Daily peritoneal administration of sodium pyrophosphate in a dialysis solution prevents the development of vascular calcification in a mouse model of uraemia

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Abstract

Background. The high rate of cardiovascular mortality in patients with end-stage renal disease (ESRD) is a significant barrier to improved life expectancy. Unique in this population is the marked development and aggressive worsening of vascular calcification (VC). Pyrophosphate (PPi), an endogenous molecule, appears to naturally inhibit soft tissue calcification, but may be depressed in chronic kidney disease (CKD) and ESRD. Although once thought to be a promising therapeutic, PPi’s very short half-life in circulation curtailed earlier studies. We tested the possibility that a slow, continuous entry of PPi into the circulation and prevention of VC might be achieved by daily peritoneal dialysis (PD).

Methods. Pharmacokinetic studies were first carried out in rats with renal impairment resulting from a 5/6 nephrectomy. Efficacy studies were then performed in the apolipoprotein E gene knockout mouse model overlaid with CKD. PPi was delivered by means of a permanent peritoneal catheter in a solution simulating PD, but without the timed removal of spent dialysate. von Kossa’s staining followed by semiquantitative morphological image processing, with separation of inside (intimal) and outside (presumed medial) lesions, was used to determine aortic root calcification.

Results. In comparison to an intravenous bolus, delivery of PPi in a PD solution resulted in a slower, extended delivery over >4 h. Next, the efficacy studies showed that a 6-day/week PD-simulated administration of PPi resulted in a dose-dependent inhibition of aortic calcification in both intimal and medial lesions. A dose-response effect on total aortic calcification was also documented, with a full inhibition seen at the highest dose. A limited peritoneal catheter-
related inflammation was observed, as expected, and included the placebo-treated control groups. This inflammatory response could have masked a lower level PPI-specific adverse effect, but none was observed.

Conclusions. Our findings suggest potential for PPI, administered during PD, to prevent the development of VC and to potentially extend the life of ESRD patients.

Keywords: cardiovascular disease; peritoneal dialysis; pyrophosphate; therapeutic; vascular calcification

Introduction

The average life expectancy of end-stage renal disease (ESRD) patients is only 4–5 years. In spite of major improvements in dialysis techniques over the last decades, the prognosis of the patients remains similar to that of many forms of metastatic cancer. The morbidity and mortality associated with cardiovascular disease which complicates ESRD is the single most important limitation to improved quality of life and survival with, depending on the age group, a 4–30 times higher relative risk of dying compared to an age-adjusted non-ESRD population[1]. The reason for this dramatic difference is incompletely understood but is likely to be multifactorial.

In the search for intermediate surrogate outcome factors, vascular calcification (VC) is considered by many as a valuable parameter thought to reflect mortality risk. VC mainly occurs in large arterial vessels, such as the aorta, as well as in coronary arteries. The degree of VC is an independent and strong predictor of death in ESRD [2, 3]. In contrast to the non-ESRD population, where calcification of the vessel wall mainly occurs later in life in the intima, VC in ESRD patients may occur in the intima, the media or in both. Whereas intima calcification reflects atheromatous lesions, media calcification (also called Mönckeberg’s calcification) appears mainly linked to degenerative changes within the smooth muscle and elastic fiber layer of the vessel. It is generally associated with increased vessel stiffness. The medial type calcification appears to be primarily made up of hydroxapatite. It is at present thought to result from an active process mediated first by pathologic vascular smooth muscle cells (VSMC) that are subsequently transformed into bone-forming osteoblast-like cells [4, 5]. This change in phenotype and function appears to be driven by the uraemic environment, often in association with inflammation, and is influenced by a number of chronic kidney disease (CKD)-related factors including hypocalcaemia and hyperphosphataemia [5–7]. Although the progression of VC appears to be accelerated in most dialysis patients [8, 9], current studies indicate that this process begins during earlier stages of CKD [10]. Currently available treatments have only limited effects on this process, and there are no drugs available that have been specifically designed and proven to prevent or reverse VC.

Studies proving a causal link between VC and the increase in mortality associated with ESRD are currently lacking. However, it is reasonable to assume that VC and the associated vessel stiffness are important mediators of the frequent increase in systemic blood pressure, left ventricular mass and cardiomyopathy in ESRD patients and which have all been shown to be directly involved in mortality risk. This assumption is supported by a wealth of literature showing a close association between severe VC and mortality in ESRD [2, 3].

Fleisch et al. [11] established almost 50 years ago that the molecule pyrophosphate (PPI), a critical element in bone mineralization, is key to inhibiting the process of hydroxyapatite formation and calcification in soft tissues, whereas it is not normally active in bone due to its rapid degradation by the enzyme tissue non-specific alkaline phosphatase (TNAP) [12]. They and others have subsequently demonstrated an inhibitory role for this endogenous molecule in tissue and organ culture models of VC [11, 13]. This role is further supported by the finding that patients with inherited mutations that inactivate the enzyme ecto-nucleotide pyrophosphate/phosphodiesterase 1, which generates PPI, develop a condition known as idiopathic infantile arterial calcification [14]. In this rare disease, calcification in the form of hydroxyapatite deposition occurs in large muscular arteries and is associated with a stenosing, fibroproliferative medial smooth muscle cell-mediated process. Death typically occurs in the first year, generally as a result of ischaemic cardiomyopathy and other complications of obstructive arteriopathy including renal artery stenosis.

Despite these striking observations, the potential usefulness of PPI administration for the treatment or prevention of VC has not been clearly demonstrated or actively pursued. This is presumably because the extremely short serum half-life of PPI, as reported 30 years ago in dogs, was thought to be a limitation to its clinical usefulness [15]. Instead, longer active PPI analogues such as the bisphosphonates, were deemed necessary to develop. While a number of these analogues are currently used for the treatment of high turnover osteoporosis, their use in ESRD is contraindicated since the patients cannot excrete the drug and, unlike PPI, they are not broken down by the ubiquitous circulating PPI degradative enzymes such as alkaline phosphatase. Their accumulation can lead to softening of bone due to reduced bone turnover [16].

We reasoned that because small molecules present in the peritoneal dialysis (PD) solution are slowly transported into the plasma during the dwell time required for PD, this could provide an opportunity for a slow, daily or multiple-daily administration of PPI, potentially creating an ideal platform for a consistent regulated delivery of this molecule. Previous studies on VC in ESRD have been hampered by the lack of suitable models. Most experimental models produce exclusively intimal calcification with, either no or only inconsistent medial calcification, some animals demonstrating no VC at all. We have recently described a model whereby the apolipoprotein E gene knockout (apoE−/−) mouse is overlaid with a partial nephrectomy, thereby producing chronic kidney failure and aggravating the hypercholesterolaemia [17]. Under these conditions, aortic root lesions consisting of calcified atheromatous plaques (intimal calcification) and in addition calcified lesions outside of the plaques (medial calcification) are consistently produced. We therefore used this model to test the hypothesis that PPI, when delivered on a daily basis in a PD solution designed to maintain stability of the molecule, may prevent the progression of VC associated with the uraemic state.
Dialysis solution PPI prevents VC

Materials and methods

Preparation of peritoneal solutions

The PPI-containing solution was prepared and sterilized first in a two-chamber configuration, slightly modified from that described for Physio-neal with 4.3% w/v dextrose (Baxter Healthcare Corporation, Round Lake, IL). The PPI was contained in the buffered chamber, and when combined with the glucose chamber prior to administration, it produced a pH of 7.4 ± 0.2. The stability of PPI, in the single part and upon mixing, was determined at the time of production, as well as through the time period of administration. The concentration of PPI remained stable (± 6%) during the experimental study period. Control placebo solutions were prepared in an identical manner but lacked the added PPI.

Animals and experimental in life procedures

Pharmacokinetic study in rats. A study was carried out in male Sprague Dawley® rats, obtained from Charles River Laboratories (Portage, MI) and maintained by Preclinical Resources (Baxter Healthcare Corporation) to determine plasma bioavailability of 135P-labelled PPI ([135P]PPI) after intravenous (IV) or intraperitoneal (IP) administration. Partial nephrectomy was performed by the vendor, involving surgical removal of one kidney followed by a second removal of the other kidney. The experiment was initiated 4 weeks after surgery, at which time the rats had reached ~55% renal impairment, as determined by clinical chemistry methods. Animals were maintained on a standard rodent meal (Harlan Teklad Global Diets 2018C) and drinking water ad libitum, except during dosing and sample collection. Rats received a single IV dose of saline (2.25 mM PPI, 4 mL/kg) or an IP injection of a buffered PD solution (150 μM PPI, 60 mL/kg). In both cases, the total dose was 2 mg/kg PPI, containing 50 μCi 32P-PPI. Plasma was collected at various time intervals through 24 h post-dose to determine the pharmacokinetics of PPI. Samples were tested by two methods utilizing liquid chromatography for total radioactive counts with additional analysis by high-performance liquid chromatography with radioactive detection for separation of PPI from Pi and other phosphate-containing compounds. The limit of detection of PPI in plasma in our assay was <0.04 μg/mL or <0.2 μM. Basic pharmacokinetic parameters were determined based upon concentrations of PPI using a non-compartmental model.

VC study in apoE−/− mice. Homozygous apolipoprotein E knockout (apoE−/−) mice were purchased from Charles Rivers Breeding Laboratories (Charles Rivers, Lyon, France). The mice were housed at the animal facility of Amiens Medical and Pharmacology School in polycarbonate cages in a pathogen-free, temperature-controlled (25°C) room, with a strict 12-h light/dark cycle and free access to lab chow and water. All animals were handled in accordance with French legislation and the protocol was approved by an institutional animal care committee. We created CKD in 8-week-old female apoE−/− mice and assigned them randomly to four groups as follows:

1. non-CKD—apoE−/− animals treated with dialysis solution alone (control group, six mice),
2. CKD/ apoE−/− animals treated with dialysis solution alone (with no PPIs—CKD-placebo group, eight mice),
3. CKD/ apoE−/− animals treated by low dose (30 μM or 0.33 mg/kg/day) PPI in dialysis solution (CKD-PPI low dose group, eight mice),
4. CKD/ apoE−/− animals treated high dose (150 μM or 1.66 mg/kg/day) PPI in dialysis solution (CKD-PPI high dose group, eight mice).

We used a two-step procedure to create CKD in the mice at 10 weeks of age. Briefly, at the age of 8 weeks, cortical electrocauterization was applied to the right kidney through a 2-cm flank incision, and contralateral total nephrectomy was performed through a similar incision 2 weeks later. Special care was taken to avoid damage to the adrenal glands. Other mice underwent a two-step procedure of sham operations with decapsulation of both kidneys, with a 14-day interval between the two operations. Blood samples were collected 2 weeks after nephrectomy, and IP catheters implanted at this time. Animals of the CKD group with urea levels >20 mM (normal mouse serum urea concentration, <12 mM) were subsequently randomized to three CKD subgroups: two CKD subgroups were treated with PPI at two different doses, (one IP injection per day for 6 days), and one CKD subgroup received the dialysis solution alone for a time period of 8 weeks. At the end of the study, each mouse was anesthetized with IP injection of ketamine/xylazine (100/20 mg/kg) and sacrificed. Whole blood was collected via cardiac puncture. Subsequently, through the same puncture, a solution of 4% phosphate-buffered saline was perfused with physiological pressure. The heart with the aortic root was then separated from ascending aorta, as reported previously [18]. Cryosections of the aortic root tissue were used for quantification of VC and of atherosclerotic lesions. The longitudinal aorta was stored at −80°C and subsequently used for biochemical quantification of calcium content.

Peritoneal catheter implant and use in mice. A customized IP catheter (MicroPort (S-ISC) catheter, Kent Scientific, Torrington, Connecticut) was implanted into the mice. The animals were anesthetized with ketamine/xylazine (100, 20 mg/kg). For the IP catheter, we performed an incision in the skin in the right flank of the animal. The skin was separated from the muscle layer below. Through another incision in this second layer, the end of the catheter was introduced into the peritoneal cavity. The port was placed at the subcutaneous space of the mouse’s back. During the first week post surgery, 0.2 mL saline was instilled in order to prevent catheter trapping. Thereafter, during the experimental procedure, dialysis solution alone (control and placebo groups) or with PPI was instilled 6 days/week during 8 weeks.

Experimental diets in mouse efficacy studies. The powder diet (Harlan Teklad Global Diet 2918; Harlan Teklad, Gannat, France) used for all animal groups (i.e. non-CKD and CKD mice) and contained 18.9% protein, 6% (w/w) fat, 1.01% calcium, 0.65% phosphorous (calcium/phosphorus ratio 1.55) and 1.54 IU/g vitamin D3.

Serum biochemistry

Blood was sampled via retro-orbital venous sinus puncture at time points of randomization and via cardiac puncture at time of sacrifice. Collected blood was put into chilled dry tubes, spun in a refrigerated centrifuge for 15 min and serum was stored at −80°C. Serum levels of urea, total calcium, phosphorus, triglycerides and total cholesterol were measured using Hitachi 917 autoanalyzer (Roche, Meylan, France) as described previously.

Quantitative and qualitative evaluation of calcification in aortic root lesions

We performed von Kossa staining in 7-μ thick cryosections of aortic root tissue in order to evaluate plaque and non-plaque calcification. This location allows the distinction of intimal and medial-like calcification, respectively. Data were first expressed as the relative proportion of the calcified area to the total surface area covering either the inside or the outside of atherosclerotic lesions. Data were expressed as the relative proportion of calcified area to total surface area of either atherosclerotic lesions or vessel area outside atheromatous plaques using Histolab software, as reported previously [19].

Biochemical quantification of thoracic aorta calcium content

The samples were weighed (fresh weight). Then, they were divided into small pieces and placed in an oven at 110°C over 3–4 days until optimal desiccation. In the following step, the samples were re-weighed (to determine dry weight) and digested with the help of 12 N HCl for 1 week until complete tissue lyses. Later, samples were diluted to reach 0.6 N HCl and homogenized with a tissue homogenizer. Homogenates were then suitable for biochemical assessment of calcium content.

Calcium content was determined with the o-cresolphthalein complexone method. The principle of this method is based on the purple-colored complex formed by calcium with o-cresolphthalein complexone in an alkaline medium. The optical density (OD) of the colour was measured with a spectrophotometer at 565 nm. The samples’ ODs were compared to a curve calibrated with calcium standards. Finally, the precise calcium quantity was calculated taking into account the final digestion volume normalized by dry weight.

Quantification of atherosclerotic lesions

Aortic root lesion area was determined from 7-μ thick serial aortic root cryosections followed by oil red O staining and analysis of intimal area atherosclerosis, as described previously [17].

Histological evaluation of peritoneal-associated tissue

Tissue samples at the termination of the experiment were removed, fixed in 10% neutral buffered formalin, trimmed, processed, embedded in paraffin and sectioned. Haematoxylin- and eosin (H&E)-stained slides were prepared and examined by light microscopy. Microscopic observations were subjectively graded based on the relative severity of the change: Grade 1 = minimal, Grade 2 = mild, Grade 3 = moderate and Grade 4 = marked. For the diaphragm, as an example, minimal involvement was that confined to the...
subserosal, mild severity involved <25% of the thickness of the diaphragm, moderate severity involved 25–50% of the thickness of the diaphragm and marked severity would involve >50% of the thickness of the diaphragm.

Statistical analysis
Results were expressed either as means ± SDs or SEM as indicated in the text, table or figure. Comparisons among group means were performed by one-way analysis of variance. Inter-group differences were considered to be significant when \( P < 0.05 \).

Results

PPi pharmacokinetics in rats
To first examine the hypothesis that delivery of PPi via daily PD should lead to an increase in plasma PPi concentrations over a time period sufficient to prevent soft tissue calcification, pharmacokinetic studies were carried out in rats using \(^{32}\text{P}\)-labelled PPi. Partially nephrectomized rats, monitored to confirm renal impairment, were then injected with either (i) an IV bolus of PPi (2.25 mM) of 4 mL total volume per kilogram of saline or (ii) an IP injection of a 60 mL/kg PD-solution containing PPi (150 \( \mu \text{M} \)), approximating a volume utilized in PD. In both groups, each rat had a total PPi exposure of 2 mg/kg. The IV bolus delivery resulted in an immediate and quite large peak plasma concentration (~50 \( \mu \text{M} \)). Thereafter, \(^{32}\text{P}\)-PPi was rapidly metabolized within ~1-h post-injection (Figure 1). With PD delivery, there was a similarly rapid appearance of plasma \(^{32}\text{P}\)-PPi. In contrast to IV delivery, levels peaked at a much lower concentration (~1 \( \mu \text{M} \)); however, it remained elevated for several hours. A comparison of calculated pharmacokinetic parameters following both IV and PD delivery is shown in Table 1.

Serum biochemistry
Body weight and serum biochemistry characteristics of apoE\(^{-/-}\) mice at the 2-week post-nephrectomy time point, that is, at the time of randomization, are shown in Table 2. Four CKD mice died following surgical procedure. As expected, mice having undergone nephrectomy (CKD groups) showed a significant elevation of serum urea (3-fold greater than normal), total calcium, triglycerides and total cholesterol, as compared to sham surgery group. The body weight of the animals and serum phosphorus levels remained unchanged. There were no significant differences between the CKD groups in any of the parameters examined, indicating successful randomization.

Table 3 shows the same parameters as above at the time of sacrifice (that is, after 8 weeks of treatment). Blood samples were collected 24 h after the last treatment. Based on the pharmacokinetic findings above, this was at a time when total PPi levels, altered by exogenous treatment, had returned to normal. Serum urea, total calcium and total cholesterol were all significantly elevated in the CKD group. None were altered by long-term treatment with PPi. Serum triglycerides were also elevated at the end of the study period, but unlike the values observed at the time of randomization, the difference between the CKD and the non-CKD groups was no longer significant.

PPi treatment and aortic calcification
We next determined the effect on VC of PPi treatment via PD delivery. Figure 2 shows qualitative data based on von Kossa staining of aortic root, with representative sections for each animal group. As expected, overall calcification in the non-CKD group was limited to sparse foci, whereas the CKD-placebo group exhibited extensive calcification across the aortic root. The extensive calcification caused by CKD was markedly reduced by low dose PPi–PD treatment and nearly completely prevented in animals treated with high dose PPi–PD (Figure 2). The effect on calcification inside the lesions was separately analysed and compared to that outside the lesions, the latter being considered to be predominantly medial-type calcification. The area of
aortic root calcification measured inside the atherosclerotic lesions was found to be greatly increased (~5-fold) in animals with CKD, compared with the calcified area in sham operated (non-CKD) group (Figure 3A). A strong dose-dependent blockade of intimal calcification was obtained by the treatment with PPi–PD. The solution containing the high dose of PPi completely prevented (P < 0.0002) the increase in intimal calcification due to CKD, whereas the solution with the low PPi dose inhibited ~50% (P < 0.04) of the calcification inside the lesions. The calcification outside the lesions (medial-like) was also elevated in the CKD group, and this calcium phosphate deposition was totally blocked as well (P < 0.004), even at the low PPi dosage (P < 0.005) (Figure 3B).

Total aorta calcium was also biochemically determined at sacrifice. Figure 4 shows that CKD induced a significant increase (by ~65%) in total calcium content, compared to that in mice from the sham group. Treatment with the highest PPi–PD dose (150μM) was capable of completely preventing the increase of aortic calcium, resulting in mean values slightly below those of the sham group (P < 0.029). A dose effect was observed, since the lower PPi dose (30μM) produced a less robust reduction in calcification, although the difference with respect to that of the CKD group did not reach the level of statistical significance (P < 0.089) (Figure 4).

**Table 1.** Calculated pharmacokinetic parameters: IV versus PD delivery

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nephrectomized (5/6 Nx)</th>
<th>IV</th>
<th>PD</th>
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<tbody>
<tr>
<td>Cmax (µg/mL)</td>
<td>8.81</td>
<td>0.285</td>
<td></td>
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<tr>
<td>t1/2 (h)</td>
<td>0.203</td>
<td>4.66</td>
<td></td>
</tr>
<tr>
<td>AUCl∞ (h × µg/mL)</td>
<td>1.23</td>
<td>0.989</td>
<td></td>
</tr>
<tr>
<td>Bioavailability (%)</td>
<td>100</td>
<td>80</td>
<td></td>
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</tbody>
</table>

**Table 2.** Total body weight and serum biochemistry 2 weeks after nephrectomy or sham operation (time point of randomization)

<table>
<thead>
<tr>
<th></th>
<th>Sham placebo (n = 6)</th>
<th>CKD placebo (n = 6)</th>
<th>CKD PPi LD (n = 7)</th>
<th>CKD PPi HD (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>20 ± 0.89</td>
<td>19.71 ± 0.95</td>
<td>19.13 ± 0.83</td>
<td>19.25 ± 1.28</td>
</tr>
<tr>
<td>Urea (mM)</td>
<td>9.58 ± 1.62</td>
<td>33.8 ± 11.27</td>
<td>31.97 ± 7.07</td>
<td>31.9 ± 5.67</td>
</tr>
<tr>
<td>Total calcium (mM)</td>
<td>2.11 ± 0.07</td>
<td>2.46 ± 0.069</td>
<td>2.46 ± 0.074</td>
<td>2.50 ± 0.156</td>
</tr>
<tr>
<td>Phosphorus (mM)</td>
<td>2.60 ± 0.41</td>
<td>2.59 ± 0.22</td>
<td>2.38 ± 0.17</td>
<td>2.40 ± 0.28</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>1.25 ± 0.30</td>
<td>2.48 ± 0.779</td>
<td>2.10 ± 0.89</td>
<td>1.99 ± 0.66</td>
</tr>
<tr>
<td>Total cholesterol (mM)</td>
<td>5.99 ± 1.39</td>
<td>14.54 ± 2.11</td>
<td>12.9 ± 2.97</td>
<td>13.3 ± 1.90</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD. CKD, chronic kidney disease; LD, low dose; HD, high dose.

**Discussion**

We began this study with pharmacokinetic experiments designed to examine the possibility that when delivered via a PD solution, exogenous PPi might enter the circulation slowly, and rather than being quickly degraded in the peritoneum, provide for continued administration with the potential for daily elevation of levels. The similarity in systemic availability of PPi following IV and IP administration in the rat indicates that PPi was almost completely absorbed for the IP dose, with minimal degradation in the peritoneum. In the present study, maximum concentrations of PPi in plasma were lower and the time to reach these maximal values were prolonged following IP administration in comparison to those determined following an equivalent dose via IV administration. Consequently, detectable levels of PPi remained in plasma following IP delivery through 8-h post-administration, whereas measurable levels of PPi in plasma following IV administration of an equivalent dose fell below detection within 1-h post-dose. These results clearly indicate that there were substantial differences in the PPi plasma levels and pharmacokinetics following IP and IV administration.

**PPi treatment and potential toxicology**

In order to determine if PPi treatment might exert any toxic effects, tissues and/or organs (heart, kidney, liver and parietal peritoneum) were sampled at sacrifice and examined by light microscopy after H&E staining and scored on a 1–4 scale as described in the Materials and Methods section. Subacute to chronic inflammation of the serosa of the liver, spleen and/or parietal peritoneum was present in mice from all groups including the sham control and placebo groups, that is both in the absence and the presence of PPi treatment (Table 4). The serosal reaction was characterized by expansion of the submesothelium with a mixed inflammatory cell infiltrate including mononuclear cells, neutrophils and plasma cells. In the diaphragm (parietal peritoneum), the inflammation extended from the peritoneum into the underlying interstitial space between muscle fibres (Figure 5). The presence of serosal inflammation in all animal groups indicated that these changes were likely the result of a response to the permanent catheter as a foreign body, perhaps complicated by chronic exposure to the peritoneal solution, and were not due to drug treatment.

We began this study with pharmacokinetic experiments designed to examine the possibility that when delivered via a PD solution, exogenous PPi might enter the circulation slowly, and rather than being quickly degraded in the peritoneum, provide for continued administration with the potential for daily elevation of levels. The similarity in systemic availability of PPi following IV and IP administration in the rat indicates that PPi was almost completely absorbed for the IP dose, with minimal degradation in the peritoneum. In the present study, maximum concentrations of PPi in plasma were lower and the time to reach these maximal values were prolonged following IP administration in comparison to those determined following an equivalent dose via IV administration. Consequently, detectable levels of PPi remained in plasma following IP delivery through 8-h post-administration, whereas measurable levels of PPi in plasma following IV administration of an equivalent dose fell below detection within 1-h post-dose. These results clearly indicate that there were substantial differences in the PPi plasma levels and pharmacokinetics following IP and IV administration.
The rapid disappearance of PPi following IV administration was not unlike that reported by Jung et al. [15], 30 years ago. The current results supported our hypothesis and the subsequent part of the study, that is, the efficacy testing of this administration in apoE<sup>-/-</sup> mice as a model of VC in ESRD.

We next showed that PPi in micromolar concentrations, when delivered daily (six times per week) in a PD solution, is able to totally prevent the development of aortic calcification in an appropriate mouse model of ESRD. We think that this is a suitable, and perhaps the most relevant, small animal model of the cardiovascular disease observed in ESRD patients. In this model, the uraemic state is created in an environment of moderate hyperlipidaemia and results in the consistent production of atherosclerosis at the thoracic aorta site, together with marked calcium deposition.

Table 3. Total body weight, serum biochemistry and atherosclerotic plaque lesion area at sacrifice<sup>a</sup>

<table>
<thead>
<tr>
<th></th>
<th>Sham placebo (n = 5)</th>
<th>CKD placebo (n = 6)</th>
<th>CKD PPi LD (n = 7)</th>
<th>CKD PPi HD (n = 7)</th>
</tr>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>21.8 ± 1.23</td>
<td>20 ± 1.71</td>
<td>21.1 ± 1.34</td>
<td>20.47 ± 0.8</td>
</tr>
<tr>
<td>Urea (mM)</td>
<td>7.46 ± 1.43</td>
<td>25.83 ± 0.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.84 ± 0.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.73 ± 1.44&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>T-Calcium (mM)</td>
<td>2.18 ± 0.14</td>
<td>2.54 ± 0.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.35 ± 0.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.40 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phosphorus (mM)</td>
<td>2.43 ± 0.32</td>
<td>2.25 ± 0.25</td>
<td>2.38 ± 0.30</td>
<td>2.34 ± 0.29</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>1.38 ± 0.62</td>
<td>1.63 ± 0.55</td>
<td>1.67 ± 0.55</td>
<td>1.31 ± 0.53</td>
</tr>
<tr>
<td>T-Cholesterol (mM)</td>
<td>9.72 ± 1.32</td>
<td>14 ± 0.84&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.15 ± 1.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.80 ± 1.44&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Atherosclerotic plaque lesions at aortic root (%)</td>
<td>8.19 ± 8.99</td>
<td>9.11 ± 5.59</td>
<td>10.96 ± 4.64</td>
<td>10.28 ± 8.84</td>
</tr>
</tbody>
</table>

<sup>a</sup>Results are expressed as mean ± SD; T, total. CKD, chronic kidney disease; LD, low dose; HD, high dose.

<sup>b</sup>Versus sham placebo (P < 0.05).

<sup>c</sup>Versus CKD placebo (P < 0.05).

Fig. 2. Qualitative analysis of calcification at the aortic root and effect of long-term PD administration of PPi in mice. Shown are the results of von Kossa silver staining representative of each treatment group. In the left column, areas of calcification can be visualized in black against a violet background. The right column shows representative results following morphological image processing; areas of calcification are visualized in white and the remaining tissue in black. CKD: chronic kidney disease; low dose: PPi low dose group (30 μM or 0.33 mg/kg/day); high dose: PPi high dose group (150 μM or 1.66 mg/kg/day).
resembling hydroxyapatite both within the intima and outside the intima, the latter considered as medial calcification [17, 19] Our finding that a PD solution containing 150 μM PPI was able to completely block total aortic calcification indicates that, under these conditions, it is an effective treatment for preventing both medial and intimal calcium phosphate deposition. The blockade of aortic calcification was less marked when the PPI dose was reduced to the lower 30 μM concentration, indicating a dose–response effect. This then suggests a potentially effective range for consideration in future human studies.

Our study allowed the visualization, by von Kossa silver nitrate staining, of calcified lesions at the aortic root site, along with the biochemical quantification of aortic calcium content, enabling the assessment of potential protective effects of PPI treatment separately on the development of the atherosclerotic lesion itself and the calcification within the lesions as well as outside the lesions. We found, like in previous studies using the same animal model, that at the aortic root site, the marked hyperlipidaemia caused by apo-

lipoprotein E deletion produced severe atherosclerotic lesions in non-uraemic apoE−/− mice such that they were not further increased by the superimposition of uraemia in mice with CKD [17]. However, at the aortic root site, the extent of calcification both inside and outside the atheromatous lesions was greatly increased by the uraemic state. Although PPI treatment was unable to reduce the size of the atherosclerotic lesions at that anatomical site, it did totally block calcification both within and outside the plaques when administered at high dose. Of interest, both the lower and the higher PPI concentrations were able to completely prevent the development of calcification outside the intimal lesions. This could mean that one PPI treatment dosage might suffice to totally block the progression of overall vessel calcification. This view is supported by the finding of a striking reduction in the biochemically measured total calcium content of the aorta.

The ability of PPI to block the progression of VC may not be surprising given that a number of previous in vitro and in vivo studies have shown a potential for this compound to inhibit the mineralization process in various forms of human disease [13, 15, 20, 21]. However, it became clear in early studies that since PPI half-life in the circulation is very brief, supraphysiological doses are likely be required to be effective in vivo [15]. Under the condition of thrice weekly conventional haemodialysis this would likely require unrealistically high IV doses in a non-hospital setting. This remains to be tested. However, the present study employs a PD delivery and shows the efficacy of this method of administration, producing clear results with only slightly increased near physiological serum PPI levels.

The mechanism of the PPI-induced blockade of aortic calcification has not been addressed in our study. However, published data indicate that PPI may act through multiple routes to exert this action. One mode by which PPI has been reported to inhibit VC is via reduction of hydroxyapatite crystal formation. Work by Fleisch et al. [22] suggests that PPI is able to inhibit hydroxyapatite crystal growth, even in

### Table 4. Peritoneal histopathological evaluation

<table>
<thead>
<tr>
<th>Individual animal histopathology observations</th>
<th>Severity grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observation</td>
<td>0</td>
</tr>
<tr>
<td>Liver, inflammation, capsule</td>
<td>Non-CKD/apo E−/− control group</td>
</tr>
<tr>
<td>CKD/apo E−/− control group</td>
<td>2</td>
</tr>
<tr>
<td>CKD/apo E−/− treated with low-dose PPI</td>
<td>0</td>
</tr>
<tr>
<td>CKD/apo E−/− treated with high-dose PP</td>
<td>1</td>
</tr>
<tr>
<td>Spleen, inflammation, capsule</td>
<td>CKD/apo E−/− control group</td>
</tr>
<tr>
<td>Non-CKD/apo E−/− control group</td>
<td>2</td>
</tr>
<tr>
<td>CKD/apo E−/− treated with low-dose PPI</td>
<td>0</td>
</tr>
<tr>
<td>CKD/apo E−/− treated with high-dose PP</td>
<td>2</td>
</tr>
<tr>
<td>Diaphragm, inflammation, peritoneum</td>
<td>Non-CKD/apo E−/− control group</td>
</tr>
<tr>
<td>CKD/apo E−/− control group</td>
<td>2</td>
</tr>
<tr>
<td>CKD/apo E−/− treated with low-dose PPI</td>
<td>1</td>
</tr>
<tr>
<td>CKD/apo E−/− treated with high-dose PP</td>
<td>2</td>
</tr>
</tbody>
</table>

*Values are the result of scoring of individual animals on haematoxylin- and eosin-stained slides under light microscopy. Grade 1 = minimal, Grade 2 = mild, Grade 3 = moderate, Grade 4 = marked, Grade 0 = inflammation absent.*
supersaturated solution of calcium and phosphate. The extent to which PPI is able to inhibit crystal growth is dependent on solution temperature. It is statistically significant at 37°C. Garimella et al. [23] examined the effect of PPI on hydroxyapatite formation in in vitro cultures of matrix vesicles from bone growth plates. They found no difference in the intensity of mineral deposition but did find that the addition of PPI prevented the formation of a highly crystalline hydroxyapatite solid. In addition, studies have shown that PPI limits calcium deposition in in vitro cultures of VSMCs [24], calvarial cells [25], and cultured aortic rings [13].

Fig. 5. Haematoxylin/eosin staining of mouse parietal peritoneum (diaphragm) for histopathology: representative section. A total of six to seven animals from each group, and multiple sections from each tissue sample were examined. Shown is a randomly selected section from the control, CKD/apoE−/− (no PPI) group. There was no significant difference observed between any of the groups (see Table 4).

We did not examine the possibility of a PPI effect on normal bone metabolism in this study. Extracellular matrix (ECM) mineralization is a physiological process in bone, but a pathological process in soft tissues. The mechanisms determining the spatial restriction of ECM mineralization to bone under physiological conditions are understood only partially. Murshed et al. [29] showed that a normal extracellular phosphate concentration is required for bone mineralization and that lowering this concentration prevents mineralization of any ECM. However, simply raising extracellular phosphate concentration is not sufficient to induce pathological mineralization; this is because of the presence of all ECM of PPI, an inhibitor of mineralization. ECM mineralization occurs only in bone because of the exclusive co-expression in osteoblasts of type I collagen and TNAP, an enzyme that cleaves PPI. This dual requirement explains why the ectopic expression of TNAP in cells producing fibrillar collagen is sufficient to induce pathological mineralization. Thus PPI at supra-physiologic concentrations should, at least in theory, have limited, or no, effect on bone since it would be metabolized immediately by TNAP. The situation is different for bisphosphonates. These drugs are non-hydrolyzable analogues of PPI and therefore not sensitive to TNAP. They would thus inhibit bone formation and mineralization and inhibit VC as well. This is the likely explanation for why bisphosphonates, although successful at preventing vascular soft tissue calcification in rats, did not find their way into clinical practice for this indication [30]. Work is in progress in our laboratory to confirm the lack of skeletal side effects of PPI at supraphysiological doses.

In conclusion, PPI, when delivered in a PD solution, is able to prevent in a dose-dependent manner the development of aortic calcification in an appropriate mouse model of CKD and ESRD. This efficacy is obtained without apparent toxicity. Although the mechanisms behind these effects remain to be explored, this study suggests a potentially effective dose range for consideration in future human studies.

References