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The risk of nephrolithiasis is causally related to inactive matrix Gla protein, a marker of vitamin K status: a Mendelian randomization study in a Flemish population

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ABSTRACT

Background. Vitamin K (VK)-dependent γ-glutamate carboxylation and serine phosphorylation activate matrix Gla protein (MGP) to a potent locally acting inhibitor of calcification. Nephrolithiasis represents a process of unwanted calcification associated with substantial mortality and high recurrence rates. We hypothesized that the risk of nephrolithiasis increases with VK shortage, as exemplified by higher plasma levels of desphospho-uncarboxylated MGP (dp-ucMGP).

Methods. In 1748 randomly recruited Flemish individuals (51.1% women; mean age 46.8 years), we determined dp-ucMGP and the prevalence of nephrolithiasis at baseline (April 1996–February 2015) and its incidence during follow-up until March 2016. We estimated the multivariable-adjusted relative risk associated with the doubling of dp-ucMGP, using logistic or Cox regression. We did a Mendelian randomization analysis using four *MGP* genotypes as instrumental variables.

Results. With adjustments applied for sex, age and 24-h urinary volume and calcium excretion, the odds of having prevalent nephrolithiasis [$n = 144$ (8.2%)] associated with dp-ucMGP was 1.31 [95% confidence interval (CI) 1.04–1.64; $P = 0.022$]. dp-ucMGP levels were associated ($P \leq 0.001$) with *MGP* variants *rs2098435*, *rs4236* and *rs2430692*. In the Mendelian analysis, the causal odds ratio was 3.82 (95% CI 1.15–12.7; $P = 0.029$). The incidence of nephrolithiasis over 12.0 years (median) was 37 cases (0.2%). With similar adjustments as before, the hazard ratio in relation to dp-ucMGP was 2.48 (95% CI 1.71–3.61; $P < 0.001$). Additional adjustment for a nephrolithiasis propensity score produced consistent results.

Conclusion. Higher levels of inactive dp-ucMGP may be causally associated with the risk of nephrolithiasis. Whether or not VK deficiency plays a role in these observations remains to be firmly established.

Keywords: calcification, matrix Gla protein, nephrolithiasis, population science, vitamin K

INTRODUCTION

Vascular smooth muscle cells and the endothelium synthesize matrix Gla protein (MGP), a small secretory protein (11 kDa) that contains five γ-carboxyglutamate (Gla) amino-acid residues [1]. Activation of MGP requires two post-translational modifications: vitamin K (VK)-dependent γ-glutamate carboxylation and serine phosphorylation [1]. Once activated, MGP is a potent inhibitor of vascular calcification [1]. The inactive

form, desphospho-uncarboxylated MGP (dp-ucMGP), reflects poor VK status [1]. In the Flemish Study on Environment, Genes and Health Outcomes (FLEMENGHO), plasma dp-ucMGP predicted total and cardiovascular mortality [2]. In a multi-ethnic study, including Flemish people and white and black South Africans, estimated glomerular filtration was inversely correlated with dp-ucMGP, thereby extending the protective role of activated MGP from the macrocirculation to a microcirculatory trait [3].

Nephrolithiasis represents a non-vascular process of unwanted calcification associated with substantial morbidity and high recurrence rates [4]. MGP is expressed in the kidney, where it directly binds to crystals [5]. Two case–control studies found an association between nephrolithiasis and genetic variation in the *MGP* gene [6, 7]. In rat models of nephrolithiasis, lack of MGP expression was associated with crystal formation in injured renal tubules [8], whereas its expression was upregulated in response to calcium oxalate [8, 9]. In the FLEMENGHO cohort [2], we tested the hypothesis that VK deficiency as reflected by circulating dp-ucMGP levels might be associated with the prevalence and incidence of nephrolithiasis. To test causality, we used genetic variation in the *MGP* gene as the instrumental variable.

MATERIALS AND METHODS

Study population

FLEMENGHO complies with the Helsinki Declaration for research in human subjects [10]. The ethics committee of the University of Leuven approved the study. As described in previous publications [11, 12], from August 1985 to November 1990, a random sample of the households living in a geographically defined area of Northern Belgium was investigated with the goal to recruit an equal number of participants in each of six subgroups by sex and age (20–39, 40–59 and ≥60 years). All household members ≥20 years of age were invited, provided that the quota in their sex–age group had not yet been satisfied. From June 1996 until January 2004, recruitment of families continued using the former participants (1985–1990) as index persons and also including teenagers. The participation rate at enrolment was 78.0%. The participants were repeatedly followed up. In all study phases, we used the same standardized methods to measure clinical and biochemical variables, administer questionnaires and determine the incidence of fatal and non-fatal outcomes [11, 12]. At each contact, participants gave or renewed informed written consent.

Of 3343 enrolled participants, 2947 had serum and plasma samples and aliquots of 24-h urine collections available in the FLEMENGHO biobank. The date of blood collection ranged from 29 April 1996 until 12 February 2015 [interquartile range (IQR) 12 March 1998–26 September 2002; median, 10 December 1998] (Figure 1). Of the remaining 2947 participants, 969 were not eligible for analysis because, as adolescents <18 years of age, they were at very low risk of nephrolithiasis [13–15] ($n = 184$) or because dp-ucMGP ($n = 540$) or required covariables [serum calcium ($n = 141$), serum uric acid ($n = 60$) or 24-h urinary calcium ($n = 44$)] had not been measured. We excluded a further 230 participants because they were on warfarin at the time of the dp-ucMGP measurement ($n = 12$), because their dp-ucMGP level was more than 3 standard deviations (SDs) of the population mean ($n = 13$), because their 24-h urine collection was inaccurate according to previously published criteria ($n = 39$) [16], or because they had been lost to follow-up ($n = 166$). Thus the number of participants statistically analysed totaled 1748, of which 1466 had their MGP genotype determined and were included in the Mendelian randomization analysis.

Measurements at baseline

In the current study, baseline refers to the date on which blood was sampled for measurement of dp-ucMGP. Trained nurses measured blood pressure after participants had rested for at least 5 min in a seated position. Blood pressure was the average of five consecutive auscultatory readings obtained with a standard mercury sphygmomanometer. Hypertension was a blood pressure of at least 140 mmHg systolic or 90 mmHg diastolic or the use of antihypertensive drugs. The nurses also administered questionnaires inquiring into each participant's medical history, smoking and drinking habits and intake of medications. Body mass index (BMI) was weight in kilograms divided by height in meters squared.

Participants collected a 24-h urine sample in wide-neck polyethylene containers for measurement of volume, creatinine, sodium, potassium and calcium. Within 2 weeks of the urine collection, at the time of the clinical examination, a venous blood sample was obtained after the participants had been fasting for 6–8 h. Blood samples were analysed for serum creatinine, total calcium, magnesium, uric acid, γ-glutamyltransferase (index of

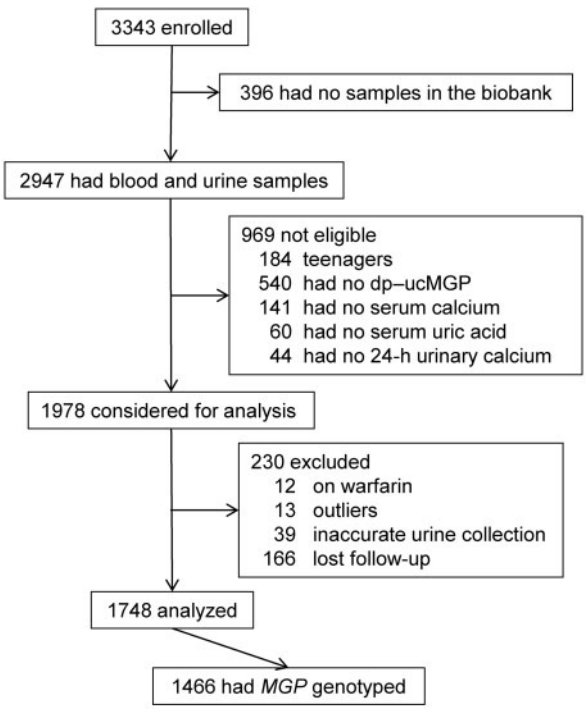

FIGURE 1: Flow chart of participants.

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alcohol intake) and plasma glucose, using automated methods in certified laboratories. Estimated glomerular filtration rate (eGFR) was derived from serum creatinine, according to the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [17]. We staged CKD according to the National Kidney Foundation [Kidney Disease Outcomes Quality Initiative (KDOQI)] guideline [18] as eGFR $\geq$90, 89–60, 59–30, 29–15 and $<$15 mL/min/1.73 m^2 for stages 1, 2, 3, 4 and 5, respectively. dp-ucMGP was measured using enzyme-linked immunosorbent assay (ELISA) kits at VitaK (Maastricht University, Maastricht, The Netherlands) [19]. The concentration of dp-ucMGP was assessed using the inaKtif MGP iSYS kit (Immunodiagnostic Systems, Boldon, UK), which is a dual-antibody test based on the sandwich ELISA developed by VitaK. In a previous study we demonstrated that our MGP assay was not sensitive to long-term storage of the plasma samples [3].

Genotyping

MGP (4738 base pairs) maps to a genomic area on chromosome 12 (p13.1–p12.3) characterized by high linkage disequilibrium (Supplementary data, Figure S1). We selected four tagging single-nucleotide polymorphisms (SNPs; *rs2098435, rs4236, rs1800802* and *rs2430692*) that are in high linkage disequilibrium ($r^2 > 0.80$) with 223 other SNPs covering the entire gene with extension into the 3′ and 5′ flanking regions (Supplementary data, Tables S1 and S2). After DNA extraction from peripheral blood [20], SNPs were genotyped using the TaqMan OpenArray Genotyping System (Life Technologies, Foster City, CA, USA). All DNA samples were loaded at 50 ng/mL and amplified according to the manufacturer's instructions. For analysis of the genotypes, we used autocalling methods as implemented in the TaqMan Genotyper software version 1.3 (Life Technologies). Next, genotype clusters were evaluated manually with the call rate set at $>$0.90. Sixteen duplicate samples gave 100% reproducibility for the four *MGP* SNPs. The overall call rate was 98%.

Ascertainment of events

At annual intervals we ascertained the vital status of all participants via the National Population Registry (Brussels, Belgium). We obtained the International Classification of Disease codes for the immediate and underlying causes of death from the Flemish Registry of Death Certificates. For 1748 participants followed up until 10 March 2016, we collected information on the incidence of non-fatal endpoints, including nephrolithiasis, via one or more structured telephone interviews ($n = 324$), via contact with the participant's general practitioner ($n = 992$) or via face-to-face follow-up visits with repeated administration of the same standardized questionnaire as was used at baseline ($n = 1487$). The number of face-to-face follow-up visits was one in 616 participants, two in 371, three in 329 and four or more in 171 participants. Starting from October 2014, after we had obtained ethical clearance, we also consulted the digital medical records of ambulatory and hospitalized patients receiving care at the University Hospitals Leuven or at three regional hospitals located in the catchment area of the study. This generated additional information on non-fatal outcomes in 1174 study participants (67.2%). In 1388 participants, confirmatory information on non-fatal outcomes was available from two or more of the aforementioned sources. Trained nosologists used the International Classification of Diseases to code nephrolithiasis. Investigators (A.H., T.K. and J.A.S.) blinded with regard to the dp-ucMGP levels adjudicated all prevalent, recurrent and incident nephrolithiasis cases against the medical records of general practitioners or hospitals up to 10 March 2016. Cases were symptomatic patients with nephrolithiasis or ureterolithiasis, who often had been hospitalized for diagnosis and treatment or were followed up in day care. None of the cases rested on incidental findings in asymptomatic patients. We did not include bladder stones in the study endpoint.

Statistical analysis

For database management and statistical analysis, we used the SAS system, version 9.4 (SAS Institute, Cary, NC, USA). Significance was a two-tailed α-level of ≤ 0.05. Means and proportions were compared using the large-sample z-test or analysis of variance and Fisher's exact test, respectively. We normalized the distributions of dp-ucMGP and γ-glutamyltransferase by a logarithmic transformation.

We computed relative risk in relation to dp-ucMGP using multivariable-adjusted logistic and Cox regression as appropriate in cross-sectional and prospective analyses, respectively. The characteristics considered as covariables were sex, age, BMI, mean arterial pressure, serum total calcium, magnesium, uric acid, γ-glutamyltransferase (marker of alcohol consumption), total cholesterol, plasma glucose, 24-h urinary volume, sodium, potassium, calcium, smoking and antihypertensive drug treatment by main drug classes, i.e. diuretics (thiazides [21], loop diuretics and aldosterone antagonists), renin–angiotensin system inhibitors (β-blockers, angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor blockers) and vasodilators (calcium channel blockers and α-blockers). As reported previously [22], the covariables retained for adjustment were sex, age and the 24-h urinary volume and calcium excretion. In addition, we computed a propensity score as the dp-ucMGP level predicted by other covariables of potential physiological relevance, including BMI, mean arterial pressure, plasma glucose, serum total cholesterol and creatinine, intake of thiazides and a history of diabetes mellitus. Because thiazide diuretics reduce urinary calcium excretion [21], we only included this class of diuretics in the propensity score.

We tested the Hardy–Weinberg equilibrium in unrelated participants, using the exact statistics available in the PROC ALLELE procedure of the SAS package. To assess the causality of the association between the prevalence of kidney stones and dp-ucMGP, we applied a Mendelian randomization approach using the *MGP* genotypes as instrumental variables. Mendelian randomization is a method that allows testing for a causal effect in observational data in the presence of confounding factors by using genetic variants that are a proxy for environmentally modifiable exposures as instruments [23]. While accounting for family clusters in a two-stage least square procedure [2], we first predicted log dp-ucMGP from sex, age, 24-h urinary calcium and volume and the genetic variants of MGP using the PROC MIXED procedure. In the second step, we regressed the risk of nephrolithiasis on the dp-ucMGP levels predicted in the first step, using the PROC GENMOD procedure. The Mendelian

randomization approach controls for unmeasured confounders and reverse causality that may distort the directly assessed association between outcome and the exposure of interest (measured dp-ucMGP) [24]. We assessed the strength of the *a priori* defined instrumental variables, the *MGP* genotypes, using the *F*-statistic [25].

RESULTS

Characteristics of participants

All 1748 participants (Figure 1) were white Europeans, of whom 893 (51.1%) were women. The study population consisted of 279 singletons and 1469 related subjects, belonging to 318 single-generation families and 155 multigeneration pedigrees. Age averaged (±SD) 46.8 ± 15.4 years, eGFR 90.8 ± 20.4 mL/min/1.73 m^2, blood pressure 125.9 ± 16.1 mmHg systolic and 77.9 ± 10.4 mmHg diastolic and BMI 25.8 ± 4.3 kg/m^2. Among all participants, 541 (31.0%) had hypertension, of whom 295 (54.5%) were on antihypertensive drug treatment, 438 participants (25.1%) reported smoking and 762 (43.6%) reported alcohol intake.

Table 1 lists the characteristics of participants by tertiles of the distribution of dp-ucMGP. Across increasing categories of dp-ucMGP, more participants had hypertension or diabetes mellitus or were on antihypertensive drug treatment ($P < 0.001$), whereas fewer reported smoking ($P < 0.001$). Furthermore, age, BMI, blood pressure, plasma glucose, total cholesterol, serum magnesium, uric acid and γ-glutamyltransferase all increased with higher categories of dp-ucMGP ($P \leq 0.005$). According to the KDOQI criteria [18], 958 (54.8%) participants had stage 1 CKD, 643 (36.8%) stage 2, 139 (8.0%) stage 3, 6 (0.3%) stage 4 and 2 (0.1%) stage 5. Across increasing categories of dp-ucMGP (Table 2), eGFR decreased ($P < 0.001$) and the prevalence of CKD increased ($P < 0.001$).

Prevalence of nephrolithiasis

Among 1748 participants, 144 (8.2%) had a history of nephrolithiasis at baseline. Compared with participants without kidney stones, participants with kidney stones included fewer women (38.9 versus 52.2%; $P = 0.002$), had higher serum uric acid (315 versus 293 µmol/L; $P = 0.003$), serum magnesium (0.84 versus 0.82 mmol/L; $P = 0.027$) and 24-h urinary calcium (4.70 versus 3.87 mmol; $P < 0.001$), but lower eGFR (86.1 versus 91.2 mL/min/1.73 m^2; $P = 0.004$). Furthermore, although formal significance was not reached, participants with kidney stones had a lower 24-h urinary volume (1.49 versus 1.59 L; $P = 0.083$) but higher 24-h urinary sodium output (170 versus 158 mmol; $P = 0.085$) than participants without kidney stones. None of the other covariables was different between participants with kidney stones and participants without kidney stones. The

Table 1. Baseline characteristics of participants by tertiles of the dp-ucMGP distribution

Characteristics	Category of dp-ucMGP (µg/L)			P-value
	<2.99	2.99–4.88	≥4.88	
All patients in category [*n* (%)]	580	587	581	
Women	301 (51.9)	294 (50.1)	298 (51.3)	0.82
Smokers	185 (31.9)	167 (28.4)	86 (14.8)***	<0.001
Drinking alcohol	264 (45.5)	251 (42.8)	247 (42.5)	0.52
Hypertension	123 (21.2)	158 (26.9)*	260 (44.8)***	<0.001
Antihypertensive treatment	55 (9.5)	88 (15.0)**	152 (26.2)***	<0.001
Diabetes mellitus	9 (1.6)	13 (2.2)	36 (6.2)***	<0.001
Mean (±SD) of characteristic				
Age (years)	41.2 ± 13.0	45.1 ± 14.5***	54.0 ± 15.6***	<0.001
Body mass index (kg/m^2)	24.4 ± 3.6	25.6 ± 4.1***	27.4 ± 4.6***	<0.001
Systolic blood pressure (mmHg)	122.3 ± 13.7	124.9 ± 15.3**	130.6 ± 17.8***	<0.001
Diastolic blood pressure (mmHg)	76.7 ± 9.9	77.4 ± 10.7	79.6 ± 10.4***	<0.001
Serum total cholesterol (mmol/L)	5.26 ± 1.02	5.34 ± 1.04	5.60 ± 1.06***	<0.001
Blood glucose (mmol/L)	4.90 ± 1.12	5.07 ± 1.33*	5.24 ± 1.62*	<0.001
Serum total calcium (mmol/L)	2.38 ± 0.11	2.38 ± 0.14	2.37 ± 0.17	0.44
Serum magnesium (mmol/L)	0.817 ± 0.08	0.819 ± 0.08	0.831 ± 0.08**	0.005
Serum uric acid (µmol/L)	281.0 ± 74.4	290.7 ± 82.5*	315.2 ± 87.8***	<0.001
24-h urinary volume (L)	1.55 ± 0.66	1.61 ± 0.72	1.58 ± 0.61	0.34
24-h urinary sodium (mmol)	160.6 ± 76.3	163.2 ± 81.8	156.1 ± 79.0	0.30
24-h urinary potassium (mmol)	67.8 ± 29.9	67.1 ± 27.6	64.8 ± 27.4	0.17
24-h urinary calcium (mmol)	3.96 ± 1.92	4.05 ± 2.10	3.79 ± 2.05*	0.10
Geometric mean (IQR) of characteristic				
γ-glutamyltransferase (units/L)	17.0 (11.5–23.4)	18.6 (12.9–25.7)**	20.9 (14.1–28.8) ***	<0.001
dp-ucMGP (µg/L)	1.88 (1.60–2.60)	3.85 (3.43–4.42) ***	6.85 (5.56–7.86) ***	<0.001

Baseline refers to the date of blood collection for dp-ucMGP measurement. To convert dp-ucMGP from µg/L into pmol/L, multiply by 94.299. Hypertension was a blood pressure of ≥140 mmHg systolic or ≥90 mmHg diastolic or the use of antihypertensive drugs. Diabetes mellitus was a fasting glucose level >126 or a random glucose level >200 mg/dL (7.0 or 11.1 mmol/L, respectively), or the use of antidiabetic agents.

P-values denote the significance of the difference in prevalence or means across tertiles of the distribution of dp-ucMGP. Significance of the difference with the adjacent lower tertile: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

covariables retained in the stepdown logistic regression procedure were sex, age and 24-h urinary volume and calcium excretion. The mutually adjusted odds ratios (ORs) were 0.68 [95% confidence interval (CI) 0.47–0.98; P = 0.036] for being female, and per 1 SD increment, 1.58 (95% CI 1.36–1.83; P < 0.001) for age (+15.4 years), 0.75 (95% CI 0.61–0.92; P = 0.0055) for urinary volume (+0.67 L/24 h) and 1.47 (95% CI 1.24–1.73; P < 0.001) for urinary calcium excretion (+2.03 mmol/24 h).

As listed in Table 3, the OR expressing the crude risk of nephrolithiasis associated with a doubling of dp-ucMGP was 1.50 (95% CI 1.21–1.85; P < 0.001). With adjustments applied for sex, age and 24-h urinary volume and calcium excretion (Table 3), the OR was 1.31 (95% CI 1.04–1.64; P = 0.022). In a model additionally adjusted for a propensity score generated from BMI, mean arterial pressure, plasma glucose, serum total cholesterol and creatinine, use of thiazide and a history of diabetes mellitus, the OR was 1.29 (95% CI 1.02–1.62; P = 0.035).

Incident and recurrent nephrolithiasis

Over a median follow-up of 12.0 years (5th–95th percentile interval: 1.4–18.1 years), 37 patients (10 women and 27 men) experienced incident ($n = 16$) or recurrent ($n = 21$) nephrolithiasis. Face-to-face follow-up visits, telephone interviews, medical records held by general practitioners or hospitals or a combination of these sources identified 6 (37.5%), 0, 1 (6.2%), 12 (75.0%) and 3 (18.8%) of 16 patients with incident nephrolithiasis. For the 21 cases of recurrent nephrolithiasis, these numbers were 14 (66.7%), 1 (4.8%), 6 (28.6%), 11 (52.4%) and 8 (38.1%), respectively. The overall incidence rate was therefore 1.91 per 1000 person-years of follow-up (95% CI 1.29–2.53). Figure 2 shows the sex- and age-adjusted cumulative incidence of nephrolithiasis by tertiles of dp-ucMGP distribution. Compared with the low tertile, the incidence was slightly higher in the middle tertile (P = 0.11) and significantly higher (P = 0.007) in the top tertile of the dp-ucMGP distribution.

The hazard ratio (HR) expressing the crude risk of recurrent combined with incident nephrolithiasis ($n = 37$) associated with a doubling of dp-ucMGP was 2.21 (95% CI 1.50–3.27; P < 0.001; Table 3). In adjusted and fully adjusted models, these HRs were 2.48 (95% CI 1.71–3.61; P < 0.001) and 2.46 (95% CI 1.68–3.60; P < 0.001), respectively. The adjusted and fully adjusted HRs derived for the 16 participants with a first episode of nephrolithiasis were 2.41 (95% CI 1.44–4.04; P < 0.001) and 2.41 (95% CI 1.43–4.06; P = 0.001), respectively.

Mendelian randomization study

In 536 unrelated founders, the four SNPs covering the entire *MGP* gene (Supplementary data, Tables S1 and S2 and Figure S1) complied with Hardy–Weinberg equilibrium (P ≥ 0.34; Supplementary data, Table S3). While accounting for family clusters and with adjustments applied for previously identified covariables [2], including age, BMI, smoking and drinking (Supplementary data, Table S4), dp-ucMGP was significantly

Table 2. Renal function by tertiles of the dp-ucMGP distribution

Characteristics	Category of dp-ucMGP (μg/L)			P-value
	<2.99	2.99–4.88	≥4.88	
All patients in the category (*n*)	580	587	581	
Stage of chronic kidney disease [*n* (%)]				<0.001
1	377 (65.0)	352 (60.0)	229 (39.4)	
2	188 (32.4)	193 (32.9)	262 (45.1)	
3	14 (2.4)	40 (6.8)	85 (14.6)	
4	1 (0.17)	2 (0.34)	3 (0.52)	
5	0 (0)	0 (0)	2 (0.34)	
Mean (±SD) of the characteristic				
Serum creatinine (μmol/L)	77.5 ± 17.3	79.8 ± 17.9	85.2 ± 35.0***	<0.001
eGFR (mL/min/1.73 m^2)	96.8 ± 17.8	92.5 ± 19.8***	83.1 ± 21.2***	<0.001

eGFR determined according to the CKD-EPI equation.
CKD was staged according to the KDOQI guideline as eGFR ≥90, 60–89, 30–59, 29–15 and <15 mL/min/1.73 m^2 for stage 1, 2, 3, 4 and 5, respectively.
P-values denote the significance of the difference in prevalence or means across tertiles of the dp-ucMGP distribution. Significance of the difference with the adjacent lower tertile:
*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

Table 3. Relative risks associating nephrolithiasis with circulating matrix Gla protein

Level of adjustment	Prevalence (*n* cases/*n* at risk; 144/1748)		Incidence (*n* cases/*n* at risk; 37/1748)		Incidence (*n* cases/*n* at risk; 16/1748)	
	Odds ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
Unadjusted	1.50 (1.21–1.85)	<0.001	2.21 (1.50–3.27)	<0.001	2.03 (1.12–3.68)	0.020
Adjusted	1.31 (1.04–1.64)	0.022	2.48 (1.71–3.61)	<0.001	2.41 (1.44–4.04)	<0.001
Fully adjusted	1.29 (1.02–1.62)	0.035	2.46 (1.68–3.60)	<0.001	2.41 (1.43–4.06)	0.001

Odds ratios express the risk of the prevalent nephrolithiasis and hazards ratios the risk of incident nephrolithiasis associated with a doubling of dp-ucMGP. Prevalence includes 144 participants with a history of nephrolithiasis at baseline. Incidence includes 21 patients with recurrent nephrolithiasis and 16 with a first episode of nephrolithiasis. Adjusted models accounted for sex, age and 24-h urinary volume and calcium excretion. Fully adjusted models additionally accounted for a propensity score generated from BMI, mean arterial pressure, plasma glucose, serum total cholesterol and creatinine, use of thiazides and a history of diabetes mellitus.

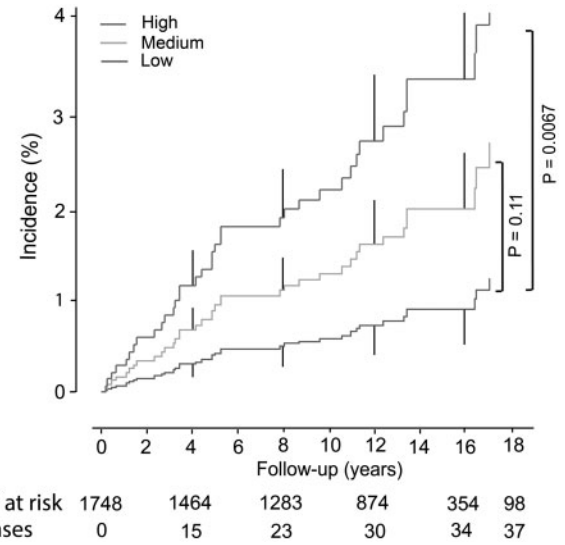

FIGURE 2: Sex- and age-adjusted cumulative incidence of nephrolithiasis by tertiles of the distribution of dp-ucMGP. P-values refer to the differences between the low and the middle and top tertiles of dp-ucMGP distribution. Vertical lines denote the standard error. The median follow-up was 12.0 years.

($P \leq 0.001$) associated with *rs2098435*, *rs4236* and *rs2430692* in 1466 participants with available *MGP* genotypes. Major allele carriers had higher levels, whereas there was no association between dp-ucMGP and *rs1800802* ($P = 0.21$). The *F*-statistics for *rs2098435, rs4236* and *rs2430692* were 24.5, 26.0, and 30.4, respectively. These SNPs explained 1.13, 1.24 and 1.60% of the variance in dp-ucMGP. Sensitivity analyses in 536 unrelated founders were confirmatory (Supplementary data, Table S4). In a model adjusted for sex, age and 24-h urinary volume and calcium excretion, the causal OR relating the prevalence of nephrolithiasis ($n = 144$) to the instrumental variable was 3.82 (95% CI 1.15–12.7; $P = 0.029$). In a sensitivity analysis with the 16 new cases added, the causal OR was 3.79 (95% CI 1.29–11.2; $P = 0.016$).

DISCUSSION

We hypothesized that MGP plays a role in the pathogenesis of kidney stones and that the risk of nephrolithiasis increases with VK shortage, as exemplified by higher plasma levels of dp-ucMGP. Our key findings can be summarized as follows: (i) for a doubling of dp-ucMGP, the odds of nephrolithiasis increased by 31% in the cross-sectional analysis; (ii) the Mendelian randomization analysis suggested that this association was causal and (iii) in the longitudinal analysis spanning 12 years of follow-up (median), the risk of having recurrent or new nephrolithiasis increased 2.5-fold for a doubling of the baseline dp-ucMGP level. To our knowledge, our current study is the first population survey assessing the risk of nephrolithiasis in relation to dp-ucMGP, a biomarker of VK status [1]. In a randomized controlled trial, supplementation with VK_2 180 μg daily compared with placebo reduced plasma dp-ucMGP by 50% [26].

Several of our observations are in line with the literature and support the validity of our study endpoint. In the USA, the prevalence of nephrolithiasis is 8.8% [27] and the annual incidence is 0.5% [28]. In our current study, these estimates were of similar magnitude: 8.2 and 0.2%, respectively. Second, nephrolithiasis is more common in men than women throughout most of adult life except in the sixth decade, when the incidence decreases in men, but increases in women [4]. In our study, women had a 32% lower risk of prevalent kidney stones than men. The most important pathophysiological factors for calcium nephrolithiasis are low urinary volume and hypercalciuria [4]. In our study, 1 SD increments in 24-h urinary volume and calciuria at baseline were associated with 25% lower and 47% higher risk of nephrolithiasis, respectively.

Several experimental studies support our current epidemiological observation of an association between kidney stones and MGP [8, 9, 29, 30]. Gao *et al.* [9] exposed cultured renal tubular cells (NRK-52E) to oxalate and to calcium oxalate monohydrate crystals. Within 3 h after exposure to calcium oxalate monohydrate, the MGP mRNA levels increased ≥70-fold, whereas in the presence of the non-crystalline oxalate MGP, mRNA expression time-dependently rose, reaching a maximum of a 20-fold increase after 24 h. Khan *et al.* [29] replicated the aforementioned findings using Madin–Darby canine kidney tubular cells. Immunohistochemical studies by the same authors demonstrated that in hyperoxaluric Sprague Dawley rats, renal tubular epithelial cells exposed to oxalate or calcium oxalate expressed MGP and that the endothelium of the peritubular microvessels participated in MGP generation. Along similar lines, Lu *et al.* [8] reported that in hyperoxaluric Sprague Dawley rats, MGP is polarly expressed at the apical membrane of tubular epithelial cells in the ascending thick limbs of Henle's loop and the distal convoluted tubule and in stone-forming rats also in the medullary collecting duct. Multilaminated crystals developed in injured renal tubules that lacked MGP expression [8]. Goiko *et al.* [30] synthesized peptides corresponding to the phosphorylated and γ-carboxylated sequences of human MGP in both post-translationally modified and non-modified forms. Depending on the amino acid sequence, these peptides inhibited nucleation, growth or both of hydroxyapatite and calcium oxalate monohydrate crystals.

Association studies linking kidney stones to genetic variation in *MGP* support the causality inferred from our Mendelian randomization study [6, 7]. In 122 Japanese patients with kidney stones and 125 controls, Gao *et al.* [6] investigated 19 SNPs in *MGP*, including *rs4236* and *rs1800802*. Compared with minor allele *rs4236* carriers (*G*; prevalence 24.3%), major allele homozygotes (*AA*; prevalence 75.7%) had a 1.82-fold increased risk of kidney stones (95% CI 1.00–3.22; $P = 0.047$). In contrast, *rs1800802* was not associated with the risk of nephrolithiasis ($P = 0.56$). In keeping with this case–control study [6], we noticed that the *A* allele of *rs4236* was associated with higher levels of inactive dp-ucMGP, whereas such association was not present for the *T/C* (major/minor allele) polymorphism at *rs1800802* (Supplementary data, Table S4). In a Chinese study of 354 cases and 374 controls, Lu *et al.* [7] confirmed that the risk of nephrolithiasis was associated with *rs4236*, but not with *rs1800802*. Compared with minor allele *rs4236* carriers

(*G*; prevalence 29.8%), major allele homozygotes (*AA*; prevalence 70.2%) had a 1.39-fold (95% CI 1.01–1.92; P = 0.042) increased risk of kidney stones. SNP *rs4236* is located in exon 4 of the human *MGP* gene. Substitution of *A* by *G* leads to an amino acid change from threonine to alanine in the COOH terminus of MGP (Arg-Lys-Arg-Arg-Gly-Thr-Lys) and, because of the ensuing alteration in charge or conformation, probably affects the post-translational modification or function of MGP [6].

Calcareous stones are by far the most common kidney nephroliths, accounting for >80% of cases [4, 31–33]. Among 2800 patients examined at a renal stone clinic in Southampton [34], most stones were calcium oxalate (89%), with a 1:4 ratio of calcium oxalate to mixed calcium oxalate phosphate stones. Pure calcium phosphate stones were rare (2%). In Sweden, ∼85% of all stones formed in the urinary tract could be classified as calcium stones, including calcium oxalate and calcium phosphate [35]. These observations suggest that active MGP might prevent nephrolithiasis by inhibition of the deposition of calcium crystals. From a mechanistic viewpoint, another line of research to be pursued is to what extent other VK-independent mechanisms might protect against nephrolithiasis and make up for a deficit in activated MGP. Osteopontin (OPN) is expressed in the human kidney [36], does not require VK for its activation and inhibits the formation of insoluble calcium salts [37, 38]. Hyperoxaluric mice upregulate OPN expression in response to intratubular deposition of calcium oxalate [37]. Mice deficient in MGP alone ($MGP^{-/-}OPN^{+/+}$) develop arterial calcification with upregulation of OPN expression in calcified arteries, whereas mice lacking both MGP and OPN ($MGP^{-/-}OPN^{-/-}$) showed accelerated and enhanced medial calcification [38]. In contrast, according to the US Food and Drug Administration (www.ehealthme.com/ds/warfarin%20sodium/kidney%20stones), the incidence of kidney stones on treatment with warfarin is as low as 0.27% and therefore of the same order of magnitude as observed in the general population [28]. Nephroliths in patients on anticoagulant therapy are more likely to be haematin rather than calcium stones [39].

Our current study should be interpreted within the context of its possible limitations. First, although in line with the literature [4, 28], the number of incident cases in our study was relatively small. However, even with this small number of incident cases, the association of incident nephrolithiasis was strong, with a more than 2-fold increase in the risk with a doubling of the baseline dp-ucMGP level. Moreover, the cross-sectional analysis with 144 participants with kidney stones was concordant. Second, in the Mendelian randomization analysis, we used four SNPs in the *MGP* gene. The current literature does not provide much information on the frequency and functionality of these genetic markers in ethnic groups different from white Flemish (Supplementary data, Table S4), Japanese [6] or Han Chinese [7]. In spite of the consistency in the allelic frequencies of *rs4236* and *rs1800802*, our current genetic results should be extrapolated to other ethnicities with caution.

Notwithstanding these potential limitations, our current observations highlight potentially important clinical implications. The literature shows an association of CKD with nephrocalcinosis [40] or nephrolithiasis [41–43]. In a study conducted at the University Hospitals Leuven, the prevalence of nephrocalcinosis in kidney donors and in patients with CKD stages 1, 2, 3–4 and 5 amounted to 4.6, 14.3, 20.2 and 54.0%, respectively [40]. In multivariable-adjusted analyses of the Third National Health and Nutrition Examination Survey, estimated eGFR in stone formers with a BMI of $\geq$27 kg/m^2 was 3.4 mL/min/1.73 m^2 lower than that of similar non-stone formers (P = 0.005), whereas such an association was not found among leaner individuals [41]. The authors attributed their observations to changes in acid–base and mineral metabolism [40] or to decreased renal function [40]. As highlighted by the data in Table 2, we propose that VK deficiency might be the mechanism linking CKD and nephrolithiasis, either by protection of the renal microcirculation [3] or by other unknown mechanisms. This hypothesis might be relevant in view of the high recurrence rate of nephrolithiasis [14, 44]. Ferraro *et al.* [44] systematically reviewed the recurrence of idiopathic calcium kidney stones in 2168 patients enrolled in 21 randomized controlled trials [median follow-up 3.2 years (range 0.5–9.7)]. They reported that the median recurrence of kidney stones was 15 per 100 person-years (range 0–110) [44]. The recurrence of nephrolithiasis entails substantial costs [15], in particular in middle-aged economically active people, and is associated with a high risk of cardiovascular events [45, 46]. Finally, more than one-third of our study participants had a dp-ucMGP level higher than optimal for the prevention of macrovascular complications (4.6 μg/L) [2]. Our findings therefore suggest that increasing the dietary intake of VK, either by supplementation or by increasing the intake of nutrients rich in VK, such as leafy vegetables and fermented foods [47], might prevent the formation of kidney stones. To translate our present findings into clinical application, a randomized clinical trial of VK substitution to prevent stone recurrence is a research priority. It would also test the causality suggested by our Mendelian randomization analysis.

The three key assumptions underlying a Mendelian randomization analysis [23] were met: (i) the phenotype (dp-ucMGP) was associated with the instrumental variables (*MGP* genotypes), (ii) the *MGP* genotypes were not associated with the confounders (Supplementary data, Table S5) and (iii) and the instrumental variable (*MGP* genotypes) should only be related to the outcome under study (nephrolithiasis) through its association with the phenotype (dp-ucMGP). However, in addition to violation of the key assumptions, the instrumental variables might be weak, leading to imprecise biased estimates or decreased power. In our current analyses, the *F*-statistics relating plasma dp-ucMGP levels to the three SNPs in *MGP* were highly statistically significant, although the explained variance ranged from 1.13 to 1.60%. *F*-values >10 signify sufficient strength to ensure the validity of the instrumental variable [48].

In conclusion, higher levels of inactive dp-ucMGP are associated with the risk of nephrolithiasis. This association might be causal, as evidenced by our Mendelian randomization analysis and by previously published genetic association studies [6, 7]. Further studies should clarify the underlying molecular pathways and substantiate the speculation that VK supplementation might promote renal health by reducing the risk of nephrolithiasis.

SUPPLEMENTARY DATA

Supplementary data are available online at http://ndt.oxfordjournals.org.

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CONFLICT OF INTEREST STATEMENT

N.A.E.D. and C.V. are employees of the R&D Group VitaK. The other authors declare no conflict of interest.

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Supplemental Information

This appendix formed part of the original submission and has been peer reviewed. Supplement to: The risk of nephrolithiasis is causally related to inactive Matrix Gla Protein, a marker of vitamin K status: a Mendelian randomization study in a Flemish population.

Table of Contents

Table S1. Four common tagging SNPs in the *MGP* gene that are in high linkage disequilibrium (r^2>0.80) with 223 proxy SNPs (starts)

SNP ID	bp	Location	MAF	Tagged SNP
rs2098435	15004589	Flanking-3'	0.45	rs2080545, rs2098434, rs7309504, rs6488717, rs6488716, rs10772811, rs7316763, rs2430724, rs4414276, rs115201791, rs7953256, rs12314055, rs116843477, rs10846064, rs7953763, rs12320644, rs12298507, rs2430729, rs10846068, rs7971527
rs4236	15035081	Coding	0.41	rs1985583, rs7132884, rs6488725, rs34464763, rs6488724, rs11056227, rs4763403, rs4763402, rs1544687, rs11835413, rs2900342, rs725444, rs10772814, rs10846074, rs2430731, rs6488723, rs6488722, rs6488721, rs6488720, rs2098436, rs3940337, rs12320004, rs7307883, rs7294526, rs2445409, rs4764129, rs11056209, rs7294463, rs7305000, rs7301929, rs10846067, rs7965885, rs6488715, rs6488714, rs2430725, rs7966998, rs2445411, rs3887182, rs11056202, rs11276, rs12226970, rs12829642, rs2430723, rs7311924, rs10772809, rs10772808, rs4764128, rs1001096, rs12811845, rs2287227, rs2287226, rs11056199, rs2430737, rs7310951, rs2445410, rs11056203, rs10846066, rs7980899, rs725445, rs2024505, rs2430687, rs2430689, rs2430690, rs3088189, rs10846075, rs4763404, rs1800801, rs10492151, rs4763401, rs767842, rs10846065, rs67615407, rs12307494, rs11056198, rs2430740, rs12811790, rs11056230, rs10492150, rs12305441, rs12426616, rs1861698, rs12822851, rs1049897, rs12300129, rs9668569, rs58120888, rs7965448, rs12311463, rs67482087, rs67436073, rs11056233, rs7135211, rs7306888, rs4581512, rs12316046, rs12322273, rs11615259, rs55785959, rs11614333, rs11610597, rs4764134, rs61922970, rs61922971, rs10772807, rs4290285, rs75293129, rs11056201

Table S1. Four common tagging SNPs in the *MGP* gene that are in high linkage disequilibrium (r^2>0.80) with 223 proxy SNPs (ends)

SNP ID	bp	Location	MAF	Tagged SNP
rs1800802	15038919	Flanking-5'	0.20	rs11056207, rs11056223, rs11056197
rs2430692	15065823	Flanking-5'	0.44	rs2430691, rs7310026, rs7308508, rs2216292, rs2445388, rs2193357, rs2193358, rs918121, rs918122, rs918123, rs1861695, rs10772816, rs10772817, rs10772818, rs10846082, rs1005577, rs2430704, rs6488727, rs4272830, rs4140767, rs7487408, rs2098433, rs11056243, rs11056244, rs2430706, rs2430707, rs2445370, rs12311015, rs4764135, rs4764136, rs2430690, rs2445382, rs2430689, rs2430687, rs2024505, rs2193356, rs2445384, rs10772819, rs2430695, rs2445380, rs10047541, rs2445379, rs2256576, rs2193360, rs2417403, rs2417404, rs2445374, rs12312821, rs2430700, rs2430701, rs11056242, rs12302886, rs11056246, rs4764137, rs4764138, rs11056247, rs34356871, rs10492149, rs4764139, rs4764140, rs4764141, rs3748295, rs3748296, rs7295194, rs12320865, rs7295480, rs10772821, rs10846087, rs10846088, rs11056248, rs4763406, rs12298173, rs7134290, rs2430702, rs12811790, rs3887182, rs11056202, rs11276, rs12226970, rs12829642, rs2430723, rs7311924, rs10772809, rs10772808, rs4764128, rs1001096, rs12811845, rs2287227, rs2287226, rs11056199, rs3748297, rs2256577, rs2193359

SNP ID is a GenBank ID number (NCBI). Position and location type were taken from the human genome sequence assemblies (NCBI Build 37.3). Allele frequencies were calculated in the present population. Proxy SNPs present an r^2>0.80 with selected MGP markers. SNP, single-nucleotide polymorphism; bp, base pairs; MAF, minor allele frequency.

Table S2. Distance, r^2 and *D'* for paired *MGP* SNPs

SNP1	SNP2	Distance	r^2	*D'*
rs2098435	*rs4236*	30492	0.659	1
rs2098435	*rs2430692*	61234	0.500	0.774
rs2098435	*rs1800802*	34330	0.004	0.070
rs4236	*rs2430692*	30742	0.791	1
rs4236	*rs1800802*	3838	0.176	1
rs1800802	*rs2430692*	26904	0.018	0.286

The distance, r^2 and *D'* were calculated using the 1000 genomes panel as reference.

Table S3. Allele and genotype frequencies in 536 unrelated founders

Gene	Allele		Genotype			P
rs2098435	*A*	*G*	*AA*	*AG*	*GG*	
n = 530	486 (45.8)	574 (54.2)	113 (21.3)	260 (49.1)	157 (29.6)	0.78
rs4236	*A*	*G*	*AA*	*GA*	*GG*	
n = 536	652 (60.8)	420 (39.2)	193 (36.0)	266 (49.6)	77 (14.4)	0.34
rs1800802	*C*	*T*	*CC*	*TC*	*TT*	
n = 532	178 (16.7)	886 (83.3)	14 (2.6)	150 (28.2)	368 (69.2)	0.78
rs2430692	*A*	*G*	*AA*	*AG*	*GG*	
n = 525	617 (58.8)	433 (41.2)	177 (33.7)	263 (50.1)	85 (16.2)	0.44

Values indicate number of alleles or genotypes (%). P-values test departure from Hardy–Weinberg equilibrium.

Table S4. Association of dp-ucMGP levels with *MGP* genotypes

Genotypes	1466 Participants		536 Unrelated Founders	
	Estimate±SE	P	Estimate±SE	P
rs2098435				
AA	0.91 (0.89–0.93)	<0.001	0.92 (0.89–0.96)	<0.001
AG	0.99 (0.97–1.01)	0.22	0.97 (0.95–1.002)	0.08
GG	1.11 (1.09–1.14)	<0.001	1.11 (1.08–1.15)	<0.001
rs4236				
AA	1.11 (1.09–1.14)	<0.001	1.12 (1.08–1.15)	<0.001
GA	1.00 (0.99–1.02)	0.60	0.98 (0.95–1.01)	0.28
GG	0.90 (0.87–0.92)	<0.001	0.91 (0.88–0.95)	<0.001
rs1800802				
CC	0.97 (0.92–1.02)	0.19	0.98 (0.90–1.06)	0.64
TC	1.01 (0.98–1.04)	0.54	1.00 (0.95–1.05)	0.90
TT	1.02 (0.998–1.05)	0.09	1.02 (0.98–1.07)	0.33
rs2430692				
AA	1.11 (1.09–1.13)	<0.001	1.12 (1.09–1.16)	<0.001
AG	1.01 (0.99–1.03)	0.16	0.99 (0.96–1.02)	0.40
GG	0.89 (0.87–0.91)	<0.001	0.90 (0.87–0.94)	<0.001

Estimate (± SE) for genotypic effects were derived by deviation-from-mean coding in 1466 participants, while accounting for family cluster and in 536 unrelated founders. All models included age, body mass index, smoking and drinking as covariables.

Table S5. Association of *MGP* genotypes with confounding factors

Characteristic	***rs4236***			***rs2098435***			***rs2430692***		
	AA	*G*-allele carriers	P	*GG*	*A*-allele carriers	P	*AA*	*G*-allele carriers	P
Number with genotype	548	918		406	1037		513	917	
Women (n [%])	285 (52.0)	463 (50.4)	0.56	212 (52.2)	528 (50.9)	0.66	266 (51.8)	463 (50.5)	0.62
Age (years)	45.5±15.4	45.6±15.4	0.89	45.7±15.6	45.6±15.4	0.87	45.2±15.3	45.8±15.4	0.42
24-h urinary volume (L)	1.55±0.69	1.60±0.66	0.16	1.52±0.65	1.59±0.67	0.090	1.54±0.70	1.59±0.66	0.13
24-h urinary calcium (mmol)	3.85±2.06	3.97±2.02	0.26	3.74±2.01	3.98±2.02	0.042	3.88±2.08	3.97±2.02	0.40
Propensity score	2.535±0.09	2.541±0.08	0.15	2.537±0.09	2.540±0.08	0.58	2.536±0.09	2.541±0.08	0.28

Values are number of participants (%) or mean±SD. P values denote the significance of the difference between major allele homozygotes and minor allele carriers.

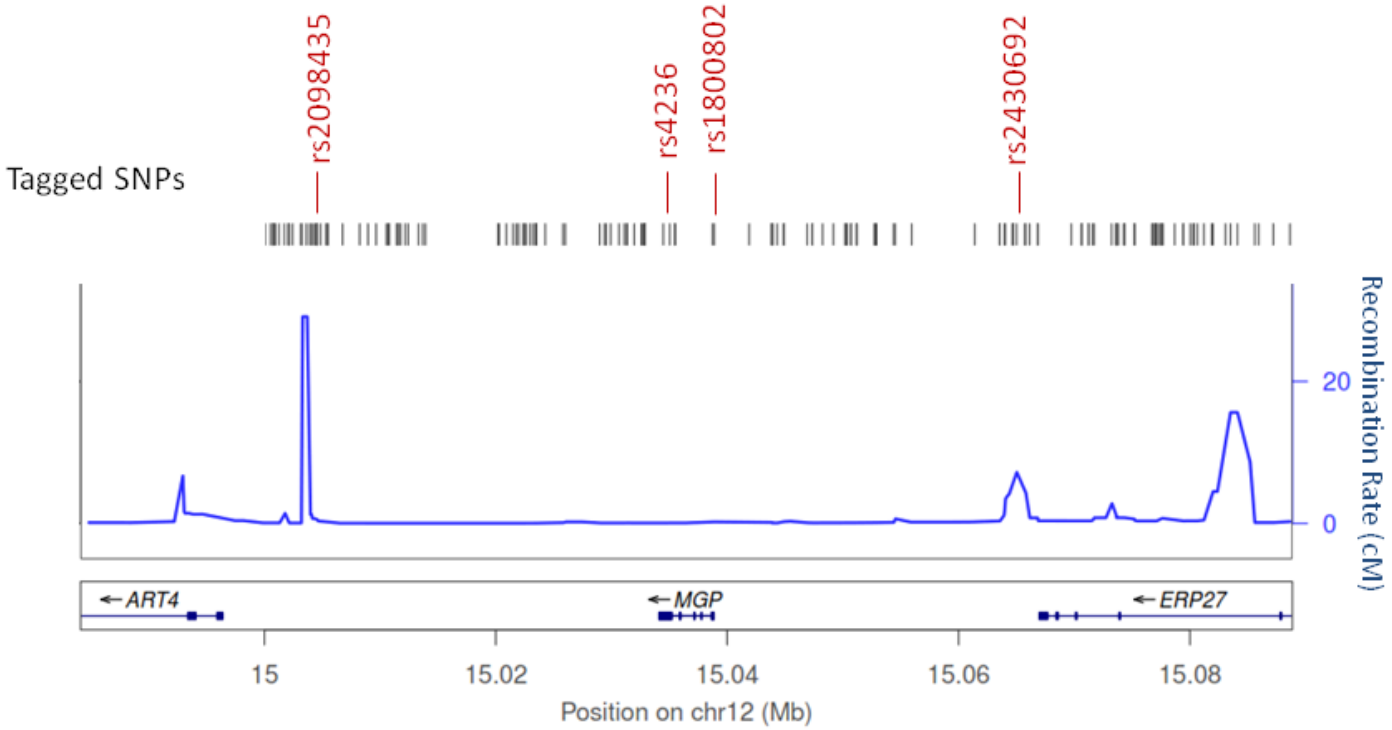

Figure S1.

The x-axis represents the physical position on the chromosome (build 37, hg19). The y-axis and the line indicate the recombination rate. We selected four SNPs (rs number and position given) that are in high linkage disequilibrium (r^2>0.80) with 223 proxy SNPs denoted by vertical lines. More information is available in Supplementary Table 1.