus, along with GSTA1, and GSTT1, GSTM1 exibits phospholipid hydroperoxide glutathione peroxidase activity [230]. In addition to its catalytic site, GSTM1 has also a functional noncatalytic domain that inhibits activation of the apoptosis signalling-regulating kinase 1 (ASK1)-p38 signalling pathway [231]. Under conditions of cellular stress, GSTM1 dissociates from ASK1, which leads to apoptosis [232].

In humans, GSTM1 is one of the five different Mu-classes identified so far [233]. All five classes are positioned on chromosome 1p13.3. Between 30 and 50 percent of different human population are homozygous for *GSTM1-null* genotype, thus completely lacking the GSTM1 enzyme [234].

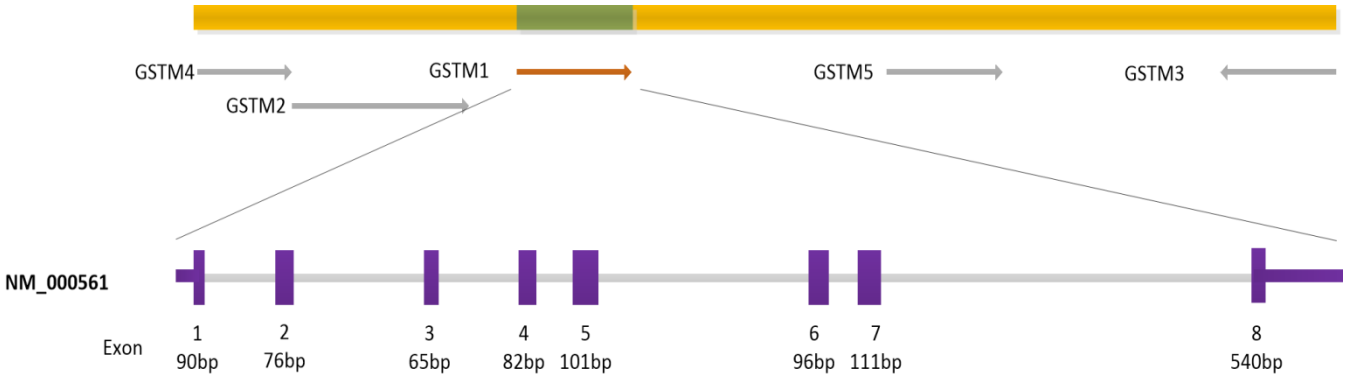



**Figure 18.** *GSTM1* gene, adopted from *Suvakov, 2016*

The potential detrimental effect of GSTM1 deficiency is reflected in the accumulation of toxic metabolites and reactive oxygen species, thereby influencing susceptibility to both ESRD and CVD. Suvakov et al., showed the association of *GSTM1-null* genotype with the increased risk of ESRD development, as well as, shorter overall and cardiovascular survival after 3 and 5 years of follow up [146,235,236]. Individuals with *GSTM1-null* genotype were shown to have significantly increased risks for developing coronary artery disease/atherosclerosis [237,238], stroke [239,240] and hypertension [241]. In the African American Study of Kidney Disease and Hypertension (AASK) participants homozygous or heterozygous for *GSTM1* deletion had, respectively, a 1.7- or 2-fold increased risk for the composite outcome expressed as a decline in GFR, dialysis onset or overall mortality [242]. Similarly, in the Atherosclerosis Risk in Communities (ARIC) study, *GSTM1* deletion was associated with both kidney failure and heart failure, independent of traditional risk factors [243].

The proposed link between *GSTM1* deletion and oxidative stress in ESRD patients was supported by results of Suvakov et al. that demonstrated elevated levels of several byproducts of protein and lipid oxidative damage in ESRD patients with *GSTM1-null* genotype when compared to those with *GSTM1-active* genotype [126]. Noteworthy, haemodialysed patients with *GSTM1-null* genotype had higher concentrations of AOPPs [124], which effects on endothelial dysfunction have been already discussed in the previous paragraphs. Studies conducted on CKD animal models also confirmed that *GSTM1* deletion results in increased oxidative stress *in vivo*. GSTM1 knockout mice displayed higher urinary 8-isoprostane levels and higher superoxide radicals in the kidney tissue [244]. On the contrary, transgenic overexpression of GSTM1 in rats decreased renal levels of MDA and oxidized/reduced glutathione ratio [245]. *In vitro* studies supported aforementioned findings. *GSTM1* knockdown in vascular smooth muscle cells (VSMCs), led to increased ROS production [246] and 4-HNE adducts levels [242]. Conversely, overexpression of GSTM1 in VSMCs reduced 4-HNE adduct levels [242].

Besides its role in cellular protection against ROS, GSTM1 has been connected with modulation of inflammation in CKD as well. For instance, in human cohorts with ESRD, the positive association between *GSTM1* deletion and ICAM-1 and VCAM-1 levels has been shown [124]. Similar results were reported on GSTM1 knock out mice, which displayed higher levels of CXCL-1, MCP-1, and IL-6 proinflamatory cytokines than wild type counterparts [244].

In summary, the influence of GSTM1 deletion on susceptibility to CVD among dialyzed patients may be at least partially explained by excessive oxidative stress and increased inflammation in patients with *GSTM1-null* genotype. However, only two studies examining the specific contribution of this gene in the vasculature pathology were those conducted on VSMCs. The results of these investigations showed that VSMCs respond to a lack of GSTM1 enzyme by increased oxidative stress, cell proliferation and migration [246]. Given that medial vascular smooth muscle cells proliferation and migration into the arterial intima are one of the main events in the development of atherosclerosis, it was proposed that GSTM1 deletion may be directly involved in atherogenic process through enhanced susceptibility to vascular remodelling [246]. However, having in mind *in vivo* studies that showed elevated circulating levels of molecules associated with endothelial dysfunction such as ICAM-1, VCAM-1 in humans [124] and MCP-1 in mice [244] with GSTM1deletion, it is reasonable to assume that GSTM1 reduction potentiates atherogenic processes in uremic conditions through its influence on endothelial cells as well. This hypothesis will be further investigated in this thesis.

## 1.7 Summarizing contemporary findings and paving the way for future research directions in ESRD

Well recognized causes of CKD, such as hypertension and diabetes, converge at the final common pathway – oxidative stress mediated loss of renal function. Both excessive production of ROS and impaired antioxidant function are found in CKD patients, and are especially pronounced in its final stage – ESRD. Interestingly, not all patients with this

disease suffer the same extent of oxidative stress, and thus their course of the disease and prognosis differs. Therefore, it could be hypothesised that the individual susceptibility towards ESRD may rely on functional variations of genes encoding antioxidant regulatory and catalytic proteins, such as Nrf2, SOD2 and GPX1.

Studies investigating the role of SNPs of *Nrf2* rs6721961, *SOD2* rs4880, and *GPX1* rs1050450 on CKD susceptibility and prognosis are scarce. So far, several studies have shown an association of low-activity *SOD2 Val* allele or *GPX1 Leu* allele with increased risk of developing CKD, as well as with a greater decline in GFR, indicating a faster progression of CKD in these patients [67,169,182,247]. However, some studies reported only weak or no association between these polymorphisms and CKD [169,176]. Only two studies examined the impact of *SOD2* and *GPX1* polymorphisms or *Nrf2* polymorphism on ESRD risk [145,175]. Both studies were conducted on Asian population and reported no significant association. Nevertheless, the influence of these polymorphisms on the risk of ESRD in Caucasians has not been investigated so far. While it is assumed that high oxidative burden in ESRD patients may rely on polymorphisms of antioxidant regulatory and catalytic proteins, there is no data in the literature regarding the impact of *Nrf2*, *SOD2* and *GPX1* polymorphisms on levels of oxidative stress biomarkers in patients on haemodialysis.

Excessive oxidative stress and inflammation in HD patients have been linked to poor prognosis, as well as increased cardiovascular morbidity and mortality. Nevertheless, there is no data on the association of antioxidant *SOD2* and *GPX1* gene polymorphisms with survival in these patients, while the impact of *Nrf2* polymorphism on ESRD patients' survival was examined only in one study in Asian population [145]. One of the Nrf2 target genes is *GSTM1.* GSTM1 also belongs to enzymes with antioxidant activity. HD patients carrying the *GSTM1-null* genotype have significantly higher overall and cardiovascular mortality over a five-year follow-up, compared to patients with *GSTM1-active* genotype [236]. However, the long-term effect of *GSTM1* polymorphism on ESRD patients' prognosis is still lacking. Given that the antioxidant system is comprised of a network of different interactions, it is unfeasible to identify a distinct variable which could be used as a single marker of disease. Therefore, biomarkers that have roles in the processes underpinning the pathogenesis of ESRD should be observed in combination, in order to determine the best predictive algorithm of long term survival in ESRD patients. So far, there are no studies that examined whether the combination of antioxidant *Nrf2*, *SOD2*, *GPX1* and *GSTM1* gene polymorphisms could be included in the panel designed to predict prognosis in ESRD patients. Moreover, since the individual prognostic impact of several biomarkers of protein and lipid oxidative damage has been confirmed, it can be assumed that the combination of these biomarkers may have a stronger prognostic potential compared to that of each individual biomarker. This biomarker panel could be precise enough to provide distinctive *strata* of patients.

Poor prognosis and premature death in ESRD is frequently attributed to cardiovascular complications. Studies aiming to discover parameters that will reflect the extent of cardiovascular damage and consequent prognosis in uremic patients, found that several inflammatory and oxidative stress biomarkers could have a predictive value. For instance, circulating levels of biomarkers of endothelial dysfunction can reflect the extent

of endothelial injury in uremic patients, and hence indicate the severity of CVD. Notably, it has been recently shown that patients with *GSTM1-null* genotype on haemodialysis have significantly elevated markers of endothelial dysfunction, besides a higher cardiovascular mortality [124]. Therefore, it is important to elucidate the molecular mechanisms through which this genotype leads to the development of endothelial dysfunction in uremic conditions *in vitro*, using endothelial cell line (e.g. HUVECs). This type of research can have clinical significance in assessing the prognosis and application of individualized therapy in patients on haemodialysis.

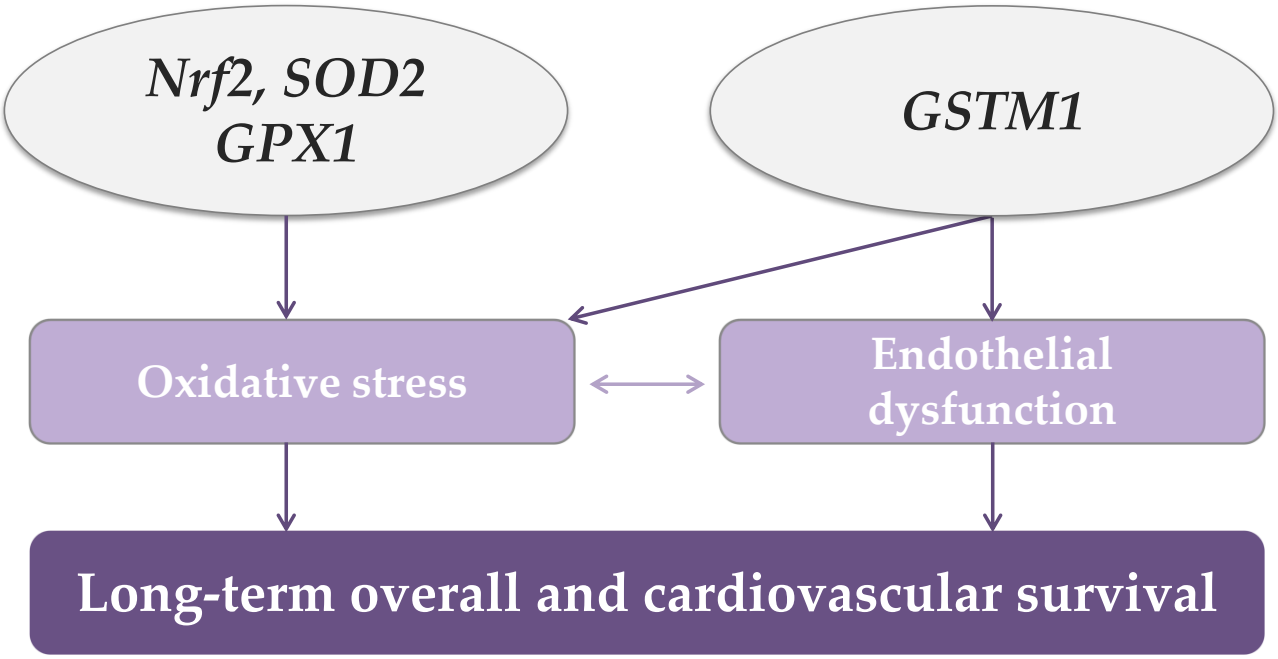



**Figure 19.** The proposed association between regulatory and antioxidant gene polymorphisms with oxidative stress, endothelial dysfunction and prognosis in ESRD

# 2. AIMS

1. To assess the association of *Nrf2* (rs6721961), *SOD2* (rs4880) and *GPX1* (rs1050450) gene polymorphisms with the risk of end-stage renal disease development.

2. To analyse the association of *Nrf2* (rs6721961), *SOD2* (rs4880) and *GPX1* (rs1050450) gene polymorphisms with the plasma levels of oxidative stress byproducts and soluble adhesion molecules in the end-stage renal disease patients.

3. To examine the prognostic role of gene polymorphisms of *Nrf2* (rs6721961), *SOD2* (rs4880) and *GPX1* (rs1050450), together with *GSTM1* deletion polymorphism, on 8-year overall and cardiovascular survival in the end-stage renal disease patients.

4. To explore the prognostic role of oxidative stress byproducts and adhesion molecules, together with *GSTM1* deletion polymorphism, on 8-year overall and cardiovascular survival in end-stage renal disease patients.

5. To elucidate the effects of GSTM1 knockdown and the uremic serum on oxidative stress and the expression of a panel of inflammatory markers in human umbilical vein endothelial cells.

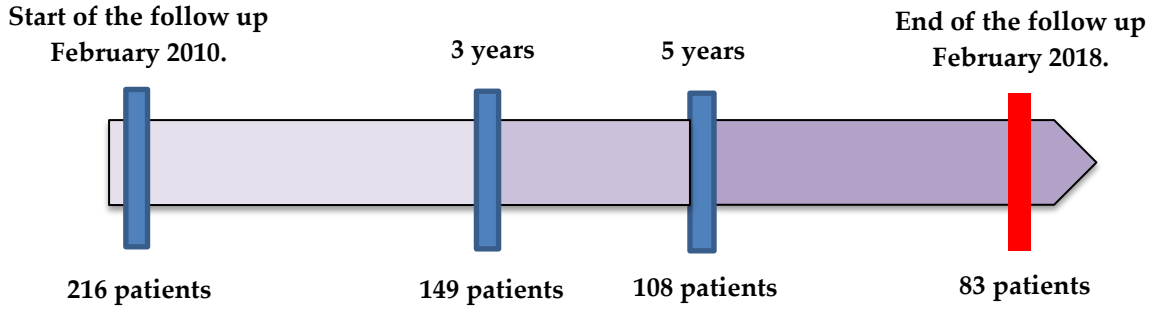
# 3. MATERIAL AND METHODS

## 3.1 Study subjects

This study was comprised of 630 participants: 256 ESRD patients from three dialysis centres (Centre for the Renal Diseases, Zvezdara University Medical Centre, Belgrade; Department of Nephrology and Haemodialysis, University teaching hospital Zemun, Belgrade; Special Hospital for Balkanic Endemic Nephropathy, Lazarevac) and 374 controls with normal renal function admitted to the Clinic of Urology, Clinical Centre Serbia during the same period of time for either routine checkup or nephrolithiasis.

The inclusion criteria for patients were as follows: over 21 years of age, ongoing hemodialysis treatment (3 times a week, for at least 3 months prior to the study onset) and being able and willing to provide informed consent. Exclusion criteria were: previously registered malignancy, infectious co-morbidity evaluated on the basis of C-reactive protein values, HIV, HBV or HCV infections and if patients did not want to participate in the study. The inclusion criteria for the control group were: normal kidney function verified by the blood levels of urea and creatinine and being able and willing to provide informed consent.

A part of this study was conducted as a prospective cohort study which involved 216 ESRD patients. Patients were recruited in February 2010 and followed until February 2018. The outcome was defined as death or the end of the follow-up period. Subjects' data were censored if patients dropped out from the study or if they underwent kidney transplantation. Overall and cardiovascular survival was registered 36, 60 and 96 months from the time of the study onset. Cardiovascular cause of death was defined as myocardial infarction, cerebral vascular insult, heart failure and sudden cardiac death. Overall mortality included aforementioned cardiovascular causes of death with additional non-cardiovascular causes: cachexia, gastro-intestinal bleeding, infections, malignant diseases and unknown causes.




All collected blood samples used in this study were the part of the biobank of the project of the Ministry of Education and Science, Republic of Serbia (No 175052) entitled „Glutathione S-transferases in susceptibility to disease". This study was approved by the Ethical Committee of the Faculty of Medicine, University of Belgrade (No 1550/V-30) and conducted in accordance with the Helsinki Declaration from 2013.

## 3.2 Analysis of the *Nrf2, SOD2, GPX1,* and *GSTM1* genotypes

### 3.2.1 Blood samples

Ethylene diamine tetraacetic acid (EDTA) blood samples were obtained at the study onset. Whole blood, taken for the purpose of the DNA isolation was transported on ice and stored at -20°C at the Institute of Medical and Clinical Biochemistry, Faculty of Medicine, University of Belgrade, Serbia.

### 3.2.2 DNA extraction

A total DNA was purified from leukocytes of 200μl EDTA-anticoagulated peripheral blood using *QIAamp DNA Mini Kit* (*Qiagen, Inc., Chatsworth, CA*). Manufacturer's protocol was comprised of 4 steps: 1) lyses (lysis buffer destroyed cell membranes while proteinase K removed histones and other proteins); 2) DNA was adsorbed onto the *QIAamp* silica membrane during a centrifugation; 3) DNA bound to the *QIAamp* silica membrane was washed in 2 centrifugation steps with *Buffer AW1* and *Buffer AW2*; 4) Purified DNA, free of protein, nucleases and other contaminants or inhibitors, was eluted from the *QIAamp Mini spin* column in a concentrated form in *AE Buffer* and stored at -20$^0$C. DNA concentration and purity were determined spectrophotometrically at 230, 260, 280 and 320 nm using *GeneQuantpro* (*Biochrom, Cambridge, England*).

### 3.2.3 Determination of *Nrf2* polymorphism

The analysis of *Nrf2* polymorphism (rs6721961) was performed by confronting 2-pair primers (CTPP) Polymerase Chain Reaction (PCR) method [145]. Components of the PCR reaction mixture (2μL genomic DNA, 10μl PCR master mix, 7μl water) with primers were used to amplify the DNA fragment of the *Nrf2*gene. The PCR reaction was performed in *Mastercycler gradient thermal cycler* (*Eppendorf, Hamburg, Germany*). Primers sequences and PCR protocol details are given in the Table 5. After electrophoresis on a 2% agarose gel with *SYBR® Safe DNA Gel Stain (Invitrogen Corporation, Carlsbad, CA, USA*), DNA fragments of 282, 213 and 205 bp were visualized using *Chemidoc* (*Biorad, Hercules, California, USA*).

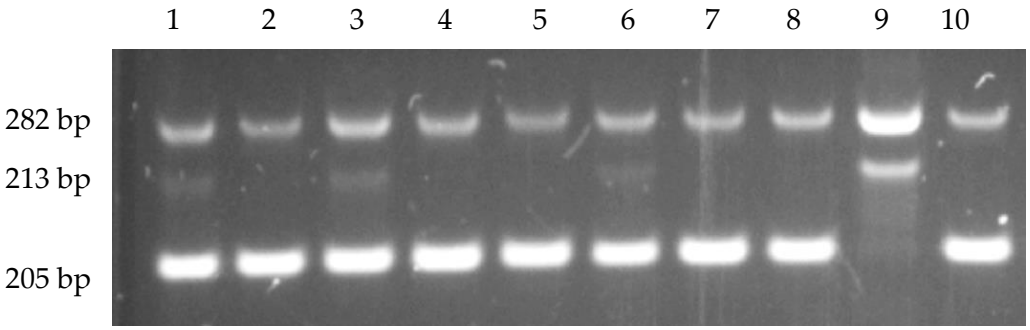



**Figure 20.** 2% agarose gel electrophoresis: PCR products of the *Nrf2* gene. Lanes 1,3 and 6 represent *C/A* genotype (282 bp, 213 bp, 205 bp); lanes 2,4,5,7,8 and 10 comprise 282 bp and 205 bp bands and represent *C/C* genotype; Lane 9 represents *A/A* genotype (282 bp, 213 bp)

### 3.2.4 Determination of *SOD2* polymorphism

To determine *SOD2* (rs4880) polymorphism, the real-time PCR (qPCR) was performed on *Mastercyclerep realplex* (*Eppendorf, Hamburg, Germany*). *TaqMan® SNP Genotyping Assays* (*Life Technologies, Applied Biosystems, Carlsbad, CA, USA, assay* ID: C_8709053_10) was used (transition substitution: A/G; context sequence [VIC/FAM]: CTGCCTGGAGCCCAGATACCCCAAA [A/G] CCGGAGCCAGCTGCCTGCTGGTGCT). 5µl of each DNA sample, along with positive and negative controls, was added to 96-well plate and vaporized at 65°C for 30 min. Afterwards, PCR reaction mixture (0.125 µl *TaqMan* probe, 2.5 µl *HotStart PCR master mix*, 2.375 µl water) was added in each well. Initial denaturation step (95˚C, 4 min) was followed by 40 cycles (95˚C for 15s and 60˚C for 1min). The results were visualized using *Mastercycler® ep realplex* software (*Eppendorf, Hamburg, Germany*).

### 3.2.5 Determination of *GPX1* polymorphism

*GPX1* (rs1050450) polymorphism was determined by PCR- Restriction Fragment Length Polymorphism (PCR - RFLP). Components of the PCR reaction mixture (2µL genomic DNA, 10µl PCR master mix, 7µl water) with primers were used to amplify the DNA fragment (128 bp) of the *GPX1*gene. The PCR reaction was performed in *Mastercycler gradient thermal cycler* (*Eppendorf, Hamburg, Germany*). Primers sequences and PCR protocol details are given in the Table 5. Enzymatic digestion of amplified sequence was performed overnight at 30°C using Apa1 restriction enzyme *(Thermo Fisher Scientific, Waltham, Massachusetts, USA)*. The restriction fragments of 128, 67 and 61 bp were visualized after using on-chip electrophoresis by 2100 Bioanalyzer (*Agilent Techologies*).

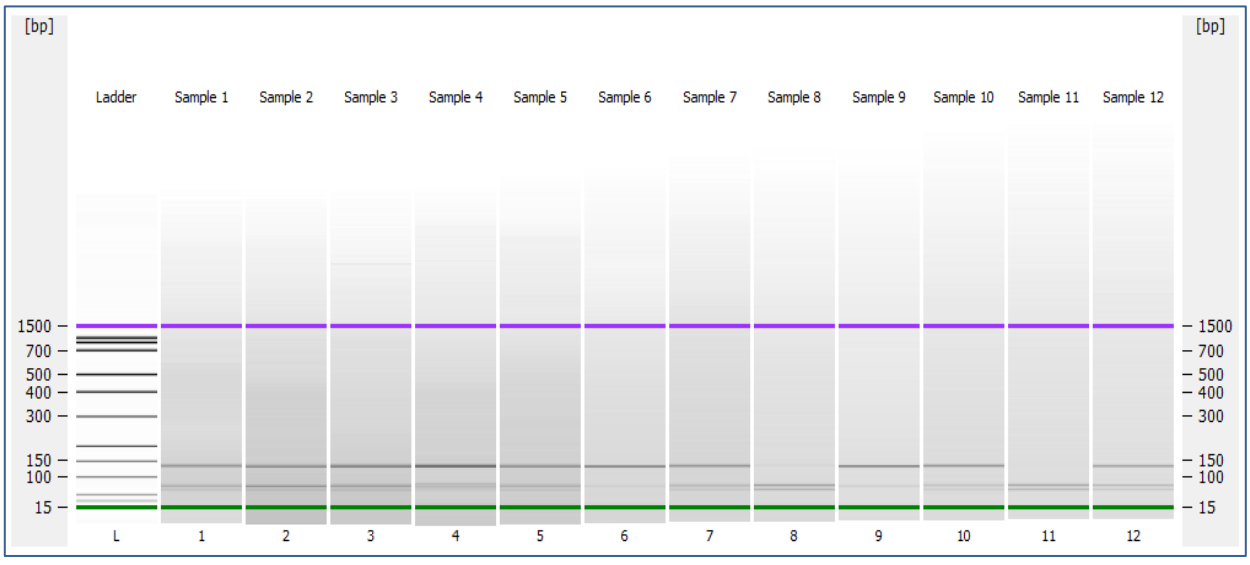



**Figure 21**. On-chip electrophoresis: PCR-RFLP restriction products of the *GPX1* gene. Lanes 1-5, 7, 10 and 12 represent *Pro/Leu* genotype (128 bp, 67 bp, 61 bp); lanes 6 and 9 comprise 128 bp band and represent *Leu/Leu* genotype; lanes 8 and 11 contain two restriction fragments (67 bp and 61 bp) and indicate *Pro/Pro* genotype; L: DNA ladder

### 3.2.6 Determination of *GSTM1* polymorphism

*GSTM1* deletion polymorphism was assessed by multiplex PCR. *CYP1A1* gene was used as an internal control, which confirmed presence of DNA in each sample. Components of the PCR reaction mixture were as follows: 1.5 µl genomic DNA, 6.25 µl PCR master mix, 2.65 µl water, *GSTM1* and *CYPA1* primers. The PCR reaction was carried out in *Mastercycler gradient thermal cycler* (*Eppendorf, Hamburg, Germany*) through several steps described in the Table 5. PCR products stained with *SYBR® Safe DNA Gel Stain (Invitrogen Corporation, Carlsbad, CA, USA*) were separated on a 2% agarose gel and visualized on *Chemidoc* (*Biorad, Hercules, California, USA*). The presence of the 215 bp band referred to *GSTM1-active* genotype and the absence of the band indicated *GSTM1-null* genotype. The assay does not distinguish heterozygous from homozygous referent genotype.

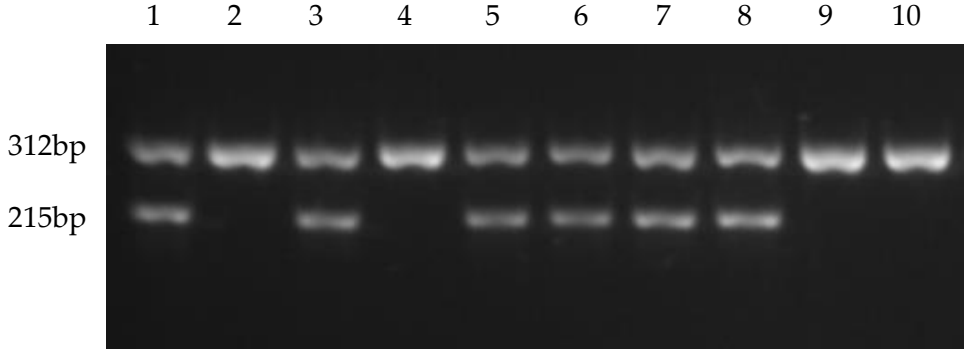



**Figure 22.** 2% agarose gel electrophoresis: PCR products of the *GSTM1* gene Lanes 1, 3, 5, 6, 7 and 8 are represent *GSTM1-active* genotype (both homozygous and heterozygous; 215bp) and lanes 2, 4, 9 and 10 represent *GSTM1-null* genotype. PCR products of *CYP1A1* gene (312 bp) are shown in upper line.

**Table 5:** The PCR genotyping conditions

| Gene | Primer sequence | PCR protocol | PCR products |
|---|---|---|---|
| *Nrf2* rs6721961 | F1: 5′CCCTGATTTGGAGGTGCAGAACC-3′<br>F2: 5′-GGGGAGATGTGGACAGCG-3′<br>R1: 5′-GCGAACACGAGCTGCCGGA-3′<br>R2: 5′-CTCCGTTTGCCTTTGACGAC-3′ | CTPP PCR 33 cycles<br>Denature: 95˚C for 10 min followed by 95˚C for 1 min. Annealing: 64˚C for 1 min. Extension: 72˚C for 1 min. Final extension: 72˚C for 5 min. | *C/C:* 282 bp, 113 bp<br>*A/A*: 282 bp, 205 bp<br>*C/A:* 282 bp, 205 bp,113 bp |
| *GPX1* rs1050450 | F: 5′-GCCGCCGCTTCCAGACCAT-3′<br>R: 5′-CCCCCCGAGACAGCAGCACT-3′ | RFLP PCR 30 cycles<br>Denature: 95˚C for 5 min followed by 95˚C for 20s. Annealing: 66˚C for 40s. Extension: 72˚C for 40s .Final extension: 72˚C for 5 min. | *Pro/Pro:* 67 bp, 61 bp<br>*Leu/Leu*: 128 bp,<br>*Pro/Leu:* 67 bp, 61 bp, 128 bp |
| *GSTM1* deletion | F: 5′-GAACTCCCTGAAAAGCTAAAGC-3′<br>R: 5′-CCCCCCGAGACAGCAGCACT-3′ | Multiplex PCR 33cycles<br>Denature: 94˚C for 3 min followed by 94˚C for 30s. Annealing: 59˚C for 30s. Extension: 72˚C for 45s .Final extension: 72˚C for 4 min. | *GSTM1-active*: 215bp band<br>*GSTM1-null:* no band |
| *CYP1A1* positive control | F: 5′-GAACTGCCACTT CAGCTGTCT-3′<br>R: 5′-CAGCTGCATTTGGAAGTGCTC-3′ | | 312 bp band |

## 3.3 Analysis of oxidative stress biomarkers in plasma of ESRD Patients

### 3.3.1 Plasma separation

Venous blood samples (5 ml) were taken from ESRD patients just before the start of a hemodialysis session, prior to the administration of heparin. Blood was collected in standard sterile polystyrene vacuum tubes with EDTA. Following centrifugation (3600 rpm for 10 min) the plasma was stored in aliquots at -80°C until use. Plasma concentration of oxidative stress byproducts was analysed according to the methods given in the Table 6.

### 3.3.2 Measurement of lipid peroxidation markers (MDA and MDA adducts)

Malondialdehyde (MDA) concentration was measured according to the colorimetric method of Dousett et al. [248] using thiobarbituric acid reactive substances (TBARS). MDA conjugates with TBARS in acidic conditions forming red colored MDA-TBA compound which has a light absorption peak at 532 nm and molar absorption coefficient of $1.56 \times 10^5$ l/(mol x cm). MDA values were expressed as mmol/l.

MDA protein adducts (MDAadd) were determined by enzyme immunoassay (*OxiSelect™ ELISA kits, Cell Biolabs*) according to the manufacturer protocol. Briefly, 50 µl of plasma samples or MDA-BSA standards were added to the wells of the MDA conjugate coated plate. After 10 min incubation, an anti-MDA polyclonal antibody was added, followed by a horse radish peroxidase (HRP)- conjugated secondary antibody. Absorbance of each well was read at 450 nm on *Sunrise absorbance micro plate reader (TECAN, Switzerland*). The content of MDA-add in plasma samples was determined by comparison with a predetermined MDA-BSA standard curve. Detection limit of the kit was 2 pmol/mg.

### 3.3.3 Measurement of protein oxidative damage (protein thiol groups, protein carbonyls, nitrotyrosine, and AOPP)

Protein thiol groups (P-SH) were assayed spectrophotometrically according to the method previously described by Jocelyn [249]. P-SH reduce DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)] making yellow colored 5-thio-2-nitrobenzoic acid ($TNB^-$). TNB has molar extinction coefficient of $13.6 \times 10^3$ lmol/1cm at 412 nm wavelength. This assay was performed in dark, as the light can reduce DTNB reagent nonspecifically. Thiol groups were expressed as µmol/g.

Protein carbonyls were quantified by enzyme immunoassay (*OxiSelect™ ELISA kits, Cell Biolabs*). Namely, 100 µL of 10 µg/ml protein samples or BSA standards were added to the 96-well protein binding plate. The protein carbonyls present in the sample or standard are derivatized to DNPH (dinitrophenylhydrazine) and probed with an anti-DNP antibody, followed by an HRP-conjugated secondary antibody. Absorbance was read at 450 nm on *Sunrise absorbance micro plate reader (TECAN, Switzerland*). The protein carbonyl content in plasma samples was determined by comparing with a previously plotted standard curve and expressed as nmol/mg.

Nitrotyrosine was assessed by competitive enzyme immunoassay (*OxiSelect™ ELISA kits, Cell Biolabs)* following the manufacturer's protocol. In a first step, 50 μl of each sample or nitrated BSA standard were added to a nitrated BSA preabsorbed EIA plate. After a brief incubation, an anti-nitrotyrosine antibody was added, followed by an HRP-conjugated secondary antibody. The protein nitrotyrosine content in all samples was determined using the standard curve. Results were determined at 450 nm on *Sunrise absorbance micro plate reader (TECAN, Switzerland*). and expressed as nmol/l.

Advanced oxidation protein products (AOPP) were determined spectrophotometrically by modified method of Witko-Sarsat et al. [109]. Plasma (200 μl) samples diluted in PBS (1:5) or chloramine-T standard solutions (0 to 100 μmol/l), were mixed with 20 μl of acetic acid. 10 μl of 1.16 M potassium iodide were then added, followed by 20 μl of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm using *STASAR, GILFORD III-Lighting spectrophotometer.* AOPP concentrations were expressed as μmol/l.

### 3.3.4 Measurement of total-oxidant status

Total oxidant status (TOS) was determined spectrophotometrically using method described by Erel [250]. Briefly, oxidants present in plasma samples oxidize the ferrous ion–o-dianisidine complex to ferric ion. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The absorbance of this complex, at 560 nm, was related to the total amount of oxidants present in the sample. Results were expressed in micromolar hydrogen peroxide equivalent per liter (μmol $H_2O_2$Equiv./l), as the different concentrations of $H_2O_2$ were used to plot the standard curve.

### 3.3.5 Measurement of prooxidant-antioxidant balance

Prooxidant-antioxidant balance (PAB) was assayed by modified method of Alamdari et al. [251]. This method is based on two different oxidation–reduction reactions which take place simultaneously: 1) enzymatic reaction where peroxides oxidize 3,3′5,5′-tetramethylbenzidine (TMB) chromogen to a blue coloured cation and 2) chemical reaction where antioxidants (uric acid) reduce the TMB cation to a colorless compound. The photometric absorbance is compared with the absorbances given by a series of standard solutions that are made by mixing varying proportions (0–100%) of $H_2O_2$ with uric acid. These two compounds are representatives of oxidants and antioxidants as they do not interact with each other, and do not neutralize the activity of each other. 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 prooxidants is determined as a value of $H_2O_2$ concentration expressed in μmol x $l^{-1}$. The PAB values of plasma samples were expressed in arbitrary HK units (Hamidi-Koliakos) based on the percentage of $H_2O_2$ evaluated in standard solution.

**Table 6**. Analysis of oxidative stress byproducts, TOS and PAB in plasma of ESRD patients

| Parameter | Method | Units |
|---|---|---|
| MDA | Spectrophotometric method of *Dousett et al.* | mmol/l |
| MDAadd | ELISA *OxiSelect™ ELISA kits, Cell Biolabs* | pmol/mg |
| P-SH | Spectrophotometric method of *Jocelyn* | μmol/g |
| Protein carbonyls | ELISA *OxiSelect™ ELISA kits, Cell Biolabs* | nmol/mg |
| Nitrotyrosine | ELISA *OxiSelect™ ELISA kits, Cell Biolabs* | nmol/l |
| AOPP | Spectrophotometric method of *Witko-Sarsat et al.* | μmol/l |
| TOS | Spectrophotometric method of *Erel* | μmol $H_2O_2$Equiv./L |
| PAB | Spectrophotometric method of *Alamdari et al.* | HK units |

MDA, malondialdehyde; MDAadd, malondialdehyde adducts; PSH, protein thiol groups; AOPP, advanced oxidation protein products; TOS, total oxidant status; PAB, prooxidant antioxidant balance.

## 3.4 Analysis of circulating adhesion molecules in plasma of ESRD patients

### 3.4.1 Measurement of human soluble VCAM-1

Human soluble Vascular Cell Adhesion Molecule 1 (VCAM-1) was determined by a solid-phase sandwich ELISA kit (*Novex, Life Technologies*). According to the manufacturer's protocol, 100 μl of standards, controls, or diluted plasma samples were added to antibody coated 96-wells plate. Consecutively, 50 μl of anti-sVCAM-1 Biotin Conjugate solution was added into each well, except in the chromogen blanks. After 2 h incubation at 37°C, solution was aspirated and wells were washed. At the final step, Streptavidin-HRP conjugated secondary antibody was added to each well, followed by TMB and Stop solution. Absorbance was read at 450 nm on *Sunrise absorbance micro plate reader (TECAN, Switzerland*). sVCAM concentrations were read from the standard curve and expressed as ng/ml.

### 3.4.2 Measurement of human soluble ICAM-1

Human soluble Intercellular Adhesion Molecule 1 (ICAM-1) was assayed by commercial solid-phase sandwich ELISA (*Thermo Fisher Scientific, Waltham, Massachusetts, USA*). In brief, standards, controls and plasma samples were added to the antibody pre-coated wells of the supplied microplate. After 4 washings of the plate, biotinylated antibody was added, followed by a Streptavidin-HRP conjugated secondary antibody. After incubation, washing steps were performed in order to rid the microplate of unbound substance. Afterwards, a TMB substrate solution and Stop solution were added. Absorbance was read at 450 nm and 550 nm on *Sunrise absorbance micro plate reader (TECAN, Switzerland*). Values measured on 550 nm were subtracted from those on 450 nm to correct the optical imperfections in the microplate. sICAM-1 concentrations were read from the standard curve and expressed as pg/ml.

## 3.5. *In vitro* analysis of endothelial dysfunction in uremic conditions

### 3.5.1 Human umbilical vein endothelial cells culture

To determine the role of *GSTM1* genotype on endothelial dysfunction in uremic conditions, commercial HUVECs were used.

HUVECs (*ATCC Manassas, Virginia, USA, kindly donated by Professor Andriana Margariti*) were routinely cultivated in 75 $cm^2$ ventilated flasks coated with 0.2% gelatine in a MV2 growth medium (*Endothelial Cell Growth Medium MV2, PromoCell, Germany*) supplemented with 5% foetal calf serum (FCS), epidermal growth factor 5 ng/ml, basic fibroblast growth factor (FGF) 10 ng/ml, insulin-like growth factor 20 ng/ml, VEGF 0.5 ng/ml, ascorbic acid 1 μg/ml and hydrocortisone 0.2 μg/ml. Cells were maintained at 37°C in a humidified atmosphere with 5 % $CO_2$. After reaching 80-90% confluence, cells were passaged using 0.05 % trypsin EDTA (*Sigma-Aldrich, UK*). Cells were seeded in triplicates in a gelatine-coated 96-well plates (5000 cells/well) for viability assays or into gelatine-coated 6-well plates (150 000 cells/well) for Western blot analysis, oxidative stress measurements, and assessment of cytokines expression. Cells were used for experiments up to passage 14.

Cell culturing experiments were carried out in a class II microbiological safety cabinet, in aseptic conditions. Cell culture media, media supplements and plastics were purchased as sterile, stored in accordance with manufacturer's guidelines. All solutions required to be sterile were passed through a syringe-driven sterilising filter (0.2 μm pores). Glassware was washed and autoclaved prior to use.

### 3.5.2 HUVECs treatments with human sera

HUVEC treatments were consisted of human sera obtained from healthy volunteers (control serum, n=10) and patients on haemodialysis (uremic serum, n=30). The inclusion and exclusion criteria for the participants were as described above (Page 36). The blood was taken from patients prior to haemodialysis session in the Centre for the Renal Diseases, Zvezdara University Medical Centre, Belgrade and immediately transferred on ice to the Institute of Medical and Clinical Biochemistry, Medical Faculty, University of Belgrade. Serum was obtained from blood samples after centrifugation (3600 rpm for 10 min). Serum samples were transported on dry ice from the Institute of Medical and Clinical Biochemistry, Medical Faculty, University of Belgrade to the School of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast, Northern Ireland, where all analysis on cell cultures were carried out.

### 3.5.3 The viability assay

To establish the optimal dose and time for the cell treatments, cell viability was assessed by a colorimetric method based on measuring mitochondrial dehydrogenase activity, using *MTS Cell Proliferation Assay Kit* (*Abcam, UK*). This method is based on the

reduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) compound by viable cells to formazan salt that are soluble in cell culture media. This conversion is carried out by NADPH-dependent dehydrogenase enzymes in metabolically active cells.

The protocol was comprised of several steps. Firstly, 5000 cells/well were cultured in a 96-well plate in a final volume of 200 μl/well. After 24 h, growth media was discarded and cells were treated with media (as a control), 10%, 20%, 30% control/uremic serum for 4 h and 6 h. Afterwards, 20 μl of MTS reagent was added into each well and incubated for 2 h at 37°C in standard culture conditions. The formazan salt produced by viable cells was quantified by measuring the absorbance at 490 nm on *FLUOstar® Omega plate reader (BMG Labtech, Germany)*, and the colour intensity correlated with the number of living adherent cells. Neither the increase of serum percentage nor the duration of incubation time influenced significantly the cell viability (Figure 34), hence treatments with the 30% human sera for 6 h were chosen.

### 3.5.4 GSTM1 knockdown using siRNA

To silence GSTM1 protein expression in HUVECs, 150 000 cells/well were seeded in 6-well plates in 2 ml MV2 growth medium and allowed to attach overnight. The following day, the transfection using GSTM1 small interfering RNA *(*siRNK*) (Termo Fisher Scientific, UK)* and DharmaFECT transfection reagent (*GE Healthcare*, *Chicago, Illinois, USA)* was performed.

In order to test the most effective dose and time for GSTM1 knockdown, cells were treated with 20 nM, 50 nM and 100 nM small interfering RNA (siRNA) for 72 h and 96 h. According to the Western blot analysis, 96-hour treatment with 100 nM siRNA appeared to be the most effective, and all following experiments were performed using that dose and time for GSTM1 knockdown (Figure 33).

Concisely, 100 μM GSTM1 siRNA stock solution was diluted to 2 μM siRNA in nuclease free water. Furthermore, 2 μM GSTM1 siRNA and transfection reagent were diluted in 2 separate tubes: Tube 1 contained 100 μl of 2 μM siRNA mixed with the same ammount of serum free media (the total volume was 200 μl/well); Tube 2 contained 2 μl transfection reagent and 198 μl serum free media (the total volume was 200 μl/well). Tubes were incubated for 5 min at room temperature inside the cell culture fume hood after which, the content of Tube 1 was added to Tube 2 and further incubated for 20 min. Meanwhile, cell media was aspirated and 1600 μl of the complete media was added to each well. After 20 min incubation, 400 μl of the transfection solution was added to each well. Cells were incubated for 96 h at 37°C in a humidified atmosphere with 5 % $CO_2$. The silencing effect was confirmed by Western blot.

Following the results obtained from our optimisation protocols, all further treatments of $GSTM1^{+/+}$ HUVECs and HUVECs silenced for *GSTM1* gene ($GSTM1^{+/-}$) consisted of 30% control or uremic serum for 6 h (Figure 23), after which cytokine expression and oxidative stress measurements were performed.

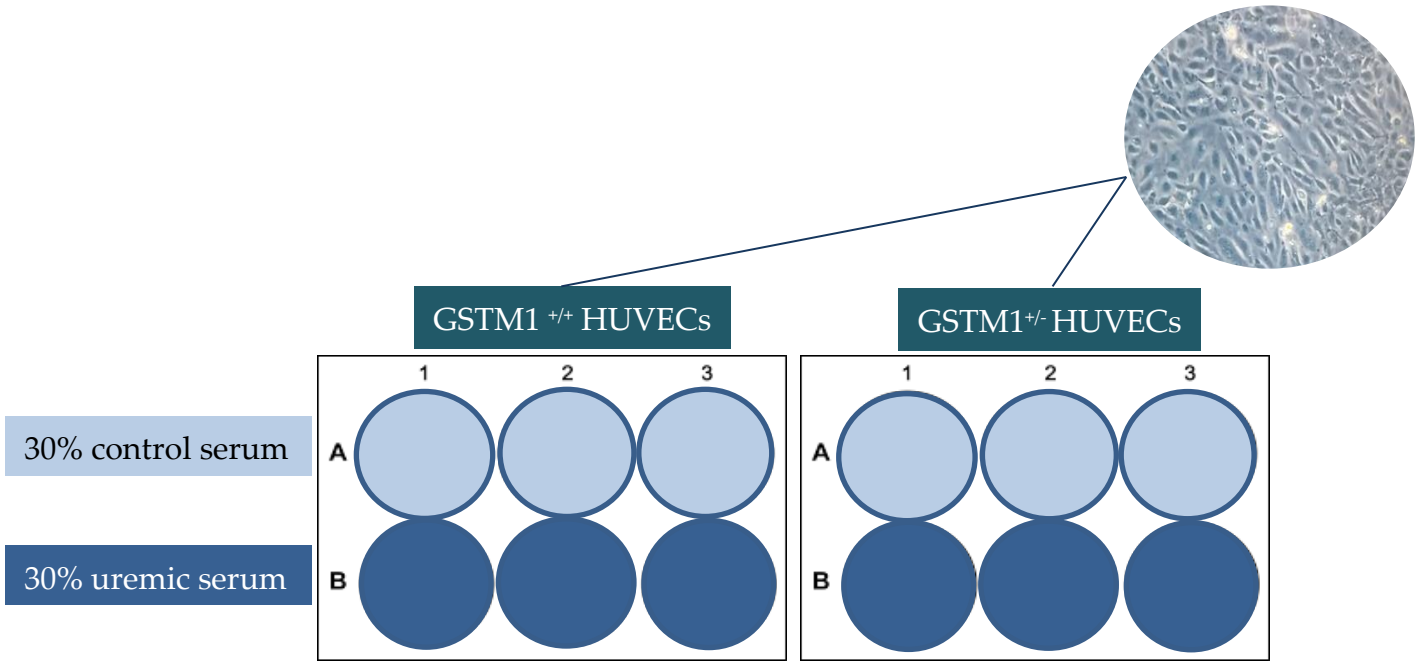



**Figure 23.** HUVECs pre-treatments and treatments

### 3.5.5 Western blot analysis

#### 3.5.5.1 Cell protein extraction

After the incubation time with GSTM1 siRNA expired, media was aspirated from all wells, and cells were washed with PBS. Cells were then scraped using a cell scraper in a 200 µl RIPA buffer (50 mM Tris-HCL pH 8.0, 150 mM NaCl, 1% IGEPAL, 0.5% Sodium Deoxycholate, 10% SDS) supplemented with protease inhibitor cocktail (*Roche, UK*). Cells were incubated for 20 min and vortexed every 5 min before centrifugation (15 000 rcf for 15 min at 4°C). The supernatant was transferred into an eppendorf tubes and stored at -80°C.

#### 3.5.5.2 Protein quantification

Proteins were quantified using *Bicinchoninic Acid Protein Assay kit (BCA, Thermo Fisher Scientific, UK)* according to the manufacturer's protocol. This assay is based on reduction of $Cu^{2+}$ to $Cu^{1+}$ by proteins in an alkaline environment which is followed by colorimetric detection of the cuprous cation ($Cu^{1+}$) by BCA.

In brief, 10 µl of each protein sample, BSA standard (0,5-2 mg/ml) and negative controls were loaded in duplicate on a 96-well plate. In the following step, 200 µl working reagent was added to each well, and the plate was incubated for 30 min at 37 °C. Absorbance was read at 562 nm on a *FLUOstar® Omega plate reader* (*BMG Labtech, Germany*). Protein concentrations were obtained from a BSA protein standard curve (Figure 24).

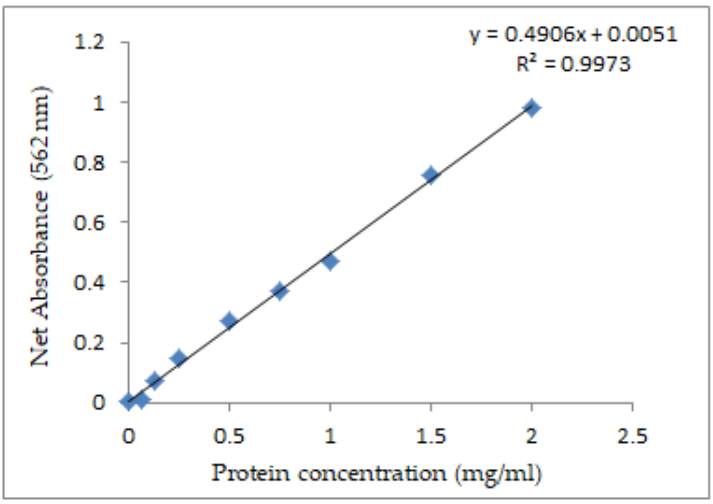



**Figure 24.** Standard curve of net absorbance versus protein concentration; Equation: y = a + b*x; y- net absorbance at 562nm; a-slope; b-intercept; x- protein concentration in mg/ml; Adj. $R^2$=0.9973

### 3.5.5.3 Western blotting

Cell protein extraction and quantification were followed by final preparation of protein samples for loading on sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). Namely, an equivalent amount of protein per sample was mixed with lithium dodecyl sulphate loading dye (*Invitrogen, USA*). In order to reduce and denature proteins reducing sample agent (*Invitrogen, USA*) was added and samples were boiled for 10 min at 95°C.

The same amounts of proteins (15 μg) were loaded on SDS-PAGE together with *Page ruler plus* (*Bio-Rad, UK*) protein ladder as a guide for molecular weight of proteins. Acrylamide stacking gel (4 %) and acrylamide resolving gel (10%) were prepared and poured into a ready-to-use cassette (*Novox, USA*). Electrophoresis (80 V for 10 min, then 100 V for 90 min) was performed on *PowerPac (Invitrogen, UK)*. In the following step, proteins were transferred from polyacrylamide gel onto a nitrocellulose membrane (*Bio-Rad, UK*) at a voltage of 120V for 90 min. Protein transfer was confirmed by *Ponceau* S staining (*Cell Signalling Technology, UK*).

To prevent non-specific binding of antibodies, membranes were blocked in 5 % non-fat milk *(Bio-Rad, UK)* diluted in Tris-buffered saline with 0.1 % Tween-20 (TBS-T) for 1 h at room temperature. Membranes were then incubated overnight at 4°C with constant shaking in primary antibodies: GSTM1 monoclonal mouse *(R&D Systems, Minneapolis, USA)* diluted 1:1000 in 3 % milk, monoclonal mouse β-actin (*Thermo Fisher Scientific, UK*) diluted 1:10 000 in 3 % milk, monoclonal mouse anti-ICAM1 *(Santa Cruz, USA)* and polyclonal goat anti-VCAM1 *(Santa Cruz, USA)* diluted 1:200 in 5 % milk. The following day, membranes were washed with TBS-T and incubated with specific HRP-conjugated secondary antibodies: anti-mouse 1:5000 (*Abcam, UK)* or anti-goat 1:1000 (*RayBiotech, USA*) for 1 h at room temperature. In the last step, the secondary antibodies were discarded, membranes were washed and then treated with *West Femto Maximum sensitivity substrate (Thermo Fisher Scientific, UK).* Chemiluminescent bands were visualised on the *G-box (Kodak, UK) or ChemiDoc (BioRad, USA).* Densitometry analysis was performed using

*ImageJ* software (*National Institutes of Health, Bethesda, USA*). Protein expression was adjusted to housekeeping protein, β-actin.

### 3.5.6 Analysis of oxidative distress in HUVECs

In order to estimate whether GSTM1 knockdown influences oxidative distress in HUVECs in uremic conditions, the following parameters were measured: SOD and GPX enzyme activity, MDA levels and total ROS production. All analyses were performed in cell lysates prepared as described above (Page 45), except the total ROS which was assayed by flow cytometry.

#### 3.5.6.1 Measurement of SOD activity

The SOD enzyme activity was assessed by the method of Misra and Fridovich [252]. This method is based on the ability of SOD to inhibit the autoxidation of adrenaline at pH 10.2. Adrenaline is quite stable in acid solutions but oxidized in a growing manner as the pH rises. The autoxidation of adrenaline is initiated by traces of heavy metals present as contaminants in the used reagents. The production of coloured adrenochrome in reaction mixtures (10 mmol/l adrenaline disolved in 20 mmol/l HCl, 1 mmol/l EDTA, and 0,05 mmol/l sodium carbonate at pH 10.2) with cell protein extracts (sample) or without them (control) was followed at 480 nm. Activity of SOD was expressed as the percentage of inhibition of adrenaline autoxidation.

#### 3.5.6.2 Measurement of GPX activity

GPX activity was assessed as reported by Günzler and Flohe [85]. The reaction mixture was consisted of: 870 μl Tris Buffer pH 7.6, 20 μl of glutatione reductase (GR), GSH, NADPH, t-butyl-hydroperoxide (t-BOOH) and cell protein extract. GPX activity was assayed by the subsequent oxidation of NADPH at 340 nm with t-butyl-hydroperoxide as substrate. One unit of enzyme activity was expressed as mmol NADPH oxidized per minute, assuming $6.22x10^3$/l/mol/cm to be the molar absorbance of NADPH at 340 nm.

#### 3.5.6.3 Measurement of MDA levels

MDA levels in cell lysates were measured using the competitive ELISA kit *(Elabscience, Wuhan, China).* According to the protocol provided with commercial kit, 50 μl of standards, samples and blanks were added in duplicate to each well of MDA pre-coated ELISA plate with consecutive addition of 50 μl biotinylated antibody. During the reaction, MDA in the sample or standard competes with a fixed amount of MDA for sites on the biotinylated detection antibody specific to MDA. After the 45 min incubation time, wells were washed in order to eliminate excess conjugate and unbound sample or standard and HRP-conjugated antibody was added. The colour change was measured spectrophotometrically at a wavelength of 450 nm. The concentration of MDA in the

samples was then determined by comparing the OD of the samples to the standard curve and expressed as ng/$10^6$ cells.

#### 3.5.6.4 The total ROS measurement

To assess the total ROS production, 2',7'-dichlorodihydrofluorescein diacetate ($H_2$DCFDA; *Invitrogen, California, USA*) stain was used. $H_2$DCFDA is a chemically reduced form of fluorescein used as an indicator for ROS. Upon cleavage of the acetate groups by intracellular esterases and oxidation, non-fluorescent $H_2$DCFDA is converted to highly fluorescent 2',7'-dichlorofluorescein (DCF).

As for the other experiments, HUVECs were seeded in 6-well Petri dishes (150 000 cells/well) and transfected with GSTM1 siRNA. After 90 h incubation, the transfection solution was discarded, and cells were incubated for the next 6 h with the 30% control serum or uremic serum. Cells were then trypsinased, resuspended in 5 ml flow cytometry buffer (1% FBS in PBS) and incubated with 5 ul $H_2$DCFDA stain for 30 min at 37°C. Following the centrifugation at 400 g for 8 min, supernatants were removed, and cells were allowed to recover for 15 min at 37°C in 1 ml of MV2 growth media. In the final step, cells were resuspended in 500 μl FACS buffer. 5 μl *7-AAD-viability staining solution (eBioscience, San Diego, USA*) was added prior to measurement on the *Attune NxT Acoustic Focusing Flow Cytometer (Invitrogen, California, USA).* The results were analysed using *FlowJo, ver. 10.4 (Stanford Jr. University, USA).*

### 3.5.7 Analysis of cytokines expression in HUVECs

To explore the silencing effect of GSTM1 on endothelial inflammation in uremic conditions, 105 inflammatory markers were assessed simultaneously in cell protein extracts using *Proteome Profiler™ Human XL Cytokine Array Kit (R&D Systems, UK).*

For this purpose, four different treatments were performed in triplicates: $GSTM^{+/+}$ control/uremic serum, $GSTM^{+/-}$ control/uremic serum. After the incubation time expired, tratments were aspirated from all wells, and cells were washed with PBS. Cells were then scraped using a cell scraper in a lysis buffer 17 *(R&D Systems, UK),* supplemented with 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 10 μg/mL pepstatin. Cell lysates were obtained after centrifugation at 14 000 g for 5 min.

Pooled cell lysates (n=3/group) were probed on four separate nitrocellulose membranes and incubated over-night. Each membrane contained capture and control antibodies spotted in duplicate, which allowed simultaneous measuring of 105 cytokines expression. The following day membranes were washed, and further incubated for 1 h with a cocktail of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagents are then applied, and chemiluminescent spots were visualised on the *G-box (Kodak, UK).*

Results were quantified using *HLimage++ software* and normalised to the reference spots positioned at three of the corners of each blot *(Western Vision Software, US).* Results were presented as a graded heat map using *GraphPad Prism* software.

## 3.6 Data analysis

### 3.6.1 Statistical analysis

The analyses of demographic and clinical characteristics of study subjects, association of assessed gene polymorphisms with the risk of ESRD development and biomarkers of oxidative damage, the influence of oxidative stress parameters and endothelial dysfunction molecules on ESRD patients' survival, together with statistical analyses on cell cultures, were performed using *Statistical Package for the Social Sciences (SPSS) ver. 17.0 (Chicago, IL).*

Differences between the groups were compared using $\chi 2$ test for categorical variables. $\chi 2$ test was also used in order to test the deviation of the genotype distribution from Hardy–Weinberg equilibrium. All categorical variables were presented using frequency (n, %) counts.

After initial test for normality (*Kolmogorov–Smirnov test*), Student's t-test, Mann-Whitney, ANOVA with Bonferroni *post hoc* correction or Kruskal-Wallis tests were used to compare continuous variables, where appropriate. Depending on normality, all continuous variables were expressed as mean ± standard deviation (SD) or median with interquartile range (IQR).

Binary and multinomial logistic regressions were used to assess the contribution of the gene polymorphisms to the ESRD risk. Odds ratio (OD) with 95% confidence interval (CI) was computed after adjusting for age and gender as the possible confounders.

The predictive value of eight biomarkers of oxidative stress (SH-groups, carbonyls, nitrotyrosine, AOPP, MDA, MDA-adducts, TOS, PAB) and endothelial dysfunction (sICAM-1, sVCAM-1) measured in plasma of ESRD patients was evaluated using three Cox proportional hazards models. In these models adjustments were made for confounding variables: in Model 1 for demographic characteristics (cause of ESRD, age and gender); in Model 2 for clinical and laboratory parameters (BMI, smoking status, creatinine, cholesterol, CRP, serum albumin) and in Model 3 for comorbidities (diabetes, hypertension, myocardial infarction, peripheral vascular disease and cerebrovascular disease). Hazard ratios (HR) with 95% CI were calculated after adjusting for confounders presented in aforementioned models. ROC curves were used to obtain cut-off values of parameters oxidative stress (AOPP, PAB) and endothelial dysfunction (sICAM-1, sVCAM-1). Cut of values of thiol groups, protein carbonyls, nitrotirosine, MDA, MDAadd and TOS were determined based on median values. Patients were subsequently stratified into a group with low (below the cut-off) or a group with high (above the cut-off value) levels of a parameter observed. The cumulative overall and cardiovascular survival over the 8-year follow up was analysed by the Kaplan-Meier method.

### 3.6.2 Time to event modelling

Time-to-event modelling was used to evaluate the influence of *Nrf2*, *SOD2*, *GPX1* and *GSTM1* polymorphisms on overall and cardiovascular survival at three time points (3, 5,

and 8 years). The analysis was performed using MonolixSuite (version 2018R2, France, Lixoft SAS, 2018) with the stochastic approximation expectation maximization (SAEM) algorithm and the Markov Chain Monte Carlo procedure [253]. In addition, the R (version 3.5.1, The CRAN project) with dplyr (version 0.7.5), ggplot2 (version 2.2.1) and Rsmlx (version 1.1.0) packages was used. In order to describe the survival curve, multiple hazard functions were tested. Based on the lowest Akaike (AIC) and Bayesian information criterion (BIC) values, the best fit for hazard model was chosen.

The effects of patient's characteristics, *SOD2*, *GPX1*, *Nrf2*, and *GSTM1* polymorphisms, and their combinations, were investigated in a stepwise manner. In order to determine significant covariates, we used Pearson correlation analyses and the Wald test. Covariate model was built based on standard modelling procedures [254–256]. Covariates were introduced sequentially in the model when they produced a statistically significant reduction ($p < 0.05$) in the objective function value (OFV). The evaluation of the model was performed using bootstrap analysis (N = 500 replicates) to determine standard errors in the parameters' estimates. Moreover, visual predictive checks (VPCs) were performed on 1000 simulations to evaluate the adequacy of the survivor functions and enable the comparison of the simulated Kaplan–Meier curves (derived from simulated events) with the observed Kaplan–Meier estimates. The final survivor functions were used to calculate the median probability of survival at three time points (3, 5, and 8 years).

Level of statistical significance was set at $p<0.05$.

# 4. RESULTS

## 4.1 Demographic and clinical characteristics of the study group

Demographic and clinical characteristics of the study group are presented in Table 7. As shown in the table, 256 patients did not differ significantly from 374 controls in terms of age (62.41 ± 11.91 vs. 61.09 ± 10.78 years respectively) and gender: patient group was comprised of 147 males (57%) and 109 females (43%) while 199 males (53%) and 175 females (47%) were included in the control group. Diabetes was diagnosed in 27 (13%) patients while neither participant from the control group had this disease. As expected, differences between these groups were observed in hypertension, BMI and biochemical serum parameters.

**Table 7.** Demographic and clinical characteristics of ESRD patients and controls

| Variable | Controls | Patients | p |
|---|---|---|---|
| Age (years) | 61.09 ± 10.78 | 62.41 ± 11.91 | 0.155 |
| Gender, *n* (%) | | | |
| Male | 199 (53) | 147 (57) | |
| Female | 175 (47) | 109 (43) | 0.297 |
| Hypertension, *n* (%) [a] | | | |
| No | 245 (69) | 42 (20) | |
| Yes | 109 (31) | 171 (80) | <0.001 |
| Diabetes, *n* (%) [a] | | | |
| No | 281 (100) | 188 (87) | |
| Yes | 0 (0) | 27 (13) | <0.001 |
| BMI (kg/m$^2$) [a] | 26.17 ± 4.27 | 24.57 ± 4.07 | <0.001 |
| Biochemical serum parameters [a] | | | |
| Urea (mmol/L) | 5.36 ± 1.99 | 23.92 ± 5.01 | <0.001 |
| Creatinine (μmol/L) | 82.09 ± 15.04 | 856.93 ± 233.63 | <0.001 |
| Albumin (g/L) | 43.93 ± 3.79 | 38.61 ± 4.40 | <0.001 |
| Total cholesterol (mmol/L) | 4.34 ± 0.99 | 4.63 ± 1.14 | <0.003 |
| TAG (mmol/L) | 1.61 ± 0.59 | 2.06 ± 1.34 | <0.001 |
| Haemoglobin (g/L) | 142.14 ± 17.23 | 105.17 ± 14.76 | <0.001 |
| Haematocrit (%) | 41.00 ± 6.36 | 31.55 ± 4.56 | <0.001 |
| Serum iron (μmol/L) | 19.00 ± 4.32 | 11.29 ± 5.97 | <0.001 |
| Ferritin (ng/mL) | 56.44 ± 28.26 | 377.15 ± 258.81 | <0.001 |

All results are presented as mean ± SD or percentage. [a] Based on the data available.

The distribution of the underlying ESRD etiology was as follows: hypertensive nephrosclerosis (*n*=94, 37%), glomerulonephritis (*n*=34, 13%), diabetic nephropathy (*n*=24, 9%), polycystic renal disease (*n*=18, 7%), pyelonephritis (*n*=17, 7%), Balkan endemic nephropathy (*n*=62, 24%), obstructive nephropathy (*n*=3, 1%), and unknown (*n*=4, 2%).

## 4.2 The association of *SOD2*, *GPX1* and *Nrf2* polymorphisms with the risk of ESRD development

In the analysis of given polymorphisms (*SOD2* rs4880, *GPX1* rs1050450, *Nrf2* rs6721961), only *SOD2* reached statistically significant association with the risk of ESRD development (Table 8). Namely, *SOD2 Val/Val* carriers were more frequent among patients than in controls (32% vs. 22%), as well as, in two fold higher risk of ESRD development than *Ala/Ala* homozygotes (OR=2.01, 95%CI=1.28-3.16, p=0.002). By contrast, the low-activity genotypes of *GPX1* and *Nrf2* gene did not seem to influence the risk of ESRD development individually. The percentage of low-activity homozygotes for either *GPX1* or *Nrf2* gene was the same among patients and controls (12% and 2% respectively).

**Table 8.** The association of *SOD2, GPX1* and *Nrf2* polymorphisms with the risk of ESRD development

| Genotypes | Controls, *n* (%) | Patients, *n* (%) | OR (95% CI) | p |
|---|---|---|---|---|
| *SOD2* rs4880 | | | | |
| *Ala/Ala* | 113 (32) | 56 (23) | 1.0[a] | |
| *Ala/Val* | 167 (46) | 111 (45) | 1.31 (0.88–1.97) | 0.180 |
| *Val/Val* | 79 (22) | 77 (32) | 2.01 (1.28–3.16) | 0.002 |
| *GPX1* rs1050450 | | | | |
| *Pro/Pro* | 158 (42) | 101 (40) | 1.0[a] | |
| *Pro/Leu* | 164 (45) | 122 (48) | 1.22 (0.86–1.72) | 0.271 |
| *Leu/Leu* | 43 (12) | 32 (12) | 1.17 (0.69–1.98) | 0.558 |
| *Nrf2* rs6721961 | | | | |
| *C/C* | 241 (71) | 185 (73) | 1.0 [a] | |
| *C/A* | 94 (27) | 64 (25) | 0.87 (0.59–1.26) | 0.461 |
| *A/A* | 7 (2) | 4 (2) | 0.75 (0.21–2.61) | 0.649 |

Adjustments: age, gender, [a] Reference category, OR, odds ratio; CI, confidence interval; *n*, number of participants. For *SOD2* rs4880, genotyping was successful in 244 of 256 patients and 359 of 374 controls. For *GPX1* rs1050450, genotyping was successful in 255 of 256 patients and 365 of 374 controls. For *Nrf2* rs6721961, genotyping was successful in 253 of 256 patients and 342 of 374 controls.

Although *GPX1* and *Nrf2* polymorphisms alone did not show statistically significant association with the ESRD risk of development, they had a great impact when combined with *SOD2* polymorphism. Namely, individuals who carried both *GPX1* and *SOD2* low-activity genotypes (*GPX1 Leu/Leu* and *SOD2 Val/Val*) were at the highest risk of ESRD development (OR=3.27, 95%CI=1.21-8.82, p=0.019) (Table 9). Interestingly, *SOD2* low-activity genotype (*Val/Val*) with more active, *C/C* form of *Nrf2* gene, was also associated with an increased risk of ESRD development (OR =1.79, 95%CI=1.14-2.82, p=0.011).

**Table 9.** The association of combined *SOD2, GPX1* and *Nrf2* genotypes with the risk of ESRD development

| Genotypes | Controls, *n*(%) | Patients, *n*(%) | OR (95% CI) | p |
|---|---|---|---|---|
| *SOD2* and *GPX1* | | | | |
| *Ala/Ala+Val/Ala / Pro/Pro+Pro/Leu* | 230 (68) | 151 (62) | 1.0 [a] | |
| *Ala/Ala+Val /Ala / Leu/Leu* | 36 (10) | 16 (7) | 0.69 (0.37-1.28) | 0.239 |
| *Val/Val / Pro/Pro+Pro/Leu* | 67 (20) | 63 (26) | 1.49 (0.99-2.24) | 0.051 |
| *Val/Val / Leu/Leu* | 6 (2) | 13 (5) | 3.27(1.12-8.25) | 0.019* |
| *Nrf2* and *SOD2* | | | | |
| C/C / *Ala/Ala +Val/Ala* | 183 (55) | 122 (50) | 1.0[a] | |
| C/C / *Val/Val* | 49 (15) | 56 (23) | 1.80 (1.14-2.82) | 0.011* |
| C/A+A/A / *Ala/Ala +Val/Ala* | 76 (23) | 44 (18) | 0.85 (0.55-1.32) | 0.465 |
| C/A+A/A / *Val/Val* | 22 (7) | 21 (9) | 1.46 (0.76-2.80) | 0.256 |
| *Nr2* and *GPX1* | | | | |
| C/C / *Pro/Pro+Pro/Leu* | 202 (60) | 158 (63) | 1.0[a] | |
| C/C / *Leu/Leu* | 34 (10) | 26 (10) | 0.96 (0.55-1.67) | 0.876 |
| C/A+A/A / *Pro/Pro+Pro/Leu* | 94 (28) | 64 (25) | 0.84 (0.57-1.24) | 0.391 |
| C/A+A/A / *Leu/Leu* | 5 (2) | 4 (2) | 1.04 (0.27-3.97) | 0.951 |

Adjustments: age, gender, [a] Reference category, OR, odds ratio; CI, confidence interval; *n*, number of participants. For *SOD2* rs4880, genotyping was successful in 244 of 256 patients and 359 of 374 controls. For *GPX1* rs1050450, genotyping was successful in 255 of 256 patients and 365 of 374 controls. For *Nrf2* rs6721961, genotyping was successful in 253 of 256 patients and 342 of 374 controls.

## 4.3 The association *of SOD2, GPX*1, and *Nrf2* polymorphisms with byproducts of oxidative damage

High oxidative burden in ESRD patients leads to extensive damage of biological macromolecules. In this study, we investigated individual susceptibility of ESRD patients towards the panel of oxidative stress byproducts. In order to achieve this, we analyzed the association between polymorphisms of antioxidant regulatory and catalytic proteins (Nrf2, SOD2 and GPX1) and plasma levels of oxidatively damaged macromolecules (proteins and lipids), prooxidant-antioxidant balance and total oxidant status in ESRD patients.

### 4.3.1 The association of *SOD2, GPX1,* and *Nrf2* polymorphisms with biomarkers of protein oxidative damage

Along with aforementioned results which point to the influence of *SOD2* polymorphism on the risk of ESRD development, this polymorphism also showed multiple associations with biomarkers of oxidative damage.

*SOD2* polymorphism had a significant impact on the protein oxidative damage byproducts (Table 10). Namely, *SOD2 Val/Val* homozygotes had lower concentration of protein thiol groups when compared to *SOD2 Ala* carriers (p=0.049). The content of protein carbonyl groups was also higher in *SOD2 Val/Val* carriers in comparison to *SOD2 Ala/Ala*

homozygotes (p=0.037). *GPX1* and *Nrf2* polymorphisms did not show significant association with the protein thiol and carbonyl groups levels.

The assessment of two additional markers of protein oxidative damage (nitrotyrosine and AOPP) showed trends toward increase in *SOD2 Val/Val* homozygotes as well. Nitrotyrosine levels were 19% higher in *SOD2 Val/Val* homozygotes compared to SOD2 *Ala* carriers (p=0.129). Both of the assessed parameters were lower in ESRD patients with *Nrf2 C/A* or *A/A* genotypes in comparison to *C/C* homozygotes; however, only nitrotyrosine levels showed a statistically significant decrease of 19% (p=0.045). No significant associations were observed between *GPX1* polymorphism and nitrotyrosine or AOPP levels in our cohort of ESRD patients.

**Table 10.** The association of *SOD2, GPX1,* and *Nrf2* polymorphisms with byproducts of protein oxidative damage

| Genotypes | | PSH µmol/g | Carbonyls nmol/mg | AOPP µmol/l | Nitrotyrosine nmol/l |
|---|---|---|---|---|---|
| *SOD2* | *Ala/Ala* [a] | 6.9 [5.4-8.9]<br>100% | 2.14±0.13<br>100% | 64.3 [56.7-69.1]<br>100% | 64.5 [46.1-91.2]<br>100% |
| | *Val/Ala* | 6.7 [5.5-8.3]<br>97% | 2.30±0.23<br>107% | 61.1 [46.8-74.3]<br>95% | 59.3 [45.2-87.5]<br>95% |
| | *Val/Val* | 6.1 [5.3-7.3]<br>88% | 2.32±0.26*<br>108% | 64.6 [48.1-80.8]<br>100% | 72.3 [46.1-101.2]<br>112% |
| | *Ala/Ala+Val/Ala*[a] | 6.7 [5.5-8.5]<br>100% | 2.23±0.21<br>100% | 62.7 [48.4-73.3]<br>100% | 60.3 [46.1-87.5]<br>100% |
| | *Val/Val* | 6.1 [5.3-7.3]*<br>91% | 2.32±0.26<br>104% | 64.6 [48.1-80.8]<br>103% | 72.3 [46.1-101.2]<br>119% |
| *GPX1* | *Pro/Pro*[a] | 6.1 [5.1-8.0]<br>100% | 2.27±0.23<br>100% | 63.8 [46.5-73.7]<br>100% | 64.5 [50.1-93.1]<br>100% |
| | *Pro/Leu* | 6.5 [5.5-8.3]<br>106% | 2.29±0.24<br>101% | 63.1 [49.3-74.4]<br>99% | 64.5 [46.1-93.1]<br>100% |
| | *Leu/Leu* | 6.6 [5.5-7.3]<br>108% | 2.19±0.19<br>96% | 67.5 [48.2-85.1]<br>106% | 60.2 [44.7-78.7]<br>93% |
| | *Pro/Pro+Pro/Leu* | 6.3 [5.4-8.1]<br>100% | 2.28±0.23<br>100% | 63.5 [48.6-74.3]<br>100% | 64.5 [46.1-93.1]<br>100% |
| | *Leu/Leu* | 6.6 [5.5-7.3]<br>105% | 2.19±0.19<br>96% | 67.5 [48.2-85.1]<br>106% | 60.3 [44.7-78.7]<br>93% |
| *Nrf2* | *C/C*[a] | 6.3 [5.5-8.0]<br>100% | 2.24±0.22<br>100% | 65.7 [48.8-75.7]<br>100% | 64.5 [50.1-93.1]<br>100% |
| | *C/A+A/A* | 6.4 [5.5-8.2]<br>102% | 2.34±0.25<br>104% | 56.7 [44.6-70.5]<br>86% | 52.3 [38.5-87.5]*<br>81% |

All values are presented as mean ± SD or median with interquartile range (IQR). [a] referent genotype. PSH, protein thiol groups; AOPP, advanced oxidation protein products; * $p < 0.05$ when compared to the referent genotype. 100% presented values of plasma protein oxidation byproducts measured in ESRD patients with referent *SOD2, GPX1* or *Nrf2* genotypes

### 4.3.2 The association of *SOD2, GPX1,* and *Nrf2* polymorphisms with biomarkers of lipid oxidative damage

In accordance with aforementioned results, both assessed byproducts of lipid oxidative damage (MDA and MDA adducts) were elevated in *SOD2 Val/Val* homozygotes (Table 11). MDA levels were 18% higher in patients homozygous for *SOD2 Val* allele when compared to *SOD2 Ala/Ala* homozygotes (p=0.036). Similarly, patients with *SOD2 Val/Val* genotype had 9% higher levels of MDAadd in comparison to ESRD patients carrying at least one *SOD2 Ala* allele (0.046).

Regarding the influence of *GPX1* and *Nrf2* polymorphisms on lipid oxidative damage byproducts, the ESRD patients carrying low activity genotypes of these genes had slightly higher MDA (13% and 9%, respectively) levels in comparison to homozygotes for referent genotypes (Table 11). However, these changes remained statistically insignificant.

**Table 11.** The association of *SOD2, GPX1,* and *Nrf2* polymorphisms with biomarkers of lipid oxidative damage

| Genotypes | | MDA (mmol/l) | MDAadd (pmol/mg) |
|---|---|---|---|
| *SOD2* | *Ala/Ala* [a] | 2.17±0.78<br>100% | 40.28±8.04<br>100% |
| | *Val/Ala* | 2.37±0.72<br>109% | 38.93±9.42<br>97% |
| | *Val/Val* | 2.57±0.79*<br>118% | 43.17±10.15<br>107% |
| | *Ala/Ala+Val/Ala*[a] | 2.29±0.75<br>100% | 39.49±8.84<br>100% |
| | *Val/Val* | 2.57±0.79*<br>112% | 43.17±10.15*<br>109% |
| *GPX1* | *Pro/Pro*[a] | 2.33±0.79<br>100% | 40.17±7.63<br>100% |
| | *Pro/Leu* | 2.34±0.74<br>100% | 41.82±11.00<br>104% |
| | *Leu/Leu* | 2.65±0.81<br>114% | 38.91±8.19<br>97% |
| | *Pro/Pro+Pro/Leu* | 2.33±0.77<br>100% | 41.04±9.54<br>100% |
| | *Leu/Leu* | 2.65±0.81<br>113% | 38.91±8.19<br>97% |
| *Nrf2* | *C/C*[a] | 2.17±0.78<br>100% | 40.28±8.04<br>100% |
| | *C/A+A/A* | 2.37±0.72<br>109% | 38.93±9.42<br>97% |

All values are presented as mean ± SD. [a] referent genotype. MDA, malondialdehyde; MDAadd, malondialdehyde adducts; * $p < 0.05$ when compared to the referent genotype. 100% presented values of plasma lipid oxidation byproducts measured in ESRD patients with referent *SOD2, GPX1* or *Nrf2* genotypes

### 4.3.3 The association of *SOD2, GPX1,* and *Nrf2* polymorphisms with TOS and PAB

Along with measurements of protein and lipid oxidative damage byproducts, the additive effect of different oxidant molecules was further assessed, and presented through the levels of TOS and PAB (Table 12). Once again, the *SOD2* polymorphism had the most prominent effect on these parameters. TOS and PAB concentrations were increased in patients with *SOD2 Val/Val* genotype (33% and 27% respectively), but only elevation of PAB reached statistical significance (p=0.044). Other two polymorphisms did not significantly influence the TOS and PAB levels in ESRD patients, although the increase of TOS by 52% in *GPX1 Leu/Leu* homozygotes can be noted.

**Table 12**. The association of *SOD2, GPX1,* and *Nrf2* polymorphisms with TOS and PAB

| Genotypes | | TOS (μmol $H_2O_2$ equiv./l ) | PAB (HK units) |
|---|---|---|---|
| ***SOD2*** | *Ala/Ala* [a] | 18.6 [13.7–34.1]<br>100% | 142.2 [71.8–184.4]<br>100% |
| | *Val/Ala* | 18.2 [12.8–50.8]<br>98% | 113.9 [61.8–218.3]<br>80% |
| | *Val/Val* | 24.5 [12.8–56.3]<br>131% | 160.4 [105.1–251.5]<br>113% |
| | *Ala/Ala+Val/Ala*[a] | 18.4 [13.3–48.3]<br>100% | 126.6 [67.7–211.0]<br>100% |
| | *Val/Val* | 24.5 [12.8–56.3]<br>133% | 160.4 [105.1–251.5] *<br>127% |
| ***GPX1*** | *Pro/Pro*[a] | 17.8 [14.0–41.0]<br>100% | 153.8 [83.8–233.0]<br>100% |
| | *Pro/Leu* | 19.1 [12.8–53.2]<br>107% | 143.0 [71.7–226.0]<br>93% |
| | *Leu/Leu* | 27.2 [16.8–47.5]<br>152% | 128.6 [68.4–191.8]<br>84% |
| | *Pro/Pro+Pro/Leu* | 18.2 [13.2–48.4]<br>100% | 146.8 [78.2–229.9]<br>100% |
| | *Leu/Leu* | 27.2 [16.8–47.5]<br>145% | 128.6 [68.4–191.8]<br>88% |
| ***Nrf2*** | *C/C*[a] | 22.0 [13.3–53.6]<br>100% | 142.6 [83.0–216.9]<br>100% |
| | *C/A+A/A* | 16.2 [13.2–28.3]<br>73% | 159.4 [72.8–252.6]<br>112% |

All values are presented as median with interquartile range (IQR). [a] referent genotype. TOS, total oxidant status; PAB, prooxidant antioxidant balance;. * $p < 0.05$ when compared to the referent genotype. 100% presented values of plasma TOS and PAB measured in ESRD patients with referent *SOD2, GPX1* or *Nrf2* genotypes

## 4.4 The association of *SOD2*, *GPX1*, and *Nrf2* polymorphisms with circulating soluble adhesion molecules

In inflammatory and pro-oxidant environment in patients with ESRD, endothelium responds by expressing cellular adhesion molecules (ICAM-1 and VCAM-1) that facilitate the adhesion of leukocytes to the endothelial cells. In this study, we assessed the association between the concentrations of circulating sICAM-1 and sVCAM-1 adhesion molecules, determined in plasma of ESRD patients, and polymorphisms of antioxidant regulatory and catalytic genes (Nrf2, SOD2 and GPX1). The results presented herewith show that the variant homozygotes of all assessed genes have slightly elevated levels of sICAM-1 in comparison to ESRD patients carrying at least one referent allele, although statistically insignificant (Table 13). The assessed polymorphisms did not have an influence on sVCAM-1 levels.

**Table 13**. The association of *SOD2*, *GPX1*, and *Nrf2* polymorphisms with sICAM-1 and sVCAM-1

| Genotypes | | sICAM-1 (pg/ml) | sVCAM-1 (ng/ml) |
|---|---|---|---|
| *SOD2* | *Ala/Ala* [a] | 87.8 [71.4–95.8]<br>100% | 650.5 [479.5–687.5]<br>100% |
| | *Val/Ala* | 85.8 [74.2–100.5]<br>98% | 649.6 [600.2–688.3]<br>99% |
| | *Val/Val* | 92.1 [71.0–117.0]<br>107% | 647.7 [578.2–685.2]<br>99% |
| | *Ala/Ala+Val/Ala* [a] | 86.4 [73.8–99.3]<br>100% | 650.4 [556.2–688.0]<br>100% |
| | *Val/Val* | 92.1 [71.0–117.0]<br>107% | 647.7 [578.2–685.2]<br>99% |
| *GPX1* | *Pro/Pro* [a] | 93.3 [74.5–111.4]<br>100% | 642.6 [544.2-689.0]<br>100% |
| | *Pro/Leu* | 81.8 [72.24–104.8]<br>88% | 651.4 [601.7-675.9]<br>101% |
| | *Leu/Leu* | 92.0 [70.1–99.4]<br>98% | 654.3 [480.8-720.7]<br>102% |
| | *Pro/Pro+Pro/Leu* | 87.8 [73.4–106.8]<br>100% | 649.1 [578.9-682.3]<br>100% |
| | *Leu/Leu* | 92.0 [70.1–99.4]<br>104% | 654.3 [480.8-720.7]<br>101% |
| *Nrf2* | *C/C* [a] | 87.0 [71.3–101.9]<br>100% | 647.7 [548.0-686.4]<br>100% |
| | *C/A+A/A* | 92.7 [75.7–112.5]<br>106% | 656.7 [620.6–688.3]<br>101% |

All values are presented as median with interquartile range (IQR). [a] referent genotype. sICAM, soluble Intercellular Adhesion Molecule 1; sVCAM, soluble Vascular Cell Adhesion Molecule 1. 100% presented values of plasma sICAM-1 and sVCAM-1 measured in ESRD patients with referent *SOD2*, *GPX1* or *Nrf2* genotypes

## 4.5 The influence of *SOD2, GPX1, Nrf2* and *GSTM1* polymorphisms on ESRD patient survival

In this study multiple associations between polymorphisms of antioxidant, *SOD2, GPX1* and *Nrf2* genes with the risk of ESRD development and byproducts of oxidative damage were observed. In a further course of this thesis we aimed to examine their prognostic role on 8-year overall and cardiovascular survival in ESRD patients. As we previously described the strong impact of *GSTM1* polymorphism on oxidative phenotype, [126], and 5-year survival in ESRD patients' cohort [124], we wanted to further deepen our investigation by assessing whether it can be included in the panel of genes designed to predict 8-year survival in ESRD patients.

During the 8-year follow-up period, overall mortality rate was 66,8% (133 patients). Mean follow up period for survivors was 55.38±33.21 months. Causes of death were as follows: cardiovascular disease (n=73), cachexia (n=2), infection or sepsis (n=12), gastrointestinal bleeding (n=2), chronic obstructive lung disease (n=1), malignant disease (n=7) and unknown (n=36). Time-to-event modeling was preformed to evaluate overall and cardiovascular survival at three time points (3, 5 and 8 years of follow up). Uniform hazard model best fitted the overall survival (Figure 25), while the cardiovascular survival data was best explained by Weibull hazard model (Figure 27).

### 4.5.1 The influence of *SOD2, GPX1, Nrf2* and *GSTM1* polymorphisms on overall survival

In the first step of covariate analysis, patients' age and *GSTM1* polymorphism were identified as the most significant predictors of overall survival ($p<0.001$ and $p<0.05$ respectively). The model of the overall survival was further improved by addition of the combination of *Nrf2* and *GPX1* polymorphisms ($p<0.05$), as two-level categorical covariates in the model. In the following steps, inclusion of remaining covariates did not improve the overall survival model. In the backward steps, no covariate was excluded from the full model.

Details of the model building are given in the Table 14. Notably, in unicovariate analysis combined *SOD2* and *GPX1* polymorphisms significantly decreased OFV value ($p<0.05$) as the second step of covariates testing. However, their prognostic impact on the overall survival was less significant than age, *GSTM1* polymorphism and *GPX1/Nrf2* combined genotypes.

Developed final hazard model for the overall survival function was calculated as follows:

$$h_t = \frac{1}{Te - t}, \text{where } Te = 139 \cdot \left(\frac{AGE}{65}\right)^{-2.27} \cdot \begin{cases} e^{0.765}, & if\ Nrf2\ C/C + GPX1Leu/Leu \\ e^{-0.357}, & if\ GSTM1\ null\ genotype \end{cases}$$

**Table 14**. Summary of covariate testing in overall survival model building

| | *p*-value | | | |
|---|---|---|---|---|
| **Covariate** | **Base** | **Uni-Covariate** | **Bi-Covariate** | **Three-Covariate** |
| Age (years) | <0.001 | + | + | + |
| *GPX1 (Pro/Pro and Pro/Leu vs Leu/Leu)* | n.s. | n.s. | n.s. | n.s. |
| *GSTM1 (null vs active)* | <0.05 | <0.05 | + | + |
| *Nrf2 (C/A and A/A vs C/C)* | n.s. | n.s. | n.s. | n.s. |
| *SOD2 (Val/Ala vs Val/Val)* | n.s. | n.s. | n.s. | n.s. |
| *SOD2_GPX1* | n.s. | <0.05 | n.s. | n.s. |
| *SOD2_GSTM1* | n.s. | n.s. | n.s. | n.s. |
| *SOD2_Nrf2* | n.s. | n.s. | n.s. | n.s. |
| *GPX1_Nrf2* | n.s. | <0.05 | <0.05 | + |
| *GPX1_GSTM1* | n.s. | n.s. | n.s. | n.s. |
| *Nrf2_GSTM1* | n.s. | n.s. | n.s. | n.s. |

n.s. – not significant; + - covariate included in the model.

The estimated parameters of the hazard function and standard errors of the corresponding parameters of the final model are presented in Table 15.

**Table 15**. Final population parameter values for the overall survival model

| **Parameter** | **Estimated value** | **Standard error** |
|---|---|---|
| $T_{pop}$ | 139 | 17.8 |
| Age (years) effect on T | -2.27 | 0.5 |
| *GSTM1* (*null*) effect on T | -0.357 | 0.168 |
| *Nrf2+GPX1* (*C/C+Leu/Leu*) effect on T | 0.765 | 0.347 |
| Variance of T | 0.649 | 0.113 |

$T_{pop}$ – population scale parameter indicating time at which survival equals 0.

The empirical and model-predicted Kaplan-Meier plots of the survival probability, with regard to covariates included in the survival model, suggest that this model is suitable to predict the survival in our cohort of ESRD patients (Figure 25). Overall survival in ESRD patients was dependent on previously established parameters (*GSTM1* polymorphism and age (Figure 25, B&C)) and the combination of the best survival genotypes of the *Nrf2* (*C/C*) and *GPX1* (*Leu/Leu*) (Figure 25, D).

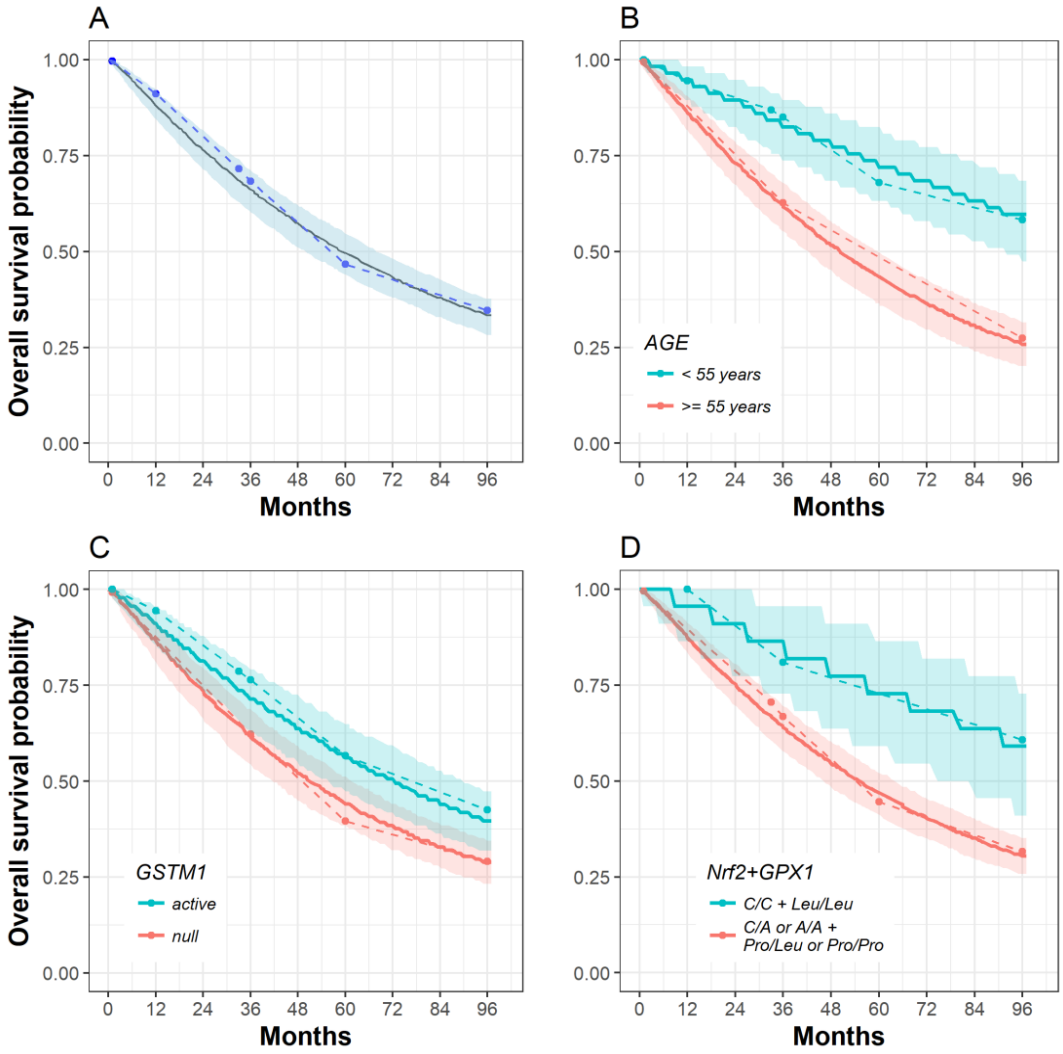



**Figure 25.** Kaplan-Meier curves for the overall survival of: observed events (dashed line) including censored data (circle), and survival model with uniform hazard estimates (solid line) and its 90% prediction interval (shaded area). No covariate stratification (A); Stratification by final survival model covariates: age (B), *GSTM1 active* genotype is associated with longer overall survival (C), "best survival" genotypes of the *Nrf2 (C/C)* and *GPX1 (Leu/Leu)* are associated with longer overall survival (D).

The greatest benefit of the model is in its predictability. Hence, it can be used to analytically calculate the probabilities of the survival using patients' characteristics (age together with assesed polymorphisms). Table 16 shows the probabilities of the overall survival at three time points (3, 5 and 8 years). Thus, average patient aged 55, carrier of the "best survival" genotypes (*GSTM1-active* and *Nrf C/C+GPX1 Leu/Leu*) has much better probability of 8-year overall survival when compared to age-matched carrier of *GSTM1-null* and *Nrf C/A* or *A/A + GPX1 Pro/Leu* or *Pro/Pro* genotypes (75.43% vs. 12.48%). This difference is even more pronaunced in older patients, since 70 years old patient with the aforementioned "best survival genotypes" had 54.14% probability to survive in comparison to age-matched carrier of *GSTM1-null* and *Nrf C/A* or *A/A + GPX1 Pro/Leu* or *Pro/Pro* genotypes whose probability to survive was estimated to be ≈0.

**Table 16.** Model-based prediction of overall survival

| Patients' characteristics | | | Probability (%) to survive at least | | |
|---|---|---|---|---|---|
| **AGE (years)** | ***GSTM1 genotype*** | ***Nrf2+GPX1 genotype*** | **3 years** | **5 years** | **8 years** |
| 40 | *active* | *C/C + Leu/Leu* | 95.91 | 93.10 | 88.73 |
| | | *C/A or A/A + Pro/Leu or Pro/Pro* | 91.02 | 84.59 | 74.25 |
| | *null* | *C/C + Leu/Leu* | 94.11 | 90.00 | 83.52 |
| | | *C/A or A/A + Pro/Leu or Pro/Pro* | 86.92 | 77.28 | 61.40 |
| 55 | *active* | *C/C + Leu/Leu* | 91.40 | 85.27 | 75.43 |
| | | *C/A or A/A + Pro/Leu or Pro/Pro* | 80.62 | 65.75 | 40.80 |
| | *null* | *C/C + Leu/Leu* | 87.49 | 78.31 | 63.23 |
| | | *C/A or A/A + Pro/Leu or Pro/Pro* | 71.23 | 48.16 | 12.48 |
| 70 | *active* | *C/C + Leu/Leu* | 84.68 | 73.22 | 54.14 |
| | | *C/A or A/A + Pro/Leu or Pro/Pro* | 64.29 | 35.21 | 1.14 |
| | *null* | *C/C + Leu/Leu* | 77.42 | 59.79 | 30.42 |
| | | *C/A or A/A + Pro/Leu or Pro/Pro* | 45.88 | 6.71 | ≈0 |

The empirical Kaplan-Meier plots for the *SOD2*, *GPX1* and *Nrf2* polymorphisms, which were not included in the model, are given in Figure 26. These results show the separation of survival curves, with longer survival of ESRD patients with referent genotypes of *SOD2* and *Nrf2* genes, however these changes did not reach statistical significance. On the other hand, patients with *GPX1 Leu/Leu* genotype had significantly longer overal survival after 8 year follow up, than those carrying *GPX1 Pro/Leu* or *Pro/Pro* genotypes (p=0.038).

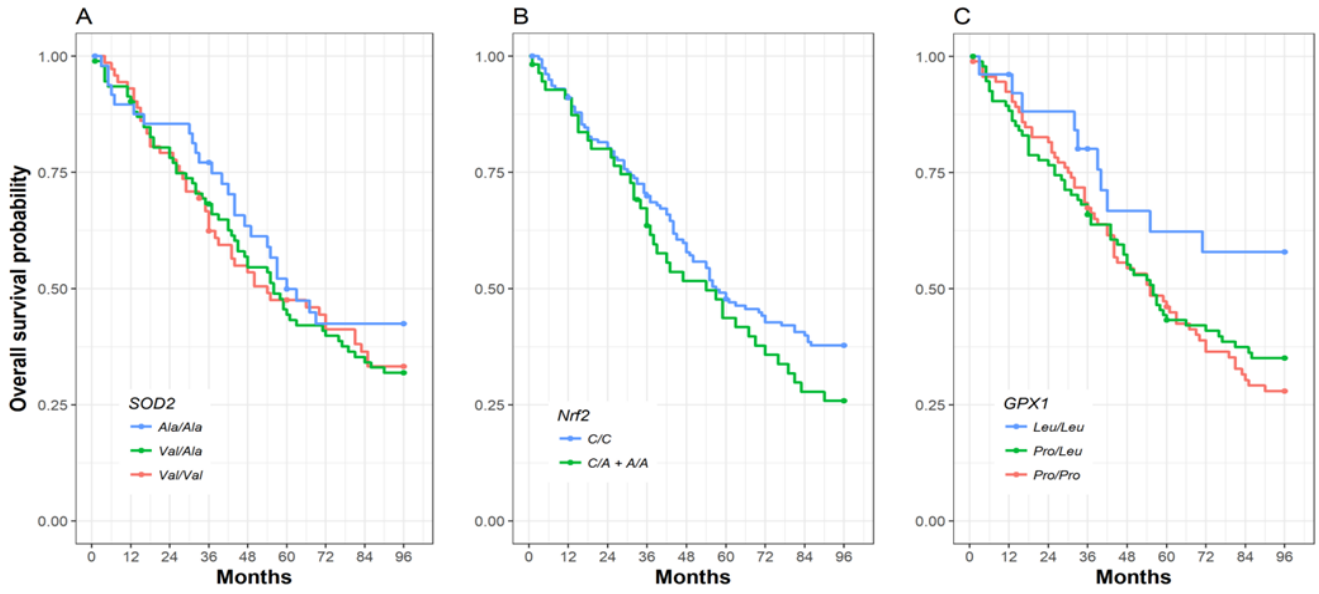



**Figure 26**. Empirical Kaplan-Meier curve (lines) and censored data (circle) for overall survival data based *SOD2* (A), *Nrf2* (B) and *GPX1* (C) genotype.

### 4.5.2 The influence of *SOD2, GPX1, Nrf2* and *GSTM1* polymorphisms on cardiovascular survival

After the stepwise covariate inclusion methodology was applied, developed cardiovascular survival model included combined impact of patients' age as a continuous variable, and polymorphisms of *GSTM1* and *GPX1* as two-level categorical variables. As expected, patients' age had the greatest prognostic influence on the survival (decreasing OFV by 18.28 units, p<0.005). The model of the cardiovascular survival was further improved by the addition of *GSTM1 (*p<0.01) and *GPX1 (*p<0.05) polymorphisms that in total reduced OFV for additional 12.11 units. In the backward steps, no covariate was excluded from the full model.

Detailes of the model building are given in Table 17. It shows that in uni-covariate analysis, combination of *SOD2* or *Nrf2* with *GSTM1* produced statistically significant reduction in OFV (p<0.05) as well. Moreover, the separation in the empirical Kaplan-Meier plots for the individual *SOD2* and *Nrf2* polymorphisms exists (Figure 28), indicating that this should be further examined. However, their prognostic impact on the cardiovascular survival was less significant than age, *GSTM1* and *GPX1* polymorphisms.

The final hazard model for the cardiovascular survival data was calculated as follows:

$$h_t = \frac{1.64}{Te} \cdot \left(\frac{t}{Te}\right)^{0.64}, \text{ where } Te = 169 \cdot \left(\frac{AGE}{65}\right)^{-2.61} \cdot \begin{cases} e^{0.849}, & if GPX1\ Leu/Leu\ genotype \\ e^{-0.632}, & if\ GSTM1\ null\ genotype \end{cases}$$

**Table 17.** Summary of covariate testing in cardiovascular survival model building

| | ***p*-value** | | | |
|---|---|---|---|---|
| **Covariate** | **Base** | **Uni-Covariate** | **Bi-Covariate** | **Three-Covariate** |
| Age (years) | <0.005 | + | + | + |
| *GPX1 (Pro/Pro* and *Pro/Leu vs Leu/Leu)* | n.s. | n.s. | <0.05 | + |
| *GSTM1 (active vs null)* | n.s. | <0.01 | + | + |
| *Nrf2 (C/A and A/A vs C/C)* | n.s. | n.s. | n.s. | n.s. |
| *SOD2 (Val/Ala vs Val/Val)* | n.s. | n.s. | n.s. | n.s. |
| *SOD2_GPX1* | n.s. | n.s. | n.s. | n.s. |
| *SOD2_GSTM1* | n.s. | <0.05 | n.s. | n.s. |
| *SOD2_Nrf2* | n.s. | n.s. | n.s. | n.s. |
| *GPX1_Nrf2* | n.s. | n.s. | n.s. | n.s. |
| *GPX1_GSTM1* | n.s. | n.s. | n.s. | n.s. |
| *Nrf2_GSTM1* | n.s. | p<0.05 | n.s. | n.s. |

n.s. – not significant; + - covariate included in the model.

The estimated parameters and standard errors of the corresponding parameters of the final models are reported in Table 18.

**Table 18.** Final population parameter values for the cardiovascular survival model

| Parameter | Estimated value | Standard error |
|---|---|---|
| $T_{pop}$ | 169 | 34 |
| Age effect on T | -2.61 | 0.796 |
| *GPX1 (Leu/Leu)* effect on T | 0.849 | 0.457 |
| *GSTM1* (*null*) effect on T | -0.632 | 0.227 |
| $p_{pop}$ | 1.64 | 0.357 |
| Variance of T | 0.954 | 0.205 |

$T_{pop}$ – population scale parameter indicating time at which survival equals 0.4; $p_{pop}$ – population shape parameter.

Good concordance was achieved between the empirical and the median of the model-predicted Kaplan-Meier curves. Empirical Kaplan-Meier curve lies within the simulation prediction interval indicating there is no model misspecification (Figure 27). Variant *GPX1* (*Leu/Leu*) genotype, together with *GSTM1* polymorphism and age contributed to longer cardiovascular survival.

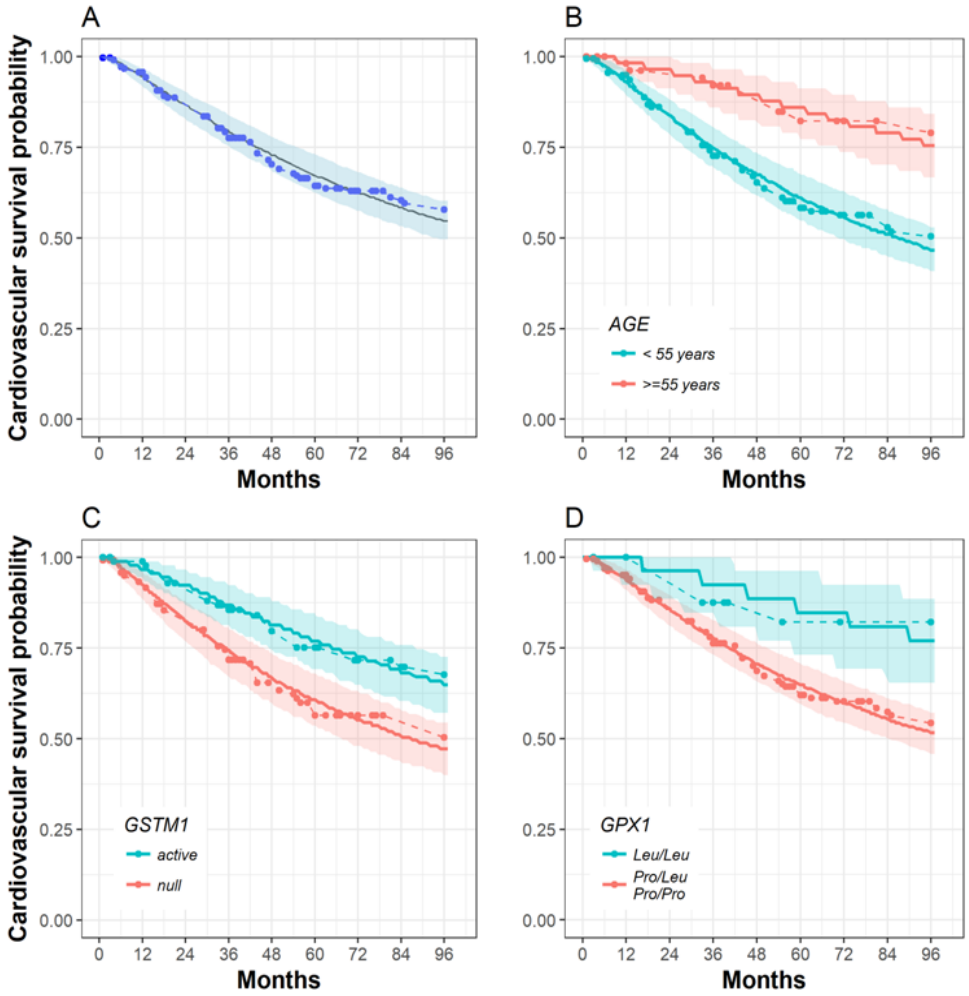



**Figure 27.** Kaplan-Meier curves for the cardiovascular survival of: observed events (dashed line) including censored data (circle), and survival model with Weibull hazard estimates (solid line) and its 90% prediction interval (shaded area). No covariate stratification (A); Stratification by final survival model covariates: age (B), *GSTM1 active* genotype is associated with longer cardiovascular survival (C), *GPX1* genotype(*Leu/Leu*) genotype contributes to longer cardiovascular survival (D).

The probabilities of the cardiovacular survival at three time points (3, 5 and 8 years) with respect to combination of *GSTM1* and *GPX1* polymorphisms are presented in Table 19. Results clearly indicate age and *GSTM1-null* genotype together with *GPX1 Pro/Leu* or *Pro/Pro* genotypes, as the main determinants of shorter cardiovascular-specific survival. For instance, patient aged 55, carrier of best survival genotype (*GSTM1-active* and *GPX1 Leu/Leu*) has much better probability of 8-year cardiovascular survival when compared to age-matched carrier of *GSTM1-null* and *GPX1 Pro/Leu* or *Pro/Pro* genotypes (92.42% *vs.* 40.88%). This difference is even greater in older patients, since 70 years old patient with the aforementioned best survival genotype had 80.14% probability to survive in comparison to age-matched carrier of *GSTM1-null* and *GPX1 Pro/Leu* or *Pro/Pro* genotypes whose probability of cardiovascular survival was estimated to be only 8.11%.

**Table 19**. Model-based prediction of cardiovascular survival

| **Patients' characteristics** | | | **Probability (%) to survive at least** | | |
|---|---|---|---|---|---|
| **AGE (years)** | ***GSTM1 genotype*** | ***GPX1 genotype*** | **3 years** | **5 years** | **8 years** |
| *40* | *active* | *Leu/Leu* | 99.60 | 99.07 | 98.00 |
| | | *Pro/Leu Pro/Pro* | 98.39 | 96.31 | 92.20 |
| | *null* | *Leu/Leu* | 98.87 | 97.40 | 94.47 |
| | | *Pro/Leu Pro/Pro* | 95.52 | 89.95 | 79.54 |
| *55* | *active* | *Leu/Leu* | 98.43 | 96.42 | 92.42 |
| | | *Pro/Leu Pro/Pro* | 93.85 | 86.35 | 72.81 |
| | *null* | *Leu/Leu* | 95.65 | 90.23 | 80.07 |
| | | *Pro/Leu Pro/Pro* | 83.60 | 66.11 | 40.88 |
| *70* | *active* | *Leu/Leu* | 95.67 | 90.27 | 80.14 |
| | | *Pro/Leu Pro/Pro* | 83.67 | 66.22 | 41.03 |
| | *null* | *Leu/Leu* | 88.26 | 74.92 | 53.58 |
| | | *Pro/Leu Pro/Pro* | 60.49 | 31.29 | 8.11 |

The empirical Kaplan-Meier plots for the *SOD2* and *Nrf2* polymorphisms, which were not included in the model, are given in Figure 28. Acording to the results, *SOD2 Ala* homozygotes had the longest cardiovascular survival, which gradually decreased with an addition of each low activity, *SOD Val* allele (p=0.076). Regarding *Nrf2* polymorphism, clear separation of survival curves was observed after 5 years of follow up. Namely, ESRD patients with *Nrf2 C/C* genotype had a trend towards longer survival than those with either *C/A* or *A/A* genotypes (p=0.097).

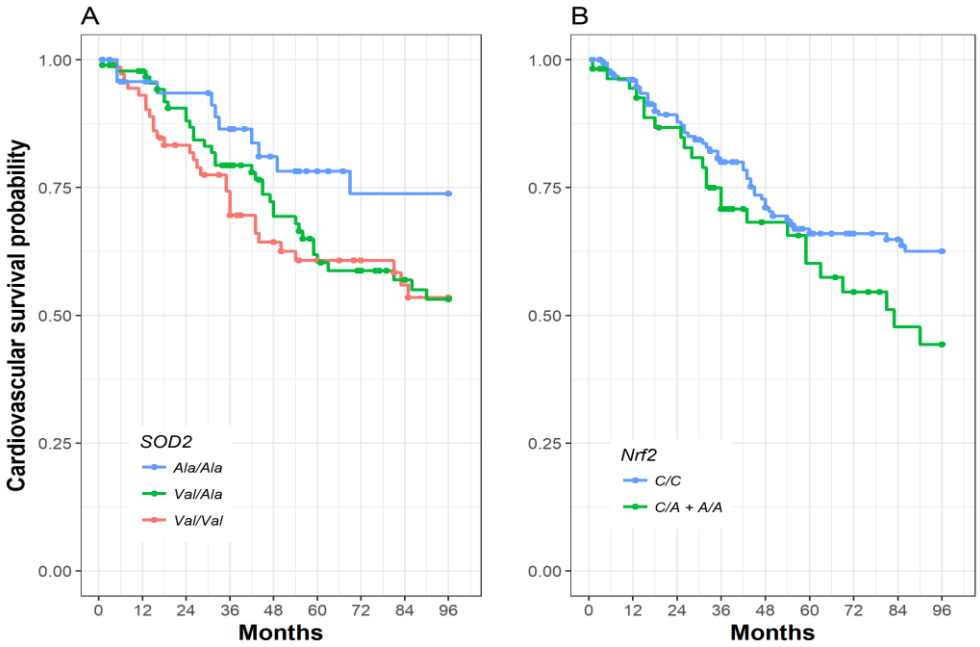



**Figure 28**. Empirical Kaplan-Meier curve (lines) and censored data (circle) for cardiovascular survival data based *SOD2* (A), and *Nrf2* (B) genotype.

## 4.6. The influence of biomarkers of oxidative stress and endothelial dysfunction on long term survival of ESRD patients

In the further course of this study, the influence of biomarkers of oxidative stress (thiol groups, carbonyls, nitrotyrosine, AOPP, MDA, MDA-adducts, TOS, PAB) and endothelial dysfunction (sICAM-1, sVCAM-1) on 8-year survival of ESRD patients was analysed. Patients were stratified into two groups based on the cut-off value of the observed parameter. Cut-off values for AOPP, PAB, sICAM-1 and sVCAM-1 were obtained from the ROC curves and area under the curve (AUC), 95% CI, sensitivity and specificity, as well as the calculated statistical significance are given in the Table 20. Cut-off values for other analysed biomarkers of oxidative stress were median values of these parameters. Kaplan-Meier survival analysis and calculation of HR after adjusting for confounders (presented in three models as described in the Method section) were consequently performed. According to the results, AOPP, PAB, MDA, sICAM-1 and sVCAM-1 had a substantial predictive power of a long term, 8-year survival (Table 21).

**Table 20.** Cut-off points and AUC values

| Parameter | Cut-off value | AUC overall survival | AUC cardiovascular survival |
|---|---|---|---|
| AOPP | 60.9 μmol/L | 0.6 (95% CI 0.50–0.68, $p$ = 0.052, specificity: 0.51, sensitivity: 0.62) | 0.64 (95% CI 0.55–0.73, $p$ = 0.004, specificity: 0.51, sensitivity: 0.72) |
| PAB | 130.76 HK units | 0.6 (95% CI 0.51–0.70, $p$ =0.036, sensitivity: 0.62, specificity: 0.56) | 0.61 (95% CI 0.51–0.70, $p$ = 0.035, sensitivity: 0.65, specificity: 0.51) |
| sICAM-1 | 77.56 ng/mL | 0.63 (95% CI 0.51–0.75, $p$ = 0.052, specificity 0.42, sensitivity 0.69) | 0.63 (95% CI 0.51–0.75, $p$ = 0.052, specificity 0.42, sensitivity 0.69) |
| sVCAM-1 | 644.09 ng/mL | 0.64 (95% CI 0.55–0.73, $p$ = 0.003, specificity: 0.61, sensitivity: 0.62) | 0.61 (95% CI 0.51–0.70, $p$ = 0.033, specificity: 0.53, sensitivity: 0.69) |

ESRD patients with AOPP levels above the cut-off value of 60.9 µmol/L had shorter cardiovascular survival (log-rank 5.316, $p$ = 0.021, Figure 29 A), which stayed significant even after adjusting for the confounders in Models 1–3 (Table 21). AOPP did not show statistically significant impact on overall survival (Figure 29 A), Table 21). Patients with high PAB levels (over 130.76 HK units) had significantly shorter both overall and cardiovascular survival (log-rank 9.689, $p$ = 0.002 and log-rank 6.321, $p$ = 0.012 respectively, Figure 29 B). Models 1–3 lead to modest change in survival compared to non-adjusted model (Table 21).

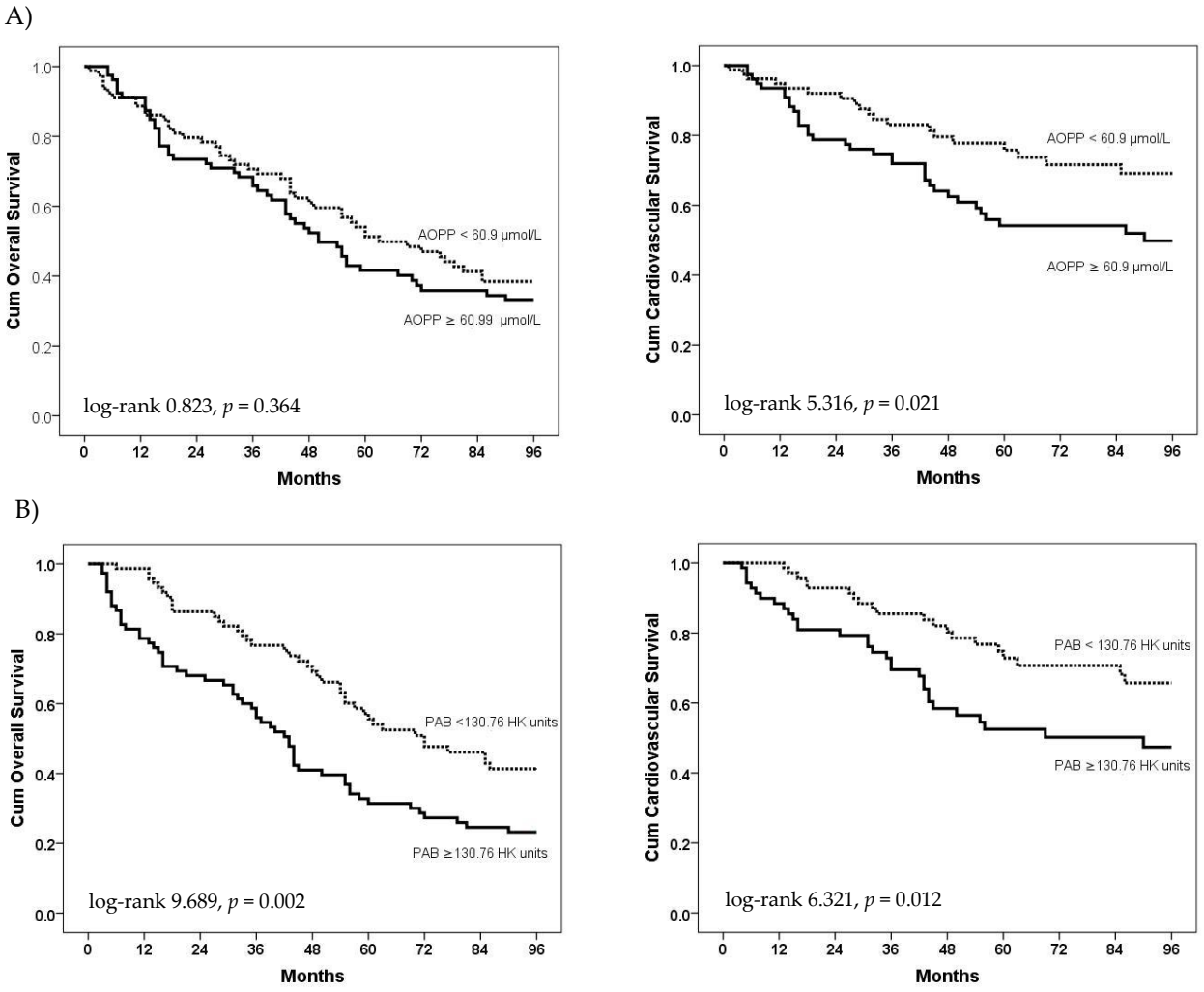



**Figure 29.** Kaplan-Meier survival curves for 8-year overall and cardiovascular survival of ESRD patients dichotomized by cut-off values of AOPP A) and PAB B).

ESRD patients with concentrations of MDA above the median (2.33 µmol/L) had a trend towards shorter overall and cardiovascular survival (Breslow: 3.766, $p$ = 0.052 and Breslow: 3.218, $p$ = 0.073, respectively) compared to the patients with lower MDA concentrations (Figure 30). Moreover, Cox regression analysis showed that the patients with high levels of MDA had higher risk of overall (HR 1.5, 95% CI 1.0-2.1, $p$ = 0.051) and cardiovascular mortality (HR 1.6, 95% CI 1.0-2.8, $p$ = 0.061) in Model 1 (Table 21).

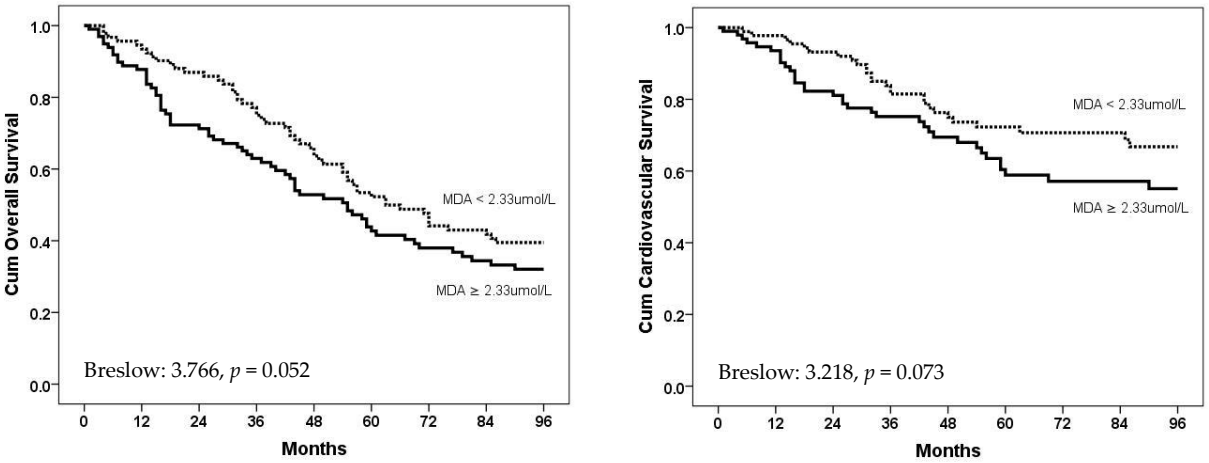



**Figure 30.** Kaplan-Meier survival curves for 8-year overall and cardiovascular survival of ESRD patients dichotomized by cut-off values of MDA.

Regarding the prognostic role of cell adhesion molecules in ESRD patients, increased sVCAM-1 levels (above 644.09 ng/mL) were associated with shorter overall (log-rank 6.41, $p$ = 0.011) and cardiovascular survival (log-rank 5.96, $p$ = 0.015) (Figure 31 A). Adjusting for the confounders in Models 1–3 did not abolish the predictive value of sVCAM-1 (Table 21). On the other hand, ESRD patients with high levels of sICAM-1 (above 77.56 ng/mL) had a trend towards shorter overall and cardiovascular survival (log-rank 2.21, $p$ = 0.137; and log-rank 2.87, $p$ = 0.090, respectively) (Figure 31 B). Notably, high sICAM-1 levels were associated with increased risk of cardiovascular mortality (HR 1.8, 95% CI 1.1–3.1, $p$ = 0.033, Table 21).

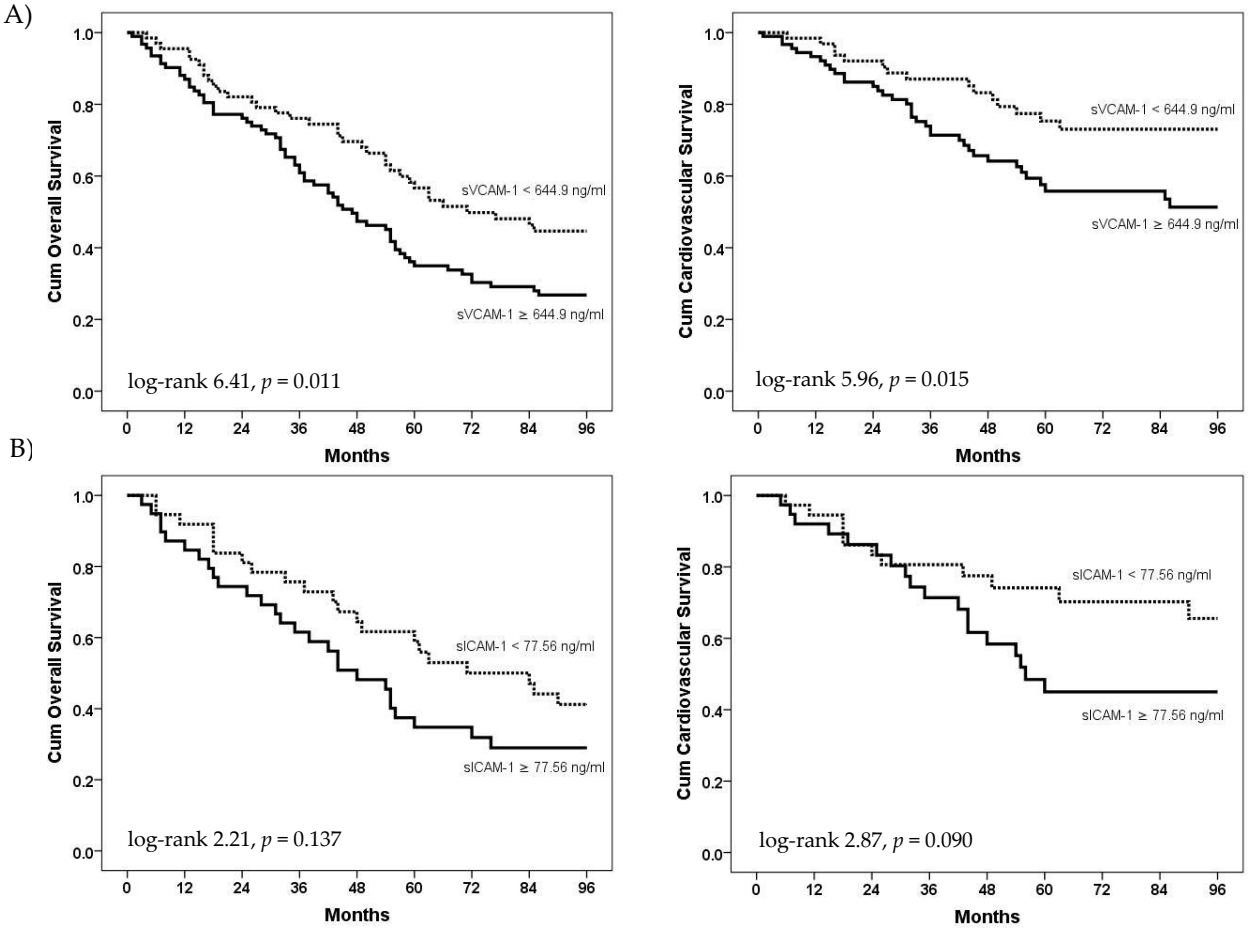



**Figure 31.** Kaplan-Meier survival curves for 8-year overall and cardiovascular survival of ESRD patients dichotomized by cut-off values of sVCAM-1 A) and sICAM-1 B).

**Table 21.** Biomarkers of oxidative stress and endothelial dysfunction as predictors of 8-year overall and cardiovascular survival in patients with ESRD

| Parameter | Models | Overall mortality | | | Cardiovascular mortality | | |
|---|---|---|---|---|---|---|---|
| | | Hazard ratio | 95% CI | p-value | Hazard ratio | 95% CI | p-value |
| AOPP | Unadjusted | 1.2 | 0.8-1.8 | 0.368 | 1.9 | 1.1-3.4 | 0.024 |
| | Model 1 | 1.4 | 0.9-2.1 | 0.108 | 2.3 | 1.3-4.1 | 0.005 |
| | Model 2 | 1.1 | 0.8-1.7 | 0.559 | 1.8 | 1.0-3.2 | 0.044 |
| | Model 3 | 1.3 | 0.9-1.9 | 0.226 | 2.0 | 1.1-3.6 | 0.016 |
| PAB | Unadjusted | 1.9 | 1.3-2.8 | 0.002 | 2.0 | 1.2-3.6 | 0.014 |
| | Model 1 | 1.8 | 1.2-2.8 | 0.005 | 1.8 | 1.0-3.3 | 0.040 |
| | Model 2 | 1.7 | 1.1-2.6 | 0.009 | 1.9 | 1.0-3.3 | 0.035 |
| | Model 3 | 2. 3 | 1.5-3.5 | <0.001 | 2.4 | 1.3-4.3 | 0.004 |
| MDA | Unadjusted | 1.3 | 0.9-1.9 | 0.128 | 1.5 | 0.9-2.5 | 0.101 |
| | Model 1 | 1.5 | 1.0-2.1 | 0.051 | 1.6 | 1.0-2.8 | 0.061 |
| | Model 2 | 1.3 | 0.9-1.8 | 0.198 | 1.5 | 0.9-2.5 | 0.141 |
| | Model 3 | 1.4 | 0.9-2.0 | 0.100 | 1.5 | 0.9-2.6 | 0.119 |
| sVCAM-1 | Unadjusted | 1.7 | 1.1-2.5 | 0.013 | 2.1 | 1.1-3.8 | 0.017 |
| | Model 1 | 1.8 | 1.2-2.8 | 0.006 | 2.1 | 1.1-3.8 | 0.020 |
| | Model 2 | 1.7 | 1.1-2.6 | 0.012 | 2.0 | 1.1-3.8 | 0.022 |
| | Model 3 | 1.6 | 1.1-2.5 | 0.023 | 2.0 | 1.1-3.8 | 0.022 |
| sICAM-1 | Unadjusted | 1.5 | 0.9-2.7 | 0.142 | 1.8 | 1.1-3.1 | 0.033 |
| | Model 1 | 1.1 | 0.6-2.2 | 0.742 | 1.7 | 0.9-2.9 | 0.081 |
| | Model 2 | 1.5 | 0.8-2.7 | 0.222 | 2.2 | 1.2-3.8 | 0.008 |
| | Model 3 | 1.3 | 0.9-2.1 | 0.184 | 1.8 | 1.0-3.2 | 0.045 |

AOPP, advanced oxidation protein products; PAB, prooxidant-antioxidant balance; MDA, malondialdehyde; sVCAM-1, soluble vascular cell adhesion molecule; sICAM-1, soluble intercellular adhesion molecule; ESRD, end-stage renal disease; CI, confidence interval; Adjustments: Model 1 (cause of ESRD, age and gender); Model 2 (BMI, smoking status, creatinine, cholesterol, CRP, serum albumin), Model 3 (diabetes, hypertension, myocardial infarction, peripheral vascular disease and cerebrovascular disease).

As shown in the Table 22, other estimated biomarkers of oxidative stress (thiol groups, protein carbonyls, nitrotirosine, MDAadd and TOS) did not have significant prediction value. Only a trend towards decreased cardiovascular mortality was observed in patients with high thiol groups (HR: 0.6, 95%CI=0.4-1.1, p=0.083) and increased cardiovascular mortality in patients with high TOS (HR: 1.7, 95%CI=1-3, p=0.050) in the Model 1.

**Table 22.** Biomarkers of oxidative stress that did not shown a predictive value in terms of 8-year overall and cardiovascular survival in patients with ESRD

| Parameter | Models | Overall mortality | | | Cardiovascular mortality | | |
|---|---|---|---|---|---|---|---|
| | | Hazard ratio | 95% CI | p | Hazard ratio | 95% CI | p |
| PSH | Model 1 | 0.8 | 0.6-1.2 | 0.257 | 0.6 | 0.4-1.1 | 0.083 |
| | Model 2 | 0.8 | 0.6-1.2 | 0.293 | 0.7 | 0.4-1.1 | 0.109 |
| | Model 3 | 0.8 | 0.6-1.2 | 0.316 | 0.7 | 0.4-1.1 | 0.650 |
| Carbonyls | Model 1 | 0.9 | 0.5-1.6 | 0.627 | 1.1 | 0.4-2.8 | 0.867 |
| | Model 2 | 0.8 | 0.4-1.5 | 0.407 | 1.1 | 0.4-2.8 | 0.857 |
| | Model 3 | 0.8 | 0.4-1.5 | 0.468 | 1.1 | 0.4-2.9 | 0.795 |
| Nitrotyrosine | Model 1 | 0.9 | 0.6-1.4 | 0.774 | 1.0 | 0.6-1.8 | 0.975 |
| | Model 2 | 0.9 | 0.6-1.4 | 0.857 | 1.1 | 0.6-1.9 | 0.799 |
| | Model 3 | 0.9 | 0.6-1.4 | 0.685 | 1.1 | 0.6-1.9 | 0.854 |
| MDA adducts | Model 1 | 1.2 | 0.7-1.8 | 0.548 | 0.9 | 0.5-1.7 | 0.731 |
| | Model 2 | 1.1 | 0.7-1.7 | 0.765 | 0.9 | 0.4-1.7 | 0.642 |
| | Model 3 | 1.1 | 0.7-1.8 | 0.569 | 0.9 | 0.5-1.8 | 0.922 |
| TOS | Model 1 | 1.1 | 0.7-1.7 | 0.587 | 1.7 | 1.0-3.0 | 0.050 |
| | Model 2 | 0.9 | 0.6-1.4 | 0.774 | 1.4 | 0.8-2.4 | 0.219 |
| | Model 3 | 1.1 | 0.8-1.6 | 0.592 | 1.6 | 0.9-2.7 | 0.104 |

PSH, protein thiol groups; TOS, total oxidant status, CI, confidence interval; Adjustments: Model 1 (cause of ESRD, age and gender); Model 2 (BMI, smoking status, creatinine, cholesterol, CRP, serum albumin), Model 3 (diabetes, hypertension, myocardial infarction, peripheral vascular disease and cerebrovascular disease).

### 4.6.1 Evaluating a risk prediction score of a panel of six biomarkers in patients with ESRD

According to the aforementioned results, we were able to determine several parameters with strong predictive value in terms of 8-year overall and cardiovascular survival in ESRD patients. Among the examined gene polymorphisms, *GSTM1-null* genotype showed the most promising prognostic potential. Moreover, out of ten biomarkers of oxidative stress and endothelial dysfunction analysed, five of them appeared to be significant predictors of patients' survival. Given that the ESRD is a multi-factorial disease involving multiple processes which are difficult to capture through a single biomarker, we aimed to determine whether a panel of 6 biomarkers (*GSTM1* genotype, AOPP, PAB, MDA, sVCAM-1, sICAM-1) may have enhanced prognostic potential compared to that of each individual biomarker.

Patients were stratified into a three groups in order to analyse prediction of overall survival: 1) The lowest risk group included patients with score zero (combination of *GSTM1-active* genotype and low levels of AOPP, PAB, MDA, sICAM- 1 and sVCAM-1); 2) The intermediate-risk group included patients with scores 1–2 (1 or 2 positive biomarkers). 3) The highest risk group included patients with score 3 or more (combination of *GSTM1-null* genotype and/or high levels of AOPP, PAB, MDA, sICAM- 1 and sVCAM-1). In order to analyse prediction of cardiovascular survival, due to lower number of outcomes, patients were stratified into 2 risk categories (low and high risk). The lowest risk group included patients with 0, 1 or 2 positive biomarkers, while high-risk group included patients with 3 or more positive biomarkers.

As shown in the Figure 32, ESRD patients within high-risk group (score ≥3) had significantly shorter overall survival compared to low-risk patients (score 0). Median overall survival in the high-risk group was 42 months, in the intermediate-risk group was 85 months, while in low risk group median was not reached (log-rank 22.430, $p < 0.001$). The cardiovascular survival was also significantly lower in patients within the high-risk group (score ≥3) compared to patients in the low-risk group (score 0). According to the Kaplan-Meier survival analysis, median cardiovascular survival for high-risk patients was 59 months while those in low-risk group did not reach the median (log-rank 21.133, $p < 0.001$).

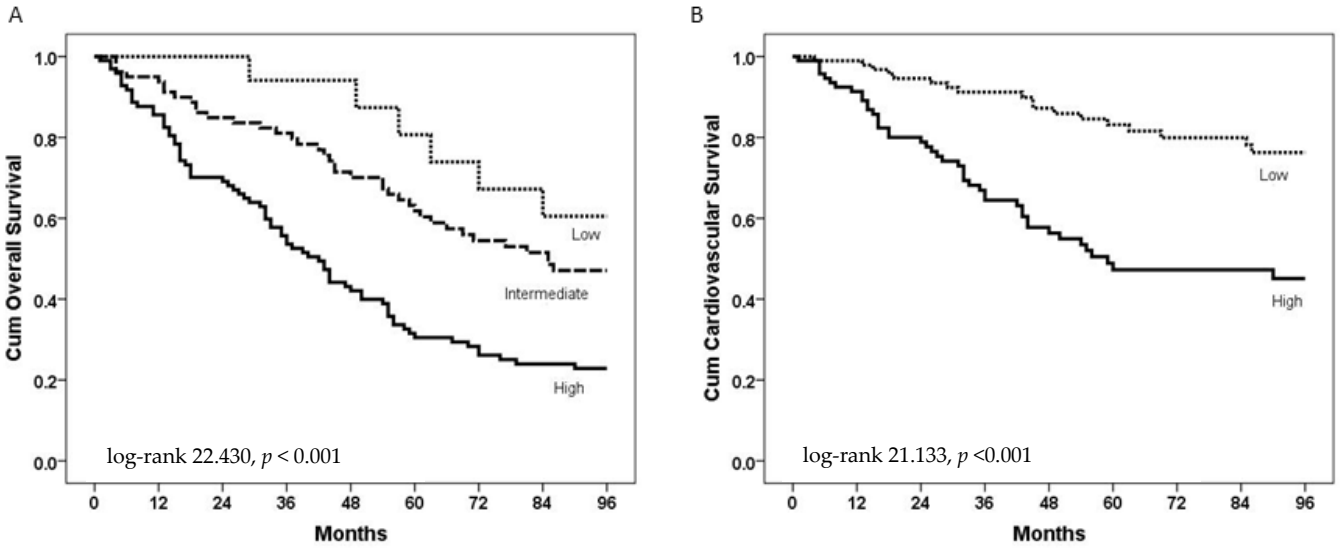



**Figure 32.** Kaplan-Meier survival analysis using 6 different biomarkers (GSTM1 genotype, plasma levels of AOPP, PAB, MDA, sVCAM-1 and sICAM-1 above the cut-off level). Patients were stratified into a three risk groups in order to analyse prediction of overall survival, and into a two groups for cardiovascular survival risk analysis.

## 4.6 The influence of GSTM1 knockdown and uremic serum on redox homeostasis and cytokine expression in human umbilical vein endothelial cells

According to the results obtained in this study, *GSTM1-null* genotype was the most significant independent predictor of 8-year overall and cardiovascular survival. Building upon previous findings that indicate the significance of the GSTM1 deletion on the oxidative phenotype of dialyzed patients and their susceptibility to CVD [126,236], and results presented herewith on its influence on cardiovascular prognosis, we aimed to elucidate the specific contribution of GSTM1 deletion on endothelial dysfunction in the uremic milieu. In order to achieve this, we analyzed oxidative stress and expression of a panel of 105 inflammatory markers in HUVECs silenced for the GSTM1 gene.

### 4.6.1 GSTM1 knockdown in HUVECs

In order to assess the role of GSTM1 knockdown on endothelial dysfunction, HUVECs were treated with 100 nM GSTM1 siRNA ($GSTM1^{+/-}$) or DharmaFECT transfection reagent as a control ($GSTM1^{+/+}$). After 96 h proteins were extracted and western blotting was performed to confirm diminished GSTM1 expression. HUVECs treated with GSTM1 siRNA demonstrated ~90% reduction in GSTM1 protein levels compared to the control (Figure 33, n=3, $p<0.001$).

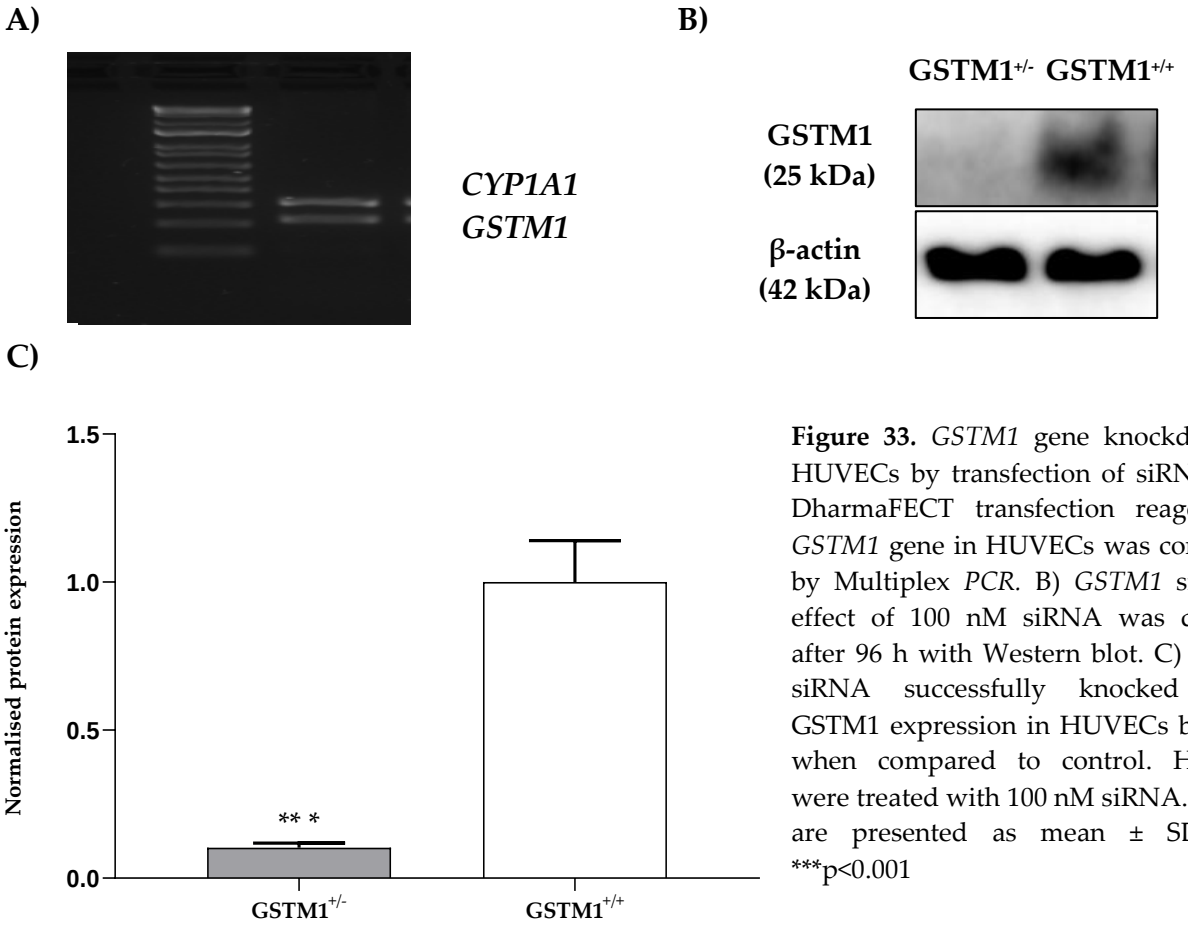



**Figure 33.** *GSTM1* gene knockdown in HUVECs by transfection of siRNA with DharmaFECT transfection reagent. A) *GSTM1* gene in HUVECs was confirmed by Multiplex *PCR*. B) *GSTM1* silencing effect of 100 nM siRNA was detected after 96 h with Western blot. C) GSTM1 siRNA successfully knocked down GSTM1 expression in HUVECs by ~90% when compared to control. HUVECs were treated with 100 nM siRNA. Results are presented as mean ± SD, n=3, ***$p<0.001$

### 4.6.2 The viability of HUVECs incubated in control and uremic serum

To establish the optimal dose and time for the cell treatments, MTS viability assay was performed in HUVECs incubated in medium, 10%, 20%, 30% control or uremic serum for two different periods of time (4 h and 6 h). Neither the increase in serum percentage nor the duration of incubation time influenced significantly the cell viability (Figure 34). The viability of cells incubated in 30% uremic serum for 6 h was decreased less than 5% compared to the cells incubated in growth medium and this was not statistically significant. Therefore, all further HUVECs treatments were consisted of 30% control and uremic serum and lasted for 6 h, after which cytokine expression and oxidative stress measurements were performed.

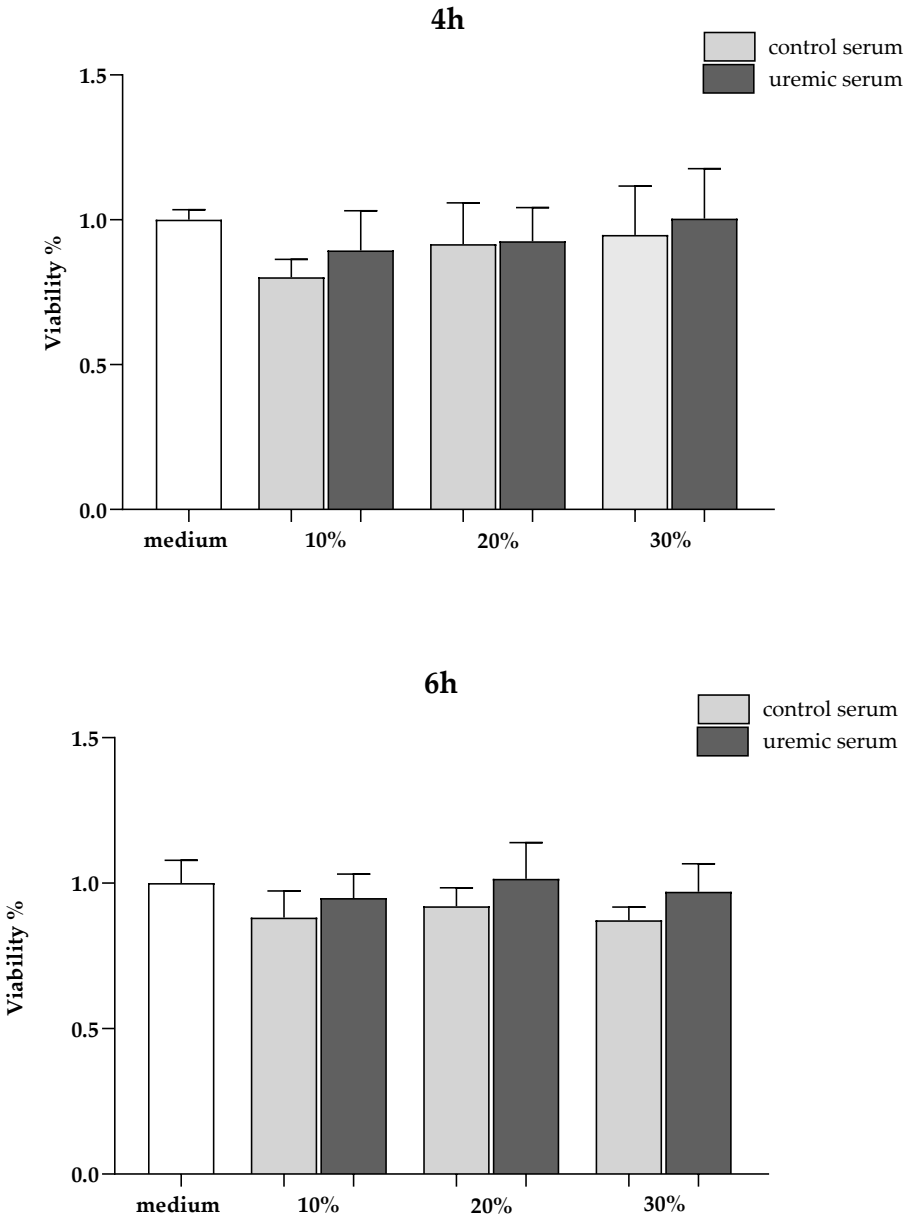



**Figure 34.** The viability of HUVECs. HUVECs (n=6) were incubated in medium; 10%, 20%, 30% control and uremic serum for: A) 4 h and B) 6 h. Cell viability was determined by MTS test.

### 4.6.3 The influence of GSTM1 knockdown on oxidative stress parameters in HUVECs incubated in uremic serum

To determine whether the uremic serum and GSTM1 knockdown could directly cause differences in the activity of antioxidative enzymes (SOD and GPX), as well as, the total ROS production and MDA levels, GSTM1$^{+/+}$ and GSTM1$^{+/-}$ HUVECs were incubated in the control or uremic serum containing media.

As shown in the Figure 35 A&B, the incubation of HUVECs with uremic serum led to a significant decrease in the activity of SOD and GPX antioxidant enzymes in GSTM$^{+/+}$ HUVECs compared to control serum conditions ($p<0.05$). However, the silencing of GSTM1 gene did not show statistically significant effect on these parameters in any of the observed settings.

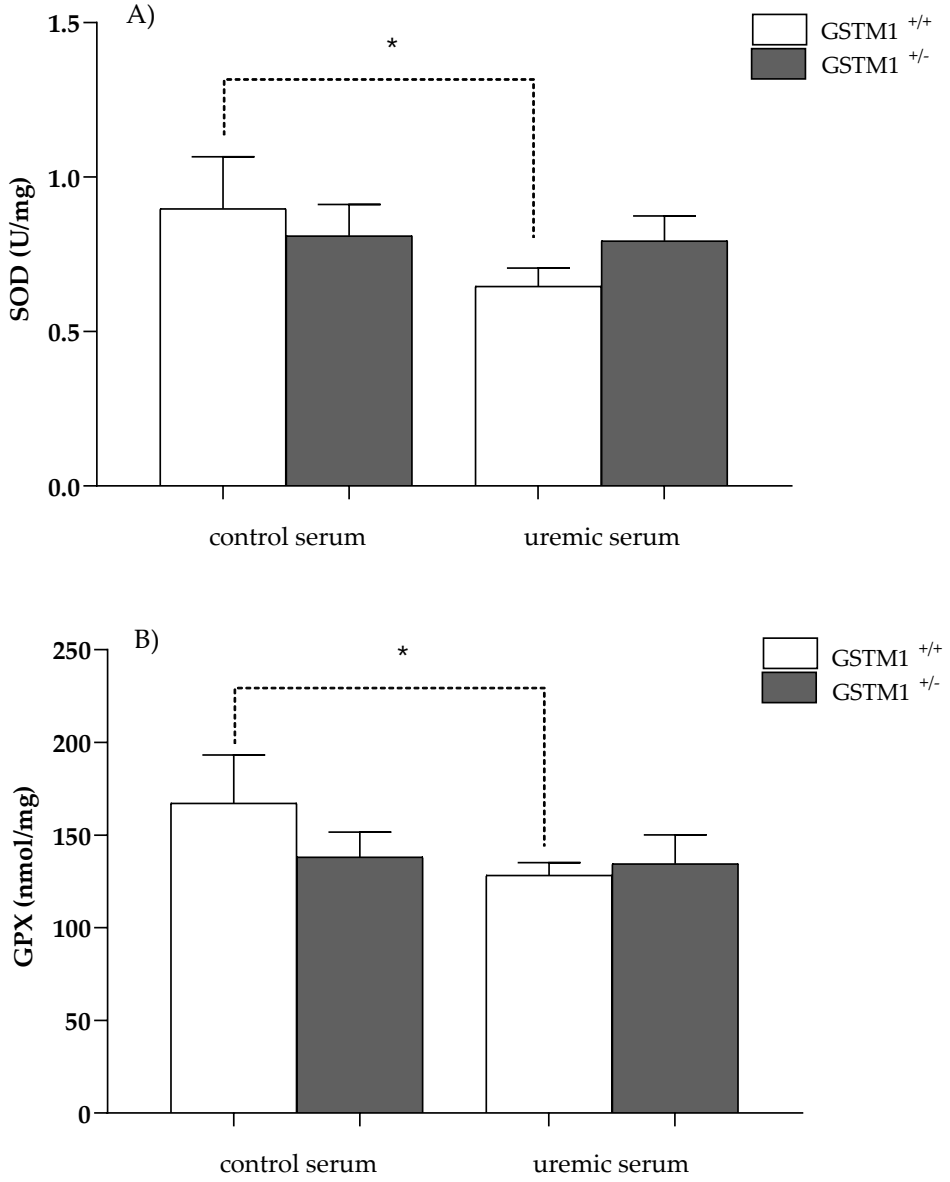



**Figure 35.** The influence of uremic serum and GSTM1 knockdown on the activity of antioxidant enzymes in HUVECs. GSTM1$^{+/+}$ and GSTM1$^{+/-}$ HUVECs were incubated in 30% control and 30% uremic serum containing media for 6 h. A) SOD activity and B) GPX activity were determined by spectrophotometry. Results are presented as mean ± SD, n=3. * $p<0.05$

With respect to oxidative stress byproducts, the exposure to uremic serum, led to an increase of lipid oxidative stress byproducts - MDA ($p<0.05$) in GSTM1$^{+/+}$ HUVECs compared to their counterparts incubated in control serum (Figure 36, A)). Similarly, GSTM1 knockdown showed a trend towards increased MDA levels in GSTM1$^{+/-}$ HUVECs compared to GSTM1$^{+/+}$ HUVECs incubated in control serum ($p=0.053$, Figure 36, A)). However, neither uremic serum or the GSTM1 knockdown had statistically significant impact on total ROS in HUVECs (Figure 36, B)).

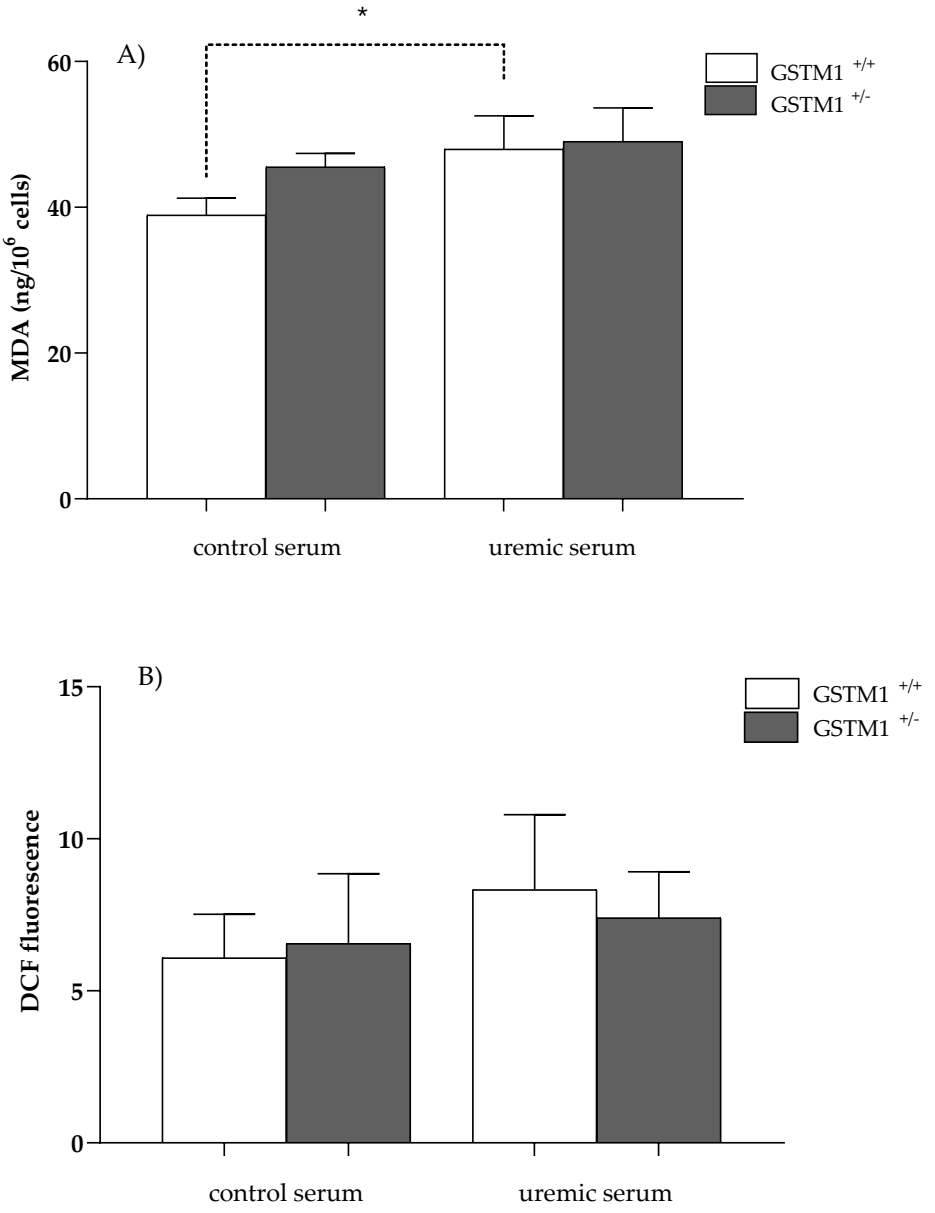



**Figure 36.** The influence of uremic serum and GSTM1 knockdown on oxidative stress byproducts in HUVECs. GSTM1$^{+/+}$ and GSTM1$^{+/-}$ HUVECs were incubated in 30% control and 30% uremic serum containing media for 6 h. A) MDA levels were determined by ELISA.. B) A total ROS were determined by flow cytometry after DCF staining. Results are presented as mean ± SD, n=3. * $p<0.05$

### 4.6.4 The influence of uremic serum and GSTM1 knockdown on cytokine expression in HUVECs

To investigate the potential role of uremic serum and GSTM1 downregulation on endothelial cell inflammation, we assessed the relative expression of over 100 cytokines in protein extracts from GSTM1$^{+/+}$ and GSTM1$^{+/-}$ HUVECs incubated in sera obtained from HD patients or healthy volunteers. Pooled protein lysates (n=3/group) from four different groups of treated cells (GSTM1$^{+/+}$ control serum; GSTM1$^{+/+}$ uremic serum; GSTM1$^{+/-}$ control serum; GSTM1$^{+/-}$ uremic serum) were probed on four separate nitrocellulose membranes to measure cytokine expression, and the array key and original blots are presented in the Table 23 and Figure 37 respectively.

**Table 23.** Array key

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Reference | | Adiponectin | | Apo A-I | | Angiogenin | | ANGPT1 | | ANGPT2 | | BAFF | | BDNF | | C5/C5a | | CD14 | | CD30 | | Reference | |
| B | | | CD40 L | | CHI3L1 | | Compl.F D | | CRP | | Cripto-1 | | Cystatin C | | Dkk-1 | | DPPIV | | EGF | | EMMPRIN | | | |
| C | | | ENA-78 | | Endoglin | | Fas Ligand | | FGF basic | | FGF-7 | | FGF-19 | | Flt-3 L | | G-CSF | | GDF-15 | | GM-CSF | | | |
| D | GRO-α | | Somatotropin | | HGF | | ICAM-1 | | IFN-γ | | IGFBP-2 | | IGFBP-3 | | IL-1α | | IL-1β | | IL-1ra | | IL-2 | | IL-3 | |
| E | IL-4 | | IL-5 | | IL-6 | | IL-8 | | IL-10 | | IL-11 | | IL-12 p70 | | IL-13 | | IL-15 | | IL-16 | | IL-17A | | IL-18 BPa | |
| F | IL-19 | | IL-22 | | IL-23 | | IL-24 | | IL-27 | | IL-31 | | IL-32 | | IL-33 | | IL-34 | | IP-10 | | I-TAC | | Kallikrein 3 | |
| G | Leptin | | LIF | | Lipocalin-2 | | MCP-1 | | MCP-3 | | M-CSF | | MIF | | MIG | | MIP-1α/1β | | MIP-3 α | | MIP-3 β | | MMP-9 | |
| H | MPO | | Osteopontin | | PDGF-AA | | PDGF-AB/BB | | Pentraxin-3 | | PF4 | | RAGE | | RANTES | | RBP-4 | | Relaxin-2 | | Resistin | | SDF-1α | |
| I | Serpin E1 | | SHBG | | ST2 | | TARC | | TFF3 | | TfR | | TGF-α | | THBS1 | | TNF-α | | uPAR | | VEGF | | | |
| J | Reference | | | | Vit D BP | | CD31 | | TIM-3 | | VCAM-1 | | | | | | | | | | | | NC | |

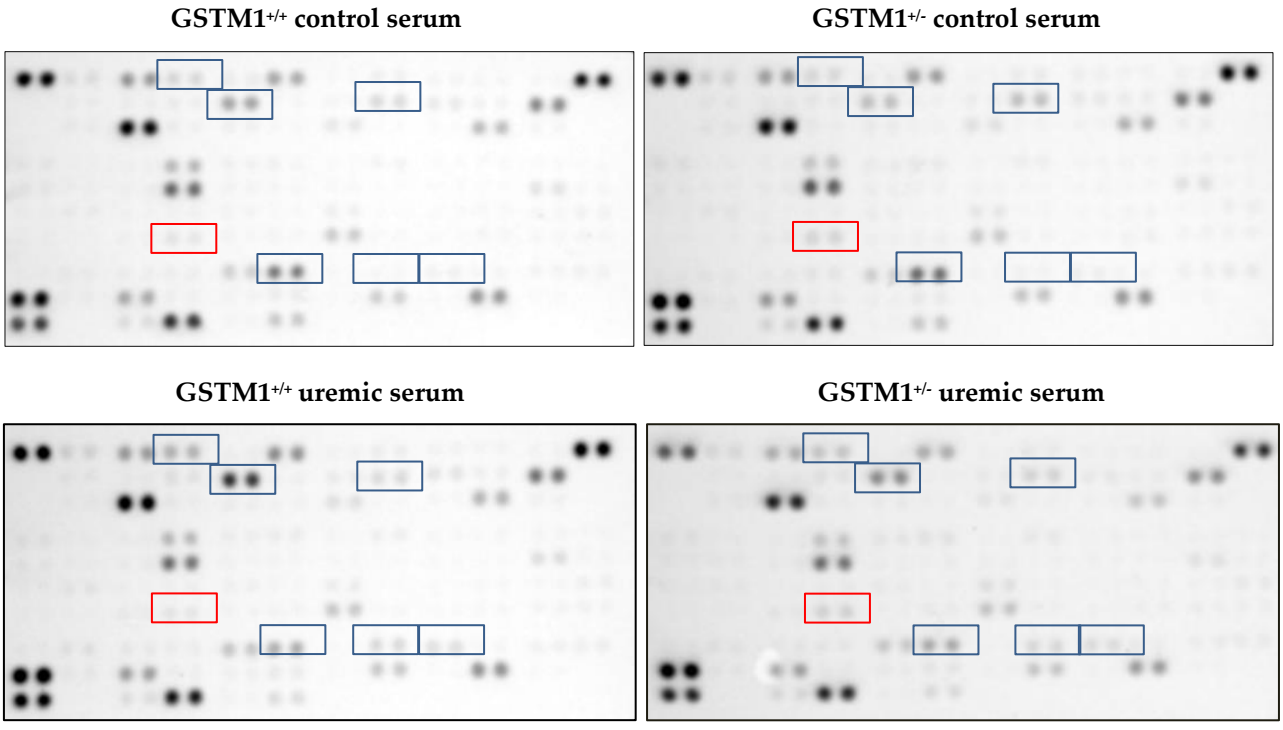



**Figure 37.** Proteome Profiler Human XL Cytokine Array and original blots. Cytokines that exhibited ≥2-fold change in uremic serum when compared to control serum treatment are marked blue (vertical comparison between images). Cytokines that exhibited ≥2-fold change in GSTM1$^{+/-}$ cells when compared to GSTM1$^{+/+}$ are marked red (horizontal comparison between images).

The quantified densities of spots were normalised to the reference spots positioned at three of the corners of each blot. The relative expression of 103 inflammatory cytokines is presented as a graded heat map (Figure 38) where the lowest expressions of proteins are coloured green, and the highest expression black. The most highly expressed proteins were serpin, endoglin and CD31 (data not shown due to their impact on the heat map legend).

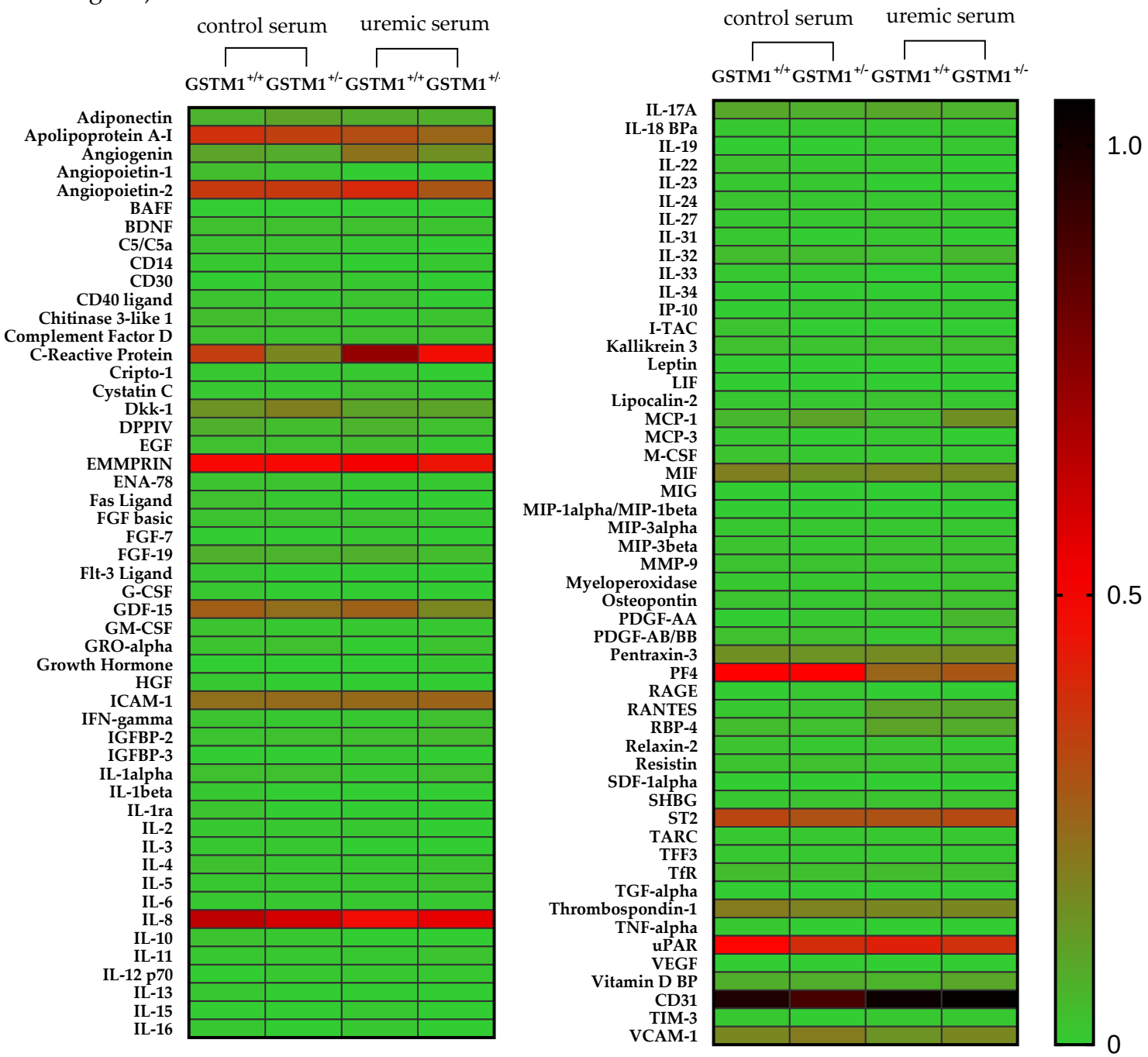



**Figure 38.** Expression of a panel of cytokines determined by Proteome Profiler Human XL Cytokine Array. HUVECs (n=3/group, pooled), transfected with GSTM1 siRNA and GSTM1$^{+/+}$ HUVECs were incubated in 30% control or uremic serum containing media for 6 h. Heatmap represents pixel densities of spots normalized by respective reference spots.

Additionally, the proteins whose expression crossed the threshold of two fold change in response to uremic serum treatment or GSTM1 knockdown are presented as bars (Figure 39).

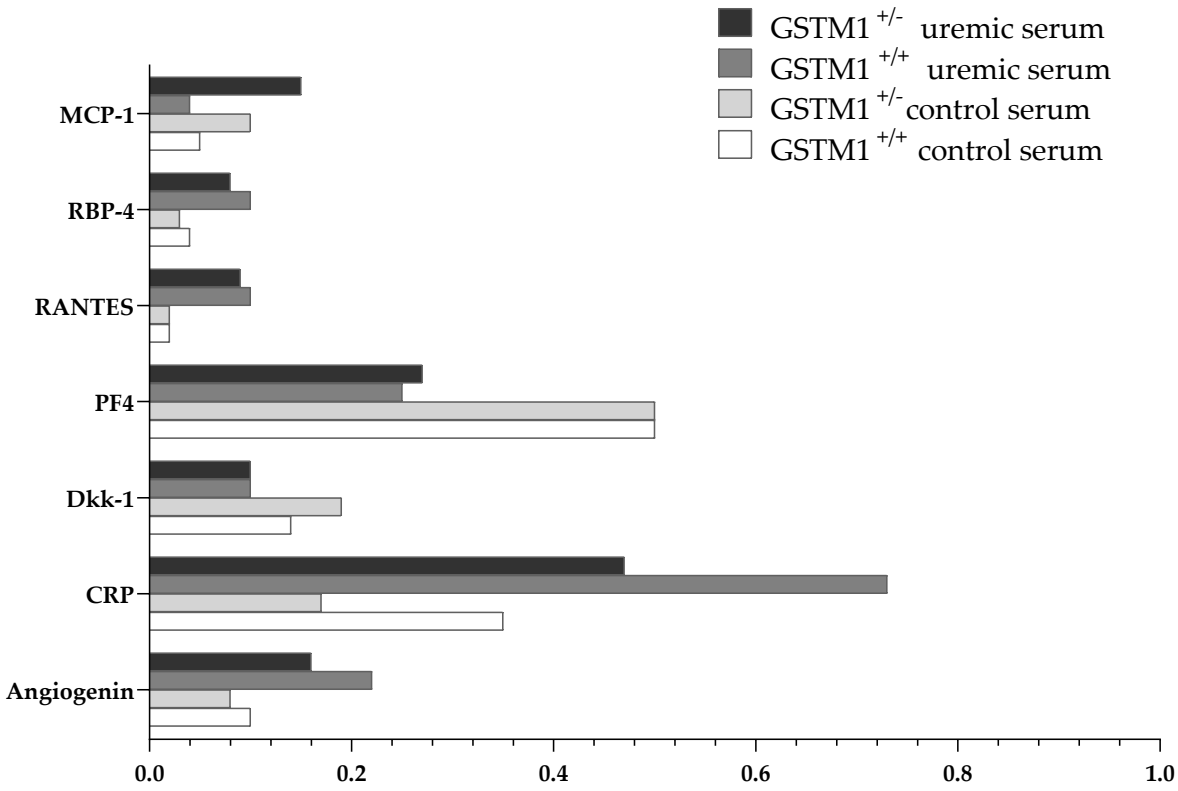



**Figure 39.** The relative expression of **c**ytokines that exhibited at least 2 -fold change in response to GSTM1 knockdown or uremic serum treatment

According to our analysis, the expression of the majority of enlisted proteins was affected by uremic serum treatment (Figure 37, Figure 38 and Figure 39). Namely, incubation in uremic serum resulted in over 2 fold higher expression of retinol-binding protein 4 (RBP4), regulated on activation, normal T cell expressed and secreted (RANTES), C-reactive protein (CRP) and angiogenin (Figure 39) compared to the HUVECs incubated in control serum. On contrary, the expression of dickkopf-1 (Dkk-1) and platelet factor 4 (PF4) was supressed by uremic serum treatment. Interestingly, the expression of only one protein crossed the set threshold in a response to GSTM1 knockdown. The MCP-1 expression increased 2-fold in GSTM1$^{+/-}$ HUVECs incubated in control serum and 3.8-fold in GSTM1$^{+/-}$ HUVECs incubated in uremic serum compared to corresponding GSTM1$^{+/+}$ HUVECs.

### 4.6.5 The influence of uremic serum and GSTM1 knockdown on ICAM-1 and VCAM-1 expression in HUVECs

According to the proteome array analysis, ICAM-1 and VCAM-1 expression were elevated in HUVECs silenced for GSTM1 gene, although they were not presented in bar graphs since they did not reach the set 2-fold change. Based on the results of our study showing that soluble ICAM-1 and VCAM-1 have a strong predictive role in terms of 8-year cardiovascular survival in ESRD patients, and that our previous analysis showed that their levels might be dependent on *GSTM1* genotype in these patients [257], we further tested the ICAM-1 and VCAM-1 expression by the Western blot (Figure 40 and Figure 41).

The incubation of HUVECs with uremic serum led to a significant increase in ICAM-1 expression in GSTM$^{+/-}$ HUVECs compared to control serum conditions ($p<0.05$) (Figure 40). GSTM1 knockdown also led to rise of ICAM-1 expression in HUVECs incubated in control serum and even more pronounced rise in HUVECs incubated in uremic serum ($p<0.05$).

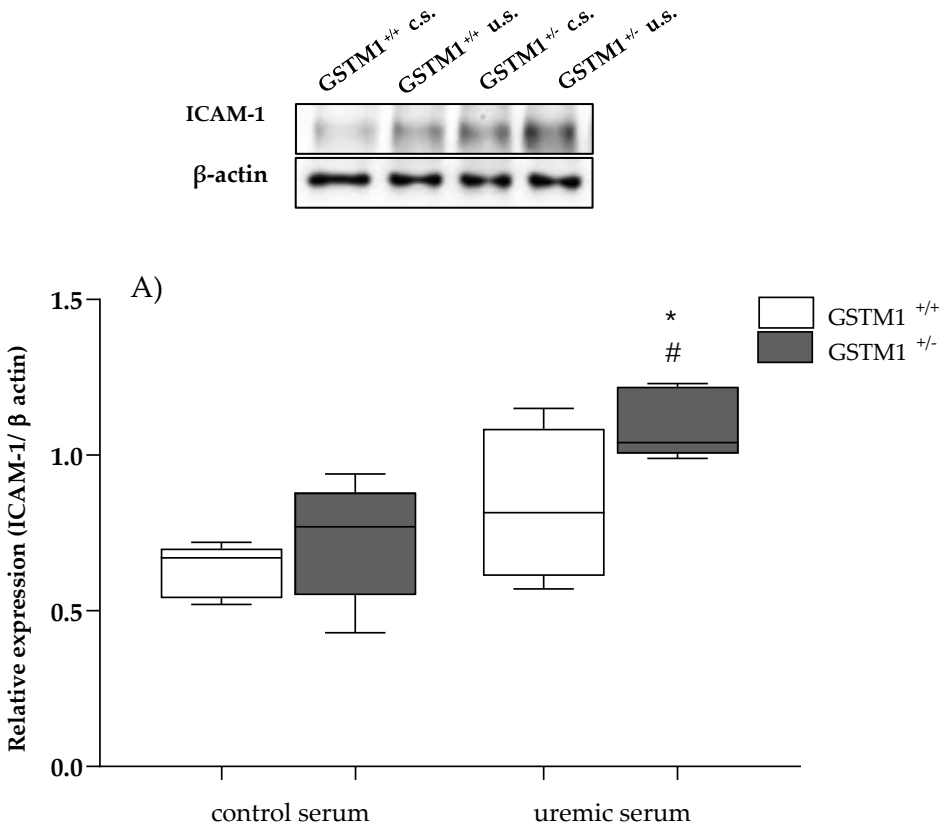



**Figure 40.** The influence of uremic serum and GSTM1 knockdown on ICAM-1 expression in HUVECs. GSTM1$^{+/+}$ and GSTM1$^{+/-}$ HUVECs were incubated in 30% control (c.s.) or 30% uremic serum (u.s.) – containing media for 6 h. ICAM-1 expression was determined by Western blot. Results are presented as median with interquartile range, n=5. *$p<0.05$ GSTM1$^{+/-}$ HUVECs compared to GSTM1$^{+/+}$ HUVECs.; # $p<0.05$ GSTM1$^{+/-}$ HUVECs in uremic serum compared to GSTM $^{+/-}$ HUVECs in control serum.

VCAM-1 expression in HUVECs was not affected by the uremic serum treatment (Figure 41). GSTM1 knockdown led to a trend in increase of VCAM-1 expression in HUVECs incubated in control serum as well as in uremic serum treatment when compared to HUVECs with normal levels of GSTM1, however it did not reach statistical significance.

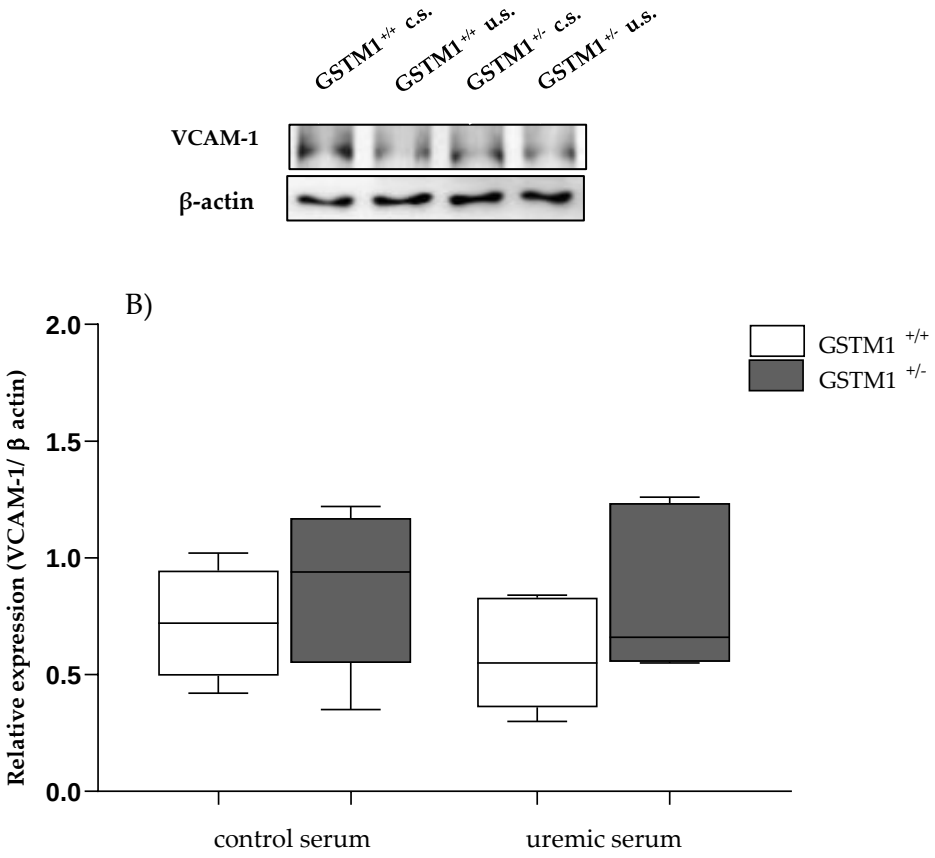



**Figure 41.** The influence of uremic serum and GSTM1 knockdown on VCAM-1 expression in HUVECs. GSTM1$^{+/+}$ and GSTM1$^{+/-}$ HUVECs were incubated in 30% control (c.s.) or 30% uremic serum (u.s.) – containing media for 6 h. VCAM-1 expression was determined by Western blot. Results are presented as median with interquartile range, n=5.

# 5. DISCUSSION

This thesis presents a comprehensive research on the significance of *Nrf2* rs6721961, *SOD2* rs4880, *GPX1* rs1050450 and *GSTM1* deletion antioxidant gene polymorphisms in ESRD. We assessed the association of these polymorphisms with the risk of ESRD development in a cohort of 630 participants from Serbia. Furthermore, we analysed the functional significance of these polymorphisms by determining their association with plasma levels of eight byproducts of oxidative stress, including two circulating adhesion molecules. The next step in our analysis included the examination of the predictive power of two biomarker panels in terms of the 8-year overall and cardiovascular survival in ESRD patients. The first biomarker panel was comprised of a specific combination of aforementioned genes. The second biomarker panel was consisted of a combination of byproducts of oxidative stress, circulating adhesion molecules and *GSTM1* deletion polymorphism. The final part of this thesis examined the specific contribution of GSTM1 deletion on endothelial dysfunction in uremic milieu. In order to achieve this, we employed a series of human umbilical vein endothelial cell - based *in vitro* experiments and analyzed oxidative stress and expression of a panel of over a hundred inflammatory markers in HUVECs silenced for the GSTM1 gene.

Progressive deterioration of renal structure and function, along with the associated cardiovascular complications of ESRD, is largely attributed to oxidative stress. It has been suggested that excessive oxidative stress in ESRD patients could be associated with specific genetic patterns [20,126,258]. Notably, gene polymorphisms of regulatory and catalytic antioxidant proteins result in alteration in their proteins' activity profile, hence affecting individual's antioxidant capacity [151,165,183,185]. Therefore, it can be hypothesized that individual susceptibility towards ESRD lies in functional variations of genes encoding antioxidant regulatory and catalytic proteins, including *Nrf2, SOD2* and *GPX1.*

The results obtained in this thesis confirm the association of antioxidant gene polymorphisms with the risk of ESRD development. *SOD2 Val*/*Val* genotype had an independent effect on the risk of ESRD development, which was even more pronounced in combination with GPX1 *Leu*/*Leu* genotype. The combination of *SOD2 Val*/*Val* genotype with *Nrf2 C*/*C* genotype significantly elevated the risk of the ESRD as well.

The most notable finding concerning the risk of ESRD development in this thesis was the one showing the association of *SOD2 Val*/*Val* genotype with increased susceptibility to ESRD development. This finding is in line with several studies concluding that *Val*/*Val* genotype of *SOD2* gene increases the risk of diabetic nephropathy in both T1DM and T2DM patients [171–173]. Furthermore, in a study of Crawford et al, patients carrying *Val* allele had a more rapid decline in eGFR suggesting faster progression of CKD in these patients [169]. Our results, along with aforementioned findings of other investigators, support the hypothesis that diminished SOD2 antioxidant activity contributes to the risk of diseases that have been linked to oxidative stress, such as ESRD. Concisely, the *Val* allele of *SOD2* rs4880 gene results in reduced expression of SOD2,

production of unstable mRNA, as well as impaired import of SOD2 enzyme into the mitochondra, all of which leads to impaired antioxidant defence [165,168].

The polymorphism of another key antioxidant, *GPX1*, enzyme also results in impaired catalytic GPX1 enzyme activity [102]. The influence of *GPX1* rs1050450 SNP on the CKD risk has been recently studied [169]. The authors showed that the *GPX1* genotype frequencies did not differ between CKD patients and controls. Similarly, we found no independent influence of *GPX1* polymorphism on the risk of ESRD. However, in our study, *GPX1* polymorphism enhanced the effect of *SOD2* polymorphism on the risk of ESRD development. This was exemplified by the finding that the individuals homozygous for low-activity alleles of both genes had 3 times higher risk of ESRD, compared to the 2-fold increase found in *Val*/*Val SOD2* individuals. This result aligns with the study of Chao et al. that showed *GPX1 Leu*/*Leu* genotype elevating the risk of ESRD only when combined with another, PPAR-*γ G*/*G* genotype [175]. These findings demonstrate that the antioxidant system is comprised of a range of various interactions, thus eliminating the possibility of identifying a distinct variable, which could be used as a single marker of disease. This is also substantiated by the fact that, in our study, *Nrf2* had no independent influence on the ESRD risk, or on other assessed biomarkers of oxidative stress, or patient survival. And yet, *Nrf2* had a significant role when observed in combination with other genes. Concerning the risk of ESRD development, individuals homozygous for referent, *C*, allele of *Nrf2* gene and low-activity, *Val* allele of *SOD2* gene exhibited greater susceptibility towards ESRD development. This finding might suggest that the impact of *Nrf2* gene and its transcript depends on the gene variant which is the final *Nrf2* target. Therefore, we could assume that the intensified transcription of the less active form of SOD2 enzyme results in poor antioxidant defence, leading to higher susceptibility towards ESRD.

In a further course of this thesis, we aimed to examine whether polymorphisms of *Nrf2*, *SOD2* and *GPX1* genes, together with *GSTM1* deletion polymorphism, could be included in the panel designed to predict 8-year overall and cardiovascular survival in ESRD patients. According to the results obtained in this study, *GSTM1-null* genotype was the most significant independent predictor of long-term overall and cardiovascular survival. In addition to *GSTM1* polymorphism and age, a combination of the *Nrf2 C*/*C* and *GPX1 Leu*/*Leu* genotypes contributed to longer overall, while variant *GPX1 Leu*/*Leu* genotype contributed to longer cardiovascular survival.

Multiple studies reported significant association between *GSTM1-null* genotype and faster progression of kidney disease [242], as well as higher mortality rate among ESRD patients [236,258]. Our previously published data is consistent with other investigators, showing the higher overall and cardiovascular mortality in dialyzed patients with *GSTM1-null* genotype over a three and five-year follow-up [124,126]. The results presented in this thesis confirm the effect of *GSTM1* polymorphism on ESRD patients' prognosis even after 8-year follow up. This is the first study that examined the influence of *GSTM1* polymorphism on the 8-year survival in patients on dialysis. The poorer prognosis in ESRD patients lacking the GSTM1 enzyme has a biologically plausible explanation. Given that the GSTM1 enzyme has antioxidant function, GSTM1 deficiency is associated with decreased antioxidant protection in individuals with *GSTM1-null*

genotype. The connection between GSTM1 deletion and oxidative stress in ESRD patients was supported by our previous findings that demonstrated elevated levels of several byproducts of protein and lipid oxidative damage in ESRD patients with *GSTM1-null* genotype [126]. Therefore, the detrimental effect of GSTM1 deficiency is reflected in the increased susceptibility to oxidative and carbonyl stress in ESRD patients, thereby resulting in worse prognosis in these patients.

This study is also the only one examining the potential prognostic influence of *GPX1* rs1050450 polymorphism on ESRD patients' survival. Unexpectedly, low activity, *GPX1 Leu*/*Leu*, genotype contributed to longer overall, as well as cardiovascular survival in ESRD patients. GPX1 regulates cellular oxidant status through elimination of hydroperoxides via oxidation of glutathione. Therefore, more active GPX1 contributes to further decrease of already depleted stores of reduced glutathione in ESRD, thus diminishing cellular non-enzymatic antioxidant protection. Studies examining the association between GPX1 polymorphism and the susceptibly to CVDs and survival, reported conflicting results. Even though there are a few studies which suggest the adverse influence of *GPX1 Leu* allele on CVDs, such as coronary heart disease, calcification of coronary arteries and increased intima-media thickness [82], there are several studies conversely reporting that *Leu* allele may be protective, as well [82,259,260]. It has been shown that *Leu* allele may reduce the risk of thoracic aortic aneurysm in patients with hypertension [82,260]. Moreover, Soerensen et al. showed that low activity *GPX1 Leu* allele was associated with longer survival in the oldest population [259]. Based on these findings it is tempting to speculate that certain level of hydrogen peroxide and physiological ROS dependent signalling, in accordance with the latest definition of oxidative eustress, might have beneficial effects, especially on cardiovascular function. Moderate levels of ROS were reported to have positive effects on endothelial or cardiomyocyte homeostasis [261]. Overall, there is likely a delicate balance between the beneficial and deleterious effects of ROS, with complex signalling mechanisms that need to be explored further to provide better understanding and enable clinical translation.

When assessing the potential prognostic value of *Nrf2* rs6721961 polymorphism in ESRD patients, our results showed that *Nrf2 C*/*C* genotype had an impact on longer overall survival after being combined with *GPX1 Leu*/*Leu* genotype. Nevertheless, no individual association of *Nrf2* polymorphism was observed in relation to the risk, overall and cardiovascular survival among these patients. These results are similar to those obtained in a study of Shimoyama et al. using Japanese cohort of 216 ESRD patients and 464 controls [145]. In their investigation, the *Nrf2* rs6721961 polymorphism did not have an impact on the risk of ESRD development or overall/cardiovascular survival. The only significant association was found between other *Nrf2* polymorphism, *G*/*A* rs35652124 and cardiovascular survival in dialysed patients [145]. Despite recent progress in elucidation of *Nrf2* polymorphisms in the course of kidney deterioration towards ESRD, this field is still unexplored. Our result on the importance of *Nrf2* in survival of ESRD patients could suggest that ESRD patients with diminished Nrf2 expression and function (as present in variant, *Nrf2 A* carriers) may benefit from novel therapeutic agents capable of inducing Nrf2 activation, such as bardoxolone methyl. Measurable improvement in clinical parameters of CKD patients on bardoxolone methyl's therapy were significant increase in

GFR, decrease in serum creatinine and blood urea nitrogen, as well as an increase of creatinine clearance [160,161]. These effects could be attributed to bardoxolone methyl's activation of the Nrf2 pathway, and subsequent restoration of redox imbalance [160,161].

Finally, according to our results, *SOD2* polymorphism did not have significant prognostic impact on ESRD patients' survival either individually or in combination with other assessed polymorphisms. Nevertheless, a trend towards longer cardiovascular survival was observed in patients homozygous for more active, *SOD2 Ala* allele. This trend is in concordance with several studies examining the relationship between this *SOD2* SNP and CVD. The authors reported a positive association between low activity *SOD2 Va*l allele and greater carotid artery intima-media thickness and higher LDL levels among large cohort of 989 hypertensive individuals and controls [262]. Moreover, in the study of Gottlieb et al., *SOD2 Val* allele correlated with higher levels of oxLDL [263]. Notably in a study by Crawford et al., *SOD2 Val* allele accounted for 78% of patients with CKD of vascular origin [169].

In the era of precision medicine, the future of biomarker application in ESRD lies in the multimarker panel strategy, which would include specific combination of biomarkers that reflect different pathophysiological processes underlying ESRD. To the best of our knowledge, these are the first survival models regarding *SOD2, Nrf2* and *GPX1* polymorphisms for ESRD patients in Caucasian population. In addition, our investigation offers one more valuable predictive algorithm of ESRD patients' survival that includes several oxidative stress byproducts and markers of endothelial dysfunction.

Heightened oxidation markers levels of lipids, proteins, and DNA have been indubitably shown when CKD patients were compared with healthy controls. Reduced plasma protein thiols are universal finding in patients with CKD, independent of the degree of renal failure [12,90]. Increased plasma carbonyls were also found in ESRD patients [101,102]. Patients with advanced CKD have elevated plasma levels of AOPP and AOPP plasma levels were proposed as markers of the CKD progression [109,111]. Plasma levels of markers of lipid peroxidation, MDA and isoprostanes showed an opposing relationship with GFR in CKD patients [12,116–118]. Nevertheless, the data regarding the prognostic role of the oxidative stress biomarkers in ESRD patients is scarce.

In this thesis, the predictive value of eight biomarkers of oxidative stress (thiol groups, carbonyls, nitrotyrosine, AOPPs, MDA, MDA-adducts, TOS, PAB) and endothelial dysfunction (sICAM-1, sVCAM-1) measured in plasma of ESRD patients was evaluated using Kaplan-Meier survival analysis and three Cox proportional hazards models. In these models adjustments were made for confounding variables (Model 1 for cause of ESRD, age and gender; Model 2 for BMI, smoking status, creatinine, cholesterol, CRP, serum albumin; Model 3 for diabetes, hypertension, myocardial infarction, peripheral vascular disease and cerebrovascular disease). According to data obtained in this study, AOPPs were significant predictors of cardiovascular survival. PAB was significant predictor of both overall and cardiovascular survival. Moreover, ESRD patients with high concentrations of MDA had a trend towards shorter overall and cardiovascular survival compared to the patients with lower MDA concentrations. Increased sVCAM-1 levels were associated with shorter overall and cardiovascular survival, while high sICAM-1 levels were associated with increased risk of cardiovascular mortality. These results are in

concordance with the previous data of our research group showing that byproducts of oxidative stress (AOPP, PAB and MDA) and endothelial dysfunction (sICAM-1 and sVCAM-1) were significant predictors of 5-year survival in ESRD patients [124].

Elevated plasma AOPP level has been reported either in patients with AKI or CKD [47,264]. At the same time, AOPP has been recently implicated as a risk factor for atherosclerotic cardiovascular events in healthy individuals, as well as in dialysed patients [206]. Moreover, Kaneda et al. reported increased levels of AOPPs in HD and CAD patients and showed that the AOPP levels were independent predictors of CAD [265]. In addition, a positive association between AOPP levels and carotid intima-media thickness was also observed in HD patients [266]. Prognostic significance of AOPPs in terms of cardiovascular survival in dialysed patients in our study is in concordance with other investigators. Interestingly, the mechanisms by which AOPPs might contribute to endothelial dysfunction have been proposed. *In vitro* study of Guo et al. demonstrated that AOPPs contribute to accelerated vascular disease via promoting oxidative stress and inflammation [47]. AOPPs may bind to the endothelial RAGE receptor, which subsequently induces ROS production by activation of NADPH oxidase [47]. Moreover, the interaction between AOPPs and RAGE activates downstream signalling pathways involving ERK 1/2, p38 MAPK, and NF-κB [47]. This cascade of events results in overexpression of ICAM-1 and VCAM-1 adhesion molecules [47]. In summary, high levels of AOPPs were found in both CKD and CVD, and were linked to poorer cardiovascular survival in ESRD. Molecular basis underpinning AOPPs-mediated vascular injury has also been observed. Therefore, our data together with findings from other studies suggest that AOPPs could be a potential targets for future intervention in ESRD patients with CVD comorbidities.

Apart from proteins, polyunsaturated fatty acids are susceptible to oxidative modifications, as well. The impact of oxidized lipids in vascular diseases has been extensively studied. Several cardiovascular events are associated with increasing serum levels of MDA. These include hypertension, acute myocardial infarction and CAD. A large multi-centric study following a cohort of 700 patients with stable CAD, reported a strong predictive power of MDA serum levels in relation to adverse cardiovascular events [267]. Besides the potential of MDA as a marker of CVDs progression, MDA was implicated as a marker for CKD onset, progression and prognosis. According to the several studies, elevated MDA levels positively correlated with the CKD progression [12,116–118] and cardiovascular risk [123]. In our study, ESRD patients with high MDA levels had a trend towards shorter cardiovascular survival compared to the patients with lower MDA concentrations, which is in line with the literature.

Furthermore, a significant prognostic value of PAB was also demonstrated in this thesis. Patients with high PAB levels had almost a 2-fold higher risk of overall as well as cardiovascular mortality. Data regarding the association of PAB levels with either susceptibility to CKD or CVD, or their prognosis are limited. Several studies reported an association between PAB and CVDs, showing that PAB levels were elevated 12-h post myocardial infarction [268] and that PAB might be associated with CAD [269,270]. The significant prognostic potential of PAB on 5-year survival previously reported by our group [124] has been confirmed on 8-years follow-up in this thesis. This effect remained

significant even after adjusting for cofounders like demographic, laboratory characteristics and comorbidities. Our results outline the importance of PAB measurement in prediction of ESRD patients' prognosis and suggest that this field should be further examined.

In addition to biomarkers of oxidative stress, we also examined the predictive value of markers of endothelial dysfunction, sICAM-1 and sVCAM-1. These molecules have an essential role in the pathogenesis of atherosclerotic cardiovascular disease. Elevated levels of sICAM-1 and sVCAM-1 have been reported previously in CKD patients, both on conservative treatment and HD [211,271]. Apart from its significance in prediction of the risk for cardiovascular events in the general population, sICAM-1 levels were found to be an independent predictor of mortality in pre-dialysis and HD patients as well [272–275]. In our study, high sICAM-1 levels were associated with increased risk of cardiovascular mortality which is in line with the literature. sVCAM-1 had a more pronounced predictive effect in our study, since elevated sVCAM-1 levels influenced higher overall and cardiovascular mortality. This is in concordance with the study of Papagianni et al. that reported that sVCAM-1 was an independent predictor of 5-year overall and cardiovascular survival [275]. There are several studies, however, showing no association between soluble cell adhesion molecules and mortality in patients with CKD [276,277]. Moreover, our preliminary data after 5-year follow-up, showed significant influence of sVCAM-1 levels on HD patients' survival, while only a trend was observed with sICAM-1 [124]. As such, in order to reach a clearer picture, more data should be gathered, preferably with larger sample size and longitudinal study designs.

After the analysis, we ascertained biomarkers that significantly predicted survival. These biomarkers (*GSTM1* genotype, high AOPP, PAB, MDA, sICAM-1, sVCAM-1) were further combined into a scoring model that stratified patients into high and low mortality risk. According to our results, significantly shorter overall and cardiovascular survival was observed in patients with the highest score.

As discussed in previous paragraphs in this thesis, high levels of oxidative stress byproducts are found in ESRD patients, and may have a role in predicting survival. The etiology of enhanced oxidative damage in ESRD patients could be linked to patients' genetic predisposition, apart from the already established factors such as uremic state and dialysis procedure [126]. Our research group has previously shown that the elevation of these biomarkers in ESRD patients can be dependent on the polymorphisms of GST genes [126]. Members of GST enzyme superfamily are able to detoxify accumulated uremic toxins and exhibit antioxidant activity [227,228,230]. Nevertheless, it has not been investigated yet if polymorphisms of enzymes front-line protection against ROS - SOD2 and GPX1 together with Nrf2, can influence individual susceptibility towards the increase of panel of oxidative stress biomarkers assessed in this study. We hypothesized that patients carrying high-activity alleles of antioxidant genes *SOD2, GPX1,* as well as *Nrf2* have better protection against ROS. Therefore, a possible mechanistic explanation for the reported influence of these polymorphisms on either risk and/or prognosis in ESRD patients may lie in their direct impact on oxidative stress. However, only *SOD2* polymorphism exerted notable influence on these parameters in our study.

In addition to having high independent impact on ESRD risk, *SOD2* polymorphism significantly correlated with oxidative stress byproducts. Regarding byproducts of protein

oxidative damage, we showed significant differences in terms of thiol groups and protein carbonyls. To the best of our knowledge, only one study investigated the association between *SOD2* rs4880 polymorphism and protein oxidative stress byproducts in kidney pathology [173]. In that study, variant *SOD2 Val/Val* genotype correlated with higher plasma AOPP concentration and lower SOD activity, among 310 T1DM patients who developed diabetic nephropathy [173]. Additionally, a haplotype analysis of four *SOD2* polymorphisms rs4880, rs574613, rs2758329 rs8031, in the same study showed an association of other *SOD2* risk alleles with higher AOPP concentrations [173]. The absence of significant association of AOPP with *SOD2* rs4880 polymorphism in our study was probably due to several limitations such as small cohort and heterogeneous etiology of ESRD (our study had a small percentage of diabetic patients).

Variant *SOD2* genotype correlated with the byproducts of lipid oxidative damage (MDA and MDAadd) in plasma of ESRD patients. It has been recently reported that the major plasma aminothiols (homocysteine, cysteinylglycine, cysteine, glutathione), together with AOPP and MDA detrimentally affected patients with kidney diseases, such as autosomal dominant polycystic kidney disease (ADPKD) and IgA nephropathy (IGAN) [278]. Along with these markers of oxidative stress, the same investigation included the assessment of ten SNPs of antioxidant enzymes with *SOD2* rs4880 between them. However, investigators did not correlate *SOD2* polymorphism with aforementioned biomarkers, not reporting on its possible influence on CKD [278]. Therefore, we were the first group to explore the association between *SOD2* rs4880 polymorphism and oxidative phenotype among ESRD patients. Our study design indicates that the influence of *SOD2* polymorphism on the susceptibility to ESRD development might be mediated by the increased oxidative stress injury found in patients with low activity *SOD2 Val* allele.

Regarding *GPX1* polymorphism, we did not show any statistically significant association with oxidative phenotype. The only study correlating other *GPX1* gene polymorphisms (rs3448, rs1987628, rs9819758, rs8179164) and biomarkers of oxidative stress (AOPP and isoprostanes) is the investigation of Mohammedi et al [279]. In this study, *T* allele of *GPX1* rs3448 gene had an impact on higher susceptibility to ESRD among diabetic patients, as well as, higher levels of AOPP and isoprostanes [279]. Their analysis of other three investigated *GPX1* polymorphisms showed no association with ESRD incidence among T1DM patients nor with oxidative stress parameters, which is in line with our study.

*Nrf2* polymorphism did not correlate with the levels of oxidative stress byproducts in ESRD in our study. Having in mind that this *Nrf2* polymorphism associates with attenuated binding of Nrf2 to the ARE, which results in decreased Nrf2-dependent antioxidant and detoxifying gene transcription, this finding was surprising [151,153]. Studies performed on CKD animal models reported a decline in nuclear Nrf2 in kidneys, in contrast to expected reactive upregulation [280]. As there are conflicting findings in the literature, more data is needed to clarify the role of *Nrf2* polymorphism on the progression of kidney deterioration.

The impact of *GSTM1* polymorphism on biomarkers of oxidative stress and endothelial dysfunction in this ESRD patients' cohort has been previously reported [126]. Building upon previous notions that indicate the significance of the GSTM1 deletion on

the oxidative phenotype of dialyzed patients, including their susceptibility to CVD, as well as their poorer cardiovascular prognosis [126,236], we aimed to elucidate the specific contribution of GSTM1 deletion on endothelial dysfunction in the uremic milieu.

In the final part of our investigation, HUVECs were silenced for *GSTM1* gene and treated with 30% control or uremic serum for 6 h, after which oxidative stress measurements (SOD and GPX1 activity, MDA levels and ROS) and relative expression of 105 cytokines were analysed. According to our results, uremic serum caused redox imbalance in HUVECs, characterised by a decrease in SOD and GPX antioxidant activity and increase in MDA levels, independently of the GSTM1 knockdown. Uremic serum treatment also led to perturbed expression of inflammatory cytokines. Under uremic conditions, angiogenin, ICAM-1, RANTES, CRP and RBP4 were upregulated, while Dkk-1 and PF4 expression was decreased. Notably, the reduction in GSTM1 in HUVECs led to an increase in MCP-1 expression. In addition, GSTM1 knockdown led to increased expression of a cell adhesion molecule ICAM-1 in HUVECs, while the trend towards increased VCAM-1 expression was observed.

Endothelial cells are permanently exposed to uremic toxins in ESRD, which exert a pletora of detrimental changes on endothelium. Collectively, these changes are characterised by reduced vasodilation and the promotion of proliferation, coagulation, monocyte adhesion and oxidative stress [209]. Oxidative stress plays a pivotal role in the uremic toxins-mediated development of endothelial dysfunction. The pro-oxidant role of uremic toxins in endothelium has been confirmed by several *in vitro* studies (Table 1S, Supplement). It was previously demonstrated that the sources of excessive ROS production in HUVECs under uremic conditions include the activation of NADPH oxidase, eNOS uncoupling and mitochondrial dysfunction [43,45,220,281]. Increased oxidative stress in HUVECs treated with uremic serum or isolated uremic toxins are also based on impaired cellular enzymatic antioxidant defence, characterised by decreased SOD and GPX expression and/or activity [45,53]. According to the data obtained in this thesis, uremic serum treatment caused a decrease in both SOD and GPX antioxidant capacity, which in turn increased oxidative damage of lipids. Enhanced oxidant injury of endothelial macromolecules under uremic conditions has been also reported by the other research groups [45,53]. Besides, our *in vitro* findings are in line with well documented increase in MDA levels [116–118] and decrease in extracellular antioxidant capacity in CKD patients [66–68,89,90]. Therefore, our results provide further evidence that endothelial cells contribute to systemic oxidative stress in uraemia.

Given the role of GSTM1 deletion on the susceptibility to ESRD and poor prognosis in these patients, we hypothesised that the lack of GSTM1 activity in endothelial cells will make them more vulnerable and prone to oxidative damage. Interestingly, while ROS and MDA levels did not differ between $GSTM1^{+/+}$ cells and cells silenced for GSTM1 ($GSTM1^{+/-}$) under uremic conditions, HUVECs silenced for GSTM1 had a higher ROS and especially MDA levels ($p=0.05$) under control serum conditions. This is in line with the studies reporting that GSTM1 knockdown in VSMCs led to increased ROS and HNE levels [242,246].

The absence of the influence of GSTM1 knockdown on the extent of oxidative stress under uremic conditions is likely due to downregulation of GSTM1 in $GSTM1^{+/+}$ cells via

downregulated Nrf2, or by the compensatory upregulation of Nrf2 in GSTM1$^{+/-}$ cells. The resulting effect of both scenarios is the same antioxidant protection in GSTM1$^{+/+}$ and GSTM1$^{+/-}$ cells, and thus the same level of oxidative stress in HUVECs. In view of the fact that the GSTM1 is a known Nrf2 target [229], and that the diminished Nrf2 activity was found in HUVECs under uremic milieu [45], it is possible that uremic serum led to a decrease in GSTM1 expression in GSTM1$^{+/+}$ HUVECs via downregulation of Nrf2. This may suggest that GSTM1$^{+/+}$ cells in the uremic milieu were left without GSTM1 protection. On the other hand, GSTM1 knockdown in GSTM1$^{+/-}$ HUVECs might have led to compensatory upregulation of Nrf2 and Nrf2 target antioxidant genes, thus having the same overall antioxidant protection as GSTM1$^{+/+}$ cells. Indeed, it has been reported that GSTM1 knockdown in VSMCs resulted in a significant reactive increase in Nrf2 levels [242]. Thus, while Nrf2 regulates the transcription of GSTM1, the authors suggested that GSTM1 in turn might regulate the expression of Nrf2 post-transcriptionally [242]. This may lead to a compensatory upregulation of the antioxidant enzymes under the conditions of reduced GSTM1. In this line, our results showed the slight upregulation of SOD and GPX antioxidant activities in HUVECs silenced for GSTM1, although this was not statistically significant. Since our study is the only one examining the impact of GSTM1 on oxidative stress in endothelium, it is necessary to further explore whether the GSTM1 deletion contributes to endothelial dysfunction in uraemia via perturbations of redox balance.

Regarding the expression of cytokines, incubation in uremic serum resulted in higher expression of angiogenin, ICAM-1, RANTES, CRP and RB4, while the expression of Dkk-1 and PF4 was suppressed. These changes should be interpreted in the context of complex pathophysiology of ESRD patients' vasculature, often leading to arteriosclerosis and atherosclerosis. In the development of arteriosclerosis in CKD, a key role is played by a disturbance of calcium and phosphate homeostasis, which results in hypocalcaemia, hyperphosphatemia and secondary hyperparathyroidism and together with uremic toxins, contributes to accelerated calcifications of arteries. One of the proteins involved in mineralization or calcification of arterial walls is Dkk-1 [282]. Dkk-1 is well known as a protein associated with impaired osteoblast activation and the bone loss [283]. The expression of Dkk-1 is not restricted to bone, as it has been also found in endothelium and other tissues [282]. So far, several studies implicated that Dkk-1 has a protective role against arterial calcifications [284–286]. On the other hand, it has been suggested that reduced Dkk-1 could promote mineralization or calcification [284]**.** This is substantiated by the fact that suppressed Dkk-1 expression has been found at sites of aortic calcification in mice [287]. According to the data obtained in this thesis, uremic serum treatment diminished the expression of Dkk-1 in HUVECs. Our results are in line with other authors that reported a negative association between circulating Dkk-1 levels and arterial stiffness in pre-dialysis CKD [288] and arterial calcified plaques in T2DM [285]. Although our findings should be confirmed in larger clinical settings, these results point to the potential future therapies that may target Dkk-1 downregulation in ESRD.

Atherosclerosis is primarily a disorder of the intima of medium diameter arteries, characterized by plaque formation, narrowing and occlusion of the blood vessels [289]. It is believed that the mechanism of atherosclerosis in ESRD patients includes the same

events as in the rest of the population without CKD. However, the rate of progression of atherosclerosis as well as the degree of oxidative modifications, expression of adhesion molecules, formation of foam cells and proliferation of smooth muscle cells is more intense in CKD patients [201]. In our study uremic serum treatment altered the expression of molecules known to stimulate cell proliferation (angiogenin) and monocyte adhesion (ICAM-1, RANTES and PF4) in HUVECs. Notably, these processes have essential roles in the pathophysiology of atherosclerosis.

Angiogenin was first identified as a potent inducer of neovascularization [290]. The role of angiogenin has been implicated in promoting angiogenesis by activating endothelial and vascular smooth muscle cells, as well as in cell migration, invasion, and formation of tubular structures [291]. Noteworthy, high angiogenin levels were found in CKD patients and increase significantly with CKD progression [292]. In our study, uremic serum caused an increase in angiogenin expression in HUVECs. So far, high angiogenin levels have been linked with increased arterial stiffness in patients with CKD [292]. Moreover, positive association between angiogenin levels and acute coronary syndrome, as well as peripheral occlusive arterial disease were found [293,294]. Therefore, bearing in mind the aforementioned properties of angiogenin, our results indicate that endothelium might contribute to accelerated atherosclerosis in uremic conditions by upregulating angiogenin expression.

ICAM-1 and VCAM-1 are involved in firm adhesion of leukocytes to endothelium, which is the early event in the development of atherosclerosis. ICAM-1 is constitutively expressed by resting endothelial cells. On the other hand VCAM-1 is weakly expressed by the resting endothelium. The expression of these molecules is strongly enhanced by cytokine stimuli. In our study uremic serum treatment caused an increase in ICAM-1 expression in HUVECs, while VCAM-1 expression remained the same as in control serum conditions.This is aligned to the study of Tumur et al. et al who reported that uremic toxin IS significantly increased ICAM-1 expression in HUVECs, while this effect on VCAM-1 was slower [295]. In contrast, the data obtained in the clinical settings of CKD patients showed the increase in both aforementioned adhesion molecules [296]. The absence of a upregulation of VCAM-1 in HUVECs as a response to uremic serum treatment might be explained by a short incubation time, which might not be sufficient for VCAM-1 upregulation.

Endothelial cells in proinflammatory environment produce chemokines such as RANTES. RANTES is involved in transmigration and arrest of monocytes onto activated endothelium; hence it has been recognized as an important mediator of atherogenic processes [297]. Our study showed for the first time that RANTES expression might be upregulated *in vitro* upon exposure to uremic serum. Its importance in renal disease was indicated in a study of renal transplants undergoing rejection where high amounts of RANTES bound to the vascular endothelium were observed. On the other hand, this effect was not present in normal renal tissues [298]. In our study, RANTES was expressed only in uremic serum incubated HUVECs, while the HUVECs incubated in control serum did not have detectable expression of this protein. The only adhesion molecule whose expression was decreased upon uremic serum stimulation in our study was PF4. However, in the view of the fact that PF4 inhibits progenitor cell proliferation and

angiogenesis [299], its decreased expression in HUVECs might result in atherosclerosis promotion, and therefore seems biologically plausible.

Uremic serum treatment also led to the upregulation of two acute-phase reactants in HUVECs, RBP4 and CRP in this study. RBP4 is a protein primarily secreted by the liver and adipose tissue, and its well established function is retinol transport in the circulation [300]. Notably, elevated levels of RBP4 isoforms were reported in CKD patients [301]. Similarly, Thawnashom et al. showed that RBP4 levels in T2DM patients might depend on the level of eGFR; individuals with eGFR <60 ml/min/1.73 $m^2$ had higher RBP4 levels [302]. Elevated RBP4 levels have been also associated with the higher degree of carotid intima-media thickness in T2DM patients [303]. It seems that RBP4 stimulates the expression of proinflammatory cytokines and adhesion molecules in HUVECs, thus inducing inflammation and accelerating endothelial dysfunction [304]. In relation to CRP, uremic serum treated HUVECs had over 2-fold rice in CRP expression compared to HUVECs incubated in control serum. Our study confirms previous findings that endothelial cells are one of the sources of CRP production [305]. In addition to being a marker of inflammation, a growing body of evidence suggests that CRP may directly participate in the development of atherosclerotic vascular disease. Therefore, elevated CRP levels have emerged as one of the most powerful independent predictors of cardiovascular disease. To the best of our knowledge this is the first result on upregulated CRP expression in HUVECs upon exposure to uremic serum.

It is likely that the inflammatory response in HUVECs to uremic injury could be a result of induction of overlapping signalling pathway. Indeed, the promoters of RANTES, ICAM-1, CRP and RBP4 genes comprise binding sites for the NF-κB transcription factor [295,306–308]. Multiple studies consistently reported that excessive ROS production in uremic serum treated HUVECs leads to activation of NF-*κ*B signalling pathway [44,50,221,225]. Having in mind that uremic serum promoted oxidative stress in HUVECs in this study, consequent activation of NF-κB signalling pathway might be one of the possible mechanisms for the upregulation of inflammatory molecules in HUVECs. This is should be explored in the future studies.

Finally, the GSTM1 knockdown in HUVECs led to an increased expression of endothelial adhesion molecules MCP-1, and ICAM-1. Particularly, the MCP-1 expression increased over 2-fold in response to GSTM1 knockdown in HUVECs incubated in either control or uremic serum. The highest expression of MCP-1 was observed in uremic serum treated $GSTM1^{+/-}$ HUVECs, which might suggest an additive effect of uraemia and GSTM1 deletion on MCP-1 upregulation. The increase in the expression of MCP-1 mRNA and protein in HUVECs treated with uremic toxins has been reported previously [220]. With respect to the role of MCP-1 in attracting monocytes to the site of vascular injury, our findings offer one of the mechanistic clues for higher risk of CVDs in individuals lacking GSTM1, which is even more prominent in uremic milieu in ESRD patients. The data obtained in this study is in accordance with the study of Gigliotti et al. who recently reported that GSTM1 knock out mice had a significant rise in renal expression of MCP-1 [244]. MCP-1 is also an important factor in the pathogenesis and progression of renal failure [309]. Higher urinary MCP-1 levels were found in CKD patients and correlated with kidney damage [309]. Although the precise mechanism of GSTM1-mediated

regulation of MCP-1 remains unknown, it is important to note that GSTM1 has a functional noncatalytic domain that inhibits activation of the ASK1-p38 signalling pathway [231]. Terada et al. reported that ASK1 directly regulates MCP-1 expression [310]. Moreover, p38 MAPK - mediated regulation of MCP-1 expression has also been confirmed in HUVECs [311]. Thus, it might be postulated that the lack of GSTM1 protein in HUVECs silenced for the GSTM1 gene results in higher expression of MCP-1 due to the lack of ASK1 inhibition. Similarly, it is possible that another two upregulated proteins, ICAM-1 and VCAM-1, in a response to GSTM1 knock down might be associated with ASK1 signalling pathway [312]. Further studies are needed to confirm our hypothesis.

In summary, the results of our *in vitro* investigation confirmed the previous findings that the endothelium responds to uremic conditions by increased oxidative stress and inflammation. Notably, we reported for the first time that the expression of Dkk-1, angiogenin, RANTES, PF4, RBP4 and CRP might be altered in HUVECs upon exposure to uremic serum. Interestingly, this study describes a novel function of endothelial GSTM1 in the regulation of monocyte migration and adhesion, through its role in upregulaton of MCP-1. In addition, HUVECs silenced for the GSTM1 gene exhibited higher expression of ICAM-1 than $GSTM^{+/+}$ cells further strengthening its potential biomarker role as a predictor of CVD in ESRD patients. The association of the GSTM1 knockdown with the upregulation of adhesion molecules might be at least partly responsible for the increased susceptibility of ESRD patients with *GSTM1-null* genotype to CVD.

# 6. CONCLUSION

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

1. *SOD2* rs4880, *GPX1* rs1050450 and *Nrf2* rs6721961 polymorphisms influence the risk of ESRD development individually and/or in combination.

- *SOD2* polymorphism has an individual impact on ESRD development. Individuals with SOD2 *Val*/*Val* genotype have a 2 fold higher risk of ESRD development than *Ala*/*Ala* homozygotes.
- Individuals who carry both *GPX1* and *SOD2* low-activity genotypes (*GPX1 Leu*/*Leu* and *SOD2 Val*/*Val*) are at a 3.3 fold higher risk of ESRD development compared to those with at least one protective *GPX1* and *SOD2* allele (*GPX1 Pro*/*Pro+Pro*/*Leu* / *SOD2 Ala*/*Ala+Ala*/*Val*).
- *SOD2 Val*/*Val* genotype with *Nrf2 C*/*C* genotype is associated with 1.79 increased risk of ESRD development.

2. *SOD2* polymorphism has a significant impact on the protein (thiol groups, carbonyl groups) and lipid (MDA, MDAadd) oxidative stress byproducts in ESRD patients.

3. *Nrf2* rs672961, *SOD2* rs4880, *GPX1* rs1050450 and *GSTM1* deletion polymorphisms, biomarkers of oxidative stress and biomarkers of endothelial dysfunction have significant prognostic potential in terms of 8-year overall and cardiovascular survival in ESRD patients.

- *GSTM1-null* genotype is the strongest independent predictor of 8-year overall and cardiovascular survival.
- Combination of the best survival genotypes including the *Nrf2 C*/*C* and *GPX1 Leu*/*Leu*, together with *GSTM1-active* genotype and age contributes to longer overall survival.
- *GPX1 Leu*/*Leu* genotype, together with *GSTM1-active* genotype and age is associated with longer cardiovascular survival.
- PAB levels are significant predictors of both overall and cardiovascular survival, while AOPP levels are significant predictors of cardiovascular survival.
- Increased sVCAM-1 levels are associated with shorter overall and cardiovascular survival, while high sICAM-1 levels are associated with increased risk of cardiovascular mortality.

- When six biomarkers (*GSTM1* genotype, high AOPP, PAB, MDA, sICAM-1, sVCAM-1) are combined into a scoring prognostic model, a significantly shorter overall and cardiovascular survival is observed in patients with the highest score.

4. Uremic serum treatment and/or GSTM1 knockdown induces redox imbalance accompanied with altered expression of a series of cytokines involved in arteriosclerosis and atherosclerosis in HUVECs.

- HUVECs treated with uremic serum exhibit impaired redox balance characterized by decreased antioxidant (SOD and GPX) enzyme activities and enhanced lipid peroxidation, independently of the GSTM1 knockdown.
- Uremic serum treatment alters the expression of a series of inflammatory cytokines involved in either arteriosclerosis and/or atherosclerosis in HUVECs, including Dkk-1, angiogenin, PF4, ICAM-1, RANTES, RB4 and CRP.
- GSTM1 knockdown in HUVECs leads to upregulation of molecules involved in monocyte adhesion to endothelium (ICAM-1) and transmigration across the vascular endothelium (MCP-1).

New developments in the field of antioxidant polymorphisms in ESRD patients could lead to better stratification of ESRD patients based on a prognostic panel of antioxidant genes and biomarkers of oxidative stress, and provide a more personalised medicine approach for the need of targeted antioxidant therapy in these patients. The association of the GSTM1 knockdown with the upregulation of adhesion molecules might be at least partly responsible for the increased susceptibility of ESRD patients with *GSTM1-null* genotype to CVD.

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# SUPPLEMENT

**Table 1S.** The effects of uremic serum and isolated uremic toxins on HUVECs

| Reference | Material and methods | Results |
|---|---|---|
| [221] | HUVECs were isolated and confirmed by FACS specific markers: CD31, CD144, vWF, CD141<br>**Transfection:** AhR siRNA<br>**Treatments:** IAA (5-50μM)<br>**Analysis:**<br>**RT-PCR**: COX-2, Cyp1A1, Cyp1B1, AHRR, AHR, IL-8,IL-6, ICAM-1, VCAM-1, MCP-1, NF-kB, p50, p65<br>**WB**: COX-2, phospho-p38, p38, phospho-ERK1/2, ERK1<br>**FACS:** ROS<br>**ELISA**: PGE2 | -IAA icreased: COX-2, IL-8, ICAM-1,MCP-1, IL-6 mRNA and PGE2 levels in HUVECs<br>-IAA activated an AhR/p38 MAPK/NF-kB signalling pathway that induced COX-2 up-regulation<br>-IAA increased ROS after 4h and up to 24h |
| [43] | HUVECs were isolated and confirmed as described [221]<br>**Treatments:** IS (25-250 μg/ml), 5h ± inhibitors of:<br>XO (allopurinol), NO synthase (L-NAME), NAD(P)H oxidase (apocynin or DPI), mitochondrial electron transport chain (rotenone) ± antioxidants vitamin E, vitamin C, N-acetyl cysteine<br>**Analysis:**<br>**FACS:** ROS<br>**RT-PCR**: eNOS, iNOS<br>**ELISA**: GSH levels | - IS increase ROS production in HUVECs<br>-Apocynin inhibited IS -induced ROS production in HUVEC<br>- IS did not alter expression of eNOS mRNA<br>- iNOS was not detected in HUVECs<br>- IS decreased total GSH levels in higher concentrations<br>- The increase of ROS was inhibited by apocynin (71%), viamin C (90%), NAC (65%) and vitamin E (46%) |
| [220] | Commercial HUVECs and HASMC<br>**Transfection:** Nox4 siRNA<br>**Treatments:** PCS (10-1000 μmol/L), 1h ± inhibitors of:<br>NAD(P)H oxidase (DPI), PI3K (wortmannin), PKC (calphostin C), OAT (probenecid)<br>**Analysis:**<br>**ELISA:** MCP-1, ALP activity<br>**FACS:** ROS<br>**RT-PCR**:MCP-1, ALP, OPN, Cbfα1<br>**WB:** Nox4, OPN | -PCS led to ROS increase in HUVEC and HASMC, which was inhibited by all inhibitors suggesting the contribution of NADPH oxidase system and cellular uptake of PCS via OAT<br>-PCS increased Nox4<br>-1000μmol/L PCS resulted in MCP-1 mRNA increase and MCP-1 protein secretion by 2.5 times (this effect was suppressed by probenecid)<br>- PCS increased mRNA levels of ALP, OPN and Cbfa1 in HASMC and ALT activity (this effect was suppressed by probenecid)<br>-Nox4 knockdown suppressed PCS-induced ROS generation in HUVEC or HASMC but had no effect on the basal ROS levels |
| [212] | HUVECs were isolated and confirmed as described [221]<br>**Treatments:** Pre- or post- HD serum (dilutions 5% - 20% v/v), 24-72h<br>**Analysis:**<br>**Cell viability assay:** MTT, Crystal violet<br>**Migration assay**<br>**FACS:** cell apoptosis assay annexin V FITS kit<br>**Endothelial wound repair**<br>**Gelatin zymography**: activity of MMP-2 and MMP-9<br>**WB**: MMP-2,-9, TIMP-1,-2<br>**RT-PCR**: MMP-2,-9, TIMP-1,-2 | - Increased concentrations of pre-HD serum resulted in reduced proliferation of endothelial cells compared to the relative concentrations of post-HD serum<br>- Pre- or post-HD sera led to a time dependent increase in the percentage of apoptotic cells. This effect was more pronounced in pre- HD serum cultured cells<br>- Pre-HD serum had a small positive effect on the migration of HUVEC. Post-HD serum had a dose-dependent induction on the migration of endothelial cells<br>- Endothelial wound repair in monolayers exposed to pre-HD serum was lower than in cells exposed to post-HD serum<br>- Pre-HD serum increased MMP-9 activity and MMP-2 protein levels after 24 hours compared to post-HD serum treatment<br>- Post-HD serum increased TIMP-1 and TIMP-2 protein levels compared to pre-HD serum treatment<br>- Pre- or post-HD sera induced the expression of both collagen IV and elastin. This increase was more pronounced with the post-HD serum |
| [224] | HUVECs were isolated and confirmed by morphology and immunocytochemistry with anti-CD31 monoclonal antibody<br>**Treatments:** 10% control/uremic serum containing media, 6h and 12h<br>**Analysis:**<br>**Cell viability assay:** Trypan blue, MTT<br>**ELISA**: SDF-1 (stromal cell-derived factor-1), IL-8 | -Incubation with 10% uremic serum containing media resulted in lower expression of SDF-1 after 6h and increased expression of IL-8 after 12h, when compared to the 10% control serum containing media |

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| [313] | HUVECs were isolated and confirmed as described [221]<br>**Treatments:** PCS (10-50μg/ml) and IS (25-125 μg/ml), 24h<br>**Analysis:**<br>**Endothelial cell proliferation assay** was assessed by BrdU incorporation into cellular DNA<br>**Cell viability assay:** Tripan blue<br>**FACS:** cell apoptosis assay annexin V FITS kit<br>**Endothelial wound repair** | - PCS inhibited endothelial proliferation in a dose-dependent manner<br>- IS inhibited endothelial proliferation regardless IS dose<br>- PCS and IS did not induce endothelial apoptosis<br>- PCS and IS reduced endothelial wound repair |
| [44] | Commercial HUVECs and THP-1 cells<br>**Transfection**: pNF-kB-Luc (NF-kB firefly luciferase cDNA construct) and pRL-TK (thymidine kinase-Renilla luciferase construct)<br>**Treatments:** TNF-α 4h ± IS (2mmol/l), 20h ± inhibitors of: NAD(P)H oxidase (apocynin), XO (allopurinol), mitochondrial electron transport chain (rotenone), OAT (probenecid) ± antioxidants: N-acetyl cysteine<br>**Analysis:**<br>**Monocyte adhesion assay**<br>**WB:** ICAM-1, VCAM-1, E-selectin, JNK, p-JNK, p38, p-p38,MAPK, ERK1/2, p-ERK1/2, p65 , p-p65, NF-kB | -IS enhanced monocyte adhesion to TNF- α activated HUVECs<br>-IS enhanced TNF- α-induced E-selectin expression in HUVEC, but not ICAM-1 and VCAM-1 expression<br>-Blocking with antibody against E-selectin, but not against ICAM-1 and VCAM-1 inhibited IS-enhanced leukocyte adhesion induced by TNF- α<br>-JNK signaling pathway was involved in IS-enhanced leukocyte-endothelial interactions<br>-IS enhanced JNK and p38 MAPK (but not ERK1/2) phosphorilation in HUVEC in presence of TNF-α<br>- NF-kB signaling pathway was involved in IS-enhanced leukocyte-endothelial interactions<br>-Apocynin and N-acetyl cysteine reduced IS-induced THP-1 cell adhesion to HUVEC and E-selectin expression. Allopuriol and rotenone had no effect.<br>-Probenecid inhibited the IS mediated increase in THP-1 adhesion and E-selectin expression but did not suppress ICAM-1 and VCAM-1 expression |
| [46] | HUVECs isolated and confirmed [221] and commercial THP-1 cells<br>**Treatments:** PCS (1-100μg/ml), 4h or 24h ± TNF-α or IL-1β<br>**Analysis:**<br>**Quantification of cell adhesion molecules expression:** quantitative immunofluorescent indirect assay<br>**FACS**: ICAM-1, VCAM-1, E-selectin<br>**Monocyte adhesion assay**<br>**RT-PCR:** ICAM-1, VCAM-1 | - PCS alone had no effect on endothelial adhesion molecules, but TNF-α and IL-1β induced a strong increase in ICAM-1 and VCAM-1 expression on endothelial surface<br>- PCS inhibited cytokine – stimulated expression of ICAM-1 and VCAM-1 but not E-selectin<br>- PCS reduced cytokine induced THP-1 adhesion to endothelial cells |
| [222] | HUVECs and RBC were isolated as described [314]<br>**Treatments:** control/uremic RBC (10% haematocrit), 3h ,6h ,12h ,18h ,24h ± inhibitor of the MAPK pathway ( PD98059)<br>**Analysis:**<br>**WB**: ICAM-1, VCAM-1, MAPK, p-MAPK, Akt, p-473 Akt, p-Ser eNOS 1177<br>**RT-PCR**: ICAM-1, VCAM-1<br>**U937 cell adhesion assay** | -Uremic RBC caused time-dependent increase in VCAM-1 and ICAM-1 protein expression (12h,24h 2x↑) when compared to control-RBC<br>-After 6h of incubation, uremic RBC increased VCAM-1 and ICAM-1 mRNA when compared to control-RBC (mRNA peaked after 6h and returned at baseline levels after 18-24h).<br>- Uremic RBC caused greater MAPK phosphorylation than control-RBC<br>- Inhibitor of MAPK pathway ( PD98059) profoundly inhibited the uremic-RBC mediated increase of VCAM-1 and ICAM-1 mRNA and protein expression<br>- Uremic RBC caused almost complete inhibition of Akt phosphorylation and reduced eNOS activation<br>- Uremic RBC caused increase in monocyte adhesion compared with C-RBC |
| [315] | Commercial HUVECs<br>**Treatments:** incubation with 11 isolated uremic toxins at their mean and maximum serum concentrations found in hemodyalised patients<br>**Analysis:**<br>**FACS: ROS** | - At mean serum concentrations IS induced ROS production in HUVECs most intensely, followed by CMPF<br>- PCS did not induce any ROS production in HUVEC<br>-At their maximum concentration, IS showed the most intense ROS production in HUVECs, followed by indoxyl glucuronide |
| [281] | Commercial HUVECs<br>**Treatments:** IS (0-250μg/ml), 48h ± N-acetyl cysteine, vitamin C<br>**Analysis:**<br>**Cell viability assay:** MTT, Crystal violet<br>**FACS:** ROS, MMP<br>**RT-PCR:** mitochondrial DNA (mt-DNA) copy number<br>**Fluorescent microscope:** Mitochondrial mass | -Increasing doses of IS corresponded to decreased cell viability and higher levels of ROS<br>- MMP was reduced in IS treated HUVECs. Vitamin C and NAC was able to counteract the effect of IS<br>- mtDNA copy number and mitochondrial mass were dramatically reduced in IS treated HUVECs when compared to untreated controls. These effects could be reversed by NAC and vitamin C |

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| [47] | HUVECs were isolated and confirmed as described [316]<br>**Treatments:** AOPP-HSA (50-400μg/ml), AOPP-F (fraction formed *in vivo*- uremic serum, 50-200μg/ml), AGEs-HAS (2-10 μg/ml), 5-30min ± inhibitors of:<br>XO (allopurinol), NO synthase (L-NAME), NAD(P)H oxidase ( apocynin, DPI), mitochondrial electron transport chain (rotenone), PKC (Go6983), and $O_2^-$ scavenger (bovine cytosolic Cu/Zn SOD, c-SOD)<br>**Analysis:**<br>**FACS: ROS**<br>**Lucigenin-enhanced chemiluminiscence:** analysis of NAD(P)H oxidase $O_2^-$ production<br>**Microplate based assay**: binding AOPPs to RAGE<br>**WB:** $p47^{phox}$, $p22^{phox}$, NF-kB/p65, ERK1/2, p-ERK1/2, p38, p-p38, JNK 1/2, p- JNK 1/2, ICAM-1, VCAM-1<br>**Immunofluorescence:** NF-kB nuclear translocation<br>**RT-PCR:** ICAM-1, VCAM-1<br>**Immunostaining:** ICAM-1, VCAM-1 | -AOPP-HSA bounded to RAGE and inhibited the binding of soluble RAGE with AGEs<br>-AOPP-HSA and AOPP-F induced ROS production in HUVEC. ROS production was dependent on interaction of AOPPs with RAGE<br>-AOPP-HSA induced ROS production was suppressed only by NADPH oxidase inhibitors (DPI, apocynin)<br>-AOPP-HSA activated NAD(P)H oxidase through ligation of RAGE<br>- MAPK activation was mediated by by interaction of AOPPs with RAGE and regulated by $O_2^-$ activated NAD(P)H oxidase<br>- AOPP-HSA had no effect on JNK<br>-AOPPs activated NF-kB through RAGE-mediated signals<br>-AOPPs induced ICAM-1 and VCAM-1 expression at gene and protein levels through RAGE-mediated signalling pathway. This effect of AOPPs was prevented by blocking RAGE |
| [50] | Commercial HUVECs and THP-1 cells<br>**Treatments:** 0-20% control/uremic human serum containing media , 24h ± 4-PBA or PDTC<br>**Analysis:**<br>**Cell viability assay:** MTT<br>**FACS:** cell cycle<br>**RT-PCR:** GRP78, $P^{21}$, NF-kB, MCP-1, VEGF<br>**WB:** GRP78, p-ERK, MCP-1, Nf-kB, $P^{21}$<br>**ELISA:** VEGF, MCP-1<br>**Monocyte adhesion assay** | -Uremic serum (2.5-10%) treated HUVECs had higher proliferation than control serum treated HUVECs .The higher concentrations than 10% of uremic serum could significantly inhibit cell proliferation in dose-dependent manner<br>-In uremic serum group mRNA and protein levels of NF-kB, MCP-1 and VEGF were significantly increased when compared to control serum group<br>-Uremic serum can activate endoplasmic reticulum stress (ER) in HUVECs, measured through increased GRP78 (a chaperon released from ER membrane) mRNA and protein expression<br>-p-ERK expression also increased in U.S. group<br>-Treatment with 4-PBA significantly suppressed ER stress, NF-kB expression, MCP-1 and VEGF expression , as well as uremic serum-induced HUVEC proliferation<br>-Treatment with 4-PBA reduced the migration of THP-1 monocytes, which was previously induced by incubation with uremic serum |
| [225] | Commercial HUVECs and THP-1 cells<br>**Transfection:** RAGE siRNA, adenovirus expressing KLF2, GFP or IkBα dominant-negative mutant<br>**Treatments:** uremic porcine serum (0-100%), 24-48h or individual protein-bound uremic toxins: CML-BSA (5.4mg/L), IS (23.1mg/L), PCS (20.9mg/L)<br>**Analysis:**<br>**Cell viability assay:** MTT<br>**RT-PCR:** KLF2<br>**WB:** KLF2, anti-p65<br>**Monocyte adhesion assay**<br>**Fluorescent probes: ROS** | -Increasing concentrations of uremic serum decreased cell viability.<br>-KLF2 expression was reduced after incubation with 10% uremic serum for 48h. After 24h of incubation with 10% dialysed serum, KLF2 expression returned to baseline levels.<br>-CML-BSA and PCS reduced KLF2 expression, and upregulated the expression of RAGE<br>-RAGE knockdown normalized KLF2 expression<br>-CML-BSA increased ROS production and monocyte adhesion. This effect was reversed by KLF2 overexpression<br>-Activation of NF-kB by RAGE is responsible for supressing KLF2 |
| [223] | Commercial HUVECs<br>**Treatments:** 10% control/uremic human serum containing media, 6-24h HUVEs were treated with uremic serum obtained from ESRD patients with hypertension or diabetes or both<br>**Analysis:**<br>**Cell viability assay:** Trypan blue<br>**RT-PCR**: **:** VEGF, SDF-1, MCP-1<br>**ELISA:** VEGF, SDF-1, MCP-1 | -The mRNA expression of VEGF, SDF-1 and MCP-1 was higher in HUVECs treated with uremic serum than in those treated with control serum at all time points<br>-The peak of VEGF mRNA expression was at 6 h after treatment with uremic serum, then decreased after that.<br>- MCP-1 mRNA expression was higher in cells treated with uremic serum at all points when compared with cells treated with healthy serum, those treated with uremic serum from hypertensive patients showed the highest levels of MCP-1 mRNA expression at all time points. MCP-1 protein expression was higher in |

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| | | endothelial cells treated with uremic serum<br>- SDF-1 mRNA expression showed a time-dependent increase for all cohorts that peaked at 24 h after treatment with uremic serum. Similar to its mRNA expression, SDF-1 protein showed a time-dependent increase that peaked at 24 h after treatment |
| [53] | Commercial HUVECs<br>**Treatments:** IS (61 μg/mL), PCS (40 μg/mL), 0-24h ± Açaí seed extract (ASE)<br>**Analysis:**<br>**Cell viability:** Trypan blue<br>**Migration assay**<br>**RT-PCR**: **:** ICAM, VCAM, TNF-alpha<br>**ELISA:** TNF-alpha<br>**WB:** SOD2, CAT, GPX<br>**Spectrophotometrically**: SOD, GPX, CAT activities**,** protein carbonylation | - IS and PCS reduced cell viability and migratory capacity in endothelial cells. ASE prevented cell death and restored the migratory capacity in cells exposed to IS<br>- IS and PCS induced ICAM-1, VCAM-1 and MCP-1 expression. ASE prevented uremic toxins' effects on pro-inflammatory cytokine expression<br>- IS and PCS treated cells had higher TNF-α levels then control cells, which was reversed by ASE<br>- In cells treated with both uremic toxins (individualy or in combination), the expression of SOD enzyme was significantly lower when compared to control cells<br>-The expression of GPX in cells treated with PCS was lower when compared to control cells<br>-Endothelial cells treated with PCS or IS+ PCS showed a significantly higher SOD activity when compared to control cells<br>- IS+ASE treated cells a greater GPx activity when compared to other groups treated with uremic toxins<br>-Catalase activity was lower in cells treated with IS and IS+ PCS compared to ASE group.<br>- Cells treated simultaneously with both toxins showed a significantly higher amount of protein carbonylation when compared to the other groups |
| [45] | Commercial HUVECs<br>**Transfection:** Nrf2 siRNA 48h<br>**Treatments:** control/uremic serum 24h ± pterostilbene (PT)<br>**Analysis:**<br>**Cell proliferation:** Cell counting kit-8<br>**Lucigenin-derived chemiluminescence method:** Superoxide anions level and NAD(P)H oxidase activity<br>**Commercial kits:** LDH, MDA, nitrotyrosine levels, SOD, CAT activities NO production, eNOS activity, $H_2O_2$ assay<br>**RT-PCR:** Keap-1, Nrf2, TNF-α, IL-1β, VCAM-1, MCP-1<br>**ELISA:** TNF-α, IL-1β, VCAM-1, MCP-1<br>**WB:** eNOS, p-eNOS, iNOS, Keap-1, Nrf2, HO-1 | - Uremic serum inhibited the proliferation and increased the LDH release in HUVECs. Pretreatment with PT declined these changes<br>- In HUVECs treated with uremic serum activities of SOD and CAT were decreased, but the levels of MDA, $H_2O_2$ , superoxide anion and NAD(P)H oxidase activity were increased<br>-HUVECs treated with US exhibited higher NO and nitrotyrosine content than those in the control group, and these changes were reversed by PT.<br>-The reduced eNOS activity in response to uremic serum was restored by PT pretreatment.<br>-The phosphorylation level of eNOS was markedly decreased, while iNOS expression was significantly elevated in uremic serum -treated HUVECs, and these changes were reversed by PT<br>-Incubation of HUVECs with US resulted in significant increase of proinflammatory cytokines IL-1β, TNF-α, MCP-1 and VCAM-1 expression of at both protein and mRNA level<br>- Uremic serum -incubated HUVECs displayed the decreased total Nrf2, HO-1 expression and increased Keap1 expression at both protein and mRNA level, which were rectified by PT. |

Abbreviations: HUVEC, human umbilical vein endothelial cells; HASMC, human aortic smooth muscle cells; THP-1, human monocyte cell line; IAA, indole-3 acetic acid; PCS, p-cresol sulfate; IS, indoxyl sulfate; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid; DPI, diphenylene iodonium; NAC, N-acetyl-L-cysteine; 4-PBA, chemical chaperone 4-phenylbutyric acid; PDTC, pyrrolidine dithiocarbamate; CRP, C-reactive protein; AhR, aryl hydrocarbon receptor; COX, cyclooxygenase.; OAT, organic anion transporter; MCP-1, monocyte chemotactic protein-1; ALP, alkaline phosphatase; Cbfa1, core-binding factor alpha 1; OPN, osteopontin; ROS, reactive oxygen species; TIMPS, tissue inhibitors of metaloproteinases; SDF-1, stromal cell-derived factor-1; MMP, mitochondrial membrane potential

**LIST OF ABREVIATIONS**

| | |
|---|---|
| AASK | African American Study of Kidney Disease and Hypertension |
| ADMA | Asymmetric dimethylarginine |
| AGEs | Advanced glycation end products |
| AKI | Acute kidney injury |
| ALI | Acute lung injury |
| AOPPs | Advanced oxidation protein products |
| AREs | Antioxidant response elements |
| ARIC | Atherosclerosis Risk in Communities |
| ASK1 | Apoptosis signaling-regulating kinase 1 |
| BMI | Body Mass Index |
| BSA | Bovine serum albumin |
| BEN | Balkan Endemic Nephropathy |
| bZIP | Basic-region leucine zipper |
| CAD | Coronary artery disease |
| CKD | Chronic kidney disease |
| CMPF | 3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid |
| CNC | Cap “n” collar |
| CRP | C-reactive protein |
| CsA | Cyclosporin-A |
| CTPP | Confronting 2-pair primers |
| CVD | Cardiovascular disease |
| CVI | Cerebrovascular insult |
| Dkk-1 | Dickkopf-1 |
| DM | Diabetes mellitus |
| DNPH | Dinitrophenylhydrazine |
| DTNB | 5,5′-dithiobis-(2-nitrobenzoic acid) |
| DNP | Dinitrophenyl |
| DNA | Deoxyribonucleic acid |
| ED | Endothelial dysfunction |
| EDTA | Ethylene diamine tetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| eGFR | Estimated glomerular filtration rate |
| EMPs | Endothelial microparticles |
| eNOS | Endothelial nitric oxide synthase |
| ESRD | End-stage renal disease |
| GBD | Global Burden of Disease |

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| GFR | Glomerular filtration rate |
| GN | Glomerulonephritis |
| GPX | Glutathione peroxidase |
| GR | Glutathione reductase |
| GSH | Glutathione |
| GSH | Glutathione |
| GSSG | Oxidized glutathione |
| GST | Glutathione S-transferase |
| $H_2O_2$ | Hydrogen peroxide |
| HD | Hemodialysis |
| HRP | Horse radish peroxidase |
| HK units | Hamidi-Koliakos |
| HNE | Hydroxynonenal |
| HOCl | Hypochlorous acid |
| HT | Hypertension |
| HUVECs | Human umbilical vein endothelial cells |
| I/R | Ischemia/reperfusion |
| ICAM | Intracellular cell adhesion molecules |
| IkB$\alpha$ | NF-kB inhibitory protein |
| IL | Interleukin |
| IS | Indoxyl sulphate |
| KDIGO | Kidney Disease Improving Global Outcomes |
| Keap1 | Kelch-like ECH-associated protein 1 |
| LDL | Low-density lipoprotein |
| MAPK | Mitogen-activated protein kinases |
| MBD | Mineral bone disorder |
| Misc | Miscellaneous |
| MCP-1 | Monocyte chemoattractant protein-1 |
| MDA | Malondialdehyde |
| MDAadd | Malondialdehyde adducts |
| MMPs | Matrix metalloproteinases |
| MPO | Myeloperoxidase |
| MTS | Mitochondrial targeting sequence |
| NAC | N-Acetylcysteine |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| Neh | Nrf2-ECH homology domains |
| NF-kB | Nuclear factor kB |
| NO | Nitric oxide |
| NOX | NADPH -oxidases |
| Nrf2 | Nuclear factor erythroid 2–related factor 2 |

| | |
|---|---|
| $O_2^-$ | Superoxide anion radical |
| OFV | Objective function value |
| $ONOO^-$ | Peroxynitrite |
| OS | Oxidative stress |
| Ox-LDL: | Oxidized LDL |
| PAB | Prooxidant-antioxidant balance |
| PCR | Polymerase Chain Reaction |
| PCS | P-cresyl sulphate |
| PF4 | Platelet factor 4 |
| PMNs | Polymorphonuclear white blood cells |
| pmp | Per million population |
| PN | Pyelonephritis |
| PKD | Polycystic kidney disease |
| PSH | Thiol groups |
| PUFAs | Polyunsaturated fatty acids |
| RBC | Red blood cell |
| RBP4 | Retinol binding protein 4 |
| RANTES | Regulated on activation, normal T cell expressed and secreted |
| RFLP | Restriction Fragment Length Polymorphism |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| RRT | Renal replacement therapy |
| SDF-1 | Stromal cell-derivedfactor-1 |
| Sec | Selenocysteine |
| Se-H | Selenol |
| Se-OH | Selenenic acid |
| Se-SG | Glutathiolated selenol |
| siRNA | Small interfering RNA |
| SNP | Single nucleotide polymorphism |
| SOD | Superoxide dismutase |
| TBARS | Thiobarbituric acid-reactive substances |
| TMB | 3,3´5,5´-tetramethylbenzidine |
| $TNB^-$ | 5-thio-2-nitrobenzoic acid |
| TNF | Tumor necrosis factor |
| TOS | Total oxidant status |
| VCAM | Vascular cell adhesion molecules |
| VEGF | Vascular endothelial growth factor |
| VPCs | Visual predictive checks |
| VSMCs | Vascular smooth muscle cells |

| | |
|---|---|
| XDH | Xanthine dehydrogenase |
| XO | Xantine oxidase |
| 8-OHdG | 8-Hydroxy-2′-deoxyguanosine |

**BIOGRAPHY**

Dr Đurđa Jerotić (maiden name Jovanović) was born in Jagodina in 1991, Serbia, where she completed her elementary and high school education with honors. In 2010, she started her studies at Faculty of Medicine, University of Belgrade. Over the course of her studies, she was engaged as a student teaching assistant at the Institute of Anatomy (2011-2012), Institute of Histology and Embryology (2012-2013) and at the Institute of Medical and Clinical Biochemistry (2013-2016), at the Faculty of Medicine, University of Belgrade. As a member of Professional and Scientific Students' Research Center (CSNIRS) at her alma mater (2014-2016), she was also involved in organising 56$^{th}$ and 57$^{th}$ Serbian Students' Congresses of Biomedical Sciences. During her studies, she received awards for the best students of Faculty of Medicine, University of Belgrade in 2010/2011, 2012/2013 and 2013/2014 academic year. She was also granted by Ministry of Education and Technological Development of the Republic of Serbia (2011-2016), The City of Belgrade Scholarship for the best students from Belgrade, and Republic of Serbia's Young Talents Fund "Dositeja" Scholarship for the best students in their final year of studies. Dr Jerotić spent 4 years working on research projects as an undergraduate student at the Institute of Medical and Clinical Biochemistry (2012-2016) and presented the results of her work in multiple National and International Congresses. She graduated on the Faculty of Medicine, University of Belgrade with average grade of 9.86/10.0.

In 2016, dr Jerotić joined the research group headed by Professor dr Tatjana Simić at the Institute of Medical and Clinical Biochemistry, Faculty of Medicine, University of Belgrade as a Research Assistant, on project No. 175052 entitled "The role of glutathione S-transferase polymorphisms in susceptibility to disease development", financed by the Serbian Ministry of Education and Science. In 2016/2017 she enrolled in her PhD studies at the Faculty of Medicine, University of Belgrade - Tumor biology and oxidative diseases module, which she finished with the average grade of 10.0/10.0. In 2018, she became a Teaching Associate in the field of Medical Biochemistry. During the school year 2018/2019, Đurđa Jerotić started her residency in Laboratory Medicine and in June 2020 she was promoted to a Teaching Assistant.

In 2017, dr Jerotić received ERASMUS+ grant and spent three months in Clinical Biochemisty and Human Nutrition laboratory in University of Perugia, Italy, involved in a project entitled "Functional proteomics of Glutathione S-transferase interactome in human hepatocarcinoma" headed by Professor Francesco Galli. A year after, in 2018, she was awarded by FEBS grant and worked for 6-weeks in Centre for Experimental Medicine at the School of Medicine, Dentistry and Biomedical Sciences in Queen's University of Belfast, mentored by dr Lana McClements. In collaboration with colleagues, her work on this project resulted in data which was included in her PhD thesis.

Dr Đurđa Jerotić is the author/co-author of 13 *in extenso* papers published in journals indexed in the Science Citation Index (SCI). In three articles she is the first author.

She is married and has a daughter.

Прилог 1.

# Изјава о ауторству

Име и презиме аутора Бурђа Јеротић

Број индекса 5080/2016

**Изјављујем**

да је докторска дисертација под насловом

**ASSOCIATION OF *NRF2, SOD2* AND *GPX1* GENE POLYMORPHISMS WITH MARKERS OF OXIDATIVE STRESS AND PROGNOSIS IN PATIENTS WITH END STAGE RENAL DISEASE**

**(POVEZANOST POLIMORFIZAMA *NRF2, SOD2 I GPX1* GENA SA POKAZATELJIMA OKSIDATIVNOG DISTRESA I PROGNOZOM KOD BOLESNIKA SA TERMINALNOM BUBREŽNOM SLABOŠĆU)**

- резултат сопственог истраживачког рада;
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**Потпис аутора**

У Београду, 11. 6. 2021.

Бурђа Јеротић

Прилог 2.

# Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора: Ђурђа Јеротић

Број индекса: 5080/2016

Студијски програм: Биологија тумора и оксидативна обољења

Наслов рада: ПОВЕЗАНОСТ ПОЛИМОРФИЗАМА *NRF2, SOD2 И GPX1* ГЕНА СА ПОКАЗАТЕЉИМА ОКСИДАТИВНОГ ДИСТРЕСА И ПРОГНОЗОМ КОД БОЛЕСНИКА СА ТЕРМИНАЛНОМ БУБРЕЖНОМ СЛАБОШЋУ

Ментор: проф. др Марија Матић

Коментор: проф. Лана МекКлементс

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла ради похрањена у **Дигиталном репозиторијуму Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског назива доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

**Потпис аутора**

У Београду, 11. 6. 2021.

Ђурђа Јеротић

Прилог 3.

# Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

ПОВЕЗАНОСТ ПОЛИМОРФИЗАМА *NRF2, SOD2 И GPX1* ГЕНА СА ПОКАЗАТЕЉИМА ОКСИДАТИВНОГ ДИСТРЕСА И ПРОГНОЗОМ КОД БОЛЕСНИКА СА ТЕРМИНАЛНОМ БУБРЕЖНОМ СЛАБОШЋУ

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигиталном репозиторијуму Универзитета у Београду и доступну у отвореном приступу могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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У Београду, 11. 6. 2021.

Бурђа Јрашић