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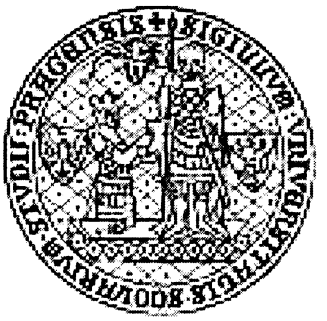

DIPLOMA THESIS

2007 **Alexandra Germanová**

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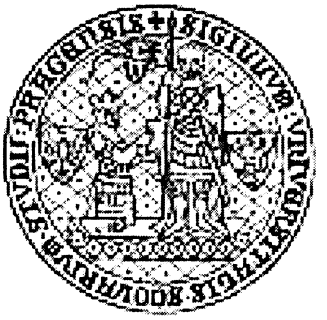

Diploma Thesis

Polymorphisms of Glyoxalase I

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Prague, 2007

I certify that I worked out this diploma thesis single-handedly under the supervision of my thesis supervisor Doc. MUDr. Marta Kalousová, Ph.D. and my thesis consultant RNDr. Marie Jáchymová, Ph.D. and I certify that I have properly cited all used sources.

Prague, 4th May 2007

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Contents

Abbreviations

3-DG-Arg-imidazolon	3-deoxyglucosone-arginine-imidazolon
Å	aangstroem (angstrom) 1Å=0.1 nm
AGEs	advanced glycation end products
ANOVA	analysis of variance
bp	basepair(s)
BSA	bovine serum albumin
CEL	N^{ε}-carboxy-ethyllysine
CML	N^{ε}-carboxy-methyllysine
CoA	coenzym A
ddNTP	dideoxyribonucleoside triphosphate
dNTP	deoxyribonucleoside triphosphate
ds-DNA	double-stranded deoxyribonucleic acid
EDTA	ethylenediamine-tetraacetic acid
ELISA	enzyme linked immunosorbent assay
EtBr	ethidium bromide
FXIII	Factor XIII - fibrin stabilizing factor
GHS	glutathione
GLO I	glyoxalase I
GLO II	glyoxalase II
GOLD	glyoxallysine dimer
HbA1c	hemoglobin A1c, glycated hemoglobin
HD	hemodialysis
HLA-DR	human leukocyte antigen-DR
HWE	Hardy-Weinberg equilibrium
ICAM-1	intercellular adhesion molecule-1
IL-1	interleukin-1
k_{cat}	molecular activity
kDa	kilodalton (1kDa = 1000 grams per mole)

K_m	Michaelis constant
LDL	low density lipoprotein
MOLD	methylglyoxallysine dimer
m-RNA	messenger ribonucleic acid
NEB 4 buffer	New England Biolabs 4 buffer
NF-κB	nuclear factor-κB
PAI-1	plasminogen activator inhibitor 1
PCR	polymerase chain reaction
RAGE	receptor for advanced glycation end products
RE	restriction enzyme
RFLP	restriction fragment length polymorphism
rpm	rotation per minute
SD	standard deviation
SDS	sodium dodecyl sulphate
SLS	salmiak lysis buffer
SNP	single nucleotide polymorphism
sRAGE	soluble receptor for advanced glycation end products
TNF	tumor necrosis factor
TNM classification	tumour nodes metastasis classification
TRIS	2-amino-2-hydroxymethyl-1,3-propanediol
UTR	untranslated region
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor

1 Introduction

Advanced glycation end products are the consequence of increased glucose level, oxidative and carbonyl stress and cause pathological changes in the organism. Glyoxalase I has a very important role in the degradation of theirs precursors. It is supposed that apart of other mechanisms, genetic aspects of advanced glycation end products and their metabolism and action may contribute to the development of several diseases.

1.1 Advanced glycation end products

Advanced glycation end products (AGEs) were described for the first time by French chemist Maillard in 1912 [1]. These compounds were first studied by food chemists, but as later research shows, AGEs are involved in the pathogenesis of several diseases, such as diabetes mellitus, cardiovascular diseases, chronic renal failure [2]. Advanced glycation end products are studied also in connection with cancer [3,4] and neurodegenerative diseases [5]. Their formation as well as their total amount in the organism is influenced genetically [6]. Because of that, more knowledge in this field of study can help to clear up the mechanism of pathogenesis of these diseases and prosper with its treatment.

1.1.1 General characteristic of advanced glycation end products

Advanced glycation end products are wide group of heterogeneous compounds, which are able to modify biological macromolecules both in plasma and tissues. Although only few of them were described yet, there are some of them characterised as model AGEs. The best known are: pentosidine, N^{ε}-carboxy-methyllysine (CML), N^{ε}-carboxy-ethyllysine (CEL), glyoxallysine dimer (GOLD), methylglyoxallysine dimer (MOLD), imidazolone (3-deoxyglucosone-arginine-imidazolon) and pyralline [7,8,9] (Figure 1-1, page 7).

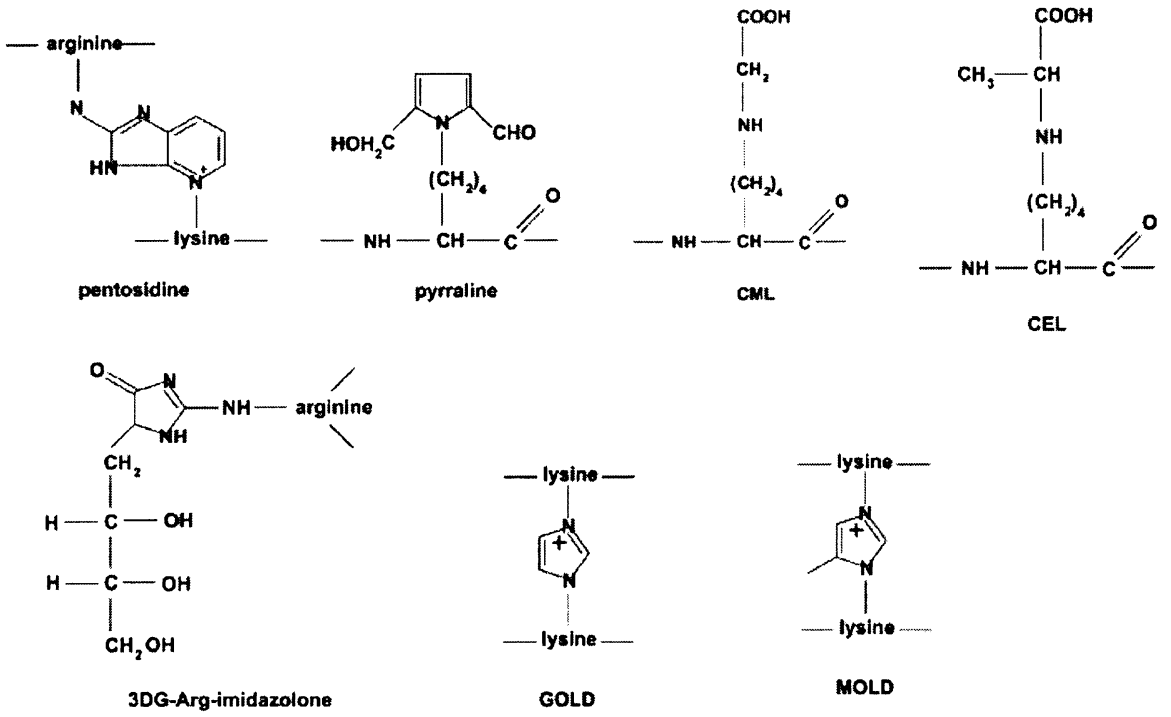


Figure 1-1. Structure of advanced glycation end products [from 10].

1.1.2 Formation of advanced glycation end products

There are several factors affecting the amount of advanced glycation end products in the organism. Their formation as well as their degradation is complex processes. First, the most common way of formation AGEs, is Maillard reaction (non-enzymatic glycation) [1]. Oxidative and carbonyl stress also increases advanced glycation end products levels in organism [11, 12]. In addition, very important role play also exogenous sources of AGEs (food [13] and tobacco smoke [14]).

1.1.2.1 Maillard reaction – the way of advanced glycation end product formation

First step, which takes about hours, is condensation of an aldehyde group of a reducing sugar with an ε-amino group (lysine residues) of protein, and the reversible formation of a Schiff base (Figure 1-2, page 8). This phase depends on the extracellular and intracellular glucose concentration [10]. Schiff base then rearranges into a more stable ketoamine, usually called Amadori product [15]. These compounds can be determined as

fructosamine (mainly glycated albumin) and glycated hemoglobin [10]. Conversion of Schiff base into Amadori product takes about days and it's partially reversible.

In the late phase, during weeks or months, independently of the sugar concentration, via condensation, dehydration, fragmentation and cyclization, advanced glycation end products arise [16]. AGEs can be formed from various sugars, both intra- and extracellular, e.g. glucose, fructose, glucose-6-phospate, glyceraldehyde-3-phospate [10, 17].

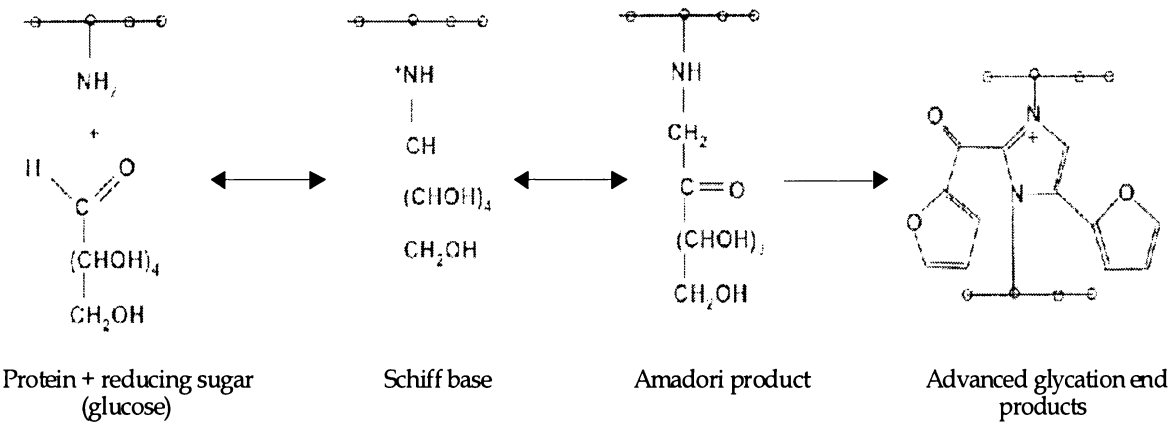


Figure 1-2. Maillard reaction [modified after 10].

1.1.2.2 Other ways of advanced glycation end products formation

Both oxidative and carbonyl stress are involved in AGEs formation.

Oxidative stress is characterised as an imbalance between reactive oxygen and nitorgen species or free radicals and antioxidants in favor of free radicals. In this case, physiological conditions of cells are disturbed. Autooxidation of sugars as well as others intermediates of Maillard reaction can give *reactive carbonyl compounds*, which are significant precursors of advanced glycation end products (Figure 1-3, page 9). Last but not least, lipoperoxidation of polyunsaturated fatty acids can also result in reactive carbonyl compounds formation [6, 10, 17].

Carbonyl stress is characterised as a reactive carbonyl compounds overload, which can be caused by increased formation and/or decreased clearance or detoxification of reactive carbonyl compounds [18]. Both oxidative and carbonyl stress are related. Reactive carbonyl compounds are highly reactive aldehydes or α-oxo-aldehydes derived from carbohydrates, ascorbate, lipids and aminoacids both by oxidative and non-oxidative pathways [10, 17] (Figure 1-4, page10).

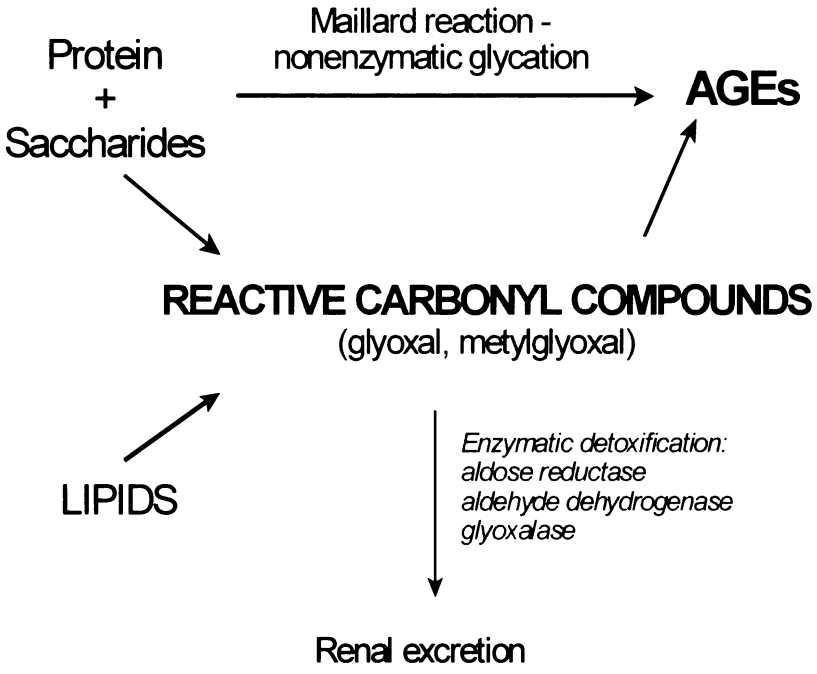


Figure 1-3. Formation of advanced glycation end products (AGEs) and reactive carbonyl compounds [from 10].

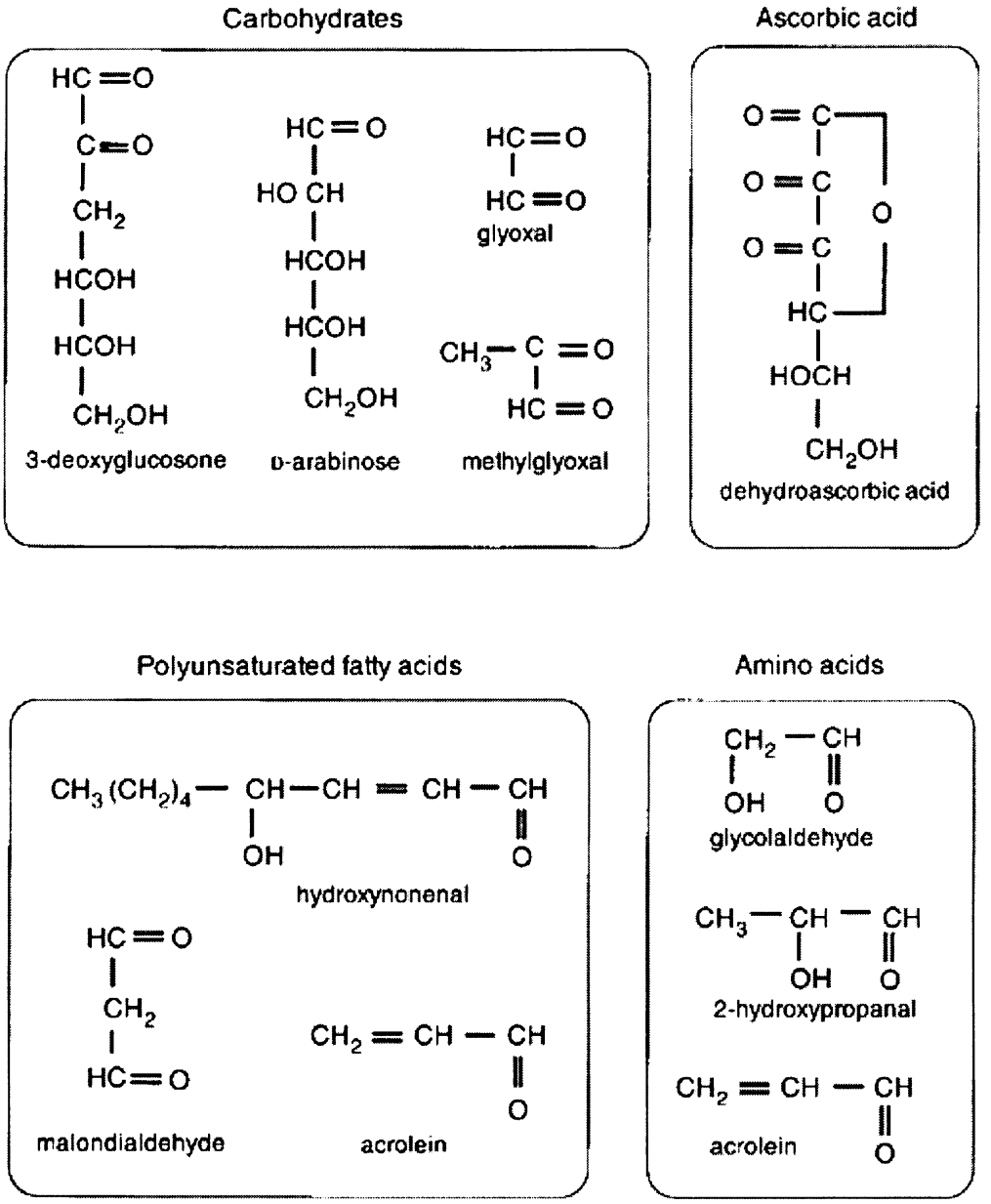


Figure 1-4. Structure of reactive carbonyl compounds derived from carbohydrates, ascorbate, lipids and amino acids [from 19].

1.1.3 Effects of advanced glycation end products in the organism

The yellow-brown pigmentation, linkage to the proteins, cross-linking ability and the reaction with AGE specific receptors are the main attributes of most of advanced glycation

end products [10]. Only some of them have characteristic fluorescence – excitation at 350 nm and emission at 430 nm (e.g. pentosidine).

AGEs can directly damage the structure of extracellular matrix, change its physical and chemical properties and metabolism [20]. Intracellular accumulation of advanced glycation end products is involved also in abnormal cross-linking formation, importantly at collagen level, which is very relevant mechanism of AGEs damage [21].

Each of AGEs-modified proteins has unique physical-chemical properties [17]:

a) increase resistance to enzymatic and thermic digestion,
b) relatively high acid-degradation stability,
c) variations in charges,
d) variations in solubility,
e) cross-linking,
f) decrease pI value.

Generally, increased level of advanced glycation end products causes modification of biological molecules, such as proteins, lipids and also nucleic acids. The glycation of DNA gives rise to characteristic nucleotide adducts, which are associated with mutagenesis and carcinogenesis [6].

The effect of advanced glycation end products on the cells is mediated also indirectly via their interaction with specific receptors. Several receptors for advanced glycation end products have recently been identified: RAGE, the best characterized receptor for AGEs, which is linked to cell activation, induction of oxidative stress and inflammatory response [22]; AGER1, a 50-kDa protein involved in ligand endocytosis and progressing [23]; AGER2, an 80-90 kDa protein involved in early signalling [24] and AGER3, contributing to both removal and cell activation [25].

RAGE – multiligand receptor consisting of the extracellular ligand-binding domain and transmembrane domain, can be expressed on the surface of various cells (e.g. monocytes, macrophages, mesanglial cells, neurons, endothelial cells, smooth muscle cells and fibroblasts). After AGE-RAGE interaction NF-κB is activated and this result to the production of cytokines, growth factors (TNF, IL-1) and adhesion molecules (ICAM-1, VCAM-1), increased vascular permeability and further toxic effects [17].

Soluble form of RAGE (sRAGE), primarily localised in blood, is a naturally occurring inhibitor of pathological effect mediated via RAGE. It is a RAGE isoform

lacking transmembrane domain and in humans it result from alternative splicing of RAGE mRNA [26].

1.1.4 Degradation of advanced glycation end products

Advanced glycation end products levels in the body reflect a balance between their formation and catabolism through tissue degradation and renal elimination. Tissue degradation occurs through endocytosis by macrophages and other cellular systems via receptor-specific or nonreceptor pathways. The subsequent proteolysis of advanced glycation end products determines the formation of low molecular weight AGE-peptides, which are excreted into the urine [21]. Specific enzymes, such as *aldose reductase, aldehyde dehydrogenase* or *glyoxalase,* have also important role in degradation of AGE-precursors, reactive carbonyl compounds [6, 10, 17] (Figure 1-3).

Glyoxalase system is one of the most important system of these involved in enzymatic degradation of precursors of AGEs. Its basic substrates are glyoxal and methylglyoxal, which are the most frequent precursors of advanced glycation end products in intracellular space and plasma [6]. For more details about glyoxalase system see 1.2, page 14.

1.1.5 Clinical backround of advanced glycation end products

Advanced glycation end products are generated under physiological conditions and their amount increases with aging. They have been implicated in the pathogenesis of several diseases, e.g. atherosclerosis, diabetes mellitus, chronic renal failure, neurodegenerative diseases (Alzheimer disease) and cancer.

Diabetes mellitus. After AGEs were first described and studied in food chemistry, they were shown to have a relation to the development of complications in diabetic patients [27, 28]. Diabetes mellitus is associated with hyperglycemia and thus with accelerated non-enzymatic glycation [20] and also with oxidative and carbonyl stress [19]. In diabetic patients, advanced glycation end products accumulate in tissues and take part in the development of diabetic complications – nephropathy, neuropathy, retinopathy and angiopathy. They cause damage to biological membranes and endothelium. Moreover, they

modify LDL particles and together with vascular damage, they are involved in the acceleration of atherosclerosis [20], disorder with common occurence in diabetic patients.

Chronic renal failure. In patients with decreased renal function, serum advanced glycation end product are elevated several fold more than in patients with normal renal function [10]. Pentosidine and carboxy-methyllysine are markedly elevated in both plasma proteins and skin collagen of uremic patients. Thus, carbonyl stress as well as oxidative stress in uremia may contribute to the long-term complications associated with chronic renal failure and dialysis, such as dialysis-related amyloids and accelerated artherosclerosis [19].

Cancer. Advanced glycation end products are studied also in connection with cancer. Both oxidative and carbonyl stress may contribute to the process of carcinogenesis [3,4]. Glyoxal and methylglyoxal induce multi-base deletions and base-pair substitutions [6]. Methylglyoxal also produces an increase in sister-chromatid exchanges, which correlates to mutagenic activity and stimulates synthesis of VEGF (vascular endothelial growth factor) and induces the apoptosis of T-cells. Glyoxal activates some proteinkinases (e.g. c-src) and elevates intracellular tyrosine phosporylation [29]. Tumours increasingly uptake glucose and in tumour tissue the glycolysis is more frequented, thus it can lead to the incerased amount of glycated proteins. Presence of advanced glycation end products (CML, argpyrimidine) was demonstrated in several human cancer tissues [30].

1.2 Glyoxalase system

The glyoxalase system is present in the cytoplasm of cells and cellular organelles, particularly mitochondria. Many researches suggest it's ubiquitous [31, 32]. This enzyme system is expressed in a variety of tissues including in normal and malignant tissue, in embryos and differentiated tissue, and in infected and noninfected tissues [32]. It's among the earliest expressed during embryogenesis and development and persists through maturation, adult life and senescence [33].

1.2.1 Glyoxalase pathway

The glyoxalase system catalyses the alteration of reactive acyclic α-oxoaldehydes to the corresponding α-hydroxyacids. It comprises two enzymes [34]: glyoxalase I (GLO I), glyoxalase II (GLO II) and a catalytic amount of reduced glutathione GHS (threepeptid γ-L-glutamyl-L-cysteinylglycine) as a cofactor [31, 35].

Glyoxalase I catalyses the isomeration of the hemithioacetal, formed non-enzymatically from α-oxoaldehyde and GHS [36], into the thioester of GHS and the corresponding α-hydoxyacid (S-2-hydroxyacylglutathione). Glyoxalase II catalyses the hydrolysis of this compund to the corresponding α-hydroxyacid and reforms GHS [37,38] (Figure 1-5, page 15).

1.2.2 Glyoxalase I (EC 4.4.1.5)

Glyoxalase I (isomerase) is a member of the vicinal oxygen chelate superfamily which also includes as different enzymes as *extradiol dioxygenase* (oxidative cleavage of C-C bond), *fosfomycin resistance protein* (nucleophilic substitution), *methylmalonyl-CoA epimerase* (epimerizations) *and bleomycin rezistance protein* (no enzyme, sequestration).

These proteins share the same βαββ-fold that provide a metal coordination site. All enzymes of this superfamily use a divalent metal ion to perform catalysis [39].

Glyoxalase I activity is present in all tissues of prokaryotic and eukaryotic organisms. Up to now, more than sixty glyoxalase I sequences have been reported: human, murine,

yeast, plant, protozoal, fungal and bacterial. Except yeast, all enzymes are dimeric. The yeast enzyme is monomer, but with two copies of a segment equivalent to the monomer of the human enzyme [40]. The sequence identity of human glyoxalase I with the bacterial enzyme (*Pseudomonas putida*) is 55% [40] and with the yeast enzyme between residues 1-182 and 183-326 (*Saccharomyces cerevisiae*) is 47%, suggesting glyoxalase I of different origins may have arisen by divergent evolution from a common ancestor [41]. For more details about human glyoxalase I see 1.3, page 17.

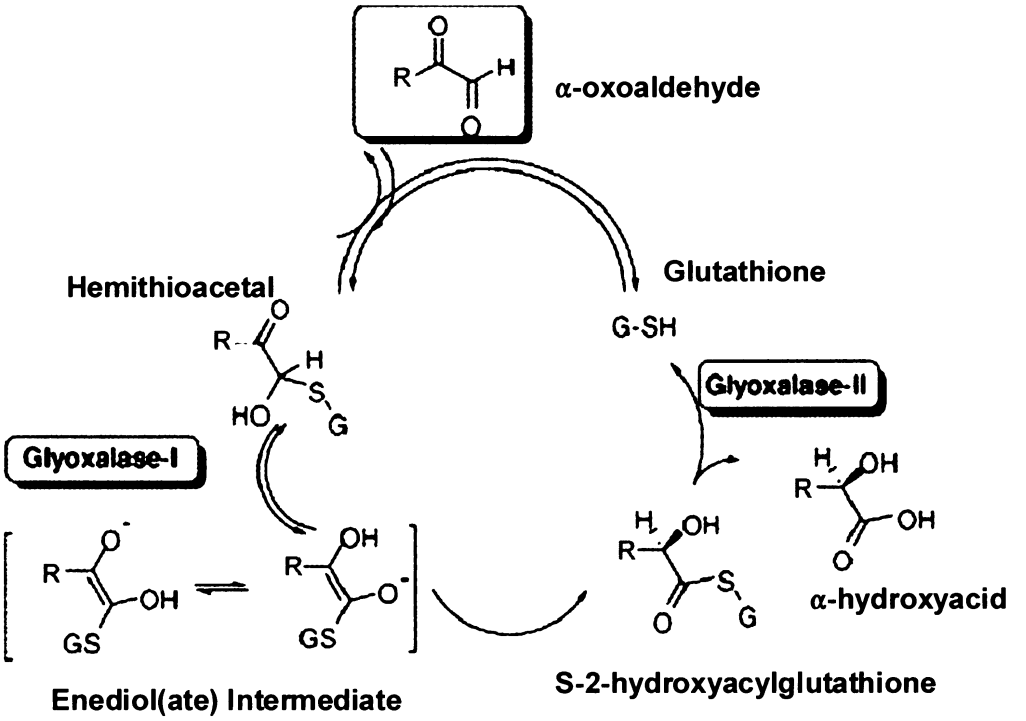


Figure 1-5. Glyoxalase pathway [modified after 42].

1.2.3 Glyoxalase II (EC 3.1.2.6)

Glyoxalase II catalyses the conversion of S-2-hydroxyacylglutathione derivates to an α-hydroxyacid and reforms GHS, consumed in the glyoxalase I - catalysed reaction step.

Glyoxalase II is a member of the zinc metalloenzymehydrolase family having a β-lactamase fold [43]. It's present both in cytosol and in mitochodndria of human cells. Human glyoxalase II is a 260 amino acid protein, with isoelectric point 8.5. The enzyme is a monomer with a molecular mass of 29 kDa. The gene for glyoxalase II is situated on chromosome 16, and there is essentially only one phenotype exppressed [44], although a second rare variant was described in certain population [45].

1.3 Human glyoxalase I

Human glyoxalase I is a 184 amino acid protein with a molecular weight 42 kDa (sequence) or 46 kDa (gel filtration) and an isoelectric point of 4.8-5.1 [40]. The N-terminal Met is removed in post-translational processing by methionine aminopeptidase. There are four possible phosporylation sites – two serines and two threonines (residues 108-111), but no phosphorylation of enzyme have been reported [46].

Glyoxalase I activity is present in all human tissues. Specific activities of fetal tissues are about 3 times higher than corresponding adult tissues. There is about 0.2 μg of enzyme per gram of protein in human tissues and blood cells [40].

Methylglyoxal is the main physiological substrate for glyoxalase I. It is formed as a by-product from the triose-phospate isomerase reaction in glycolysis [47], from acetone in ketone body mechanism [48] and from aminoacetone in threonine catabolism [49]. Glyoxal, another significant substrate, is formed by lipid peroxidation and degradation of glucose and glycated proteins [50]. Both glyoxal and methylglyoxal are elevated also by oxidative and carbonyl stress and they are the most frequent precursors of AGEs of intracellular space and plasma.

Methylglyoxal (glyoxal) is converted into S-D-lactoylglutathione (S-Glycolyl-glutathione) and then hydrolysed to D-lactate (glycolate), respectively.

For the methylglyoxal-glutathione hemithioacetal and human glyoxalase I, Michaelis constant K_m is 71-130 μM and molecular activity (turnover number) k_{cat} is (7-11) x 10^4 min^{-1} [40].

1.3.1 Structure of glyoxalase I

Structure of human glyoxalase I in complex with S-benzyl-glutathione was resolved by X-ray crystallography at 2.2 Å resolution [51]. This enzyme is composed of two identical monomers. Each monomer is built up from two domains (residues 31-104 and 124-183), linked by a 20 residue connection, and from a long N-terminal arm (Figure 1-6A, page 18). Every domain contains common motif of βαββββ topology. The active site

is situated in a barrel, which is formed from the two subunits interacting mutually in an anti-parallel fashion, thus the active site provide only active dimer [38] (Figure 1-6B).

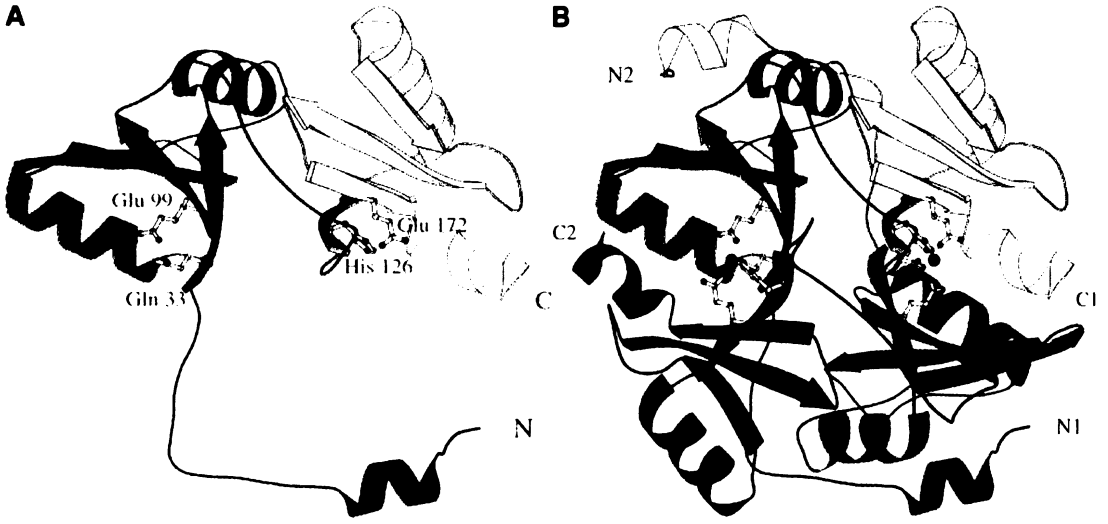


Figure 1-6. Human glyoxalase I.: A) monomer, B)dimer [from 38].

Zinc binding site of glyoxalase I

Each subunit of the dimeric enzyme contains one zinc ion, Zn^{2+}, necessary for its catalytic function. The apoenzyme is catalytically inactive. Significant catalytic activity was demonstrated also with some others divalent ions, like Mg^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, and Ca^{2+} [52, 53].

The metal ion is ligated in the active site by two residues from each domain – Gln 33 and Glu 99 from the first domain and His 126 and Glu 172 from the second domain. Depending on whether an inhibitor or a product is bound to the active site, one or two water molecules might be ligated to the zinc ion to complete octahedral coordination [38, 54, 55, 56].

Glutathione binding site of glyoxalase I

The interactions of glutathione conjugate with glyoxalase I are markedly different from those seen in other enzymes binding glutathione (for example, glutathione transferase), where there are salt links and hydrogen bonds to the polar groups of all three residues of a tripeptide [57]. In glyoxalase I, the only polar interaction with the protein involve the γ-glutamyl residue of glutathione, although they are highly specific [38].

1.3.2 Reaction mechanism of glyoxalase I

Glyoxalase I catalyse the isomeration of hemithioacetal, which is formed spontaneously from α-oxoaldehyde and GHS, into S-2-hydroxyacylglutathione (Figure 1-5, page 15).

The mechanism proposed for the glyoxalase I reaction involves base-catalysed shielded-proton transfer from C-1 to C-2 of the hemithioacetal, bound in the active site, to form a *cis*-ene-diolate intermediate, and rapid ketonization to the thioester product [38]. The main catalytic role of the Zn ion is to electrostatically stabilize the ene-diolate intermediate and reduce the activation free energy of proton transfer [55] (Figure 1-7).

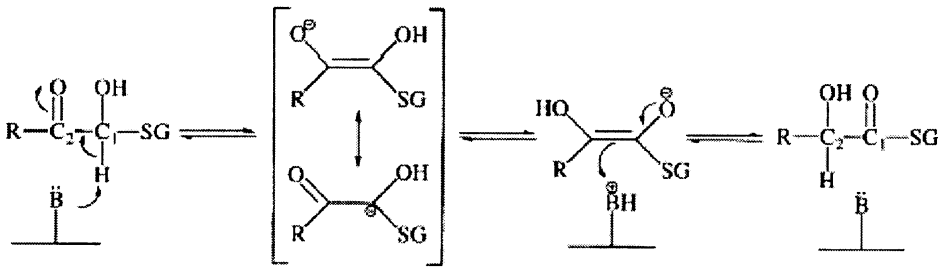


Figure 1-7. Catalytic mechanism of glyoxalase I. A shielded base (B) removes the proton from C1 atom of the hemithioacetal and then reprotonates C2 [from 51].

The mechanism of reaction depends on the stereospecifity of substrate. Both S and R substrate give the same product, indicating stereospecific proton transfer.

In S reaction, Glu-172 abstracs the proton from C1 and leaves the zinc center, and then zinc-bound enediolate intermediate is creating. In the second step, Glu-172 delivers proton to C2 to create a S-2-hydroxyacylglutathione derivate (Figure 1-8, page 21).

In R reaction, Glu-99 is the abstracting base, but cannot be a delivering base, since this would yield to imprecise product. Thereby C2 must receive the proton again from Glu-172 [55, 56] (Figure 1-9, page 22).

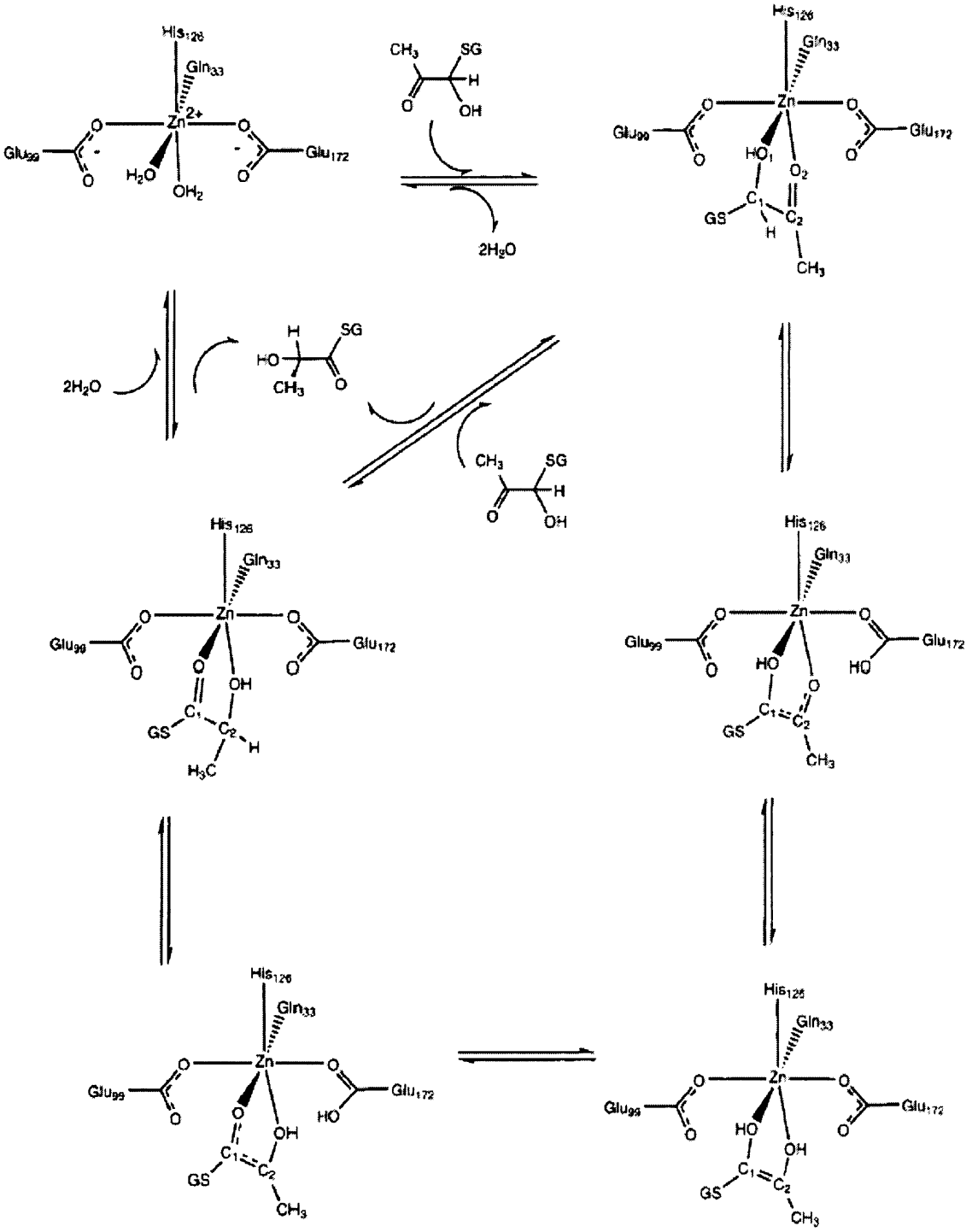


Figure 1-8. Proposed reaction mechanism for the S enantiomer of the substrate – methylglyoxal [from 55].

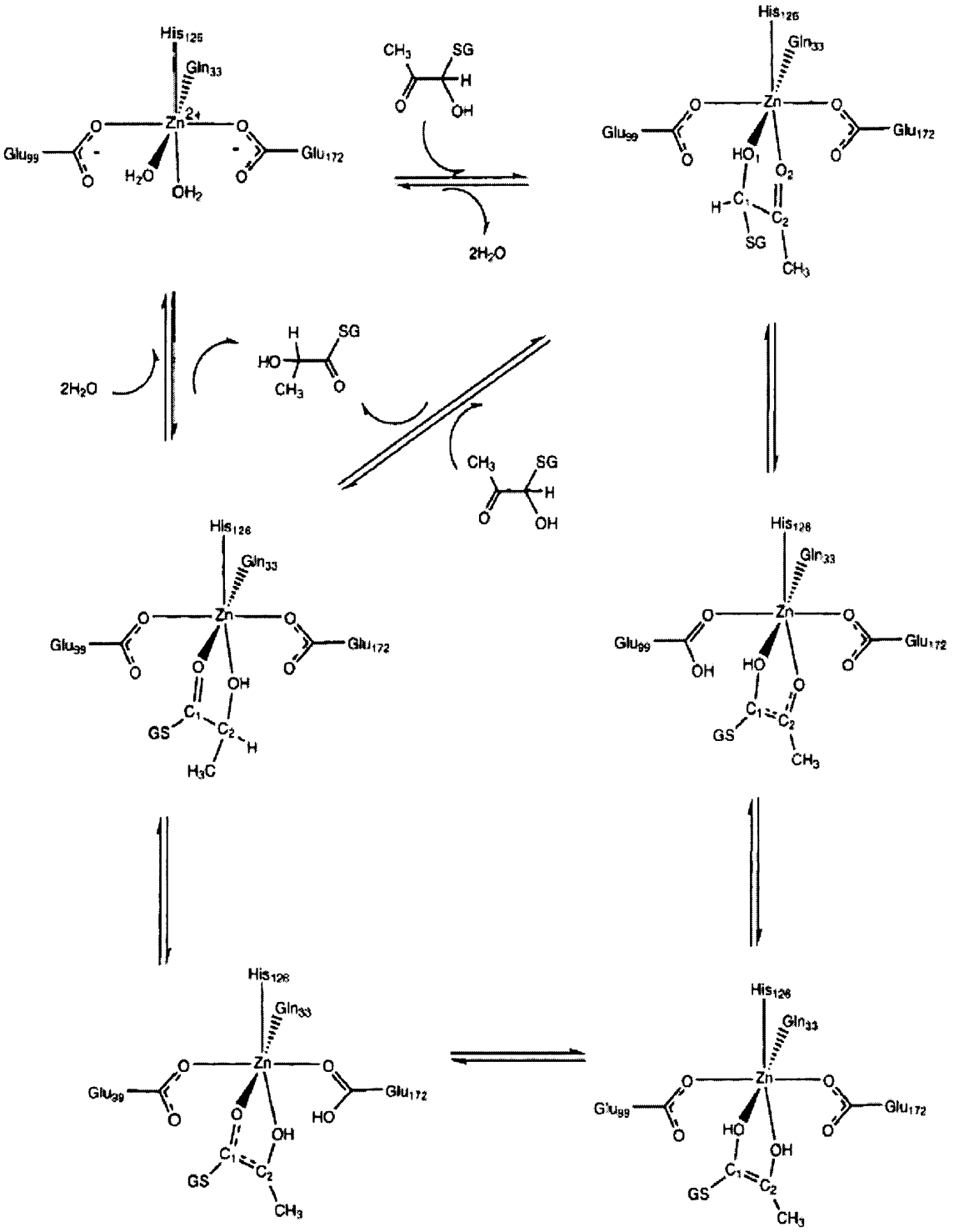


Figure 1-9. Proposed reaction mechanism for the R enantiomer of the substrate-methylglyoxal [from 55].

1.3.3 Genetic characterization of glyoxalase I

Gene for glyoxalase I is situated on chromosome 6 (locus 6p21,3 – 6p21,2), close to the major histocompatibility complex HLA-DR [31]. It contains 6 exons, comprising 27,215 bp from the translation start site to the poly(A)site [32]. The gene promoter contains insulin response element and metal response element, indicating, that $ZnCl_2$ and insulin regulate the activity of the enzyme [58].

There are several variations in the sequence of glyoxalase I gene and some of them cause amino acid change in the sequence of protein. Five single nucleotide polymorphisms (SNPs) in the exon areas, which are registered in SNPs database of National Center for Biotechnology Information, induce amino acid replacement [59] (For more details see 1.4.2, page 24).

In 1975, Kompf et al. [60] first described three phenotypes of human glyoxalase I, GLO 1-1 (homozygote CC), GLO 1-2 (heterozygote AC) and GLO 2-2 (homozygote AA), representing the homozygous and heterozygous expression of a diallelic gene, GLO^1 (allele C) and GLO^2 (allele A). Glyoxalase I alleles are in a simple co-dominant manner, with characteristic phenotypic expression present in all tissues. As Kim et al. [61] reported, difference in the allele expression products relate with the amino acid change at position 111 in the sequence of protein. Adenine/cytosine variation in the position 20 203 from the translation start site cause glutamic acid/alanine alteration in the position 111 in the protein sequence. This polymorphism was studied in several disorders yet (See 1.4.2.1, page 26).

The population genetics of GLO^1 allele frequency shows that this frequency is highest in native tribes in Alaska, and decreases geographically South and East to Europe and South America, through Africa, the Middle East and India, to the very low GLO^1 allele frequencies of the Far East and Oceania [62].

Recent research suggests six different transcripts of glyoxalase I, but they vary only in their 3‘ or 5‘ sequence. No insertion or deletion of strings of amino acids within the body of the protein was reported, thus it seems, glyoxalase I transcript does not report to an alternative exon splicing [32].

1.4 Genetic polymorphism

Already in 1940 Ford et al. defined *genetics polymorphism* as the occurrence in the same population of more than one allele in the same locus in at least 1% of a population. We can observe polymorphism in DNA, RNA or peptide sequence (biochemical polymorphism). There is also chromosome polymorphism (wild variety of modification in chromosomal structure) [63].

1.4.1 Single nucleotide polymorphism SNP

The simplest type of genetics polymorphism is a *single nucleotide polymorphism*. In this case, there is a variation only in a single nucleotide in nucleotide sequence.

SNPs may fall within coding sequences of genes, noncoding regions of genes, or in the intergenic regions between genes. Single nucleotide polymorphisms within a coding sequence will not necessarily change the amino acid sequence of the protein that is produced, due to the degeneracy of the genetic code. A SNP in which both forms lead to the same polypeptide sequence is termed *synonymous* (a silent mutation), if a different peptide is produced they are *nonsynonymous*. SNPs that are not in protein coding regions may still have the consequences for gene splicing, transcription factor binding, the sequence of non-coding RNA, or stability of protein.

Single nucleotide polymorphisms make up 90% of all human genetic variations, and SNPs with a minor allele frequency of ≥ 1% occurs every 300-1000 bases along the human genome [64].

1.4.2 Single nucleotide polymorphisms of glyoxalase I gene

The high gene to SNP ratio in regions 6p21.3 and 6p21.2 (approximating the GLO I locus) suggest it is highly conserved and nucleotide variants are clinically disadvantageous.

Gale [32] identified 70 single nucleotide polymorphisms of the glyoxalase I gene using the methods of bioinformatics:

- six within the first 4.5 kb of 5‘ UTR, one of them within the 5‘ non-coding region of the primary transcript (-7 C to T, from the translation start site)

- sixty within the introns
- three within the 3‘ UTR
- one within the coding sequence: A20 203C variation in DNA sequence causes an amino acid change at position 111 (Glu111Ala) in the amino acid sequence.

In SNPs database of National Center for Biotechnology Information [59], there are 121 SNPs of GLO I gene registered:

- eight SNPs in the exons (Table 1-1)
- eleven SNPs in untranslated regions
- 102 SNPs in the introns

Table 1-1. Single nucleotide polymorphisms of glyoxalase I within the exons [from 59].

Exon			Nt change	AA change	AA position
Exon 1	nonsynonymous	wild-type	C	Cys [C]	19
		mutation	A	Tyr [Y]	
Exon 2	nonsynonymous	wild-type	C	Leu [L]	31
		mutation	T	Leu [L]	
Exon 3	nonsynonymous	wild-type	A	Tyr [Y]	71
		mutation	G	Cys [C]	
	synonymous	wild-type	C	Ile [I]	81
		mutation	T	Ile [I]	
	nonsynonymous	wild-type	C	Pro [P]	82
		mutation	A	His [H]	
Exon 4	nonsynonymous	wild-type	A	Glu [E]	111
		mutation	C	Ala [A]	
	nonsynonymous	wild-type	C	Pro [P]	122
		mutation	A	Thr [T]	
	synonymous	wild-type	A	Gly [G]	124
		mutation	T	Gly [G]	

It was already mentioned, that the nucleotide variation within the 3‘ or 5‘ UTRs may affect glyoxalase I expression, stability or posttranscriptional regulation, but still the most important polymorphisms are those, which cause an amino acids replacements. Only two SNPs of glyoxalase I gene have been studied in association with selected disorders yet (See 1.4.2.1).

1.4.2.1 Glyoxalase I and the single nucleotide polymorphism Glu111Ala

Polymorphism Glu111Ala was first described by Kompf et al. in 1975 in red blood cells [65]. This mutation is relatively common in the West and has been studied in many populations [62]. It is caused by nucleotide variation (wild type adenine to cytosine) in position 20 203 from the translation start site (position 419 in m-RNA).

Glyoxalase I was studied in several human malignances, such as in colon tumor tissue [66], neoplastic lung tissue [67] and in tumor urgenital tissue [68].

Samadi et al. [69] investigated glyoxalase I phenotypic expression in the prostatic tissue from patients with prostate cancer and the significantly high frequency ($P<0.0001$) of the GLO 2-2 (homozygote AA) phenotype was detected, suggesting it is a potential risk factor for prostate cancer.

Another study of glyoxalase I gene frequencies has shown, that there is a significant excess of glyoxalase homozygote GLO 1-1 (homozygote CC) and a deficiency of types GLO 2-1 (heterozygote AC) and GLO 2-2 (homozygote AA) in insulin-dependent diabetic patients [70].

Politi et al. [71] tested the hypothesis, that Glu111Ala is related with panic disorder. The result of the study did not point toward a major role of this polymorphism in the pathogenesis of panic disorder. Nevertheless, it was found to be associated with the diagnosis of specific type of panic disorder with about 50% incidence within the population with this disease.

The Glu111Ala was also studied as a possible autism susceptibility factor. Results show decrease (38%) in glyoxalase I activity and accumulation of advanced glycation end products in autism brains. In addition, this study suggests, the homozygosity for A20 203 resulting in Glu111 is a predisposing factor of autism [72].

Chen et al. [73] studied this polymorphism in context of Alzheimer’s disease, but no relationship between this polymorphism and disease was observed.

A significant association was found between Glu111Ala and pro-thrombic factor concentrations, so it may alter susceptibility to macrovascular complications [74].

Glyoxalase I deficiency is associated with unusually high plasma levels of advanced glycation end products in a hemodialysis patients [75]. Till now, no study resolving glyoxalase I polymorphisms in connection with uremic complications was published.

1.4.2.2 Single nucleotide polymorphism –7 C to T

This polymorphism may be functionally important because it approximates minimal promoter [58] and DNA-dependent RNA polymerase site. The C allele was predicted to bind CG-factor, a transcription factor that represses transcription, whereas the T allele did not, which may lead elevated AGEs levels through reduced glyoxalase I expression [76].

SNP –7 C to T was studied only in connection with pro-thrombic factors and the result shows the relevant importance to these factors [74].

2 Aim of study

1. ***To establish the methods for the single nucleotide polymorphisms detection:***

   - To establish the method for detection of Glu111Ala polymorphism of the glyoxalase I gene.
   - To establish the method for detection of –7 C to T (from the translation start site) polymorphism of the glyoxalase I gene.

2. ***Clinical application of the established method:***

   - To study Glu111Ala polymorphism in hemodialysis patients.
   - To study Glu111Ala polymorphism in patients with breast cancer.
   - To study Glu111Ala polymorphism in healthy controls for comparison.

3 Materials and methods

3.1 Materials

3.1.1 Chemicals and other materials

a) for isolation of DNA

Sodium chloride, G.R. (Penta, Czech republic), *Ammonium chloride, G.R.* (Fluka, Germany), *Ammonium hydrogen carbonate, G.R.* (Fluka, Germany), *Magnesium chloride hexahydrate, G.R.* (Fluka, Germany), *Kalium chloride, G.R.* (Lachema, Czech republic), *Ethylenediamine-tetraacetic acid* EDTA, *G.R.* (Sigma, USA), *Sodium dodecyl sulphate* SDS, *G.R.* (Ampresco®, USA), *2-amino-2-hydroxymethyl-1,3-propanediol* TRIS, *G.R.* (MP Biomedicals, USA), *Hydrogen chloride, G.R.* (Lachema, Czech republic), *Sodium hydroxide, G.R.* (Lachema, Czech republic), *Ethanol*, 96perc, (v/v) (Penta, Czech republic), *Protienkinase K* (Roche, Germany)

b) for PCR-RFLP analysis and DNA sequence analysis

Isopropanol G.R. (Penta, Czech republic), *Ethanol*, 96perc, (v/v) (Penta, Czech republic), *Sodium acetate G.R.* (Merck, Germany), *Sucrose G.R.* (Ampresco®, USA), *Tetrabromophenolsulfonephthalein - Bromophenol blue* (Lachema, Czech republic), *Boric acid G.R.* (Penta, Czech republic), *2-amino-2-hydroxymethyl-1,3-propanediol* TRIS *G.R.* (MP Biomedicals, USA), *Ethylenediamine-tetraacetic acid* EDTA *G.R.* (Sigma, USA), *Ethidium bromide* (Ampresco®, USA), *Agarose* (Serva, Germany), *MetaPhor® Agarose* (Cambrex, USA), *PCR Core Kit* (Roche, Germany), *Taq DNA polymerase* (Fermentas, USA), *O'GeneRuler^{TM} 50bp DNA ladder* (Fermentas, USA), *O'GeneRuler^{TM} 100bp DNA ladder* (Fermentas, USA), *Primers for PCR* and *DNA sequence analysis* (Generi Biotech, Czech republic), *Restriction enzymes Tsp RI, Bts I* (BioLabs, USA), *Restrictiom enyzme Bsm AI* (Fermentas, USA), *Genome Lab^{TM} DTCS – Quick Start Kit* (Beckman Coulter, USA), *Genome Lab^{TM} Separation gel – LPA I.* (Beckman Coulter, USA), *QIAquick Gel Extraction Kit* (QIAGEN, USA)

3.1.2 Instruments

- Water treatmet: Direct-Q® 3 UV, Milipore, France; Meladest® 65, Melag, Germany
- Centrifuges: Rotana 460R, Hettich, Germany; Universal 32 R, Hettich, Germany
- Thermal Cycler: DNA Engine Dyad^{TM} Peltier Thermal Cycler, Bio-Rad, USA
- DNA sequencing: CEQ^{TM} 8000 Genetic Analysis System, Beckman Coulter, USA
- Horisontal nucleid acid electrophoresis tank Minigel 2, Apelex, France
- Electrophoresis power supply Apelex PS 1006, France
- UV transilluminator TS-312A Spectroline®, USA
- Microwave oven: Micromaxx® MM 41580, Germany
- Photometer: Biophotometer 6131, Eppendorf®, Germany
- Cuvettes: UVette®, Eppendorf®, Germany
- PCR cooler, Eppendorf®, Germany
- Precision weighing balance Snug 600, AdamLab, UK
- Vortex: Biovortex V-1 plus, Biosan, Latvia
- Sample incubator: Dry-block® heater, DB-2A Techne, USA
- Corning® filter systems, USA

3.2 Methods

All the procedures I used to do in the medical examination gloves and all used materials and working place were sterile.

3.2.1 Isolation of DNA

Isolation of DNA was performed by modified salting out procedure after Miller et al. [77].

3.2.1.1 Principle of isolation of DNA

Red blood cell lysis in the presence of salmiak lysing buffer (SLS) and nuclear cell lysis in the presence of sodium dodecyl sulfate (SDS) and proteinase K followed by salting-out of residual polypeptides by dehydration and precipitation with saturated sodium chloride solution. In final phase, precipitated DNA is dissolved by ethanol.

3.2.1.2 Solutions for isolation of DNA

All used solutions, except ethanol, 10 M NaOH and 2 M HCl, were filtered by filter system Corning and stored at 2-8°C. Ethanol was stored at –20°C before use.

- **0.5 M EDTA** (stock solution, 500 ml): 39.05 g EDTA, 5 ml 10 M NaOH , add re-distilled water into volume 500 ml
- **10 M NaOH** (100 ml): 40 g NaOH, add re-distilled water into volume 100 ml
- **SLB** (stock solution, 500 ml): 41.45 g NH_4Cl, 3.95 g NH_4HCO_3, 1 ml 0.5 M EDTA, add re-distilled water into volume 500 ml
- **TK-1 buffer** (2000 ml, pH 7.6): 1.488 g KCl, 2.422 g TRIS, 4.064 g $MgCl_2.6H_2O$, 8 ml 0.5 M EDTA, 2 M HCl for pH arrangement, add re-distilled water into volume 2000 ml
- **2 M HCl** (100 ml)**:** 8.5 ml HCl, add re-distilled water into volume 100 ml
- **4 M NaCl** (200 ml): 46.75 g NaCl, add re-distilled water into volume 200 ml

- **Lysis buffer for nuclear cells** (500 ml, pH 8.2): 50 ml 4 M NaCl, 5 ml 1 M TRIS-HCl (pH 8.2), 2 ml 0.5 M EDTA, add re-distilled water into volume 500 ml
- **1 M TRIS-HCl** (100 ml, pH 8.2): 12.1 g TRIS, 2 M HCl for pH arrangement, add re-distilled water into volume 100 ml
- **Proteinase K dissolvent solution** (200 ml)**:** 10 ml 10% SDS, 0.8 ml 0.5 M EDTA, add re-distilled water into volume 200 ml
- **10% SDS w/v**, keep in laboratory temperature
- **Proteinase K working solution** (50 ml): 100 mg proteinase K, 50 ml proteinase K dissolvent solution; keep 600 µl aliquotes at –70°C
- **96% ethanol**
- **70% ethanol**
- **6 M NaCl** (100 ml): 35.1 g NaCl, add re-distilled water into volume 100 ml
- **TE buffer** (40 ml): 400 µl 1 M TRIS-HCl (pH 7.6), 80 µl 0.5 M EDTA, add re-distilled water into volume 40 ml

3.2.1.3 Working procedure of isolation of DNA

The red blood cell lysis was done according to following steps:

1. Dilute blood sample (7 ml, EDTA) in 50 ml sterile centrifugation tube with 40 ml cooled 10 times diluted SLB buffer. Incubate the tube for 30 minutes at 0°C before centrifugation (1500x g, 30 minutes, 10°C). Remove supernatant, add 40 ml cooled 10 times diluted SLB buffer again and repeat the procedure.
2. After supernatant separation, add 30 ml TK-1 buffer to the tube and shake vigorously. Centrifuge 20 minutes at 3000 rpm (10°C), for the red blood cell membranes elimination. Repeat this step three times, until the nuclear cell pellet is purely white.

The isolation of DNA was done accorging to following steps:

3. Resuspend the leukocyte pellet with 3 ml of lysis buffer for nuclear cells and shake vigorously. Incubate the cell lysates overnight at 37°C with 600 µl of proteinase K working solution and 200 µl 10% SDS.

4. After digestion is complete, add 1 ml of 6 M NaCl to the mixture and shake vigorously for 15 second. Centrifuge 30 minutes at 6000 rpm at laboratory temperature.
5. Leave the precipitated protein pellet at the bottom of the tube and transfer the supernatant containing the DNA to another 50 ml tube. At least, add 3 volumes of frozen (-20°C) 96% ethanol and invert the tube several times until the DNA is precipitated (Figure 3-1).
6. Remove the precipitated DNA strands with the sterile glass hooklet and rinse it with the frozen (-20°C) 70% ethanol.
7. Transfer the glass hooklet with the dry DNA to the sterile cryo-micro tube containing 1000 µl TE buffer. The DNA is dissolved at laboratory temperature overnight.

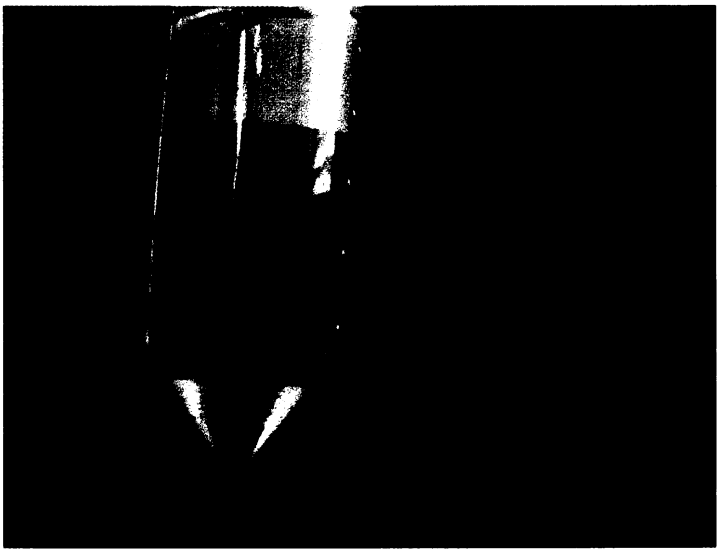


Figure 3-1. Precipitated DNA.

3.2.2 Concentration of DNA in samples

The concentration of isolated DNA was measured by Biophotometer 6131 (Eppendorf).

3.2.2.1 Principle of the concentration determination

The concentration of DNA is determined by the spectrophotometric analysis. The measured value of ultraviolet (UV) absorbance at 260 nm is transfered into concentration: one degree of optical density equates to the 50 μg/ml ds-DNA. As an indication of the purity of the nucleic acid sample which has been measured, the absorbance at 230, 280 and 320 nm as well as the ratios A_{260}/A_{280} and A_{260}/A_{230} are displayed in addition to the concentration result and the absorbance at a wavelength of 260 nm. With pure samples, the absorbance at 320 nm should be approximately zero and the ratios A_{260}/A_{280} and A_{260}/A_{230} shoud be approximately 2.

3.2.3 PCR-RFLP analysis

SNP Glu111Ala. This polymorphism is caused by the adenine (wild-type)/cytosine variation in the position 20 203 from the translation start site (position 419 in m-RNA), which leads to the glutamic acid (wild-type)/alanine alteration at the position 111 in the sequence of the enzyme glyoxalase I.

I have established the method for polymorphism detection based on PCR-RFLP analysis. The variance of adenine to cytosine leads to the cancellation of restriction site of the restriction enzyme Bsm AI and thus, this SNP can be detected pursuant to the length of the DNA fragments varies.

SNP -7C to T (from the translation start site) is localized in the promoter area of the GLO I gene and it does not lead to the amino acid variation, but the nucleotide variation leads to the creation of restriction site for the restriction enzymes Tsp RI and Bts I. This polymorphism, as well as SNP Glu111Ala, was analysed by PCR-RFLP method and the results were confirmed by DNA sequence analysis (See 3.2.4, page 41).

3.2.3.1 Principle of PCR-RFLP analysis

First step of PCR-RFLP analysis is polymerase chain reaction

Polymerase chain reaction (PCR), invented by Kary B. Mullis (awarded the 1993 Nobel Price for chemistry for PCR), is a technique to exponentially amplify in vitro a small quantity of a specific nucleotide sequence in the presence of template sequence, two oligonucleotide primers that hybridize to the opposite strands and flank the region of interest in the target DNA, a thermostable DNA polymerase, a dNTPs and a Mg^{2+} ions. The reaction is cycled (circa 30times) involving template denaturation (95°C), primer annealing (temperature depends on T_m of primers), and the extension of the annealed primers by DNA polymerase (70-72°) until enough copies are made for further analysis (Figure 3-2, page 36).

PCR has become one of the most widely used techniques in molecular biology. It is a rapid and simple mean of producing relatively large numbers of copies of DNA molecules from minute quantities of the source DNA material.

Second step of PCR-RFLP analysis is restriction fragment lenght polymorphism analysis

Restriction fragment length polymorphism (RFLP) analysis uses restriction enzymes (RE) to cut DNA at specific 4-6 bp recognition (restriction) sites. Variations in DNA sequence may generate or remove restriction sites result in changes in fragment numbers or lengths in restriction of the digested DNAs. After PCR, the amplified DNA sample is digested with one or more RE's and resulting fragments are separated according to the molecular size using gel electrophoresis.

Presence and absence of fragments resulting from changes in recognition sites of RE's are used in identifying species or populations and analysing single nucleotide polymorphisms as well as the other DNA mutations (deletions, insertions).

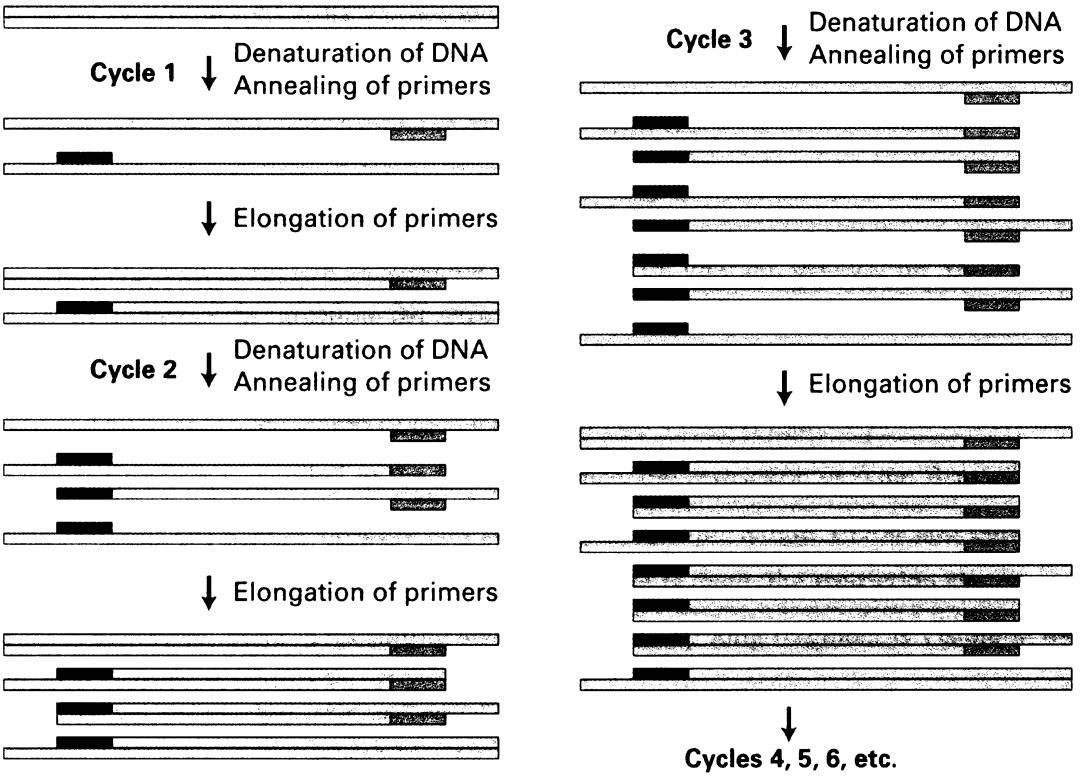


Figure 3-2. Polymerase chain reaction [from 78].

Third step of PCR-RFLP analysis is agarose gel electrophoresis

Agarose gel electrophoresis is a method used in biochemistry and molecular biology to separate DNA or RNA molecules by size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field. Shorter molecules move faster and migrate further than longer ones. But conformation of the DNA molecule is also a factor. To avoid this problem linear molecules are usually separated, usually DNA fragments from a restriction digest, linear DNA PCR products, or RNAs. Loading buffers are added to the DNA in order to visualize it and sediment it in the gel well. Negatively charged indicators keep track of the position of the DNA during electrophoresis. Molecular size standards are used to estimate the fragment size.

Ethidium bromide (EtBr) is the most common dye used for agarose gel electrophoresis. It fluoresces under UV light when intercalated into DNA (or RNA). By

running DNA through an EtBr-treated gel and visualizing it with the UV light, distinct bands of DNA become visible.

PCR-RFLP analysis of Glu111Ala polymorphism

The SNP Glu111Ala can be detected by PCR-RFLP method, because the variance of adenine to cytosine in position 20 203 from the translation start site leads to the cancellation of the restriction site of the restriction enzyme Bsm AI and thus, this SNP can be detected pursuant to the length of the DNA fragments varies (Figure 3-3).

If adenine is incorporated in the DNA sequence, the restriction enzyme Bsm AI cuts the 203 bp long DNA PCR product and two fragments with length 143bp and 60bp are visible in the UV light after electrophoresis. If cytosine is there, the restriction site for RE Bsm AI is cancelled, there is no cutting and a single 203 bp long fragment is visible in the UV light.

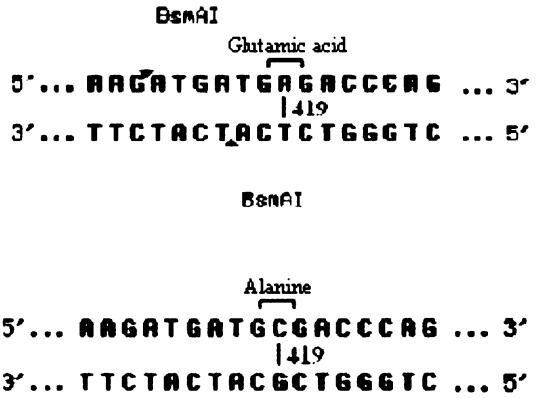


Figure 3-3. The presence and absence of the restriction site of RE Bsm AI.

PCR-RFLP analysis of -7C to T polymorphism

The cytosine/thymine change in the position –7 from the translation start site causes creation of the restriction site for the restriction enzymes Tsp RI and Bts I. Both enzymes were used for the analysis of this SNP.

Both enzymes cut the 155 bp long DNA PCR product if a nucleotide thymine is incorporated in the DNA and form two fragments with lengths 59 and 96 bp.

3.2.3.2 Solutions for PCR-RFLP analysis

- **TBE buffer** (stock solution, 1000 ml): 54 g TRIS, 4.6 ml 0.5M EDTA, 25 g H_3BO_3, add re-distilled water into volume 1000 ml
- **Loading buffer:** 4 g sucrose, 2.5 mg Bromphenol blue, 10 ml TBE buffer
- **Ethidium bromide** (100 ml): 50 mg Ethidium bromide, add re-distilled water into volume 100 ml

3.2.3.3 Working procedure of PCR-RFLP analysis

PCR was done according to following steps:

1. Dilute the templates DNA to concentration 20 ng/µl in the sterile tubes.
2. In a thin-walled PCR tube (on ice), add all reagents of PCR mix, except template DNA (Table 3-1 and 3-2, page 39).
3. Gently vortex the mixture to produce a homogeneous sample and make the 20 µl aliquotes in PCR strips.
4. Add 5 µl (100 ng) of diluted DNA into PCR strip and mix well.
5. Centrifuge briefly to collect the sample at the bottom of the strip.
6. Place samples in a thermal cycler and start PCR (for PCR conditions, see Table 3-3, page 39).

The primers used in PCR were predicted by Primer3 Input (http://-frodo.wi.mit.edu/) (Table 3-4, page 40).

Table 3-1. PCR reagents for one DNA sample (Glu111Ala).

SNP Glu111Ala		
Reagents	**Concentration**	**Volume [µl]**
Sterile water		11.2
PCR buffer (Roche)	10x	2.5
dNTP stock solution (Roche)	10 mM of each dNTPs	1.0
forward primer GLY1	5µM	2.5
reverse primer GLY 2	5µM	2.5
Taq DNA polymerase (Roche)	5U/ µl	0.3
DNA template	100ng	5.0
Total volume		**25**

Table 3-2. PCR reagents for one DNA sample (-7C to T).

SNP -7C to T		
Reagents	**Concentration**	**Volume [µl]**
Sterile water		9.7
PCR buffer (Fermentas)	10x	2.5
dNTP stock solution (Roche)	10mM of each dNTP	1.0
forward primer GLY3	5µM	2.5
reverse primer GLY4	5µM	2.5
$MgCl_2$ (Fermentas)	25 mM	1.5
Taq DNA polymerase (Fermentas)	5U/ µl	0.3
DNA template	100ng	5.0
Total volume		**25**

Table 3-3. PCR conditions.

	SNP Glu111Ala		*SNP –7C to T*	
Step	**Temperature (°C)**	**Time**	**Temperature (°C)**	**Time**
1	92	5 min	95	3 min
2	94	30 sec	95	25 sec
3	60	30 sec	55	40 sec
4	72	1 min	72	1min
5	29x step 2-4		32x step 2-4	
6	68	5 min	72	7 min

Table 3-4. Primers for PCR and DNA sequence analysis.

Primer	5'...Sequence... 3'	Product size	SNP
GLY 1 GLY2	GCA GGG GTT AGG CCA ATT AT CAG GCA AAC TTA CCG AAT CC	203 bp	Glu111Ala *PCR, Sequenation*
GLY3 GLY4	GCG TAG TGT GGG TGA CTC CT TGT GCC CAC CTT GGT ACT G	155 bp	-7C to T *PCR*
GLY3 GLY5	GCG TAG TGT GGG TGA CTC CT TAT CCC TCT TCC CAT CAC ACT C	283 bp	-7C to T *Sequenation*

RFLP was done according to following steps:

1. In a sterile 1.5 ml tube (on ice), add all reagents of mix (Table 3-5).
2. Gently vortex the mixture and add 2.31 µl of mixture to each strip with amplified DNA sample.
3. Incubate overnight at 37°C (Bsm AI) or at 65°C for 6 hours (Tsp RI, Bts I).

Restriction enzymes, as well as DNA fragment sizes after cutting, were predicted by NebCutter V2.0 (http://tools.neb.com/NEBcutter2/index.php).

Table 3-5. Reagents of the restriction mixture.

	SNP Glu111Ala		***SNP –7C to T***	
		Volume [µl]		**Volume [µl]**
NEB 4 buffer		1.83		1.83
BSA		0.18		0.18
RE	***Bsm AI***	0.3	***Tsp RI, Bts I***	0.3
Total		**2.31**		**2.31**

Agarose gel electrophoresis was done accoring to following steps:

Products were separated by electrophoresis in 3% agarose gel and visualized in UV light in the presence of ethidium bromide.

1. Add 40 ml of 10 times diluted frozen TBE buffer to the 1.2 g of frozen agarose. Swirl to mix.
2. Bring the solution to the boil to dissolve the agarose in a microwave oven.
3. Add 40 µl of ethidium bromide and swirl to mix.
4. Pour the gel slowly into the smaller tank. Push any bubbles away to the side using a disposable tip. Insert the combs.
5. Leave to set for 30 minutes at laboratory temperature and 20 minutes in cold.
6. Pour 210 ml of 10 times diluted TBE buffer into the bigger gel tank, add 210 µl of ethidium bromide and submerge the smaller tank with gel.
7. Take out the combs.
8. Add 4 µl of the loading buffer to the each strip.
9. Load the samples and finish with a final lane of 50 or 100 bp marker.
10. Close the gel tank, switch on the power-source and run the gel first at 30-40 V (5 minutes), then at 70-90 V (5 minutes) and finally at 150V.
11. Put the gel under the UV light to visualize the DNA fragments.

3.2.4 DNA sequence analysis

Both Glu111Ala and –7C to T (from the translation start site) polymorphisms were analysed also by DNA sequence analysis for confirmation of the PCR-RFLP results.

3.2.4.1 Principle of DNA sequence analysis

DNA sequencing is the process of determination of the nucleotide order of a given DNA fragment. Currently, most DNA sequencing is performed using the chain termination method developed by Frederic Sanger. This technique uses sequence-specific termination of a DNA synthesis reaction using modified nucleotide substrates – dideoxyribonucleoside triphosphates (ddNTPs). These molecules, in contrast to normal deoxyribonucleotides

(dNTPs), lack a 3' hydroxyl group, so they cannot form a phosphodiester bond with the next incoming dNTP and the truncated daughter fragments discontinued with labelled ddNTPs are formed. The *'dye terminator sequencing'* method is performed in a single reaction. This is accomplished by labelling each of the ddNTPs with a separate fluorescent dye, which fluoresces at a different wavelength. The daughter fragments are then size-separated by electrophoresis in a capillary filled with a polyacrylamide gel and the order of appearance of each of the four different fluorescent dyes at the end of the gel is recorded. This method now is used for the majority of sequencing reactions.

DNA sequence analysis of Glu111Ala and –7C to T polymorphisms

First, the DNA samples were amplified by PCR and separated in 2% agarose gel, then cut and cleaned out of it. Pure DNA products were used for DNA sequencing reaction, in which truncated daughter fragments discontinued with labelled ddNTPs were formed. Ethanol precipitation followed for DNA sequencing reaction reagents elimination. DNA samples were then loaded into sequencing instrument and nucleotide sequence of DNA fragments was determined.

3.2.4.2 The working procedure of DNA sequence analysis

Both PCR and agarose gel electrophoresis working procedures were described in part 3.2.3.3, page 38. The PCR products were separated by electrophoresis in 2% agarose gel and 283bp (-7C to T analysis) or 203bp (Glu111Ala analysis) bands were cut out of it with sterile scalpel in UV light and purified from the gel in TBE buffer. Primers for PRC reaction were also predicted by Primer3 Input (See Table 3-4, page 40).

DNA extraction from agarose gel was done according to following steps:

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a 1.5 ml tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).
3. Incubate at 50°C for 1-2 minutes (or until the gel slice had completely dissolved). Mix by vortexing the tube.
4. After the gel slice has dissolved completely, add 1 gel volume of isopropanol to the sample and mix.

5. Place a spin column in a provided 2 ml collection tube.
6. To bind DNA, apply the sample to the column and centrifuge at 13 000 rpm for 1 minute (22°C).
7. Discard flow-through and add 0.5 ml of Buffer QG to the column and centrifuge (13 000 rpm, 1 minute, 22°C).
8. To wash, add 0.75 ml of Buffer PE to the column and centrifuge (13 000 rpm, 1 minute, 22°C).
9. Discard the flow-through and centrifuge (13 000 rpm, 1 minute, 22°C).
10. Place the column into a clean 1.5 ml microcentrifuge tube.
11. To elute the DNA, add 25 µl of warm water to the centre of the column membrane and centrifuge at 13 000 rpm for 1 minute (22°C).
12. Repeat the step 11.

DNA sequencing reaction was done according to following steps:

1. In a sample plate, add all reagents of sequencing reaction (Table 3-6) according to DNA band intensity in control 2% agarose electrophoresis.
2. Mix reaction components thoroughly and centrifuge briefly.
3. Start thermal cycling program (Table 3-7, page 44).

Table 3-6. Reagents of DNA sequencing reaction.

	Weak band	*Intensive band*
Re-distilled water	2-4 µl	0-5 µl
Pure DNA template	1-3 µl	5-10 µl
Sequencing primer 1.6µM	1 µl	2 µl
Quick Start Master Mix	4 µl	8 µl
Total	**10 µl**	**20 µl**

Table 3-7. Conditions of the DNA sequencing reaction.

	Temperature (°C)	Time
1	96	20 sec
2	50	20 sec
3	60	4 min
4	32x step 2-4	

Ethanol precipitation was done according to following steps:

1. Add 2 µl of 3 M sodium acetate and 50 µl of 96% ethanol to each sample.
2. Mix thoroughly the sample plate and keep it in the laboratory temperature for 15 minutes.
3. Centrifuge at 4000 rpm for 35 minutes (4°C).
4. After centrifugation, turn the sample plate immediately on the chemical pulp and centrifuge at 500 rpm at 4°C for 1 minute.
5. Add 70 µl of 70% ethanol to each sample and mix the sample plate.
6. Centrifuge at 3500 rpm at 4°C for 15 minutes.
7. After centrifugation, turn the sample plate on the chemical pulp and centrifuge at 500 rpm at 4°C for 1 minute and 30 seconds.
8. Repeat the steps 5.-7.
9. Keep in laboratory temperature until samples are dry (circa 10 minutes).

Sample preparation for the loading into the instrument was done according to following steps:

1. Resuspend the samples in 20 µl of the Sample Loading Solution.
2. Overlay each of the resuspended samples with one drop of light mineral oil.
3. Load the sample plate into the instrument and start the DNA sequence analysis.

3.3 Parametres of clinical study

This study involved two groups of patients - hemodialysis patients and patients with breast cancer. Healthy subjects served as a control group for comparison.

The study was performed in adherence to the principles of the Declaration of Helsinki and approved by the Institutional Ethical Committee. All patients have given their informed consent prior to entering the study.

3.3.1 Hemodialysis patients and control group

A total of 214 unrelated Caucasian patients on chronic hemodialysis from six dialysis centres, all of them in stable clinical status, were enrolled in the study.

The studied group of hemodialysis (HD) patients considered of 119 men (56%) and 95 women (44%), mean age 63.1±13.4 years. The majority of the patients were dialysed 3 times a week for 4 hours and their dialysis treatment lasted for 1-4 years.

In this group, the measured value of hemoglobin was 106±12.9 g/l ($p<0.001$) and creatinine 757±204 μmol/l ($p<0.001$). 31% of patients suffered from diabetes mellitus and 41% had dyslipidemia. Case history of 87% patients included hypertension, cardiovascular disease was present in 61%.

3.3.2 Patients with breast cancer and control group

119 unrelated Caucasian women were examined and followed in the Department of Oncology of the General Faculty Hospital in Prague. All women were after the breast surgery and after the end of adjuvant chemotherapy.

Mean age of women with breast cancer was 60.6 ± 9.9 years. Patients were divided based on clinical stage (TNM classification – size of tumor, involvement of lymphatic nodes, presence of distant metastases), histological grading (degree of tumor differentiation) and presence of estrogen receptor (Table 3-8).

98 patients were in the remission, 7 suffered from the stable disease and 1 with progressed disease. 9 patients died and rest were lost from follow-up.

All patients had creatinine less than 110 μmol/l and were not diabetics.

Table 3-8. Characteristics of patients with breast cancer.

Division based on clinical stage (TNM classification) *(number of patients)*				
Stage 0	Stage I	Stage II	Stage III	Stage IV
5	42	56	15	1
Division based on histological grade *(number of patients)*				
Grade I		Grade II	Grade III	
19		56	31	
Division based on positivity of estrogen receptor *(number of patients)*				
Estrogen receptor positive		Estrogen receptor negative		
92		23		

Stage 0 – micro carcinoma, stage I – localized carcinoma, stage II - locally progressive carcinoma, stage III - advanced locally progressive carcinoma, stage IV - carcinoma with metastases.

3.3.3 Control groups

89 unrelated Caucasian healthy subjects served as controls for hemodialysis patients. The group considered of 31 men (35%) and 58 women (65%), mean age 56.6±8.3 years.

58 women from this group served as controls for the patients with breast cancer, mean age 55.9±5.0 years.

3.3.4 Samples

In hemodialysis patients, blood was collected via puncture of the arteriovenous fistule before starting the dialysis session and prior to heparin administration. In patients with breast cancer and control subjects, blood was collected after overnight fasting via puncture of the cubital vein, simultaneously with blood collection for routine control examinations. For DNA analysis, blood was collected into tubes containing EDTA, which were stored at –4°C. Isolation of DNA was performed by myself within 1 week (See 3.3.1). For additional biochemical analysis, blood was collected into tubes without anticoagulant, centrifuged for

10 minutes at 3000 rpm and serum was frozen at –80°C. Analysis of all samples was performed within 6 months after collection.

3.3.5 Biochemical analyses

sRAGE was measured with sandwich ELISA (enzyme linked immunosorbent assay) using standard kits Quantikine (RD System, USA) according to the manufacturer's protocol.

Advanced glycation end product related florescence was measured spectrophotometrically (excitation 350 nm, emission 430 nm) [79, 80].

Other routine biochemical parameters were determined with standard clinical-chemistry methods recommended by the IFCC (International Federation of Clinical Chemistry).

All biochemical analyses were done in the Institute of Clinical Chemistry and Laboratory Diagnostics, 1st Faculty of Medicine and General University Hospital, Charles University, Prague.

3.3.6 Statistical analysis

The results of biochemical parameters are expressed as mean ± standard deviation. The unpaired t-test and Wilcoxon test were used for the evaluation of the differences between groups. Concerning the evaluation of the glyoxalase I gene polymorphism, the significance of differences from Hardy-Weinberg equilibrium as well as those in the genotype distribution and/or allelic frequencies among group were tested using the χ^2 test (done by myself). For finding differences among subgroups, ANOVA (analysis of variance) test and Kruskal-Wallis test were used (done in co-operation with a professional statistician).

All results were considered as statistically significant at $p<0.05$.

4 Results

4.1 Isolation of DNA and concentration of DNA in samples

Isolation of DNA as well as measurement of the concentration of the DNA was done according to working procedures in the parts 3.2.1, page 32 and 3.2.2, page 34.

DNA was isolated and its concentration was measured together from 214 blood samples from hemodialysis patients, 119 blood samples from patients with breast cancer and 89 blood samples from healthy controls.

The values of DNA concentration varied from 100 to 400 ng/μl and purity of isolated DNA was approximately 1.8-2.0.

4.2 Analysis of SNP Glu111Ala

I have established the method for polymorphism detection based on PCR-RFLP analysis. For the results confirmation, DNA sequence analysis was used.

PCR-RFLP analysis was done according to working procedure in part 3.2.3, page 38. Working procedure of DNA sequence analysis is described in part 3.2.4, page 42. Results of these methods as well as results from biochemical analyses (see 3.3.5, page 47) were used for detailed statistical analysis. Figure 4-1 depicts the DNA fragments after restriction enzyme cutting and agarose electrophoresis representing three possible genotypes: heterozygote and two homozygotes. Figure 4-2 shows the results of DNA sequence analysis – the same genotypes: heterozygote AC and two homozygotes AA and CC.

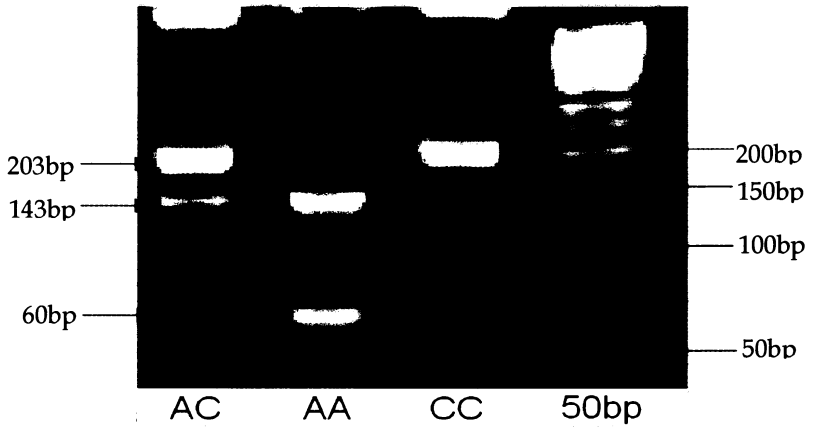


Figure 4-1. SNP Glu111Ala: heterozygote AC and two homozygotes AA and CC (PCR-RFLP analysis).

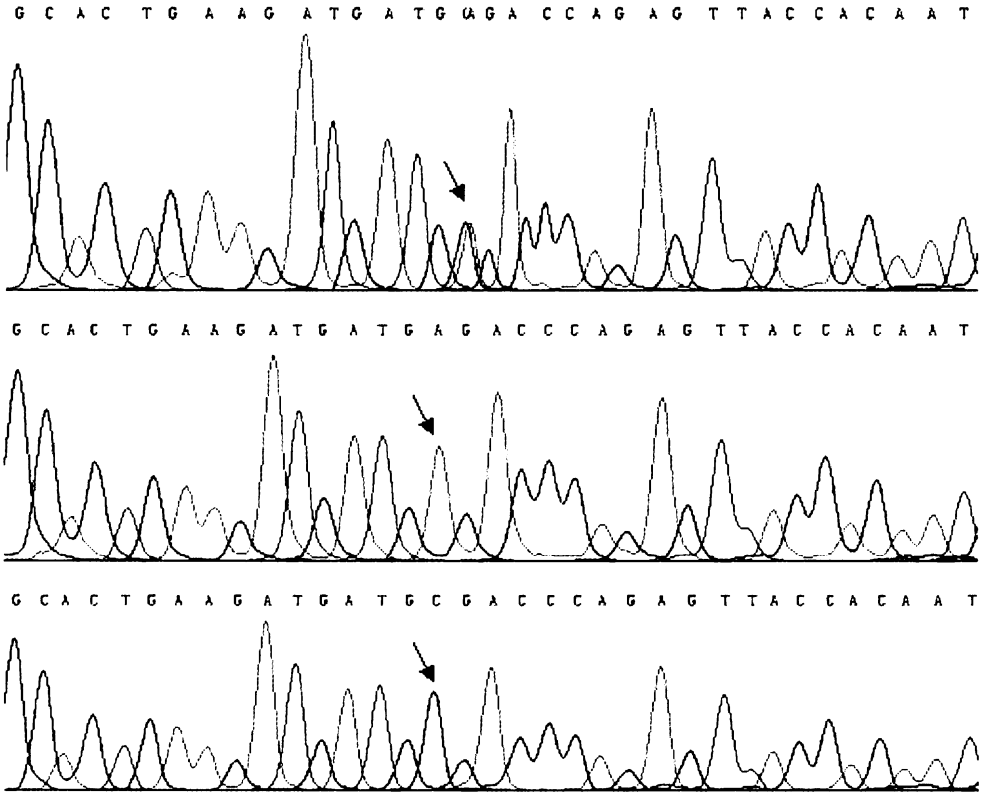


Figure 4-2. SNP Glu111Ala: heterozygote AC and two homozygotes AA and CC (DNA sequence analysis).

4.2.1 Hemodialysis patients

Allelic and genotype frequencies

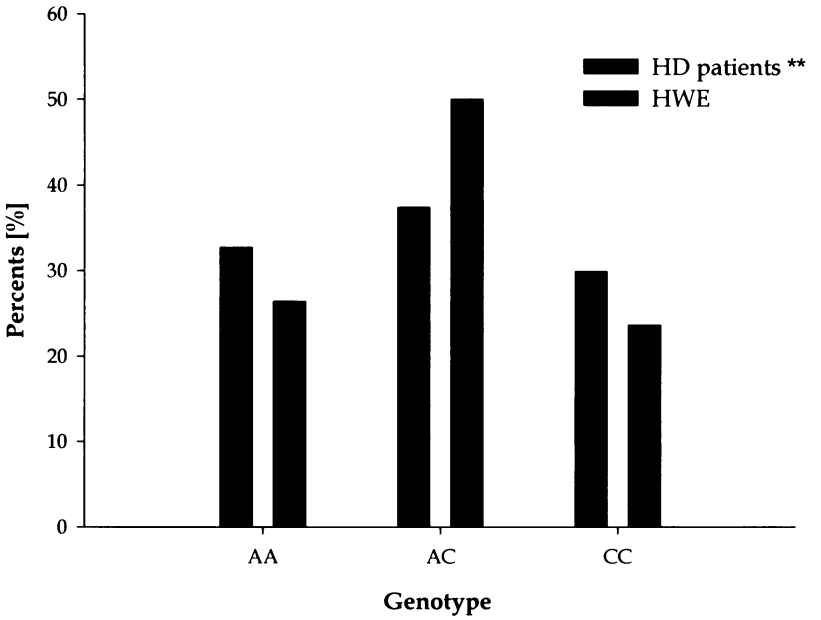
Allelic as well as genotype frequencies did not differ between HD patients and controls but were not in Hardy-Weinberg equilibrium. The mutated C allele was found in 48.6% in HD patients and 47.8% in controls while A allele was present in 51.4% in HD patients and 52.2% in controls, respectively. Genotype frequencies are shown in Table 4-1 (See also Graph 4-1 and 4-2).

Allelic as well as genotype frequencies did not differ between men and women.

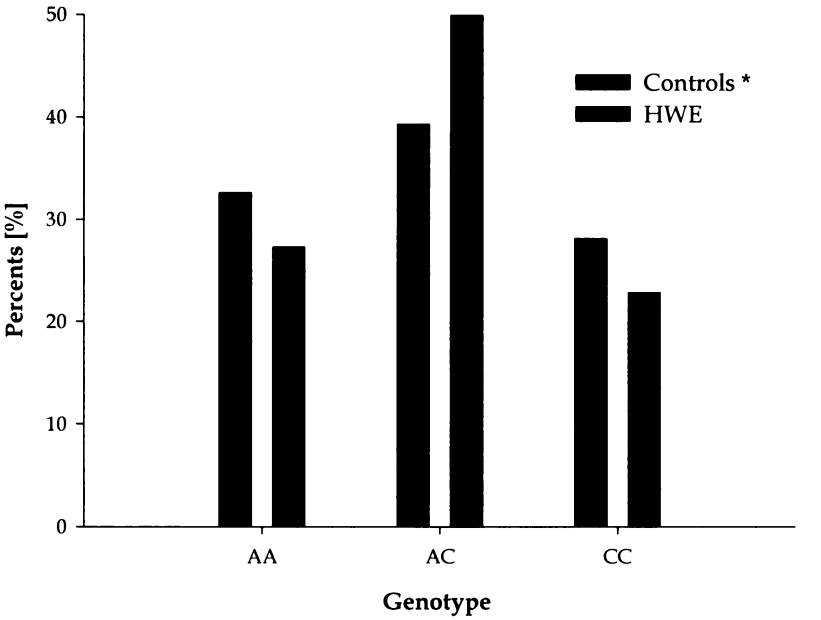
Table 4-1. Genotype frequencies of Glu111Ala polymorphism of the glyoxalase I gene in hemodialysis patients and controls – determined frequencies and expected frequencies according to Hardy-Weinberg equilibrium.

	AA	*AC*	*CC*	
HD patients	32.7%	37.4%	29.9%	**
HWE	26.4%	50.0%	23.6%	
Controls	32.6%	39.3%	28.1%	*
HWE	27.3%	49.9%	22.8%	

*$p<0.05$, **$p<0.001$ vs HWE.



Graph 4-1. HD patients genotype frequencies in comparison to HWE (to Table 12, **p<0.001 vs HWE).



Graph 4-2. Controls genotype frequencies in comparison to HWE (to Table 12, *p<0.05 vs HWE).

Comparison of hemodialysis patients with healthy controls

HD patients had significantly higher level of sRAGE and AGE-related fluorescence compared to healthy controls (Table 4-2).

Table 4-2. Laboratory characteristics of HD patients, comparison with healthy controls.

Characteristic	***HD patients***	***Controls***	
Number of patients (men/women)	214 (119/95)	89 (31/58)	*
Age [years]	63.1±13.4	56.6±8.3	**
Glucose [mmol/l]	6.2±2.3	5.2±0.9	**
HbA1c [%] *(N=64)*	5.0±1.8	n.a.	
AGE-related fluorescence [AU]	$11.2\pm2.7\times10^5$	$3.8\pm1.9\times10^5$	**
sRAGE [pg/ml]	3427±1508	1758±637	**

Data are expressed as mean±standard deviation, *$p<0.005$, **$p<0.001$ HD patients versus controls, n.a. - not assessed.

Genotype/phenotype associations

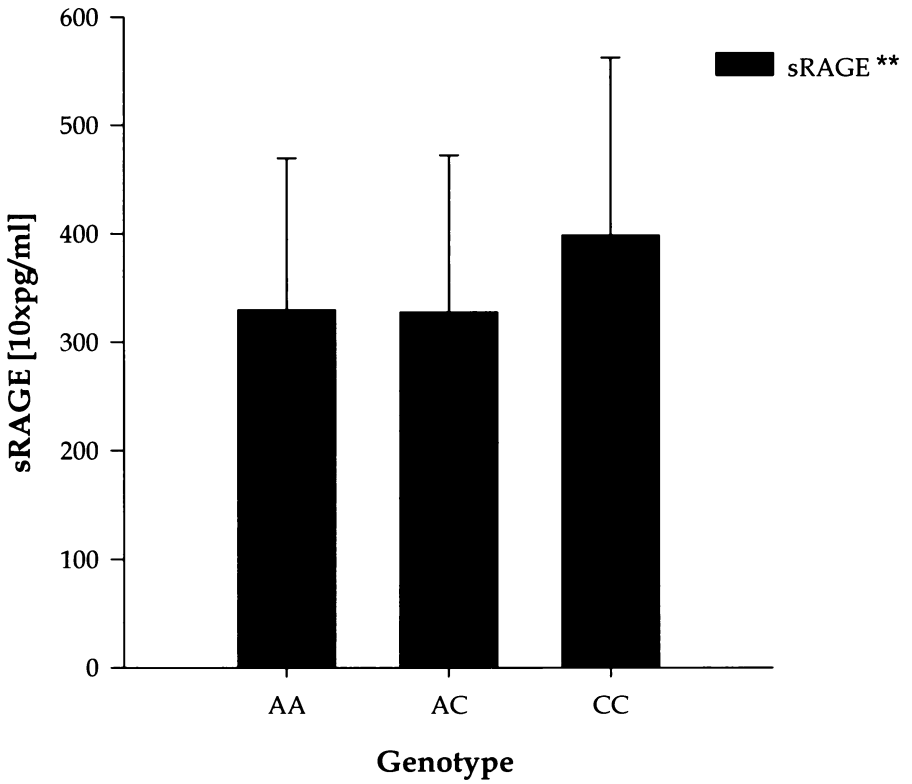
The studied SNP was not associated with serum levels of glucose, AGE-related fluorescence and with HbA1c. However, it was significantly linked with serum sRAGE levels, which sensitively reflects the AGE-burden of the organism (highest in CC variant, $p<0.01$) and with the AGEs/sRAGE ratio (lowest in CC variant, $p<0.05$) (Table 4-3, Graph 4-3 and 4-4).

In the CC variant, significantly higher prevalence of cardiovascular disease was found while the prevalence of hypertension, diabetes mellitus and dyslipidemia did not differ between genotypes (Table 4-4).

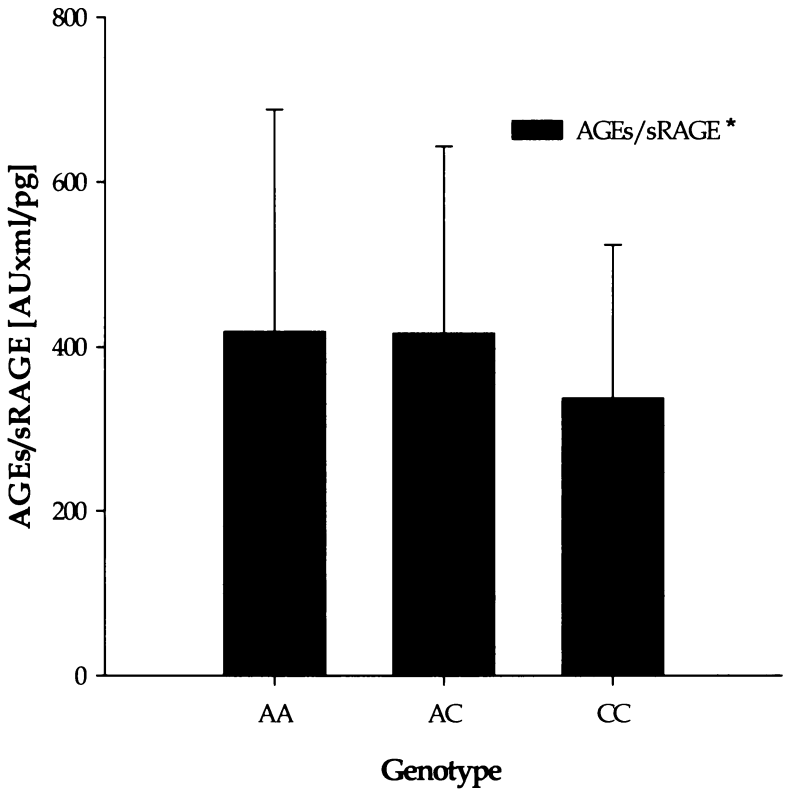
Table 4-3. Levels of laboratory parameters in each genotype of Glu111Ala polymorphism of the glyoxalase I gene in hemodialysis patients *(N=214).*

Parameter	***AA****(N=70)*	***AC****(N=80)*	***CC****(N=64)*	
Glucose [mmol/l]	5.9±1.7	6.1±2.4	6.7±2.5	
HbA1c [%]	4.9±1.4	5.6±2.4	5.2±1.5	
AGEs fluorescence [AU]	$1.1\pm0.3x10^6$	$1.1\pm0.2x10^6$	$1.1\pm0.2x10^6$	
sRAGE [pg/ml]	3297±1398	3277±1445	3986±1638	**
AGEs/sRAGE [Auxml/pg]	419±269	417±226	338±186	*

Data are expressed as mean±SD, *p<0.05, **p<0.01 CC versus AA and AC.



Graph 4-3. sRAGE levels in each genotype of SNP Glu111Ala in hemodialysis patients. (Data are expressed as mean±SD, **p<0.01 comparison among genetic variants, CC versus AC and AA.)



Graph 4-4. AGEs/sRAGE levels in each genotype of SNP Glu111Ala in hemodialysis patients. (Data are expressed as mean±SD, *p<0.05 comparison among genetic variants, CC versus AC and AA.)

Table 4-4. Prevalence of cardiovascular disease, hypertension, diabetes mellitus and dyslipidemia in each genotype of Glu111Ala polymorphism of the glyoxalase I gene in hemodialysis patients *(N=214).*

Disease/genotype	***AA****(N=70)*	***AC****(N=80)*	***CC****(N=64)*	
Cardiovascular disease	54%	54%	77%	*
Hypertension	89%	86%	86%	
Diabetes mellitus	26%	28%	41%	
Dyslipidemia	39%	48%	36%	

*p<0.01 comparison among genetic variants, CC versus AA and AC.

4.2.2 Patients with breast cancer

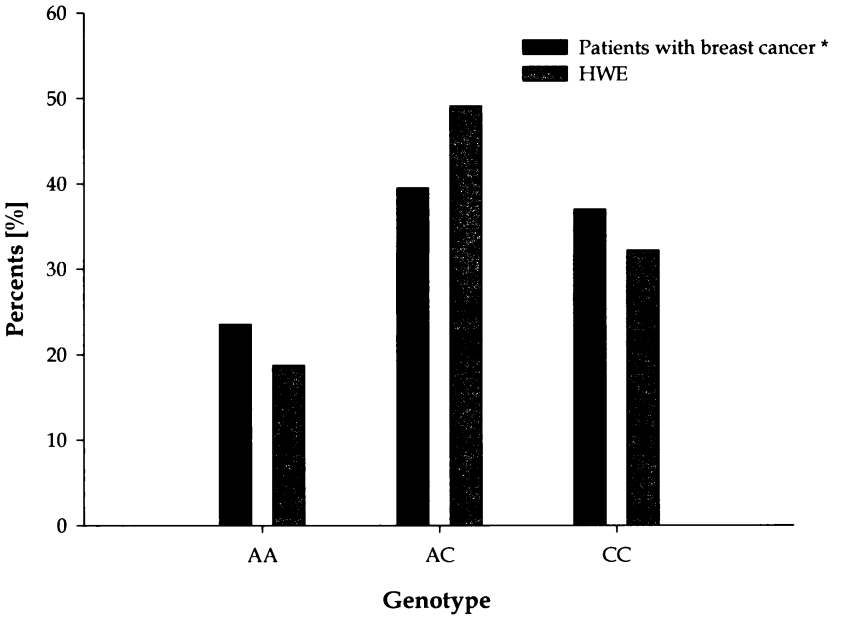
Allelic and genotype frequencies

Allelic as well as genotype frequencies were similar in patients with breast cancer and controls but patients with breast cancer were not in Hardy-Weinberg equilibrium. The mutated C allele was found in 56.7% in patients with breast cancer and 51.7% in controls while A allele was present in 43.3% in patients with breast and 48.3% in controls, respectively. Genotype frequencies are shown in Table 4-5, Graph 4-5 and 4-6.

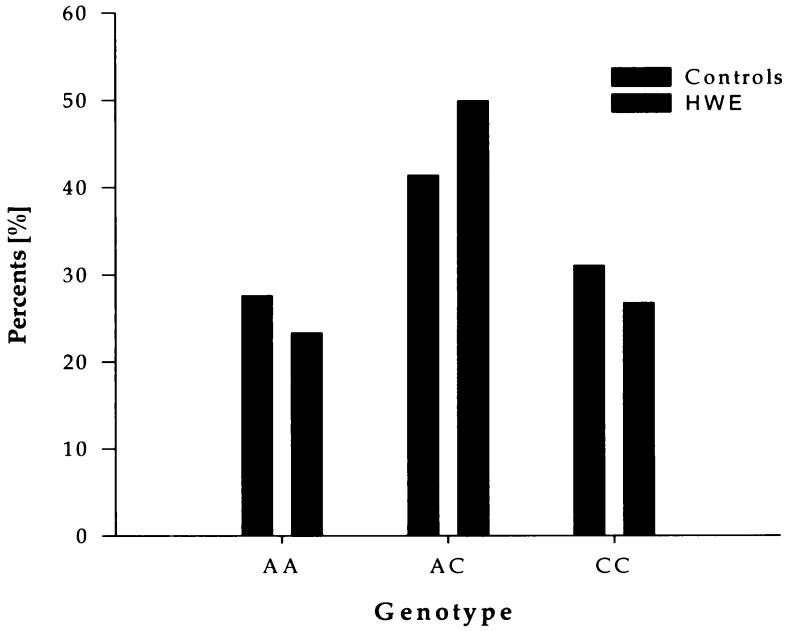
Table 4-5. Genotype frequencies of Glu111Ala polymorphism of the glyoxalase I gene in patients with breast cancer and controls – determined frequencies and expected frequencies according to Hardy-Weinberg equilibrium

	AA	*AC*	*CC*	
Patients with breast cancer	23.5%	39.5%	36.9%	*
HWE	18.7%	49.1%	32.2%	
Controls	27.6%	41.4%	31.0%	
HWE	23.3%	49.9%	26.8%	

*$p<0.05$ vs HWE



Graph 4-5. Patients with breast cancer genotype frequencies in comparison to HWE (to Table 15, *p<0.05 vs HWE).



Graph 4-6. Controls genotype frequencies in comparison to HWE (to Table 15).

Comparison of patients with breast cancer with healthy controls

Patients with breast cancer had significantly higher level of AGEs and AGEs/sRAGE rate compared to healthy controls. Level of soluble receptor for advanced glycation end products did not differ in patients with breast cancer compared to healthy subjects (Table 4-6).

Table 4-6. Laboratory characteristics of patients with breast cancer, comparison with healthy controls.

Characteristic	***Patients with breast cancer***	***Controls***	
Number of patients	119	58	
Age [years]	60.6±9.9	55.9±5.0	*
AGEs [AU]	407 346±118 330	343 884±70 198	*
sRAGE [pg/ml]	1601±826	1788±516	
AGEs/sRAGE [AUxml/pg]	319.8±209.3	213.1±84.2	**

Data are expressed as mean±SD, *$p<0.05$, ** $p<0.001$ patients with breast cancer versus controls.

Genotype/phenotype associations

After patients were divided to the genotype types, the level of AGEs and sRAGE as same as in all group of patients was found.

Significant changes in patients in clinical stage III were found. (Table 4-7, Graph 4-7).

In subgroups of patients based on histological grade were no significant differences of the distribution of genotypes or allels compared to the healthy controls.

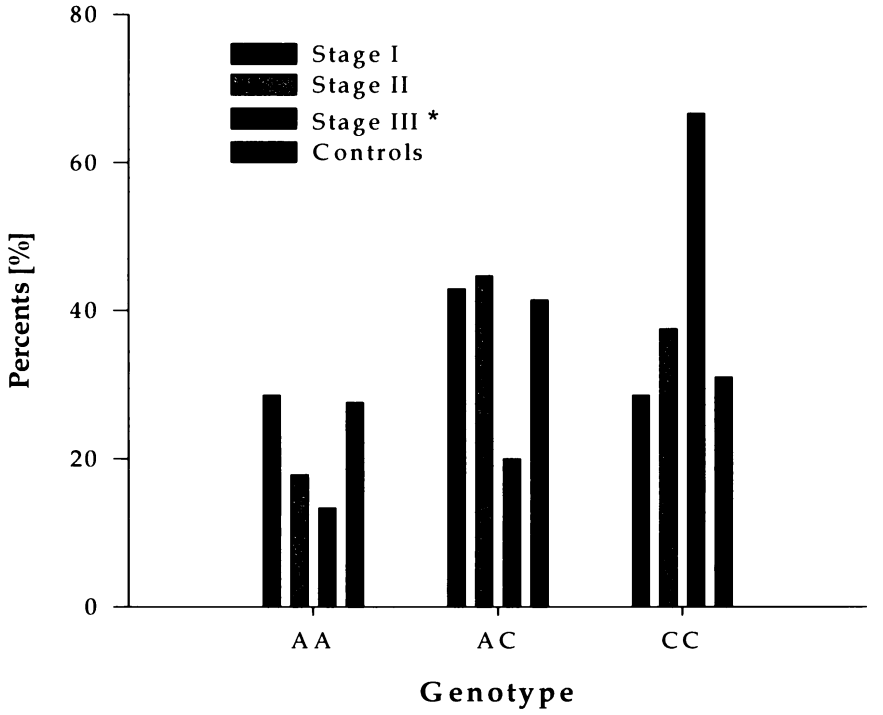
Patients with negative estrogen receptor had higher frequency of CC genotype. The distribution of C allele was also significantly increased (Table 4-8, Graph 4-8).

Controls with genotype AA and AC had significantly lower AGEs/sRAGE rate than patients with the same genotypes and whole group of patient as well. (Table 4-9 and Graph 4-9).

Table 4-7. Genotype and allelic frequencies in subgroups of patients with breast cancer divided based on clinical stage.

	Genotype			*Alleles*		
	AA	**AC**	**CC**	**A**	**C**	
Stage I	28.6%	42.8%	28.6%	50.0%	50.0%	
Stage II	17.9%	44.6%	37.5%	40.2%	59.8%	
Stage III	13.3%	20.0%	66.7%	23.3%	76.7%	*
Controls	27.6%	41.4%	31.0%	48.3%	51.7%	

*p<0.05 vs controls

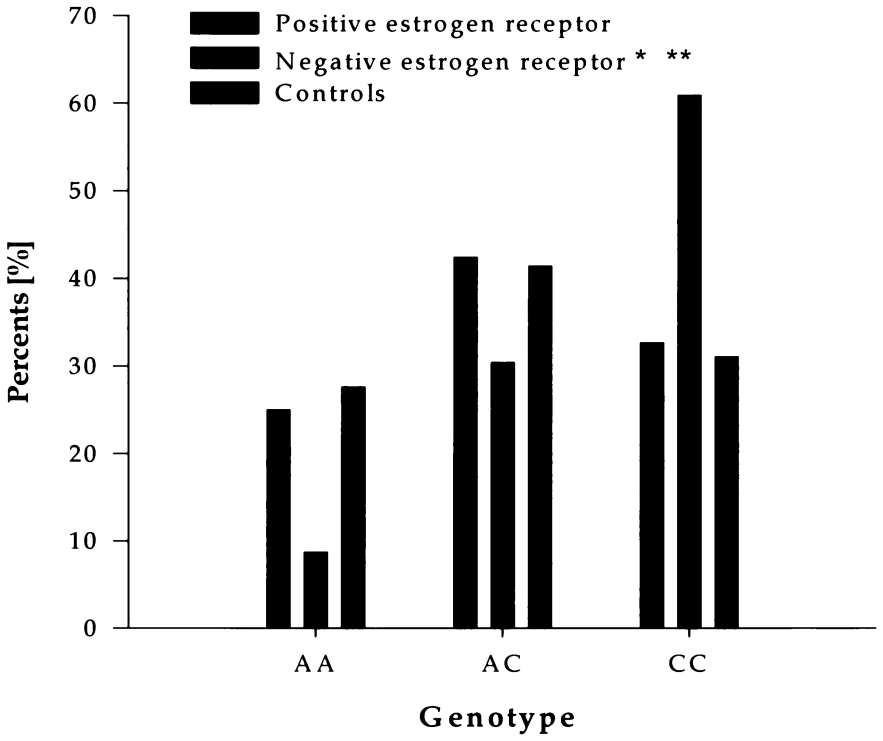


Graph 4-7. Genotype frequencies in subgroups of patients with breast cancer divided based on clinical stage (*p<0.05).

Table 4-8. Genotype and allelic frequencies in subgroups of patients with breast cancer divided based on presence of estrogen receptor.

	Genotype			*Alleles*		
	AA	**AC**	**CC**	**A**	**C**	
Estrogen receptor negative	8.7%	30.4%	60.9%	23.9%	76.1%	* **
Estrogen receptor positive	25.0%	42.4%	32.6%	46.2%	53.8%	
Controls	27.6%	41.4%	31.0%	48.3%	51.7%	

*p<0.05 vs controls, **<0.01 vs patient with positive estrogen receptor.

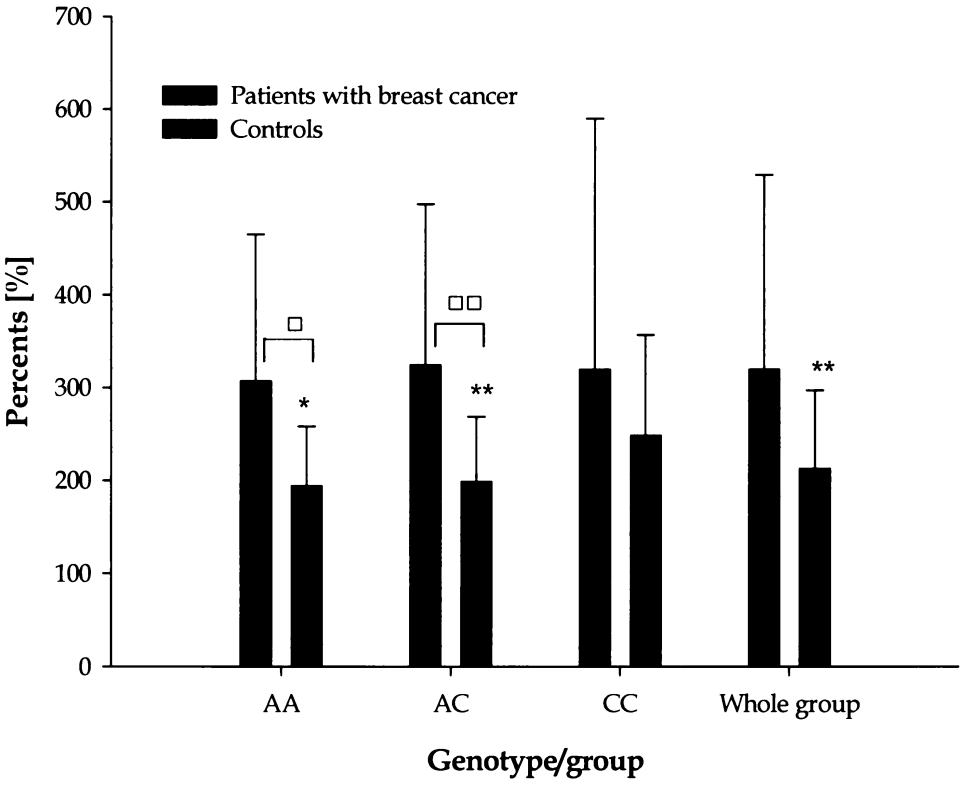


Graph 4-8. Genotype frequencies in subgroups of patients with breast cancer divided based on presence of estrogen receptor (*p<0.05 vs controls **<0.01 vs patient with positive estrogen receptor).

Table 4-9. AGEs/sRAGE rates in patients with breast cancer and controls in each genotype and whole group.

	AGEs/sRAGE rate [Auxml/pg]			
	AA	**AC**	**CC**	**Whole group**
Patients with breast cancer	307.2±157.6 □	324.7±172.8 □□	319.7±269.9	319.8±209.3
Controls	194.5±63.9*	198.8±69.9**	248.7±108.2	213.1±84.2*

*p<0.01 vs patients with breast cancer, **p<0.001 vs patients with breast cancer, □ <0.005 vs AA controls, □□<0.005 vs AC controls.



Graph 4-9. AGEs/sRAGE rates in patients with breast cancer and controls in each genotype and in whole group (*p<0.01 vs patients with breast cancer, **p<0.001 vs patients with breast cancer, □ <0.005 vs AA controls, □□<0.005 vs AC controls).

4.3 Analysis of SNP -7C to T

I have established the method for this polymorphism detection based on PCR-RFLP analysis. For the results confirmation, DNA sequence analysis was used. Figure 4-3 depicts the DNA fragments after restriction enzyme cutting and agarose electrophoresis representing three possible genotypes: heterozygote and two homozygotes. Figure 4-4 shows the results of DNA sequence analysis – the same genotypes: heterozygote TC and two homozygotes TT and CC.

The working procedures of the PCR-RFLP analysis and DNA sequence analysis are described in part 3.2.3, page 38 and 3.2.4, page 42.

This polymorphism was analysed only in a subgroup of studied subject (50 hemodialysis patient and 18 controls). Because of the small analysed groups, the results are expressed only in percents of genotype representation (Table 4-10).

Allelic as well as genotype frequencies did not differ between HD patients and controls. The mutated C allele was found in 51.0% in HD patients and 52.8% in controls while T allele was present in 49.0% in HD patients and 47.2% in controls, respectively.

Table 4-10. Genotype frequencies of –7 C to T polymorphism of the glyoxalase I gene in HD patients and controls.

	CC	*TC*	*TT*
Patients with breast cancer	26.0%	50.0%	24.0%
Controls	22.2%	61.1%	16.7%

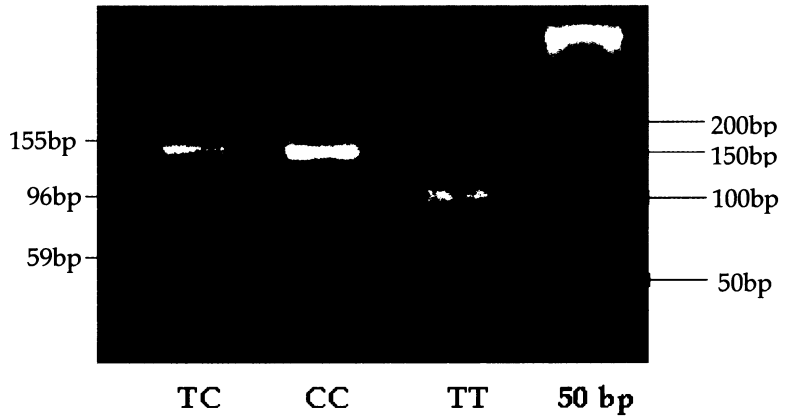


Figure 4-3 SNP –7 C to T: heterozygote TC and two homozygotes CC and TT (PCR-RFLP analysis, RE Bts I).

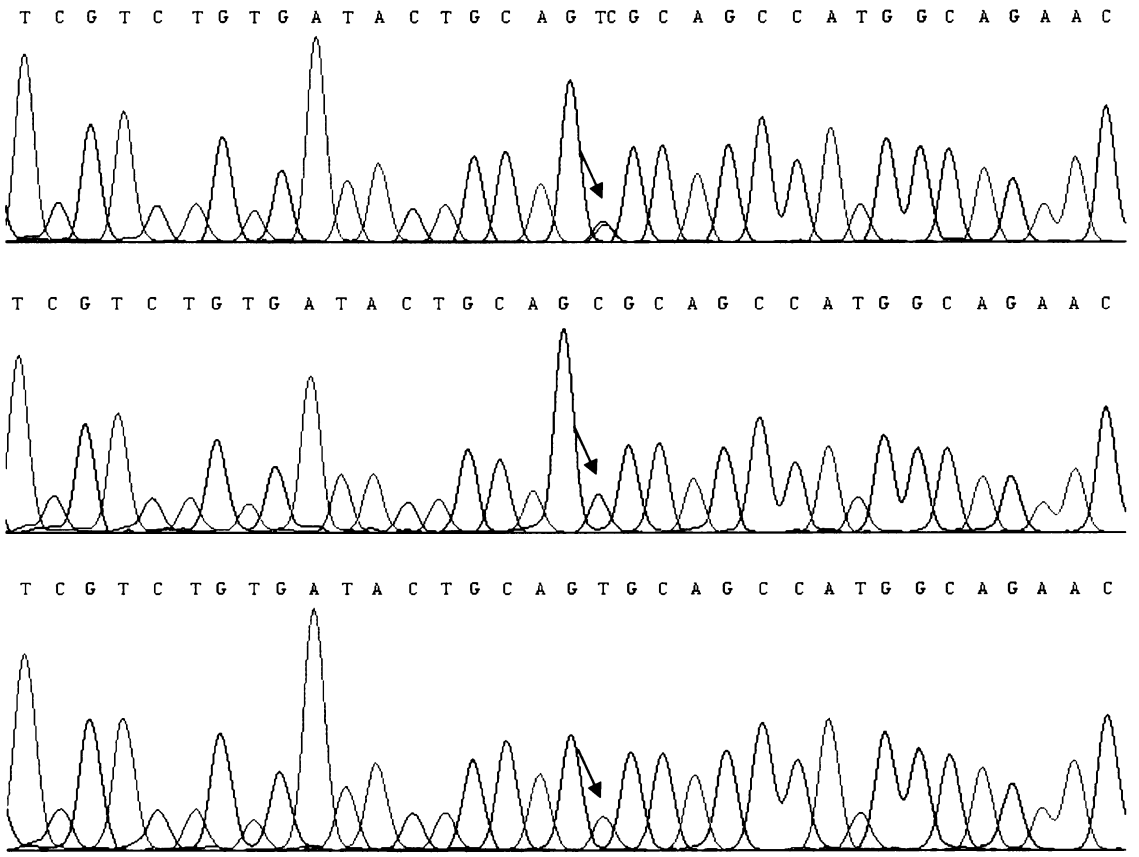


Figure 4-4. SNP –7 C to T: heterozygote TC and two homozygotes CC and TT (DNA sequence analysis).

5 Discussion

Over the last 20 years, biomedical researchers have developed a number of techniques for SNP detection. Many common diseases in humans are not caused by a genetic variation within a single gene but are influenced by complex interactions among multiple genes as well as environmental and lifestyle factors. Although both environmental and lifestyle factors add tremendously to the uncertainty of developing a disease, it is currently difficult to measure and evaluate their overall effect on a disease process. Therefore, we can refer mainly to a person's genetic predisposition, or the potential of an individual to develop a disease based on genes and hereditary factors.

Genetic factors may also confer susceptibility or resistance to a disease and determine the severity or progression of disease. Because genetic factors also affect a person's response to drug therapy, DNA polymorphisms such as SNPs will be useful in helping researchers determine and understand why individuals differ in their abilities to absorb or clear certain drugs, as well as to determine why an individual may experience an adverse side effect to a particular drug [59].

One of the targets of this study was to establish the methods for the analysis of the single nucleotide polymorphisms Glu111Ala and –7C to T.

PCR-RFLP analysis is suitable for analysis of polymorphisms, that cause the restriction site creation/cancellation with the base-exchange. The single nucleotide polymorphism Glu111Ala (A20 203C; A419C in mRNA position) is one of such polymorphisms because it leads to the cancellation of the restriction site for the restriction enzyme Bsm AI and thus, it could be analysed by PCR-RFLP analysis. The primers, restriction enzyme and PCR condition were chosen by myself with the aid of competent web applications. Very important conditions for rewarding the result of the analysis were both good proceeds of DNA from PCR and accuracy of restriction enzyme cutting without production of nonspecific fragments. Both conditions were fulfilled. DNA samples from 214 hemodialysis patients, 119 patients with breast cancer and 89 healthy controls were analysed by this method.

In addition, the results were confirmed by DNA sequence analysis. This method is very precise, but also very expensive, the DNA sample preparation is difficult and takes a

long time. Thus, this method served only as a control method. 40 DNA samples were analysed by this method and the results from PCR-RFLP were confirmed.

This polymorphism was studied and described in literature several times until now. Junaid et al. [72] and Politi et al. [71] used for analysis of this SNP PCR-RFLP method. There were some differences in PCR conditions from those used in my working procedure and for restriction analysis endonuclease SfaNI was used. Gale et al. [74] availed the PCR-RFLP analysis too. This time, restriction enzyme Bsm AI was used and PRC conditions were except small differences as same as PCR conditions using by me. Only Chen et al. [73] used a different method for this SNP detection - pyrosequencing. It's the method of DNA sequencing based on a chemiluminescent enzymatic reaction. Each time a nucleotide is incorporated into the growing chain a cascade of enzymatic reactions is triggered which results in a light signal.

In this study, the SNP Glu111Ala was analysed by PCR-RFLP method established by me. The method is quite fast, cheaper than sequence analysis and gives good results.

Polymorphism –7C to T approximates minimal promoter [58] and DNA-dependent RNA polymerase site. The mutant C allele was predicted to bind CG-factor (GCF), a transcription factor that represses transcription. This polymorphism was analysed also by PCR-RFLP analysis. The primers, restriction enzyme and PCR condition were chosen by myself with the aid of competent web applications.

Although it was found the optimal temperature for primer annealing by PCR with temperature gradient, some of samples gave very low proceeds of amplified DNA. One of the reason can be a troublesome linkage of primers, which can be caused by unmapped deletions or other mutations in 5'UTR area. First restriction enzyme Tsp RI used in restriction analysis was objectionable, the cutting of DNA after digestion with 3U of RE (16 hours, 65°C – conditions recommended by the manufacturer) was not complete. 4U of RE and the same conditions gave the same results. Second endonuclease Bts I used in analysis worked well. Of course, the results from PCR-RFLP analysis were confirmed by the DNA sequence analysis. All 68 DNA samples analysed by PCR-RFLP were also analysed by sequenation for entire confirmation of the results. Considering difficult method together with problems with method optimalization, only several analyses were done. Thus, we have obtained the results only form small subgroup of HD patients and controls. Moreover, the analysis of this polymorphism was over the beginning aims of this study.

This polymorphism was described in literature only once. Gale et al. [74] analysed this SNP by PCR-RFLP, using 0.5U of the restriction endonuclease Hha I. PRC conditions were except small differences the same as PCR conditions using by me.

In this study, the method for SNP –7C to T analysis was established. Because of difficultness of the method, together with problems with method optimalization, I have analysed DNA samples only in subgroup of 50 HD patients and 18 controls.

Next of my targets was to study Glu111Ala single nucleotide polymorphism of the glyoxalase I gene in hemodialysis patients in relationship to their clinical status and laboratory characteristics.

This is the first study showing the association of Glu111Ala polymorphism of glyoxalase I gene with the prevalence of cardiovascular disease and with the sRAGE level in hemodialysis patients.

Only two SNPs of glyoxalase I gene have been studied in association with selected disorders yet – Glu111Ala and –7 C to T (from the translation start site). For the Glu111Ala polymorphism, a relationship to the prevalence of insulin dependent diabetes mellitus [70] and pro-trombotic factors [74] was demonstrated, however, no association with the risk for Alzheimer´s disease in the overall population was shown [73].

A possible role of glyoxalase in vascular damage was referred by Miyata et al. [75]. He reported a case of a Japanese woman on chronic hemodialysis with multiple cardiovascular complications in absence of predisposing risk factors but with a deficiency of glyoxalase I, and an unusually high level of advanced glycation end products - pentosidine and carboxymethyllysine. No genetic mutation in the coding region of the glyoxalase I gene was detected, however, a possible mutation in the promoter/enhancer region was not excluded. Studying Glu111Ala polymorphism of the glyoxalase I gene, which leads to glutamic acid/alanine alteration in the position 111 in the protein sequence of the enzyme [61], we demonstrate a higher prevalence of cardiovascular disease in the mutated homozygotes (CC), although the prevalence of risk factors (hypertension, diabetes mellitus and dyslipidemia) does not differ among genotypes. Glyoxalase I and polymorphisms of its gene should be thus taken into consideration when evaluation possible factors and mechanisms related to cardiovascular disease.

Both AGEs and RAGE take part in the pathogenesis of vascular complications [81, 82, 83], but only serum levels of the protective sRAGE were linked to the studied SNP, while the levels of fluorescent AGEs and their precursors were not. Similarly, in

another study, neither pentosidine nor carboxymethyllysine levels usually present in hemodialysis patients were correlated with the level of glyoxalase I activity [75]. sRAGE sensitively reflects the AGE-burden of the organism and seems to represent a more useful marker than fluorescent AGEs since AGEs are a heterogeneous group of compounds and some of them are non-fluorescent. Additionally, other detoxification mechanisms of AGEs might be important as well. However, when AGEs and sRAGE were evaluated together, the relationship to the SNP was still significant, which points out the outstanding significance of sRAGE.

Our analysis revealed a significant deviation of allele distribution from Hardy-Weinberg equilibrium, which is in line with findings in other populations [73, 84]. This deviation was found both in old as well as in a young population [73], suggesting the existence of selection forces.

In conclusion, we demonstrate for the first time the association of Glu111Ala polymorphism of glyoxalase I gene with sRAGE levels and show the genetic predisposition to vascular complications in hemodialysis patients.

Finally, my aim was to study Glu111Ala polymorphism and to find significant association with presence of breast cancer, or connection to clinical characteristics of patients.

Glyoxalase I was showed to play an important role in pathogenesis of cancer. Samadi et al. [69] investigated co-occurrence of polymorphism Glu111Ala and risk of prostate cancer and concluded that GLO 2-2 (homozygote AA) phenotype was potential risk factor for human prostate cancer. Until now, no study of SNP Glu111Ala in connection to breast cancer was published.

Allelic as well as genotype frequencies were similar in patients with breast cancer and controls, but patients with breast cancer were not in Hardy-Weinberg equilibrium, which is in line with findings in other populations [73, 84]. This deviation was found both in old as well as in a young population [73], suggesting the existence of selection forces. The whole group of controls (men and women) was not in Hardy-Weinberg equilibrium too, but as controls for this group of patients served only women, thus the deviation was not such significant. Relatively small group of controls *(N=58)* was another problem for evaluation of this deviation.

Significantly higher frequencies of CC genotype in subgroup of patients with negative estrogen receptor were found compared to healthy controls and subgroup of

patients with positive estrogen receptor as well. The role of estrogen in proper function of Glyoxalase I is important, however the mechanism is still unknown. Rulli et al. [85] studied the effect of estrogen on glyoxalase I activity in estrogen dependent and estrogen independent breast cancer tissue and demonstrated estrogen induced proliferation and over expression of glyoxalase I in estrogen dependent cancer tissue. Contrary, the study showed that blockade of estrogen receptor by tamoxifen, stopped proliferation and decreased expression of the enzyme in the same estrogen positive cancer tissue, however mechanism of regulation of glyoxalase I gene transcription was not clear, since the enzyme gene promoter does not contain estrogen response element.

Patients with negative estrogen receptor are considered to have worse prognosis, so we might conclude, that genotype CC is associated with the group and worse prognosis.

The subgroup of patients in clinical stage III had higher frequency of CC genotype, compared to healthy control, and compared to the rest of patients as well. We may hypothesize that CC genotype is associated with quicker progresses of disease and thus sooner development of higher clinical stage. Contrary, the potential risk factor in human prostate cancer is believed to be AA genotype [69].

In this study, we demonstrate the association of SNP Glu111Ala with progression of the breast cancer and presence of the estrogen receptor, but the further investigation with larger amount of subjects is needed to confirm our results.

6 Conclusions

- I have established the method for detection of SNP Glu111Ala of the glyoxalase I gene based on PCR-RFLP analysis. Together 422 DNA samples were analysed by this method and result were confirmed by DNA sequence analysis. The method can be well-used in clinical medicine.

- I have established the method for detection of SNP –7C to T of the glyoxalase I gene based on PCR-RFLP analysis. The results were confirmed by DNA sequence analysis. Because of difficultness of the method, together with problems with method optimalization, I have analysed DNA samples only in small subgroup of 50 HD patients and 18 controls.

- The SNP Glu111Ala was studied in hemodialysis patients and in healthy controls for comparison. Allelic as well as genotype frequencies did not differ between HD patients and controls but were not in Hardy-Weinberg equilibrium. The studied SNP was not associated with serum levels of glucose, AGE-related fluorescence and with HbA1c. However, it was significantly linked with serum sRAGE levels, which sensitively reflect the AGE-burden of the organism and with the AGEs/sRAGE ratio. In the CC variant, significantly higher prevalence of cardiovascular disease was found while the prevalence of hypertension, diabetes mellitus and dyslipidemia did not differ between genotypes.

- The SNP Glu111Ala was studied in patients with breast cancer and in healthy controls for comparison. Allelic as well as genotype frequencies were similar in patients with breast cancer and controls but patients with breast cancer were not in Hardy-Weinberg equilibrium. The group of patients with negative estrogen receptor and patients in clinical stage III had higher frequency of CC genotype, that why we concluded that this genotype could be negative prognostic factor, but further study is needed.

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