Ph.D. theses summary

Pathogenesis of the chronic complications of type 2 diabetes mellitus, chronic kidney disease and ageing. The role of oxidative stress, endothelial dysfunction and the renin-angiotensin system.

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ABBREVIATIONS

ACE, angiotensin converting enzyme
ADMA, asymmetric dimethylarginine
AGE, advanced glycation end products
ATP, adenosine triphosphate
CBB, Coomassie brillant blue
CKD, chronic kidney disease
CONTR, group of control subjects
DM, diabetes mellitus
DM CAT, group of cataract patients with type 2 diabetes mellitus
DOPA, 3-,4-dihydroxy-phenylalanine
EDTA, ethylenediamine-tetraacetic acid
eNOS, endothelial nitric oxide synthase
Fe^{2+}, ferrous iron, Fe(II)
Fe^{3+}, ferric iron, Fe(III)
$Fe(NH_4)(SO_4)_2$, ferri-ammonium-sulfate
Fex, fractional excretion
γ-GT, γ-glutamyl transferase
GPX, glutathione peroxidase
GSH, reduced glutathione
H_2O_2, hydrogen peroxide
Hb A_{1c}, hemoglobin A_{1c}
HPLC, high performance liquid chromatography
L-NMMA, L-N-monomethyl-arginine
m-Tyr, meta-tyrosine
non-DM CAT, group of cataract patients without diabetes mellitus
$\cdot O_2^-$, superoxide free radical
·OH, hydroxyl free radical
o-Tyr, ortho-tyrosine
p-Tyr, para-tyrosine
PAGE, polyacrylamide gel-electrophoresis
PCR, polymerase chain reaction
Phe, phenylalanine
RAGE, receptor of AGEs
SD, standard deviation
SDS, sodium dodecylsulphate
SDMA, symmetric dimethylarginine
SOD, superoxide dismutase

1. INTRODUCTION

Pathogenesis of the chronic complications of type 2 diabetes mellitus (DM), ageing and chronic kidney disease (CKD) is multifactorial; it may involve environmental factors, inflammation, an unfavorable genetic background, metabolic disturbances, free radical-processes etc. Some of these factors will be discussed below.

1.1. Oxidative stress

Oxidative stress is defined by the imbalance of free radicals and antioxidants. This can be the consequence of an increased radical production and/or a decreased antioxidant capacity. Oxidative stress has been implied for a long time as a key process in the development of complications of diabetes mellitus and chronic renal disease. The imbalance between free radicals/reactive oxygen species (superoxide anion [$\cdot O_2^-$], hydrogen peroxide [H_2O_2], hydroxyl free radical [$\cdot OH$] etc.) and antioxidant systems gives rise to free radical-mediated damage. Metal-catalyzed oxidation reactions – like the Fenton reaction, where hydrogen peroxide is cleaved to $\cdot OH$ and hydroxyl anion – play an important role in the generation of oxidative stress.

In diabetes mellitus (DM) high glucose concentration evokes oxidative stress among others due to the damaged intracellular metabolism, the poliol-pathway and non-enzymatic glycation reactions. In non-diabetic subjects, free radicals formed during ageing processes may also contribute to age-related protein damage.

In renal disease, there is an increased rate of oxidative stress processes as a consequence of multiple causes. The inflammatory processes in the background of the kidney disease may contribute to oxidative stress. There is also an activation of the renin-angiotensin system involving a high activity of the angiotensin-converting enzyme (ACE). The decrease of glomerular filtration leads to the accumulation of small molecular weight substances such

as advanced glycation endproducts (AGEs), pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α. The AGE-receptor of AGE (RAGE) interaction and cytokines activate inflammatory cells; that leads to respiratory burst, release of free radical species, chemoattractants. Hemodialysis also contributes to oxidative stress.

1.1.1. The role of iron

The classical Fenton-reaction has been described as a cleavage reaction of H_2O_2 catalyzed by the redox cycling of ferrous iron. To avoid Fenton-like reactions, iron is usually stored in a form of iron-protein complexes, such as transferrin or ferritin. The non-protein-bound forms of iron may also be complexed by other substances as citrate, organic phosphates. These chelators may either enhance or suppress redox-activity of iron.

1.1.2. Antioxidant enzymes

The cytosolic Cu/Zn-superoxide dismutase (SOD) and the mitochondrial Mn-SOD transform $\cdot O_2^-$ to H_2O_2. The catalase enzyme is responsible for the detoxification of the ROS hydrogen peroxide to biologically inactive water molecules. One of the peroxidases is the glutathione peroxidase (GPX), a 80 kDa selenium-containing enzyme, which uses reduced glutathione (GSH) for the reduction H_2O_2. The gamma-glutamyl transferase enzyme (γ-GT) helps the utilization of extracellular GSH by degradation of extracellular GSH and transport of the produced free amino acids that are then used for intracellular GSH re-synthesis.

1.1.3. Conversion-reactions of phenylalanine

Aromatic side chain amino acids are susceptible to oxidative stress, and their products are stable. The essential amino acid phenylalanine (Phe) is converted to the physiologic, semi-essential para-tyrosine (p-Tyr) by the phenylalanine hydroxylase enzyme. No other isoform of tyrosine is formed in this enzymatic reaction. In the presence of hydroxyl free radical, Phe can be hydroxylated in para, meta and ortho positions. Thus, para-, meta- and ortho-tyrosine (p-,

m-, o-Tyr) are all formed in this free radical-reaction. In a further enzymatic or hydroxyl free radical-driven reaction 3,4-dihydroxi-phenylalanine (DOPA) is formed from p-Tyr. However, DOPA may be degraded to DOPA-quinones due to further free radical-caused damage.

1.1.4. The effects of the L-arginine analogues

The methylated L-arginine analogues L-N-monomethylarginine (L-NMMA) and asymmetrical dimethylarginine (ADMA) are competitive inhibitors of the endothelial nitric oxide (eNOS) enzyme, and may lead to an increased adhesion of inflammatory cells to the endothelium, an increased platelet aggregation and activation, i.e. to endothelial dysfunction.

2. AIMS

2.1. *In vitro* experiments on Phe-Tyr conversion[I,II]

- Aims were to analyze the kinetics of the *in vitro* Phe-Tyr conversion in a hydroxyl free radical producing system containing ferric iron and hydrogen peroxide;
- to measure the role of the redox cycle of iron in the conversion reaction;
- to measure the amount of the Tyr (p-, m- and o-Tyr) isomers produced in the *in vitro* Phe-Tyr conversion reaction;
- to detect the effect of iron chelators and pH on the production of p-, m- and o-Tyr.

2.2. Clinical study of patients with type 2 diabetes mellitus and/or chronic kidney disease on the urinary and plasma levels and renal excretion of p- and o-Tyr[III]

- Aims were to measure p-, m-, o-Tyr and Phe concentrations both in urine and in plasma samples of controls, of patients with type 2 diabetes mellitus or chronic kidney disease or a combination of both;
- to investigate the correlation between the levels of urinary 8-epi-prostaglandin-$F_{2\alpha}$, a marker of lipid peroxidation, with the urinary level of the hydroxyl free radical marker, o-Tyr;
- to examine the renal handling of the physiologically produced and the hydroxyl radical-derived Tyr isomers.

2.3. Clinical study of cataract patients with and without type 2 diabetes mellitus[IV]

- Aims were to compare the composition of proteins of non-cataractous lenses, cataract lenses of patients with and without type 2 diabetes mellitus;
- to compare protein content of the soluble fraction of the lens and the total lens homogenate in cataractous and non-cataractous lenses;
- to compare protein composition of the soluble fractions and the total lens homogenates;
- to measure markers of the oxidative post-translational modification of Phe residues (m-, o-Tyr and DOPA) in lens proteins of the soluble fraction and the total lens homogenates;
- to measure Phe content of the soluble fraction and the total lens homogenates.

2.4. Clinical study of the connection between ACE gene polymorphism and clinical parameters of patients with type 2 diabetes mellitus[V,VII,VIII]

- Aims were to investigate of a possible connection between the ACE gene I/D polymorphism and carbohydrate metabolism, target organ damage and other clinical parameters;
- to examine if the use of ACE inhibitory drugs is in connection with the glycemic state of the type 2 diabetic patients.

2.5. Clinical study on glutathione peroxidase enzyme gene-polymorphism and L-arginine analogues[VI]

- Aim of the study was to compare serum levels of L-NMMA, L-ADMA and L-SDMA in groups of type 2 diabetic patients with different glutathione peroxidase genotypes.

3. METHODS

3.1. *In vitro* experiments on Phe-Tyr conversion in a Fenton-like metal-catalyzed oxidation reaction

Spectrophotometric method: To detect *in vitro* Tyr production, fluorescent spectrophotometry in a Hitachi F-4500 spectrophotometer (Hitachi Inc, Tokyo, Japan) was used, excitation wavelength was set to 275 nm, the emission wavelength was set to 305 nm, and the slit was 10 nm for both excitation and emission. Materials and their concentrations were as follows: Phe, 100 μmol/l and 1 mmol/l; desferrioxamine, 2 mmol/l; H_2O_2 1 mmol/l; ferric-ammonium-sulfate $[Fe(NH_4)(SO_4)_2]$ 25, 50, and 100 μmol/l; ethylene-diamine-

tetraacetic acid (EDTA), 1 mmol/l; citric acid, 1 mmol/l. $Fe(NH_4)(SO_4)_2$ was used as the source of ferric iron, therefore it will be referred to as ferric iron below. We also planned to measure the effect of ATP chelation on the reaction, but the fluorescence of ATP interferred with the spectrometric measurement. To investigate whether superoxide free radical is produced in the reaction, and whether it plays a role in the Phe-Tyr conversion, superoxide dismutase (SOD, 0.8 to 100 U/ml) was used. Also heat-inactivated (95 °C, 50 minutes, 100 U/ml) SOD was tested

HPLC method: HPLC measurements were carried out from the abovementioned experimental sets. The analysis was performed using a Shimadzu Class LC-10 AD_{VP} HPLC system equipped with an RF-10 A_{XL} fluorescent detector. The analysis was performed using a Licrospher C-18 ODS column, in an isocratic run using an aqueous solution of 1 % acetic acid and 1 % sodium-acetate as the mobile phase. Fluorescence wavelengths were as above.

3.2. Clinical study of patients with type 2 diabetes mellitus and/or chronic renal failure on the plasma and urinary p- and o-Tyr levels and renal excretion[III]

All listed clinical studies listed below were approved by the Ethical Board of the Medical Faculty of the University of Pécs. In a cross-sectional clinical study four groups of patients were investigated: *(1)* a group of non-diabetic control subjects without chronic kidney disease (CONTR, n = 14), *(2)* a group of patients with Stage III chronic kidney disease (CKD, n = 12), *(3)* type 2 diabetic patients (DIAB, n = 17), and *(4)* patients with type 2 diabetes and Stage III. chronic kidney disease (DIAB-CKD, n = 19). The groups did not show any significant difference as regards their age or gender. There was no significant difference between the CKD and the DIAB-CKD patients regarding the severity of their renal impairment. The DIAB and the DIAB-CKD groups did not differ in their fructosamine and hemoglobin A_{1c} levels. There was no significant difference between the groups in the parameters of liver function and serum lipids. Twenty-four hour collected urine and

heparinized fasting plasma samples were obtained, and routine clinical parameters were also measured. Trichloroacetic acid-deproteinized 24 hour collected urine or freshly obtained fasting heparinized plasma samples were used for the analysis on the HPLC device. The run was performed as described earlier. Urinary 8-epi-prostaglandin-$F_{2\alpha}$ levels were determined by a competitive ELISA assay kit (Oxis).

3.3. Clinical study of cataract patients with and without type 2 diabetes mellitus[IV]

In a cross-sectional study we examined human lenses that were removed during extracapsular cataract surgery. Cadaver eyes enucleated for corneal transplantation served as controls. Three groups were formed, *(1)* a group of non-diabetic non-cataractous controls (CONTR), *(2)* patients with cataract and type 2 diabetes mellitus (DM CAT), and *(3)* a group of patients with cataract without diabetes (non-DM CAT). There was no difference between the age of the CONTR and the DM CAT groups; however, patients in the non-DM CAT group were significantly older than both the CONTR and the DM CAT groups.

All cataractous lenses were removed under the same circumstances by the same surgeon using a phacoemulsification technique. The water-soluble and the water-insoluble components of the lenses were separated from each other by centrifugation (13,000 rpm, 30 min). Soluble protein fractions were gained as the supernatant of equal amounts of the homogenates of each sample. The same samples were used for the investigation of protein distribution, for protein electrophoresis and high performance liquid chromatographic analysis. Sample protein content of total homogenates and supernatants was measured according to Bradford. Separation of proteins was performed by sodium-dodecylsuphate polyacrylamide gel-electrophoresis (SDS-PAGE) according to Laemmli in a 12.5 % gel in a Mini Protean 3 Cell. Low molecular weight proteins were used as markers. Detection of protein fractions was performed by silver post-intensification according to Willoughby

following the traditional Coomassie brilliant blue (CBB) R-250 staining. For the HPLC-based amino acid analysis, the samples were hydrolyzed in the presence of desferrioxamine and butylated hydroxytoluene overnight, at 120 °C using 12 N hydrochloric acid. The hydrolyzate was filtered and injected onto the HPLC column, the run was carried out as earlier. The amino acid concentrations were corrected for protein content or Phe concentrations.

3.4. Clinical study on connections between the angiotensin-converting enzyme gene polymorphism and clinical parameters of patients with type 2 diabetes mellitus[V]

In this clinical study, n = 145 patients with type 2 diabetes mellitus were investigated. ACE gene polymorphism was determined using a sensitized touchdown polymerase chain reaction (PCR) method from dezoxyribonucleotic acid isolated by salting out from peripheral blood. Plasma glucose, HbA_{1c}, serum fructosamine, total cholesterol, triglyceride, HDL and LDL cholesterol, γ-GT activity, creatinine, blood urea nitrogen, serum bilirubin and albuminuria were determined using standard laboratory methods.

3.5. Clinical study on the plasma levels of L-arginine analogues in patients with type 2 diabetes mellitus and different glutathione peroxidase genotypes[VI]

The studied DNA sequence was first amplified using PCR, then a single nucleotide polymorphism detector device (ABI PRISM SNaPshot Multiplex, Applied Biosystem) was used for genotyping. Plasma samples were de-proteinized using sulphosalicylic acid, amino acids were derivatized to fluorescent substances using ortho-phtalaldehyde and mercaptoethanol. Addition of orthophosphoric acid was added to stop the reaction. The analysis was carried out on a Yamamura C_{18} ODS column of the Shimadzu HPLC device in a gradient run. The eluents were 10 mmol/l potassium-phosphate buffer + 10 % acetonitryl and a 40 % acetonitryl + 30 % methanol solution. The amino acids were detected at 350 nm excitation and 450 nm emission wavelengths.

3.6. Statistical analysis

In the case of all studies, distribution of the data was tested. Normally distributed data were characterized by mean +/- SD, and parametric tests (ANOVA, Student's t-test, Pearson correlation) were used. Non-normally distributed data were characterized by median +/- interquartile rage, and non-parametric tests (Kruskal-Wallis, Mann-Whitney u-test, non-parametric correlations) were used. Statistical tests were carried out using SPSS.

4. RESULTS AND DISCUSSION

4.1. *In vitro* model of phenylalanine-tyrosine conversion in a metal-catalyzed oxidation reaction (Publications I-II)

The spectrofluorimetric method was adequate to detect Tyr production in the *in vitro* system. Addition of H_2O_2 to Phe results in Tyr production; however the increase of fluorescence in the absence of iron was not significant. Increasing concentrations of ferric iron lead to a concentration-dependent increase of Tyr production, which increase could be totally inhibited by pre-incubation with the iron redox-cycle inhibitor, desferrioxamine. This fact indicates that the redox-cycling of iron is needed for Tyr production.

The effect of iron chelators and pH: EDTA caused a slow, time-dependent increase in Tyr fluorescence. In the presence of citrate, a faster increase in the initial phase and a slower increase in the second phase could be observed. Citrate led to Tyr production in the absence of H_2O_2, as well, which is a consequence of the autoxidation of citrate.

The citrate and EDTA experiments were repeated using a Sørensen phosphate buffer (pH 7.4) as vehicle. In the presence of the phosphate buffer, lower Tyr production could be measured in the Phe-Fe-EDTA-H_2O_2 and Phe-Fe-citrate-H_2O_2 systems, as well as in the Phe-Fe-citrate system.

The effect of SOD: Addition of SOD decreases Tyr production in a concentration-dependent manner. Fifty minute heating (at 95 °C) completely abolishes the effect of SOD, and Tyr production is similar to the system containing no SOD. Thus, the reaction series involves the presence of $\cdot O_2^-$, as well.

4.2. *In vitro* production of para-, meta- and ortho-tyrosine as a consequence of hydroxyl free radical attack. HPLC studies. (Publications I-II)

In the presence of the hydroxyl free-radical producing system, well detectable amounts of p-, m- and o-Tyr were produced. Production of m-Tyr was highest, while that of p-Tyr was the lowest. The amount of the Tyr isomers produced in this reaction was in the range between 300 and 500 nmol/l. The addition of desferrioxamine significantly prevented p-, m- and o-Tyr production. There was no significant change of the concentration of Phe. The observation that Phe levels were not changed significantly is probably a consequence of the fact that the conversion rate of Phe to Tyr is only approx. 1:10,000, i.e. there is an abundance of Phe.

The effect of iron chelators and pH: In the absence of H_2O_2, only the Fe-citrate complex lead to marked Tyr production and Phe consumption compared to the control state. In the reaction mixtures containing H_2O_2, ATP decreased, while EDTA and citrate increased p-, m- and o-Tyr production. The results are similar to the fluorimetric data, except for the lack of significance in the case of Phe+Fe+EDTA+H_2O_2. Upon data of the literature, this is the consequence of an interference of EDTA with the fluorometric detection of Tyr. This hypothesis is confirmed by a low initial Tyr-fluorescence in the EDTA samples.

Presence of the phosphate buffer lead to lower p-Tyr production in the Phe-Fe-EDTA-H_2O_2, in the Phe-Fe-citrate-H_2O_2 and in the Phe-Fe-citrate systems. If there is an abundance of H^+ ions, OH^- ions will be captured and turned to H_2O, therefore the Fenton reaction is shifted even more to the right, and more $\cdot OH$ radicals will be formed. This can be inhibited by the phosphate buffer. In metabolic disturbances e.g. in DM, accumulation of citrate, low

levels of ATP and a shift towards acidosis could contribute to ferric iron-derived oxidative damage. Citrate is also of interest, as it can be induced by hyperglycemia, furthermore accumulation of citrate-iron complexes has been shown to develop in hemochromatosis patients. Upon our results, and data of the literature, the hypothesized reaction series is as follows:

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + \cdot O_2^- + 2\,H^+ \quad (1)$$

$$Fe^{3+} + \cdot O_2^- \leftrightarrows Fe^{2+} + O_2 \quad (2)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH \quad (3)$$

The ·OH produced in reaction (3) is able to chemically modify Phe:

$$3\,\cdot OH + 3\,Phe \rightarrow p\text{-}Tyr + m\text{-}Tyr + o\text{-}Tyr \qquad \text{(not 1:1:1 stochiometrically)} \quad (4)$$

4.3. Results of the high performance liquid chromatographic detection of para- and ortho-Tyr in urine and plasma samples, and of the measurement of urinary 8-epi-prostaglandine F_{2a} levels (Publication III.)

Para-tyrosine: We found that the CKD group had a significantly lower plasma level of p-Tyr than the CONTR and the DIAB groups. Also, the DIAB-CKD group had a lower plasma p-Tyr level than the DIAB group. We obtained similar results when correcting data to the plasma Phe levels. Phe levels did not differ among the patient groups.

Urinary levels of p-Tyr were corrected for urinary concentrations of creatinine. Urinary concentrations of creatinine did not differ in the groups. The CONTR group had a higher urinary p-Tyr/creatinine ratio than the CKD group. Also, the DIAB group had a higher p-Tyr/creatinine value than the CKD or the DIAB-CKD groups. When daily p-Tyr excretion was calculated, we found that the CKD and the DIAB-CKD groups had a lower p-Tyr excretion than the DIAB group. Plasma levels of p-Tyr correlated with urinary levels of p-Tyr.

The CKD and the DIAB-CKD group had a lower p-Tyr clearance than the DIAB group. We found no difference comparing the patient groups with the CONTR group. To

check the effect of the renal impairment on the handling of p-Tyr, fractional excretion (Fex) was calculated. There was no difference between the groups with regard to the Fex of p-Tyr.

Latest data of the literature imply that renal impairment causes a decreased renal phenylalanine-hydroxylase enzyme activity. In our case, the p-Tyr Fex values were all below 100 % (range for all groups: 0.24 - 20.67 %), indicating that p-Tyr is effectively retained by the kidney. A decreased synthesis and not an increased loss of p-Tyr is indicated by the fact that patients with CKD had decreased plasma p-Tyr levels with a Fex similar to the CONTR group and low urinary p-Tyr levels.

Ortho-tyrosine: The plasma o-Tyr level of the CKD and the DIAB-CKD groups tended to be higher than in the CONTR group, but the observed difference was not significant. We obtained similar results when correcting serum o-Tyr data to the serum Phe levels. The urinary o-Tyr/creatinine ratio was higher in the three groups of patients than in the CONTR group, and higher in the DIAB-CKD group than in the DIAB group. The three groups of patients had significantly higher daily o-Tyr excretion than the CONTR group. The daily o-Tyr excretion of the two diabetic groups was also higher than in that of the CKD group. The correlation between plasma levels of o-Tyr and urinary levels of o-Tyr for all groups was not significant. As a summary, we found an additive effect of diabetes and kidney failure on the renal excretion of the hydroxyl radical marker o-Tyr.

There was no significant difference in the o-Tyr clearance among the groups. However, we found that the DIAB and the DIAB-CKD groups had a significantly higher Fex of o-Tyr than the CONTR group. The median Fex of o-Tyr exceeded 100 % in both diabetic groups. Such a Fex value of a substance could be a consequence of two processes, i.e. active tubular secretion of the substance, or production of the substance *in loco* in the kidney. Upon our data, we cannot distinguish which process is likely to be superior. We could speculate that

glucose-derived oxidative stress on tubular cells may increase the production and in this way Fex of o-Tyr in the DIAB and the DIAB-CKD groups.

The Fex of o-Tyr was significantly higher than the Fex of p-Tyr in the control group, this suggests that p-Tyr is more efficiently (approx. ten times) retained by the kidney than o-Tyr, even in the control group. There is a different renal handling of p- and o-Tyr even though the only difference between the two amino acids is the location of the hydroxyl group. This might be an adaptive process, as the physiological Tyr isoform is retained, while the pathological isoform is excreted.

8-epi-prostaglandin-$F_{2\alpha}$: Urinary 8-epi-prostaglandin-$F_{2\alpha}$/creatinine ratio did not correlate with plasma o-Tyr, plasma o-Tyr/Phe ratio, urinary o-Tyr/creatinine ratio, urinary o-Tyr excretion, o-Tyr clearance nor with Fex of o-Tyr. The lack of correlation probably indicates a different origin of 8-epi-prostaglandin-$F_{2\alpha}$ and o-Tyr.

4.4. Results of the study on human cataract lenses (Publication IV.)

Protein distribution: We found that the protein distribution between the soluble and non-soluble phases is different, i.e. the ratio of soluble proteins is lower in cataractous lenses compared to the CONTR group. There was no significant difference between the DM CAT and non-DM CAT groups, both had equally low soluble protein content, corresponding to the fact that the development of cataract involves insolubility of the proteins.

ELFO analysis: In each sample, there were several visible bands in the 20-30 kDa area (where the lens crystallins can be found), but the pattern and the intensity of the bands was different, as in the total homogenates of cataractous lenses two additional bands also become quantitatively dominant. These bands are either not present in the supernatants and the control sample, or they show less intensity. This result shows that there is a difference in the low molecular weight proteins between cataractous and non-cataractous lenses. Moreover,

it is worth mentioning that in case of all total homogenates we observed staining, which indicates the presence of high molecular weight aggregates. Such aggregates are thought to be partially responsible for the decreased transparency of the lens, and their production indicates insolubility of the lens proteins, as confirmed by our protein solubility measurements, too.

HPLC analysis: The concentration of total lens homogenates was set to 1 mg/ml using the homogenizing buffer, while in the lens supernatants of the three groups there were higher protein concentrations (medians: 1.45 - 2.50 mg/ml). Amino acid concentrations of the solutes were thus corrected for sample protein content.

In our experimental system, the amount of p-Tyr was not measured, as its concentration exceeds that of DOPA, o-Tyr and m-Tyr by approx. three orders of magnitude. The amount of m-Tyr was below detection limit in a majority of supernatant samples. By comparing the groups in this regard, we found that detectability of m-Tyr was higher in the supernatants of the non-DM CAT (18/22 cases, detectable/all cases) group than in the CONTR (7/17 cases) and the DM CAT (10/20 cases) groups (chi^2-test $p<0.05$). In the total homogenates we found that detectability of m-Tyr was higher in the DM CAT (20/20 cases) and non-DM CAT groups (22/22 cases) than in the CONTR (11/17 cases) group. In case of the total homogenates, chi^2-test could not be performed for m-Tyr detectability and the patient groups because of mathematical reasons (expected number of cases per cell was too low). In the case of m-Tyr, statistics could only be calculated for those samples, where m-Tyr was detectable.

Inter-group comparisons: Concentration of the amino acids was expressed as their ratio to sample protein content, i.e. DOPA/protein, m-Tyr/protein, o-Tyr/protein and Phe/protein ratios were calculated. In the supernatants, DOPA and o-Tyr could be well detected; however in a majority of the supernatant samples, m-Tyr was below the detection limit. We have found in our abovementioned study that m- and o-Tyr are produced nearly

equimolarly *in vitro*, but it is reported in the literature that the concentration of o-Tyr *in vivo*, in different biological samples can exceed that of m-Tyr by up to ten times, such as seen in our samples.

When comparing the three groups, we found that there was no difference between DOPA/protein, m-Tyr/protein and o-Tyr/protein ratios of the *supernatants* of the three groups. This result suggests that the soluble proteins – that can be found in the lens supernatants – contain equal amounts of the oxidized amino acids.

The Phe/protein ratio in the *supernatants* of the DM CAT samples was not significantly lower than that of the CONTR samples. However, in the supernatants of the non-DM CAT group we found a significantly lower Phe/protein ratio than in the CONTR group.

In the lens *total homogenates*, DOPA and o-Tyr could be measured, as well. In the majority of total homogenates of the CONTR samples, m-Tyr was below the detection limit, while it could be detected well in the cataractous samples. There was no difference between DOPA/protein ratios of the total homogenates of the three groups. However, m-Tyr/protein and o-Tyr/protein of both DM CAT and non-DM CAT groups was higher than that of CONTR samples.

In the *total homogenates*, there was no difference in the Phe content of among the groups. The difference between Phe content of proteins of the supernatant and the total homogenate was calculated for all groups by dividing Phe/protein of the supernatant with Phe/protein of the total homogenate: the median ratio of the CONTR group was 67%, and there was a lower ratio in the DM CAT and non-DM CAT groups (26% and 30%, respectively, $p<0.05$ vs. CONTR, for both).

Intra-group comparisons of supernatants and total homogenates: We found that the total homogenates and supernatants of CONTR samples did not differ in their DOPA/protein ratio, however, in the DM CAT and non-DM CAT groups there was a higher DOPA/protein ratio in

total homogenates compared to supernatants. m-Tyr could be measured well in total homogenates of DM CAT and non-DM CAT lenses. The m-Tyr/protein ratio of the total homogenates was higher in all three groups than that the m-Tyr/protein ratio of the supernatants. Compared to the supernatants, the o-Tyr/protein ratio was higher in the total homogenates of the DM CAT and non-DM CAT groups, but not the CONTR. In all three groups, the Phe/protein ratio of the total homogenates was higher than that of the supernatants.

In the HPLC studies, we have observed the accumulation of the oxidized amino acids in the proteins of total homogenates of the cataractous samples, which process is not accompanied by an accumulation of the same hydroxyl radical markers in the supernatant of the cataract samples. These two findings provide an indirect proof that the accumulation of the oxidative stress markers occurs in the non-water soluble phase of the cataractous lens proteins.

If there is a connection between the Phe-oxidation and insolubility of lens proteins is unclear, it is possible that they are two outcomes of the same pathologic alterations. However, from the literature we know that some Phe side-chains of lens crystallins do play an important role in the chaperone function of crystallins thus in lens protein solubilisation. We know that single point-mutations of these Phe side-chains may completely abolish the chaperone function. Hydroxyl radical-related modification of the side chains of the Phe amino acids in key positions for the chaperone function of crystallins might also influence protein solubility and may also this way contribute to cataract formation.

The different Phe content of the proteins of the supernatants and the total homogenates, and the lower Phe content in the cataractous supernatants – versus that of the controls – is probably not a consequence of the consumption of Phe by the hydroxyl radical-derived processes, as there is a difference of approximately three-four orders of magnitude

between protein Phe content and protein m- and o-Tyr content (Phe vs. m-Tyr and o-Tyr, approx. 1000 µmol/g vs. 20 nmol/g and 200 nmol/g). Moreover, we measured a lower Phe/protein ratio in all supernatants without the accumulation of the oxidized amino acids. There was a further decrease of Phe content in the non-DM CAT group vs. controls in the supernatants. This is not accompanied by an increase in the amount of oxidized amino acids, either. Thus, the difference in the Phe content might rather suggest that different types of proteins can be found in the water-soluble and in the water-insoluble lens components.

4.5. Results of the study on the connection between angiotensin-converting enzyme genotype and clinical characteristics in patients with type 2 diabetes mellitus (Publication V., VII, VIII.)

There was no difference between patients with II (n = 27), ID (n = 68) or DD (n = 50) genotype concerning major clinical characteristics. When grouping the patients according to their ACE genotype, we found that serum fructosamine levels were significantly higher in the DD group than in the II group. Grouping patients according to the presence or absence of the D allele (II vs. ID+DD genotypes), we found significantly higher fructosamine level in the ID+DD group than in the II group. Using median test and grouping patients to a below-the-median and above-the-median group, we found a significant connection between serum fructosamine level and the ACE gene polymorphism.

We also intended to find out, if the inhibition of the renin-angiotensin system by an ACE inhibitor agent is accompanied by a different glycemic profile. We found that patients treated by ACE inhibitors had a significantly lower fructosamine level than patients not receiving this type of drug.

When grouping the patients according to the ACE genotype, we found that daily albuminuria was highest in the patients with DD genotype. When examining glycation parameters, a significant correlation between serum fructosamine levels, blood glucose and

Hb A_{1c} was found. Both plasma glucose and fructosamine correlated well with the albuminuria, however Hb A_{1C} did not.

We investigated the activity of the gamma glutamyl transferase (γ-GT) enzyme, an enzyme that plays a part among others in the antioxidant defense system. Levels of the γ-GT enzyme correlated well with C-reactive protein and blood urea nitrogen, a marker of renal function, (corrected for age).

However, there was no significant positive correlation between serum bilirubin (a marker of liver damage) and γ-GT. Furthermore, there was no difference in serum bilirubin levels between the groups with different ACE genotypes. Despite these facts, the role of non-alcoholic steatosis hepatis (NASH) cannot be totally ruled out. Based on these results, however, we might suggest considering γ-GT in non-liver damaged diabetic patients also as a marker of microinflammation and oxidative stress.

4.6. Results of the study on glutathione peroxidase genotype and serum levels of L-arginine analogues (Publication VI.)

The genotype distributions and the allele frequencies in our patients (n=148) showed no significant difference from the Hardy-Weinberg equilibrium. There was no difference between two groups with and without the presence of the P allele (P/P and P/L vs L/L) regarding plasma L-SDMA levels. However, patients with L/L genotype had significantly lower L-NMMA, L-ADMA level than the patients having the P allele.

These results might suggest that increased oxidative stress as a consequence of the GPX-1 genotype may lead to an elevation of serum levels of L-Arg analogues. As the groups compared were matched for blood pressure, we cannot state that the higher level of the L-Arg analogues in our patients could lead to the development of hypertension.

5. CONCLUSIONS

According to results of our *in vitro* studies, fluorescent measurement techniques (both spectrofluorimetry and HPLC) proved to be useful in monitoring Phe to Tyr conversion in a free-radical producing system. The redox cycling of iron and the production of $\cdot O_2^-$ proved to be necessary for the amino acid modification. Tyr production was influenced by the presence of iron-chelators, as well. ATP inhibited, citrate and EDTA increased the Phe-Tyr conversion. Also pH of the solute is of critical importance, the use of a phosphate buffer can decrease Tyr production. Citrate is able to autoxidize and initiate the reaction in the absence of H_2O_2, too.

We found that the physiological p-Tyr is retained by both the healthy and the damaged kidney. Also, its production is decreased in renal impairment. According to our findings and data of the literature, measurement of urinary and plasma o-Tyr is a simple and valuable method for the indirect detection of hydroxyl free radical production. In patients with type 2 diabetes mellitus with or without renal failure, the renal excretion of o-Tyr is enhanced as a consequence of an increased renal tubular secretion or *in loco* renal o-Tyr production.

In another study, we found indirect evidence for the accumulation of oxidized amino acids in the non-water soluble phase of lens proteins, the presence of high molecular weight aggregates in cataractous total homogenates, and a decrease of protein concentration in the water-soluble phase of cataractous lenses. The accumulation of oxidized amino acids and the oxidative modification of Phe residues may be a link between protein damage and lens opacification.

We detected a worse glycemic status and a larger albuminuria in DM patients with a disfavourable ACE genotype, a better glycemia in those patients that were treated with an ACEI. We also detected higher levels of L-Arg analogues in DM patients with a less beneficial genotype of the antioxidant GPX-1 enzyme.

6. LIST OF THE Ph.D. THESES:

1. The *in vitro* phenylalanine-to-tyrosine conversion reaction can be used a marker of the *in vitro* hydroxyl free radical-derived damage to the essential amino acid phenylalanine during a metal-catalyzed oxidation reaction.
2. All three isoforms of tyrosine i.e. para-, meta- and ortho-tyrosine are formed in the Fenton-like metal-catalyzed oxidation reaction. The oxidation of phenylalanine may be prevented by the use of antioxidants, and it is influenced by the presence of iron complexes and pH.
3. The hydroxyl free radical marker ortho-tyrosine may accumulate in the urine of patients with type 2 diabetes mellitus and/or chronic renal failure.
4. The renal transport of ortho-tyrosine differs from that of para-tyrosine, and there is an increased secretion or *in loco* production of ortho-tyrosine in the kidney of patients with type 2 diabetes mellitus.
5. Ortho-, meta-tyrosine and dihydroxyphenylalanine accumulate in the non-water soluble fraction of lens proteins of patients with diabetic and non-diabetic cataract.
6. The phenylalanine content of the water-soluble components of cataractous lenses differs from that of the total lens homogenates.
7. The renin-angiotensin system i.e. the D allele of the angiotensin converting enzyme influences the carbohydrate metabolism, and may lead to an increased oxidative stress in patients with type 2 diabetes mellitus.
8. The antioxidant glutathione peroxidase enzyme may influence the serum levels of L-arginine analogues and it may this way prevent endothelial dysfunction in patients with type 2 diabetes mellitus.

7. List of abstracts and publications used for the theses

This thesis is based on the following *congress abstracts*, indicated by numbering in the text:

I. **G. Molnár,** Z. Wagner, L. Wagner, P. Degrell, Z. Matus, B. Kocsis, B. Laczy, J. Nagy, I. Wittmann: Ferric iron and its complexes induce para-, meta – and ortho-tyrosine formation from phenylalanine in the presence of hydrogen peroxide. Role of the superoxide free radical in the reaction. Abstract, *Diabetologia 46 (S2): A403 (2003),* **imp. fact.: 5.689**

II. **Molnár G.,** Wagner Z., Wagner L., Degrell P., Matus Z., Kocsis B., Laczy B., Nagy J., Wittmann I.: (Production of the superoxide free radical is needed for the hydroxylation of phenylalanine in the Fe [III]- hidrogen peroxyde system. Role of the Fe [III]-complexes) – Hungarian. Abstract, *Hypertonia és Nephrologia 7 (S3): 88 (2003)*

This thesis is based on the following *publications*, as indicated by numbering in the text:

III. **G. A. Molnár,** Z. Wagner, L. Markó, T. Kőszegi, M. Mohás, B. Kocsis, Z. Matus, L. Wagner, M. Tamaskó, I. Mazák, B. Laczy, J. Nagy, I. Wittmann: Urinary ortho-tyrosine excretion in diabetes mellitus and renal failure: evidence for hydroxyl radical production. *Kidney International 68 (5):2281-2287 (2005),* **imp. fact.: 4.927**

IV. **G. A. Molnár,** V. Nemes, Zs. Biró, A. Ludány, Z. Wagner, I. Wittmann: Accumulation of the hydroxyl free radical markers meta-, ortho-tyrosine and DOPA in cataractous lenses is accompanied by a lower protein and phenylalanine content of the water-soluble phase. *Free Radical Research 39 (12): 1359-1366 (2005),* **imp. fact.: 2.323**

V. **Molnár G.A.,** Wagner Z., Wagner L., Melegh B., Kőszegi T., Degrell P., Bene J., Tamaskó M., Laczy B., Nagy J., Wittmann I.: [Effect of ACE gene polymorphism on carbohydrate metabolism, on oxidative stress and on end-organ damage in type-2 diabetes mellitus] – Hungarian. *Orvosi Hetilap 145 (16): 15-19 (2004)*

VI. T. Szelestei, S. Bähring, Z. Wagner, A. Aydin, **G. A. Molnár**, J. Nagy, I. Wittmann: Serum levels of L-arginine analogues and glutathione peroxidase and catalase gene variants in Type 2 diabetes mellitus patients. *Diabetic Medicine 22: 356-357 (2005),* **imp. fact.: 2.725**

VII. I. Wittmann, **G.A. Molnár**, P. Degrell, Z. Wagner, M. Tamaskó, B. Laczy, P. Brasnyó, L. Wagner, J. Nagy: Prevention and treatment of diabetic nephropathy. *Diabetes Research and Clinical Practice 68 (S1): S36-42 (2005),* **imp. fact.: 1.236,** citation: **1**

VIII. Z. Wagner, M. Molnár, **G. A. Molnár**, M. Tamaskó, B. Laczy, L. Wagner, B. Csiky, A. Heidland, J. Nagy, I. Wittmann: Serum Carboxymethyl-lysine Predicts Mortality in Hemodialysis Patients. *Americal Journal of Kidney Diseases 47: 294-300 (2006.),* **imp. fact.: 4.412** (in 2005), citations: **6**

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