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*Published in:*  
Nephrology Dialysis Transplantation

*DOI:*  
[10.1093/ndt/gfz190](https://doi.org/10.1093/ndt/gfz190)

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*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2020

[Link to publication in University of Groningen/UMCG research database](#)

### *Citation for published version (APA):*

Ter Braake, A. D., Eelderink, C., Zeper, L. W., Pasch, A., Bakker, S. J. L., de Borst, M. H., Hoenderop, J. G. J., & de Baaij, J. H. F. (2020). Calciprotein particle inhibition explains magnesiummediated protection against vascular calcification. *Nephrology Dialysis Transplantation*, 35(5), 765-773. [gfz190]. <https://doi.org/10.1093/ndt/gfz190>

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# Calciprotein particle inhibition explains magnesium-mediated protection against vascular calcification

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## ABSTRACT

**Background.** Phosphate (Pi) toxicity is a strong determinant of vascular calcification development in chronic kidney disease (CKD). Magnesium ( $Mg^{2+}$ ) may improve cardiovascular risk via vascular calcification. The mechanism by which  $Mg^{2+}$  counteracts vascular calcification remains incompletely described. Here we investigated the effects of  $Mg^{2+}$  on Pi and secondary crystalline calciprotein particles (CPP2)-induced calcification and crystal maturation.

**Methods.** Vascular smooth muscle cells (VSMCs) were treated with high Pi or CPP2 and supplemented with  $Mg^{2+}$  to study cellular calcification. The effect of  $Mg^{2+}$  on CPP maturation, morphology and composition was studied by medium absorbance, electron microscopy and energy dispersive spectroscopy. To translate our findings to CKD patients, the effects of  $Mg^{2+}$  on calcification propensity ( $T_{50}$ ) were measured in sera from CKD patients and healthy controls.

**Results.**  $Mg^{2+}$  supplementation prevented Pi-induced calcification in VSMCs.  $Mg^{2+}$  dose-dependently delayed the maturation of primary CPP1 to CPP2 *in vitro*.  $Mg^{2+}$  did not prevent calcification and associated gene and protein expression when added to already formed CPP2. Confirmatory experiments in human serum demonstrated that the addition of 0.2 mmol/L  $Mg^{2+}$  increased  $T_{50}$  from healthy controls by  $51 \pm 15$  min ( $P < 0.05$ ) and CKD patients by  $44 \pm 13$  min ( $P < 0.05$ ). Each further 0.2 mmol/L addition of  $Mg^{2+}$  led to further increases in both groups.

**Conclusions.** Our results demonstrate that crystalline CPP2 mediates Pi-induced calcification in VSMCs. *In vitro*,  $Mg^{2+}$  delays crystalline CPP2 formation and thereby prevents Pi-induced calcification.

**Keywords:** calciprotein particle, calcification propensity, chronic kidney disease, magnesium, vascular calcification

## INTRODUCTION

Vascular calcification contributes to cardiovascular morbidity and mortality in chronic kidney disease (CKD) [1]. Currently treatment options are limited and persisting vascular calcification remains a clinical problem. In past years, several epidemiological studies have shown that a lower serum magnesium ( $Mg^{2+}$ ) status is independently associated with an increased risk of vascular calcification and cardiovascular mortality in CKD patients [2–4]. Therefore it is hypothesized that  $Mg^{2+}$  could be an effective tool to limit vascular calcification [5]. However, the mechanisms underlying the potent anti-calcification properties of  $Mg^{2+}$  are incompletely understood.

Hydroxyapatite- and protein-containing calciprotein particles (CPPs) are major drivers of calcification [6, 7]. The transition from calcium ( $Ca^{2+}$ )- and phosphate (Pi)-containing amorphous or primary CPP1 towards crystalline or secondary CPP2 is key in the development of vascular smooth muscle cell (VSMC) calcification [6, 8]. Due to disturbances in the bone-mineral axis, resulting from an elevated Pi and decreased calcification inhibitors such as matrix gla protein (MGP) and fetuin-A, high Pi levels crystallize into CPP2 [7, 9]. These events result in active reprogramming of VSMCs, which in turn enhances the calcification process by up-regulating osteogenic genes, producing excess extracellular matrix, undergoing apoptosis and releasing pro-calcific exosomes [10–12].

Since VSMCs may activate osteogenic pathways that contribute to calcification, the capacity of  $Mg^{2+}$  to inhibit vascular calcification may rely on direct modulation of these cellular processes [13]. Indeed, multiple studies demonstrate that  $Mg^{2+}$  did not inhibit calcification upon transient receptor potential melastatin 7 blocking, an abundant  $Mg^{2+}$  channel in VSMCs, implying that intracellular  $Mg^{2+}$  prevents calcification [14, 15]. In addition,  $Mg^{2+}$  supplementation *in vitro* correlates with

reduced expression of pro-calcification genes and with increased expression of calcification protectors [16–18]. On the contrary, in the extracellular compartment,  $Mg^{2+}$  has potent anti-crystallization properties, which have been shown to be of importance in its capacity to prevent VSMC calcification *in vitro* [19–22].

The importance of CPP2 in the development of vascular calcification has been exploited in a novel diagnostic tool where the intrinsic capacity of patient serum to prevent the transition from CPP1 to CPP2, or calcification propensity of the serum, can be measured using the  $T_{50}$  test [7, 23, 24]. The identification of factors affecting  $T_{50}$  is of interest in the context of clinical management of vascular calcification, as these factors may influence the development and progression of vascular calcification in renal disease patients [23, 25–27].  $T_{50}$  is correlated with cardiovascular mortality and is affected by Pi [23]. Therefore, whether Pi toxicity resulting in increased risk for vascular calcification is determined by the presence of soluble Pi or that Pi toxicity is mediated by crystallization in CPP2 is important to consider.

In this study we aimed to delineate the mechanisms that explain the effects of  $Mg^{2+}$  on VSMC calcification. In our study we induced VSMC calcification using both Pi and CPP2, which allows comparison of the direct and indirect effects of  $Mg^{2+}$  supplementation on VSMC calcification. Using scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDX), we studied CPP transition, morphology and composition in the presence and absence of  $Mg^{2+}$  *in vitro*. Furthermore, we studied the influence of  $Mg^{2+}$  on calcification propensity in serum from CKD patients and healthy controls *ex vivo*.

## MATERIALS AND METHODS

### Cell culture

Human aortic VSMCs (hVSMCs) were purchased from the American Type Culture Collection (Manassas, VA, USA, lot no. PCS-100-012) and cultured in Dulbecco's Modified Eagle Medium (DMEM; Lonza, Basel, Switzerland) consisting of 20% (v/v) foetal bovine serum (FBS; BioWest, GE Healthcare, Little Chalfont, UK), 2 mmol/L L-glutamine and 10  $\mu$ g/mL ciprofloxacin at 37°C in a humidified incubator containing 5% carbon dioxide ( $CO_2$ ; v/v). Cells were used until the 10th passage. For calcification experiments, cells were cultured in 12-well plates and switched to calcification medium at subconfluence. Calcification medium consisted of 5% FBS (v/v) and 3 mmol/L Pi ( $NaH_2PO_4$ ), and 2 mmol/L  $Mg^{2+}$  when indicated. Cells were cultured for up to 8 days. The medium was changed every 2–3 days.

### CPP synthesis, isolation and induction of calcification

Phenol red-free DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) was supplemented with 3.5 mmol/L  $NaH_2PO_4$ , 1 mmol/L calcium chloride, 10% FBS (v/v), 2 mmol/L L-glutamine and 10  $\mu$ g/mL ciprofloxacin and stored in a humidified incubator at 37°C containing 5%  $CO_2$  (v/v) for 14 days. For isolation of CPP2, CPP-containing media were centrifuged at 16 000 g for 30 min at room temperature. The

pellet was resuspended in DMEM containing 10  $\mu$ g/mL ciprofloxacin and stored at 4°C. The  $Ca^{2+}$  content of the CPP2 suspension was measured using the *o*-cresolphthalein complexone method as described elsewhere [19]. Cells were treated with CPP2 at a final concentration ranging from 5 to 100  $\mu$ g/mL  $Ca^{2+}$  (5–100 CPP). Cells were cultured in their designated media for up to 4 days without a change of media.

### Analysis of VSMC calcification

Quantification of total cellular  $Ca^{2+}$  deposition using the *o*-cresolphthalein complexone method and alizarin red staining were performed as previously described [19].

### Gene expression analysis

Total RNA was isolated from hVSMCs using TRIzol (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions. Genomic DNA was removed by DNase treatment prior to complementary DNA synthesis from 1  $\mu$ g total RNA (Promega, Madison, WI, USA). The primers used for polymerase chain reaction (PCR) amplification were equally efficient (Supplementary data, Table S1). Reverse transcription quantitative PCR was executed in duplicate using IQ SYBRGreen Mix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol in a Bio-Rad thermocycler. The expression levels of genes of interest were normalized to *GAPDH* expression levels.

### Protein expression analysis

To prepare total lysate of hVSMCs, the hVSMC monolayer was scraped in a 1% (v/v) Triton-X100 lysis buffer containing protease inhibitors. The protein concentration was determined using the Pierce BCA Kit according to the manufacturer's instructions (Thermo Fisher Scientific Waltham). Subsequently samples consisting of equal amounts of protein were denatured in Laemmli buffer containing 10 mmol/L dithiothreitol and applied to sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Blots were incubated at 4°C overnight with primary antibodies against osteopontin (OPN; 1:1000; R&D Systems, Minneapolis, MN, USA; #MAB14331-100), transgelin (SM22 $\alpha$ ; 1:5000; Abcam, Cambridge, UK; #ab14106) and MGP (1:500; Proteintech, Rosemont, IL; #10734-1-AP). Blots were subsequently incubated with horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature. Band intensity was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and expression was corrected for  $\beta$ -actin and expression levels per group were normalized to the control.

### Electron microscopy

CPPs were transferred onto copper tape, coated with carbon and used for SEM analysis (GeminiSEM, Zeiss, Oberkochen, Germany) and EDX for elemental analysis (QUANTAX 200, Bruker, Billerica, MA, USA). Images were obtained using an Everhart–Thornley secondary electron detector (Bruker) at 5 kV for morphological observations and 20 kV for microelemental analyses. For transmission EM (TEM), the CPP solution was transferred onto a Formvar-coated copper grid and air

dried. TEM was performed on a JEOL JEM 1400 microscope (JEOL USA, Peabody, MA, USA) with an accelerating voltage of 60 kV. Images were acquired at 15 000-fold magnification (Gatan, Pleasanton, CA, USA).

### CPP maturation assay

CPP2 were generated in phenol red-free DMEM (Gibco) containing 10% FBS and  $MgCl_2$  was added to reach 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 mmol/L. As a reference, medium containing 10% FBS (v/v) was included. CPP2 maturation was monitored by daily measurement of the absorption at 570 nm for 14 days using a Benchmark Plus Microplate Spectrophotometer System (Bio-Rad) [6]. A separate group where  $MgCl_2$  was added only after absorption exceeded 0.15 (after 5 days) was included to study the effects of  $Mg^{2+}$  on already formed CPP2 and monitored until complete ripening after 14 days. To study the effects of high Pi on the inhibition of CPP2 by 2.0 mmol/L  $Mg^{2+}$ , CPP2 maturation was monitored in the presence of 4 and 5 mmol/L Pi (final concentration) in addition to the standard CPP mixture in a separate experiment. To test a potential role for  $Mg^{2+}$  in altering the pH of the CPP mixtures, pH was measured for the CPP mixtures without  $Mg^{2+}$  and with 2.0 mmol/L  $Mg^{2+}$  at 0, 7 and 14 days of incubation.

### Calcification propensity ( $T_{50}$ test) in serum samples supplemented with $Mg^{2+}$

Serum samples collected prior to transplantation from renal transplant recipients (CKD patients) and donors (healthy controls) of the TransplantLines cohort study (METc UMCG No. 2014/077, NTC03272841) were used in this study [28]. Blood from 10 subjects per group was collected in 4 mL serum collection tubes and centrifuged at 1300 g for 10 min at room temperature. Serum was stored at  $-80^{\circ}C$  until analysis. The  $T_{50}$  test (calcification propensity) has been described previously [24]. Serum was subjected to the  $T_{50}$  test without any additions (baseline) and after addition of solutions with  $MgCl_2$  resulting in increased concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 mmol/L. After 570 min, the test was terminated. Other laboratory measurements were performed during routine clinical visits prior to serum collection for this study (Supplementary data, Table S2).

### Statistical analysis

Parametric data were analysed by one-way analysis of variance (ANOVA) with Tukey's *post hoc* test to correct for multiple comparisons using PRISM software (GraphPad Software, San Diego, CA, USA). Non-parametric data as identified by the Shapiro–Wilk test for normality were analysed using Kruskal–Wallis analysis with Dunn's correction for multiple comparisons. For grouped analysis, data were analysed using a two-way ANOVA. All data are shown as mean  $\pm$  standard deviation (SD). P-values  $<0.05$  were considered statistically significant.

## RESULTS

### $Mg^{2+}$ prevents high Pi-induced VSMC calcification

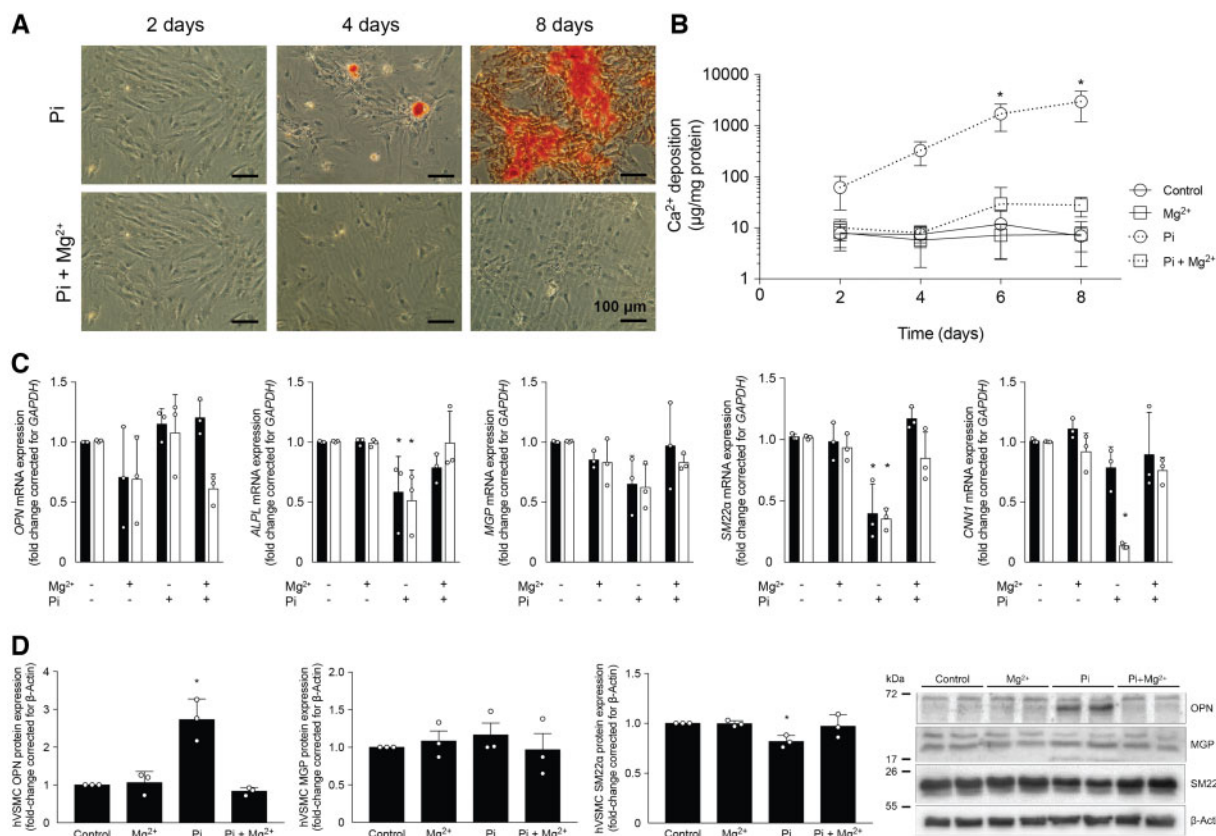
To study the preventive effect of  $Mg^{2+}$  on *in vitro* calcification, VSMCs were cultured in Pi medium and supplemented

with  $MgCl_2$  (Figure 1). VSMCs cultured in high Pi showed calcification, as visualized by the alizarin red staining (Figure 1A) and as quantified by the *o*-cresolphthalein complexone method for  $Ca^{2+}$  (Figure 1B). VSMCs cultured in high Pi supplemented with  $Mg^{2+}$  stained negative for alizarin red and  $Ca^{2+}$  deposition was reduced by  $>100$ -fold versus high Pi-treated VSMCs after 8 days ( $28 \pm 12$  versus  $2990 \pm 1800 \mu g Ca^{2+}/mg$  protein;  $P < 0.05$ ). The preventive effect of  $Mg^{2+}$  on hVSMC calcification was preserved at 4 and 5 mmol/L Pi (Supplementary data, Figure S1). To evaluate the effect of  $Mg^{2+}$  on genetic changes induced by calcification in VSMCs, the gene expression of calcification inducers alkaline phosphatase (*ALP*) and *OPN* and calcification inhibitor *MGP* in addition to contractile markers calponin (*CNN1*) and *SM22 $\alpha$*  were studied (Figure 1C). Compared with control VSMCs, VSMCs cultured in 3 mmol/L Pi had a 40–60% reduction in *ALP* and *SM22 $\alpha$*  gene expression after both 6 and 8 days of incubation ( $P < 0.05$ ). *MGP* mRNA expression remained stable. After high Pi treatment for 8 days, VSMCs expressed only  $\sim 20\%$  of *CNN1* gene expression compared with control VSMCs ( $P < 0.05$ ). Pi-treated VSMCs did not change *OPN* gene expression.  $Mg^{2+}$  supplementation in addition to high Pi preserved the levels of *MGP*, *ALP*, *SM22 $\alpha$*  and *CNN1* gene expression versus control VSMCs. VSMCs treated with 2 mmol/L  $Mg^{2+}$  alone and in combination with 3 mmol/L Pi showed a non-significant downregulation of *OPN* after 8 days. On a protein level, *OPN* was increased 2-fold by Pi treatment ( $P < 0.05$ ). Furthermore, *SM22 $\alpha$*  protein expression was decreased after 8 days of Pi treatment. In high Pi-treated hVSMCs supplemented with  $Mg^{2+}$ , both the increased *OPN* and decreased *SM22 $\alpha$*  protein expression were normalized. *MGP* protein expression remained stable in all treatment groups (Figure 1D).

### CPPs induce VSMC calcification independent of $Mg^{2+}$

To be able to distinguish between intracellular and extracellular actions of  $Mg^{2+}$ , the effects of  $Mg^{2+}$  on CPP2-induced VSMC calcification were assessed (Figure 2). Alizarin red staining (Figure 2A) and  $Ca^{2+}$  deposition measurements (Figure 2B) showed increased calcification after 24 h in VSMCs treated with 50 CPP2 ( $488 \pm 62$  versus  $2 \pm 2 \mu g Ca^{2+}/mg$  protein;  $P < 0.05$ ).  $Mg^{2+}$  supplementation to CPP2-treated VSMCs did not reduce  $Ca^{2+}$  deposition compared with CPP2-only treated VSMCs after 24 h ( $488 \pm 62$  versus  $565 \pm 62 \mu g Ca^{2+}/mg$  protein) and 4 days ( $657 \pm 132$  versus  $783 \pm 142 \mu g Ca^{2+}/mg$  protein, respectively). CPP2 treatment decreased *CNN1* gene expression by almost 50% after both 2 days and 4 days (Figure 2C;  $P < 0.05$ ). CPP2 treatment resulted in downregulation of *OPN* and *ALP* after 2 days, which seemed to be prevented by  $Mg^{2+}$ . *SM22 $\alpha$*  mRNA expression was decreased after 2 days of CPP treatment and after 4 days of  $Mg^{2+}$ -supplemented CPP2 treatment. Protein concentrations of *OPN* were increased  $\sim 4$ -fold in both CPP2-treated hVSMCs with and without 2 mmol/L  $Mg^{2+}$  (Figure 2D). *SM22 $\alpha$*  protein expression showed a non-significant trend towards a reduction in CPP2-treated cells independent of  $Mg^{2+}$ . *MGP* protein expression was stable in all treatment groups. To test whether  $Mg^{2+}$  might prevent CPP-induced calcification at lower CPP2





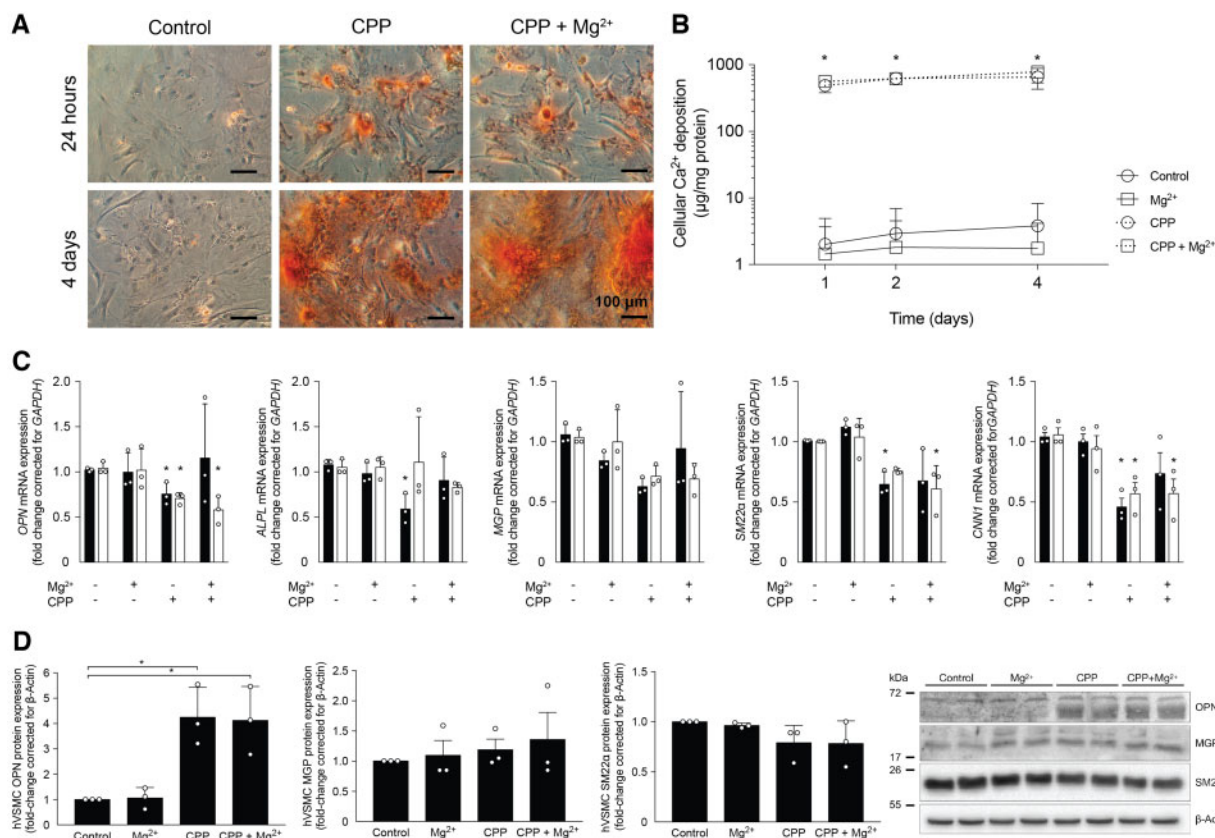
**FIGURE 1:** Mg<sup>2+</sup> prevents Pi-induced calcification in hVSMCs. (A) Alizarin red staining was negative in Mg<sup>2+</sup>-supplemented Pi cultures, indicating the absence of calcification after 2, 4 and 8 days. (B) Ca<sup>2+</sup> deposition on VSMCs was increased in Pi-treated cells compared with controls, but was absent after 2.0 mmol/L Mg<sup>2+</sup> supplementation. (C) Pi-induced calcification decreased ALP and non-significantly decreased calcification inhibitor MGP and VSMC contractile genes SM22α and CNN1 after 6 days (black bars) and 8 days (white bars) of incubation, which was prevented by Mg<sup>2+</sup>. (D) Protein expression of OPN, SM22α and MGP was measured and quantified. Data are shown as fold changes compared with the day-matched controls and presented as the mean of three separate experiments, consisting of three replicate cultures ± SD. \*P < 0.05 versus day-matched control.

concentrations, Mg<sup>2+</sup> was supplemented to VSMCs treated with a broad range of CPP2 dosages (Figure 3). Both 2 and 5 mmol/L Mg<sup>2+</sup> did not reduce VSMC Ca<sup>2+</sup> deposition at any of the CPP2 dosages, ranging from 5 to 100 CPP. Compared with CPP2 treatment containing no additional Mg<sup>2+</sup>, supplementation of 5 mmol/L Mg<sup>2+</sup> resulted in increased Ca<sup>2+</sup> deposition at 25, 50 and 75 CPP2 [82 ± 5% versus 99 ± 16% at 75 CPP (P < 0.05), respectively, where 100 CPP2 with no additional Mg<sup>2+</sup> is set to 100% calcification].

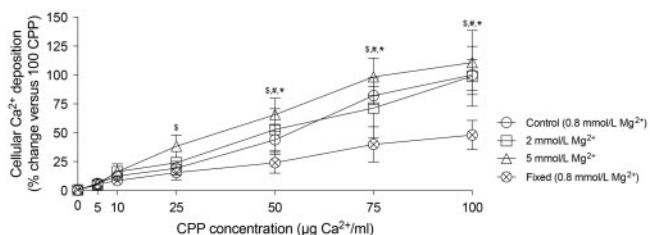
### Mg<sup>2+</sup> dose-dependently prevents secondary CPP maturation

To study the action of Mg<sup>2+</sup> on CPP transition *in vitro*, CPP medium was incubated to form CPP1 and CPP2 in the presence of a range of MgCl<sub>2</sub> concentrations. CPP medium showed an initial increase in absorbance reflecting CPP1 formation after 1 day (absorbance change from 0.042 to 0.055) and the second increase in absorbance reflecting CPP2 formation after 3 days (absorbance change from 0.055 to 0.138; Figure 4A). Mg<sup>2+</sup> supplementation resulted in a similar increase in absorbance after 1 day of culture (absorbance change of supernatant from 0.042 to 0.057 for 2.0 mmol/L Mg<sup>2+</sup>). Mg<sup>2+</sup> final concentrations of 1.6,

1.8 and 2.0 mmol/L preserved the CPP1 state for 14 days (final absorbance 0.077). A final concentration of 1.0, 1.2 and 1.4 mmol/L delayed the second increase in absorbance by 1, 7 and 11 days, respectively. In the presence of 4 and 5 mmol/L Pi, 2 mmol/L Mg<sup>2+</sup> inhibited CPP1 to CPP2 transition (Supplementary data, Figure S1). Increasing Mg<sup>2+</sup> to a concentration of 2.0 mmol/L to CPP medium after CPP2 had formed (on Day 5) did not result in changes in the absorption of the CPP medium (final absorbance 0.170 versus 0.174). After 14 days, CPP2 and CPP2 supplemented with Mg<sup>2+</sup> were isolated and analysed by EM and EDX to study CPP characteristics (Figure 4B–G). Mg<sup>2+</sup> supplementation did not affect the pH of the CPP mixture (Supplementary data, Figure S2). SEM revealed similar morphology of the CPP2 independent of Mg<sup>2+</sup> treatment (Figure 4B and E). EDX peaks were present at energy levels corresponding to enriched Pi and Ca<sup>2+</sup> (Figure 4C and F). Quantification of the peaks showed that Mg<sup>2+</sup>-supplemented CPP2 contains 37.1% oxygen, 18.7% Pi and 39.4% Ca<sup>2+</sup>, which did not significantly differ from CPP2 without Mg<sup>2+</sup> supplementation (Figure 4D and G). In addition, there was a slight trend towards increased Na<sup>+</sup>, Cl<sup>-</sup> and Mg<sup>2+</sup> content in Mg<sup>2+</sup>-supplemented CPP2, but this was not statistically significant.



**FIGURE 2:** Mg<sup>2+</sup> does not prevent CPP2-induced calcification in hVSMCs. (A) Alizarin red staining and (B) Ca<sup>2+</sup> deposition quantification show that CPP2-induced calcification is not prevented by 2.0 mmol/L Mg<sup>2+</sup> after 24 h and 4 days. (C) Gene expression related to calcification after CPP2 treatment was measured after 2 days (black bars) and 4 days (white bars) and remained unchanged by Mg<sup>2+</sup>. (D) Protein expression of OPN, SM22α and MGP was measured and quantified. Data are shown as fold changes compared with the day-matched controls and presented as the mean of three separate experiments, consisting of three replicate cultures ± SD. \*P < 0.05.



**FIGURE 3:** High Mg<sup>2+</sup> concentration stimulates CPP2-induced calcification. Ca<sup>2+</sup> deposition was measured after incubation of VSMCs for 24 h with varying CPP2 concentrations in the presence of 0.8 (control), 2 and 5 mmol/L Mg<sup>2+</sup>. Mg<sup>2+</sup> did not prevent CPP2-induced calcification at any CPP concentration. At 5 mmol/L, Mg<sup>2+</sup> increased cellular Ca<sup>2+</sup> deposition at CPP2 concentrations of 25, 50 and 75 µg/mL Ca<sup>2+</sup> versus CPP2 treatment without additional Mg<sup>2+</sup>. Data are shown as fold changes compared with 100 CPP (set at 100%). Presented data are the mean of three separate experiments consisting of three replicate cultures ± SD. P < 0.05 for CPP2 incubated with \*0.8, #2 and \$5 mmol/L Mg<sup>2+</sup> versus CPP2 concentration-matched formalin-fixed cultures.

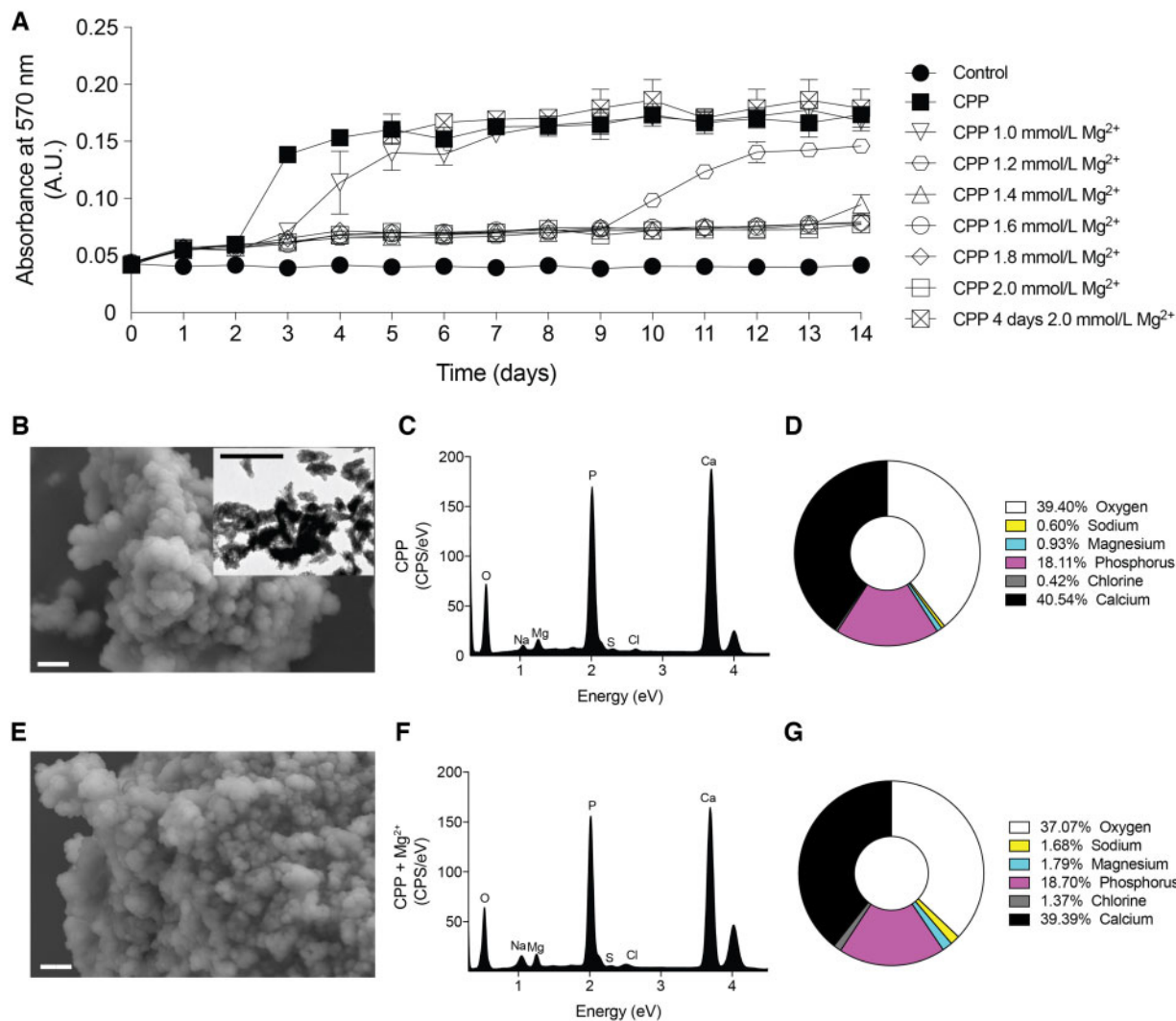
### Mg<sup>2+</sup> improves calcification propensity in serum from CKD patients and healthy controls

To examine the effects of Mg<sup>2+</sup> on serum calcification propensity in CKD patients, the serum of CKD patients and

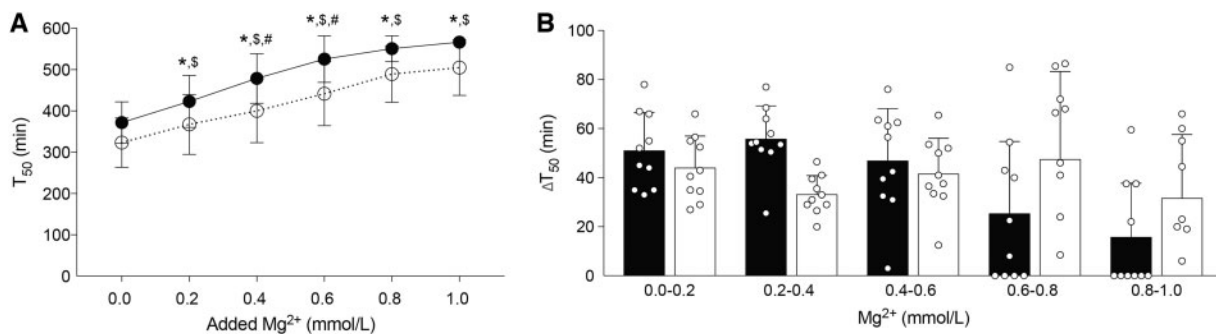
healthy controls was supplemented with increasing concentrations of Mg<sup>2+</sup> *ex vivo* and subsequently T<sub>50</sub> was measured. Serum Mg<sup>2+</sup> concentration was not different between the groups (0.8 ± 0.2 versus 0.9 ± 0.5 mmol/L). There was a non-significant trend towards a lower baseline T<sub>50</sub> in CKD patients compared with controls (372 ± 50 versus 323 ± 57 min). The addition of 0.2 mmol/L Mg<sup>2+</sup> significantly improved T<sub>50</sub> in donor patients by 51 ± 16 min and CKD patients by 44 ± 13 min (Figure 5A; P < 0.05). Each increment of 0.2 mmol/L addition of Mg<sup>2+</sup> led to a similar increase of ~40 min in CKD patients and 50 min in healthy controls (Figure 5B).

## DISCUSSION

In this study we demonstrate that Mg<sup>2+</sup> prevents high Pi-induced VSMC calcification. The preventive effect of Mg<sup>2+</sup> is mediated via delayed CPP2 formation, because Mg<sup>2+</sup> is unable to prevent calcification once CPP2 has formed. Specifically, we show that Mg<sup>2+</sup> does not prevent CPP1 formation. Rather, Mg<sup>2+</sup> prevents the transition from CPP1 to CPP2. In the sera of CKD patients and healthy controls, a small increase in Mg<sup>2+</sup> of 0.2 mmol/L improved T<sub>50</sub>, reflecting a decreased calcification propensity. In addition, our results indicate that crystalline maturation of Pi towards CPP2 is an essential step in VSMC calcification.



**FIGURE 4:** Mg<sup>2+</sup> dose-dependently inhibits the transition from CPP1 to CPP2 and does not affect CPP2 morphology after nucleation. (A) Absorbance of the CPP mixture containing different Mg<sup>2+</sup> concentrations was measured at 570 nm as a readout for CPP1 (~0.05 AU) to CPP2 (~0.15 AU) transition. This graph is representative of five independently executed experiments each consisting of three replicates. Data are expressed as mean ± SD. A final concentration of 2.0 mmol/L Mg<sup>2+</sup> after CPP2 (E–G) formation did not alter morphology (SEM) or composition (EDX) compared with control CPP2 (B–D). A TEM picture was inserted in B to confirm CPP2. Scale bars correspond to 300 nm (white) and 500 nm (black).



**FIGURE 5:** Mg<sup>2+</sup> dose-dependently increases T<sub>50</sub> in CKD patient serum. Serum T<sub>50</sub> is increased upon Mg<sup>2+</sup> supplementation of serum from both healthy controls (solid line) and CKD patients (dotted line, A). (B) Change in T<sub>50</sub> similar in CKD patients after each addition of Mg<sup>2+</sup> compared with healthy controls. Black and white bars represent healthy controls and CKD patients, respectively. Data are shown as the mean of 10 patients in each group and are expressed as mean ± SD. P < 0.05 \*for healthy controls versus baseline; \$for CKD patients versus baseline and #for CKD patients versus healthy controls at specific Mg<sup>2+</sup> concentration.



The mechanism by which  $Mg^{2+}$  prevents high Pi-induced calcification in CKD models is incompletely understood. Using different *in vitro* models, we demonstrate that  $Mg^{2+}$  only prevents Pi-induced VSMC calcification prior to formation of CPP2. After CPP2 formation,  $Mg^{2+}$  supplementation did not alter crystal composition and morphology and did not prevent calcification of VSMCs. In addition,  $Mg^{2+}$  supplementation did not modify CPP2-induced expression profiles of osteogenic or VSMC contractility genes and proteins. In contrast,  $Mg^{2+}$  potently prevents Pi-induced calcification through indirect pathways, as an increased concentration of  $Mg^{2+}$  was insufficient to prevent VSMC calcification induced by CPP2. We have shown previously in a bovine model of VSMC calcification that  $Mg^{2+}$  blocks  $Ca^{2+}$ -Pi crystal formation preceding Pi-induced calcification and independent of transcriptional changes in bovine VSMCs [19]. Other studies have identified the potent anti-crystallization properties of  $Mg^{2+}$  [20, 22, 29]. In aqueous solutions,  $Mg^{2+}$  is known to stabilize amorphous  $Ca^{2+}$ -Pi particles and delay crystal nucleation [30]. Contrarily, another study suggested that the preventive action of  $Mg^{2+}$  on VSMC calcification did not depend on the inhibition of CPP2 maturation [31]. For instance, in the study by Louvet *et al.* [31], hydroxyapatite crystals were generated in the presence of hVSMC with 1% FBS only, whereas we used 5% FBS [31]. In low serum conditions, the availability of calcification inhibitors such as fetuin-A is lower, resulting in a milieu that is more prone to calcification. Thus the inhibitory effects of  $Mg^{2+}$  on CPP2 formation may potentially be overruled as Pi can precipitate more readily [24].

In our study,  $Mg^{2+}$  blocked calcification induced by high Pi, which is consistent with previous studies [15, 16, 18, 32]. When comparing our results, it is important to note that the reported experimental setups have used high Pi to induce VSMC calcification and have supplemented  $Mg^{2+}$  immediately in order to prevent calcification [15, 16, 18, 32]. Under these circumstances, it is possible that  $Mg^{2+}$  blocked crystal or CPP2 maturation that would otherwise have occurred naturally. Therefore inhibition of Pi-induced calcification may have been secondary to the prevention of CPP2 formation. With high Pi, medium crystals form spontaneously over time. The cellular effects of CPP2-induced calcification are already present 24 h after induction, while those of high Pi-induced calcification only occur after ~1 week. Accordingly, it was shown that crystalline Pi-containing particles induce calcification and VSMC transdifferentiation rather than soluble Pi or amorphous  $Ca^{2+}$ -Pi particles (or CPP1) [6, 8, 11].

Calcification propensity ( $T_{50}$ ) is a risk factor and indicator for cardiovascular morbidity and mortality in CKD patients [23, 33, 34]. To translate the effects of  $Mg^{2+}$  on CPP2 maturation to a more clinical setting, we investigated the effects of stepwise  $Mg^{2+}$  additions to serum from renal disease patients and healthy controls on  $T_{50}$ . Our results demonstrate that  $Mg^{2+}$  increased  $T_{50}$  by ~50 min per 0.2 mmol/L  $Mg^{2+}$  increase in serum from CKD patients and healthy controls. Interestingly, the dose-dependent effects of  $Mg^{2+}$  on calcification propensity are similar in both groups. These *ex vivo* results are consistent

with a previous study in patients on dialysis [35, 36]. It was demonstrated that  $Mg^{2+}$  effectively and promptly increased  $T_{50}$  by 72 min once dialysate  $Mg^{2+}$  was adjusted from 0.5 to 1.0 mmol/L, which resulted in a serum increase of 0.36 mmol/L [36]. Dialysate  $Mg^{2+}$  supplementation increased  $T_{50}$  without modifying intracellular  $Mg^{2+}$  concentrations, consistent with the notion that serum  $Mg^{2+}$  is key to modify calcification propensity [36]. The ability of  $Mg^{2+}$  to potently modify  $T_{50}$  is of great clinical interest. Given the sensitivity of  $T_{50}$  for  $Mg^{2+}$ , this relationship should be clinically exploited. Further clinical studies should investigate whether *ex vivo* determinations of calcification propensity could be used to define optimal dialysate or oral  $Mg^{2+}$  concentration for CKD patients.

Our results indicate that cellular Pi toxicity leading to calcification is determined by the presence of crystalline Pi in the form of CPP2. Efforts to prevent vascular calcification are currently aimed at lowering serum Pi concentrations in CKD patients. Importantly, management of serum Pi by Pi binders is insufficient to decrease the risk for cardiovascular disease and vascular calcification in CKD [37]. Instead, determinants affecting the transition from Pi towards crystalline Pi (CPP2) potentially prove to be more clinically relevant.  $Mg^{2+}$  prevented VSMC calcification *in vitro* despite high Pi concentrations and improved calcification propensity in humans without normalizing serum Pi concentrations [19, 36]. In addition, increased serum  $Mg^{2+}$  (>1.27 mmol/L) neutralizes the association between serum Pi concentration and the risk for cardiovascular mortality [38]. Therefore  $Mg^{2+}$  potentially disarms Pi toxicity in CKD without changing the soluble Pi concentration itself. As such, Pi toxicity, and therefore calcification risk, should be clinically determined based on Pi crystallinity.

This is the first study that uses CPP2-induced VSMC calcification to distinguish intracellular effects from extracellular effects of  $Mg^{2+}$ . In addition, our approach includes *in vitro* models and an *ex vivo* study coupling the effects of  $Mg^{2+}$  to calcification propensity. Although we demonstrate that  $Mg^{2+}$  limits VSMC calcification primarily through the inhibition of CPP2 maturation already at small incremental concentrations, we cannot exclude potential contributing effects of intracellular pathways related to calcification. While the results presented here need to be verified *in vivo*, our conclusions may have important implications for clinical interpretation of the effects of  $Mg^{2+}$  on calcification. Recently a longer-term randomized clinical study using oral magnesium oxide supplements showed halted progression of coronary artery calcification in predialysis CKD patients [39]. Another study is currently being initiated (ClinicalTrials.gov identifier NCT02542319) [40]. These studies are a major step towards improving cardiovascular outcomes in CKD patients using  $Mg^{2+}$  supplements.

In conclusion, our study demonstrates that  $Mg^{2+}$  prevents hVSMC calcification by inhibition of CPP2 maturation *in vitro*. In serum from CKD patients,  $Mg^{2+}$  increased  $T_{50}$  in a dose-dependent manner. Increasing serum  $Mg^{2+}$  may be a promising treatment to target pathological CPP2 maturation in CKD-induced vascular calcification.



## SUPPLEMENTARY DATA

Supplementary data are available at [ndt online](https://ndt.online).

## ACKNOWLEDGEMENTS

The authors thank Huib Croes and Caro Bos (both from the Radboud University Medical Center, Nijmegen, The Netherlands) and Dr Swapna Karthik (Calciscon, Bern, Switzerland) for their wonderful technical assistance. The authors also thank Dr Jan-Luuk Hillebrands (University Medical Center Groningen, Groningen, The Netherlands) for sharing his expertise related to calciprotein particle synthesis.

## FUNDING

This research was funded by grants from the Netherlands Organization for Scientific Research (NWO Veni 016.186.012 and VICI 016.130.668) and the Dutch Kidney Foundation (Kolff 14OKG17 and 15OP02). This work was furthermore supported by the NIGRAM2+ consortium, funded by Health Holland (LSHM17034) and the Dutch Kidney Foundation (16TKI02).

## AUTHORS' CONTRIBUTIONS

A.D.t.B., J.H.F.d.B., C.E., J.G.J.H. and M.H.d.B. were involved in the conception and design of the experiments. A.D.t.B., C.E., A.P. and L.W.Z. performed experiments and analysed data. S.J.L.B. recruited patients and founded the TransplantLines cohort study. A.D.t.B. drafted the manuscript. A.D.t.B., J.H.F.d.B., C.E., J.G.J.H., A.P. and M.H.d.B. critically revised the manuscript. All authors approved the final version of the manuscript.

## CONFLICT OF INTEREST STATEMENT

M.H.d.B. has served as a consultant or received honoraria (to employer) from Amgen, Bayer, Kyowa Kirin Pharma, Sanofi Genzyme and Vifor Fresenius Medical Care Renal Pharma. A.P. is the inventor of the T<sub>50</sub> test and co-founder, shareholder and employee of Calciscon.

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Received: 9.5.2019; Editorial decision: 27.8.2019