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.

(Ph.D. theses)

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ABBREVIATIONS

ACE, angiotensin converting enzyme

AGE, advanced glycation end products

ALE, advances lipoxidation end products

ADMA, asymmetric dimethylarginine

APOP, advanced protein oxidation product

ATP, adenosine triphosphate

AUC, area-under-the-curve

BHT, buthylated hydroxytoluene

CBB, Coomassie brillant blue

CKD, chronic kidney disease

CONTR, group of control subjects

CRP, C-reactive protein

CSE, carbonyl stress end products

DBPTB, N-phenyacyl-4,5-dimethyl-thiazolium bromide

DDAH, dimetyhlarginine dimethylaminohydrolase

DM, diabetes mellitus

DM CAT, group of cataract patients with type 2 diabetes mellitus

DNA, dezoxyribonucleotic acid

DOPA, 3-,4-dihydroxy-phenylalanine

DPTC, 4,5-dimethyl-3-phenacylthiazolium chloride

EDTA, ethylenediamine-tetraacetic acid

EGTA, ethylene glycol – bis (2-amino-ethylether)-tetraacetic acid

ELFO, electrophoresis

eNOS, endothelial nitric oxide synthase

ESR, electron spin resonance

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

GSSG, oxidized glutathione

$Hb\ A_{1c}$, hemoglobin A_{1c}

H_2O_2, hydrogen peroxide

HoCys, homocysteine

HPLC, high performance liquid chromatography

iNOS, inducible nitric oxide synthase

K-W test, Kruskal-Wallis test

L-NMMA, L-N-monomethyl-arginine

m-Tyr, meta-tyrosine

M-W U test, Mann-Whitney U test

NEG, non-enzymatic glycation

nNOS, neuronal nitric oxide synthase

NO, nitric oxide

NOS, nitric oxide synthase

non-DM CAT, group of cataract patients without diabetes mellitus

$\cdot O_2^-$, superoxide free radical

$\cdot OH$, hydroxyl free radical

ONOO, peroxynitrite

OPA, ortho-phtalaldehyde

o-Tyr, ortho-tyrosine

PAGE, polyacrylamide gel-electrophoresis

PCR, polymerase chain reaction

Phe, phenylalanine

PMSF, phenylmethanesulfonyl fluoride

PTB, 3-phenacylthiazolium bromide

p-Tyr, para-tyrosine

RAGE, receptor of AGEs

RNS, reactive nitrogen species

ROS, reactive oxygen species

SAM, S-adenosine methionine

SD, standard deviation

SDMA, symmetric dimethylarginine

SDS, sodium dodecylsulphate

SEM, standard error of the mean

SOD, superoxide dismutase

Tyr, tyrosine

1. Abstract

Background: Pathogenesis of the chronic complications of type 2 diabetes mellitus (DM), ageing and chronic kidney disease (CKD) is multifactorial; it involves environmental factors, inflammation, an unfavorable genetic background, metabolic disturbances, oxidative stress processes etc.

Aims: Aims of our studies were ***i)*** to investigate the phenylalanine-to-tyrosine conversion in a Fenton-like process, the effect of iron chelators on the reactions; ***ii)*** to measure oxidative stress products in the urine and serum of patients with DM and/or CKD; ***iii)*** to detect oxidized amino acids in cataractous lenses and to find a clue for a possible connection between the accumulation of oxidized amino acids and insolubilization of lens proteins; ***iv)*** to investigate the influence of the polymorphism of the angiotensin converting enzyme (ACE) gene and ***v)*** antioxidant enzymes on the metabolic status of patients with DM.

Methods: ***ad i)*** A ferric iron - H_2O_2 - phenylalanine (Phe) *in vitro* system was used to evoke hydroxyl free radical damage. The production of the autofluorescent amino acid, tyrosine (Tyr) was monitored spectrofluorimetrically, at wavelengths of 275 nm of excitation and 305 nm of emission. The role of Fe^{3+} and of the superoxide free radical in the reaction was studied using desferrioxamine and superoxide dismutase (SOD), respectively. The effect of iron chelators (EDTA, citric acid) was also investigated. To measure the production of different Tyr isomers i.e. para-Tyr (p-Tyr), meta-Tyr (m-Tyr) and ortho-Tyr (o-Tyr), and the consumption of Phe, experiments were carried out using a C_{18}-column- and fluorescent detector-equipped HPLC system, as well. ***ad ii)*** The same HPLC system was used to measure p-Tyr and o-Tyr in deproteinized plasma and 24 h-collected urine samples of patients. By measuring plasma and urinary concentrations of creatinine and urine output, as well, clearance and fractional excretion for p- and o-Tyr could be calculated. ***ad iii)*** Cataractous lens proteins were divided to water-soluble and non-soluble fractions; subsequently, the

water-soluble phase and the total lens homogenates underwent electrophoretic analysis, and after acidic hydrolysis oxidized amino acids (DOPA, m-Tyr and o-Tyr) were determined. In two cross-sectional studies, connections between ***ad iv)*** ACE gene or ***ad v)*** glutathione peroxidase gene polymorphism, endothelial dysfunction and clinical parameters of patients with DM were investigated.

Results: ***ad i)*** In a Fenton-like reaction, a marked Tyr production was detected, that could be inhibited using desferrioxamine or SOD. Complexation of ferric iron by citrate and EDTA increased the p-, m-, o-Tyr production, and the use of a phosphate buffer had an inhibitory effect. ***ad ii)*** We found low plasma levels and urinary clearance of p-Tyr in patients with CKD compared to controls. Urinary excretion and fractional excretion of o-Tyr was increased in patients with DM and DM+CKD. ***ad iii)*** Cataractous lenses had less soluble protein than control lenses; the electrophoretic distribution of lens proteins was also different. The water-soluble lens components of cataractous lenses had lower levels of DOPA, m-Tyr and o-Tyr than the total homogenates. The Phe content of soluble lens proteins was lower in cataractous than in non-cataractous lenses. ***ad iv)*** Patients with DM having one or two D alleles of the ACE gene had higher fructosamine levels, albuminuria, gamma-glutamyl trasferase activity than patients with the II genotype. ***ad v)*** Patients with DM having P allele of the glutathione peroxidase gene, had higher levels of methylated arginine analogues.

Key words: angiotensin converting enzyme gene polymorphism, ageing, albuminuria, L-asymmetric dimethyl-arginine (ADMA), cataract, chronic kidney disease, 3,4-dihydroxy-phenylalanine (DOPA), 8-epi-prostaglandin-$F_{2\alpha}$, ferric iron, fractioned excretion, fructosamine, glutathione peroxidase (GPX-1) gene polymorphism, hydroxyl free radical, hypertension, iron chelators, meta-tyrosine, L-N-monomethyl-arginine (L-NMMA), ortho-tyrosine, oxidative stress, para-tyrosine, phenylalanine, protein solubility, L-symmetric dimethyl-arginine (SDMA), type 2 diabetes mellitus

2. Összefoglalás

Háttér: A 2-es típusú cukorbetegség (DM), az öregedés és a krónikus vesebetegség (CKD) patogenezise multifaktoriális; környezeti tényezőket, gyulladásos folyamatokat, előnytelen genetikai hátteret, metabolikus zavarokat, oxidatív stresszt stb. foglal magában.

Céklitűzések: Vizsgálataink célkitűzései voltak: ***1)*** megvizsgálni a fenilalanin-tirozin átalakulást egy Fenton-szerű folyamatban és vas-kalátorok hatását ezen reakcióra; ***2)*** oxidatív stressz termékeket meghatározni DM és/vagy CKD betegek vizeletében és szérumában; ***3)*** cataractás lencsékben oxidált aminosavakat kimutatni, és lehetséges kapcsolatot keresni az oxidált aminosavak felszaporodása és a lencsefehérjék oldhatatlanná válása között; megvizsgálni ***4)*** az angiotenzin-konvertáló enzim (ACE) gén és ***5)*** antioxidáns enzimek gén-polimorfizmusának hatását DM betegek anyagcsereparamétereire.

Módszerek: ***ad 1)*** Egy ferri vas - H_2O_2 - fenilalanin (Phe) *in vitro* rendszert használtunk a hidroxil szabadgyök okozta károsodás létrehozásásra. Az autofluoreszcens aminosav, a tirozin (Tyr) mennyiségét spectrofluorimetriás módszerrel követtük, 275 nm-es excitációs és 305 nm-es emissziós hullámhosszok mellett. A Fe^{3+}-nak és a szuperoxid szabadgyöknek a reakcióban játszott szerepét dezferoxaminnal illetve szuperoxid dizmutázzal (SOD) igazoltuk. Megvizsgáltuk továbbá vaskelátorok (EDTA, citrát) hatását is. Az egyes Tyr izomerek, így a para-Tyr (p-Tyr), a meta-Tyr (m-Tyr) és az orto-Tyr (o-Tyr) termelődésének, valamint a Phe fogyásának meghatározására a kísérleteket egy C_{18}-as kromatográfiás oszloppal és fluoreszcens detektorral felszerelt HPLC rendszerrel is kiértékeltük. ***ad 2)*** Ugyanezen HPLC-rendszert használtuk a p-Tyr és az o-Tyr mennyiségének meghatározására betegek fehérjementesített plazmájában és 24h-s gyűjtött vizeletében. A plazma és a vizelet kreatinin-koncentrációjának és a vizeletmennyiségnek meghatározásával a p- és az o-Tyr clearance-e és frakcionált exkréciója is meghatározható volt. ***ad 3)*** Cataractás lencsefehérjéket választottunk szét vízoldékony és vízoldhatatlan

frakciókra, majd a vízoldékony fázisból és a teljes lencsehomogenátumokból elektroforetikus vizsgálatot és savas hidrolízis után oxidált aminosavak (DOPA, m-Tyr és o-Tyr) mennyiségének meghatározását végeztük el. Két keresztmetszeti vizsgálatban kapcsolatot kerestünk ***ad 4)*** az ACE gén és ***ad 5)*** a glutation peroxidáz gén polimorfizmusa, az endotéldiszfunkció és a DM betegek más klinikai paraméterei között.

Eredmények: ***ad 1)*** Egy Fenton-szerű reakcióban jelentős Tyr-termelődést mutattunk ki, ami dezferoxaminnal vagy SOD-dal gátolható volt. A ferri vas citrát és EDTA révén történő komplexálása fokozta a p-, a m- és az o-Tyr termelődését, míg foszfátpuffer használata gátló hatással volt a reakcióra. ***ad 2)*** CKD betegekben a kontrollokhoz képest alacsonyabb plazma szinteket és clearance-t találtunk a p-Tyr esetében. Az o-Tyr ürítése és frakcionált exkréciója magasabb volt DM és DM+CKD betegek esetében. ***ad 3)*** A cataractás lencsékben kevesebb volt a vízoldékony fehérje, mint a control lecsékben, és a lencsefehérjék elektroforetikus megoszlása is eltérő volt. A cataractás lencsék vízoldékony lencsekomponenseiben alacsonyabb volt a DOPA, a m-Tyr és az o-Tyr mennyisége a totál homogenátumokhoz képest. A vízoldékony lecsefehérjék Phe-tartalma alacsonyabb volt a cataractás lencsékben, mint a nem-cataractás lencsékben. ***ad 4)*** Az egy vagy két D alléllel rendelkező DM betegek fruktózaminszintje, albuminuriája, gamma-glutamil transzferáz enzim aktivitása magasabb volt az II genotípusú betegekéhez képest. ***ad 5)*** A glutation peroxidáz gén P alléljét hordozó DM betegekben magasabb volt a metilált arginin-analógok szintje.

Kulcsszavak: 2-es típusú diabétesz, angiotenzin-konvertáló enzim gén-polimorfizmus, öregedés, albuminuria, aszimmetrikus dimetilarginin (ADMA), cataracta, 3,4-dihidroxi-fenil-alanin (DOPA), 8-epi-prosztaglandin-$F_{2\alpha}$, fehérje oldékonyság, fenilalanin, ferri vas, frakcionált exkréció, fruktózamin, glutation peroxidáz (GPX-1) gén-polimorfizmus, hidroxil szabadgyök, hipertónia, krónikus vesebetegség, meta-tirozin, L-N-monometil-arginin (L-NMMA), orto-tirozin, oxidatív stressz, para-tirozin, szimmetrikus dimetil-arginin (SDMA), vaskelátorok

3. 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**

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), No. of citations: **2**

4. 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. These factors will be discussed below.

4.1. Oxidative stress, carbonyl stress processes and metabolic disturbances

4.1.1. 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.[1,2] The imbalance between free radicals and antioxidant systems gives rise to free radical-mediated damage. Metal-catalyzed oxidation reactions, like the Fenton reaction, where hydrogen peroxide is cleaved to hydroxyl free radical and hydroxyl anion, play an important role in the generation of oxidative stress.[3] In DM there is an increased oxidative stress e.g. due to the high glucose concentrations. In CKD, there is a microinflammatory state, caused by uremic toxins, an enhanced formation and a decreased clearance of pro-inflammatory cytokines (e.g. TNF-α, IL-12) and an accumulation of advanced glycation end products.[4]

4.1.2. Free radicals and reactive oxygen/nitrogen species

Free radicals are characterized by the presence of an unpaired electron that makes these molecules highly unstable. Unpaired electrons tend to pair formation, thus free radicals are chemically highly reactive and have a short half-lifetime. As a consequence of their low concentrations and their short lifetime, they cannot be easily detected. Some of the free-radical-derived or -related substances (e.g. hydrogen peroxide, singlet oxygen) do not have any unpaired electrons, despite they are highly reactive. These materials are therefore no real free radicals, they are called reactive oxygen/nitrogen species (ROS/RNS).[5]

Detection of free radicals may require direct or indirect methods. The direct detection implies electron spin resonance spectroscopy (ESR) and the use of spin traps. These latter molecules trap the free radicals, and they form an adduct with a much longer lifetime. Triggering and relaxation of these free-radical adducts can be measured by ESR providing information on the amount and structure of the radicals.[6,7]

An indirect possibility is to detect the free-radical-induced damage to molecules rather than the presence of the free radicals themselves. Such methods do not require an ESR device, but are less specific than the ESR measurements. Indirect methods for free-radical detection include the measurement of consumption of antioxidants (e.g. Total Antioxidant Status, TAS or Total Antioxidant Capacity, TAC), that of thiobarbituric acid-reactive substances, lipidperoxidation products (e.g. isoprostanes) or amino acid oxidation products etc.[8,9]

4.1.3. Free radical-reactions

4.1.3.1. Formation of the reactive oxygen species, reduction of molecular oxygen

Molecular oxygen has an electron pair on its outer orbital, therefore it is stable. The yield of free radicals requires activation of the oxygen molecule either via excitation (singlet oxygen), reduction (superoxide free radical, hydrogen peroxide, hydroxyl free radical),

molecular scission (oxygen atom), or oxidation (molecular oxygen ion). The most common way is the partial reduction of oxygen.

The oxygen molecule can be reduced in a four-electron reaction to chemically inert water *(Figure 1)* e.g. in the mitochondria during terminal oxidation by cytochrome enzymes. However, by a single-electron reduction of oxygen, superoxide free radical ($\cdot O_2^-$) is formed. Such a partial reduction can take place when oxygen-containing solutions are excited by ionizing radiation or ultrasound. Other, physiologically more relevant mechanisms involve "autoxidation" of reducing sugars, other non-enzymatic chemical reactions, and enzymatic reactions such as the xanthine-oxidase and the NADPH-oxidase reactions.

The $\cdot O_2^-$ has a short lifespan, it becomes dismutated i.e. further reduced to form hydrogen peroxide (H_2O_2). The reaction may occur spontaneously, but it may be catalyzed by the superoxide dismutase enzyme (SOD), as well. Although H_2O_2 is not a free radical, it is still reactive; therefore it belongs to the ROS, and it may give rise to the production of even more reactive substances, as the hydroxyl free radical (·OH). The SOD-reaction leads to a formation of a ROS from a free radical, i.e. a chemically less active substance is formed, therefore both isoforms of SOD, the cytosolic Cu/Zn-SOD and the mitochondrial Mn-SOD are regarded as antioxidant enzymes.

·OH is the most reactive of all free radical species, therefore has the shortest life-span. It is produced by the cleavage reactions of H_2O_2, such as the Haber-Weiss and Fenton reactions. ·OH may directly damage biologically important macromolecules, while itself becomes further reduced to water in these reactions.

As reactions of H_2O_2 can lead to ·OH formation it is important that there exist some routes by which H_2O_2 may be degraded before yielding ·OH. These reactions are catalyzed by the catalase enzyme and the peroxidases such as glutathione peroxidase.[5,10]

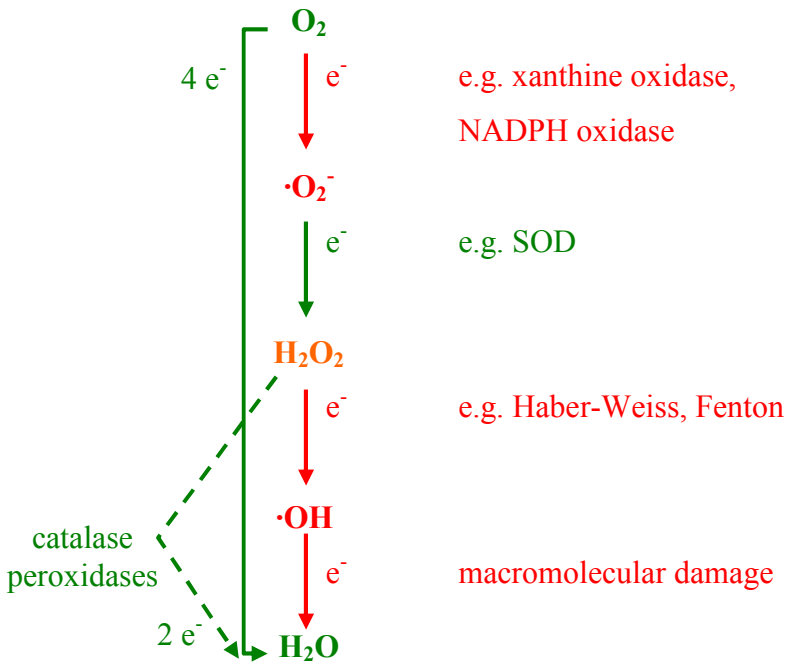

Figure 1
Schematic drawing of the reduction of molecular oxygen, and the formation of reactive oxygen species (ROS). Free radicals and pathways leading to the formation of free radicals or radical-related damage are represented in red color; H_2O_2 as a non-radical ROS in orange, while non-ROS molecules and antioxidant pathways appear in green.

4.1.3.2. The Haber-Weiss reaction:

In this reaction, H_2O_2 will be split to the more active free radical species, ·OH and to OH^-.[10]

$$\cdot O_2^- + H_2O_2 \rightarrow O_2 + \cdot OH + OH^-$$

4.1.3.3. The Fenton and Fenton-like reactions

The classical Fenton-reaction has been described as a cleavage reaction of H_2O_2 catalyzed by the redox cycling of ferrous iron. The simplified equation is:

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

Ferric iron and iron complexes are also often used in *in vitro* experiments to model free radical production in Fenton-like reactions. 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.[10]

4.1.3.4. Damage of biologically important macromolecules as a consequence of free radical-attack

Free radicals are, as a consequence of their unique chemical structure highly reactive, they can attack biologically active molecules, such as lipids, carbohydrates, nucleic acids, proteins and amino acids.

Peroxidation of partially unsaturated fatty acids, membrane lipids may involve enzymatic and non-enzymatic pathways, as well. Oxidized metabolites of the arachidonic acid are formed mainly in enzymatic reactions, they are highly important messengers. The non-enzymatic reactions are fast, and unlike the enzymatic reactions, they are not that strictly controlled. Cyclic endoperoxides and lipid hydroperoxides are formed during the lipid-peroxidation, which give rise to even smaller advanced lipid peroxidation end products (ALE) such as malondialdehyde, glyoxal, methylglyoxal, acrolein.[9,11]

There is also a free-radical-related damage of nucleic acids and nucleotides mainly by ·OH which may lead to strand-breaks, hydroxylation of nucleotide bases (e.g. to 5-hydroxy-methyl-uracyl and 8-hydroxy-uracyl), formation of thymidine-dimers and consequently mutations. These alterations may have a role in the pathogenesis of malignancies.[12]

Free radical-related damage to proteins may give rise to advanced protein oxidation products (APOP). Oxidative stress-related modification of amino acids shall be discussed in detail in chapter 3.4.7.1.1.

4.1.3.5. Damage to nitric oxide and formation of peroxynitrite

Nitric oxide (NO) is a potent vasodilator, an endothelium-dependent relax factor, which is also able to decrease platelet aggregation, leukocyte adhesion and vascular smooth

muscle cell proliferation. Thus, NO is anti-thrombotic, protects against vascular remodeling and decreases blood pressure. NO is formed by the nitric oxide synthase (NOS) enzyme, that has three isoforms, the inducible NOS (iNOS) and the constitutively expressed endothelial (eNOS) and neural (nNOS) isoforms. The NOS enzyme cleaves the amino acid L-arginine (L-Arg) to NO and citrulline in a NADPH-dependent reaction. NO has a short lifespan, and it has been shown that it has an unpaired electron, i.e. it is one of the free radical species, as well. Decreased NO levels are described in kidney diseases, probably as a mediator and/or consequence of renal damage.[13]

In the presence of $\cdot O_2^-$, the two free radicals (NO and $\cdot O_2^-$) may react with each other and form a substance that is called peroxynitrite (ONOO). The properties of ONOO are the opposite of that of NO, namely ONOO is vasoconstrictor, pro-thrombotic and induces remodeling etc. In some pathological states NOS enzyme activity may be uncoupled from the L-Arg to NO conversion, in these states the redox reactions still take place, and the enzyme produces $\cdot O_2^-$. This may further contribute to ONOO production.[14]

4.1.4. The antioxidant defense system

4.1.4.1. Scavenger molecules

Scavengers are antioxidant molecules that directly react with ROS and thereby prevent free radical-related damage. Scavengers need to be present in a high concentration to exert their antioxidant function, as they react stochiometrically with the radicals. They are grouped to intracellular and extracellular scavengers. The intracellular ones include reduced glutathione (GSH), ascorbic acid, vitamin E, ubiquinol etc. On the other hand, uric acid, bilirubin, serum albumin and others belong to the extracellular scavengers. It is important to take into consideration that the antioxidant or pro-oxidant function of a molecule depends on the redox potential of the molecule and that of other molecules in the environment. For

example ascorbic acid – besides being a scavenger – may also give rise to the formation of free radicals in autoxidation processes. Scavengers may be specific for the free radical they are capable scavenging.[5]

4.1.4.2. Antioxidant enzymes

4.1.4.2.1. Superoxide dismutase

The cytosolic Cu/Zn-SOD and the mitochondrial Mn-SOD catalyze the following reaction:

$$2 \cdot O_2^- + 2 H^+ \rightarrow H_2O_2 + O_2$$

In a first step, the transition metals of the SOD enzyme (Cu or Mn) oxidize the first $\cdot O_2^-$ to oxygen, while they become reduced. In the second step, hydrogen ions are used to reduce the second $\cdot O_2^-$, while the transition metal becomes re-oxidized.[5]

4.1.4.2.2. Catalase

The catalase enzyme is responsible for the detoxification of the ROS hydrogen peroxide to biologically inactive water molecules:

$$2 H_2O_2 \longrightarrow 2 H_2O + O_2$$

In the prosthetic group of the catalase molecule, a redox-active Fe^{3+} can be found in a protoporphyrin ring that has a role similar to the transition metals found in SOD.[5]

4.1.4.2.3. Peroxidases

Like the catalase enzyme, peroxidases also work on the detoxification of H_2O_2 but they utilize organic or inorganic molecules (AH_2 in the equations below) for the reduction of H_2O_2. They have a heme prostatic group just like catalase.[5] The catalyzed reaction is:

$$H_2O_2 + AH_2 \longrightarrow 2 H_2O + A$$

(AH_2 = reduced substrate, A = oxidized substrate)

The probably most important type of the peroxidases is the glutathione peroxidase (GPX), a 80 kDa selenium-containing enzyme, which uses GSH for the reduction H_2O_2. In the reaction disulphide-cross-linked oxidized glutathione (GS-SG) is formed.

$$2\ GSH + H_2O_2 \longrightarrow GS\text{-}SG + 2\ H_2O$$

GPX-1 is located in the cytoplasm and/or the mitochondrion of cells of the kidney, liver and erythrocytes; GPX-2 in the cytosol of hepatocytes and colon cells; GPX-3 extracellularly in the plasma, in the kidney; GPX-4 in testicular cells and renal epithelial cells. GPX-5 has been described in mouse epididymis.[15,16,17,18] Altered function of glutathione peroxidases has been described to play a role among others in allergic diseases, inflammatory diseases in the development of cancer and neurodegenerative diseases.[18] 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. Elevated serum levels of γ-GT indicate an adaptation to oxidative stress.[19]

4.1.5. Diabetes mellitus, oxidative stress, carbonyl stress and ageing

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.[20,21]

4.1.5.1. Non-enzymatic glycation and carbonyl stress

In the non-enzymatic glycation reaction (NEG) reducing sugars or carbonyls bind to the amino ($–NH_2$) group of amino acids and other molecules. The extent of endogenous NEG process is related to duration and magnitude of hyperglycemia in diabetic patients. This reaction is reversible and this way a Schiff-base is formed. The Schiff-base product is turned

into an Amadori-product (1-amino-1-deoxiketose) in just a few days' time. As the reaction proceeds, advanced glycation end products (AGE) are formed.[22] Accumulation of the AGEs in the proteins depends on the turnover of the protein, because the chemically modified proteins are degraded and get replaced by intact proteins. Therefore, proteins with a slow turnover (e.g. type I and type IV collagen, matrix proteins, the low density lipoprotein, fibrin and crystalline proteins) are particularly prone for the glycation process. These malfunctioning glycated proteins may accumulate in the tissues, thus contribute to diabetic complications such as the diabetic neuro-, nephro-, retino- and macroangiopathy, and other conditions like Alzheimer's disease or cataract formation.[23]

Glycated proteins may bind to high affinity receptors of AGEs (RAGE). The binding of AGEs and APOPs to the RAGE activates complex pathways involving the extracellular signal-regulated kinase 1/2 (Erk 1/2), the jun-N-terminal kinase (JNK), the $p38^{MAPK}$, and the NFκB pathway.[24] These changes in the monocytes and macrophages and may lead to respiratory burst, the release of pro-inflammatory cytokines (TNF-α, IL-1, INF-γ, PDGF), free radicals and chemoattractants. This way, AGEs may lead to a state with an increased oxidative stress.[25]

4.1.5.2. Glucose autoxidation

The autoxidation of glucose and other reducing sugars may be a part of the NEG reaction. In this reaction, the glucose molecule becomes oxidized, while it may reduce molecular oxygen to $\cdot O_2^-$. The reaction is not a real "autoxidation", as it is not driven by the reaction kinetics alone, but it requires the presence of transition metals – such as iron or copper – as catalysts of the reaction. Not only glucose, but also other reducing sugars, such as fructose, ascorbic acid or citric acid may undergo an autoxidation, as well.[26]

4.1.5.3. The poliol pathway

The poliol pathway is a series of biochemical reactions that becomes important in pathophysiological situations, namely in hyperglycemia. In a normoglycemic state the activity of the pathway is rather low; as the affinity of the first enzyme of the pathway, aldose reductase is low towards glucose. In these cases glucose is metabolized in the normal metabolic reactions. But in hyperglycemia, the expression of the enzymes of the poliol pathway increases, thus fructose and sorbitol become formed. The reactions involve the oxidation of NADPH to $NADP^+$ that eventually may lead to a dramatic decrease in NADPH levels. In another part of the reaction, NAD^+ is reduced to NADH, therefore NADH will accumulate. Another consequence is the accumulation of the osmotically active poliols (such as sorbitol) that leads to an increase of osmotic pressure inside the cells.

Sorbitol also interferes with the Na^+-K^+ ATPase, intracellular Na^+ concentration will increase, cells will swell and furthermore the high Na^+ concentration will lead to the accumulation of Ca^{2+}. The accumulation of Ca^{2+} may eventually lead to toxic damage of the mitochondria, a decrease of ATP production, an uncoupling of the mitochondrial breathing chain, and to increased level of mitochondrial free radical production i.e. oxidative stress. NADPH is required for the NOS reaction, and as NADPH will be consumed in the poliol pathway reactions, L-Arg will not be turned into the potent vasodilator, NO. As a consequence, endothelial dysfunction may also develop.[21,25]

4.1.5.4. Hyperglycaemic pseudohypoxia

The NAD^+/NADH ratio plays a crucial role in the sensation of hypoxia in the cells. As a consequence of the poliol pathway, the NAD^+/NADH ratio will decrease, similarly as in hypoxia. This state is therefore called pseudohypoxia, and leads to the inhibition of some enzymes (e.g. isocitrate dehydrogenase, glycerin-aldehyde-3-phosphate dehydrogenase), and this way to metabolic disturbances.[27]

4.1.6. Renal disease and oxidative stress

In renal disease, there is an increased rate of oxidative stress processes as a consequence of multiple causes. In early kidney disease, in a state with a normal clearance, the inflammatory processes in the background of the kidney disease may contribute to oxidative stress, measured e.g. as decreased GSH, increased oxidized glutathione (GSSG) and increased lipid peroxide content of red blood cells.[28,29] With the progression, the decrease of glomerular filtration leads to the accumulation of small molecular weight substances such as AGEs[30], pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α. The AGE-RAGE interaction and cytokines activate inflammatory cells; that leads to respiratory burst, release of free radical species, chemoattractants. Later, in hemodialyzed patients, an interaction between leukocytes and the partially bioincompatible surface of the dialyzing membrane together with a potential bacterial contamination of the dialyzing fluid also leads to inflammation and oxidative stress.[4,31] Hemodialysis may also lead to the transient decrease in the amount of dialyzable antioxidants, which process is only later accompanied by an increase in levels of antioxidant proteins (heme oxigenase-1, ferritin), making these patients in a certain time-window susceptible to ROS.[32]

4.1.7. Ageing and oxidative stress

Ageing itself is a physiological process, but there are some pathological conditions that are connected with an increased rate of ageing of tissues such as atherosclerosis, Alzheimer's disease, Parkinson's disease, cataract formation, dialysis-related amyloidosis. During the life of the cell, modified proteins, nucleic acids and lipids accumulate as a consequence of oxidative stress and carbonyl stress processes. They may eventually lead to apoptosis of the cell.[33] Such an increased oxidative stress is thought to play a role in the ageing diseases listed above.[34] A state with an increased rate of ageing is DM. Both carbonyl stress and oxidative stress are increased and may lead to a cumulative damage of cell

components.[35] Therefore, some diabetic complications are also seen as a derivative of a fast rate of ageing.

4.2. Oxidative derivates of phenylalanine and tyrosine

4.2.1. Conversion-reactions of phenylalanine

Amino acid oxidation-products are stable and specific markers of free radical production.[36] Reactive oxygen species such as superoxide and hydroxyl free radicals damage protein-bound or free amino acids.[37,38] Aromatic side chain amino acids are susceptible to oxidative stress, and their products, e.g. ortho-tyrosine, meta-tyrosine and dityrosine are stable.[1]

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 *(Figure 2)*.

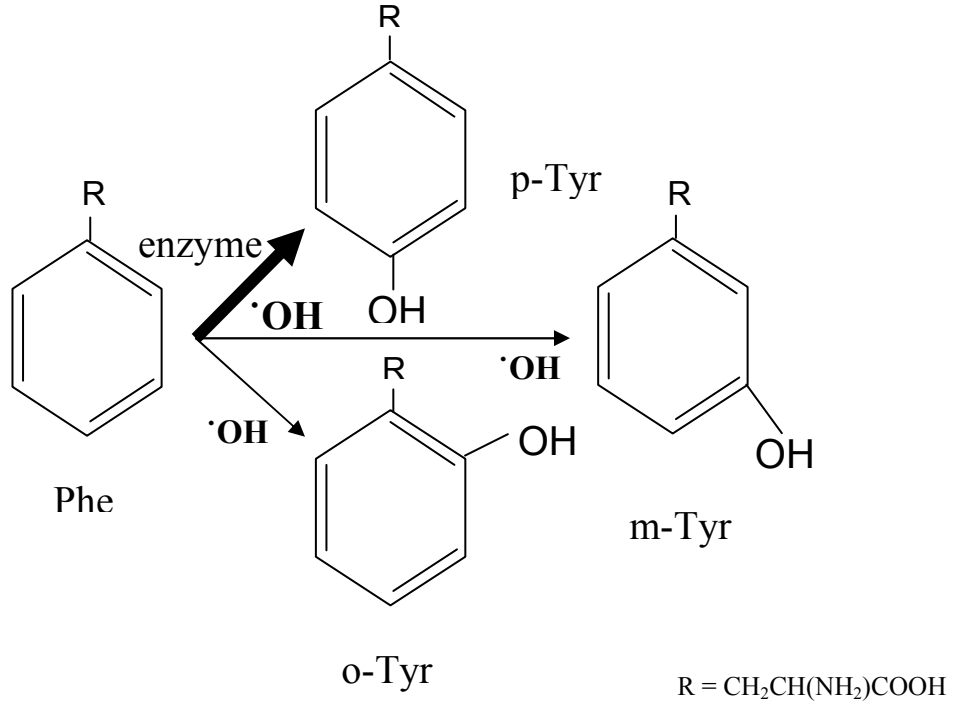

Figure 2

Conversion reactions of phenylalanine to para-, meta- and ortho-tyrosine. The wide arrow (➔) represents the enzymatic reaction, while the narrow arrow (→) shows the reactions in the presence of hydroxyl free radical.
Abbreviations: Phe, phenylalanine; p-Tyr, para-tyrosine; m-Tyr, meta-tyrosine; o-Tyr, ortho-tyrosine; ˙OH, hydroxyl free radical.

Consequently, p-Tyr may be formed both physiologically and in the oxidative stress processes, while m- and o-Tyr are specific hydroxyl free radical markers. 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.[38,39]

Amino acids are filtered in the glomeruli and reabsorbed from the primary filtrate in the renal tubules. The route of reabsorption depends on the nature and chemical structure of the amino acid. Both Phe and p-Tyr are reabsorbed via the transporters of the neutral amino acids. This process is of high efficacy, with only 1% of the filtered amino acids escaping with the urine. Beside filtration, there are other sources of urinary amino acids such as tubular secretion at the apical membrane and tubular brush border peptidases that split oligopeptides to amino acids. The fractional excretion (Fex) of p-Tyr and Phe varies at around 1% (mean Fex of p-Tyr, 1.18%; mean Fex of Phe, 0.9%) in humans.[40]

4.2.2. Significance of oxidative phenylalanine derivates

o- and m-Tyr have been used as markers of hydroxyl free radical formation among others in sera of euthyroid subjects after triiodothyronin administration[41], in cataractous lenses[42] and in the plasma after myocardial ischemia-reperfusion.[43] In the cataractous lens, protein-bound DOPA is regarded as a marker of hydroxyl radical-mediated damage, as well.[42]

Accumulation of o- and m- Tyr has been widely described as correlating with an increase of malondialdehyde formation[44], an increase in NADPH oxidase activity and an

increased level of oxidized fatty acids[45], a decrease in reduced glutathione levels[46] etc. Oxidized amino acid derivatives are superior to other oxidative stress markers (e.g. lipid peroxidation products, glycoxidation products), as they are reliable, specific, highly stable markers and are not produced in sample preparation procedures.[47] Moreover, o-Tyr can be detected well through its autofluorescence.[37]

4.3. Cataract in diabetes mellitus and ageing

4.3.1. Importance of cataract formation in the population

Cataract formation and the subsequent loss of vision is a major health problem even in the most developed countries, there are more than 20 million people worldwide who have lost their vision upon cataract disease.[48] The prevalence increases with age, in the population with over 80 years of age, it may reach up to over 80%.[49]

4.3.2. Characteristics of the human lens

The lens is a flexible, normally transparent biconvex-shaped disc, and it is located between the aqueous humor and the vitreous body. It is an organ that is unique in many regards: it has an approx. 35 % cellular protein concentration necessary for the high refractory index needed for focusing the light; and it is avascular i.e. possesses no direct blood supply. Because of the avascularity and the lens capsule it is a relatively closed system.[50,51] Despite the high protein concentration, the lens proteins stay soluble because of the highly organized structure that makes the lens transparent for the visible light.[50]

The epithelial cells of the lens proliferate in the equatorial, germinative region, and migrate backwards, they turn to fiber cells. The latter produced fiber cells ’drive’ the old ones towards the inner part of the lens. Therefore, the oldest cells are located in the lens nucleus, while the youngest ones in the cortex of the lens.[52] A characteristic feature of the lens

proteins is a very low turnover. As a consequence of this, damages due to the physico-chemical effects of the environment cumulate in the lens, and finally they lead to lens opacification.[51]

4.3.3. Causes and common mechanisms of cataract formation

The cataractogenic effects of ultraviolet light, ionizing radiation, pharmacological agents, smoking and diabetes mellitus (DM) are well-known[53,54,55], but the exact pathogenesis of cataract formation is a complex process with many still unknown components. It is known that, as a consequence of the physico-chemical impacts, the dynamic interactions between water, inorganic ions and proteins (representing 99 % of the dry weight) disintegrate, and the balance between the tissue and the environment suffers disturbances.[56] On the level of proteins, irreversible damages evolve, which affect the cytoskeletal system of the lens, as well as the crystalline-type proteins. Glycation, carbonylation, formation of cross-links, oxidation of thiol-groups and formation of aggregates finally lead to insolubilisation of proteins[57,58,59], and therefore opacity develops.

4.3.4. Crystalline-type proteins

Crystallins represent the major class of water-soluble lens proteins. There are two major groups of crystallins, the ubiquitous and the taxon-specific types. As the name states, taxon-specific crystallins can only be found in selected species, while the ubiquitous forms are species-independent. The ubiquitous forms are classified to three subgroups: alpha-, beta- and gamma-crystallins (α-, β-, γ-crystallins).[48]

4.3.4.1. Post-translational modification of amino acids in proteins

The post-translational modification of proteins and the structural alteration and damage of amino acids is of importance, as the amino acid sequence i.e. the primary structure of proteins is one of the major determinants of protein structure and function. The alteration of the primary structure may lead in several cases to a change in steric conformation, to the change of the secondary, tertiary and quaternary protein structure. This may lead to altered hydrophilic and hydrophobic interactions, the interaction of proteins and water molecules can suffer disturbances, and this process may finally lead to the insolubilization, the precipitation of the proteins.[60]

4.3.4.1.1. Amino acid oxidation

Many reactive oxygen or nitrogen species (ROS or RNS) may lead to amino acid oxidation, among others superoxide free radical ($\cdot O^{2-}$), hydrogen peroxide (H_2O_2), hydroxyl free radical (·OH), hipochloric acid (HOCl), peroxynitrite (ONOO).

Oxidation products of amino acids with an aliphatic side-chain are the instable hydroperoxides, alcohols (e.g. 3-hydroxy-Val, 4-hydroxy-Val and the also physiologically produced 4-hydroxy-Pro) and reactive carbonyl species.

Oxidation products of amino acids with a hetero-atom containing side-chain: e.g. the oxidative stress marker 3-hydroxy-Lys, the also physiologically produced 4- and 5-hydroxy-Lys (from Lys); hydroperoxides and 4-hydroxy-Glu (from Glu); 5-hydroxy-2-aminovalerianic acid (from Arg); Met-sulphoxide (from Met). Cys residues may become cross-linked by disulphide bond, which type of modification of γS-crystallins is known to play a role in precipitation of lens proteins.[38,48]

Oxidation products of amino acids with an aromatic side chain include cyclic indols (from Trp); Asn or Asp through 2-oxo-His (from His); phenylacetic acid, phenylpiruvic acid, p-, m- and o-Tyr (from Phe, *see chapter 3.3.1*); DOPA isomers, dityrosine cross-chains, 3-NO-Tyr and 3-Cl-Tyr (from Tyr).[38]

4.3.4.1.2. Glycation

The amino groups of proteins are susceptible to reactions with reducing sugars or autoxidized sugars. These processes are further enhanced in the presence of ROS. The glycation reactions lead to the accumulation of reactive carbonyls, advanced glycation end products (AGEs), to protein chain cross-linking and/or fragmentation.[39]

4.3.4.1.3. Deamidation

A modification of amino-group containing amino acid side-chains (Gln, Asn) in lens crystallins is deamidation. Deamidation of Asn occurs spontaneously, while the reaction in the case of Gln can be catalyzed by the transglutaminase enzyme. This enzyme can be found in the human lens, as well, and catalyses *in vitro* deamidation of Glu side-chains of lens crystallins.[48]

4.3.4.1.4. Other modifications

Other post-translational modifications of the amino acid residues in crystallins include phosphorylation, adduct-formation (e.g. by reactive carbonyls), and chain fragmentation. Approximately 30 % of the lens proteins are phosphorylated; this modification plays either a role in regulatory mechanisms or contributes to tertiary-quaternary protein structures.[48]

4.3.4.1.5. Defense against oxidative modification by side-chain methylation

Some Cys residues in human γB-, γC-, γD- és γS-crystallins proved to be methylated. This chemical modification prevents the oxidative modification of Cys, and protein cross-links, probably also playing a role in the solubilization of native crystallins.[48]

4.4. Methylated L-arginine-derivates and endothelial dysfunction

4.4.1. Production and metabolism of L-NMMA, ADMA and SDMA

Methylated arginine analogues are produced by the proteolysis of post-translationally modified proteins. Proteins arginine residues may be methylated to L-N-monomethylarginine (L-NMMA) by protein arginine methyltransferases (PMRTs). Further methylation of L-NMMA may occur either asymmetrically by the type 1 PMRTs producing protein-bound asymmetric dimethylarginine (ADMA), or by the type 2 PMRTs leading to the production of the symmetric SDMA. The arginine-methylated proteins undergo a fast degradation leading to the formation of non-protein-bound, free L-NMMA, ADMA and SDMA.

A majority of the arginine analogues is broken down by the dimethylarginine dimethylaminohydrolase (DDAH) enzyme. The remaining amino acid analogues leave the cells via cationic amino acid transporters, enter the blood. If they are not excreted via glomerular filtration, they may reach the target organ i.e. the endothelium.[61,62]

4.4.2. The effects of the L-arginine analogues

L-NMMA and ADMA are competitive inhibitors of the eNOS enzyme. By binding to NOS, L-NMMA and ADMA are able to compete with the binding of L-Arg to the enzyme, an effect that can be override by high levels of L-Arg. Therefore, in a state of high ADMA and L-NMMA levels, less NO is formed by the endothelium, leading to an increased adhesion of inflammatory cells to the endothelium, an increased platelet aggregation and activation, i.e. to endothelial dysfunction.

Plasma ADMA levels have a narrow distribution in the general population; therefore small alterations may have a high relevance[61]. Elevated ADMA plasma levels have been described among others in patients with hypertension, DM, impaired glucose tolerance, CKD, dyslipidemia, hyperhomocysteinemia and ischemic heart disease.[63]

4.5. Genetic polymorphisms

4.5.1. ACE gene polymorphism

Angiotensin-converting enzyme (ACE) gene insertion/deletion (I/D) polymorphism was first described as a predictor of hypertension[64] and cardiovascular morbidity.[65] A characteristic feature of the polymorphism is that with increasing number of D alleles, also circulating ACE activity and angiotensin-II (At-II) levels increase (II<ID<DD).[66] It is known that in pathological states of high At-II levels – e.g. renal artery stenosis –, there is a high level of oxidative stress, and at the same time one can find an impaired endothelium-dependent vasodilation also in arteries besides the renal artery.[67] In DM, there is a high intrarenal At-II concentration[68], at the same time marked oxidative stress is also present.[69] Many authors have observed more target organ damage, a higher cardiovascular mortality[70], a faster clinical[71] and pathological[72] progression of diabetic nephropathy in case of the ID or DD genotype.

4.5.2. Glutathione peroxidase gene polymorphism

The GPX enzyme is an important antioxidant enzyme. Its role is underlined e.g. by the observation that K.O. mutation of the GPX gene leads to an increased supceptibility of the brain to ischaemia-reperfusion injuries. The enzyme activity is influenced by many factors such as dietary vitamine intake, alcohol consumption, smoking etc.[73] Forsberg and co-workers have found a possible gene polymorphism of the GPX gene upon an EST Blast analysis, and they verified the presence of the polymorphism using a SSCP (single-strand conformational polymorphism) analysis and direct sequencing.[74] The C/T substitution of the 593rd base pair has been found to lead to a Pro/Leu amino acid exchange in position 197. The significance of the Pro/Leu polymorphism is still controversial; one study verified, however another one did not verify a lower enzyme activity of the carriers of the T allele; some studies

found a connection between the GPX-1 genotype and the risk of lung cancer, however in some studies the TT genotype was associated with an increased, in others with a decreased risk of lung cancer compared to the CC genotype.[75] The possible role of GPX-1 in the development of cardiovascular diseases is still unclear.

5. AIMS

5.1. *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.

5.2. 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.

5.3. 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.

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

- 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.

5.5. 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.

6. METHODS

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

6.1.1. Analysis of the *in vitro* Tyr production using fluorescent spectrophotometry[I,II]

We planned to investigate Tyr production in an *in vitro* system containing ferric iron, Phe and H_2O_2. The aromatic side-chain of the Tyr molecule has autofluorescent properties with an absorption maximum at 275 nm, and an emission maximum at 305 nm. To detect Tyr production, fluorescent spectrophotometry in a Hitachi F-4500 spectrophotometer (Hitachi Inc, Tokyo, Japan) was used. We used quartz cuvettes for the measurements, 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.

6.1.1.1. Materials used for the *in vitro* spectrofluorimetric measurements

All materials were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). 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. Water-based solutions were used, and they were prepared daily. The H_2O_2 solution was handled on ice prior to use, to prevent its degradation. Ferric iron is insoluble in water, thus it was dissolved in solutions of Phe or iron chelators to prevent its precipitation. As buffers may be contaminated by traces of iron, and as buffering substances may influence availability of ferric iron, buffer solution was used in selected cases only (as indicated).

6.1.1.2. Tyr production in the *in vitro* system, the effect of the concentration of ferric iron on the Phe-Tyr conversion

The total Tyr production may be studied using fluorescent spectophotometry. The reaction system contained Phe, ferric iron and H_2O_2. Concentration dependency of ferric iron and the effect of the addition of desferrioxamine, a potent iron chelator were investigated. Desferrioxamine irreversibly complexates ferric iron and prevents its oxidative cycling.[5]

In the experiments, 100 μmol/l Phe was pre-incubated with ferric iron (0, 25, 50 or 100 μmol/l) to ensure water-solubility of ferric iron. Out of the Phe-iron mixture, 1 ml was pipetted into a quartz cuvette, then baseline was recorded for one minute. The reaction was started by the addition of 1 mmol/l H_2O_2. In the experiments with desferrioxamine, the iron chelator was pre-incubated with the Phe-iron solution in a concentration of 2 mmol/l. The control mixture contained Phe and 100 μmmol/l ferric iron only.

6.1.1.3. The effect of reversible iron-chelators on the *in vitro* Tyr production

The effect of two reversible iron chelators, EDTA and citrate was studied, as well. A Phe solution (100 μmol/l) was pre-incubated with 100 μmol/l ferric iron and 1 mmol/l EDTA or citrate. After recording baseline for one minute, reaction was started by the addition of 1 mmol/l of H_2O_2. The control sample contained Phe, ferric iron and H_2O_2 only. We also planned to measure the effect of ATP chelation on the reaction, but the fluorescence of ATP interferred with the spectrometric measurement.

6.1.1.4. The effect of superoxide dismutase on Tyr production

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) was used.

Different concentrations (between 0.8 and 100 U/ml) of SOD were used, to check for concentration dependency of the effect of SOD. The reaction mixture contained 100 µmol/l ferric salt, 1 mmol/l EDTA, 1 mmol/l Phe and SOD (in the given concentrations). Reaction was started always by the addition of 1 mmol/l of H_2O_2. To investigate if the effect of SOD is due to its enzymatic function, heat-inactivated (95 °C, 50 minutes) SOD in the concentration of 100 U/ml was also tested. The spectofluorimetric measurement was carried out as described before.

6.1.2. Analysis of the *in vitro* production of Tyr isomers using HPLC with fluorescent detection[I,II]

Using the spectofluorimetric method, only the total Tyr production can be detected. However, this type of measurement does not provide any data on the production of the single Tyr isomers, i.e. p-, m- and o-Tyr. To obtain these data as well, HPLC measurements were carried out. The analysis was performed using a Shimadzu Class LC-10 AD_{VP} HPLC system (Shimadzu USA Manufacturing Inc., Canby, OR, USA) equipped with a Shimadzu RF-10 A_{XL} fluorescent detector (Shimadzu USA Manufacturing Inc., Canby, OR, USA). The amino acids (p-, m-, o-Tyr, Phe) were measured upon their autofluorescence, the Tyr isoforms at 275 nm excitation and 305 nm emission wavelengths, while Phe at 258 nm excitation and 288 nm emission wavelengths. 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. External standard calibration and measurement of areas-under-the-curve (AUC) were used to calculate the exact concentrations of the investigated amino acids.

First, elution time of Phe, p-, m- and o-Tyr was determined by running an aqueous solution of each amino acid, as well as amino acid mixtures. *Figure 3* shows an original registrate of a run of the amino acid mixture containing 350 nM p-, m-, o-Tyr-t and 1 mM Phe. The AUC is proportional to the concentration of the amino acid in the solution.

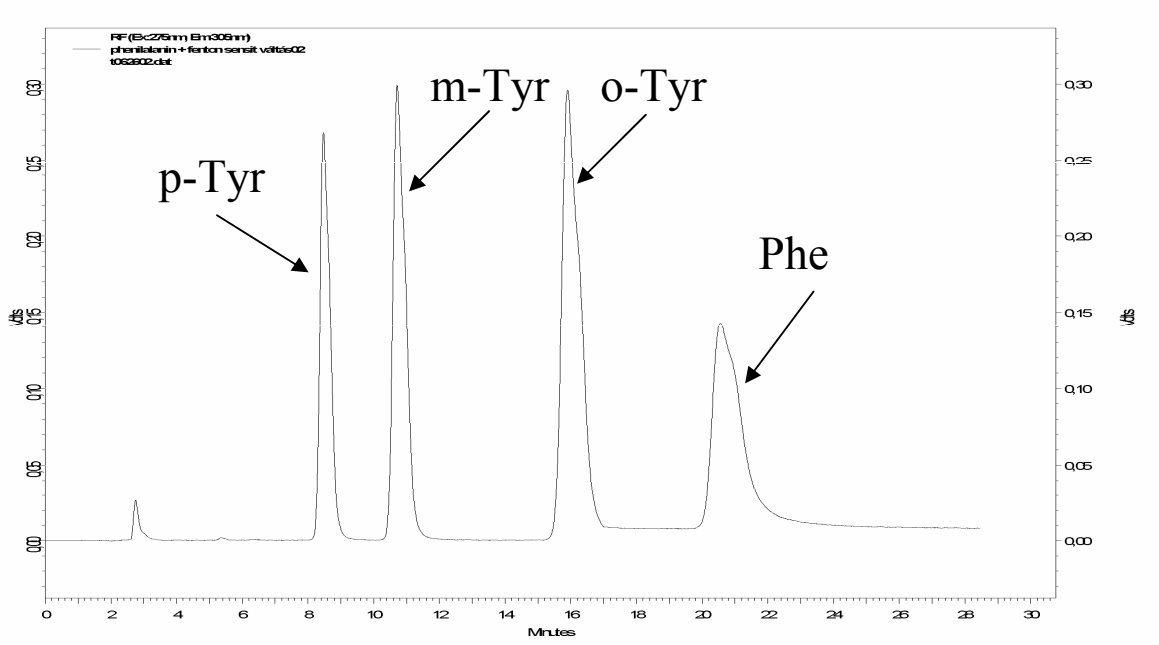

Figure 3
Original record showing the results of the HPLC run of a solution containing 350 nM p-, m-, o-Tyr and 1 mM Phe. The peaks correspond to p-Tyr, m-Tyr, o-Tyr and Phe, respectively. The method contained a change of sensitivity before the Phe peak; therefore the peak of Phe is lower than that of the Tyr isomers despite the higher concentration.

Subsequently, upon measurements of amino acid standard solutions (12.5, 25, 50, 100, µmol/l of p-Tyr and Phe; 50, 100, 200 and 400 nmol/l of m- and o-Tyr was used), calibration curves have been drawn for p-, m-, o-Tyr and Phe, respectively. These curves were used for the external standard calibration.

6.1.2.1. Materials used for the *in vitro* HPLC measurements

p-, m- Tyr, Phe and ATP were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA), while o-Tyr from ICN Biochemicals Inc. (Aurora, OH, USA). The substances were used in the following concentrations: Phe, 0.125, 0.25, 0.5, 1 mmol/l; m- and o-Tyr, 50, 100, 200, 400 nmol/l; p-Tyr, 12.5, 25, 50, 100 µmol/l; desferrioxamine, 2 mmol/l; H_2O_2, 1 mmol/l; ferric-ammonium-sulfate ($FeNH_4SO_4$) 100 µmol/l, adenosine triphosphate (ATP) 1 mmol/l. All amino acid solutions were aqueous. To avoid precipitation of the ferric salt, first Phe and ATP have been dissolved, the ferric salt was only added later so that ATP and Phe were able

to complexate Fe^{3+} and thus prevent its precipitation. When analyzed with HPLC, the presence of ATP does not disturb the detection, as it elutes much faster than the Tyr isomers (see *Figure 11*). Fresh H_2O_2 solution was prepared daily and handled on ice to prevent its degradation.

6.1.2.2. Production of p-, m- and o-Tyr and the effect of desferrioxamine

Production of Tyr isomers was studied in an *in vitro* system containing 1 mmol Phe, 100 µmol/l ferric salt, 1 mmol/l ATP and 1 mmol H_2O_2. The reaction was initiated by the addition of H_2O_2 and the HPLC analysis was carried out after 1 hour incubation. We also investigated the effect of the addition of 2 mmol/l of the iron-chelator desferrioxamine on the reaction. In this case, the reaction was initiated by H_2O_2, as well. After an hour of incubation, 20 µl of the solution was used for the analysis in each case. A 1 mmol/l Phe solution served as control.

6.2. 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

6.2.1. Groups in the study of patients with type 2 diabetes mellitus and/or chronic renal failure[III]

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). Medians of measured and predicted clearance (*Table 2*) in the CKD and DIAB-CKD groups were in the range of Stage III CKD (moderate decrease of

GFR) according to the *Definition and Classification of the Stages of Kidney Disease* by the National Kidney Foundation (i.e. 30 - 59 ml/min)[76]. Diagnoses of the patients with chronic kidney disease were chronic pyelonephritis (n = 4), polycystic kidney disease (n = 3), nephrosclerosis (n = 2), IgA nephropathy (n = 1), minimal change disease (n = 1) and renal vasculitis (n = 1). The groups did not show any significant difference as regards their age (p = 0.426) or gender (p = 0.723).

There was no significant difference between the CKD and the DIAB-CKD patients regarding the severity of their renal impairment (serum creatinine, p = 0.795; measured creatinine clearance, p = 0.272; predicted creatinine clearance [Cockroft-Gault], p = 0.820). The DIAB and the DIAB-CKD groups did not differ in their fructosamine (p = 0.797) and hemoglobin A_{1c} levels (p = 0.298). There was no significant difference between the groups in the parameters of liver function (e.g. serum ALAT, lactic acid dehydrogenase, and alkaline phosphatase activities), serum total cholesterol, HDL and LDL cholesterol (data not shown). Important clinical characteristics of the four groups are shown in *Table 1*.

Table 1 Clinical characteristics of the four groups of patients in the study of human urine and plasma samples

	CONTR	CKD	DIAB	DIAB-CKD
Number of cases	14	12	18	20
Gender (male/female)	4/10	5/7	7/11	8/12
Age (years)	61 (54-65)	54 (51-65)	62 (57-69)	69 (59-73)
Blood urea nitrogen (mmol/l)	4.8 (4.1-6.0)	12.3[a] (10.2-15.8)	7.3 [b] (6.5-9.5)	16.2[a c] (13.1-23.9)
Serum creatinine (µmol/l)	79 (70-90)	190[a c] (147-255)	81 (68-88)	196[a c] (173-257)
Creatinine clearance - measured (ml/min)	109 (83-140)	31[a c] (16-33)	109 (93-124)	38[a c] (23-41)
- predicted (Cockroft) (ml/min)	80 (71-87)	35[a c] (30-56)	109 (90-144)	38[a c] (25-53)
Fructosamine (µmol/l)	-	-	267 (243-304)	259 (235-367)
Hemoglobin A_{1c} (%)	-	-	8.65 (8.22-9.59)	7.13 (6.23-9.01)

Abbreviations are: CONTR, group of healthy control subjects; CKD, group of patients with Stage III. chronic kidney disease; DIAB, group of diabetic patients; DIAB-CKD, group of patients with diabetes and Stage III. chronic kidney disease. Data are shown as median and (inter-quartile range). The Mann-Whitney-U test was only used when the Kruskall-Wallis test for all groups was significant ($p < 0.05$); [a], $p < 0.05$ vs. control subjects; [b], $p < 0.05$ vs. CKD patients; [c], $p < 0.05$ vs. type 2 diabetic patients.

Twenty-four hour collected urine and heparinized fasting plasma samples were obtained, and routine clinical parameters were also measured. As diet may influence plasma o-Tyr levels, fasting plasma samples were obtained. For determination of laboratory parameters, standard methods were used. The study was approved by the Ethical Committee of the Medical Faculty of the University of Pécs. All patients and controls gave informed consent.

6.2.2. Determination of non-protein bound ortho- and para-tyrosine in urine and fasting plasma samples[III]

A modification of the method described by Ishimitsu et al[77] was used for the analysis. Briefly, from the 24 hour collected urine or freshly obtained fasting heparinized plasma samples, aliquots of 250 µl were taken and handled on ice. 125 µl of 60 % trichloroacetic acid was added to the samples, vortexed, and incubated 30 minutes on ice to precipitate protein content. To remove the precipitate, samples were then centrifuged at 15,000 rpm for 10 minutes in Eppendorf tubes. The supernatant was filtered through a 0.2 µm syringe filter (Millipore Co., Billerica, MA, USA), and 20 µl was injected into the manual injector of the HPLC device. The run was performed as described earlier. In some cases standard peak-addition was also used to verify the elution time of the substances.

6.2.3. Determination of urinary 8-epi-prostaglandin-F2α[III]

Urinary 8-epi-prostaglandin-$F_{2\alpha}$ levels were determined by a competitive ELISA assay kit purchased from Oxis Research (Oxis Health Products, Portland, Oregon, USA).

6.2.4. Statistical analysis[III]

As most data were of non-normal distribution, we used the non-parametric Kruskall-Wallis test and Mann-Whitney U test and Spearman's rho correlation. Because of the non-normal distribution, median and the inter-quartile range were used to characterize distribution of the data. To reduce false positive results, multiple comparisons were carried out using Kruskall-Wallis and median-test, while the Mann-Whitney U test was only used for two-group comparisons, where p values < 0.05 were regarded as statistically significant.

6.3. Clinical study of cataract patients with and without type 2 diabetes mellitus

6.3.1. Patient groups and control lenses[IV]

In a cross-sectional study we examined human lenses that were removed during extracapsular cataract surgery, upon informed consent. Cadaver eyes enucleated for corneal transplantation served as controls. The study was approved by the Ethical Board of the Medical Faculty of the University of Pécs.

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. This finding is not surprising, as progredient phase of senile cataract (i.e. in the non-DM CAT group) develops at older age than diabetic cataract of the same phase (i.e. in the DM CAT group). Main clinical characteristics of the patients are shown in *Table 2*.

Table 2 Clinical characteristics of the patients in the study of human lenses

	CONTR	DM CAT	Non-DM CAT
Number of cases	n = 17	n = 22	n = 20
Age (years)	62 (48 - 73)	67 (64 - 78)	75[a b] (71 - 82)
Hb A_{1c} (%)		6.74 (6.35 - 7.64)	
Fructosamine (μmol/l)		272 (227 - 334)	
Fasting plasma glucose (mmol/l)		8.10 (7.03 - 9.23)	

Abbreviations: CONTR, group of control subjects; DM CAT, group of cataract patients with type 2 diabetes mellitus; non-DM, group of cataract patients without diabetes mellitus; Hb A_{1c}, hemoglobin A_{1c}; [a], $p < 0.05$ vs. CONTR; [b], $p < 0.05$ vs. DM CAT. Data are given as median (inter-quartile range).

6.3.2. Cataract surgery and preparation of control lenses[IV]

All cataractous lenses were removed under the same circumstances by the same surgeon. Surgery was carried out under topical anaesthesia through a 3.2 mm long clear corneal incision after dilation of the pupil. Capsulorhexis was performed under the infusion of a viscoelastic material and was followed by hydrodissection and rotation of lens nucleus. The lens nucleus was removed by a "divide and conquer" phacoemulsification technique, the cortex by irrigation-aspiration. The posterior capsule was polished and vacuum-cleaned when needed, and a foldable artificial lens was implanted into the capsular bag under viscoelastic material, after wound enlargement. Viscoelastic material was then washed out thoroughly and operation was finished with subconjunctival gentamycine and dexamethasone injection. Cadaver lenses were extracted from the eye, decapsulated, and were subsequently transported in a buffered medium. Further process was carried out without delay.

6.3.3. Protein solubility and distribution[IV]

The specimen from the surgical medium was extracted immediately after surgery by centrifugation for 30 minutes at 13,000 rpm. The obtained pellet was resuspended in a Tris-buffered solution (pH 6.8) containing EDTA (1 mmol/l), ethylene glycol-bis(2-aminoethylether)-tetraacetic acid (EGTA, 4 mmol/l) and phenylmethanesulfonyl fluoride

(PMSF, 0.2 mmol/l). After this, both the cataractous and the control samples were treated and homogenized the same way. Assessment of protein concentration of the total homogenate was carried out according to Bradford from aliquot amounts of samples following repeated homogenization.[78]

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 protein concentration of the supernatants was assessed according to Bradford, as well. The ratio of soluble proteins was calculated as percent of the total protein content. Until further process, the homogenates and supernatants were stored at – 70 °C. The same samples were used for the investigation of protein distribution, for protein electrophoresis and high performance liquid chromatographic analysis.

6.3.4. Gel-electrophoretic studies[IV]

Separation of proteins was performed by sodium-dodecylsuphate polyacrylamide gel-electrophoresis (SDS-PAGE) according to Laemmli.[79] Comparative PAGE was performed both from proteins of the total homogenate and the supernatant proteins. Laemmli buffer was added to proteins. 3-3 µg protein per line was analyzed 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[80] following the traditional Coomassie brilliant blue (CBB) R-250 staining. After documentation of the electrophoretograms, the stained gels were assessed by direct densitometry.

6.3.5. High performance liquid chromatographic analysis of lens samples[IV]

The measurement of amino acid composition of lens proteins was carried out in a high performance liquid chromatographic (HPLC) analysis by modification of the method

described previously in *Chapter 5.2.2*. In well-closing, O-ring protected polypropylene tubes 200 µl of the samples was measured, subsequently 4 µl of 400 mmol/l desferrioxamine (final concentration: 3.6 mmol/l) and 40 µl of 500 mmol/l butylated hydroxytoluene (final concentration: 45 mmol/l) were added to avoid possible free radical formation during hydrolysis. Then 200 µl of 12 N hydrochloric acid was added, and we performed an overnight acid hydrolysis of the proteins at 120 °C.

The hydrolyzate was then filtered through a 0.2 µm filter (Millipore Co., Billerica, MA, USA) and 20 µl of the filtrate was injected onto the HPLC column of a Shimadzu Class LC-10 ADVP HPLC device (description see above) using a Rheodyne manual injector. Quantitative analysis of the amino acids was carried out using a Licrospher-C_{18} silica column (Merck) upon their autofluorescence. Tyr isoforms and DOPA were measured at 275 nm excitation and 305 nm emission, while Phe at 258 nm excitation and 288 nm emission wavelengths using a Shimadzu RF-10AXL fluorescent detector. Therefore no pre-column or post-column staining or derivatization was required. An isocratic HPLC run was performed, the eluent contained 1 % acetic acid, 1 % sodium-acetate and 98 % distilled water. The area-under-the-curve (AUC) was determined for the amino acids, and exact concentrations were calculated using external standard calibration. In some cases the elution time of the substances was also verified by standard peak-addition method. The amino acid concentrations were corrected for protein content or Phe concentrations.

6.3.6. Statistical analysis[IV]

Statistical analysis was performed using the SPSS 10.0 software (SPSS Inc., IE, USA). As most data were of non-normal distribution, we used median and inter-quartile range to characterize distribution of the data, for the same reason Kruskal-Wallis test and Mann-Whitney U-tests were used for between-group comparisons.

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

6.4.1. Clinical characteristics of the patients[v]

Clinical features of our patients (n = 145, patients with type 2 diabetes mellitus) participating in the cross-sectional study are shown in *Table 4*. A part of the patients was treated using oral hypoglycemic agents (n = 39), while the other ones were treated with insulin (n = 106). ACE gene polymorphism was determined in collaboration using a sensitized touchdown polymerase chain reaction (PCR) method[81] 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.

Table 4 Clinical parameters of patients (n = 145) involved in the study.

	Median (minimum-maximum)
Age (years)	64 (29-82)
Duration of diabetes (years)	13.5 (0-34)
Age at the diagnosis of diabetes (years)	48 (24-78)
Body mass index (kg/m^2)	30.1 (15.9-47.8)
Hemoglobin A_{1c} (%)	7.0 (5.2-16.3)
Fructosamine (μmol/l)	319 (180-640)
Random plasma glucose (mmol/l)	8.4 (3.2-21.2)
Serum total cholesterol (mmol/l)	5.5 (2.3-11.5)
Serum LDL cholesterol (mmol/l)	3.3 (0.9-6.2)
Serum HDL cholesterol (mmol/l)	1.14 (0.6-2.7)
Serum triglyceride (mmol/l)	1.9 (0.4-18.3)
Blood urea nitrogen (mmol/l)	7.3 (3.4-31.4)

Serum creatinine (μmol/l)	88 (42-387)
Glomerular filtration rate (ml/min)†	76 (15-261)
Albuminuria (mg/day)	15 (3-1334)
Serum γ-glutamyl transferase activity (U/l)	28 (7-238)
Systolic blood pressure (mmHg)	135 (100-190)
Diastolic blood pressure (mmHg)	70 (60-110)
Number of antihypertensive drugs used (n)	4 (0-7)

Abbreviations: HDL, high density lipoprotein; †, GFR was estimated upon the Cockroft-Gault formula.

6.4.2. Statistical analysis[V]

The parameters with a non-normal distribution were characterized by median and range. Because of the non-normal distribution we used non-parametric tests such as the Mann-Whitney U-test, median-test, Kendall's tau-b correlation for the statistical analysis of the data (SPSS v. 10.1, Chicago, Ill., USA). To reduce false positive results, multiple comparisons were carried out using Kruskal-Wallis- and median-test, while Mann-Whitney U-test was only used for two-group comparisons.

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

6.5.1. Determination of GPX genotype[VI]

The GPX genotyping has been carried out in collaboration in Berlin, Germany. 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.

6.5.2. HPLC measurement of the L-arginine analogues[VI]

Upon informed consent, plasma samples were obtained from the patients in a fasting state. To avoid degradation of the L-arginine analogues, plasma samples were handled on ice until further processing.

In contrast to Phe and the Tyr isoforms, neither one of the L-arginine analogues possesses fluorescent properties, therefore pre-column derivatization was required to be able to use fluorescence for detection. Amino acids were stained using ortho-phtalaldehyde (OPA), which substance binds to the amino groups of the amines – if there is an abundance of thiol groups in the reaction mixture –, and is thereby converted to a fluorophore.

To obtain non-protein bound amino acids, following procedure was carried out: 10 mg sulphosalicylic acid was dissolved in 500 µl of distilled water, and then 500 µl of the plasma was added. Twenty microliters of 5 µmol/l homotyrosine (internal standard) and 80 µl of distilled water were added. Then samples were kept 10 minutes on ice to precipitate protein content. The precipitate was removed by centrifugation (4,500 g, 4 °C, 10 min). Fivehundred milliliters of the supernatant were pipetted into an Eppendorf tube, and 500 µl 0.05 mol/l potassium-tetraborate solution (pH 9.46) was added. For the derivatization, an OPA-mercaptoethanol solution was prepared by dissolving 10 mg OPA in 200 µl methanol, adding 10.6 µl mercaptoethanol and 1800 µl of the borate buffer. For the pre-column derivatization, 20 µl of the OPA-mercaptoethanol was added to 80 µl of the borate-buffered plasma. Sixty seconds were allowed for staining, then 20 µl of 1 % orthophosphoric acid was added to stop the reaction. The solution was then filtered through a 0.2 µm syringe filter (Millipore Inc. Co., Billerica, MA, USA) and 20 µl was injected into the Shimadzu HPLC device.

The analysis was performed on a Shimadzu Class 10aVP device (specification see above) using a Yamamura C_{18} ODS column (YMC Europe GmbH., Schermbeck, Germany), in a gradient run. The eluents were 10 mmol/l potassium-phosphate buffer + 10 % acetonitryl

(Eluent A) and a 40 % acetonitryl + 30 % methanol solution (Eluent B). The time program was as follows:

Time (min)	0.01	2.5	17.5	22.5	25	27.5	30	45
A conc. (%)	90	90	80	71.8	20	20	90	90
B.conc. (%)	10	10	20	28.2	80	80	10	10

The amino acids were detected with the fluorescent detector at 350 nm excitation and 450 nm emission wavelengths. Standard peak-addition method using the addition of 80 µl of the standard solutions (containing L-NMMA, L-ADMA, L-SDMA and homoarginine) to pooled plasma was performed. AUC were measured, exact concentrations were determined using AUCs and a calibration curve.

6.5.3. Statistical analysis[VI]

As data were of normal distribution, they were characterized by mean ± SD. The groups were compared using a Student t-test of the SPSS software.

7 RESULTS

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

7.1.1. Kinetics of the phenylalanine-to-tyrosine conversion, and the concentration-dependent effect of iron on the conversion reaction[I,II]

The spectrofluorimetric method was adequate to detect Tyr production in the *in vitro* system. We performed a six minute time scan. Results are shown on *Figure 4*. The curves represent the following *in vitro* systems: (1) Phe (dark blue line), + (2) Phe + H_2O_2 (black line), (3) Phe + 25 µmol/l ferric iron + H_2O_2 (light blue line), (4) Phe +50 µmol/l ferric iron + H_2O_2 (red line), (5) Phe + 100 µmol/l ferric iron + H_2O_2 (green line), (6) Phe + 100 µmol/l ferric iron + 2 mmol/l desferrioxamine + H_2O_2 (dark blue line). The control curve (dark blue line, Phe only) did not show any time-dependent elevation. Addition of H_2O_2 resulted in a slight elevation of the curve (black line), the addition of ferric iron caused a concentration-dependent increase in Tyr-fluorescence. The pre-incubation of the system with desferrioxamine totally inhibited Tyr production, this curve is running on the baseline together with the control line, they cannot be separated from each other that is, why only one dark blue line is visible.

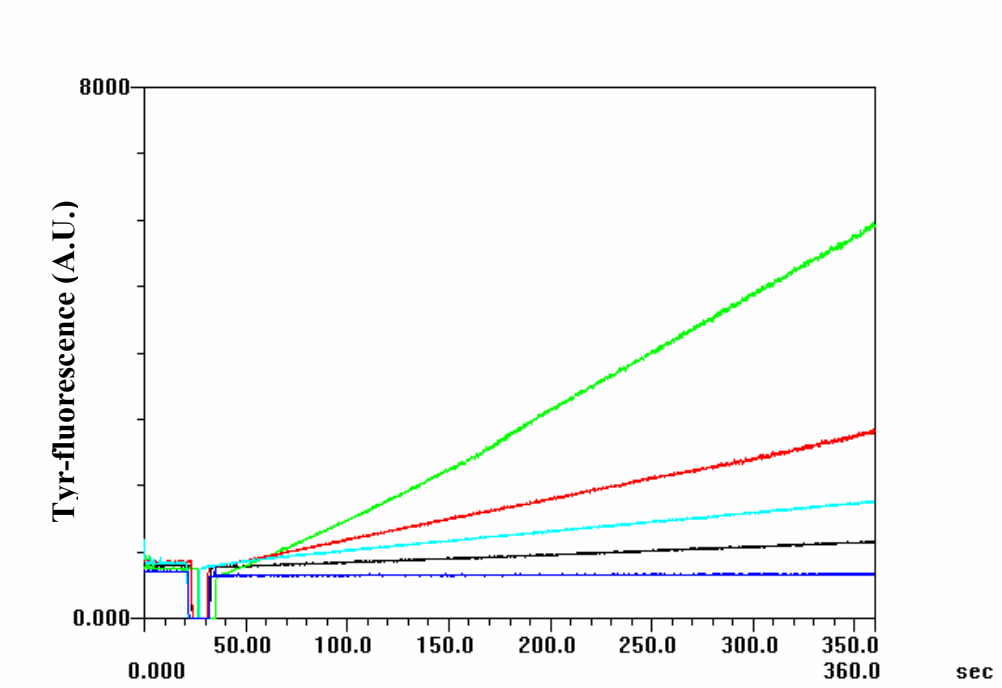

Figure 4
Tyrosine production in an *in vitro* system containing phenylalanine with or without the presence of ferric iron and hydrogen peroxide.
Dark blue curve (—), phenylalanine (Phe) only (control); black curve (—), Phe + H_2O_2; light blue curve (—), Phe + 25 µmol ferric iron + H_2O_2; red curve (—), Phe + 50 µmol ferric iron + H_2O_2; green curve (—), Phe + 100 µmol ferric iron + H_2O_2; dark blue curve (—) (running together with the control), Phe + 100 µmol ferric iron + H_2O_2 + desferrioxamine.

Figure 5 shows the results of the statistical evaluation of the spectrophotometric results. We have observed that, addition of H_2O_2 to Phe results in Tyr production; however the increase in fluorescence was not significant. Increasing concentrations of ferric iron lead to a concentration-dependent increase of Tyr production, which increase could be totally inhibited by the iron redox-cycle inhibitor, desferrioxamine.

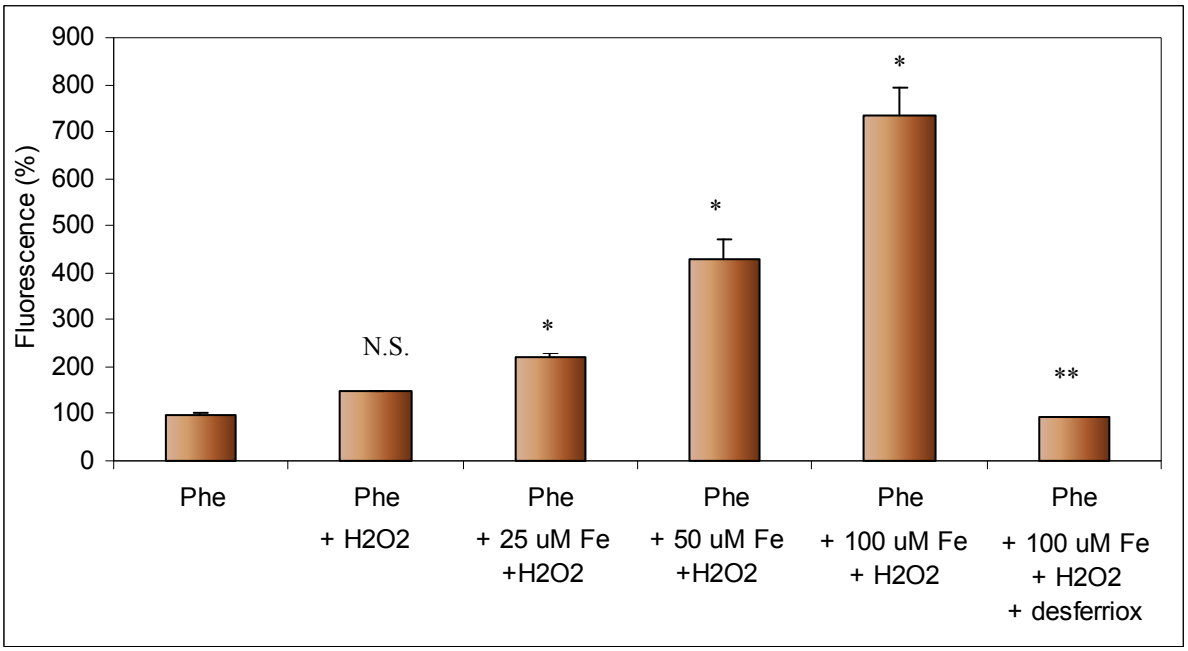

Figure 5
Tyrosine production in an *in vitro* system containing phenylalanine with or without the presence of ferric iron and hydrogen peroxide.
Abbreviations: Phe, phenylalanine; H_2O_2, hydrogen peroxide; Fe, ferric iron; desferriox, desferrioxamine; *, $p < 0.05$ vs. Phe only, **, $p < 0.05$ vs. Phe+100µmol/l Fe + H_2O_2.

7.1.2. Iron complexes and tyrosine production[II]

We further investigated the effect of iron chelators on Tyr production. Spectrofluorimetric measurements are represented on *Figure 6*. Iron chelators EDTA and citrate influenced the kinetics of Tyr production. 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. The effect of ATP could not be analyzed on a spectrofluorimeter, as ATP itself is excited at this wavelength, and absorbs light inhibiting this way the detection of Tyr molecules.

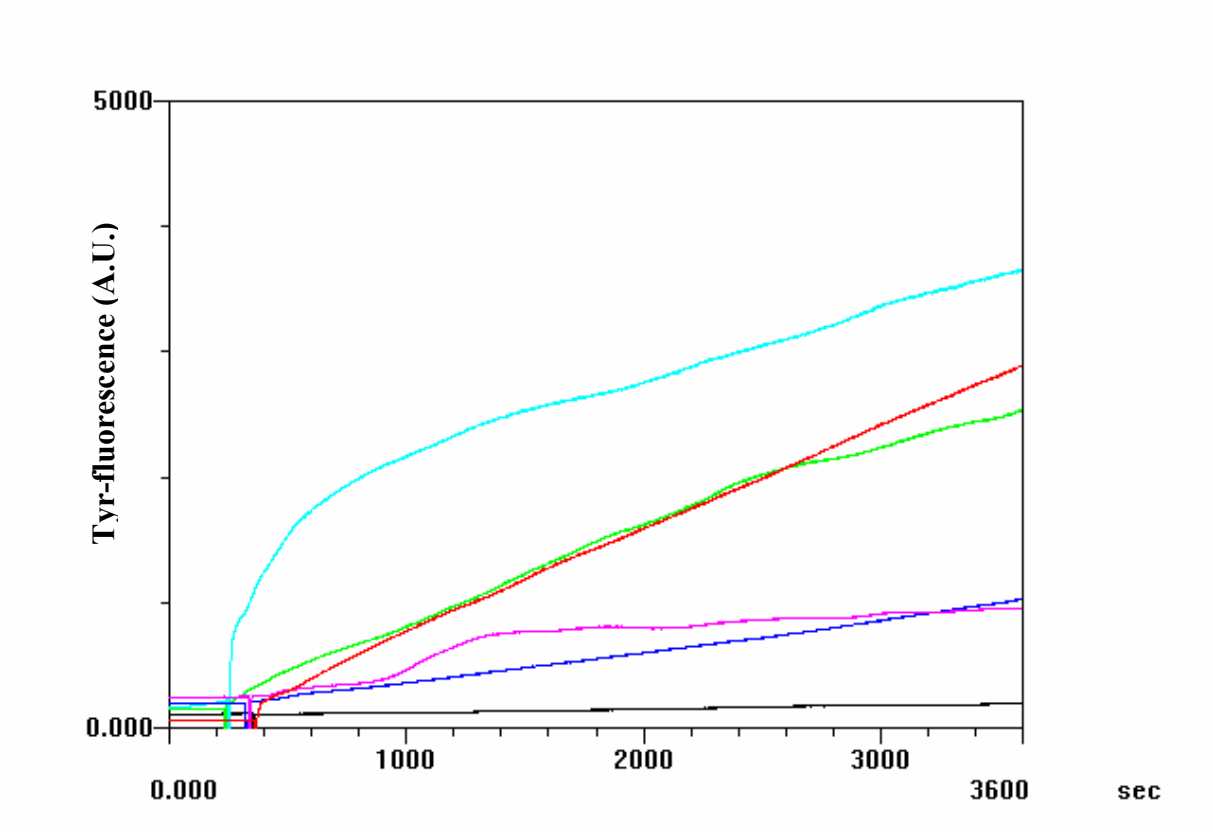

Figure 6
The effect of iron chelators on the phenylalanine-to-tyrosine conversion.
Black curve (—), Phe+Fe+H_2O; green curve (—), Phe+Fe+H_2O_2; light blue curve (—), Phe+Fe+citrate+H_2O_2; red curve (—), Phe+Fe+EDTA+H_2O_2; magenta curve (—), Phe+Fe+citrate+H_2O_2+PB; dark blue curve (—), Phe+Fe+EDTA+ H_2O_2+PB
Abbreviations: EDTA, ethylenediamine-tetraacetic acid; Fe, ferric iron; H_2O, distilled water; H_2O_2, hidrogen proxide; PB: phosphate buffer; Phe: phenylalanine; Tyr, tyrosine

Figure 7 shows that there was a marked Tyr production in the presence of ferric iron, Phe and H_2O_2. The extent of Tyr production was not significantly higher in the presence of EDTA, but was higher in the presence of citrate compared to Phe+Fe+H_2O_2 alone.

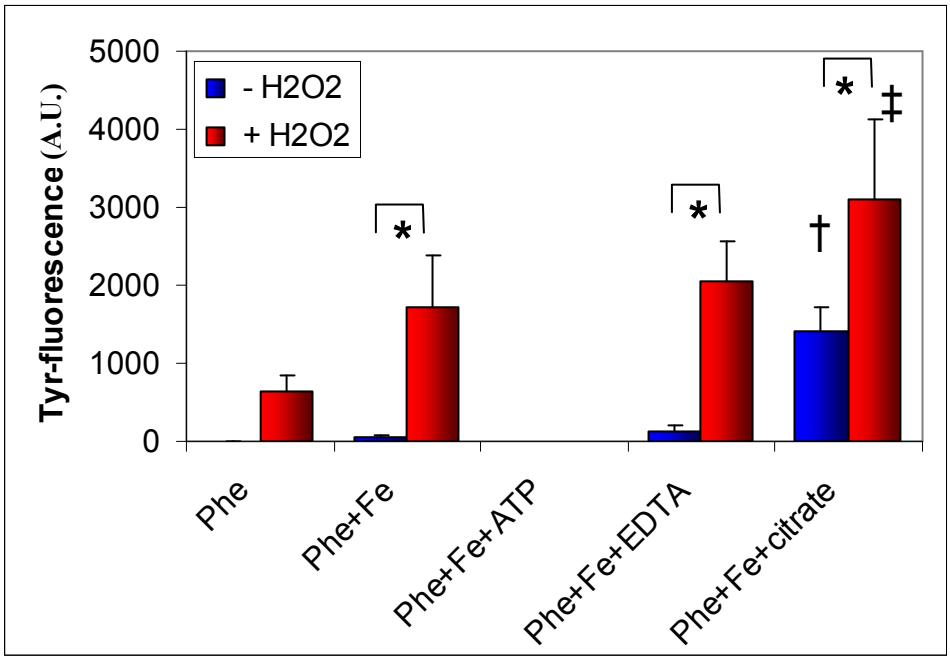

Figure 7
Phenylalanine-to-tyrosine conversion in the presence of ferric iron and iron complexes.
The blue bars (■) represent the reaction mixture in the absence of H_2O_2, the red bars (■) represent the reaction mixture in the presence of H_2O_2.
Abbreviations: Fe, ferric iron; ATP, adenosine triphosphate; EDTA, ethylenediamine-tetraacetic acid; †, $p<0.05$ vs. Phe+Fe; ‡, $p<0.05$ vs. Phe+Fe+H_2O_2; *, $p<0.05$ vs. in the absence of H_2O_2.

In the classical Fenton reaction, besides ·OH, also OH^- is formed. Therefore, the reaction should be sensitive to pH. Especially in the case of citrate, the acidic pH could lead to the consumption of OH^-, thereby shifting the reaction to the right, promoting free radical formation. Therefore, the citrate and EDTA experiments were repeated using a Sørensen phosphate buffer (pH 7.4) as vehicle, as shown on *Figure 8*:

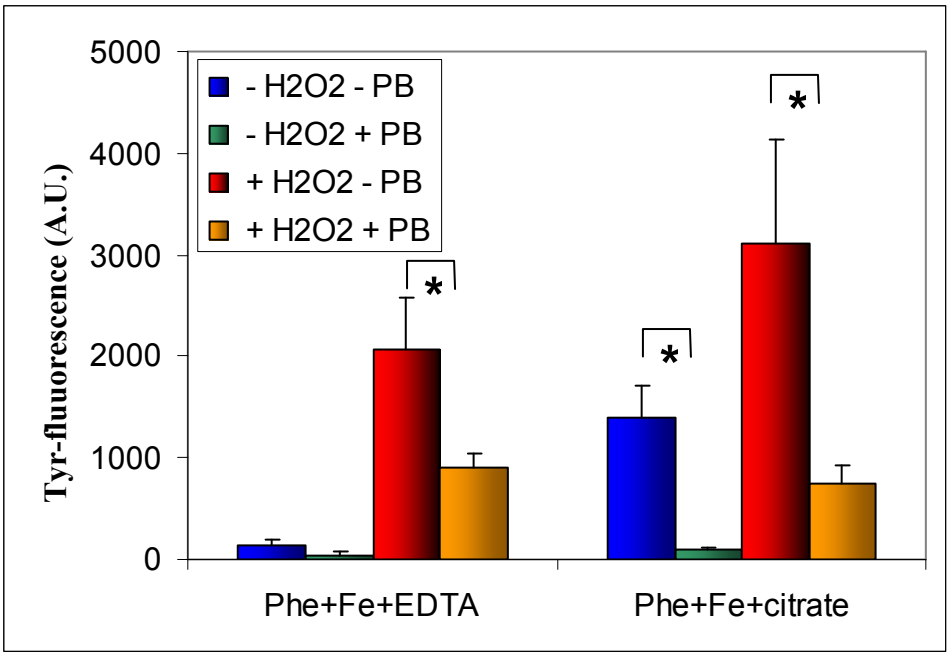

Figure 8
The effect of pH on tyrosine production in the *in vitro* reaction.
The blue bars ( ) represent the reaction mixture in the absence of H_2O_2, the red bars ( ) represent the reaction mixture in the presence of H_2O_2. The green bars ( ) and the orange bars ( ) represent the systems depicted in blue and red, repectively, but with a Sørensen phosphate buffer (pH 7.4) as vehicle.

As seen on *Figure 8*, 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.

7.1.3. The effect of SOD on Tyr production[I,II]

To investigate, whether the superoxide free radical may play a role in this Fenton-like reaction, SOD was added to the reaction mixture. To ensure that the effect of the addition of SOD is a consequence of its enzymatic property, also heat-inactivated SOD was tested.

Addition of SOD decreases Tyr production in a concentration-dependent manner *(Figure 9)*. Fifty minute heating (at 95 °C) completely abolishes the effect of SOD, and Tyr production is similar to the system containing no SOD.

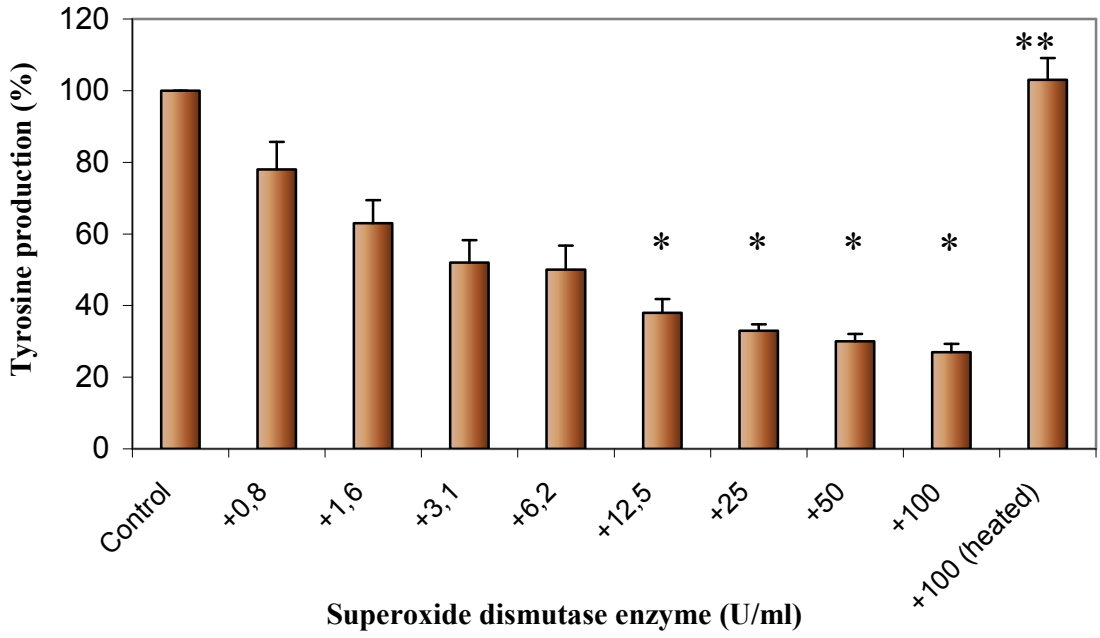

Figure 9
Inhibitory effect of SOD on the Phe-Tyr conversion. The Tyr production of the 'control' state (containing no SOD) was regarded as 100%.
Abbreviations: *, $p < 0.05$ vs. Control; **, $p < 0.05$ vs. +100 U/ml SOD.

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

7.2.1. Para-, meta-, ortho-tyrosine production in the presence of the ferric iron – hydrogen peroxide system. The effect of desferrioxamine on the reaction.[I,II]

In chapter 4.1. we demonstrated the increase of tyrosine-like fluorescence as a consequence of hydroxyl free radical attack on Phe. This method however does not provide information on the *in vitro* production of the different Tyr isomers. Therefore, experiments were repeated, the Tyr isomers and Phe were separated by HPLC. *Figure 10* demonstrates two chromatograms, ***i)*** the black curve shows that in the absence of hydrogen peroxide and ferric iron no Tyr isomers are produced, there is a single peak corresponding Phe; ***ii)*** the red curve shows that in the presence of Phe, adenosine triphosphate (ATP), ferric iron and

hydrogen peroxide, all three isomers of Phe are formed. The first peak is that of ATP, the amino acids are represented by the peaks as follows: p-Tyr, m-Tyr, o-Tyr, Phe.

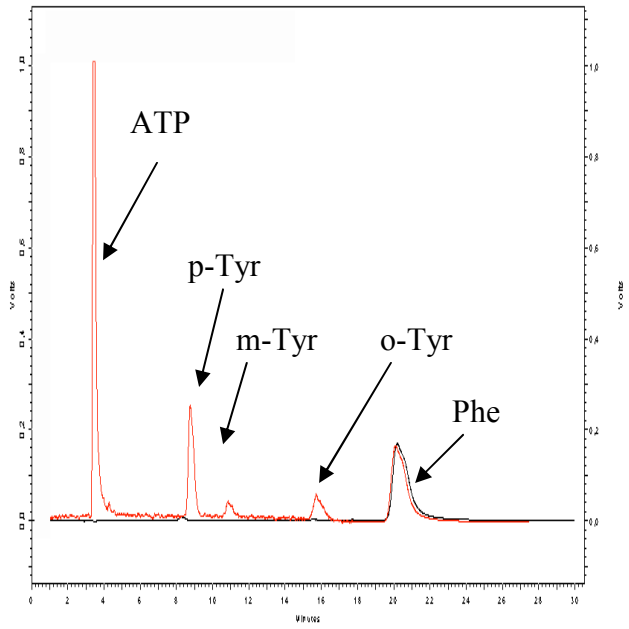

Figure 10
Para-, meta- and ortho-tyrosine production in the *in vitro* system.
The chromatograms represent the phenylalanine-containing solution (black curve) and the phenylalanine – ferric iron – hydrogen peroxide – ATP containing solution (red curve). Abbreviations: ATP, adenosine triphosphate; p-Tyr, para-tyrosine; m-Tyr, meta-tyrosine; o-Tyr, ortho-tyrosine; Phe, phenylalanine.

Experiments were carried out in triplicates, and repeated in the presence of desferrioxamine, as well. Amounts of the produced amino acids, p-Tyr, m-Tyr, o-Tyr, and the concentrations of Phe in the experiments are represented on *Figure 11, panels A, B, C, D,* respectively.

The structure of all four diagrams is similar, i.e. the first bar represents the concentration of the amino acid in the phenylalanine-containing solution, the second bar shows the concentration of the amino acid in the phenylalanine – ferric iron – hydrogen peroxide – ATP containing solution, the third bar indicates the concentration of the amino acid in the solution that was pre-incubated with the iron-chelator desferrioxamine. Here we

draw the attention that the units on the y axis of the first three diagrams *(Figure 11, panels A, B and C)* are represented as nmol/l, while the units on *Figure 11, panel D* are given in mmol/l.

The diagrams show, that in the control state, when the solute did not contain the hydroxyl free radical producing *in vitro* system, no p-Tyr, no m-Tyr and no o-Tyr was present. 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 *(Figure 11, panels A, B, C)*. Neither the presence of the free radical-producing system, nor the addition of desferrioxamine caused a significant change of the concentration of Phe *(Figure 11, panel D)*.

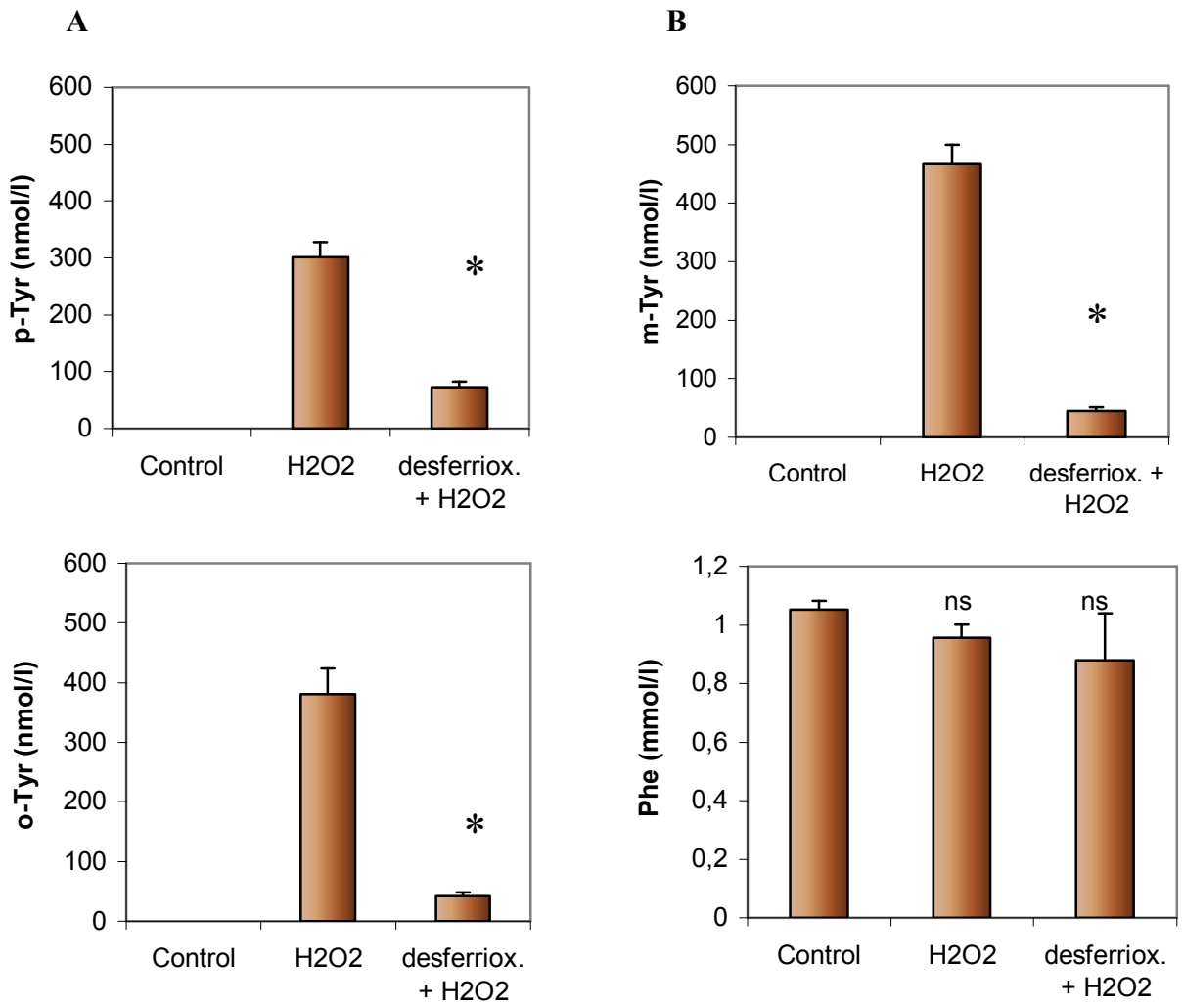

Figure 11
Concentrations of p-Tyr (Panel A), m-Tyr (B), o-Tyr (C) and Phe (D) before and after the addition of the *in vitro* hydroxyl free radical-producing system (1 mmol/l H_2O_2 and 100 µmol/l Fe^{3+}, 1 mmol/l ATP) to the 1 mmol/l Phe solution. The effect of pre-incubation with desferrioxamine (2 mmol/l).
Abbreviations: p-Tyr, para-tyrosine; m-Tyr, meta-tyrosine; o-Tyr, ortho-tyrosine; Phe, phenylalanine; H_2O_2, hydroxyl free radical producing *in vitro* system; *, $p < 0.001$ (vs. H_2O_2).

7.2.2. The effect of iron chelators on the *in vitro* reaction[II]

The effect of iron chelators on the production of Tyr isomers in the *in vitro* reaction was studied by the use of HPLC. The reaction mixtures depicted on *Figure 7* were immediately transferred to the HPLC device, and analyzed for Tyr isomers and Phe content. Production of all Tyr isomers increased in the presence of EDTA and citrate + H_2O_2 *(Figure 12)*, while the Tyr production was decreased by the phosphate buffer (*Figure 12)* and ATP *(not shown).*

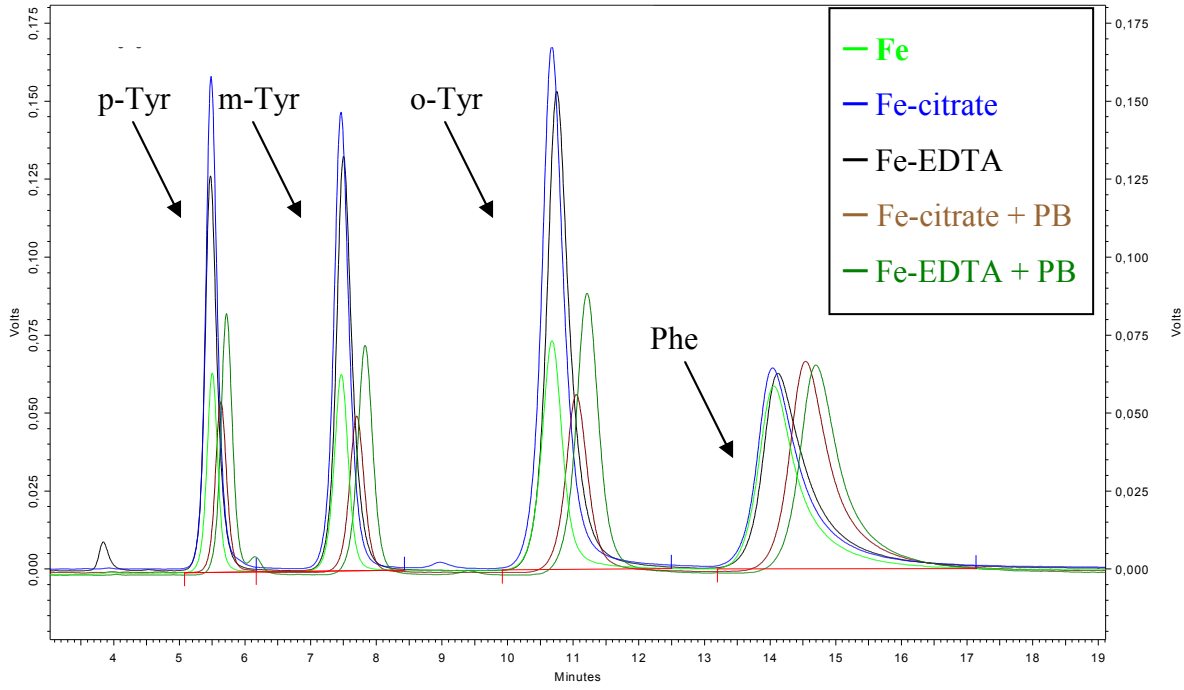

Figure 12
Effect of iron chelators and pH on the *in vitro* reaction.
Original record from the HPLC device; the curves represent chromatograms of the *in vitro* system in the presence of (i) Phe + Fe + H_2O_2 (**light green**), (ii) Phe + citrate + H_2O_2 (**blue**), (iii) Phe + Fe-EDTA + H_2O_2 (**black**), (iv) Phe + Fe-citrate + H_2O_2 + phosphate buffer (**brown**) and (v) Phe + Fe-EDTA + H_2O_2 + phosphate buffer (**dark green**).

Abbreviations: Fe, ferric iron; EDTA, ethylene-diaminetetraacetic acid; p-Tyr, para-tyrosine; m-Tyr, meta-tyrosine; o-Tyr, ortho-tyrosine; PB, phosphate buffer; Phe, phenylalanine.

Experiments were carried out in triplicates, the amounts of the amino acids have been measured upon the AUCs. Numerical results of the three sets of experiments are shown on *Figure 13*, where data are represented as mean ± SD.

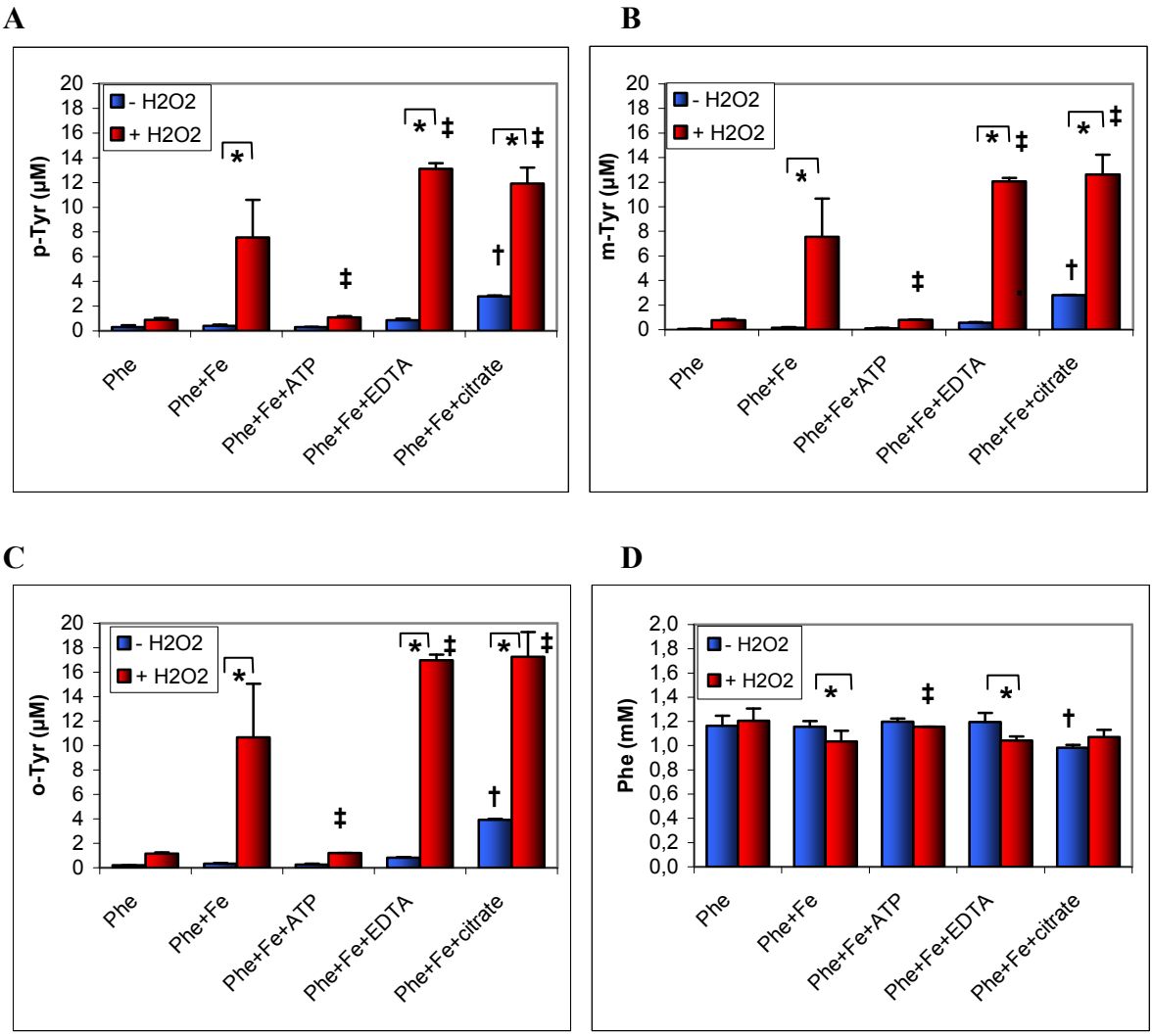

Figure 13
The amount of the produced para-, meta- and ortho-tyrosine and the remaining phenylalanine in the *in vitro* reactions. The effect of iron chelators on the reaction.
The blue bars (■) represent the reaction mixture in the absence of H_2O_2, the red bars (■) represent the reaction mixture in the presence of H_2O_2.
Abbreviations: p-Tyr, para-tyrosine; m-Tyr, meta-tyrosine; o-Tyr, ortho-tyrosine; Phe, phenylalanine; Fe, ferric iron; ATP, adenosine triphosphate; EDTA, ethylenediamine-tetraacetic acid; †, $p<0.05$ vs. Phe+Fe; ‡, $p<0.05$ vs. Phe+Fe+H_2O_2; *, $p<0.05$ vs. in the absence of H_2O_2.

As seen on *Figure 13*, the results are similar to the fluorimetric data, except for the lack of significance in the case of Phe+Fe+EDTA+H_2O_2. 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 compared to Phe-Fe-H_2O_2. Phe consumption in the reaction mixtures was lower in the Phe-Fe-ATP-H_2O_2 system than in the Phe-Fe-H_2O_2 system.

The experiments with the Sørensen phosphate buffer (pH 7.4) as vehicle were analyzed by HPLC, too, as shown on *Figure 12 and 14*:

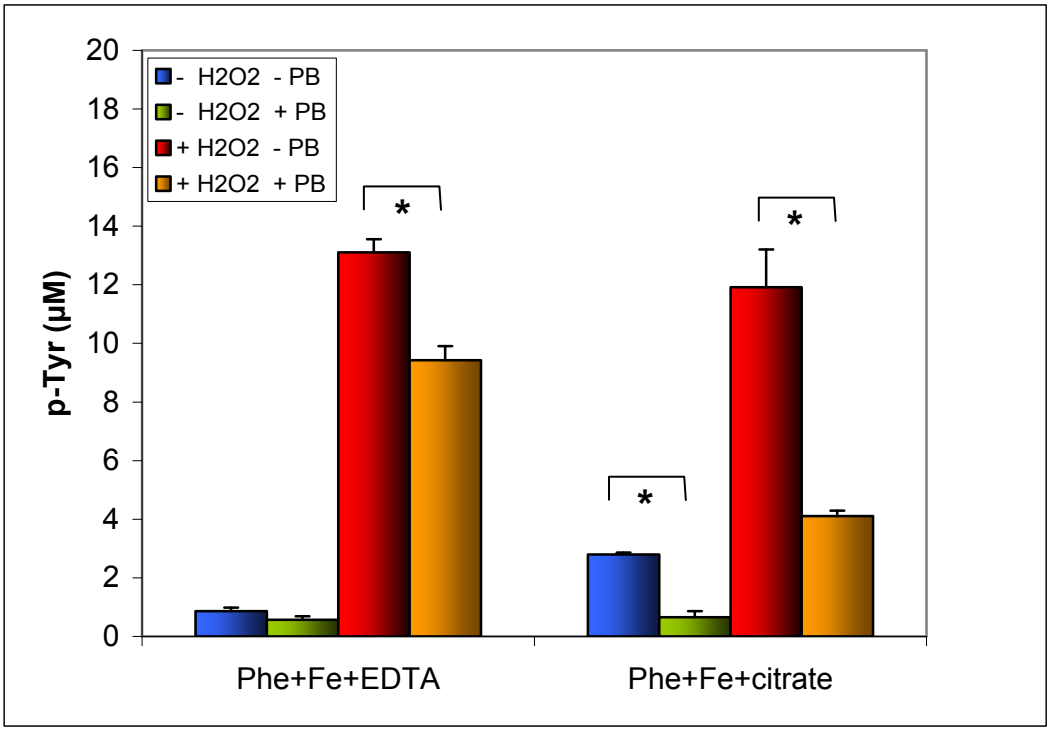

Figure 14
The effect of pH on tyrosine production in the *in vitro* reaction.
The blue bars (■) represent the reaction mixture in the absence of H_2O_2, the red bars (■) represent the reaction mixture in the presence of H_2O_2. The green bars (■) and the orange bars (■) represent the systems depicted in blue and red, repectively, but with a Sørensen phosphate buffer (pH 7.4) as vehicle.
Abbreviations: p-Tyr, para-tyrosine; Fe: ferric iron; EDTA, ethylenediamine-tetraacetic acid; H_2O_2: hydrogen peroxide; PB: phosphate buffer; *, $p<0.05$ vs. without phosphate buffer.

Results shown on *Figure 14* correspond well to the fluorimetric data, i.e. lower p-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 in the presence of the phosphate buffer.

7.3. 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.)

7.3.1. Detection of the single amino acids[III]

Both o- and p-Tyr could be well detected in the urine samples (*Figure 15*). Urinary Phe levels were below detection limit in the majority of cases, and so urinary Phe levels were not measured. In the plasma samples, p-, o-Tyr and Phe were present, all at well measurable concentrations. m-Tyr was either not detectable or co-eluted with another substance (proven with standard peak-addition), therefore it was not determined.

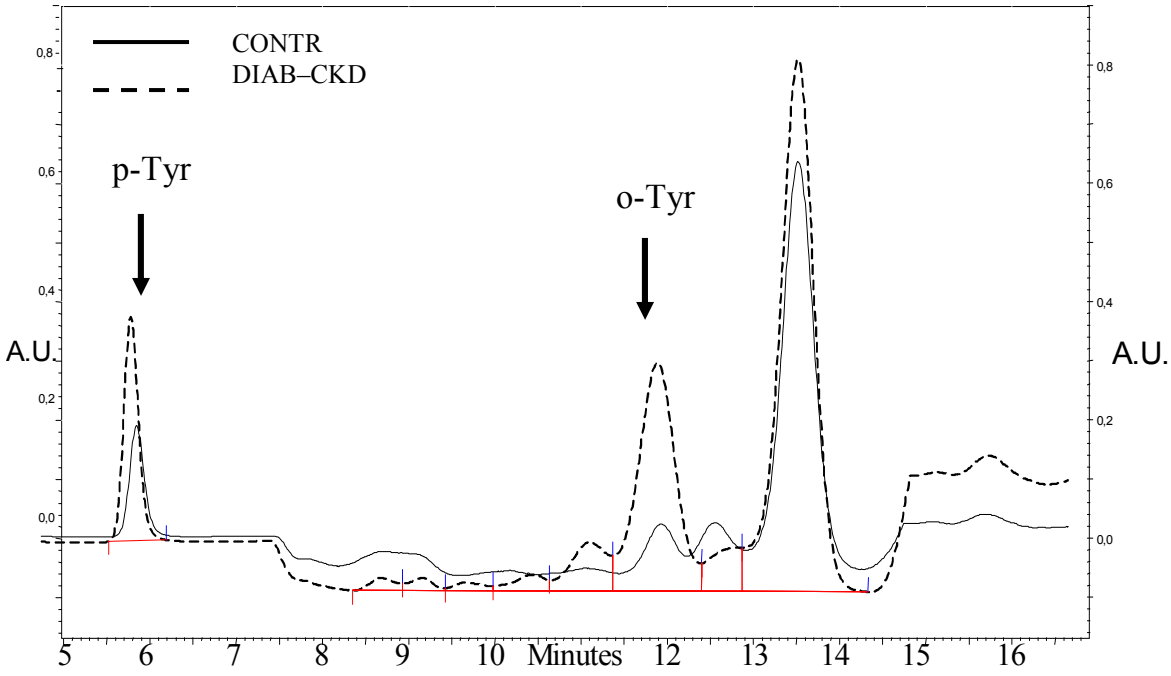

Figure 15
Chromatogram of urine samples of a patient from the control group (——) and a patient with diabetes and chronic kidney disease (– – –).
Abbreviations: p-Tyr, para-tyrosine; o-Tyr, ortho-tyrosine.

To test the reproducibility of the method, inter-assay variations of nine urine and plasma samples from three repeated measurements were calculated. Between the analyses, the samples were stored at - 20 °C; being frozen and thawed for each measurement. The tests were performed on three different days, using the same equipment, and each time the whole sample-handling procedure was carried out. The average inter-assay CVs in our study were 7.8 % for urinary p-Tyr concentration and 7.7 % for urinary o-Tyr concentration. Averages of CVs were 7.1 % for plasma p-Tyr concentration and 6.3 % for plasma o-Tyr concentration.

Median concentration of 8-epi-prostaglandin-$F_{2\alpha}$ in our study population was 1049 ng/mmol creatinine, which corresponds to the range described in the literature (e.g. 10 - 1600 ng/mmol creatinine in an analysis of the Framingham study group).[Keaney2003]

7.3.2. Plasma levels and urinary excretion of para-tyrosine[III]

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 *(Table 4)*. We obtained similar results when correcting data to the plasma Phe levels *(data not shown)*. Phe levels did not differ among the patient groups (median plasma Phe levels were 40.50 µmol/l for the CONTR group, 27.52 µmol/l for the CKD group, 31.08 µmol/l for the DIAB group, 30.03 µmol/l for the DIAB-CKD group, $p = 0.448$).

Urinary levels of p-Tyr were corrected for urinary concentrations of creatinine. Urinary concentrations of creatinine did not differ in the groups *(data not shown)*. 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 *(Table 4)*. 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 *(Table 4)*. Plasma levels of p-Tyr correlated with urinary levels of p-Tyr ($r = 0.468$, $p = 0.001$).

7.3.3. Renal clearance and fractional excretion of para-tyrosine[III]

The CKD and the DIAB-CKD group had a lower p-Tyr clearance than the DIAB group (0.34 [0.12 - 0.64] and 0.46 [0.18 - 0.74] vs. 1.13 [0.58 - 1.64] ml/min, $p < 0.05$). We found no difference when comparing the patient groups with the CONTR group (CONTR, 0.63 [0.26 - 0.99] ml/min). 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 *(Table 4)*.

7.3.4. Plasma levels and urinary excretion of ortho-tyrosine[III]

The CKD and the DIAB-CKD groups tended to have a higher plasma o-Tyr level than the CONTR group, but the observed difference was not significant ($p = 0.286$, *Table 4*). We obtained similar results when correcting data to the serum Phe levels (*data not shown*).

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 the CKD group (*Table 4*). The correlation between plasma levels of o-Tyr and urinary levels of o-Tyr was not significant ($r = 0.219$, $p = 0.130$).

Urinary 8-epi-prostaglandin-$F_{2\alpha}$/creatinine ratio did not correlate with plasma o-Tyr ($r = 0.04$), plasma o-Tyr/Phe ratio ($r = -0.06$), urinary o-Tyr/creatinine ratio ($r = 0.12$), urinary o-Tyr excretion ($r = 0.16$, $p > 0.05$ for all).

7.3.5. Renal clearance and fractional excretion of ortho-tyrosine[III]

There was no significant difference in the o-Tyr clearance among the groups (CONTR, 12.21 [2.13 - 21.26]; CKD, 5.92 [2.64 - 59.68]; DIAB, 122.34 [47.01 - 204.86]; DIAB-CKD, 29.14 [8.94 - 76.22] ml/min, p = 0.070).

In the case of o-Tyr, 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. The Fex of o-Tyr was significantly higher than the Fex of p-Tyr in the control group *(Table 4)*.

Urinary 8-epi-prostaglandin-$F_{2\alpha}$/creatinine ratio did not correlate with o-Tyr clearance nor with Fex of o-Tyr (r = 0.01 and r = - 0.167, respectively; p > 0.05 for all).

Table 4 Plasma level and urinary excretion para- and ortho-tyrosine

	CONTR	CKD	DIAB	DIAB-CKD
Plasma p-Tyr (µmol/l)	55.96 (35.62 - 56.97)	28.45[a c] (25.60 - 34.42)	46.11 (42.09 - 49.31)	32.46[c] (29.15 - 39.01)
Urine p-Tyr/creatinine (µmol/mmol)	4.27 (3.03 - 5.23)	1.80[a c] (1.58 - 1.97)	5.31 (4.26 - 9.28)	1.95[c] (1.45 - 3.90)
Urinary p-Tyr excretion (µmol/day)	23.62 (16.79 - 81.57)	18.78[c] (5.10 - 40.98)	68.78 (43.84 - 113.63)	20.44[c] (9.11 - 29.74)
p-Tyr Fex (%)	0.67 (0.56 - 0.79)	1.36 (1.09 - 2.14)	0.98 (0.73 - 1.35)	1.06 (0.71 - 3.54)
Plasma o-Tyr (µmol/l)	0.022 (0.013 - 0.054)	0.050 (0.024 - 0.145)	0.023 (0.015 - 0.029)	0.054 (0.019 - 0.378)
Urine o-Tyr/creatinine (µmol/mmol)	0.034 (0.0001 - 0.035)	0.175[a] (0.056 - 0.481)	0.291[a] (0.103 - 0.330)	0.479[a c] (0.367 - 0.701)
o-Tyr excretion (µmol/day)	0.24 (0.00 - 0.35)	1.22[a] (0.94 - 1.83)	3.41[a b] (2.72 - 4.99)	4.03[a b] (2.58 - 6.51)
oTyr Fex (%)	7.86[d] (3.81 - 12.08)	27.28 (8.55 - 373.02)	125.29[a] (69.32 - 140.35)	111.89[a] (68.79 - 185.90)

Abbreviations: CONTR, group of healthy control subjects; CKD, group of patients with Stage III. chronic kidney disease; DIAB, group of diabetic patients; DIAB-CKD, group of patients with diabetes and Stage III. chronic kidney disease; p-Tyr, para-tyrosine; o-Tyr, ortho-tyrosine; Fex, fractional excretion. Data are shown as median (inter-quartile range). The Mann-Whitney-U test was only used when the Kruskal-Wallis test for all groups was significant ($p < 0.05$). [a], $p < 0.05$ vs. control subjects; [b], $p < 0.05$ vs. CKD patients; [c], $p < 0.05$ vs. type 2 diabetic patients; [d], $p < 0.05$ vs. p-Tyr Fex.

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

7.4.1. Protein distribution of normal human and cataract lenses[IV]

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 *(Figure 16)*.

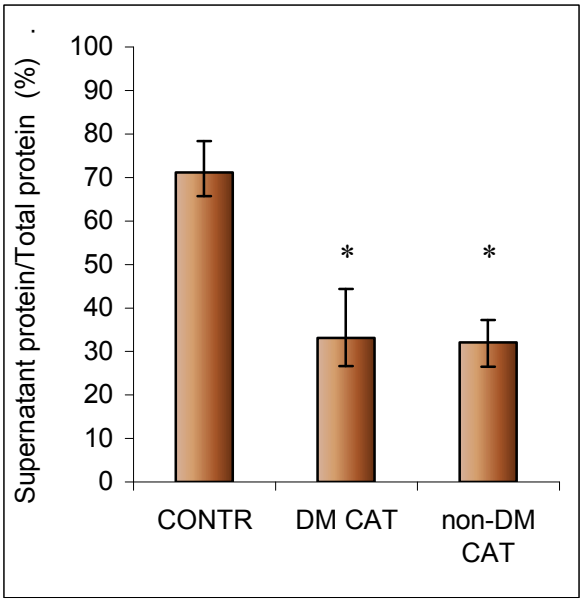

Figure 16
Protein distribution between the supernatant fractions and the total homogenates calculated by the ratio of protein contents of the supernatant and total homogenates.
Abbreviations: CONTR, group of control subjects; DM CAT, group of cataract patients with type 2 diabetes mellitus; non-DM CAT, group of cataract patients without diabetes mellitus. Values are represented as median (inter-quartile range).
*, $p<0.05$ versus CONTR.

7.4.2. Results of the electrophoretic analysis of lens samples[IV]

Proteins were separated depending on their molecular weight in each sample with comparative electrophoretic (ELFO) studies. Equal protein amounts were compared. This way, similarities and differences between protein composition were well comparable. Analysis was performed both from total and soluble protein fractions. Among lens proteins,

the presence of proteins in the range between 20-30 kDa was characteristic for each sample. According to densitometric measurements, this group represented approx. 30 % of the electrophoretized proteins of CONTR samples.

Figure 17 shows electrophoretograms of representative samples. The differences among the protein pattern of the 20-30 kDa group are striking. In each sample, there are several visible bands in this area, but the pattern and the intensity of the bands is different. Among the visible bands, the two bands nearest to the 20 kDa marker (*marked with white arrowheads in lane 6*), are present in each sample, and they show a high intensity in each sample. However, in the total homogenates of cataractous lenses *(lanes 3 and 5)*, close to the 30 kDa marker, two additional bands – *marked with white arrows in lanes 3 and 5* – also become quantitatively dominant. These bands are either not present in the supernatants and the control sample, or they show less intensity.

Moreover, it is worth mentioning that in case of all total homogenates we observed staining, which indicates the presence of high molecular weight aggregates, at the dividing line between stacking and separating gel and in the stacking gel itself *(see the > 94 kDa area on Figure 17)*. Proteins in the stacking gel are mainly stained in the total homogenate of the cataractous samples, and less in the control sample *(see lanes 3 and 5 vs. lane 1)*. The presence of high molecular weight aggregates is less characteristic for the supernatants *(compare total homogenates vs. lens supernatants; i.e. lane 1 vs. 2, lane 3 vs. 4, lane 5 vs. 6; Figure 17)*.

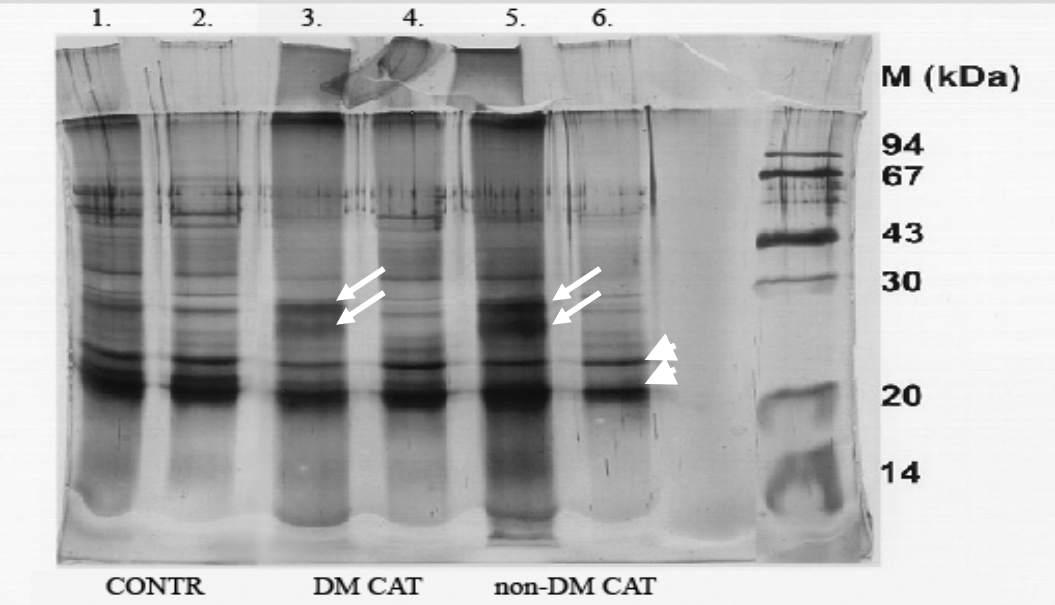

Figure 17
Sodium dodecylsulphate-polyacrylamide electrophoretogram (SDS-PAGE) of proteins of representative lens samples.
Bands 1-2., CONTR samples; bands 3-4., DM CAT samples; bands 5-6., non-DM CAT samples; bands 1., 3., 5., total homogenates; bands 2., 4., 6., supernatants.
Abbreviations: CONTR, group of control subjects; DM CAT, group of cataract patients with type 2 diabetes mellitus; non-DM CAT, group of cataract patients without diabetes mellitus.
Arrows and arrowheads: see explanation in the text.

7.4.3. Results of the HPLC analysis of lens homogenates[IV]

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.

Using the HPLC method with fluorescent detection, the amount of DOPA, p-Tyr, m-Tyr, o-Tyr as well as the parent amino acid Phe can theoretically be measured in the same run of the samples. 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. Two representative chromatograms are shown on *Figure 18:*

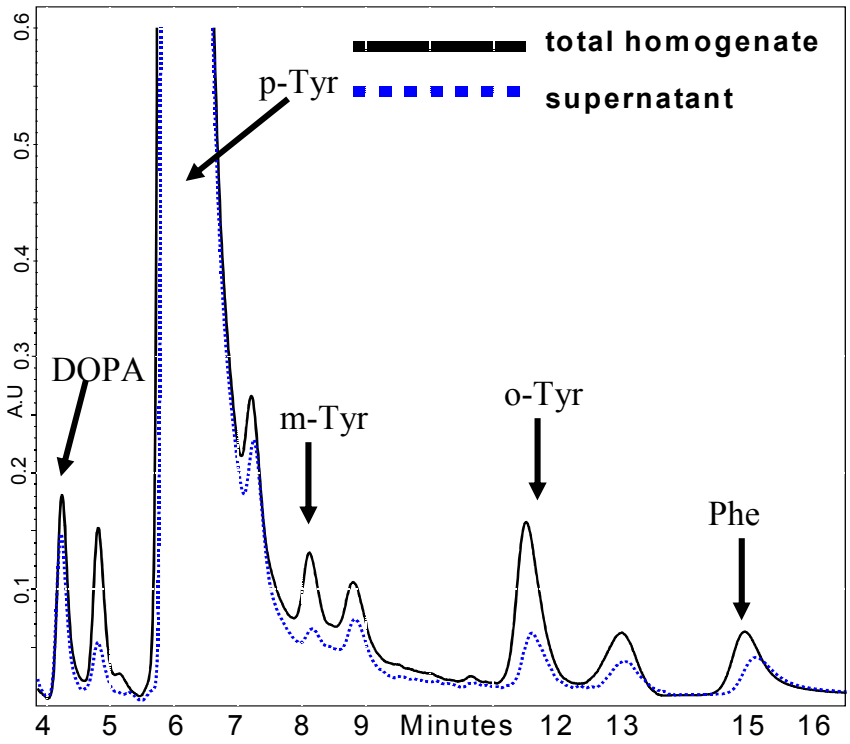

Figure 18
HPLC registrate of the total homogenate (continuous line, 1 mg/ml protein) and the supernatant lens fraction (dotted line, 2.5 mg/ml protein) of a cataract patient with type 2 diabetes mellitus.
Abbreviations: DOPA, 3,4-dihydroxy-phenylalanine; p-Tyr, para-tyrosine; m-Tyr, meta-tyrosine; o-Tyr, ortho-tyrosine; Phe, phenylalanine.

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, descriptive and comparative statistics could only be calculated for those samples, where m-Tyr was detectable *(Table 5)*.

7.4.4. Inter-group comparisons[IV]

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 lens supernatants, DOPA and o-Tyr could be well detected; however in a majority of the supernatant samples, m-Tyr was below the detection limit. 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. The Phe/protein ratio 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 *(Table 5)*.

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).

7.4.5. Intra-group comparisons of supernatants and total homogenates[IV]

Besides the inter-group comparison, also intra-group comparison of supernatants and total homogenates of the same samples was carried out. In the CONTR, DM CAT and non-DM CAT groups, composition of total homogenates was compared to that of the supernatants.

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 *(Table 5)*.

Table 5 Amino acid contents of the supernatant samples and the total homogenates corrected for sample protein content

		CONTR	DM CAT	Non-DM CAT
Supernatant samples	DOPA/protein (nmol/g)	413.56 (79.01 - 1505.84)	305.53 (196.99 - 409.63)	188.61 (114.00 - 245.42)
	m-Tyr/protein† (nmol/g)	1.06 (0.77 - 3.01)	3.20 (1.22 - 4.74)	2.46 (1.54 - 4.47)
	o-Tyr/protein (nmol/g)	30.96 (3.39 - 37.08)	22.43 (7.82 - 87.99)	18.66 (11.17 - 121.84)
	Phe/protein μmol/g	633 (299 - 819)	382 (332 - 512)	252[a b] (197 - 306)
Total homogenates	DOPA/protein (nmol/g)	1100.35 (289.34 - 1767.30)	904.12[c] (738.49 - 1287.17)	1346.23[c] (428.44 - 1738.49)
	m-Tyr/protein† (nmol/g)	3.41[c] (3.02 - 4.61)	15.34[a c] (2.39 - 29.65)	20.28[a c] (7.18 - 57.29)
	o-Tyr/protein (nmol/g)	38.61 (12.47 - 51.64)	217.58[a c] (22.76 - 919.10)	211.78[a c] (51.76 - 1128.03)
	Phe/protein (μmol/g)	1102[c] (601 - 1246)	1187[c] (956 - 1622)	967[c] (626 - 1336)

Abbreviations: CONTR, group of control subjects; DM CAT, group of cataract patients with type 2 diabetes mellitus; non-DM, group of cataract patients without diabetes mellitus;

DOPA, 3,4-dihydroxy-phenylalanine; m-Tyr, meta-tyrosine; o-Tyr, ortho-tyrosine; Phe, phenylalanine; [a], $p < 0.05$ vs. CONTR; [b], $p < 0.05$ vs. DM CAT; [c], $p < 0.05$ vs. supernatant; [†]: amount of m-Tyr in some samples was below detection limit, statistics were only calculated for those samples, in which m-Tyr was in the detection range. Values are represented as median (inter-quartile range).

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

7.5.1. Main clinical characteristics of the patients with different genotypes[V]

There was no difference between patients with II (n = 27), ID (n = 68) or DD (n = 50) genotype concerning age, duration of diabetes, age at the diagnosis of diabetes, body mass index, Hb A_{1c}, plasma glucose level, systolic blood pressure, diastolic blood pressure, serum total cholesterol, triglyceride, HDL and LDL cholesterol, blood urea nitrogen, creatinine, uric acid, calculated glomerular filtration rate *(results not shown in graph)*.

7.5.2. Evidence for connections between the angiotensin-converting enzyme gene and the glycemic state[V]

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 *(Table 6)*.

Table 6 Serum fructosamine level in the patient groups with different ACE genotypes.

Genotype	Fructosamine (μmol/l)
II (n = 28)	294 (197 - 460)
ID (n = 71)	310 (180 - 640)
DD (n = 53)	331[a] (218 - 581)

Abbreviations: [a], $p<0.05$ vs. II. Data are expressed as median (range).

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, *(Table 7)*.

Table 7 Serum fructosamine level and number of antihypertensive drugs used in the groups formed by patients with II and ID/DD genotype.

Genotype	Fructosamine (μmol/l)
II	294 (197 - 460)
ID + DD	328 (180 - 640)[a]

Abbreviations: [a], $p < 0.05$ vs. II group

Using median test and grouping patients to a below-the-median and above-the-median group, we found a significant connection ($p < 0.05$) between serum fructosamine level and the ACE gene polymorphism *(Table 8)*.

Table 8 Number of patients with fructosamine above and below the median in the patient groups with different genotypes.

Genotype	Above the median (> 319 μmol/l)	Below the median (< 319 μmol/l)
II	8	20
ID	35	36
DD	33	20

7.5.3. Glycemic state and inhibition of the renin-angiotensin system[V]

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 *(Table 9)*.

Table 9 Serum fructosamine levels in patients on ACEI therapy and in patients without ACEI therapy.

Therapy	Fructosamine (μmol/l)
ACEI +	305 (197 - 581)
ACEI -	349[a] (251 - 640)

Abbreviations: [a], $p < 0.05$ vs. ACEI+; ACEI+, patients treated with ACEI; ACEI-, patients not treated with ACEI. Data are expressed as median (range).

7.5.4. Endothelial dysfunction[v]

When grouping the patients according to the ACE genotype, we found that daily albuminuria was highest in the patients with DD genotype *(Table 10)*.

Table 10 Levels of albuminuria in the patient groups with different ACE genotypes (for statistical analysis, the natural logarithm of albuminuria – ln albuminuria – was used).

Genotype	Albuminuria (mg/day)
II	13 (3 - 447)
ID	12 (3 - 1334)
DD	25[a] (4 - 932)

Abbreviations: [a], $p<0.05$ vs., ID. Data are expressed as mean (range).

When examining glycation parameters, a significant correlation between serum fructosamine levels, blood glucose and Hb A_{1c} was found *(Table 11)*. Both plasma glucose and fructosamine correlated well with the albuminuria, however Hb A_{1C} did not *(Table 11)*.

Table 11 Correlation significance *(p)* levels between metabolic parameters and a marker of the endothelial dysfunction.

	Fructosamine	Glucose	Albuminuria
Hb A_{1c}	<0.001	<0.001	N.S.
Fructosamine		<0.001	0.006
Glucose			0.005

Abbreviations: N.S., non-significant.

7.5.5. Angiotensin-converting enzyme genotype, gamma glutamyl transferase activity and other clinical parameters[V]

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 *(Table 12)*.

Table 12 Levels of serum γ-GT activity and albuminuria in the patient groups with different ACE genotype.

Genotype	γ-GT activity (U/l)
II	28 (11-124)
ID	25 (7-238)
DD	43[a b] (12-200)

Abbreviations: [a], $p < 0.05$ vs. II; [b], $p < 0.05$ vs., ID. Data are expressed as mean (range).

Levels of the γ-GT enzyme correlated well with C-reactive protein ($p<0.05$) and blood urea nitrogen, a marker of renal function, ($p<0.05$, 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 (II: 7.2 (2.5-13.5); ID: 6.05 (2.7-19.3); DD: 7.2 (2.0-11.6) mmol/l; Kruskal-Wallis test: $p = 0.587$; median-test: $p = 0.330$).

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

7.6.1. Results of the genetic analysis[VI]

The genotype distributions (P/P, n = 73; P/L, n = 64; L/L, n = 11) and the allele frequencies (P, 70.9 %; L, 29.1 %) showed no significant difference from the Hardy-Weinberg equilibrium.

7.6.2. Plasma levels of L-arginine analogues in the patient groups with different genotypes[VI]

Patients were grouped according to the presence or absence of the GPX P allele, one group consisted of the patients with P/P and P/L genotypes, the other group contained the patients with L/L genotype. The two groups were matched for age, gender, duration of diabetes, HbA_{1c} level, BMI, blood pressure and serum creatinine levels, albuminuria (data not shown). There was no difference between the two groups 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 *(Table 13)*.

Table 13 Plasma L-arginine analogue levels of type 2 diabetic patients with different GPX genotypes.

Genotype	P/P or P/L n = 137	L/L n = 11
Plasma L-NMMA (µmol/l)	0.046 ± 0.024	0.033 ± 0.019[a]
Plasma L-ADMA (µmol/l)	0.63 ± 0.27	0.42 ± 0.19[a]
Plasma L-SDMA (µmol/l)	0.67 ± 0.48	0.52 ± 0.28

Abbreviations: L-NMMA, L-N-monomethylarginine; L-ADMA, L-asymmetric dimethylarginine; L-SDMA, L-symmetric dimethylarginine; [a], $p < 0.05$ vs. the P/P or P/L group.

8 DISCUSSION (Publications I-VIII.)

Diabetes mellitus and chronic kidney diseases are major health problems that may affect a large proprotion of the population. Much is known about the pathogenesis of these diseases and of that of their complications, and these processes seem to be multifactorial. There is evidence for the role of the genetic background, of the environmental factors and of intrinsic factors such as altered metabolic pathways. Therefore we investigated oxidative stress processes, glycation, reactive carbonyl compounds, genetic alterations, and their potential role in the development of the complications of DM and CRF, and their applications as markers in type 2 DM mellitus and/or CRF.

8.1. *In vitro* experiments on Phe-Tyr conversion in a Fenton-like metal-catalyzed oxidation reaction[I,II]

In *in vitro* experiments, we investigated p-, m- and o-Tyr production as a consequence of free radical attack on phenylalanine due to a free radical-producing *in vitro* system. In our system, the production of Tyr from Phe indirectly proved the presence of the oxidizing free radical. Both the spectrofluorimetric and the HPLC method proved to be applicable for the measurement of Tyr production. In the ferric iron - H_2O_2 - Phe system, the redox cycling of iron proved to be eligible for free radical production. This process was influenced by iron chelators, i.e. ATP decreased, EDTA and citrate increased the Phe oxidation.

Iron is known to play a crucial role in many processes that include the mitochondrial electron-transport chains, oxygen transport in the red blood cells etc. In the body, both ferrous and ferric forms of iron may be present, mainly in a protein-coupled form or as iron-complexes. Ferrous iron is highly redox-active and it is turned to ferric iron in numerous redox reactions, including the Fenton reaction, as well. In the classical Fenton reaction, the ferrous iron – ferric iron conversion catalyzes the cleavage of the reactive oxygen species,

H_2O_2 to ·OH and OH^- anion. The ·OH anion is one of the most active free radicals with the shortest lifetime. To avoid the high reactivity of ferrous iron, it is present in the body attached to proteins (cytochrome enzymes, hemoglobin and hem-oxidases).[83] The ferric form of iron is mainly bound in the form of ferritin- or transferring-iron complexes. By the modification (glycation or oxidation) of the apoproteins, non-protein-bound ferric iron may be released from the complexes.[84] In our sets of experiments, we investigated the reactions of the redox-active ferric iron and its complexes, as this form of iron may be of real pathophysiological relevance.[85]

It is well-known, that oxidative stress is able to modify the chemical structure of amino acids[86,37]; e.g. in the presence of ·OH, Tyr isomers can be produced from Phe. These conversions can be used as an indirect marker of the attack of free radicals to amino acids.[44]

In our *in vitro* system containing Phe, ferric iron and hydrogen peroxide, we used a spectrophotometric method to detect total Tyr production that could be used as an indirect proof for free radical attack on the aromatic ring of Phe. A marked increase in Tyr-fluorescence could be observed in the reaction. Pre-incubation of the solution with the irreversible iron-chelator desferrioxamine prevented Tyr production, this fact indicates that the redox-cycle of iron is needed for hydroxyl free radical production.

The spectrofluorometric method measures the increase in total Tyr fluorescence, and it provides no data on the relative amounts of the Tyr isomers i.e. p-, m- and o-Tyr. Furthermore, any compound (e.g. ATP) that is present in the reaction mixture and absorbes light at the given wavelength (e.g. 275 nm), interferes with a spectrofluorimetric detection, as these molecules absorb a part of light that cannot excite the fluorophore thus leading to lower fluorescence. Therefore, an HPLC method was used to separate the three isomers from each other and from Phe. In this system, also Fe-ATP as Fenton-reactant could be used. Retention times of the amino acids with our HPLC systems correspond to the data of the literature[77].

The increase in the amount of all three isomers as well as the change of Phe could be detected in a single HPLC run.

There is a clear difference in the order of magnitude of the amount of the substrate Phe (approx. 1 mmol/l) and the Tyr isomers (approx. 200-300 nmol/l) produced in our *in vitro* system, indicating that approximately one of 10,000 Phe molecules is converted to either p-, m- or o-Tyr in the Fenton-like reaction with Fe-ATP. The production of Tyr isomers requires oxidation of Phe by a free radical species formed in the reaction, but the exact chemical nature of the free radical formed in this reaction requires is unknown. Most likely it is a hydroxyl free radical, but some authors rather suggest the presence of a ferryl or perferryl radical or a 'crypto •OH' radical.[10] Electron spin resonance spectroscopic measurements would be needed to provide more data.

The exact chemical reaction in the Fenton-like system is also unclear, John M.C. Gutteridge suggests in a review a simplified equation of ferric-salt-derived radical formation:

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2\cdot + H^+ \quad (1)$$

In further, he states that this simple equation would not really explain all characteristics of Fenton-like reactions in the presence of ferric salts and H_2O_2. He says that these reactions must be rather more complex and include $\cdot O^{2-}$, as addition of SOD nearly completely blocks the reaction. He does not, however provide an exact series of reactions.[10]

In the case of iron-complexes, the reaction way may be different, e.g. Gutteridge and co-workers[87] suggest the following reaction scheme for Fe^{3+}-EDTA-derived oxidative damage:

$$Fe^{3+}\text{–EDTA} + H_2O_2 \rightarrow Fe^{3+}OOH^-\text{–EDTA} + H^+ \quad (2)$$

$$Fe^{3+}OOH^-\text{–EDTA} + H_2O_2 \rightarrow FeO^{2+}\text{–EDTA} + HO_2\cdot + H_2O \quad (3)$$

$$FeO^{2+}\text{–EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{–EDTA} + HO_2\cdot + OH^- \quad (4)$$

$$HO_2\cdot \rightarrow H^+ + \cdot O_2^- \quad (5)$$

$$Fe^{3+}\text{–EDTA} + \cdot O_2^- \leftrightarrows Fe^{2+}\text{–EDTA} + O_2 \quad (6)$$

$$Fe^{2+}\text{–EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{–EDTA} + OH^- + \cdot OH \quad (7)$$

In other publications[86,89,87], Barry Halliwell and John M.C. Gutteridge suggest that Fe^{3+} could catalyze $\cdot O^2$ formation from H_2O_2 (8), then reactions equivalent to (6) and (7) would continue leading to ·OH formation.

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + \cdot O_2^- + 2\ H^+ \quad (8)$$

$$Fe^{3+} + \cdot O_2^- \leftrightarrows Fe^{2+} + O_2 \quad (9)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH \quad (10)$$

The ·OH produced in reaction (10) is able to chemically modify Phe:

$$3\ \cdot OH + 3\ \text{Phe} \rightarrow \text{p-Tyr} + \text{m-Tyr} + \text{o-Tyr} \quad (11)$$

This series of reactions should be sensitive to the effect of SOD because of the presence of $\cdot O_2^-$ in equation (9), and in fact, we managed to verify this specific SOD-sensitivity in our *in vitro* experiments, as the increase in Tyr-fluorescence could be blocked using active SOD, but could not be blocked using heat-inactivated SOD.

8.1.1. The effect of iron chelators on the *in vitro* reaction[I,II]

Iron chelators are known to alter the redox reactions of iron, e.g. desferrioxamine is supposed to irreversibly chelate ferric iron and inhibit its redox cycling.[90] For other complexants, the case is not that simple. There are theoretical determinants of chelator effect, most important of which is the chemical structure of the chelator. Chelators with an oxygen atom as Fe-ligand (e.g. EDTA, citrate, ADP, ATP), should theoretically decrease the reduction potential of iron i.e. stabilize the Fe^{3+} form. On the other hand, nitrogen or sulphur-containing chelators (e.g. histamine) should increase the reduction potential of iron i.e. stabilize the Fe^{2+} form. The detected reactivity of iron-complexes however, also depends on the detection system.[91] We have chosen a hydroxylation assay of Phe as a detection system because of the physiological relevance of the conversion. In an *in vitro* study on the

conversion of radiolysis-derived $\cdot O_2^-$ in the presence of Fe^{3+}-chelates, the rate of conversion was: Fe-EDTA > Fe-citrate >> Fe-pyrophosphate > Fe-lactate > Fe-ATP.[92] In our experiments, the case is more complex, as Fe is required for $\cdot O_2^-$ generation, i.e. there is supposed to be a Fe^{3+} to Fe^{2+} and a subsequent Fe^{2+} to Fe^{3+} conversion, too. However, we have found similar results, i.e. in the HPLC detection both the ferric iron-EDTA complex and the ferric iron-citrate complex lead to a higher Tyr production than ferric iron alone (i.e. complexed by Phe). On the other hand, ATP-chelated iron was less effective in the Phe-to-Tyr conversion. This result is confirmed by the literature, as ATP should promote lipid peroxidation processes rather than OH formation.[93] Moreover, in the presence of citrate we could observe a marked Tyr in the absence of H_2O_2, as well. A higher Tyr production was concomitted by a higher Phe consumption in the Fe-chelate experiments.

Results in the spectrofluorimetric and HPLC detection were similar, except for the Fe+EDTA+Phe+H_2O_2 system. Here, we detected only a slight increase in fluorescence in the spectrofluorimeter (similar to Fe+Phe+H_2O_2), while the HPLC analysis of the reaction mixtures at the end-point revealed high p-, m- and o-Tyr concentrations (similar to Fe+citrate+Phe+H_2O_2, compare *Figures 8 and 14*). In the background of this difference we hypothesize the interference of the Fe-EDTA complex with the Tyr detection at 275 nm excitation and 305 nm emission wavelengths. Confirming this hypothesis, we found that not only the increase in fluorescence but also the initial fluorescence was lower in these samples. Literature data on the optical absorbance spectrum of EDTA-complexes are not detailed, but according to available data, the ferric-EDTA complex should have a rather broad-shaped absorption peak around 260 nm.[94] These facts also confirm that the HPLC provides more reliable end-point data than the spectrofluorimeter, because of lower probability of optical interference. However, it provides no kinetic data unlike the spectrofluorimetric

measurements. Thus, best results can be achieved with a combined measurement and comparison of the two methods.

The citrate-complex of Fe^{3+} is of special interest, as it has probably the most pathophysiological relevance. From clinical data we know that, there is an accumulation of a Fe-citrate complex in the blood of hemochromatosis patients.[95] Citrate is also an important intermediate of the Szent-Györgyi-Krebs cycle; it has been shown in mouse embryos that hyperglycemia leads to activation of the poliol pathway, thus to an increase in NADH, inhibition of some of the enzymes of the Szent-Györgyi-Krebs cycle and finally to the accumulation of intermediates such as citrate.[96] Another point is that citrate autoxidizes easily, thereby it could reduce Fe^{3+} to form Fe^{2+}, accelerating the formation of ROS, as verified by our data i.e. the Tyr production in the absence of H_2O_2.

8.1.2. The effect of a phosphate buffer on the *in vitro* reaction[II]

The pH of the reaction mixture (especially in the case of citrate and EDTA) may be of critical importance in Fenton-like reactions, as in the classical form of the Fenton reaction, OH^- is formed. If there is an abundance of H^+ ions, OH^- ions will be captured and turned to H_2O, therefore equation (10) is shifted even more to the right, and more ·OH radicals will be formed.[92] It has been verified e.g., that free radical formation due to the autoxidation of Fe^{2+} is faster at acidic pH than at the physiological pH. Moreover, this process was also affected by buffers, as Tris inhibited, while phosphate (also a potent iron chelator) and bicarbonate increased the autoxidation of Fe^{2+}.[91] In our data, the use of a phosphate buffer solution (pH 7.4) as a vehicle decreased Tyr production compared to H_2O in the case of Fe-citrate and Fe-EDTA, as well. In metabolic disturbances e.g. in DM, the accumulation of citrate, the lower levels of ATP and a shift towards acidosis could thus contribute to ferric iron-derived oxidative damage.

Upon our experimental data and data of the literature, we could finally conclude that m- and o-Tyr could be used as markers of *in vitro* free radical-related damage. Therefore, we tested if detection of these substances could be used in clinical studies, as well.

8.2. Clinical study on the plasma and urinary p- and o-Tyr levels and renal excretion[III]

In a cross-sectional study, we determined urinary and plasma p- and o-Tyr levels in diabetes and renal failure. We proved that our method was suitable for the measurement of both urinary and plasma non-protein bound p- and o-Tyr with a good reproducibility. The plasma p-Tyr, o-Tyr and Phe levels corresponded with the range described in the literature. Ishimitsu et al. using this method found mean p-, o-Tyr and Phe serum concentrations of 52 µmol/L, 17 nmol/L and 58 µmol/L in healthy persons[77], while medians of our results for the CONTR group were 56 µmol/L (p-Tyr), 22 nmol/L (o-Tyr), 40 µmol/L (Phe), respectively.

From data of an animal experiment,[98] we know that serum levels of non-protein-bound o-Tyr reach highest concentrations at 15 minutes after oral, intramuscular or intraperitoneal Phe administration. Therefore, changes of free o-Tyr levels might be indicative of short-term free radical processes. The turnover of protein-bound o-Tyr is slower than that of free o-Tyr, thus we also tried to detect m- and o-Tyr in plasma protein hydrolysates. We found that in the plasma protein hydrolysates m- and o-Tyr did not resolve well from other peaks, therefore they could not be measured in plasma proteins using our method. According to the literature, the concentration of non-protein bound free o-Tyr is 4.03 times higher than the concentration of protein-bound o-Tyr in the plasma.[99] A major aim of this study was mainly to obtain information on the renal handling of p-Tyr and o-Tyr. To be able to calculate renal clearance and Fex of the substances, we needed to measure the non-protein bound form of p-Tyr and o-Tyr.

8.2.1. Plasma and urinary p-Tyr levels and renal excretion[III]

It is known that in uremia there is an impaired amino acid metabolism.[100,101] Through the amino acid analysis of uremic and azotemic sera, lower p-Tyr concentrations were found than in healthy controls (CKD vs. controls: 26 vs. 46 µmol/L[102], and 27 vs. 54 µmol/L.[103] Our corresponding data were 28 vs. 56 µmol/L. Latest data of the literature imply that renal impairment causes a decreased renal phenylalanine-hydroxylase enzyme activity.[104] 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 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.

8.2.2. Plasma and urinary o-Tyr levels and renal excretion[III]

Free radical-derived damage has been suspected to have a biologically relevant role in the pathogenesis of complications of diabetes or chronic uremia.[11] Thus, identification of well detectable and stable markers of oxidative damage is of special interest. Among the oxidative stress markers, F_2-isoprostanes are specific to lipid peroxidation processes, and they are also generated during sample storage and handling.[36] On the contrary, o-Tyr is specific for the hydroxyl free radical-derived damage of Phe. The fact that, the 8-epi-prostaglandin-$F_{2\alpha}$/creatinine ratio did not correlate with the parameters of o-Tyr excretion, is probably due to the different origin of 8-epi-prostaglandin-$F_{2\alpha}$ and o-Tyr. In our study, we observed an additive effect of diabetes and kidney failure on the excretion of the hydroxyl radical marker o-Tyr.

The abundance of plasma Phe (27.52 µmol/L Phe vs. 0.05 µmol/L o-Tyr in the CKD group) makes it unlikely that the amount of Phe would be rate-limiting in the o-Tyr formation. Plasma o-Tyr/Phe ratio and plasma o-Tyr concentration showed the same tendencies. This

also supports, that o-Tyr levels would rather reflect hydroxyl radical formation than the changes in Phe levels.

The lack of correlation between urinary excretion and plasma level of o-Tyr may indicate that the urinary excretion of o-Tyr is not determined by glomerular filtration alone, but by active renal transport processes, as well. Our data also confirm that the renal handling of o-Tyr is different among the patient groups. The median of fractional excretion of o-Tyr was above 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 superior. In diabetes, concentration of glucose in the urine may rise to 100-200 mmol/L (non-published data). 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.

Our data also suggest that p-Tyr is more efficiently (approx. ten times) retained by the kidney than o-Tyr, even in the control group (*Table 4*), which indicates 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 may be an adaptive process, as the physiological Tyr isoform is retained, while the pathological isoform is excreted.

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

In another study, the amount of the same markers, along with DOPA was investigated in cataractous and non-cataractous lens samples. We also investigated the protein distribution, ELFO picture of the same lenses. This way we could compare the lens composition of the non-cataractous controls, patients with diabetic cataract and senile cataract. By comparing the water-soluble supernatant and the total lens homogenate, we intended to find connection between water-solubility and lens composition.

8.3.1. Protein solubility and electrophoretic properties[IV]

According to our findings, protein content of the soluble fraction was lower than that of the total homogenates, and the soluble protein content of cataractous lenses showed a significant decrease when compared to controls. Between the different types of cataracts (DM CAT and non-DM CAT), we have not found any significant difference.

Analyzing equal amounts of proteins, control samples were the most heterogeneous. The range between 20 and 30 kDa deserves special interest. Protein fractions in the 20 kDa area are presumably alpha-crystalline subunits. Comparing control fractions with the cataractous samples the most striking differences are observed in the 20-30 kDa range. In the range of the medium weight, soluble proteins of control samples show a rich pattern, while the same fractions of cataractous lenses show a poor pattern. It is worth mentioning that in the high molecular weight region, we could identify the presence of high molecular weight aggregates in the total homogenates. This fraction is less characteristic for the lens supernatants. Our studies on proteins confirm literary data that refer to crystalline insolubilisation and cytoskeletal reconstruction during cataractogenesis.[105,48]

8.3.2. High performance liquid chromatographic analysis of lens samples[IV]

The HPLC method used in our study is capable of detecting the autofluorescent amino acids in the homogenates of cataractous lenses. The presence of the Phe-derived free radical marker amino acids DOPA and o-Tyr could be verified and their concentrations could be measured and compared in the supernatants and the total lens homogenates. The amount of m-Tyr was below detection limit in a high proportion of the CONTR total samples, and could not be determined in a larger proportion of the supernatant samples. It is well-known[37], and in other experiments we have also found (*Chapter 6.2.1.*), that oxidized Phe derivatives are formed in nearly equimolar concentrations *in vitro*, but their formation might be different in

vivo. Therefore there can be concentration differences between m-Tyr and o-Tyr of up to ten times.[37]

It is widely accepted and in fact recommended to correct the amount of the oxidized amino acid to the amount of the parent amino acid (in our case to Phe).[37] However, we found that there is a significant difference in Phe content of the proteins in the lens supernatant and in the proteins of the total homogenates. We also found that Phe content of the supernatants changes from controls to cataract samples. If we calculate the ratio of oxidized amino acid/Phe, it may be influenced to a larger extent by the difference in the amount of Phe than by the amount of the oxidized amino acids. Therefore, we used the amino acid/protein ratio for comparisons. In our study, the o-Tyr/Phe ratio for the total homogenate of control lenses vs. senile cataractous lenses (0.034 vs. 0.69 nmol o-Tyr/μmol Phe) roughly corresponds the range described in the literature, i.e. 0.18 vs. 0.65 nmol o-Tyr/μmol Phe[42] and 0.5 vs. 1.8 nmol o-Tyr/μmol Phe[37] (*data not shown in table*).

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.

The Phe/protein ratio is influenced by the consumption of Phe by only 0.1-0.01 %, while the Phe content of the DM CAT and non-DM CAT samples is 40% and 60% lower than that of the control samples (*Table 5,* supernatant data). 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, *Table 5*). 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 (*Table 5*). These facts further support that the difference in Phe content is not a consequence of the oxidation of Phe. 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.

Alpha- crystallins represent approximately 40% of the soluble human lens proteins. These proteins usually form well-structured multimolecular aggregates, the structure is mainly stabilized by hydrophobic inter-subunit interactions. There are two classes of α–crystallins, the αA- and αB-crystallins, the αA type is rather lens-specific, the αB type is ubiquitous. Although both types contain the "α-crystalline domain", that is a characteristic of small heat shock proteins (sHsp), they possess different functions as molecular chaperones.

The β-crystallins are lens-specific, they usually form oligomers made up by β-crystalline hetero- or homodimers. The majority of γ-crystallins (except for γS) is lens-specific, as well, but unlike β-crystallins, they are represented in a monomeric form.

Despite the high protein concentration, crystallins normally do not precipitate in the lens, but stay water-soluble, this way contributing to lens transparency. There are two major factors accounting for the stability of the crystalline-solution, one is energetical, the other one is chemical.

The high viscosity of the protein solution, the macromolecular "crowding", the inter- and intramolecular interactions, exclusion of water molecules due to hydrophobicity of the inner core and hydrophylicity of the outer surface of crystalline multimers leads to a stable

state with a low free energy, as a consequence of this, an activational energy is needed for protein precipitation.[48]

The other factor is the interaction of crystalline proteins, and their chaperone function. Chaperones in general are proteins that are able to bind denaturated protein chains, and either re-fold them into the native tertiary and quaternary structure, or promote their regulated break-down. A part of the chaperones can be induced by heat-shock, hence they are called heat-shock proteins (Hsp). Some crystallins are indeed Hsp's, and they may have an important role as chaperones. For example, α-crystallins can stabilize other crystallins (β- and γ-), other, non-crystalline proteins, and themselves, as well.[48] There are specific structures in crystallins which have a strong influence on chaperone function, e.g. the N-terminal domain, the N-terminal arm, the C-terminal arm and the 71-88 amino acids of αA-crystallins.[48]

It is known that amino acid composition of lens proteins is heterogenous among different parts of the lens, and in fact, the Phe and Tyr content may be used to distinguish between α, β and γ crystalline proteins and to monitor maturation of lens proteins.[106] Our data obtained by calculating the supernatant Phe/total homogenate Phe ratio, also indicate that proteins in the supernatants and the total homogenates are different, as reported by the literature.[107]

The major components of lens proteins are crystallins that play an important role in keeping the solution of high-protein concentration transparent. Investigation of m- and o-Tyr content of the lens proteins deserves special interest because of two reasons: *i.* The oxidized derivatives of Phe are selective markers of the presence of hydroxyl free radical. The accumulation of m- and o-Tyr is shown to run parallel to the accumulation of other oxidative stress-related processes, such as disulphide-bond formation, glycoxydation and the presence of cross-links[39] which are shown to play a role in the insolubilisation of lens proteins. *ii.* m- and o-Tyr are also noteworthy because they are markers of the oxidative modification of an

important amino acid, Phe. Phe is shown to play a pivotal role in the solubility and chaperone function of crystallins.[108,109] On the one hand, there is a highly conserved SRLFDQFFG sequence in the N-terminal domain of αA- as well as αB-crystalline and other heat shock proteins. The Phe (F) residues together with the Leu (L) residue form a hydrophobic core that have been shown to play a crucial role in inter-subunit interactions and oligomer formation, stabilization of secondary, tertiary and quaternary protein structure and chaperone function of α-crystallins.[108] It has also been proven that there is another Phe in a key position, namely the ^{71}Phe of αA-crystalline. Site-directed, selective mutagenesis of this single amino acid has been shown to completely abolish chaperone function of αA-crystalline.[109] 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.

8.4. Clinical study on the angiotensin-converting enzyme gene polymorphism and clinical parameters of patients with type 2 diabetes mellitus[V,VII,VIII]

8.4.1. ACE genotype and glycation processes[V]

In the study on ACE genotype in patients with DM, we found that patients with an unfavorable ACE genotype had higher fructosamine levels, albuminuria and needed more antihypertensive drugs than patients with a more beneficial genotype. In these patients, the more active RAAS may lead to endothelial dysfunction (i.e. more severe hypertension and albuminuria) in part directly through the action of higher Ang II levels. But it is also known that in the presence of D allele, one has to concern an increased insulin resistance and an increased prevalence of the metabolic syndrome. The insulin resistant state accompanying the D allele leads to a worse glycemic state as described by a higher Hb A_{1c} and plasma glucose level in the study of Lee et al[110], and a higher fructosamine level in our study. This process

makes the patients susceptible to the exhaustion of the beta cells, thus they may need earlier insulin therapy *(data not shown)*. The worse glycemic state may also contribute to the development of endothelial dysfunction, as shown by the correlations between albuminuria, fructosamine (a marker of the glycation of albumin) and plasma glucose levels. Others have also found that the ACE genotype influences target-organ damage.[111] The lack of correlation between Hb A_{1c} levels and albuminuria may be explained by the fact that Hb A_{1c} indicates long-term intracellular AGE-derived protein damage. The other two factors (fructosamine and glucose) are indicative of short-term extracellular glycation state, that may contribute more to albumin glycation and endothelial damage, thus Hb A_{1c} may be rather a "marker", why fructosamine and glucose rather "makers" of the glycation-derived damage.

8.4.2. The significance of the γ-GT activity enzyme activity[V]

In our investigation the ACE genotype also showed a connection with γ-GT. In patients with D allele – who have a worse metabolic state with more glycation product (fructosamine) formation – there is a higher γ-GT activity. Data of the literature show that the elevation of the γ-GT level may indicate adaptation to oxidative stress triggered by glycation products. Oxidative stress activates antioxidant defense systems, a major part of which utilizes GSH to inactivate free radicals. The substrate needed to regenerate the antioxidant capacity are provided in part by the γ-GT enzyme.[19]

The question may arise, whether the difference in γ-GT observed in relation to the ACE polymorphism may be a consequence of the so-called non-alcoholic steatosis hepatis (NASH) that is common in type 2 diabetes. To exclude this possibility we also measured the serum bilirubin level, as a marker of hepatic damage. There was no significant positive correlation between serum bilirubin and γ-GT in our study, and patients with different ACE genotype did not show any difference in the serum bilirubin levels. Despite these facts, the

role of 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.

8.4.3. The role of AGEs in the pathogenesis of the complications of DM[VII,VIII]

From results of our study, we might conclude that the DD genotype of ACE polymorphism might cause a worse controllable hypertension and metabolic state. The worse carbohydrate metabolism leads to the accumulation of AGEs that may lead to an increased oxidative stress state. Hypertension, the glycation products and oxidative stress may all lead to endothelial damage that is indicated by increased albuminuria. These data underline the importance of diabetes-related AGEs in the development of late diabetic complications.

Besides the endogenous AGEs formed in NEG reactions, there is another source of AGEs, as they may be exogenously produced and taken up by the body via inhalation (from cigarette smoke) or the digestion process (from food). AGEs in food may be produced easily when heating food rich in carbohydrates and proteins. This occurs either in the case of food processing by the manufacturer or in the kitchen during normal cooking or frying procedures. These exogenously and endogenously produced AGEs together determine the amount of AGEs in the body, and both sources contribute target-organ damage, as shown by data of our workgroup, indicating that food-derived AGEs may increase albuminuria and that high levels of circulating AGEs in a hemodialyzed, partly smoking patient population lead to a higher mortality of the patients.[112,113,114]

8.4.4. Oxidative stress, AGEs, reactive carbonyl species and the kidney[VII,VIII]

There is a multiple connection between glycation processes and oxidative stress. In the NEG reactions, reactive carbonyl intermediates such as glyoxal, methylglyoxal, 3-deoxy-glucosone are formed.[115] These will bind to the $-NH_2$ group of proteins, i.e. precede with the

glycation reactions and cause AGE-modification of proteins. Similar reactive carbonyl molecules may be produced by lipid peroxidation (e.g. malondialdehide, acrolein, hydroxynonenal), modification of amino acids (e.g. glycolaldehide, 2-hydroxy-propanal, acrolein), auotoxidation of ascorbate (e.g. dehydroascorbate, L-threose, 3-deoxy-xylosone).

These carbonyls have multiple effects; they are precursors of advanced glycation-, glycoxydation- and lipidperoxydation end products, as well. The carbonyls and oxidative stress are inter-related, as carbonyls may evoke oxidative stress (e.g. via RAGE activation), while oxidative stress also may enhance formation of carbonyl molecules. In the end, carbonyl stress end-products (CSE) are formed (e.g. N-e-carboxymethyllysine, carboxyethyllysine, pentosidine).[11] CSE is a category that includes a part of AGEs and ALEs, as well.

The connection between AGEs and the kidney is complex, too. In tissues with a higher protein turnover, high molecular weight (HMW) AGEs may be broken down to form low molecular weight (LMW) AGE-peptides and small AGE molecules. The molecular size of these LMW AGEs are is smaller than the cut-off of the glomerular endothelial barrier, thus they can be excreted in the kidney. In fact, the kidney is the only elimination route of AGEs. Therefore, decrease of GFR in chronic kidney diseases may contribute to higher serum AGE levels because of a decreased excretion. The accumulation of AGEs, however, may lead to further endothelial damage and to further deterioration of kidney function, which leads to further AGE accumulation, i.e. to the development of a vitious cycle.[30]

8.5. Clinical study in patients with type 2 diabetes mellitus and different glutathione peroxidase genotypes[VI]

In a study on the GPX-1 genotype and clinical parameters of patients with type 2 DM, we found higher levels of ADMA and L-NMMA in patients with the unfavorable genotype predisposing to increased susceptibility to oxidative stress. There is an approximately 1.5x

difference between the two groups, and according to a review by Teerlink[61], such a difference may already regarded as biologically significant because of the narrow distribution of serum ADMA and L-NMMA in the general population. 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. But it is a risk factor that could be, in interaction with other risk factors, provoke a raise in blood pressure.

8.5.1. Oxidative stress and endothelial dysfunction[VI]

Upon literature data, it seems that there is a potentially strong link between oxidative stress, ADMA, homocysteine (HoCys) and endothelial dysfunction. ADMA and HoCys are inter-related. On the one hand, the source of the methyl group to be transferred to Arg to form ADMA is S-adenosine-methionine (SAM). In this reaction, SAM is turned to HoCys. Therefore, HoCys is formed as a byproduct during the synthesis of ADMA. On the other hand, HoCys increases oxidative stress via increasing $\cdot O_2^-$ production, uncoupling of the NOS, inhibition of antioxidant enzymes (e.g. GPX).[116,117] The increased oxidative stress state leads to a higher rate of protein degradation, this way protein-bound ADMA will be released to form free ADMA. Oxidative stress also inhibits the function of DDAH, the inhibition of break-down further contributes to the accumulation of ADMA. Thus, it is feasible that in states that predispose to high HoCys levels and/or oxidative stress, endothelial dysfunction develops.

8.5.2. Other genetic factors

Other genetic factors influencing the risk of development of chronic complications of DM and CRF include hemochromatosis and methylene-tetrahidrofolate reductase gene

polymorphisms such as HFE-Cl87G and MTHFR-C677T, respectively. In an earlier study of our workgroup, patients with type 2 diabetes mellitus and HFE-Cl87G or MTHFR-C677T mutations had elevated urinary albumin excretion, and more cardiovascular events compared to control patients.[118] Methylene-tetrahidrofolate reductase may influence endothelial function and oxidative stress via its effect on Ho-Cys metabolism, as discussed above. On the other hand, as seen in the *in vitro* experiments, iron may have an important role in free radical formation; therefore iron overload may lead to ROS-related cellular damage. As previously mentioned, the high formation of free radicals in the presence of the Fe-citrate complex is in a possible connection with hemochromatosis, too, as a marked accumulation of a Fe-citrate complex as non-transferrin-bound iron has been described in patients with hemochromatosis.[95]

8.6. Summary

Possible common pathogenetic pathways in the development of the chronic complications of type 2 DM, CRF and ageing, together with potential intervention sites are depicted on *Figure 19.*

Concluding, we may say that the pathogenesis of the chronic complications of DM, CKD and ageing is a complex process, involves among others free radical reactions, the RAAS, AGE formation, inflammatory processes, endothelial dysfunction; therefore, to avoid or postpone these complications, a complex therapy is needed, depending on genetic, metabolic and other characteristics of the individual patient.

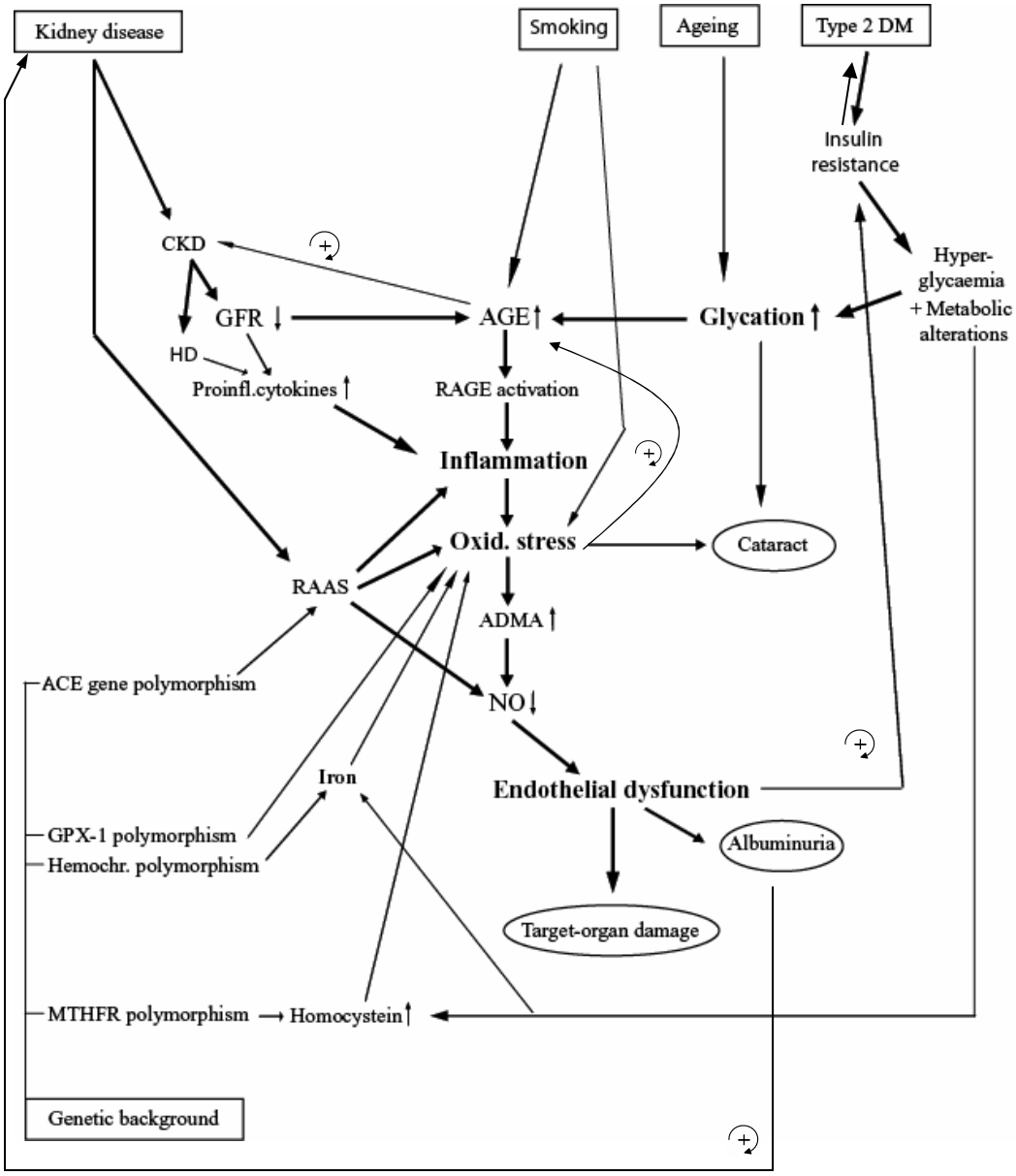

Figure 19
A schematic drawing on the hypothesized or known common ways in the pathogenesis of the complications of chronic kidney disease, type 2 diabetes mellitus, ageing and smoking, with the influence of the genetic background. The diagram is based on literature data and on publications of the workgroup of I. Wittmann, J. Nagy, L. Wagner, Z. Wagner, I. Mazák, T. Kovács, T. Szelestei, P. Degrell, T. Vas, G.A. Molnár, M. Tamaskó, B. Laczy, L. Markó and their collaborators.
Abbreviations: DM, diabetes mellitus; CKD, chronic kidney disease; GFR, glomerular filtration rate; AGE, advanced glycation end products; HD, hemodialysis; RAGE, receptor of AGE; RAAS, renin-angiotensin-aldosterone system; ADMA, asymmetric dimethylarginine; ACE, angiotensin-converting enzyme; L-Arg, L-arginine; NO, nitric oxide; GPX, glutathion peroxidase; HFE, hemochromatosis gene; MTHFR, methylene tetrahidrofolate reductase; ⊕, positive feedback loop.

9 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, the need of more antihypertensive agents 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.

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11 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.

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Ez a dokumentum és az eredmények messze nem csak az én munkám, hanem mindannyiunk, mindannyiótok munkájának is köszönhetők. Örülök, hogy az elmúlt években egy fiatalabb és tapasztaltabb kollégákból, klinikusokból, kutatókból, asszisztensekből, együttműködő partnerekből, TDKs hallgatókból álló kutatócsoport tagja lehettem. Mindannyiuknak hálás vagyok segítségükért, néhányukat név szerint is megemlítve:

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