Lanthanum carbonate, like sevelamer-HCl, retards the progression of vascular calcification and atherosclerosis in uremic apolipoprotein E-deficient mice

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Abstract

Background. Atherosclerosis and vascular calcification (VC) progression in chronic kidney disease is favored by disturbances of mineral metabolism. We compared the effect of phosphate binder lanthanum (La) carbonate with sevelamer-HCl on atherosclerosis, VC and bone structure and function in mice with chronic renal failure (CRF).

Methods. Apolipoprotein E-deficient (apoE−/−) mice were randomized to one non-CRF and three CRF groups, fed with standard diet (one non-CRF and one CRF) or diet supplemented with either 3% lanthanum carbonate (La3%) or 3% sevelamer-HCl (Sev3%).

Results. Both La3% and Sev3% supplemented CRF mice displayed a decrease of serum phosphorus, calcification at both intimal and medial aortic sites and atherosclerosis. This was associated with a reduction of plaque Type I collagen expression by both binders and of positive nitrotyrosine staining in response to sevelamer-HCl only. Increased mineral apposition and bone formation rates in unsupplemented CRF mice were reduced by Sev3% but not by La3%.

Conclusions. The beneficial effects of La carbonate and sevelamer-HCl on the progression of VC and atherosclerosis in CRF mice could be mainly due to a decrease in phosphate retention and likewise a reduction of arterial Type I collagen expression. The effect of La carbonate differed from that of sevelamer-HCl in that it did not appear to exert its vascular effects via changes in oxidative stress or bone remodeling in the present model.

Keywords: apoE−/− mouse; atherosclerosis; bone histomorphometry; CRF; vascular calcification

Introduction

Patients with chronic kidney disease (CKD) generally suffer from a variety of complications, including accelerated cardiovascular disease. The latter is the most common cause of morbidity and mortality in these patients. It is due to numerous causes, including atherosclerosis, arterial stiffening and calcification [1]. The vascular disease is favored by classical risk factors such as age, diabetes, hypertension and smoking. In addition, several other disturbances, which are more specifically associated with the uremic state, also play an important role in the pathogenesis of the rapidly progressing lesions of the arterial wall. Among these is the mineral and bone disorder associated with chronic kidney disease (CKD–MBD), as reflected by serum biochemical changes including calcium, phosphorus, parathyroid hormone (PTH), vitamin D metabolites and fibroblast growth factor 23 (FGF 23), as well as by abnormalities of bone structure and function. The latter are the result of mineral and endocrine disturbances that determine the different types of renal osteodystrophy [2, 3]. Recent reports point to an interaction between mineral metabolism imbalance, vessel wall changes and altered bone turnover [4–6]. This has led to the creation of the acronym CKD–MBD.

It is important to optimize therapeutic approaches aimed at controlling this disorder. The introduction of novel phosphate binders into clinical practice allows the control of hyperphosphatemia in the absence of calcium or aluminium overload. To date, it is widely accepted that an adequate control of serum phosphorus is important in the clinical management of patients with CKD. Possible measures include dietary phosphate restriction, use of oral phosphate
binders, control of secondary hyperparathyroidism and adequate dialysis therapy in patients with CKD Stage 5D. Two reports have shown that the calcium-free phosphate binder sevelamer-HCl is able to slow the rapid progression of arterial calcification in chronic dialysis patients better than calcium carbonate/calcium acetate [7], despite similar control of hyperphosphatemia. This superiority, although subject to ongoing discussion, has been ascribed to properties other than phosphate binding alone [8].

Lanthanum carbonate, which has been introduced into clinical practice more recently, has been found to be as effective as calcium carbonate and sevelamer-HCl in decreasing serum phosphorus at equivalent doses, both in laboratory animals with chronic renal failure (CRF) and in patients with end-stage renal disease (ESRD) [9]. However, experience with the effect of lanthanum carbonate on vascular calcification (VC) in patients with CKD is still lacking. Therefore, we have undertaken the present experimental study, using our model of apolipoprotein E-deficient (apoE−/−) mice with superimposed CRF and accelerated arterial calcification and atherosclerosis [10]. We have previously shown in this model that other phosphorus-lowering medications including sevelamer-HCl [11] and the calcimimetic R568 [12] were able to attenuate these CRF-induced vascular changes. We now have undertaken to compare in this apoE−/− CRF mouse model the effects of lanthanum carbonate to those of sevelamer-HCl on the development of arterial wall calcifications and atherosclerosis and also on bone structure and turnover.

Materials and methods

Animals, experimental procedure and diet

Homozygous apoE−/− female mice were initially purchased from Charles River Breeding Laboratories (Wilmington, MA) and subsequently bred in the animal facility of Necker Medical School, Paris. All procedures were in accordance with National Institutes of Health (NIH) guidelines for the care and use of experimental animals (NIH publication No. 85-23). The mice were housed in polycarbonate cages, five mice per cage, in a pathogen-free temperature-controlled (25°C) facility with a strict 12-h light/dark cycle and free access to powder diet and water. The diet (2918; Harlan Teklad Global Diet, Harlan, UK) contained 18.9% protein, 6% fat, 1.01% calcium, 0.65% phosphorus, with calcium/phosphorus ratio of 1.55 and 1 IU/g vitamin D3. This is a regular mouse diet, which is widely used in animal facilities and whose phosphate content is not considered to be high.

We used a two-step procedure to create CRF [10, 11, 13] using 8-week-old female apoE−/− mice. Briefly, at the age of 8 weeks, we applied cortical electrocautery to the right kidney and performed left nephrectomy 2 weeks later. Blood samples were taken 2 weeks after nephrectomy. At 12 weeks of age, all apoE−/− mice with established CRF, as confirmed by serum urea levels >20 mmol/L (versus serum urea concentration of 7–11 mmol/L in control mice), were randomly assigned to three groups, with 15 animals per group. One control CRF mouse group was on standard 2918 diet, whereas the other two CRF mouse groups were allocated to La carbonate 3%-supplemented 2918 Diet (CRF La3%) or sevelamer-HCl 3%-supplemented 2918 Diet (CRF Sev3%). Control 8-week-old female non-CRF apoE−/− mice underwent a two-step procedure sham operation. In order to mark de novo-formed bone tissue, calcine (20 mg/kg) was firstly injected intraperitoneally 7 days before sacrifice and then demeclocycline (50 mg/kg) 2 days before sacrifice. At the end of the 8-week study period, mice were sacrificed at 20 weeks of age, that is 10 weeks after creation of CRF and 8 weeks of experimental diet. Each mouse was anesthetized with ketamine/xylazine (100 mg/kg, 20 mg/kg) and blood was collected via cardiac puncture. Subsequently, through the same puncture, a solution of phosphate-buffered saline was infused with physiological pressure through the heart apex and then the heart and aorta were dissected down to the renal arteries and removed. The heart with the aortic root was separated from more distal aorta for immunohistochemical analysis and quantitative calcification and atherosclerosis assessment, as described previously [10, 11]. The rest of the aorta was used for quantification of atherosclerotic lesions [11, 13, 14]. Finally, the left femur was dissected free of soft tissue and immersed in 70% ethanol.

Serum biochemistry

Serum urea, calcium and phosphorus were measured using a Hitachi 917 autoanalyzer (Roche, Meylan, France) as described previously [14, 15, 16, 17]. Serum intact PTH (PTH) was measured using an ELISA kit from Alpco (Salem, NH). We also determined circulating serum amyloid A (SAA) protein using enzyme immunoassay for mouse serum (ELISA kit MG45182; IBL Hamburg) in order to evaluate the degree of inflammation [11].

Protein profiling by MALDI-TOF mass spectrometry

We used an MB-WCX profiling kit (Magnetic Beads Weak Cation eX-change chromatography; Bruker-Daltonics, Bremen, Germany) designed for enrichment and purification of serum proteins and peptides with positively charged functional groups. Briefly, 5 μL of serum was mixed with 20 μL of conditioned MB-WCX. After incubation and agitation, the MB-WCX samples were washed two times with a solution provided by the vendor. Cation desorption was performed using 5 μL of elution solution. The eluate was mixed with 5 μL of stabilization solution and then diluted 10-fold in ω-cyano-4-hydroxyquinamic acid (Bruker) solution (0.3 g/L in ethanol/acetone v/v, 2:1). The final sample mixture (1.0 μL) was then spotted on the target plate AnchorChip 600 (Bruker) and analyzed by Matrix-Assisted Laser Desorption/Ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) (Autoflex®, Bruker). Ionization was achieved by irradiation with a nitrogen-operating laser at 20 Hz. Ions were accelerated at +20 kV with 170 ns of pulsed ion extraction delay. Spectra were obtained automatically in linear positive mode using FlexControl v2.2 (Bruker). The software tool AutoXecute acquisition control was used for automated data acquisition. The analysis included five initial shots at laser power of 30% prior to acquisition of spectra with 1000 shots at a variable laser power of 25–40%. The spectra obtained were then classified into the four designed groups and analyzed using the dedicated software ClinPro Tools v2.2 (Bruker). This software performs automatic normalization, baseline subtraction, peak detection and recalibration of the spectra. An average spectrum was generated for each group. QuickClassifier algorithm with analysis of variance (ANOVA) was used to find the statistically relevant peaks characteristic of the different groups.

Quantification of atherosclerotic lesions

Aortic atherosclerotic lesion area was assessed using two independent methods: evaluation of aortic root lesion area and thoracic aorta lesion area. Aortic root lesion area was determined from serial 7-μm aortic root sections followed by Oil red O staining and analysis of intimal area as previously described. For the evaluation of atherosclerotic plaque area in thoracic aorta, the entire aorta was opened longitudinally to use ‘en face’ method [11, 14]. Briefly, the aortas were carefully freed of connective and adipose tissue under a dissection microscope, opened longitudinally and stained with Oil red O. Lesion quantification was made by Histolab software (Microvision Instruments, Evry, France) as described previously. The extent of atherosclerosis was expressed as the percentage of surface area of the aorta covered by lesions [11, 14].

Quantitative and qualitative evaluation of aortic calcification

We performed von Kossa staining in 7-μm cryosections of aortic tissue to evaluate calcium-phosphate deposits inside and outside atheromatous plaques in apoE−/− mice. These locations of calcification are supposed to reflect intima and media calcification. The precision and accuracy of this method using semiautomatic measurement software have been reported elsewhere. In a recent personal study, we compared this imaging method with a direct biochemical measurement of aorta calcium content and found a significant correlation between it and calcium–phosphate deposits inside and outside atheromatous plaques, (r = 0.66, P = 0.038 and r = 0.77, P = 0.003, respectively). Data were expressed as the percentage of calcified area to total surface area of either atherosclerotic lesions or vessel area outside atheromatous plaques as described previously [10, 11, 13].

Quantification of Type I collagen content in aortic root lesions

To determine the Type I collagen content in atheromatous lesions, we performed an immunohistochemical analysis using an anti-Type I collagen
La carbonate in uremic apolipoprotein E-deficient mice

antibody [Affinity Purified Anti-Collagen Type I (rabbit) from Rockland, ref No. 600-401-103-0.1 lot # 20288]. Aortic sections were first fixed in room temperature acetone for 10 min and placed in bath of wash buffer. The sections were then preincubated in peroxidase blocking solution (Dako, Glostrup, Denmark) for 5 min before incubation for 15 min at room temperature with Purified Anti-Collagen Type I. Antibodies were used in diluents with background reducing components (Dako). After repeat rinsing with Tris buffer, the sections were treated with peroxi-
dase-labeled streptavidin (Dako) for 15 min followed by reaction with diaminobenzidine/hydrogen peroxidase as chromogen-substrate, which results in a brown-colored precipitate at the antigen site. Negative controls included omission of the primary antibody. At the end, the sections were counterstained with haematoxylin. Image of the best aortic root section per slide with three visible aortic valve leaflets was captured on ×25 magnifi-
cation on a microcomputer equipped with a Sony tube camera. This tissue was quantified by computerized image analysis Histolab software (Microvision Instruments).

Quantification of nitrotyrosine staining in aortic root lesions

The presence in atheromatous lesions of nitrotyrosine, a marker for oxi-
dative stress, was assessed using the immunostaining technique as de-
scribed previously [11, 14].

Bone histomorphometry

Undecalciﬁed femurs were embedded in methyl methacrylate. Five-
micrometer-thick longitudinal sections were cut on a microtome (Poly-
cut S; Leika, Heidelberg, Germany) and stained with toluidine blue (pH 6.4). Static and dynamic parameters of bone structure, formation and resorption were measured at the distal metaphyses (magnification ×250), 195 μm from the growth plate, in a total of 20 ﬁelds using an OsteoMeasure morphometry system (Osteometrics, Atlanta, GA) as described previously [15]. Dynamic bone parameters were obtained from unstained 10 μm sections, which were examined by ﬂuorescent light microscopy (Nikon, Tokyo, Japan). The mineral apposition rate was expressed in micrometers per day, and bone formation rate was expressed per unit of bone surface per day. The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research. Ten to 15 mice per group were analyzed.

Statistical analysis

Results were expressed as mean ± SEM. Differences between groups were analyzed by one- and two-variable ANOVA, Fisher test and χ² test as appropriate. Animals in CRF groups with serum urea <20 mmol/L at sacriﬁce were excluded from the statistical analysis.

Results

Body weight and serum biochemistry

As expected, at the time of sacriﬁce, that is 10 weeks after creation of CRF and 8 weeks of experimental period, serum urea concentrations were signiﬁcantly higher in CRF than in non-CRF mice (Table 1). Mean body weight decreased in CRF mice receiving La3%- or Sev3%-supplemented 2918 diet as compared to non-CRF mice (Table 1). Serum calcium and calcium × phosphorus (CaXP) product were signiﬁcantly increased in apoE−/− CRF mice receiving 2918 diet alone although serum phosphorus was comparable (Table 1). Serum phosphorus and CaXP product were signiﬁcantly reduced by either La3% or Sev3% treatment at the time of sacriﬁce, with no change in serum total calcium (Table 1). Serum iPTH levels were numerically higher in Untreated CRF mice than in non-CRF mice but the difference was not statistically signiﬁcant. CRF mice on La3%- or Sev3%-supplemented 2918 diet had serum iPTH levels comparable to those of non-CRF mice. SAA levels showed no signiﬁcant difference between groups. Mortality was low during the study and similar among CRF groups. Some deaths occurred as a consequence of either surgical manipulation or retro-bulbar sinus blood sampling. There were no deaths in the non-CRF group and no deaths due to CRF within the limits of the observation time.

Quantification of aorta calcification

As expected, CRF mice exhibited a signiﬁcant increase of plaque and non-plaque VC, compared with their control non-CRF littermates (Figure 1A and B). CRF mice exposed to either La3%- or Sev3%-supplemented 2918 diet exhibited a signiﬁcant similar decrease of plaque and non-plaque VC, compared with their control CRF littermates receiving 2918 diet alone (Figure 1A and B).

Quantification of atherosclerotic lesions

As expected, CRF mice exhibited a signiﬁcant increase of plaque lesion area in longitudinal thoracic aorta, compared with their control non-CRF littermates (Figure 2). CRF mice exposed to either La3%- or Sev3%-supplemented 2918 diet exhibited a signiﬁcant similar decrease of plaque lesion area in longitudinal thoracic aorta, compared with their unsupplemented CRF littermates (Figure 2). There was no difference of the extension of plaque lesions at aortic root sites in non-CRF mice with that of control CRF mice and of CRF mice exposed to either La3%- or Sev3%-supplemented 2918 diet, respectively (data not shown).

Quantification of Type I collagen expression and positive nitrotyrosine staining in aortic root lesions

CRF was associated with an increase of Type I collagen expression in atherosclerotic plaques. Exposure to either La3%- or Sev3%-supplemented 2918 diet led to a decrease of Type I collagen expression in aortic plaques of CRF mice as compared to unsupplemented control CRF animals (Table 2, Figure 3). Positive nitrotyrosine staining in atheromatous lesions was also increased in CRF mice as compared with non-CRF mice. Sev3% supplementation led to a signiﬁcant decrease in this expression. Such an effect was not observed in response to La3% supplementation (Table 2).

ClinProt serum proﬁling

Serum proﬁles, as analyzed by MALDI-TOF MS in the mass range of 1–10 kDa, were determined for each of the four mouse groups. A total of 117 peptides in common to the four groups were detected and compared by ‘Quick Classiﬁer’ algorithm. Among these, 38 peaks were found to be signiﬁcantly different between non-CRF and CRF control/ CRF phosphate binder-supplemented mice. The clustering analyses generated for these four groups are represented by reporting the distribution of the intensity of the two most signiﬁcantly different peaks in each single sample. In these analyses, the two automatically selected peaks were 6118 m/z (P < 0.01) and 1327 m/z (P < 0.001) (Figure 4). These latter peptide peaks were signiﬁcantly modiﬁed by CRF state and at least one peak (i.e. 1327 m/z) was back to non-CRF status level after Sev3% supplementation, whereas both of them remained at CRF status level after La3% supplementation (Figure 4).
Histomorphometric analysis of femoral bone

Table 3 and Figure 5 show that, at the time of sacrifice, CRF apoE−/− mice had higher bone volume (BV/TV), trabecular thickness and trabecular number than control non-CRF mice. CRF apoE−/− mice exposed to either La3% or Sev3% also had higher bone volume (BV/TV), trabecular thickness and trabecular number as compared to control non-CRF mice. This gain in bone mass was associated with an increase in trabecular thickness and a significant decrease in trabecular separation in all CRF mouse groups (Table 3 and Figure 5).

Bone remodeling and mineralization parameters

Osteoblast surface was significantly higher in CRF apoE−/− mice as compared to control non-CRF mice. Bone formation rate and mineral apposition rate were increased in CRF mice compared with control non-CRF mice. Both dynamic parameters were found to be normalized in Sev3% but not in La3%-supplemented CRF mice (Table 3, Figure 5). The state of CRF was also associated with an increase in osteoid thickness, with a further augmentation in La3%-supplemented CRF mice. However, osteoid volume and osteoid surface were not modified by CRF and were comparable in all four groups. Mineralization lag time was decreased in CRF mice on Sev3% and La3% exposure (Table 3).

Adjusted apposition rate (Aj AR), however, was not altered in any of the four groups. Finally, neither CRF nor the phosphate binders modified any of the measured bone resorption parameters (Table 3).

### Table 1. Body weight and serum biochemistry at the time of sacrifice a

<table>
<thead>
<tr>
<th></th>
<th>Non-CRF 2918b</th>
<th>CRF 2918c</th>
<th>CRF 2918+La d</th>
<th>CRF 2918+Sev e</th>
<th>Global P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g) (n, 10, 15, 13, 15)</td>
<td>22.6 ± 0.7</td>
<td>21.2 ± 0.3</td>
<td>20 ± 0.6b</td>
<td>20.2 ± 0.4b</td>
<td>NS</td>
</tr>
<tr>
<td>Urea (mmol/L) (n, 10, 13, 11, 13)</td>
<td>8.5 ± 0.3</td>
<td>32.4 ± 2.5b</td>
<td>32.9 ± 2.1b</td>
<td>34.6 ± 3.1b</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Calcium (mmol/L) (n, 9, 13, 10, 12)</td>
<td>2.5 ± 0.02</td>
<td>2.7 ± 0.05b</td>
<td>2.8 ± 0.01b</td>
<td>2.8 ± 0.01b</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Phosphorus (mmol/L) (n, 9, 11, 10, 11)</td>
<td>2.4 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>1.9 ± 0.1bc</td>
<td>2.04 ± 0.1bc</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ca × P (mmol/L²) (n, 9, 11, 10, 11)</td>
<td>6.03 ± 0.33</td>
<td>6.50 ± 0.36b</td>
<td>5.10 ± 0.32bc</td>
<td>5.60 ± 0.27bc</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>iPTH (pg/mL) (n, 10, 13, 13, 15)</td>
<td>45.3 ± 6.2</td>
<td>124.6 ± 73.9</td>
<td>44.7 ± 14.3</td>
<td>40.5 ± 8.7</td>
<td>NS</td>
</tr>
<tr>
<td>SAA (µg/mL) (n, 10, 15, 13, 15)</td>
<td>63.8 ± 22.2</td>
<td>80.3 ± 20.4</td>
<td>95.5 ± 23.6</td>
<td>49.4 ± 15.6</td>
<td>NS</td>
</tr>
</tbody>
</table>

aValues are expressed as means ± SEM. n stands for number of mice. Data were analyzed by ANOVA; P stands for global P of ANOVA; superscript letters show significant difference to corresponding group (P < 0.05). CRF indicates chronic renal failure.

bMouse groups: non CRF 2918, apo E−/− non CRF group receiving 2918 control diet.

cMouse groups: CRF 2918, apo E−/− CRF group receiving 2918 control diet.

dMouse groups: CRF 2918+La, apo E−/− CRF group receiving La carbonate 3% in the 2918 diet.

eMouse groups: CRF 2918+Sev, apo E−/− CRF group receiving sevelamer-HCl 3% in the 2918 diet.
Discussion

Our study led to three major findings. First, both La carbonate and sevelamer-HCl administration decreased serum phosphorus and retarded the progression of VC and atherosclerosis in uremic apoE/−/− mice. These effects of the two phosphate binders were comparable. Second, a similar reduction of CRF-induced aortic Type I collagen expression in atherosclerotic plaques was observed with either binder. However, La carbonate did not reduce aortic nitrotyrosine staining, in contrast to sevelamer-HCl. Third, bone volume and turnover of apoE/−/− mice, which were increased in the state of CRF, remained unchanged under lanthanum carbonate treatment, although bone formation rate and mineral apposition rate were normalized by sevelamer-HCl.

The present study shows for the first time that the phosphate binder La carbonate is capable of preventing both uremia-enhanced VC and atherosclerosis in apoE/−/− mice. These effects are comparable to those of sevelamer, as previously reported by us for sevelamer-HCl [11] and by Mathew et al. for sevelamer carbonate [7]. They were observed along with a decrease of serum phosphorus and Ca×P product, like in sevelamer-HCl-exposed mice. We must point out that in the present experimental series, serum phosphate was not increased in CRF mice compared with non-CRF mice although we did observe slight degrees of hyperphosphatemia in CRF mice of previous series using the same experimental model [10–12]. Possible explanations for this apparent discrepancy are different degrees of CRF severity and different types of dietary phosphate and protein sources not indicated by the animal food provider since the protein source may play an important role [18]. This is reminiscent of patients with CKD Stages 3–5, with highly variable degrees of hyperphosphatemia from one patient to the other.

Phosphate retention and hyperphosphatemia associated with CRF have long been known to play a major role in
promoting VC. In recent years, the underlying mechanisms have been characterized at the cellular and molecular level, including transformation of vascular smooth muscle cells (VSMCs) to an osteochondrogenic phenotype in response to increases in phosphorus concentration in the culture medium [16, 17]. Therefore, the beneficial effect of La carbonate on VC could be explained, like that of sevelamer, by improved control of phosphate retention. The fact that either binder was able to reduce the progression of both aortic calcification and atherosclerosis to a similar extent could indicate that for this beneficial effect to occur phosphate binding is more important than other, phosphate binding-independent actions. Recently, Neven et al. [19] reached similar conclusions in rats, showing that 2% La carbonate reduced VC in the adenine model of CRF. However, other mechanisms of actions for the observed vascular effects of La carbonate have to be considered. They include a direct calcium channel blocking effect of the La3+ ion [20], similar to the action of classic calcium channel blocking agents [21, 22], and a direct or indirect activation of the calcium-sensing receptor [23]. The latter effect has been observed at La concentrations in the nanomolar range, compatible with serum La levels of uraemic animals or dialysis patients treated with La carbonate [19, 24]. The hypothesis of a direct activation of the calcium-sensing receptor would be compatible with our recent observation of an inhibition of VSMC calcification in vitro by the calcimimetic R568 [12].

Fig. 4. Serum profiling by MALDI-TOF MS and spectra analysis by ClinPro Tools software for peptides. (A) Cluster analysis revealing the two more significant peptides: 1327 m/z and 6118 m/z. (B) peptide profiles for 1327 m/z and (C) peptide profiles for 6118 m/z. Mouse groups as in legend to Figure 1; apo E−/− non-CRF group, red colour; apo E−/− CRF group, green colour; apo E−/− CRF group on La3%, blue colour and apo E−/− CRF group on Sev3%, yellow colour. Data were analyzed by ANOVA (means ± SDs), global ANOVA P < 0.05.
Other indirect or direct effects on VSMC are potential mechanisms worth considering. They could consist in an interference with VSMC transformation into osteoblast-like cells and/or the synthesis or activity of local or circulating calcification inhibitors such as matrix Gla-protein (MGP), osteoprotegerin (OPG), osteopontin (OPN), fetuin-A, FGF23 and klotho [25]. Thus, La acetate has recently been shown to reduce aortic messenger RNA expression of OPN and the Na/Pi co-transporter Pit 1 and to increase aortic gamma-carboxylated Gla-protein expression in rats with vitamin D3/nicotine-induced arterial calcification [26], in addition to lowering serum phosphorus and alkaline phosphatases. Note that in this study, the effects of La acetate, not La carbonate, have been tested.

An attenuation of oxidative stress and/or inflammation is yet another potential mechanism of action. However, we did not observe a decrease in aortic nitrotyrosine staining in response to La carbonate treatment, in contrast to the effect of sevelamer-HCl, as already reported by us previously [11]. We also did not observe a reduction in serum SAA levels, a systemic marker of inflammation, with either La carbonate or sevelamer-HCl treatment. Our finding of increased Type I collagen expression in the aorta of CRF mice and its inhibition by both La carbonate and sevelamer-HCl, together with less aortic calcification, represents as yet another potential mechanism of action. It is in line with the observation by Murshed et al. [27] in bone that for mineralization to occur not only is phosphate required but also Type I collagen. It remains to be explained how the two calcium-free phosphate binders bring about the observed decrease in Type I collagen content. However, it is possible that both drugs, by reducing serum phosphorus levels, were able to block VSMC transdifferentiation to osteoblast-like cells capable to produce osteogenic matrix including Type I collagen.

Our finding of increased Type I collagen expression in the aorta of CRF mice and its inhibition by both La carbonate and sevelamer-HCl, together with less aortic calcification, represents as yet another potential mechanism of action. It is in line with the observation by Murshed et al. [27] in bone that for mineralization to occur not only is phosphate required but also Type I collagen. It remains to be explained how the two calcium-free phosphate binders bring about the observed decrease in Type I collagen content. However, it is possible that both drugs, by reducing serum phosphorus levels, were able to block VSMC transdifferentiation to osteoblast-like cells capable to produce osteogenic matrix including Type I collagen.

Exploring still other potential mechanisms, we proceeded to proteomic analysis of mouse sera in the present study. We found several peptides to be significantly modified by CRF. The height of one of the identified peptide peaks was significantly reduced toward normal by sevelamer-HCl (i.e. 1327 m/z). In contrast, no peptide peak was modified by La carbonate. This preliminary observation only suggests a global effect. Peptide peak sequencing is underway. Together with the attenuation of oxidative stress by sevelamer, this finding might support the hypothesis that La carbonate is a pure phosphate binder, whereas sevelamer exerts other actions such as cholesterol lowering [8].

Of note, not all phosphate binders decrease both aortic calcification and atherosclerosis in this CRF model. Thus, calcium carbonate did not affect atheroma progression, although it reduced arterial calcification [13]. One important difference with respect to calcium-free binders is the hypercalcaemic effect of calcium-containing binders.

### Table 3. Bone histomorphometry parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non-CRF 2918b (n = 9–10)</th>
<th>CRF 2918c (n = 12–13)</th>
<th>CRF 2918 + La()d (n = 11–12)</th>
<th>CRF2918 + Sev()e (n = 10–13)</th>
<th>Global P</th>
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<tbody>
<tr>
<td><strong>Structure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Bone volume (BV/TV, %)</td>
<td>5.79 ± 0.47</td>
<td>8.42 ± 0.50()b</td>
<td>9.53 ± 0.81()b</td>
<td>8.9 ± 0.67()b</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Trabecular thickness (Th.Th, µm)</td>
<td>27.54 ± 3.96</td>
<td>32.63 ± 3.97()b</td>
<td>33.23 ± 4.38()b</td>
<td>32.78 ± 3.03()b</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Trabecular number (Tb.N/mm)</td>
<td>2.04 ± 0.38</td>
<td>2.55 ± 0.43()b</td>
<td>2.90 ± 0.68()b</td>
<td>2.71 ± 0.61()b</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Trabecular separation (Th.Sp, µm)</td>
<td>456.01 ± 73</td>
<td>353 ± 52.84()b</td>
<td>329.35 ± 25()b</td>
<td>336.3 ± 66.3()b</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Bone formation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteoblast surface (Ob.S/BS, %)</td>
<td>9.94 ± 6.12</td>
<td>17.83 ± 8.17()b</td>
<td>17.55 ± 6.81()b</td>
<td>11.9 ± 7.83()b</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Mineral apposition rate (MAR, µm/day)</td>
<td>0.52 ± 0.17</td>
<td>0.75 ± 0.17()b</td>
<td>0.82 ± 0.26()b</td>
<td>0.58 ± 0.19()d</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mineralizing surface (MS/BS, %)</td>
<td>9.74 ± 3.72</td>
<td>13 ± 4.93</td>
<td>11.06 ± 3.69</td>
<td>9.48 ± 3.73</td>
<td>NS</td>
</tr>
<tr>
<td>Bone formation rate (BFR, µm3/µm3/day)</td>
<td>0.053 ± 0.034</td>
<td>0.104 ± 0.046()b</td>
<td>0.105 ± 0.038()b</td>
<td>0.061 ± 0.032()d</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Osteoid volume (OV/BV, %)</td>
<td>1.02 ± 0.7</td>
<td>1.83 ± 0.81</td>
<td>2.21 ± 1.027</td>
<td>1.82 ± 0.39</td>
<td>NS</td>
</tr>
<tr>
<td>Osteoid surface (OS/BS, %)</td>
<td>12.68 ± 7.4</td>
<td>22.4 ± 9.72</td>
<td>21.1 ± 7.11</td>
<td>17.15 ± 10.9</td>
<td>0.067</td>
</tr>
<tr>
<td>Osteoid thickness (O.Th, %)</td>
<td>1.02 ± 0.35</td>
<td>1.40 ± 0.2()h</td>
<td>1.7 ± 0.37()h</td>
<td>1.61 ± 0.41()h</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Adjusted apposition rate (AJ AR, µm/day)</td>
<td>0.5 ± 0.21</td>
<td>0.49 ± 0.29</td>
<td>0.48 ± 0.18</td>
<td>0.41 ± 0.26</td>
<td>NS</td>
</tr>
<tr>
<td>Mineralization lag time (MLT, days)</td>
<td>2.4 ± 1.26</td>
<td>3.79 ± 1.26</td>
<td>4.08 ± 1.73()b</td>
<td>4.72 ± 2.35()b</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Bone resorption</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eroded surface (ES/BS, %)</td>
<td>6.96 ± 2.96</td>
<td>6.66 ± 4.16</td>
<td>6.1 ± 0.38</td>
<td>5.35 ± 0.69</td>
<td>NS</td>
</tr>
<tr>
<td>Osteoclast surface (OC/BS, %)</td>
<td>1.46 ± 0.60</td>
<td>2.31 ± 1.19</td>
<td>1.61 ± 0.59</td>
<td>1.56 ± 1.03</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Mouse groups as in Table 1. Data were analyzed by ANOVA (means ± SEMs); P stands for global P of ANOVA. CRF indicates chronic renal failure; superscript letters show significant difference to corresponding group (P < 0.05). n stands for number of mice.*
La carbonate exhibited no changes in static or dynamic bone parameters. Similarly, sevelamer-HCl treated mice displayed no change in static bone parameters. However, this drug induced normalization of dynamic parameters, namely bone formation and mineralization. To explain the absence of an improvement in structural parameters with sevelamer-HCl, we hypothesize that the observation time period was too short to allow for the detection of bone structure changes. Of note, La carbonate also did not modify bone structure [34] or dynamic bone parameters [35] in chronic hemodialysis patients. However, the percentage of patients with improvement of bone formation rate or activation frequency was significantly greater after 1 year of La carbonate treatment than after 1 year of standard therapy [35]. Sevelamer-HCl induced an increase in bone formation rate but no overall change in the incidence of dynamic bone in hemodialysis patients compared with calcium-containing phosphate binder [36]. It also prevented trabecular bone loss in such patients [37]. In line with these findings, Sampath et al. [38] have shown recently that sevelamer is capable of restoring bone volume and improving bone microarchitecture and strength in aged ovariectomized rats with normal kidney function, possibly by modulating oxidative stress-induced systemic inflammation. Whether this observation in animal models is relevant and transposable to CKD patients remains to be evaluated.

In conclusion, both La carbonate and sevelamer-HCl retard the progression of VC and atherosclerosis in uremic apoE−/− mice. These effects could be mainly explained by the phosphorus lowering action of both binders. In addition, whether direct local effects of absorbed La also play a role remains to be seen. The often claimed inverse association between changes in bone and vascular structure, which was not apparent in the present study, requires further exploration.

References
1. Drueke TB, Massy ZA. Atherosclerosis in CKD: differences from the general population. Nat Rev Nephrol 2010; 6: 723–735
5. Mathew S, Lund RJ, Strebeck F et al. Reversal of the adynamic bone disorder and decreased vascular calcification in chronic kidney
La carbonate in uremic apolipoprotein E-deficient mice

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