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Deletion of the P2Y2 receptor aggravates internal elastic lamina calcification in chronic kidney disease mice through upregulation of alkaline phosphatase and lipocalin-2

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Abbreviations

ACTA2: actin alpha 2
ALPL: alkaline phosphatase
BMP2: bone morphogenetic protein 2
CKD: chronic kidney disease
COL3A1: collagen type III alpha 1 chain
FAAS: flame atomic absorption spectrometry
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
IL-6: interleukin 6
LCN2: lipocalin-2
P2Y2R: purinergic receptor P2Y2
Runx2: runt-related transcription factor 2
Sox9: SRY-box transcription factor 9
TGFb1: transforming growth factor beta 1
TNAP: tissue non-specific alkaline phosphatase
VSMC: vascular smooth muscle cells
Abstract

Calcification of the medial layer, inducing arterial stiffness, contributes significantly to the cardiovascular mortality in patients with chronic kidney disease (CKD). Extracellular nucleotides block mineralization of arteries by binding to purinergic receptors including the P2Y$_2$ receptor. This study investigates whether a deletion of the P2Y$_2$ receptor influences the development of arterial media calcification in CKD mice. Animals were divided in (i) wild type mice with normal renal function (control diet) (n = 8), (ii) P2Y$_2$R$^{-/-}$ mice with normal renal function (n = 8), (iii) wild type mice with CKD (n = 27) and (iv) P2Y$_2$R$^{-/-}$ mice with CKD (n = 22). To induce CKD, animals received an alternating (0.2-0.3%) adenine diet for seven weeks. All CKD groups developed a similar degree of chronic renal failure as reflected by high serum creatinine and phosphorus levels. Also, the presence of CKD induced calcification in the heart and medial layer of the aortic wall. However, deletion of the P2Y$_2$ receptor makes CKD mice more susceptible for the development of calcification in the heart and aorta (aortic calcium scores (median ± IQR), CKD-wild type: 0.34±4.3 mg calcium/g wet tissue and CKD-P2Y$_2$R$^{-/-}$: 4.0±13.2 mg calcium/g wet tissue). As indicated by serum and aortic mRNA markers, this P2Y$_2$R$^{-/-}$ mediated increase in CKD related arterial media calcification was associated with an elevation of calcification stimulators, including alkaline phosphatase and inflammatory molecules interleukin-6 and lipocalin 2. The P2Y$_2$ receptor should be considered as an interesting therapeutic target for tackling CKD related arterial media calcification.

Keywords: arterial media calcification, chronic kidney disease, purinergic signaling, acute phase signaling, alkaline phosphatase
Introduction

Chronic kidney disease (CKD) is a worldwide and high burden disease with its primary causes being hypertension and diabetes. CKD patients are living longer due to the improved hospital care and availability of renal replacement techniques. Still, a doubling of the number of people receiving kidney replacement therapy (≈2.5 million) is projected for 2030 [1]. Also, CKD is a risk multiplier for the development of cardiovascular complications such as atherosclerotic plaques, thrombotic events, vascular inflammation and cardiovascular calcification. Approximately 40-50% of CKD patients stage 4 and 5 die due to cardiovascular events. Moreover, the cardiovascular mortality of young end-stage CKD patients (25-34 years) equals that of the ≈85-year-old general population [2]. Cardiovascular calcification or the deposition of calcium-phosphate crystals (often as hydroxyapatite) has been found at four distinct sites throughout the vasculature being (i) arterial intima (atherosclerotic plaque calcification), (ii) the medial layer in the arterial wall (arterial media calcification), (iii) aortic valve and (iv) the cutaneous blood vessels (calciphylaxis) [3].

Arterial media calcification, also called Mönckeberg’s arteriosclerosis, introduces a degree of vascular stiffness favoring cardiovascular events such as left ventricular hypertrophy and hypertension [4]. Arterial media calcification can be found in 50% of the CKD patients [5], which most probably is even an underestimation as no effective treatment is available and therefore CKD patients are not routinely screened for arterial media calcifications. Currently, therapies focus on treating the risk factors for arterial media calcification including phosphate binders and calcimimetics to control circulating phosphate and calcium levels in CKD patients [6]. Both hyperphosphatemia and –calcemia, typically observed in CKD patients, trigger i) a passive precipitation of calcium-phosphate in the medial layer of the arterial wall and (ii) an active cell regulated mechanism by which vascular smooth muscle cells (VSMCs) undergo a phenotypic switch into osteo-/chondrogenic like cells [7,8]. Furthermore, in CKD patients levels of circulating calcification inhibitors (i.e. pyrophosphate, matrix gla protein, fetuin-A) and calcification stimulators (i.e. uremic toxins, inflammatory cytokines) are outbalanced [7]. It is furthermore important to mention that the prominent molecular mechanisms during development of pathological arterial media calcification are comparable to those observed during physiological bone mineralization indicating a high resemblance between both processes [9]. Multiple studies have shown the involvement of purinergic
signalling in inhibiting bone mineralization [10-13], opening a research avenue to evaluate the potential therapeutic effect of modifying purinergic signalling in the treatment of arterial media calcification.

Purinergic signalling (through purines and pyrimidines) finds it role in many cellular processes such as proliferation, differentiation and death [14]. In 1972, Geoffrey Burnstock revealed, after molecular cloning experiments, the different subtypes of purinergic receptors. A distinction of two broad family types of purinergic receptors is made, being P1 and P2 receptors. The G protein-coupled P1 receptor family are activated by adenosine and contains four subtypes A1, A2A, A2B and A3. The P2 receptor family compromises eight G protein-coupled P2Y receptors (P2Y1,2,4,6,11,12,13,14) and seven ligand-gated ion channels P2X receptors (P2X1-7) [15,16]. Patel et al. revealed that ATP and UTP induced inhibitory effects on VSMC calcification by activating P2 receptors [17]. Additionally, a 3-fold increase in the mRNA expression levels of P2X1, P2X2, P2X4, P2X5, P2X6 and P2Y2 receptors was observed in calcifying VSMCs compared to control VSMCs [17] suggesting a potential role of these receptors in ectopic mineralization. Interestingly, regulating the activity of the P2Y2 receptor influenced the calcification process in other types of arterial calcification, i.e. aortic valve calcification [18] and intima/atherosclerotic calcification [19]. Recently, it has been shown that treatment with a P2Y2R specific agonist, 2-thioUTP, halted the progression of calcification in mouse and human valve aortic interstitial cells by downregulating fibrotic genes (COL3A1, TGFb1, ACTA2) [20]. In line with these results, Bouchareb et al. showed that 2 months of 2-thioUTP treatment reduced the aortic valve mineral content through upregulation of carbonic anhydrase inducing acidification of the extracellular space [18]. Furthermore, apolipoprotein E knockout mice, susceptible to arterial intima calcification, with a P2Y2 receptor knockout background developed significantly more extensive calcification in the intima layer of the vessel wall as compared to apolipoprotein E knockout mice expressing the P2Y2 receptor [19]. These studies already point out that targeting the P2Y2 receptor would be a promising way to halt cardiovascular calcifications. However, so far, the role of the P2Y2 receptor in arterial media calcification has not yet been investigated in vivo. Hereto, we evaluate whether deletion of the P2Y2 receptor in a CKD mouse model aggravates the development of arterial media calcifications.
Material and Methods

Animal experiment

All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals 85–23 (1996) and approved by the University of Antwerp Ethics Committee (Permit number: 2017-05, Approval date: 23/02/2017). Animal experiments have been performed according to ARRIVE guidelines. Animals were housed eight per cage, exposed to 12-h light/dark cycles, and had free access to food and water. Mice lacking the P2Y$_2$R gene (P2Y$_2$R$^{-/-}$) were received from Jackson Laboratories (Bar Harbor, Maine, USA). Knockout mice were bred from homozygote (P2Y$_2$R$^{-/-}$) breeding pairs. Wild types with same genetic background (C56BL6J), gender (male) and age (8 weeks old) were obtained from Charles River Laboratories (Wilmington, Massachusetts, USA).

Animals were divided in (i) wild type mice with normal renal function (control diet) ($n = 8$), (ii) P2Y$_2$R$^{-/-}$ mice with normal renal function ($n = 8$), (iii) wild type mice with CKD ($n = 26$) and (iv) P2Y$_2$R$^{-/-}$ mice with CKD ($n = 22$). To induce chronic renal failure, animals received an alternating adenine diet including 1 week 0.3% adenine diet – 3 weeks 0.2% adenine diet – 2 weeks 0.3% adenine diet – 1 week 0.2% adenine diet. Male mice were used in this experiment, since female animals are more resistant against the development of CKD [21,22] and cardiovascular disease [23]. After 7 weeks animals were sacrificed through exsanguination through the retro-orbital plexus after anesthesia with 60 mg/kg ketamine (Pfizer, Puurs, Belgium) and 16 mg/kg xylazine (Bayer Animal Health, Monheim, Germany) via intraperitoneal injection. Before the planned sacrifice, three wild type and four P2Y$_2$R$^{-/-}$ mice with CKD died due to the severity of chronic renal failure.

Analysis of Biochemical Parameters

Phosphorus levels in serum samples were obtained with the Ecoline S Phosphate kit (Diasys, Holzheim, Germany). Serum creatinine levels were determined by use of the Mouse Creatinine Kit (Crystal Chem. Inc., Illinois, USA). Serum urea nitrogen concentrations were analyzed by BUN colorimetric detection kit (Thermofisher Scientific, Massachusetts, USA). Alkaline phosphatase levels in serum samples were
investigated through the colorimetric assay kit SensoLyte pNPP alkaline phosphatase (Anaspec, California, USA). Serum FGF23 (KAINOS Laboratories, Tokyo, Japan) and lipocalin-2 (RayBiotech Life, Georgia, USA) levels were measured by means of an ELISA kit.

**Quantification of Adenine Crystals in the Kidney**

At euthanasia, one slice of the left kidney was fixed in neutral buffered formalin for 4 hours and embedded in a paraffin block. Four-micrometer sections were stained with periodic acid–Schiff to quantify renal adenine crystals. The percentage of the area covered by adenine crystals was determined using Axiovision image analysis software (Release 4.5; Carl Zeiss, Oberkochen, Germany) by calculating the ratio of the number of pixels taken by the crystals to the number of pixels taken by the total renal tissue.

**Quantification of Cardiovascular Calcification**

The degree of cardiovascular calcification was analyzed by measuring (i) the calcium content in the heart and thoracic aorta through flame atomic absorption spectrometry (FAAS) (Perkin-Elmer, Wellesley, MA, USA) and (ii) percentage calcified area on Von Kossa stained sections of the abdominal aorta. The heart and thoracic aorta was weighed on a precision balance followed by a digestion in 65% HNO$_3$ at 60 °C for 6 h. After appropriate dilution in 0.1% La(NO$_3$)$_3$ to eliminate chemical interference, the calcium content in the digests was measured with FAAS and expressed as mg calcium/g wet tissue. Furthermore, the abdominal aorta was fixed in neutral buffered formalin for 90 min. Approximately, 3-5 tissue parts of 2 to 3 mm were cut and embedded upright in a paraffin block. Aortic sections (5 µm thick) were stained with Von Kossa and counterstained with hematoxylin and eosin. To calculate the percentage calcified area, Axiovision image analysis software (Release 4.5; Carl Zeiss, Oberkochen, Germany) defined the ratio of the number of pixels taken by Von Kossa positive area versus the number of pixels taken by the total tissue area.
Quantitative Real Time PCR

The mRNA transcript expression of GAPDH (Mm99999915_g1), actin alpha 2 (ACTA-2, Mm00725412_s1), SRY-box transcription factor 9 (Sox-9, Mm00448840_m1), alkaline phosphatase (ALPL, Mm00475834_m1), interleukin-6 (IL-6, Mm00446190_m1) and lipocalin-2 (LCN2, Mm01324470_m1) was determined in the distal part of the abdominal aorta. Total mRNA was extracted using Isolate II RNA mini Kit (Meridian Bioscience, Ohio, USA). Real-time polymerase chain reaction (PCR) with a Quant Studio 3 detection system (Applied Biosystems) based on Taqman fluorescence methodology was used for mRNA quantification. Taqman probe and primers were purchased as Taqman gene expression assays-on-demand from Thermo Fisher Scientific. Each gene was tested in triplicate and normalized to the expression of the housekeeping transcript GAPDH.

Bone histomorphometry

The proximal part of the tibia was fixed in 70% ethanol overnight at 4°C, dehydrated and embedded in 100% methylmethacrylate (Merck, Hohenbrunn, Germany). A Goldner staining was performed on tibia sections (5 µM thick) to determine bone formation and -resorption parameters, including osteoid area and eroded perimeter. The Axiovision image analysis software (Release 4.5, Carl Zeiss, Oberkochen, Germany) was used.

Statistical Analysis

Statistical comparisons were made by non-parametric testing (Prism 8.1.1, GraphPad Software Inc., California, USA). Statistical difference between groups at one time-points was tested via a Mann-Whitney U test. The p-value was adjusted by post-hoc Bonferroni correction. To investigate the relationship between aortic calcification and aortic mRNA expression profiles, a Spearman rho univariate correlation analysis was performed. Representative data are presented as median and individual values and considered significant when p-value ≤ 0.05.
Results

No difference in chronic renal failure and mineral metabolism markers between wild type and P2Y2R\(-/-\) mice

Serum creatinine and urea nitrogen levels were significantly increased after 7 weeks administration of an adenine diet to induce CKD compared to mice on a standard chow diet, with no differences between wild type and P2Y2R\(-/-\) mice (Figure 1A-B). The formation of adenine crystals in the renal tissue was quantified by PAS staining. A significant and comparable increase in % of adenine crystal surface was found for both wild type and P2Y2R\(-/-\) mice with CKD versus mice on a standard chow diet (Figure 2).

A two-fold increase of serum phosphorus levels was observed in the mice with CKD versus mice on a standard chow diet. However, no difference for serum phosphorus levels between wild type and P2Y2R\(-/-\) mice with CKD was found. In accordance, the phosphaturic hormone FGF-23 levels in serum were significantly elevated in the groups with CKD, reflecting an altered mineral metabolism in these mice (Figure 1C-D).

The development of cardiovascular calcification was significantly more severe in P2Y2R\(-/-\) mice with CKD versus wild type mice.

The presence of chronic renal failure triggered the development of calcification in the aorta and heart. Moreover, a significant 3-fold increase in mean calcium content of the aortic and heart tissue was found in P2Y2R\(-/-\) mice with CKD versus wild type mice with CKD (Figure 3). In agreement, Figure 4 shows that the percentage of positive Von Kossa staining in the aorta and heart was elevated in P2Y2R\(-/-\) mice with CKD versus wild type mice with CKD. Microscopic images of Von Kossa stained aortic sections revealed that the calcifications were situated in the media layer of the vessel wall, close to the internal elastic lamina.

Deletion of the P2Y2R induces a significant increase in osteo/chondrogenic markers alkaline phosphatase and Sox-9

Both deletion of the P2Y2 receptor and CKD induce a significant increase in serum alkaline phosphatase. In accordance, P2Y2R\(-/-\) mice with CKD (6.05 ± 2.54 U ALPL/mg protein; median ± IQR) were the group with the highest alkaline phosphatase activity.
(significantly higher than wild type CKD-mice; 4.31 ± 1.04 U ALPL/mg protein, median ± IQR). Serum alkaline phosphatase levels correlate strongly with aortic calcium scores assessed by FAAS (Spearman r = 0.85, P-value < 0.0001). Subsequently, the mRNA transcripts of aortic alkaline phosphatase showed a trend towards elevated upregulation in P2Y$_2$R$^{-/-}$ mice versus wild type, whether or not in the presence of CKD (Figure 5A-B). In line with these results, a significant increase in Sox-9 mRNA transcript was observed in the aortic tissue of P2Y$_2$R$^{-/-}$ mice without CKD. Furthermore, the mRNA expression of ACTA-2, a contractile VSMC marker, showed a decreasing trend in wild type mice with CKD and became significant for P2Y$_2$R$^{-/-}$ mice with CKD (Figure 5C-D).

**Deletion of the P2Y$_2$R induces a significant increase in inflammation markers IL-6 and LCN2**

Figure 6 shows that the mRNA expression of inflammatory marker genes IL-6 and LCN2 was up to 7-fold higher in the aortic tissue of P2Y$_2$R$^{-/-}$ mice with CKD versus the wild type mice with CKD. In accordance, circulating levels of serum LCN2 were highest in P2Y$_2$R$^{-/-}$ mice with CKD. Subsequently, a positive, significant correlation was found between IL-6 and aortic calcium scores (Spearman r = 0.53, P-value < 0.05) as well as between LCN2 and aortic calcium scores (Spearman r = 0.69, P-value < 0.001). Also, serum phosphorus levels correlated strongly with both LCN2 mRNA expression (Spearman r = 0.70, P-value < 0.001) and serum LCN2 levels (Spearman r = 0.66, P-value < 0.001).

**No difference in bone formation and -resorption between wild type and P2Y$_2$R$^{-/-}$ mice with chronic renal failure.**

The presence of a severe CKD stage in the mice led to a clear effect on bone formation and -resorption. As figure 7 shows osteoid (or unmineralized bone) area was significantly decreased while eroded perimeter increased in mice with CKD, without significant differences between wild type and P2Y$_2$R$^{-/-}$ mice.
The present study revealed that deletion of the P2Y₂ receptor makes mice with CKD more susceptible for the development of calcification in the heart and media layer of the aortic wall. Administration of adenine for seven weeks induced typical clinical features of CKD in the wild type and P2Y₂R⁻/⁻ mice as evidenced by an increase in serum creatinine and urea nitrogen levels as well as an altered mineral homeostasis characterized by abnormal levels of phosphorus and phosphaturic hormone FGF-23. Because the progression of arterial media calcification greatly depends on the severity of renal failure and/or a disordered bone metabolism, we investigated whether P2Y₂ receptor deficiency influenced renal function and/or bone metabolism. Others have demonstrated that deletion of the P2Y₂ receptor in subtotal nephrectomized mice aggravates renal injury as creatinine clearance was significantly more decreased as compared to wild type, subtotal nephrectomized mice [24]. The P2Y₂ receptor also facilitates renal sodium and water reabsorption in mice [25]. Conversely, the present study revealed that absence of the P2Y₂ receptor did not worsen renal function in adenine-induced CKD mice based on serum creatinine and urea nitrogen levels and semi-quantification of adenine-induced renal crystals. Furthermore, previous studies have found that P2Y₂R⁻/⁻ mice show small alterations in their bone phenotype at the age of 16 to 24 weeks [12]. In the present study, mice were at the age of 15 weeks when tibias were collected and did not yet show alterations in their bone phenotype. However, a clear effect on bone formation/-resorption was found for mice with CKD regardless of the P2Y₂ receptor deletion. Taken together, our data suggest that P2Y₂ receptor deficiency induced deleterious effects on the vessel wall independently of an effect on renal function or bone metabolism.

Interestingly, in line with the results of Orriss et al. [12], we found an association between P2Y₂ receptor deficiency and an increase in serum ALPL levels. Four types of alkaline phosphates exist including germcel, placental, intestinal and tissue non-specific alkaline phosphatase (TNAP) [26]. In human plasma, 95% of the ALPL activity is attributed to the TNAP isozyme, mainly expressed by the liver and bone [27]. In the bone, TNAP is produced by the osteoblasts to maintain adequate physiological bone mineralization by enabling the hydrolysis of the important calcification inhibitor pyrophosphate into inorganic phosphate. However, treatment of hypophosphatasia patients to normalize systemic TNAP levels did not correct their symptoms [28], whilst
treating them with bone-targeted TNAP replacement therapy turned out to be successful [29]. In other words, it seems that the physiological role of local ALPL levels is more important than that of systemic/circulating ALPL. Furthermore, in case of CKD related arterial calcification, both serum (systemic) and aortic (local) TNAP levels associate with arterial calcification development [30]. This is in agreement with results of the present study showing that both systemic and local aortic ALPL (mRNA) levels increase in mice with a P2Y2 receptor deficiency. During transdifferentiation of VMSC into osteoblast-like cells, ALPL expression is induced favoring the arterial media calcification process [7]. Moreover increased aortic ALPL expression precedes initial calcium deposition and upregulation of other bone-related markers genes (i.e. Runx2, BMP2, Fetuin-A) in uremic rats [31]. In accordance herewith, we found an upregulation of the osteo/chondrogenic marker gene Sox-9 in P2Y2R−/− mice, pointing out that Sox-9 might also be classified as an early inducer of arterial calcification. Indeed, our research group has recently shown that healthy rats exposed to a nitric oxide synthase inhibitor, L-NAME, had significantly elevated Sox-9 mRNA transcripts in the aortic tissue versus control rats. Likewise, L-NAME aggravated the development of arterial media calcification when rats were exposed to an additional pro-calcifying factor (i.e. warfarin) [32]. Interestingly, the P2Y2 receptor promotes nitric oxide synthesis to maintain vascular tonus [33]. Taken together, we suggest that inadequate signaling via the P2Y2 receptor favors a predisposition for the development of ectopic calcifications due to an upregulation of ALPL and Sox-9 mRNA expression in the vessel wall. However, findings of the present study show that P2Y2R−/− mice do not spontaneously develop arterial media calcification unless, an additional trigger is present, in this case chronic renal failure.

Beside its mineralizing effect, ALPL has also anti-inflammatory actions by dephosphorylating inflammation triggering moieties (i.e. lipopolysaccharides) [34]. In septic mice, ALPL enzyme activity was increased in the parenchyma of the brain [35]. Therefore, we investigated whether P2Y2R−/− mediated rise of serum ALPL activity could be attributed to the presence of an inflammatory environment in the mice. The present study showed that the P2Y2R−/− mice with CKD experienced an up to 7-fold increase in inflammatory gene expression levels of IL-6 and LCN2 in the aortic tissue. These results are in agreement with the data from Husseini et al., who showed that treating calcifying aortic valve interstitial cells with a P2Y2 receptor agonist (2-thioUTP)
significantly decreased IL-6 transcripts [36]. Moreover, it has been shown that LCN2 (also called neutrophil gelatinase-associated lipocalin (NGAL)) is associated with high IL-6 levels in atherosclerotic lesions [37] and promotes IL-6 release in human macrophages, VSMCs and endothelial cells [38]. LCN2 is a member of the acute phase response proteins [39]. Our research group has shown that the inflammation pathway 'acute phase response signaling' plays a crucial causal role in the onset and development of uremic toxin induced arterial calcification in rats [40]. Also, others have recently demonstrated that acute phase proteins amyloid A and LCN2 drive phenotypic switching of VSMCs in atherosclerotic plaques [41,42]. In this context is the strong correlation between the aortic calcium scores and the inflammatory markers LCN2 and IL-6, as found in the present study, of particular interest.

Nonetheless, Patel et al. suggested that the P2Y2 receptor did not play a major role in in vitro VSMC calcification as ATP and UTP (P2Y receptor agonists) continue to inhibit calcification in VSMC cultures from P2Y2R−/− mice [17]. Their results suggested that these agonists block VSMC calcification through purinergic receptor independent actions including the production of a hydrolysis product, pyrophosphate. Pyrophosphate is well-known and potent calcification inhibitor [43]. The study of Patel et al. [17] points out the issue of translation of in vitro results towards the in vivo setting in arterial calcification experiments. An important drawback of the in vitro setup is the lack of communication between different cell types such as VSMCs and endothelial cells. Regarding to this, it is worth mentioning that the P2Y2 receptor is expressed by endothelial cells and regulates its nitric oxide release [33]. Interestingly, our research group has recently observed that a reduced basal nitric oxide production stimulates arterial media calcification in warfarin exposed mice [44]. Thus, arguing that the P2Y2 receptor might mediate its effects on arterial media calcification through affecting the behavior of endothelial cells or other vascular cells rather than VSMCs. In light of this, the use of a mouse model with specific P2Y2 receptor deletion in the endothelial cell or VSMC, instead of a global knockout model, is of particular interest.

The use of a global P2Y2R knock-out model can be seen as a drawback of the present study. Nonetheless, we suggest that P2Y2 receptor deficiency in non-arterial cells (i.e. osteoblasts, kidney cells) probably did not contributed to the higher degree of arterial media calcification in the global P2Y2R−/− CKD mouse model. Since our results show a similar degree of CKD and bone disease in wild type and P2Y2R−/− mice. However the
use of a specific VSMC or endothelial cell P2Y<sub>2</sub>R<sup>-/-</sup> mice should enable definite prove for this. In the present study, based on the Von Kossa stained aortic tissue sections, aortic calcifications were situated in the internal elastic lamina and thus in close proximity to the endothelium. Interestingly, several genetically defined arterial calcification diseases (i.e. Buerger's disease, idiopathic infantile arterial calcification, diffuse arterial calcified elastopathy, and pseudoxanthoma elasticum) and a case-report of HIV-positive patients with coronary calcifications are characterized by calcifications limited primarily to the internal elastic lamina of large- and medium-sized arteries [45]. Internal elastic lamina calcification is an underestimated type of arterial calcification in which endothelial dysfunction, inflammation and P2Y<sub>2</sub> receptor signaling might play a role.

In conclusion, deletion of the P2Y<sub>2</sub> receptor leads to a predisposition of the development of internal elastic lamina calcification in mice probably due to increased circulating and local levels of the calcification stimulator ALPL. Moreover, absence of this receptor aggravated the degree of arterial calcification in mice with chronic renal failure which was associated with the induction of aortic expression of the pro-inflammatory cytokine IL-6 and the acute phase protein LCN2. The P2Y<sub>2</sub> receptor could be an interesting therapeutic target for tackling, not only CKD related arterial media calcification but also other types of cardiovascular calcification such as intimal atherosclerotic calcification and aortic valve calcification.
Data Availability Statement

Included in article: The data that support the findings of this study are available in the methods and/or supplementary material of this article.

Conflict of Interest statement

All authors have nothing to disclose.

Author contributions

Opdebeeck B. carried out the experiments, analyzed the data, drafted and revised the paper; Huysmans I. carried out the experiments; Orriss I., D’Haese P. and Verhulst A. contributed to the study design and interpretation of the results and revised the manuscript; all authors approved the final version of the manuscript and accept responsibility for the integrity of the data analysis.

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References


**Figure legends**

**Figure 1:** Chronic renal failure and mineral metabolism markers were indifferent between wild type versus P2Y$_2$R$^{-/-}$ mice with CKD. Chronic renal failure markers (A) serum creatinine levels and (B) serum urea nitrogen levels. Mineral metabolism markers (C) serum phosphorus levels and (D) serum FGF23 levels. Data presented as individual values (dots) and median (grey line), * P-value < 0.05, ** P-value < 0.005, *** P-value < 0.001 and **** P-value < 0.0001.
Figure 2: No difference in the degree of adenine crystals formation between CKD groups. (A) % adenine crystal area with data presented as individual values (dots) and median (grey line) and (B) representative PAS stained renal sections for visualization of adenine crystals (arrows) of wild type, wild type with CKD, P2Y2R<sup>-/-</sup> and P2Y2R<sup>-/-</sup> with CKD mice.
Figure 3: Deletion of the P2Y$_2$ receptor increases calcium scores in the aorta and heart tissue. Calcium content of (A) aorta and (B) heart. Data presented as individual values (dots) and median (grey line), * P-value < 0.05, ** P-value < 0.005 and **** P-value < 0.0001.
Figure 4: Aortic calcification are situated in the media layer of the arterial wall. The % calcified surface of (A) aorta and (B) heart. Data presented as individual values (dots) and median (grey line), * P-value < 0.05. (C) Representative Von Kossa stained aortic sections of wild type, P2Y$_2$R$^{-/-}$, wild type with CKD and P2Y$_2$R$^{-/-}$ with CKD mice. Arrows point towards calcification sites.
Figure 5: Deletion of the P2Y2 receptor leads to increased osteo/chondrogenic markers. The osteo/chondrogenic markers (A) serum alkaline phosphatase (ALPL) activity, (B) aortic mRNA transcripts ALPL and (C) aortic mRNA transcripts Sox-9. The vascular smooth muscle cell marker (D) aortic mRNA transcripts ACTA-2. Data presented as individual values (dots) and median (grey line), * P-value < 0.05 and ** P-value < 0.005.
Figure 6: Deletion of the P2Y$_2$ receptor during chronic renal failure augments mRNA expression of inflammatory genes in the aortic tissue. mRNA expression profiles in the aortic tissue of (A) interleukin 6 (IL-6) and (B) lipocalin-2 (LCN2) as well as (C) serum LCN2 levels. Data presented as individual values (dots) and median (grey line), * P-value < 0.05 without bonferonni correction, *** P-value < 0.001 and **** P-value < 0.0001.
Figure 7: No difference in bone formation and resorption between wild type and P2Y₂R⁻/⁻ mice with chronic renal failure. The percentage of (A) osteoid area and (B) eroded perimeter. Data presented as individual values (dots) and median (grey line), * P-value < 0.05, *** P-value < 0.001. (C) Representative Goldner stained tibia sections of wild type, P2Y₂R⁻/⁻, wild type with CKD and P2Y₂R⁻/⁻ with CKD mice. Small arrows point towards osteoid or unmineralized bone. Long arrows point towards eroded areas.