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# THE ASSOCIATION OF GLUTATHIONE TRANSFERASE A1, M1, P1 AND T1 GENE POLYMORPHISMS WITH THE RISK OF RENAL CELL CARCINOMA DEVELOPMENT AND PROGRESSION

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# POVEZANOST POLIMORFIZAMA GENA ZA GLUTATION TRANSFERAZE A1, M1, P1 I T1 SA RIZIKOM ZA NASTANAK I PROGRESIJOM KARCINOMA BUBREŽNOG PARENHIMA

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*Ko ne zna, a ne zna da ne zna – opasan je, izbegavajte ga!*
*Ko ne zna, a zna da ne zna – dete je, naučite ga!*
*Ko zna, a ne zna da zna – spava, probudite ga!*
*Ko zna i zna da zna – mudar je, sledite ga!*

***Konfučije***

***Mojim roditeljima***
***Mojoj Keki***

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**Mark Twain**

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## ABSTRACT

### THE ASSOCIATION OF GLUTATHIONE TRANSFERASE A1, M1, P1 AND T1 GENE POLYMORPHISMS WITH THE RISK OF RENAL CELL CARCINOMA DEVELOPMENT AND PROGRESSION

**Vesna M. Ćorić**

**Background**: Cytosolic glutathione S-transferases (GSTs) might affect both the development and the progression of renal cell carcinoma (RCC) due to their dual functionality. The aim of this study was to evaluate specific role of *GST* gene variants (*GSTA1, GSTM1, GSTT1* and *GSTP1*) as determinants of risk in patients with renal cell carcinoma, independently or simultaneously with recognized RCC risk factors, as well as to discern whether phenotype changes reflect genotype-associated risk. Furthermore, we evaluated the effect of *GST* gene variants on postoperative prognosis in RCC patients. Special attention was paid to the most frequent type of RCC, clear renal cell carcinoma (ccRCC).

**Methods**: *GST* genotypes were determined in 305 RCC patients and 326 matched-controls in whom overall survival was evaluated as well. The levels of benzo(a)pyrene diolepoxide (BPDE)-DNA adducts and 8-hydroxy-2'-deoxyguanosine (8-OHdG) were determined by ELISA method. The expression of GSTM1 and GSTP1 protein level, as well as the level of regulatory (ASK1, JNK1/2) and executor (Caspase-3) apoptotic molecules in ccRCC tissue samples were analyzed by method of immunoblot. The presence of GSTM1:ASK1/ GSTP1:JNK1/2 protein:protein interactions was determined by means of immunoprecipitation.

**Results**: Significant association between *GST* genotype and risk of overall RCC and ccRCC development was found for *GSTM1-null* and *GSTP1-variant* genotypes, independently ($p<0.05$). Furthermore, 22% of all recruited ccRCC patients were carriers of combined *GSTM1-null/GSTT1-active/GSTA1-low activity/GSTP1-variant* genotype, exhibiting 9.32-fold elevated ccRCC risk compared to the reference genotype combination ($p=0.041$). Significant association between *GST* genotype and ccRCC risk in smokers was found only for the *GSTP1* genotype, while *GSTM1-null/GSTP1-variant/GSTA1 low-activity* genotype combination was present in 94% of smokers with ccRCC, increasing the risk of ccRCC up to 7.57 ($p=0.026$). Furthermore, ccRCC smokers with *GSTM1-null* genotype had significantly higher concentration of BPDE-DNA adducts in comparison with *GSTM1-active* cRCC smokers (p=

0.050). No association was found between *GST* gene variants and the level of 8-OHdG. However, *GSTM1-null* genotype was the most frequent in grade II (G2) RCC and ccRCC tumors. Survival analysis indicated shorter overall survival for the whole group of RCC and ccRCC patients with *GSTM1-active* genotype ($p<0.05$). Furthermore, overall RCC and ccRCC patients with *GSTM1-active* genotype had a significantly higher hazard ratio ($p<0.05$), analyzed in all three regression models, compared to the carriers of *GSTM1-null* genotype. Finally, the presence of GSTM1:ASK1, as well as GSTP1:JNK1/2 protein:protein interactions was found in all ccRCC tissue samples studied.

**Conclusions**: Certain *GST* polymorphisms might be associated with the risk of RCC, with special emphasis on *GSTM1-null* and *GSTP1-variant* genotypes. Combined *GSTM1-null/GSTT1-active/GSTA1 low activity/GSTP1-variant* genotypes might be considered a "risk-associated genotype combination" in ccRCC. On the other hand, *GSTM1-null* genotype is associated with favorable postoperative prognosis in RCC. The possible molecular mechanism underlying the role of GST proteins in RCC progression might be the presence of GSTM1:ASK1 and GSTP1:JNK1 protein:protein interactions. Hence, determination of *GSTM1* genotype might serve as a valuable indicator in both RCC risk assessment and postoperative prognosis.

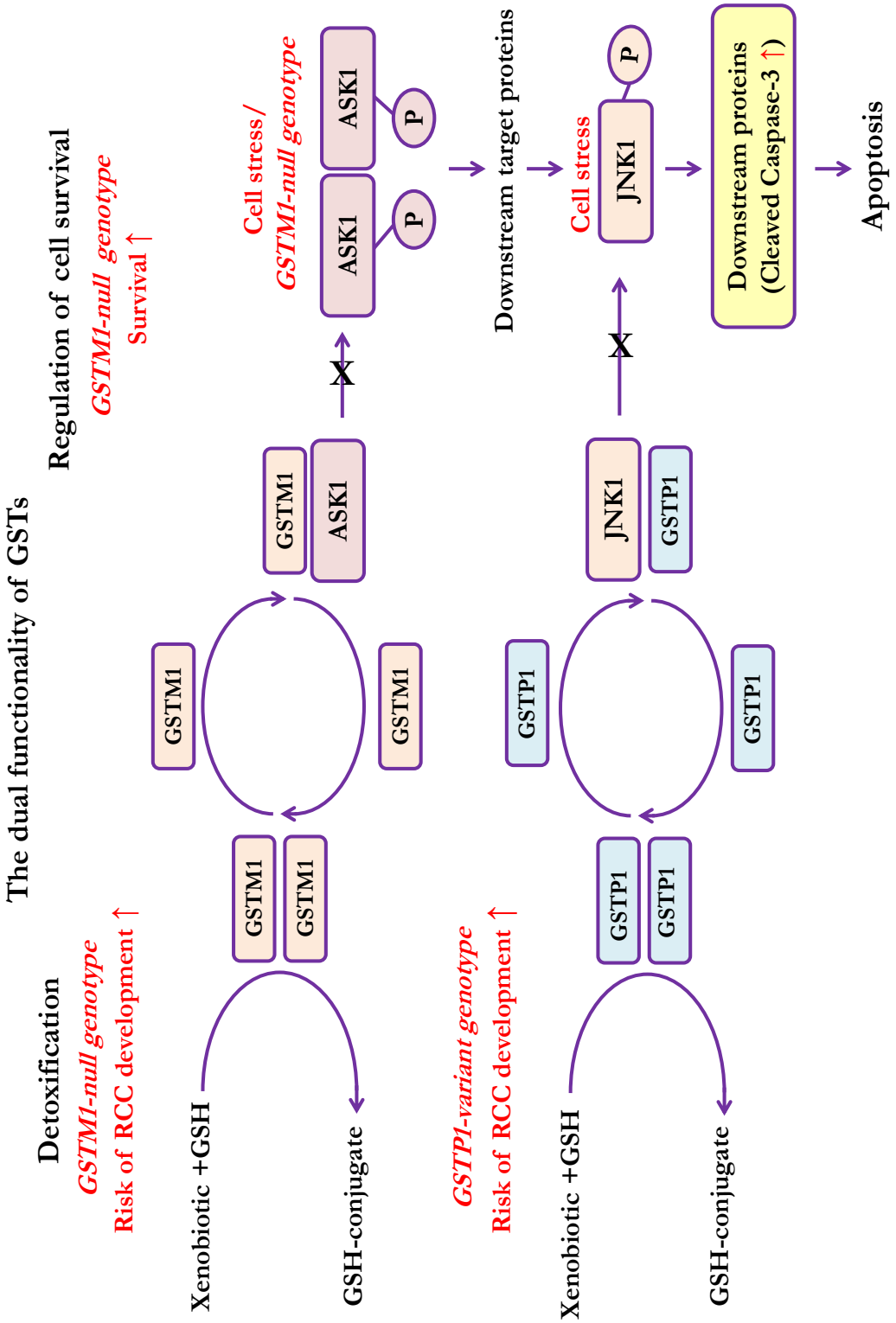
**Key words:** GST, RCC, risk, prognosis, survival, BPDE, 8-OHdG, MAPK, protein expression;

**Scientific Field:** Medicine

**Scientific Dicipline:** Medical and clinical biochemistry

**UDC:**

**Graphical abstract**



## SAŽETAK

## POVEZANOST POLIMORFIZAMA GENA ZA GLUTATION TRANSFERAZE A1, M1, P1 I T1 SA RIZIKOM ZA NASTANAK I PROGRESIJOM KARCINOMA BUBREŽNOG PARENHIMA

**Vesna M. Ćorić**

**Uvod**: Zbog uloga koje poseduju, citosolne glutation S-transferaze (GST) mogu biti značajne kako u nastanku, tako i u progresiji karcinoma bubrežnog parenhima (KBP). U ovoj studiji je ispitivana uloga pojedinih *GST* genskih varijanti (*GSTA1, GSTM1, GSTT1* i *GSTP1*) u nastanku KBP, nezavisno ili udruženo sa poznatim faktorima rizika za nastanak ovog karcinoma, kao i moguća povezanost fenotipskih karakteristika tumora sa odgovarajućim genotipom. Pored toga, ispitivan je i potencijalni prognostički značaj polimorfne ekspresije GST proteina kod bolesnika sa KBP. Posebna pažnja je posvećena najučestalijem podtipu KBP, svetloćelijskom karcinomu bubrežnog parenhima (sKBP).

**Materijal i Metode**: Polimorfizam GSTa je određivan kod 305 pacijenata sa KBP i kod 326 kontrola, uparenih po godinama i polu. Pored fenotipskih karakteristika tumora, u grupi pacijenata sa KPB je praćeno i preživljavanje. Nivoi benzo(a)piren diolepoksid (BPDE)-DNK-konjugata, kao i nivoi 8-hidroksi-2-deoksiguanozina (8-OHdG) su određivani ELISA metodom. Ekspresija GSTM1 i GSTP1 proteina, kao i ekspresija regulatornih (ASK1, JNK1/2) i egzekutornih (Caspaza 3) apoptotskih molekula u uzorcima tumorskog tkiva je analizirana metodom imunoblota. Prisustvo GSTM1:ASK1, odnosno GSTP1:JNK1/2 protein:proteinske interakcije je ispitivano metodom imunoprecipitacije.

**Rezultati**: Uočen je značajan efekat *GSTM1-nultog* i *GSTP1-varijantnog* genotipa na rizik za nastanak KBP ($p<0.05$). Pored toga, 22% svih pacijenata sa sKBP su bili nosioci kombinovanog *GSTM1-nultog/GSTT1-aktivnog/GSTA1-genotipa smanjene aktivnosti/GSTP1-varijantnog* genotipa i bili su u 9.32 - puta većem riziku za nastanak sKBP u poređenju sa nosiocima referentnog genotipa (*GSTM1-aktivni/GSTT1-nulti/GSTA1-aktivni/GSTP1-referentni* genotip) ($p=0.041$). Uočen je efekat *GSTP1-varijantnog* genotipa na rizik za nastanak KBP kod pušača, dok je kombinacija *GSTM1-nulti/GSTP1-varijantni/GSTA1-genotip smanjene aktivnosti* bila prisutna u 94% pušača sa sKBP, povećavajući rizik od nastanka sKBP na 7.57 puta ($p=0.026$).

Takođe, pušači sa sKBP, nosioci *GSTM1-nultog* genotipa su imali značajno više nivoe BPDE-DNK-konjugata u poređenju sa nosiocima *GSTM1-aktivnog* genotipa (p=0.050). Ipak, nije nađena asocijacija između nosilaca različitih *GST* genskih varijanti po pitanju nivoa 8-OHdG. Kada je ispitivana povezanost fenotipskih karakteristika tumora sa *GST* genotipom, uočeno je da je *GSTM1-nulti* genotip bio najučestaliji kod pacijenata sa G2 gradusom tumora. Rezultati ispitivanja prognostičke uloge GST u KBP su pokazali da je kumulativna verovatnoća preživljavanja pacijenata sa KBP i sKBP, nosilaca *GSTM1-aktivnog* genotipa bila manja u odnosu na ispitanike sa *GSTM1-nultim* genotipom (p<0.05). Štaviše, pacijenti sa KBP i sKBP, nosioci *GSTM1-aktivnog* genotipa imali su statistički značajno veći rizik od smrtnog ishoda u sva tri ispitivana modela u odnosu na nosioce *GSTM1-nultog* genotipa (p<0.05). Konačno, prisustvo GSTM1:ASK1, kao i GSTP1:JNK1/2 protein:proteinske interakcije je potvrđeno u svim analiziranim uzorcima tumorskog tkiva sKBP.

**Zaključci**: Određeni polimorfizmi *GST* mogu imati značajan efekat na rizik za nastanak KBP, sa posebnim naglaskom na *GSTM1-nulti i GSTP1-varijantni* genotip. Kombinovani *GSTM1-nulti/GSTT1-aktivni/GSTA1 genotip smanjene aktivnosti/GSTP1-varijantni* genotip može se smatrati "rizičnim genotipom" za nastanak sKBP. Sa druge strane, *GSTM1-nuti* genotip je udružen sa boljom postoperativnom prognozom pacijenata sa KBP, a u osnovi prognostičke uloge GST u KBP bi bilo prisustvo GSTM1:ASK1 i GSTP1:JNK1 protein:proteinskih intrakcija. Stoga, određivanje *GSTM1* genotipa može predstavljati koristan pokazatelj u proceni rizika za nastanak KBP i postoperativnu prognozu pacijenata sa ovim karcinomom.

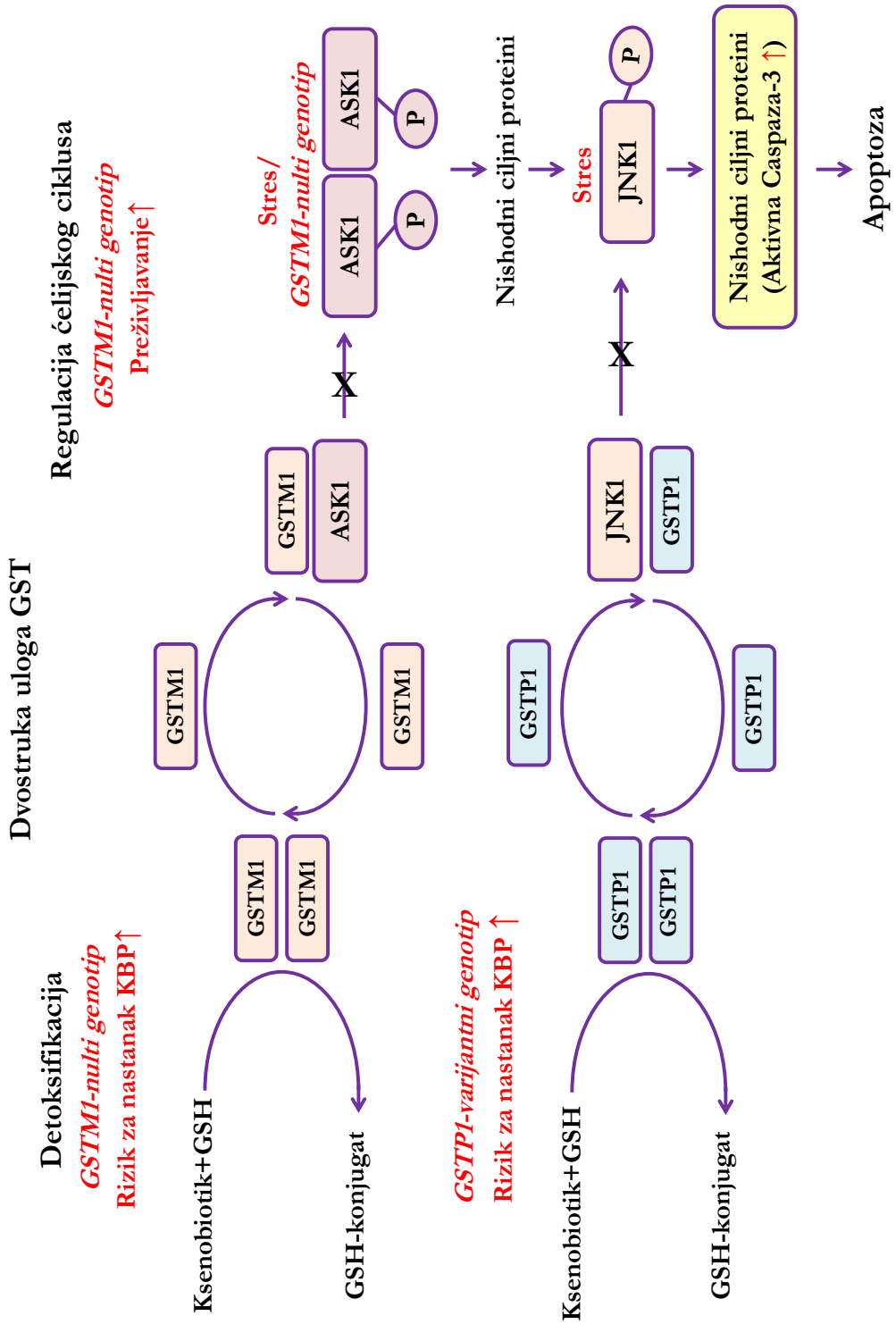
**Ključne reči:** GST, KBP, rizik, prognoza, preživljavanje, BPDE, 8-OHdG, MAPK, ekspresija proteina;

**Naučna oblast:** Medicina

**Uža naučna oblast:** Medicinska i klinička biohemija

**UDK:**

**Grafički abstrakt**



## Contents

# INTRODUCTION

## 1.1 Renal cancer

Nowadays, renal masses are being increasingly detected as unexpected findings on diagnostic abdominal imaging, usually performed for non-kidney related clinical conditions, and may comprise a simple renal cyst that require no treatment or even follow-up (Ellimoottil et al., 2014). However, the majority of cases comprise benign renal lesions (angiolipomas or oncocytomas) or malignant renal lesions, that are usually subjected to further procedures and interventions (Shah et al., 2010).

### 1.1.1 Incidence

Representing 2-3% of all malignancies, kidney cancer and renal pelvis cancer are among top ten most common cancers in the world (Ferlay et al., 2015). Renal cell carcinoma (RCC) is the predominant form of kidney malignancy, comprising various morphological variants of RCC types with specific chromosomal alterations and molecular pathway abnormalities (B. Escudier et al., 2014; Ljungberg et al., 2015). On the other hand, urothelial carcinoma, arising in the renal pelvis, accounts for less than 10% of histologically confirmed kidney carcinomas.

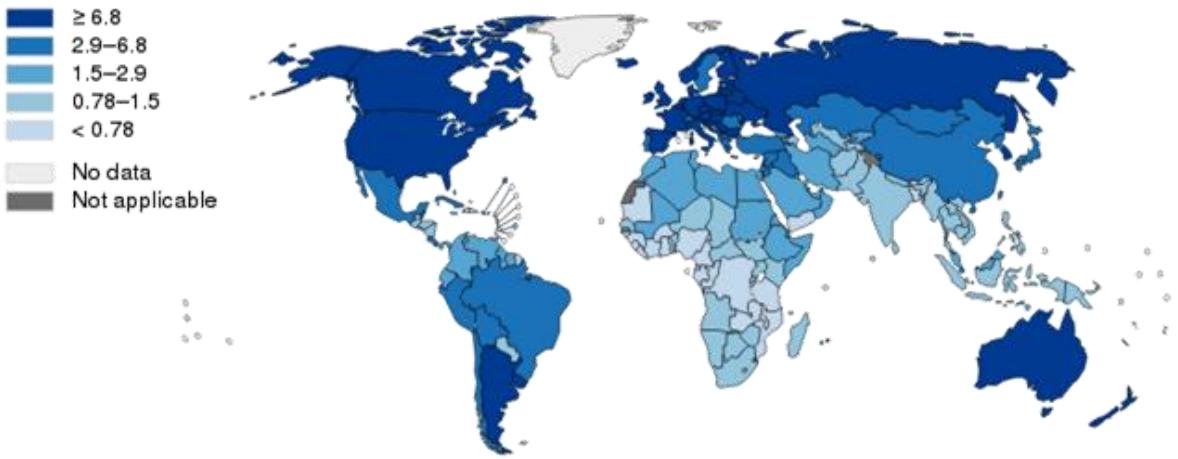



**Figure 1**. Estimated age-standardized rates of kidney incidence cases in the World, for both sexes, cancer, worldwide; Adopted from *Ferlay et al., 2015,* available at http://globocan.iarc.fr/

The incidence of RCC varies between nations. Over the course of the last few decades, the incidence has increased in many parts of the world, probably due to the improved tumor detection with diagnostic abdominal imaging (Mathew et al., 2002), being the highest in Europe, North America and Australia (Petejova and Martinek, 2016). The global incidence rate reported in 2012 was 6.0/100.000 for men and 3.0/100.000 for women (Figure 1) (Ferlay et al., 2015). Similarly, the incidence in Serbia in 2013 was reported as 6.1 (men) and 3.0 (women) per 100.000 people (*Cancer Incidence and Mortality in Central Serbia 2013*, 2015). Approximately, there is a 1.5:1 predominance in men over women, with peak incidence of RCC occurring between 60 and 70 years of age (Ljungberg et al., 2015).

### 1.1.2 Diagnostics

Most RCCs are asymptomatic in early stages. It seems that the use of high-resolution cross-sectional imaging modalities over the last few decades has led to the increase in incidental detection of renal masses, often characterized as small and low-graded (Gill et al., 2010). Nowadays, between 48-66% of such RCCs are detected incidentally (Krabbe et al., 2014). Still, many renal masses remain asymptomatic until the late stages of the disease. The classic triad of flank pain, gross haematuria and palpable abdominal mass is now rare (6-10%) and correlates with advanced disease and subtypes associated with poor prognosis (Patard et al., 2005). Paraneoplastic syndromes (hypertension, anemia, weight loss as most common) are found in approximately 30% of patients with symptomatic RCC (Sacco et al., 2009). Symptoms caused by metastatic RCC, such as bone pain, deterioration of performance status, or persistent cough are now observed in few patients (Kim et al., 2003).

Most renal masses can be detected accurately using imaging alone. The most traditional approach for both detecting and characterizing renal masses are ultrasonography, computed tomography and magnetic resonance imaging, which alone can classify renal masses as cystic or solid (Ljungberg et al., 2015). Renal biopsies are increasingly being used for the following purposes: (1) for histological diagnosis of radiologically indeterminate renal masses to avoid surgery in the event of benign lesions; (2) to select patients with small renal masses for surveillance approaches; (3) to obtain histology before ablative treatments; and (4) to select the most suitable medical and surgical treatment strategy in the setting of metastatic RCC, as

explained in details by several papers on RCC management (Abel et al., 2010; Leveridge et al., 2011; Schmidbauer et al., 2008).

### 1.1.3 Pathology assessment

Adult kidney cancers that originate from renal parenchyma, in particular form the nephrons, are mainly adenocarcinomas, also known as renal cell carcinoma (RCC), whereas those that arise from the collecting system are mainly transitional cell carcinomas (TCC).

2004 WHO classification, that was modified by the International Society of Urological Pathology (ISUP) Vancouver Classification in 2012, recognizes three major histological subtypes of RCC: (1) clear cell RCC (ccRCC), which arises from the proximal convolute tubule and is the most frequent subtype of sporadic RCC in adults (70-85%), followed by (2) papillary (pRCC), comprising type I and II (10-15%, of which 60–70% are type I) and (3) chromophobe RCC (chRCC, 4-5%) (Srigley et al., 2013).

Less common cancers include papillary adenoma, multilocular cystic clear-cell carcinoma, hybrid oncocytic chromophobe tumor, carcinoma of the collecting ducts of *Bellini*, renal medullary carcinoma, carcinoma associated with neuroblastoma and mucinous tubular and spindle-cell carcinoma. The ISUP Vancouver Consensus Statement added five new epithelial tumor subtypes of renal cell carcinoma: tubulocystic, acquired cystic disease associated clear-cell, tubulopapillary, microphthalmia family translocation, and hereditary leiomyomatosis– renal cell carcinoma syndrome-associated (Srigley et al., 2013).

The particular information on the RCC subtype is of the utmost importance. Various subtypes exhibit different biological behavior, prognosis and treatment option, with ccRCC still being the most aggressive, with the highest rate of invasion, metastasis and mortality (Protzel et al., 2012). On the other hand, if sarcomatoid or rhabdoid differentiation occurs in any subtype, it is usually associated with highly aggressive behavior and poor prognosis (Eble et al., 2006).

Besides RCC subtype and the presence of sarcomatoid features, histological diagnosis includes the evaluation of nuclear grade, vascular invasion, tumor necrosis and invasion of the collecting system and peri-renal fat. Until recently, *Fuhrman* nuclear grade has been the most widely accepted grading system (Fuhrman et al., 1982). However, at the 2012 ISUP conference, a simplified, nuclear grading system, based only on the size and the shape of nucleoli, was

proposed and will replace the *Fuhrman* grading system (Srigley et al., 2013). Moreover, new Union for International Cancer Control tumor–node–metastasis staging system should be used (Table 1.) (Edge and American Joint Committee on Cancer, 2010).

### 1.1.4 Treatment

Surgical resection appears to remain as the standard of care, in case of both radical and partial nephrectomy. Meanwhile, cryoablation and radiofrequency ablation, where tumor is destroyed by low or high temperatures, has gained vast attention. Concerning the management of small, incidentally detected renal masses, another emerging approach is "watchfull waiting". However, given their unproven long-term efficacy, the renal ablation and "watchfull waiting" have not established themselves as standards of care, but are, for the timebeing, reserved for elderly and/or patients with comorbidities and small renal masses with limited life expectancy (Ljungberg et al., 2015).

**Table 1**. Staging of RCC Union for International Cancer Control (UICC) tumor–node–metastasis (TNM) classification of malignant tumors; Adopted *from Edge and American Joinr Committee on Cancer, 2010*

| **T** | **Primary tumor** |
|---|---|
| Tx | Primary tumor cannot be assessed |
| T0 | No evidence of primary tumor |
| T1 | Tumor ≤7 cm in greatest dimension, limited to the kidney |
| T1a | Tumor ≤4.0 cm |
| T1b | Tumor >4.0 cm but ≤7.0 cm |
| T2 | Tumor >7.0 cm in greatest dimension, limited to the kidney |
| T2a | Tumor >7 cm but ≤10 cm |
| T2b | Tumor >10 cm, limited to the kidney |
| T3 | Tumor extends to major veins or perinephric tissues but not into the ipsilateral adrenal gland and not beyond Gerota's fascia |
| T3a | Tumor grossly extends into the renal vein or its segmental (muscle-containing) branches, or tumor invades peri-renal and/or renal sinus fat (peri-pelvic) but not beyond Gerota's fascia |
| T3b | Tumor grossly extends into the vena cava below the diaphragm |
| T3c | Tumor grossly extends into the vena cava above the diaphragm or invades the wall of the vena cava |
| T4 | Tumor invades beyond Gerota's fascia (including contiguous extension into the ipsilateral adrenal gland) |

**Table 1, *continued***. Staging of RCC, Union for International Cancer Control (UICC) tumor–node–metastasis (TNM) classification of malignant tumors; *from Edge and American Joinr Committee on Cancer, 2010*

| **N** | **Regional lymph nodes** | | |
|---|---|---|---|
| Nx | Regional lymph nodes cannot be assessed | | |
| N0 | No regional lymph node metastasis | | |
| N1 | Metastasis in regional lymph node(s) | | |
| **M** | **Distant metastases** | | |
| cM0 | Clinically no distant metastasis | | |
| cM1 | Clinically distant metastasis | | |
| pM1 | Pathologically proven distant metastasis, e.g. needle biopsy | | |
| **Stage grouping** | | | |
| Stage I | T1 | No | M0 |
| Stage II | T2 | No | M0 |
| | T3 | Any | M0 |
| Stage III | T1-T3 | N1 | M0 |
| | T4 | Any | M0 |
| Stage IV | Any | Any | M1 |

### 1.1.5 Mortality

Although most of the incidentally detected renal lesions have been characterized as small and low-grad tumors, about 20-30% of all patients are diagnosed with metastatic disease (mRCC) due to the early hematologic dissemination (Siegel et al., 2014). Moreover, about 20% of all patients undergoing nephrectomy will develop mRCC during follow-up (Ljungberg et al., 2011; Petejova and Martinek, 2016). The most frequent sites of metastatic disease are lungs, bone and brain. However, adrenal glands, contralateral kidney and liver might be involved as well (Bianchi et al., 2012).

According to the US National Cancer Institute data, the 5-yr overall survival for all RCC subtypes has increased since 1970's and reached 73%, probably due to the increase in the number of incidentally detected RCC cases and the introduction of targeted therapies (Wahlgren et al., 2013). Still, patients with metastatic disease have a median survival of around 13 months. The 5-year survival rate in such patients is below 10%, probably due to the chemotherapy and radiotherapy resistance, as well as low efficacy and toxicity of

immunotherapy (Klinghoffer et al., 2009; Ljungberg et al., 2015). Reported mortality rates worldwide are 2.5/100.000 in men and 1.2/100.000 in women (Figure 2) (Ferlay et al., 2015), whereas in Serbia the rates are higher with 6.1/100.000 in men and 3.0/100.000 in women, as reported in 2013 (*Cancer Incidence and Mortality in Central Serbia 2013*, 2015).

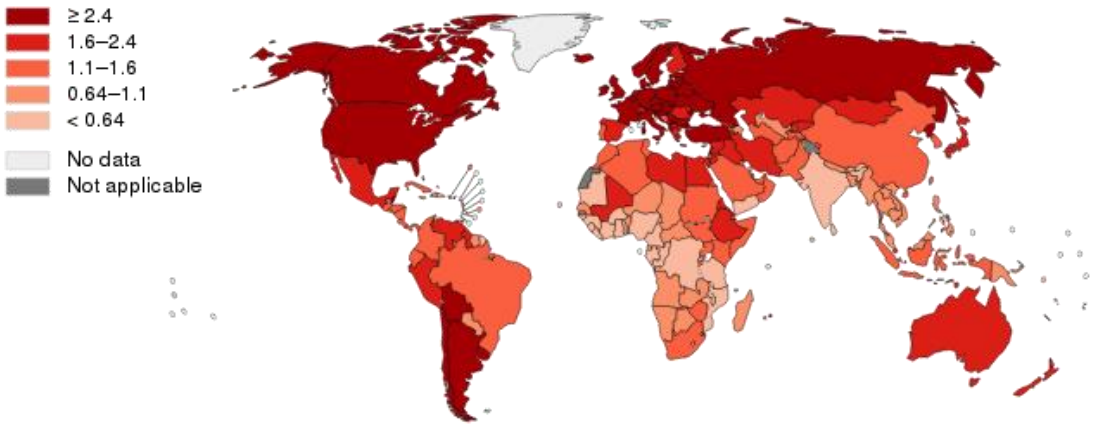



**Figure 2.** Estimated age-standardized kidney cancer mortality rates in the World for both sexes in 2012; Adopted from *Ferlay et al, 2015*, available at http://globocan.iarc.fr/

### 1.1.6 Etiology

A number of predisposing conditions are known to increase the risk of RCC development. Recognized risk factors include cigarette smoking, obesity and hypertension (Capitanio and Montorsi, 2016; B Escudier et al., 2014; Ljungberg et al., 2015; Petejova and Martinek, 2016; Terris et al., 2016). RCC also appears to be more frequent in patients with renal failure, acquired cystic disease and tuberous sclerosis (B. Escudier et al., 2014).

Several other suspected risk factors for RCC have been evaluated, such as high intake of dairy products and low consumption of fruits and vegetables, lack of physical activity, low socioeconomic status, treatment of hypertension with thiazide diuretics, family history of disease and multi-parity (Hunt et al., 2005).

#### 1.1.6.1 Cigarette smoking

The most well recognized risk factor for the development of RCC is cigarette smoking. In 2004, both the International Agency for Research on Cancer (IRAC) and the U.S. Surgeon General concluded that there are sufficient data to support causality between smoking and the development of RCC, due to the known carcinogenic effects of numerous tobacco components (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2004; Office of the Surgeon General (US) and Office on Smoking and Health (US), 2004).

The association between RCC and cigarette smoking is well established, although reported risk for ever-smokers compared with never-smokers is moderate. Namely, a comprehensive meta-analysis of 19 case-control and 5 cohort studies has demonstrated that the cigarette smoking exerts a modest, but significant increase in risk for developing RCC (RR=1.38 (95%CI: 1.27-1.50)) for both sexes (Hunt et al., 2005). Additionally, two studies reported that the risk for RCC increased in a dose-dependent manner and was, to a certain degree, reversible with prolonged smoking cessation (>10 years) (Hunt et al., 2005; Parker et al., 2003). These findings corresponded with results of later study of *Theis et al.* who have yet again reported the associations between cigarette smoking and RCC, as well as the protective effects for smoking cessation (Theis et al., 2008). Moreover, it seems that there is even evidence to support the fact that never-smokers, exposed to environmental tobacco smoke at home or work, are also at increased risk of RCC (Hu et al., 2005; Theis et al., 2008).

A large body of epidemiological literature evaluated the risk factors for RCCs development, however, without respect to RCC subtype. The results of recent prospective study suggested that smoking, the greatest modifiable risk factor for RCC, increases the risk of certain common RCC subtypes (ccRCC and pRCC) but not the others (chRCC) (Patel et al., 2015). What is more, RCC patients who are current smokers or have a history of tobacco exposure seem to exhibit more aggressive forms of RCC and with worst cancer-specific/overall survival (Terris et al., 2016).

Mechanisms by which tobacco might contribute to RCC develompent are unclear and likely multifactorial. Cigarette smoke is a rich source of free radicals, which are believed to be responsible for initiation of many tumors by inducing DNA damage that accumulates in the cells. In addition to free radicals, more than 60 carcinogens have been found in cigarette smoke. Among these, sufficient evidence of carcinogenicity was found for polycyclic aromatic

hydrocarbons (PAHs) such as benzo(a)pyrene and aromatic amines (such as 4-amino biphenyl) (International Agency for Research on Cancer and International Agency for Research on Cancer, 1993). Particular interest has been given to the most abundant, benzo(a)pyrene (B(a)P) and its carcinogenic metabolites, stereoisomers of 7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE) (Alexandrov et al., 2002). The (+)-antiisomer [(+)-anti-BPDE] seems to be the most potent carcinogen of all PAH diol epoxides (Slaga et al., 1979). Namely, BPDE is known as 'the bullet of the smoking gun', leaving its fingerprints in the blood of smokers, in the form of adducts with either serum albumin or DNA (Alexandrov et al., 2002; Ketterer, 1996). PAH BPDE-DNA adduct are shown to induce mutation(s), predominantly in the form of G to T transversions (Figure 3). For instance, the levels and the distribution of BPDE-DNA adducts in lung tumor tissue, obtained from smokers, are found to closely correlate with the gene sites containing the highest rate of p53 mutations, which was not the case for the population of non-smokers (Hollstein et al., 1991).

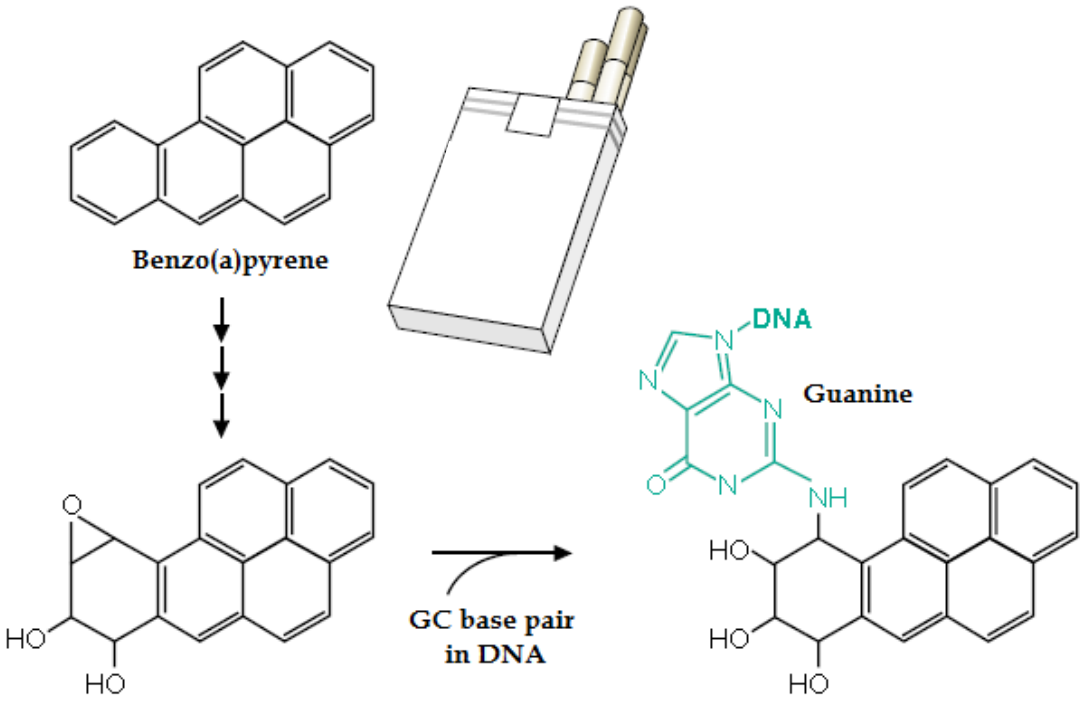



**Figure 3**. Oxidation of benzo(a)pyrene and covalent binding to DNA. Adapted from *Lieberman et al., 2013*;

Another major component of tobacco, nicotine, might also contribute to RCC tumorigenesis by stimulating pathological angiogenesis. Nicotine is known to increase endothelial cell number, capillary network formation and angiogenic response in neoplasia, mediated in part by the vascular endothelial growth factor protein, commonly up-regulated in ccRCC (Heeschen et al., 2001). However, in pRCC angiogenesis appears not to have a major role in tumorigenesis and, therefore, is not likely to be linked with smoking (Ooi et al., 2011).

#### 1.1.6.2 Obesity

Excess bodyweight, whether in people who are overweight (defined as a body-mass index, BMI of 25 to 29·9 kg/m²) or obese (BMI of 30 kg/m² or greater), is increasingly being recognized as an important risk factor for some common cancers (Renehan et al., 2008). In case of RCC, excess body weight has been recognized as a risk factor in several case control and cohort studies (Beebe-Dimmer et al., 2012; Bergström et al., 2001; Chow et al., 2000; Leiba et al., 2013; Pischon et al., 2006), following a dose dependent response pattern (Adams et al., 2008; Pischon et al., 2006). Namely, it has been suggested that overweight and obese patients had an increased risk of RCC, by 24% for men and 34% for women, for every 5 kg/m$^2$ increase in BMI (Renehan et al., 2008). Moreover, a quantitative summary analysis of 14 studies reported a relative risk for both sexes of 1.07 (95% CI: 1.05-1.09) per unit of increase in BMI (corresponding to 3 kg body weight increase for a subject of average height) (Bergström et al., 2001). In a large cohort of Norwegian man and woman, *Bjørge et al* have established remarkably similar results with calculated relative risk of 1.05 per unit increase in BMI (Bjørge et al., 2004). Several studies indicated that excess bodyweight in late adolescence is associated with an increased risk of RCC (Beebe-Dimmer et al., 2012; Leiba et al., 2013). Nevertheless, it seems that obesity is a favorable factor in terms of prognosis of RCC, despite its contribution to increased RCC risk (Haferkamp et al., 2008; Kamat et al., 2004).

Several mechanisms, explaining the increased risk of RCC development in obese patients have been proposed, however, direct evidence in humans is limited. These include chronic renal tissue hypoxia, insulin resistance and a compensatory hyperinsulinemia, altered endocrine milieu and production of adipokines, obesity-induced inflammatory response, as well as lipid peroxidation and oxidative stress, as shown in Figure 4 (Klinghoffer et al., 2009).

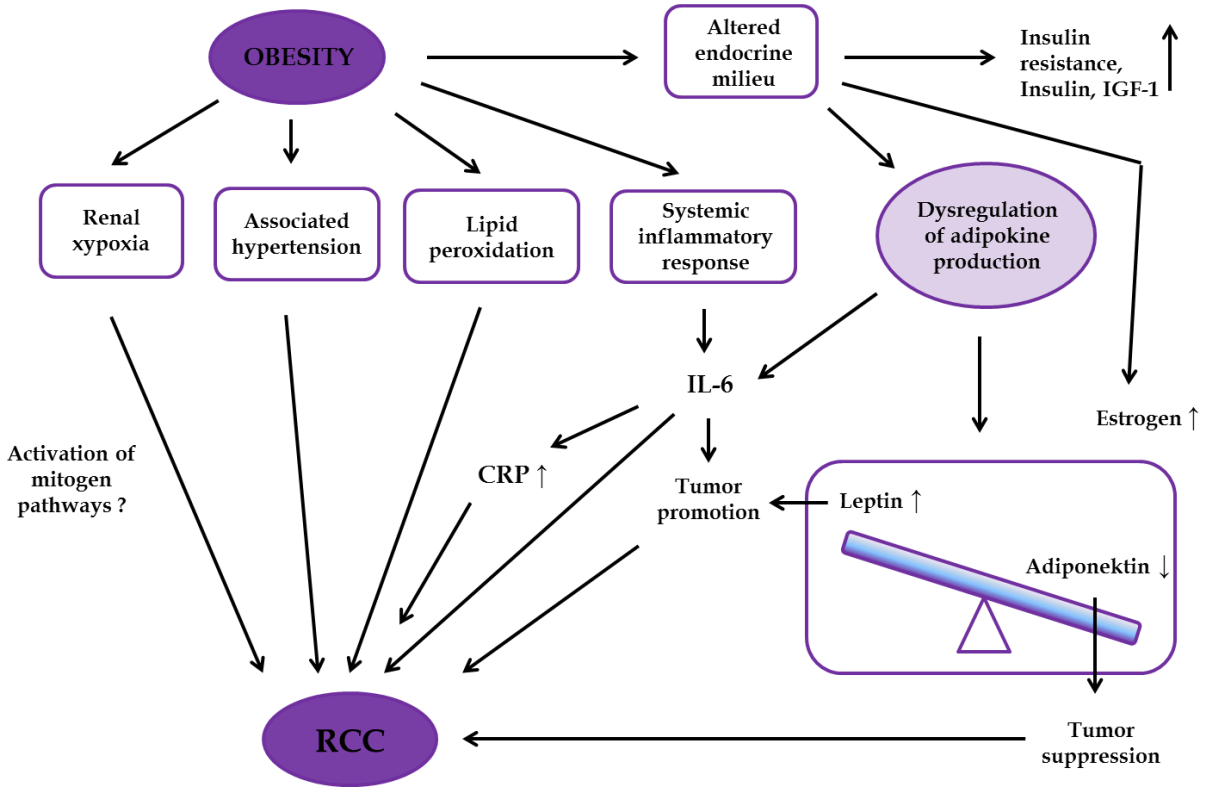



**Figure 4.** Potential obesity-related mechanisms responsible for RCC development; Adopted from *Klinghoffer et al, 2009*. Abbreviations: IGF-1- insulin growth factor; IL-6- interleukin 6; CRP- c reactive protein; RCC- renal cell carcinoma.

### 1.1.6.3 Hypertension

The effect of hypertension, or its treatment, on the risk of RCC has been evaluated in a number of studies, which provided several lines of evidence supporting the fact that history of long-term hypertension is associated with the increased risk of RCC (Brennan et al., 2008; Chow et al., 2000; Corrao et al., 2007; Vatten et al., 2007; Weikert et al., 2008). Namely, one of the largest European prospective studies demonstrated two- to three-fold increased risk for RCC development in patients with both elevated systolic and diastolic blood pressure, that was independent of gender, BMI, smoking and the use of antihypertensive therapy (Weikert et al., 2008). Multiple studies, comprising patients from both Europe and the USA, reported the same results, emphasizing the dose-response association of increasing RCC risk with rising blood pressure (Chow et al., 2000; Vatten et al., 2007; Weikert et al., 2008).

Furthermore, data from two cohort studies highlighted the hypothesis that underlying disorder of hypertension, rather than its treatment, increases the risk of RCC, while effective

blood pressure control may modify the risk (Chow et al., 2000; Weikert et al., 2008).

Uncontrolled hypertension can lead to a number of diseases, many of which are recognized as predisposing conditions for the RCC development, such as end-stage renal disease. Although several theories have been proposed to explain the association between hypertension and RCC, neither experimental studies, nor epidemiologic investigations have elucidated the biological mechanism underlying the observed association. It is presumed that renal carcinogenesis in part may be promoted by the increased formation of reactive oxygen species, as well as dysregulated lipid peroxidation in the proximal renal tubules of both hypertensive and obese individuals (Gago-Dominguez et al., 2002). Moreover, there is evidence suggesting that the mechanism behind hypertension and other recognized risk factors of kidney cancer, such as smoking and obesity, may include tissue hypoxia (Sharifi and Farrar, 2006). Many other mechanism have been proposed, such as abnormality in the apoptotic process, mitogenic effect of angiotensin II, catecholamines and vasopressin, potential carcinogenetic mechanisms of diuretics, etc. (Corrao et al., 2007). However, more detailed experimental approaches are necessary to elucidate the potential mechanism behind hypertension associated RCC risk.

#### 1.1.6.4 Genetic factors

A number of familial syndromes with well-identified causative genetic mutations (such as *Von Hipper-Lindau disease*, etc), strongly predispose affected individuals to the development of RCC. Still, these hereditary syndromes comprise only a small portion of RCC cases, with majority of cases developing sporadically (Pfaffenroth and Linehan, 2008).

Although recognized risk factors for RCC development (smoking, obesity and hypertension) are rather common in general population, only a small group of exposed people will eventually develop RCC. This suggests that the development of RCC can be partially explained by genetic variations among the populations (Yang et al., 2013). Namely, it is well established that health maintenance, as well as disease development is highly influenced by gene-environment interaction (Figure 5). In particular, the exposure to the same environmental factors does not warrant the same effect on different individuals, due to the presence of a number of genetic variations, significantly contributing to inter-individual differences in susceptibility to disease development (Hollman et al., 2016; Yadav et al., 2014).

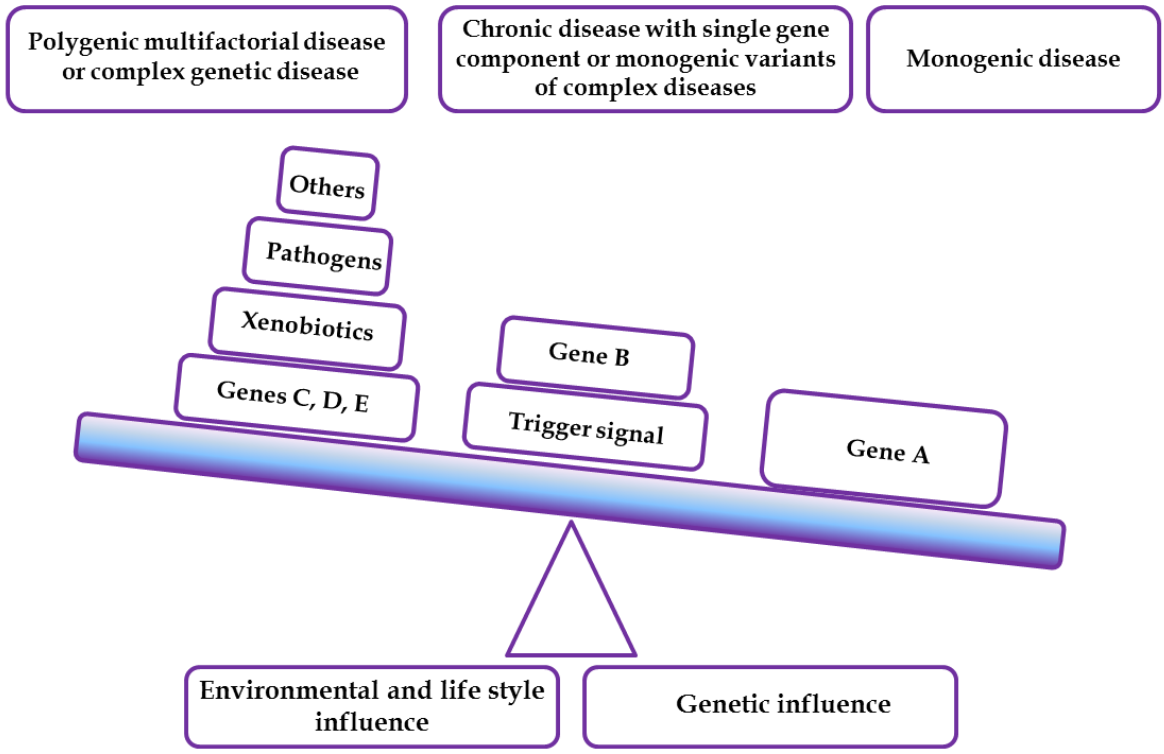



**Figure 5**. Genetic variants and environmental factors determine the risk of a disease; Adopted from *Di Pietro et al*, 2010.

## 1.2 GUTATHIONE S- TRANSFERASES (GSTs)

A growing number of genes encoding enzymes involved in biotransformation and cellular defense (such as cytochrome P450, UDP-glucuronosyltransferase, sulfotransferase, etc.) has been identified, leading to increased knowledge of the allelic variants in genes that may result in a differential susceptibility to environmental and oxidative stress (Board and Menon, 2013; Hollman et al., 2016). In humans, cellular detoxification system is divided into three phases: Phase I (comprising reactions of oxidation, reduction and hydrolysis), Phase II (conjugation) and Phase III (exeretrion). Glutathione transferases (EC 2.5.1.18), also referred to as glutathione S-transferases or GSTs, are multifunctional enzymes involved in number of catalytic and non-catalytic processes, however, traditionally recognized as principal Phase II enzymes. Namely, GSTs are known for their ability to catalyze the nucleophilic addition of the glutathione (GSH) to a wide variety of nonpolar compounds of exogenous and endogenous origin, with electrophilic functional groups, rendering the products more water-soluble and

facilitating their elimination from the cell by Phase III enzymes (Figure 6) (Di Pietro et al., 2010; Hayes et al., 2005; Tew and Townsend, 2012; Wu and Dong, 2012).

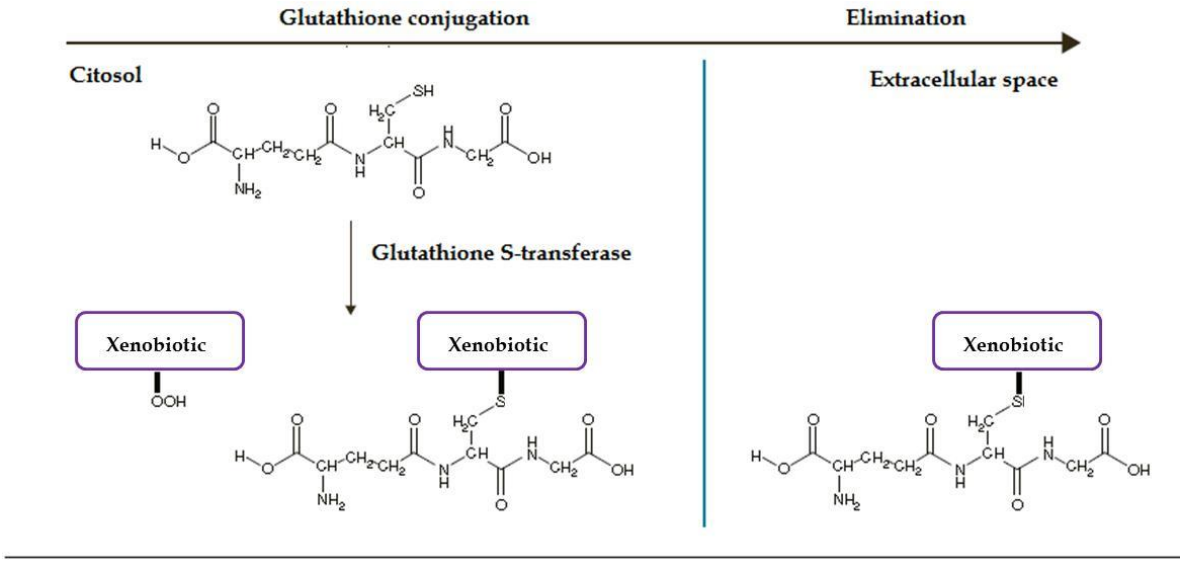



**Figure 6.** Conjugation of xenobiotic to GSH-conjugates, catalyzed by GSTs; Adopted from *Di Pietro et al., 2010*

There are three major families of GST proteins: (1) cytosolic GSTs - constituting the largest family, (2) mitochondrial and (3) microsomal, also known as Membrane Associated Protein in Eicosanoid and Glutathione metabolism, (MAPEG) (Hayes et al., 2005). Cytosolic GST enzymes are further categorized in 7 classes, designated by the names of the Greek letters and abbreviated in Roman capitals: alpha, A (five members), mu, M (five members), pi, P (one member), theta, T (two members), zeta, Z (one member), omega, O (two members), and sigma, S (one member) subfamilies (Figure 7), based on their amino acid sequence identity (Mannervik et al., 2005). Namely, members of the same class possess more than 40% amino acid sequence identity (sometimes more than 90%) and less than 25% sequence identity between classes (Hayes et al., 2005).

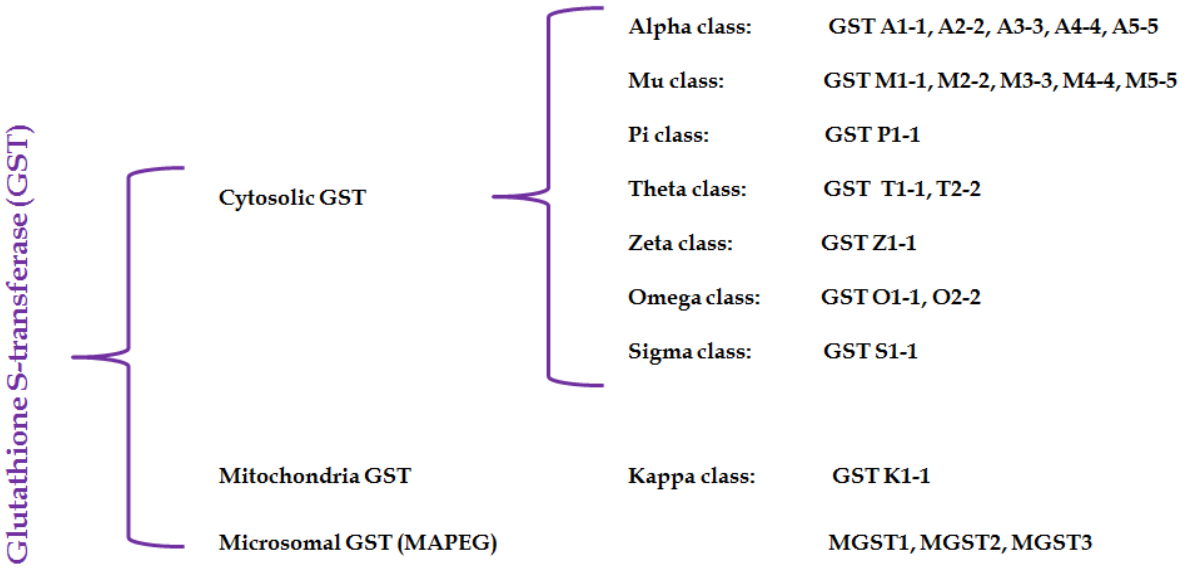



**Figure 7**. Classification of human glutathione transferases (GSTs) based on amino acid sequence identity. Adopted from *Wu and Dong,* 2012.

### 1.2.1 Structure and substrate specificity

Soluble GSTs function as approximately 50 kDA dimers, constituted as homodimers or heterodimers being formed from subunits within a given GST isoenzyme class (Hayes and Pulford, 1995) Although it is still unclear whether the dimeric structure is relevant for all biologicals function of the GSTs, evidence suggests that the dimeric structure increases the stability and provides a proper structure of the active site, at least for the efficient catalysis (Abdalla et al., 2002; Armstrong, 2010). On the other hand, there are *in vivo* evidence of monomer-dimer equilibrium of GSTs that are involved in protein:protein interactions, having the capacity to dissociate into monomers in order to form heterodimers with other monomeric proteins, such as mitogen activated protein kinases (Adler et al., 1999; Cho et al., 2001).

A single GST unit consists of an N-terminal α/β-domain (or *G-domain*) and an all-α-helical domain (or *H-domain*) (Wu and Dong, 2012). The *G-domain* is conserved throughout all classes as it represents the binding site for tripeptide cofactor - glutathione (GSH, γ-glutamyl-cysteinyl-glycine). Cytosolic GSTs seem to be subdivided on the basis of different mode of GSH activation (Atkinson and Babbitt, 2009). Namely, the sulfur atom of GSH is located at the N-terminal end and is invariably hydrogen bonded to a catalytic residue in the protein. This hydrogen bond interaction plays a crucial role in GST catalysis by stabilizing the activated

GSH (thiolate anion, GS–). Moreover, different subgroups of GST contain various catalytic residues interacting and subsequently activating the GSH: (1) *tyrosine* in the alpha-, mu-, pi-, and sigma class of GSTs, (2) *serine* in the theta- and zeta-class of GSTs, and (3) *cysteine* in the omega-class of GSTs (Armstrong, 2010).

The *H-domain* is not conserved by nature and varies greatly in shape, size and chemical character (hydrophobicity) across classes, enabling numerous electrophilic compounds to bind to it in a non-specific binding mode (Oakley, 2011). For instance, GSTA1-1 is known for its 'promiscuous substrate selectivity', being able to catalyze various, structurally unrelated compounds such as steroids, benzo(a)pyrene diol epoxides, as well as products of lipid degradation (Wu and Dong, 2012). Contrary to alpha GSTs, mu-class members have larger active site and are able to bind many bulkier electrophilic agents such as aflatoxin B1-epoxides and benzpyrene diols (Wu and Dong, 2012). Among their substrates, GSTs conjugate even the signaling molecules such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 and 4-hydroxynonenal and therefore might modulate a number of signaling pathways (Hayes et al., 2005). The list of some of the recognized GST substrates is presented in Table 2, available in numerous studies on xenobiotics substrate specificity of GSTs (Armstrong, 2010; Hayes et al., 2005). It is noteworthy to mention that humans express a large number of different GSTs with overlapping substrate specificities, resulting in difficulty to identify isoenzymes solely on their catalytic properties (Habig et al., 1974; Hayes et al., 2005).

For example, polyaromatic hydrocarbons (PAH) represent an important class of environmental pollutants and are shown to be substrates for multiple classes of GST (Table 2.). As indicated in Table 2, the diol epoxides of PAHs are catalyzed predominantly by GSTs, with the Mu class showing the highest activity towards most PAH epoxides, especially (+)-anti-BPDE (Hayes and Strange, 2000; Sundberg et al., 2002, 1998), followed by GSTP1-1 and GSTA1-1 (Figure 8) (Sundberg et al., 2002).

**Table 2**. Recognized substrates of cytosolic GST; Adopted from *Armstrong, 2010; Hayes et al., 2005*

| GST enzyme | GST preferred substrate |
|---|---|
| GSTA1 | '5-ADD, BCDE, BPDE, Busulfan, Chlorambucil, DBADE, DBPDE, BPhDE, N-a-PhIP |
| GSTM1 | trans-4-phenyl-3-buten-2-one, BPDE, CDE, DBADE, trans-stilbene oxide, styrene-7,8-oxide |
| GSTT1 | BCNU, butadiene epoxide, $CH_2Cl_2$, EPNP, ethylene oxide |
| GSTP1 | acrolein, base propenals, BPDE, CDE, Chlorambucil, COMC-6, EA, Thiotepa |

Abbreviations: 5-ADD, 5-androstene-3,17-dione; BCDE, benzo[g]chrysene diol epoxide; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; BPDE, benzo(a)pyrene diol epoxide; BPhDE, benzo[c]phenanthrene diol epoxide; CDE, chrysene-1,2-diol 3,4-epoxide; COMC-6, crotonyloxymethyl-2-cyclohexenone; DBADE, dibenz[a,h]anthracene diol epoxide; DBPDE, dibenzo[a,l]pyrene diol epoxide; EA, ethacrynic acid; EPNP, 1,2-epoxy-3-(p-nitrophenoxy)propane; N-a-PhIP, N-acetoxy-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine.

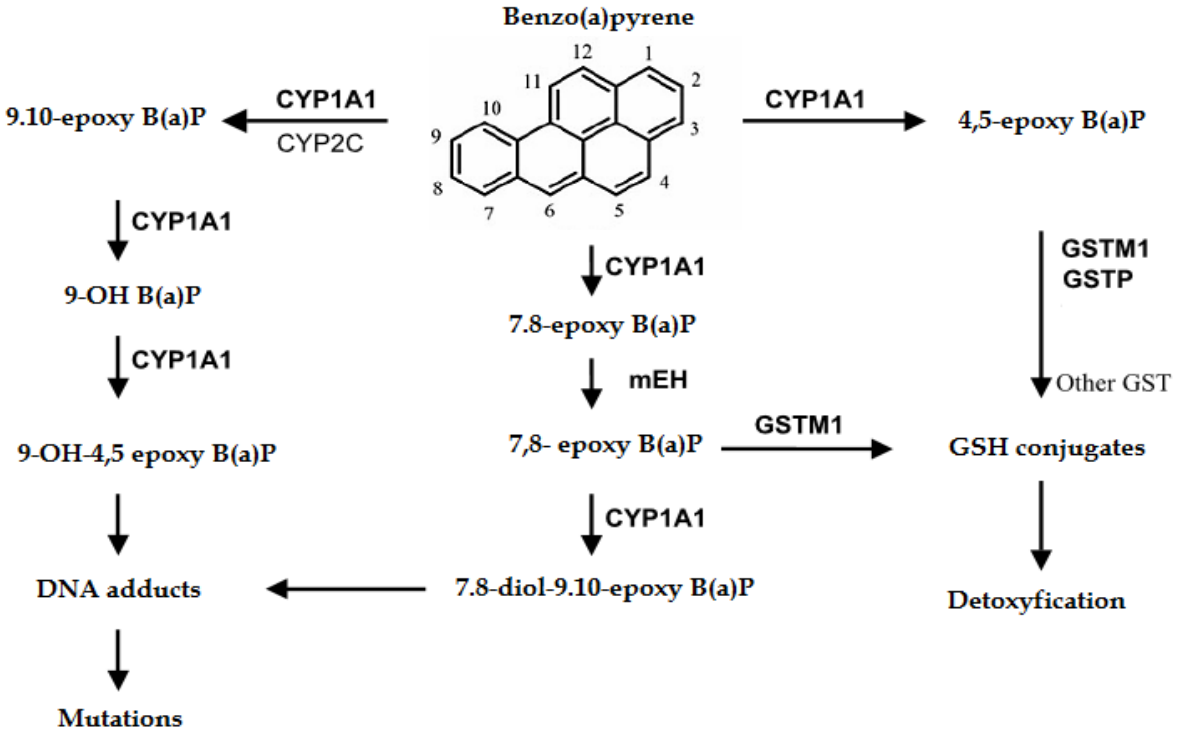



**Figure 8.** The metabolism of B(a)P; Adopted from *Lodovicici et al., 2004*; Abbreviations: B(a)P: benzo(a)pyrene; mEH: microsomal epoxide hydrolase

### 1.2.2 Functions of GSTs

GSTs comprise a set of cellular proteins (GSTome) with various catalytic and non-catalytic functions (Grek et al., 2013; Wu and Dong, 2012).

#### 1.2.2.1 Metabolism of xenobiotics by GSTs

Namely, GST have been described as one of the most important enzymes involved in cell detoxification processes as they are crucial for their role in metabolizing both exogenous (chemical carcinogens, environmental pollutants and even antitumor agents) and endogenous electrophilic compounds (Hayes et al., 2005). Strong electrophiles arise from both xenobiotic and endobiotic compounds, being a result of mixed-function oxidation activity of cytochrome P-450 in Phase I reactions (Dourado et al., 2008).

The primary metabolic role of GST is to detoxify such reactive electrophiles, by catalyzing reaction of conjugation with GSH (Figure 7). The reaction catalyzed by GST consists of the nucleophilic addition of the sulfur thiolate of GSH to a wide range of compounds, containing electrophilic atoms of carbon, sulfur, nitrogen and phosphorous (Dourado et al., 2008). Namely, upon GSH activation, the nucleophilic sulfur atom attacks the electrophilic toxic compound present in the *H-site*, usually producing a less toxic compound, a glutathione S-conjugate (GSH conjugate). The formation, processing and transport of GSH conjugates takes place in a number of organs (liver, biliary tract, gastrointestinal tract, and kidney) and involves considerable inter- and intra-organ cooperativity (Commandeur et al., 1995; Hinchman and Ballatori, 1994). Namely, upon formation, GSH conjugates are recognized and exported from the cell by ATP-dependent transmembrane pumps, such as P-glycoproteins and multidrug resistance-related proteins (MRP1, MRP2) (Haimeur et al., 2004; Paumi et al., 2001) and consequently subjected to metabolism of mercapturic acid via formation of the *S*-cysteine conjugates (Figure 9). The kidney is shown to play a very important role in the metabolism of both GSH conjugates and *S*-cysteine conjugates to their corresponding mercapturic acids (Commandeur et al., 1995), followed by the excretion in the urine (Egner et al., 2008) or bile (Teichert et al., 2009) in Phase III. Of note, GST effectiveness depends on the combined actions of glutamate cysteine ligase and GSH synthase to supply GSH and, on the other hand, the actions of transporters to remove GSH conjugates from the cell (Di Pietro et al., 2010).

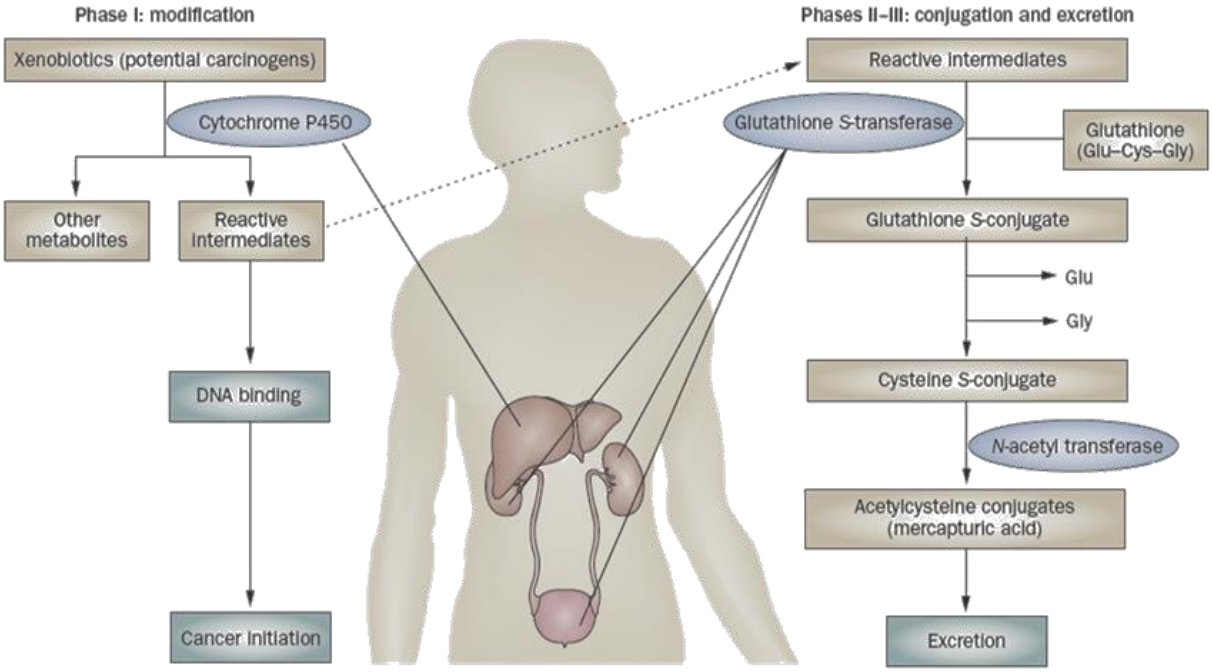



**Figure 9**. The metabolism of xenobiotics and the formation of GSH conjugates and their subsequent metabolism towards mercapturic acid; Adopted from *Simic et al., 2009*, available at http://www.nature.com/nrurol/journal/v6/n5/fig_tab/nrurol.2009.49_F2.html;
Abbreviations: Cys- Cysteine, Glu- glutamate, Gly- glycine

However, not all reactions catalyzed by GST enzymes result in detoxification of a foreign compound, rendering it less reactive and more soluble. Namely, in certain instances some GSTs are involved in reactions of xenobiotic bio-activation, resulting in a GSH conjugate that is even more reactive than the parent compound. A growing number of evidence supports the aforementioned phenomenon, where mutagens, carcinogens and even some prodrugs are metabolically activated by conjugation with GSH (Guengerich, 2005; Kurtovic et al., 2008). Mono- and dihaloalkanes (Guengerich et al., 2003; Wheeler et al., 2001) as well as polyhalogenated alkenes (Armstrong, 2010) are some of the examples of compounds in which GSH conjugates still contain a potent electrophilic center and are capable of modifying important cell macromolecules, such as DNA (Hayes et al., 2005). There is evidence that this is particularly true for the kidney. Namely, in the compelling example of GSH addition to methylenechloride, catalyzed by the *theta* class of GST enzymes, highly electrophilic compound is yield and rapidly hydrolyzed to even more toxic formalaldehyde (Ahmed and

Anders, 1976; Meyer et al., 1991) that might contribute to the process of the target-organ toxicity and possible renal tumorigenicity (Anders and Dekant, 1998).

#### 1.2.2.2 Metabolism of endogenous compounds by GSTs

Being a multifunctional group of enzymes, GSTs are involved in intracellular binding and transport of hydrophobic compounds (Hayes and Pulford, 1995), catalysis of key steps in the synthesis of leukotrienes, prostaglandins (Inoue et al., 2003) and steroid hormones (Tars et al., 2010), as well as the degradation of tyrosine (Hayes et al., 2005).

Moreover, GSTs are involved in the inactivation and reduction of endogenous reactive by-products generated during oxidative stress. Namely, an increase in the intracellular levels of reactive oxygen/nitrogen species (ROS/RNOS), including oxygen and nitrogen-free radicals, leads to membrane dysfunction, DNA damage and inactivation of proteins. It is presumed that chronic oxidative stress has numerous pathological consequences, including the development of complex diseases, such as cancer. ROS/RNOS are shown to damage cellular constituents, not only in a direct way, but also indirectly through the production of reactive secondary metabolites. Polyunsaturated fatty acids in cell membranes are particularly sensitive to the process of lipid peroxidation, resulting in the production of short-living lipid hydroperoxides that tend to break down to yield more reactive secondary electrophiles, some of which are genotoxic (Marnett et al., 2003). Phospholipid, fatty acid and cholesterol hydroperoxides are substrates for several GSTs, especially for the members of class *alpha* class enzymes (Seeley et al., 2006). Among the substrates for GSTA4-4 are products of lipid peroxide oxidation, acrolein and 4-hydorxynonenal (4-HNE) (Hubatsch et al., 1998). Furthermore, the oxidation of nucleotides yields base propenals and hydroperoxides that are detoxified by GSTs.

Overall, it seems that some GST isoenzymes exhibit selenium independent glutathione peroxidase activity and along with other antioxidant enzymes provide a certain shield against a range of harmful electrophiles, produced during redox imbalance (Hayes and McLellan, 1999). Another interesting implication of GSTs in the protection against oxidative damage and the control of redox signaling pathway function is their capability of S-glutathionylation. Namely, S-glutathionylation is a posttranslational protein modification, characterized by the conjugation of GSH to low PKa cysteine sulfydryl or sulfonic-acid moieties in target proteins, in response to endogenous oxidative or nitrosative stress-mediated signaling events or from exposure to

external environmental drug treatment (Tew et al., 2011). Moreover, there is a growing body evidence delineating the importance of S-glutathionylation in the regulation of protein structure and function (Tew et al., 2011).

#### 1.2.2.3 The role of GSTs in the regulation of cell signaling

In addition to their role in the biotransformation reactions, there is some evidence which clearly indicates the involvement of GST in the cellular survival, proliferation and apoptosis as well, by the means of protein:protein interactions with the signaling molecules. (Board and Menon, 2013; Laborde, 2010; McIlwain et al., 2006; Tew and Townsend, 2012). Namely, GSTs are shown to negatively regulate protein kinases such c-Jun NH2-terminal kinase (JNK1) and apoptosis signal-regulating kinase 1 (ASK1).

The first example of GST-mediated kinase regulation was the discovery of the GSTP1:JNK1 complexes (Adler et al., 1999). Namely, it seems that under physiological conditions, a portion of GSTP1 is bound to kinase JNK1, regulating the level of JNK1 activity. However, in case of increased ROS content, the GSTP1:JNK1 complex dissociates which in turn leads to the association of GSTP1 into oligomers (Figure 10, A). Now activated, JNK1 induces a chain of events, starting from the phosphorylation of its substrate, the transcription factor c-Jun, and resulting in apoptosis (Adler et al., 1999; Board and Menon, 2013). A similar pattern was observed in the case of GSTP1 interaction with Factor 2 bound to the TNFα receptor (TRAF2). On one hand, the dissociation of this particular complex results in the oligomerization of the GSTP1 and on the other, in differentiation/proliferation or apoptosis, depending on the severity of oxidative stress. It is noteworthy to mention that the catalytic activity of GSTP1 is not affected by the involvement in protein:protein interactions, suggesting that the active site of GSTP1 is not engaged in this process (Tew and Townsend, 2012).

Another example of protein:protein interaction, similar to the one of GSTP1, is a complex between mitogen activated kinase (MAPK) ASK1 and GSTM1-1, found to be important for the maintenance of the normal level of p38 phosphorylation (Figure 10, B) (Cho et al., 2001). Namely, ASK1 is MAPK kinase kinase (MAPK3) that activates JNK1 and p38 pathways, leading to cytokine and stressed-induced apoptosis (Ichijo et al., 1997). Environmental stress causes the disruption of the complex of GSTM1:ASK1, which accumulates GSTM1 into oligomers, while ASK1 is being activated (Dorion et al., 2002). This

dissociation results in a subsequent activation of JNK1 and p38-dependent signal pathways, ultimately leading to stress-induced apoptosis. Similarly to GSTP1, this role of GSTM1 seems to be independent of the GST enzyme activity (Cho et al., 2001).

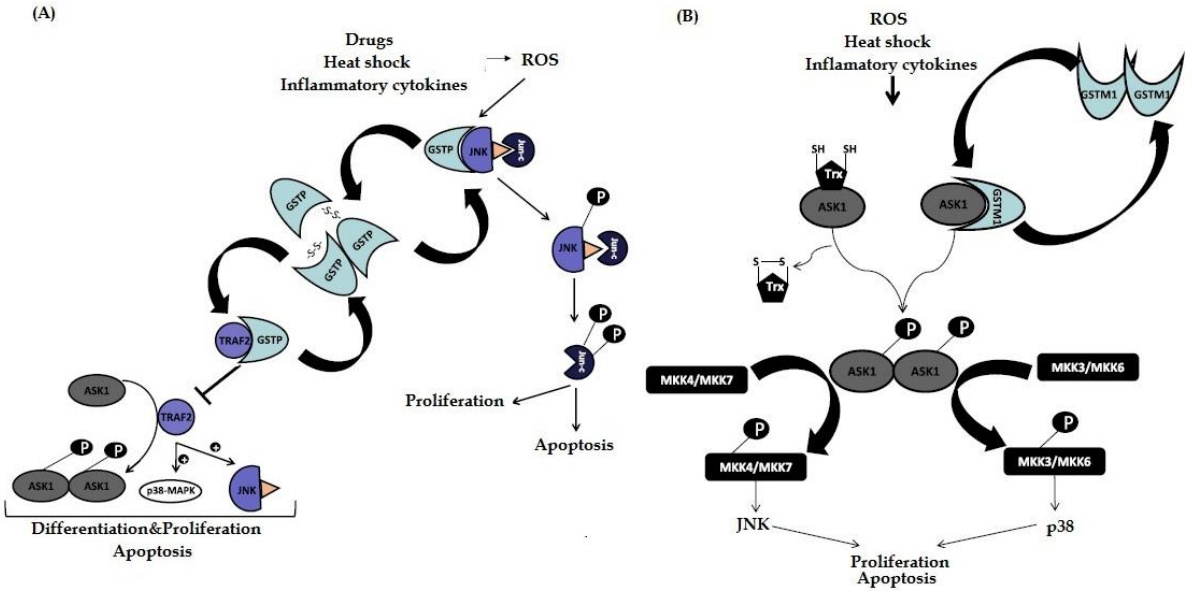



**Figure 10**. Various interactions between GSTs and protein kinases, implicated in stress-signaling pathway: (A) protein:protein interaction of GSTP1:JNK1, (B) protein:protein interaction of GSTM1:ASK1; Adopted from *Board and Menon, 2013*; Abbreviations: ROS- reactive oxygen species; JNK1: c-Jun NH2-terminal kinase; ASK1- apoptosis signal-regulating kinase 1; TRAF2- factor 2 bound to the TNFα receptor;

Finally, even GSTA1 possesses the capacity of forming protein:protein complexes with JNK1. Namely, the homology between GSTA and GSTP family members may explain why GSTA1 by a similar mechanism can also suppress JNK1 signaling, caused by inflammatory cytokines or oxidative stress. It seems that enhanced GSTA1-1 expression significantly decreases the number of cells subjected to the apoptosis due to inhibition of JNK1-dependent phosphorylation of c-jun and the activation of Caspase-3 (Romero et al., 2006).

### 1.2.3 Genetic variations in human GSTs

GSTs are members of a multi-gene family. The most studied GSTs are encoded by clusters of paralogous genes on a given chromosome (Hollman et al., 2016). Apart from variations found between GST classes, a substantial genetic heterogeneity was found within

classes, because of gene duplications, deletions and single nucleotide polymorphisms in both coding and non-coding gene regions. Many of the variations found within genes encoding for human cytosolic GSTs make a direct impact on the protein structure, function and expression, reshaping their substrate specificity and diversity as well (Board and Menon, 2013). Some of the most frequently studied *GST* polymorphisms are present in Table 3.

**Table 3**. Polymorphism in human cytosolic GST; Adapted from *Hayes et al., 2005*

| Class | Allele | Nucleotides in gene at variable position | Protein affected |
|---|---|---|---|
| Alpha | *GSTA1**A | −631T, −567T, −69C, −52G | "Reference" protein levels |
| | *GSTA1**B | −631G, −567G, −69T, −52A | Low protein levels |
| Mu | *GSTM1**A | 519G | $Lys^{173}$ |
| | *GSTM1**B | 519C | $Asn^{173}$ |
| | *GSTM1**0 | Gene deletion | No protein |
| | *GSTM1**1x2 | Gene duplication | Overexpression of GSTM1 protein |
| Theta | *GSTT1**A | 310A | $Thr^{104}$ |
| | *GSTT1**B | 310C | $Pro^{104}$ |
| | *GSTT1**0 | Gene deletion | No protein |
| Pi | *GSTP1**A | 313A, 341C, 555C | $Ile^{105}$, $Ala^{114,}$ $Ser^{185}$ |
| | *GSTP1**B | 313G, 341C, 555T | $Val^{105}$, $Ala^{114,}$ $Ser^{185105}$ |
| | *GSTP1**C | 313G, 341T, 555T | $Val^{105}$, $Val^{114,}$ $Ser^{185}$ |
| | *GSTP1**D | 313A, 341T | $Ile^{105}$, $Val^{114}$ |

Abbreviations: Lys- lysine; Asn- asparagine; Thr- threonine; Pro- proline; Ile- isoleucine; Ala- alanine; Ser- serine; Val- valine;

Vast majority of polymorphisms identified within genes encoding for cytosolic GSTs comprise single nucleotide substitution or variation (SNP). Generally, SNPs are divided into synonymous and non-synonymous. Synonymous polymorphisms (sSNP) occur due to the nucleotide change resulting in the amino acid substitution, however, without affecting protein function. On the other hand, missense, nonsense and frameshift changes represent non-synonymous mutations (nsSNP) that generate a significant change in terms of structure and consequently function of translated protein. Several lines of evidence indicate that out of 237 coding nsSNP found in *GSTs* genes (Table 4) (Yadav et al., 2014), certain nsSNPs seem to exert deleterious effects, leading to carcinogenesis and the development of other non-malignant diseases (Hayes and Strange, 2000; McIlwain et al., 2006). Moreover, due to the functional significance of *GST* polymorphisms, *Hollman et al.* have recently suggested a

classification of diseases that are highly related to SNPs found in GSTs. The five proposed categories are: (1) cancers (2) inflammatory or immune-mediated disorders (3) neurological disorders (4) aging-related or metabolic disorders and (5) reproductive disorders. Environmental toxins are the most important affecting factors for all five categories of diseases and are the causing factors for all cancers, inflammatory or immune-mediated disorders, as well as reproductive disorders (Hollman et al., 2016).

**Table 4**. Genes encoding for major cytosolic GSTs and their SNP distribution; Adapted from *Yadav et al., 2014*

| Gene Family | Genome Location | Total SNP | nsSNP | sSNP | 3'UTR | 5'UTR | iSNP |
|---|---|---|---|---|---|---|---|
| *GSTM* | Chr1 | 1072 | 92 | 53 | 55 | 8 | 864 |
| *GSTA* | Chr6 | 1702 | 98 | 43 | 34 | 21 | 1506 |
| *GSTP* | Chr11 | 180 | 17 | 6 | 3 | 6 | 148 |
| *GSTT* | Chr22 | 239 | 30 | 11 | 3 | 5 | 192 |
| Total | | 3193 | **237** | 113 | 95 | 40 | 2710 |

Abbreviations: nsSNP:-non-synonymous SNP; sSNP- synonymous SNP; 3'UTR- 3' untranslated region; 5'UTR- 5' untranslated region; iSNP -intronic SNP.

Deletional polymorphisms of genes encoding for human cytosolic GSTM1 and GSTT1 are rather common in human population. They result in a *null genotype*, characterized by a general lack in enzymatic activity. In last two decades, the impact of GSTM1 and GSTT1 deficiency was the subject of a vast number of molecular epidemiological studies that tried to elucidate if the association between some diseases known to be caused by environmental agents and *GST* gene polymorphisms does exist.

#### 1.2.3.1 Glutathione S-transferase M1

GSTM1 protein is composed of 218 amino acids, organized as either homo or heterodimers with calculated molecular mass of 25.712 Da for each subunit. Isoelectric point for this protein is around: at pH 6.6 (Mannervik, 1985).

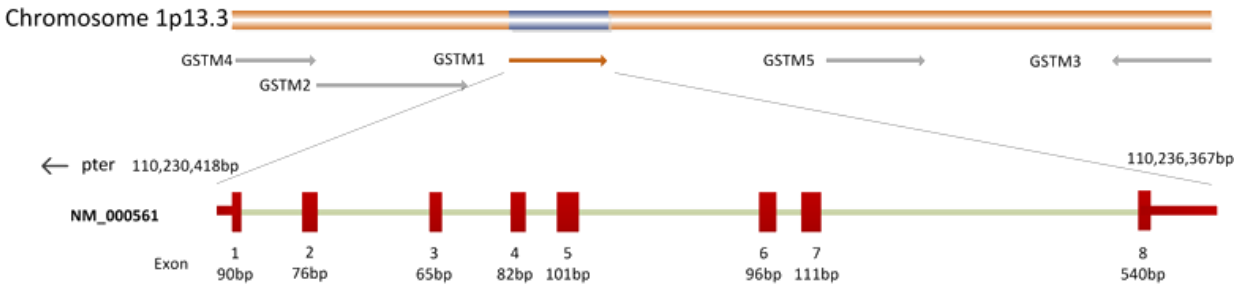



**Figure 11.** The location of *GSTM1* gene on chromosome 1; Adopted from *Pejovic-Milovancevic et al., 2010*

In humans, GSTM1 gene is located on chromosome 1p13.3 within 100-kb gene cluster (*5'-GSTM4-GSTM2-GSTM1-GSTM5-GSTM3-3'*) (Pearson et al., 1993). Moreover, within *GST* mu cluster, two-tandem *GSTM1* genes, situated between the *GSTM2* and *GSTM5* genes, were discovered (McLellan et al., 1997). Precisely, the GSTM1 gene is composed of 8 exons, spanning a region of 21,244 bases. It comprises four different alleles, resulting in several M1 class polymorphisms, marked as *GSTM1**0, *GSTM1**A, *GSTM1**B and *GSTM1**1x2 (Table 3) (Board, 1981; Wu et al., 2012).

*GSTM1**0 (*GSTM1-null)* allele is the result of a 20-kb segment deletion (Xu et al., 1998), hence homozygotes for *GSTM1-null* allele produce no GSTM1 protein. This deletion results in a novel 7.4-kb HindIII fragment with the loss of 10.3- and 11.4-kb HindIII fragments. The end-points of the polymorphic *GSTM1* deletion are: the left repeated region 5 kb downstream from the 3'-end of the *GSTM2* gene and 5 kb upstream from the beginning of the *GSTM1* gene; the right repeated region 5 kb downstream from the 3'-end of the *GSTM1* and 10 kb upstream from the 5'-end of the *GSTM5* gene (Pejovic-Milovancevic et al., 2016; Xu et al., 1998) (Figure 11). The prevalence of *GSTM1* deletion polymorphisms seems to vary across ethnic groups, from 18% to 66% (median, 50%) in Caucasians and 38%-58% in Asians (Wu et al., 2012).

*GSTM1**A and *GSTM1**B differ by a single base in exon 7 (Seidegård and Pero, 1988). *GSTM1**A and *GSTM1**B arise from a C/G substitution at base position 534, resulting in an interchange of Lys/Asn at amino acid 173 (Widersten et al., 1991), which does not appear to affect enzyme function (Table 3). However, the substitution causes the formation of homodimers (*GSTM1A**1A, *GSTM1B**1B) or heterodimers (*GSTM1A**1B).

Finally, in Saudi Arabian population, a unique *GSTM1* variant *GSTM1**1x2, containing a duplicated *GSTM1* gene has been identified (Evans et al., 1996) presumably leading to ultra-rapid enzyme activity of GSTM1 protein (McLellan et al., 1997).

Among all *GSTM1* polymorphisms, the highest attention was given to *GSTM1* deltional polymorphism. The GSTM1 deficiency appears to be associated with a modest but significant risk of several types of cancers, such as lung (Ye et al., 2006), colon (Economopoulos and Sergentanis, 2010) and bladder (Matic et al., 2013), as well as various response rates to some chemotherapeutics (Ambrosone et al., 2001). The underlying mechanism conferring an increased risk of cancer in *GSTM1-null* carriers would be that such individuals are more susceptible to chemical-induced carcinogenesis due to the diminished activity of xenobiotic-metabolizing defense system (Di Pietro et al., 2010). Moreover, monomeric form of GSTM1 is shown to negatively regulate kinase-dependent proliferation pathways by forming protein:protein complexes with MAPK kinase kinase ASK1 (Cho et al., 2001; Dorion et al., 2002). Slower tumor progression, as well as impaired clinical response to therapy in a variety of tumor types has been associated with altered GSTM1 expression. Namely, it has been speculated that carriers of *GSTM1-null* genotype exhibit better survival, possibly due to the decreased level of apoptotic activity in tumor tissue (De Martino et al., 2010; McIlwain et al., 2006).

#### 1.2.3.2 Glutathione S-transferase T1

The gene for *GSTT1* is situated at chromosome 22 (22q11.23), and composed of 5 exons, spanning a region of 8,179 bases (Figure 12). In the case of *GSTT1*, gene homozygous deletion, termed "*GSTT1-null*" genotype is present in ~20% of Caucasians, leading to the lack of *GSTT1* enzyme activity (Wiencke et al., 1995). Namely, a 54251bp fragment comprising the gene for *GSTT1*, is found to be deleted from chromosome 22, most likely by a homologous recombination event between two highly homologous sequence stretches that flank *GSTT1* (Pejovic-Milovancevic et al., 2016; Sprenger et al., 2000).

Another *GSTT1* polymorphism was found in exon 3 of *GSTT1* gene, resulting in two different variants: *GSTT1**A and *GSTT1**B. The substitution of threonine at residue 104 to a proline causes a decrease in catalytic activity due to the conformational change located in the middle of alpha-helix 4 (Alexandrie et al., 2002).

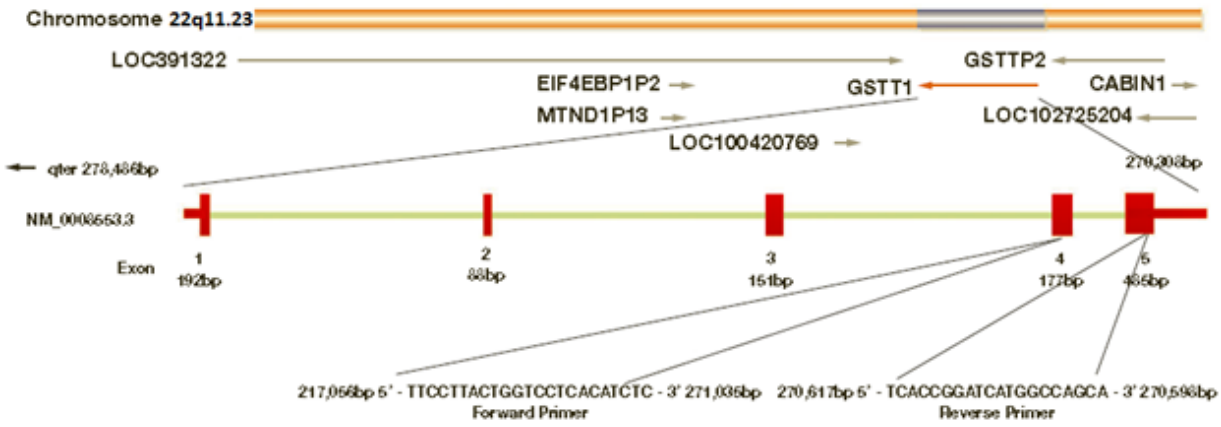



**Figure 12**. The location of *GSTT1* gene on chromosome 22; adopted from *Pejovic-Milovancevic et al., 2016*

GSTT1-1 has been highly conserved during evolution and played a major role in phase-II biotransformation of a number of drugs and industrial chemicals, e.g. cytostatic drugs, hydrocarbons and halogenated hydrocarbons. However, it seems that when it comes to gene-environment interactions, GSTT1 deficiency may be either deleterious or beneficial depending upon circumstances. Apparently, GSTT1 may play a role as a risk modifier only in the case of subjects exposed to relevant substrates (Buzio et al., 2003). Namely, as mentioned earlier, members of the GST *theta* class are involved in GSH conjugation of certain halo- and dihaloalkanes, producing even more toxic reactive intermediates (Guengerich et al., 2003). On the other hand, GSTT1 is capable of detoxifying compounds such as methyl bromide (Pemble et al., 1994), ethylene dibromide (Ploemen et al., 1995) and ethylene oxide (Yong et al., 2001).

#### 1.2.3.3 Glutathione S-transferase A1

Gene cluster for members of GST *alpha* is localized on chromosome 6p12.1-6p12.2, covering more than 400 kb of genes GSTA1-GSTA5 (Morel et al., 2002) (Figure 13). GSTA1 is dominantly expressed in the liver. However, GST *alpha* expression seems to be modified by genetic polymorphism. *GSTA1* polymorphism is represented by three apparently linked single nucleotide polymorphisms in an SP1-responsive element within the proximal promoter (G-52A, C-69T and T-567G), plus at least four SNPs further upstream and a silent SNP A-375G (Figure 13).

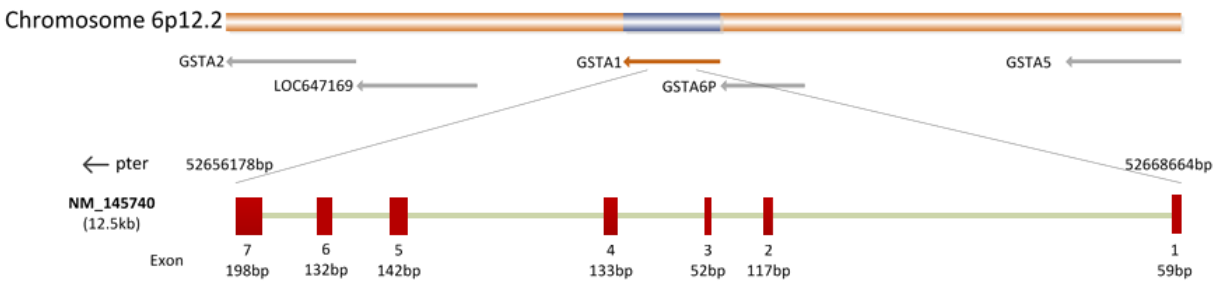



**Figure 13.** The location of *GSTA1* gene on chromosome 6; adopted from *Savic-Radojevic and Radic, 2014*

Two variants, *GSTA1**A (-567T, -69C,-52G) and *GSTA1**B (-67G, -69T, -52A), have been named according to the linked functional SNPs. The frequency of the *GSTA1**A haplotype ranges between 0.6 and 0.85 in African, Asian and European populations. It seems that aforementioned substitutions result in differential expression with lower transcriptional activation of variant *GSTA1**B than common *GSTA1**A allele (Coles and Kadlubar, 2005). Namely, it has been shown that the mean expression of GSTA1 in liver samples of *GSTA1**A homozygotes was approximately 4-fold higher than that of *GSTA1**B homozygotes (Coles et al., 2001). On the other hand, no difference in expression was found in pancreatic tissue, suggesting that the effect of this polymorphism might be tissue specific (Coles and Kadlubar, 2003). Moreover, *Chuang et al.* have clearly demonstrated that GSTA1 is a biomarker for clear cell RCCs by means of several molecular techniques, whereas the same marker was absent in other renal tumors (Chuang et al., 2005).

GSTA1, along with GSTA2, catalyzes the glutathione conjugation of a wide range of electrophiles, possess glutathione dependent steroid isomerase activity and GSH-dependent selenium independent peroxidase activity (Coles and Kadlubar, 2005). It has been suggested that this genetic variation of GSTA1 can change an individual's susceptibility to carcinogens and toxins, as well as affect the efficacy of some drugs (Coles and Kadlubar, 2003). However, the results of these studies have been rather conflicting.

#### 1.2.3.4 Glutathione S-transferase P1

GSTP1 gene is located on the chromosome 11q13 (Figure 14). Two common functional variants have been found within these gene, resulting in a interchange in amino acids 105 (Isoleucine, Ile to Valine,Val) and 114 (Alanine, Ala to Valine) (Ali-Osman et al.,

1997). Four haplotypes have been identified: *GSTP1**A *(**Ile105+*Ala114), GSTP1**B *(**Val105+*Ala114), GSTP1**C *(**Val105+*Val114)* and *GSTP1**D *(**Ile105+*Val 114)* (Watson et al., 1998). The allele frequencies for *GSTP1**A, *B, and *C in Caucasian populations are found to be 0.685, 0.262, and 0.0687, respectively (Garte et al., 2001).

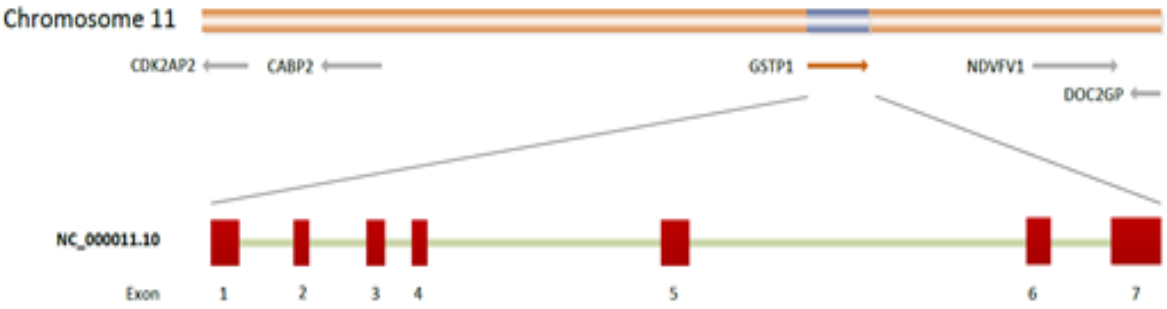



**Figure 14.** The location of *GSTP1* gene on chromosome 11; adopted from *Simic, 2016*

Far more studied substitution is the *Ile105Val, occurring due to the nucleotide substitution A1404G at exon 5, base pair 313, contributing to the architecture of the hydrophobic substrate binding H-site and different substrate specificity (Reinemer et al., 1992). For instance, GSTP1 variants exhibit significantly different rates of conjugating activity towards (+)-anti-BPDE, with higher turnover for isoform *GSTP1**Val105 than for isoform *GSTP1**Ile105, due to the more favorable substrate-binding setting in the H-site (Hu et al., 1997). This finding was further supported by the results of *Sundberg et al.*, who additionally suggested that higher risk for tumor formation by PAH in homozygous carriers of *GSTP1**Val105 is not caused by a lower catalytic efficacy of the corresponding enzyme, but proposed a display of other reasons (Sundberg et al., 1998).

Throughout the literature, GSTP1 genotype has been associated with differences in cancer susceptibility, chemotherapeutic response and overexpression in variety of tumors. However, several lines of evidence indicate that the underlying mechanism might not be solely based on the polymorphic expression of GSTP1. Namely, the fact that GSTP is shown to be a potent inhibitor of signaling molecules, such as JNK1, via formation of protein:protein interactions (Adler et al., 1999; Tew et al., 2011), seems to affect survival and/or apoptotic pathways, related to the observed drug resistance (Adler et al., 1999; McIlwain et al., 2006;

Townsend et al., 2005). It has been suggested that the upregulation of GSTP found in many drug-resistant tumors such as ovarian, lung, breast colon and hematological cancers (Laborde, 2010), might cause negative regulation of JNK1 through aforementioned protein:protein interactions and subsequently suppress apoptotic pathways, conferring resistance to drug-induced death (Board and Menon, 2013).

## 1.3 GENETIC POLYMORPHISM OF GLUTATHIONE TRANSFERASES IN PATIENTS WITH RENAL CELL CARCINOMA

Mounting evidence suggests that members of the subfamily of cytosolic GSTs possess roles far beyond the classical GSH-dependent enzymatic conjugation of electrophilic metabolites and xenobiotics. Namely, a well-known homo- and hetero-dimeric forms of GSTs seem to exist in a redox-sensitive dynamic equilibrium with its monomeric forms, that are capable of forming protein:protein interactions with other cellular proteins (Tew et al., 2011). The latest interpretation of such protein:protein interactions emphasizes that multiple signaling and regulatory functions of GSTs coexist with the classical enzymatic and small molecule binding role of these proteins (Bartolini and Galli, 2016). Such vast capabilities of GSTs become very important when it comes to tumorigenesis and even therapy resistance.

The impact of GST functional human polymorphisms on cancer susceptibility as well as therapeutic outcomes has been extensively studied in the context of its Phase II detoxification properties. However, the role of these polymorphisms as mediators of protein: protein interactions in better understanding of the cancer progression is yet to be determined.

In case of RCC, some studies suggest that cytosolic GSTs might be implicated not solely in the development, but also in the progression of RCC (Ahmad et al., 2012; De Martino et al., 2010; Salinas-Sánchez et al., 2012; Sweeney et al., 2000). GSTs are involved in the biotransformation of several compounds recognized as risk factors for RCC (Hayes and Strange, 2000). The main site for the initial glutathione conjugation of toxic compounds is generally assumed to be the liver, followed by a mandatory transfer of conjugates to the kidney (Simic et al., 2009). However, the initial bio-activation step of some nephrocarcinogens can take place in the kidney itself (Green et al., 1997). The potential genotoxicity of carcinogens depends on the biotransformation capacity of renal tissue. Prominent genetic heterogeneity, resulting from the gene deletions, as well as from SNPs in the coding and non-coding regions

of *GST* genes, might affect GST isoenzyme profiles in renal parenchyma and therefore serve as a valuable indicator for predicting the risk of cancer development (Di Pietro et al., 2010).

There is a growing body of evidence suggesting that during the tumor development, important changes appear in the cellular redox homeostasis as well. This can be particularly true for the kidney due to its high metabolic activity and oxygen demands (Simic et al., 2009). Moreover, it is believed that renal cell carcinoma also belongs to tumors in which significant changes occur in cellular redox balance. This can be partially explained by the fact that all three recognized risk factors for RCC development (obesity, hypertension and smoking) have been linked with increased endogenous formation of reactive oxidants. Thus, in RCC, marked oxidative alterations of lipids, proteins and DNA have been found (Pljesa-Ercegovac et al., 2008). Although a broad range of DNA products are produced during oxidative damage to DNA (Valavanidis et al., 2009), 8-hydroxy-2'-deoxyguanosine (8-OHdG) is the most widely used fingerprint of radical attack towards DNA (Figure 15). Results obtained from several studies indicated the significant role of 8-OHdG as a potential biomarker in risk assessment and prognosis of various diseases associated with oxidative stress mechanisms, such as cancers, aging and degenerative diseases (Valavanidis et al., 2009). However, despite the fact that some GST isoenzymes might be involved in regulation of cellular redox homeostasis by their nonselenium-dependent GPX activity, their role in the DNA antioxidant protection and corresponding level of 8-OHdG has not been assesed in such context in RCC.

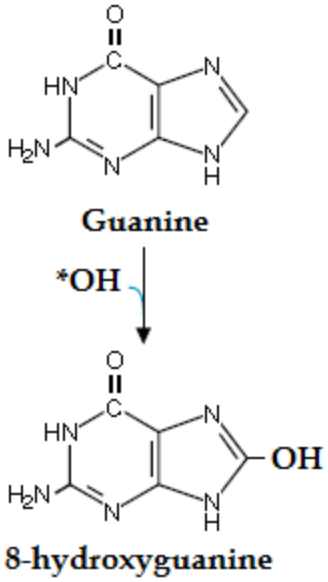



**Figure 15.** The structure of 8-hydorxyguanine (8-OHdG)

Furthermore, DNA adducts associated with tobacco smoking have been suggested as a marker of biologically effective dose of tobacco carcinogens that might improve individual cancer risk prediction (Wiencke, 2002). As previously mentioned, cigarette smoke is a rich source of both free radicals, shown to induce DNA damage that accumulates in cells, but also more than 60 carcinogens, such as PAHs. Both free radicals and reactive PAH metabolites are detoxified by GSTs (Filiadis and Hrouda, 2000; Jung and Messing, 2000). So far, the relationship between *GST* genotype and BPDE-DNA adduct formation in determining the risk for RCC has not been evaluated in patients with RCC.

Although the associations between the certain *GST* genotypes and RCC risk has been debated in a certain number of published literature (Abid et al., 2016; Cheng et al., 2012; Huang et al., 2015; Jia et al., 2014; Liu et al., 2012; Yang et al., 2013), the data on the prognostic value of GST polymorphism in patients with RCC are scarce (De Martino et al., 2010), probably due to the fact that the molecular mechanism supporting the role of GSTs in RCC progression has not been clarified as yet. Since GSTP1 was shown to be an endogenous inhibitor of JNK1, whereas GSTM1 negatively regulates ASK1, both might contribute to the lower tissue apoptotic activity. In the setting of the tumor tissue, the aforementioned interactions might correlate with high-graded tumors. So far, there are no data which would indicate the significance of this protein:protein interactions in human RCC in terms of tumor progression. What is more, it is still unclear whether the polymorphic expression of GSTs may influence the activity of apoptotic signal pathways in RCC progression.

## 2 THE OBJECTIVES

Due to the potential functional significance of common polymorphisms in genes encoding for cytosolic glutathione transferase A1, M1, T1 and P1 in both onset and prognosis of RCC, the aim of our investigation was:

**1.** To evaluate the role of *GSTA1 (*rs3957357*), GSTM1, GSTP1 (*rs1695*)* and *GSTT1* gene polymorphisms in susceptibility to development of renal cell carcinoma, as well as, to investigate if there is a combined effect of genotype and the recognized renal cell carcinoma risk factors (smoking, obesity, hypertension)

**2.** To evaluate whether the presence of *GSTA1, GSTM1, GSTP1* and *GSTT1* gene variants is associated with higher levels of byproducts of oxidative DNA damage (8-OHdG) and increased formation of BPDE-DNA adducts, as well as, clinical characteristics of the tumor (tumor stage, pT and grade, G)

**3.** To evaluate whether polymorphic expression of GST protein might have a prognostic role in patients with renal cell carcinoma

**4.** To evaluate the association between GSTM1 and GSTP1 expression and expression of regulatory (ASK1, JNK1/2) and executor (Caspase-3) apoptotic molecules in human ccRCC tissue samples, as well as, the presence of GSTM1:ASK1 and GSTP1:JNK1/2 protein:protein interactions

# 3 MATERIALS AND METHODS

## 3.1 Design

Case-control study

## 3.2 Study population

Incident RCC cases, treated and followed at the Clinic of Urology, Clinical Center of Serbia, Belgrade, between 2011 and 2014, were enrolled into this study. The case group comprised a total of 305 subjects (201 men, 104 women; average age 59.25±11.47 years) with histologically confirmed diagnosis of RCC according to the 2004 WHO classification of Tumors (Eble et al., 2006), modified by ISUP Vancouver Classification (Srigley et al., 2013) and 2009 TNM classification system (Sobin et al., 2010). Inclusion criteria for the RCC patients were:

- The presence of malignantly enhanced lesion confirmed by imaging (ultrasound/computed tomography/magnetic resonance imaging),
- Confirmed histological diagnosis (RCC type, nuclear grade, sarcomatoid features, vascular invasion, tumor necrosis and invasion of the collecting system and peri-renal fat)
- Patients with nephrectomy (partial or total)
- Male or female, age ≥ 18 years old
- Subject's willingness to provide written informed consent

Cases were excluded if they had a previous positive history of cancer.

The control group initially comprised 454 individuals (233 men, 221 women, average age 60.40±12.31 years) who had undergone surgery for benign conditions, unrelated to both non-malignant and malignant urological condition at the same clinical center. The initial control group was further matched to RCC patients according to gender and age, and finally included 326 individuals (209 men, 117 women; average age 60.75±11.52 years) with no previous personal history of cancer. Inclusion criteria for the controls were:

- The verified absence of malignantly enhanced lesions
- Male or female, age ≥ 18 years old
- Subject's willingness to provide written informed consent

Controls were excluded if they had a previous personal history of cancer.

Responce rate was 93% and the most common reason for no participation was personal.

The basic demographic data and recognized risk factors for RCC (smoking history, obesity and hypertension) were obtained from the study subjects using the structured questionnaire (Djukic et al., 2013) composed at the Institute of Epidemiology, Faculty of Medicine University in Belgrade, during the time of blood collection. In our study, obese patients were defined as individuals with BMI above 25kg/$m^2$ and smokers as individuals who reported every day smoking during a minimum of 60-day period prior to their enrollment in the study. Further on, participants were asked about the number of cigarettes smoked per day and duration of smoking. All collected data referred to a time period prior to the diagnosis of RCC for the cases, and a corresponding period for the controls.

### 3.3 Ethics

The study was approved by the Institutional Ethical board (October 13$^{th}$, 2011, approval number 29/X-3, Faculty of Medicine, University of Belgrade, Serbia) and was performed in accordance with principles of Helsinki declaration from 2013. Informed written consent was obtained from all recruited subjects.

### 3.4 Materials

#### 3.4.1 Blood and plasma samples

EDTA blood was collected from each patient treated at the Clinic of Urology, Clinical Center of Serbia, Belgrade. 400μl of the whole blood was taken for the purpose of the DNA isolation and the rest was centrifuged for 10 min at 3600 rpm/4°C for plasma separation. In order to prevent the auto-oxidation of the plasma sample, 10μl of butilated hydroxytoluen (10 mmol/L) was added per ml of plasma.

EDTA blood samples were stored at -20°C whereas plasma samples were kept at -80 °C at the Institute of Medical and Clinical biochemistry, Faculty of Medicine, University of Belgrade, Serbia.

### 3.4.2 Tissue samples

Tumor and respective non-tumor samples (n=20) were taken during partial or total nephrectomy from patient treated at the Clinic of Urology, Clinical Center of Serbia, Belgrade. All tissue samples had their RCC type, nuclear grade, sarcomatoid features, vascular invasion, tumor necrosis and invasion of the collecting system and peri-renal fat confirmed. The samples were stored in *RNAlater RNA Stabilization* reagent (*Qiagen, Chatsworth, California, USA*) at -20$^0$C and kept at the Institute of Medical and Clinical biochemistry, Faculty of Medicine, University of Belgrade, Serbia.

## 3.5 Methods

### 3.5.1 DNA isolation

Total DNA was isolated from 200μl of the whole peripheral blood and up to 25mg of non-tumor kidney tissue samples, using *QIAamp DNA Blood Mini Kit* (*Qiagen, Chatsworth CA, USA*) according to the manufacture's protocol. Namely, the *QIAamp DNA* purification procedure comprised 5 steps and was carried out using *QIAamp Mini spin columns* with a small chance of sample-to-sample cross-contamination. In the first step, optimized detergent buffers and enzyme *Proteinase K* (600 mAU/ml, 40 mAU/mg protein) were used to lyse samples and stabilize DNA. In the case of non-tumor kidney tissue samples, no mechanical homogenization was necessary, as the tissue samples were lysed enzymatically during prolonged incubation with *Proteinase K*. In the second step, DNA was adsorbed onto the *QIAamp silica membrane* during a brief centrifugation. The lysate buffering conditions are adjusted to allow optimal binding of the DNA to the *QIAamp* silica membrane. In the following two steps, the DNA bound to the *QIAamp* membrane was washed with *Buffer AW1* and *Buffer AW2* in 2 centrifugation without affecting DNA binding. The washing steps can be repeated as the residual contaminants can inhibit genotyping and other downstream enzymatic reactions. Purified DNA was eluted from the *QIAamp Mini spin* column in a concentrated form in *AE Buffer*. Isolated DNA, free of protein, nucleases and other contaminants or inhibitors, was stored at -20$^0$C for later use. DNA concentration and purity were determined spectrophotometrically at 230, 260, 280 i 320 nm using GeneQuant*pro* (*Biochrom, Cambridge, England*).

### 3.5.2 Genotyping

Genotyping was performed blinded to the case-control status and blinded quality control samples were inserted to validate genotyping identification procedures. Concordance for blinded samples was 100%. All assays performed contained positive and negative controls. All primers used are synthesized and bought from *Metabion International AG* (*Planegg, Germany*) (Table 5).

**Table 5.** The PCR genotyping conditions

| Gene | Primer sequences | PCR protocol | Gel electrophoresis results |
|---|---|---|---|
| ***GSTA1 C69T (rs3957357)*** | F, 5'- GCATCAGCTTGCCCTTCA -3',<br>R, 5'- AAACGCTGTCACCGTCCTG -3' | Denature: 94˚C for 4mins<br>Followed by 94˚C for 20s<br>Annealing: 58˚C for 20s<br>Extension: 72˚C for 40s<br>#cycles: 33<br>Final extension: 72˚C for 5 mins | *Eam1104I* incubation at 37˚C overnight<br>*GSTA1**CC- 400bp<br>*GSTA1**CT- 400bp, 308bp and 92bp<br>*GSTA1**TT- 308bp and 92bp |
| ***GSTM1*** | F, 5'-GAACTCCCTGAAAAGCTAAAGC-3',<br>R, 5'-GTTGGGCTCAAATATACGGTGG-3' | Multiplex PCR:<br>Denature: 94˚C for 4mins<br>Followed by 94˚C for 30s<br>Annealing: 59˚C for 30s<br>Extension: 72˚C for 45s<br>#cycles: 30<br>Final extension: 72˚C for 5mins | *GSTM1-active*: 215bp band<br>*GSTM1-null:* no band |
| ***GSTT1*** | F, 5'-TTCCTTACTGGTCCTCACATCTC-3',<br>R, 5'-TCACCGGATCATGGCCAGCA-3' | | *GSTT1-active*: 481bp band<br>*GSTT1-null-*: no band |
| ***CYP1A1*** | F, 5'-GAACTGCCACTT CAGCTGTCT-3'<br>R, 5'-CAGCTGCATTTG GAAGTGCTC-3' | | 312bp band |

#### 3.5.2.1 Genotyping of *GSTM1* and *GSTT1*

The DNA sequences of *GSTM1* and *GSTT1* were analyzed by multiplex polymerase chain reaction (PCR) in *Mastercycler gradient thermal cycler* (*Eppendorf, Hamburg, Germany*) according to the method by *Abdel-Rahman et al.* (Abdel-Rahman et al., 1996) (Table 5).

The multiplex PCR technique used to detect homozygous deletions of *GSTM1* and *GSTT1* included primers for *GSTM1*, *GSTT1* and *CYP1A1* housekeeping

gene, used as an internal control for amplifiable DNA (Table 5). Isolated DNA (~50ng) was amplified in a total volume of 25 μl reaction mixture containing 7.5 pmol of each primer, 12.5μl of *MasterMix (*0,05U/μL Taq DNK polymerase, 4 mmol $MgCl_2$ i 0,4 mmol of dNTP) and water (*Thermo Fisher Scientific, Waltham, Massachusetts, USA*). Amplified PCR products (GSTM1: 215 bp, GSTT1: 481 bp, CYP1A1: 312 bp) were electrophoresed (125V constant, 0.27A, 50W) on 2% agarose gel, stained with *SYBR® Safe DNA Gel Stain* (*Invitrogen Corporation, Carlsbad, CA, USA*) and visualized on *GL200 Camera* (*Gel Logic Imaging System, Kodak*) or on *Chemidoc (Biorad, Hercules, CA, USA*) (Figure 16).

Since the assay does not distinguish heterozygous or homozygous wild-type genotypes and therefore detects the presence (at least one allele present, homozygote or heterozygote) or the absence (complete deletion of both alleles, homozygote) of the genotype, the *active* genotype were detected according to presence of the particular band (*GSTM1-active*: 215 bp, *GSTT1-active:* 481 bp) and the absence of these bands was indicative of the *null* genotypes.

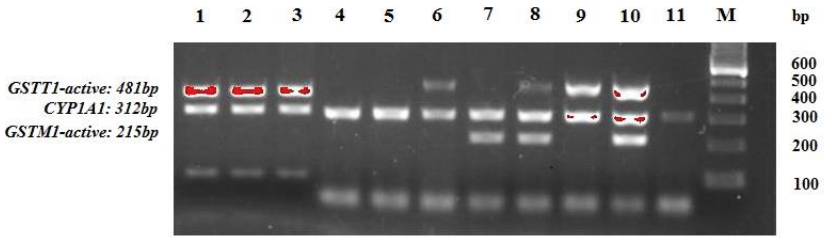



**Figure 16.** 2% agarose gel electrophoretogram: PCR products of the *GSTM1* and *GSTT1* genes. Lanes 8 and 10 comprise PCR products of patients with the *GSTT1-active/GSTM1-active* genotype (481 bp and 215 bp bands, respectively); lanes 1, 2, 3, 6 and 9 comprise PCR products of patients with the *GSTT1-active/GSTM1-null* genotype (481 bp bands); Lane 7 comprises PCR products of patients with *GSTT1-null/GSTM1-active* genotype (215bp bands); Lines 4, 5 and 11 indicate patients with *GSTT1-null/GSTM1-null* genotype; 312bp band represents the *CYP1A1* housekeeping gene, used as internal control for amplifiable DNA; M, DNA marker

### 3.5.2.2 The genotyping of *GSTA1**C69T (rs3957357)

The analysis of the SNP *GSTA1**C69T (rs3957357) was performed using PCR-restriction fragment length polymorphism (RFLP) according to the method by *Ping et al* (Table 5) (Ping et al., 2006). A 400 bp fragment was amplified in a total volume of 20μl reaction mixture containing 12.5pmol of each primer, 10μl of *MasterMix* and water (*Thermo Fisher*

*Scientific, Waltham, Massachusetts, USA*) and subjected to the PCR protocol indicated in the Table 5. For RFLP analysis, 5µl of PCR product was digested overnight at 37°C with 2U of restriction enzyme *EarI* and 1x*Tango Buffer* (*Thermo Fisher Scientific, Waltham, Massachusetts, USA*) in total volume of 15µl. DNAse free water was used as the negative control. Digested products (*GSTA1**CC: 400bp, *GSTA1**CT: 400bp + 308 pb + 92bp and *GSTA1**TT: 308bp+92 bp) were separated on 3% agarose gel (125V constant, 0.27A, 50W) and stained with *SYBR® Safe DNA Gel Stain (Invitrogen Corporation, Carlsbad, CA, USA*) and visualized on *GL200 Camera* (*Gel Logic Imaging System, Kodak*) or on *Chemidoc (Biorad, Hercules, CA, USA*) (Figure 17).

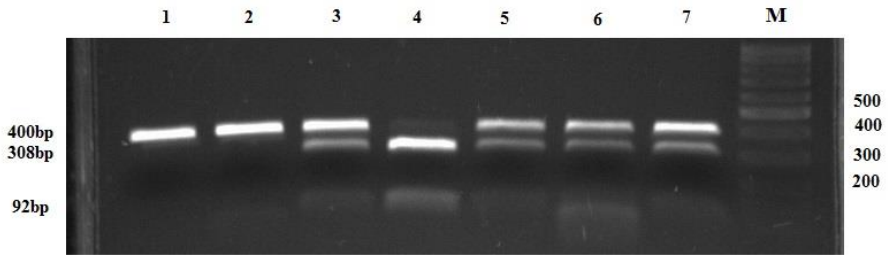



**Figure 17**. 3% agarose gel electrophoretogram: PCR-RFLP restriction products of the *GSTA1* gene. Lanes 1 and 2 comprise PCR products of patients with the *GSTA1**CC genotype (400 bp bands); lanes 3, 5, 6 and 7 comprise PCR-RFLP restriction products of patients with the *GSTA1**CT genotype (400bp, 308bp, 92bp bands); Lane 4 comprises RFLP-PCR restriction products of patients with *GSTA1**TT genotype (308bp, 92bp bands); M, DNA marker; N, negative control without a DNA content.

### 3.5.2.3 The genotyping of *GSTP1** Ile105Val (rs1695)

For analyses of SNP polymorphism *GSTP1**Ile105Val (transition substitution: A/G; context sequence [VIC/FAM]: CGTGGAGG ACCTCCGCTGCAA ATAC [A/G] TCTCCCTC ATCTACACCAACTATGT), a 5´ nuclease TaqMan® SNP Genotyping Assays (*Life Technologies, Applied Biosystems, Carlsbad, CA, USA, assay ID: C__3237198_20* ) was used for amplifying and detecting respective SNP alleles in purified genomic DNA samples according to the manufactures' instructions.

Reaction mixture, containing *MasterMix*, water (*Thermo Fisher Scientific, Waltham, Massachusetts, USA*) and fluorescence probes were pipetted by *EppMotion* automated liquid

handling system (*Eppendorf, Hamburg, Germany*). PCR amplification and the plotted fluorescence signal endpoint reading was performed on *Mastercycler ep realplex* (*Eppendorf, Hamburg, Germany*).

### 3.5.3 Determination of BPDE-DNA adducts and 8-OHdG levels by enzyme linked immunosorbent assays (ELISA) method

The level of benzo(a)pyrene diol epoxide DNA adducts (BPDE-DNA) was determined using the standard method *OxiSelect BPDE-DNA Adduct ELISA Kit (Cell Biolabs, Inc., San Diego, California, USA)* according to the manufactures' instructions. Namely, isolated DNA samples were diluted to a concentration of 2 μg/mL. BPDE-DNA standards and DNA samples were adsorbed onto a 96-well DNA high-binding plate. The BPDE-DNA adducts present in the sample or standard were probed with an *anti-BPDE-I antibody*, followed by the incubation with a horse radish peroxidase (HRP) conjugated secondary antibody. Sample/standard absorbance was read at 450/620nm wavelength on *LKB 5060-006 Micro Plate Reader (Vienna, Austria)*. The BPDE-DNA adduct content in samples was determined by comparing with a standard curve that was prepared from predetermined BPDE-DNA standards (Figure 18). The results were expressed as ng/ml of BPDE-DNA adducts.

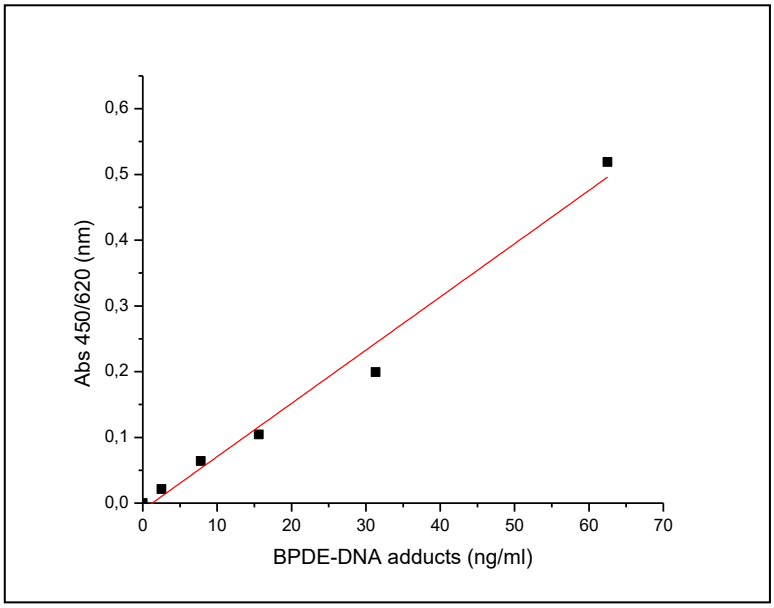



**Figure 18**. BPDE-DNA ELISA Standard Curve. Equation: y = a + b*x; y- Abs450/620 (nm); a-slope; b-intercept; x- BPDE-DNA adduct level in ng/ml; Adj. R Square: 0.9892

The quantitative measurement of 8-hydroxy-2'-deoxyguanosine (8-OHdG) was determined using the standard method *OxiSelect Oxidative DNA Damage ELISA* kit *(Cell Biolabs, Inc., San Diego, California, USA)*, according to the manufacture's protocol. Namely, 50µl of plasma samples or 8-OHdG standards were added to an 8-OHdG/BSA conjugate pre-absorbed microplate. After a brief incubation, an *anti-8-OHdG* monoclonal antibody was added, followed by an horse radish peroxidase (HRP) conjugated secondary antibody. Sample/standard absorbance was read at 450/620nm wavelength on *LKB 5060-006 Micro Plate Reader (Vienna, Austria)*. The 8-OHdG content in plasma samples was determined by comparison with predetermined 8-OHdG standard curve (Figure 19). The results were expressed as ng/ml of 8-OHdG.

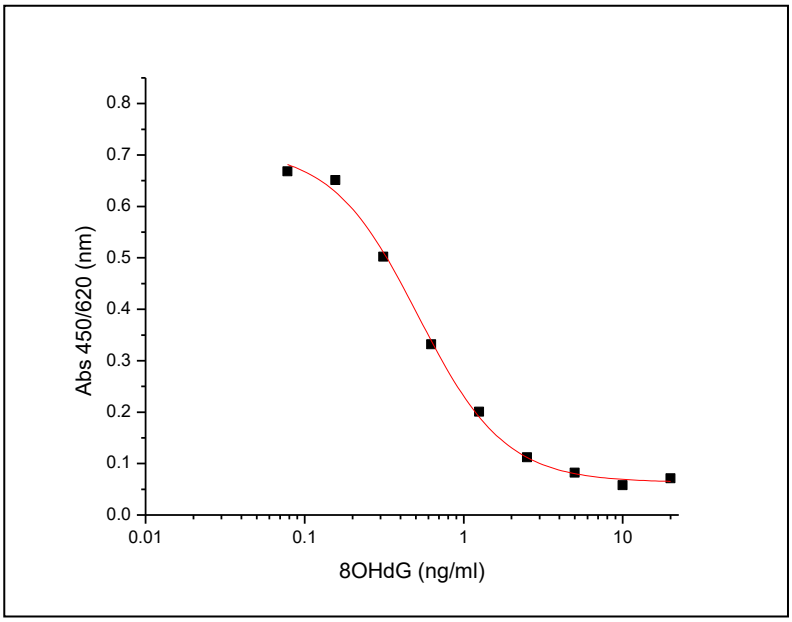



**Figure 19**. 8-OHdG ELISA Standard Curve. Equation: y = 0.7118 + (0.06325 - 0.7118) * x^1.5955 / (0.5166^1.5955 + x^1.5955), y- absorbance at 450/620, x-concentration of 8-OHdG; Adj. R-Square: 0.9936

### 3.5.4 Identification of GSTM1, GSTP1, ASK1, JNK1/2 and Cleaved Caspase-3 by the method of immunoblot

#### 3.5.4.1 Tissue sample preparation

ccRCC tumor and respective non-tumor kidney tissue samples (n=20) were homogenized in ice cold tissue lysis buffer (50mM Tris, 200mM NaCl, 1mM dithiothreitol, pH

7,8) containing protease and phosphatase inhibitors (*Sigma-Aldrich, St. Louis, Missouri, USA*). After homogenization, samples were centrifuged at 3000rpm/4°C for 10 min. Obtained supernatants were separated and centrifuged at 36100rpm/4°C for 60 min.

### 3.5.4.2 Pooling of non-tumor kidney tissue samples for Western blot analysis

In order to reduce biological variance, two separate pools of non-tumor kidney tissue samples were prepared according to the *GSTM1* genotype: (1) *GSTM1-active* pool, containing non-tumor kidney tissue samples of RCC patients carrying the *GSTM1-active* genotype and (2) *GSTM1-null* pool containing non-tumor kidney tissue sample of ccRCC patients carrying *GSTM1-null* genotype. The pools were prepared by mixing the equal parts of 6 different samples.

### 3.5.4.3 Protein quantification

Protein quantification was performed in obtained cytosols and pools using *Bicinchoninic Acid Protein Assay* kit (*BCA-1, Sigma-Aldrich, St. Louis, Missouri, USA*) on a 96-well plate. The assay is based on the formation of a $Cu^{+2}$-protein complex under alkaline conditions, followed by reduction of the $Cu^{+2}$ to $Cu^{+1}$. The amount of reduction is proportional to content of the protein present. Protein concentrations were calculated from a BSA protein standard curve (Figure 20) and expressed as μg/μl.

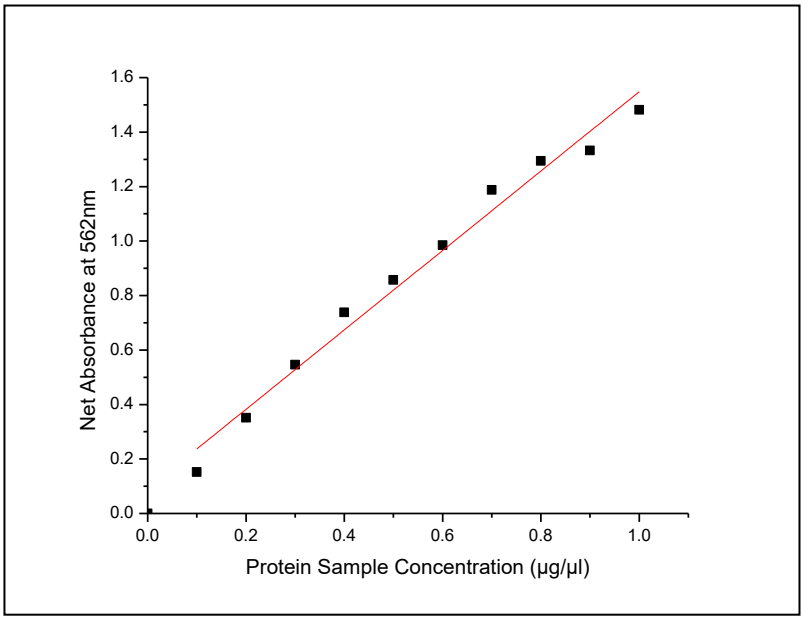



**Figure 20.** Standard curve of net absorbance versus protein sample concentration; Equation: y = a + b*x; y- net absorbance at 562nm; a-slope; b-intercept; x- protein sample concentration in μg/μl; Adj. R Square: 0.9879

### 3.5.4.4 Western blot analysis

Obtained ccRCC cytosols and pools of non-tumor kidney tissue samples were subjected to Sodium Dodecyl Sulfate-Polyacrilamide Gel Electrophoresis (SDS-PAGE) and immunoblot analysis for the identification of ASK1, JNK1/2, GSTM1, GSTP1 and Cleaved Caspase-3 according to the method of *Laemmli et al.* (Laemmli, 1970) and *Towbin et al* (Towbin et al., 1979).

Polyacrilamide gels were designed as percent solutions (Table 6) and made according to the given formulation from $dH_20$, 30% acrylamide mix (*Biorad, Hercules, CA, USA*), Tris Buffer, 10% Sodium Dodecyl Sulfate (SDS, *Merck Millipore, Darmstadt, Germany*), 10% amonium per-sulphate (APS, *Thermo Fisher Scientific, Waltham, Massachusetts, USA*) and tetramethylethylenediamine (TEMED*, Biorad, Hercules, CA, USA*), depending on the investigated protein molecular weight. In order to reduce and denaturate the proteins, the one part of a loading buffer, containing 2 x *Laemmeli* buffer (*Biorad, Hercules, CA, USA*) and dithiothreitol (DTT, *SERVA Electrophoresis GmbH, Heidelberg, Germany*) in a final concentration of 50mM, was mixed with the one part of the sample. The mixture was boiled at $95^0$C for 5 minutes. Finally, 50μg of total protein was loaded per SDS-PAGE gel. In order to determine the protein size and to monitor the progress of an electrophoresis run, the appropriate molecular weight marker was used (*PageRuler™ Prestained Protein Ladder, 10 to 180 kDa, Thermo Fisher Scientific, Waltham, Massachusetts, USA*)

Electrophoresis (200V constant, at 4°C) was performed using *Biorad Mini-PORTEAN Tetra Cell* (*Biorad, Hercules, CA, USA*) followed by transfer onto nitrocellulose membranes (100V constant, at 4°C) using *Biorad Criterion™ blotter system (Biorad, Hercules, CA, USA)*. Protein transfer was confirmed by *Ponceau* S staining (*Sigma-Aldrich, St. Louis, Missouri, USA*).

Membranes were blocked overnight with constant shaking at 4°C in 3% milk/1% BSA in Tris-buffered saline with 0.1% Tween20 (TTBS, *Sigma-Aldrich, St. Louis, Missouri, USA*). Primary antibodies against ASK1 ( *Santa Cruz, Dallas, Texas, USA*), JNK1/2 (*Sigma-Aldrich, St. Louis, Missouri, USA*), GSTM1 ( *Santa Cruz, Dallas, Texas, USA*), GSTP1 (*Abcam, Cambridge, UK*), Cleaved Caspase-3 (*Cell Signaling, Danvers, Massachusetts, USA*) and β-actin (*Sigma-Aldrich, St. Louis, Missouri, USA*) were diluted in 1% BSA in phosphate-buffered saline, added to the membranes and incubated for 3h at room temperature on orbital shaker. Afterwards,

membranes were washed and incubated with appropriate HRP conjugated secondary antibodies (1:2000, *Sigma-Aldrich, St. Louis, Missouri, USA*) for 1hr at room temperature. Finally, the membranes were treated with chemiluminescence detection substrate (*Invitrogen Corporation, Carlsbad, CA, USA*) for 1min and exposed to X-ray films (*Amesham Hyperfilm ECL, GE Healthcare, Buckinghamshire, England*).

Densitometry analysis of the scanned X-ray was performed using open source image processing program Image J (*National Institutes of Health, Bethesda, USA*).

**Table 6**. Electorphoresis and blot conditions according to the protein size and primary antibody type

| Protein | Size of the protein (kDa) | Gel percentage (%) | Type of primary antibody | Dilution of the primary antibody (R) | Type of the secondary antibody |
|---|---|---|---|---|---|
| ASK1 | 165 | 8% | monoclonal anti-mouse | 1:500 | anti-mouse developed in goat |
| JNK1/2 | 46/55 | 10% | monoclonal anti-rabbit | 1:1000 | anti-rabbit developed in goat |
| GSTM1 | 26 | 12% | polyclonal anti-rabbit | 1:500 | anti-rabbit developed in goat |
| GSTP1 | 23 | 12% | polyclonal anti-rabbit | 1:500 | anti-rabbit developed in goat |
| Cleaved Caspase-3 | 17/19 | 14% | monoclonal anti-rabbit | 1:1000 | anti-rabbit developed in goat |
| B-globin | 42 | 8-14% | monoclonal anti-mouse | 1:2000 | anti-mouse developed in goat |

### 3.5.5 Immunoprecipitation and identification of GSTM1-1:ASK1 and GSTP1-1:JNK protein:protein interactions by the method of immunoblot

Immunoprecipitation experiments were performed using Catch *and Release® v2.0 High Throughput (HT) Immunoprecipitation* Assay Kit-*96 well* (*Upstate Biotech Inc. for Merck Millipore, Darmstadt, Germany*) according to the manufactures' instructions. Namely, 96-well filter plate was pre-coated with *20% w/v slurry resign* and *Affinity Ligand*. Cytosols containing 1μg/μl of total cell proteins were incubated either with 2μg of the primary antibody against GSTM1 (rabbit, *Santa Cruz, Dallas, Texas, USA*) or GSTP1 (rabbit, *Abcam, Cambridge, UK*), followed by

repeating washing steps. Finally, samples were re-suspended in 30μl of *2xLamelly buffer* (*Biorad, Hercules, CA, USA*), heated at 90°C for five minutes and collected by centrifugation at 1500rpm for one minute. Supernatant fraction was further subjected to SDS-PAGE and *Western* blot analysis in oreder to investigate the existence of GSTM1:ASK1 or GSTP1:JNK1 protein:protein interactions, respectively, according to the previously described protocols (Laemmli, 1970; Towbin et al., 1979).

### 3.5.6 Statistical analysis

In this study, the data of continuous variables were expressed as mean ± standard deviation (SD) or median (minimum-maximum) whereas categorical variables were presented using frequency (n, %) counts. Distribution was tested by using *Kolmogorov–Smirnov*. Differences in investigated parameters were assessed by using *ANOVA* test for continuous data with normal distribution and *Mann–Whitney* rank-sum test for continuous data with non-normal distribution. Finally, $\chi 2$ test was used for categorical variables. *Hardy-Weinberg* equilibrium was tested for each polymorphism calculating $\chi 2$ test for the patients and the controls separately.

The genetic variants and their risk for disease were computed by odds ratios (OR) and 95% confidence intervals (CI) by logistic regression analysis. OR was adjusted by variables indicating recognized risk factors for RCC, as potential confounders, as well as by age and gender.

The effect of four *GST* genotypes on overall survival, defined as time from nephrectomy to the date of death or last follow-up (November 1$^{st}$ 2015.) was evaluated in overall group of RCC patients, as well as ccRCC patients. The follow-up data were available in 285 patients with RCC and 219 patients with ccRCC. The loss of 20 ccRCC patients' occurred due to the loss of their contact information. Median follow-up was 43 (1-125) months.

Survival analysis was performed using the *Kaplan-Meier* method to estimate the cumulative survival probability. The long-rank test was performed for the assessment of differences in survival according to the different categories of variables.

The predictive value of different *GST* genotypes in overall mortality were assessed by the *Cox* proportional hazard regression models, adjusted by covariates in three different models: Model 1 was adjusted to age and gender, Model 2 to the covariates from Model 1 and recognized risk factors for RCC development (pack-years for smoking, obesity, hypertension)

and Model 3 to the covariates from Model 2 and clinical characteristics of tumor (tumor type, grade G, pT stage). Calculations were performed using the SPSS software version 17.0 (*Chicago, IL, USA*). P value of ≤0.05 will considered to be statistically significant.

The association between GSTM1-1 and ASK1 with Cleaved Caspase-3 expression was analyzed using *Pearson's* or *Spearman's* coefficient of linear correlation.

# 4 RESULTS

## 4.1 *GST* polymorphism in patients with renal cell carcinoma (RCC)

Selected baseline characteristics of 305 patients with RCC and 326 respective controls are shown in Table 7. As indicated, no statistical difference was found regarding age, gender and obesity (p>0.05) between RCC patients and controls. However, hypertensive subjects exhibited almost 3-fold increased risk for RCC in comparison with normotensive subjects (OR=2.86, 95%CI: 1.65-4.93, p<0.001). Moreover, our results have shown that smoking alone was associated with an increased risk of RCC (OR=1.50, 95%CI: 1.02-2.20, p=0.037).

**Table 7**. Baseline characteristic of 305 RCC patients and 326 age and gender matched controls

| | RCC patients | Controls | OR (95%CI) | p-value |
|---|---|---|---|---|
| **Age** (years)[a] | 59.25 ± 11.47 | 60.75±11.52 | / | 0.104 |
| **Gender**, n (%) | | | | |
| Male | 201 (66) | 209 (64) | 1.00[i] | |
| Female | 104 (34) | 117(36) | 0.90 (0.49-1.64)[b] | 0.736 |
| **Hypertension**, n (%)[h] | | | | |
| No | 113 (44) | 216 (71) | 1.00[i] | |
| Yes | 142 (56) | 86 (29) | 2.86 (1.65-4.93)[c] | <0.001 |
| **Obesity**, n (%)[h] | | | | |
| BMI < 25 | 87 (35) | 109 (37) | 1.00[i] | |
| BMI > 25 | 163 (65) | 188 (63) | 0.88 (0.52-1.48)[d] | 0.633 |
| **BMI (**kg/m$^2$**)**[a] | 26.75±4.29 | 26.45±3.78 | / | 0.391 |
| **Smoking**, n (%)[h] | | | | |
| Never | 107 (41) | 156 (49) | 1.00[i] | |
| Ever[e] | 153 (59) | 162 (51) | 1.50 (1.02-2.20)[f] | 0.037 |
| **Pack-years**[g] | 32 (1.35-145.0) | 30 (1.00-120.00) | / | 0.125 |

[a]Mean ±SD; [b] OR, odds ratio adjusted to age, pack-years, BMI (body mass index), hypertension; [c]OR, odds ratio adjusted to age, gender, pack-years, BMI (body mass index); [d]OR, odds ratio adjusted to age,gender, pack-years, hypertension; [e]minimum of 60-day period any time prior to the study onset; [f]OR, odds ratio adjusted to age, gender, BMI, hypertension; [g]Median (Min-Max); [h]Based on the data available; [i]Reference group. CI, confidence interval;

As presented in Table 8, the high majority of cases suffered from clear cell RCC (78%) and the tumor grade II (G2, 53%), while stages pT1 and pT3 (42% and 44%, respectively) were shown to be the most frequent.

**Table 8.** Clinical characteristics of patients' tumor

| **Tumor type** | **RCC patients, n (%)** |
|---|---|
| Clear cell RCC | 239 (78) |
| Papillary RCC | 39 (13) |
| Chromophobe RCC | 19 (6) |
| Collecting Duct RCC | 5 (2) |
| Other | 3 (1) |
| **Tumor grade**, n (%)[a] | |
| Grade I, G1 | 33 (13) |
| Grade II, G2 | 139 (53) |
| Grade III, G3 | 77 (30) |
| Grade IV, G4 | 11 (4) |
| **pT stage**, n (%)[b] | |
| pT1 | 120 (42) |
| pT2 | 35 (12) |
| pT3 | 127 (44) |
| pT4 | 7 (3) |

[a,b] Data available data on patients' tumor grade and pT stage, depending on the type of surgery and pathohistology diagnostics;

### 4.1.1 The association of *GST* genotypes with RCC risk

As shown in Table 9, higher frequency of *GSTM1-null* carriers was observed in RCC patients (55%) with an adjusted OR of 1.85 (95%CI: 1.10-3.09, p=0.020), indicating a significant association of the *GSTM1-null* genotype with RCC risk. However, *GSTT1-active* and *GSTA1-CC* (*active*) genotypes did not seem to enhance the risk for RCC (OR=0.99, 95%CI: 0.55-1.77, p=0.977 and OR=1.05, 95%CI: 0.61-1.79, p=0.865, respectively). By contrast, *GSTP1 IleVal+ValVal* (*variant)* genotype was notably over-represented among cases (75%) compared to controls (57%). Moreover, the risk of RCC was elevated (OR=3.86, 95%CI: 2.11-7.05, p<0.001) among carriers of the *GSTP1 IleVal+ValVal* (*variant)* genotype compared to the carriers of the *GSTP IleIle (wild-type*) genoype.

**Table 9.** *GST* genotypes in relation to the risk of RCC

| ***GST* genotype** | **RCC patients n, %** | **Controls n, %** | **OR (95%CI)[e]** | **p- value** |
|---|---|---|---|---|
| ***GSTM1*** | | | | |
| *active*[a] | 136 (45) | 163 (50) | 1.00[f] | |
| *null*[b] | 169 (55) | 163 (50) | 1.85 (1.10-3.09) | 0.020 |
| ***GSTT1*** | | | | |
| *null*[b] | 79 (26) | 89 (27) | 1.00[f] | |
| *active*[a] | 226 (74) | 237 (73) | 0.99 (0.55-1.77) | 0.977 |
| ***GSTA1* (rs 3957357)** | | | | |
| *CC (active)* | 94 (31) | 134 (41) | 1.00[f] | |
| *CT+TT (low activity)*[c] | 211 (69) | 192(59) | 1.05 (0.61-1.79) | 0.865 |
| ***GSTP1* (rs1695)** | | | | |
| *IleIle (wild-type)* | 74 (25) | 141 (43) | 1.00[f] | |
| *IleVal+ValVal (variant)*[d] | 227 (75) | 185 (57) | 3.86 (2.11-7.05) | <0.001 |

[a]*Active*, if at least one active allele present; [b]*Null* if no active alleles present; [c]*Low activity*, if at least one *T* allele present. [d]*Variant*, if at least one *Val* allele present; [e]OR, odds ratio adjusted to age, gender, pack years, BMI, hypertension; CI, confidence interval; [f]Reference group; Deletion *GSTM1* and *GSTT1* genotypes were investigated in 305 cases and all recruited controls. SNP polymorphism *GSTA1**C69T and *GSTP1**Ile105Val were analyzed in 305 and 301 RCC cases, respectively, and all recruited controls.

In order to test the effect of gene-gene interaction for all four *GST* genotypes, both crude and adjusted odds ratio was calculated (Table 10). Interestingly, a significant association, in terms of RCC risk, was found when the combined effect of *GSTM1* and *GSTP1* genotypes was assessed alone (*GSTM1-active/GSTP1 wild-type vs. GSTM1-null/GST-variant*: adjusted OR=9.41, 95%CI: 3.40-26.04, p<0.001) or together with other *GST* genotypes (*GSTM1-active/GSTA1-active/GSTP1 wild-type vs. GSTM1-null/GSTA1-low activity/GSTP1-variant*, adjusted OR=14.28, 95%CI: 2.82-72.54, p=0.001 or *GSTM1-active/GSTT1-null/GSTP1 wild-type vs. GSTM1-null/GSTT1-active/GSTP1-variant*, adjusted OR=18.10, 95%CI: 1.75-186.66, p=0.015). Ultimately, when all four *GST* genotypes were analyzed, the number of RCC patients, carrying presumably "the risk-associated genotype" combination *GSTM1-null/GSTT1-active/GSTA1-low activity/GSTP1-variant* (62 out of 305), prevailed over the number of RCC patients with *GSTM1-active/GSTT1-null/GSTA1-active/GSTP1wild type* genotypes (1 out of 305). According to our results, patients with "the risk-associated genotype" combination were at 15-fold higher risk for RCC development (crude OR=15.12, 95%CI: 1.86-122.64, p=0.011; OR adjusted to age and gender 15.70, 95%CI: 1.91-128.58, p=0.010) compared to the reference genotype.

**Table 10.** Combined effect of *GST* genotypes on risk of RCC

| Combined Genotype | RCC patients, n % | Controls, n% | Crude OR (95%CI) | p-value | Adjusted OR (95%CI) | p-value |
|---|---|---|---|---|---|---|
| ***GSTM1/GSTP1 genotype*** | | | | | | |
| *GSTM1-active*[a]*/GSTP1-wild type* | 29 (19) | 70 (43) | 1.00 [e] | | 1.00 [e] | |
| *GSTM-null*[b]*/GSTP1-variant*[c] | 122 (81) | 92 (57) | 3.20 (1.92-5.33) | <0.001 | 9.41 (3.40-26.04) [f] | <0.001 |
| ***GSTM1/GSTA1/GSTP1 genotype*** | | | | | | |
| *GSTM1-active*[a]*/GSTA1-active/GSTP1-wild type* | 11 (11) | 36 (38) | 1.00 [e] | | 1.00 [e] | |
| *GSTM-null*[b]*/GSTA1-low activity*[d]*/GSTP1-variant*[c] | 87 (89) | 59 (62) | 4.82 (2.27-10.23) | <0.001 | 14.28 (2.81-72.54) | 0.001 |
| ***GSTM1/GSTT1/GSTP1 genotype*** | | | | | | |
| *GSTM1-active*[a]*/GSTT1-null*[b]*/GSTP1-wild type* | 3 (3) | 16 (19) | 1.00 [e] | | 1.00 [e] | |
| *GSTM-null*[b]*/GSTT1-active/GSTP1-variant*[c] | 90 (97) | 67 (81) | 7.16 (2.00-25.38) | 0.002 | 18.10 (1.75-186.6) | 0.015 |
| ***GSTM1/GSTT1/GSTA1/GSTP1 genotype*** | | | | | | |
| *GSTM1-active*[a]*/GSTT1-null*[b]*/GSTA1-active/ GSTP1-wild type* | 1 (2) | 10 (19) | 1.00 [e] | | 1.00[g] | |
| *GSTM-null*[b]*/GSTT1-active*[a]*/GSTA1-low activity*[d]*/ GSTP1-variant*[c] | 62 (98) | 41 (81) | 15.12 (1.86-122.6) | 0.011 | 15.70 (1.91-128.5)[g] | 0.010 |

[a]*Active*, if at least one active allele present; [b]*Null*, if no active alleles present; [c]*Variant*, if at least one *Val* allele present; [d]*Low activity*, if at least one *T* allele present; [e]Reference group; [f]OR odds ratio adjusted to age, gender, pack years, BMI, hypertension; [g]OR odds ratio adjusted to age and gender; CI, confidence interval;

Finally, we decided to test the cumulative effect of *GST* risk-associated genotypes (*GSTM1-null, GSTT1-active, GSTA1-low activity* and *GSTP1-variant*) on the risk of RCC development. As indicated in the Table 11, a trend in OR was observed (OR=4.52, 95%CI: 0.54-37.63, p=0.162 in patients with one risk-associated genotype, OR=8.64, 95%CI: 1.08-68.92, p=0.042 in RCC patients with 2 risk-associated genotypes, OR=10.70, 95%CI: 1.91-128.58, p=0.025 in patients with 3 risk-associated genotypes), with the highest OR present in RCC patients with "the risk-associated genotype" combination: *GSTM1-null/GSTT1-active/GSTA1-low activity/GSTP1-variant* (OR=15.70, 95%CI: 1.91-128.58, p=0.010).

**Table 11**. Cumulative effect of *GST* risk-associated genotypes on the risk of RCC development

| Number of risk-associated *GST* genotypes | RCC patients n, % | Controls n, % | OR (95%CI)[a] | p- value |
|---|---|---|---|---|
| 0 | 1 (1) | 10 (3) | 1.00[b] | |
| 1 | 22 (7) | 50 (15) | 4.52 (0.54-37.63) | 0.162 |
| 2 | 94 (31) | 112 (34) | 8.64 (1.08-68.92) | 0.042 |
| 3 | 122 (41) | 113 (35) | 10.70 (1.34-85.18) | 0.025 |
| 4 | 62 (21) | 41 (13) | 15.70 (1.91-128.58) | 0.010 |

*0:* Reference genotype combination (*GSTM1-active/GSTT1-null/GSTA1 active/GSTP1-wild type)*; 1, 2, 3, 4: 1, 2, 3, 4: The number of the present risk-carrying genotypes: either one of each risk-carrying, or two of each risk-carrying, or three of each risk-carrying or all four risk-carrying GST genotypes (*GSTM1-null* or *GSTT1-active* or *GSTA1 low-activity* or *GSTP1-variant*); [a]OR odds ratio adjusted to age, gender; CI, confidence interval; [b]Reference group;

### 4.1.2 The association between *GST* genotypes and the levels of 8-OHdG in RCC patients

Since oxidative stress might be implicated in the development of RCC, we determined the levels of 8-OHdG (ng/ml) in RCC patients, stratified by the presence or absence of the recognized risk factors for RCC development and analyzed them with respect to various *GST* genotypes (Tables 12-14). Although the obtained values were slightly higher in RCC patients with „risk-associated genotypes" compared to the values observed in RCC patients carrying genotypes not associated to RCC risk, the results did not reach the statistical significance (p>0.05).

**Table 12**. The association between *GST* genotypes and the levels of 8-OHdG (ng/ml) in RCC patients stratified according to the smoking status

| Genotype | | Smoking | 8-OHdG (ng/ml) [e] |
|---|---|---|---|
| ***GSTM1*** | *GSTM1-active*[a] | No | 1.21 (0.49-3.72) |
| | *GSTM1-null*[b] | Yes[f] | 1.32 (0.52-4.92) |
| | | p-value | 0.811 |
| ***GSTT1*** | *GSTT1-null*[b] | No | 1.36 (0.77-3.72) |
| | *GSTT1-active*[a] | Yes[f] | 1.32 (0.52-3.14) |
| | | p-value | 0.983 |
| ***GSTA1*** **(rs 3957357)** | *GSTA1-active* | No | 1.35 (0.39-4.92) |
| | *GSTA1-low activity*[c] | Yes[f] | 1.36 (0.49-6.52) |
| | | p-value | 0.892 |
| ***GSTP1 (rs1695)*** | *GSTP1-wild type* | No | 1.21 (0.69-2.24) |
| | *GSTP1-variant*[d] | Yes[f] | 1.24 (0.39-3.98) |
| | | p-value | 0.794 |

[a]*Active*, if at least one active allele present; [b]*Null* if no active alleles present; [c]*Low activity*, if at least one *T* allele present. [d]*Variant*, if at least one *Val* allele present; [e]Mean (Min-Max) values; [f]minimum of 60-day of smoking period any time prior to the study onset

**Table 13.** The association between *GST* genotypes and the levels of 8-OHdG (ng/ml) in RCC patients stratified according to the BMI category

| Genotype | | Obesity | 8-OHdG (ng/ml) [e] |
|---|---|---|---|
| ***GSTM1*** | *GSTM1-active*[a] | No | 1.21 (0.49-2.51) |
| | *GSTM1-null*[b] | Yes[f] | 1.28 (0.52-2.81) |
| | | p-value | 0.962 |
| ***GSTT1*** | *GSTT1-null*[b] | No | 1.14 (0.91-4.92) |
| | *GSTT1-active*[a] | Yes[f] | 1.24 (0.52-3.14) |
| | | p-value | 0.984 |
| ***GSTA1*** **(rs 3957357)** | *GSTA1-active* | No | 1.14 (0.49-6.51) |
| | *GSTA1-low activity*[c] | Yes[f] | 1.28 (0.39-7.29) |
| | | p-value | 0.345 |
| ***GSTP1*** ***(rs1695)*** | *GSTP1-wild type* | No | 1.21 (0.62-4.92) |
| | *GSTP1-variant*[d] | Yes[f] | 1.24 (0.39-7.29) |
| | | p-value | 0.982 |

[a]*Active*, if at least one active allele present; [b]*Null* if no active alleles present; [c]*Low activity*, if at least one *T* allele present. [d]*Variant*, if at least one *Val* allele present; [e]Mean (Min-Max) values; [f]Body mass index above 25kg/m$^2$

**Table 14.** The association between *GST* genotypes and the levels of 8-OHdG (ng/ml) in RCC patients stratified according to the level of blood pressure

| Genotype | | Hypertension | 8-OHdG (ng/ml)[e] |
|---|---|---|---|
| ***GSTM1*** | *GSTM1-active*[a] | No | 1.21 (0.49-3.98) |
| | *GSTM1-null*[b] | Yes | 1.36 (0.52-2.81) |
| | | p-value | 0.999 |
| ***GSTT1*** | *GSTT1-null*[b] | No | 1.43 (0.79-4.92) |
| | *GSTT1-active*[a] | Yes | 1.44 (0.52-3.14) |
| | | p-value | 0.498 |
| ***GSTA1*** **(rs 3957357)** | *GSTA1-active* | No | 1.08 (0.49-1.69) |
| | *GSTA1-low activity*[c] | Yes | 1.24 (0.39-7.29) |
| | | p-value | 0.342 |
| ***GSTP1*** ***(rs1695)*** | *GSTP1-wild type* | No | 0.94 (0.62-4.92) |
| | *GSTP1-variant*[d] | Yes | 1.14 (0.39-7.29) |
| | | p-value | 0.425 |

[a]*Active*, if at least one active allele present; [b]*Null* if no active alleles present; [c]*Low activity*, if at least one *T* allele present. [d]*Variant*, if at least one *Val* allele present; [e]Mean (Min-Max) values

### 4.1.3 The association between *GST* genotypes and grade and stages of RCC

In order to discern whether the *GST* polymorphisms might affect the RCC tumor progression, we assessed the association between *GST* polymorphisms and grade and stages of RCC included in the study (Figures 21-24). The results concerning the *GSTM1-null* genotype showed significant difference with respect to tumor grade (p=0.016). Namely, *GSTM1-null* genotype was the most frequent in grade II tumors (G2, Figure 21A). Unfortunately, there was no statistical difference found for *GSTT1*, *GSTA1* and *GSTP1* genotypes (Figure 21B and Figure 22).

Regarding the tumor stage, no association was observed between any of the analysed *GST* genotypes and RCC stage (Figure 23 and Figure 24, p>0.05).

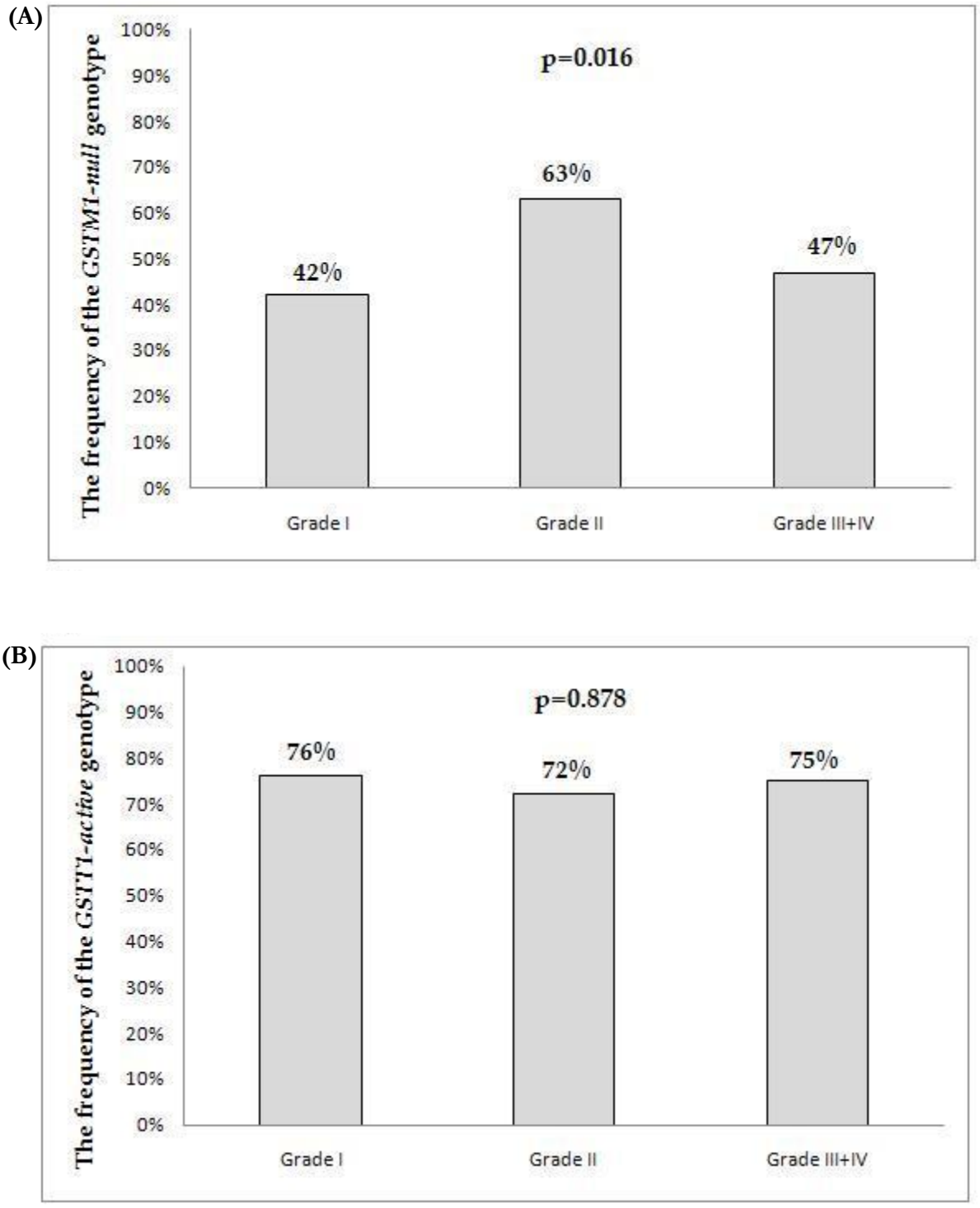



**Figure 21**. The frequency of *GST* deletional risk-associated genotypes in RCC patients stratified according to tumor grade: (A) *GSTM1-null* genotype; (B) *GSTT1-active* genotype

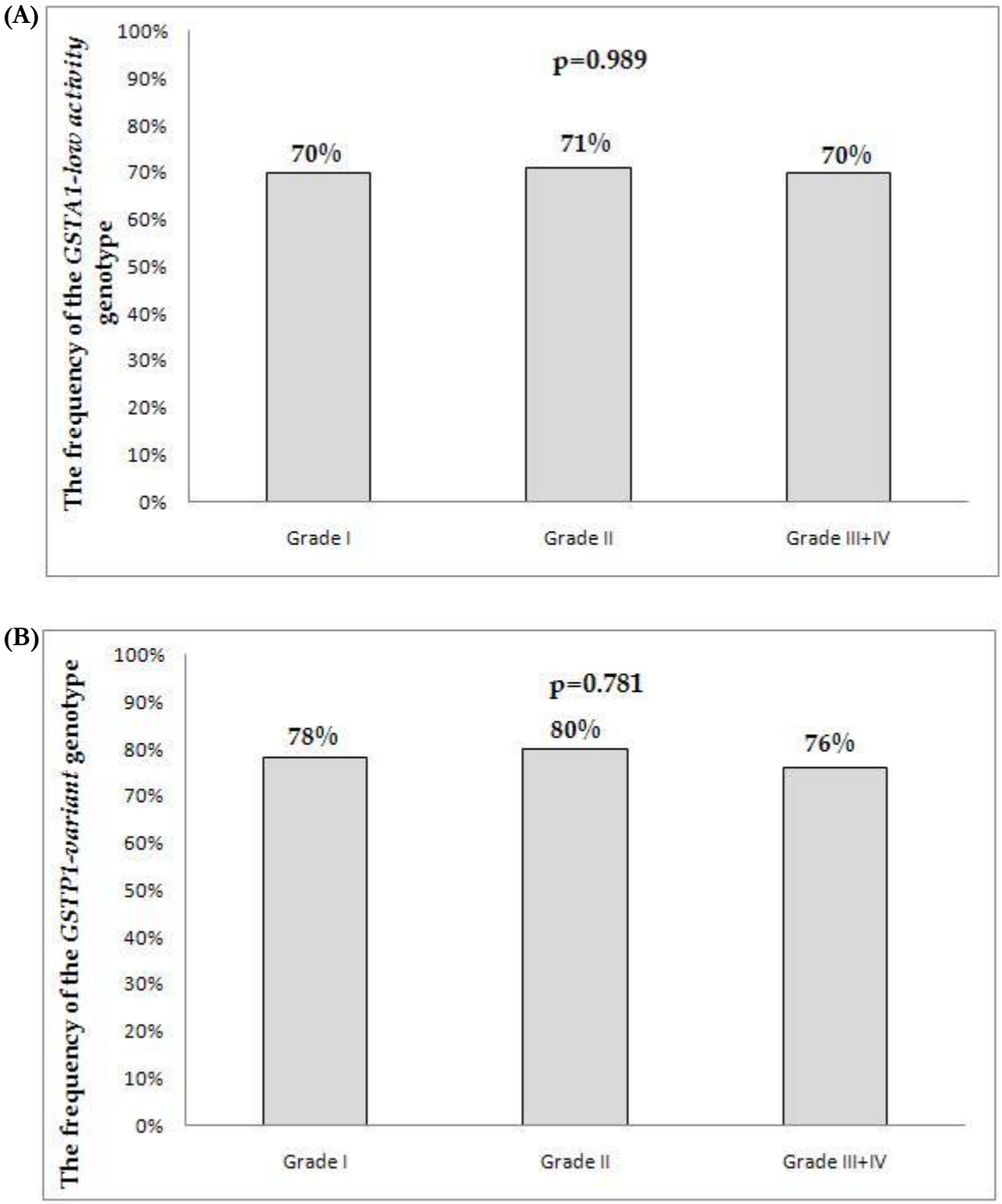



**Figure 22**. The frequency of *GST* SNP risk-associated genotypes in RCC patients stratified according to tumor grade: (A) *GSTA1-low activity* genotype; (B) *GSTP1-variant* genotype

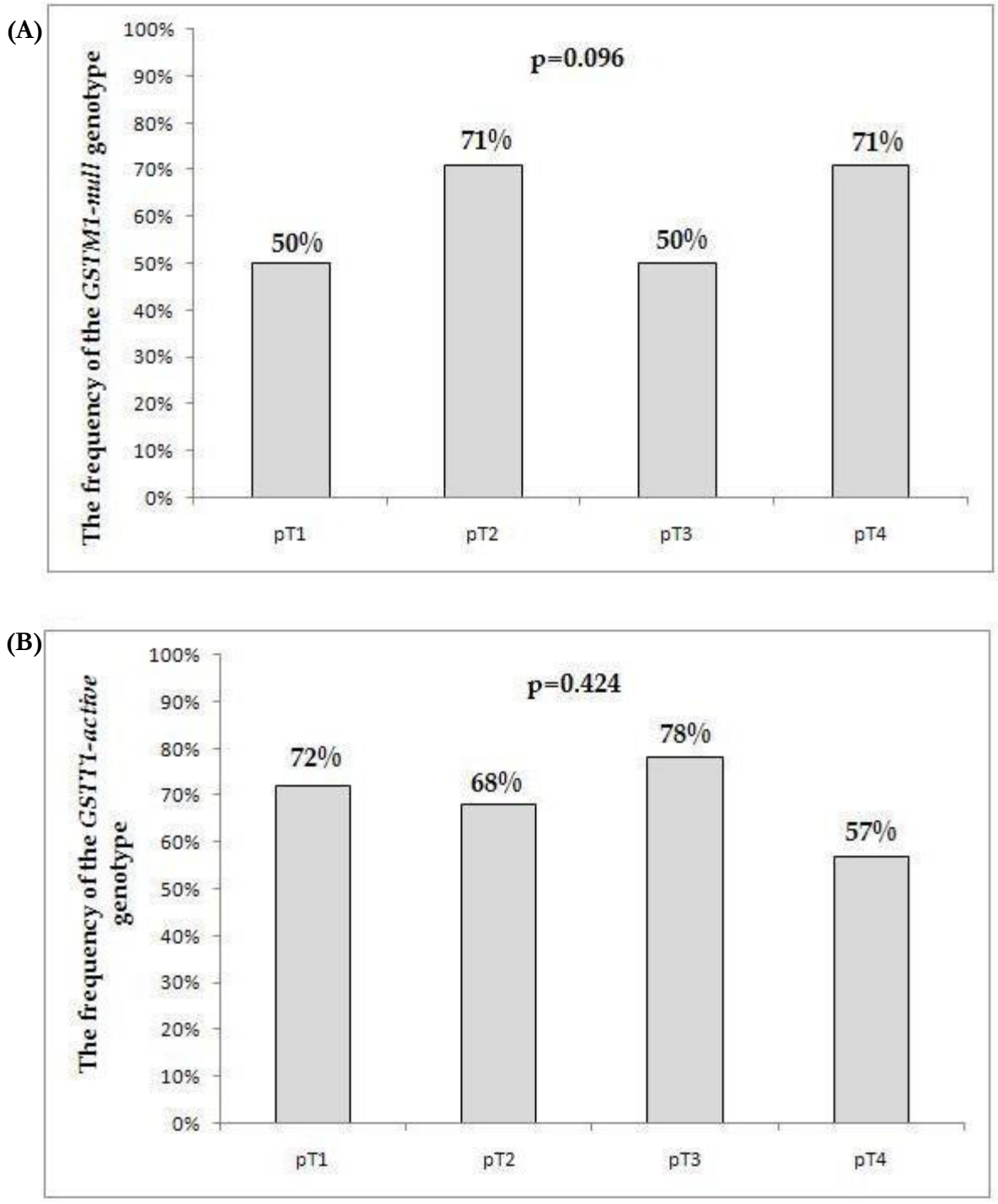



**Figure 23**. The frequency of *GST* deletional risk-associated genotypes in RCC patients stratified according to tumor stage: (A) *GSTM1-null* genotype; (B) *GSTT1-active* genotype

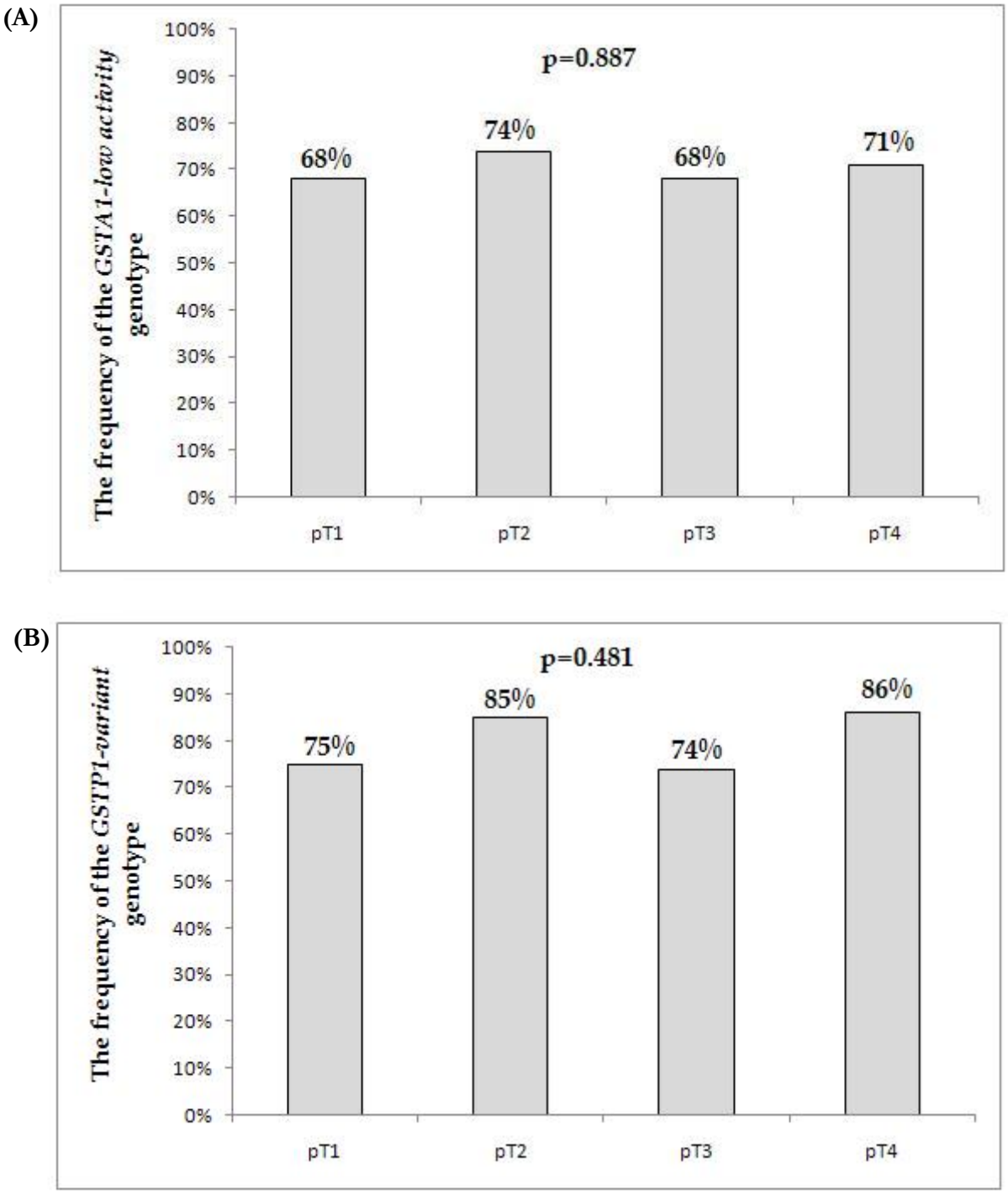



**Figure 24**. The frequency of *GST* SNP risk-associated genotypes in RCC patients stratified according to tumor stage: (A) *GSTA1-low activity* genotype; (B) *GSTP1-variant* genotype

### 4.1.4 The effect of *GST* genotypes on overall survival in RCC patients

In the group of 285 patients with RCC included in the follow-up, there were 85 (30%) deaths during the mean follow-up of 46.64±28.13 months (ranging from 1-125) months.

In Table 15, the frequencies of *GST* genotypes in living and deceased RCC patients are shown. Significantly higher frequency was observed only for the *GSTM1-null* genotype in living RCC patients (60%) in comparison with the frequency of *GSTM1-null* genotype in deceased RCC patients (45%, p=0.024). However, the obtain frequencies of other analyzed *GST* genotypes were not statistically significant (p>0.05).

*Kaplan-Meier* survival analysis indicated shorter overall survival (Log Rank: p=0.021) only for the patients with *GSTM1-active* genotype, compared to carriers of *GSTM1-null* genotype (Figure 25). On the other hand, *Kaplan-Meier* analysis did not demonstrate a significantly shorter time towards the death in patients carrying any other analyzed *GST* genotype (p>0.05, Figure 26-28).

**Table 15**. The frequency of the *GST* genotypes stratified according to the follow-up status

| ***GST* genotype** | **Living patients n, %** | **Deceased patients, n %** | **p-value** |
|---|---|---|---|
| ***GSTM1*** | | | |
| *active*[a] | 80 (40) | 45 (55) | |
| *null*[b] | 119 (60) | 37 (45) | 0.024 |
| ***GSTT1*** | | | |
| *null*[b] | 53 (27) | 20 (25) | |
| *active*[a] | 146 (73) | 62 (75) | 0.697 |
| ***GSTA1*** (rs 3957357) | | | |
| *CC (active)* | 67 (33) | 25 (30) | |
| *CT+TT (low activity)*[c] | 132 (67) | 57 (70) | 0.606 |
| ***GSTP1*** (rs1695) | | | |
| *IleIle (wild-type)* | 53 (27) | 17 (21) | |
| *IleVal+ValVal (variant)*[d] | 143 (73) | 65 (79) | 0.269 |

[a]*Active*, if at least one active allele present; [b]*Null* if no active alleles present; [c]*Low activity*, if at least one *T* allele present.
[d]*Variant*, if at least one *Val* allele present; Number of diseased patients n=85

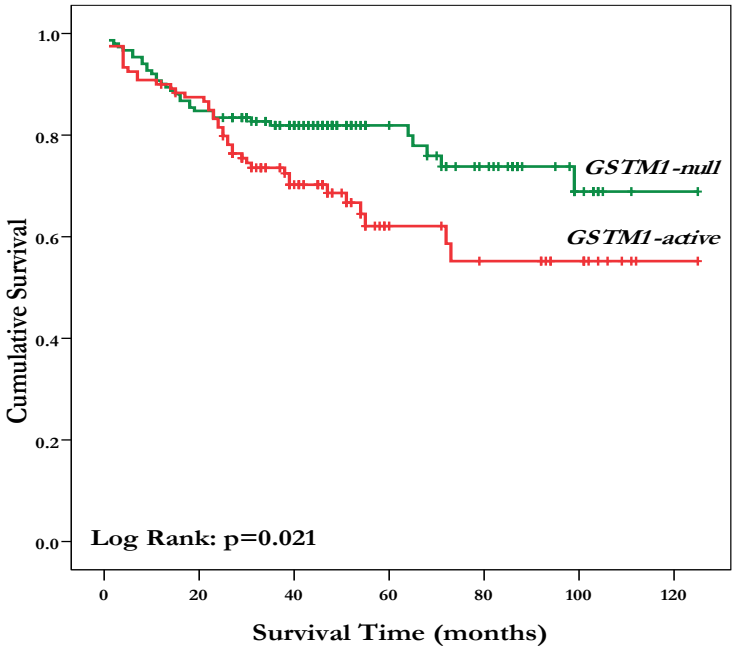



**Figure 25.** *Kaplan-Meier* Survival Curves for overall mortality according to *GSTM1* polymorphism; *Active*, if at least one active allele present; *Null* if no active alleles present

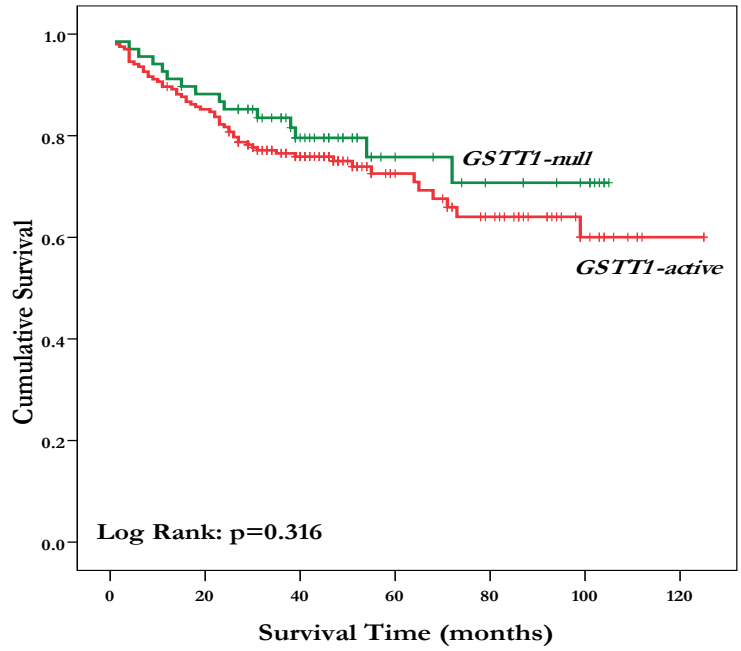



**Figure 26.** *Kaplan-Meier* Survival Curves for overall mortality according to *GSTT1* polymorphism; *Active*, if at least one active allele present; *Null* if no active alleles present

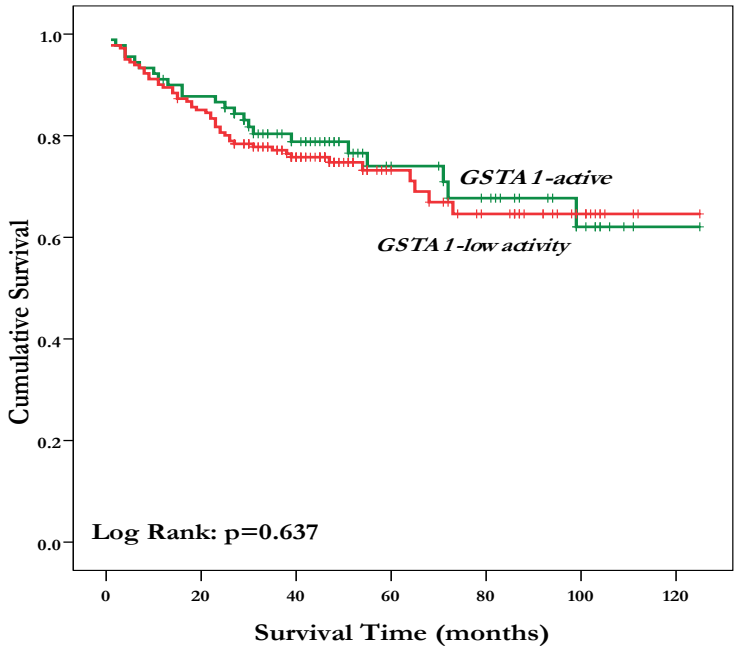



**Figure 27.** *Kaplan-Meier* Survival Curves for overall mortality according to *GSTA1* polymorphism; *Low activity*, if at least one *T* allele present;

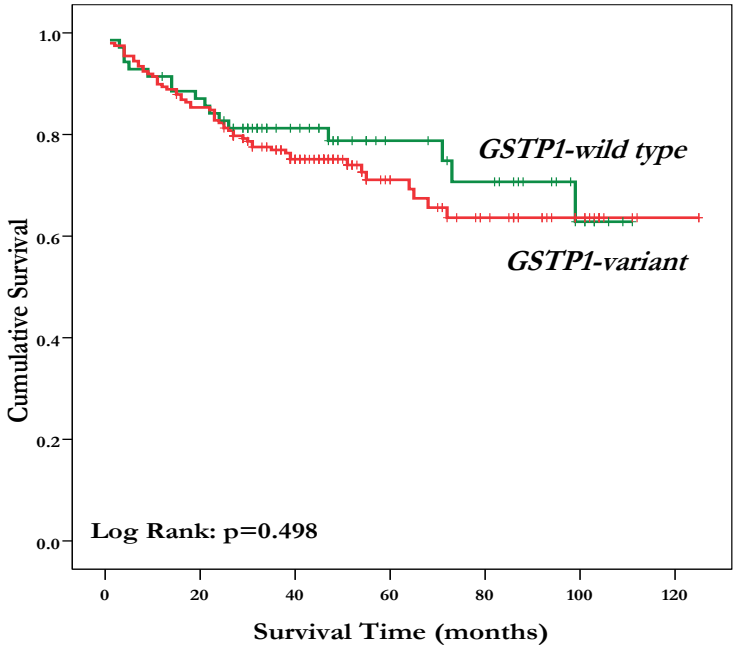



**Figure 28.** *Kaplan-Meier* Survival Curves for overall mortality according to *GSTP1* polymorphism; *Variant*, if at least one *Val* allele present

Tables 16-18 summarize the associations between different *GST* genotypes and overall mortality, adjusted by covariates in three different models. According to our results of multivariate *Cox* analysis, the *GSTM1-active* genotype was confirmed to be an independent predictor of higher risk of overall mortality in RCC patients. Namely, this genotype had a significant multivariable adjusted HR in all three models: in Model 1 HR=1.60 (95%CI: 1.00-2.56, p=0.047, Table 16), in Model 2 HR=3.45 (95%CI: 1.61-7.42, p=0.001, Table 17) and in Model 3 HR=3.09 (95%CI: 1.19-8.02, p=0.020, Table 18). A similar approach was used for analyzing the predictive value of other *GST* genotypes, yet only *GSTP1-variant* demonstrated HR of 1.68 in Model 2 (95%CI: 0.55-5.14, p=0.359, Table 17) and 1.70 in Model 3 (95%CI: 0.38-7.41; p=0.488, Table 18), without reaching statistical significance.

**Table 16**. *GST* polymorphisms as predictors for overall mortality in RCC patients, Model 1

| **Model 1** | |
|---|---|
| HR (95% CI) | p-value |
| **Risk for overall mortality comparing *GSTM1-active*[a] genotype to *GSTM1-null*[b] genotype carriers** | |
| 1.60 (1.00-2.56) | 0.047 |
| **Risk for overall mortality comparing *GSTT1-active*[a] genotype to *GSTT1-null*[b] genotype carriers** | |
| 1.10 (0.62-1.97) | 0.741 |
| **Risk for overall mortality comparing *GSTA1-low activity*[c] genotype to *GSTA1-active* genotype carriers** | |
| 1.18 (0.72-1.95) | 0.504 |
| **Risk for overall mortality comparing *GSTP1-variant*[d] genotype to *GSTP1-wild type* genotype carriers** | |
| 1.23 (0.71-2.13) | 0.447 |

Model 1 adjusted to age and gender; [a]*Active*, if at least one active allele present;[b]*Null*, if no active alleles present; [c]*Low activity*, if at least one *T* allele present; [d]*Variant*, if at least one *Val* allele present; HR, hazard ratio; CI, confidence interval

**Table 17**. *GST* polymorphisms as predictors for overall mortality in RCC patients, Model 2

| **Model 2** | |
|---|---|
| HR (95% CI) | p-value |
| **Risk for overall mortality comparing *GSTM1-active*[a] genotype to *GSTM1-null*[b] genotype carriers** | |
| 3.45 (1.61-7.42) | 0.001 |
| **Risk for overall mortality comparing *GSTT1-active*[a] genotype to *GSTT1-null*[b] genotype carriers** | |
| 0.75 (0.33-1.73) | 0.511 |
| **Risk for overall mortality comparing *GSTA1-low activity*[c] genotype to *GSTA1-active* genotype carriers** | |
| 1.03 (0.47-2.22) | 0.940 |
| **Risk for overall mortality comparing *GSTP1-variant*[d] genotype to *GSTP1-wild type* genotype carriers** | |
| 1.68 (0.55-5.14) | 0.359 |

Model 2 adjusted to the covariates from Model 1 and recognized risk factors for RCC development (packyears, BMI, hypertension); [a]*Active*, if at least one active allele present; [b]*Null*, if no active alleles present; [c]*Low activity*, if at least one *T* allele present; [d]*Variant*, if at least one *Val* allele present; HR, hazard ratio; CI, confidence interval

**Table 18**. *GST* polymorphisms as predictors for overall mortality in RCC patients, Model 3

| **Model 3** | |
|---|---|
| HR (95% CI) | p-value |
| **Risk for overall mortality comparing *GSTM1-active*[a] genotype to *GSTM1-null*[b] genotype carriers** | |
| 3.09 (1.19-8.02) | 0.020 |
| **Risk for overall mortality comparing *GSTT1-active*[a] genotype to *GSTT1-null*[b] genotype carriers** | |
| 0.97 (0.39-2.43) | 0.961 |
| **Risk for overall mortality comparing *GSTA1-low activity*[c] genotype to *GSTA1-active* genotype carriers** | |
| 0.91 (0.38-2.16) | 0.839 |
| **Risk for overall mortality comparing *GSTP1-variant*[d] genotype to *GSTP1-wild type* genotype carriers** | |
| 1.70 (0.37-7.38) | 0.488 |

Model 3 adjusted to the covariates from Model 2 and clinical characteristics of tumor (tumor type, grade, pT stage); [a]*Active*, if at least one active allele present;[b]*Null*, if no active alleles present; [c]*Low activity*, if at least one *T* allele present; [d]*Variant*, if at least one *Val* allele present; HR, hazard ratio; CI, confidence interval

### 4.2 *GST* polymorphism in patients with clear cell renal cell carcinoma (ccRCC)

Since clear renal cell carcinoma (ccRCC) represents approximately 80% of all RCC and seems to be the most aggressive RCC subtype, we further focused on the group of ccRCC patients that were included in this study.

Baseline characteristics of patients with ccRCC and respective controls are shown in Table 19. As shown, ccRCC patients and controls did not differ in terms of age, gender, obesity and smoking ($p>0.05$). However, more than half of the cases (52%) suffered from hypertension compared to controls (26%).

**Table 19.** Baseline characteristic of 199 patients with ccRCC and 274 age and gender matched controls

| Variable | ccRCC patients | Controls | OR (95%CI) | p-value |
|---|---|---|---|---|
| **Age** (years)[a] | 58.09 ± 11.51 | 59.77±10.96 | / | 0.113 |
| **Gender**, n (%) | | | | |
| Male | 133 (67) | 184 (67) | 1.00 (reference group) | |
| Female | 66 (33) | 90 (33) | 1.01 (0.68-1.49) | 0.942 |
| **Hypertension**, n (%) | | | | |
| No | 75 (48) | 191 (74) | 1.00 (reference group) | |
| Yes | 82 (52) | 67 (26) | 3.07 (1.61-5.84)[b] | 0.001 |
| **Obesity**, n (%) | | | | |
| BMI below 25 | 63 (40) | 92 (38) | 1.00 (reference group) | |
| BMI above 25 | 96 (60) | 153 (62) | 0.80 (0.43-1.46)[c] | 0.478 |
| **BMI (**kg/m$^2$**)**[a] | 26.41±4.49 | 26.36±3.49 | / | 0.893 |
| **Smoking**, n (%) | | | | |
| Never | 67 (41) | 126 (48) | 1.00 (reference group) | |
| Ever[d] | 97 (59) | 138 (52) | 1.44 (0.90-2.29)[e] | 0.125 |
| **Pack-years**[f] | 30 (1.35-141.00) | 30 (0.10-88.00) | / | 0.242 |
| **Fuhrmangrade**, n (%) | | | | |
| Grade I, G1 | 21 (13) | / | / | |
| Grade II, G2 | 89 (55) | / | / | |
| Grade III, G3 | 48 (29) | / | / | |
| Grade IV, G4 | 5 (3) | / | / | |
| **pTstage**, n (%) | | | | |
| pT1 | 78 (42) | / | / | |
| pT2 | 23 (13) | / | / | |
| pT3 | 78 (42) | / | / | |
| pT4 | 5 (3) | / | / | |

[a]Mean ±SD; [b]OR, odds ratio adjusted to age, gender, pack-years, BMI (body mass index); [c]OR, odds ratio adjusted to age, gender, pack-years, hypertension; [d] Every day smoking during a minimum of 60-day period prior to the study onset; [e]OR, odds ratio adjusted to age, gender, BMI, hypertension; CI, confidence interval; [f]Median (Min-Max).

### 4.2.1 The association of *GST* genotypes with ccRCC risk

The frequency of *GSTM1-null* genotype was higher (56%) in ccRCC patients than in controls (50%) and individuals with *GSTM1-null* genotype were at 2.07-fold higher risk of ccRCC development (95%CI: 1.11-3.84, p=0.021) (Table 20). On the other hand, no significant association with ccRCC risk was found for *GSTT1-active* genotype (OR=1.08, 95%CI: 0.52-2.27, p=0.822). Regarding SNP polymorphisms, the obtained results showed the lack of the *GSTA1**C69T polymorphism effect on ccRCC risk, since the carriers of *GSTA1**CT+TT *(low activity)* genotype were not at increased risk of ccRCC in comparison with individuals with *GSTA1**CC (*active)* genotype (OR=1.19, 95%CI: 0.63-2.25, p=0.580). Interestingly, carriers of *GSTP1**IleVal+ValVal (*variant)* genotype (77% of ccRCC patients compared to 58% of controls) were at 3.14-fold increased risk of ccRCC (95%CI: 1.54-6.43, p<0.001) (Table 20).

**Table 20.** *GST* genotypes in relation to the risk of ccRCC

| ***GST* genotype** | **ccRCC patients n, %** | **Controls n, %** | **OR (95%CI)**[e] | **p- value** |
|---|---|---|---|---|
| ***GSTM1*** | | | | |
| *Active*[a] | 87 (44) | 137 (50) | 1.00 (reference group) | |
| *Null*[b] | 109 (56) | 137 (50) | 2.07 (1.11-3.84) | 0.021 |
| ***GSTT1*** | | | | |
| *Null*[b] | 44 (22) | 71 (26) | 1.00 (reference group) | |
| *Active*[a] | 152 (78) | 203 (74) | 1.08 (0.52-2.27) | 0.822 |
| ***GSTA1* (rs 3957357)** | | | | |
| *CC* | 65 (33) | 112 (41) | 1.00 (reference group) | |
| *CT+TT (low activity)*[c] | 132 (67) | 162 (59) | 1.19 (0.63-2.25) | 0.580 |
| ***GSTP1* (rs1695)** | | | | |
| *IleIle (wild-type)* | 44 (23) | 115 (42) | 1.00 (reference group) | |
| *IleVal+ValVal (variant)*[d] | 150 (77) | 159 (58) | 3.14 (1.54-6.43) | <0.001 |

[a]*Active*, if at least one active allele present; [b]*Null* if no active alleles present; [c]*Low activity*, if at least one *T* allele present. [d]*Variant*, if at least one *Val* allele present; [e]OR, odds ratio adjusted to age, gender, pack-years, BMI, hypertension; CI, confidence interval

Combined effect on ccRCC risk was tested for all genotypes (Table 21). No significant association, in terms of ccRCC risk, was established when the combined effect of any two of *GSTM1, GSTA1* and *GSTT1* genotypes were assessed (p>0.05).

**Table 21**. Combined effect of *GST* genotypes on risk of ccRCC

| ***GST* genotypes** | ***GSTM1*** | | ***GSTA1*** | | ***GSTP1*** | |
|---|---|---|---|---|---|---|
| | *Active*[a] | *Null*[b] | *Active*[a] | *Low-activity*[c] | *Wild-type* | *Variant*[d] |
| ***GSTA1* (rs3957357)** | | | | | | |
| *Active*[a] | | | | | | |
| Ca (%)/Co(%) | 29(15)/54(20) | 35(18)/58(21) | - | - | - | - |
| OR (95%CI)[e] | 1.00[f] | 2.14 (0.67-6.86) | - | | - | |
| *p-value* | - | 0.192 | - | - | - | - |
| *Low-activity*[c] | | | | | | |
| Ca/Co | 58(30)/83(30) | 73(37)/79(29) | - | - | - | |
| OR (95%CI)[e] | 1.03 (0.41-2.61) | 2.00 (0.79-5.07) | - | - | - | - |
| *p-value* | 0.941 | 0.145 | - | - | - | - |
| ***GSTP1* (rs1695)** | | | | | | |
| *Wild-type* | | | | | | |
| Ca (%)/Co(%) | 17(9)/55(20) | 27(14)/60(22) | 17(9)/54(20) | 26(13)/61(22) | - | |
| OR (95%CI)[e] | 1.00[f] | 5.63 (1.11-28.53) | 1.00[f] | 3.40 (0.72-16.00) | - | - |
| *p-value* | - | 0.032 | - | 0.127 | - | - |
| *Variant*[d] | | | | | | |
| Ca (%)/Co(%) | 67(35)/82(30) | 81(42)/77(28) | 46(24)/58(21) | 103 (54)/101(36) | - | - |
| OR (95%CI)[e] | 4.40 (1.17-16.46) | 11.23 (2.62-48.08) | 5.39 (1.42-20.39) | 4.93 (1.48-16.43) | - | - |
| *p-value* | 0.026 | <0.001 | 0.015 | 0.009 | - | - |
| ***GSTT1*** | | | | | | |
| *Active*[a] | | | | | | |
| Ca (%)/Co(%) | 67(34)/102(37) | 85(44)/101(36) | 53(27)/82(30) | 99(51)/121(44) | 37(19)/85(31) | 113(59)/118(43) |
| OR (95%CI)[e] | 1.57 (0.49-5.01) | 2.78 (0.80-9.64) | 3.32 (0.72-15.22) | 2.62(0.65-10.58) | 2.47 (0.37-10.37) | 4.10 (0.95-17.35) |
| *p-value* | 0.435 | 0.103 | 0.128 | 0.175 | 0.348 | 0.052 |
| *Null*[b] | | | | | | |
| Ca (%)/Co(%) | 20(10)/35(13) | 24(12)/36(14) | 11(6)/30(11) | 32(16)/41(15) | 7(4)/30(11) | 35(18)/41(15) |
| OR (95%CI)[e] | 1.00[f] | 3.36 (0.77-14.66) | 1.00[f] | 3.57 (0.72-17.72) | 1.00[f] | 4.47 (0.72-27.43) |
| *p-value* | - | 0.101 | - | 0.113 | - | 0.105 |

[a]*Active*, if at least one active allele present. [b]*Null*, if no active alleles present. [c]*Low activity*, if at least one *T* allele present. [d]*Variant*, if at least one *Val* allele present; [e]OR, odds ratio adjusted to age, gender, pack-years, BMI, hypertension; CI, confidence interval; Ca, number of ccRCC patients; Co, controls; [f]Reference group

On the other hand, a statistically significant association between *GST* polymorphism and susceptibility to ccRCC was found when the effect of *GSTP1-variant* genotype was analyzed in combination with the other three genotypes (*GSTP1-variant* and *GSTM1-null* OR=11.23, 95%CI: 2.62-48.08, p<0.001; *GSTP1-variant* and *GSTA1low-activity* OR=4.93, 95%CI: 1.48-16.43, p=0.009; *GSTP1-variant* and *GSTT1-active* OR=4.10, 95%CI: 0.95-17.35, p=0.052).

The cumulative effect of the suggested "risk-associated" *GST* genotypes in RCC was analyzed in patients with ccRCC, in comparison with the reference genotype combination (Table 22). Indeed, the obtained results were similar to results in the RCC group and showed a trend in OR that was statistically significant only in the case of the previously termed "risk-associated genotype combination" of all four *GST* genotypes. Namely, we found striking evidence in favor of increased susceptibility to ccRCC in carriers of combined *GSTM1-null, GSTT1-active, GSTA1 low-activity* and *GSTP1-variant* genotype. It is important to note that such individuals comprised 22% of all recruited ccRCC patients, in comparison with 12% of control subjects, and exhibited 9.32-fold elevated risk compared to carriers of combined *GSTM1-active, GSTT1-null, GSTA1-active and GSTP1 wild-type* genotypes (95%CI: 1.08-80.18, p=0.041, Table 22).

**Table 22.** Cumulative effect of *GST* risk-associated genotypes on the risk of ccRCC development

| Number of "risk-associated *GST* genotypes" | RCC patients n, % | Controls n, % | OR (95%CI)[a] | p- value |
|---|---|---|---|---|
| 0 | 1 (1) | 7 (3) | 1.00[b] | |
| 1 | 11 (6) | 42 (15) | 1.79 (0.19-16.18) | 0.605 |
| 2 | 57 (30) | 90 (33) | 4.47 (0.53-37.51) | 0.167 |
| 3 | 79 (41) | 101 (37) | 5.21 (0.62-43.48) | 0.127 |
| 4 | 43 (22) | 34 (12) | 9.32 (1.08-80.18) | 0.041 |

*0:*Reference genotype combination (*GSTM1-active/GSTT1-null/GSTA1 active/GSTP1-wild type)*; 1, 2, 3, 4: The number of "risk-associated genotypes": either *GSTM1-null,GSTT1-actie,GSTA1low-activity or GSTP1-variant;* [a]OR odds ratio adjusted to age, gender; [b]Reference group; CI, confidence interval;

### 4.2.2 Modifying effect on risk of ccRCC conferred by recognized risk factors for ccRCC development

Results on the modifying effect on risk of ccRCC conferred by recognized risk factors for ccRCC development are shown in Tables 23-25. As presented, significant modifying effect on risk of ccRCC conferred by hypertension had individuals with either *GSTM1-null, GSTT1-active, GSTA1 low-activity* or *GSTP1-variant* genotype. Namely, they were at increased risk of ccRCC when compared

to the normotensive *GSTM1-active, GSTT1-null, GSTA1-active* or *GSTP1 wild-type* individuals (OR=6.01, 95%CI: 2.48–14.52, p<0.001; OR=3.27, 95%CI: 1.20–8.91, p=0.020; OR=3.85, 95%CI: 1.54–9.58, p=0.004 and OR=8.29, 95%CI: 3.12–22.04, p<0.001 respectively, Table 23). Another factor that contributed significantly to risk of ccRCC, only in carriers of *GSTP1-variant* genotype, was smoking (OR=3.70, 95%CI: 1.75-7.83, p=0.001, Table 25).

**Table 23.** Modifying effect on risk of ccRCC development conferred by hypertension

| Genotype vs Hypertension | | ccRCC patients n, % | Controls n, % | OR (95%CI)[e] | p-value |
|---|---|---|---|---|---|
| ***GSTM1*** | *Active*[a]*/Normotensive* | 37 (44) | 98 (72) | 1.00[f] | |
| | *Null*[b]*/ Hypertensive* | 47 (56) | 37 (28) | 6.01 (2.48-14.52) | <0.001 |
| ***GSTT1*** | *Null*[b]*/Normotensive* | 57 (75) | 141 (90) | 1.00[f] | |
| | *Active*[a]*/ Hypertensive* | | 14 (10) | 3.27 (1.20-8.91) | 0.020 |
| ***GSTA1 (rs3957357)*** | *Active*[a]*/Normotensive* | 27 (33) | 72 (66) | 1.00[f] | |
| | *Low activity*[c]*/ Hypertensive* | 56 (67) | 36 (34) | 3.85 (1.54-9.58) | 0.004 |
| ***GSTP1 (rs1695)*** | *Wild-type/Normotensive* | 19 (22) | 79 (68) | 1.00[f] | |
| | *Variant*[d]*/ Hypertensive* | 69 (78) | 36 (32) | 8.29 (3.12-22.04) | <0.001 |

[a]*Active*, if at least one active allele present; [b]*Null*, if no active alleles present; [c]*Low activity*, if at least one *T* allele present; [d]*Variant*, if at least one *Val* allele present; [e]OR, odds ratio adjusted to age, gender, pack-years, BMI; CI, confidence interval; [f]Reference group

**Table 24.** Modifying effect on risk of ccRCC development conferred by obesity

| Genotype vs Obesity | | ccRCC patients n, % | Controls n, % | OR (95%CI)[e] | p-value |
|---|---|---|---|---|---|
| ***GSTM1*** | *Active*[a]*/Non-obese* | 25 (35) | 50 (37) | 1.00[f] | |
| | *Null*[b]*/ Obese* | 47 (65) | 84 (63) | 1.78 (0.71-4.5) | 0.212 |
| ***GSTT1*** | *Null*[b]*/Non- obese* | 45 (38) | 69 (38) | 1.00[f] | |
| | *Active*[a]*/ Obese* | 74 (62) | 115 (62) | 0.83 (0.26-2.26) | 0.759 |
| ***GSTA1 (rs3957357)*** | *Active*[a]*/Non-obese* | 19 (23) | 48 (32) | 1.00[f] | |
| | *Low activity*[c]*/Obese* | 62 (77) | 103 (68) | 1.39 (0.54-3.78) | 0.518 |
| ***GSTP1 (rs1695)*** | *Wild-type/Non-obese* | 10 (12) | 35 (28) | 1.00[f] | |
| | *Variant*[d]*/Obese* | 74 (88) | 89 (72) | 2.21 (0.79-6.13) | 0.125 |

[a]*Active*, if at least one active allele present; [b]*Null*, if no active alleles present; [c]*Low activity*, if at least one *T* allele present; [d]*Variant*, if at least one *Val* allele present; [e]OR, odds ratio adjusted to age, gender, pack-years, hypertension; CI, confidence interval; [f]Reference group

**Table 25.** Modifying effect on risk of ccRCC development conferred by smoking

| Genotype vs Smoking status | | ccRCC patients n, % | Controls n, % | OR (95%CI)[e] | p-value |
|---|---|---|---|---|---|
| ***GSTM1*** | *Active[a]/Non-smoker* | 37 (29) | 50 (47) | 1.00[f] | |
| | *Null[b]/Smoker* | 57 (71) | 58 (53) | 1.39 (0.63-2.34) | 0.553 |
| ***GSTT1*** | *Null[b]/Non-smoker* | 15 (17) | 36 (25) | 1.00[f] | |
| | *Active[a]/Smoker* | 71 (83) | 108 (75) | 1.75 (0.82-3.75) | 0.144 |
| ***GSTA1 (rs3957357)*** | *Active[a]/Non-smoker* | 25 (27) | 61 (40) | 1.00[f] | |
| | *Low-activity[c]/Smoker* | 67 (73) | 92 (60) | 1.65 (0.87-3.13) | 0.122 |
| ***GSTP1 (rs1695)*** | *Wild-type/Non-smoker* | 16 (17) | 57 (42) | 1.00[f] | |
| | *Variant[d]/Smoker* | 77 (83) | 85 (58) | 3.70 (1.75-7.83) | 0.001 |

[a]*Active*, if at least one active allele present; [b]*Null*, if no active alleles present; [c]*Low activity*, if at least one *T* allele present; [d]*Variant*, if at least one *Val* allele present; [e]OR, odds ratio adjusted to age, gender, BMI, hypertension; CI, confidence interval; [f]Reference group

### 4.2.3 *GST* genotypes and ccRCC risk in smokers

Further on, we focused on the population of smokers with ccRCC (Table 26 and Table 27). Significant association between *GST* genotype and the risk of ccRCC in smokers was found only for the *GSTP1* genotype. Namely, smokers with *GSTP1-variant* genotype were at 2.87-fold higher risk of developing ccRCC (OR=2.87, 95%CI: 1.45-5.69, p=0.002, Table 26) than smokers carrying *GSTP1-wild type* genotype. Although *GSTM1-null* genotype did not, at least independently, significantly affect the risk of ccRCC in smokers (OR=1.71, 95%CI: 0.95-3.06, p=0.072, Table 27), when present in combination with *GSTP1-variant* genotype, it contributed in a way that smokers with *GSTM1-null/GSTP1-variant* genotype exhibited 5.4–fold increased risk of ccRCC (95%CI: 1.74-16.98, p=0.004, Table 27) in comparison with carriers of *GSTM1-active/GSTP1-wild type* genotype. In this manner, we confirmed the results obtained on the whole study population. The observed effect of combined *GSTM1-null/GSTP1-variant* genotype on ccRCC risk was even more pronounced when *GSTA1 low-activity* genotype was included. Namely, *GSTM1-null/GSTP1-variant/GSTA1-low activity* combination of genotypes, which was present in 94% of smokers with ccRCC, as opposed to 70% in controls, increased the risk of ccRCC up to 7.57 (95%CI: 1.26-45.30, p=0.026, Table 27).

**Table 26**. The association between individual *GST* genotypes and the levels of BPDE-DNA adducts in ccRCC smokers

| ***GST* genotypes in smokers** | **ccRCC smokers n,%** | **Controls smokers n,%** | **OR (95%CI)[e]** | **p-value** | **BPDE-DNA adducts in ccRCC smokers (ng/ml)[g]** | **p-value** |
|---|---|---|---|---|---|---|
| ***GSTM1*** | | | | | | |
| *Active*[a] | 37 (39) | 80 (58) | 1.00[f] | | 2.13 (1.39-5.22) | |
| *Null*[b] | 57 (61) | 58 (42) | 1.71 (0.95-3.06) | 0.072 | 2.74 (1.64-17.93) | 0.050 |
| ***GSTT1*** | | | | | | |
| *Null*[b] | 23 (24) | 30 (22) | 1.00[f] | | 2.81 (1.51-8.55) | |
| *Active*[b] | 71 (76) | 108 (78) | 1.07 (0.53-2.15) | 0.849 | 2.37 (1.39-17.93) | 0.324 |
| ***GSTA1* (rs3957357)** | | | | | | |
| *Active* | 23 (25) | 46 (33) | 1.00[f] | | 2.50 (1.39-8.55) | |
| *Low-activity*[c] | 67 (75) | 92 (67) | 1.21 (0.65-2.25) | 0.537 | 2.50 (1.39-17.93) | 0.612 |
| ***GSTP1* (rs1695)** | | | | | | |
| *Wild-type* | 16 (17) | 53 (38) | 1.00[f] | | 2.50 (1.76-6.95) | |
| *Variant*[d] | 77 (83) | 85 (62) | 2.87 (1.45-5.69) | 0.002 | 2.44 (1.39-17.39) | 0.655 |

[a]*Active*, if at least one active allele present; [b]*Null*, if no active alleles present; [c]*Low activity*, if at least one *T* allele present; [d]*Variant*, if at least one *Val* allele present; [e]OR odds ratio adjusted to age, gender, BMI, hypertension; CI, confidence interval; [f]Reference group; [g]Median (min-max);

The levels of BPDE-DNA adducts were initially compared between non-smokers (2.19 ng/ml (1.39-8.67)) and smokers (2.50 ng/ml (1.39-17.93)) with ccRCC, however, no statistical significance was reached (p=0.402). In an attempt to discern whether phenotype changes reflect genotype-associated risk of ccRCC, comparative analysis of BPDE-DNA adduct levels in ccRCC smokers who were carriers of *active/wild-type* versus *null/low-activity/variant* genotype, individually or in combination, was performed (Table 26 and Table 27). These results have shown that smokers with *GSTM1-null* genotype had significantly higher concentration of BPDE-DNA adducts (2.74 ng/ml (1.64-17.93)) in comparison with *GSTM1-active* smokers (2.13ng/ml (1,39 – 5.22), p=0.050, Table 26).

**Table 27**. The association between combined *GST* genotypes and the levels of BPDE-DNA adducts in ccRCC smokers

| ***GST* genotypes in smokers** | **ccRCC smokers n,%** | **Controls smokers n,%** | **OR (95%CI)[e]** | **p-value** | **BPDE-DNA adducts in ccRCC smokers (ng/ml)[g]** | **p-value** |
|---|---|---|---|---|---|---|
| ***GSTM1/ GSTP1*** | | | | | | |
| *GSTM1-active[a] / GSTP1 wild-type* | 5 (10) | 27 (46) | 1.00[f] | | 2.00 (1.76-2.75) | |
| *GSTM1-null[b] / GSTP1-variant[d]* | 45 (90) | 32 (54) | 5.44 (1.74-16.98) | 0.004 | 2.50 (1.64-17.93) | 0.347 |
| ***GSTM1/GSTA1/GSTP1*** | | | | | | |
| *GSTM1-active[a] / GSTA1-active/ GSTP1 wild-type[d]* | 2 (6) | 10 (30) | 1.00[f] | | 2.37 (2.01-2.75) | |
| *GSTM1 null[b] / GSTA1-low activity[c] / GSTP1-variant[d]* | 32 (94) | 23 (70) | 7.57 (1.26-45.30) | 0.026 | 2.44 (1.64-17.93) | 0.813 |

[a]*Active*, if at least one active allele present; [b]*Null*, if no active alleles present; [c]*Low activity*, if at least one *T* allele present; [d]*Variant*, if at least one *Val* allele present; [e]OR odds ratio adjusted to age, gender, BMI, hypertension; CI, confidence interval; [f]Reference group; [g]Median (Min-Max)

### 4.2.4 The association between *GST* genotypes and tumor grade and stages of ccRCC

The analysis of *GST* polymorphisms as potential prognostic marker of ccRCC was performed as presented in Figures 29-32. Once again, the results concerning the *GSTM1-null* genotype showed significant difference with respect the tumors grade (Figure 29A, p=0.045). Namely, *GSTM1-null* genotype was again found to be the most frequent in grade II tumors (G2). Similarly to the results of obtained on the whole group of RCC patients, there were no statistical difference found for other analyzed *GST* genotypes (Figures 30-32).

Regarding the tumor stage, no association between *GST* polymorphisms and ccRCC stage was observed.

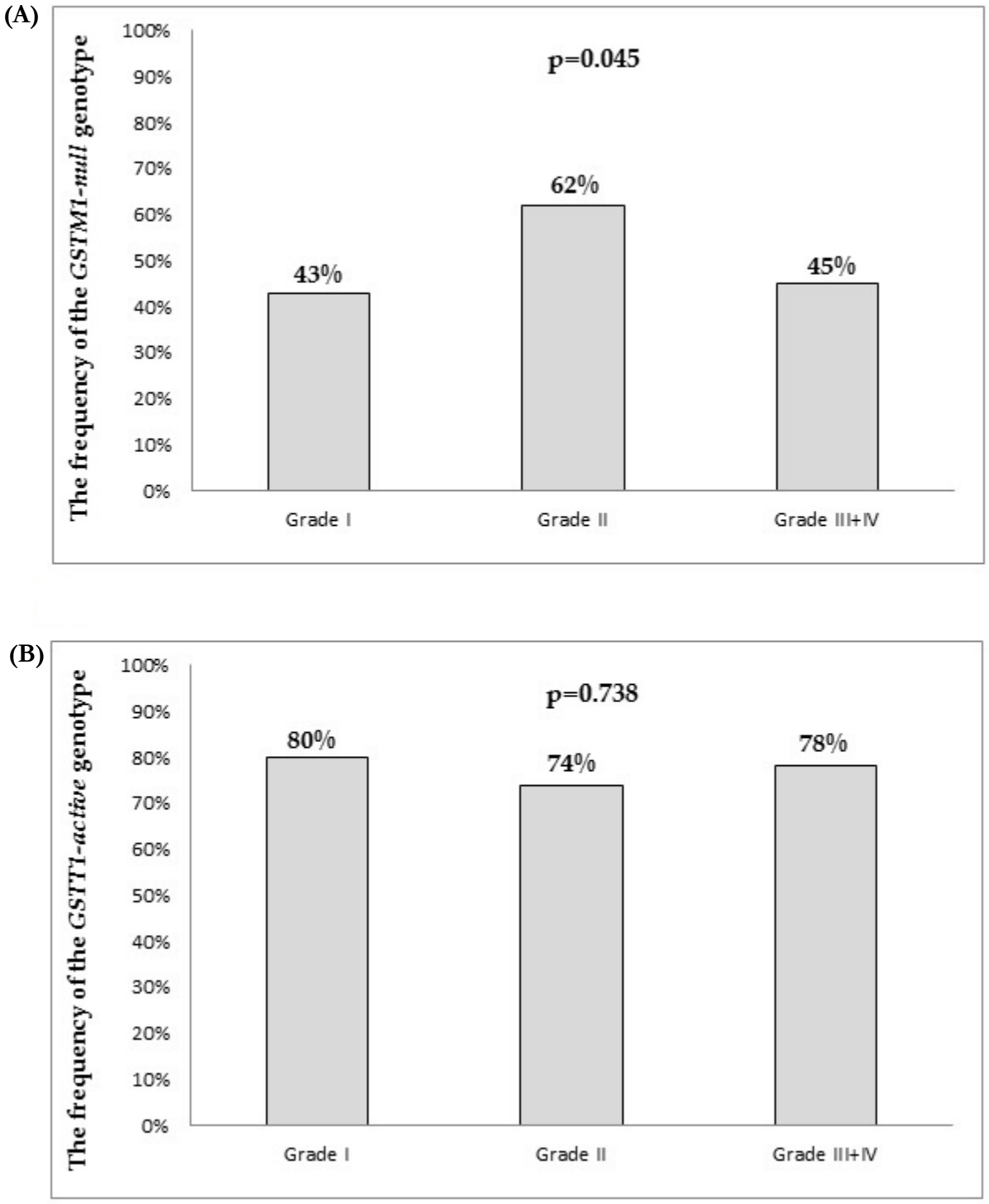



**Figure 29**. The frequency of *GST* deletional risk-associated genotypes in ccRCC patients stratified according to tumor grade: (A) *GSTM1-null* genotype; (B) *GSTT1-active* genotype

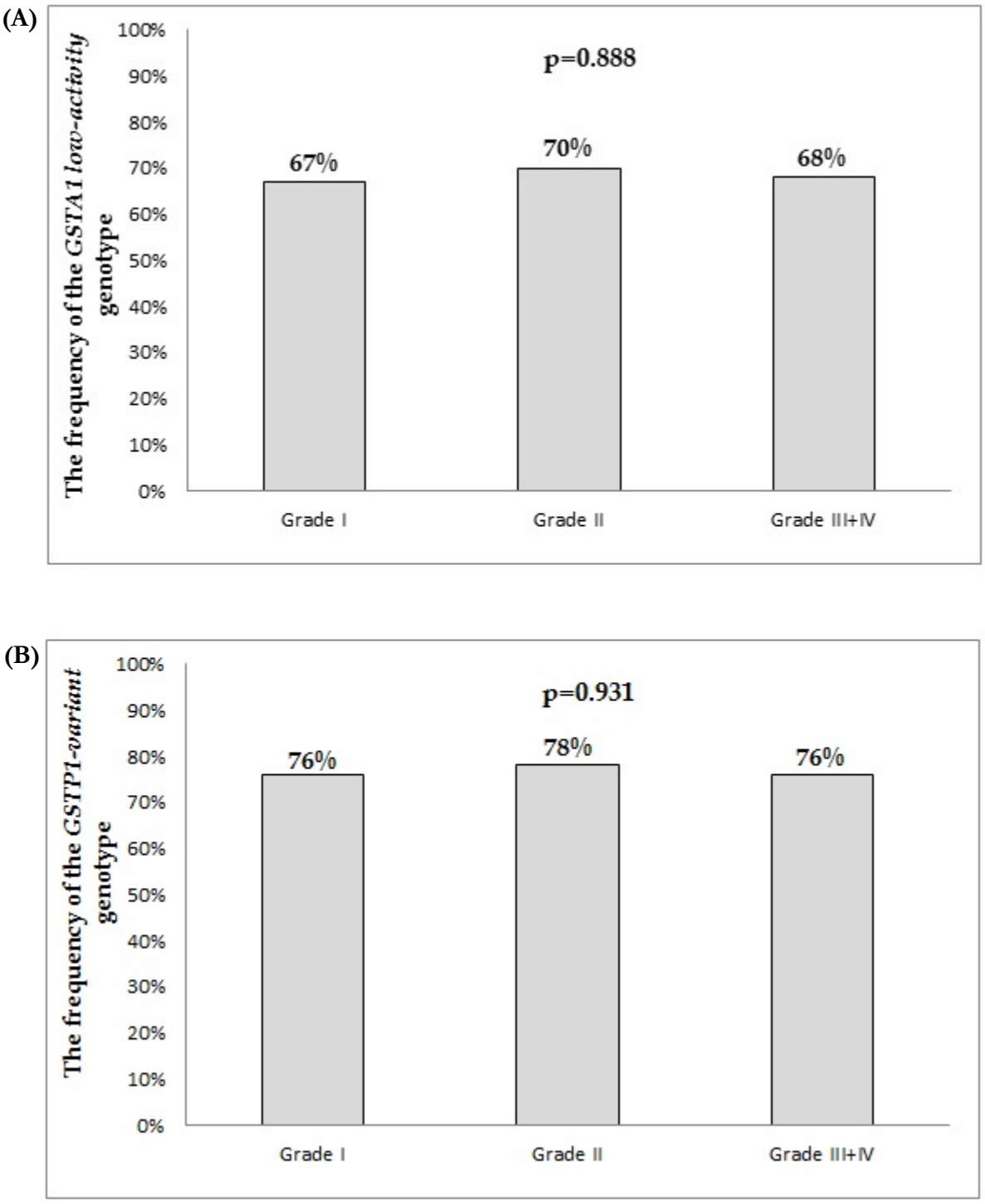



**Figure 30**. The frequency of *GST* SNP risk-associated genotypes in ccRCC patients stratified according to tumor grade: (A) *GSTA1-low activity* genotype; (B) *GSTP1-variant* genotype

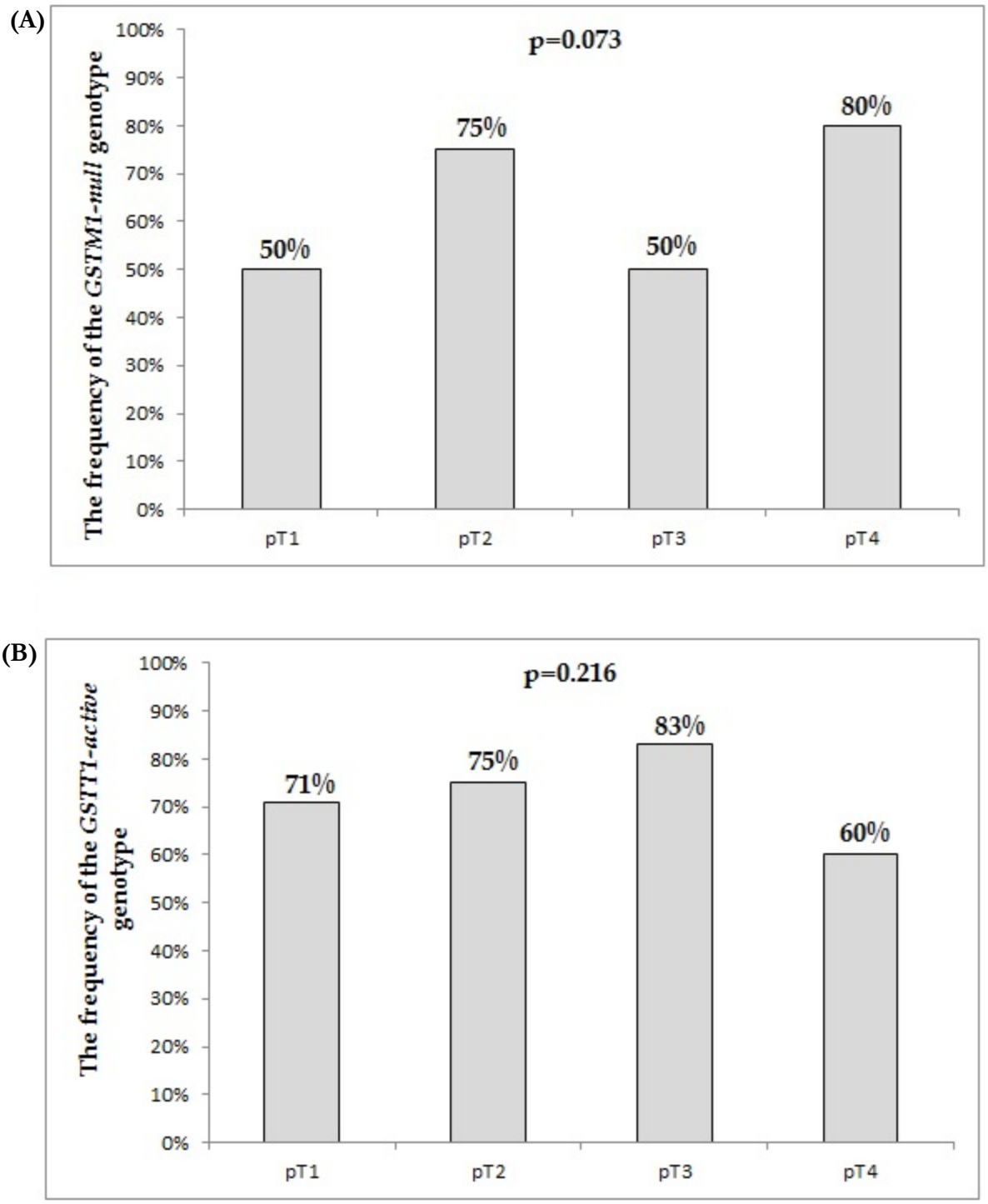



**Figure 31**. The frequency of *GST* deletional risk-associated genotypes in ccRCC patients stratified according to tumor stage: (A) *GSTM1-null* genotype; (B) *GSTT1-active* genotype

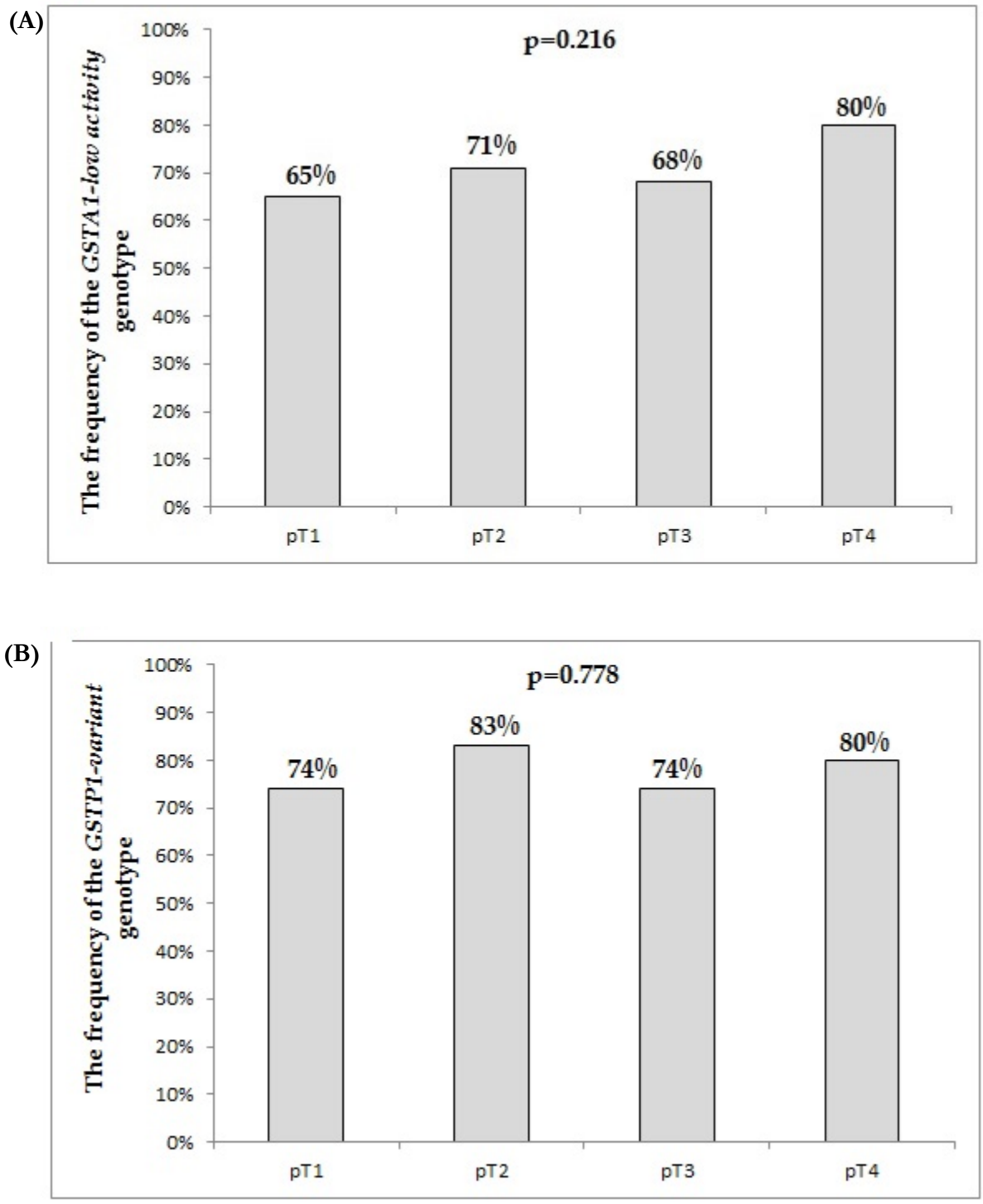



**Figure 32**. The frequency of *GST* SNP risk-associated genotypes in ccRCC patients stratified according to tumor stage: (A) *GSTA1-low activity* genotype; (B) *GSTP1-variant* genotype;

### 4.2.5 The effect of *GST* genotypes on overall survival in ccRCC patients

In the group of 219 patients with ccRCC that were included in the follow-up, there were 60 (27%) deaths during the mean follow-up of 48.09±0.9 (ranging from 1-125) months.

In Table 28, the frequencies of *GST* genotypes in living and deceased in patients with ccRCC are shown. Significantly higher frequency was observed only for the *GSTM1-null* genotype in living ccRCC patients (60%) in comparison with the frequency of *GSTM1-null* genotype in deceased ccRCC patients (42%, p=0.016). However, the obtain frequencies of other analyzed *GST* genotypes were not statistically significant (p>0.05, Table 28).

**Table 28**. The frequency of the *GST* genotypes stratified according to the follow-up status

| ***GST* genotype** | **Living patients n, %** | **Diseased patients, n %** | **p-value** |
|---|---|---|---|
| ***GSTM1*** | | | |
| *active*[a] | 64 (40) | 35 (58) | |
| *null*[b] | 95 (60) | 25 (42) | 0.016 |
| ***GSTT1*** | | | |
| *null*[b] | 41 (25) | 13 (22) | |
| *active*[a] | 118 (75) | 47 (78) | 0.528 |
| ***GSTA1* (rs 3957357)** | | | |
| *CC (active)* | 57 (36) | 19 (32) | |
| *CT+TT (low activity)*[c] | 102 (64) | 41 (68) | 0.562 |
| ***GSTP1* (rs1695)** | | | |
| *IleIle (wild-type)* | 43 (27) | 12 (20) | |
| *IleVal+ValVal (variant)*[d] | 114 (73) | 48 (80) | 0.263 |

[a]*Active*, if at least one active allele present; [b]*Null* if no active alleles present; [c]*Low activity*, if at least one *T* allele present.
[d]*Variant*, if at least one *Val* allele present; Number of diseased patients n=60

In a similar way to the whole group included in the study, *Kaplan-Meier* survival analysis indicated shorter overall survival (Log Rank: p=0.026) only for the patients with *GSTM1-active* genotype, compared to ccRCC carriers of *GSTM1-null* genotype (Figure 33). On the other hand, Kaplan-Meier analysis did not demonstrate a significantly shorter time towards the death in ccRCC patients carrying any other analyzed *GST* genotype (p>0.05, Figures 34-36).

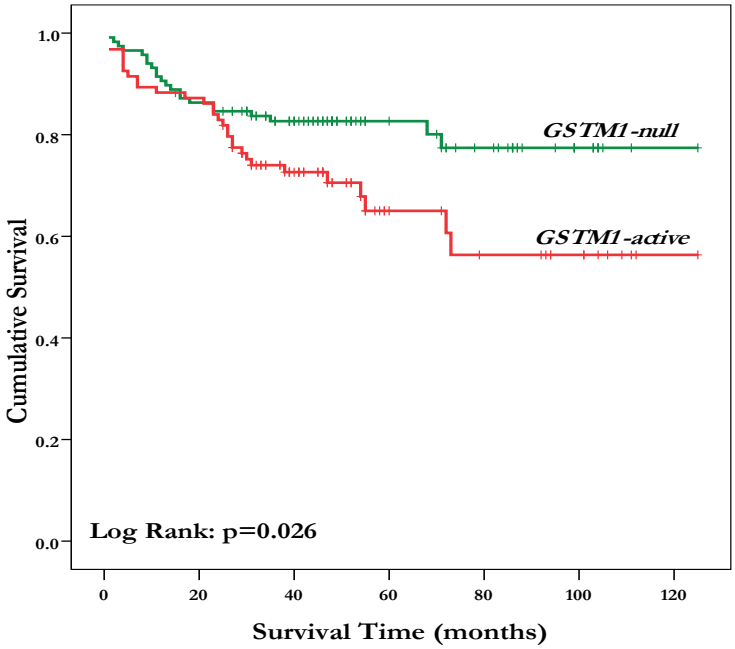



**Figure 33.** *Kaplan-Meier* Survival Curves for overall mortality in ccRCC patients according to *GSTM1* polymorphism; *Active*, if at least one active allele present; *Null* if no active alleles present

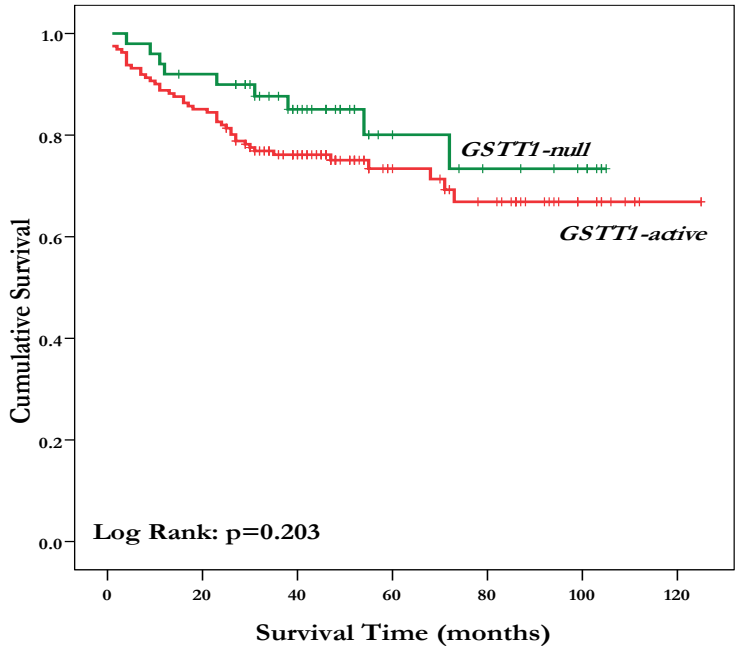



**Figure 34**. *Kaplan-Meier* Survival Curves for overall mortality ccRCC patients according to *GSTT1* polymorphism; *Active*, if at least one active allele present; *Null* if no active alleles present

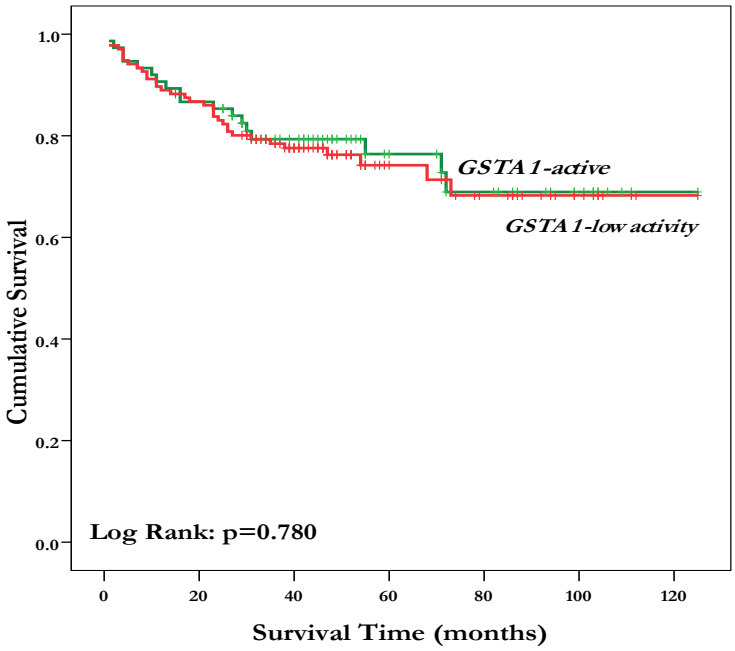



**Figure 35**. *Kaplan-Meier* Survival Curves for overall mortality ccRCC patients according to *GSTA1* polymorphism; *Low activity*, if at least one *T* allele present

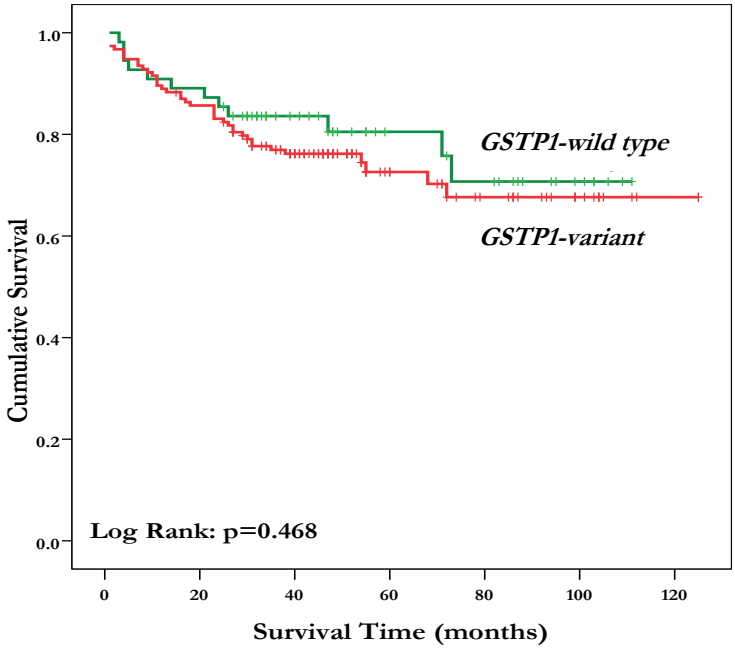



**Figure 36.** *Kaplan-Meier* Survival Curves for overall mortality ccRCC patients according to *GSTP1* polymorphism; *Variant*, if at least one *Val* allele present.

Tables 29-31 summarize the associations between different *GST* genotypes and overall mortality for ccRCC, adjusted by covariates in three different models, analogously to the RCC group.

**Table 29**. *GST* polymorphisms as predictors for overall mortality in ccRCC patients, Model 1

| **Model 1** | |
|---|---|
| HR (95% CI) | p-value |
| **Risk for overall mortality comparing *GSTM1-active*[a] genotype to *GSTM1-null*[b] genotype carriers** | |
| 1.68 (0.96-2.94) | 0.065 |
| **Risk for overall mortality comparing *GSTT1-active*[a] genotype to *GSTT1-null*[b] genotype carriers** | |
| 1.22 (0.58-2.56) | 0.588 |
| **Risk for overall mortality comparing *GSTA1-low activitc*[c] genotype to *GSTA1-active* genotype carriers** | |
| 1.12 (0.63-2.00) | 0.681 |
| **Risk for overall mortality comparing *GSTP1-variant*[d] genotype to *GSTP1-wild type* genotype carriers** | |
| 1.28 (0.67-2.46) | 0.444 |

Model 1 adjusted to age and gender; [a]*Active*, if at least one active allele present; [b]*Null*, if no active alleles present; [c]*Low activity*, if at least one *T* allele present; [d]*Variant*, if at least one *Val* allele present; HR, hazard ratio; CI, confidence interval

**Table 30**. *GST* polymorphisms as predictors for overall mortality in ccRCC patients, Model 2

| **Model 2** | |
|---|---|
| HR (95% CI) | p-value |
| **Risk for overall mortality comparing *GSTM1-active*[a] genotype to *GSTM1-null*[b] genotype carriers** | |
| 4.28 (1.72-10.63) | 0.002 |
| **Risk for overall mortality comparing *GSTT1-active*[a] genotype to *GSTT1-null*[b] genotype carriers** | |
| 1.04 (0.37-2.91) | 0.937 |
| **Risk for overall mortality comparing *GSTA1-low activity*[c] genotype to *GSTA1-active* genotype carriers** | |
| 0.81 (0.32-2.05) | 0.669 |
| **Risk for overall mortality comparing *GSTP1-variant*[d] genotype to *GSTP1-wild type* genotype carriers** | |
| 1.95 (0.51-7.39) | 0.327 |

Model 2 adjusted to the covariates from Model 1 and recognized risk factors for RCC development (packyears, BMI, hypertension); [a]*Active*, if at least one active allele present; [b]*Null*, if no active alleles present; [c]*Low activity*, if at least one *T* allele present; [d]*Variant*, if at least one *Val* allele present; HR, hazard ratio; CI, confidence interval

**Table 31**. *GST* polymorphisms as predictors for overall mortality in ccRCC patients, Model 3

| **Model 3** | |
|---|---|
| HR (95% CI) | p-value |
| **Risk for overall mortality comparing *GSTM1-active*[a] genotype to *GSTM1-null*[b] genotype carriers** | |
| 5.93 (1.64-21.50) | 0.007 |
| **Risk for overall mortality comparing *GSTT1-active*[a] genotype to *GSTT1-null*[b] genotype carriers** | |
| 1.30 (0.41-4.07) | 0.644 |
| **Risk for overall mortality comparing *GSTA1-low activity*[c] genotype to *GSTA1-active* genotype carriers** | |
| 0.67 (0.24-4.81) | 0.432 |
| **Risk for overall mortality comparing *GSTP1-variant*[d] genotype to *GSTP1-wild type* genotype carriers** | |
| 1.85 (0.32-10.61) | 0.487 |

Model 3 adjusted to the covariates from Model 2 and clinical characteristics of tumor (grade, pT stage); [a]*Active*, if at least one active allele present; [b]*Null*, if no active alleles present; [c]*Low activity*, if at least one *T* allele present; [d]*Variant*, if at least one *Val* allele present; HR, hazard ratio; CI, confidence interval

We managed to confirm the results obtained on the whole group included in the study, as the *GSTM1-active* genotype was proved to be an independent predictor of higher risk of overall mortality in ccRCC patients as well. Namely, this genotype had a significant multivariable adjusted HR in Model 2 HR=4.2 (95%CI: 1.72-10.63, p=0.002, Table 30) and in Model 3 HR=5.93 (95%CI: 1.64-21.50, p=0.007, Table 31). A similar approach was used for analyzing the predictive value of other *GST* genotypes, however, the results did not reach statistical significance although 2-fold higher HR was observed for *GSTP1-varinat* genotype in Model 2 (HR=1.95, 95%CI: 0.51-7.39, p=0.327, Table 30) and Model 3 (HR=1.85, 95%CI: 0.32-10.61, p=0.487, Table 31).

### 4.3 GSTM1 and GSTP1 protein expression in pools of non-tumor kidney tissue samples and ccRCC tissue samples

After the fact that the *GSTM1* and *GSTP1* variants have conferred a significant risk towards RCC development, GSTM1 and GSTP1 protein expression was determined in pools of non-tumor kidney tissue samples and ccRCC tissue samples, with respect to tumor grade.

GSTM1 protein has been shown to carry out regulatory functions in terms of cell survival and/or ASK1-dependent apoptotic cell death, depending on the strength and duration of the cell stress. Owning to the fact that *GSTM1* deletion polymorphisms is found in 50% of White population, cytosols - obtained from pools of non-tumor kidney tissue samples and RCC tissue samples, were subdivided according to the *GSTM1* genotype, for the purpose of the determination of the ASK1 and Cleaved Caspase-3 protein expression. The following immnoblot analysis has confirmed the absence of GSTM1 protein in samples of *GSTM1-null* individuals and, vicevrsa, the presence of GSTM1 protein in *GSTM1-active* individuals (Figure 37). Furthermore, the densitometry analysis has shown the change in GSTM1 expression with ccRCC grade advancement. Namely, a significantly lower expression of the GSTM1 protein was found across ccRCC tumor grades in comparison with non-tumor kidney tissue pools ($p<0.001$, Figure 37B).

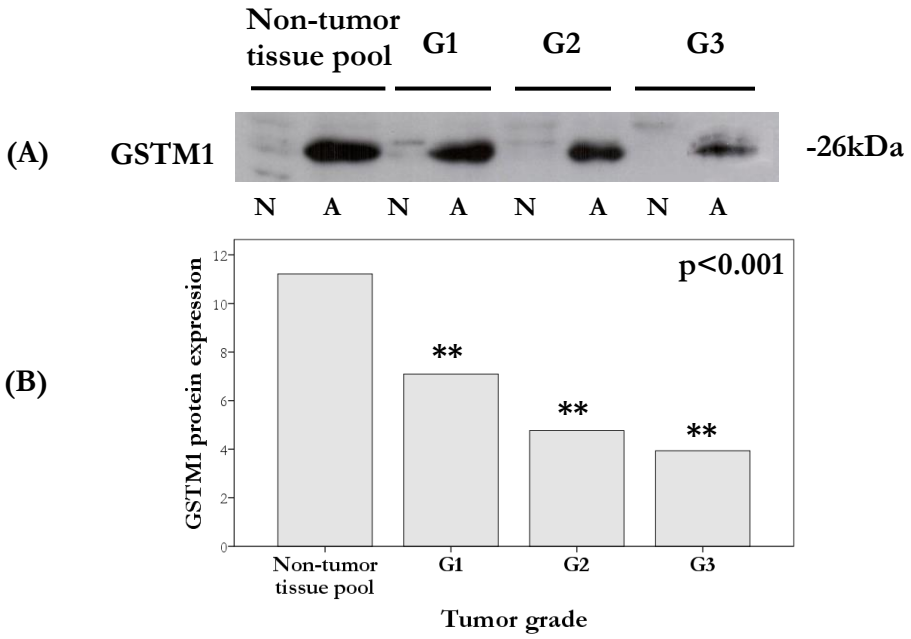



**Figure 37.** Expression of GSTM1 protein (26kDa) in pool of non-tumor kidney tissue samples, as well as in ccRCC tissue samples (G1-G3) according to *GSTM1* genotype; N- *GSTM1-null* genotype; A-*GSTM1-active* genotype; G1- tumor grade I; G2- tumor grade II; G3- tumor grade III

Sevelral studies have demonstrated that GSTP differential expression in tumor tissue seems to correlate with the tumor grade and heightened drug resistance. Since RCC is known for its chemotherapy and radiotherapy resistance, we determined the GSTP1 protein expression in pools of non-tumor kidney tissue samples, as well as in ccRCC tissue samples, irrespective of the *GSTP1* genotype (Figure 38). In this case, no statistical difference was found for GSTP1 protein levels in ccRCC tissue samples ($p>0.05$). However, a trend was observed indicating the gradual increase of GSTP1 protein expression across tumor grade (Figure 38B). No statistical significance in terms of GSTP1 protein level has been found between the pools of non-tumor kidney tissue samples and ccRCC tissue samples (Figure 38B, $p>0.05$).

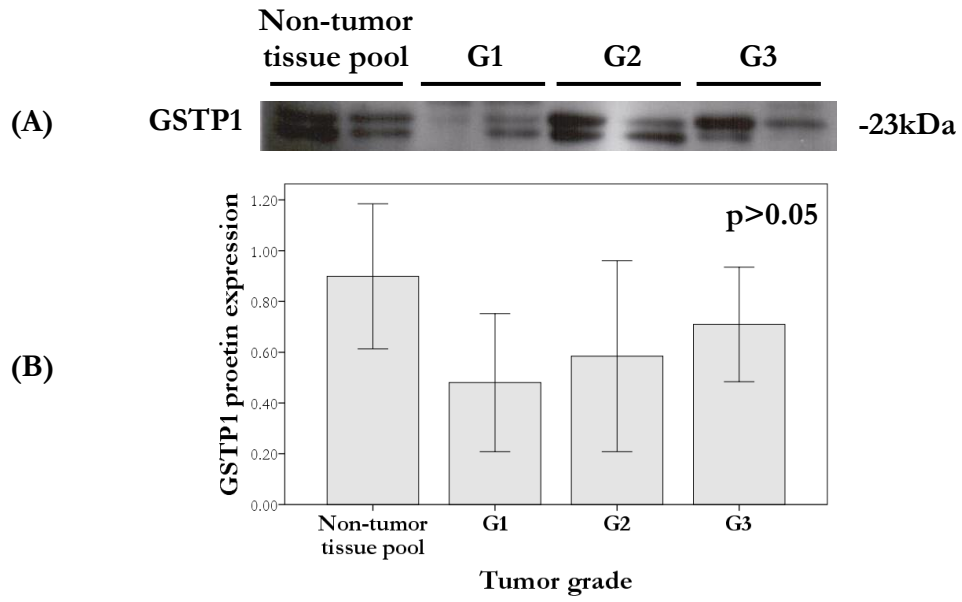



**Figure 38.** Expression of GSTP1 (23kDa) protein in pool of non-tumor kidney tissue samples, as well as in ccRCC tissue samples (G1-G3); G1- tumor grade I; G2- tumor grade II; G3- tumor grade III

### 4.4 Expression of regulatory apoptotic moleculs (ASK1 and JNK1/2) in pools of non-tumor kidney tissue samples and ccRCC tissue samples

Several lines of evidence suggest that monomeric forms of GSTM1 and GSTP1 proteins seem to negatively regulate aforementioned kinase-dependent proliferation pathways, by forming protein:protein complexes with regulatory MAP kinases, such as ASK1 and JNK1, respectively. Therefore, we further investigated the exspresion of ASK1 and JNK1/2 in obtained pools of non-tumor kidney tissue samples and ccRCC tissue samples.

Our resuts indicated a significantly lower protein level of ASK1 in G2 and G3 tumor tissue samples compared to the ASK1 protein levels found in pools of non-tumor kidney tissue samples, as well as G1 grade ccRCC tissue samples (Figure 39B, $p<0.05$).

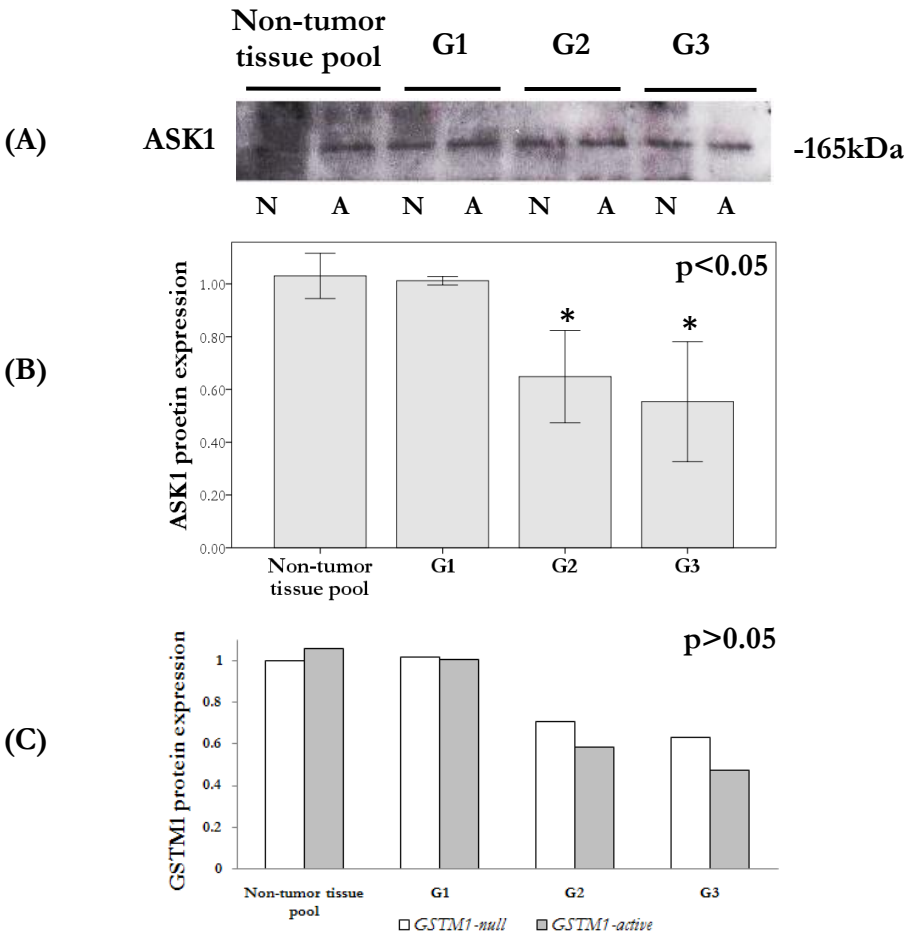



**Figure 39.** Expression of ASK1 (165kDa) protein in pool of non-tumor kidney tissue samples, as well as in ccRCC tissue samples (G1-G3), independently or according to the *GSTM1* genotype; N-*GSTM1-null* genotype; A-*GSTM1-active* genotype; G1- tumor grade I; G2- tumor grade II; G3- tumor grade III

Further on, we determined the impact of GSTM1 genotype on ASK1 protein levels. No statistical significance in ASK1 expression was found when the *GSTM1-null* tumor samples were compared to *GSTM1-active* tumor samples within different tumor grades (Figure 39C, $p>0.05$).

On the other hand, the JNK1/2 expressed was evidently higher in non-tumor and G1 tumor tissue, compared to G2 and G3 tumor tissue samples, yet the obtain results were not statistically significant (Figure 40B, p>0.05).

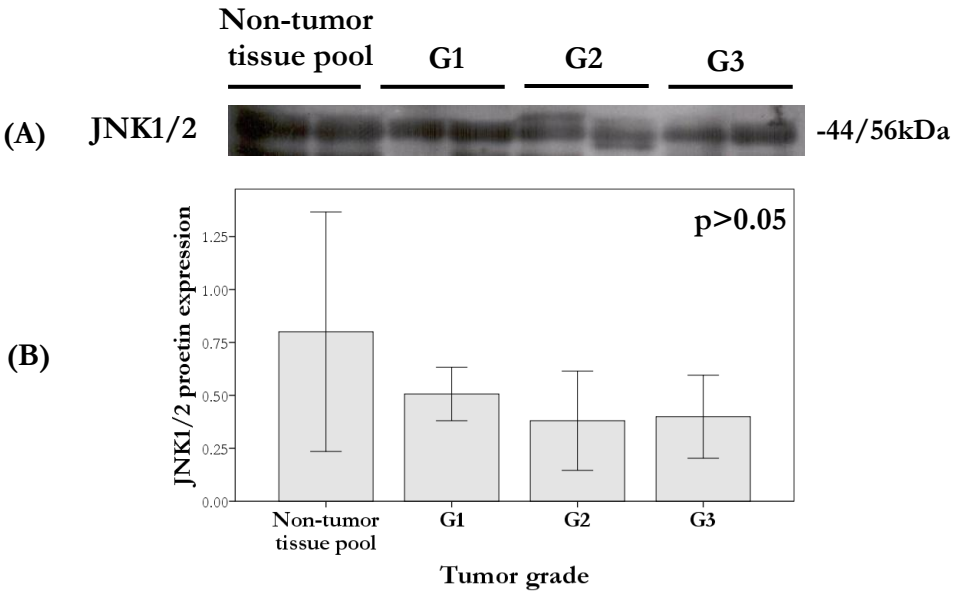



**Figure 40.** Expression of JNK1/2 (44/56kDa) protein in pool of non-tumor kidney tissue samples, as well as in ccRCC tissue samples (G1-G3); G1- tumor grade I; G2- tumor grade II; G3- tumor grade III

### 4.5 Expression of executor apoptotic molecul (Cleaved Caspase-3) in pools of non-tumor kidney tissue samples and ccRCC tissue samples

Caspase-3, an executor caspase, represents the most downstream enzyme in the apoptosis-inducing protease pathway and is shown to correlate with the level of apoptosis in the best manner. As ASK1-dependent apoptotic pathway is mainly medicated by cytochrome c release from mitochondria and activation of Caspase-3 activity, we determined the expression of Cleaved Caspase, the Caspaase-3 activated form, in both ccRCC tumor and adjacent non-tumor tissue samples, with respect to tumor grade and *GSTM1* genotype.

In our study, the expression of Cleaved Caspase-3 gradually decreased across tumor grade, reaching the statistical significance only in G3, when compared to the pool of non-tumor kidney tissue samples (Figure 41B, p<0.05). Although a trend of decrease in Cleaved Caspase-3 expression

was evident across tumor grades and the protein level was obviously lower in *GSTM1-active* individuals, the results were not statistically significant (Figure 41C, p>0.05).

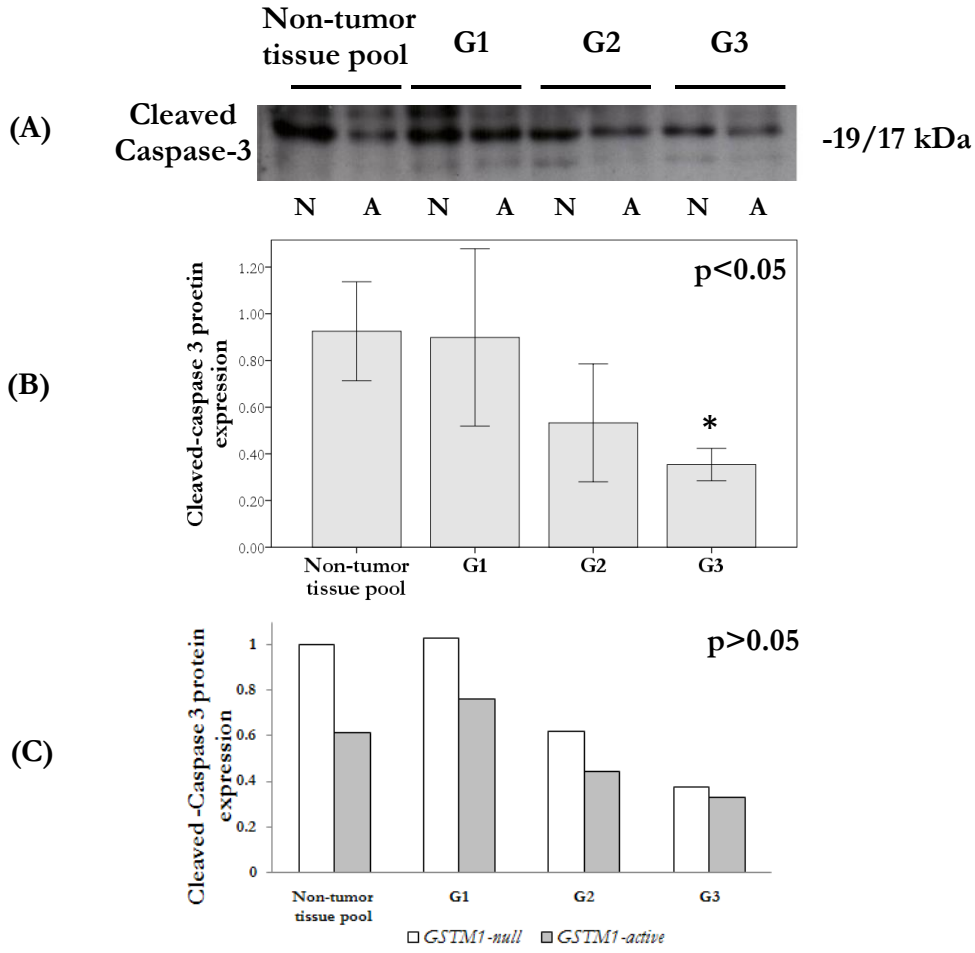



**Figure 41.** Expression of Cleaved Caspase-3 (19/17kDa) protein in pool of non-tumor kidney tissue samples, as well as in ccRCC tissue samples (G1-G3) independently or according to the *GSTM1* genotype; N- *GSTM1-null* genotype; A-*GSTM1-active* genotype

### 4.6 Correlation between GSTM1/GSTP1 and regulatory (ASK1) and executor (Cleaved Caspase-3) apoptotic molecules

A strong positive correlation (correlation coefficient, r>0.5) was found GSTM1 and Cleaved Caspase-3 expression (r=0.81, p=0.014), as well as ASK1 and Cleaved Caspase-3 expression (r=0.93, p=0.001). On the other hand, weak positive correlation (correlation coefficient, r<0.3) was found between GSTP1 and Cleaved Caspase-3 expression (r=0.024, p=0.999) (Table 32).

**Table 32.** Correlation between GSTM1/GSTP1 and regulatory (ASK1) and executor (Caspase-3) apoptotic molecules

| Correlation | Correlation coefficient (r) | p-value |
|---|---|---|
| GSTM1 and Cleaved Caspase-3 expression | 0.81 | 0.014 |
| ASK1 and Cleaved Caspase-3 expression | 0.93 | 0.001 |
| GSTP1 and Cleaved Caspase-3 expression | 0.024 | 0.999 |

### 4.7 The analysis of GSTM1:ASK1 and GSTP1:JNK1/2 protein:protein interactions in ccRCC tissue samples

In order to clarify the potential molecular mechanism underlying the role of GSTM1 in RCC progression, the presence of GSTM1:ASK1 protein:protein interaction was analyzed in specimens of tumor tissue obtained from 20 patients with ccRCC. Tumor tissue samples were divided in three groups, according to the tumor's grade (n=6 of grade I, G1, n=7 of grade II, G2, n=7 of grade III, G3). Protein immunoprecipitation was performed and followed by Western blot analysis. Indeed, the presence of GSTM1:ASK1 protein:protein interaction was confirmed in all ccRCC samples studied (Figure 42).

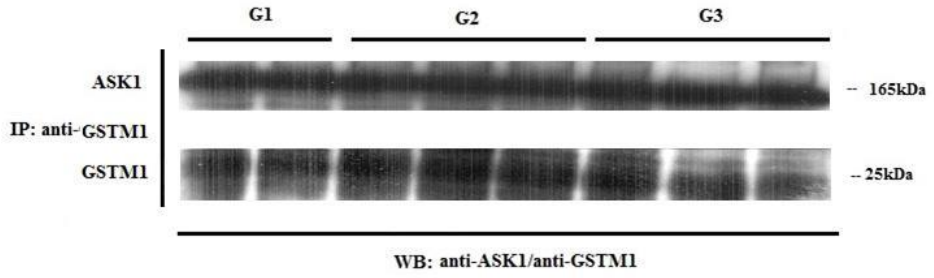



**Figure 42**. Cytosols obtained from ccRCC tissue homogenates were immunoprecipitated with anti-GSTM1 antibody. The samples were subjected to SDS-PAGE electrophoresis on 10% gel, followed by incubation with the primary antibodies against GSTM1 and ASK1; G1- tumor grade I; G2- tumor grade II; G3- tumor grade III. IP- immunoprecipitation; WB- Western blot

Since it is already known that GSTP1 may act as an inhibitor of apoptosis by means of controlling JNK catalytic activity, we analyzed if the potential presence of GSTP1:JNK1/2

complexes in specimens of tumor tissue obtained from 20 patients with ccRCC. As described previously, tumor tissue samples were divided in three groups, according to the tumor's grade (n=6 of grade I, G1, n=7 of grade II, G2, n=7 of grade III, G3). Protein immunoprecipitation, followed by Western blot analysis, showed the presence of JNK1/2/GSTP1 complexes in all ccRCC samples studied (Figure 43).

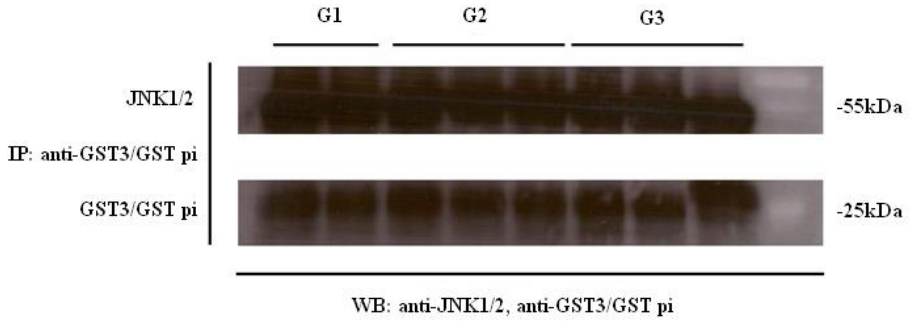



**Figure 43**. Cytosols obtained from ccRCC tissue homogenates were immunoprecipitated with anti-GST3/GST pi antibody. The samples were subjected to SDS-PAGE electrophoresis on 10% gel, followed by incubation with the primary antibodies against GST3/GST pi and JNK1/2; G1- tumor grade I; G2- tumor grade II; G3- tumor grade III. IP- immunoprecipitation; WB- Western blot

In this manner, we confrmed that GSTM1 and GSTP1 proteins do form protein:protein complexes with regulatory MAP kinases, ASK1 and JNK1, respectively and in that way seem to negatively regulate kinase-dependent proliferation pathways.

## 5 DISCUSSION

Prominent genetic heterogeneity, resulting from either gene deletions or SNPs in the coding and non-coding regions of *GST* genes, might serve as a valuable indicator of cancer risk assessment (Di Pietro et al., 2010; Hollman et al., 2016; Josephy, 2010). Moreover, there is a growing body of evidence that GSTs may participate in tumor progression and affect patients' survival by regulating a number of cellular processes via protein:protein interactions as endogenous negative regulators of protein kinases (Board and Menon, 2013; Laborde, 2010; McIlwain et al., 2006; Pajaud et al., 2012; Tew and Townsend, 2012).

In this study, we noted an increased risk of both overall RCC and ccRCC development in carriers of *GSTM1-null* and *GSTP1-variant* genotypes, which was even more pronounced when they were in combination. Furthermore, 22% of all recruited ccRCC patients were carriers of combined *GSTM1-null/GSTT1-active/GSTA1 low-activity/ GSTP1-variant* genotype, which might be considered as "risk-associated genotype combination". On the other hand, an unfavorable postoperative prognosis was found for both overall RCC and ccRCC carriers of the *GSTM1-active* genotype. The molecular mechanism underlying the role of monomeric GSTM1 and GSTP1 forms in RCC progression might be explained by the presence of GSTM1:ASK1 and GSTP1:JNK1/2 protein:protein interactions.

Due to a complex process of RCC development and progression, so far, little progress has been made towards the development of specific biomarkers that would be useful for the following RCC clinical scenarios: (a) identifying subjects who have or are very likely to develop RCC, (b) identifying renal masses that can be placed under active surveillance prior to resection, as opposed to tumors of advanced pathologic stage, and (c) precise more accurate prognosis of patients with developed RCC. In recent years, attention has been reverted towards genetic variants, such as deletional and SNP polymorphisms, often referred to as "*quantitative trait loci*", that could contribute a small, but significant risk not only for the development, but also for the progression of complex disorder such as cancer (Foulkes, 2009). Hence, the panel of candidate genes, with respect to their different cellular functions, are currently being investigated as a part of potential future marker profile for renal cell carcinoma, including carcinogen detoxification genes, such as GSTs (Protzel et al., 2012). This xenobiotic-metabolizing enzymes play an important role in cellular protection from chemical stress, by conjugating reduced glutathione to a variety of electrophilic xenobiotics or products of oxidative stress (Hayes et al., 2005; Oakley, 2011). Presumably, *GST* genotyping could

identify individuals in whom this process is diminished, due to complete lack or alteration in GST enzyme activity.

The *GSTM1-null* genotype was the focus of numerous investigations attempting to elucidate the effect of GSTM1 deficiency and susceptibility to cancers. The underlying hypothesis of these studies is that homozygous deletion of GSTM1 may leave susceptible tissues unprotected from somatic DNA mutations, due to impaired ability to detoxify electrophilic carcinogen, and may place *GSTM1-null* individuals at increased cancer risk. Many of these studies failed to demonstrate such an association, including the impact of *GSTM1-null* genotype on RCC development, which has been further evaluated in numerous meta-analysis (Abid et al., 2016; Cheng et al., 2012; Huang et al., 2015; Jia et al., 2014; Liu et al., 2012; Yang et al., 2013). However, this refers only to *GSTM1-null* genotype as individual risk factor for RCC, while it has been suggested that when present together with polymorphisms in certain genes related to activation or detoxification of renal carcinogens (CYP1A1, GSTT1, GSTP1, NAT2), *GSTM1-null* genotype is associated with increased risk of RCC (De Martino et al., 2010). Still, our study did demonstrate that the *GSTM1-null* was a significant genetic risk factor for RCC development, which was further in complete agreement with our findings regarding the role of *GSTM1* polymorphism in ccRCC development. A possible explanation for discrepancy in obtained results might be attributed to the evident lack of patients' individual-level data that are necessary for adjusted risk analysis, as well as to differences in the study populations. To our knowledge, the present study is the first that was conducted on a significant number of RCC cases (comprising patients with clear cell RCC, papillary RCC, chromophobe RCC, collecting duct RCC, etc.) and respective controls, with available detailed individual information regarding demographic data, clinical characteristics of tumor and lifestyle factors, used for adjusted risk analysis.

Another widely investigated *GST* polymorphism, *GSTT1*, seems to be even more controversial (Cheng et al., 2012; Salinas-Sánchez et al., 2012; Yang et al., 2013). GSTT1 deficiency is the result of a gene deletion. After the discovery that GSTT1 can activate some compounds to even more reactive intermediates (Brüning et al., 1997; Guengerich, 2005; Thier et al., 2003), the *GSTT1* deletion polymorphism was the subject of many studies, some of which tried to determine if the presence of the *GSTT1-active* genotype was associated with RCC development, independently or in combination with high and long-term exposure to certain environmental or occupational hazards (Buzio et al., 2003; Karami et al., 2008; Longuemaux et al., 1999). Apparently, GSTT1 may play a role as a risk modifier only in the case of subjects exposed to relevant substrates (Buzio et al., 2003).

On one hand, GSTT1 is involved in detoxification of ethylene oxide, formed endogenously from ethene, which is present at high levels in cigarette smoke (Hayes and Pulford, 1995). On the other hand, there is also evidence that *GSTT1-active* individuals, occupationally exposed to relevant compounds, such as halo- and dihaloalkanes, may produce potentially nephrotoxic glutathione conjugated compounds resulting in toxic effects and selective vulnerability of the tubular renal epithelium (Buzio et al., 2003; Hayes and Pulford, 1995; Meyer et al., 1991) Thus, the knowledge of specific environmental compounds, involved in the carcinogenesis process, is of quite importance for evaluating the relationships between *GSTT1* genotype and cancer susceptibility. In case of RCC, *Buzio et al.* (Buzio et al., 2003) and *Karami et al.* (Karami et al., 2008) reported an increased risk of this tumor in *GSTT1-active* individuals, occupationally exposed to pesticides produced from halogenated compounds. Moreover, *Brüning* et al.(Brüning et al., 1997) suggested that high occupational exposure to the solvent trichloroethene also increased the risk for RCC among *GSTT1-active* individuals. Regarding the present study, it has been shown that *GSTT1-active* genotype did not, at least independently, contribute to RCC susceptibility. Although the structured questioners used in this study comprised a question regarding the occupational title that was held for more than one year, we considered it a poor tool for estimating the exposure levels to specific agents. Nevertheless, our results are in agreement with the 2 most recent meta-analysis (Jia et al., 2014; Yang et al., 2013) who found no association between the *GSTT1* polymorphism and RCC risk in the overall and European populations.

In contrast to other GSTs, only one polymorphism has been found in the coding region of *GSTA1* gene, whereas several SNPs have been identified in 5' non-coding promoter region of *GSTA1* gene, among them *GSTA1**C69T (rs3957356). Although it has been suggested that GSTA1 expression is dominantly observed in clear cell RCC (Simic et al., 2009), whereas completely absent in chromophobic type of this tumor (Liu et al., 2007), the data on the potential role of *GSTA1* SNP in both onset and prognosis of RCC are limited (Searchfield et al., 2011). This is even more surprising, considering the fact that GST *alpha* is predominantly expressed in the proximal convoluted tubule, the main site of nephrotoxins and renal carcinogens toxicity (Dekant, 1993), and from which clear cell RCC originates (Simic et al., 2001, 2001). The results obtained in this study showed no significance in terms of increased risk for RCC and even ccRCC development in carriers of *GSTA1-low activity* genotype (*CT+TT*), at least when analyzed independently. The possible explanation would be that the effect of this particular polymorphism may be masked by the activity of others GSTs, due to the overlapping substrate specificities.

GSTP1 SNP (rs1659) is one of the most extensively studied *GST* polymorphisms. This SNP encodes the *Ile105Val* substitution, which influences *Ile105* and *Val105* variants' catalytic efficacy (Dusinská et al., 2001; Hu et al., 1997) and has been investigated not only in terms of cancer susceptibility, but also in relation to drug resistance (Laborde, 2010; Townsend et al., 2005; Townsend and Tew, 2003). Some of the available data suggest that active, yet functionally different GSTP1 isoenzymes may play a key role in the metabolism of environmental carcinogens, hence affecting the risk of RCC (Longuemaux et al., 1999). However, certain meta-analyses on *GST* polymorphisms in RCC did not report any significant individual association between *GSTP1* genotype and RCC risk (Jia et al., 2014; Yang et al., 2013). Once again, the overall sample size, as well as the lack of some important information, such as pathological status, environmental exposure and lifestyle habits, prevented the authors of these meta-analysis from determining the association between *GSTP1* variants and RCC risk. Nevertheless, it has been already established that in comparison with normal kidney tissue, RCC contains significantly lower GSTP1 activity (Longuemaux et al., 1999), suggesting that GSTP1 might act as local modifier of renal cancer tumorigenesis. Our results on RCC patients indeed demonstrated a significantly increased risk for cancer development in patients carrying *GSTP1-variant* (*ValVal*) genotype, which was in line with the results obtained on the subpopulation of ccRCC patients. This particular association was even more pronounced when the combined effect of *GST* polymorphism and the susceptibility to overall RCC and ccRCC dvelopment was assessed in various genetic models, comprising *GSTP1-variant* genotype. What is more, the relevance of the patients' clinical data, available for the adjusted risk analysis in this study, enhanced the association, as demonstrated by the obtained adjusted ORs, that was calculated next to the crude ORs.

The simultaneous identification of all *GST* genotypes appears to be of particular importance for reliable interpretation of the role of the *GST* family in RCC development, as the impaired activity of the single given GST may be compensated by other isoforms, due to the overlapping substrate specificities. Interestingly, we found that 22% of all ccRCC patients in our study were carriers of combined *GSTM1-null/GSTT1-active/GSTA1 low-activity/GSTP1-variant* genotype, suggesting that this particular combination of *GST* genotypes might be considered as „risk-associated genotype combination" in RCC. Moreover, a trend in OR was observed when the cumulative effect of *GST* genotypes on the risk of both overall RCC and ccRCC development was analyzed. So far, only a small number of case-control studies have assessed the combined effect of *GST* polymorphism and the susceptibility to RCC. *Ahmad et al.* (Ahmad et al., 2012) have found that

three-way combination of *GSTM1-null*, *GSTT1-null* and *GSTP1-variant* genotypes resulted in 4.5-fold increase in RCC risk, which partially agrees with our results, while *Sweeny et al.* (Sweeney et al., 2000) have shown an elevated risk of RCC in carriers of *GSTT1-null* genotype in all combinations of *GSTM1* and *GSTP1* genotypes. The effect of gene-gene interaction was further analyzed in a couple of meta-analysis, however, most of the genetic models showed no significant association between combined effect of *GSTs* polymorphism and RCC risk (Jia et al., 2014; Yang et al., 2013).

Cigarette smoking, hypotension and obesity are considered as recognized risk factors for RCC development, accounting for about half of the cases diagnosed in the USA (Sweeney et al., 2000). In this study, we observed an increase in RCC risk among individuals with smoking habits and hypertension, which lends support to previous findings in the literature (Brennan et al., 2008; Hunt et al., 2005; Theis et al., 2008; Weikert et al., 2008). However, our study failed to show any association between obesity and RCC. This apparent lack of correlation can be possibly attributed to the fact that our recruited controls were hospital based and to the prevalence of overweight individuals that might have been higher.

Cigarette smoking is a very well recognized risk factor for RCC development. Since GSTs are involved in the metabolism of carcinogens in cigarette smoke, *GST* genotype and smoking may have a synergic effect on RCC risk. Moreover, the results of recent prospective study suggested that smoking increases the risk of certain common RCC subtypes, such as ccRCC and pRCC, but not the chRCC (Patel et al., 2015). The data on *GSTM1* and *GSTT1* genotypes among RCC cases and controls, stratified by smoking status, were available only in 2 studies (Karami et al., 2008; Sweeney et al., 2000) and were further analyzed in meta-analysis of *Jia et al.* No significant association between tested *GST* genotypes and RCC risk among smokers was found, most probably due to the small sample size (Jia et al., 2014). In our study, the significant association between *GST* genotype and the risk of ccRCC in smokers was found only for the *GSTP1* genotype. Moreover, our results have shown that the *GSTM1-null/GSTP1-variant/GSTA1 low-activity* genotype combination was present in 94% of smokers with ccRCC, increasing the risk of ccRCC up to 7.57. Unfortunately, none of the ccRCC smokers in our study had the four-way „risk-associated genotype combination". Interestingly, we were the first to show that ccRCC smokers with *GSTM1-null* genotype had significantly higher concentration of BPDE-DNA adducts compared to *GSTM1-active* ccRCC smokers. The hypothesized role of GSTM1 deficiency in carcinogenesis suggests that DNA damage may occur at a higher rate in carriers of *GSTM1-null* genotype than in *GSTM1-active* individuals (Rebbeck, 1997). A number of studies consistent with this hypothesis have been conducted in

various pathologies but RCC, some reporting an increased levels of various DNA adducts in *GSTM1-null* individuals, due to their inability to eliminate potentially harmful active carcinogenic compounds (studies discussed in (Alexandrov et al., 2002; Rebbeck, 1997). This seems to be biologically plausible, especially in the case of compounds that are known to be metabolized by GSTM1 and are involved in the carcinogenic process. Namely it has been shown that GSTM1 influences sensitivity to chemical carcinogenesis in individuals exposed to widespread environmental contaminants such as benzo(a)pyrene, styrene-7,8-oxide, and trans-stilbene oxide (Rebbeck, 1997). In particular, tobacco smoke, dietary habits and indoor ambient air are important sources of exposure to benzo(a)pyrene (B(a)P), already implicated as one of the components of tobacco smoke related to the induction of lung cancer in smokers (Watanabe et al., 2009). In humans, B(a)P undergoes two successive oxygenation reactions, mediated predominantly by CYP1A1, ultimately leading to BPDE production. This highly mutagenic compound is predominately metabolized by GSTM1 followed by GSTP1 and GSTA1, with the different variants showing different substrate specificity (Alexandrov et al., 2002; Sundberg et al., 2002, 1998). The study of *Lodovici et al* have characterized subjects with *GSTM1-null* and *GSTP1 wild-type* as carriers of "high-risk" genotypes, associated with higher levels of BPDE-DNA adducts (Lodovici et al., 2004). Indeed, sever lines of evidence suggest that *GSTP1**Val allelic variant of the enzyme possesses greater catalytic activity for BPDE and other PAH diol epoxides (Alexandrov et al., 2002; Sundberg et al., 2002, 1998). Although *GSTM1-null* ccRCC smokers in this study had significantly higher levels of BPDE DNA-adducts, our results failed to demonstrate any significant correlation between *GSTP1* polymorphism and BPDE-DNA adducts, suggesting that some other GSTP1 substrates might also be important in RCC development. Other factors, such as CYP1A1 expression, DNA repairing processes, as well as cell turnover could also affect the level of such DNA adducts and should be taken in consideration as well (Alexandrov et al., 2002).

Regarding the modifying effect on risk of ccRCC conferred by hypertension, it seems that this recognized risk factor contributed to genotype-associated ccRCC risk in all examined polymorphisms, once again with special emphasis on *GSTM1-null* and *GSTP1-variant* genotypes. Still, as hypertension alone was significantly associated with ccRCC development, the obtained results indicated a possible effect of exposure rather than the effect of the polymorphism itself. However, the obtained results might also be explained by the significant antioxidant role of GSTs, since oxidative stress is recognized as an important pathogenetic factor in the hypertension development (Polimanti et al., 2013). In this line, it has been suggested that the determination of *GST* genotypes

might be helpful in identifying individuals at high-risk for hypertension, especially in the case of *GSTA1* polymorphism (Oniki et al., 2008).

We found no significant association in terms of modifying effect on risk of ccRCC conferred by obesity. The literature data regarding the GST-BMI interaction seem to be scarce. *Sweeney at al.* have found an association between *GSTT1-null* genotype and RCC risk that seemed to vary by BMI, with the strongest association among subjects in the lowest tertile of BMI, who were otherwise at low risk of RCC (Sweeney et al., 2000). According to the authors, the possible mechanism might involve reactive oxidants, where the protection by GSTT1 is important among individuals with low levels of exposure, but becomes over-whelmed and does not the affect risk among the highly exposed. However, it is possible that the GSTT1-BMI interaction was a chance finding (Sweeney et al., 2000).

Interestingly, all of the three recognized risk factors for RCC development (smoking, hypertension and obesity) have been linked with increased endogenous formation of reactive oxidants and lipid peroxidation, as possible underlying mechanisms. Moreover, it has already been shown that changes in redox status in RCC tumor tissue occur as a consequence of decreased enzyme antioxidant capacity, together with altered GST *alpha* expression, contributing to both RCC development and tumor growth (Pljesa-Ercegovac et al., 2008). Some of the GST enzymes are shown to possess catalytic activity towards phospholipids, hydroperoxide, supporting the fact that GSTs may prevent DNA damage from lipid peroxides formed endogenously due to redox imbalance (Ahmad et al., 2012; Hurst et al., 1998; Seeley et al., 2006). Presumably, the presence of polymorphisms in *GST* genes may result in a lower antioxidant capacity of GST enzymes and higher level of oxidant DNA damage (Sweeney et al., 2000). Therefore, we determined the levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), the most widely used fingerprint of radical attack towards DNA, in RCC patients that were stratified by the presence or absence of the recognized risk factors for RCC development, and analyzed them with respect to various *GST* genotypes. Although the acquired values were slightly higher in RCC patients with „risk-associated genotypes“ compared to the values observed in RCC patients with reference genotype, obtained results did not reach the statistical significance.

When assessing the potential prognostic value of *GST* polymorphism in RCC patients, our result indicated that carriers of *GSTM1-null* genotype had significantly better survival compared to the carriers of *GSTM1-active* genotype. Moreover, *GSTM1-null* genotype independently predicted favorable postoperative prognosis for RCC patients when the association between different *GST*

genotypes and overall mortality, adjusted by covariates in three different models, was analyzed. Finally, the frequency of the *GSTM1-null* genotype was significantly higher among living overall RCC and ccRCC patients in comparison with the deceased. Indeed, our results on the role of *GSTM1-null* genotype in RCC patients survival are in complete agreement with previous results of *De Martino et al.* who have also reported that the *GSTM1-null* genotype was associated with a higher survival rate in RCC patients (De Martino et al., 2010). Moreover, these findings are consistent with a decreased death rate that was found for *GSTM1-null* women, however, diagnosed with breast cancer (Ambrosone et al., 2001). One of the possibly most clinically important observation regarding the GSTM1 deficiency is that it significantly contributed to survival of patients undergoing chemotherapy for childhood leukemia (Board and Menon, 2013).

Unfortunately, no statistically significant results were obtained for other investigated *GST* polymorphisms in terms of postoperative prognosis and the risk of overall mortality, although 1.7-fold increased risk was found for carriers of *GSTP1-variant* (*ValVal*) genotype in two investigated models. Previous data on the prognostic role of GSTs seem to been limited only to *GSTM1* polymorphism. Yet another study investigated whether the expression of GST α and GST π has any prognostic value in RCC patients, however, without assessing the effect of most common *GST* polymorphisms. Survival analysis was restricted to ccRCC and indicated a better survival of patients with ccRCC tumors expressing GST α (Searchfield et al., 2011).

Our results on the association between *GST* polymorphisms and tumor grade /clinical TNM stage of RCC have shown significant correlation only for the *GSTM1* genotype. The association of the *GSTM1-null* with tumor grade II (G2) was noted for RCC and for its most frequent subtype, ccRCC. The results of this study are in agreement with the findings of *De Martino et al.* who have also reported that the *GSTM1-null* genotype was associated with a lower tumor grade (De Martino et al., 2010). On the other hand, two case-control studies have demonstrated an association of *GSTT1-null* genotype with more advanced clinical TNM stages and tumor grades in RCC patients, which were further confirmed in a very recent meta-analyisis of *Huang et al* (Huang et al., 2015). According to the recent study by *Tan et al*, another GST μ class member, GSTM3 and its SNP *rs1332018* variant, has proved to be not only a significant risk factor, but also significant prognostic factor for RCC development (Tan et al., 2013).

The possible molecular mechanism underlying the role of GSTM1 protein in RCC progression might be explained through the effect of GSTM1 on one of the major signal transduction pathway, which includes a cascade of three classes of protein kinases: mitogen-activated

protein kinase kinase kinase (MAP3K), MAPK kinase (MAP2K) and MAPK. This evolutionally conserved intracellular system is known for regulating the cellular stress response (Shiizaki et al., 2013). First in line is the apoptosis signal-regulating kinase 1 (ASK1), a MAP3K family member, that can activate the well-known MAPK signaling cascades, the c-Jun $NH_2$-terminal kinase/stress-activated protein kinase (JNK/SAPK) and the p38 (Ichijo et al., 1997), both involved in cellular stress-induced apoptosis (Ichijo et al., 1997; Shiizaki et al., 2013; Tobiume et al., 2001). Namely, ASK1 is activated by various types of stress, including oxidative stress, endoplasmatic reticulum stress, anti-cancer drugs and calcium overload, as well as by receptor mediated inflammatory signals such as TNF-α and lipopolysaccharide (Matsuzawa et al., 2010). In unstimulated cell, ASK1 is constitutively engaged in forming high molecular mass complex, termed ASK1 signalosome, that consists of homo-oligomeric ASK1, its negative regulator thioredoxin (Trx) and other mostly unidentified components (Noguchi et al., 2005). What is more, the formation of an even higher mass complex of ASK1 (>3000kDa) seems to be induced under ROS and TNF-α stimulation (Shiizaki et al., 2013). There, Trx disassociates from ASK1, while tumor necrosis factor-receptor associated factor-2/6 (TRAF2/TRAF6) are recruited to signalosome, positively regulating ASK1 activity by facilitating the auto-phosphorilation of ASK1 (Noguchi et al., 2005).

The redox regulatory protein Trx is one of the most studied negative regulators of ASK1 (Fujino et al., 2007; Matsuzawa et al., 2010; Shiizaki et al., 2013). However, many studies have revealed that a variety of antioxidant enzymes and molecular chaperons also inhibit the activation of ASK1 (Matsuzawa et al., 2010). According to the results obtained booth *in vivo* and *in vitro,* mouse GSTMu1-1 physically interacts with ASK1 as well, functioning as another ASK1 negative regulator. Namely, *Cho et al.* have shown that under unstimulated conditions, mGSTM1-1 block ASK1 oligomersiation, suppresses ASK1 mediated activation of JNK/SAPK signaling cascade and represses ASK1-dependent apoptotic cell death, independently of its transferase activity (Cho et al., 2001; Dorion et al., 2002). Interestingly, it has been shown that the same region of ASK1 seems to be engaged in protein:protein interactions with either GSTM1-1 or Trx, suggesting the presence of a pool of GSTM1:ASK1-1 and ASK1:Trx complexes under unstressed conditions (Pajaud et al., 2012). Various types of cell stress (heat shock or reactive oxygen species) can result in the release of ASK1 from GSTM1:ASK1-1 or ASK1:Trx complexes, ASK1 oligomerisation, autophosphorylation and subsequently the activation of the p38 signal pathway (Dorion et al., 2002).

The results of this study, for the first time, have confirmed the association between GSTM1 and ASK1 in human ccRCC tissue samples. Our next goal was to assess the significance of this

protein:protein interaction and see if the polymorphic expression of GSTM1 may influence the ASK1-dependent apoptosis. As ASK1-JNK/p38 apoptotic pathway is mainly medicated by cytochrome c release from mitochondria and activation of Caspase-3 activity (Hatai et al., 2000), we determined the expression of ASK1 and Cleaved Caspase-3 in both ccRCC tumor and adjacent non-tumor tissue samples, all stratified according to the *GSTM1* genotype. Caspase-3, an executor caspase, represents the most downstream enzyme in the apoptosis-inducing protease pathway and is shown to correlate with the level of apoptosis in the best manner. Cleaved Caspase-3 is its activated form, responsible for the actual destruction of the cell, cleaving multiple structural and repaired proteins (Pljesa-Ercegovac et al., 2011). As expected, a trend of decrease in ASK1 and Cleaved Caspase-3 expressions was evident with grade advancement. Moreover, although there was no statistically significant difference in the protein level between the carriers of two *GSTM1* genotypes, the level of both ASK1 and Cleaved Caspase-3 was obviously lower in *GSTM1-active* individuals across all tumor grades, supporting the possible role of GSTM1 as yet another negative regulator of ASK1-dependent apoptosis mediated by mitochondria-dependent caspase activation. The ASK1-JNK/p38 pathway was found to be of quite importance for the occurrence of the apoptosis in RCC cells. Namely, *Hassan et al.* demonstrated that upon the induction of ASK1-JNK/p38 pathway by anti-Fas monoclonal antibody, the activation of Fas-Associated protein with Death Domain (FADD)-Caspase-8-Bid signaling occurred in studied RCC cell lines, resulting in the translocation of both Bax and Bak proteins, and subsequently mitochondrial dysregulation that is characterized by the loss of mitochondrial membrane potential, cytochrome c release and cleavage of Caspase-9, Caspase-3 and Poly (ADP-ribose) polymerase (PARP) (Hassan et al., 2009). Indeed, our results indicated a strong positive correlation between the expression of ASK1 and Cleaved Caspase-3.

In summary, it can be speculated that RCC patients with *GSTM1-null* genotype, and consequently deficient GSTM1, may have higher ASK1 activity, resulting in increased apoptotic activity in the tumor. On the other hand, patients with *GSTM1-active* genotype may have increased tumor proliferation due to decreased ASK1-dependent apoptotic activity, leading to RCC progression and poorer survival. The mentioned events may be even more evident when stressors, such as heat shock or reactive oxygen species, activate ASK1 (McIlwain et al., 2006).

Monomeric form of GSTP1 protein seem to be involved in protein:protein with JNK1 or MAPK8, which has been demonstrated to promote one of the most important regulatory functions of GSTP1 - stress-dependent regulation of cell cycle progression through either differentiation, proliferation, senescence or apoptotic responses (Bartolini and Galli, 2016). Namely, it is known that

by means of protein:protein interactions, GSTP1 directly targets at least three proteins in one response-pathway: TRAF2 and its downstream targets, JNK and ASK1. Structural aspects of these interactions remain poorly characterized. Earliest and better characterized physical interactions of GSTP1 includes the one with kinase JNK1 (Adler et al., 1999). So far, it is known that four putative domains are involved in the interaction between GSTP1 subunits and JNK. Two of these are involved in GSTP1 binding to JNK, whereas the other two affect phosphorylation of JNK (Adler and Pincus, 2004). It is speculated that the proposed mechanisms by which GSTP1 inhibits activation of JNK comprises either blocking phosphorylation of JNK or the promotion of dephosphorylation of phosphorylated JNK (Adler and Pincus, 2004). The particular GSTP1:JNK1 interaction has been reported in human leukemia, hepatic carcinoma, bladder cancer and neuroblastoma cells (Pljesa-Ercegovac et al., 2010). Moreover, the results of this study have confirmed the presence of GSTP1:JNK1/2 complex in all investigated RCC samples. This protein:protein interaction seems to be of particular importance as it represents the new, functional link between upregulated GSTP1 and malignant phenotype. In particular, the overexpression of GSTP1 was found in not only in tumors but in drug resistant cells, correlating with tumor stage and grade (Simic et al., 2009; Townsend and Tew, 2003). For a long time, the elevated expression of the GSTP1 in tumors has been frequently associated with detoxification reactions. However, GSTP1 overexpression has been determined even in cases when there was no evidence that the selecting drug was a substrate for this enzyme. Indeed, the signaling and regulatory functions observed between the monomeric forms of GSTP1s and other cellular proteins, seems to perfectly coexist with the classical detoxification function of dimeric GSTP1, thus linking the GSTP-mediated detoxification and signaling response to electrophilic stressors (Bartolini and Galli, 2016). Namely, upregulation of GSTP in tumors would result in an increase in inactive sequestered JNK subsequently suppressing apoptotic signaling pathways and conferring resistance to drug induced cell death. This would explain the correlation observed between increased GSTP1 expression in tumors and survival. Identified protein:protein or metabolic/redox interactions of the monomeric and oligomeric forms of GSTP proteins suggest that the role of GSTP as regulatory protein becomes very important in tumorogenesis and drug resistance. In theory, in case of excess ROS generation following drug administration, GSTP1 would dissociate from JNK and trigger a cascade of signaling events starting with the activation of c-Jun via phosphorylation, leading subsequently to proliferation or apoptosis (Board and Menon, 2013).

In this study, the protein level of GSTP1 increased with tumor grade advancement whereas the expression of JNK1/2 differed between low and high graded samples. However, the effect of polymorphic expression of GSTP1 on the JNK1-dependent apoptosis was not estimated. So far, only one study of *Thevenin* et al. have shown that *GSTP1* variant *Val* allele is a better JNK1 inhibitor, hence with the greater antiapoptotic effect than the wild-type *Ile* allele (Thévenin et al., 2011).

This study has several limitations that need to be addressed. The PCR analysis of *GSTM1* and *GSTT1* genotypes does not positively distinguish between homozygous wild type from heterozygous individuals which may not reflect the true underlying genetic model and thus may not provide a valid and accurate estimate of the genetic risk. Furthermore, the case-control design was used for estimating of associations between *GST* genotypes and risk of RCC and therefore the selection bias might influence the results. Additionally, recall bias regarding the recognized risk factors for RCC development might have influenced the results as well. Furthermore, the data on environmental or occupational exposure were not validated, hence not used in the analysis of the obtained results. Our control group was hospital-based and relatively small. Therefore, the use of population controls may have been more appropriate. In this line, the study subjects were white only; therefore the possible effect of ethnicity could not be evaluated. Moreover, the BPDE-DNA adduct levels were not determined in control population.

# 6 CONCLUSIONS

Based on the provided results and discussion, the following conclusions can be drawn:

- The results of this study have confirmed that smoking and hypertension are independent risk factors for RCC development:
    - Smokers exhibit 1.5-fold increased risk for RCC development compared to non-smokers
    - Hypertensive patients exhibit 3-fold increased risk for RCC development compared to normotensive patients
- *GSTM1-null,* as well as *GSTP1-variant* genotypes represent independent risk factors for RCC development:
    - Carriers of *GSTM1-null* genotype exhibit 2-fold increased risk for overall RCC and specifically ccRCC development compared to the carriers of *GSTM1-active* genotype
    - Carriers of *GSTP1-variant* genotype exhibit 3-ford increased risk for overall RCC and specifically ccRCC development compared to the carriers of *GSTP1-wild type* genotype
- Combined *GSTM1-null/GSTT1-active/GSTA1- low activity/GSTP1-variant* genotype might be considered as "risk-associated genotype combination" in both overall RCC and ccRCC patients:
    - There is a dominant combined effect of *GSTM1-null/GSTP1-variant* genotypes on overall RCC and ccRCC risk development
    - There is a cumulative effect of "risk-associated genotype combination" on overall RCC and ccRCC risk development
- Significant modifying effect on ccRCC risk develompent conferred by hypertension was observed in carriers of *GST* variant genotypes*:*
    - Hypertensive patients with *GSTM1-null* genotype exhbit 6-fold increased risk of ccRRC development in comparison with normotensive patients with *GSTM1-active* genotype
    - Hypertensive patients with *GSTT1-active* genotype exhbit 3-fold increased risk of ccRRC development in comparison with normotensive patients with *GSTT1-null* genotype

- Hypertensive patients with *GSTA1-low activity* genotype exhbit 4-fold increased risk of ccRRC development in comparison with normotensive patients with *GSTA1-active* genotype
- Hypertensive patients with *GSTP1-variant* genotype exhbit 8-fold increased risk of ccRRC development in comparison with normotensive patients with *GSTP1-wild type* genotype

- The lack of modifying effect on ccRCC risk develompent conferred by obesity was observed in carriers of *GST* variant genotypes
- Significant modifying effect on ccRCC risk develompent conferred by smoking was observed in carriers of *GST* variant genotype.:
  - Smokers with *GSTP1-variant* genotype exhibit 4-fold increased risk of ccRCC development when compared to the non-smokers with *GSTP1-wild type* genotype
  - Smokers with *GSTP1-variant* genotype exhibit 3-fold increased risk of ccRCC development when compared to the smokers with *GSTP1-wild type* genotype
  - Smokers with combined *GSTM1-null/GSTP1-variant* genotype exhibit 5-fold increased risk of ccRCC development when compared to the smokers with *GSTM1-active/GSTP1-wild type* genotype
  - Smokers with combined *GSTM1-null/GSTP1-variant/GSTA1-low activity* genotype exhibit 7-fold increased risk of ccRCC development when compared to the smokers with *GSTM1-active/GSTP1-wild type/GSTA1-active* genotype
- The presence of *GSTM1-null* genotype is associated with significantly higher levels of BPDE-DNA adduct formation in ccRCC smokers
- The presence of *GST* variants is not associated with higher levels of byproducts of oxidative DNA damage (8-OHdG) in RCC patients, even when stratified according to the presence of recogized risk factors for RCC development
- The presence of *GSTM1* genotype is associated with RCC tumor grade:
  - *GSTM1-null* genotype was the most frequent in grade II (G2) RCC and ccRCC tumors
  - No association was observed between *GSTA1, GSTT1* and *GSTP1* genotypes and overall RCC and ccRCC stage

- *GSTM1* genotype represents a significant prognostic factor in overall RCC and specifically ccRCC patients:
    - Significantly higher frequency of the *GSTM1-null* genotype was observed in living overall RCC and ccRCC patients in comparison with the frequency of *GSTM1-null* genotype in deceased overall RCC and ccRCC patients included in the follow-up
    - Shorter overall survival was found for the whole group of RCC, as well as ccRCC patients with *GSTM1-active* genotype, compared to the RCC and ccRCC carriers of *GSTM1-null* genotype
    - *GSTM1-active* genotype was confirmed to be an independent predictor of higher risk of overall mortality compared to the *GSTM1-null* genotype, in the whole group of RCC and ccRCC patients, when analyzed in 3 different proportional hazard regression models
- The expression pattern of the analyzed GSTM1 and GSTP1 protein, as well as regulatory (ASK1, JNK1/2) and executor (Cleaved-Capsase 3) apoptotic molecules varied with tumor grade:
    - Significantly lower expression of the GSTM1 protein was found in ccRCC tissue samples with higher grade in comparison with non-tumor kidney tissue pool
    - Significantly lower expression of ASK1 was found in G2 and G3 ccRCC tissue samples
    - A gradual increase of GSTP1 protein expression across tumor grade was observed in ccRCC tissue samples
    - JNK1/2 expressesion was higher in non-tumor and G1 tumor tissue, compared to G2 and G3 in ccRCC tissue samples
    - The expression of Cleaved Caspase-3 gradually decreased across tumor grade in ccRCC tissue samples
- The supposed molecular mechanism underlying the prognostic role of GSTM1 protein is the negative regulation of apoptotic signaling pathways through sequestration of signaling ASK1 kinase by GSTM1 protein:
    - The presence of GSTM1:ASK1 protein:protein interaction was found in all ccRCC samples studied

- A strong positive correlation was found between GSTM1 and expression of executor (Cleaved Caspase-3) apoptotic molecules
- Cleaved Caspase-3 protein levels were lower in *GSTM1-active* individuals across tumor grade in comparison with Cleaved Caspase-3 protein levels in *GSTM1-null* individuals

- Non-catalytical regulatory role of GSTP1 protein was confirmed in all ccRCC tissue samples:
  - The presence of GSTP1:JNK1/2 complexes was found in all ccRCC tissue samples
- Overall, *GST* variants may contribute to individual RCC risk assessment in terms of both development and postoperative prognosis

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## LIST OF ABREVIATIONS

4-HNE: 4-hydorxynonenal

8-OHdG: 8-hydroxy-2'-deoxyguanosine

APS: amonium per-sulphate

ASK1: apoptosis signal-regulating kinase

B(a)P: benzo(a)pyrene

BCA: bicinchoninic acid

BMI: bodi mass index

BPDE: benzo(a)pyrene diolepoxide

BSA: bovine serum albumine

Ca: cases

ccRCC: clear renal cell carcinoma

chRCC: chromophobe renal cell racinoma

CI: confidence interval

Co: controls

DNA: deoxyribonucleic acid

DTT: dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

ELISA: enzyme linked immunosorbent assays

FAAD: Fas-Associated protein with Death Domain

GST: glutathione S-transferase

GSH: glutathione

HRP: horseradish peroxidase

IRAC: International Agency for Research on Cancer

ISUP: International Society of Urological Pathology

JNK1: c-Jun NH2-terminal kinase

MAPK: mitogen activated kinase

mRCC: metastatic renal cell carcinoma

OR: odds ratio

PAH: polyaromatic hydrocarbons

PARP: Poly (ADP-ribose) polymerase

PCR: polymerase chain reaction

pRCC: papillary renal cell carcinoma

RCC: renal cell carcinoma

RFLP: restriction fragment length polymorphism

RNOS: reactive nitrogen oxygen species

ROS: reactive oxygen species

RR: relative risk

SAPK: stress-activated protein kinase

SDS: sodium dodecyl sulfate

SDS-PAGE: sodium dodecyl sulfate-polyacrilamide gel electrophoresis

SNP: single nucleotide polyorphism

TCC: transitional cell carcinoma

TNM: tumor–node–metastasis

TRAF2: factor 2 bound to the TNFα receptor

Trx: thioredoxin

UICC: Union for International Cancer Control

UDP: Uridine diphosphate

WHO: World health organisation

## BIOGRAPHY

Teaching Assistant Vesna Ćorić, MD was born in Belgrade in 1985, where she completed her elementary and high school education with honors. In 2004, 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 Human Genetics (2005-2006), Institute of Medical Pathology (2007-2008) and at the Institute of Medical and Clinical Biochemistry (2007-2010), at the Faculty of Medicine, University of Belgrade. She received the Scholarship from Ministry of Education of Serbia (2005-2010) and Scholarship Endowment of *Dragoljub Marinkovic,* managed by the University of Belgrade (2008-2010). In 2007, Vesna Ćorić joined the research group headed by Professor dr Tatjana Simić, Vice Dean for Research at Faculty of Medicine, University of Belgrade. She took a special interest in studying the potential role of glutathione S-transferase polymorphisms in patients with renal cell carcinoma. She presented the results of her experimental work at numerous foreign and domestic conferences and won two prizes for best oral presentations. She graduated from the School of Medicine University of Belgrade in 2010, with average grade 9.31/10.00.

In 2010/2011, Vesna Ćorić enrolled in her PhD studies at the School of Medicine, University of Belgrade – Molecular Medicine module. In 2011, she started working at the Institute of Medical and Clinical Biochemistry as a Research Trainee, 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 2012, she became a Teaching Associate in the field of Medical Biochemistry. During the school year 2013/2014, Vesna Ćorić started her residency in Laboratory Medicine and in September 2014 she was promoted to a Teaching Assistant. She participated in numerous domestic and foreign conferences. Dr Ćorić attended the „SFRR-IUBMB Advanced School 2012" summer school in Spetses (Greece), where she won the prize for best poster presentation. In 2013 she spent 3 months in the ERAWEB (Erasmus Mundus – Western Balkans) academic exchange program in Torino (Italy) at the Medical Faculty, University of Torino, where she worked on a project entitled „Optimization of oncological therapy: new drugs against Multidrug Resistance", headed by Professor dr Chiara Riganti. From 2014-2016 Dr Ćorić acted as a PhD representative within the Executive board of ORPHEUS (Organization of PhD education in Biomedicine and Health Sciences in the European System) and during that time she organized

several workshops on PhD education. She participated in establishing the first PhD candidates' association at the School of Medicine, University of Belgrade.

Vesna Ćorić is the author/co-author of 15 *in extenso* papers published in journals indexed in the Science Citation Index (SCI). Her papers have been cited 76 times according to SCOPUS, and her h index is 4.

**Prilog 1.**

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Broj upisa: MM10/10

Studijski program: Molekularna medicina

Naslov rada **„ASSOCIATION OF GLUTATHIONE TRANSFERASE A1, M1, P1 AND T1 GENE POLYMORPHISMS WITH THE RISK OF RENAL CELL CARCINOMA DEVELOPMENT AND PROGRESSION"**

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