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만성신장질환 환자에서 심혈관계 질환의 예후 예측인자로서의 마그네슘

Magnesium as a predictor of cardiovascular disease in pre-dialysis CKD patients

2022년 8월

서울대학교 대학원
임상의과학과
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지도교수 오 국 환

이 논문을 의학박사 학위논문으로 제출함
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Abstract

Magnesium as a predictor of cardiovascular disease in pre-dialysis CKD patients

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Background: There are few large-scale studies of the association between magnesium (Mg) and cardiovascular (CV) outcomes in pre-dialysis chronic kidney disease (CKD) patients. The effect of Mg on vascular calcification through the NF-κB pathway has not been elucidated. Therefore, I investigated the effects of Mg on CV outcomes in a large-scale cohort of pre-dialysis CKD patients and explored the mechanism of Mg in vascular calcification through NF-κB.

Methods: I investigated the association between serum Mg and CV outcomes in a prospective, multi-center cohort of pre-dialysis CKD patients (n=1,646). Patients were divided into three groups according to serum Mg concentration. The primary endpoint was composite outcome, defined as either a CV event and/or all-cause death. Secondary outcomes were coronary artery calcification (CAC) progression and arterial stiffness progression as assessed by mean brachial-ankle pulse wave velocity (baPWV). Experiments were conducted with primary human aortic vascular smooth muscle cells (VSMCs).

Results: During 7,368 person-years of follow up, the primary outcome

occurred in 153 patients (20.8 per 1,000 person-years). In a multivariable cause-specific model, patients in the hypomagnesemia group (serum Mg <1.8 mg/dL) were at elevated risk of the composite outcome (hazard ratio (HR) 2.53 [1.12−5.69]; *P*=0.024; serum Mg 1.8−2.2 mg/dL as the reference group). Patients with hypomagnesemia also had exhibited risk of progression to CAC and arterial stiffness. In cultured VSMCs, treatment with $MgCl_2$ blunted phosphate-induced calcification, osteo-chondrogenic signaling, and NF-κB activation.

Conclusions: Low Mg level is a predictor of cardiovascular outcomes in pre-dialysis CKD patients. Mg supplementation ameliorates phosphate-induced osteochondrogenic transdifferentiation of VSMCs and vascular calcification through suppression of NF-κB activation.

Keyword: cardiovascular disease; death; vascular calcification; magnesium; chronic kidney disease

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Chapter 1. General introduction

1.1. Study Background

1.1.1 Vascular calcification as a risk factor for cardiovascular disease in chronic kidney disease

Cardiovascular disease (CVD) is the leading cause of death in patients with chronic kidney disease (CKD) (1). Traditional risk factors for CVD are diabetes mellitus (DM), hypertension, age, and dyslipidemia (2). However, vascular calcification is a non-traditional risk factor associated with CKD that increases the risk of CVD even after adjustment for traditional risk factors (3). As CKD progresses, so does the risk of vascular calcification due to dysregulation of mineral and bone metabolism (4).

1.1.2 Mechanism of the progression of vascular calcification in chronic kidney disease

Under normal physiology, active inducers and active inhibitors of vascular calcification are balanced. However, in CKD, active inducers such as hyperphosphatemia, hypercalcemia, inflammatory cytokine, and oxidative stress increase and active inhibitors such as Matrix Gla protein, Fetuin-A, and bone morphogenetic protein 7 (BMP7) decrease (5). Therefore, as CKD progresses, vascular calcification progresses. In the process of vascular calcification, there are inorganic components such as amorphous calcium phosphate and cellular components such as apoptosis, osteochondrogenic differentiation, matrix vesicle release, and elastin degradation (5, 6). When phosphate (P) is taken up into

vascular smooth muscle cells (VSMC), it causes Runt-related transcription factor 2 (RUNX2) expression and osteogenic gene expression. In addition, P causes apoptosis in VSMCs. VSMCs undergo apoptosis and phenotypic changes. These cause deposition of calcium phosphate nanocrystals onto the vessel wall. When VSMCs endocytose nanocrystals, lysosomal degradation occurs in the cell and calcium and P increase within the intracellular milieu (6). To compensate for this, VSMCs release matrix vesicle loaded with Ca and P. However, if the increase in intracellular Ca and P is overloaded compared with the compensation, apoptosis occurs and the resulting apoptotic bodies and matrix vesicles form nanocrystals, accelerating calcification (6). Use of non-calcium containing P binders to prevent vascular calcification has been shown to improve cardiovascular (CV) and overall outcomes in advanced CKD patients (7), suggesting that the prevention of vascular calcification improves CV outcomes.

1.1.3 Magnesium and cardiovascular disease

Magnesium (Mg) affects cardiac conduction and myocardial contraction by regulating ion channels and the Na/K ATPase pump in cardiac myocytes and pacemaker cells (8). Mg can also cause arrhythmias; a lower serum Mg is associated with a slower heart rate and prolonged QT-interval (9). Therefore, Mg is used as a treatment for arrhythmias such as torsade de pointes (10). The relationship between Mg and vascular calcification has been investigated. Experimental studies have demonstrated that Mg inhibits calcification through cellular and molecular mechanisms (11, 12). Mg suppresses calcification induced by phosphorus (11) and up-regulates expression of the Matrix-Gla protein, an

inhibitor of vascular calcification. Mg also inhibits transcription factors that mediate osteochondrogenic differentiation of vascular smooth cells via transient receptor potential melastin 7 cation channels (12). In addition, Mg prevents apoptosis, which causes calcification (11). Mg is considered a calcification inhibitor (11, 12).

1.1.4 Magnesium in CKD

Renal handling of Mg plays an important role in Mg homeostasis. In order to maintain Mg in the normal range until CKD stage 3, reduced renal function is compensated for by increasing fractional Mg excretion (13). However, when CKD progresses below glomerular filtration rate (GFR) 30 ml/minute, the compensatory mechanism is insufficient and hypermagnesemia might occur (14). In addition, hypermagnesemia was associated with all-cause mortality and CV event (15), it was a common view to monitor hypermagnesemia in CKD patients. However, it has recently been reported that hypomagnesemia is not rare in CKD patients (16). Hypomagnesemia is commonly observed in patients with DM (17) and is also caused by medication such as diuretics, proton pump inhibitor and calcineurin inhibitors (18, 19). In addition, vitamin D deficiency also cause hypomagnesemia (20). These risk factors of hypomagnesemia are associated with patients with CKD, therefore, it is important to monitor hypomagnesemia in patients with CKD.

1.1.5 Previous studies on hypomagnesemia and cardiovascular diseases in CKD patients

Epidemiologic studies have shown that low Mg values in the general population

are associated with an increased risk of all-cause mortality and CVD (9). However, the association between Mg and CVD and all-cause mortality in CKD patients remains controversial (21-29). Most studies in CKD patients were conducted in those with end-stage renal disease (ESRD) undergoing dialysis (22, 23, 27-29). When conducting Mg-related studies, it is necessary to distinguish between pre-dialysis CKD and dialyzed patients because of the concentration of Mg in dialysis fluid (30). The few studies of pre-dialysis CKD patients that have been performed have reported an inverse relationship between Mg and CVD or all-cause death (21, 24-26, 31). However, these studies were small-scale (24, 25, 31) or limited to specific CKD stages (24, 31)

1.1.6 The role of NF-κB in vascular calcification

Nuclear factor kappa B (NF-κB) was discovered in 1980 by Sen and Baltimore (32). Phosphorylation-dependent ubiquitination and degradation of IκBα causes nuclear translocation of NF-κB, which acts as a transcription factor (33, 34). Activated NF-κB subunits, including p65/*RelA*, migrate to the nucleus and initiate gene transcription (35). As a transcription factor, NF-κB plays an important role in immunity, inflammation, cell proliferation, differentiation, and survival (36). In particular, NF-κB acts as a major regulator of osteochondrogenic transdifferentiation, leading to vascular calcification (37). In hyperphosphatemia, NF-κB is activated (37). NF-κB stimulates VSMC mineralization (33, 38), causes upregulation of RUNX2, which is an osteogenic transcription factor (39), and increases expression of MSX2, which induces expression of alkaline phosphatase (ALP), a key molecule in VSMC mineralization (40). Collectively, activation of

NF-κB causes vascular calcification. In other words, when NF-κB is inhibited, hyperphosphatemia-induced vascular calcification can be prevented (33, 37, 41).

1.1.7 Previous studies related to magnesium and NF-κB

Previous studies have shown the effect of Mg on NF-κB. Mg inhibited NF-κB activation and decreased inflammatory cytokine production in neonate cells (42). Mg therapy also inhibited NF-κB protein production in rats (43). Human umbilical vein endothelial cells cultured in low Mg led to activation of NF-κB (44), which increased the secretion of the important factors in atherogenesis such as interleukin 8 and platelet-derived growth factor BB, suggesting that low Mg cause endothelial dysfunction through pro-inflammatory and pro-atherogenic processes. Mg decreased NF-κB activation and reduced cytokine production in mesenchymal stem cells (45). Mg also inhibited macrophage-induced inflammation and enhanced chondrogenic differentiation in these cells. Collectively, previous studies showed that Mg inhibited NF-κB activation. Meanwhile, to the best of my knowledge, none of the studies on Mg in the NF-κB pathway were conducted with VSMCs.

1.2. Purpose of Research

In the present study, I investigated the association between Mg and the composite outcome of either cardiovascular event or all-cause death in pre-dialysis CKD patients from the KoreaN cohort study for Outcome in patients With CKD (KNOW-CKD). I also analyzed the association between Mg and coronary artery calcification (CAC) progression, which is a strong predictor of future cardiac events in CKD patients (46). I intend to show the mechanism by which Mg inhibits

vascular calcification through the suppression of NF-κB.

Chapter 2. Low magnesium predicts cardiovascular outcomes in pre-dialysis CKD patients

2.1. Materials and Methods

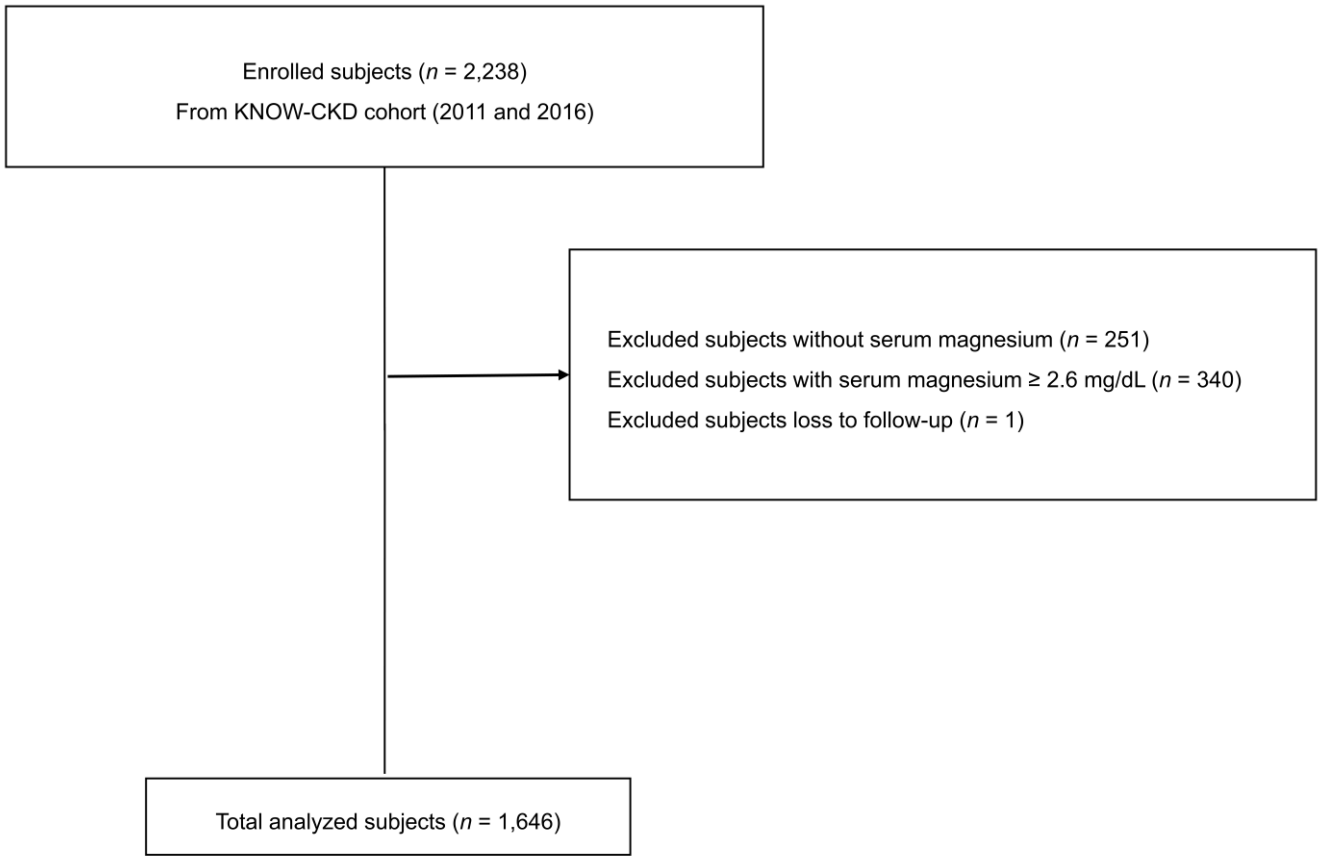
2.1.1 Study participants

The KNOW-CKD is an ongoing, nationwide, multicenter, prospective cohort study involving nine tertiary hospitals in Korea (47). Briefly, pre-dialysis CKD patients (n=2,238) with all stages of CKD aged 20-75 years were enrolled from 2011 to 2016. Patients with a history of dialysis, any organ transplant, heart failure (NYHA function class III or IV), liver failure, history of cancer, a single kidney, or pregnant at the time of the study were excluded. The study complies with the Declaration of Helsinki and was approved by the institutional review boards of the participating centers: Seoul National University Hospital (H-1704-025-842), Seoul National University Bundang Hospital (B-1106/129-008), Yonsei University Severance Hospital (4-2011-0163), Kangbuk Samsung Medical Center (2011-01-076), Seoul St. Mary's Hospital (KC11OIMI0441), Gil Hospital (GIRBA2553), Eulji General Hospital (201105-01), Chonnam National University Hospital (CNUH-2011-092), and Busan Paik Hospital (11-091).

Subjects with an unmeasured serum Mg level (n=251) were excluded at study entry. Given that my aim was to investigate the risk of a low level of Mg, we excluded subjects with serum Mg ≥2.6 mg/dL (n=340), as this has been shown to be associated with increased CV mortality and all-cause mortality (15). Finally, after excluding one subject lost to follow-up (n=1), 1,646 patients were analyzed

(Figure 2.1.1). Subjects were classified into the following groups according to serum Mg level: hypomagnesemia (<1.8 mg/dL), normomagnesemia (1.8-2.2 mg/dL), or hypermagnesemia (>2.2 mg/dL).

Figure 2.1.1 Flow diagram of participants involved in analyzing primary outcome



2.1.2 Data collection and laboratory measurements

Data regarding demographics, past medical history, and medications were obtained through self-reports and medical records. Baseline laboratory data were serum Mg, serum P, calcium, alkaline phosphatase (ALP), intact parathyroid hormone (iPTH), 25-hydroxy vitamin D, total CO_2, hemoglobin, albumin, total cholesterol, triglycerides, high-sensitivity C-reactive protein (hs-CRP), and hemoglobin A1c (Hb A1c) levels. Measured 24-hour urine protein also was included. Baseline serum Mg was measured by colorimetry (ADVIA Chemistry XPT, Siemens, Erlangen, Germany) with an assay range of 0.4-5.0 mg/dL at a central laboratory (Lab Genomics, Seoul, Republic of Korea). Mg level was measured to the nearest tenth. An isotope dilution mass spectrometry-calibrated method was used to measure serum creatinine at the central laboratory. Estimated GFR (eGFR) was estimated by the Chronic Kidney Disease Epidemiology Collaboration (48). Corrected calcium was defined by the following formula: serum total calcium (mg/dL) + 0.8 × [(4 – serum albumin (g/dL)] [if serum albumin <4.0 g/dL] (49). CVDs as the baseline comorbid condition were defined as coronary artery disease, peripheral vascular disease, cerebrovascular disease, congestive heart failure, or arrhythmia at the time of study enrollment. Uncontrolled hypertension was defined as systolic blood pressure (SBP) ≥130 mmHg or diastolic blood pressure (DBP) ≥80 mmHg (50). Obesity was defined as body mass index (BMI) ≥25 kg/m^2 (51).

2.1.3 Coronary artery calcification measurements

Electrocardiography-gated multi-slice computed tomography scanning of the thorax was conducted according to standard protocols. CAC score was determined

as described by Agatston et al. (52). A committee of investigators reviewed all data. CAC was assessed at study entry and 4 years.

2.1.4 Pulse wave velocity measurements

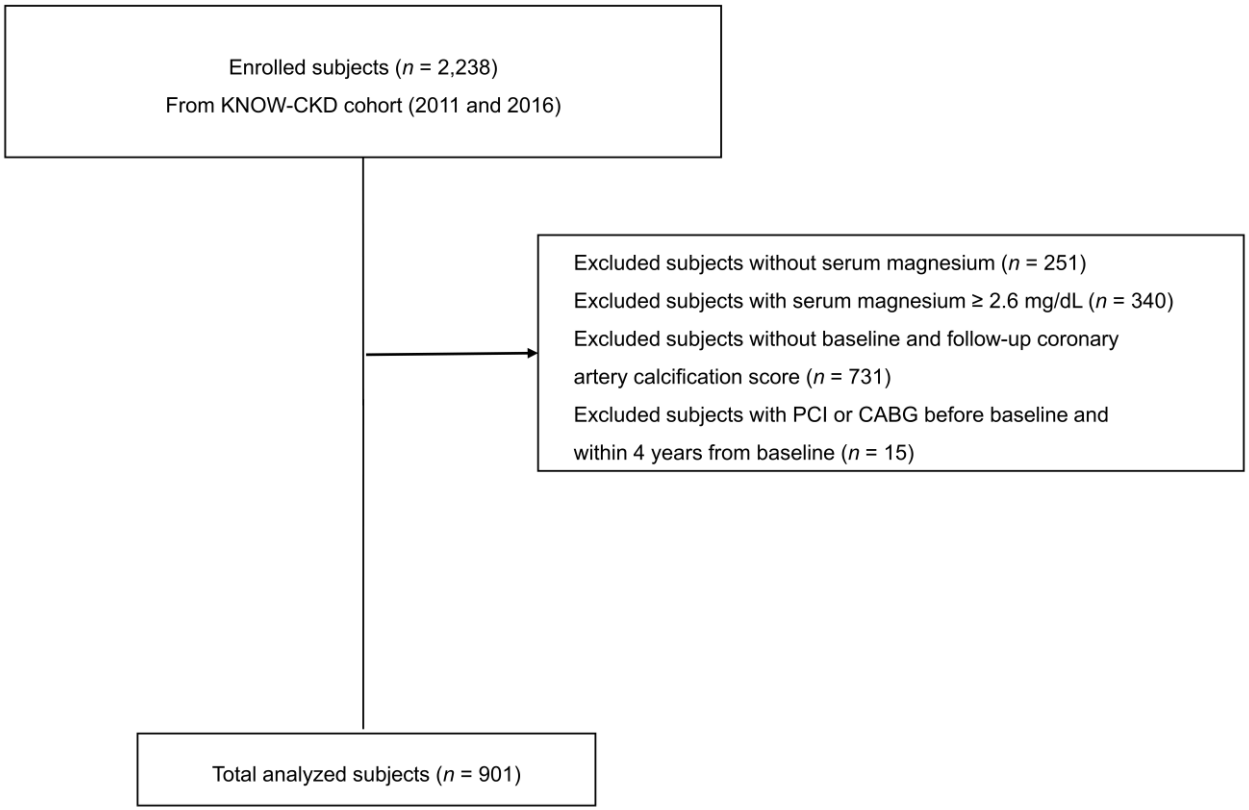
Brachial-to-ankle pulse wave velocity (baPWV), which is a marker of arterial stiffness (53), was measured using a VP-1000 analyzer (Collin co., Komaki, Japan). Subjects rested in the supine position for 5 minutes and were examined. Cuffs were placed on the brachia and ankles. Waveforms of bilateral brachial and posterior-tibial arterial pressures were stored for 10 seconds by extremity cuffs connected to a plethysmographic sensor and an oscillometric pressure sensor wound around both arms and ankles. Electrodes placed on both wrists were used to obtain an electrocardiogram. S1 and S2 heart sounds were detected by a microphone on the left edge of the sternum in the third intercostal space. The time interval between the wave front of the brachial waveform and that of the ankle waveform was the time interval between the brachium and ankle and was measured using a VP-1000 analyzer. Distance between baPWV sampling points was adjusted for patient height. Distances from the suprasternal notch to the brachium and from the suprasternal notch to the ankle were calculated. I used the mean value of the right baPWV and left baPWV. PWV was assessed at study entry and at 4 years.

2.1.5 Outcomes

The primary outcome was the composite of the first occurrence of a cardiovascular event and the onset of all-cause death. CV events were defined as myocardial infarction, unstable angina, coronary revascularization, stroke, heart failure, and

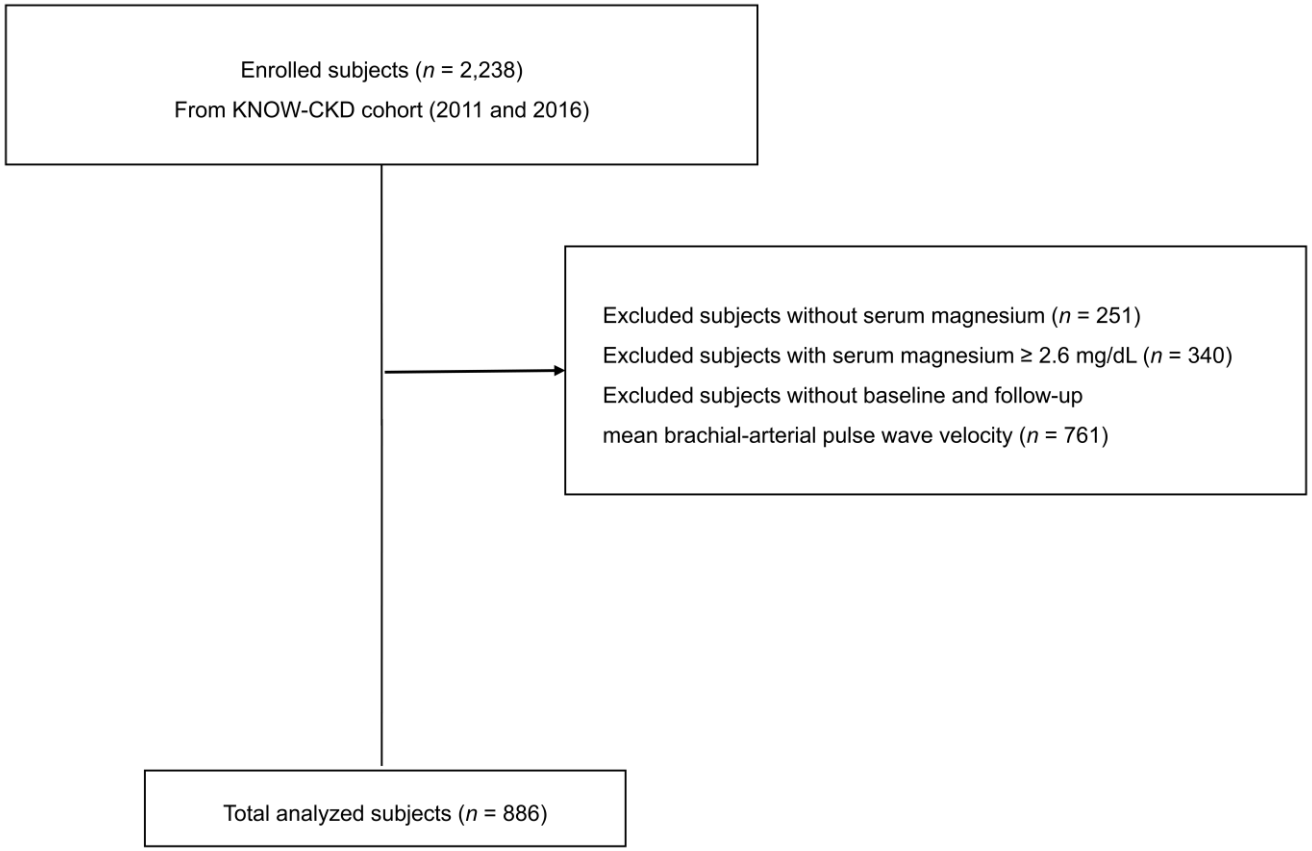
other CV events (symptomatic arrhythmia, peripheral artery disease, cerebral aneurysm with coiling or stent, aggravated valvular heart disease, abdominal aortic aneurysm, and pericardial disease). Survival time was defined as the time from enrollment to occurrence of the event or the last follow-up. Subjects who were lost to follow-up were censored at the time of the last study visit. Secondary outcomes were CAC progression, which was defined as a difference ≥2.5 between the square root (√) of the baseline and follow-up CAC scores (54), and arterial stiffness progression, which was defined as a change (%) in mean PWV ≥10% between baseline and the 4 year follow-up (55). Specifically, to analyze CAC progression, I included only subjects for whom both baseline and follow-up CAC scores were measured (n=916) and excluded those (n=15) who underwent coronary revascularization during the 4-year follow-up. Finally, 901 subjects were included in the analysis of CAC progression (Figure 2.1.2). To analyze arterial stiffness progression, I included subjects for whom both baseline and follow-up mean baPWVs were measured. A total of 886 subjects was included in the final analysis of arterial stiffness progression (Figure 2.1.3).

Figure 2.1.2 Flow diagram of participants involved in analyzing coronary artery calcification progression



PCI, percutaneous coronary intervention; CABG, coronary artery bypass graft surgery

Figure 2.1.3 Flow diagram of participants involved in arterial stiffness progression



2.1.6 Statistical analysis

Categorical variables are expressed as number and percentage of participants. Continuous variables are described as mean ± standard deviation or as median with interquartile range (IQR). The Shapiro-Wilk test was used to test normality. Baseline clinical characteristics were compared according to groups of serum Mg. When analyzing the association between Mg and outcomes, the second group of Mg was used as the reference. To analyze *P* values for trends, linear-by-linear association was used for categorical variables, and the Jonckheere-Terpstra test was used for continuous variables. To explore the associations between Mg and the composite of the first occurrence of a CV event and the onset of all-cause death, cause-specific hazard models were used. The composite renal outcome, which was defined as a halving of GFR or the occurrence of ESRD before the composite outcome, was considered a competing risk. ESRD was defined as initiation of renal replacement therapy. Multiple covariates were used in these models. In model 1, demographic information [age, sex, and BMI] was adjusted. In model 2, risk factors associated with CVD and vascular calcification (eGFR, total cholesterol, albumin, phosphorus, and corrected calcium) were further adjusted. In model 3, mean arterial pressure (MAP) additionally was adjusted. Finally, in model 4, DM also was adjusted. Results from multivariable models are presented as hazard ratios (HRs) and 95% confidence intervals (95% CIs). Cumulative composite outcomes of CV event and all-cause death were derived using the cumulative incidence function for competing risk. Fine-Gray subdistribution hazard models were used to confirmed the association observed in the primary analysis. The same multivariable models were used. To explore the association between Mg and progression of

vascular calcification or arterial stiffness, odds ratios (ORs) of CAC progression and arterial stiffness progression were calculated using binary logistic regression. Multivariable models were used. Results from multivariable logistic regression models are presented as ORs and 95% CIs. Cubic spline curves were used to show the association between Mg as a continuous variable and the ORs for CAC progression and arterial stiffness progression. All statistical analyses were performed using SPSS software (version 25; IBM Corp., Armonk, NY, USA) or R software (version 4.0.4; www.r-projectorg; R Foundation for Statistical Computing, Vienna). *P* values <0.05 were considered significant.

2.2. Results

2.2.1 Baseline characteristics at study entry

Table 2.2.1 shows the baseline characteristics at study entry according to serum Mg. Among the study population, median age was 54.0 [IQR 45.0-63.0] years, and 61.3% of subjects were male. Median Mg was 2.2 [IQR 2.1-2.3] mg/dL, and median P was 3.6 [IQR 3.2-4.0] mg/dL. Baseline CAC scores were 0 in 49.6% of subjects, between 0 and 100 in 27.5% of subjects, and ≥100 in 22.9% of subjects. Male sex was predominant in the hypomagnesemia group, which also included more patients with diabetes and more current smokers. In addition, levels of P were lowest in the hypomagnesemia group, while calcium, triglyceride, Hb A1c, and proteinuria levels were highest in this group.

Table 2.2.1 Baseline participant characteristics at study entry

Variable	Serum magnesium (mg/dL)				*P for trend*
	Total (n =1,646)	<1.8 (n = 37)	1.8-2.2 (n = 914)	>2.2 (n = 695)	
Age (year)	54.0 [45.0;63.0]	54.0 [45.0;61.0]	54.0 [44.0;62.0]	55.0 [45.0;64.0]	0.056
Male (%)	1,009 (61.3)	27 (73.0)	580 (63.5)	402 (57.8)	0.007
Etiology of CKD (%)					0.243
Glomerulonephritis	607 (36.9)	13 (35.1)	346 (37.9)	248 (35.7)	
Diabetic nephropathy	359 (21.8)	7 (18.9)	197 (21.6)	155 (22.3)	
Hypertensive nephropathy	292 (17.7)	6 (16.2)	151 (16.5)	135 (19.4)	
Polycystic kidney disease	288 (17.5)	3 (8.1)	157 (17.2)	128 (18.4)	
Others	100 (6.1)	8 (21.6)	63 (6.9)	29 (4.2)	
eGFR (ml/min per 1.73 m^2)	50.4 [31.2;76.5]	47.8 [37.0;76.4]	52.6 [33.5;79.2]	46.5 [28.7;72.3]	0.004
Coronary artery calcification score (AU) (%)					0.459
0	789 (49.6)	17 (48.6)	442 (49.9)	330 (49.3)	
0< <100	437 (27.5)	11 (31.4)	249 (28.1)	177 (26.4)	
≥100	364 (22.9)	7 (20.0)	194 (21.9)	163 (24.3)	
Mean PWV (m/s)	14.5 [12.9;17.0]	15.6 [13.3;17.4]	14.5 [13.0;16.9]	14.4 [12.8;17.0]	0.510
Systolic blood pressure (mmHg)	127.0 [118.0;136.0]	127.0 [118.0;138.0]	127.0 [118.0;136.0]	126.0 [117.0;136.0]	0.447
Diastolic blood pressure (mmHg)	77.0 [70.0;84.0]	75.0 [67.0;82.0]	78.0 [70.0;85.0]	76.0 [69.0;82.0]	0.004
Pulse pressure (mmHg)	49.0 [42.0;57.0]	51.0 [43.0;57.0]	49.0 [42.0;58.0]	50.0 [42.0;57.0]	0.442
Body mass index (kg/m^2)	24.4 [22.4;26.5]	24.9 [22.2;26.8]	24.5 [22.7;26.7]	24.3 [22.1;26.4]	0.140
Use of medication					

Variable	Serum magnesium (mg/dL)				*P for trend*
	Total (n =1,646)	<1.8 (n = 37)	1.8-2.2 (n = 914)	>2.2 (n = 695)	
Vitamin D agonists (%)	26 (5.7)	0 (0.0)	13 (5.6)	13 (5.8)	0.810
Phosphate binders (%)	137 (29.8)	1 (20.0)	76 (32.9)	60 (26.8)	0.223
Diuretics (%)	485 (31.3)	13 (35.1)	262 (30.4)	210 (32.3)	0.609
Diabetes (%)	538 (32.7)	17 (45.9)	309 (33.9)	212 (30.5)	0.047
Cardiovascular disease (%)	251 (15.2)	7 (18.9)	141 (15.4)	103 (14.8)	0.577
Smoking					0.033
Never	863 (52.5)	14 (37.8)	461 (50.4)	388 (56.0)	
Former	504 (30.7)	12 (32.4)	290 (31.7)	202 (29.1)	
Current	277 (16.8)	11 (29.7)	163 (17.8)	103 (14.9)	
Laboratory findings					
Magnesium (mg/dL)	2.2 [2.1;2.3]	1.7 [1.6; 1.7]	2.1 [2.0; 2.2]	2.4 [2.3; 2.5]	<0.001
Phosphate (mg/dL)	3.6 [3.2; 4.0]	3.3 [2.9; 3.6]	3.6 [3.2; 4.0]	3.7 [3.2; 4.1]	<0.001
Calcium (mg/dL)	9.0 [8.8;9.2]	9.1 [8.8; 9.4]	9.0 [8.8; 9.3]	9.0 [8.7; 9.2]	0.001
Alkaline phosphatase (IU/L)	68.0 [54.0;91.0]	80.0 [56.0;93.0]	67.0 [52.0;90.0]	69.0 [55.0;91.5]	0.106
Intact parathyroid hormone (pg/ml)	47.0 [30.9;75.2]	54.5 [26.2;92.6]	45.9 [29.0;72.6]	49.3 [33.0;78.0]	0.260
25-OH Vitamin D (ng/mL)	16.5 [12.1;23.0]	15.9 [12.7;26.0]	16.6 [12.0;23.2]	16.2 [12.2;22.4]	0.787
Total CO_2 (mEq/L)	26.0 [24.0;28.0]	26.0 [24.0;28.0]	26.3 [24.0;28.0]	26.0 [23.0;28.1]	0.096
Hemoglobin (g/dL)	12.9 [11.5;14.5]	12.0 [11.0;13.4]	13.0 [11.5;14.6]	12.9 [11.4;14.4]	0.046
Albumin (g/dL)	4.2 [4.0; 4.5]	4.2 [3.8; 4.4]	4.2 [4.0; 4.5]	4.3 [4.0; 4.5]	0.276
Total cholesterol (mg/dL)	172.0 [147.0;198.0]	178.0 [148.0;203.0]	172.0 [147.0;199.0]	171.0 [148.0;197.0]	0.378
Triglycerides (mg/dL)	134.0 [91.0;196.0]	181.0 [113.0;294.0]	137.0 [91.5;206.0]	128.0 [90.0;179.0]	<0.001
hs-CRP (mg/L)	0.6 [0.2; 1.7]	0.7 [0.2; 1.8]	0.6 [0.2; 1.8]	0.6 [0.2; 1.4]	0.292
Hb A1c (%)	6.4 [5.7; 7.4]	6.8 [6.1; 7.3]	6.6 [5.8; 7.5]	6.3 [5.6; 7.1]	0.002

Variable	Serum magnesium (mg/dL)				*P for trend*
	Total (n =1,646)	<1.8 (n = 37)	1.8-2.2 (n = 914)	>2.2 (n = 695)	
Proteinuria (mg/day)	540.1 [160.9;1448.8]	921.6 [487.5;3034.0]	579.1 [160.0;1692.0]	500.0 [154.7;1239.7]	0.001

CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; PWV, pulse wave velocity; hs-CRP, high-sensitivity C-reactive protein; Hb A1c, hemoglobin A1c

2.2.2 Serum Mg and the composite outcome of cardiovascular event and all-cause death

During 7,368 person-years of follow-up, the primary outcome occurred in 153 patients (20.8 per 1,000 person-years). The incidence rate of the composite outcome from the lowest to the highest Mg group was 47.4, 20.2, and 20.2 per 1,000 person-years, respectively. Adjusted HRs (95% CIs) for the composite outcome (compared with the normomagnesemia group) were 2.53 (1.12-6.69) and 0.93 (0.66-1.32) for the hypomagnesemia and hypermagnesemia groups, respectively (Model 4 in Table 2.2.2). Cumulative composite outcomes tended to be higher in the hypomagnesemia group (Figure 2.2.1).

Table 2.2.2 Cause-specific hazard models for the composite outcome of cardiovascular event and all-cause death

Serum magnesium (mg/dL)	Number of participants	Number of events (%)	Model 1		Model 2		Model 3		Model 4	
			HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
<1.8	37	7 (18.9)	2.44 (1.10−5.41)	0.028	2.75 (1.23−6.16)	0.013	2.73 (1.22−6.12)	0.014	2.53 (1.12−5.69)	0.024
1.8−2.2	914	83 (9.1)	1 (Reference)		1 (Reference)		1 (Reference)		1 (Reference)	
>2.2	695	63 (9.1)	0.94 (0.67−1.30)	0.717	0.93 (0.67−1.31)	0.715	0.93 (0.66−1.30)	0.689	0.93 (0.66−1.32)	0.717

HR, Hazard ratio; CI, confidence interval.

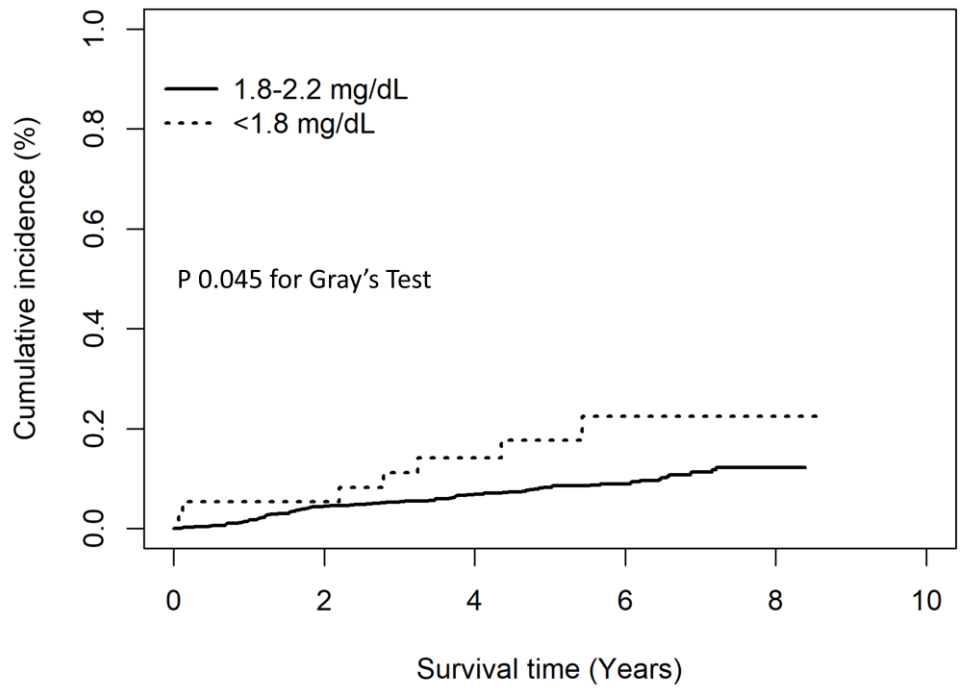
Model 1: adjusted for age, sex, and body mass index

Model 2: Model 1 plus baseline estimated glomerular filtration rate, serum total cholesterol, albumin, phosphorus, and corrected calcium

Model 3: Model 2 plus mean arterial pressure

Model 4: Model 3 plus diabetes mellitus

Figure 2.2.1 Cumulative incidence of the composite outcome of cardiovascular event and all-cause death according to serum magnesium concentration



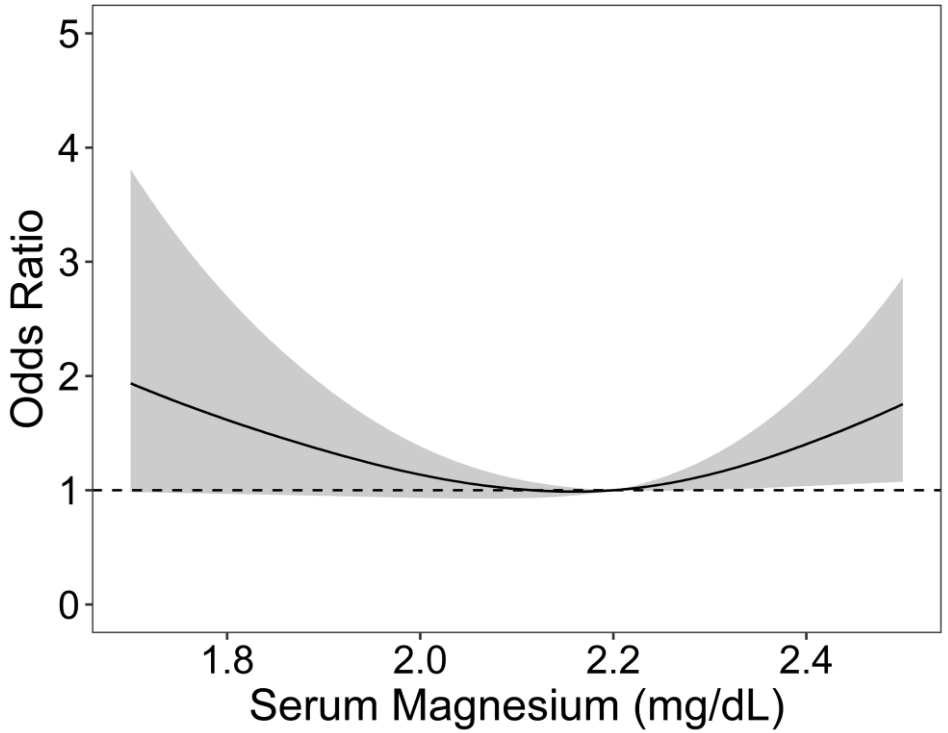
2.2.3 Serum Mg and coronary artery calcification progression

CAC was measured both at study entry and at 4 years. CAC progressed in 11 (45.8%), 155 (31.2%), and 133 (35.0%) patients in the lowest to the highest groups of Mg, respectively. The cubic spline curve showed high risk for CAC progression in the hypomagnesemia category (Figure 2.2.2).

2.2.4 Serum Mg and arterial stiffness progression

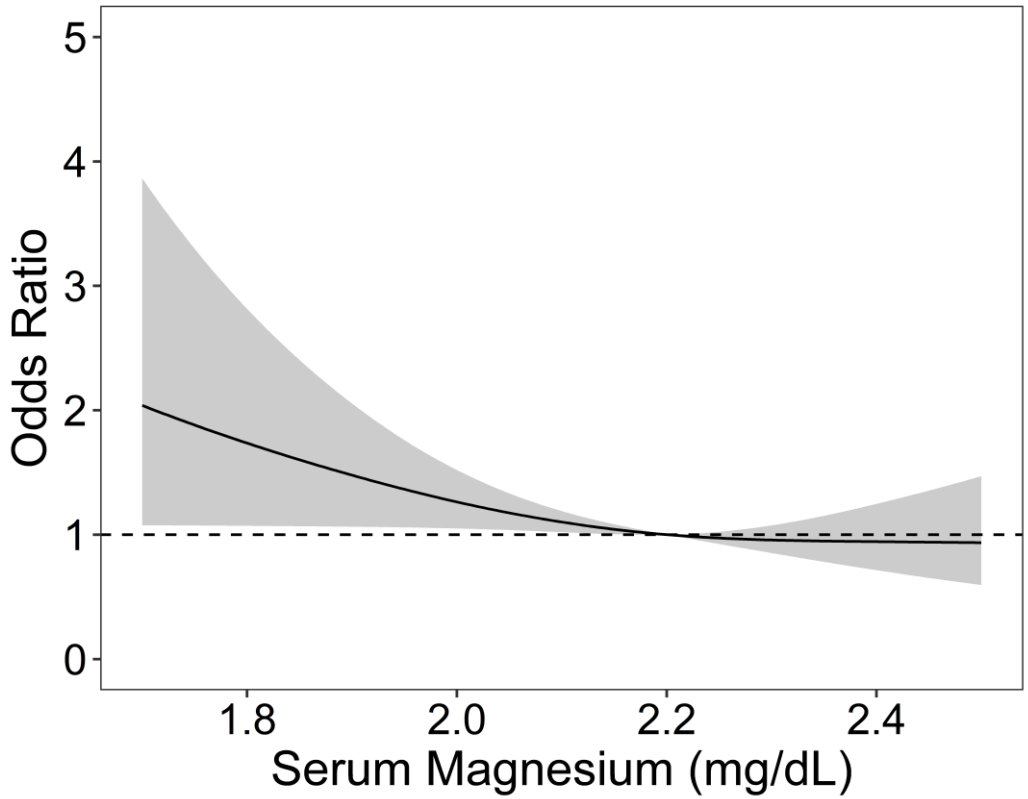
Pulse wave velocity was measured both at study entry and at 4 years. Arterial stiffness progressed in 10 (52.6%), 216 (44.1%), and 158 (41.9%) patients from the lowest to the highest groups of Mg, respectively. The cubic spline curve showed high risk for arterial stiffness progression in the hypomagnesemia category (Figure 2.2.3).

Figure 2.2.2 Relationship between magnesium and coronary artery calcification progression



Multivariable models were adjusted for age, sex, body mass index, baseline estimated glomerular filtration rate, serum total cholesterol, albumin, phosphorus, corrected calcium, mean arterial blood pressure, diabetes mellitus, use of lipid lowering agents, and baseline coronary artery calcification.

Figure 2.2.3 Relationship between magnesium and arterial stiffness progression



Multivariable models were adjusted for age, sex, body mass index, baseline estimated glomerular filtration rate, serum total cholesterol, albumin, phosphorus, corrected calcium, mean arterial blood pressure, and diabetes mellitus.

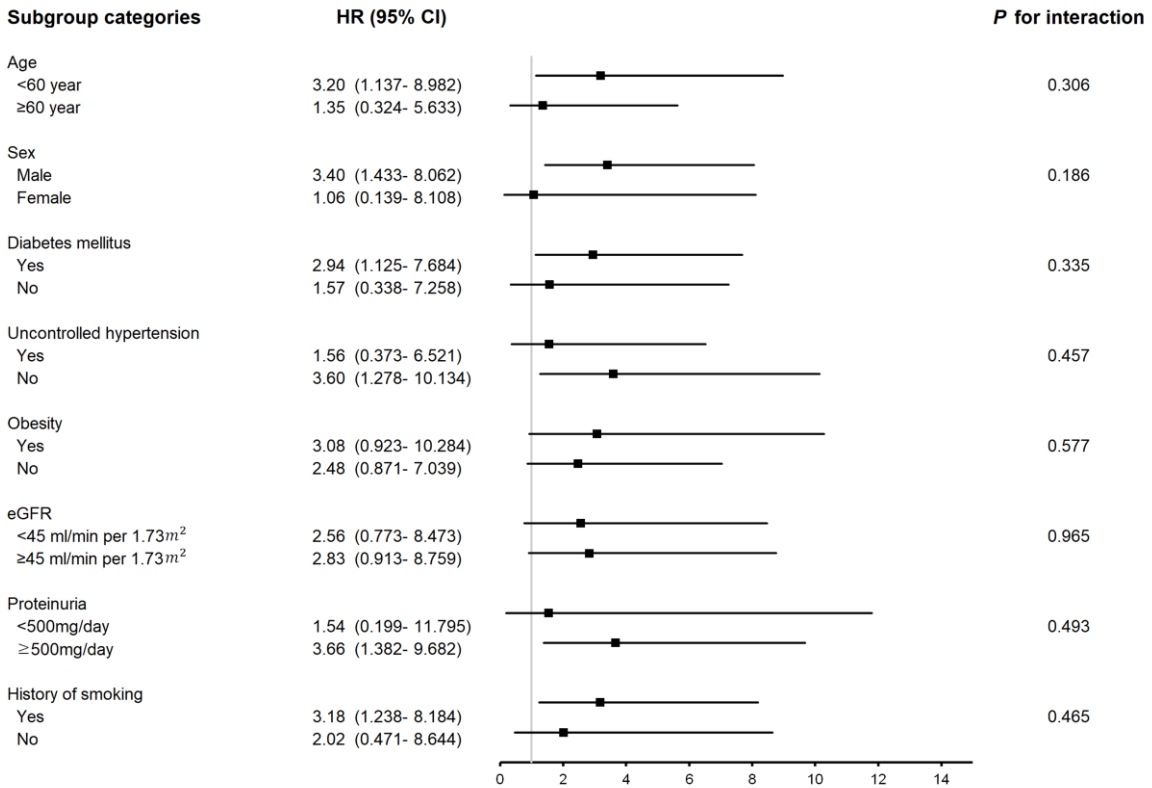
2.2.5 Subgroup analyses

To identify modification effects of subgroups on the association between Mg and the composite of the first occurrence of a CV event and/or all-cause death, we stratified subjects by age (<60 or ≥60 years old), sex (male or female), DM (yes or no), uncontrolled hypertension (yes or no), obesity (yes or no), eGFR (<45 or ≥45 ml/min per 1.73 m^2), proteinuria (<500 or ≥500 mg/day), and history of smoking (yes or no). The association between a low Mg level (Mg <1.8 mg/dL) and the composite outcome was analyzed using Model 4 in all subgroups. *P* values for interaction were >0.05 for age, sex, DM, uncontrolled hypertension, obesity, eGFR, proteinuria, and smoking subgroups, suggesting that the composite outcome risk associated with Mg was evident regardless of these factors (Figure 2.2.4). Subgroup analyses indicated that low Mg was associated with higher composite outcome risk in men, patients <60 years of age, and those with DM, controlled hypertension, overt proteinuria, or a history of smoking.

2.2.6 Sensitivity analyses

The association between Mg and a composite of the first occurrence of a CV event and all-cause death was confirmed using Fine-Gray subdistribution hazard models. Adjusted HRs (95% CIs) of the composite outcome (compared with that of the normomagnesemia group) were 2.51 (1.16-5.42) and 0.96 (0.68-1.35) for the hypomagnesemia and hypermagnesemia groups, respectively (Model 4 in Table 2.2.3).

Figure 2.2.4 Subgroup analyses of the association between magnesium and the composite outcome of cardiovascular event or all-cause death



HR, hazard ratio; CI, confidence interval

Table 2.2.3 Fine-Gray subdistribution hazard models for the composite outcome of cardiovascular event and all-cause death

Serum magnesium (mg/dL)	Number of participants	Number of events (%)	Model 1		Model 2		Model 3		Model 4	
			HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
<1.8	37	7 (18.9)	2.41 (1.08−5.36)	0.030	2.65 (1.19−5.85)	0.016	2.62 (1.19−5.80)	0.016	2.51 (1.16−5.42)	0.019
1.8−2.2	914	83 (9.1)	1 (Reference)		1 (Reference)		1 (Reference)		1 (Reference)	
>2.2	695	63 (9.1)	0.91 (0.65−1.27)	0.588	0.97 (0.69−1.36)	0.868	0.95 (0.68−1.34)	0.808	0.96 (0.68−1.35)	0.824

HR, Hazard ratio; CI, confidence interval.

Model 1: adjusted for age, sex, and body mass index

Model 2: Model 1 plus baseline estimated glomerular filtration rate, serum total cholesterol, albumin, phosphorus, and corrected calcium

Model 3: Model 2 plus mean arterial pressure

Model 4: Model 3 plus diabetes mellitus

Chapter 3. Magnesium inhibits phosphate-induced vascular calcification through suppression of NF-κB

3.1. Materials and Methods

3.1.1 Cell culture and treatment

Primary human aortic VSMCs were obtained from Lonza Inc. (Walkersville, MD, USA) and grown for five passages in SmBM smooth muscle cell basal medium with the SmGM-2 smooth muscle SingleQuots kit (Lonza Inc., Walkersville, MD, USA). VSMCs were transferred to Dulbecco's modified Eagle's medium with 10% fetal bovine serum and treated with 3.5 mM inorganic phosphate (a mixture of Na_2HPO_4 and NaH_2PO_4, pH 7.3) and/or 1.0 mM and 2.0 mM $MgCl_2$ (Sigma, St. Louis, MO, USA) for 7 days and 14 days. The medium was changed every 2 days.

3.1.2 Staining of cell matrix calcium deposition

Alizarin red S (ScienCell, Carlsbad, CA, USA) was used to detect calcium deposition in the cell matrix. After 14 days of treatment, cells were washed with phosphate-buffered saline (PBS), fixed with 4% formaldehyde for 15 minutes, rinsed with deionized water three times, stained with 2% alizarin red staining solution for 30 minutes, and rinsed with deionized water.

3.1.3 Quantification of intracellular calcium content

After 7 days of culture, cells were decalcified by 24-h incubation in HCl (0.6 M). The calcium content in the medium was determined using a calcium colorimetric

assay kit (Biovision Inc., Milpitas, CA, USA).

3.1.4 Western blot analysis

Western blot was performed using anti-NF-κB p65 (1:1000; Cell Signaling Technology), anti-phospho-NF-κB p65 (1:1000; Cell Signaling Technology), anti-IκBα (1:1000; Cell Signaling Technology), anti-phospho-IκBα (1:1000; Cell Signaling Technology), anti-RUNX2 (1:1000; Cell Signaling Technology), or anti-β-actin (1:10000 ; Sigma Aldrich) antibodies and then secondary anti-rabbit IgG, HRP-linked (1:3000; Cell Signaling Technology) and secondary anti-mouse IgG, HRP-linked (1:3000; Cell Signaling Technology) antibodies. Antibody binding was detected with ECL detection reagent (Promega Corp.). The western blot was quantified with ImageJ software (National Institutes of Health).

3.1.5 Statistical analysis

Data were expressed as scatterdot plots and arithmetic mean±standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism software (ver. 9.0; GraphPad Inc., La Jolla, CA, USA). Two groups were compared using unpaired two-tailed t tests. $P<0.05$ was considered statistically significant.

3.2. Results

3.2.1 Effect of magnesium on phosphate-induced vascular calcification of VSMCs

The first series of experiments explored the effect of Mg on calcification of primary human aortic VSMCs under elevated phosphate conditions (Figure 3.2.1).

In VSMCs, treatment with phosphate medium was followed by a significant increase in calcium deposition (Figure 3.2.2). On the other hand, treatment with Mg and P was followed by a decrease in calcium deposition. The same pattern was observed when intracellular calcium content was quantified (Figure 3.2.3).

3.2.2 Effect of magnesium on phosphate-induced osteoinductive signaling and calcification of VSMCs

The expression of RUNX2, an osteogenic marker, significantly increased in high-phosphate medium (Figure 3.2.4). On the other hand, $MgCl_2$ treatment significantly blunted the expression of phosphate-induced osteogenic markers in VSMCs.

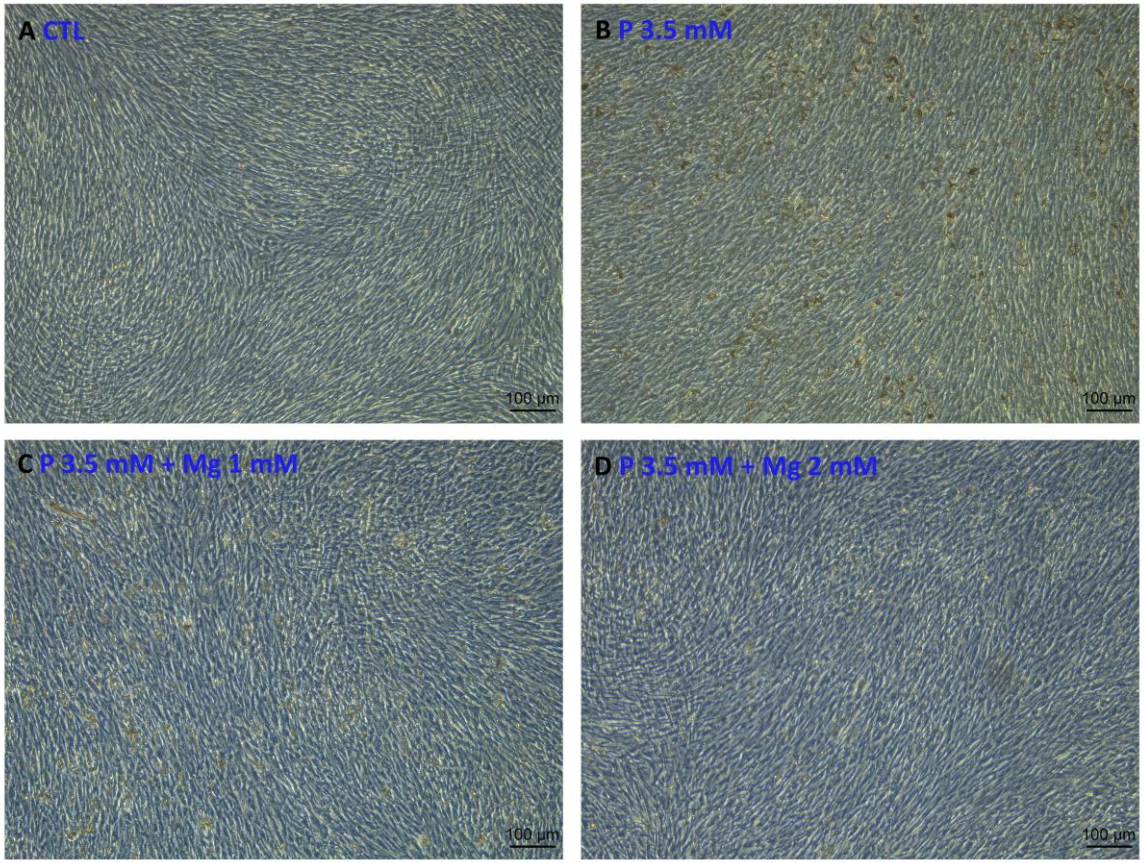
3.2.3 Effect of magnesium on phosphate-induced NF-κB activation in VSMCs

To explore the underlying mechanisms of the protective effects of Mg under high phosphate conditions, I investigated the activation of NF-κB. As shown in Figure 3.2.5, phosphate treatment significantly increased IκBα phosphorylation, and NF-κB phosphorylation showed an increasing trend. In contrast, $MgCl_2$ treatment significantly blunted both IκBα and NF-κB phosphorylation (Figure 3.2.5).

3.2.4 Changes in expression levels of phosphorylation of NF-κB over time

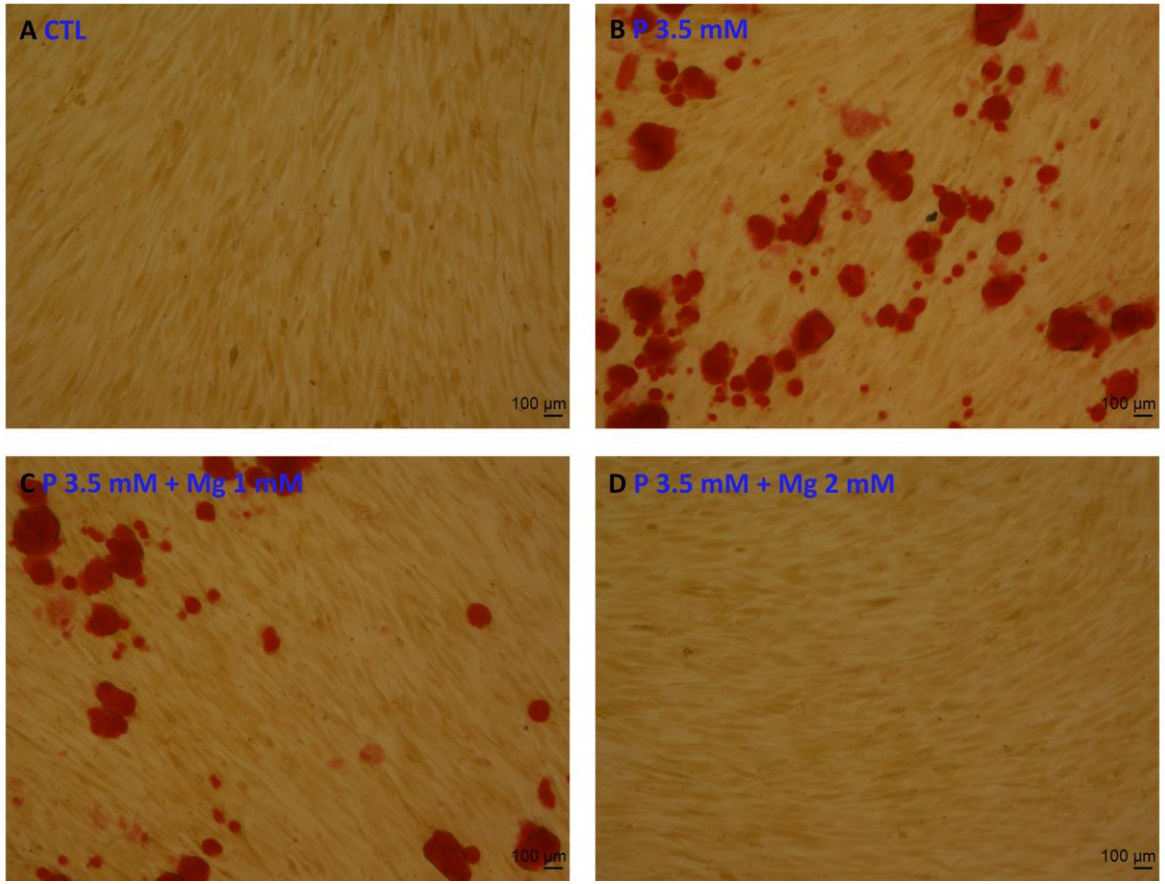
Western blot analyses were performed with different incubation periods to confirm changes in the expression levels of phosphorylation of NF-κB over time (Figure 3.2.6). The phospho-NF-κB/NF-κB/β-actin ratio was highest on day 2 and decreased over time (Figure 3.2.7). $MgCl_2$ treatment showed a tendency to blunt NF-κB phosphorylation, and the degree of this was greatest on day 2.

Figure 3.2.1 Human aortic vascular smooth muscle cells after treatment for 14 days with control or with high-phosphate medium with or without additional treatment with 1 or 2 mM $MgCl_2$



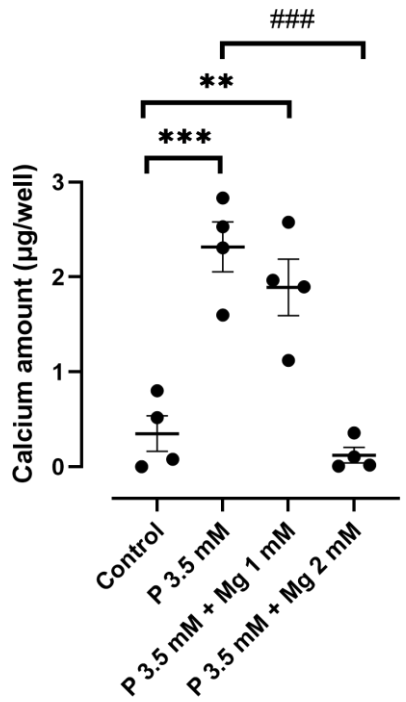
CTL, control

Figure 3.2.2 Alizarin red staining of human aortic vascular smooth muscle cells after treatment for 14 days with control or with high-phosphate medium with or without additional treatment with 1 or 2 mM $MgCl_2$



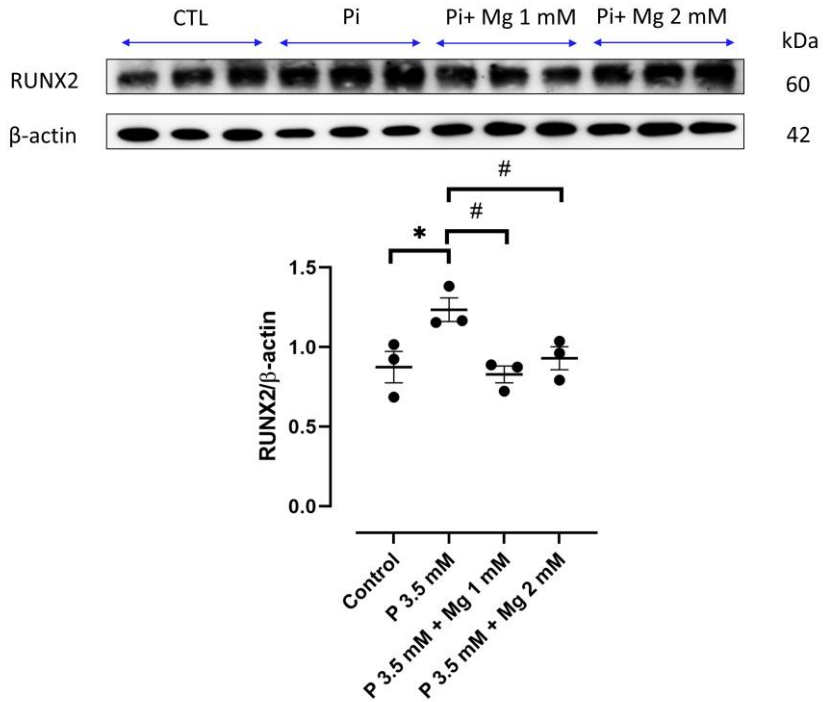
CTL, control

Figure 3.2.3 Scatterdot plots of arithmetic mean±SEM calcium content in human aortic vascular smooth muscle cells after treatment for 7 days with control or with high-phosphate medium with or without additional treatment with 1 or 2 mM $MgCl_2$



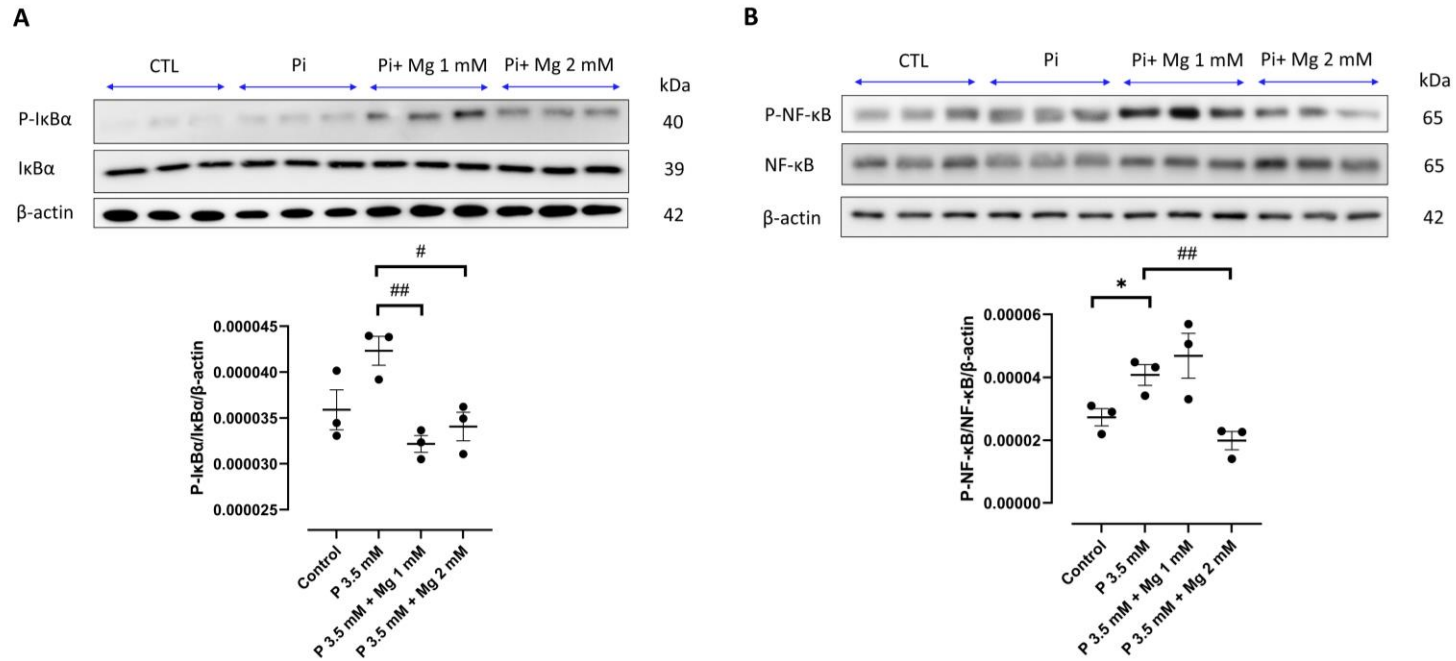
$^{**}P<0.01$; $^{***}P<0.001$ statistically significant versus control treated human aortic VSMCs; $^{\#\#\#}P<0.001$ statistically significant versus human aortic VSMCs treated with phosphate

Figure 3.2.4 Representative original Western blots and arithmetic mean±SEM of normalized RUNX2/β-actin protein ratio in human aortic vascular smooth muscle cells after treatment for 14 days with control or high-phosphate medium with or without additional treatment with 1 or 2 mM $MgCl_2$



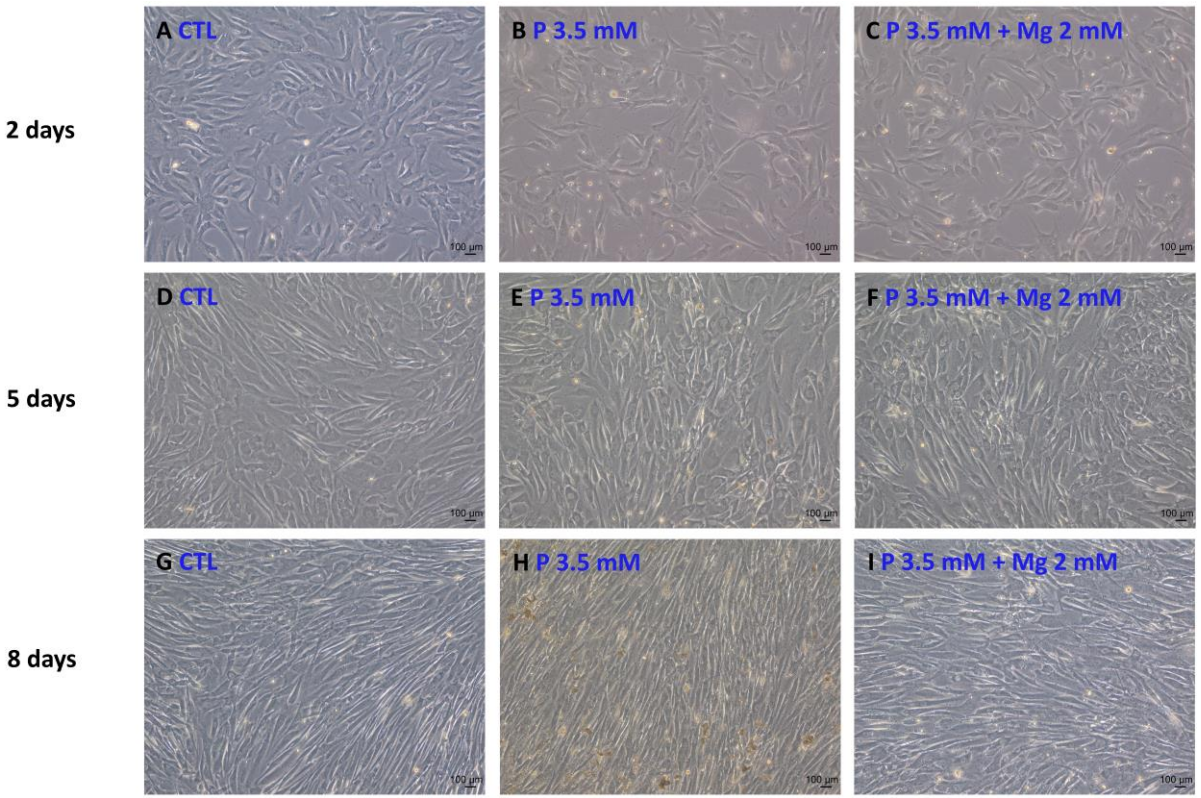
CTL, control; Pi, phosphate; $^{*}P<0.05$ statistically significant versus control treated human aortic VSMCs; $^{\#}P<0.05$ statistically significant versus human aortic VSMCs treated with phosphate

Figure 3.2.5 Representative original Western blots and arithmetic mean±SEM of normalized phospho-IκBα/IκBα/β-actin protein ratio (A) and normalized phospho-NF-κB/ NF-κB/β-actin protein ratio (B) in human aortic vascular smooth muscle cells after treatment for 14 days with control or high-phosphate medium with or without additional treatment with 1 or 2 mM $MgCl_2$



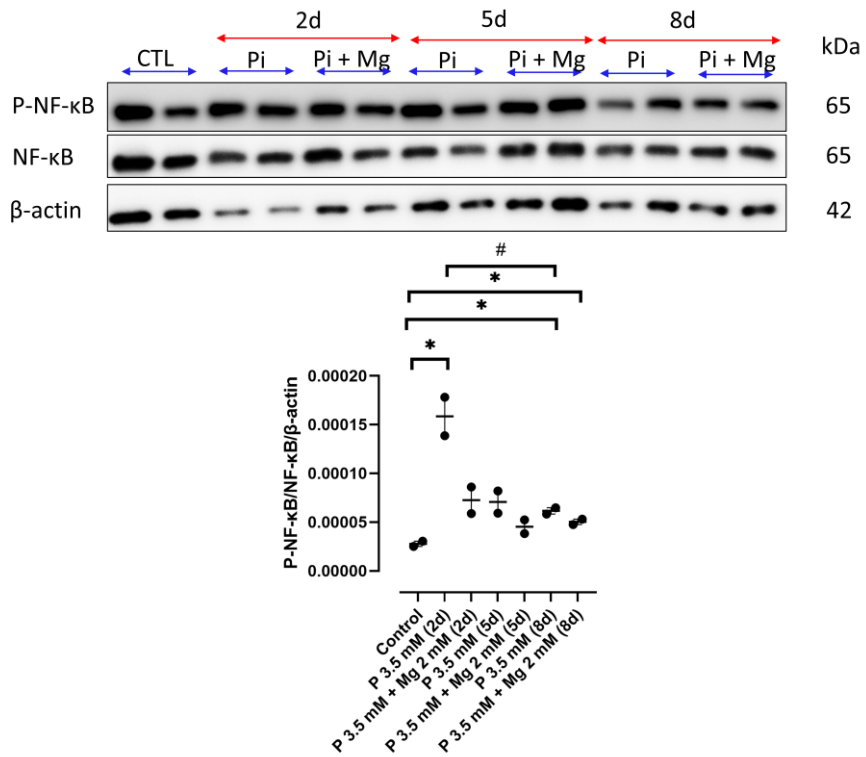
CTL, control; Pi, phosphate; $^{*}P<0.05$ statistically significant versus control treated human aortic VSMCs; $^{\#}P<0.05$; $^{\#\#}P<0.01$ statistically significant versus human aortic VSMCs treated with phosphate

Figure 3.2.6 Human aortic vascular smooth muscle cells after treatment for 2 days (A-C), 5 days (D-F), and 8 days (G-I) with control or with high-phosphate medium with or without additional treatment with 2 mM $MgCl_2$



CTL, control

Figure 3.2.7 Representative original Western blots and arithmetic mean±SEM of normalized phospho-NF-κB/ NF-κB/β-actin protein ratio over time



CTL, control; Pi, phosphate; d, days; $^{*}P<0.05$ statistically significant versus control treated human aortic VSMCs; $^{\#}P<0.05$ statistically significant versus human aortic VSMCs treated with phosphate (2d)

Chapter 4. General discussions and conclusions

4.1. Discussion

In the present study, I found that lower Mg level was associated with a composite outcome of a CV event and/or all-cause death. This finding was confirmed through sensitivity analysis, and the association was evident regardless of confounders in subgroup analysis. Lower Mg level was associated with progression of CAC as a major surrogate marker of adverse CV outcomes. I also showed that lower Mg was associated with arterial stiffness progression as assessed by mean baPWV. In addition, Mg inhibited phosphate-induced calcification of human aortic VSMCs through suppression of NF-κB.

In patients with CKD, hypermagnesemia has traditionally been considered an issue, because when renal function deteriorated, the compensatory mechanism to maintain Mg in the normal range became inadequate, and then hypermagnesemia might occur (13, 14). However, there are several risk factors of hypomagnesemia which are closely associated with CKD such as vitamin D deficiency, DM, and medication (17-20). Hypomagnesemia is not rare in patients with CKD (16). Therefore, hypomagnesemia has been considered as an important issue in CKD.

Previous studies have shown that Mg can be used to treat arrhythmias such as torsade de pointes (10) or heart failure (56). In experimental studies, Mg has been shown to function as a calcification inhibitor *in vitro* (11, 12) and *in vivo* (57). A few clinical studies have shown an inverse association between Mg and

vascular calcification (58). Some clinical studies suggested that Mg was protective against CVD and all-cause mortality in the general population (9). However, in patients with CKD, no consensus conclusion was reached as to whether Mg would benefit CVD and all-cause mortality (21-29). In ESRD patients on dialysis, Mg concentration in the dialysis fluid is an important factor affecting Mg balance (30). Therefore, when conducting Mg-related studies in CKD patients, the pre-dialysis CKD population should be distinguished from ESRD patients receiving dialysis treatment. However, most previous studies were conducted in ESRD patients on dialysis (22, 23, 27-29). To date, only a few studies of pre-dialysis CKD patients have been performed (21, 24, 25, 31), and three of these four studies found no association between low Mg level and CVD (24, 25, 31). The fourth study reported that hypermagnesemia (>2.2 mg/dL), not hypomagnesemia, predicted CVD and all-cause mortality (31). Furthermore, with the exception of one study (25), no association between low Mg and all-cause mortality was found (24, 31). However, most of these studies were restricted to patients with advanced CKD, making it difficult to generalize the results to the entire CKD population. In addition, the small number of CKD subjects analyzed (n< 1,000) decreased statistical power to detect significant relationships. However, a recent analysis of 3,867 CKD patients from the Chronic Renal Insufficiency Cohort (CRIC) study (21) reported that, although Mg is not associated with the risk of composite CV events (myocardial infarction, cerebrovascular accident, heart failure, and peripheral artery disease), low Mg does increase the risk of atrial fibrillation and all-cause death. Collectively, it is unclear whether low Mg can predict CV outcomes in pre-dialysis CKD patients. No longitudinal studies have been performed to analyze Mg and CAC

progression in pre-dialysis CKD patients. The current study, to the best of my knowledge, is the first longitudinal study to investigate the associations between Mg and CVD and all-cause mortality as well as CAC progression in pre-dialysis CKD patients.

In my study, risk of CV events and/or all-cause mortality in the hypomagnesemia group of Mg (<1.8 mg/dL) was 1.71 times higher than that in the normomagnesemia group. Risk of CAC progression was also higher in the hypomagnesemia group. Several possible mechanisms explain these findings. The first possible mechanism that can explain this is endothelial dysfunction due to Mg deficiency (59). Low Mg level exerts oxidative stress on endothelial cells (60), resulting in pro-inflammatory and pro-thrombotic processes (61). These processes contribute to endothelial dysfunction and atherosclerosis. Indeed, Mg deficiency has been shown to be related to endothelial dysfunction in humans (62). Mg deficiency can cause vascular stiffness (63), which can be caused by endothelial dysfunction. We showed that low Mg was associated with arterial stiffness progression as evaluated by change in mean baPWV over time. Vascular stiffness was shown to be associated with CV events and vascular calcification in a clinical study (64). Second, hypomagnesemia is associated with dyslipidemia (65), which is a risk factor for atherosclerosis. This has been shown in a clinical study of pre-dialysis CKD patients (66) as well as in an experimental study (65). Third, vitamin D deficiency can be found frequently in CKD (67). Vitamin D deficiency reduces gastrointestinal absorption of Mg (20) and increases the risk of vascular calcification (68). Fourth, hypomagnesemia is associated with insulin resistance (69), which is associated with an increased risk of vascular calcification

progression (70). Fifth, Mg supplementation can improve blood pressure (71), which is a traditional risk factor for both CVD and vascular calcification (2). Finally, my experiment showed that Mg inhibits the phosphorylation of IκBα through regulation of NF-κB, thereby inhibiting vascular calcification. NF-κB is the key regulator of vascular calcification (37), and activated NF-κB subunit migrates to the nucleus and act as a transcription factor of osteogenic transdifferentiation (35). NF-κB causes upregulation of RUNX2, which is an osteogenic transcription factor (39), and this was also confirmed by my study. Activated NF-κB induces expression of ALP through upregulation of MSX2, leading to VSMC mineralization (40). Previous studies showed that when NF-κB was inhibited, hyperphosphatemia-induced vascular calcification was also inhibited (33, 37, 41); the present study also showed that hyperphosphatemia-induced vascular calcification was blunted through suppression of NF-κB by Mg.

Among the causes of hypomagnesemia is malnutrition (72). Therefore, it is important to evaluate nutritional status when evaluating Mg status. Nutritional evaluation such as nutritional risk score (73) or nutritional risk index (74) can be performed when overall nutritional status is evaluated through surveys such as food diary or food questionnaire. However, this information was not available in the datasets used in this study. Instead, nutrition status can be evaluated using BMI or serum albumin, and, as shown in Table 2.2.1, there was no difference in BMI or albumin according to Mg level. In addition, when performing primary analysis, albumin and, BMI were adjusted, and the association between hypomagnesemia and primary outcome remained (Table 2.2.2).

Although baseline serum Mg was measured only once during primary analysis in

the present study, a previous study compared serum Mg values at baseline and 3 years later with fair agreement (75). The CRIC study also showed that there was no significant difference in median values of serum Mg at baseline and 1 year later (21). In addition, another study performed time-varying survival analysis through serial measurement of serum Mg (22). Collectively, these studies showed that lower serum Mg was associated with mortality or CV events.

A strength of this study is that it is the first large-scale, multi-center longitudinal study to investigate the association between Mg and CVD and all-cause death as well as CAC progression, which is a strong predictor of future cardiac events (46), in pre-dialysis CKD patients. My study was not limited to a specific CKD stage but included non-dialyzed CKD patients with all stages of the disease. Also, my study showed for the first time that Mg inhibited NF-κb, thereby inhibiting vascular calcification. Limitations of my study include that it was observational. Therefore, hidden unmeasured confounders might have been present in my analyses. Because Mg deficiency can be attributed to malnutrition (30), which is a risk factor for CVD in CKD (76), it is important to adjust for nutritional status. In this study, nutritional status was not assessed by food diary or food frequency questionnaire. For this reason, I include serum albumin and BMI as covariates in my analyses. In addition, in subgroup analysis, the *P* for interaction of BMI was insignificant. This suggested that BMI was not an effect modifier. Second, Mg was measured only once at study entry.

4.2. Conclusions

In conclusion, this study showed that hypomagnesemia is associated with CVD and

all-cause death and with the progression of CAC and arterial stiffness over time. The protective effect of Mg is at least in part caused by inhibition of NF-κB activity via suppression of phosphorylation of IκBα. My findings suggest that Mg is a predictor of CV outcomes in pre-dialysis CKD patients.

As a clinical suggestion based on my study and previous studies (21, 77-79), it is recommended to avoid hypomagnesemia (serum Mg <1.8 mg/dL) in patients with pre-dialysis CKD to improve CV outcomes. Further clinical studies are needed that can confirm these findings and their clinical implications.

Bibilography

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초 록

서론: 투석을 하지 않는 만성신장질환 환자에서 마그네슘과 심혈관계 질환의 관련성을 분석한 대규모의 연구는 드물다. 또한 마그네슘이 NF-κB를 억제하여 혈관석회화에 영향을 미치는 기전은 밝혀지지 않았다. 따라서, 이 연구는 대규모의, 투석을 하지 않는 만성신장질환 환자들을 대상으로 마그네슘이 심혈관계 질환에 미치는 영향에 대해서 분석하고 마그네슘이 NF-κB를 억제하여 혈관석회화를 억제하는 기전을 밝히고자 한다.

방법: 투석을 하지 않는 만성신장질환 환자로 구성된 전향적, 다기관 코호트를 이용하여 1,646명의 환자에서 혈청 마그네슘과 심혈관계 질환의 관련성을 분석하였다. 환자는 혈청 마그네슘 농도에 따라 세 그룹으로 나누었다. 일차 결과 변수는 심혈관계 질환이나 모든 원인의 사망으로 정의된 복합 결과 변수로 정의하였다. 이차 결과 변수는 관상동맥혈관 석회화의 진행과 평균 상완-발목 맥파 속도로 평가한 동맥경화도의 진행이었다. 실험은 인간의 대동맥 혈관 평활근 세포로 진행하였다.

결과: 중앙값 6년의 추적기간 동안, 153명 (9.3%)의 환자에서 심혈관계 질환이나 모든 원인의 사망이 발생하였다. 다변수 모델에서 저마그네슘혈증 환자군 (혈청 마그네슘 <1.8 mg/dL)은 정상 혈중 마그네슘 환자군 (혈청 마그네슘 1.8−2.2 mg/ dL)에 비해서 심혈관계 질환이나 모든 원인의 사망의 발생의 위험이 높았다 (위험비 2.53 [1.12−5.69]; *P*=0.024). 저마그네슘혈증 환자군은 관상동맥혈관 석회화의 진행과 동맥경화도의 진행의 위험이 높았다. 배양한 대동맥 혈관 평활근 세포에서, 마그네슘을 처리하

면 NF-κB이 억제되면서 인산염으로 유도된 석회화가 억제되었다.

결론: 저마그네슘혈증은 투석을 하기 전 만성신장질환 환자에서 심혈관계 질환의 예측인자이다. 마그네슘은 NF-κB를 억제하여 인산염으로 유도된 혈관석회화를 억제한다.

주요어: 심혈관계 질환; 사망; 혈관 석회화; 마그네슘; 만성신장질환

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