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Genetická predispozice a nové biochemické markery u vybraných stavů v porodnictví

Genetic backround and new biochemical markers in pathological pregnancy

Disertační práce

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ABSTRAKT

Receptor pro produkty pokročilé glykace (RAGE), jeho solubilní forma (sRAGE) a glyoxalasa 1 jsou důležitou součástí patogeneze mnoha chronických nemocí.

Cílem dizertační práce bylo objasnit význam sRAGE, čtyř polymorfizmů genu pro *RAGE* a jednoho polymorfizmu genu *glyoxalasy 1* ve fyziologickém i patologickém těhotenství.

Koncentrace sRAGE v séru byla stanovena u zdravých těhotných žen (N=120), u žen s hrozícím předčasným porodem (N=99), s preeklampsií (N=35), s růstovou retardací plodu (IUGR) (N=22) a s těhotenskou cholestázou (ICP) (N=14). Ve stejné skupině zdravých těhotných žen a žen s patologickým těhotenstvím byly stanoveny čtyři *RAGE* polymorfizmy (*RAGE* -429T/C, -374A/T, *RAGE* Gly82Ser (557G/A), *RAGE* 2184A/G) a jeden polymorfizmus glyoxalasy 1 *GLO 1* Glu111Ala (419A/C).

Hladina sRAGE v séru zdravých těhotných žen byla signifikantně nižší v porovnání s netěhotnými kontrolami. Hladina sRAGE se v průběhu těhotenství měnila, byla nízká v 1.trimestru, stoupala v 2.trimestru a zase klesala ve 3.trimestru a před porodem.

Těhotné pacientky s předčasným porodem měly významně nižší sérové koncentrace sRAGE v porovnání s pacientkami s hrozícím předčasným porodem či v porovnání se zdravými kontrolami. Hladina sRAGE negativně korelovala s hladinou leukocytů u předčasného porodu. Pacientky s preeklampsií měly signifikantně vyšší sérovou koncentraci sRAGE ve srovnání se zdravými těhotnými ženami. Hladina sRAGE pozitivně korelovala s proteinurií, se sérovou hladinou kyseliny močové a kreatininu. Sérové koncentrace sRAGE nebyly změněny u pacientek s IUGR či ICP. Hladina sRAGE pozitivně korelovala se sérovou koncentrací kyseliny močové a kreatininu u pacientek s IUGR. Hladina sRAGE rovněž pozitivně korelovala se sérovou koncentrací alaninaminotransferázy (ALT) u pacientek s ICP. U sledovaných skupin nebyly nalezeny žádné rozdíly v genotypových či alelických frekvencích studovaných polymorfizmů genu pro *RAGE* a genu *glyoxalasy 1*.

Tyto výsledky částečně přispívají k pochopení patogeneze některých patologických stavů v těhotenství, což může být důležité při odhalování rizikových pacientek a zajištění adekvátní prenatální péče. Pro potvrzení našich výsledků je však nezbytné provést další studie s větším počtem subjektů, zejména pacientek s preeklampsií, IUGR a ICP.

Klíčové slova: receptor pro produkty pokročilé glykace, RAGE, solubilní receptor pro produkty pokročilé glykace, sRAGE, glyoxalasa I, předčasný porod, preeklampsie, nitroděložní růstová retardace, těhotenská cholestáza

ABSTRACT

Receptor for advanced glycation end products (RAGE), its soluble form (sRAGE) and glyoxalase 1 (GLO 1) are important part of pathogenesis of many chronic diseases.

The aim of this thesis was to elucidate role of sRAGE, four chosen *RAGE* polymorphisms and one *GLO 1* polymorphism in physiologic pregnancy and in pregnancy with complications.

Serum sRAGE levels were determined in healthy pregnant women (N=120) and in pregnancies complicated with threatening preterm labor (N=99), preeclampsia (N=35), intrauterine growth restriction (IUGR) (N=22) and intrahepatic cholestasis of pregnancy (ICP) (N=14). Four *RAGE* polymorphisms (*RAGE* -429T/C, -374A/T, *RAGE* Gly82Ser (557G/A), *RAGE* 2184A/G) and one *glyoxalase 1* polymorphism *GLO 1* Glu111Ala (419A/C) were studied in the same population of healthy pregnant women and women with pathological pregnancy.

Serum sRAGE levels are low in comparison to non-pregnant controls, but they vary during the physiologic pregnancy. Serum sRAGE levels are low in the 1st trimester, increased in the 2nd trimester and again decreased in the 3rd trimester. Women with premature labor have significantly decreased serum sRAGE levels in comparison to women with threatening premature labor and in comparison to healthy pregnant women. sRAGE correlates negatively with leukocyte count in preterm labor. Patients with preeclampsia have significantly increased serum sRAGE levels compare to healthy pregnant controls. sRAGE correlates positively with proteinuria, with serum uric acid and creatinine level. Serum sRAGE levels are not affected in patients with IUGR or ICP. sRAGE correlates positively with serum uric acid and creatinine level in patients with IUGR. sRAGE correlates negatively with serum alanine amino transferase (ALT) level in patients with ICP. There are no differences in genotype or allelic frequencies of studied RAGE and glyoxalase 1 polymorphisms among studied groups.

These results partly elucidate pathogenesis of pathological pregnancies. It might help to uncover high risk patients and provide them early adequate prenatal care. Further studies with larger studied group (especially patients with preeclampsia, IUGR, ICP) are still needed to confirm results.

Key words: receptor for advanced glycation end products, RAGE, soluble receptor for advanced glycation end products, sRAGE, glyoxalase 1, preterm labor, preeclampsia, intrauterine growth restriction, intrahepatic cholestasis of pregnancy

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CONTENTS

ABBREVIATIONS

Aβ	Amyloid Beta Peptide
ACBC4	ATP-binding Cassette, Sub-family B, Member 4
ABCB11	ATP-binding Cassette, Sub-family B, Member 11
ADAM 10	A Disintegrin and Metalloproteinase Domain containing Protein 10
AGE - R1	Advanced Glycation End Products Receptor 1
AGE - R2	Advanced Glycation End Products Receptor 2
AGE - R3	Advanced Glycation End Products Receptor 3
ALT	Alanineaminotransaminase
ANOVA	Analysis of Variance
APP	Amyloid Precursor Protein
ARE	Anti-oxidant Response Element
AST	Aspartateaminotransaminase
Bad	Bcl-2 Associated Death Promoter
Bax	Bcl-2 Associated X Protein
BSEP	Bile Salt Export Pump
Cdc42	Cell Division Control Protein 42
CEL	N^{ε}-Carboxy-Ethyllysine
CML	N^{ε}-Carboxy-Methyllysine
COX-2	Cyclooxygenase 2
cRAGE	cleaved Receptor for Advanced Glycation End Products
CREB	cAMP Response Element-binding Protein
CRP	C - reactive protein
DAMP	Damage Associated Molecular Pattern Molecule
dNTP	Deoxyribonucleotide Triphosphate
EDTA	Ethylenediamine-tetraacetic acid
EGF	Epidermal Growth Factor
ERK 1/2	Ras-extracellular Signal-regulated Kinase 1/2
EN-RAGE	Extracellular Newly identified RAGE binding Protein

esRAGE	endogenous soluble Receptor for Advanced Glycation End Products
EtBr	Ethidium Bromide
FEEL - 1	Fasciclin, Epidermal Growth Factor like, Laminin-type EGF-like, Link Domain containing Scavenger Receptor 1
FEEL - 2	Fasciclin, Epidermal Growth Factor like, Laminin-type EGF-like, Link Domain containing Scavenger Receptor 2
FH-1	Formin Homology 1 domain
GLO 1	Glyoxalase 1
GLO 2	Glyoxalase 2
GOLD	Glyoxallysine Dimer
GSH	Glutathione
GSSG	Glutathione Disulfide
HELLP	Hemolysis, Elevated Liver Enzymes, Low Platelets
HLA-A	Human Leukocyte Antigen A
HLA-B	Human Leukocyte Antigen B
HLA-G	Human Leukocyte Antigen G
HMGB1	High Mobility Group Box-1 Protein
HWE	Hardy-Weinberg equilibium
ICAM-1	Intercellular Adhesion Molecule 1
ICP	Intrahepatic Cholestasis of Pregnancy
IFCC	International Federation of Clinical Chemistry
IGF-1	Insulin like Growth Factor 1
IL-1	Interleukin 1
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IL-10	Interleukin 10
iNOS	inducible Nitric Oxide Synthase
IUGR	Intrauterine Growth Restriction
JAK	Janus Kinase
JNK	c-jun-NH2-terminal Kinase

LOX -1	Lectin-like Oxidized Low Density Lipoprotein Receptor 1
LPS	Lipopolysaccharide
MAP	p38 Mitogen Activated Protein
mDia-1	mammalian Dhiaphanous Protein 1
MDR3	Multi Drug Resistance Protein 3
MHC	Major Histocompatibility Complex
MMP 8	Matrix Metalopeptidase 8
MMP 9	Matrix Metalopeptidase 9
MOLD	Methylglyoxallysine Dimer
NDRG1	N-myc-Downstream Regulated Gene 1
NF-κB	Nuclear Factor-kappaB
NMDA	N-Methyl-D-Aspartate
Nrf2	Nuclear Factor-erythroid 2 p45 Subunit-related Factor 2
p53	Tumor Protein 53
PAMP	Pathogen Associated Molecular Pattern Molecule
PCR	Polymerase Chain Reaction
PDGF	Plate Derived Growth Factor
PLGF	Placental Growth Factor
PPROM	Preterm Premature Rupture of Membranes
RAAS	Renin Angiotensin Aldosterone System
Rac1	Ras - related C3 Botulinum Toxin Substrate 1
RAGE	Receptor for Advanced Glycation End Products
RFLP	Restrictrion Fragment Length Polymosphism
rpm	rotations per minute
SAPK	Stress Activated Protein Kinase
sAPPα	soluble Amyloid Precursor Protein α
sAPPβ	soluble Amyloid Precursor Protein β
sFLT-1	soluble Fms-like Tyrosine Kinase 1
SDS	Sodium dodecyl sulphate
SGA	Small for Gestational Age
SH3 domain	SRC Homology 3 domain

SLB	Salmiak Lysis Buffer
SNP	Single Nucleotide Polymorphism
SR - A	Macrophage Scavenger Receptor class A
SR - B	Macrophage Scavenger Receptor class B
sRAGE	soluble Receptor for Advanced Glycation End Products
STAT	Signal Transducer and Activator of Transcription
STAT-3	Signal Transducer and Activator of Transcription 3
STD	Sexually Transmitted Diseases
Taq	*Thermus Aquatic* DNA polymerase
TGF-1β	Transforming Growth Factor 1β
TLR 4	Toll-like Receptor 4
TLR 2	Toll-like Receptor 2
TNF-α	Tumor Necrosis Factor α
TRIS	2-Amino-2-Hydroxymethyl-1,3-Propanediol
UDCA	Ursodeoxycholic Acid
VCAM -1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor

1 Introduction

Pregnancy is an important part of human life. The development of an embryo in the uterus and its interaction with the mother's body is a complex process. Many aspects of human pregnancy have already been discovered and described, but many others remain unclear.

Physiological pregnancy is associated with enhanced oxidative stress, and its importance in pathological pregnancy is considerably higher.

1.1 Receptor for Advanced Glycation End Products

The receptor for advanced glycation end products, RAGE, was first described by Neeper et al. in 1992 (Neeper M. et al., 1992). It was first described as a receptor for advanced glycation end products, a heterogeneous group of compounds (See 1.1.4.1) however other ligands for RAGE were described later (See 1.1.4).

1.1.1 Structure of the Receptor for Advanced Glycation End Products

The receptor for advanced glycation end products, RAGE, is a transmebrane protein receptor, located in the cellular surface of various types of cells. Mature RAGE protein consists of 404 amino acids. The molecular weight of the RAGE protein is variable, between 45 and 55 kDa and depends on the different glycosylation state of the protein. RAGE has an extracellular domain consisting of 321 amino acids (amino acids 23-317), a single membrane-spanning domain (amino acids 343-363) and a C terminal intracellular domain comprising 41 amino acids (amino acids 364-404). N terminal signal peptide (amino acids 1-22) is cleaved after glycine 22. See Figure 1.1.

Further analyses of RAGE showed 3 immunoglobulin-like domains localized in the extracellular domain. These domains are defined by a set of conserved cysteine residues. The first extracellular RAGE domain (amino acids 23-116) has a similar pattern to the variable immunoglobulin domain (V-like domain). It contains 2 potential N-linked glycosylation sites. The second and third extracellular domains (amino acids 124-221, resp. 227-317) resemble a constant immunoglobulin domain (C2-like domains). Cysteine from the sequence of amino acids thyroxine - serine - cysteine -valine - alanine - threonine - histidne (YSCVATH motif) located at position 299

of the domain, forms an intradomain disulfide bond with a near -COOH terminal cysteine. The pattern "(phenylalanine- thyroxine) - x - cysteine - x - (valine-alanine) - x - histidine" [(F, Y)xCx(V, A)xH], predicates an immunoglobulin / major histocompatibility complex signature, especially an immunoglobin constant type domain (Neeper M. et al., 1992).

The C-terminal intracytoplasmatic domain has a unique structure, it has no specific structural motif characteristic for receptor signaling and it lacks protein-tyrosine or serine/threonine kinase activity. The N-terminal segment of the cytoplasmatic tail of RAGE folds into an alpha-turn followed by an unstructured well conserved part (Rai V. et al., 2012). One side of the alpha-turn is polar and hydrophilic, and its primary structure is well conserved. In vitro studies revealed the importance of two amino acids, arginine at position 5 and glutamine at position 6, which are part of the alpha-turn and are relevant for the interaction between the RAGE cytoplasmatic tail and the only known cytosolic binding partner, protein mammalian Diaphanous-1 (mDia-1). The study of Rai et al. (Rai V. et al., 2012) showed that the mutation of these two amino acids into two alanines inhibits RAGE – ligand cell signaling.

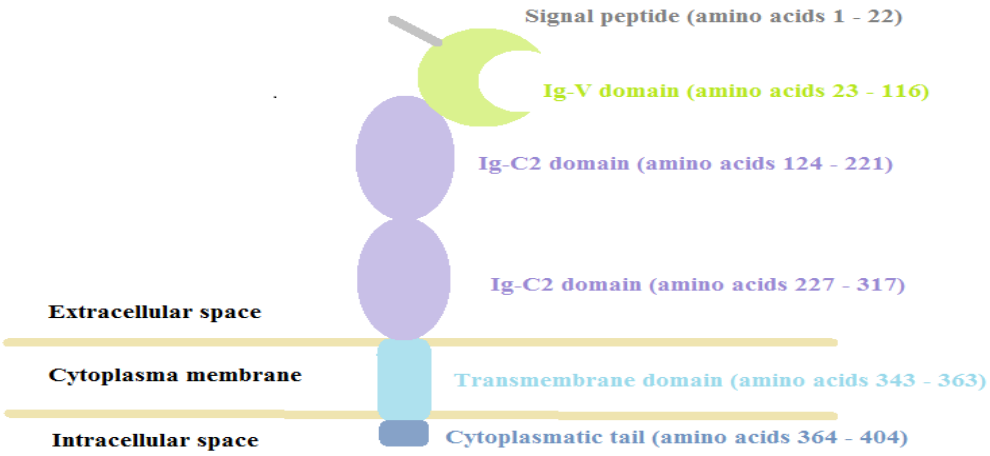


Figure 1.1 Structure of the receptor for advanced glycation end products

The single cytosolic binding partner of the RAGE, mDia-1, was identified by Hudson et al. in 2008 (Hudson BI. et al., 2008). mDia -1 belongs to the formin protein family. It contains the formin homology 1 domain (FH-1 domain), characterized by polyproline stretches. The polyproline repeats are able to interact with several specific protein motifs which are included in cell signaling.

Thus, Dia – 1 interacts with the SRC homology 3 domain (SH3 domain), the WW domain and other domains specific for signaling proteins. mDial-1 is a potent actin and microtubule polymerization factor.

1.1.2 Signaling of the Receptor for Advanced Glycation End Products

RAGE signaling pathways are not completely revealed. However, it is known that the RAGE – ligand interaction results in the activation of several signal transduction pathways dependent on the ligand type, environment and cell type. RAGE signaling pathways include reactive oxygen species, Ras-extracellular signal-regulated kinase 1/2 (ERK 1/2) (Huttunen HJ. et al., 2002), cell division control protein 42/Ras related C3 botulinum toxin substrate 1 (Cdc42/Rac1) (Huttunen HJ. et al., 1999), stress-activated protein kinase/c-jun-NH2-terminal kinase (SAPK/JNK), p38 mitogen-activated protein (MAP) kinase (Taguchi A. et al., 2000), p21/ras, rhoGTPases, phosphoinositol-3 kinase and Janus kinase/signal transducer and the activator of transcription (JAK/STAT) pathway (Huang JS. et al., 2001), with important downstream consequences, such as the activation of nuclear factor-kappaB (NF-κB) (Yan SD. et al., 1994), cAMP response element-binding (CREB) protein (Huttunen HJ. et al., 2002) or the signal transducer and activator of transcription 3 (Stat-3) (Huang JS. et al., 2001).

1.1.3 Effect of the Receptor for Advanced Glycation End Products activation

RAGE-ligand interaction leads to the activation of several signaling pathways (Huttenen HJ. et al., 1999, Huttenen HJ. et al., 2002, Taguchi A. et al., 2000, Huang JS et al., 2001) (See 1.1.2). The activation of the receptor finally results in the expression of genes for enzymes such as cyclooxygenase-2 (COX-2) (Shanmugan N. et al., 2003), inducible nitric oxide synthase (iNOS) (Sousa MM. et al., 2001), genes for cytokines and growth factors like tumor necrosis factor - α (TNF-a), interleukin - 1 (IL-1), (Wang Z. et al., 2002), plate-derivated growth factor (PDGF) (Yamamoto Y. et al., 1996), insulin like growth factor - 1 (IGF-1) (Pugliese, 1997) or interferon γ (Imani F. et al., 1993). RAGE-ligand interaction also promotes the expression of genes for adhesion molecules, e.g. intercellular adhesion molecule - 1 (ICAM-1) and vascular cell adhesion molecule - 1 (VCAM-1) (Schmidt AM. et al., 1995). Furthermore, NF-κB activated by RAGE downregulates

apoptosis-promoting genes, such as the gene for tumor protein 53 (p53), Bcl-2 associated X protein (Bax) and Bcl-2 associated death promoter (Bad) (Zamora R. et al., 2005). RAGE activation induces macrophages, stimulates cells proliferation, up-regulates pro-thrombotic pathways (Yamagishi S. et al., 1996) and increases the synthesis of collagen IV, fibronectin and proteoglycans (Pugliese, 1997). RAGE activation also suppresses the creation of reduced gluthathione and thus causes increased intracellular oxidative stress (Chen J. et al, 2010). The depletion of reduced gluthation reduces the activity of glyoxalase 1, an enzyme, which prevents the formation of AGEs (See 1.2).

RAGE - ligand activation causes a positive feed-forward loop, in which the stimuli activate NF-κB, which induces RAGE expression, followed again by NF-κB activation.

Shortly, RAGE activation results in proinflammatory, proliferative and anti-apoptoic cell responses.

1.1.4 Receptor for Advanced Glycation End Products Ligands

RAGE is a multiligand receptor which recognizes and binds a broad repertoire of ligands. The most important are advanced glycation end products, a family of S100/calgranulins proteins, aphotherin and amyloid β peptide, however, RAGE also recognizes molecules on the surfaces of bacteria, prions and leukocytes.

1.1.4.1 Advanced Glycation End Products

Advanced glycation end products, AGEs, were the first ligands described for RAGE. AGEs are a wide group of heterogeneous compounds which can cause tissue damage directly, by modifying the biological structure and changing their physical and chemical properties or indirectly, via binding to RAGE.

AGEs are mostly products of the non-enzymatic glycation, Maillard reaction, which was first described in 1912 by Maillard. The first step involves the condensation of an aldehyde group of a reducing sugar and a primary ε-amino group (lysine residues) of protein which forms through a carbinolamine intermediate reversible Schiff base. The reaction is dependent on sugar concentration. Schiff base then rearranges into a more stable ketoamine, usually called Amadori product (Amadori M., 1929), at room temperature and physiologic pH. The production of Amadori products is quicker in the presence of phosphate ions and at an alkaline pH (Isbell H., Frush H.,

1958). The conversion is partially reversible. Even if Amadori products are more stable than Schiff bases, they can undergo enolization, and depending on pH, can produce furfural derivates or reductones via dicarbonyl intermediates. Intermediate steps include condensation, dehydratation and cyclization. Amadori products can also form dicarbonyls through retro-aldol fragmentation in the presence of metal ion or through oxidative fission. Dicarbonyl compounds are very reactive and critical for the production of AGEs. All these reactions are independent of the sugar concentration. The precise mechanism of the production of AGEs is still not completely clear and probably involves complex parallel and sequential reactions (Zeitsch K., 2000, Kurata T., Otsuka Y, 1998, Davidek T. et al., 2006, Cammerer B. et al., 1999, Watkins NG. et al., 1985) (See Figure 1.2).

Except for the described Maillard reaction, oxidative and carbonyl stress are considerable sources of AGEs together with exogenous sources in "western" food and in smoking.

AGEs are a diverse group of compounds. Their common properties include a yellow-brown pigmentation, linkage to proteins, the ability of cross-linking and reaction with AGE specific receptors. They have specific characteristic fluorescence excitation at 350 nm and emission at 430 mm, however some of them are non-florescent (N^{ε}-carboxy-methyllysine, methylglyoxallysine dimer, glyoxallysine dimer). Nowadays, more than 20 AGEs have been identified and characterized, such as N^{ε}-carboxy-methyllysine (CML), pentosidine, glyoxallysine dimer (GOLD), methylglyoxallysine dimer (MOLD), imidazolone (3-deoxyglucosone-arginine-imidazolon), N^{ε}-carboxy-ethyllysine (CEL) and pyralline (Wells-Knecht KJ. et al., 1996, Dyer DG. et al., 1991, Ahmed MU. et al., 1986)

An organism has several ways to detoxify AGEs. However, the most important is the glyoxalase system which decreases serum levels of methylglyoxal and glyoxal, the precursors of the biggest pool of AGEs in vivo (Ranganathan S. et al., 1995). For more information see chapter 1.2. The other enzymes, such as aldose reductase and aldehyde dehydrogenase, also take part in the degradation of AGEs precursors. Tissue AGEs are eliminated mostly via endocytosis by macrophages or by endothelial cells, fibroblasts or mesangial cells via receptor specific and non-receptor systems. After endocytosis AGEs undergo proteolysis, which forms low molecular weight AGE-peptides. These are eliminated from an organism by the kidney (Vlassara H., 2001, Urbarri J. et al, 2003).

AGEs can cause damage directly by changing the properties of an extracellular matrix. They bind to the proteins and change their physical and chemical characteristics, increase resistance to

termic and enzymatic digestion, increase acid-degradation stability, change the charges and solubility of molecules, and they cause cross-linking of molecules (Kalousová M. et al, 2004).

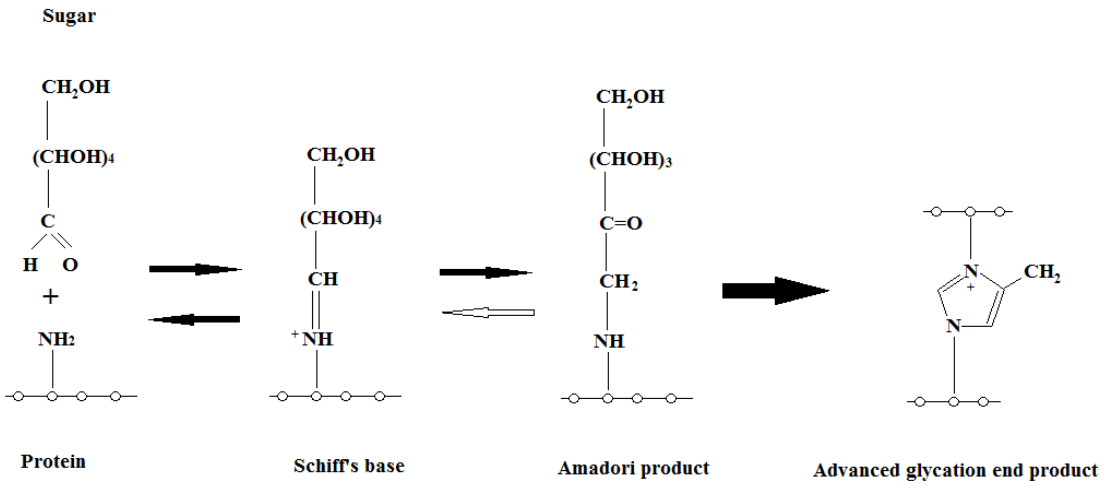


Figure 1.2 Maillard reaction

AGEs cause indirect damage by binding to specific receptors. Several receptors which bind AGEs are identified. Beside RAGE, the most known receptors are: AGE - R1 (type I integral membrane protein), which takes part in the endocytosis of AGEs, AGE - R2 (AGE inducible tyrosine-phosphorylated protein), which is involved in cell signaling, and AGE - R3 (Galectin 3), important for the detoxification of AGEs. Other AGEs binding receptors are: the macrophage scavenger receptor class A (SR-A), class B (SR-B1), the lectin-like oxidized low density lipoprotein receptor-1 (LOX-1), the fasciclin, epidermal growth factor (EGF)-like, laminin-type EGF-like, link domain containing scavenger receptor 1 and 2 (FEEL-1, FEEL-2), megalin and the toll-like receptor 4 (TLR4) (Vlassara H, 2001).

AGEs have different potential to trigger the most important AGE receptor, RAGE, e.g. CML is a strong inducer of RAGE signaling, while pyrraline and pentosidine do not start RAGE signaling (Herold K. et al., 2007).

AGEs play an important role in the pathogenesis of many diseases. Their importance in the pathophysiology of diabetes mellitus and its complications is well known (Makino H. et al, 1996). Nevertheless, they are part of the pathogenesis of hypertension and cardiovascular diseases (Soldatos G., Cooper ME., 2006), chronic inflammatory diseases (Miyata T. et al., 1998)

neurodegenerative diseases (Takeuchi et al., 2004), cancer (Stopper H. et al., 2003) and they accumulate in patients with chronic renal diseases (Makita Z et al., 1991).

The role of advanced glycation end products was also studied in pathological pregnancy. Chekir et al. showed the accumulation of AGEs in placentas of patients with preeclampsia (Chekir, 2006). The study by Harsem et al. proved elevated serum AGEs levels in patients with gestational diabetes. However, patients with preeclampsia did not show increased serum AGEs levels (Harsem NK. et al., 2008).

1.1.4.2 S100 Proteins

S100 proteins are calcium binding proteins. They were first isolated from bovine brain in 1965 by Moore. They were called S100 proteins because they are soluble in 100% saturated ammonium sulfate at a neutral pH (Moore BW et al., 1965). Most of them contain approximately 100 amino acids, with two calcium binding EF-hand motifs and a central hinge region between them, which is distinct for every member of this protein family and in some cases, is followed by a family specific carboxy terminal tail. The EF-hand is composed of helix-loop-helix motifs. S100 proteins are distinguished from other EF-hand proteins via the manner in which they bind ions of Ca^{2+} at their N-terminal binding sites. Their ability to bind transition metals like copper, manganese and zinc differs them from other EF-hand proteins (Donato R., 2001, Heizmann CW et al., 2002). S100 proteins form either homodimers or higher order oligomers. Only some of them form heterodimers.

S100 proteins are expressed in a cell-specific manner and they act intracellularly as well as extracellularly. S100 proteins are expressed in the heart and skeletal muscle (S100A1), the lung and kidney (S100A2), neurons (S100A4, S100B), fibroblasts, epithelial cells (S100A6), keratinocytes (S100A7), granulocytes, monocytes, macrophages (S100A8/A9) and in the placenta (S100P).

Intracellularly, S100 proteins control various types of cell activity in a Ca^{2+} dependent manner. At low intracellular Ca^{2+} levels, they exist in a closed relatively hydrophilic conformation, at high Ca^{2+} levels, they bind ions of Ca^{2+} and expose hydrophobic sites which allow them to interact with specific target proteins, including membranes, cytoskeleton, transcription factors, enzymes, channels, receptors and signaling molecules (Donato R., 2001)

The extracellular acting of S100 proteins differs. Extracellularly S100 proteins have al Ca^{2+} binding sites occupied. Their ability to interact is dependent on the properties of the binding transition metals and the potential of oligomerisation. Extracellular S100 proteins exist in a hydrophobic state. They either interact with the lipid phase of the cell membrane and change membrane channel activity (Zolese G., 1988) or they affect membrane receptors. S100 proteins interact with G-protein coupled receptors, scavenger receptors, the toll-like receptor and RAGE. RAGE can bind several S100 proteins, including S100A1, S100A2, S100A5, S100A6, S100A7, S100A8/A9, S100A11, S100A12, S100A13 and S100B (Donato R., 2007).

Intracellular S100 proteins regulate a lot of cellular activities. They are part of the cell cycle, cell differentiation and survival, apoptosis, cell motility, intracellular Ca^{2+} metabolism and the transduction of intracellular signals. They also take part in learning and memory, phototransduction and innate and adaptive immunity (Arcuri C. et al., 2005, Liu J. et al., 2011, Brozzi F et al., 2009, Donato R., 2001). They take part in many pathological conditions, like inflammation (Donato R., 2001), cancer (Donato R, 2001, Donato R., 2007), diabetes and its complications (Kosaki A. et al., 2004), atherosclerosis (Averill MM et al., 2011) and neurodegeneration and mood disorders (Steiner J. et al., 2010).

1.1.4.2.1 S100A12 (EN-RAGE)

S100A12 or EN-RAGE (extracellular newly identified RAGE binding protein) is a member of the S100 protein family. EN-RAGE is expressed in neutrophils and it constitutes 5% of all cytosolic proteins in resting neutrophils. However, the expression of EN-RAGE is stimulated in activated granulocytes and monocytes under inflammatory conditions.

The mature EN-RAGE protein consists of 91 amino-acids and a missing N-terminal methionine, with typical secondary and tertiary structure of S100 proteins with the presence of 2 EF-hand calcium binding domains and hinge region. The 15 amino-acid long C-terminal sequence is equivalent to calcitermin, peptide with antibacterial and antifungal properties. It is located in the human respiration secret (Cole AM. et al., 2001). The sequence has a putative zinc binding domain and helical conformation (Cole AM et al., 2001). Amino acids 3-39 are probably needed for binding to RAGE, because the residues share homology with the RAGE binding motif of another RAGE

ligand, a high-mobility group box-1 protein (HMGB-1). EN-RAGE binds calcium, copper and zinc ions.

Intracellular EN-RAGE exists as an antiparalel homodimer, extracelullar EN-RAGE forms homodimers and hexamers. EN-RAGE is secreted via an alternative pathway bypassing the classic Golgi route.

Data about intracellular EN-RAGE protein targets are limited. The best known extracellular target protein is RAGE (Donato R., 2007). EN-RAGE binding to the VC1 domain of RAGE triggers a signal cascade of MAP-kinase, leading to NF-κB activation. The process results in the secretion of cytokines TNF-α and interleukin-1β (IL-1β) and the expression of adhesion molecules ICAM-1 and VCAM-1. EN-RAGE thus mediates pro-inflammatory action on neutrophils, phagocytes, lymphocytes and endothelial cells (Yang Z. et al., 2001).

It was believed for a long time that extracellular EN-RAGE interacts only with RAGE. Recently, new evidence has shown that EN-RAGE interacts with the non-RAGE cell surface receptor on mast cells and monocytes. The G-protein-coupled receptor is likely to be responsible for the non-RAGE cells effect (Yan WX., 2008).

Regarding EN-RAGE properties and effects, the role of EN-RAGE has been studied in the pathogenesis of many proinflammatory states, such as inflammatory bowel disease (de Jong NS. et al., 2006), rheumatoid arthritis (Wittkowski H. et al., 2007), Kawasaki disease (Foell D. et al., 2003), 2005), in atherosclerosis (Kim JK et al., 2012), hypertension and diabetes mellitus (Kosaki A. et al., 2004).

The serum level of EN-RAGE was also measured in women with preeclampsia and it was shown that EN-RAGE serum levels are elevated in preeclampsia (Naruse K et al., 2012).

1.1.4.3 High-mobility Group Box protein 1

High-mobility Group Box protein 1 (HMGB1) was first purified in 1973. It was named according to its electrophoretic mobility in polyacrylamide gel. HMGB1 is highly conserved protein through species and it is found in all eukaryotic cells (Goodwin GH et al., 1973).

HMGB1 consists of 215 amino-acids, with a molecular weight of 25 000 Da. The protein has two DNA binding domains, called boxes A and B, and a C-terminal domain. A and B boxes are formed of 80 amino-acids, arranged in three α-helices. The boxes have an unusual r - like shape,

containing two shoulders of which one is slightly shorter. These domains are strongly positively charged. The C-terminal domain contains 30 repetitive aspartic and glutamic acid residues and is strongly negatively charged (Tsuda K et al., 1988). See Figure 1.3.

HMGB1 was firstly described as a non-histone nuclear protein, which binds double stranded DNA. It is able to alter DNA. HMGB1 participates in the regulation of chromatin structure, transcription, DNA damage repair and recombination (Calogero S. et al., 1999, Lange SS. et al., 2009). It regulates gene expression by modulating the compactness of chromatin fiber. HMGB-1 decreases the compactness of chromatin and thus improves the access of transcriptional factors to the DNA strands. Histones and HMGB1 compete for the same binding sites (Bonaldi T et al., 2002, Jayaraman L. et al., 1998).

However, HMGB1 has an extracellular function as well. HMGB-1 is released from cells of the innate and adaptive immune system after stimulation by various signals, such as lipopolysaccharide (LPS), IL-1β, TNF-α (Bianchi ME., Manfredi AA., 2007), via secretory lysosomes (Bonaldi T. et al., 2003), but it can be passively released from necrotic or damaged cells (Rovere-Querini et al., 2004) in contrast to apoptotic cells which modify their chromatin so HMGB-1 can not be released (Scaffidi et al., 2002).

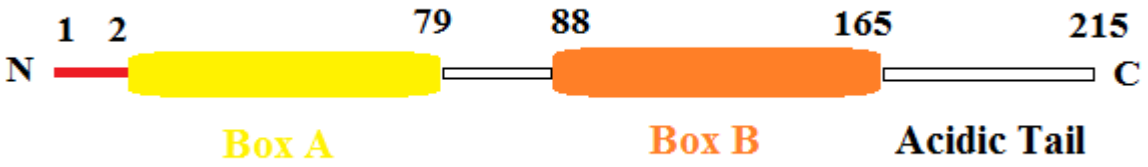


Figure 1-3. Structure of HMGB1 protein

HMGB1 binds to several pattern-recognition receptors such as Toll-like receptor 2 (TLR 2), Toll-like receptor 4 (TLR 4) and RAGE. The HMGB-1 interaction with TLR 2 or TLR 4 activates several different protein-kinase signaling pathways, including JNK, MAP and ERK1/2. The HMGB-1 – RAGE interaction activates two different signaling pathways. One of them includes

small GTPases (Rac1, Cdc-42) and causes the reorganization of cell the cytoskeletal network. The other one includes MAP kinase, which subsequently activates NF-κB (Huttunen HJ. et al., 1999).

Studies show that HMGB1 does not act like a signal molecule alone. It interacts with other molecules including DNA, RNA, LPS and IL -1β. HMGB1 complex with DNA or RNA binds and signals via RAGE. HMGB-1 associated with IL -1β signals through the IL-1 receptor or associates with LPS and triggers TLR 4. HMGB1 – nucleosome complex activates preferentially TLR 2 (Sha Y. et al., 2008)

HMGB1 is considered as a damage-associated molecular pattern molecule, DAMP, molecules which activate and intensify immune response after cell exposure to various damage types, including infections as well as different kinds of trauma (radiation, cytotoxic agents). DAMPs are subdivided into endogenous molecules, called alarmins and exogenous molecules, called pathogen–associated molecular pattern molecules or PAMPs.

HMGB1, as alarmin, has a wide spectrum of proinflammatory effects. It stimulates the maturation of immune cells (dendritic cells, T-cells), their migration to the site of injury, the release of cytokines and others inflammatory mediators (Kokkola R. et al., 2005). HMGB-1 also stimulates the production of oxygen species by neutrophils. HMGB-1 stimulation of endothelial cells causes the release of Il-8, TNF- α, MCP- 1) and the expression of ICAM-1 and VCAM -1 on the endothelial cell surface (Messmer D. et al., 2004, Dumitriu IE et al., 2005, Sha Y. et al., 2008).

HMGB-1 takes part in promoting tissue repair and regeneration. It induces stem cell migration to the site of inflammation, as well as it promotes mesangioblasts and endothelial proliferation and migration through the endothialial layer of vessels (Mitola S., 2006). The topical administration of HMGB1 to the wounds of diabetic mice led to accelerated wound healing, the HMGB1 inhibitor also suppressed wound healing in non-diabetic mice (Straino S. et al., 2008).

HMGB1 was first considered a late mediator of sepsis (Wang H. et al., 1999), however, latter studies showed its importance in the pathogenesis of several other pathological states, such as autoimmune diseases (Kokkola R. et al., 2002), cancer and the development of metastases (Kuniyasu H. et al., 2003) and in trauma and sterile inflammation (Peltz ED. et al., 2009, Kohno T et al., 2009).

The role of HMGB-1 was also studied in physiological and pathological pregnancy. HMGB1 is expressed in almost all cells of a term placenta and its higher expression was identified in placentas of women suffering from preeclampsia (Holmlund U. et al., 2007). Nevertheless, serum

HMGB1 levels of patients with preeclampsia do not differ from the serum HMGB1 level of healthy pregnant women. Romero et al. (Romero R. et al., 2012) showed elevated HMGB1 levels in the amniotic fluid of pregnant women with preterm labor regardless of the state of amniotic membranes.

1.1.4.4 Amyloid Beta Peptide

Amyloid beta peptide (Aβ) is a peptide with a molecular weight of approximately 4000 Da and usually contains 36-43 amino acids. Aβ is amphiphilic, the first 28 amino acids are polar, the rest comprise non-polar hydrophobic amino acids, isoleucine and alanine. This primary structure is responsible for their tendency to aggregate (Jarrett JT. et al., 1993). Aβ has a β-sheet-like secondary structure. The unfolded Aβ monomers form dimers, trimers or higher order oligomers, which can subsequently form insoluble fibrils and plaques.

Aβ originates from amyloid precursor protein (APP). It is a ubiquitous single transmembrane glycoprotein, which is mainly expressed in the cells of the central nervous system. Its physiological function is not quite clear, but it is known that APP participates in the regulation of the synaptic function and neuronal activity (Priller C. et al., 2006), the regulation of cholesterol metabolism (Grimm MO., 2005) and is part of the iron cell export system (Duce JA. et al., 2010). APP undergoes proteolytic cleavage of its extracellular domain by two possible pathways. The α-secretase cleavage of APP prevents the production of Aβ because the α-secretase cleavage site is located within Aβ at the bond Lys16-Leu17. The α-cleavage produces a large soluble ectodomain of APP called soluble amyloid precursor protein α (sAPPα). The production of sAPPα is constitutive and APP is primarily processed by this secretase. Aβ is produced as APP undergoes the other possible cleavage pathway via β-secretase. It acts at two possible cleavage sites, known as β-sites, Asp1 and Glu11. Upon the β-cleavage, APP releases a soluble ectodomain called soluble amyloid precursor protein β (sAPPβ), which in comparison to sAPPα is missing its carboxy-terminal part, Aβ1-16. 99 amino-acids C-terminal fragment within the membrane, which is created at β-cleavage, is subsequently processed by γ-secretase to produce 36-43 amino acid long fragments of Aβ.

Current studies focus on Aβ, especially on Aβ42 (42 amino acids) mostly as a cause of Alzheimer disease, and its pathological effects. However, Aβ also has a physiological function in organisms. It is involved in kinase activation, the protection against metal-induced oxidative

damage, the regulation of cholesterol transport, the formation of an ion channel and anti-microbial activity.

Aβ interacts with a various number of cell surface proteins, including APP, N-methyl-D-aspartate (NMDA) receptors, integrins, α7nicotinic acetylcholine receptors, p75 neurotrophin receptors, RAGE, serpin-enzyme complex receptors, insulin receptors, scavenger receptors on microglial cells and heparine sulphate proteoglycans (Deane R. et al., 2004).

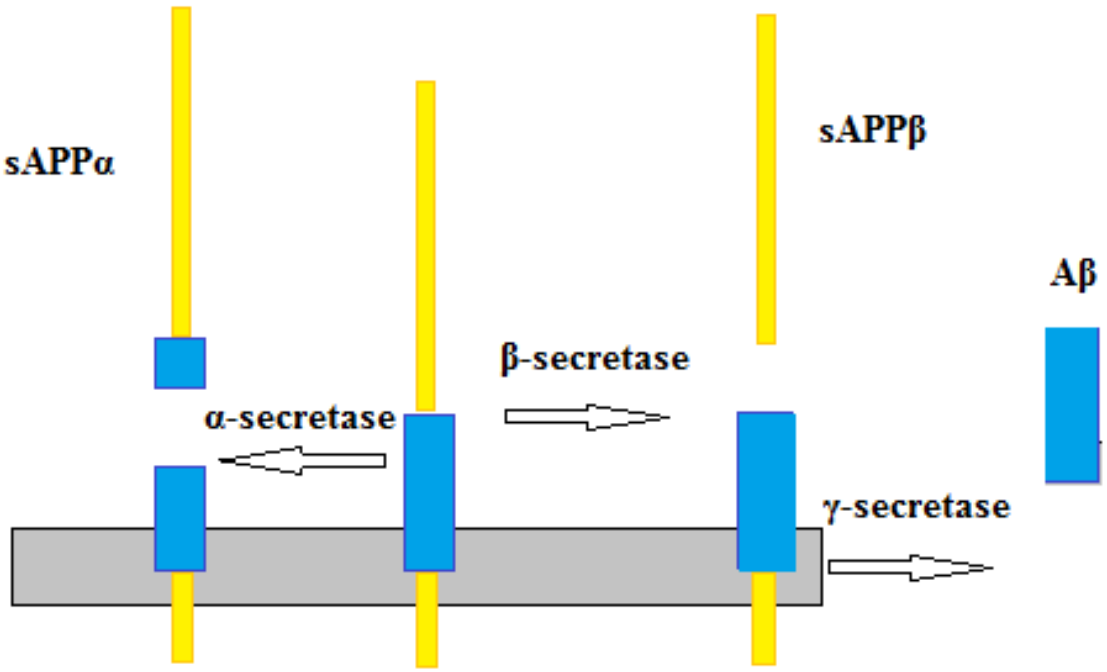


Figure 1.4 Formation of Amyloid Beta Peptide

Aβ oligomers can incorporate the membranes and form calcium channels and thus, deregulate cell homeostasis.

The interaction between RAGE and Aβ results in the activation of NF-κB, the generation of oxidative stress, the expression of adhesion molecules, VCAM, ICAM, cytokines and endothelin-1

causing inflammation. The activation of RAGE induces the transcytosis of Aβ from plasma to the brain, which may promote apoptosis (Deane R. et al., 2004).

As said before, Aβ is mostly studied due to its important role in the pathogenesis of Alzheimer's disease. Today there is no research which studies Aβ and its role in physiological or pathological pregnancy.

1.1.5 Receptor for Advanced Glycation End Products Expression

RAGE expression in organism, both in a constitutive or inducible manner, depends on the development stage and cell type.

During the embryonic stage, RAGE is constitutively expressed in high levels in all cells, especially in cells of the central nervous system. RAGE is down-regulated in most cells during adult life. It is constitutively highly expressed in the lungs and skin of adults, a low constitutive expression is found in the spleen, thymus, pancreas, leukocytes, small intenstine, liver, testis, ovaries, kidneys and colon (in descending order) (Demling R et al., 2006). Monocyte/macrophages, fibroblasts, endothelial cells, smooth muscle cells and neural cells do not express RAGE in significant levels upon physiological condition. However, RAGE can be expressed in these cells after stimulation.

1.1.6. Genetics of Receptor for Advanced Glycation End Products

The human *RAGE* gene is located on chromosome 6 (6p21.3) in the major histocompatibility complex (MHC) class III region. It is 3354 bp long and is composed of a 5' flanking region, 11 exons, 10 introns and a short 3' UTR part. At the 5' end multiple transcription start sites were identified, at positions -11, -13, -31 and -121 and at the '3 end, only a polyadenylation signal was found. Upon transcription an mRNA of approximately 1.4 kB arises.

According to the database of polymorphism from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=177), in May 2014, there were 188 known single nucleotide polymorphisms (SNP) of the RAGE gene. SNP is the simplest polymorphism, where only one nucleotide in the DNA is changed, inserted or deleted. Depending on the location and nucleotide type, SNP can cause a change of amino acid in the peptide chain -

non-synonymous SNP, or it doesn't cause a change of amino acid - synonymous SNP. SNPs cause 90% of all genetic mutations.

Out of 188 SNPs of the RAGE gene:

- 60 SNPs are in exons
- 62 SNPs are in introns
- 3 SNP is in the 5' UTR part
- 3 SNPs are in the 3' UTR part
- 47 SNPs are in the5' flanking region
- 13 SNPs are in the 3' flanking region

In exons, there are 49 non-synonymous SNPs and 11 synonymous SNPs (See Table 1.1)

Table 1.1 Single nucleotide polymorphism in exons of gene for RAGE

Exon	Non-synonymous SNP		Synonymous SNP		Deletions	
2	Ala28Gly	rs386544768	Gly18Gly	rs372263884		
	Ala28Val	rs17846804	Gly22Gly	rs368584948		
	Arg29Trp	rs144337279	Leu49Leu	rs370229513		
	Pro33Leu	rs199964705				
	Cys38Trp	rs201829223				
	Arg48GTrp	rs375902201				
	Arg48Gln	rs35030981				
	Trp51Ter	rs35802968				
3	Trp72Ter	rs200718197	Gly70Gly	rs143397572	Gln67Pro	rs35334561
	Arg77Cys	rs80096349	Asn81Asn	rs375871446		
	Gly82Ser	rs2070600	Pro87Pro	rs142500219		
	Lys110Arg	rs147376814	Val89Val	rs35795092		
	Arg114Ter	rs373195347				
	Arg114Gln	rs17846806				
	Gln119Ter	rs142731923				
5	Val141Met	rs372397484				
	Cys144Trp	rs138726985				
	Ala152Thr	rs368175894				
	Leu159Phe	rs188057660				
6	Arg179Gly	rs370118672	Glu175Glu	rs148384214		
	Arg198Gln	rs375056984	Arg198Arg	rs368048335		

	Phe206Ser	rs373910069		
	Arg216Ter	rs202226593		
	Arg218Trp	rs149408505		
7	Val241Leu	rs17846799		
	Gly246Asp	rs150282885		
	Ala248Gly	rs376859625		
	Val261Asp	rs201178949		
	Trp271Arg	rs140930365		
8	Glu290Gln	rs201866140		
	Pro293Leu	rs201967398		
	Cys301Ser	rs138178120		
	Gly309Arg	rs138186526		
	Arg314His	rs77170610		
10	Gly346Glu	rs149093508	Leu343Leu	rs143357175
	Ala355Thr	rs145090083		
	Arg368His	rs373730068		
	Arg368Cys	rs367834402		
	Arg369Gln	rs386580498		
	Arg369Gly	rs149968577		
	Arg369Gln	rs3176931		
11	Arg385His	rs373746820	Glu383Glu	rs191750543
	Asn389His	rs370335494		
	Ser391Leu	rs147340908		
	Glu393Lys	rs376556772		
	Pro394Thr	rs144228131		
	Gly397Arg	rs200524532		
	Gly403Glu	rs140366801		

Most of these SNPs seem to be very rare in population (Kaňková K. et al., 2001), so only a small number of them came to the center of attention.

Two polymorphisms in the promoter region of the *RAGE* gene, -429 T/C and -374 T/A, are some of the most studied. The -374 T/A polymorphism in the regulatory site causes decreased binding of the negative nuclear factor and thus, increased gene transcription (Hudson BI. et al., 2001). The -429 T/C polymorphism also causes increased transcriptional activity of RAGE in vitro, however, data about the behavior of this polymorphism in vivo are not so clear (Hudson BI. et al., 2001). The studies pointed to the importance of -374T/A polymorphism in cardiovascular diseases (Falcone C., 2004) and in diabetes and its complication (Kawai T. et al., 2013). The minor allele

-429C of the *RAGE* -429T/C polymorphism has a higher incidence in patients with diabetes type I and is linked to insulin resistance (Sullivan CM. et al., 2005). *RAGE* -429 T/C polymorphism is associated with systemic lupus erythematosus and lupus nefritis (Martens HA. et al., 2012). *RAGE* -429CC genotype is also considered a prognostic factor in chronic hemodialysis patients, while it is related to a higher mortality of these patients (Kalousová M. et al., 2010).

Polymorphism Gly82Ser (557G/A) is located in exon 3. Changing guanine to adenine causes the substitution of glycine to serine in position 82 within the V domain of RAGE. The mutation leads to N-glycosylation at Asn81 and results in the enhanced binding of S100A12 and AGEs. The minor allele is also associated with lower sRAGE levels (Park SJ. et al., 2011). The significance of polymorphism was shown in many pathological states, such as diabetes and its complications (Kaňková K. et al., 2001) or Alzheimer's disease (Li K. et al., 2010).

RAGE polymorphisms 1704 G/T, 2184 A/G and 2245G/A are located in intron 8. The way of their action is not clear, but it is supposed they are involved in alternative splicing of RAGE mRNA (Kaňková K. et al., 2001), while alternative splicing involves the region between intron 7 and 9. *RAGE* 2184A/G polymorphism is associated with lupus erythomathosus and lupus nephritis (Martens HA. et al., 2012), non-diabetic microvascular dermatoses (Kaňková K. et al., 2001), breast cancer (Tesařová P. et al., 2007) and oxidative status (Kaňková K. et al., 2001). *RAGE* 2184GG genotype is as well as the above mentioned *RAGE* -429CC genotype associated with higher mortality in hemodialysis patients (Kalousová M. et al., 2010).

Latest studies revealed that RAGE mRNA can undergo alternative splicing to produce 19 known RAGE mRNA splice variants, except for full length RAGE mRNA. The expression of the full-length variant is significantly dominant. The RAGEv_1 splicing mRNA variant coding soluble receptor for advanced glycation end products is also expressed in a significant amount. The other splice variants are expressed minimally (Hudson BI. et al., 2008, Sterenczak KA. et al., 2009).

1.1.7 Soluble Receptor for Advanced Glycation End Products

The soluble receptor for advanced glycation end products (sRAGE) is a RAGE which is missing the transmembrane domain and is located extracellularly. Soluble RAGE is produced by alternatively splicing of RAGE mRNA and actively released - endogenous soluble RAGE

(esRAGE) or it is cleaved from the full length RAGE on cell surfaces by enzymes - cleaved RAGE (cRAGE)

As mentioned above, esRAGE is a product of alternative splicing of the RAGE DNA. It is a product of the RAGE_v1 mRNA splice variant, which includes part of intron 9 with exon 9 and exon 10 is excluded. Upon translation of the mRNA, a 347 amino acid long protein is formed, with a 22 amino acid long signal sequence, however, it misses the transmembrane domain. The protein is N-glycosylated in Golgi aparatus and after is released extracellulary (Hudson BI. et al., 2008)

cRAGE is produced by the shedding of full length RAGE on cell surfaces with proteases. Two proteases are responsible for the cleavage of cRAGE, the membrane sheddase ADAM 10 (A disintegrin and metalloproteinase domain-containing protein 10), an enzyme belonging to the zinc-dependent metzicin family of metalloproteases and gelatinase MMP 9 (matrix metalopeptidase 9), an enzyme belonging to zinc-dependent metallopeptidases. cRAGE is cleaved between amino acid 315 and 332 of full - length RAGE. Shedding occurs constitutively, however, it can be induced by RAGE-ligand binding (Metz VV. et al., 2012, Zhang L. et al., 2008). Soluble RAGE detected in organism is mostly cRAGE, esRAGE represents only a minority of sRAGE. It is supposed that a significant part of the circulating sRAGE in organism is produced by the cleavage of full-length RAGE in the lungs, where RAGE is highly expressed during adult life (Demling R. et al., 2006).

sRAGE missing transmembrane domain might act as a decoy by biding RAGE ligands and not triggering RAGE signal pathways. esRAGE and cRAGE do not differ functionally. sRAGE is nowadays widely studied as a possible biomarker in the diagnosis and prognosis of chronic diseases, and its therapeutic implications are considered.

1.1.8 The Receptor for Advanced Glycation End Products and Diseases

Regarding RAGE characteristics, the role of RAGE and sRAGE has been studied in the pathogenesis of many diseases. As it was first described as a receptor for AGEs, primarily it was studied in the pathogenesis of diabetes mellitus and its complications (Hori O. et al., 1996, Vlassara H., Bucala R., 1996).

After defining other RAGE ligands, the spectrum of studied pathological states became wider. Its significance was proven in the pathogenesis of cardiovascular diseases (Sakaguchi T. et al., 2003, Krum H., Liew D., 2003), atherosclerosis (Basta G., 2008), chronic inflammatory diseases

like inflammatory bowel diseases (Dabritz J. et al., 2011), auto-immune diseases like systemic lupus erythematosus (Martens HA. et al., 2012) or polymyositis (Haslbeck KM. et al., 2005), chronic renal diseases (Kalousová M . et al., 2006), neurodegenerative diseases, especially in Alzheimer disease (Lue LF. et al., 2009), cancer and the development of metastases (Bartling B. et al., 2005).

1.1.9 The Receptor for Advanced Glycation End Products in Pathological Pregnancy

RAGE came to the center of attention in 2003, when Cook et al. (Cooke CL. et al., 2003) showed a higher expression of RAGE in the placentas of patients with preeclampsia.

Since then, sRAGE has been studied in the amniotic fluid of women with intraamniotic infection or inflammation. However, its levels were not influenced (Buhimschi AI. et al.., 2007). These results were inconsistent with the research of Romero et al. (Romero R. et al., 2008) who showed that sRAGE amniotic fluid levels are influenced by the gestational age of fetus and the labor, and that the concentration of sRAGE in amniotic fluid is increased in patients with intraamniotic infection, however, patients with clinical signs of chorioamnionitis at term had decreased sRAGE amniotic fluid levels (Romero R. et al., 2012).

Elevated serum or amniotic fluid sRAGE, resp. esRAGE levels were shown in patients with preeclampsia (Fasshauer M. et al., 2008, Kwon JT. et al., 2011, Oliver EA. et al., 2011).

Altered serum sRAGE levels were also found in pregnant women with diabetes mellitus type 1 (Pertynska-Marczewska M. et al., 2008).

Lately, maternal serum sRAGE levels were used to predict the adverse outcome of prematurely born infants, showing that very low maternal sRAGE levels are associated with a high risk of an adverse event - neonatal sepsis (Bastek JA. et al., 2012).

1.2 Glyoxalase 1 (EC 4.4.1.5)

Glyoxalase 1 (GLO 1) is part of the glyoxalase system. It is responsible for the detoxification of AGEs precursors, such as methylglyoxal. The glyoxalase system catalyses the transformation of reactive acyclic α-oxoaldehydes to α-hydroxyacids. It consists of glyoxalase 1, glyoxalase 2 (GLO 2) and reduced glutathione - threepeptide γ-L-glutamyl-L-cysteinylglycine (GSH) as a cofactor (Thornalley PJ., 1993).

Glyoxalase 1 catalyses the isomeration of the hemithioacetal, which is formed non-enzymatically from α-oxoaldehyde and GSH, into the S-2-hydroxyacylglutathione derivates. Glyoxalase 2 then catalyses the hydrolysis of S-2-hydroxyacylglutathione into the corresponding α-hydroxyacid and reforms GSH (Thornalley PJ., 1993).

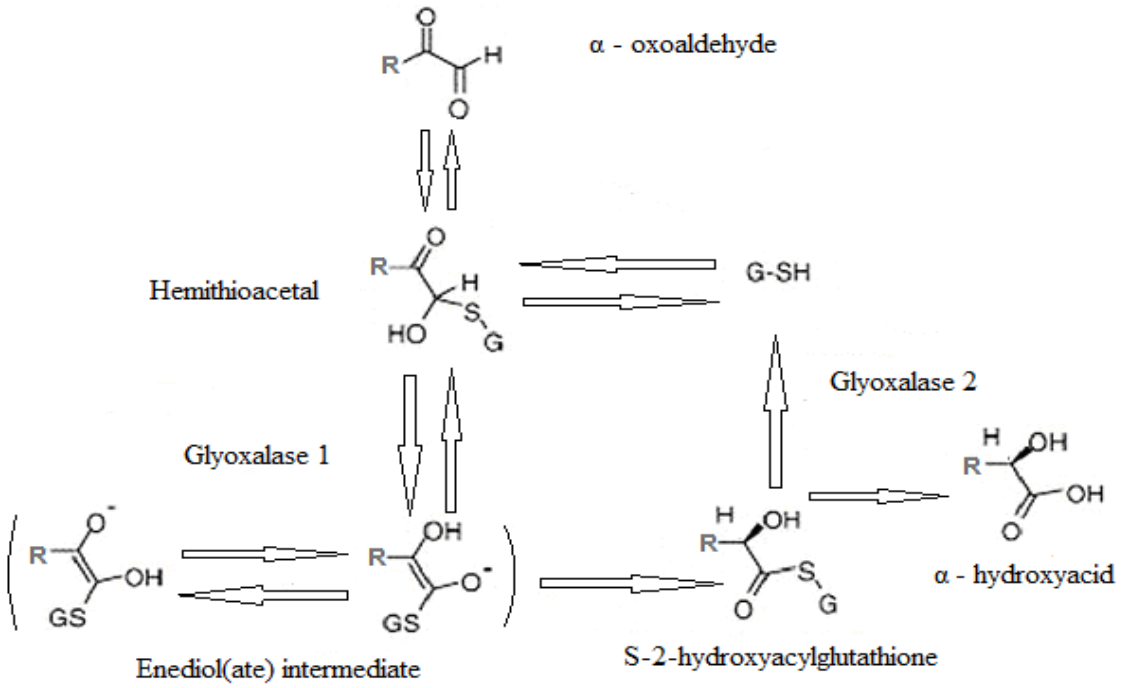


Fig. 1.5 Glyoxalase system

1.2.1 Characterisation of Glyoxalase 1

Glyoxalase 1 (isomerase) is a member of the vicinal oxygen chelate superfamily which also includes enzymes such as extradiol dioxygenase (oxidative cleavage of C-C bond), methylmalonyl-CoA epimerase (epimerisations), fosfomycin resistance protein (nucleophilic substitution) and bleomycin resistance protein (no enzyme, sequestration).

The proteins are characterized with the same βαβββ-fold that provide a metal coordination site. All enzymes of the superfamily use a divalent metal ion to perform catalysis.

Glyoxalase 1 is a ubiquitous enzyme, whose activity is present in all prokaryotic and eukaryotic organisms. All known glyoxalases, except for yeast one, are dimeric. Yeast glyoxalase I exists as a monomer. Glyoxalase 1 is present in the cell cytoplasm and cellular organelles, particularly in mitochondrias. It is expressed during embryonic and fetal development and persists in adult life and senescence. Glyoxalase 1 activity is present in all human tissues. Glyoxalase 1 activity in the fetal tissues is about three times higher than in the corresponding adult tissues.

1.2.2 Structure of Glyoxalase 1

Glyoxalase 1 is a 183 amino acid long protein with a molecular weight of 42 kDa and an isoelectric point of 4.8-5.1 Glyoxalase 1 consists of two domains (amino residues 31-104 and 124 - 183) and an N terminal arm. The domains are connected by 20 amino acid long linkage. The domains constrain the common motif of βαβββ topology. The Glyoxalase 1 has four possible phosphorylation sites – two serines and two threonines (residues 108-111), but the phosphorylation of enzyme has not been reported.

The functional enzyme exists as a homodimer. The active site is situated in the dimer interface, in a barrel, which is formed by two subunits interacting mutually in anti-parallel fashion (Cameron AD. et al., 1997).

Glyoxalase 1 contains a zinc binding site as well as a glutathione binding site. The zinc binding site is situated in the middle of the barrel. The zinc ion is bound to the site with four amino acid residues, Gln33 and Glu99 from the first domain and His126 and Glu171 from the second domain. The amino acids, together with two water molecules, form octahedral co-ordination (Cameron AD. et al., 1997). The zinc ion is important for the catalytic function. Glyoxalase 1 is inactive without a zinc ion. However, glyoxalase I can be activated also by Mg^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+} and Ca^{2+} ions (Uotila L., Koivusalo M., 1975).

The interaction of glutathione conjugate with glyoxalase 1 is quite different from those in other enzymes binding glutathione. In glyoxalase 1 very specific interactions with the protein involve the γ-glutamyl residue of glutathione (Cameron AD. et al., 1997).

1.2.3 Reaction Mechanism of Glyoxalase 1

Glyoxalase 1 catalyses the isomeration of hemithioacetal which is formed spontaneously from α-oxoaldehyde and GHS into S-2-hydroxyacylglutathione.

The mechanism proposed for the glyoxalase 1 reaction involves a 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 ketonisation to the thioester product (Cameron AD. et al., 1997). The main catalytic role of the Zn ion is to electrostatically stabilize the ene-diolate intermediate and to reduce the activation free energy of proton transfer (Himo F., Siegbahn PE. et al., 2001)

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

1.2.4 Glyoxalase 1 Substrates

The main substrate for glyoxalase 1 is the methylglyoxal, which is formed as a by-product of the triose-phospate isomerase reaction, either in glycolysis or from acetone in the ketone body mechanism or from aminoacetone in threonine catabolism.

Another significant substrate is glyoxal formed by lipid peroxidation and the degradation of glucose and glycated proteins. Glyoxal and methylglyoxal are elevated by oxidative and carbonyl stress and they are the most frequent precursors of intracellular space and plasma AGEs.

Methylglyoxal (glyoxal) is converted into S-D-Lactoyl-glutathione (S-Glycolyl-glutathione) and then hydrolysed to D-lactate (glycolate) (Thornalley PJ., 1993).

1.2.5 Glyoxalase 1 Function and Regulation

Glyoxalase 1 is a ubiquitous enzyme, whose full function is not fully understood. Nowadays, it is believed that its main function is the detoxification of α-ketoaldehydes, the by-products of glycolytic pathways. The role of glyoxalase 1 in the regulation of cell growth has also been proposed, but not clearly confirmed (Klapos MP., 1999).

The activity of glyoxalase 1 is regulated by cell redox state. Glutathionylation of the enzyme by GSSG causes a reversible inhibition of glyoxalase 1 activity (Birkenmeier G. et al., 2010).

Glutathionylation causes oxidation of 139Cys and thus, changes the glyoxalase 1 function. The mechanism of glutathionylation is responsible for AGEs-induced inhibition of glyoxalase 1 activity, while elevated AGEs levels cause reactive oxygen species production and the depletion of GSH.

1.2.6 Genetics of the Glyoxalase 1

The human glyoxalase 1 gene is situated on chromosome 6 (locus 6p21.3 – 6p21.2), close to the MHC complex HLA-DR. It is 27,215 bp long and consists of 5' UTR flanking region, 6 exons, 5 introns and a 3' UTR part. The gene promoter contains insulin, testosterone and metal response element.

According to the database of polymorphism the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=2739) in May 2014 there were 733 known single nucleotide polymorphisms of the glyoxalase 1 gene.

Out of 733 SNPs of glyoxalase 1 gene:

- 32 are in exons
- 609 are in introns
- 10 in the 5' UTR region
- 27 in the 3' UTR region
- 41 in the 5' flanking region
- 14 in the 3' flanking region

7 exons SNPs are synonymous and the rest are non-synonymous (See Table 1.2).

Despite the numerous amounts of described SNPs, the studies have mainly focused on only two of them, Ala111Glu in the exon 4 and -7C/T in the promoter region of the Glyoxalase 1 gene.

The polymorphism Glu111Ala (or 419A/C) is located in exon 4. It causes the nucleotide change (adenine to cytosine) in position 419. The SNP is relatively common and has been studied in many populations. Recent studies have shown that this SNP causes a change of amino acid in the side chain of the monomer. The change can induce the hyperfosphorylation of protein and together with conformational changes, decreases the activity of the enzyme (Barua M. et al., 2011). The importance of the polymorphism was described in diabetes (McCann Vj. et al, 1981), in hemodialysis patients with chronic renal failure (Kalousová M. et al., 2010), in psychiatric diseases, especially in autism (Junaid MA. et al., 2004) and in cancer (Germanová A. et al., 2009).

-7 C/T *GLO 1* polymorphism might be important because it is located in the proximity of the minimal promoter and the DNA-dependent RNA polymerase site. It is supposed that the C allele binds the GC-factor, the repressive transcription factor, and the T allele does not bind it, which may lead to a reduced glyoxalase 1 expression (Gale CP., Grant PJ., 2004). -7 C/T *GLO 1* polymorphism is associated with a prothrombotic state (Gale CP. et al., 2004)

Table 1.2 Exon SNPs of glyoxalase 1

Exon	Non-synonymous SNP		Synonymous SNP		Deletions	
1	Gly10Ser	rs113061497				
	Thr12Lys	rs140507823				
	Cys19Tyr	rs17855424				
	Cys20Arg	rs147217818				
	Cys20Tyr	rs377328531				
	Ser26Asn	rs377238075				
2	Gln34Ter	rs143771246	Leu31Leu	rs4451130	Gln34Ter	rs143771246
	Arg38Ter	rs139437714				
	Thr56Met	rs150725457				
3	Tyr71Cys	rs11544283	Thr56Thr	rs11544281		
	Tyr75Ser	rs141160784	Ile81Ile	rs11544284		
	Glu76Val	rs369375025	Ala92Ala	rs182114911		
	Pro82His	rs11544279				
	Ala92Val	rs371576917				
	Thr98Ile	rs372282719				
4	Gly106Ser	rs372375649	Gly124Gly	rs1130534		
	Glu111Ala	rs 4746	Phe125Phe	rs112857632		
	Asp121Glu	rs143083347				
	Pro122Thr	rs11544280				
	Arg123Gln	rs376095398				
5	Gly126Val	rs145158912				
	Ile128Thr	rs141465532				
	Val135Gly	rs190185384				
	Val150Leu	rs145173943				
6			Tyr170Tyr	rs150395428		

Recent studies found six transcript variants of glyoxalase 1, however, they vary only in their 3' or 5' sequence and no insertion or deletion in the peptide string was described. No alternative splicing of glyoxalase 1 transcript has been reported until now (Gale CP. et al., 2004).

Newer studies focus on alterations in the expression of the glyoxalase 1 gene. The expression of glyoxalase 1 is up-regulated after cell stimulation with testosterone or estrogen (Antognelli C. et al., 2007), which influences the gene promoter. Xue et al. (Xue M. et al., 2012) described an anti-oxidant response element (ARE) located in the un-translated region of the exon 1. Transcriptional factor Nrf2 (nuclear factor-erythroid 2 p45 subunit-related factor 2), which is activated by methylglyoxal, stimulates the transcription of glyoxalase 1 through the ARE.

On the other hand, down-regulation of the gene expression could be evocated by metformin or troglitazone, however, the mechanism is not fully elucidated (Helgager J. et al., 2010, Dong L. et al., 2012). Furthermore, HMGB1 binding to RAGE decreases the expression of glyoxalase 1 through Nrf2 (Thornalley PJ., 2009).

1.2.7 Glyoxalase 1 and Diseases

The role of glyoxalase 1 in the pathogenesis of many diseases has already been described. The importance of glyoxalase 1 in diabetes, mainly in the development of a diabetic complication like neuropathy or retinopathy, was described by Skapare et al. (Skapare E. et al., 2013). The study pointed out a decreased activity of glyoxalase 1 in patients with diabetes mellitus type 1 or 2 suffering from diabetic nephropathy.

On the contrary, the polymorphisms of glyoxalase 1 are not associated with vascular diseases like hypertension or atherosclerosis (Engelen L. et al., 2009). However, the study by Kalousová et al. showed the association of the *GLO 1* 419A/C polymorphism with vascular complication in chronic hemodialysis patients (Kalousová M. et al., 2008). The genotype *GLO 1* 419 CC is also associated with the increased mortality rate in hemodialysis patients (Kalousová M. et al., 2010).

The study by Sidoti et al. (Sidoti A. et al., 2007) described the association of the 419A/C polymorphism with an increased risk of multiple sclerosis development.

The role of glyoxalase 1 has been widely studied in psychiatric diseases. Several studies did not find an association between *GLO 1* 419A/C polymorphism and Alzheimer's disease (Chen F. et al., 2004) nor with panic attack diseases (Politi P. et al., 2006) nor autism (Sacco R. et al., 2007).

However, the study by Junaid et al. (Junaid MA et al., 2004) focused on the activity of glyoxalase 1 in the brain of patients with autism and proved its decreased activity. Consequently, the study found an association between decreased glyoxalase activity and *GLO 1* 419A/C polymorphism and marked the polymorphism as the susceptibility factor for autism. The association of *GLO 1* 419A/C polymorphism with decreased activity of the glyoxalase 1 was later confirmed by Barua (Barua M. et al., 2011).

The importance of glyoxalase 1 in the pathogenesis of cancer is clearly proven. Glyoxalase 1 is over-expressed in many types of cancer (Antognelli C et al., 2006, Wang Y. et al., 2012, Fonsecha-Sancez MA et al., 2012). The Glyoxalase 1 protects cancer cells from toxic metabolites, arising from increased cancer metabolism and today, it is supposed that this over-expression of glyoxalase 1 in tumor tissues is partly responsible for chemotherapy resistance (Tsuruo T. et al., 2003), but the precise mechanism is not understood.

1.2.8 Glyoxalase 1 and Pregnancy

The role of glyoxalase 1 in the physiological pregnancy has not yet been studied. The only study concerning glyoxalase 1 and pathological pregnancy showed decreased activity of glyoxalase 1 in patients with preeclampsia (Sankaralingam S. et al., 2009).

1.3 Pathological Pregnancy

1.3.1 Premature Labor

Premature labor is labor which occurs between the 24^{th} and the 37^{th} week of pregnancy. The incidence of premature labor varies between countries, however, it tends to be rising. 7% of all labors are preterm in the Czech Republic. Premature labors are responsible for one million neonatal deaths worldwide annually. Preterm labors cause serious morbidity of preterm born infants, such as severe intraventricular hemorrhage, periventricular leukomalacia, necrotising enterocolitis, bronchopulmonary dysplasia, myocardial dysfunction and neonatal sepsis.

Nowadays, the premature labor is considered a multifactor process, in which infection, inflammation, uteroplacental ischemia, hormone metabolism disorders, impaired maternal tolerance

of a fetus, uterine distension and cervical incompetence or allergies take part. However the influence of life-style factors and genetic factors are discussed as well.

The role of infection and inflammation in the pathogenesis of preterm labor is generally accepted. However, the infection needs a specific genetic background to cause preterm labor. The significance of cytokines, prostaglandins and matrix-metalloproteinases in preterm labor has already been confirmed (Gomez R. et al., 1997, Romero R. et al., 1998). Anti-inflammatory interleukin-10 (Il-10) is considered a key factor for pregnancy "maintenance" (Gotsh F. et al., 2008). The transforming gowth factor 1β (TGF-1β), pro-inflammatory cytokine, which takes part in the initiation of inflammation, is studied as an important factor for the immunological tolerance of fetus as an allograft. Elevated interleukin 6 (Il-6) and matrix metalopeptidase 8 (MMP-8) in the amniotic fluid are signs of the intraamniotic infection and threatening preterm labour. Romero et al. showed that TNF-α polymorphism, together with bacterial vaginosis, increases the risk of premature labor ten times (Romero R. et al., 2004).

Uteroplacental ischemia can also lead to premature labor. Congenital or acquired trombophilic states, with excessive micro-coagulation, have a potential effect on the placental micro-circulation. The endothelial dysfunction initiates a cascade of biochemical processes leading to premature labor. The most important trombophilic states are Leiden mutation, the mutation of prothrombin and the mutation of methylentetrahydrofolate reductase (Arias F. et al., 1998).

Lately studies have focused on altered maternal immunological functions, while the fetus is considered an allograft and premature labor or miscarriage in the wider sense are considered impaired maternal tolerance for the fetus. The essential mechanism of the mother - fetus tolerance is the balance between the up- and down- regulation of the Major Histocompatibility Complex (MHC) antigens. Human leukocyte antigen A (HLA-A) and B (HLA-B) are down-regulated by the trophoblast and Human Leukocyte Antigen G (HLA-G) protects the fetus against the maternal immune response (Szekeres-Bartho J., 2002). Latest studies have focused on the galectin-1, member of the family of lectins, which bind beta-galactoside. Galectin-1 has an essential function in reproduction and pregnancy. Animal studies have shown that galectin-1 deficient mice developed normally and did not show any phenotypic abnormality, however, the mice had a higher rate of allogeneic fetal losses but comparable a rate of syngeneic fetal losses. Moreover, the administration of recombinant galectin-1 prevented allogeneic fetal losses (Blois SM. et al., 2007).

The importance of progesterone in premature labor has been widely studied. It has been found that a functional withdrawal of progesterone can contribute to the process of premature labor. A clear mechanism of progesterone action is not understood, but it is evident that progesterone has anti-inflammatory effects, decreases uterine sensitivity to estrogens and thus down-regulates oxytocin receptors on the uterine myometrial cells' surface (Sfakianaki AK., Norwitz ER., 2006).

The symptoms of threatening preterm labor are uterine contractions every 15 minutes, progressive cervical dilatation, preterm premature rupture of membranes (PPROM) and bleeding.

Laboratory elevated leukocyte count or C-reactive protein can be found, but these laboratory markers are not specific for preterm labor. Today, there is no specific laboratory marker for threatening preterm labor.

Ultrasound investigation is useful for the objective assessment of cervical length. Ultrasound cervicometry is an available imaging method which can contribute to the prediction of threatening preterm labor. A uterine cervix shorter than 25 mm before the 32^{nd} week of pregnancy is an indication of vaginal administration of progesterone. Moreover, the combination of a short cervix on ultrasound cervicometry (less than 25 mm) and positive fibronectin in the vaginal fluid (more than 50 μg/ml) is an indication of the preventive application of oxytocine antagonists, while the risk of premature labor is considered to be 30 %.

The detection of threatening premature labor allows obstetricians to use therapy to prolong the pregnancy. Tocolytics, antibiotics, progesterone and corticosteroids are most often used for the treatment with the aim of prolonging the pregnancy or preparing the infant for labor. Unfortunately, the treatment is not fully successful and there is no primary prevention for preterm labor, so preterm labor remains a health and socioeconomic problem in majority of developed countries.

1.3.2 Hypertensive Disorders, Gestational Hypertension, Preeclampsia

Hypertensive disorders in pregnancy are characterized as elevated systolic blood pressure, above 140 mm Hg and elevated diastolic blood pressure, above 90 mm Hg. Gestational hypertension, also called pregnancy induced hypertension, preeclampsia, chronic hypertension, and preeclampsia superposed on chronic hypertension belongs to hypertensive disorders. The incidence of hypertensive disorders in pregnancy is 6 - 8 % and more often affects primiparous women (10 -

14%) than multiparous women (5 - 7%). Hypertensive disorders during pregnancy, especially preeclampsia, are the main cause of the maternal mortality.

Pathogenesis of hypertension and especially of preeclampsia has been studied intensively and several pathogenesis mechanisms have been proposed, however a clear mechanism is not yet known.

The placenta, not the fetus plays the central role in the pathogenesis of preeclampsia. studies show that preeclampsia can appear in patients with a hydatidiform mole without an embryo (Maynard SE., Karumanchi SA., 2011) and it can also persist in women with extrauterine pregnancy after the removal of fetus but not placenta (Maynard SE. et al., 2005). All cases of preeclampsia disappear after the delivery or removal of the placenta.

Impaired placental invasion to maternal spiral arterioles in the uterine myometrium, between the 10^{th} and 20^{th} week of pregnancy, is the first step in the pathogenesis of preeclampsia. Normal placental invasion creates high caliber conduit vessels from small resistance vessels in decidua using cytokines, growth factors, transcription factors and immunological factors.

Several studies have shown changes in the renin-angiotensin-aldosterone system (RAAS) and both the circulating and local (placental), are involved in the pathogenesis of preeclampsia. Most RAS components are decreased in preeclampsia, however, the activity of angiotensin receptor 1 is increased (Yang J. et al., 2013).

The role of angiogenetic factors in the pathogenesis of preeclampsia is inalienable. Vascular endothelial growth factors (VEGF) and placental growth factor (PLGF) are crucial in the remodeling process of maternal arterioles in decidua. They are released to the maternal body by circulating trophoblast cells. A soluble receptor for VEGF, known as soluble fms-like tyrosine kinase-1 (sFLT-1), is up-regulated in the preeclampsia and functions as a trap for VEGF and PLGF (Lam C. et al., 2005). As a result, decreased levels of PLGF are considered marker of subclinical preeclampsia. PLGF decreases weeks before the clinical onset of preeclampsia (Lam C. et al., 2005).

Increased oxidative stress also takes part in the pathogenesis of diseases. It is well documented that product levels of lipid peroxidation are increased and anti-oxidants concentrations are low in preeclampsia (Serdar Z. et al., 2003). Regarding the impaired activity of superoxide dismutase in preeclampsia, superoxide reacts with nitric oxide and forms peroxynitrite. Thus, the

deficiency of nitrite oxide triggers endothelial dysfunction in preeclampsia (Sankaralingam S. et al., 2006).

Clinical symptoms of hypertensive disorders in pregnancy include hypertension, i.e. systolic blood pressure over 140 mm Hg and diastolic blood pressure over 90 mm Hg. Edemas, rising body weight or headaches signalize preeclampsia. Right epigastrium pain, heavy frontal headache, visual problems and vomiting are considered prodromes of eclampsia, epileptic like seizures.

Patients with gestational hypertension do not have special laboratory findings, however patients with preeclampsia have proteinuria (over 300 mg per 24 hours) and elevated serum uric acid levels. Higher hemoglobin levels and increased hematocrit are signs of hemo-concentration. Lower serum total protein and albumin levels could be found during laboratory examination in preeclampsia. Laboratory findings in eclampsia do not differ from preeclampsia findings.

Ultrasound examination of patients with hypertension disorders is focused on the evaluation of fetus well-being. Ultrasound biometry, flowmetry (aimed at arteria cerebri media and arteria umbilicalis) and amniotic fluid index are used for the assessment of chronic or acute fetal hypoxia.

Antihypertensive agents, especially alpha-2-mimetics (methyldopa), a calcium channel blocker (amplodipine) or a beta-1-blocker (metoprolol) are used in the management of hypertensive disorders in pregnancy. The infusion of magnesium sulphate is used for the treatment and prevention of eclampsia. However, the agents are used to attenuate symptoms and give time for the right timing of labor. The only cure is the termination of pregnancy.

Studies all over the world are trying to find a suitable marker for screening of preeclampsia. Doppler ultrasound of arteria uterina is recommended, as well as some biochemical markers (Pregnancy associated plasma protein - A, PLGF, activin A, inhibin A, placental protein 13, sEndoglin), but any of these are used in medical practise.

1.3.3 Intrahepatic Cholestasis of Pregnancy

Intrahepatic cholestasis of pregnancy (ICP) is characterized by elevated liver enzymes during pregnancy, after other causes of increased liver enzymes are excluded. Serum bile acids are also increased. ICP is more often called obstetric hepatosis in the Czech Republic. The incidence of the disease in European countries, as well as in North America and Australia, is less than 1% (Germain AM. et al., 2002), however, it is more common (13-27%) in south American countries like Bolivia

or Chile (Riely CA., Bacq Y., 2004). ICP is not life-threatening and it does not cause permanent health problems in pregnant women, but it can be very harmful for the fetus, causing preterm labor, fetal stress and sudden intrauterine death, the mechanism of which is not well understood (Williamson C. et al., 2004).

The pathogenesis of ICP is multifactorial and not fully uncovered. The contribution of hormonal, environmental and genetic factors is described.

The spectrum of progesterone metabolites is different in patients with ICP to those of healthy patients (Meng LJ. et al., 1997). These metabolites influence bile acids transport (Vallejo M. et al., 2006).

The environmental influence was studied due to the fact that the incidence of ICP is higher during the winter months. Selenium, a cofactor of some liver enzymes, has a serum level and peroral intake lower in patients with ICP (Kauppila A. et al., 1987).

However, it seems that the genetic predisposition is the most important factor. Mutations of genes encoding proteins which take part in the bile acids transport have been extensively studied, especially gene ATP-binding cassette, sub-family B, member 4 (ABCB4) and gene ATP-binding cassette, sub-family B, member 11 (ABCB11) genes. The ABCB4 gene codes multi-drug resistance protein 3 (MDR3). MDR3 is a transmembrane lipid transporter of phosphatidylcholine. It translocates phosphatidylcholine into the intrahepatic bile duct. The ABCB11 gene codes bile salt export pump protein (BSEP), liver-specific enzyme transporting bile acids. Mutations of both genes were described in the ICP (Anzivino C. et al., 2013).

ICP presents with pruritus, mostly located on palms or soles, but it can affect all body parts. Jaundice is rare. Symptoms like anorexia, tiredness, dark urine and steatorrhea occur rarely.

In laboratory examination, elevated liver enzymes, alanine amino transaminase (ALT) and aspartate amino transaminase (AST) are found. γ-glutamyl transpeptidase and bilirubin are usually in a normal range. The most important finding is elevated total bile acids in serum. However, this examination is not routine in many hospitals in the Czech Republic. Urine analysis shows an elevated excretion of bile acids, especially of primary bile acids.

Ultrasound examination is used for liver and gall bladder assessment. In a few patients with ICP gall bladder stones could be found. More often ultrasound is used for the assessment of the wellbeing of fetus by biometry and flowmetry.

Ursodeoxycholic acid (UDCA), a hydrophilic bile acid, is used as a first line therapy for ICP. UDCA stimulates bile excretion by post-transcriptional regulation of BSEP and MDR3. S-adenosyl-L-methionine is also used in treatment however, its effect has not been proven.

1.3.4 Intrauterine Growth Restriction

Intrauterine growth restriction (IUGR) is defined as the growth of a fetus which does not achieve its genetically determined potential. IUGR is often confused with small for gestational age (SGA), a small fetus which grows according to its genetic potential. The incidence of intrauterine growth restriction is about 10%, but is more common in developing countries. IUGR increases the risk of intrauterine death and can have postnatal health consequences, such as impaired psycho-motoric development, early development of diabetes and arterial hypertension in adult life.

IUGR is a likely symptom of other diseases. Most often it is caused by fetal, placental or maternal diseases. Chromosomal abnormalities (Down syndrome, Edward's syndrome) or other genetic syndromes (Beckwith-Wiedemann syndrome) are considered as fetal reasons of IUGR. Placental insufficiency is most often caused by preeclampsia. Chronic maternal diseases, like hypertension, chronic renal diseases, autoimmune diseases and diabetes mellitus as well as smoking or inadequate nutrition of the mother are also causes of IUGR.

Ultrasound scan is essential for the diagnosis of IUGR. Most fetuses are under the 3rd percentile according population to the biometry graph. However, it is necessary to repeat biometry in 14 days to exclude diagnosis SGA. The ultrasound flowmetry of arteria uterina, ductus venosus and arteria cerebri media are important for the assessment of the biophysical profile of the fetus.

Laboratory examination is important for the evaluation of the maternal health condition, but usually does not say much about the fetal health state.

There is no specific treatment for IUGR, even though several have been tried. Ultrasound is done regularly, mainly for the establishment of the fetal state and the correct timing of labor.

2 Aim of the Study

The importance of receptor for advanced glycation end products, soluble receptor for advanced glycation end products and glyoxalase 1 has been proven in the pathogenesis of chronic diseases such as inflammatory diseases, diabetes mellitus, cardiovascular diseases, chronic renal diseases and cancer. Common sign of these diseases is altered oxidative status and microinflammation. Oxidative stress and microinflammation is also typical for physiologic pregnancy and even more for pregnancy complication. The aim of the work was to explore role of RAGE, sRAGE and glyoxalase 1 in physiologic and pathologic pregnancy.

1. Assessment of sRAGE serum levels

- To study sRAGE serum levels and their dynamics during physiologic pregnancy.
- To study sRAGE serum levels in pregnant women with threatening preterm labor and compare them with women with physiologic pregnancy.
- To study sRAGE serum levels in pregnant women with other pregnancy induced diseases and compare them with women with physiologic pregnancy.

2. Assessment of RAGE polymorphisms - rs1800625 RAGE -429 T/C, rs1800624 RAGE -374 A/T, rs2070600 RAGEGly82Ser (557 G/A), rs3134940 RAGE 2184 A/G and Glyoxalase 1 polymorphism - rs4746 GLO 1 Glu111Ala (419A/C)

- To study these SNPs in pregnant women with threatening preterm labor.
- To study these SNPs in pregnant women with other pregnancy induced diseases.
- To study these SNPs in healthy pregnant controls for comparison.

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals and Other Materials

sRAGE assessment

Human RAGE Quantikine ELISA Kit (R&D Systems, USA)

Isolation of the DNA

Ammonium chloride, G.R. (Fluka, Germany)

Ammonium hydrogen carbonate, G.R. (Fluka, Germany)

Magnesium chloride hexahydrate, G.R. (Fluka, Germany)

Sodium chloride, G.R. (Penta, Czech republic)

Ethanol, 96perc, (v/v) (Penta, Czech republic)

Kalium chloride, G.R. (Lachema, Czech republic)

Hydrogen chloride, G.R. (Lachema, Czech republic)

Sodium hydroxide, G.R. (Lachema, Czech republic)

Sodium dodecyl sulphate (SDS), G.R. (Ampresco®, USA)

Ethylenediamine-tetraacetic acid (EDTA), *G.R.* (Sigma, USA)

2-amino-2-hydroxymethyl-1,3-propanediol TRIS, *G.R.* (MP Biomedicals, USA)

Proteinkinase K (Roche, Germany)

PCR-RFLP analysis

Ethanol, *96perc, (v/v)* (Penta, Czech republic)

Boric acid G.R. (Penta, Czech republic)

Tetrabromophenolsulfonephthalein - Bromophenol blue (Lachema, Czech republic)

2-amino-2-hydroxymethyl-1,3-propanediol (TRIS) G.R. (MP Biomedicals, USA)

Ethylenediamine-tetraacetic acid (EDTA) G.R. (Sigma, USA)

*Sucrose G.R. (*Ampresco®, 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 (Generi Biotech, Czech republic)

Restriction enzymes Mfe, AluI, BsmFI (BioLabs, USA)

Restrictiom enyzme BsmAI (Fermentas, USA)

3.1.2 Instruments

- Water treatment: 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)
- Horisontal nucleid acid electrophoresis tank Minigel 2 (Apelex, France)
- Electrophoresis power supply Apelex PS 1006 (Apelex, France)
- UV transilluminator TS-312A Spectroline® (Spectroline, USA)
- Microwave oven: Micromaxx® MM 41580, (Micromaxx, Germany)
- PCR cooler (Eppendorf®, Germany)
- Vortex: Biovortex V-1 plus (Biosan, Latvia)
- Sample incubator: Dry-block® heater DB-2A (Techne, USA)
- Corning® filter systems (Corning Inc., USA)
- Photometer: Biophotometer 6131 (Eppendorf®, Germany)
- Cuvettes: UVette® (Eppendorf®, Germany)

3.2 Methods

All the procedures, except for sRAGE assessment, were done in medical examination gloves and all used materials and work places were sterile.

3.2.1 sRAGE assessment

For sRAGE serum level determination a commercial ELISA kit, Human RAGE Quantikine ELISA Kit (R&D Systems, USA), was used.

3.2.1.1. Principle of the Method

sRAGE is assessed by the quantitative sandwich enzyme immunoassay. The plate is pre-coated by monoclonal antibodies specific for the extracellular domain of RAGE. The RAGE in the samples is bound to the plate by the antibodies. After washing away the rest of the sample, enzyme linked-polyclonal antibodies specific for the extracellular domain of RAGE are added. After incubation, unbounded enzyme linked-polyclonal antibodies are washed out. Consequently, the substrate for the enzyme is added and the color develops in intensity directly proportional to the sRAGE concentration. The intensity of the color is measured photometrically.

3.2.1.2 Reagents

RAGE microplate (a polystyrene microplate with 96 wells precoated with the monoclonal antibody specific for human RAGE)
RAGE conjugate (a polyclonal antibody specific for human RAGE conjugated to horseradish peroxidase with preservatives)
RAGE standard (recombinant human RAGE in a buffer with preservatives)
Assay Diluent RD 1-60 (a buffered protein base with preservatives and blue dye)
Calibrator Diluent RD6-10 (a buffered protein base with preservatives)
Wash Buffer Concentrate (a 25-fold concentrated solution of buffered surfactant with preservatives)
Color Reagent A (stabilized hydrogen peroxide)
Color Reagent B (stabilized tetramethylbenzidine)
Stop Solution (2N sulfuric acid)

3.2.1.3 Working Procedure

1. All reagents are brought to room temperature.
2. 20 ml of wash buffer concentrate is added to 480 ml of distilled water.
3. The RAGE standard is reconstituted by adding 1 ml of distilled water, forming a stock solution of 50 000 pg/ml. Afterwards, the stock solution and the Calibrator Diluent RD6-10 are used to form dilution series (5000, 2500, 1250, 625, 313, 156, 78 pg/ml). The Calibrator Diluent is zero standard.

4. 100 µl of the Assay Diluent RD1-60 is added to each well.
5. 50 µl of standards, samples and controls are added to each well and are incubated for 2 hours at room temperature.
6. Each well is aspirated and then washed out using 400 µl of the wash buffer. The process is repeated 3 times. Consequently, the plate is inverted against a clean paper towel.
7. 200 µl of the RAGE Conjugate is added to each well and incubated for 2 hours at room temperature.
8. Aspirating and washing out is repeated as in step 6.
9. The color reagents A and B are mixed together to form a Substrate Solution 15 minutes prior use.
10. 200 µl of the Substrate Solution is added to each well and incubated for 30 minutes. Wells are protected from light.
11. 50 µl of the Stop Solution is added to each well.
12. For the determination of the optical density, a microplate reader set to 450 nm with correction set to 540 nm is used.

3.2.2 Isolation of DNA

A modified salting-out method was used for DNA extraction (Miller SA. et al., 1988).

3.2.2.1 Principle of the Method

DNA is extracted from erythrocytes after their cells' membrane lysis in the presence of salmiak lysing buffer (SLB) and nuclear membrane lysis in the presence of sodium dodecyl sulfate (SDS) and proteinase K (Miller SA. et al., 1988). The residual peptides are salted out (dehydration and precipitation) by a saturated solution of sodium chloride. Afterwards, the extracted DNA is dissolved in ethanol.

3.2.2.2 Solutions

- *0.5M EDTA* (stock solution, 500ml): 39.05 g EDTA, 5ml 10M NaOH and re-distilled water added to a volume of 500ml
- *10M NaOH* (100ml): 40 g NaOH and re-distilled water added to a volume of 100ml

- *SLB* (stock solution, 500ml): 41.45 g NH_4Cl, 3.95 g NH_4HCO_3, 1ml 0.5 M EDTA and re-distilled water added to a volume of 500ml
- *TK-1 buffer* (2000ml, pH 7.6): 1.488g KCl, 2.422g TRIS, 4.064g $MgCl_2.6H_2O$, 8ml 0.5M EDTA, 2M HCl for pH arrangement and re-distilled water added to a volume of 2000ml
- *2M HCl* (100ml)**:** 8.5 ml HCl and re-distilled water added to a volume of 100ml
- *4M NaCl* (200ml): 46.75 g NaCl and re-distilled water added to a volume of 200ml
- *Lysis buffer for nuclear cells* (500ml, pH 8.2): 50 ml 4M NaCl, 5ml 1M TRIS-HCl (pH 8.2), 2ml 0.5M EDTA and re-distilled water added to a volume of 500ml
- *1M TRIS-HCl* (100ml, pH 8.2): 12.1g TRIS, 2M HCl for pH arrangement and re-distilled water added to a volume of 100ml
- *Proteinase K dissolvent solution* (200ml)**:** 10ml 10% SDS, 0.8ml 0.5M EDTA and re-distilled water added to a volume of 200ml
- *10% SDS w/v*, kept at laboratory temperature
- *Proteinase K working solution* (50ml): 100mg proteinase K, 50ml proteinase K dissolvent solution; 600 µl aliquotes kept at –70°C
- *96% ethanol*
- *70% ethanol*
- *6M NaCl* (100ml): 35.1 g NaCl and re-distilled water added to a volume of100ml
- *TE buffer* (40ml): 400µl 1M TRIS-HCl (pH 7.6), 80 µl 0.5 EDTA and re-distilled water added to a volume of 40ml

The filter system Corning was used to filter each solution except for ethanol, 10 M NaOH and 2M HCl. The solutions were stored at a temperature of 2-8°C. Ethanol was stored at a temperature of -20°C before use.

3.2.2.3 Working Procedure

1. Blood sample (7ml, EDTA) is overflowed into a 50ml sterile centrifugation tube and the tube is filled in with 40ml of cooled SLB buffer diluted10 times. The tube is incubated for 30 minutes at a temperature of 0°C. Centrifugation (3000 rotations per minutes (rpm), 10°C) takes 30 minutes. The supernatant is carefully removed and 40ml of cooled SLB buffer diluted 10 times is added again and the whole procedure is repeated once more.

2. After the supernatant is removed, again, 30ml of TK-1 buffer is added to the tube. The tube is shaken vigorously. Centrifugation takes 20 minutes (3000 rpm, 10°C). Red blood cell membranes are eliminated during this step. This step is repeated at least three times, until a pure white nuclear cell pellet is gained.
3. The white nuclear pellet is re-suspended with 3ml of lysis buffer for nuclear cells. The tube is shaken properly. The tubes are incubated overnight at 37°C with 600µl of proteinase K working solution and 200µl of 10% SDS.
4. After overnight digestion, 1ml of 6M NaCl is added to the mixture and shaken vigorously for approximately 15 seconds. The subsequent centrifugation takes 30 minutes at 6000rpm at laboratory temperature.
5. The precipitated protein pellet, arisen after centrifugation, is at the bottom of the tube, so the supernatant containing DNA is moved to another 50 ml sterile tube. Frozen (-20°C) 96% ethanol is added to the tube with the supernatant. The tube is twisted until the DNA is precipitated.
6. Precipitated DNA strands are taken by the sterile glass hooklet and cleaned with the frozen (-20°C) 70% ethanol.
7. The glass hooklets with the dry DNA are put into the sterile cryo-micro tube containing a 1000µl TE buffer. The DNA is dissolved at laboratory temperature overnight.

3.2.3 DNA Concentration Assessment

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

3.2.3.1 Principle of the Method

The DNA concentration is assessed by spectrophotometric analysis. Measured values of ultraviolet (UV) absorbance at 260 nm are transferred into concentrations: one degree of optical density matches to 50µg/ml ds-DNA. The absorbance at 230, 280 and 320 nm as well as ratios A_{260}/A_{280} and A_{260}/A_{230} serves as an indicator of the nucleic acid purity. The absorbance at 320 nm is approximately zero and the ratios A_{260}/A_{280} and A_{260}/A_{230} are approximately two in pure samples.

3.2.4 Polymerase Chain Reaction

3.2.4.1 Principle of the Method

Polymerase chain reaction (PCR) was invented by Kary B. Mullis in 1985. Since then PCR has become one of the most widely used techniques in molecular biology. It is a simple and very sensitive method to produce relatively large numbers of copies of DNA molecules from a minimum amount of DNA.

The technique is used for exponential amplification of a small quantity of a specific nucleotide sequence in the presence of a DNA template, thermostable DNA polymerase, deoxyribonucleotide triphosphates (dNTPs), Mg^{2+} and two specific primers, which define the beginning and the end of the produced copy of DNA.

PCR has the following parts that are cycled approximately 30 times until enough copies for DNA analysis are prepared:

1. Denaturation - DNA is heated to the temperature of 94 - 95 °C for 20-30 seconds. Hydrogen bonds between complementary bases are disrupted during this phase and single strand DNA is produced.

2. Annealing - the temperature is decreased to 50 - 65 °C (the exact temperature depends on the T_m of the primers) for 20 - 40 seconds. During this phase, primers anneal to specific sites of single strand DNA and thermostable DNA polymerase binds to newly formed double strand DNA.

3. Extension - the temperature usually increases to 72 °C. It is optimal for the activity of the most frequently used DNA polymerase Taq (*Thermus Aquatic* DNA polymerase). The double strand DNA is produced from free complentary dNTPs according the template in 5' to 3'direction.

3.2.4.2 Working Procedure

1. DNA samples are diluted to the concentration of 20ng/µl in sterile tubes.
2. All reagents of the PCR mix, except for diluted DNA (See Table 3.1) are added to a 2 ml sterile tube (on ice).
3. The mixture is mixed gently and 20µl aliquotes are pipetted to the PCR strips.
4. 5µl (100 ng) of diluted DNA sample is added to the PCR strip and mixed well.
5. The PCR strips are shortly centrifuged so the samples are at the bottom of the strip.

6. The PCR strips are put in a thermal cycler and PCR is started. (For PCR conditions, see Table 3.2 and Table 3.3).

The primers used in PCR were predicted by Primer3 Input (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table 3.4).

Table 3.1 Polymerase chain reaction reagents for one sample

Reagents	**Concentration**	**Volume (ml)**
Sterile water		11.2
PCR buffer	10x	2.5
dNTP Mix solution	10 mM of every dNTP	1.0
primer sense	5µM	2.5
primer antisense	5µM	2.5
Taq DNA polymerase	5U/µl	0.3
DNA sample	100 ng	5.0

Table 3.2 PCR thermic condition for polymorphisms *RAGE* -429T/C (rs1800625), *RAGE* -374T/A (rs1800624), *RAGE* Gly82Ser (557 G/A, rs2070600)

Step	**Temperature (°C)**	**Time**
1	94	2 min
2	94	30 sec
3	59.5	45 sec
4	72	1min
5		repeat 34x steps 2-4
6	72	7 min

Table 3.3 PCR thermic condition for polymorphisms *RAGE* 2184A/G (rs13209119) and *GLO1* Glu111Ala (419A/C, rs4746)

	***RAGE* 2184A/G**		***GLO1* Glu111Ala (419A/C)**	
Step	**Temperature (°C)**	**Time**	**Temperature (°C)**	**Time**
1	94	2 min	92	5 min
2	94	25 sec	94	30 sec
3	68.4	45 sec	60	30 sec
4	72	1min	72	1 min
5		repeat 32x steps 2-4		repeat 30x steps 2-4
6	72	7 min	68	5 min

Table 3.4 Primers used for the amplification of studied polymorphism

Polymorphism	**5' ...sequence... 3'**	**Product size**
RAGE -429T/C	sense: GGG GCA GTT CTC TCC TCA CT antisense: GGT TCA GGC CAG ACT GTT GT	250 bp
RAGE -374T/A	sense: GGG GCA GTT CTC TCC TCA CT antisense: GGT TCA GGC CAG ACT GTT GT	250 bp
RAGE Gly82Ser (557G/A)	sense: GTA AGC GGG GCT CCT GTT GCA antisense: GGC CAA GGC TGG GGT TGA AGG	397 bp
RAGE 2184A/G	sense: GGCCTCAGGACCAGGGAACCTACA antisense: TTGGTCAGGCTGGTCTCGAACTCC	402 bp
GLO1 Glu111Ala (419A/C)	sense: GCA GGG GTT AGG CCA ATT AT antisense: CAG GCA AAC TTA CCG AAT CC	203 bp

3.2.5 Restriction Fragment Length Polymorphism analysis

3.2.5.1 Principle of the Method

Restriction fragment length polymorphism analysis (RFLP) is widely used for the analysis of SNPs as well as other mutations (deletion, mutation).

RFLP uses restriction enzymes binding to specific 4-6 bp long DNA restriction sites and cutting DNA. Variations in the DNA sequence cause the creation or removal of these restriction sites and thus, causes changes in the fragment numbers or lengths of the digested DNA. After PCR amplification, the DNA is digested with one or more restriction enzymes and produced fragments are separated according to molecular size using gel electrophoresis.

3.2.5.2 Working Procedure

1. All reagents are added to a sterile 1.5 ml tube one ice (see Table 3.5 and Table 3.6).
2. Gently mixed reagents are added to each strip with an amplified DNA sample.
3. Strips with amplified DNA and restriction enzymes are incubated overnight at the optimal temperature for the restriction enzymes (37°C for AluI, MfeI, BsmAI, 65°C for BsmFI).

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

3.2.6 Agarose Gel Electrophoresis

3.2.6.1 Principle of the Method

Agarose gel electrophoresis is a method used for the separation of DNA or RNA molecules by size. The negatively charged molecules move through an agarose matrix from cathode to anode in an electric field. Smaller molecules move faster and migrate further than larger ones. The movement is influenced by the DNA conformation as well as by the agar concentration. The DNA

travelling in the gel is made visible by adding loading buffers to the DNA. Molecular size standards are used to estimate the fragment size.

Ethidium bromide (EtBr) is the most common dye used for agarose gel electrophoresis. EtBr fluoresces under UV light when it is intercalated into the DNA. After electrophoresis through an EtBr-treated gel, distinct DNA bands are visible with UV light.

Table 3.5 Reagents for RFLP for *RAGE* -429T/C (rs1800625), *RAGE* -374T/A (rs1800624), *RAGE* Gly82Ser (557 G/A, rs2070600)

Polymorphism	***RAGE*-429T/C**	***RAGE* -374T/A**	***RAGE* Gly82Ser (557 G/A)**
Reagents	**Volume (µl)**	**Volume (µl)**	**Volume (µl)**
Buffer	1.83	1.83	1.83
BSA	0.18	0.18	0.18
Restriction enzyme	0.3	0.3	0.3
Restriction enzyme - type	***AluI***	***MfeI***	***AluI***

Table 3.6. Reagents for RFLP for *RAGE* 2184A/G (rs13209119) and *GLO1* Glu111Ala (419A/C, rs4746)

Polymorphism	***RAGE* 2184 A/G**	***GLO1* Glu111Ala (419A/C)**
Reagents	**Volume (µl)**	**Volume (µl)**
Sterile water	4.3	
Buffer	2.4	1.83
BSA	0.3	0.18
Restriction enzyme	1.0	0.3
Restriction enzyme - type	***BsmFI***	***BsmAI***

3.2.6.2 Solutions

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

3.2.6.3 Working Procedure

1. 40ml of frozen TBE buffer diluted 10 times is added to the 1.2g of frozen agarose and swirled.
2. The solution is boiled in a microwave oven to dissolve the agarose.
3. 40µl of EtBr is added and swirled.
4. The gel is slowly put into the smaller tank. Bubbles are put to the side using disposable tips and combs are inserted.
5. The gel is let to cool off for 30 minutes at laboratory temperature and for 20 minutes in the cold.
6. 210 ml of TBE buffer diluted 10 times is put into the bigger gel tank and 210µl of EtBr and after a smaller tank with gel is submerged.
7. The combs are taken out.
8. 4µl of the loading buffer is added to the each strip.
9. The samples are loaded and the final lane is loaded with 50 or 100bp marker.
10. The gel tank is closed, the power-source is switched on and firstly is run at 30-40 V (5 minutes), then at 70-90 V (5 minutes) and finally at 150V.
11. After electrophoresis the gel is put under UV light to make DNA fragments visible.

Products were separated by electrophoresis in 3% agarose gel.

3.2.7 Routine Laboratory Parameters

Blood count was estimated by an automated hematological Beckman Coulter LH750 Hematology analyzer (Beckman Coulter, Inc, Fullerton, CA, USA).

Routine biochemical parameters were measured by commercially available kits using the manufacturer's instructions and certified techniques

A modular analyzer (Roche Diagnostics GmbH, Mannheim, Germany) was used for the estimation of serum levels of CRP, ALT, AST, bilirubin, creatinine, uric acid and urea and for the assessment of proteinuria. C-reactive protein was measured turbidimetrically. Serum liver enzymes levels, ALT and AST, were determined by the International Federation of Clinical Chemistry (IFCC) modified method at 37 °C with the addition of pyridoxal-5-phosphate. Total bilirubin was measured using a method with stabilized diazonium salt. Serum creatinine was assessed by Jaffe's reaction. Uric acid was estimated using enzymatic methods with photometric detection. Serum urea levels were determined by enzymatic UV assay with urease. Protein in a 24 hour urine sample was determined using benzethonium chloride on the same day as collections were completed.

3.2.8 Samples

The blood was collected via a puncture of the cubital vein simultaneously with blood collection for routine examinations. The blood was collected after overnight fasting. The blood count and routine biochemical parameters were established in fresh samples. For DNA analysis, blood was collected in tubes containing EDTA and stored at –4°C. The isolation of the DNA was performed within 1 week. For sRAGE analysis, blood was collected in tubes without anticoagulant, centrifuged for 10 minutes at 3000 rpm and serum was frozen at –80°C. The analysis of all samples was performed within 6 months after collection.

3.2.9 Statistical Analysis

The results of biochemical parameters are expressed as the mean ± the standard deviation. Continuous variables were compared with a one-way ANOVA (analysis of variance) test or a Kruskal-Wallis test and an unpaired t-test and Wilcoxon test, where appropriate. Associations between parameters were determined using the Pearson and Spearman correlation coefficients, according to the data distribution. For the testing of the Hardy-Weinberg equilibrium, an χ^2 test or Fischer's exact test was used. Single polymorphism associations were assessed using the χ^2 test and

the Fisher's exact test. Haplotype analysis was used for a more detailed description. For statistical analysis, the software "Prism 5", GraphPad Software Inc, was used. All tests used were two-sided and all results were considered statistically significant at $p<0.05$.

4 Study Population

The study population consisted of 120 healthy pregnant women and 164 pregnant women suffering from some pregnancy induced diseases.

120 Caucasian healthy pregnant women were examined and followed up at the Department of Gynecology and Obstetrics of the General Teaching Hospital in Prague. The women did not suffer from any internal nor pregnancy induced diseases. All of them delivered at term. The mean age of controls was 30±4 years. The blood for sRAGE examination and genetic studies was taken just once during the pregnancy, together with other routine blood examinations. At the time of blood collection, 27 women were in the 1st trimester of the pregnancy (9 ±1 weeks of pregnancy), 25 women were in the 2nd trimester (24±3 weeks of pregnancy) and 68 women were in the 3rd trimester (36±2 weeks of pregnancy). For biochemical characteristics of healthy pregnant controls see Table 4.1

In total 164 pregnant women of Caucasian origin with symptoms of threatening preterm labor, hypertensive disorders, IUGR and ICP were examined and followed up at the Department of Gynecology and Obstetrics of the General Teaching Hospital in Prague and consequently enrolled in our study.

99 pregnant patients with symptoms of threatening preterm labor were enrolled in the study. The mean age of the patients was 31±5 years. 50 patients presented with regular contraction every 15 minutes or less, 36 patients presented with preterm premature rupture of membranes (PPROM), 50 patients had cervical dilatation and 15 patients presented with vaginal bleeding. The mean week of pregnancy at the time of the enrollment to the study was 29±3 weeks. The mean week of delivery was 32±5 weeks. 75 patients with symptoms of threatening preterm labor delivered within 24 hours after blood collection for analysis. 42 patients suffered from some sexually transmitted diseases (STD) - ureaplasma, mycoplasma or chlamydia infection. Patients with threatening preterm labor did not suffer from any other pregnancy induced diseases or internal diseases.

35 pregnant patients suffering from preeclampsia were followed up and enrolled in the study. The mean age of the patients was 31±4 years. The mean week of pregnancy at the time of enrollment into the study was 35±4 weeks. The mean week of labor was 37±4 weeks. Hypertension in pregnancy was diagnosed in previously normotensive patients with two repeated systolic blood pressure above 140 mm Hg and diastolic blood pressure above 90 mm Hg. All patients had

proteinuria above 300 mg per 24 hours or developed such proteinuria during the follow-up after enrollment into study. None of the patients developed neurological symptoms (eclampsia) or symptoms of HELLP syndrome (Hemolysis, Elevated Liver enzymes, Low Platelets). Patients who suffered from other pregnancy induced diseases or internal diseases were excluded from the study.

22 pregnant patients with IUGR were followed up and enrolled in the study. The mean age of the patients was 29±4 years. The mean week of pregnancy at the time of enrollment to the study was 33±4 weeks. The mean week of delivery was 35±4. IUGR was diagnosed as the retardation of fetal growth under the 3rd percentile of regional growth curves. The diagnosis "small for gestational age" was ruled out by repeated ultrasound biometry within 14 days. Patients suffering from other pregnancy induced diseases including hypertensive disorders or internal diseases were excluded from the study subgroup.

14 pregnant patients with a diagnosis of intrahepatic cholestasis of pregnancy were followed up at the Department of Gynecology and Obstetrics and included in the study. The mean age of the patients was 32±4 years. The mean week of pregnancy at the time of enrollment to the study was 36±3, the mean week of labor was 38±2. Patients, who had elevated liver enzymes, after the exclusion of other reasons of hepatopathy, were enrolled into study. The assessment of the bile acids was not routinely available during the duration of the study. Patients suffering from other pregnancy induced diseases or internal diseases were excluded from the study subgroup.

For further biochemical characteristics of the studied subgroups, see Table 4.1

None of the patients suffered from gestational diabetes, diabetes mellitus type 1 and 2 or chronic renal diseases. Women with multiple pregnancies were excluded from the study.

All diagnoses were in accordance with the recommendations of the Czech Gynecological and Obstetrical Society and the recommendation of Čech et al. (Čech E. et al., 2006).

For the analysis of the sRAGE dynamics in physiologic pregnancy the population from the study Germanová A. et al., 2010 was used. The population consisted of all of the above described healthy pregnant women except for 41 healthy controls in the 3rd trimester, who joined the study as last ones.

For the analysis of the sRAGE concentrations in pathological pregnancy the above described population was used. The study population is identical with the population from Germanová A. et al., 2012.

For genetic analysis all pregnant subjects were used. The study population is identical with the population from the study Germanová A. et al., 2012.

24 non-pregnant healthy subjects served as controls for sRAGE analysis. The mean age of subjects was 28±5 years.

The study was performed in accordance with the principles of the Declaration of Helsinki and approved by the local Ethical Committee. All patients gave their informed consent prior to entering the study.

Table 4.1 Biochemical characteristics of pregnant women with pathological and physiological pregnancy

Parameter	Preterm Labor N=99	Preeclampsia N=35	IUGR N=22	ICP N=14	Pregnant controls N=120	P
BMI	25.2±3.8	27.9±3.7*,#	26.2±3.7	27.7±3.7*,#	24.8±3.6	<0.05
Leu (x10^9/l)	12.6±4.6 *	9.6±2.8#	10.4±3.3	9.6±2.8	10.4±2.4	<0.05
Neu relat. (%)	77.3±9.3*	66.2±11.2#	68.6±8.9#	70.8±8.7	71.5±8.5	<0.05
CRP (mg/l)	15.9±25.1+, °	8.6±14.9	5.7±4.8	5.6±6.2	n.a.	<0.05
Urea (mmol/l)	2.2±1.2	4.8±4.6 #	3.2±0.9	3.1±1.1	n.a.	<0.05
Creatinine (µmol/l)	51.1±10.7	65.4±13.4#,+	55.9±14.2	56.8±9.9	n.a.	<0.05
Uric acid (µmol/l)	214.8±53.2 +	390.3±80.3#, +, °	308.2±77.7	264.9±72.0	n.a.	<0.05
Bilirubin (µmol/l)	7.9±3.3	6.66±3.99°	6.6±2.7°	10.9±4.1	n.a.	<0.05
ALT (µkat/l)	0.34±0.18°	0.39±0.38°	0.41±0.91°	2.9±1.9	n.a.	<0.05
AST (µkat/l)	0.36±0.15°	0.50±0.28°	0.43±0.65°	1.6±1.0	n.a.	<0.05

* p<0.05 vs. controls

# p<0.05 vs. preterm labor

+ p<0.05 vs. IUGR

° p<0.05 vs. ICP

n.a. - not assessed

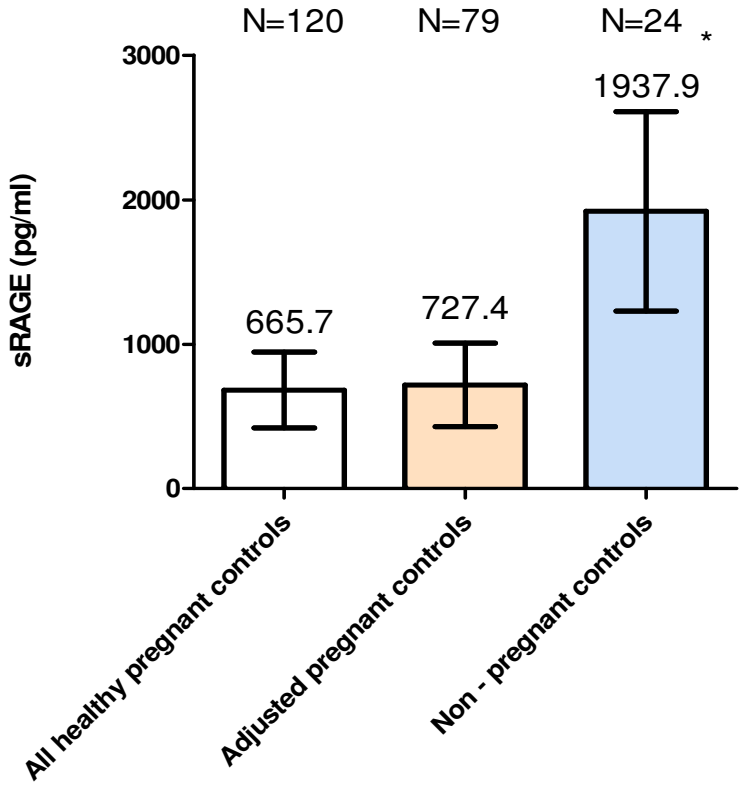
5 Results

5.1 sRAGE

5.1.1 sRAGE in Physiological Pregnancy

The mean sRAGE serum level during physiological pregnancy is significantly decreased in comparison to the mean sRAGE serum level in non - pregnant controls (665.7±263.1 pg/ml vs. 1937.9±690.8 pg/ml, $p<0.05$). However, sRAGE serum levels vary during physiological pregnancy. So, using an approximately equal number of subjects from each trimester (the study population from the study Germanová A. et al., 2010 - 27 in the 1st trimester, 25 in the 2nd trimester and 27 in the 3rd trimester) the mean sRAGE serum concentration is 727.4±315.4 pg/ml. See Figure 5.1.

Figure 5.1 Serum sRAGE levels in healthy pregnant and non-pregnant controls

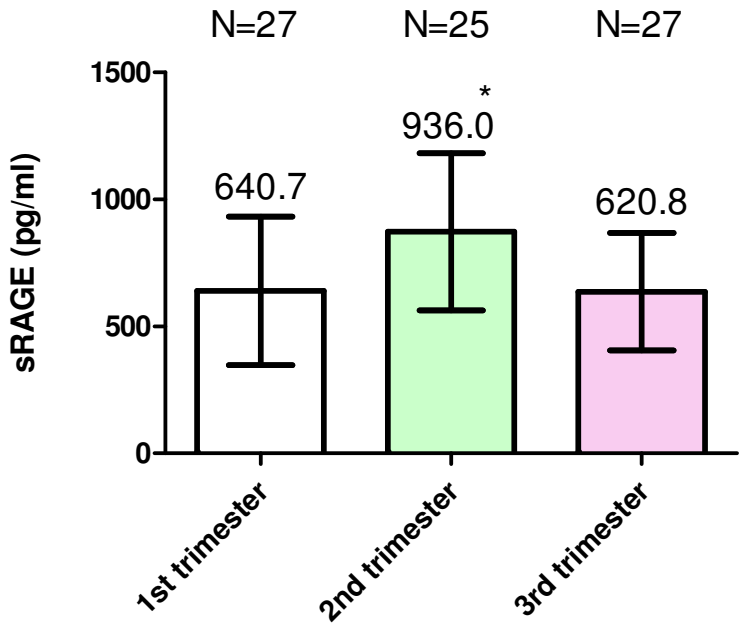


Legend: Vertical column bar graph with means and standard deviations, * $p<0.05$, in comparison to all healthy pregnant controls and adjusted healthy pregnant controls.

For further analysis only the adjusted mean of serum sRAGE levels was used.

As mentioned, the serum sRAGE level is low during the 1st trimester (640.7±291.4 pg/ml), then in the 2nd trimester, the concentration rises (936.0±331.1 pg/ml) and falls again in the 3rd trimester and before labor (620.8±225.5 pg/ml). sRAGE serum levels are significantly increased in the 2nd trimester ($p<0.05$) in comparison to the serum levels in the 1st and 3rd trimester as well as in comparison to all groups of healthy pregnant controls. See Figure 5.2.

Figure 5.2 Serum sRAGE levels during physiological pregnancy



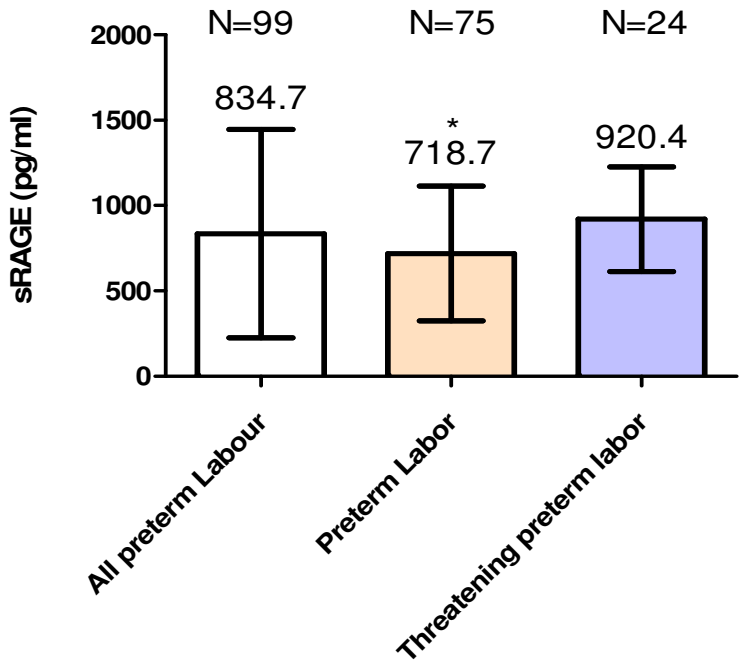
Legend: Vertical column bar graph with means and standard deviations, * $p<0.05$, in comparison to 1st trimester, 3rd trimester

5.1.2 sRAGE in Threatening Preterm Labor

The mean sRAGE serum level in patients with threatening preterm labor is significantly lower in comparison to non-pregnant controls (834.7.6±610.4 pg/ml vs. 1937.9±690.8 pg/ml, $p<0.05$). It is slightly elevated in comparison to healthy pregnant controls, but the difference is not significant (834.7.6±610.4 pg/ml vs. 727.4±315.4 pg/ml). Patients with threatening preterm labor, who delivered within 24 hours after blood collection, have significantly decreased sRAGE serum concentration in comparison to patients who delivered after 24 hours after blood collection

(718.7±395.1 vs 920.4±307.6 pg/ml, p<0.05). sRAGE serum concentrations of patients who delivered soon after enrollment into study are also significantly lower compared to healthy controls in the 2nd trimester (936.0±331.1 pg/ml, p<0.05) and do not differ significantly from the sRAGE levels in the controls in the 3rd trimester and before labor (637.1±231.7 pg/ml, p>0.05).

Figure 5.3 sRAGE serum levels in pregnant women with threatening preterm labor

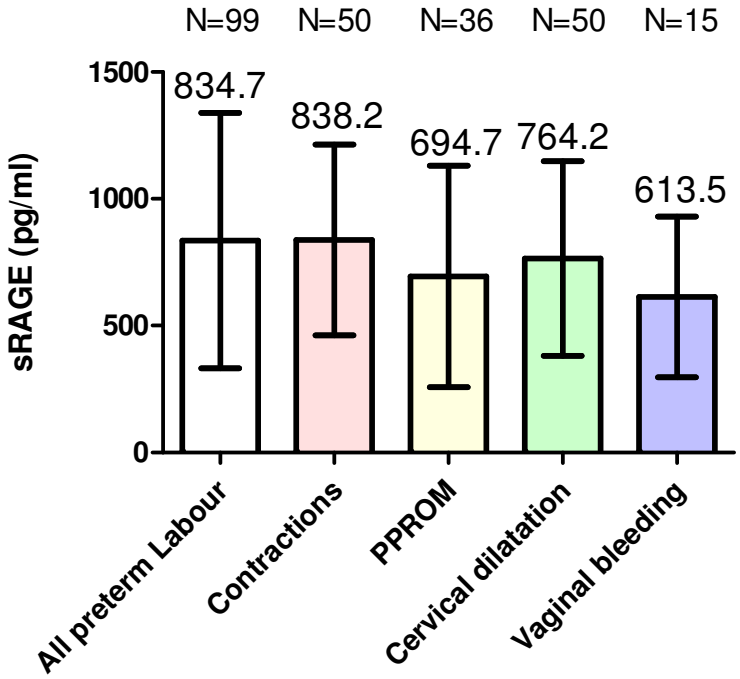


Legend: Vertical column bar graph with means and standard deviations,* p<0.05 in comparison to threatening preterm labor

There are no significant differences in sRAGE serum levels when the patients with threatening preterm labor were divided according to the symptoms (see Figure 5.4). sRAGE serum levels do not differ significantly between patients with an STD (729.4±361.0 pg/ml) and patients without STD (796.1±400.1 pg/ml).

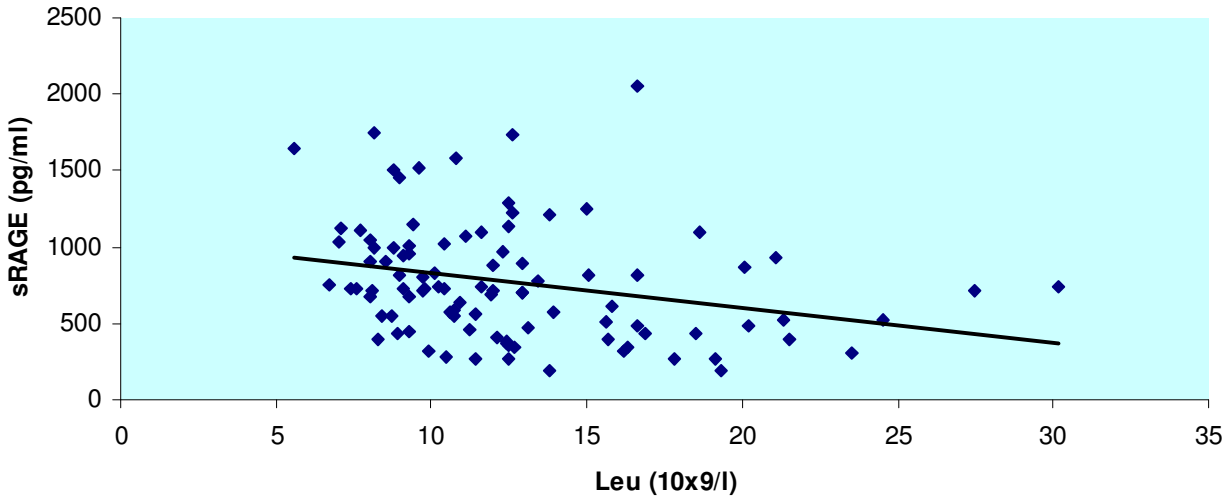
The sRAGE serum levels in preterm labor correlate negatively with leukocyte count (r= -0.34, p<0.05) and with absolute neutrophile count (r= -0.34, p<0.05) and do not correlate with other followed parameters. See Figure 5.5.

Figure 5.4 sRAGE serum levels in patients with preterm labor according to the symptoms



Legend: Vertical column bar graph with means and standard deviations

Figure 5.5 sRAGE correlation with leukocyte count in patients with preterm labor



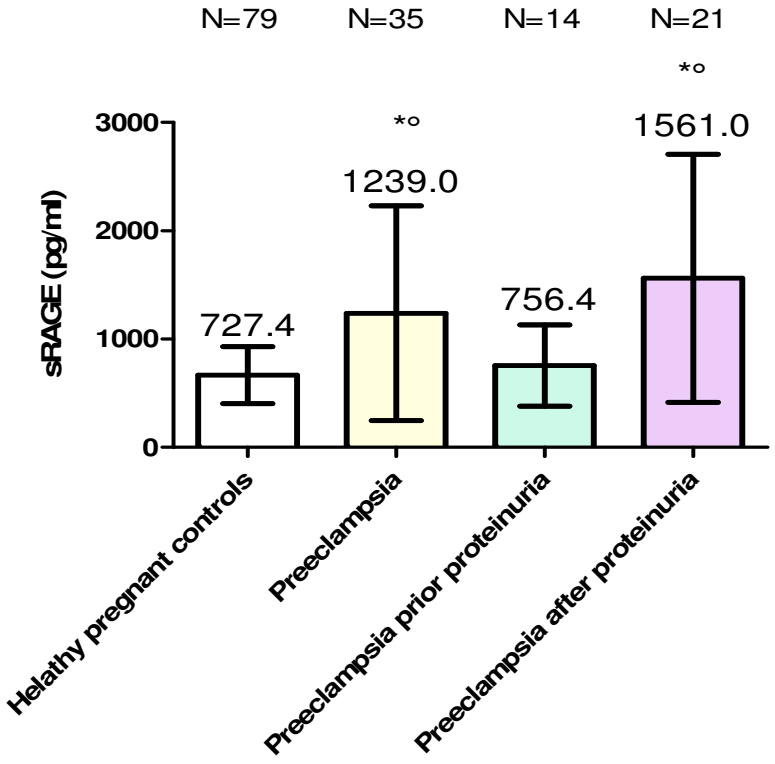
Legend: r= -0.34, p<0.05, y= -22.481x + 1051. 9

5.1.3 sRAGE in Preeclampsia

Serum sRAGE levels in preeclampsia are significantly lower in comparison to non-pregnant healthy controls (1239.0±991.8 vs. 1937.9±690.8 pg/ml, $p<0.05$), but they are significantly higher in comparison to healthy pregnant controls as well as to healthy pregnant women in the 1st, 2nd and 3rd trimester (1239.0±991.8 vs. 727.4±315.4 vs. 640.7±291.4 vs. 936.0±331.1 vs. 620.8±225.5 pg/ml, $p<0.05$)

Serum sRAGE concentrations are significantly lower in the patients with preeclampsia before the onset of proteinuria compared to patients with preeclampsia after the onset of proteinuria and compared to all patients with preeclampsia (756.4±374.5 vs. 1561.0±1144 vs. 1239.0±991.8 pg/ml, $p<0.05$). See Figure 5.6.

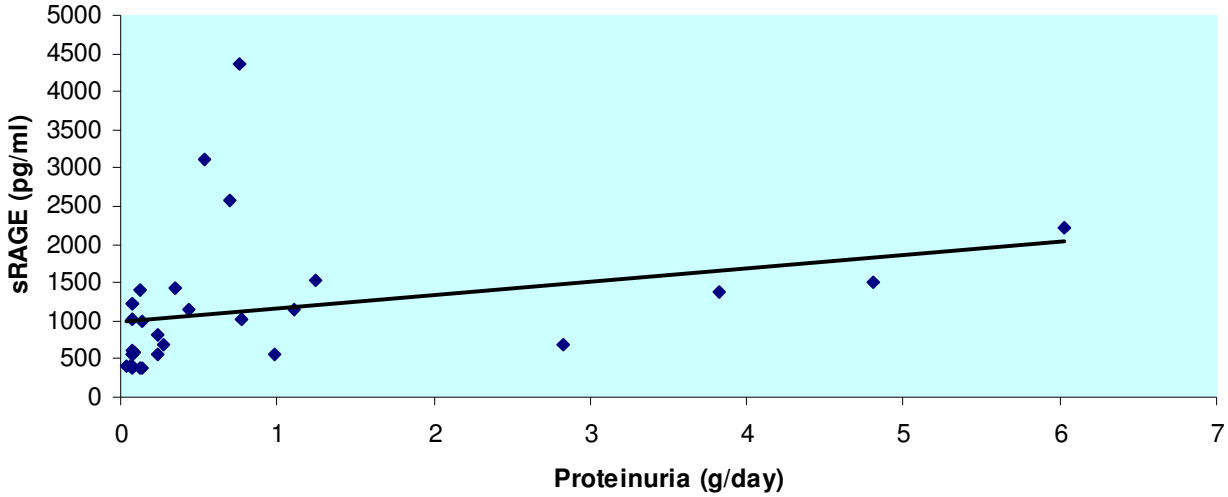
Figure 5.6 sRAGE in preeclampsia



Legend: Vertical column bar graph with means and standard deviations, * $p<0.05$ in comparison to controls, ° $p<0.05$ in comparison to preeclampsia before the onset of proteinuria

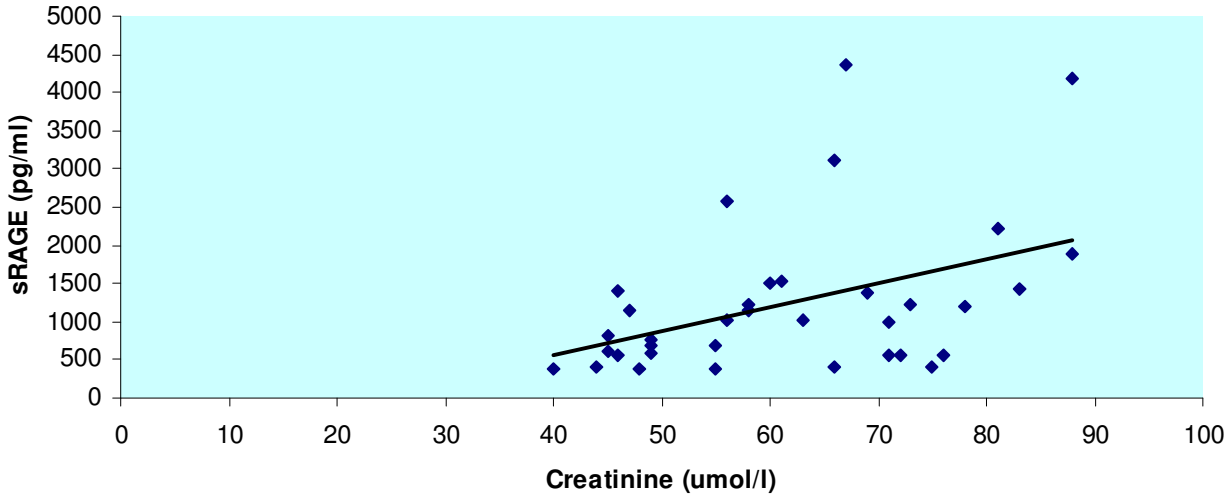
sRAGE serum levels in patients with preeclampsia correlate positively with proteinuria (grams per day) (r=0.58, p<0.05) (Figure 5.7), with creatinine serum levels (r=0.43, p<0.05) (Figure 5.8) and with uric acid serum levels (r=0.38, p<0.05) (Figure 5.9).

Figure 5.7 Correlation between sRAGE and proteinuria



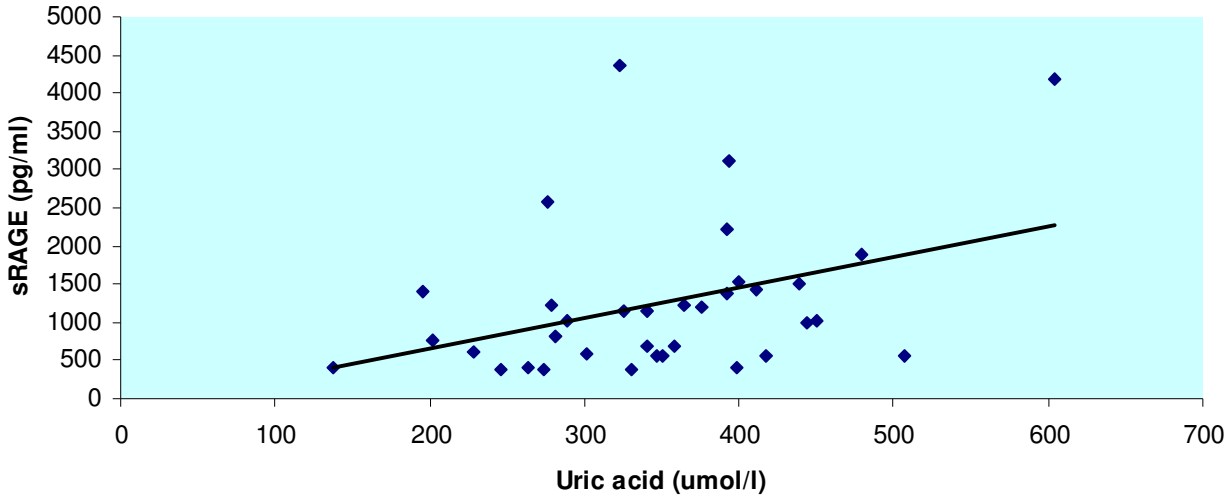
Legend: r=0.58, p<0.05, y= 173,82x + 991.18

Figure 5.8 Correlation between sRAGE and creatinine



Legend: r=0.43, p<0.05, y= 31.441x - 695.89

Figure 5.9 Correlation between sRAGE and uric acid



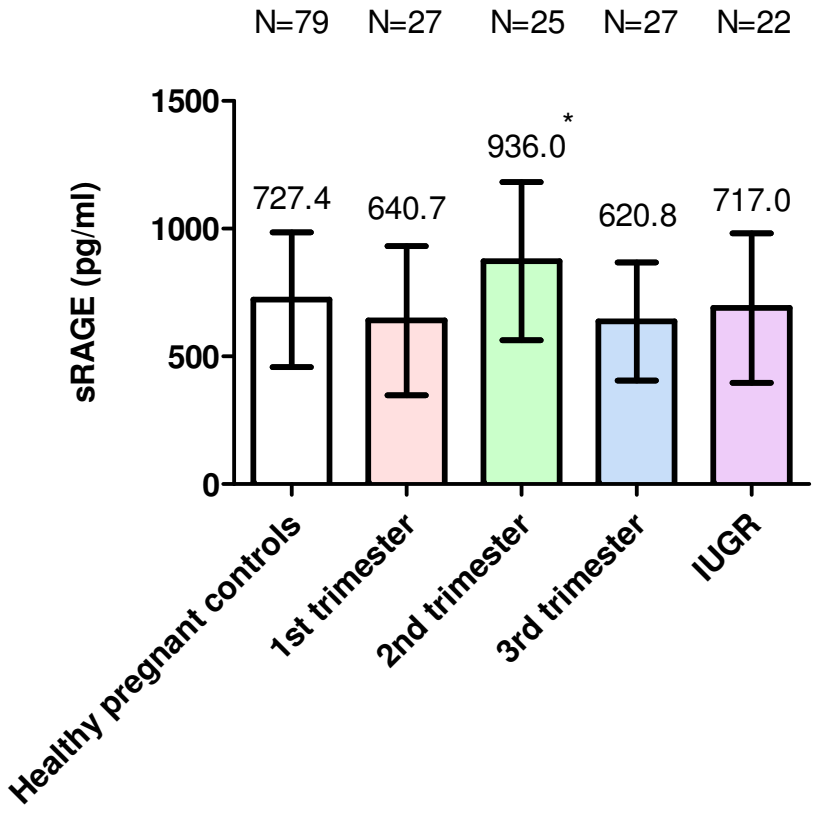
Legend: r=0.38, p<0.05, y= 3.9763x - 142.05

5.1.4 sRAGE in IUGR

sRAGE serum levels in IUGR are significantly lower in comparison to healthy non - pregnant controls (717.0±268.7 vs. 1937.9±690.8 pg/ml, p<0.05), but they do not differ significantly in comparison to healthy pregnant controls (717.0±268.7 vs. 727.4±315.4 pg/ml). See Figure 5.10.

sRAGE serum levels in IUGR correlate positively with the creatinine serum level (r=0.40, p<0.05) and with uric acid serum levels (r=0.40, p<0.05).

Figure 5.10 sRAGE levels in IUGR in comparison to healthy pregnant controls



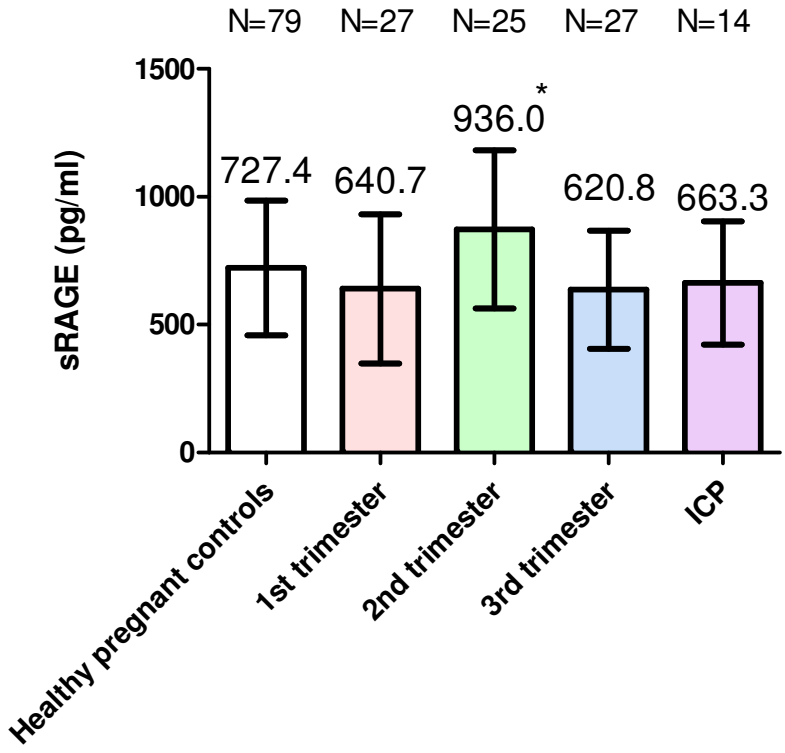
Legend: Vertical column bar graph with means and standard deviations, * $p<0.05$, in comparison to healthy pregnant controls, 1st trimester, 3rd trimester and IUGR.

5.1.5 sRAGE in ICP

sRAGE serum levels in ICP are significantly lower in comparison to healthy non - pregnant controls (663.4±241.1 pg/ml vs. .1937.9±690.8 pg/ml, $p<0.05$), but sRAGE serum levels in patients with ICP do not differ significantly from sRAGE serum levels in pregnant controls (663.3±241.2 vs. 727.4±315.4 pg/ml). See Figure 5.11.

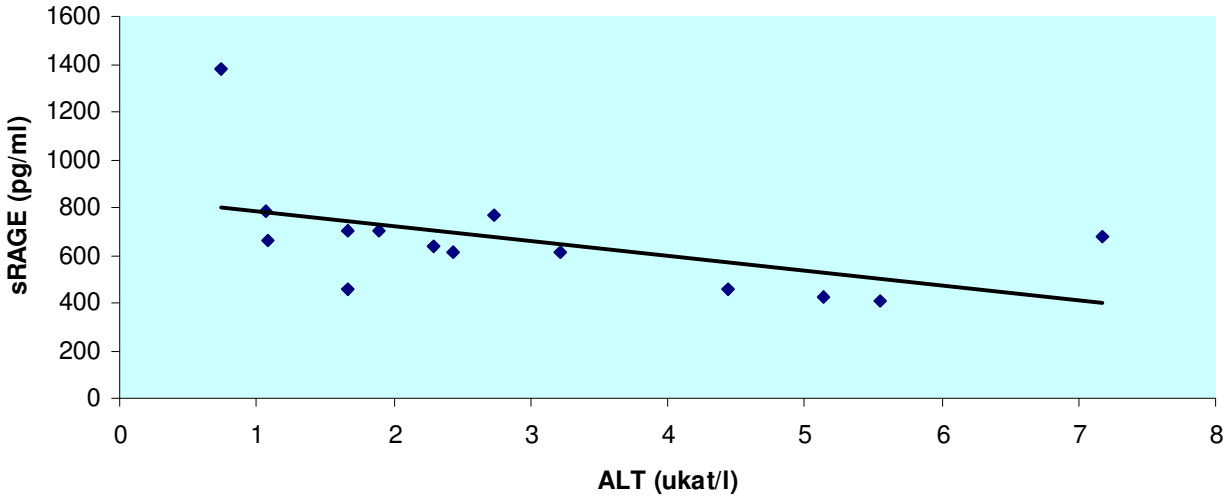
sRAGE serum levels in patients with ICP correlate negatively with ALT serum levels ($r= -0.629$, $p<0.05$, resp. $r = -0.536$, $p=0.05$, after the exclusion of extreme values of sRAGE) (See Figure 5.12). AST serum levels tend to correlate with serum sRAGE levels also, but this trend is not significant ($r= -0.529$, $p=0.06$) See Figure 5.13.

Figure 5.11 sRAGE levels in ICP in comparison to healthy pregnant controls



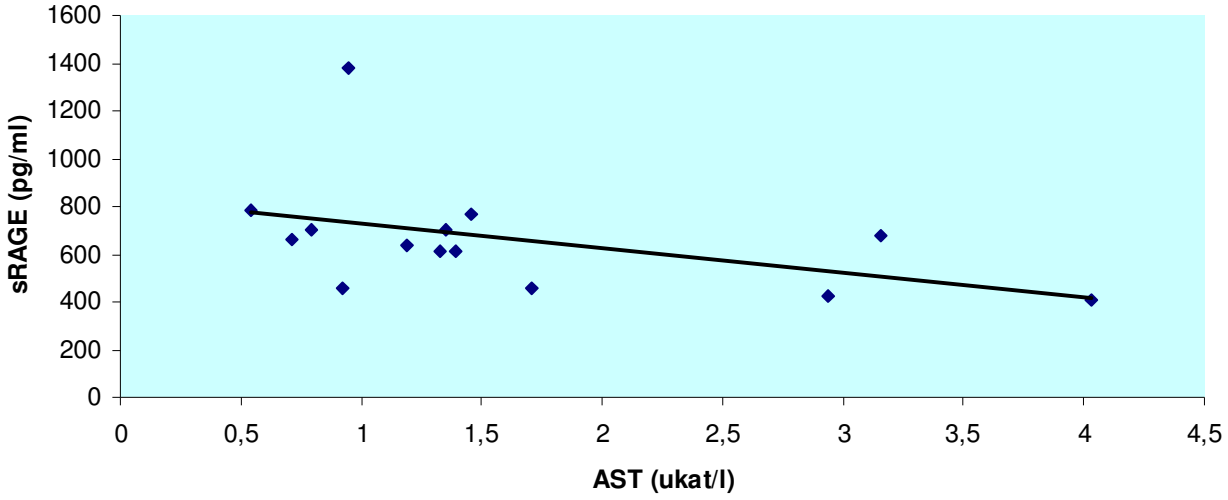
Legend: Vertical column bar graph with means and standard deviations, * $p<0.05$, in comparison to healthy pregnant controls, 1st trimester, 3rd trimester and ICP

Figure 5.12 sRAGE correlation with ALT in patient with ICP



Legend: r = -0.629, $p<0.05$, y = -62.742x + 847.6

Figure 5.13 sRAGE correlation with AST in patient with ICP



Legend: r= -0.529, p=0.06, y= -101.53x + 826.33

5.2 Genetic Analyses

5.2.1. *RAGE* Polymorphisms

5.2.1.1 *RAGE* -429 T/C Polymorphism (rs1800625)

RAGE -429 T/C polymorphism is located in the gene promoter, -429 bp from the beginning of the translation. The alteration of thymine in the wild type allele for cytosine, affects the transcriptional activity of the gene.

The primers described in the section 3.2.4.2 were used for the amplification of DNA. A 250 bp long product arose after PCR. In the presence of wild type allele T, the restriction enzyme Alu I could not cleave the PCR product. In the presence of minor allele C, 250 bp long product was cleaved by Alu I into 162 and 88 bp long fragments (see Figure 5.14)

Genotype frequencies of *RAGE* -429T/C polymorphism corresponded to the expected frequencies according to the Hardy-Weinberg equilibrium (HWE) in the control group as well as in all groups of patients (See Table 5.1).

Figure 5.14 SNP -429 T/C: heterozygote TC, homozygotes TT, CC (PCR-RFLP analysis)

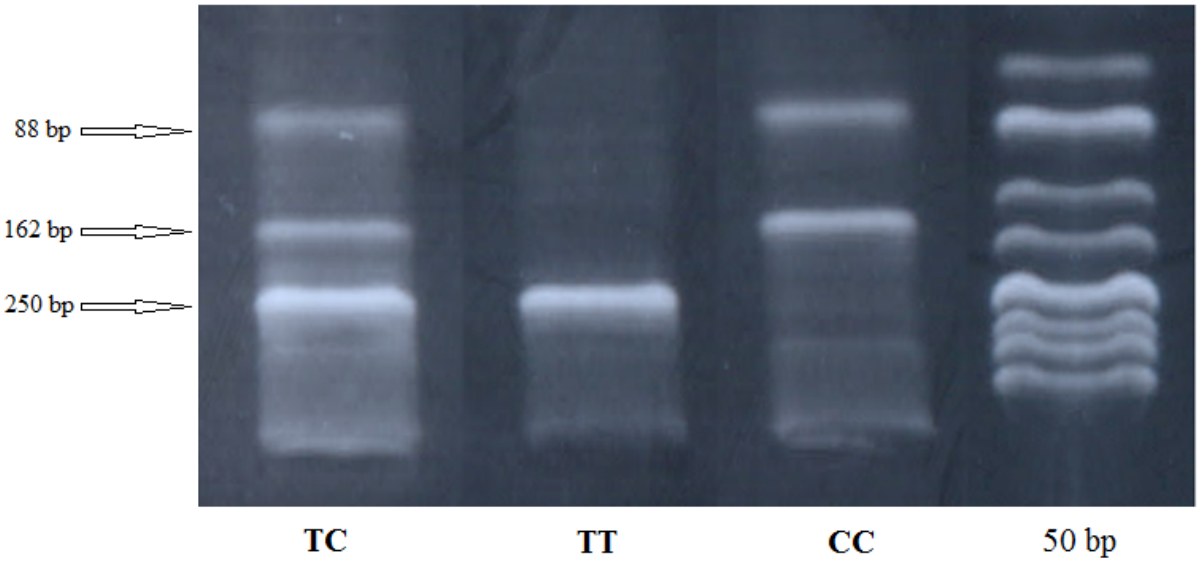


Table 5.1 Genotype frequencies of *RAGE* -429T/C polymorphism in healthy pregnant women and in women with pathological pregnancy – the determined frequencies and expected frequencies according to the Hardy-Weinberg equilibrium

Genotype	TT	TC	CC
Pathological pregnancy (N=164)	68.5%	27.3%	4.2%
HWE	67.4%	29.4%	3.2%
Healthy pregnant controls (N=120)	65.1%	32.5%	2.4%
HWE	66.2%	30.3%	3.5%

Legend: the differences are not significant

We have not described any difference in allelic or genotype frequencies among the studied groups. See Table 5.2.

Table 5.2 Allelic and genotype frequencies of *RAGE* -429T/C polymorphism in healthy pregnant women and in women with pathological pregnancy

		Threatening preterm labor N=99	Preeclampsia N=35	IUGR N=22	ICP N=14	Pregnant controls N=120
Alleles	**T**	72.9	81.0	80.5	76.5	82.1
(%)	**C**	27.1	19.0	19.5	23.5	17.9
Genotypes	**TT**	64.6	77.1	76.5	71.4	65.1
(%)	**TC**	30.3	20.0	20.6	21.4	32.5
	CC	5.1	2.1	2.9	7.2	2.4

Legend: the differences are not significant

5.2.1.2 *RAGE* -374 T/A Polymorphism (rs1800624)

RAGE -374 T/A polymorphism is located in the gene promoter, -374 bp from the translation start site. The substitution of thymine in the wild type allele for adenine effects the binding of transcriptional factors.

The primers described in section 3.2.4.2 were used for DNA amplification. PCR generated a 250 bp long product. There was no restriction site for the restriction enzyme MfeI in the presence of minor allele A. In the presence of wild type allele T a 250 bp product was cleaved by MfeI into 215 and 35 bp long fragments (see Figure 5.15).

Genotype frequencies of *RAGE* -374T/A polymorphism corresponded to the expected frequencies according to the Hardy-Weinberg equilibrium (HWE) in healthy pregnant women as well as in women with pathological pregnancy (See Table 5.3).

Figure 5.15 SNP -374 T/A: heterozygote TA, homozygotes AA, TT (PCR-RFLP analysis)

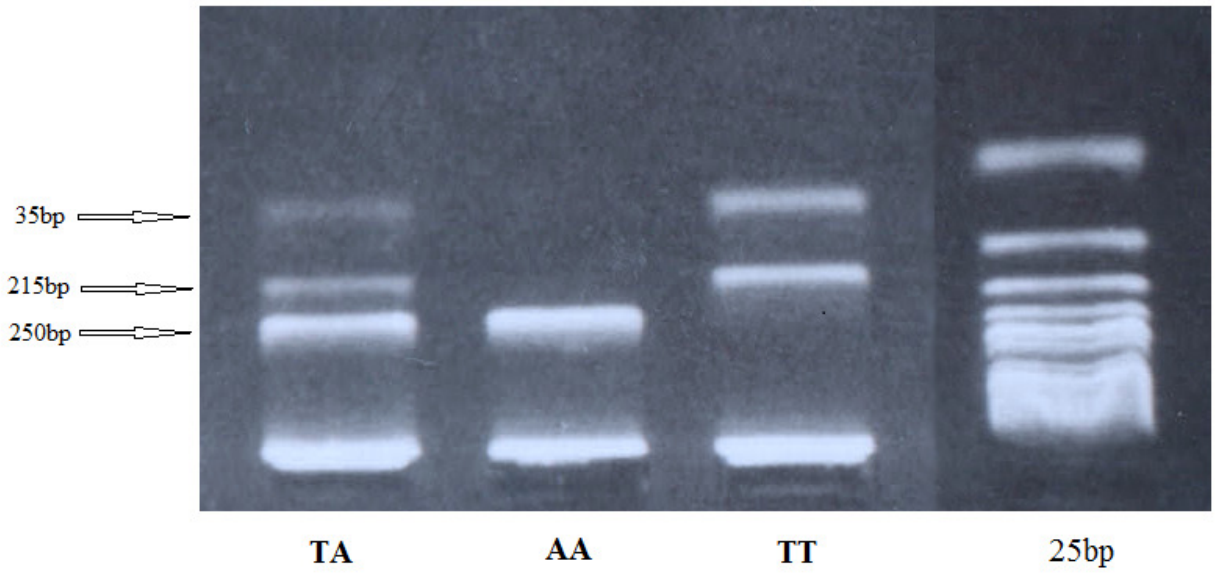


Table 5.3 Genotype frequencies of *RAGE* -374T/A polymorphism in healthy pregnant women and in women with pathological pregnancy – determined frequencies and expected frequencies according to the Hardy-Weinberg equilibrium

Genotype	TT	TA	AA
Pathological pregnancy (N=164)	39.4%	48.5%	12.1%
HWE	40.5%	46.3%	13.2%
Healthy pregnant controls (N=120)	39.7%	50.8%	9.5%
HWE	42.3%	45.5%	12.2%

Legend: the differences are not significant

We did not find any difference in allelic or genotype frequencies among studied subgroups. See Table 5.4.

Table 5.4 Allelic and genotype frequencies of *RAGE* -374T/A polymorphism in healthy pregnant women and in women with pathological pregnancy

		Threatening preterm labor N=99	Preeclampsia N=35	IUGR N=22	ICP N=14	Pregnant controls N=120
Alleles	**T**	59.5	55.8	57.7	65.0	65.1
(%)	**A**	40.5	44.2	42.3	35.0	34.9
Genotypes	**TT**	39.3	34.3	35.3	50.0	39.7
(%)	**TA**	49.5	48.6	52.9	42.9	50.8
	AA	11.2	17.1	11.8	7.1	9.5

Legend: the differences are not significant

5.2.1.3 *RAGE* Gly82Ser Polymorphism (557 G/A, rs2070600)

RAGE Gly82Ser (557 G/A) is located in the 3rd exon. The substitution of guanine (wild type allele) for adenine (mutated allele) at position 557 in mRNA causes a change in the polypeptide chain. Glycine at position 82 is substituted for serine. The ability of RAGE to bind AGEs is influenced by this change.

The primers described in the section 3.2.4.2 were used for the amplification of DNA. PCR generated a 190 bp long product. After digestion with AluI we obtained 118 and 72 bp long fragments for AA homozygous and 190 bp long fragments for GG homozygous (See Figure 5.16).

Genotype frequencies of *RAGE* Gly82Ser (557G/A) polymorphism corresponded to the expected frequencies according to the Hardy-Weinberg equilibrium (HWE) in controls and patients with pathological pregnancy (See Table 5.5).

Figure 5.16 SNP 557 G/A: heterozygote GA, homozygotes AA, GG (PCR-RFLP analysis)

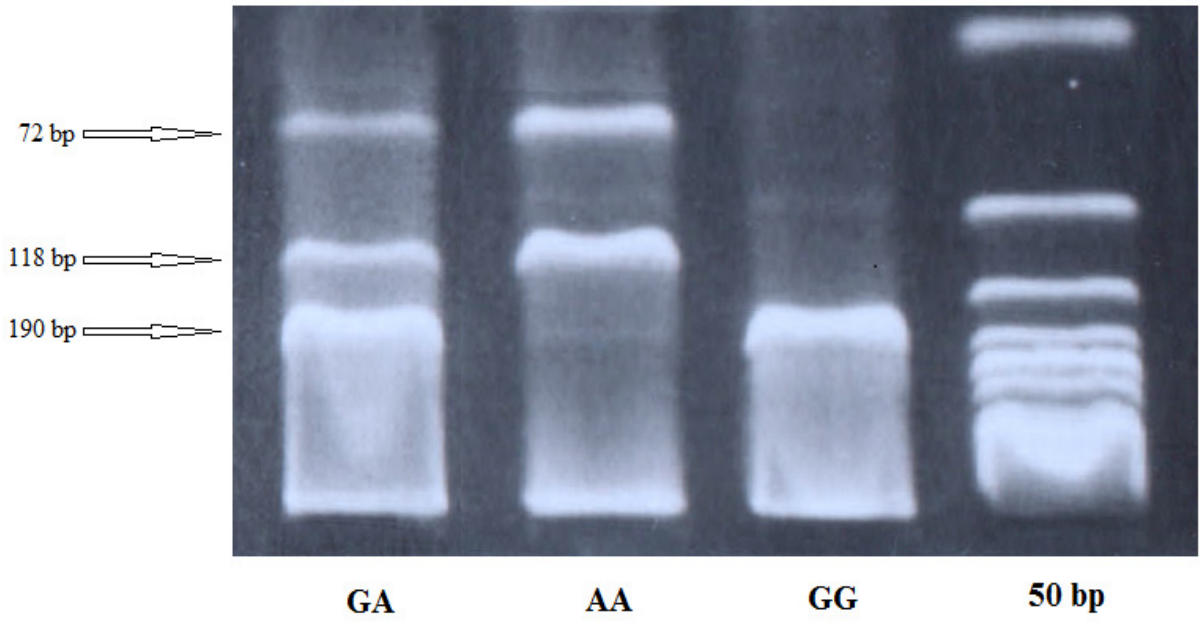


Table 5.5 Genotype frequencies of *RAGE* Gly82Ser (557G/A) polymorphism in healthy pregnant women and in women with pathological pregnancy – determined frequencies and expected frequencies according to the Hardy-Weinberg equilibrium

Genotype	GG	GA	AA
Pathological pregnancy (N=164)	96.4%	3.0%	0.6%
HWE	95.8%	4.1%	0.1%
Healthy pregnant controls (N=120)	93.7%	6.3%	0%
HWE	93.8%	6.1%	0.1%

Legend: the differences are not significant

We did not find any difference in allelic and genotype frequencies among studied subgroups (See Table 5.6).

Table 5.6 Allelic and genotype frequencies of *RAGE* Gly82Ser (557G/A) polymorphism in healthy pregnant women and in women with pathological pregnancy

		Threatening preterm labor N=99	Preeclampsia N=35	IUGR N=22	ICP N=14	Healthy controls N=120
Alleles (%)	**G**	96.1	100	97.1	93.3	96.7
	A	3.9	0	2.9	6.7	3.3
Genotypes (%)	**GG**	96.0	100	97.0	92.9	93.7
	GA	3.0	0	3.0	7.1	6.3
	AA	1.0	0	0	0	0

Legend: the differences are not significant

5.2.1.4 *RAGE* 2184 A/G Polymorphism (rs13209119)

RAGE 2184 A/G polymorphism is located in the 8th intron. The substitution of adenine (wild type allele) for guanine (mutated allele) probably influences the regulatory binding site, which participates in the production of sRAGE by alternative splicing.

The primers described in section 3.2.4.2 were used for DNA amplification. PCR generated 402 bp long products. After digestion with BsmFI we obtained 266 and 136bp long fragments for wild type allele A and 174, 136 and 92 bp long fragments for the minor allele G (See Figure 5.17).

Genotype frequencies of *RAGE* 2184A/G polymorphism corresponded to expected frequencies according to the Hardy-Weinberg equilibrium (HWE) in healthy pregnant women and also in patients with pathological pregnancy (See Table 5.7).

Figure 5.17 SNP 2184 A/G: heterozygote AG, homozygotes AA, GG (PCR-RFLP analysis)

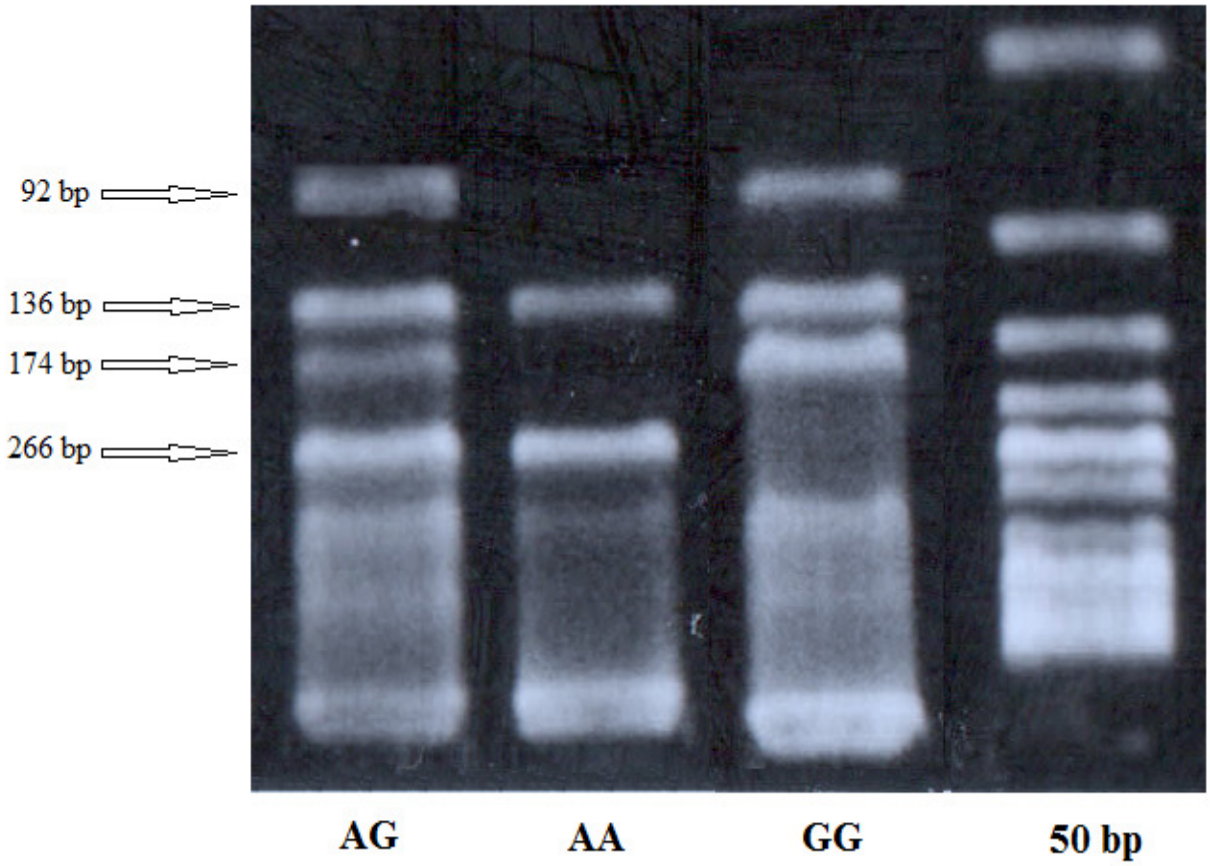


Table 5.7 Genotype frequencies of *RAGE* 2184A/G polymorphism in healthy pregnant women and in women with pathological pregnancy – determined frequencies and expected frequencies according to the Hardy-Weinberg equilibrium

Genotype	AA	AG	GG
Pathological pregnancy (N=164)	67.3%	30.3%	2.4%
HWE	67.9%	29.0%	3.1%
Healthy pregnant controls (N=120)	65.9%	30.9%	3.2%
HWE	66.2%	30.3%	3.5%

Legend: the differences are not significant

We did not find any difference in allelic and genotype frequencies among studied groups and controls (See Table 5.8).

Table 5.8 Allelic and genotype frequencies of *RAGE* 2184A/G polymorphism in healthy pregnant women and in women with pathological pregnancy

		Threatening preterm labor N=99	Preeclampsia N=35	IUGR N=22	ICP N=14	Healthy controls N=120
Alleles	**A**	73.6	80.0	75.6	77.8	81.3
(%)	**G**	26.4	20.0	24.4	22.2	18.7
Genotypes	**AA**	65.7	74.2	67.6	71.4	65.9
(%)	**AG**	30.3	25.8	32.4	28.6	30.9
	GG	4.0	0	0	0	3.2

Legend: the differences are not significant

5.2.1.5 RAGE Haplotypes

RAGE haplotype analysis did not show any significant differences in haplotype frequencies between women with pathological pregnancy and healthy pregnant controls (See Table 5.9).

Table 5.9 Haplotype frequencies of *RAGE* gene polymorphisms (*RAGE -429 T/C, -374 T/A, Gly82Ser - 557G/A, 2184 A/G*) in studied groups

Haplotypes (%)	Healthy controls N=120	Pathological pregnancy N=170	Threatening preterm labor N=99	Hypertensive disorders N=35	ICP N=14	IUGR N=22
TTGA	30.7	30.0	28.9	32.2	38.7	31.2
TTGG	10.6	10.3	10.2	9.2	9.7	10.6
TTAA	2.2	1.3	1.4	0.0	3.2	1.1
TTAG	0.0	0.2	0.3	0.0	0.0	0.0
TAGA	20.5	21.4	20.4	26.4	22.6	23.7
TAGG	5.0	4.6	4.6	4.6	0.0	5.4
TAAA	0.3	0.2	0.3	0.0	0.0	0.0
TAAG	0.0	0.0	0.0	0	0.0	0.0
CTGA	10.8	10.5	10.9	9.2	12.9	8.6
CTGG	10.5	11.2	12.0	9.2	12.9	8.6
CTAA	0.0	0.2	0.4	0.0	0.0	0.0
CTAG	0	0.2	0.4	0.0	0.0	0.0
CAGA	4.7	4.8	4.9	4.6	0.0	5.4
CAGG	4.7	5.0	5.3	4.6	0.0	5.4
CAAA	0.0	0.0	0.0	0.0	0.0	0.0
CAAG	0.0	0.0	0.0	0.0	0.0	0.0

Legend: Differences are not significant.

5.2.1.6 sRAGE and *RAGE* Polymorphisms

Focusing on the association of sRAGE serum levels with the studied RAGE polymorphisms, we detected that healthy pregnant women with genotype GA (N=7) of *RAGE* Gly82Ser (557G/A) polymorphism have significantly decreased serum sRAGE levels in comparison to healthy pregnant women with genotype GG (N=113) of *RAGE* Gly82Ser *(*557 G/A) polymorphism (483.0±104.0 vs. 692.0±262.0 pg/ml, p=0.008). We did not have any healthy pregnant control with the minor genotype AA of *RAGE* Gly82Ser (557G/A) polymorphism in our study population.

Other studied polymorphisms were not associated to altered sRAGE levels in healthy pregnant controls (See Table 5.10).

Table 5.10 sRAGE serum levels according to RAGE polymorphisms in healthy pregnant controls

Polymorphism	Genotype	N	sRAGE (pg/ml)	p
RAGE -429 T/C	TT	78	682±246	
(rs1800625)	TC	39	680±296	
	CC	3	559±58	0.607
RAGE -374 A/T	TT	46	658±262	
(rs1800624)	TA	62	695±271	
	AA	12	672±203	0.535
RAGE Gly82Ser (557 G/A)	GG	113	692±262	
(rs2070600)	GA	7	483±104	
	AA	0	-	0.008
RAGE 2184 A/G	AA	80	678±248	
(rs13209119)	AG	36	684±297	
	GG	4	623±137	0.887

A similar association was found in the pregnant women with pathological pregnancy, however, this trend was not significant (p=0.08). We only had one patient with the minor genotype

AA of *RAGE* Gly82Ser (557G/A) polymorphism, who had markedly decreased sRAGE serum level (264pg/ml). Patients with genotype GA (N=5) of *RAGE* Gly82Ser (557G/A) polymorphism had a higher sRAGE concentration than patients with genotype AA, but lower sRAGE levels than patients (N=164) with genotype GG (565.2±118.0 vs. 908.1±698.2 pg/ml, p=0.08).

Other studied polymorphisms were not associated with altered sRAGE levels in patients with patological pregnancy (See Table 5.11).

Table 5.11 sRAGE serum levels according to RAGE polymorphisms in pathological pregnancy

Polymorphism	Genotype	N	sRAGE (pg/ml)	p
RAGE -429 T/C	TT	112	956±730	
(rs1800625)	TC	45	778±607	
	CC	7	639±328	0.088
RAGE -374 A/T	TT	64	803±369	
(rs1800624)	TA	80	919±809	
	AA	20	1079±914	0.599
RAGE Gly82Ser (557 G/A)	GG	158	908±698	
(rs2070600)	GA	5	565±118	
	AA	1	264	0.085
RAGE 2184 A/G	AA	110	958±735	
(rs13209119)	AG	50	766±581	
	GG	4	712±425	0.125

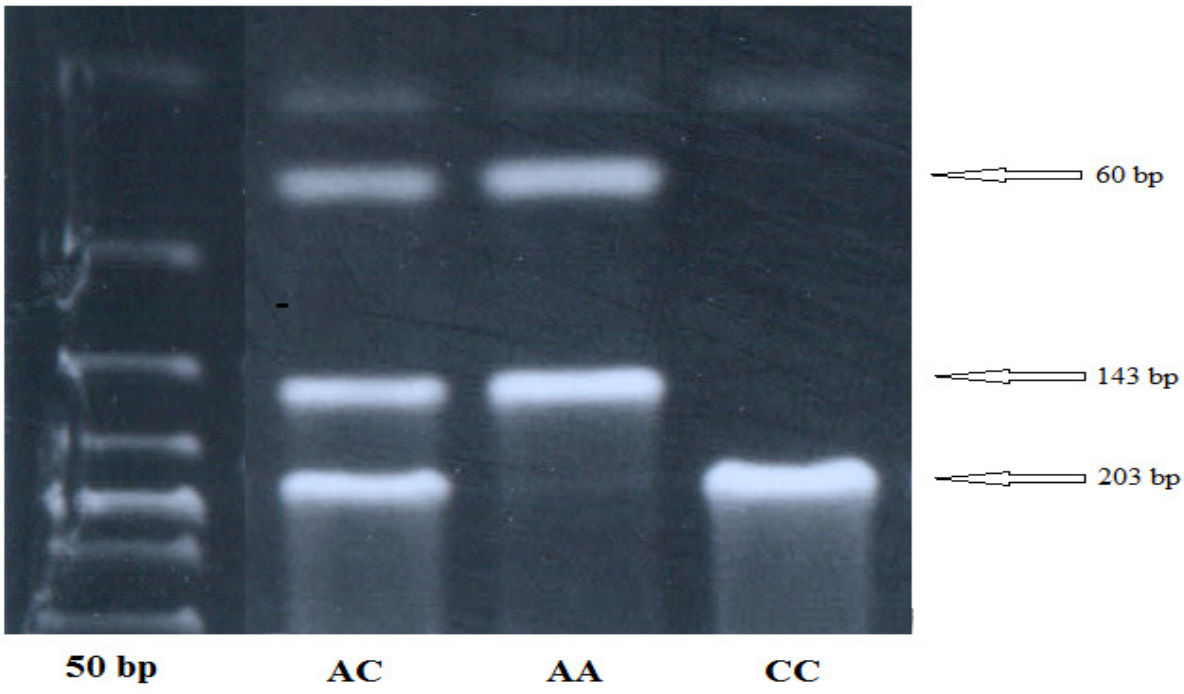
5.2.2 *Glyoxalase 1* Polymorphism

5.2.2.1 *GLO 1* Glu111Ala Polymorphism (419A/C, rs4746)

GLO 1 Glu111Ala (419A/C) polymorphism is located on the 4th exon of the gene for glyoxalase 1. It is caused by nucleotide variation, a change of wild type adenine to cytosine, in position 419 of mRNA. The substitution causes a change of glutamic acid in position 111 of the polypeptide chain for alanine. The alteration results in the decreased activity of glyoxalase 1.

The primers described in section 3.2.4.2 were used for the amplification of DNA. PCR product was 203 bp long. After digestion with BsmAI we obtained 143 and 60bp long fragments for wild type allele A and 203 bp long fragments for mutated allele C (See Figure 5.18).

Figure 5.18 SNP A419C: heterozygote AC, homozygotes AA, CC (PCR-RFLP analysis)



Genotype frequencies of *GLO 1* Glu111Ala (419A/C) polymorphism were in correspondence to the expected frequencies according to the Hardy-Weinberg equilibrium (HWE) in healthy pregnant women as well as in patients with pathological pregnancy (See Table 5.12).

Table 5.12 Genotype frequencies of *GLO 1* Glu111Ala *(*419A/C) polymorphism in healthy pregnant women and in women with pathological pregnancy – determined frequencies and expected frequencies according to the Hardy-Weinberg equilibrium

Genotype	AA	AC	CC
Pathological pregnancy (N=164)	30.9%	40.6%	28.5%
HWE	28.2%	46.0%	25.8%
Healthy pregnant controls (N=120)	34.9%	43.7%	21.4%
HWE	32.2%	49.1%	18.7%

Legend: the differences are not significant

We did not discover any significant difference in allelic or genotype frequencies among studied groups and controls (See Table 5.13).

Table 5.13 Allelic and genotype frequencies of *GLO 1* Glu111Ala (419A/C) polymorphism in healthy pregnant women and in women with pathological pregnancy

		Threatening preterm labor N=99	Preeclampsia N=35	IUGR N=22	ICP N=14	Healthy controls N=120
Alleles	**A**	48.6	57.4	52.9	57.9	56.7
(%)	**C**	51.4	42.6	47.1	42.1	43.3
Genotypes	**AA**	27.1	42.9	29.4	42.9	34.9
(%)	**AC**	41.4	34.3	50.0	35.7	43.7
	CC	31.3	22.8	20.6	21.4	21.4

Legend: the differences are not significant

5.2.2.2 sRAGE and *GLO 1* Polymorphism

GLO 1 Glu111Ala (419A/C) polymorphism is not associated with altered sRAGE serum levels in healthy pregnant controls or in patients with patological pregnancy (See Table 5.14 and 5.15).

Table 5.14 sRAGE serum levels according to *GLO1* Glu111Ala (419A/C) genotypes in healthy pregnant controls

Polymorphism	Genotype	N	sRAGE (pg/ml)	p
***GLO 1* Glu111Ala (419A/C)**	AA	38	645±237	
(rs4746)	AC	55	680±280	
	CC	27	730±255	0.412

Table 5.15 sRAGE serum levels according to *GLO 1* Glu111Ala genotypes in pathological pregnancy

Polymorphism	Genotype	N	sRAGE (pg/ml)	p
***GLO 1* Glu111Ala (419A/C)**	AA	50	927±824	
(rs4746)	AC	67	928±737	
	CC	47	808±412	0.605

6 Discussion

In this study, I have demonstrated changes of serum sRAGE levels during physiological pregnancy - lower levels in the 1st trimester, elevated levels in the 2nd trimester and decreased levels in the 3rd trimester and before labor. I have shown altered sRAGE serum levels in patients with symptoms of threatening preterm levels, especially the difference between sRAGE serum concentration between patients, who delivered prematurely immediately after enrollment into the study and patients, whose labor was postponed. I have confirmed increased sRAGE serum levels in patients with preeclampsia. I have not shown significant alteration in sRAGE serum levels in patients with intrauterine growth retardation and intrahepatic cholestasis in pregnancy.

Concerning *RAGE* polymorphisms (-429T/C, -374T/A, Gly82Ser (557G/A), 2184A/G) and *glyoxalase 1* polymorphism (Glu111Ala - 419A/C) I have not discovered any significant differences in allelic, genotype or haplotype frequencies among studied groups. However, the association of genotype 557GA of *RAGE* Gly82Ser (557G/A) polymorphism with a decreased serum sRAGE level was confirmed in accordance with previous studies.

6.1 sRAGE Analysis

6.1.1 sRAGE in Physiologic Pregnancy

Our study has shown significantly decreased serum sRAGE levels in healthy pregnant women in comparison to healthy non-pregnant controls. The mean of serum sRAGE levels in our non-pregnant population is higher than the mean serum sRAGE levels in other Czech healthy populations from other studies (Krechler T. et al., 2010, Kalousová M. et al. 2007). However, these means are still higher than the mean sRAGE serum level in physiological pregnancy and we have to consider the probable influence of gender, while the male sex is independently associated with lower sRAGE concentrations (Selvin E. et al.)

Pregnancy is a state when the women's body undergoes delicate changes in the metabolism and in immunological, hormonal and other systems. The placenta is the biggest contributor to elevated oxidative stress during pregnancy, while it contains many mitochondrias and is exposed to high oxygen partial pressure of maternal blood. Many studies have shown proof of increased

oxidative status in physiologic and pathologic pregnancy (Fialová L. et al., 2006, Arikan S. et al., 2001), as well as changes in the protective mechanisms including enzyme activity changes and plasma free radical traps (Wang Y., Walsh SW., 1996, Watson AL. et al., 1997, Kharb S., 2000). This study shows low sRAGE serum levels at the beginning of pregnancy in comparison to healthy non-pregnant women, which is likely a result of sRAGE consumption as a reaction to the increasing oxidative stress. The elevation of sRAGE concentration in the 2nd trimester might be explained by compensatory processes to accelerated oxidative stress (Toescu V. et al., 2002). In the 3rd trimester proinflammatory and inflammatory processes in myometrium, cervix, fetal membranes and placenta intensify (Norman JE. et al., 2007) and result in labor. In view of these facts, sRAGE production in the 3rd trimester is probably not sufficient and serum sRAGE levels are diminished by being used up. Nevertheless we have to count delicate changes in the mother's organism during pregnancy. All systems adapt to the needs of the developing embryo, fetus. Changes in the renal system (60% increase in glomerular filtration without changes in tubular re-absorption) have to be considered particularly as it is proven that sRAGE is present in the renal tubus and renal tubular lumen (Cheng C. et al., 2005) as well as sRAGE serum levels are significantly altered in patients with chronic kidney diseases (Kalousová M. et al., 2006).

The study (Germanová A. et al., 2010) presented for the first time the dynamics of sRAGE throughout physiological pregnancy. An earlier study by Pertynska-Marczewska (Pertynska-Marczewska M. et al., 2009) studied sRAGE serum levels in the 1st and 3rd trimester. A latter study of Kwon (Kwon JT et al., 2011), focused on levels of esRAGE during the whole normal pregnancy and showed continuously decreasing esRAGE maternal serum levels and decreasing esRAGE/sRAGE ratio throughout the pregnancy. The difference might be explained by a different population and the influence of a diverse RAGE polymorphism distribution in the population.

6.1.2 sRAGE in Pathological Pregnancy

The soluble receptor for advanced glycation end products and its association with preterm labor has already been studied. Inflammatory processes, which are nowadays considered as the main etiology factor of preterm labor, are clearly associated with sRAGE (Meijer B. et al., 2014, Bopp C. et al., 2008). The first studies pointed at the concentration of sRAGE in amniotic fluid. Buhimschi et al. (Buhimschi IA. et al., 2007) showed that sRAGE amniotic fluid levels rise with gestational

age, but they are not influenced by intraamniotic infection. On the other hand, Romero et al. (Romero R. et al., 2008) detected that sRAGE amniotic fluid levels are increased in patients with intraamniotic infection.

My study focused on sRAGE serum levels in patients with preterm labor, trying to identify a new efficient, and easily available biochemical marker of preterm labor, while up to date, there is no such marker of preterm labor. sRAGE was chosen because of its biochemical characteristics and its known association with inflammatory processes in non-pregnancy diseases (Bopp C. et al., 2008, Pullerits R. et al., 2005).

Our first results (Hájek Z. et al., 2008) showed that sRAGE serum levels are increased in patients with threatening preterm labor in comparison to healthy pregnant controls. However, the following study (Germanová A. et al., 2010) presented decreased sRAGE serum levels in patients with threatening preterm labor compared to healthy pregnant women. The difference is caused by the timing of sRAGE examination. The first study focused mainly on patients with the first symptoms of preterm labor, while the following one was aimed at patients with preterm labor in progress.

The sRAGE serum level decreases towards preterm delivery, it is lower in patients 24 hours prior to preterm labor than in patients with symptoms of threatening preterm labor who did not deliver within 24 hours. sRAGE in patients who delivered within 1 day after blood collection was significantly lower in comparison to healthy pregnant women in the 2nd trimester and similarly low as in patients in the 3rd trimester and in term labor. In physiological pregnancy, sRAGE is low in the 1st trimester and then rises in the 2nd trimester with a peak at about week 26 of pregnancy. After its level falls. The serum concentration of sRAGE immediately before preterm labor seems to be very similar to the serum concentration of sRAGE before term delivery. Preterm labor trigger factors activate similar processes that initiate term labor. Parts of both are inflammatory actions and oxidative stress (Lee SE. et al., 2008, Romero R. et al., 2006) and thus, sRAGE can be depleted towards to preterm as well as term labor. Similar results to ours showed a latter study by Bastek (Bastek JA. et al., 2012), which in accordance to our study described a significantly low serum sRAGE concentration in patients in preterm labor and moreover showed that decreased sRAGE cord blood levels were associated with a neonatal adverse outcome, especially with neonatal sepsis. The study assumed sRAGE serum concentration might be added to the clinical - biochemical model predicting preterm labor.

Focusing on inflammatory markers in women with preterm labor, our study verified a previously described negative correlation of sRAGE and leukocyte count (Kalousová M. et al., 2006), but it did not find any association with CRP.

Overall, sRAGE is significantly low in the women with preterm labor, however, inter-individual differences do not allow us to use it as the only biochemical marker of preterm labor. Nevertheless the monitoring of sRAGE dynamics in patients with symptoms of preterm labor might be useful for the recognition of real preterm labor risk.

The focus on the receptor for advanced glycation end products in preeclampsia is clearly understandable. The pathogenesis of preeclampsia is still not well understood, but its main symptoms, hypertension and proteinuria and their associations with RAGE have already been studied (Geroldi D. et al., 2005, Koyama H. et al., 2005). While sRAGE levels are decreased in patients with arterial hypertension (Geroldi D. et al., 2005, Koyama H et al., 2005), its concentration is very high in patients with proteinuria (Tan KC. et al., 2006) or chronic renal diseases (Kalousová M. et al., 2006). Several studies including ours described elevated maternal sRAGE levels in women with preeclampsia (Fasshauer M. et al., 2008, Germanová A. et al., 2010, Germanová A. et al., 2012, Oliver EA. et al., 2011, Kwon JT et al., 2011). As preeclampsia is a state defined by hypertension, we also would expect decreased sRAGE levels in preeclampsia. The fact that accelerated oxidative stress (Patill SB. et al., 2009, Siddiqui IA. et al., 2010) is typical for preeclampsia, would also support the anticipation of low sRAGE levels caused by sRAGE consumption. Nevertheless sRAGE maternal serum levels are significantly high in preeclampsia, so the role of the impaired renal function is likely to be relevant here. However, it is not clear whether this elevation is caused by the decreased elimination of sRAGE by the kidneys or up-regulation of sRAGE production. An increased esRAGE/sRAGE ratio in preeclampsia (Kwon JT. et al., 2011, Oliver EA. et al., 2011), indicates the importance of sRAGE up-regulation. Changes of the renin-angiotensin-aldosterone system (RAAS) in preeclampsia (Yang J. et al., 2013) might also contribute to the sRAGE serum levels' elevation. Angiotensin converting enzyme inhibitors increase sRAGE serum levels (Forbes JM et al., 2005). Contrary angiotensin II type I receptor antagonist telmisartan down-regulates RAGE and decreases the concentration of sRAGE (Nakamura K. et al., 2005). The obvious cross-link between RAGE axis and RAAS is currently not understood.

Our study has described the known correlation sRAGE with serum creatinine concentration (Kalousová M. et al., 2006, Fasshauer M. et al., 2008), which reflects the relation between kidney

functions and sRAGE serum levels. The correlation of sRAGE and proteinuria in preeclamptic patients could also correspond to the severity of renal impairment in preeclampsia. Similar results were described in patients with essential hypertension and albuminuria. In these patients, albuminuria was associated with elevated sRAGE serum levels (Dimitriadis K. et al., 2013).

The correlation of sRAGE with uric acid serum level displays the severity of preeclampsia. The role of uric acid in preeclampsia has been accepted for a long time. Hyperuricemia was understood only as a result of impaired kidney functions in preeclampsia. Its active participation in the pathogenesis of preeclampsia has lately been considered (Bainbridge SA., Roberts JM., 2008). During in vitro studies uric acid induces the production of platelet-derived growth factor, angiotensine II, thromoboxane and C-reactive protein (Kang DH. et al., 2005, Watanabe S. et al., 2002). It also stimulates the creation of proinflammatory cytokines (Webb R. et al., 2009). Uric acid has lately been classified as an alarmin - damage-associated molecular pattern molecule, diverse group of molecules, which can induce non-infectious inflammatory response.

Maternal serum sRAGE levels in patients with intrahepatic cholestasis have only been described by our study (Germanová A. et al., 2012). sRAGE serum levels do not differ in comparison to healthy pregnant controls. However, sRAGE negatively correlates with serum ALT levels. AST shows the same tendency to correlate with sRAGE, but this correlation is not significant ($p=0.06$). The elevation of bile acids is also typical for ICP (Geenes V., Williamson C., 2009). The secondary bile acid, deoxycholic acid, which is also elevated in patients with ICP, influences the production of one of the RAGE ligands, HMGB1 protein in epithelial cells (Fujii K. et al., 2009). Assuming that increased ALT and AST serum levels reflect increased levels of bile acids, a negative correlation of sRAGE with ALT is caused by the consumption of sRAGE. Moreover, several studies have confirmed bile acids promote oxidative stress (Bomzon A. et al., 1997).

Maternal serum sRAGE levels in women with IUGR do not differ from healthy pregnant women. Causes of intrauterine growth restriction are different and comprise fetal genetic abnormalities, placental dysfunction and maternal diseases like preeclampsia, chronic renal diseases and essential hypertension. It is believed that a common sign of IUGR is oxidative stress (Karowicz-Bilinska A. et al., 2007). The main source of oxidative stress during pregnancy is the placenta. Takagi et al. (Takagi Y. et al., 2004) showed that the concentration of oxidative damaged DNA is higher in placentas of IUGR and placentas of IUGR with preeclampsia. However, hypoxically regulated protein N-myc-downstream regulated gene 1 (NDRG1) is expressed more in placentas of

patients with preeclampsia than in placentas of IUGR (Choi SJ. et al., 2007). We could speculate that oxidative stress in IUGR is not so massive, so sRAGE levels are not affected. Moreover we have to consider that IUGR is more likely a symptom caused by a heterogeneous group of diseases, so sRAGE levels are difficult to evaluate in such small number of patients.

6.2 *RAGE* Polymorphisms Analysis

Considering all previous facts about the importance of RAGE and sRAGE in the pathogenesis of pregnancy associated diseases, the question about the significance of *RAGE* genetics and *RAGE* polymorphisms in these diseases arises. My work has focused on four *RAGE* polymorphisms and their association mainly with preterm labor. Groups of patients with hypertensive disorders, ICP and IUGR were not big enough to make a significant statistical conclusion. However, the glimpse of the connection of *RAGE* polymorphisms to these diseases was also done..

RAGE polymorphisms and their association with the susceptibility to chronic diseases have been studied intensively.

Functional *RAGE* -374 T/A and *RAGE* -429 T/C polymorphisms in the gene promoter are some of the most investigated *RAGE* polymorphisms. They are located in the gene promoter and they affect the transcriptional activity of the *RAGE* gene (Hudson BI. et al., 2001).

Studying inflammatory processes and -429T/C *RAGE* polymorphism, C allele is associated with a lower morbidity in patients with severe acute sepsis (Zeng L. et al., 2012). -429 T/C *RAGE* polymorphism is associated with the susceptibility of systemic lupus erytematodes and lupus nephritis (Martens HA. et al., 2012), however, it is not associated with Crohn's disease (Dabritz J. et al., 2011) or with multiple sclerosis (Tiszlavicz Z. et al., 2009). Generally, *RAGE* polymorphisms are linked to diabetes mellitus. The role of -429 T/C polymorphism in diabetes (Picheth G. et al., 2007) and its complications has already been described (Hudson BI. et al., 2001).

More studies until now have focused on the other *RAGE* gene promoter polymorphism -374 T/A. Several studies have affirmed the association of this polymorphism with chronic inflammatory diseases such as systemic lupus erytematodes (Martens HA. et al., 2012), Crohn's disease (Dabritz J. et al., 2011) and multiple sclerosis (Tiszlavitcz Z. et al., 2009). *RAGE* -374 T/A polymorphism and its relation to diabetes, diabetic complication is studied very often. Kawai et al. found that carriers of

the A allele have a higher risk of developing diabetes mellitus and, on top of that, male diabetic patients with essential hypertension have higher a risk of atherosclerosis and renal complication (Kawai T. et al., 2013). A study by Abdel-Azees showed similar results, which demonstrated, that diabetic patients with the A allele more often have diabetic nephropathy (Abdel-Azees HA., El-Okely AM., 2009). A finish study showed different results pointing to the AA genotype and its association with normal albumin urine excretion in diabetic patients (Pettersson-Fernholm K. et al., 2003). The meta-analytic study of Lu (Lu W., Feng B., 2010) showed the A allele of this polymorphism might be protective for vascular complication in the Caucasian diabetic population. Similarly, the A allele seems to be a protective factor for ischemic heart disease in the diabetic African-Brazilian population (dos Santos KG. et al., 2005). Also, non-diabetic patients with genotype AA of *RAGE* -374 T/A polymorphism had significantly less affected coronary arteries with severe atherosclerosis (Falcone C. et al., 2005).

RAGE Gly82Ser (557G/A) polymorphism in exon 3 is connected with amino-acid change at position 82 of the protein. Minor allele A evokes an exchange of glycine for serine. This exchange affects the N-glycosylation site, as well as it increases RAGE's ability to bind AGEs (Osawa M. et al., 2007). Several studies (Germanová A. et al., 2012, Gaens KH. et al., 2009), including ours, confirmed that decreased sRAGE levels are associated with the minor allele A also. The mechanism of this finding is currently not clear. However, we could speculate that the increased ability of RAGE to bind AGEs caused by the A allele, blocks the cleavage of cRAGE, component of sRAGE.

Again, focusing on inflammatory markers, genotype AA is associated with elevated CRP and TNF serum levels in non-obese, non-diabetic Korean subjects (Jang Y. et al., 2007). Also the A allele is more frequent in patients with rheumatoid arthritis (Hofmann MA. et al., 2002). In diabetic patients, the A allele is considered a risk factor for developing diabetic nephropathy (Prevost G. et al., 2005). Concerning cardiovascular diseases, the A allele is associated with ischemic stroke (Cui X. et al., 2013). Moreover, it is also associated with the development of Alzheimer disease (Daborg J. et al., 2010) and schizofrenia (Suchankova P. et al., 2012).

RAGE 2184 A/G polymorphism is located in intron 8. It is not clear how it influences RAGE synthesis. It is located near the splicing site, so it most likely influences alternative splicing of RAGE. Thus, it might affect sRAGE levels. The study by Kalousová et al. (Kalousová M. et al., 2007) supports this assumption. The study shows an elevated sRAGE serum concentration in hemodialyzed patients with genotype GG, however, we have to consider an altered renal function in

the study also. The allele G of this polymorphism is more often present in patients with systemic lupus erytematodes (Martens HA. et al., 2012). The GG genotype is associated with an elevated oxidative status (Kaňková K. et al., 2001). *RAGE* 2184 A/G polymorphism is associated with psoriasis and microvascular dermatoses in diabetic patients (Kaňková K. et al., 2001).

Event though there are many studies which approve the significance of *RAGE* polymorphisms in diabetes mellitus, inflammatory, cardiovascular and renal disease, the only study by Santos (Santos IC. et al., 2010) focused on gestational disease, precisely on gestational diabetes, but the study did not show any association between the studied promotor polymorphisms, *RAGE* -429 T/C and *RAGE* -374T/A, and gestational diabetes. My work (Germanová A. et al., 2012) studied four above mentioned polymorphisms in pregnancy associated diseases.

Infection and inflammatory processes are currently considered a main trigger of preterm labor. Despite the evidence that these *RAGE* polymorphisms are linked with acute or chronic inflammatory diseases, my work did not prove any association between any of the studied *RAGE* polymorphisms and preterm labor.

The connection of *RAGE* polymorphisms and hypertension is also known as the association of RAGE to proteinuria, but in our small group of patients with hypertensive disorders, we did not describe any linkage to *RAGE* polymorphisms. Another study with a larger number of subjects would be interesting to elucidate the meaning of *RAGE* polymorphisms in pathogenesis of preeclampsia, because the role of RAGE in preeclampsia is undeniable.

Our study also looked at *RAGE* polymorphisms in IUGR and did not find any association. The studied group was small, moreover, as mentioned above IUGR is often a symptom, whose etiology might be very diverse. A common sign of IUGR is oxidative stress, which is connected to *RAGE* polymorphisms (Kaňkova K. et al., 2001), so RAGE and its polymorphisms might play a partial role in the pathogenesis of IUGR.

The group of patients with ICP was even smaller. ICP is associated with oxidative stress, so RAGE might play a role in its pathogenesis, however, further study is needed to clarify this speculation.

6.3 *Glyoxalase 1* Polymorphism Analysis

The role of glyoxalase 1 in pathological pregnancy has not been discussed much, although advanced glycation products, substrates for the enzyme glyoxalase 1, have a place in the pathogenesis of pathological pregnancy. Women with gestational diabetes have elevated serum AGEs concentrations (Buongiorno AM. et al., 1997, Pertynska-Marczewska M., 2009). AGEs are also elevated in the serum and in placentas of patients with preeclampsia (Chekir C. et al., 2006). AGEs tissue levels remain elevated in preeclamptic women, even several months after delivery (Blaauw J. et al., 2006). Only the study by Sankaralingam (Sankaralingam S. et al, 2009) focused on glyoxalase 1 and its role in pathological pregnancy and showed that glyoxalase 1 is reduced in preeclampsia. Until now, besides my work, there is no other study concerning glyoxalase 1 polymorphisms and pregnancy complication.

My work has focused on the connection between *GLO1* Glu111Ala (419A/C) polymorphism and pregnancy complication. The polymorphism located in the 4^{th} exon causes amino acid change in the polypeptide chain (Glu111Ala). This change causes decreased activity of glyoxalase 1 (Barua M. et al., 2011) and an accumulation of AGEs precursors and their pathological effect. Xue et al (Xue M. et al., 2012) described the anti-oxidant response element (ARE) located in an un-translated region of exon 1. Transcriptional factor Nrf2 (nuclear factor-erythroid 2 p45 subunit-related factor 2) can activate the transcription of glyoxalase 1 through binding to ARE. Methylglyoxal, a precursor of AGEs, can activate Nrf2. On the contrary, HMGB1 binding to RAGE decreases the expression of glyoxalase 1 through Nrf2 (Thornalley PJ., 2009). Glyoxalase system, receptor for advanced glycation end products and its ligands, mainly advanced glycation end products are closely linked.

Our study did not find any connection between *GLO1* Glu111Ala (419A/C) and pregnancy complications. There was no difference in the genotype distribution between the healthy pregnant population and patients with preterm labor. There is no other study focusing on the role of advanced glycation end products or glyoxalase 1 in preterm labor. However, several studies have proved the role of the receptor for advanced glycation end products and its soluble forms in preterm delivery. Despite the evidence about the connection between the RAGE axis and the glyoxalase system, my work has not described the importance of *glyoxalase 1* Glu111Ala (419 A/C) polymorphism in preterm labor.

GLO1 419 A/C polymorphism might be associated with preeclampsia, while it was described that preeclampia is associated with elevated AGEs concentrations and decreased expression of glyoxalase 1 enzyme (Sankralingam S. et al, 2009). However, our study did not find an association between *GLO1* 419A/C polymorphism and preeclampsia, but our study group was too small for a significant statistic conclusion.

There are no studies focusing on the role of glyoxalase 1 or AGEs in IUGR or ICP. IUGR and ICP are associated with oxidative stress. The elevated activity of glyoxalase 1 decreases oxidative stress (Kim KM. et al., 2012). Our study did not find any connection between *GLO1* Glu111Ala (419A/C) polymorphism and IUGR or ICP, but both studied groups were too small, so another study to clarify these results is needed.

7 Conclusion

Receptor for advanced glycation end products, its soluble form and glyoxalase 1 take part in the pathogenesis of chronic diseases associated with altered oxidative status and microinflammation like diabetes mellitus, cardiovascular diseases, chronic renal diseases, autoimmune diseases, cancer. Oxidative stress and microinflammatory processes are as well characteristic for physiologic pregnancy and even more for pregnancy complications.

Serum sRAGE in physiologic pregnancy and pregnancy induced diseases

My research showed that serum sRAGE levels in healthy pregnant controls are significantly decreased in comparison to healthy non-pregnant subjects. However serum sRAGE levels dynamically change during physiological pregnancy. Serum sRAGE levels steeply decrease in the 1st trimester of physiologic pregnancy and continue with elevation in the 2nd trimester and a slow decrease in the 3rd trimester and before labor.

Concerning pathological pregnancy serum sRAGE levels are decreased during the preterm labor in comparison to threatening preterm labor and in comparison to healthy pregnant controls. sRAGE correlates with leukocyte count in preterm labor. Serum sRAGE concentrations are elevated in patients with preeclampsia. sRAGE positively correlates with proteinuria, serum uric acid and serum creatinine. Serum sRAGE concentrations in IUGR and ICP are not affected, but sRAGE correlates negatively with serum ALT levels in ICP and it correlates positively with serum uric acid and serum creatinine in IUGR.

Significance of RAGE and GLO1 polymorphisms in pregnant women

Four *RAGE* (*RAGE* -429T/C, *RAGE* -374 T/A, *RAGE* Gly82Ser (557G/A), *RAGE* 2184A/G) polymorphisms and one *Glyoxalase 1* polymorphism (*GLO1* Glu111Ala (419A/C)) were analyzed in this study. Despite the significant changes in sRAGE levels in pregnancy complications and association of studied polymorphisms with the susceptibility to chronic diseases, my work did not find any genotype or allelic distribution differences between women with pregnancy induced

pathologies and women with physiological pregnancy. Similarly my work did not discover any connection of *GLO1* Glu111Ala (419A/C) polymorphism with pregnancy complications.

The results of my work could bring better insight to the pregnancy and its complications. Better understanding of physiologic pregnancy and pregnancy induced diseases could improve screening of pregnancy associated diseases and prenatal care.

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LIST OF ORIGINAL ARTICLES

1. Publications related to the thesis

a) with IF

1. ***Germanová A.,*** Muravská A., Jáchymová M., Hájek Z., Koucký M., Mestek O., Zima T., Kalousová M.: Receptor for advanced glycation end products (RAGE) and glyoxalase I gene polymorphisms in pathological pregnancy. Clin Bioch., 2012; 45(16-17): 1409-1414.

IF = 2.076

2. ***Germanová A.,*** Koucký M., Hájek Z., Pařízek A., Zima T., Kalousová M.: Soluble receptor for advanced glycation end producats in physiological and pathological pregnancy. Clin. Biochem., 2010; 43(4-5): 442-446.

IF=2.019

3. Hájek Z., ***Germanová A.***, Koucký M., Zima T., Kopecký P., Vítková M., Pařízek A., Kalousová M.: Detection of feto-maternal infection/inflammation by the soluble receptor for advanced glycation end products (sRAGE): results of pilot study. J. Perinat. Med., 2008; 36(5): 399-404.

IF=1.101

2. Other publications:

a) with IF

1. Koucký M., Malíčková K., Cindrová-Davies T., ***Germanová A***., Pařízek A., Kalousová M., Hájek Z., Zima T.: Low levels of circulating T-regulatory lymphocytes and short cervical length are associated with preterm labor. J Reprod Immunol., 2014; Epub ahead of print.

IF=2.342

2. Muravská A., ***Germanová A.***, Jáchymová M., Hájek Z., Švarcová J., Zima T., Kalousová M.: Association of Pregnancy-associated plasma protein A polymorphism with preeclampsia – A pilot study. Clin Biochem., 2011; 44(17-18): 1380 - 1384.

IF=2.043

3. Germanová A., Jáchymová M., ***Germanová A***., Koucký M., Hájek Z., Zima T., Kalousová M.: Pregnancy associated plasma protein-A polymorphisms in patients with risk pregnancies. Folia Biologica, 2011; 57(2): 82 - 85.

IF=0.924

4. Koucký M., ***Germanová A.***, Kalousová M., Hill M., Cindrová-Davies T., Pařízek A., Švarcová J., Zima T., Hájek Z.: Low maternal serum matrix metalloproteinase (MMP)-2 concentrations are associated with preterm labor responce. J. Perinat. Med., 2010; 38(6): 589 - 596.

IF=1.736

5. Hill M., Pařízek A., Jirásek JE., Jirovská M., Velíková M., Dusková M., Klímková M., Pašková A., Žižka Z., ***Germanová A.***, Kalousová M., Stárka L.: Is maternal progesterone actually independent of the fetal steroids? Physiol. Res., 2010; 59 (2): 211 - 224.

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6. Germanová A., ***Germanová A.***, Tesařová P., Jáchymová M., Zima T., Kalousová M.: Glyoxalase 1 Glu111Ala polymorphism in patients with breast cancer. Cancer Invest., 2009; 27(6): 655 - 660.

IF=2.160

7. Kalousová M., Brabcová I., ***Germanová A.***, Jáchymová M., Matl I., Mestek O., Bandúr S., Zima T., Viklický O**.:** RAGE polymorphisms, renal function and histological finding at 12 months after renal transplantation. Clin. Biochem., 2009; 42 (4-5): 347 - 352.

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8. Hrazdírová L., Švabík K., Žižka Z., ***Germanová A.***, Kužel D.: Should hysteroscopy be provided for patients who have undergone instrumental intrauterine intervention after delivery? Acta Obstet Gynecol Scand., 2012; 91(4): 514 - 517.

IF=1.771

b) without IF

1. Koucký M., **Germanová A**., Hájek Z., Pařízek A., Zima T., Kalousová M., Kopecký P.: Prenatal and perinatal management of preterm labour. Prague Med Rep. , 2009; 110(4), 269 - 277.

2. Koucký M., **Germanová A**., Hájek Z., Pařízek A., Zima T., Kalousová M., Kopecký P.: Pathophysiology of preterm labour. Prague Med Rep., 2009; 110(1), 13 - 24.

SUPPLEMENTS

Supplement 1:

Germanová A., Muravská A., Jáchymová M., Hájek Z., Koucký M., Mestek O., Zima T., Kalousová M.: Receptor for advanced glycation end products (RAGE) and glyoxalase I gene polymorphisms in pathological pregnancy. Clin Bioch., 2012; 45(16-17): 1409-1414.

Supplement 2:

Germanová A., Koucký M., Hájek Z., Pařízek A., Zima T., Kalousová M.: Soluble receptor for advanced glycation end producats in physiological and pathological pregnancy. Clin. Biochem., 2010; 43(4-5): 442-446.

Supplement 3:

Hájek Z., Germanová A., Koucký M., Zima T., Kopecký P., Vítková M., Pařízek A., Kalousová M.: Detection of feto-maternal infection/inflammation by the soluble receptor for advanced glycation end products (sRAGE): results of pilot study. J. Perinat. Med., 2008; 36(5): 399-404.