

## ATHEROSCLEROSIS AND VASCULAR CALCIFICATION IN URAEMIA – A NEW EXPERIMENTAL MODEL

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**A b s t r a c t:** Cardiovascular disease (CVD) is the most frequent cause of morbidity and mortality in chronic renal failure (CRF) patients. Accelerated calcifying atherosclerosis, medial calcification, and valvular calcification are hallmarks of CVD in the dialysis population. The mechanisms by which uraemia promotes vascular calcification and the relationship between arterial wall calcification and atherosclerosis are poorly understood. We surgically induced CRF in apolipoprotein E knockout (apoE<sup>-/-</sup>) mice to study a possible acceleration of aortic atherosclerosis, the degree and type of vascular calcification as well as factors involved in the calcification process. Finally we investigated appropriate treatment measures. Atherosclerotic lesions in the thoracic aorta were significantly larger in uraemic apoE<sup>-/-</sup> mice than in non-uraemic controls. The relative proportion of the calcified area to the total surface area of both atherosclerotic lesions and lesion-free vascular tissue was increased in the aortic root of uraemic apoE<sup>-/-</sup> mice when compared with controls. The accelerated atherosclerosis was associated with an increase in aortic nitrotyrosine expression, indicating enhanced oxidative stress, and an increase in plaque collagen content, indicating changes in plaque composition. N-acetylcysteine (NAC) treatment slowed the rapid progression of atherosclerotic lesions and

reversed the increase in plaque collagen content compared with placebo treatment. NAC-treatment also reduced nitrotyrosine expression in uremic apoE<sup>-/-</sup> mice whereas the degree of macrophage infiltration was unchanged. Sevelamer treatment delayed not only vascular calcification but also atherosclerotic lesion progression in uremic apoE<sup>-/-</sup> mice. These treatment effects also were associated with diminished oxidative stress and were independent of cholesterol lowering. We anticipate that this experimental model will prove to be useful to test other treatment strategies aimed at decreasing the accelerated atherosclerosis and arterial calcification of the uraemic state.

**Key words:** atherosclerosis, vascular calcification, chronic renal failure, oxidative stress, mouse.

### *Background*

Cardiovascular disease (CVD) is responsible for more than 50% of deaths among patients with chronic renal failure (CRF) [1]. Actually, the risk of CVD in a 30-year-old CRF patient is similar to the calculated risk of a 70- to 80-year-old subject from the general population. Such a patient would suffer from an approximately 500-fold elevated mortality risk compared with the age-matched general population (US Renal Data System, 1999) [2]. In chronic kidney disease patients the prevalence of coronary artery disease is approximately 53% at the start of dialysis techniques [3] and the risk of dying from a cardiovascular event is 20-fold greater than the risk observed in the general population [4]. A high frequency of coronary artery lesions and events in CRF patients has been documented by retrospective and more convincingly by prospective studies [5]. Despite major advances in the management of cardiovascular disease, CRF patients continue to have an exceedingly high cardiovascular mortality.

Undoubtedly, CRF patients have a heavy burden of classical coronary risk factors as defined in the studies of the Framingham population, including advanced age, male gender, hypertension, dyslipidemia (in particular low HDL cholesterol), impaired insulin sensitivity, endothelial cell dysfunction, family history of coronary disease, cigarette smoking, menopause, physical inactivity and psychosocial stress. In addition, considerable attention has recently focused on so-called non-classical risk factors such as increased oxidative stress, abnormalities of calcium and phosphate metabolism and an inflammatory syndrome, since many studies have shown that classical risk factors explain only a limited proportion of the variance of CVD mortality in renal patients.

Patients with CRF also have a much higher prevalence of arterial calcification than that observed in the general population [6], and this prevalence is further increased in those with *diabetes mellitus*. It is likely that the excessive

cardiovascular calcification of such patients is a multifactorial process, including passive mechanisms such as hypercalcemia, hyperphosphatemia and lack of protective proteins, and active mechanisms such as the transformation of quiescent smooth muscle cells to actively growing cells with an osteoblast-like phenotype.

In our experimental studies performed during the last years we have tried to answer several major questions dealing with the type and speed of progression of atherosclerosis and vascular calcification in the setting of CRF.

#### *Is atherosclerosis accelerated in CRF?*

The term “accelerated atherosclerosis” was proposed by Scribner’s team in Seattle, Washington, USA 25 years ago to describe the advancing atherosclerotic disease in CRF patients [7]. Even though it has been recognized for many years that renal failure is often accompanied by calcification of arteries and other soft tissues, there has been ongoing controversy as to whether the uremic state might accelerate the progression of atherosclerosis by itself or not. Dialysis patients often present a combination of calcified atherosclerotic lesions of the intima and medial calcosis [8, 9]. Until recently, no suitable animal model was available for the study of accelerated atherosclerosis in association with CRF. In contrast to uraemic patients, uraemic rats or mice do not easily develop atheromatous lesions, being naturally better protected than humans. Therefore, we reasoned that the apolipoprotein E gene knockout (apo E<sup>-/-</sup>) mouse might constitute a more suitable model to address this issue than wild type mice [10]. Electrocoagulation of the right renal cortex followed by contra-lateral nephrectomy allowed us to develop a new uraemic mouse model characterized by moderate and stable CRF over several months. Using this model, we were able to provide evidence of accelerated atherosclerosis in uraemic mice, as compared to non-uraemic mice [16]. Two other groups developed a similar mouse model in the same time period and made comparable observations. Thus, Buzello *et al.* and Bro *et al.* showed larger atherosclerotic plaques in uraemic E<sup>-/-</sup> mice following 5/6 nephrectomy, compared with sham controls [12, 13]. Lesion formation in uraemic mice was preceded by upregulation of ICAM-1 expression in the arterial wall after 2 weeks of uremia [14] and CRF of 12-week duration was accompanied by upregulation of transcripts involved in inflammation including osteopontin, matrix metalloproteinases (MMP)-3 and -12, VCAM-1 and serum amyloid A (SAA) [15].

#### *Is atherosclerosis different in CRF?*

Extracellular matrix collagen is the primary component of atherosclerotic plaque structure. At present, there is only limited information on the impact of CRF on plaque collagen content. In our studies, the atheromatous

lesions of uraemic animals exhibited a marked increase in total collagen content although there was no evidence of increased infiltration by inflammatory cells or proteinuria levels (Figure 1) [16, 47]. The former finding is in agreement with a report by Amann *et al.*, showing a higher degree of interstitial myocardial fibrosis in uraemic than in non-uraemic rats, as well as a report by Schwartz *et al.* showing an increase in fibrosis of coronary arteries in dialysis patients [9, 17]. Both observations suggest a profibrotic effect of uraemia.

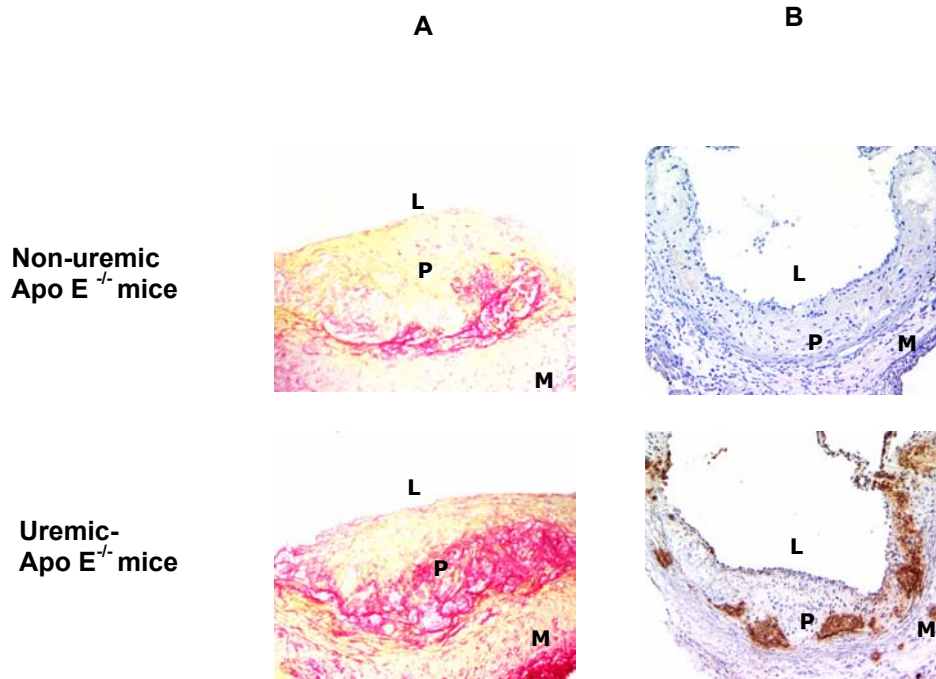


Figure 1 – Representative immunohistologic findings of aortic plaques in control vs. uremic apo E<sup>-/-</sup> mice. A. The collagen plaque content in uraemic mice was markedly increased compared with non-uraemic controls. B. Aortic nitrotyrosine expression.

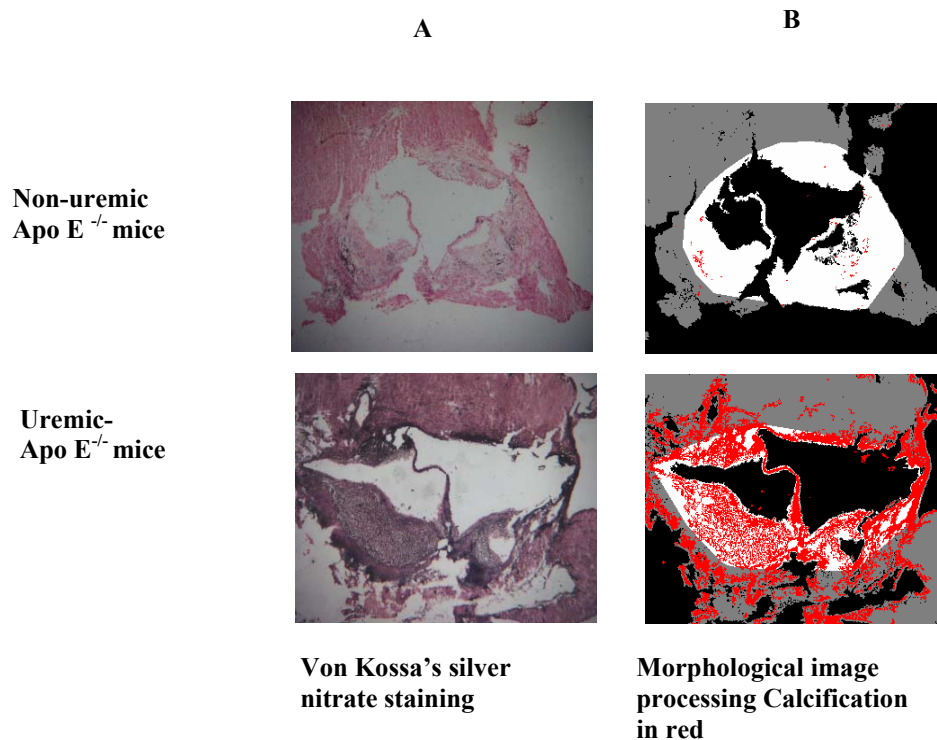
Note strong staining of nitrotyrosine in the centre of the plaque.

M, media; P, plaque; L, lumen

Slika 1 – Reprezentativni imunohistohemijski analizi na aterosklerotične plaki kaj kontrolne vs. uremične apolipoprotein

E-deficitarni gluvci. A. Procentot na kolagen kaj uremične gluvci e signifikantno zgolemen vo spredba so kontrolite. B. Eksresija na nitrotirozin vo aortata. Silno izrazen steining vo centarot na aterosklerotična lezija. M, medija; P, plaka; L, Lumen

Oxidative stress and inflammation have been recognized as playing a central role in the pathogenesis of cardiovascular disease in uraemia [18]. Evidence from reliable oxidative stress markers present in the plasma of uremic patients indicates that CRF is a pro-oxidant state [18, 19]. Until recently, biological evidence of oxidative stress in uraemic patients *in vivo* relied almost entirely on the measurement of lipid peroxidation products such as malondialdehyde and thiobarbituric acid reactive substances (TBARS) which, in general, poorly reflect the intensity of oxidative stress. In our studies, we found an increase in nitrotyrosine expression (a marker of nitrosative-oxidative stress) in the aortic wall of uraemic apoE<sup>-/-</sup> mice (Figure 1). Nitrotyrosine is an indirect marker of peroxynitrite generation that results from the reaction between nitric oxide (NO) and superoxide. Peroxynitrite further sustains oxidative injury to the endothelium and reduces NO availability.



*Figure 2 – Representative images of aorta root sections of control apoE<sup>-/-</sup> and uremic apoE<sup>-/-</sup> mice. (A) Von Kossa silver nitrate staining (calcification in black). (B) Morphologic image processing (calcification in red)*

*Slika 2 † Reprezentativni slike na sekci na aorta vo nivo*

*na aortalnite valvuli. A. Fon Kosa srebro-nitratno boewe (kalcifikaciite se odbele`ani vo crno). B. Morfolo{ka image analiza (kalcifikaciite se odbele`ani vo crveno)*

*What type of vascular calcification is promoted or modified by CRF?*

The introduction of new techniques for the quantification of vascular calcification *in vivo*, such as electron beam computed tomography (EBCT) or multislice spiral CT, has revolutionized our current thinking about the risks of vascular calcification. With these techniques coronary artery calcification (CAC) can be detected and measured as calcium “mass” or expressed as calcium “score”. In the general population, CAC is positively correlated with the atherosclerotic plaque burden [20, 21], increased risk of myocardial infarction [22, 23] and plaque instability [24, 25]. These studies suggest that the presence of CAC is sensitive and specific for detecting clinically significant coronary artery disease [26], and for identifying patients at risk of adverse cardiac events [27].

However, the EBCT technique cannot distinguish whether calcium deposits are localized in plaques (intima) or in the vascular media, respectively. Even more importantly, it also does not enable a concomitant assessment of the progression of atherosclerotic vessel wall lesions.

Enhanced medial calcification has previously been observed in uraemic rats [28] and rabbits [29]. However, in addition to media calcification, calcification of the intima was reported in only one recent study examining CRF-induced atherosclerosis in the LDL receptor deficient (LDLR<sup>-/-</sup>) mouse [30]. In this study, aortic wall calcification was evaluated by vessel tissue staining in a semi-quantitative fashion. In contrast, we were able for the first time to quantify separately calcium deposits in the intimal plaque area and the media of the aortic tissue, using a technique recently established in our laboratory [31]. In both the intimal and medial layers of the aorta, we found significant increases in calcified tissue area in uraemic apoE<sup>-/-</sup> mice, compared with non-uraemic controls (Figure 2) [16].

*Can we prevent accelerated atherosclerosis and vascular calcification in CRF?*

A prospective randomized clinical trial in chronic haemodialysis patients recently showed prevention of the progression of vascular calcification by the calcium-free and aluminum-free phosphate binder sevelamer hydrochloride, in contrast to calcium-containing phosphate binders [32]. In this study, the administration of sevelamer over a time period of 12 months effectively led to a significantly slower progression of aortic and carotid calcification measured by EBCT than the administration of calcium-containing phosphate binders. However, as mentioned above, the EBCT technique cannot distinguish whether

calcium deposits are localized in plaques (intima) or in the vascular media, respectively. Even more importantly, it also does not enable a concomitant assessment of the progression of atherosclerotic vessel wall lesions. Accordingly, we tested the hypothesis that sevelamer, a non-absorbable synthetic polymer indicated for intestinal binding of phosphate, would attenuate the progression of the CRF-associated atherosclerosis in our mouse model.

We showed for the first time that sevelamer is capable of preventing the progression of not only vascular calcification but also of atherosclerosis in uraemic apoE<sup>-/-</sup> mice [11]. Although sevelamer binds cholesterol in the intestinal lumen and thereby exerts cholesterol-lowering effects in humans, the anti-atherosclerotic effect of sevelamer in our study was observed in the absence of a change in serum total cholesterol levels. Thus, it is possible that mechanisms other than a reduction of serum cholesterol were involved in the observed anti-atherosclerotic effect of sevelamer. Interestingly, in several clinical studies high serum total cholesterol has also not been found to be a significant risk factor for mortality in dialysis patients, suggesting that other, non-traditional risk factors must be important contributors as well.

The well-known pharmacodynamic effects of sevelamer include lowering of serum phosphate as well as of the serum total and LDL-cholesterol. Little is known of possible antioxidant and/or anti-inflammatory effects of this compound. In our study, the reduction of atheromatous lesion progression in uraemic apoE<sup>-/-</sup> by sevelamer was associated with a decrease of nitrotyrosine expression within aortic lesions. The mechanisms by which sevelamer could directly modify oxidative stress, or whether it could indirectly influence local inducible nitric oxide synthase (iNOS) activity in the vessel wall, remains to be defined [33]. This raises the important question of whether sevelamer may have anti-oxidant properties mediated via other, non-lipid related mechanisms. Specifically, it is tempting to speculate that calcium deposition in the vessel wall may activate oxidative reactive processes and/or that inhibition of vascular damage by halting plaque calcification or atherosclerosis by sevelamer may attenuate the pro-oxidant response.

Two recent clinical reports suggested that antioxidant therapy might be beneficial in reducing cardiovascular events in CRF patients [34, 35]. However, a causal relationship between oxidative stress and cardiovascular disease has not yet been firmly established in such patients. Therefore, we have examined a possible direct effect of n-acetylcysteine (NAC) supplementation on uraemia-enhanced atherosclerosis in apoE<sup>-/-</sup> mice.

NAC, a thiol containing antioxidant, is currently used for the treatment of several disorders related to oxidative stress such as chronic bronchitis and acetaminophen poisoning. It has also been shown to protect renal function in patients with acute and chronic renal failure [36]. NAC exerts direct and

indirect antioxidant activity due to its sulphhydryl group, and it has particularly potent anti-myeloperoxidase product effects [37, 38]. Moreover, NAC releases cysteine after deacetylation, which in turn increases the formation of reduced glutathione peroxidase (GSH) within the intracellular pool of antioxidant molecules [39]. GSH in turn can react with peroxynitrite to form *S*-nitrosothiols, which may prevent the accumulation of peroxynitrite towards the range of toxic levels and protect against nitrosative stress [40]. GSH represents one of the most important natural antioxidant defense systems that decrease early in the course of CRF and progresses with its degree of severity [41]. NAC successfully reduces plasma malondialdehyde levels [42] and homocysteine concentration [43], and improves pulse pressure and endothelial function in CRF patients [43]. Finally, Tepel *et al.* recently showed that prolonged administration of NAC was able to reduce cardiovascular events in chronic haemodialysis patients [35]. However, whether this positive effect was related to a direct action of NAC on atherosclerosis progression is unknown.

In a second intervention study we showed that the administration of the antioxidant NAC led to a reduction of atheromatous lesion progression in our mouse model of uraemia-enhanced atherosclerosis [44]. This finding lends further support to the contention that NAC treatment has a direct positive impact on uraemia-enhanced atherosclerosis. The observed reduction was associated with a decrease of aortic nitrotyrosine expression, further supporting the hypothesis of a link between oxidative stress and atherosclerosis in CRF. This also points to the possible importance of nitrosative-oxidative stress in the accelerated atherosclerosis of CRF apoE<sup>-/-</sup> mice. In line with this contention, Xia *et al.* showed that NAC significantly decreased plasma levels of nitrate and nitrite, that is stable metabolites of NO, and reduced endothelial NO synthase protein expression in the heart and in aortic and mesenteric artery tissues of rats with streptozotocin-induced diabetes (D) [45]. Similarly, NAC increased nitric oxide and blunted the nitric oxide reduction caused by cyclosporine A, with no effect on iNOS in rat renal artery cultured cells [46]. Finally, our finding could serve as a pathogenetic explanation for the above-mentioned beneficial effect of NAC supplementation on improved cardiovascular outcome in dialysis patients [35].

### Conclusion

At present, the excessive cardiovascular mortality in CRF patients continues to represent an unmet challenge in cardiovascular medicine, and one of the major issues is a better understanding of the process of vascular calcification. Therefore, different approaches to reduce premature atherosclerosis and vascular calcification in such patients will have to be tested with respect to efficacy and practicability.



The precise mechanism of cardiovascular disease in uraemic patients remains unknown, but it is likely that a host of different factors contribute to the process. Thus, it is probably overly simplistic to implicate and target a single factor as the most important pathogenic mechanism in the development of cardiovascular calcification. Detection of risk factors for cardiovascular disease (both traditional and kidney disease related) in the early stages of renal function impairment may be necessary to achieve a significant impact on outcome.

A major limiting factor in uraemia-accelerated vascular calcification has been the lack of appropriate animal models. We propose that the CRF apoE<sup>-/-</sup> mouse, together with the uraemic LDLR<sup>-/-</sup> mouse, provides a suitable model to analyse the different molecular and cellular mechanisms responsible for this serious complication of chronic kidney disease. Moreover, it may provide a useful tool for the evaluation of new therapeutic strategies aimed at reducing the accelerated atherosclerosis and vascular calcification associated with uraemia.

Finally, large-scale intervention studies in patients with hard end-stages are urgently needed to determine whether therapeutic measures aimed at preventing cardiovascular calcification will translate into an improvement in long-term cardiovascular mortality. As our knowledge of the mechanisms of CVD in chronic kidney disease continues to expand, additional targets for intervention will be identified, and it is likely that a multifaceted therapeutic approach will be required to achieve a substantial reduction in the cardiovascular mortality of dialysis patients. Our model will be a useful and helpful model in understanding these complex interactions, and for testing the multi-strategies to prevent and treat cardiovascular disease.

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### Резиме

## АТЕРОСКЛЕРОЗА И ВАСКУЛАРНА КАЛЦИФИКАЦИЈА ВО УРЕМИЈА – НОВ ЕКСПЕРИМЕНТАЛЕН МОДЕЛ

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Кардиоваскуларната болест (КВБ) е најчеста причина за зголемиот морбидитет и морталитет кај пациенти со хронична бубрежна инсуфициенција (ХБИ). Забрзаната калцифицирачка атеросклероза, медијалната калцификација и кардијалните валвуларни калцификации се главните причини за појава на КВБ кај оваа популација. Механизмите со кои уремијата ги предизвикува и ги потенцира васкуларните калцификации, како и врската помеѓу неа и процесот на атеросклерозата се непознати.

*Експериментален модел:* Аполипопротеин Е дефицитарниот (АПО Е<sup>-/-</sup>) глушец е етаблиран модел за спонтан развој на атеросклероза. Експериментална уремија хируршки е индуцирана со кортикална електрокоагулација на едниот бубрег и нефректомија контралатерално по две недели, со цел да се испита појавата на акцелерирана атеросклероза, степенот и видот на васкуларните калцификации, како и факторите вовлечени во овие процеси. Воедно се испитуваат соодветни терапевтски мерки. Сите резултати се

споредувани со група на неуремични глумци. Резултатите потврдија дека атеросклеротичните лезии во торакалната аорта се сигнификантно поголеми кај уремичните АПО E<sup>-/-</sup> глумци во споредба со контролите. Релативната зона на васкуларна калцификација и во интимата и во медијата на аортата беше драстично поголема кај уремичните глумци отколку кај контролите. Зголемената атеросклероза беше асоцирана со зголемен стеининг на нитротирозин индицирајќи зголемен васкуларен оксидативен стрес како и зголемен процент на колаген во плаките, индицирајќи структурно ремоделирање на истите. Од друга страна, третманот со н-ацетил цистеин (НАЦ) ја стопира рапидната прогресија на атеросклеротичните лезии кај уремичните глумци во споредба со третманот со плацебо. НАЦ-третманот беше асоциран со намалување на нитротирозин стеинингот, но без промена на степенот на макрофагна инфилтрација во плаките. Третманот со севеламер хидрохлорид не само што ја намали васкуларната калцификација тука ја намали и прогресијата на атеросклерозата кај уремичните АПО E<sup>-/-</sup> глумци. Двата терапевтски пристапи беа асоцирани со намалување на васкуларниот оксидативен стрес, но не и со намалување на вкупниот холестерол. Овој експериментален модел би можел да биде корисен за тестирање на други терапевтски стратегии со цел намалување на забрзаната атеросклероза и васкуларна калцификација во состојба на уремија.

**Клучни зборови:** атеросклероза, васкуларна калцификација, хронична бубрежна инсуфициенција, оксидативен стрес, глушец.

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