Charles University in Prague
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OXIDATIVE STRESS, MICROINFLAMMATION AND CARDIOVASCULAR RISK IN PATIENTS WITH CHRONIC KIDNEY DISEASE

(PhD Thesis Summary)

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CONTENTS

1. INTRODUCTION

Cardiovascular mortality is greatly increased in chronic kidney disease (CKD) and especially end-stage renal disease (ESRD) patients in comparison with the general population (Foley *et al.* 1998, Baigent *et al.* 2000). Although the prevalence of 'traditional' cardiovascular disease (CVD) risk factors is high in these patients, the extent and severity of CVD complications is disproportionate to the underlying risk factor profile (Cheung *et al.* 2000). Consequently, there have been recent efforts to concentrate on new, 'non-traditional' CVD risk factors, such as oxidative stress, chronic low-grade inflammation, malnutrition and endothelial dysfunction in this patient population (Himmelfarb 2004).

Oxidative stress is defined as an imbalance between excessive generation of oxidant compounds and insufficient antioxidant defence mechanisms (Sies 1997). An improper, maladaptive activation of oxidative processes as well as insufficiency of the antioxidant system is chronically present in CKD patients, thus leading to cell and tissue injury (Locatelli *et al.* 2003, Canaud *et al.* 1999). Factors contributing to enhanced oxidant production in CKD patients are uremia *per se*, chronic inflammatory state, bio-incompatibility of dialysis membranes, dialysate contamination and intravenous (i.v.) administration of iron (Fe^{3+}) preparations (Locatelli *et al.* 2003, Fishbane 1999). This imbalance of pro- and antioxidant mechanisms is present from the earliest stages of CKD, but is most pronounced in chronic hemodialysis (HD) patients (Locatelli *et al.*).

Apart from enhanced oxidative stress, CKD patients are subject to chronic low-grade inflammation or 'microinflammation' (Schindler 2004). This chronic inflammatory state is caused by renal retention of pro-inflammatory cytokines (Descamps-Latscha *et al.* 1995), oxidative and carbonyl stress products accumulation (Witko- Sarsat *et al.* 1998), acidosis, volume overload (Niebauer *et al.* 1999, Dumler 2003), bioincompatibility of dialysis membranes, dialysate contamination (Schindler 2004) and frequent dialysis access site infections (Ayus *et al.* 1998, Jaar *et al.* 2000).

In CKD patients, oxidative stress and microinflammation are interconnected with endothelial dysfunction. Activated neutrophils produce myeloperoxidase (MPO), which converts hydrogen peroxide into hypochlorous acid and also functions as nitric oxide (NO·) oxidase and thus regulates vascular NO· bioavailability (Baldus *et al.* 2002). MPO also

contributes to the formation of advanced oxidation protein products (AOPP) – an acute oxidative stress marker, originally described in HD patients (Witko-Sarsat *et al.* 1996). MPO-induced oxidative injury and NO· bioavailability regulation represent a direct link between microinflammation, oxidative stress and endothelial dysfunction (Himmelfarb 2004). CKD patients have elevated levels of C-reactive protein (CRP), which represents one of the strongest predictors of cardiovascular morbidity and mortality in these patients (Arici *et al.* 2001, Stenvinkel 2001) and also in healthy individuals with a negative history of CVD (Ridker 2001, Ridker 2003), patients with known CVD (Retterstol *et al.* 2002) and in patients with acute coronary syndromes (Blake *et al.* 2003). In acute coronary syndromes, it seems that pregnancy-associated plasma protein-A (PAPP-A) could represent an even better marker than CRP (Bayes-Genis *et al.* 2001). PAPP-A is a zinc-binding metalloproteinase produced by a number of human tissues (Overgaard *et al.* 1999, Schindler *et al.* 1984), which is responsible for proteolytic cleavage of insulin-like growth factor binding protein (IGFBP)-4 and 5 and thus functions as a positive regulator of insulin-like growth factor (IGF)-I and II bioavailability (Lawrence *et al.* 1999). Abundant expression of PAPP-A was found in unstable atherosclerotic plaques and circulating PAPP-A was proposed as a candidate marker of acute coronary syndromes (Bayes-Genis *et al.* 2001). Whether circulating PAPP-A levels could be of any diagnostic or prognostic value in CKD patients' morbidity and mortality remains to be elucidated (Kalousová *et al.* Blood Purif 2004). In HD patients, there have also been found elevated serum concentrations of soluble adhesion molecules (vascular cell adhesion molecule – VCAM, intercellular adhesion molecule – ICAM-1 and E-selectin), endothelial expression of adhesion cell molecules being one of the key events in the pathogenesis of atherosclerosis (Cominacini *et al.* 1997).

As oxidative stress plays an important role in the process of atherogenesis, antioxidant therapy seems to be a promising approach to reduce CVD in CKD patients. Several studies with various antioxidants (N-acetylcystein, vitamin E-coated dialysers, oral vitamin E) have been performed with controversial results (Tepel *et al.* 2003, Tsuruoka *et al.* 2002, Boaz *et al.* 2000, Heart Protection Study Collaborative Group 2002, Mann *et al.* 2004). It is still unclear in what doses, when and whether at all antioxidants should be routinely administered to CKD patients.

2. AIMS

The aim of the study was to evaluate markers of oxidative stress, inflammation and CVD in CKD patients and to study the influence of dialysis treatment and pharmacotherapy on these parameters:

1. Study of peripheral blood neutrophil respiratory burst in HD patients:
   - Monitoring peripheral blood neutrophil respiratory burst during a HD session by chemiluminescence assessment
   - Detection of possible influence of i.v. ferric sodium-gluconate administration on peripheral blood neutrophil respiratory burst and plasma advanced oxidation protein products (AOPP) concentration during a HD session
   - Evaluation of the effect of oral vitamin E supplementation on peripheral blood neutrophil respiratory burst and plasma AOPP concentration in HD patients

2. Study of circulating pregnancy-associated plasma protein-A (PAPP-A) in CKD patients:
   - Determination and comparison of serum PAPP-A concentrations in CKD, HD and continuous ambulatory peritoneal dialysis (CAPD) patients and in healthy controls
   - Examination of possible association between PAPP-A and AOPP as a marker of oxidative stress and C-reactive protein (CRP) as an inflammatory marker

3. Investigation of plasma PAPP-A concentrations during HD session and after i.v. ferric sodium-gluconate administration.

4. Study of the effect of oral vitamin E supplementation on inflammatory and CVD markers in HD patients:
   - Detection of changes in serum PAPP-A, CRP, intercellular adhesion molecule-1 (ICAM-1) and E-selectin concentrations after 5 weeks' oral vitamin E therapy in HD patients

3. METHODS

3.1 Laboratory methods

3. 1. 1 Chemiluminescence assay

We used a modified micromethod derived from the original method described in (Metcalf *et al.* 1986).

Chemicals: Luminol (Sigma), dimethylsulphoxide (DMSO, Sigma), phorbol-myristate-acetate (PMA, Sigma).

Solutions: Hanks' balanced salt solution (HBSS) pH 7.4: prepared by dilution of 0.4g KCl, 0.06g KH_2PO_4, 8.0g NaCl, 0.35g $NaHCO_3$, 0.12g Na_2HPO_4x $12H_2O$ and 1.0g glucose in 1 l of distilled water; modified HBSS: prepared by adding 2.5ml $CaCl_2$x $2H_2O$ (14 g/l) and 5.0ml $MgSO_4$x $7H_2O$ (10 g/l) into 250ml HBSS; storage solution of luminol in DMSO (10 mmol/l); working luminol solution (1 mmol/l): prepared by mixing 900µl of modified HBSS with 100µl storage solution of luminol; phosphate buffered saline (PBS): 0.02 mol/l, pH 7.4; storage solution of PMA in DMSO (2 g/l); working PMA solution (10 mg/l): prepared by mixing 10µl of storage PMA solution with 2ml PBS and filtering the solution using 0.4µm filter); Ficoll-Paque-(tm) PLUS® solution (Pharmatech).

Material: full blood drawn on lithium heparin: 1ml per analysis, all samples were processed within 2 hours from withdrawal.

Procedure: 1ml of heparinized blood is layered on 1ml of Ficoll-Paque-(tm) PLUS ® solution and the test tube is left in vertical position at laboratory temperature for 30 minutes. After separation, neutrophils stay in a coil at the solution surface, while the rest of the cells descend lower down. The neutrophil suspension is then collected with a pipette into an Eppendorf test tube.

The test itself is performed on a microtitre plate: 50µl of neutrophil suspension is added into 100µl of modified HBSS mixed with 60µl of working luminol solution and 40µl of working PMA solution and chemiluminescence is measured immediately.

Conditions of measurement: Luminometer Thermostated LM-01T, Immunotech, Beckman Coulter Company, USA. Chemiluminescence is measured at 37°C for 45 minutes and the maximal chemiluminescence value

achieved in the measured time interval is determined. The results are expressed in relative light units (RLU), i.e. counts per minute.

The chemiluminescence value is corrected for the number of neutrophils present in the sample, determined by an automated analyzer. The result is then expressed as RLU/neutrophil count.

3. 1. 2 Advanced oxidation protein products (AOPP) assay

Spectrofotometric detection according to Witko-Sarsat (Witko-Sarsat *et al.* 1996) was used for the determination of serum AOPP levels in our modification.

Chemicals: chloramine T (Aldrich), KI 1.16 mol/l, acetic acid, Na_2HPO_4, NaH_2PO_4.

Solutions: phosphate buffered saline (PBS) 0.02 mol/l, pH 7.4.

Material: serum (or heparin plasma) - 200 µl per analysis, stored at -20° C

Procedure: 200 µl of serum (plasma) diluted 1:5 with PBS, 200 µl of chloramine T (0-100 µmol/l) for calibration and 200 µl of PBS as blank are applied on a microtitre plate. 10 µl of KI and 20 µl of acetic acid are added and absorbance is measured immediately.

Conditions of measurement: photometer Multiscan Ascent (Labsystems), absorbance is measured at 340 nm AOPP concentration is expressed as µmol/l in reference to the calibrator.

3.1.3 Pregnancy-associated plasma protein-A (PAPP-A) assay

PAPP-A was measured imunochemically, using the TRACE (Time Resolved Amplified Cryptate Emission) technology, based on non-radiating energy transfer.

Rationale: Commercial kit KRYPTOR-PAPP-A (Brahms, Cezanne) contains two different monoclonal antibodies – one is conjugated with europium cryptate and another one with flourescent agent XL 665. The antigens (PAPP-A) present in plasma samples are sandwiched between the two conjugates. The long-life fluorescent signal (wavelength 665 nm) measured during the formation of the antigen-antibody complex by the automated KRYPTOR analyzer (Cezanne), is proportional to the antigen concentration.

Material: serum (or heparin plasma) – 200 µl per analysis, stored at -20° C.

Conditions of measurement: analyzer KRYPTOR (Brahms), laser excitation at 337 nm, long-life fluorescent signal of 665 nm determined. PAPP-A concentration is expressed as mIU/l.

3.1.4 Intercellular adhesion molecule-1 (ICAM-1) assay

Soluble ICAM-1 was determined by the non-competitive enzyme-linked immunosorbent assay (ELISA) method using a standard commercial ELISA kit (BioSource International).
Reagents: murine monoclonal anti-ICAM-1 antibody-coated microwell strip, horseradish peroxidase (HRP)-conjugated murine anti-ICAM-1 antibody (HRP-conjugate), ICAM-1 standard (10 ng/ml), wash buffer (PBS with 1% Tween 20), sample diluent (buffered protein matrix), assay buffer (PBS with 1% Tween 20 and 10% bovine serum albumin), substrate solution I (tetramethylbenzidine, TMB), substrate solution II (0.02% buffered hydrogen peroxide), stop solution (phosphoric acid 1 mol/l)
Material: serum – 50 µl per analysis, stored at -20° C.
Procedure: 100 µl of serum diluted 1:100 with sample diluent (in duplicate), 100 µl of ICAM-1 standard (concentration 10 – 0.625 ng/ml) for calibration and 100 µl of sample diluent as blank are applied on the microwell strip. 50 µl of HRP-conjugate is added and the microwell strip is incubated for 2 hours at 18–25 °C. TMB substrate solution is prepared by mixing equal volumes of substrate solutions I and II. After incubation, the microwell plate is washed with the wash buffer and 100 µl of TMB substrate solution is added into each well. The microwell strip is then incubated for 15 minutes at 18-25° C. The reaction is then stopped by adding 100 µl of stop solution into each well and absorbance is measured immediately.
Conditions of measurement: photometer Multiscan RC (Lab Systems), absorbance is determined at 450 nm. ICAM-1 concentration is expressed as ng/ml.

3.1.5 Endothelial leukocyte adhesion molecule-1 (E-selectin) assay

Soluble E-selectin was determined by the non-competitive enzyme-linked immunosorbent assay (ELISA) method using a standard commercial ELISA kit (BioSource International).

Reagents: murine monoclonal anti-E-selectin antibody-coated microwell strip, horseradish peroxidase (HRP)-conjugated murine anti-E-selectin antibody (HRP-conjugate), E-selectin standard (100 ng/ml), wash buffer (PBS with 1% Tween 20), sample diluent (buffered protein matrix), assay buffer (PBS with 1% Tween 20 and 10% bovine serum albumin), substrate solution I (tetramethylbenzidine, TMB), substrate solution II (0.02% buffered hydrogen peroxide), stop solution (phosphoric acid 1 mol/l).
Material: serum – 50 µl per analysis, stored at -20° C.
Procedure: 100 µl of serum diluted 1:5 with sample diluent (in duplicate), 100 µl of ICAM-1 standard (concentration 50 – 0.8 ng/ml) for calibration and 100 µl of sample diluent as blank are applied on the microwell strip. 50µl of HRP-conjugate is added and the microwell strip is incubated for 2 hours at 18–25° C. TMB substrate solution is prepared by mixing equal volumes of substrate solutions I and II. After incubation, the microwell strip is washed with the wash buffer and 100 µl of TMB substrate solution is added into each well. The microwell strip is then incubated for 15 minutes at 18-25° C. The reaction is then stopped by adding 100 µl of stop solution into each well and absorbance is measured immediately.
Conditions of measurement: photometer Multiscan RC (Lab Systems), absorbance is determined at 450 nm. E-selectin concentration is expressed as ng/ml.

3.1.6 Vitamin E assay

Plasma α-tocopherol (vitamin E) concentration was determined by high-performance liquid chromatography (HPLC) using the ClinRep® commercial kit (Recipe Chemicals + Instruments, Germany).
***Reagents*:** mobile phase (cat. No. 22010), standard solution (cat. No. 22011), P precipitant with internal standard (cat. No. 22012), lyophilized calibrator (cat. No. 22013), sample preparation vials with reagent, lyophilized (cat. No. 22020).
***Material*:** plasma (blood drawn to ethylenediaminetetraacetic acid, EDTA) 100 µl per analysis (serum as well possible), stored at -20°C and protected against light.
***Procedure*:** 100 µl of plasma (or calibrator) is transferred into the sample preparation vial, 100 µl of P precipitant (containing 2 µg of internal standard) is added and mixed for 30 seconds on a vortex. The sample is then

centrifuged for 5 minutes at 8800 g and 20 µl of the supernatant is injected into the HPLC system.

***HPLC conditions*:** 1. Analytical column for vitamins A and E (cat. No. 22030, Recipe Chemicals + Instruments, Germany), the analysis is performed at 30° C. 2. Loop 20 µl. 3. Flow: 1.5 ml/min. 4. Detection at 295 nm, UV detector ECOM (Czech Republic). 5. ECOM HPLC system (Czech Republic). Retention time for vitamin E is approximately 7 minutes. Plasma vitamin E concentration is expressed in mg/l.

3.2 Studied groups

1) Study: Influence of parenteral iron therapy and oral vitamin E supplementation on neutrophil respiratory burst in chronic hemodialysis patients

The study was performed in a group of 7 chronic HD patients in a stable clinical status, without signs of acute inflammation, dialyzed 3 times a week for 4 hours using low-flux polysulphone dialysis membranes, all patients fulfilling the criteria of adequate dialysis (Kt/V > 1.2). The study was performed within 4 HD sessions (a total of 28 HD sessions). The patients received no antioxidants before the first 2 HD sessions. The first and the third HD session were carried out without i.v. iron administration. During the second and the fourth HD session, 62.5 mg of sodium ferric sodium-gluconate was administered as an i.v. bolus to each patient in the 65^{th} minute of the HD session. Before the last 2 HD sessions, the patients received 200mg of vitamin E per day orally for 7 days. Blood samples were taken from each patient immediately prior to starting the HD session, 60, 70 and 130 minutes after the start of the session. In each blood sample, chemiluminescence and differential blood count were assessed. Plasma AOPP concentration was measured in blood samples obtained before the start of the HD session and 130 minutes after the start of the HD session. Vitamin E serum concentration was measured before and after the 7 days vitamin E oral supplementation. The control group consisted of 14 healthy volunteers.

2) Study: Relationship of pregnancy-associated plasma protein-A to renal function and dialysis modalities

The study was performed in a group of 46 end-stage renal disease (ESRD) patients, 38 CKD patients and 25 controls:

- 36 chronic HD patients in a stable clinical status, dialyzed 3 times weekly for 4 hours, using low-flux polysulphone or diacetylcellulosic membranes, all patients fulfilling the criteria of adequate dialysis (Kt/V > 1.2).
- 10 continuous ambulatory peritoneal dialysis (CAPD) patients in a stable clinical status
- 38 CKD patients with serum creatinine concentration ranging from 89 to 790 µmol/l
- 25 healthy subjects with normal renal function

In HD patients, blood samples were taken from the arterial-venous fistula prior to starting the HD session and in the rest of the subjects they were collected via the cubital vein.

3) *Study:* Intravenous iron gluconate administration increases circulating PAPP-A in hemodialysis patients

The study was performed in a group of 20 chronic HD patients in a stable clinical status, dialyzed 3 times weekly, 4 hours per session, using low-flux polysulphone membranes, all patients fulfilling the criteria of adequate dialysis (Kt/V>1.2). The study was performed within 2 HD sessions: the first HD session was carried out without i.v. iron administration. During the second HD session, 62.5 mg of sodium ferric gluconate was administered as an intravenous bolus to each patient in the 65^{th} minute of the HD session. Blood samples were taken from each patient via the arteriovenous fistula immediately prior to starting the HD session and at 60, 130 and 240 minutes after the start of the session.

4) Study: Influence of oral vitamin E therapy on micro-inflammation and cardiovascular disease markers in chronic hemodialysis patients

The study was performed in a cohort of 29 HD patients in a stable clinical status, dialyzed 3 times weekly for 4 hours using low-flux polysulphone membranes, all patients fulfilling the criteria of adequate dialysis (Kt/V>1.2). HD patients were randomized into two groups (group A and group B) comparable by age, sex, duration of HD treatment, antihypertensive therapy and other medication. For a period of 5 weeks, patients in group A were treated with oral vitamin E (alpha-tocopherol 400mg daily). Group B patients did not receive any antioxidant supplementation. Blood samples were taken via the arterial-venous fistula prior to HD session before starting vitamin E supplementation and after 5

weeks of oral vitamin E therapy. The control group consisted of 16 age-matched healthy subjects.

3.3 Statistical analysis

The results are expressed as mean ± standard deviation (SD). Due to high interindividual variability, CRP is expressed as median (interquartile range). Nonparametric analysis of variance (Friedman's ANOVA) and nonparametric Wilcoxon matched pairs test were used for analysis of the data. Associations between analyzed parameters were assessed by Spearman's and Pearson's correlation coefficients. Comparison of independent groups was performed using unpaired Student's t-test and Mann-Whitney U test. Randomization was performed using computer software (Statistica 6.1®, Stat Soft Inc.). The results were considered statistically significant at $p < 0.05$.

4. RESULTS

4.1 Influence of parenteral iron therapy and oral vitamin E supplementation on neutrophil respiratory burst in chronic hemodialysis patients

CL value from blood samples drawn before the start of the HD session was significantly higher in HD patients than in healthy controls ($p<0.01$, ***Tab. 1***). CL decreased significantly in the 60th minute of the HD session ($p<0.05$), thereafter it remained stable. After i.v. Fe^{3+} application in the 65th minute of the session, there was a significant increase in CL at time 130 compared to CL at time 60 ($p<0.05$) (***Tab.2***). At time 70 and 130 minutes of the HD session when no vitamin E was supplemented to the patients, there was a negative correlation between the number of circulating neutrophils and *in vitro* neutrophil respiratory burst after i.v. Fe^{3+} administration ($r_{70} = -0.83$, $r_{130} = -0.96$, $p<0.05$).

After 7 days of oral vitamin E supplementation, serum vitamin E concentration in HD patients significantly increased from 12.8 ± 1.1 mg/l to 19.1 ± 6.9 mg/l ($p<0.05$). Pre-dialysis CL after the short-term vitamin E intake was significantly higher than in healthy controls and did not differ significantly from CL before the start of vitamin E supplementation (***Tab. 1***) Intra-dialysis changes in CL after short-term oral vitamin E supplementation were minimal, compared to the HD sessions without vitamin E supplementation (***Tab.2***). Plasma AOPP concentration was significantly higher in HD patients than in healthy controls and did not change significantly after short-term vitamin E supplementation (***Tab. 1***)

Tab. 1. Chemiluminescence (CL) and plasma advanced oxidation protein products (AOPP) concentration in hemodialysis (HD) patients and in healthy controls.

Parameter	Healthy controls	HD patients (before vitamin E supplementation)	HD patients (after vitamin E supplementation)
CL (RLU)	1083±325	1926±436*	1894±410*
AOPP (μmol/l)	88.9±24.8	137.5±42.7*	129.9±10.3†

$^{*}p < 0.01$ versus healthy controls, $^{\dagger}p < 0.05$ versus healthy controls

Tab. 2. Chemiluminescence (CL) changes during hemodialysis (HD) session before and after vitamin E (vit. E) supplementation.				
	Minutes of HD session			
Parameter	0	60	70	130
CL (RLU) before vit. E intake (no i.v. Fe^{3+} application)	1926±757	1220±599*	1202±637*	1083±370*
CL (RLU) before vit. E intake (HD with i.v. Fe^{3+} application in the 65th minute of HD session)	1786±309	877±292*†	945±376*†	1303±269*
CL (RLU) after vit. E intake (no i.v. Fe^{3+} application)	1894 ± 410	1587±339	1446±397	1512±346
CL (RLU) after vit. E intake (HD with i.v. Fe^{3+} application in the 65th minute of HD session)	2132±842	1489±408	1566±290	1877±629
*p < 0.05 versus time 0; †p < 0.05 versus time 130 i. v. – intravenous, Fe^{3+} - ferric sodium-gluconate (62.5 mg)				

4.2 Relationship of pregnancy-associated plasma protein-A to renal function and dialysis modalities

Serum PAPP-A concentration was significantly higher in both groups of dialyzed (HD and CAPD) patients in comparison with healthy controls. There was also a significant difference between serum PAPP-A concentration in HD and CAPD patients. Mean serum PAPP-A concentration in CKD patients not yet dialyzed did not differ significantly from healthy controls (***Tab. 3***).

In CKD patients not yet dialyzed, there was a positive correlation between serum creatinine and PAPP-A concentrations ($r = 0.68$, $p<0.05$) and a negative correlation between 24hour creatinine clearance and serum PAPP-A concentration ($r = -0.59$, $p<0.05$). After dividing the CKD group into 2 subgroups according to serum creatinine levels (S-creatinine <200 µmol/l a S-creatinine >200 µmol/l), a statistically significant correlation was found only in patients with higher creatinine levels ($r = 0.62$, $p<0.05$).

Serum AOPP concentration was significantly higher in HD, CAPD and CKD patients than in healthy controls (***Tab. 3***). Serum CRP concentration was significantly higher in HD and CAPD patients (***Tab. 3***).

Seventeen HD patients had increased CRP levels (>10,0 mg/l) and all these patients had simultaneously elevated serum PAPP-A levels >10 mIU/l. In the CAPD group, one half of the patients had elevated CRP levels and only 1 patient of this group had a simultaneously increased PAPP-A level.

Correlation between serum PAPP-A and AOPP concentrations was statistically significant in HD patients ($r = 0.49$, $p<0.05$), but this association was not observed in the groups remaining. Similarly, a significant positive correlation between PAPP-A and CRP levels was found only in HD patients ($r = 0.48$, $p<0.05$). When patients of all studied groups were taken together, the correlation coefficient between PAPP-A and CRP was 0.55 ($p<0.05$).

Tab. 3. Serum pregnancy-associated plasma protein-A (PAPP-A), advanced oxidation protein products (AOPP), C-reactive protein (CRP) and creatinine concentrations in hemodialysis (HD), peritoneal dialysis (CAPD), chronic kidney disease (CKD) patients and in healthy controls.					
Group	n	PAPP-A mIU/l[a]	AOPP µmol/l[a]	CRP mg/l[b]	S-kreatinin µmol/l[a]
HD	36	27.0± 16.5[1,5,3]	155.0±37.9[1,5,3]	10 (4.6-26.9)[1,3]	721±216[1,3]
CAPD	10	14.07± 6.73[4,2]	118.5±25.8[1]	7.7 (2.0-18.8)[8,6]	665±176[1,3]
CKD	59	9.72± 4.44	98.5±43.24[8]	0.75 (0.0-3.5)	216±142[7]
Controls	25	8.22± 2.71	78.8±23.9	1.7 (0.6-2.4)	85±25
[a] Unpaired Student's t-test (mean ± standard deviation, SD). [b] Mann-Whitney U test (median and interquartile range). [1]p<0.0001 versus controls; [2]p<0.01 versus CKD; [3]p<0.0001 versus CKD; [4]p<0.001 versus controls; [5]p<0.05 versus CAPD; [6]p<0.005 versus controls; [7]p<0.005 versus controls; [8]p<0.01 versus controls					

4.3 Intravenous iron gluconate administration increases circulating PAPP-A in hemodialysis patients

During the first HD session, which was performed without i.v. Fe^{3+} administration, there was a significant elevation of circulating PAPP-A 60 minutes after the start of the HD session, which persisted until 130 minutes after the start of the HD session. At the end of the HD session, plasma

PAPP-A concentration decreased significantly to pre-dialysis values. After correction for changes in blood volume we obtained the same results, except for serum PAPP-A concentration at time 240 of HD session: it was significantly less than the plasma PAPP-A concentration before the HD session (***Tab. 4***).

Following i.v. Fe^{3+} administration 65 minutes after the start of the HD session, there was a two-fold increase in plasma PAPP-A concentration 130 minutes after the start of the HD session. At the end of the HD session, plasma PAPP-A concentration decreased significantly, but it still remained 1.5 fold greater compared to pre-dialysis plasma PAPP-A concentration. The same results were obtained after correction of plasma PAPP-A for changes in blood volume ***(Tab. 4).***

Tab. 4. Plasma pregnancy-associated plasma protein-A (PAPP-A) concentration during 2 hemodialysis (HD) sessions, effect of intravenous (i.v.) ferric sodium-gluconate (Fe^{3+}) administration in the 65th minute of the HD session *(N=20)*.

Time of HD session (min)	PAPP-A (mIU/l)		PAPP-A/protein (mIU/g of protein)	
	HD without i.v. Fe^{3+}	HD with i.v. Fe^{3+}	HD without i.v. Fe^{3+}	HD with i.v. Fe^{3+}
0	28.9±13.3	29.0±11.2	0.44±0.22	0.43±0.18
60	34.1±10.7^{+}	36.0±9.9$^{++\,\Delta}$	0.51±0.19^{+}	0.53±0.19$^{+\,\Delta}$
130	37.7±15.2$^{+\dagger\bullet}$	79.6±28.9$^{+++\,\dagger\dagger\dagger}$	0.54±0.24$^{+\dagger\dagger\bullet}$	1.14±0.46$^{+++\,\dagger\dagger\dagger}$
240	28.5±12.4°	47.4±3.5^{+++}	0.39±0.18$^{+\circ}$	0.64±0.20^{+++}

Plasma PAPP-A concentration was divided by total protein plasma concentration for correction for hemoconcentration during HD session (expressed as mIU/g of protein). The results are expressed as mean ± standard deviation (SD).
$^{+}p < 0.05$ versus time 0; $^{++}p < 0.005$ versus time 0; $^{+++}p < 0.0005$ versus time 0; $^{\Delta}p < 0.0001$ versus time 130; $^{\bullet}p < 0.0001$ versus time 130 of HD session with i.v. Fe^{3+} application; $^{\circ}p < 0.0001$ versus time 240 of HD session with i.v. Fe^{3+} application; $^{\dagger}p < 0.005$ versus time 240; $^{\dagger\dagger\dagger}p < 0.0001$ versus time 240; $^{\dagger\dagger}p < 0.001$ versus time 240.

4.4 Influence of oral vitamin E therapy on micro-inflammation and cardiovascular disease markers in chronic hemodialysis patients

There was no significant difference between baseline vitamin E and E-selectin serum concentrations in HD patients and in healthy controls. Serum vitamin E levels were within normal ranges in both groups, showing that HD patients were replete with vitamin E at baseline. Serum ICAM-1 was higher in HD patients than in healthy controls, but this increase did not reach statistical significance (p=0.06). Baseline serum concentrations of PAPP-A and CRP were significantly higher in HD patients than in healthy controls ***(Tab 5)***.

Tab. 5. Laboratory parameters in HD patients before vitamin E supplementation and in healthy controls.

Parameter	HD patients	Controls
Vitamin E (mg/l)	11.42±3.23	11.41±1.94
ICAM-1 (ng/ml)	434.8±121.6	376.14±79.0
E-selectin (ng/ml)	54.23 ± 32.12	43.64±15.52
PAPP-A (mIU/l)	26.23±11.94^{++}	11.41±1.94
CRP (mg/l)	4.35 (1.69 – 5.69)$^{+}$	1.55 (1.00 – 3.26)

$^{+}p < 0.05$, $^{++}p < 0.001$ versus controls
ICAM-1 – intercellular adhesion molecule-1
E-selectin – endothelial leukocyte adhesion molecule-1
PAPP-A – pregnancy-associated plasma protein-A
CRP – C-reactive protein

After 5 weeks of oral vitamin E intake, serum vitamin E increased significantly in the group of HD patients treated with vitamin E and remained unchanged in the untreated group. Serum PAPP-A, CRP, ICAM-1 and E-selectin concentrations remained unchanged in both groups of HD patients ***(Tab 6)***.

Tab. 6. Effect of oral vitamin E (vit. E) supplementation on studied laboratory parameters in hemodialysis (HD) patients.

Parameter	Day 0		Day 35	
	Group A	Group B	Group A (after 5 weeks' vit. E intake)	Group B (no vit. E intake)
Vit. E (mg/l)	11.03±3.31	11.95±3.07	20.71±8.25^{+}	11.50±2.46
ICAM-1 (ng/ml)	397.8±94.0	474.4±138.0	396.4±95.9	459.0±121.4
E-selectin (ng/ml)	45.69±26.67	63.40±35.79	43.43±25.38	64.24±33.73
PAPP-A (mIU/l)	26.29±12.80	26.18±11.43	28.15±14.72	26.68±10.29
CRP (mg/l)	4.28 (1.13 – 6.92)	4.89 (1.69 – 7.84)	3.56 (2.51 – 7.04)	6.02 (2.81 – 0.2)

$^{+}p < 0.001$ versus day 0
The results are expressed as mean ± standard deviation (SD).
CRP is expressed as median (interquartile range).
ICAM-1 – intercellular adhesion molecule-1; E-selectin – endothelial leukocyte adhesion molecule-1; PAPP-A – pregnancy-associated plasma protein-A; CRP – C-reactive protein; Day 0 – before vitamin E supplementation; Day 35 – after 5 weeks' oral supplementation of 400 mg of vitamin E daily

5. DISCUSSION

5.1 Influence of parenteral iron therapy and oral vitamin E supplementation on neutrophil respiratory burst in chronic hemodialysis patients

Neutrophils are a heterogenous population, consisting of forms differing in their current state of activation. The results of our study show that HD patients' neutrophils are primed to generate free oxygen radicals when compared to those of healthy controls. This finding is in accordance with the results of other studies (**Klein *et al.* 1999**).

We observed a decrease in CL after the start of the HD session. This could be explained by neutrophil activation after their contact with the dialysis membrane, resulting in increased generation of free oxygen radicals and their partial unresponsiveness to further stimulation *in vitro*. Other possible explanation of our observation is that after the start of the HD session, the more activated, primed neutrophil population is sequestered both on the dialysis membrane and in pulmonary capillaries. Neutrophils that are afterwards detected in peripheral blood are those less activated, expressing lower number of adhesion molecules (which is the reason for lower CL value detected during the HD session). I.v. Fe^{3+} administration seems to be responsible for priming of the so far less activated part of the neutrophil population, which leads to an increased ability to generate free radicals (increase in CL in the 130th minute of the HD session, negative correlation between the number of circulating neutrophils and *in vitro* neutrophil respiratory burst after i.v. Fe^{3+} administration). This finding suggests that after i.v. Fe^{3+} administration, HD patients are exposed to enhanced oxidative stress caused by neutrophil activation.

Enhanced oxidative stress in HD patients is indicated by elevated plasma AOPP concentration in comparison with healthy controls. However, 7 days' oral supplementation of 200mg of vitamin E per day does not affect plasma AOPP concentration in HD patients. After one week oral vitamin E supplementation, there is no significant increase in CL in the 130th minute of the HD session after i.v. Fe^{3+} application. This could be partially explained by α-tocopherol-induced protein kinase C (PKC) inhibition in human neutrophils resulting in decreased generation of free radicals inducible by PKC activation (**Chan *et al.* 2001**). Vitamin E as a scavenger may

contribute to more stable CL values during the HD session following short term oral vitamin E intake observed in our study, but the total degree of oxidative stress remains unaffected.

5.2 Relationship of pregnancy-associated plasma protein-A to renal function and dialysis modalities

In this study we have found significantly increased serum PAPP-A levels in patients undergoing both HD and CAPD. The highest PAPP-A levels were observed in the HD group. CAPD patients using natural dialysis membranes (peritoneum) had significantly lower levels of PAPP-A and serum PAPP-A levels in CKD patients not yet dialyzed were even lower. CAPD patients had higher residual function than HD patients. Statistically significant positive correlation between PAPP-A and creatinine found only in the group of CKD patients not yet dialyzed suggests that serum PAPP-A levels might reflect the degree of renal damage. It seems that PAPP-A levels are mainly dependent on renal function in patients with certain degree of residual renal function, while in HD patients, so far unknown other factors (including dialysis membranes etc.) probably influence PAPP-A levels.

Similarly as Witko-Sarsat *et al.*, we describe differences between HD and CAPD patients in AOPP levels (Witko-Sarsat *et al.* 1996). Serum PAPP-A levels correlate significantly with serum AOPP as we described previously (Kalousová *et al.* 2003). Additionally, we have observed a significant positive correlation with CRP, a risk factor of cardiovascular disease - an association found previously also in patients with acute coronary syndromes (Bayes-Genis *et al.* 2001). The finding of elevated PAPP-A, CRP and AOPP levels in patients undergoing both HD and CAPD is in accordance with other findings that oxidative stress and cardiovascular risk increase with the decrease of renal function and is highest in patients on chronic HD treatment. It is possible that some common mechanism takes part in triggering synthesis of these products and so contributes to the increased cardiovascular risk of HD patients. Inflammation and oxidative stress may represent important factors that influence PAPP-A levels in these patients. Since no correlation between PAPP-A and S-creatinine was observed in HD patients, we can also hypothesize that interaction with an artificial dialysis membrane may contribute to the more pronounced PAPP-A elevation in HD patients.

The use of incompatible membranes during HD may induce increased PAPP-A synthesis, as observed in other metalloproteinases. Ebihara *et al.*

suggest that chronic stimulation of monocytes by endotoxins contaminating the dialysate or complement activation may be responsible for matrix metalloproteinase-9 (MMP-9) induction during HD session (Ebihara *et al.* 1998). We can speculate that one source of increased PAPP-A levels may be the monocyte activation during long-term HD treatment. So far we don't know the way of PAPP-A excretion, but its renal retention cannot be excluded. Similarly, increased plasma cardiac troponin T and I have been found in ESRD patients without acute coronary disease (Hafner *et al.* 1994, Li *et al.* 1995), but this increase does not correlate with serum creatinine concentration (Baum *et al.* 1996) and no explanation for this non-specific elevation of cardiac troponins has been found so far.

We can conclude that serum PAPP-A levels sensitively reflect the changes in renal function, depend on dialysis modality, and may represent a novel marker associated with inflammation and oxidative stress in ESRD patients. Similar elevation of PAPP-A in dialyzed patients (i.e. patients with increased cardiovascular risk) as in patients with acute myocardial infarction (Bayes-Genis *et al.* 2001) might indicate the link of PAPP-A to accelerated atherosclerosis and increased morbidity and mortality of dialyzed patients.

5.3 Intravenous iron gluconate administration increases circulating PAPP-A in hemodialysis patients

During the HD session when no i.v. Fe^{3+} was administered, we have observed a significant increase in circulating PAPP-A 1 hour after the start of the session and its decrease to pre-dialysis values at the end of the session. This finding is in accordance with the study of Kalousová *et al.*, where different types of dialysis membranes (polyamide and diacetate cellulosic) were used (Kalousová *et al.* Int J Artif Organs 2004). After i.v. Fe^{3+} administration, we have observed a two-fold increase in plasma PAPP-A concentration, followed by a significant decrease of circulating PAPP-A at the end of the session, with plasma PAPP-A concentration remaining 1.5-fold higher when compared to pre-dialysis values.

The initial increase in plasma PAPP-A concentration could be caused by the puncture of the arteriovenous fistula. We can also speculate that the increase of PAPP-A after the start of the HD session and after i.v. Fe^{3+} administration could be caused by acute oxidative vessel wall injury, with the release of PAPP-A from the damaged vessel wall into the circulation. It could also be explained as increased PAPP-A synthesis in response to bio-

incompatibility reactions on the dialysis membrane, similar as has been previously shown in other metalloproteinases (Ebihara *et al.* 1998). One possible source of increased PAPP-A production could be activated monocytes that are known to produce matrix metalloproteinases, involved in the process of atherosclerosis (Ebihara *et al.* 1998). I.v. Fe^{3+} administration is responsible for acute oxidative stress and acute phase reaction (Tovbin *et al.* 2002, Hořejší *et al.* 2003), associated with acute vascular damage. I.v. Fe^{3+} administration could cause further activation of monocytes with increased PAPP-A production. However, other sources of PAPP-A cannot be excluded. Possible interpretation of the decrease in plasma PAPP-A concentration at the end of the HD session could be PAPP-A metabolic degradation, clearance of PAPP-A free fragments, adsorption of PAPP-A to the dialysis membrane and possibly other mechanisms.

We can conclude that i.v. Fe^{3+} administration increases circulating levels of PAPP-A. In this way, this pro-atherosclerotic molecule related to oxidative stress might contribute to more pronounced cardiovascular complications in HD patients.

5.4 Influence of oral vitamin E therapy on micro-inflammation and cardiovascular disease markers in chronic hemodialysis patients

This study shows significantly higher baseline serum concentrations of CRP and PAPP-A in HD patients than in healthy controls, which is in accordance with our previous results (Kalousová *et al.* 2003) and suggests chronic inflammation and increased cardiovascular risk in HD patients. In this study, serum ICAM-1 concentration was higher in HD patients than in healthy controls, but this elevation - unlike in other studies (Bolton *et al.* 2001, Papagianni *et al.* 2003) - did not reach statistical significance ($p=0.06$). This could be due to the relatively small numbers of subjects included in our study and high interindividual variability of ICAM-1 serum concentrations. The finding of unchanged serum E-selectin levels in HD patients in comparison to healthy controls is in accordance with the studies mentioned above and could be due to just transitory expression of this protein on the surface of activated endothelial cells, quickly returning to basal levels, which does not exclude the possibility of E-selectin being involved in the process of atherogenesis (Carlos *et al.* 1994, Papagianni *et al.* 2003).

Although the dose and administration route of vitamin E was the same as in the SPACE study, we were unable to show evidence of any significant changes in serum concentrations in the studied parameters after 5 weeks of vitamin E supplementation. Our finding of unchanged serum CRP concentration after α-tocopherol supplementation is in accordance with the results of Himmelfarb *et al.*, who failed to show any effect on serum CRP after supplementation of 300mg α-tocopherol daily to HD patients for a period of 2 weeks (Himmelfarb *et al.* 2003). In our study, the potential beneficial effect of vitamin E supplementation on serum CRP could have been masked by the marked fluctuation of CRP levels in HD patients due to frequent silent infections, although none of the patients showed any signs of acute inflammation in the course of the study.

Failure to show beneficial effect on any of the studied parameters could be due to the relatively short course of vitamin E supplementation, despite other studies (Yukawa *et al.* 1995) had previously shown positive effect of vitamin E in short term. Another explanation is that antioxidants *in vivo* can only operate effectively if all the components of the antioxidant system are in balanced concentrations. Thus it is possible that the potential beneficial effects of vitamin E are masked by the overall imbalance of the antioxidant system (Vivekananthan *et al.* 2003). Moreover, α-tocopherol may act as an initiator of an auto-oxidation chain (Culbertson *et al.* 2001) and its pro-oxidative effects were shown in co-antioxidant-depleted HD patients (Ohkawa *et al.* 2004). It is also possible that vitamin E supplementation does not affect the parameters examined in this study and that other mechanisms play a more important role in the possible protective effect of vitamin E. There is also the possibility of vitamin E supplementation not being helpful at all, as the SPACE trial is the only one relatively large randomized trial to show benefit of vitamin E and may be just chance finding (Mann *et al.* 2004).

We can summarize that chronic micro-inflammation and increased cardiovascular risk in HD patients is documented by the significant elevation of CRP and PAPP-A when compared to healthy controls. Daily oral dose of 400mg of vitamin E is sufficient to increase serum vitamin E concentration significantly, but it does not seem to be able to reduce enhanced oxidative stress and micro-inflammation in chronic HD patients.

6. CONCLUSIONS

1. Neutrophils of chronic HD patients are primed to generate greater amounts of superoxide than those of healthy individuals. This production decreases during HD procedure and then significantly increases after intravenous ferric sodium-gluconate administration. Seven days' oral vitamin E supplementation attenuates this fluctuation of neutrophil oxidative metabolism without affecting the total degree of oxidative stress.

2. Serum PAPP-A concentration increases with the decrease of creatinine clearance and sensitively reflects changes in renal function. Serum PAPP-A concentration depends on dialysis modality: the highest PAPP-A levels are found in HD patients and this elevation is less pronounced in patients on CAPD treatment. In HD patients, there is a significant positive correlation between PAPP-A and AOPP as an oxidative stress marker and CRP as an acute phase reactant: serum PAPP-A concentration may represent a novel marker associated with inflammation and oxidative stress in these patients. Serum PAPP-A elevation in dialyzed patients, similar to that of patients with acute myocardial infarction, might indicate the link of PAPP-A to accelerated atherosclerosis and increased morbidity and mortality of dialyzed patients.

3. Plasma PAPP-A concentration increases significantly during HD session and at the end of the procedure it decreases again to pre-dialysis values. After intravenous ferric sodium-gluconate administration, there is a two-fold increase in plasma PAPP-A concentration followed by its significant decrease at the end of the session, with plasma PAPP-A levels remaining 1.5-fold higher compared to pre-dialysis values. This marked elevation of plasma PAPP-A following intravenous $Fe3^{+}$ administration may be due to iron-induced oxidative stress and suggests acute vessel wall injury following its administration.

4. Chronic inflammation and increased cardiovascular risk in HD patients is documented by significant elevation of CRP and PAPP-A when compared to healthy controls. Five weeks' treatment with the daily oral dose of 400mg of vitamin E is sufficient to increase serum vitamin E concentration significantly, but it does not seem to be able to reduce enhanced oxidative stress and micro-inflammation in chronic HD patients.

7. SUMMARY

Chronic kidney disease (CKD) patients are at significantly higher risk of atherosclerotic complications when compared to the general population. Oxidative stress and the related chronic microinflammation are possible causes of this increased cardiovascular risk, which is most pronounced in patients on chronic haemodialysis (HD) treatment. Oxidative stress in HD patients can be further increased by administration of certain drugs (especially by intravenous - i.v. iron preparations). The aim of the thesis was to investigate neutrophil respiratory burst and some molecules related to oxidative stress and chronic inflammation – pregnancy-associated plasma protein-A (PAPP-A), advanced oxidation protein products (AOPP), C-reactive protein (CRP), intercellular adhesion molecule-1 (ICAM-1) and E-selectin during HD session and to find out whether they can be pharmacologically affected by i.v. ferric sodium-gluconate and oral vitamin E administration. We focused on studying PAPP-A in chronic renal disease (CKD) patients, patients treated with continuous ambulatory peritoneal dialysis (CAPD) and chronic HD patients.

We have observed that HD patients' neutrophils are primed to generate greater quantities of superoxide than those of healthy controls. This production decreases during HD and significantly increases after i.v. administration of 62.5mg of ferric sodium-gluconate. Seven days' treatment with 200mg of vitamin E daily attenuates this fluctuation of neutrophil oxidative metabolism without affecting the total degree of oxidative stress.

We have shown that serum PAPP-A concentration increases with the decrease in creatinine clearance and it sensitively reflects the changes in renal function. Serum PAPP-A concentration depends on dialysis modality: the highest PAPP-A serum levels were observed in chronic HD patients, but they were significantly less elevated in CAPD patients. We have found a significant positive correlation between PAPP-A and AOPP as a marker of acute oxidative stress and PAPP-A and CRP as an acute phase reactant.

We have found that plasma PAPP-A concentration increases significantly during HD procedure and it decreases to pre-dialysis values at the end of the session. After i.v. iron administration, there is a two-fold increase in plasma PAPP-A concentration, followed by its decrease at the end of the session, plasma PAPP-A concentration remaining 1.5 times higher than pre-dialysis values.

We have observed that 5 weeks' oral treatment with 400mg of vitamin E is sufficient to increase serum vitamin E concentration significantly, but it does not affect PAPP-A, CRP, ICAM-1 and E-selectin serum concentrations and thus it does not seem to be able to reduce chronic inflammation in HD patients.

We can conclude that chronic micro-inflammation and increased cardiovascular risk in HD patients are documented by significant elevation of CRP, AOPP and PAPP-A when compared to healthy controls. The significant increase in plasma PAPP-A levels after i. v. iron administration is possibly due to oxidative stress induced by i. v. iron and it could indicate acute vascular damage following i.v. iron administration. Serum PAPP-A could represent a novel marker associated with inflammation and oxidative stress in end-stage renal disease (ESRD) patients. Similar elevation of serum PAPP-A in dialyzed patients as in patients with acute myocardial infarction might indicate the link of PAPP-A to accelerated atherosclerosis and thus to increased morbidity and mortality of dialyzed patients.

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