EFFECT OF RESVERATROL IN TYPE 2 DIABETES AND IN IGA NEPHROPATHY

Ph.D. theses summary

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1. Introduction

Despite of the rather high average daily fat intake in France, as compared to other European countries, epidemiological surveys document a relatively low rate of cardiovascular mortality. This phenomenon, which is often called the French paradox, is thought to be explained by a fairly high red wine consumption by the French.

Red wine is known to be rich in various polyphenolic compounds that might have a variety of health benefits. Among these polyphenols, the stilbene derivative resveratrol seems to be the most vigorously studied, which is likely due to the fact that it apparently affects a wide array of physiological and biochemical processes as shown in animal and cell-culture studies.

Resveratrol is considered to have beneficial effects on the cardiovascular system, as it has been found to improve vasodilatation, ischemic preconditioning, both of which seem to be the result of the activation of the endothelial nitric oxide synthase enzyme (eNOS), and to inhibit both platelet aggregation and vascular smooth muscle cell proliferation. In addition, resveratrol has also been demonstrated to show anti-inflammatory and anti-tumor activities, and it might even have considerable anti-aging properties as it provokes changes in cell signaling that mimics those found upon caloric restriction .

Oxidative stress is likely involved in both the development and the advancement of diabetes mellitus, as several studies demonstrate the involvement of oxygen free radicals (reactive oxygen species, ROS) in the appearance of insulin resistance, a distinctive characteristic of type 2 diabetes. In addition, oxidative stress might even play some direct role in the subsequent surfacing of other diabetes-related complications.

Resveratrol itself is an efficient antioxidant, as evidenced by both in vitro and in vivo studies, and, in parallel, it has also been shown to improve glucose homeostasis and diabetes-related impairments in diabetic animal models

Chronic kidney disease (CKD) is a worldwide health problem by contributing significantly to the increased cardiovascular morbidity and mortality, and also increasing the burden of healthcare expenses.

IgA nephropathy (IgAN) is known as the most common primary chronic glomerulonephritis. Clinical manifestations can occur in a range from very modest symptoms to the progressive renal failure. The deposition of IgA molecules in the mesangium that triggers pro-inflammatory processes is thought to have a major role leading to increased oxidative stress. It is noteworthy that poor prognosis and tendency for progression is more

pronounced in male than in female patients. In addition, it is increasingly recognized that degree of the oxidative stress somewhat also determines the long-term outcome of the disease.

CKD is characterized by increased level of oxidative stress. Resveratrol has been put forward to afford positive effects on various pathophysiological processes related to CKD by reducing oxidative stress as well as inflammatory events, such as the activation of nuclear factor erythroid 2-related factor 2 (Nrf2), the promotion of sirtuin-1 (SIRT-1) expression, or the suppression of nuclear factor κB (NF-κB).

C-C motif chemokine (eotaxin-1, CCL11) is an inflammatory biomarker, higher levels of which were found in CKD patients among other inflammatory cytokines. CCL11 may play a role in the increased cardiovascular risks. CCL11 expression could be enhanced by inflammatory cytokines. Addition of resveratrol in vitro was shown to decrease the transcription and expression of CCL11.

Previously, animal and clinical studies indicated that anti-aging alpha-Klotho has beneficial effects in CKD. Moreover, treatment with resveratrol was shown to enhance the renal expression of Klotho gene both in vitro and in vivo animal studies.

In vitro and in vivo studies showed that statin treatment may also decrease the level of the inflammatory chemokine CCL11 and may increase the level of the anti-aging protein alpha-Klotho.

Glomerular endocapillary proliferation is considered as negative prognostic sign of IgAN. Signaling pathways responsible for this phenomenon could be efficiently altered by resveratrol and other substances as detected during in silico drug screenings.

2. Aims

2.1 Study of the effect of resveratrol in type 2 diabetes

- We aimed to investigate the effects of resveratrol in controlling and/or improving insulin resistance in humans
- We also aimed to determine the effect of resveratrol on oxidative status.
- We wanted to clarify the underlying biochemical mechanisms that might – at least in part – explain the effects of resveratrol, especially in relation to type 2 diabetes.

2.2 Study of the effect of resveratrol in IgA nephropathy

- We aimed to investigate the effects of resveratrol treatment in IgAN on the renal function and albuminuria
- We also aimed to elucidate the underlying biochemical mechanisms on the oxidative stress and other clinical and laboratory parameters, as well as to explore possible mechanisms in the background

3. Patients and methods

3.1 Study of the effect of resveratrol in type 2 diabetes

All procedures involving human patients were approved by the Research Ethics Committee, University of Pécs (certificate number: 2168). Written informed consent was obtained from all participating patients. Nineteen Caucasian male patients previously diagnosed with type 2 diabetes (according to the WHO diagnostic guidelines) were included in the study. They underwent a blinded randomization into two groups: 10 patients to receive oral resveratrol twice daily (in gelatin capsules containing 5 mg resveratrol), and 9 patients for placebo (2 capsules daily, see also below).

3.1.1 Patients

All 19 patients were over 18 years of age, had normal creatinine clearance (C_{Cr} ≥ 90 ml/min) and were on either angiotensin converting enzyme inhibitor or angiotensin II receptor blocker medication. Exclusion criteria were: receiving insulin treatment, receiving corticosteroids, alcohol or drug abuse, severe liver or cardiac (New York Heart Association III-IV) disease, existing autoimmune disease, acute infection and any type of malignancy. The patients were instructed to abstain from any alcoholic beverages and foods containing substantial amounts of resveratrol (e.g. wine, red grapes, peanuts, berries).

3.1.2 Study protocol

Before the initiation of the trial all participants went through a general examination including past medical history, general physical survey, electrocardiogram, blood pressure, pulse rate and blood chemistry analysis (serum sodium, potassium, urea, creatinine, total protein, albumin, HbA_{1c}, fructosamine, alanine transaminase, aspartate aminotransferase, alkaline phosphatase, lactate dehydrogenase, gamma glutamyl transferase, bilirubin, prothrombin). The general examination was followed by a 4-week wash out period before the trial began (during which all lipid-lowering medication was ceased).

The day before the initiation of the trial (designated as baseline), 24-hour urine samples from all patients were collected, from which creatinine, albumin and ortho-tyrosine concentrations were measured. Estimated glomerular filtration rate (eGFR) values were computed according to the Cockroft-Gault equation. During the same day 24-hour blood pressure and a 48-hour continuous tissue glucose monitoring (CGM) (Medtronic MINIMED, SOF-SENSOR, MMT-7002) was initiated. This was followed by drawing blood to measure glucose, insulin, C-peptide, triglyceride, low density lipoprotein, high density lipoprotein, total cholesterol, fructosamine, high sensitivity c-reactive protein, fibrinogen, homocysteine, general blood chemistry (see also above), erythropoietin from the plasma and protein kinase B (pAkt/Akt) ratio in platelets (prepared from peripheral blood).

During the study period, the above measurements were repeated twice: at the end of the second (week 2) and the fourth week (week 4). After initiation of the CGM monitoring, the patients received a test meal (1 portion, see below), which was also followed by the collection of blood samples to determine plasma glucose, serum insulin, C-peptide and triglyceride at mins. 30, 60, 90 and 120. In addition, urine was collected to measure 4-hour creatinine clearance and albumin secretion.

3.1.3 Analytical procedures

Routine blood and urine tests were carried out by the Department of Laboratory Medicine at the University of Pécs Medical School according to standard clinical laboratory procedures. The values of $HOMA_{IR}$, $HOMA_{\beta}$, insulin sensitivity index ($ISI_{Stumvoll}$) and glucose metabolic clearance rate ($MCR_{Stumvoll}$) were calculated.

Oxidative stress was quantified by determining urinary ortho-tyrosine (o-Tyr) levels using a reverse-phase HPLC (Shimadzu LC-10 ADVP HPLC system, Shimadzu USA, OR, USA) equipped with a fluorescence detector (Shimadzu RF-10 AXL). Tyr and its isomers were excited at 275 nm and their emission was measured at 305 nm. Urinary amino acid level was normalized to urinary creatinine concentration.

Platelets were isolated from peripheral blood samples, as follows. To obtain washed human platelets, whole blood was collected from fasted (10-12 hours) patients and was mixed with trisodium citrate (9:1; 130 mM). Then the citrated blood was centrifuged at 250 g for 10 min to obtain the platelet-rich plasma by saving the supernatant into a separated polypropylene tube. The resultant platelet-rich plasma was mixed with an equal volume of washing buffer (pH: 7.4) containing 20 mM Tris-HCl and 150 mM NaCl. The platelet-rich plasma was subsequently centrifuged at 500 g for 10 min, and the platelet pellet was suspended in Hepes-Tyrode's buffer (pH: 7.4) containing (in mM) NaCl 140, KCl 4.5, $CaCl_2$ 2.5, $MgCl_2$ 1.0, glucose 11, Hepes 20, and adjusted to a final concentration of 5.0×10^8 platelets/mL. Isolated platelets were lyzed on ice for 30 min in NP40-buffer containing (in mM) Tris-HCl 20, NaCl 137, NP40 1%, glycerol 5%, and EDTA 1.0, supplemented with phosphatase and protein kinase inhibitors containing (in mM) PMSF 1.0, sodium fluoride 2.0, tetrasodium pyrophosphate 10, sodium orthovanadate 2.0, aprotinin 25, leupeptin 25. Protein concentration of the platelet lysates was determined by the Bradford assay (Bio-Rad) using bovine serum albumin as standard. All chemicals were purchased from Sigma.

The ratio of the phosphorylated form of Akt and total Akt (pAkt (Ser473)/Akt) was determined by Western blot.

Glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic peptide (GIP) and amylin were quantified by using ELISA kits commercially available from Millipore: EGLP-35K for GLP-1, EZHGIP-54K for human GIP and EZHAT-51K for human amylin.

3.1.4 Materials

Resveratrol of herbal origin (with >98% *t*-resveratrol content) and the placebo (both in gelatin capsules) was obtained from Argina Nutraceuticals (previously Admarc Nutraceuticals, Hungary) and dosed 5 mg *per* capsule. The identical placebo capsules only contained the carrier microcrystalline cellulose. Known number of (either test or placebo) capsules were boxed to check patient compliance at the end of the trial. The capsules protein-, carbohydrate- and fat-content were negligible. During the study, adverse effects of resveratrol or any signs of drug interaction have not been observed.

The test meal (Diben) was purchased from Fresenius Kabi (Germany). It was given to patients in 225 ml portions; 1 portion had an energy content of 945 kJ from 10.13 g protein, 20.81 g carbohydrate and 11.25 g fat.

3.1.5 Statistical analysis

The measured parameters (CGM data, time until maximal interstitial glucose level, $HOMA_{IR}$ values, ortho-tyrosin/urinary creatinin ratio, pAkt/Akt ratio) showed normal distribution. The data from patients of the placebo and the resveratrol groups were arranged respectively and evaluated by employing ANOVA (with Bonferoni *post hoc* test) using the program Origin (Microcal, MA, USA), and presented as mean with their standard deviation (SD). Correlation between clinical parameters was analyzed by Pearson's parametric correlation test using the program SPSS 13.0 for Windows.

As after the first two weeks into the trial no significant differences (for none of the measured parameters) between the two groups (*i.e.* resveratrol *vs.* placebo) were observed, the data of week 2 are not shown. Because of the very large individual variations even at baseline in the $HOMA_{IR}$ and ortho-tyrosine values within the same group both measures were analyzed on the individual level; *i.e.* for each participant the value measured at baseline was subtracted from that measured at week 4 (*i.e.* Δ $HOMA_{IR}$ and Δ ortho-tyrosine/creatinine ratio), then the resulting values were averaged within the respective groups.

3.2 Study of the effect of resveratrol in IgA nephropathy

Study protocol and all procedures were approved by the Research Ethics Committee, University of Pécs (certificate number: 4583). Written informed consent was obtained from all participants. Originally, our double-blind, placebo-controlled, randomized, parallel-group, prospective, 12-week long study was scheduled to involve 100 IgAN patients, however, interim analysis of 27 patients indicated that resveratrol treatment is likely to increase albuminuria, therefore, examinations were subsequently terminated. Enrolled IgAN patients were randomly assigned to receive the product containing Resveratrol (N=15; male: 9, female: 6) or Placebo N=12; male: 6, female: 6).

3.2.1 Patients

Caucasian patients with histologically determined IgAN were included. Further inclusion criteria were as follows: age > 18 yrs, CKD stage 1 and 2 (eGFR ≥ 60 ml/min/1.73 m^2); eGFR was calculated by CKD-EPI formula, treatment with angiotensin-converting enzyme-inhibitor and/or angiotensin II receptor blocker, no significant changes in proteinuria and eGFR over the past three months. The Control group involved healthy (non-IgAN) subjects. The Control group was matched with that of the IgAN patients.

Exclusion criteria implied pregnancy, lactation, acute infection, malignancy, alcohol or drug abuse, overt autoimmune disease, severe liver or cardiac (New York Heart Association III-IV) disease.

3.2.2 Study protocol

At baseline (Visit 1), urinary creatinine and albumin, ortho-tyrosine concentrations, and 24 hours creatinine clearance were measured in urine samples collected for 24 hours prior to Visit 1. First morning urine samples were also obtained from patients to assess urinary albumin concentration and albumin/creatinine ratio. Blood samples were taken to determine main laboratory parameters, including: glucose, sodium, potassium, calcium, phosphate, urea, creatinine, total protein, albumin, alanine transaminase, aspartate amino transferase, alkaline phosphatase, lactate dehydrogenase, gamma glutamyl transferase, bilirubin, blood count, IgA. Fructosamine, hemoglobin A_{1c}, insulin, C-peptide, parathyroid hormone, triglyceride, low density lipoprotein, high density lipoprotein, total cholesterol, prothrombin, high-sensitivity C-reactive protein, reticulocyte number, iron, transferrin, transferrin saturation, ferritin, erythropoietin were measured only at baseline. The levels of ortho-tyrosine, alpha-Klotho and CCL11 were also quantified.

Patients were randomly subjected to resveratrol or placebo treatment. Patients of the Resveratrol group were administered orally 2x5 mg resveratrol pills, while patients of the Placebo group were given orally 2x1 pills per day.

Aforementioned urinary and routine blood measurements were re-conducted after 6 weeks (Visit 2) and 12 weeks (Visit 3).

Medical records and physical examination were taken after 3. visit.

3.2.3 Analytical procedures

Routine blood and urinary measurements were carried out according to standard clinical laboratory procedures in the Department of Laboratory Medicine, Medical School, Univ. of Pécs (Pécs, Hungary).

Oxidative stress was determined by measuring urinary and plasma ortho-tyrosine levels as described previously (see in 3.1.3).

CCL11 levels were measured using Human CCL11/Eotaxin Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA). Alpha-Klotho levels were measured using Human soluble α-Klotho Assay Kit (Immuno-Biological Laboratories Co., Fujioka, Gunma, Japan)

3.2.4 Materials

Both resveratrol and placebo gelatin capsules were obtained from Argina Ltd. (Fót, Hungary), as described previously (see in 3.1.4.)

3.2.5 Statisctical analysis

Urinary ortho-tyrosine excretion, urinary ortho-tyrosine/creatinine ratio, eGFR, urinary albumin and albumin/creatinine ratio, CCL11 showed not normal distribution, alpha-Klotho showed normal distribution by Kruskal-Wallis test. For comparisons of Visit results either Mann-Whitney test (in case of parameters showing not normal distribution) or one-way ANOVA and *Bonferroni post hoc* test (in case of parameters showing normal distribution) was used. Mann-Whitney test was performed to compare changes at group-level. The changes of eGFR and albuminuria were compared between Resveratrol and Placebo groups, as well as between male and female patients. Results are expressed as the percentage of 6 week or 12 week, taking Baseline as 100 % due to the low number of cases and high variability of the initial data.

Results of variables showing normal distribution are expressed as mean ± SD, while median (IQR, interquartile range) values were presented for parameters with not normal distribution. Relationships between clinical variables were examined by Pearson correlation.

Statistical analyses were performed using IBM SPSS Statistics, Version 22 software.

4. Results

4.1 Study of the effect of resveratrol in type 2 diabetes

The study was completed with the full compliance of all patients enrolled. The patients that were randomly assigned to the resveratrol and the placebo group initially formed an apparently homogeneous population regarding all the assessed parameters , even considering their previous lipid medication usage. Significant differences between the two groups in the assessed clinical and biochemical parameters surfaced at the end of the fourth week of the trial (week 4).

4.1.1 Effect of resveratrol treatment on glucose levels

Some noticeable differences between the two groups (*i.e.* the placebo *vs.* the resveratrol group) emerged at week 4. While the records of the placebo group at both baseline and week 4, and those of the resveratrol group at baseline were virtually

indistinguishable, significant differences were found, when these records were statistically analyzed for either the two groups (*i.e.* the placebo *vs.* the resveratrol group) at week 4, or for the resveratrol group at baseline *vs.* week 4. For instance, the CGM records of the resveratrol group at week 4 displayed a distinct initial drop followed by a recovery, which was reflected in two statistically analyzable parameters. First, the time to the maximum glucose level significantly differed within the resveratrol group at baseline *vs.* week 4 (49.50 ± 13.83 min. *vs.* 81.25±20.49 min., p=0.006), as well as between the two groups at week 4 (81.25±20.49 min. *vs.* 58.1±18.42 min., p=0.03). Second, the extent of the initial drop (between min. 23-35 after the test meal) was again significantly different between the two groups at week 4 (6.79 ± 2.95 *vs.* 8.64 ± 4.42 mmol/l, p=0.023).

4.1.2 Effect of resveratrol treatment on insulin resistance

The changes in $HOMA_{IR}$ values at week 4 *vs.* baseline on the level of individuals were compared, then the differences were averaged within the respective group, a significant dissimilarity between the two groups emerged (resveratrol group: -1.52 ± 1.18 *vs.* placebo group: 0.04 ± 1.4 for , p=0.044). No differences were seen for $HOMA_{\beta}$ values.

4.1.3 Effect of resveratrol treatment on urinary ortho-tyrosine excretion

A significant difference between the two groups again surfaced (0.02 ± 0.046 *vs.* -0.015 ± 0.014 µmol/mmol, p=0.043), if the values of difference (*i.e.* the week 4 *minus* baseline) on the individual level were considered.

4.1.4 Effect of resveratrol treatment on pAkt/Akt ratio

Four-week long treatment with resveratrol significantly increased the ratio of phosphorylated *vs.* total Akt (pAkt/Akt) in platelets (0.78 ± 0.25 *vs.* 1.41 ± 0.36, p=0.032), while the pAkt/Akt ratio in the placebo group did not change during the trial (0.81 ± 0.54 *vs.* 0.72 ± 0.37; p=NS).

4.1.5 Effect of resveratrol treatment on incretin levels

No significant changes were found for amylin, GIP and GLP-1 throught out the trial. At week 4 the amylin level was 2.98 ± 0.69 pM in the resveratrol group and 3.11 ± 2.93 pM in the placebo group (NS); the GIP level was 38.22± 9.44 pg/ml in the resveratrol group and

36.23 ± 8.17 pg/ml in the placebo group (NS); and the GLP-1 level was 7.73±1.93 pM in the resveratrol group, and 7.21±0.72 pM in the placebo group (NS).

4.1.6 Effect of resveratrol treatment on correlation between systolic hyperbaric impact and insulin resistance

However, we have noticed that the initial negative correlation between systolic hyperbaric impact (calculated from 24-hour blood pressure monitoring) and $ISI_{Stumvoll}$ disappeared as a result of resveratrol treatment ($R_{baseline}$= -0.826, p=0.003 *vs.* $R_{week\ 4}$= -0.028, p= 0.943 for the resveratrol group) and ($R_{baseline}$= -0.757, p=0.029 *vs.* $R_{week\ 43}$= -0.716, p=0.046 for the placebo group).

Similar results were obtained, when the systolic hyperbaric impact was correlated with $MCR_{Stumvoll}$: ($R_{baseline}$= -0.834, p=0.003 *vs.* $R_{week\ 4}$=-0.023, p=0.953 for the resveratrol group) and ($R_{baseline}$= -0.757, p=0.029 *vs.* $R_{week\ 4}$= -0.717, p=0.045 for the placebo group), respectively.

4.2 Study of the effect of resveratrol in IgA nephropathy

At baseline, main clinical characteristics of male and female patients were similar between the Resveratrol group and the Placebo group.

The Control group (non-IgAN) was matched to IgAN patients regarding age (54.0 yrs (IQR:6) vs. 47.5 yrs (IQR:19);p=NS), regarding eGFR (85.18 ml/min/1.73 m2 (IQR:19) vs. 83.00 ml/min/1.73 m2 (IQR:37);p=NS), and regarding body weight (80.3 kg (IQR:20.8) vs. 83.5 kg (IQR:20.0);p=NS).

The male Control group (non-IgAN) was matched to male Resveratrol group regarding age (54.0 yrs (IQR:10) vs. 34.0 yrs (IQR:20);p=NS), regarding eGFR (87.3ml/min/1.73 m2 (IQR:47) vs. 101.0ml/min/1.73 m2 (IQR:51);p=NS), and regarding body weight (84 kg (IQR:21) vs. 87.0 kg (IQR:26.5);p=NS).

The female Control group (non-IgAN) was matched to female Resveratrol group regarding age (54.0 yrs (IQR:7) vs. 51.5 yrs (IQR:12);p=NS) , regarding eGFR (79.6 ml/min/1.73 m2 (IQR:15) vs. 79.5 ml/min/1.73 m2 (IQR:33);p=NS) , and regarding body weight (76 kg (IQR:27.5) vs. 78.9 kg (IQR:30);p=NS).

However, there was also no difference between male and female subjects in the Control group (non-IgAN) concerning age (54.0 yrs (IQR:10) vs. 54.0 yrs (IQR:7);p=NS), eGFR values (87.26 ml/min/1.73 m2 (IQR:47) vs. 79.6 ml/min/1.73 m2 (IQR:15);p=NS) and body weight (84 kg (IQR:21) vs. 76 kg (IQR:27.5);p=NS).

4.2.1 Gender-dependent effect of resveratrol treatment on urinary ortho-tyrosine excretion

At Baseline, there were no differences in urinary ortho-tyrosine concentration and ortho-tyrosine/creatinine ratio between male and female subjects in the Control (non-IgAN) group. However, male IgA patients in the Resveratrol group had higher urinary ortho-tyrosine concentration than females (534.37(911.46) nmol/l *vs.* 85.94 (189.60) nmol/l ;p=0.018), as well as urinary ortho-tyrosine/creatinine ratio compared to male (non-IgAN) controls (p=0.019) at Baseline.

At Baseline, in male IgAN patients there were no differences in the urinary ortho-tyrosine concentration (534.37(911.46) nmol/l *vs* 241.74(603.64) nmol/l, p=NS) and urinary ortho-tyrosine/creatinine ratio (90.16(193.66) nmol/mmol *vs* 60.85 nmol/mmol, p=NS)between the Resveratrol and the Placebo group.

In contrast, female IgAN patients showed comparable urinary ortho-tyrosine concentration and ortho-tyrosine/creatinine ratio with female controls at Baseline.

Resveratrol treatment after 6 week significantly decreased both urinary ortho-tyrosine concentration and urinary ortho-tyrosine/creatinine ratio in male IgAN patients (534.37(911.46) nmol/l *vs* 144.56(177.83) nmol/l; p=0.017 and 90.16(193.66) nmol/mmol *vs.* 22.80(24.42) nmol/mmol p=0.008 vs. Baseline, respectively). However, these changes were not found in female IgAN patients of the Resveratrol group.

After 12 week of resveratrol treatment, both urinary ortho-tyrosine concentration and urinary ortho-tyrosine/creatinine ratio were still substantially lower in male IgAN patients (534.37(911.46) nmol/l *vs.* 39.06(88.00) nmol/l ; p=0.001 and 90.16(193.66) nmol/mmol *vs.* 3.90(17.94) nmol/mmol; p=0.004 vs. Baseline, respectively) but were not altered in female IgAN patients of the Resveratrol group.

4.2.2 Effect of resveratrol treatment on eGFR

To demonstrate the effect of resveratrol treatment on renal function, changes of eGFR, expressed as median values of 6 week/Baseline and 12 week/Baseline ratios were calculated.

At 6 week, resveratrol treatment increased eGFR in male IgAN patients compared to the Placebo group, as indicated by higher 6 week/Baseline ratio (103,2(13) % *vs* 96 (24) %¸p=0.026). However, 12 week/Baseline ratios were similar between the Resveratrol and the Placebo group . After 3 months open label wash out period, eGFR was reverted in male IgAN patients of the Resveratrol group, as indicated by Wash out Visit/Baseline ratio compared to 6 week/Baseline ratio (p=0.028).

We found no changes of eGFR in female IgAN patients with resveratrol treatment, as indicated by comparable 6 week/Baseline (108.3(27) *vs.* 85.1(24)%, p=NS) and 12

week/Baseline ratios (102.7(14)% *vs* 97.9(20) %, between the Resveratrol and Placebo group at 6 week and 12 week.

4.2.3 Effect of resveratrol treatment on albuminuria

To demonstrate the effect of resveratrol treatment on albumin excretion, changes of urinary albumin and albumin/creatinine ratio, expressed as median values of 6 week/Baseline and 12 week/Baseline ratios were calculated.

Surprisingly, there was an increase of albuminuria in male IgAN patients after resveratrol treatment compared to the Placebo group at 6 week, as indicated by higher 6 week/Baseline ratios of the albumin/creatinine ratio (201,6(2504)% *vs* 43,9(43) %; p=0.006), and the urinary albumin concentration (452.2(1783)% *vs.* 16.7(59)%; p=0.018).

The higher 6 week/Baseline albumin/creatinine ratio remained unaltered in male IgAN patients after resveratrol treatment at 12 week, although 12 week/Baseline ratios were not statistically significant compared to the Placebo group (184.2 % *vs* 58.4(73)%; p=NS). At 12 week, resveratrol treatment resulted in higher urinary albumin concentration in male IgAN patients compared to the Placebo group, as indicated by increased 12 week/Baseline ratio (698.9(1364)% *vs*. 44.4(75,4)%, p=0,011). The changes of albumin/creatinine ratio were reversible in male IgAN patients, as indicated by open label Wash out/Baseline ratio of the Resveratrol compared to 6 week/Baseline ratio in Pacebo groups (p=NS).

Resveratrol treatment had no impact on albumin/creatinine ratio in female IgAN patients at 6 week (42.9(110)% *vs* 96.1(187)%, p=NS) or 12 week (61,4(109)% *vs* 58,4(73)%, p=NS) compared to the Placebo group, as indicated by the unaltered 6 week/Baseline and 6 week/Baseline ratios. Similarly, there were no changes in the urinary albumin concentration with resveratrol treatment in female IgAN patients at 6 week (40.0(108)% *vs.* 114.3(119) %, p=NS) or 12 week (33.5(96)% vs. 107.1(184)%, p=NS).

4.2.4 Effect of statin treatment on CCL11 and alpha-Klotho

There were regular statin-users in both the Resveratrol (N = 6) and the Placebo group (N = 4), therefore we investigated the potential modifying effect of statin treatment on CCL11 and alpha-Klotho. However, neither CCL11 nor alpha-Klotho levels showed significant differences between statin-users and non-users at baseline.

4.2.5 Effect of resveratrol treatment on CCL11 and alpha-Klotho

Resveratrol treatment had no effect on CCL11 levels irrespective of genders. The serum level of CCL11 remained unaltered in both male patients (247,25(33,13) pg/ml *vs.*

247,75(122) pg/ml, p=NS) and female patients (277,75(155,5) pg/ml *vs.* 325,5(206) pg/ml, p=NS).

The alpha-Klotho levels were also unaltered after resveratrol treatment in both male and female patients.

4.2.6 Effect of resveratrol treatment on the relationship of urinary ortho-tyrosine excretion and CCL11 levels

In male IgAN patients after resveratrol treatment, we found positive correlation between CCL11 levels and both urinary ortho-tyrosine excretion ($r = 0.829$, $p = 0.042$) and urinary ortho-tyrosine/creatinine ratio ($r = 0.829$, $p = 0.042$) at 12 week. Conversely, relationship between CLL11 levels, and urinary ortho-tyrosine concentration and urinary ortho-tyrosine/creatinine could not be observed in any female IgAN patients of the Resveratrol or the Placebo group.

5. Discussion

5.1 Study of the effect of resveratrol in type 2 diabetes

In our first double-blind, placebo controlled study, we tested the effects of trans-resveratrol on male patients with type 2 diabetes, and found that oral resveratrol in reasonably low dosages (2 x 5 mg daily) improved insulin resistance and, likely as a consequence, decreased blood glucose levels and delayed the appearance of glucose peaks after a test meal. In addition, we also found that resveratrol seemed to decrease oxidative stress and increased Akt phosphorylation. On the other hand, no resveratrol-induced changes were found in GLP-1, GIP and amylin levels. At the same time, we found no evidence that resveratrol would effect β-cell function ($HOMA_{\beta}$).

Our findings appear to be in accordance with the results of previous studies, which suggest an antioxidant role for resveratrol. For instance, resveratrol was previously found to lessen oxidative stress in isolated K562 human leukemia cells and in rats with streptozocin-induced diabetes, which might relate to resveratrol's potential to scavenge oxygen free radicals *in vitro.* On the other hand, it also seems to be capable of both decreasing the production of oxygen free radicals, and increasing certain antioxidant enzyme levels/activity.

The blood glucose reducing effect of resveratrol in diabetic rats was documented and found to be associated with a resveratrol-induced activation of Akt and eNOS. The phosphorylation of Akt is known to be an essential step of insulin signaling. Our findings with humans support the notion that the insulin-resistance lowering effect of resveratrol also occurs *via* the activation of the Akt signaling pathway.

Although at this time it is not possible to pin-point a clear cause-and-effect relationship between oxidative stress and some impairment in Akt-activating mechanisms, it is tempting to speculate that such causative coupling might exist.

It has been previously shown that a negative correlation exists between hypertension and insulin sensitivity (*i.e.* $ISI_{Stumvoll}$). Thus, it is noteworthy that the negative correlation between systolic hyperbaric impact, $ISI_{Stumvoll}$ and $MCR_{Stumvoll}$ seemed to disappear upon resveratrol treatment, suggesting that resveratrol might affect the nature of the association between insulin resistance and hypertension and might have favourable vascular effect.

5.2 Study of the effect of resveratrol in IgA nephropathy

In our second pilot study, which was terminated earlier due to the unfavorable interim analysis results showing that resveratrol treatment increased albuminuria.

Our previous report showing that male IgAN patients exerted higher level of oxidative protein damage and oxidative stress markers assessed by fluorescence method together with the initially higher urinary ortho-tyrosine excretion observed here, clearly indicate increased oxidative stress status in male IgAN patients compared to females. Our findings appear to support the notion that increased oxidative stress may be responsible, at least in part, for the poorer prognosis recognized in male IgAN patients.

Here we demonstrate that while administration of trans-resveratrol (2x5 mg per day) significantly reduced oxidative stress, it also increased eGFR and albuminuria in male IgAN patients; whereas any of these effects could not be detected in female IgAN patients. On the other hand, increased renal function and albuminuria in male IgAN patients seemed reversible effects of resveratrol treatment, as indicated by reduced eGFR and ACR values after 3 months open label wash out period.

Several reports proposed that resveratrol by suppressing oxidative stress could beneficially alter numerous pathophysiological processes in CKD. Our present data revealed to corroborate this notion only in male IgAN patients. Accordingly, we found significant relationship between urinary ortho-tyrosine/creatinine ratio and CCL11 levels after 12 weeks of resveratrol treatment in male but not in female IgAN patients. The positive correlation of ortho-tyrosine/creatinine ratio with CCL11 after resveratrol treatment suggests that altered inflammatory state could be a consequence of reduced oxidative stress.

In addition, evidences demonstrating the effects of statin treatment to decrease the level of the inflammatory chemokine CCL11 and to increase the level of the anti-aging protein alpha-Klotho could not be verified in our present study.

Increased eGFR found here with resveratrol treatment could be possibly, due to the enhancement of single nephron hyperfiltration as a result of increased renal blood flow; similar that seen in animals where resveratrol caused endothelium-dependent renal vasodilation through its antioxidant properties. Nevertheless, resveratrol treatment reduced oxidative stress associated with increased eGFR in male IgAN patients.

It is noteworthy that results of the 52-week BEAM study conducted in CKD patients with T2DM showed similar increases of eGFR and albuminuria after bardoxolone methyl treatment, which is also known to possess antioxidant and Nrf2 activator capacity. It is conceivable to suggest that there could be common mechanisms by which resveratrol and bardoxolone methyl could provoke increased urinary albumin excretion. In addition, increased proteinuria found here with resveratrol treatment was also noticed in bardoxolone methyl-treated CKD patients with T2DM in the BEACON trial. Consequently, in kidney failure augmented hyperfiltration of the residual nephrons may lead to increased proteinuria and advanced decline of the renal function.

In conclusion, our results suggest that resveratrol treatment, as bardoxolone methyl therapy was indicated earlier in CKD patients with T2DM is not recommended in patients with IgA nephropathy.

6. List of the Ph.D. theses

1. Resveratrol treatment decreases oxidative stress in T2DM patients, as assessed by measuring urinary ortho-tyrosine excretion.
2. Resveratrol treatment increases Akt phosphorylation in T2DM patients, which could play a role in the decrease of insulin resistance ($HOMA_{IR}$).
3. Resveratrol treatment do not affect incretin levels in T2DM patients.
4. Male IgAN patients show higher levels of oxidative stress than female IgAN patients or the male non-IgAN individuals as indicated by increased ortho-tyrosine excretion.
5. Resveratrol treatment afforded gender specific effect in IgAN patients. Significantly reduces oxidative stress, as indicated by decreased ortho-tyrosine excretion
6. Resveratrol treatment reverse increased eGFR and albuminuria in male IgAN patients. Any of these effects could not be detected in female IgAN patients.

7. LIST OF PUBLICATIONS

Publications related to the Ph.D. theses:

1. **P Brasnyó**, G A Molnár, M Mohás, L Markó, B Laczy, J Cseh, E Mikolás, I A Szijártó, Á Mérei, R Halmai, L G Mészáros, B Sümegi, I Wittmann. Resveratrol improves insulin sensitivity, reduces oxidative stress and activates the Akt pathway in type 2 diabetic patients. Br J Nutr. 2011 Aug;106(3):383-9. **IF: *3.013***

2. **Brasnyó P,** Molnár G A, Mohás M, Markó L, Laczy B, Cseh J, Mikolás E , Szijártó I A , Halmai R , Mészáros G L, Sümegi B, Winkler G, Wittmann I. REZVERATROL HATÁSA 2-ES TÍPUSÚ DIABETESES BETEGEK ANYAGCSERÉJÉRE. Magy. Belorv. Arch. 2012; 65: 75–81.

3. **Pál Brasnyó**, Tibor Kovács,Gergő A. Molnár, Eszter Sélley, Szilárd Kun, Tibor Vas, Boglárka Laczy, Katalin Fekete, Krisztina Kovács, László G. Mészáros, Gábor Winkler, Balázs Sümegi and István Wittmann.Resveratrol causes gender-dependent and bardoxolone methyl-like effects in patients with IgA nephropathy. Pilot study. J Nutr Food Sci 2016, 6: 442.

Chapter related to the Ph.D. theses:*:*

1. **P Brasnyó**, B Sümegi, G Winkler, I Wittmann: Resveratrol and Oxidative Stress in Diabetes Mellitus. Diabetes : Oxidative Stress and Dietary Antioxidants, edited by: Victor Preedy, Academic Press, 01/2014 : pages 99-109 (2013); ISBN: 978-0-12-405885-9

Abstracts related to the Ph.D. theses:

1. **Brasnyó P.,** Laczy B**.,** Tamaskó M., Molnár G.A., Wagner Z., Gallyas F., Wittmann I., Sümegi B.: A rezveratrol hatásainak elővizsgálata 2-es típusú diabetes mellitusos betegekben. *Diabetologia Hungarica 14(S2):27-28. (2006*)

2. **Brasnyó P**., Laczy B**.,** Tamaskó M., Molnár G.A., Wagner Z., Gallyas F., Nagy J., Wittmann I., Sümegi B.: A rezveratrol in vivo hatásai 2-es típusú diabetes mellitusos betegekben. *Magyar Belorvosi Archivum Supplementum 59. (S2):46-47. (2006*)

3. Cseh J, **Brasnyó P**, Mohás M , Laczy B , Tamaskó M, Molnár G A , Wagner Z, Sümegi B, Wittmann I: Transz-rezveratrol in vivo hatásainak vizsgálata 2-es típusú diabetes mellitusos betegekben. Előzetes eredmények. FOLIA HEPATOLOGICA (ISSN: 1419-1156) 11: (S3) pp. 12-13. (2007)

4. **Brasnyó P,** Molnár G A, Mérei Á, Cseh J, Mikolás E, Halmai R, Mészáros G L, Sümegi B, Wittmann I: Rezveratrol hatása 2-es típusú diabeteses betegekben. Új eredményeink. DIABETOLOGIA HUNGARICA (ISSN: 1217-372X) 18: (S1) pp. 63-64. (2010)

5. **P Brasnyó,** G A Molnár, M Mohás, L Markó, B Laczy, J Cseh, E Mikolás, I A Szijártó, Á Mérei, R Halmai, L G Mészáros, B Sümegi, I Wittmann.: Effect of resveratrol on insulin sensitivity, oxidative stress and Akt pathway in humans. Diabetologia (2010) 53: (Suppl1) S1-S556.

6. Kovács T, **Brasnyó P.,** Molnár G A , Sélley E, Kun Sz, Vas T, Laczy B, Fekete K, Kovács K, Mészáros G L,Winkler G, Sümegi B, Wittmann I ; Rezveratrol hatásának vizsgálata IgA nephropathiában. (Pilot Vizsgálat) . *Hypertonia és Nephrologia 2015:19(Suppl4);S1-S64.*

List of publications not related to the theses

1. Wittmann I., Molnár G A, Wagner L, Wagner Z, Tamaskó M., Laczy B, **Brasnyó P**, Halmai R, Markó L, Nagy J.: A metabolikus szindróma két koncepciójának összehasonlítása: WHO-kritériumok és ATP III-feltételrendszer *Diabetologia Hungarica 13 (4): 263-272 (2005)*

2. **Brasnyó P**, Wittmann I: A diabetes mellitus és a metabolikus szindróma kardiovaszkuláris szövődményeit vizsgáló klinikai tanulmányok tanulságai. Célértékek és kezelési stratégiák. *Granum 2011; XIV (3) : 21-24.*

3. 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 2005 Jun;68 Suppl1:S36-42. **IF: 1.236**

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