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Risk of the mechanism behind accelerated arteriosclerosis in case of patients with uremic haemodialysis

PECULIAR CARDIOVASCULAR RISK FACTORS IN CHILDREN AND ADULTS SUFFERING FROM END-STAGE RENAL DISEASE RECEIVING CHRONIC HEMODIALYSIS

Theses of Doctoral (Ph.D.) Dissertation

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INTRODUCTION

Development of hypertonia is a long process that consists of various interconnected factors. First, traditional and new-found risk factors trigger chronic changes in the endothelial function, then trigger alteration in the chemical composure and structure of the vascular wall (*stiffness*), followed by sclerosis and organ damage (nephropathy, retinopathy, myocardial- and cerebral ischaemia, or peripheral vascular diseases).

Volume-dependent (salt-sensitive) hypertension

Several epidemiologic, genetic, clinical, migration and animal experiments revealed the importance of salt intake in regulating blood pressure, exploring a strong positive correlation between salt intake and blood pressure. Research also found that low salt intake resulted in significant decrease in blood pressure, not only in hypertensive but also in normotensive patients. The kidneys' unsatisfactory sodium (Na^+) elimination ability is considered to play a key role in the development of salt-sensitive, volume-dependent hypertension.

Renal sodium retention

The following endocrine and paracrine systems play a key role in mediating renal sodium retention: endogenous ouabain (EO), renal dopaminergic system, and cardiogenic natriuretic peptide hormones (atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP)).

Connection between salt intake, plasma sodium and blood pressure

It is a generally accepted claim that continuously high salt intake causes sodium- and water-retention, which is one of the key factors causing high blood pressure. An important pathogenetic factor is the increased volume of extracellular space (ECS). It was also proved that there is a connection between high sodium intake and endothelial dysfunction, as in parallel to increased sodium-intake, endothelium-dependent vasodilation and vascular nitric oxide production will decrease. Oxidative stress, aggressive oxygen radicals, and especially the accumulation of superoxides can be identified as triggers of the abovementioned phenomenon.

Dissociation of Na^+ and volume retention

To explore the pathophysiological background of the phenomena, *Titze et al* planned a series of examinations, based on which they developed the concept of tissue-specific retention of Na^+-homeostasis. They found that in case of high-salt diet, Na^+ is, in part, stored in an osmotically inactive way, thus Na^+-retention does not automatically trigger ECS expansion, and the dissociation of Na^+ and volume retention may occur. Reversible transformation of osmotically active – inactive Na^+ provides as a puffer in terms of volume and blood pressure retention. Apart from the bone tissue, the most important Na^+-reservoir is the skin and the subcutaneous area. Glycosaminoglycans (GAGs) in the connective tissue serve as biochemical basis for Na^+-storage.

Other animal- and clinical experiments proved that with its negatively charged compartments, glycocalyx on the luminal surface of vascular endothelium is able to bind and reversibly store sodium ions. Thus, it operates as primary Na^+ puffer and strongly support the maintenance of volume balance. In case the volume of endothelial glycocalyx decreases or its integrity is damaged, its puffer function and ability to bind Na^+ decreases, thus the risk of salt-sensitive hypertension rises. The following circumstances increase patients' predisposition to the condition: hypercholesterolemia, diabetes mellitus, vascular surgeries, sepsis, chronic kidney diseases, dialysis treatment, and acute or chronic hyperglycaemia.

Evolution of preference for salt

Several examinations focused on relevant nutritional factors, especially the role of salt intake during the perinatal and infant period. Researchers found that early exposure to salt will result in children developing a long-lasting preference for salty taste and consequently their daily salt intake will be elevated. Infants who were exposed to limited salt intake had lower blood pressure, not only during the examined period but also after 7 to 15 years. Lowered blood pressure levels later in life proved to be independent of the current salt intake. Minimizing salt intake during childhood resulted in significantly lower blood pressure in later years.

Salt intake and inflammation

Low-intensity inflammation is an important pathogenetic factor behind hypertension triggered by high-salt diet. Recent studies revealed that high salt intake activates certain immune processes, resulting in the progress of auto-immune diseases. Induction and activation of T helper 17 (Th17) cells are found to be in the background of pathological progress. Another

important animal examination revealed that high salt intake triggered upregulation of inflammatory cytokines, followed by the fibrosis and thickening of the peritoneal membrane.

Researchers also revealed that cytokines produced by activated T-lymphocytes and macrophages increase the expression and activity of Na^+ transporters specific to the kidney, thus increase renal Na^+-reabsorption and cause volume excess and sodium-dependent hypertension.

Uremia and arteriosclerosis

Increased arteriosclerosis is well known by patients living with chronic renal failure. Considering the regular cardiovascular risk factors, prevalence of cardiovascular diseases is significantly higher in patients with chronic renal failure compared to the general population. Recent studies revealed that asymmetric dimethylarginine (ADMA) is in play in the development and progress of hypertension. As the increase of ADMA level of the plasma takes place before the cardiovascular disease's manifestation, it can be considered as an early biomarker of the endothelial dysfunction, and as such it may be an important predictor of cardiovascular complications of chronic kidney failure.

Other recent studies revealed that the endothelial cells and the interaction between the endothelium and the vascular smooth muscle cells controls the progress of vascular wall calcification. Activated and/or damaged endothelial cells of patients with uremia release micro particles, which promotes the development of osteogenic phenotype in endothelial progenitor cells, in vascular smooth muscle cells, and in fibroblasts. Parallel to the appearance of micro particles with endothelial origin, the number of endothelial progenitor cells responsible for the reparation of the endothelium decreases significantly. Eventually the disturbed balance between the damage and reparation of the endothelium leads to vascular calcification.

Stiffness of the artery wall can be considered as a clinical marker of arteriosclerosis, while it is also an independent risk factor of fatal- and non-fatal cardiovascular events. This is not only the case with the elderly, patients suffering from hypertension, diabetes mellitus, or end-stage renal disease, but also with the general population. Measurements examining the stiffness of the artery wall describe the structural and composite differences of the arterial wall.

A large quantity of data is available that proves how the damaged bone metabolism has significant influence on the vascular wall calcification. The deleterious interaction between

bone loss and vascular calcification results from the dysfunction of the bone-vascular axis that is amplified in chronic renal failure. A number of blocking and promoting factors in the relationship between the skeletal- and the vascular system are well-known, with a specific focus on the role of bone-related proteins, such as osteocalcine (OC), osteoprotegerin (OPG), and osteopontin (OP).

RESEARCH GOALS

Since the prevalence of cardiovascular diseases and the consequent mortality is exceptionally high in Hungary, it was important for us to examine risk factors behind this condition. We completed our examination, on one hand, with children and adolescents, having primary prevention in mind; on the other hand, we examined patients receiving haemodialysis, trusting the option of seconder prevention. Reflecting these goals, the dissertation has the following three parts (I-III.):

I. Examination of the connection between salt intake, hypertension, and target organ impairment in infants and children

Development of high blood pressure diseases and its complications can be traced back to infancy and childhood, as high amount of salt consumed with diet is extremely important in this age. However, preventive impact of potassium in the development of high blood pressure is well known. Goals of this study are to:

1. compare the amount of daily sodium and potassium intake with the measures in international recommendations (Dietary Reference Intakes, DRI);
2. examine the connection between sodium- and potassium intake and high blood pressure in function of gender, age, and body mass index (BMI);
3. compare current data with a research carried out in 1990 with the same age groups and circumstances.

II. Examination of blood pressure regulation in patients with chronic renal failure

The only goal of the examination was to define the role of asymmetric dimethylarginine (ADMA) in blood pressure regulation in patients with chronic renal failure and under haemodialysis treatment.

III. Examination of accelerated atherosclerosis in patients with chronic kidney failure

The goal of our cross-sectional study was to evaluate accelerated atherosclerosis in patients with chronic kidney failure participating in haemodialysis treatment through the examination of vascular stiffness. We aimed to identify the connection between accelerated atherosclerosis and:

1. pro-inflammatory and protective factors,
2. bone-related proteins.

I. Connection Between Hypertension and the Daily Sodium- and Potassium Intake Among Children and Adolescents in Southern Transdanubia, Hungary

Patients and Methods

Two hundred children and adolescents were enrolled in the study (100 girls and 100 boys), with the average age of 10.4 ± 3.7 years (between 1 and 18 years of age). Children's ward admission took place between 1 November 2008 and 31 January 2010. They were admitted due to elective surgery or routine clinical examination. In case of patients involved in elective surgery (eg. varicocele or testis altus, etc), the examinations were conducted one day before the surgery. None of the children suffered from renal, cardiac, or endocrine diseases, secondary hypertension, or disturbances of electrolyte and acid-base status. They consumed traditional Hungarian food, without any dietary restriction. None were prescribed medication affecting renal function or blood pressure prior to or during the study.

Anthropometric parameters were obtained by trained technicians. Body height was measured to the nearest 0.1 cm using a calibrated measuring instrument (Harpenden Stadiometer, Crymych, United Kingdom), and body weight was measured to the nearest 0.1 kg using a validated electronic scale (Seca 899, Birmingham, United Kingdom). BMI was calculated as body weight in kilograms, divided by height in meters squared (kg/m^2). Children were considered to be obese when their BMI exceeded the 95-percentile value of age and gender matched controls. Blood pressure was measured on the subdominant arm after a 5-minute rest period. Three measurements were undertaken by well-trained personnel using an automatic oscillometric device (Omron MX3, Omron Matsusaka, Japan). Mean values of systolic and diastolic blood pressure were calculated from the result of the last two examinations. The child was considered hypertensive when his systolic and/or diastolic BP exceeded the 95-percentile for age, gender, and height percentiles of matched controls.

As it is well-known, more than 90% of the sodium and 77% of the potassium consumed with food is eliminated from the body through urinary excretion. Since the electrolyte-definition based on 24-hour urine collection correlates well with dietary examinations, we assumed that a single 24-hour urine collection would provide a reliable estimate of daily sodium and potassium intake.

24-hour urine collection took place after a detailed technical information provision for participants. A specific bag was applied to collect urine of toddlers still using diapers. Urine was collected in plastic containers and was kept at 4 degrees Celsius until used. Urine collection was regarded adequate with urine production of 1 to 3 ml kg^{-1} h^{-1}. The sodium and potassium concentrations of native urine were measured by ion selective electrodes.

The study of 1990 was undertaken to estimate daily sodium intake by measuring urinary electrolyte excretion in 203 Hungarian children, age 0 to 17. Urine samples were collected over a 24-hour period. The electrolyte concentration of native urine was measured by flame photometry. We compared the self-defined, corrected sodium and potassium intake and sodium to potassium ratio measured in 2008–2010 to those of 1990.

Data were expressed as mean ± SD. Statistical analysis was prepared with SPSS v. 16 statistical computer software. For statistical evaluation, Student's unpaired t-test, linear regression analysis, and – when adjustments for confounding variables so required – multivariate regression analysis were used. P values <0.05 were considered statistically significant.

Informed consent of participating children's parents was collected.

Results

Among the 200 Hungarian children we found 32 hypertensive (16%) and 42 obese (21%) patients. The daily sodium intake was found to have significant positive correlation with age (r=0.502, p <0.0001), BMI (r=0.485, p <0.0001), and systolic blood pressure (r=0,452, p <0,0001).

The mean value of sodium intake increased steadily with age: in the 1-3 years age group it was 54.0±28.0 mmol/die (n=7), in the 4-8 age group it was 89.5±53.8 mmol/die (n=78), in the 9-13 age group it was 129.0±51.9 mmol/die (n=61), and in the 14-18 age group it was 165,4±84,2 mmol/die (n=54). These sodium intakes considerably exceeded the Adequate Intakes (AI) in each group above 4 years of age. Daily sodium excretion expressed per kg body weight

decreased progressively with age (r=0.319, p <0.0001), and no discernible difference could be detected between girls. In comparison between 1990 and 2010, data shows that sodium intake decreased by 25% among children age 1-8 but showed no difference in the 9-18 age group.

Sodium intake in the abovementioned age groups were the following (corrected values calculated from 24-hour urinary excretion): 24.1±15.3 mmol/die, 33.0±21.5 mmol/die, 44.9±22.1 mmol/die, and 53.4±23.7 mmol/die. The potassium intake was only a third of the AI among 1–8 year old children, and only about 40% of the AI among 9–18 year olds. Similarly to sodium, daily potassium intake also showed positive correlation with age (r=0.384, p <0.0001), BMI (r=0.440, p <0.0001), and systolic blood pressure (r=0.406, p <0.0001). Sodium intake per body weight (kg) decreased parallel with age. Over the last 20 years between the two studies, the potassium intake increased about 250% in 1–3 year olds, about 200% in 4–13 year olds, and about 150% in 14–18 year olds.

As a result of the parallel trend and time course of urinary sodium and potassium excretion, the sodium to potassium ratio remained practically unchanged and amounted to a value of 3.2±1.6 mmol/mmol over the age range studied here. We did not find significant differences in the sodium to potassium ratios between hypertensive and normotensive, or obese and non-obese children. The calculations show that this ratio exceeded three times the recommended values. However, it is important to mention that the sodium-potassium ratio decreased to its third compared to values from 1990.

Using multivariate regression analysis, systolic blood pressure proved to be independent of daily sodium- and potassium intake in terms of age and BMI.

Discussion

This study proved that Hungarian children's and adolescents' daily sodium intake is higher than recommended, while their daily potassium intake is lower than international recommendations. When the daily intake amount of these electrolytes was corrected for gender, age, and BMI, no influence on blood pressure was detected.

The study's strength lies in the data scope comparison, which covers 20 years. Comparing results from 1990 with our latest data, a positive tendency becomes clear: although the sodium intake remained the same (except for the 25% decrease detected in the1-8 age group), the

potassium intake showed a significant increase. Due to these trends, the sodium to potassium ratio – which is the most important risk factor for hypertension – decreased in the last 20 years.

II. Response of Asymmetric Dimethylarginine to Haemodialysis-Associated Hypotension in End-Stage Renal Disease Patients

Patients and methods

Two groups of patients were selected to participate in the research who had been undergoing haemodialysis (HD) program for at least 6 months. Group 1 consisted of 18 patients who developed hypotension during HD, while 13 patients in group 2 maintained their blood pressure at pre-dialysis level. Hypotension was defined as a drop of systolic blood pressure of 15 Hg mm or more during HD. There were no discernible differences between the two groups in age, gender, time spent on HD, and the volume of ultrafiltration. Most patients in both groups received antihypertensive treatment. Similar number of patients from both groups took ACE inhibitors, angiotensin II receptor blockers (ARB), calcium antagonists and beta-blockers. Underlying pathologies leading to end-stage renal disease (ESDR) was similar in the two groups.

For routine laboratory examinations, samples were taken before HD treatment while the patients were in stable clinical conditions. Routine tests included assessment of serum haemoglobin concentration, iron status, plasma creatinine and urea nitrogen concentration levels, protein and lipid profiles, acid-base, and electrolyte status. The examinations revealed similar results in patients with hypo- and normotension as well. Blood pressure was measured with calibrated mercury sphygmomanometer by the same trained nurse. Each value represented the average of three consecutive measurements on the same arm. Systolic and diastolic blood pressure was recorded before HD, at 20-minute intervals during HD and also after the treatment. HD was carried out with Fresenius 4008B equipment with Helixone/Fresenius polysulfone high-flux dialyzer membranes (FX50, FX60 and FX80 dialysers). Each patient was on HD three times per week. Similar dialysis fluid has been used in case of all patients (temperature 36.5 Cº, sodium concentration 138 mmol/l, calcium concentration 1,5 mmol/l, conductivity 14 mS/cm). Effective blood flow was the same in both groups (298 ± 47 and 321 ± 42 ml/min, p= 0,16). Every patient received erythropoietin treatment; and the weekly dose of epoetin-β was similar for the two groups (4.675 ± 2.750 and 6.361 ± 3.395 IU, p= 0.17).

Blood samples were taken before and immediately after HD to assess L-arginine, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) levels of the plasma. After the samples were drawn into syringes, they were immediately injected into tubes in ice water containing EDTA. Plasma was separated in a refrigerated centrifuge and stored at -20ºC until analysis. L-Arginine, ADMA and SDMA levels were measured by applying a liquid chromatography-mass spectrometry method as described previously. No correction was made for post-dialysis haemoconcentration.

Statistical analysis was carried out by SPSS 11.5 (SPSS Inc, Chicago, Ill, USA) software. Kolmogorov-Smirnov test was applied to evaluate normality of the data. Variables are presented as mean ± SD, or as median with interquartile range, in case of a skewed distribution. Statistical comparisons of groups were made by Student's t-test, Mann-Whitney test, and linear regression analysis.

Written informed consent was obtained from each participating patient. The study was approved by the institutional ethics committee.

Results

Systolic and diastolic blood pressure measured before HD was comparable both in case of the normotensive and hypotensive groups. In response to HD, blood pressure in normotensive patients remained practically unchanged, while in hypotensive patients it fell significantly by the end of HD. The difference between the groups reached the level of significance after 160 minutes of dialysis treatment.

HD-treated patients with hypotension exhibited significantly higher initial plasma L-arginine levels than those who remained normotensive. This difference in L-Arginine levels between the two groups persisted following HD, and reached statistical significance even after HD. The reduction in plasma L-Arginine levels by HD treatment was 22% in the normotensive group, and 47% in the hypotensive group.

Serum ADMA levels of ESRD patients was especially high, but decreases significantly by the completion of HD. ADMA levels of patients who experienced hypotensive episodes during HD was significantly higher than those whose blood pressure was steady throughout the treatment (before HD: 0.62 ± 0.11 μmol/l and 0.71 ± 0.13 μmol/l, p =0.04; after HD: 0.31 ± 0.11 μmol/l and 0.43 ± 0.11 μmol/l, p =0.01). Unexpectedly, we observed significantly higher ADMA levels

in hypotensive than in normotensive patients both before ($p < 004$) and after ($p < 0.01$) HD. HD resulted in a decrease of 50% of plasma ADMA levels in normotensive patients, and of 42% of hypotensive patients. Plasma SDMA levels did not differ between the two groups either before or after HD. The markedly elevated initial values were reduced by HD intervention to approximately the same extent (52% and 54%).

The paradoxically higher plasma ADMA levels in patients with hypotension both before and after HD inspired us to further explore the relationship between plasma ADMA and blood pressure. When all individual values of the two groups were evaluated and ADMA level was examined as a function of the lowest systolic and diastolic blood pressure during HD sessions, significant inverse correlation was found between pre-dialysis ADMA and systolic ($r = - 0.50$, $p < 0.01$) and diastolic ($r = - 0.59$, $p < 0.001$) blood pressure. Post-dialysis ADMA also correlated negatively with systolic ($r = - 0.49$, $p < 0.01$) and diastolic ($r = - 0.51$, $p < 0.005$) blood pressure.

Also, we found significant correlation between pre-dialysis plasma L-Arginine level and diastolic blood pressure ($r = - 0.45$, $p < 0.01$), and post-dialysis plasma L-Arginine level and systolic blood pressure ($r = - 0.40$, $p < 0.02$).

Plasma SDMA level did not correlate with the measured blood pressure level. Volume of ultrafiltration liquid did not influence the level of blood pressure, plasma L-Arginine, or dimethylarginine levels at any stage of HD treatment.

Patients with hypotension had significantly lower plasma sodium concentration compared to patients with normotension. Thus, hyponatraemia is regarded as a risk factor for the development of HD-associated hypotension. However, plasma sodium concentration proved to be independent of blood pressure and plasma ADMA levels.

Discussion

Similarly to previous findings, our study proved that in patients with ESRD on regular HD, plasma dimethylarginines are significantly increased. By the end of HD treatment, a significant decrease can be detected in the dimethylarginine level. In addition, significantly higher ADMA levels was found in patients experiencing hypotensive episodes during HD compared to those participants who managed to keep their blood pressure at pre-dialysis level. Furthermore, we found strong inverse relationship between minimum systolic and diastolic blood pressure during HD, and pre- and post-dialysis ADMA levels. This contradiction suggests

that HD-associated hypotension increases plasma ADMA to prevent further fall of the blood pressure.

Although previous research reported volume-dependent hormonal changes triggered by HD, it seems unlikely that changes in volume during HD are important factors in being prone to the disease, since both blood pressure and the ADMA level appeared to be independent of ultrafiltration volume. In fact, vasodilator hormone systems are activated before HD and supressed after the treatment. Exactly the opposite applied for vasoconstrictor hormones, as relatively low ADMA level is expected before HD which is significantly increased after treatment. Paradoxically, in our studied groups HD-related hypotensive episodes were associated with increased ADMA. The underlying mechanisms of this paradoxical reaction are unclear. We may assume that the inducible isoform of the NO synthase is upregulated during HD, and what explains blood pressure decrease during the treatment is actually the increased NO production. Excessive NO generation diminishes DDAH activity through the nitrolisation of the enzyme's active site, leading to the accumulation of ADMA and to iNO synthase inhibition. With respect to the colocalization and potential interaction of demethylating enzymes with NO synthase, the concept of local feedback regulation of NO-DDAH-ADMA-NO synthase axis has been recently put into focus. It appears to play an important role in HD-related hypotension in case of ESRD patients.

As an alternative explanation for the relationship between hypotensive episodes and ADMA, the possible role of vascular stiffness should also be considered. Previous research proved that increased ADMA level is in connection with elevated vascular stiffness, which on the other hand impairs vascular reactivity and limits the capacity to counter-regulate changes in blood pressure.

III. Influence of Pro- and Anti-inflammatory Factors and Bone-related Proteins on Arterial Stiffness in Chronic Renal Failure Patients on Haemodialysis

Patients and Methods

The first cross-sectional study of this research included 96 clinically balanced patients in chronic haemodialysis treatment (49 male, 47 female). Key disease circumstances were the following: diabetic nephropathy (24%), benign nephrosclerosis (26%), chronic

glomerulonephritis (14%), polycystic kidney disease (16%), chronic interstitial nephritis (8%), renovascular disease (1%), and other or unknown cause (11%). Majority of patients (n=84) received antihypertensive drug treatment. The control group to evaluate biochemical parameters included 20 adult individuals who did not suffer from kidney-, cardiovascular-, or metabolic disease.

The second cross-sectional study of this research included 68 (33 male, 35 female) consecutive chronic haemodialysis (HD) patients, who were in stable clinical condition during the examination. The underlying renal pathologies that progressed to ESRD were the following: diabetic nephropathy (26%), benign nephrosclerosis (23%), chronic glomerulonephritis (15%), polycystic kidney disease (13%), chronic interstitial nephritis (10%), renovascular disease (1%) and other/unknown causes (12%). Most of the patients (66) received antihypertensive therapy. 35 healthcare workers having no cardiovascular, metabolic and kidney disease served as controls for the analysis of cardiovascular and biochemical parameters.

Patients who suffered from acute myocardial infarction, previous lower limb amputation, ongoing acute infection, malignancy, pulmonary oedema, or haemodynamic instability, were closed out of the study. HD treatment in both studies was carried out in Hungary, in the FMC Dialysis Center of Pécs. Patients underwent 3 haemodialysis sessions per week, 4-hour duration each. On- line hemodiafiltration was carried out by using Fresenius 5008 B equipment with Helixone/Fresenius polysulfone high-flux dialyzer membranes. Results presented are pre-dialysis values. Body mass index (BMI) was calculated.

Blood pressure was measured using calibrated automated devices with appropriate cuff sizes (Omron MX3, Omron Matsusaka, Japan). Results presented here are pre-dialysis values. Pulse pressure and mean arterial pressure was calculated from the resulting values. Carotid-femoral PWV and augmentation index (AIx) was measured using applanation tonometry (SphygmoCor system, AtCor Medical Australia). Measurements were performed before haemodialysis sessions in supine position after at least 10-min rest in a quiet, temperature-controlled room. Measurements of the controls were performed in the morning under similar circumstances. All readings recorded met the manufacturer's quality control standards integrated into the software package.

Routine biochemical parameters were measured by standard laboratory methods. Serum concentrations of fetuin-A, α-Klotho, TNF-α, and TGF-β were measured by enzyme-linked immunosorbent assay (ELISA), with tools commercially available (IBL International GmbH,

Hamburg, Germany and BioVendor Laboratory Medicine Inc., Brno, Czech Republic). Immunometric assay was applied to define the level of serum C-reactive protein level (CRP) and 25 hydroxy vitamin D. The serum concentrations of OC, OP and OPG were measured by sandwich enzyme-linked immunosorbent assay (ELISA) (IBL International GmbH, Hamburg, Germany and BioVendor Laboratory Medicine Inc., Brno, Czech Republic).

Statistical analyses were performed using the 21.0 software of the SPSS (SPSS, Inc., Chicago, IL, USA). Normality of data distribution was tested by Kolmogorov-Smirnov test. Non-normally distributed parameters were transformed logarithmically. Correlations between continuous variables were assessed by calculation of linear regression using Pearson's test. Data were expressed as means ± S.D. in case of normal distribution, and median (lower/upper quartile) in case of non-normal distribution. Backward multiple regression analyses were performed to determine the relative contribution of selected independent variables in the constructed model to the variance of the dependent variable. Values of $P < 0.05$ were considered statistically significant.

The investigation conforms to the principles outlined in the World Medical Association Declaration of Helsinki. Informed written consent was obtained from all participants.

Results

In the first examination, pro-inflammatory (calcinogen) biomarkers (CRP, TNF-α, TGF-β1) increased significantly in ESRD patients ($p < 0.01$), while the anti-inflammatory (anti-arteriosclerotic) factors (fetuin-A: $p < 0.05$, α-Klotho: $p < 0.01$, vitamin D3: $p < 0.01$) showed significantly lower levels when compared to healthy adults.

We examined the connection between arteriosclerosis (AS) and clinical-laboratory parameters by applying univariate regression. Results showed that velocity of carotid-femoral pulse wave (cfPWV) was positively correlated with the aortic augmentation index (AIx) ($r = 0.273$, $p < 0.05$), with serum cholesterol level ($r = 0.244$, $p < 0.05$), and with fetuin-A levels ($r = 0.282$, $p < 0.05$). However, cfPWV was negatively correlated with time passed since the start of HD ($r = -0.262$, $p < 0.05$). cfPWV proved to be independent from all the other examined variables.

Correlation between parameters of arterial stiffness and clinical-laboratory factors was examined with multivariate regression analysis. Dependent variable was the augmentation

index (AIx), and the independent variables were PWV, central augmentation pressure, central systolic blood pressure, central pulse pressure, Kt/V value, and the parathormone level. Significant correlation was found only between the central augmentation index (β= 1.32, p <0.00) and the central pulse pressure (β= - 0.71, p <0.00). When considered as dependent variable, PWV was significantly influenced by fetuin-A (β= 0.24, p <0.03) and time spent in HD (β= 0.23, p <0.04).

We applied multiple linear regression to find an independent variable that is significantly correlated with the examined pro- and anti-inflammatory biomarkers. Among pro-inflammatory factors, CRP was influenced negatively by plasma vitamin D3 (β= - 0.23, p <0.01), sodium (β= - 0.26, p <0.00), and albumin (β= - 0.36, p <0.00), while it was positively influenced by BMI (β=0.76, p <0.00). When TNF-α and TGF-β1 was considered as the dependent variable, we found significant correlation only with α-Klotho (β= 0.41, p <0.00) and plasma creatinine levels (β= 0.30, p <0.00), and also with fetuin-A (β= 0.24, p <0.02) and albumin (β= 0.33, p <0.00) levels.

When we examined the protective factors with multiple regression analysis, fetuin-A showed significant negative correlation with age (β= - 0.23, p <0.05), TGF-β1 (β= - 0.23, p <0.02), and time spent with dialysis treatment (β= - 0.26, p <0.01). However, positive correlation was detected between fetuin-A and serum triglyceride level (β= 0.43, p <0.00). α-Klotho, as the dependent variable positively correlated with TNF-α (β= 0.44, p <0.00), but showed negative correlation with calcium level of the plasma (β= - 0.24, p <0.01).

In the second study, PWV and AIx increased significantly in CRF patients on HD compared to adults in the control group without major cardiovascular, renal and metabolic morbidities. Also, serum levels of the bone-related proteins OC, OPG and OP were several times higher in our uremic patients than in our controls.

Univariate linear regression analysis was applied to reveal the association between markers of vascular stiffness (VS) with the clinical and laboratory parameters. Results show that PWV correlated positively with age (r=0.411, p<0.000), whereas negatively with serum creatinine (-0.412, p<0.000), urea nitrogen (r=-0.427, p<0.000), phosphate (r=-0.325, p<0.007), potassium (r=-0.307, p<0.011) and OC (r=-0.247, p<0.049). AIx was found to relate directly to central pulse pressure (r=0.405, p<0.001), augmentation pressure (r=0.800, p<0.000), systolic blood pressure (r=0.316, p<0.000) and inversely to body height (r=-0.254, p<0.036), weight (r=-

0.277, p<0.022), heart rate (r=-0.436, p<0.000) and urea nitrogen (r=-0.321, p<0.008. PWV and AIx appeared to be independent of all other variables studied.

Out of bone-related proteins, OC was positively correlated with serum creatinine (r=0.543, p<0.000), urea nitrogen (r=0.358,p<0.004), phosphate (r=0.471,p<0.000) alkaline phosphatase (r=0.375, p<0.002), iPTH (r=0.512, p<0.000), central systolic blood pressure (r=0.348, p<0.005) and the time spent on HD (r=0.255, p<0.042). OC, however, negatively correlated with PWV (r=-0.247, p<0.049).

OPG was positively associated with age (r=0.652, p<0.000), and negatively with BMI (r=-0.313, p<0.011), body weight (r=-0.371, p<0.002) and height (r=-0.261, p<0.03). It is important to mention that there were no significant correlations between OP and any clinical or laboratory parameters routinely measured in uremia. We may assume that OC (r=0.282, p<0.024) and OPG (r=0.256, p<0.040) are related, which suggests that these distinct bone-related proteins may interact in uremic patients receiving regular HD.

Multiple linear regression models were applied to establish independent variables that have significant impact on bone-related proteins. The variance of OC was negatively influenced by PWV (β=-0.25, p<0.029) and BMI (β=-0.26, p<0.026) and positively influenced by systolic blood pressure (β=0.37, p<0.001) and hsCRP (β=0.23, p<0.049). OPG as a dependent variable correlated directly with age (β=0.69, p<0.000) and inversely with BMI (β=-0.31, p<0.001). When the model was conducted with OP as dependent variable, LDL-cholesterol proved to be the significant factor associated with OP (β=0.25, p<0.044)

Further analysis by this multiple regression model disclosed that the variance of PWV as dependent variable was only affected by age (β=0.53, p<0.001), while bone-related proteins had no significant contribution. AIx, the other measure of VS appeared to be independent of all clinical/biochemical parameters analysed.

Discussion

We confirmed that PWV, as a marker of arteriosclerosis, is influenced by serum cholesterol level, fetuin-A, and time spent in dialysis. Other factors examined in this study did not prove to significantly influence parameters of arterial stiffness. We proved the correlation

between several clinical and laboratory factors, and pro- and anti-inflammatory biomarkers. This way we emphasized that complex interconnectedness of the uremic environment, minor inflammation, and cardiovascular health.

In our cross-sectional study we examined the correlation between arterial stiffness and inflammation markers. Surprisingly, AIx and PWV both proved to be independent of pro- (CRP, TNF-α, TGF-β1) and anti-inflammatory (vitamin D3, fetuin-A, α-Klotho) biomarkers alike. However, inflammation in patients with uraemia is significantly influenced by several clinical and laboratory variable, such as lack of vitamin D, low level of serum sodium and albumin, plasma creatinine level, time spent in dialysis, BMI, and age.

The second examination confirmed that in ESRD patients on regular HD, PWV as a marker of vascular stiffness increased significantly and there was several-fold elevation in serum levels of bone- related proteins (OC, OPG, OP). As these proteins have been claimed to be implicated in the development of vascular calcification, their relationship to PWV was sought and only OC was found to correlate with PWV.

Unexpectedly, a paradoxical improvement was observed in this indirect measure of vascular calcification when PWV was analysed as a function of retention of metabolic waste products (creatinine, urea nitrogen, phosphate, potassium and iPTH). We have no apparent explanation for this observation, however, it is unlikely that these substances provide vascular protection. It is instead presumable that minor inflammation and oxidative stress outperform other factors and/or are independently associated with vascular disease. In this regard it is to be considered that inflammation and oxidative stress are both highly prevalent in patients with ESRD and have pivotal role in development of vascular calcification. Moreover, they are further amplified by HD process and ageing. Our study confirmed the importance of age by demonstrating positive association between PWV and age that remained strongly significant after adjustment for relevant confounders.

CLINICAL ASPECTS OF STUDY RESULTS, NOVEL FINDINGS

I/1. The clinical study that examined the first topic of this dissertation proved that daily sodium intake of Hungarian children and adolescents was higher, and their potassium intake was lower than the amount mentioned in international recommendations. On one hand, this is due to the

high amount of salt in ready-made foods and dishes served in school canteen, and on the other, due to Hungarian children's low fruit- and vegetable-consumption.

I/2. The current study fell short of confirming direct correlation between salt intake and high blood pressure. It is nonetheless important to mention that according to scientific literature, high sodium-intake in childhood and adolescence has long-term influence on preference for salt, high blood pressure, and development of connected diseases in later age.

I/3. We detected a positive tendency after comparing results from 1990 and 2010, as daily sodium-intake did not change significantly (except for 1-8 year olds, where a 25% decrease was measured), while potassium-intake showed significant increase. This shift caused 50% decrease in the sodium-potassium ratio in the past 20 years, which is the leading risk factor of cardiovascular diseases.

Our results also highlight that due to inappropriate dietary habits, it is necessary that Hungary promptly joins international prevention programs that promote lower sodium- and appropriate potassium intake from an early age. Such a program would decrease not only the prevalence of high blood pressure, but also age-related blood pressure increase levels, and hypertension-related morbidity and mortality among the elderly.

II. Similarly to previous findings, the second study of the dissertation proved that dimethylarginine levels in the plasma of end-stage renal disease patients participating in HD treatment increased significantly.

The results lead us to the conclusion that in case of end-stage renal disease patients participating regularly in HD, hypotensive episodes are triggered by the L-arginine-NO system. Accumulation of ADMA blocks overproduction of NO, and thus prevents further decrease of blood pressure.

The role of vascular stiffness should also be taken into consideration when examining the correlation between ADMA and hypotensive episodes. It is proven that increased ADMA level is in connection with an elevated vascular stiffness, which will thus limit the capability to regulate changes in blood pressure.

III/1. In the third study covered by this dissertation we examined the mechanism of accelerated arteriosclerosis in end-stage renal disease patients participating in chronic haemodialysis treatment. The first series of examinations covered the pro- and anti-inflammatory factors that play a role in this mechanism. Results from the first examination confirmed that pro-inflammatory cascade activates in arteriosclerosis, and pro-inflammatory biomarkers (CRP, TNF-α, TGF-β1) become more dominant than protective factors (vitamin D3, α-Klotho, fetuin-A) in patients receiving chronic HD treatment. Results show the importance of vitamin D supplements for patients receiving haemodialysis.

As this examination was cross-sectional, confirmation of the results requires further long-term, retrospective studies. In this work we selected only a few biomarkers for measurement, and further proteomic and metabolomic studies are necessary to understand the particular patomechanism of vascular damage in end-stage renal disease patients.

III/2. This study helped us confirm the role of OC in vascular calcification, although we did not find direct correlation between OP and OPG, and vascular lesion. High circulating serum level of these might be an epiphenomenon, i.e. a secondary or by-product, but it may also have a counter-regulatory role to attenuate the uremic calcification process.

To reach definitive conclusions, patients receiving HD treatment should be involved in longitudinal and prospective studies, including detailed analysis of the influence of various etiological factors and medications on various clinical and biochemical parameters.

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LIST OF PUBLICATIONS

Publications discussing the topic of this thesis – in English:

1. Vida G., Sulyok E., Lakatos O., Ertl T., Martens-Lobenhoffer J., Bode-Böger S.M.: Plasma levels of asymmetric dimethylarginine in premature neonates: its possible involvement in developmental programming of chronic diseases. Acta Paediatr. 2009; 98(3):437-41.
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Publication to be reviewed:

1. Csiky B., Peti A., Lakatos O., Gyimesi T., Sulyok E., Wittmann I.: Pro- and anti-inflammatory factors and vascular stiffness in chronic hemodialysis patients.