SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Examination of increased vascular risk factors in patients with end-stage renal disease

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UNIVERSITY OF DEBRECEN
Kálmán Laki Doctoral School

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Head of the **Examination Committee**:	László Muszbek, MD, PhD, DSc
Members of the Examination Comittee:	Attila Szabó, MD, PhD, DSc
	Mariann Harangi, MD, PhD

The Examination takes place at the Library of Division of Clinical Laboratory Science (8th floor), Institute of Laboratory Medicine, Faculty of Medicine, University of Debrecen, 26th November 2019, at 11:00 AM.

Head of the **Defense Committee**:	László Muszbek, MD, PhD, DSc
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The PhD Defense takes place at the Lecture Hall of Bldg. A, Institute of Internal Medicine, Faculty of Medicine, University of Debrecen, 26th November 2019, at 02:00 PM.

1. Introduction and literature review

1.1. Chronic kidney disease, end-stage renal disease, renal replacement therapy

Chronic kidney disease (CKD) means more and more important epidemiological problem in the developed countries. The patients can be assigned to 5 stages of CKD depending on their renal function, where the 5th stage means end-stage renal disease whith a need of renal replacement therapy. In some estimation 10 % of the population is suffering from any stage of CKD, nevertheless only a small part of them will be recognized in time and renal replacement therapies should be started with urgency in a relatively high percentage.

The stages of chronic kidney disease depends on the glomerular filtration rate (GFR). The calculation of estimated GFR (eGFR) is based on studies with high number of cases to evaluate experimental formulas, which can minimize the required measurements (e.g. blood samples, urine collection, etc.) and mostly requires easy to access antropometrical data. In 2009 the „Chronic Kidney Disease – Epidemiology" (CKD-EPI) formula was created due to a collaboration study, which can estimate the GFR up to 90 ml/min/1,73m^2 from only the measurement of the serum creatinine level and from the patients age, sex and race.

Evaluation of the CKD staging requires the exact level of the eGFR, which can be checked regularly to follow the dynamic of the changes. In CKD stage I, where the eGFR level is in the normal range, it also needs structural and/or functional disorders to be presented simultaneously to evaluate the correct staging. Dividing stage III. into 2 subgroups is based on the visible progression of the outcome, where the renal function is less, than 45 ml/min/1,73 m^2 the progression is unstoppable and the only therapeutic choice is slowing down the reduction rate. Due to the progression or any other source, when the eGFR is under 15 ml/min/1,73m^2 we call it end-stage renal disease.

For replacing the missing renal function the best choice is the kidney transplantation, but in many cases there are comorbidities beside the end stage renal disease, which can be relative or absolute contraindication for transplantation. As long as renal transplantation not an option or the graft is not functioning anymore and the eGFR level is under 10 ml/min, than dialysis should be performed. Haemodialysis and peritoneal dialysis are the options if dialysis is required. During haemodialysis the patient's blood dialysed through a semi-

permeable membrane against dialysate fluid and thanks to diffusion the small molecular weight waste products can be excreted. Haemodialysis modality itself stands for and applies diffusion and counterflow of the dialysate fluid against the blood stream. During haemodialysis the blood purification process is limited to the 10 kDa cut-off value of the membrane in pore size. In the last decades several techniques were developed to improve the clearence of higher molecular weight substances. Haemofiltration employs higher hydrostatic pressure through the semi-permeable membrane and due to convective transport waste products of wider range can be excreted with the patient's plasma. Removing at least 18 liters of fluid during a dialysis treatment lowers the cardiovascular risk és improves the survival. Sterile infusion should be administered to replace the removed fluid volume. It is important to note, that comparing to haemodialysis the removal of lower molecular weight waste products is less effective. As a compromise, but rather combining the advantages of both dialysis modality haemodiafiltration treatment was created. Haemodialysis is associated to the dialysis centers and requires the transport of the patient three times a week, while peritoneal dialysis can be performed at home making the lifestyle of the patient much easier and more free. The principle of peritoneal dialysis is the opportunity that the peritoneal membrane can be used as a semi-permeable membrane. The thin monolayer structure of cuboidal cells and basal membrane is ideal for the transport of the waste products to the dialysate fluid due to diffusion. The excess fluid from the patient is removed by osmosis due to the oncotic pressure maintained by glucose concentration of the dialysate fluid.

1.2. Risk factors of kidney diseases and kidney disease as a risk

Chronic kidney disease can be cauesd by several systemic disease, which can be also worsened by the manifestation of the kidney disease. Hypertension, atherosclerosis and diabetes are well-known conditions in the background of the development and progression of kidney disease. Hypertension and diabetes simultaneously can increase the risk of atherosclerosis and increase the speed of the progression as well, while atherosclerosis will worsen the hypertension. In all three cases the direct and indirect effects on vascular system can increase a magnitude greater the risk of morbidity and mortality of cardio- and cerebrovascular diseases. Cardiovascular mortality is still one of the leading mortality factors in developed countries, despite of the improvements in cardiovascular intervention techniques, the wider knowledge of the principles in

the developement of different diseases and the more pronounced prevention efforts.

Beside the inherited disorders such as polycystic kidney diseases, Alport's syndrome, nail patella syndrome, thin basal membrane disease, etc., the disease affecting directly the tissue of the kidney like glumerulonephritis, interstitial nephritis and tubulopathies are also playing an important role in evolving chronic kidney disease. Proteinuria can be seen as a consequence of the underlying diseases and/or the chronic kidney disease, but irrespectively of the origin it is not only a diagnostic parameter but it is also counts as an independent risk factor of cardiovascular diseases.

1.3. Vascular alterations in kidney diseases

The risk of cerebro and cardiovascular diseases deteriorates paralel with the worsening of the chronic kidney disease. Cardiovascular diseases meant to be the main cause of mortality in patients with CKD. Vascular calcification and its consequences are still one of the most thrilling clinical challenges especially in patients with ESRD, where the progression is even more pronounced. Vascular calcification can be divided mainly into 2 different entities, which can be differentiated by histological examinations. In most cases due to the several comorbidities both type can be seen simultaneously and they will worsen the patient's quality of life and will cause severe complications. Atherosclerotic lesions are associated with intimal sclerosis while Mönckeberg's type arteriosclerosis affects the smooth muscle cells in the tunica media layer of the vessel wall. Vascular calcification is concerning the the whole vasculature in a well regulated process, in which the smooth muscle cells are turning into osteoblast-like cells. In this transdifferentiation process the smooth muscle cells are loosing their specific protein pattern (e.g. alpha-actin, SM22alpha, etc.) and start expressing osteoblast specific proteins. Levels of osteocalcin, ostepontin, osteprotegerin, matrix Gla protein and alkaline phosphatase are increasing. Changes in the alkaline phophatase's level indicates and tracks the tranformation process. Several factors were identified as inducers or suppressors of vascular calcification. One of the most potent inducers is the increased phosphate level. Hyperphosphataemia and activated vitamin D treatment, which is used in the therapy of secondary hyperparathyroidism are major risk factors of vascular calcification in patients with CKD.

1.4. Hemoxigenase-ferritin system in vascular diseases

The activitation of hem-oxigenase-1/ferritin system influences favorable the high inorganic phosphate induced in vitro calcification, as our group published before. In our results the iron released from the hem group - independently of its redox state - can inhibit in a dose-dependent maner the induced accumulation of calcium in the extracellular matrix of smooth muscle cells by the increased level of inorganic phosphate. In further experiments we could identify the induced expression of ferritin heavy chain by iron as the responsible factor for the inhibitory effect. Recombinant wild type ferritin heavy chain can also inhibit the calcification process while the mutant form, which is lack of ferroxidase activity was unable to show ny inhibition proving the protective effect of the ferroxidase activity. In advanced CKD and in ESRD with renal replacement therapy hyperphosphataemia is often associated with iron deficiency. Iron deficiency together with the decreased amount of intracellular ferritin heavy chain worsen the calcification process and to overexpress FtH can inhibit the mineralization in the vascular system in patients with advanced CKD.

1.5. Alterations in the coagulation system in kidney disease

Several studies were performed to evaluate the changes in the coagulation system and the platelet function in dialysed patients. In previous paper focusing on the examination of extracorporeal circulation we succesfully identify leukocyte activation. Questions - like investigating the changes in platelet counts and function, identification of the changes toward prothrombic way in platelets, endothelial cells and the coagulation system during the treatment - are staying often unanswered. So far only one publication as a sub-study from CONTRAST study was written about comparing the platelet activation during HD and HDF treatment. The authors described increased activation associated to platelets in patients on HDF treatment, but the soluble beta-thromboglobulin levels showed decreased platelet degranulation. Thus these results between the cellular and soluble factors are not coherent we can not take a side clearly.

2. Aims

The pace of vascular calcification is more and more pronounced as the chronic kidney disease is advancing. Thanks to this the cardiovascular risk is continuously rising, which means already one of the most common cause of mortality and morbidity in patients with end-stage renal disease. Beside the inorganic phosphate many other factors are associated with the induction of calcification, such as organic phosphate and activated vitamin D. In previous experiments we were able to inhibit the extracellular mineralization by the administration of hem to the cell cultures. We could identify the induction of intracellular ferritin and more closely the key role of ferroxidase activity in the background of the inhibitory effect.

In the first part of this work our aim was the in vitro modelling of vascular calcification induced by organic phosphate and activated vitamin D. Further aims were to prove the universal inhibitory effect of ferritin and ferroxidase activity and to test alternative inducers of ferritin synthesis without increase the total iron amount in the body.

Patients on renal replacement therapy with end-stage renal disease have impaired haemostaseological parameters, which can be explained by the accumulation of uremic toxins and by the dialysis treatments. Several studies was aimed to track the changes of platelet function during dialysis treatment, but comparing the changes between different therapy modalities was never done.

In our second study we want to examine the platelet activation during dialysis treatments focusing on the soluble and surface attached factors and on the levels of cellular conjugates. Based on the findings from previous studies we want to measure the heterotypic aggregates between platelets and monocytes or neutrofil cells and soluble markers such as von Willebrand factor, P- and E-selectin.

The principle of our hypothesises and aims was to understand the pathophysiological processes in the background of the increased risk of cardiovascular diseases among patients with chronic kidney disease in a more complex way, and where it is possible we want to identify potential therapeutic approaches.

3. Materials and Methods

3.1. Cell culture

Human aortic smooth muscle cells (HAoSMC) were used from 3 different sources: Cell Applications (San Diego, CA), Lonza (Allendale, NJ), Cambrex (Wokingham, United Kingdom). Cells were from 5 independent donors. Cells were cultured in high glucose DMEM and medium was changed when signs of the decreasing pH was visible or at least every 2 days. During the experiments for the confluent cells it was enough to change the medium in every 2 days.

3.2. Alkaline phosphatase activity measurement

Cells were treated for 7 days. At the end of the experiments cells were solubilized with solubiliztion buffer (1% Triton-X 100, 0,5% Igepal CA-630, 1% Complete Mini protease inhibitor, 150 mM NaCl, 5 mM EDTA, 10 mM Tris). Alkaline phosphatase cleaves a phosphate group from p-nitrophenyl-phosphate to form p-nitrophenol, which can be measure by photometer. Maximum slope of the cinetic curves was normalized to the protein content of the cell lysate.

3.3. Alkaline phosphatase enzymeactivity staining

Cells were treated for 7 days in growing or treatment medium as the experimental protocol required. The staining process was performed by Sigma 85L3R kit according to the manufacturer's instructions.

3.4. Quantification of calcium deposition

Cells were grown on 48 well plates and were treated for 7 days. Decalcificaton was done by incubating the cells with 0.6 mM HCl for 24 hours at 37°C. We collected the supernatants and measured the raw calcium content. Cell lysate was made with solubilizing solution containing 0.1 mol/L NaOH and 0.1% SDS and the protein concentration was measured. Calcium concentration was calculated as μg calcium/mg protein from the raw calcium and protein values.

Mineralization was demonstrated with Alizarin Red staining too. Cells were stained after 7 days of treatment and representative pictures were taken.

3.5. Quantification of osteocalcin

Osteocalcin was measured from the extracellular matrix. After 7 days of treatment the extracellular matrix was dissolved then the cells were solubilized with solubilization buffer. Osteocalcin content of the samples from the extracellular matrix was measured with sandwich ELISA. Protein concentration of

the cell lysate was also performed, and the final concentration of osteocalcin was normalized to the protein content.

3.6. Measuring the protein concentration

Despite of the different types of cell solubilization methods the protein concentration was always measured with BCA reagent.

3.7. Western blot

The protein concentration was always measured for each samples for all of the experiments, therefor during the loading we could easily equalize the amount of protein in the gels. Electrophoresis was performed by 10% SDS-PAGE gel with 100 V and we let the samples run until the standard ladder was well separated. For blotting we used nitrocellulose membrane with the pore size of 0.45 μm pore and the blotting was running on 100 V for 75 minutes. Membranes were incubated with specific primary and secondary antibodies for each protein. Antigen-antibody complexes were visualized with the horseradish peroxidase chemiluminescence system in dark room with x-ray films. The loading of the samples were checked by reprobing of the membranes with glyceraldehid-3-phosphate (GAPDH). The x-ray films were analyzed with densitometry, and the results were normalized to the GAPDH values.

3.8. Quantitative RT-PCR

After 7 days of treatment cells were homogenized and the whole RNA content was isolated. cDNS was prepared with oligodT during reverse transcription. Alkaline phosphatase and GAPDH expression was detected simultaneously with multiple TaqMan Gene Expression Assay and the alkaline phosphatase mRNA concentration was normalized to GAPDH.

3.9. Ferritin heavy chain siRNA transfection

Cells were plated overnight to form 60–70% confluent monolayers, then FtH and negative control siRNA was added to the cells with serum and antibiotics free medium while the transfection was preformed by Oligofectamine. Cells were treated for additional 4 days with the ongoing inhibitory effect of siRNA.

3.10. In vivo study with different D-vitamin receptor agonists

This study included five patients with ESRD who receive intermittent hemodialysis on a regular schedule three times a week and suffer from SHPT. All patients met the criteria for paricalcitol and calcitriol medication as suggested by

the guidelines of the Hungarian Society of Nephrology. Anticoagulated blood samples were collected after the routine dialysis before and 10 minutes after the administration of drugs. The blood samples were centrifuged at 2000 g for 10 minutes. Plasma of the blood samples were collected and kept frozen until the time of experiments when they were added to the medium. Specifically cells were treated by a 50% ratio of patient's plasma and 50% of growing media without FBS for 5 days. Samples collected after the drug administration were used with or without addition of apoferritin for assessing ALP activity, thus exposure of SMCs of apoferritin lasted 5 days as well. The study design and patient care were reviewed and permission was obtained by the Ethics Committee of the University of Debrecen and the Hungarian Government.

3.11. Patient enrolment and dialysis procedures

Twenty-eight patients with ESRD were selected for an interventional longitudinal non-randomized study, each subject qualified as its own control. The Ethics Committee of the University of Debrecen approved the study protocol. The patients – after receiving detailed information about the trial – gave written informed consent. The age of participants was between 18 and 70 (13 males, 15 females, mean age 53 ± 13.8 years), and each patient was on dialysis for at least 3 months before the study was started (mean length 89.5 ± 74.5 months). The patients underwent dialysis three times a week. The same speed of blood flow (mean 366.1 ± 63.7 mL per minute) and dialysate flow (439.3 ± 76.5 mL per minute) was used in both modalities. In order to remove the excess fluid the net ultrafiltration during haemodialysis and haemodiafiltration was 2703.6 ± 1118.0 mL per session and 2432.1 ± 1014.4 mL per session, respectively. Effectiveness was calculated during each treatment employing Online Clearance Monitoring Kt/V. Haemodiafiltration was performed with the average Kt/V of 1.67 ± 0.21, while only 1 patient could not reach the target. In hemodialysis the Kt/V value was 1.48 ± 0.2 on average and 3 patients were under 1.3.

At our dialysis centre we use HDF as the standard modality, so in the beginning of our study we started the sampling with this method. To avoid any disturbing factor we waited for 2 weeks after switching to HD before measuring the platelet markers in HD treatment. The substitution fluid was manufactured on-line from ultrapure water and consisted of 138 mmol/L sodium, 2 or 3 mmol/L potassium depending on the patient's potassium value, 1.25 mmol/L calcium, 0.5 mmol/L magnesium, and 1 g/L glucose. The bicarbonate dialysis solution

contained the same amount of solutes as described above. Bicarbonate concentration of dialysate/replacement fluid was adjusted between 28–38 mmol/L to obtain plasma bicarbonate level of 20–22 mmol/L prior to dialysis. During haemodiafiltration the volume of substitution fluid was 23.4 ± 3.8 l.

3.12. Blood collection and cell counting

Blood samples were taken from the efferent line port into sodium citrated tubes at specific time points. Cell counting was carried out by automatic analyzer within two hours of blood collection and cell numbers were adjusted for the haemoconcentration by using the red blood cell counts.

3.13. Platelet poor plasma (PPP) preparation and measurement of soluble markers

Albumin levels were immediately measured for tracking the hemoconcentration during the dialysis treatment. Soluble P-selectin and E-selectin levels were measured by a commercial ELISA kit according to the manufacturer's instructions, while von Willebrand Factor (vWF) antigen levels were measured by turbidimetry. Levels of soluble proteins were adjusted for the haemoconcentration by normalizing the results for albumin values.

3.14. Flow cytometric assays

The expression of platelet surface P-selectin and leukocyte-platelet heterotypic aggregates were measured by flow cytometry. Blood samples were labelled by CD42a-FITC and CD62-PE antibodies for surface P-selectin measurement or with CD14-PE and CD42a-FITC antibodies for leukocyte-platelet heterotypic aggregates.

3.14. Statistical analysis

Kolmogorov-Smirnov test was used for the evaluation of the normality of the data. Most parameters were non-normally distributed; univariate analyses were performed by Mann–Whitney U-test or the independent samples t-test. For paired analyses, as required for the related samples, Wilcoxon signed ranks test or the paired samples t-test were performed. The Spearman's ρ value was calculated for correlation analysis.

Statistical analysis was performed using GraphPad Prism 5 and by one-way ANOVA test followed by post hoc Bonferroni's Multiple Comparison test.

4. Results

4.1. Pharmacological induction of ferritin prevents osteoblastic transformation of smooth muscle cells

Our first goal was to test the osteoblast-like transformation of human aortic SMC induced by BGP and activated vitamin D3 (Calcitriol). Both inducers have the ability to increase ALP activity in dose-dependent manner. The induction of enzyme activity was time-dependent leading to a pronounced elevation at 7 days of exposure. Western blots confirmed that the increased activity was because of the increment of ALP expression. b-glycerophosphate and calcitriol increased the enzyme level dose-dependently and the additive effect is also evident in this representative experiment.

Human aortic SMC were treated with BGP and calcitriol alone or in combination to investigate the induction of ALP in the presence or absence of apo- and holoferritin. Both forms of ferritin decreased the activity of ALP and our findings show that there were no significant differences between the two forms. As ferritin shell is made of both the H and L chains and to delineate the individual effects of each form of ferritin, we performed the following experiment using H- (FtH) and L- recombinant ferritin. Furthermore, we also utilized the H222 mutant form of FtH which is identical to the FtH, but lacks ferroxidase activity based on the mutation. Alkaline phosphatase activity assays showed that inhibition of transformation only occurred when cells were pre-incubated with FtH chain, whereas L chain and H222 (both of which lack ferroxidase activity) had no effect.

To determine the effects of exposure of cells to exogenous ferritins cells were pre-exposed to iron (in the form of ferric ammonium citrate) which induces ferritin synthesis by increasing intracellular iron. The treatment of cells with iron and subsequent induction of ferritin resulted in mitigation of BGP and calcitriol induced ALP induction. The specific involvement of ferritin in the prevention of ALP induction is supported by experiments using siRNA. Again, we exposed cells to ferric ammonium citrate, but in this case, inhibited ferritin synthesis using anti-FtH siRNA. The suppression of FtH induction abrogated the blockade of ALP induction which proceeded in cells which had not been pre-treated with BGP and calcitriol. Therefore, these results demonstrate that elevated levels of FtH - irrespective of the mode and whether endocytosis of exogenous FtH or induction of FtH synthesis caused by additional iron supplementation - directly prevents ALP expression and activity which normally occurs under conditions favouring

calcification. We also confirmed the efficiency of siRNA, and observed an approximately 80% decrease in FtH protein expression for up to 4 days after transfection.

Besides iron, which is the physiological inducer of ferritin, other agents could also be utilized to induce endogenous ferritin expression. We chose the 3H-1,2-Dithiole-3-thione (D3T). Human aortic SMC treated with increasing dose of D3T in the presence of BGP and calcitriol showed lower activity of ALP, and the decreased enzyme activity showed an inverse relationship with the increasing dosage of D3T. We confirmed induction of FtH as a dose-dependent response to D3T administration. To demonstrate that FtH induction by D3T is responsible for the inhibitory effect on ALP expression in SMC, we transfected cells with siRNA for FtH and measured ALP enzyme activity. Indeed, ALP activity was not attenuated in D3T-treated SMC grown in BGP and calcitriol containing media after silencing FtH. The efficiency of silencing FtH was approximately 70% in our experiment.

We next sought to investigate the mechanism by which FtH provides inhibitions of ALP and hence osteoblastic transformation of SMC. In vitro measurement of enzyme activity was performed for which cells were treated with BGP and calcitriol for 7 days and then solubilized. Supernatant of cell lysates were exposed to the effect of apo- and holoferritin. Levamisole, a well-known inhibitor of ALP was used to illustrate the inhibition. We found that such a treatment did not result in inhibition of ALP activity and hence results suggest that ferritin does not directly alter ALP enzyme activity. We then examined protein expression by Western blot. Cells treated with calcifying media showed increased ALP expression, whereas both apo- and holoferritin diminished the induction. Next, we examined the possibility of observed inhibitory effects at transcriptional level utilizing RT-PCR. mRNA expressions of ALP in samples were measured and normalized by GAPDH and the fold increase to growth media was calculated. We show that calcifying condition resulted in up-regulation of ALP mRNA. Results revealed that both apo- and holoferritin significantly decreased ALP mRNA levels. These results indicate that regulation of ALP expression in SMC by ferritin occurs at transcriptional level.

Ceruloplasmin is a copper-binding and carrier protein that is mainly produced by hepatocytes. However, for the purposes of our experiments it served as an important control arm as it, similar to FtH, possesses ferroxidase activity.

Ferroxidase activity of ceruloplasmin dose-dependently inhibited the ALP activity enhanced by osteoblastic inducers. The decreased activity was caused by the lowered protein expression, which was detected by Western blot analysis.

Although the expression of ALP as an early marker of mineralization was increased after exposure of human SMC to BGP and calcitriol mineralization did not occur even if cells from five independent donors were cultured for up to 3 weeks. This is in accordance with the literature - bovine SMC are mostly employed in calcification studies as they are more ready to mineralize as compared to human SMC. To demonstrate that by up-regulating ferritin by iron or D3T inhibits not only the expression of ALP, but it affects calcification as well in human SMC, we exposed human aortic SMC to 1.2 mmol/l Pi and 0.9 mmol/l calcium (calcification medium) and performed Alizarin red staining as well as measured the calcium accumulation. Granular deposits developed in cells grown in calcification medium, but not in the control culture grown in normal growth medium, as demonstrated by Alizarin red staining. We found that addition of iron or D3T to the calcification medium suppresses granular deposit development and extracellular calcium deposition in a dose-responsive manner, causing marked inhibition at concentrations of 25 and 50 μmol/l, respectively. As iron is a very potent inducer of ferritin and D3T also markedly enhances the intracellular ferritin content, next we tested whether the observed inhibitory effect of iron or D3T on calcium deposition is mimicked by apoferritin. We found that iron-free apoferritin at a dose of 2 mg/ml abolishes granule formation and inhibits calcium deposition. To confirm the inhibitory effect of iron or D3T on calcium accumulation occurs via FtH, we transfected human SMC with small interfering RNA specific to FtH. We observed an approximately 70% efficiency in FtH silencing. As we exposed cells to ferric ammonium citrate or D3T the suppression of FtH induction resulted in abrogation of the inhibition of calcium accumulation. This result indicates that the elevation of intracellular FtH by iron or D3T is the main mediator of inhibition of the calcification of extracellular matrix.

Next we investigated the presence of the bone-specific protein, osteocalcin, in the extracellular matrix. Maintaining of human SMC in calcification medium for 7 days resulted in a significant increase in osteocalcin content compared to the control. Iron or D3T decreased the up-regulation of osteocalcin in a dose-responsive manner. In addition, apoferritin also abolished expression of osteocalcin. As we silenced FtH in human SMC treated with ferric ammonium

citrate or D3T the inhibition of osteocalcin accumulation in extracellular matrix was abrogated. Taken together, intracellular ferritin induction is a major inhibitory mediator of calcification and transdifferentiation of SMC.

Vitamin D analogue paricalcitol was able to increase the activity of ALP in human aortic SMC and the inducing effect was dose-dependent. Ferroxidase activity of apo- and holoferritin inhibited the increased ALP enzyme activity induced by paricalcitol. Plasma samples from patients with ESRD and secondary hyperparathyroidism who were treated with calcitriol or paricalcitol were collected and added to human aortic SMC. After 5 days, calcitriol significantly increased enzyme activity, and apoferritin abrogated this induction. Paricalcitol also significantly induced the activity of ALP albeit to a lesser degree. Again, addition of ferritin lowered ALP activity to baseline level irrespective of the inducer.

4.2. Haemodiafiltration elicits less platelet activation compared to haemodialysis

Platelet P-selectin values were well over the reference range of <3 %, as described earlier, right from the beginning of the dialysis procedures. It needs to be emphasized that the treatment requires the dialyzer to be filled up with heparinized blood, and this is achieved by a 4–5 min roller pump-assisted extracorporeal circulation. As such, a few minutes have already elapsed when the initial sample (0 h) was collected. HD treatment resulted in higher platelet P-selectin values when compared to the HDF procedure and the difference was the largest by the end of the procedure. Although the median soluble P-selectin values were within the reference range by the end of both procedures, several results exceeded the upper reference limit. The soluble P-selectin values increased during the procedure, but significant differences were observed between the two treatment modalities at the 1 h time-point, with the values being higher during HDF.

Platelet activation was also monitored by using the indirect platelet activation tests where we examined heterotypic cell aggregates. Here, a striking elevation was observed when monocyte-platelet aggregates were analyzed compared to a mean value of 38 % obtained in controls and previously published by us and others.

The ratio of these aggregates kept increasing during the dialysis and the number of these aggregates were significantly higher in HD treatment at each analysis time point and a similar tendency could be observed in case of neutrophil-platelet aggregate ratio during dialysis. At each sampling time point HD treatment

resulted in significantly higher monocyte-platelet aggregate formation than HDF treatment.

Since endothelial injury has also been described during extracorporeal circulation we measured two endothelium associated biomarkers the soluble E-selectin and the vWF antigen. At each sampling time, there were only few soluble E-selectin values above the manufacturer suggested upper reference limit (51 ng/mL), however the median soluble E-selectin was not elevated during the procedures and there were no difference between treatment modalities. Also, no difference was observed between dialysis procedures for vWF antigen, in contrast to soluble E-selectin but vWF antigen values were elevated right from the beginning and significantly increased by the end of the HD procedure.

5. Discussion

The findings presented in the first study corroborate the inhibitory role of FtH in the inhibition of SMC mineralization and osteoblastic transformation. To induce mineralization we used BGP with or without the addition of calcitriol. These experiments confirmed previous reports identifying elevated Pi and high levels of activated vitamin-D (calcitriol) as inducers of calcification of SMC. We used ALP expression as a surrogate of SMC transition to osteoblasts as previously reported. We validated that FtH prevents calcification and osteoblastic transformation of SMC, irrespective of the inducers used in this study. In this regard, ferroxidase activity was confirmed to be essential in the mitigation of calcification. We used D3T, a well-known chemo-preventive agent that is known to induce ferritin expression. We demonstrate that induction of ferritin via D3T abrogates SMC transition to osteoblasts and therefore prevents calcification of extracellular matrix. Furthermore, we found that culture of SMC with serum samples from ESRD patients receiving calcitriol or paricalcitol leads to osteoblastic transition and we also show that addition of FtH prevents such transition.

Cardiovascular disease remains the leading cause of death in patients with advanced CKD and ESRD. Evidence suggests that adjusted cardiovascular attributed mortality is higher by about 10–20-fold in CKD patients compared to the general population. The increased prevalence of cardiovascular disease in this group of patients is, at least in part, attributable to vascular calcification. Increased prevalence of this condition in the younger patient population requiring renal replacement therapy is a strong confirmation that the uraemic milieu generates the perfect storm for initiation and acceleration of vascular calcification. Increased Pi level is a significant risk factor and has been confirmed in multiple studies to be a key regulator of vascular calcification. Other inducers of vascular calcification have also been identified. The vitamin D receptor analogues have been shown to induce mineralization of the vascular tree as well. However, different analogues have been shown to exert variable results. Importantly, calcitriol and its analogues are routinely used to manage secondary hyperparathyroidsm that is a frequent complication of end-stage kidney disease.

Ferritin is an ancient, large, spherical protein with highly conserved three-dimensional structure and is the most important intracellular iron handling machinery. The ferritins are a family of proteins similar to spherical shells,

designed to sequester and store large amounts of iron in a safe, soluble and bioavailable form. Ferritin is made of 24 subunits of two types (H, heavy and L, light chain) whose proportion depends on the iron status of the cell, the tissue and the organ. The two ferritin polypeptides are related, but FtH carries a ferroxidase activity to oxidize Fe^{2+} to Fe^{3+} allowing safe incorporation of iron into the shell. Ferritin shells can store up to 4500 iron atoms. Ferritin acts as a depot, sequestering excess iron and allowing for the mobilization of iron when needed. Our understanding of ferritin and its functions has markedly enhanced in the past decade and novel functions other than regulation of iron metabolism and homoeostasis are now attributed to ferritin. Previously, we reported that haeme and iron-mediated induction of FtH prevents calcification and osteoblastic differentiation of SMC induced by high P_i. We reported that ferroxidase activity of FtH was the key mediator of the observed inhibitory effects. Importantly, we previously found that the induction of FtH was able to actively prevent the process of transformation of SMC into osteoblast-like cells and also inhibit osteoblast activity via down-regulation of Cbfa-1, the main transcriptional factor mediating osteogenesis and responsible for the induction of osteoblast-specific gene expression. These findings were the first report to suggest a relationship between deranged iron metabolism that is a frequent complication of CKD and vascular calcification. We were hence encouraged to identify potential pharmacological agents that would be able to induce ferritin expression and examine whether such induction via chemical stimulants of ferritin would provide the same inhibitory effects. To this end we examined the compound D3T that is known to have anti-oxidant and cancer chemopreventive properties. We demonstrate that D3T-induced up-regulation of FtH provides inhibitory effects against high Pi- and calcitriol-mediated osteoblastic differentiation of SMC. It should, however, be noted that D3T is not currently approved for human use. However, it occurs naturally in cruciferous vegetables. The oral administration of D3T is being considered for development as a potential drug for the chemoprevention of hepatic and other carcinomas by the National Cancer Institute.

In conclusion, this study corroborates our previous findings and validates FtH induction as a potent inhibitor of osteoblastic transformation of SMC. Given legitimate concerns regarding utilization of excessive parenteral iron that may potentiate in excessive reactive oxygen species formation, chemical induction of FtH via D3T may be a novel preventive measure against vascular calcification. In

support of this premise, further studies to test this hypothesis in relevant animal models of vascular calcification is germane and timely given current lack of reliable preventive and/or therapeutic modalities against vascular calcification.

In summary of our platelet activation study we found novel changes in platelet activation markers during the two different dialysis treatment modalities. Platelet and soluble P-selectin values demonstrated increasing levels during the treatments, but there were a few significant differences when the two modalities were compared. Heterotypic aggregate formation was also elevated during both modalities especially in HD. Monocyte-platelet aggregates showed significantly higher levels during HD compared to HDF. Changes in neutrophil-platelet aggregates showed increasing levels in a time-dependent manner but with only lower significancy levels. Endothelial activation markers, such as E-selectin and von Willebrand factor, were observed in HD group and only vWF elevation was significant during the treatments. Individual data from our work according to previous studies and literature can make a more complex view of haemostatic abnormalities in chronic kidney diseases, since in this patient group both bleeding and thrombotic complications are observed and this dual effect is also characteristic for platelets. In CKD patients platelet dysfunction is frequently observed that may be caused by the uremic toxins, enhanced nitric oxide production and anaemia, but the platelets of dialyzed patients are constantly activated and these repeated procedures result in the ’exhausted platelet syndrome’. Even in early reports it was described, that platelet-leukocyte aggregates occur during dialysis, most likely related to the primary platelet activation. Already at this time the authors suggested this test as a biocompatibility marker. Further studies identified platelet trapping, particularly sequestration of the larger platelets in the circuit, and suggested that soluble markers like beta-thromboglobulin are suitable for detecting platelet degranulation. Another methodological approach to investigate platelet activation was published by Schoorl and colleagues, who verified platelet degranulation by light and electron microscopy. Nevertheless, in more recent reports the biocompatibility of the dialyzer membranes was monitored by the platelet count and it was concluded, that the more biocompatible newer membranes like the polysulfones do not result in marked thrombocytopenia. In our studies we found by using a highly biocompatible membrane, that the platelet count did not change dramatically however, a significant platelet count decrease was observed with both treatment modalities.

In addition to platelet activation, leukocyte activation may occur during HD and contribute to heterotypic aggregate formation. CKD patients are exposed persistently to a low-grade inflammation. The level of inflammatory markers is affected by numerous factors such as transient infections, several comorbidities, and the intermittent stimulus of the dialysis procedure. The archetype inflammatory marker, C-reactive protein, has long been shown to be able to elicit procoagulant functions by stimulating monocyte tissue factor expression. Furthermore, the binding of activated platelets, to monocytes and neutrophils, via P-selectin require P-selectin glycoprotein ligand 1 (PSGL-1) that has been shown to be redistributed by activated cytokines. We have identified that flow cytometry based assays are the most sensitive in identifying platelet and/or leukocyte activation, when comparing the two different treatment modalities. The considerable elevation in the gold-standard platelet P-selectin suggests that the very high heterotypic aggregate rate between the myeloid cells and platelets are largely due to primary platelet activation as proposed recently. This phenomenon occurs very early during dialysis as significant elevations could be observed after a few minutes of system filling compared to baseline values. Since at this early time point there is minimal recirculation this may have been caused mostly by the enhanced turbulence. In previous clinical conditions we also found augmentation compared to controls in platelet P-selectin and the monocyte-platelet ratio in obese and diabetic patients and during coronary stenting. However, the degree of the elevation of these aggregates in such patient groups were well below that observed during our present study on HD and HDF treatments. Although a previous study has postulated a role for these aggregates in the pathophysiology of vascular disorders e.g., the development of aortic stiffness, the pathomechanism is still to be elucidated. Nonetheless, soluble factors are likely to play a role in mediating platelet/leukocyte activation, since their effect may be propagated via the plasma, probably by activating complement factors. We previously identified that haemodiafiltration was found to have beneficial effect on echocardiographic markers representing left ventricular diastolic function. We investigated the changes in platelet activation markers to evaluate the effect of different types of dialysis treatments.

Although in a previous meta-analysis a similar sensitivity could be observed in vascular disorders for soluble and platelet-associated P-selectin the simultaneous investigation of these markers during dialysis treatments showed that

platelet P-selectin is having a superior sensitivity compared to soluble P-selectin. As was already suggested by Michelson probably the most sensitive platelet activation assay is the measurement of the monocyte-platelet conjugates, as platelets may lose P-selectin in the circulation. In our patient cohort, we found that such plateletleukocyte aggregates also had an excellent sensitivity as it showed nearly maximal values that can usually be observed only in agonist stimulated samples during in vitro experiments.

In contrast to platelet activation markers, the soluble endothelial cell activation marker E-selectin did not show any change in kinetics. E-selectin is synthesized de novo in stimulated endothelial cells and its expression and release requires considerable time. Unlike E-selectin, vWF is stored in the endothelial Weibel-Palade bodies and can readily be mobilized when the endothelial cells are activated. It is plausible that the elevated levels experienced during the renal replacement therapies are a result of this difference and most likely this is why in HD, vWF antigen increased significantly by the end of the procedure.

Based on these observations, we propose that the more gentle effects of HDF compared to HD may contribute to a less unfavourable vascular effect in this treatment modality. The underlying mechanism can be explained by the fundamental physical processes during dialysis treatment methods. Although both modality use the same type of dialyser, with the same pore-size, while HD employs only diffusion for excretion HDF is applying convective transport as well. Convective transport alone can increase dramatically the excretion of small to mid-size proteins with the molecular weight 5–40 kDa and its also increases the excretion of smaller molecules compared to HD. Further studies are required to determine whether the decreased platelet activation can be explained by the increased elimination of the enhancers of platelet activation during HDF.

6. Conclusion

Cardiovascular risk is elevated among chronic kidney disease patients and it is independent from the modality of renal replacement therapy. Cardiovascular risk is increasing in parallel with the progression of CKD.

There are many well-known pathophysiological factors in the background of increased CV risk, some of them are also confirmed by our research group (e.g. high inorganic phosphate, iron deficiency, etc.), while others can be found in the literature as inducers of vascular calcification (e.g. vitamin D and its analogues, coumarin derivatives, etc.).

We demonstrated an in vitro model for Mönckeberg's type mediasclerosis of vascular smooth muscle cells with activated vitamin D and beta-glycerophosphate. Induction of ferritin heavy chain expression and its ferroxidase activity inhibited succesfully the process of calcification. Among the inducers we used iron and other alternatives.

The changes in platelet count and platelet function are also crucial in the aetiology of vascular diseases. We proved that platelet activation is increased in patients during the dialysis treatments. Our research group was the first to show that haemodiafiltration modality is more favourable in this respect.

The aim of both studies was to recognize complex pathophysiological processes resulting in an increased cardiovascular risk of patients with end-stage renal disease and find new potential therapeutic strategies for everyday use.

7. List of publications:

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A PhD értekezés alapjául szolgáló közlemények

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További közlemények

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