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Decreased monocyte calcium sensing receptor expression in patients with chronic kidney disease is associated with impaired monocyte ability to reduce vascular calcification

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In chronic kidney disease (CKD), calcium-sensing receptor (CaSR) expression and function have been extensively studied in parathyroid tissue and vascular tissues. To examine whether similar changes occurred in other tissues, we measured total and surface CaSR expression in monocytes of patients with various stages of CKD and healthy volunteers respectively in cross-sectional studies. We further explored *in vitro* the impact of uremic serum on CaSR expression in monocytes (U937 and THP-1 cell lines), and whether human peripheral blood mononuclear cells or U937 and THP-1 monocytes might modify vascular calcium deposition in rat carotid arteries *in vitro*. CKD was associated with a decrease in peripheral blood mononuclear cell CaSR expression both in total and at the monocyte surface alone (43% and 34%, respectively in CKD stages 4–5). This decrease was associated with a reduction in the ability of monocytes to inhibit vascular calcification *in vitro*. Pretreatment with the calcimimetic NPSR568 of peripheral blood mononuclear cells isolated from patients with CKD significantly improved monocyte capacity to reduce carotid calcification *in vitro*. The fewer peripheral blood mononuclear cells expressing cell surface CaSR, the more calcimimetic treatment enhanced the decrease of carotid calcium content. Thus, we demonstrate that monocyte CaSR expression is decreased in patients with CKD and provide *in vitro* evidence for a potential role of this decrease in the promotion of vascular calcification. Hence, targeting this alteration or following monocyte CaSR expression as an accessible marker might represent a promising therapeutic strategy in CKD-associated arterial calcification.

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KEYWORDS: calcium-sensing receptor; chronic kidney disease; monocytes; translational medical research; vascular calcification

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Translational Statement

In chronic kidney disease (CKD) patients, monocytes are losing their “resorptive” capacity, as demonstrated in an *in vitro* functional assay. The impairment may be due at least in part to the decrease in calcium-sensing receptor expression in CKD, as preincubation of CKD stage 4–5 human monocytes with calcimimetic restores this monocyte action. The next step is to examine in hyperphosphatemic CKD patients whether restoring monocyte “resorptive” capacity can represent a new therapeutic approach aimed at slowing or protecting against vascular calcification. Our findings further suggest that monitoring blood monocyte calcium-sensing receptor expression may be beneficial in the clinical management of CKD subpopulation of patients.

The calcium-sensing receptor (CaSR), a class C G-protein coupled receptor, is expressed in several tissues, including the kidney, the parathyroid, vascular tissues, and the bone.¹ Because of its ability to sense variations of extracellular ionized calcium concentration, CaSR plays a major role in regulating parathyroid hormone secretion, calcium homeostasis (including renal tubular calcium reabsorption), and bone remodeling.

In chronic kidney disease (CKD), CaSR expression is decreased in parathyroid gland² and contributes to secondary and tertiary hyperparathyroidism.³ A CKD-associated decrease of CaSR expression has also been observed in other tissues,

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including the vasculature,⁴ heart valves,⁵ and kidney.^{6,7} The lower expression of CaSR in the vasculature is implicated in the pathogenesis of arterial calcification,⁸ whereas the decrease of renal CaSR modifies renal cation handling.⁸ Monocytes are the rare circulating cells with high CaSR expression.⁹ Several studies demonstrated that it plays a critical role in monocyte migration¹⁰ and cytokine production, such as interleukin-1 β and tumor necrosis factor- α .^{11–13} In CKD, an increase in monocyte number has been reported,¹⁴ together with a higher rate of bacterial infections.¹⁵ Because information is lacking, we asked whether monocyte CaSR expression was modulated in CKD. We quantified CaSR expression in cluster of differentiation (CD) 14⁺ peripheral blood mononuclear cells (PBMCs) from a cohort of healthy adults and patients with different stages of CKD. Moreover, we tested the *in vitro* effect of uremic serum on PBMC CaSR expression. We then investigated the role of monocyte CaSR expression in an *ex vivo* model of vascular calcification, as well as the consequences of CKD-induced CaSR variation in the same model.

METHODS

Study design

Human subjects were included in 2 parallel studies. The first study enrolled adult blood donor volunteers, as reported previously.¹⁶ Exclusion criteria were CKD, inflammatory disease, previous cardiovascular event, cancer, acute infectious disease, and primary hyperparathyroidism. All healthy volunteers were supplemented by cholecalciferol, 100,000 IU every 2 weeks, with a total dose depending on initial vitamin D status, as recommended by Groupe de Recherche et Information sur l'Ostéoporose (GRIO) guidelines¹⁷ before blood sampling.

The second study enrolled patients with CKD at the outpatient nephrology clinic of the university hospital of Amiens, France. Inclusion criteria were age 18–80 years, a confirmed diagnosis of CKD by 2 consecutive measures of CKD Epidemiology Collaboration eGFR <80 ml/min per 1.73 m² or patients receiving dialysis therapy for >3 months. Exclusion criteria were the same as those for the healthy volunteers, with the exception of CKD. Patients with a recent (<3-month) decrease of eGFR >30% were also excluded. From all CKD patients who accepted to participate in the study, blood samples were taken directly after the outpatient clinic.

The 2 pilot studies were thought to have a common secondary outcome, targeting the identification of clinical and laboratory parameters (in particular, those related to CKD) associated with the CaSR expression in monocytes.

All subjects gave their written informed consent. The study protocols were approved by a local independent ethics committee (Comité de Protection des Personnes Nord-Ouest II, Amiens, France; references 2011-A01449-32 and 2011-A01448-33, respectively; reference PI2019-8430040 for [Supplementary Figures S1–S3](#)) and the French National Agency for Medicines and Health Product Safety. The procedures followed were in accordance with the Declaration of Helsinki.

Routine biochemistry laboratory parameters and uremic toxin measurements are described in the [Supplementary Methods](#).

PBMC isolation and culture

PBMCs were isolated from 8 ml of blood sample. Samples were centrifugated on Ficoll (Histopaque 1077; reference 1077-1) at 1800

rpm for 25 minutes. Interphase-containing PBMCs were collected and washed with phosphate-buffered saline–0.5% bovine serum albumin. Cells were harvested after a last centrifugation (1800 rpm; 10 minutes) and were mixed in either 2 ml of fetal bovine serum–10% dimethyl sulfoxide for healthy volunteers or 3 ml of their own serum–10% dimethyl sulfoxide for CKD patients. Cells were then frozen progressively and were conserved for long period at –150 °C.

PBMCs were cultured in 6-well plate in Roswell Park Memorial Institute Medium (Sigma Aldrich, St. Louis, MO), supplemented with 10% fetal bovine serum, 2 mM GlutaMAX (Invitrogen, Carlsbad, CA), and 100 UI/ml penicillin-streptomycin (Sigma Aldrich). To study the effect of patient sera on CaSR expression, PBMCs were cultured for 48 hours with increasing concentration of serum (from 1% to 30%). The effects of 2 types of serum were compared: pool of serum from healthy donors (IPLA-SER-500 ml SERPIME) and pool of serum from hemodialysis patients (unused serum during routine laboratory tests, obtained in accordance with French legislation). These sera were characterized by both biochemistry laboratory using autoanalyzer techniques and quantification of uremic toxin concentrations (as described in the [Supplementary Methods](#)).

Quantification of CaSR expression in monocytes

CaSR expression was quantified by flow cytometry at the level of the monocyte membrane and in whole permeabilized monocytes. When required, permeabilization was obtained by incubation of 100 μ l of BD Cytofix/Cytoperm (BD Biosciences, Le Pont de Claix, France; 5554715) solution for 20 minutes, following manufacturer's instruction. After washes in phosphate-buffered saline–0.5% bovine serum albumin (Sigma-Aldrich), PBMCs were incubated with 10 μ l of CaSR monoclonal antibody 1:40 (5C10; ADD; Abcam, Cambridge, UK; ab19347) or with control IgG2a (X0943; DakoCytomation, Les Ulis, France) for 30 minutes at 4 °C in the dark. After washes in phosphate-buffered saline–0.5% bovine serum albumin, PBMCs were marked with phycoerythrin-conjugated IgG mouse antibodies (Polyclonal Goat Anti-Mouse Immunoglobulins/RPE [R 0480; DakoCytomation]) for 15 minutes at 4 °C in the dark. To detect monocyte population from PBMCs, a CD14 labeling was performed by incubating PBMCs with anti-CD14 antibodies conjugated to fluorescein isothiocyanate 1:4 (mouse monoclonal antibody anti-C14–fluorescein isothiocyanate 130-080-701; MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) or with monoclonal mouse IgG2a isotype control antibodies conjugated with fluorescein isothiocyanate (130-091-837; MACS; Miltenyi Biotec) for 30 minutes at 4 °C in the dark. After a last wash with phosphate-buffered saline–0.5% bovine serum albumin, surface and total CaSR expression of CD14⁺ PBMCs was then assayed by FACS Canto II (BD Biosciences). CaSR monocyte expression was performed on thawed PBMCs, by series using 20 different samples, each time in duplicate. To compare CaSR expression from different series, a minimum of 4 common samples were used between each series to apply a linear correction factor. Western blot and quantitative reverse transcription–polymerase chain reaction, also used to quantify CaSR expression, are described in the [Supplementary Methods](#).

In vitro vascular decalcification assay

Rat carotid calcification was obtained, as reported previously.¹⁸ Briefly, after careful removal of most of the carotid adventitia of 2-month-old Wistar rats, carotid artery segments (3–4 mm in length) were incubated at 37 °C and in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; D6546; Sigma-Aldrich), supplemented with 10% fetal bovine serum, 1% GlutaMAX, and 1%

Table 1 | Clinical and biochemical characteristics of healthy volunteers and patients with CKD

Characteristics	eGFR, ml/min per 1.73 m ²				P value
	Total	≥60	30–60	<30	
No.	60	20	25	15	
eGFR, ml/min per 1.73 m ²	46.9 ± 23.7	76.6 ± 12.7 ^{a,b}	42.6 ± 8.9 ^{a,c}	15.4 ± 6.7 ^{b,c}	<0.001
Serum creatinine, μmol/L	182 ± 155	88 ± 13 ^b	133 ± 24 ^c	391 ± 188 ^{b,c}	<0.001
Age, yr	59.9 ± 14.7	53.2 ± 16.6 ^a	63.9 ± 12.3 ^a	62.4 ± 13.7	0.038
Sex, male	28 (47)	9 (45)	9 (36)	10 (67)	0.167
Serum total Ca, mmol/L	2.31 ± 0.14	2.30 ± 0.08	2.34 ± 0.10	2.26 ± 0.23	0.173
Serum PO ₄ ²⁻ , mmol/L	1.10 ± 0.23	1.02 ± 0.17 ^b	1.07 ± 0.23 ^c	1.27 ± 0.20 ^{b,c}	0.002
Serum PCS, μg/ml	16.7 ± 22.1	4.7 ± 7.2 ^b	11.1 ± 12.4 ^c	41.0 ± 28.0 ^{b,c}	<0.001
Serum IS, μg/ml	5.8 ± 10.2	1.0 ± 1.6 ^b	4 ± 7.8 ^c	14.6 ± 14.1 ^{b,c}	<0.001
Serum IAA, μg/ml	0.85 ± 0.38	0.67 ± 0.24 ^b	0.84 ± 0.30	1.10 ± 0.52 ^b	0.003
Serum i-PTH, pg/ml	106 ± 117	47 ± 44 ^b	91 ± 75 ^c	216 ± 170 ^{b,c}	<0.001
Serum 25(OH) vitD ₃ , ng/ml	30.2 ± 12.3	34.4 ± 11	27.8 ± 12.6	28.8 ± 12.7	0.184
Blood Hb, g/dl	13.2 ± 1.5	14.4 ± 1.2 ^{a,b}	13.3 ± 1.0 ^{a,c}	11.7 ± 1.2 ^{b,c}	<0.001
Serum albumin, g/L	42.3 ± 4.2	44 ± 3.2	41.2 ± 4.7	42.3 ± 3.6	0.094
Serum CRP, mg/ml	6.4 ± 19.4	4.1 ± 4.6	3.5 ± 2.1	14.9 ± 39.2	0.175
Monocyte total CaSR, AU	674 ± 392	901 ± 480 ^{a,b}	589 ± 303 ^a	514 ± 253 ^b	0.004
Monocyte surface CaSR, AU	319 ± 72	365 ± 73 ^{a,b}	307 ± 60 ^a	280 ± 60 ^b	<0.001

25(OH) vitD₃, 25-hydroxy vitamin D₃; AU, arbitrary unit; Ca, calcium; CaSR, calcium-sensing receptor; CKD, chronic kidney disease; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; Hb, hemoglobin; IAA, indole acetic acid; i-PTH, intact parathyroid hormone; IS, indoxyl sulfate; PCS, paracresyl sulfate; PO₄²⁻, phosphate.

^aP < 0.05, eGFR ≥60 ml/min per 1.73 m² comparatively to 30 to 60 ml/min per 1.73 m² (Tukey post-test).

^bP < 0.05, eGFR ≥60 ml/min per 1.73 m² comparatively to <30 ml/min per 1.73 m² (Tukey post-test).

^cP < 0.05, eGFR 30 to 60 ml/min per 1.73 m² comparatively to <30 ml/min per 1.73 m² (Tukey post-test).

Healthy volunteers and CKD patients have been subdivided into 3 groups, according to their eGFR, calculated by CKD Epidemiology Collaboration formula. Values are indicated as mean ± SD or number (percentage). The P value indicated has been calculated by analysis of variance or χ² test.

penicillin/streptomycin (called hereafter complete DMEM). To induce carotid calcification, the arterial segments were exposed to complete DMEM in which inorganic phosphate (Pi) concentration was increased from 0.9 to 3.8 mM by adding Pi as NaH₂PO₄ (S5011; Sigma-Aldrich). The medium was changed every 2 days for a total of 5 days. Some carotids were exposed to monocytes (U937 and THP-1) or PBMCs during either those 5 days of culture (called hereafter “preventive model”) or an additional culture period, which started from the end of the procalcifying culture period and lasted for 5 days (called hereafter “curative model”). In the curative model, pre-calcified carotids and monocytes were cultured in complete DMEM in which the concentration of Pi remained unchanged compared with DMEM (0.9 mM). Quantification of calcium deposition was assessed, as previously described,¹⁸ with more details in the [Supplementary Methods](#).

Confocal microscopy

Localization of mineral deposition was stained by a red luminescent dye Osteosense 680 EX (Perkin-Elmer, Waltham, MA; NEV10020EX; 10 μl/ml; 24 hours at 37 °C). Monocyte cells were detected by using fluorescent PKH26 (PKH26GL; Sigma-Aldrich) staining, according to the manufacturer’s protocol. Emission at 567 nm was coded as green color to visually separate with the 680-nm wavelength of Osteosense. Cells were observed with confocal microscope, and pictures were acquired with Zeiss LSM780.

Statistical analysis

The populations of the 2 studies were regrouped and analyzed depending on their eGFR and separated into 3 groups (eGFR ≥60 ml/min, eGFR of 30–59 ml/min, and eGFR <30 ml/min). Laboratory and clinical variables were given as the mean ± SD. Qualitative variables were given as the number (frequency). Analysis of variance, followed by Tukey post-tests, was applied for quantitative variables, whereas the likelihood-ratio test was applied for qualitative variables.

To determine parameters associated with CaSR expression of monocyte (surface or total) higher than the median CaSR expression, a univariate logistic regression was performed. Variables significantly associated in univariate analysis were included in a multivariable logistic regression model using a top-down method based on the likelihood ratio.

For the *in vitro* experiments, results were represented in scatter blot with median and interquartile range. Differences between groups were analyzed using Kruskal-Wallis test, followed by Mann Whitney post-tests or Tukey post-tests. A Wilcoxon test was used for matched groups. Association between CaSR expression and calcification deposition was determined by Pearson correlation.

RESULTS

Main characteristics of the study population

The main clinical and biochemical characteristics of the study population are presented in [Table 1](#). Estimated glomerular filtration rate (eGFR) values using CKD Epidemiology Collaboration formula were equal or superior to 60 ml/min per 1.73 m² in 20 subjects, and inferior in 40 subjects. As expected, the uremic toxin serum concentrations of paracresyl sulfate (PCS), indoxyl sulfate, and indole acetic acid, as well as serum phosphate and intact parathyroid hormone levels were significantly increased in patients with CKD, whereas blood hemoglobin concentration and monocyte numbers were significantly decreased.

Monocyte CaSR expression in patients with CKD and healthy subjects

Both total and surface monocyte CaSR expressions were significantly lower in subjects with eGFR <60 ml/min per 1.73 m² compared with subjects with eGFR ≥60 ml/min per 1.73 m². Specifically, total CaSR expression was significantly decreased by

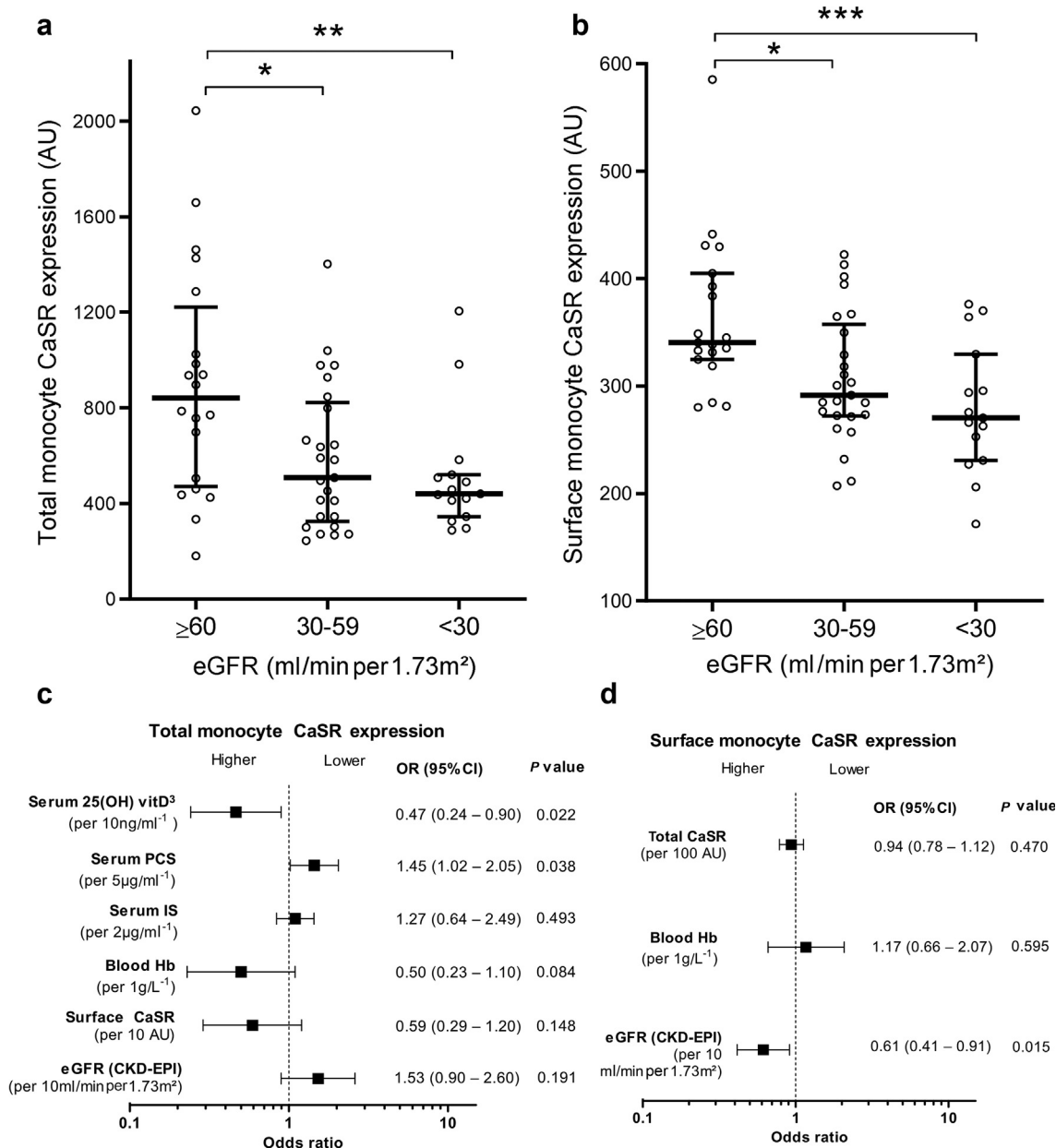


Figure 1 | Calcium-sensing receptor (CaSR) expression in cluster of differentiation 14⁺ peripheral blood mononuclear cells (PBMCs), according to estimated glomerular filtration rate (eGFR) in healthy subjects and chronic kidney disease (CKD) stage 3–5 patients (n = 60). (a,b) Protein CaSR expression was assessed by flow cytometry in PBMCs on whole monocyte or monocyte membrane. Three groups of patients were examined, according to their eGFR, as estimated by the CKD–Epidemiology Collaboration (EPI) formula. Boxplots represent median, interquartile, minimum, and maximum values. Analysis of variance *P* values were 0.0041 and 0.0007 for total and surface expression, respectively. (c,d) Association of various parameters with CaSR expression below the median, assessed using multivariable logistic regression analysis. Variables were included in the model if they were associated in univariate analysis with *P* < 0.05. Odds ratio (OR) was expressed per increment of the quantitative variables. **P* < 0.05 versus eGFR ≥60 ml/min per 1.73 m². ***P* < 0.01 versus eGFR ≥60 ml/min per 1.73 m². ****P* < 0.001 versus eGFR ≥60 ml/min per 1.73 m². 25(OH) vitD₃, 25-hydroxy vitamin D₃; AU, arbitrary unit; CI, confidence interval; Hb, hemoglobin; IS, indoxyl sulfate; PCS, paracresyl sulfate.

35% in CKD stage 3 and by 43% in CKD stages 4–5 (Figure 1a), and surface CaSR expression was decreased by 25% in CKD stage 3 and by 34% in CKD stages 4–5 (Figure 1b).

In an attempt to identify the factors involved in the decrease of monocyte CaSR expression, we analyzed several clinical and biochemical parameters. In univariate logistic regression analysis, total monocyte CaSR expression below

the median was significantly associated with eGFR (odds ratio [OR] [95% confidence interval {CI}] per 10 ml/min per 1.73 m², 0.70 [0.55–0.89]; *P* = 0.004), serum PCS (OR [95% CI] per 5 µg/ml, 1.42 [1.12–1.81]; *P* = 0.004), serum indoxyl sulfate (OR [95% CI] per 2 µg/ml, 1.54 [1.03–2.31]; *P* = 0.034), and serum 25(OH) vitamin D₃ (OR [95% CI] per 10 ng/ml, 0.50 [0.30–0.82]; *P* = 0.006). When these parameters

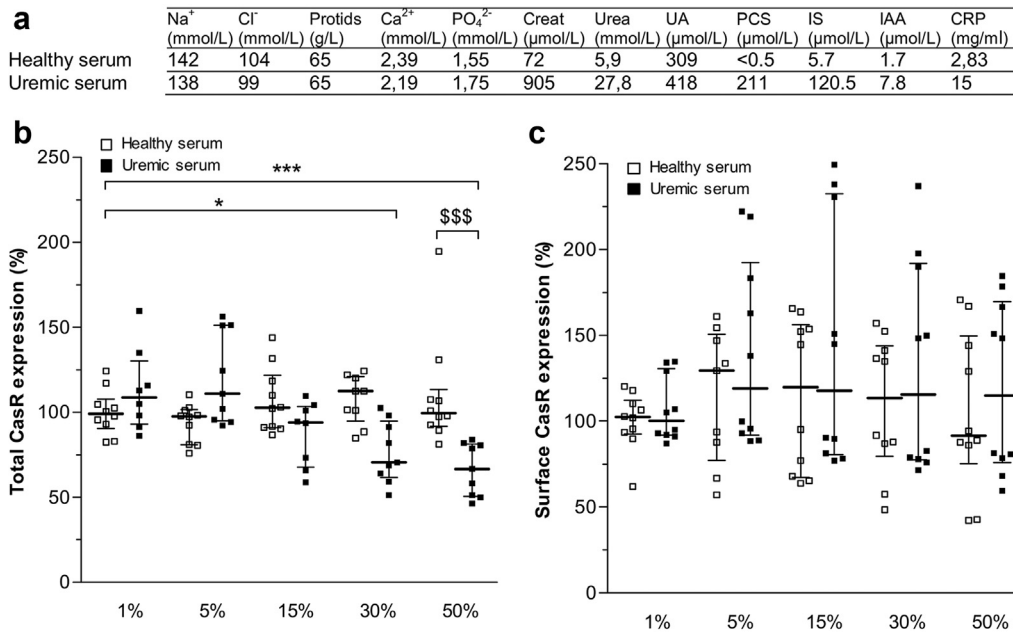


Figure 2 | Impact of uremic status on monocyte calcium-sensing receptor (CaSR) expression. (a) Biochemical parameter values of pooled sera collected from healthy donors and from chronic hemodialysis patients. (b,c) CaSR expression in cluster of differentiation 14⁺ peripheral blood mononuclear cells (PBMCs) was assessed by flow cytometry after 48 hours of incubation with increasing amounts of serum from whole monocyte or monocyte membrane. White and black bars show the results for healthy and uremic sera, respectively (mean ± SD). Experiments were performed in triplicate in PBMCs from 4 different donors. The Kruskal Wallis *P* value is <0.0001. **P* < 0.05 versus control (1% healthy serum). ****P* < 0.001 versus control (1% healthy serum). \$\$\$*P* < 0.001 (Tukey post-tests). Ca²⁺, calcium; Cl, chlorine; Creat, creatinine; CRP, C-reactive protein; IAA, indole acetic acid; IS, indoxyl sulfate; Na⁺, sodium; PCS, paracresyl sulfate; Prot, proteins; PO₄²⁻, phosphate; UA, uric acid.

were included in a multivariable model, only serum PCS and 25(OH) vitamin D₃ remained significantly associated with low CaSR expression, with OR (95% CI) per 5 μg/ml = 1.39 (1.01–1.93) (*P* = 0.046) and OR (95% CI) per 10 ng/ml = 0.54 (0.31–0.97) (*P* = 0.039), respectively (Figure 1c). In univariate logistic regression, surface monocyte CaSR expression below the median was significantly associated with eGFR (OR [95% CI] per 10 ml/min per 1.73 m², 0.63 [0.47–0.82]; *P* = 0.001), blood hemoglobin (OR [95% CI] per 1 g/L, 0.66 [0.44–0.99]; *P* = 0.044), and total monocyte CaSR expression (OR [95% CI] per 100 UA, 0.84 [0.72–0.98]; *P* = 0.029). When these variables were included in multivariable logistic regression, low surface CaSR expression of monocyte was associated only with eGFR, with OR (95% CI) per 10 ml/min per 1.73 m² = 0.61 (0.41–0.91) (*P* = 0.015) (Figure 1d).

To assess the specificity of these observations, we also performed Western blot and quantitative reverse transcription–polymerase chain reaction analysis using PBMCs isolated from stage 4–5 CKD patients (*n* = 3) as well as healthy individuals (*n* = 3). Western blot made using unsorted PBMC protein extracts demonstrated a significant reduction in CaSR expression in cells isolated from stage 4–5 CKD patients, compared with healthy individuals (Supplementary Figure S1). Reduced CaSR expression was confirmed when CD14⁺ PBMCs isolated from stage 4–5 CKD patients were assessed by flow cytometry, compared with CD14⁺ PBMCs isolated from healthy individuals. As shown

in flow cytometry histograms provided in Supplementary Figure S2, both CD14⁺ and CD14⁻ PBMCs are affected by reduced CaSR expression in CKD patients. No significant differences on CaSR mRNA expression were found between the groups of individuals tested (Supplementary Figure S3).

Impact of uremic serum on monocyte CaSR expression *in vitro*

A possible association between monocyte CaSR expression and CKD was further explored *in vitro* using pooled sera from healthy volunteers and CKD patients. As expected, serum phosphate, creatinine, urea, uric acid, PCS, indoxyl sulfate, indole acetic acid, and C-reactive protein concentrations were higher in CKD patients than healthy controls (Figure 2a). A 48-hour incubation of PBMCs in increasing amounts of uremic serum led to a dose-dependent, significant decrease in total cell CaSR expression, whereas healthy serum failed to do so (Figure 2b). However, the same incubation procedure did not significantly affect PBMC surface CaSR expression (Figure 2c).

Involvement of monocytes in vascular calcification *in vitro*

To explore potential pathophysiological consequences and considering the well-known association of soft tissue calcification with CKD¹⁹ and reduced CaSR expression,²⁰ we decided to examine a possible influence of monocyte CaSR in vascular mineralization. First, we wanted to know whether the addition of culture monocytes or PBMCs modified carotid calcification induced by phosphate (3.8 mM). We exposed rat carotids to

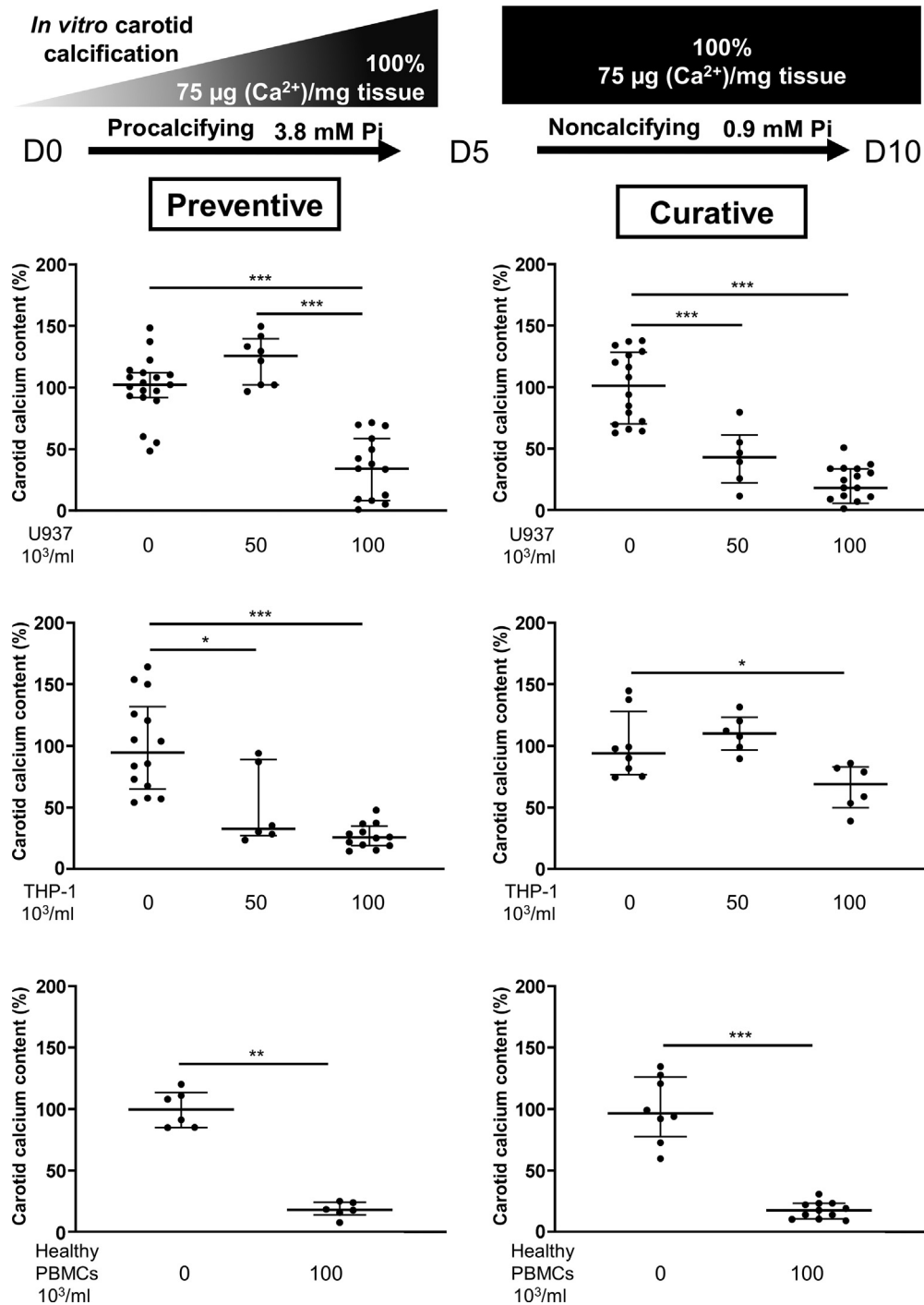


Figure 3 | Reduction of rat carotid calcium content by the addition of cell line monocytes or peripheral blood mononuclear cells (PBMCs). Monocytes (from cell lines U937 or THP-1, both obtained from American Type Culture Collection) or PBMCs (obtained from healthy donors) were added to the medium during or after initial carotid calcification. In the preventive model, rat carotid segments were exposed for 5 days to procalcifying culture conditions (3.8 mM Pi), alone or in combination with PBMCs (from $50 \times 10^3/\text{ml}$ to $100 \times 10^3/\text{ml}$). In the curative model, rat carotid segments were exposed for 5 days to procalcifying culture conditions (3.8 mM inorganic phosphate [Pi]), before being cultured for an additional 5 days in combination with PBMCs (50×10^3 cells/ml and 100×10^3 cells/ml). Calcium content was assessed at day 5 (D5) or day 10 (D10). Calcium content has been expressed as the percentage of mean calcium concentration ($\mu\text{g}/\text{mg}$), assessed when carotid segments were either not exposed or exposed to monocyte cell lines or PBMCs. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. D0, day 0.

increasing monocyte numbers, ranging from $50 \cdot 10^3$ to $100 \cdot 10^3$ cells/ml either during the mineralization process (preventive model) or for 5 days after the promineralizing culture phase

(curative model). As shown in [Supplementary Figure S4](#), carotid calcification increased to reach around $75 \mu\text{g}$ of calcium by mg of tissue after the first 5 days of culture. In the preventive

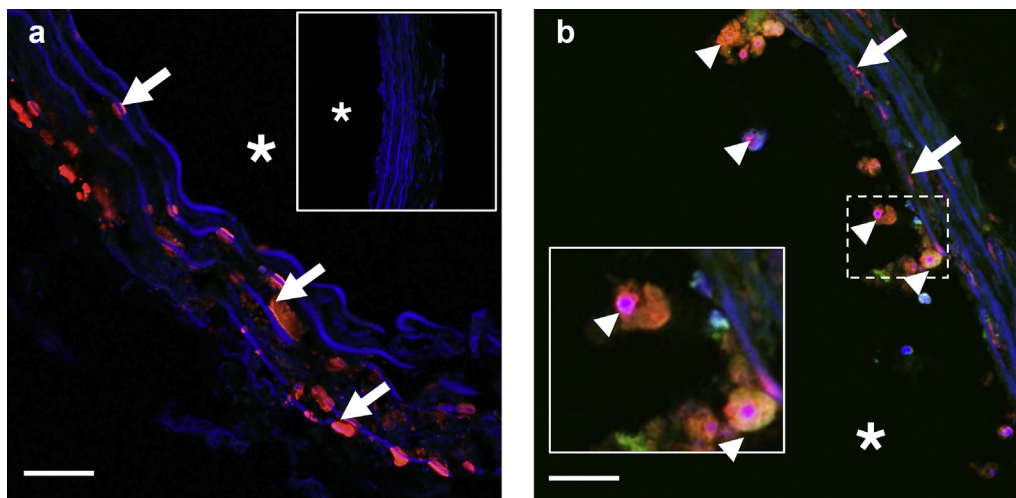


Figure 4 | Fluorescence microscopy localizing vascular calcium deposition and monocyte cells in a carotid calcification model. (a) Confocal photomicrograph of a calcified carotid segment (without monocyte addition) stained with Osteosense 680 EX. Mineral deposition appears as red fluorescent staining (arrows). In the upper right-hand corner, a representative photomicrograph shows a carotid artery segment grown for 10 days under nonprocalcifying conditions (0.9 mM inorganic phosphate) (*lumen). **(b)** Confocal photomicrograph of calcified carotid segment at day 10 of the curative model (with U-937 cells added on day 5) was stained by both Osteosense 680 EX and PKH26. PKH26 cell-membrane prestaining (green coloration) allows the localization of U-937 monocytes (arrowheads) in the vessel lumen (*), whereas Osteosense shows calcium in both the monocytes and the vessels (arrows). The lower left-hand corner shows a zoom (original magnification $\times 2$) of adherent monocyte. Bars = 100 μm . To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

model, carotid calcium content was significantly reduced by $58\% \pm 7\%$, $73\% \pm 3\%$, and $82\% \pm 3\%$ (comparatively to exposure to medium culture alone), when exposed to the presence of culture monocytes (U937 or THP-1) or PBMCs, respectively (Figure 3). Similar results were obtained in the curative model, except for calcified carotids co-cultured with THP-1 cells. The mean carotid calcium content remained unchanged (from day 5 [75.77 $\mu\text{g}/\text{mg}$] to day 10 [74.58 $\mu\text{g}/\text{mg}$]), unless carotids were co-cultured with monocytes.

Osteosense 680 EX staining and confocal microscopy allowed us to demonstrate the presence of calcium deposition in the carotids of our model (Figure 4a). Staining specificity was demonstrated by the absence of a red fluorescent signal in slices of carotids exposed to 0.9 mM of Pi for 5 days (control) and stained with Osteosense 680 EX during the last 24 hours of culture (from day 4 to day 5).

In the curative model, an additional U-937 membrane prestaining with PKH26 showed that the cells were adherent to the vascular wall (Figure 4b). The adhesion was associated with (i) an internalization of Osteosense 680 EX staining into the PKH26-positive cells and (ii) a decrease in calcium deposits in the carotid media. Interestingly, the presence of monocytes was inefficient to reduce carotid mineralization when physically separated from carotid segments (0.4- μm pore size Transwell) or when U937, THP-1, or PBMC culture supernatants were introduced in culture instead of monocytes (Supplementary Figure S5).

Association of monocyte CaSR activity or expression with monocyte ability to reduce calcification *in vitro*

Preincubation of monocytes (30 minutes) with the CaSR antagonist NPS 2143 (41 nM) significantly inhibited their

ability to reduce vascular calcification *in vitro* in both the “preventive” and “curative” models (Supplementary Figure S6). On the opposite, pretreatment of the cells for 30 minutes with the CaSR-positive allosteric modulator NPS-R568 improved the capacity of monocytes to protect against carotid calcification (Supplementary Figure S7). Taken together, these results indicate that monocytes may reduce vascular calcification *in vitro* and that the CaSR is involved in this process. To evaluate whether monocyte CaSR expression levels influence their ability to decrease vascular calcification *in vitro*, we co-cultured PBMCs from 8 CKD patients with calcified carotids (curative model). As reported in Figure 5, there was an inverse relationship between both total (Figure 5a) and surface (Figure 5b) CaSR expression on monocyte and the carotid mineral content, which reach a highly significant level ($r^2 = 0.61$; $P < 0.0001$) only with surface CaSR expression. Of note, pretreatment of the same PBMCs by a calcimimetic (NPS-R568) significantly improves CKD monocyte capacities to reduce carotid calcification (Figure 5c), but without suppressing the correlation between surface CaSR expression and calcium carotid content (Supplementary Figure S8). The less PBMCs expressed cell surface CaSR, the more NPS-R568 treatment enhanced the decrease of carotid calcium content (Figure 5d).

DISCUSSION

Our results demonstrate, for the first time that, as in parathyroid and vascular tissue,⁸ the uremic state induces a decrease of CaSR expression in blood monocytes of CKD patients. Both total and surface CaSR expression progressively decreased with the degree of severity of CKD, suggesting a potential

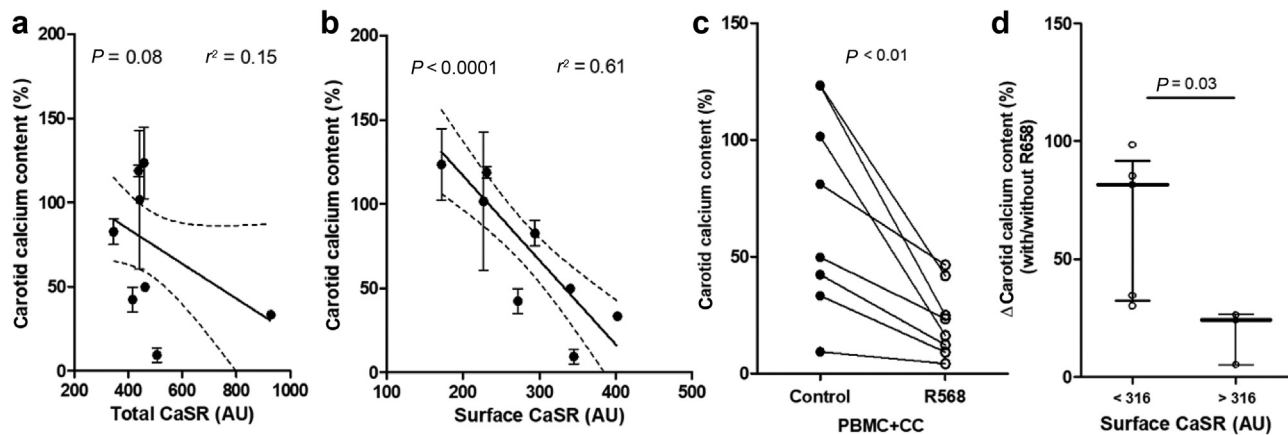


Figure 5 | Impact of decreased monocyte calcium-sensing receptor (CaSR) expression on chronic kidney disease (CKD)-associated reduction of the ability of peripheral blood mononuclear cells (PBMCs) to inhibit vascular calcification (n = 8). PBMCs from 8 patients with CKD were added to carotid segments *in vitro* in the curative model. Carotid-tissue calcium content was expressed as percentage of mean calcium concentration ($\mu\text{g}/\text{mg}$), assessed when carotid segments were not exposed to PBMCs. (a) Carotid calcium content was correlated with monocyte total CaSR expression. (b) Carotid calcium content was correlated with monocyte surface CaSR expression. (c) Carotid calcium content in the absence or presence of NPS-R568 pretreatment was matched, according to each donor, to specifically visualize the NPS-R568 effect. (d) Differences in carotid calcium content in the absence or presence of NPS-R568 pretreatment were compared between monocytes with low or sufficient CaSR expression (<316 and >316 arbitrary units [AUs], respectively). CC, calcified carotid.

pathophysiological role. Total monocyte CaSR expression was also decreased after exposure to uremic serum *in vitro*, suggesting a role of ≥ 1 circulating compounds retained in CKD, in particular uremic toxins. This is consistent with the independent association we found between total monocyte CaSR expression and serum PCS concentration in CKD patients. However, the specific cellular mechanism whereby PCS (or other uremic toxins) reduce CaSR expression in monocytes remains to be explored. The observation that surface monocyte CaSR expression is associated with CKD independently of total monocyte CaSR expression suggests that in monocytes isolated from patients with CKD, CaSR maturation, trafficking to the cytoplasmic membrane, and/or recycling are modified,^{21,22} compared with monocytes isolated from healthy individuals. Reinforcing this hypothesis, no significant differences in CaSR mRNA were found between healthy volunteers and CKD patients. These results are in agreement with mRNA-sequencing data about CD14⁺ PBMCs isolated from stage 4–5 CKD patients, indicating that the level of expression of mRNAs coding for CaSR does not seem to be affected in CD14⁺ PBMCs.²³ This is an important issue and requires further investigation.

In the present study, total serum 25(OH) vitamin D₃ was independently associated with CaSR expression, suggesting that disturbances in the vitamin D–vitamin D receptor (VDR) axis of CKD could constitute another mechanism contributing to the observed downregulation of monocyte CaSR. 25(OH) vitamin D₃ can modulate CaSR expression through direct affinity to VDR²⁴ or through local transformation toward active 1,25(OH)₂ vitamin D₃ because 1 α -25(OH) hydroxylase remains active in CKD monocytes.²⁵ We recently demonstrated that 1,25(OH)₂ vitamin D₃ was able to increase CaSR expression in monocytic U-937 cells¹⁶ as well as in vascular smooth muscle cells.²⁶ Moreover, we also found that vitamin D supplementation, given to 20 healthy volunteers

for 15 weeks, led to a 20% increase in total CaSR expression in PBMCs, although the increase failed to reach the level of statistical significance, probably because of small sample size.¹⁶ Upregulation of CaSR expression by vitamin D is not surprising because it is well known that the 2 promoters of the CaSR gene (P1 and P2) have functional vitamin D response elements. By this mechanism, vitamin D sterols upregulate CaSR expression in the parathyroid, making the gland more sensitive to extracellular calcium.²⁷ Whether active vitamin D metabolites also upregulate monocyte CaSR expression in CKD patients remains to be demonstrated.

Cardiovascular mortality increases as CKD progresses.²⁸ The increased mortality risk is associated with various factors, such as vascular calcification,^{29,30} uremic toxins, including PCS,³¹ vitamin D deficiency,^{32,33} and a proinflammatory state.^{34,35} Remarkably, we have been able to show in *in vitro* experiments that cells of different human monocyte were able (in a dose-dependent manner) not only to prevent calcium deposition, but also to resorb already formed deposits. The ability to interfere with vascular calcification was modulated according to CaSR activity and expression and required cellular contact with calcium deposits. This suggests that monocytes act through CaSR-dependent macropinocytosis and not through the secretion of soluble factor, such as proteins, enzymes, and/or cytokines. In this regard, our findings are in accordance with the recent discovery of a constitutive role of CaSR in cellular macropinocytosis³⁶ as well as in monocyte ability in rheumatoid arthritis to capture colloidal calciprotein particles through a mechanism involving CaSR and NLRP3 inflammasome activation and lysosomal activity.³⁷ The activation of the lysosomal system is consistent with an antiminerallizing process.³⁸ Although it is currently admitted that dysregulated or inappropriate environmental stimuli, including aging, atherosclerosis, CKD,

diabetes, and chronic inflammatory diseases, such as rheumatoid arthritis, in association with dysregulations of calcium and phosphate homeostasis are involved in the initiation and the development of vascular calcifications, it seems unlikely that monocytes will be affected similarly by these pathologic conditions. Therefore, further exploration of circulating monocyte “antimineralizing” capacities in various procalcifying conditions will be needed to decipher the exact role played by monocytes and CaSR activation in this process.

Unlike CaSR total monocyte expression, the decrease of surface monocyte CaSR expression correlated tightly with the loss of monocytes' ability to prevent arterial calcium deposition. This is consistent with receptor activation at the membrane and compatible with the hypothesis that a decrease in monocyte CaSR expression favors vascular calcification in CKD. However, this association needs to be further investigated in a prospective trial assessing vascular calcification by quantitative imaging procedures,³⁹ as vascular calcification can occur independently of CKD, in particular in association with age, and potentially already at early CKD stages.⁴⁰

Adding the calcimimetic NPS-R568 to the culture medium improved the calcification-inhibitory activity of monocytes isolated from CKD patients *in vitro*, suggesting potential clinical benefit. Similar inhibitory effects of calcimimetic on arterial calcification have previously been reported in chronic hemodialysis patients (ADVANCE trial).⁴¹ It is also noteworthy in this context that the calcimimetic cinacalcet has been shown to reduce the incidence of calcific arteriopathy in hemodialysis patients with moderate to severe secondary hyperparathyroidism.⁴² The cardioprotective effects of cinacalcet are still controversial. They have at least been shown in some subpopulations, as for instance in people of younger age with moderate secondary hyperparathyroidism, and in patients without diabetes compared with those with diabetes.⁴³ This is compatible with a more marked inhibitory effect of NPS-R568 in patients with reduced surface monocyte CaSR expression. Considering our results in this context, monocyte CaSR expression may represent a potential therapeutic target or at least a biological marker to determine subgroups of calcimimetic responders.

Given the pleiotropic biological effects of monocyte CaSR, it could play a role in other complications associated with CKD. The CaSR is known to synergize with chemokines to favor monocyte migration¹⁰ and to exert both proinflammatory^{11,12} and anti-inflammatory effects.⁴⁴ Thus, on the one hand, the CaSR antagonist NPS 2143 was able to protect against lipopolysaccharide-induced acute lung injury *in vivo*.¹³ On the other hand, monocyte CaSR activation has been shown to switch the proinflammatory M1 macrophage phenotype to the anti-inflammatory M2 phenotype.⁴⁴ CKD is also characterized by a higher rate of bacterial infection, the mechanisms of which are not fully elucidated.¹⁵ Recently, Canton *et al.* showed that the CaSR signals constitutive macropinocytosis in macrophages through phosphatidylinositol 3-kinase, a mechanism supporting the uptake of antigens for subsequent presentation.³⁶ Beyond reduction of calcification management, the observed decrease of

monocyte CaSR in CKD may result in the loss of sentinel ability that would contribute to immune dysfunction. Finally, it is well known that the CaSR plays an important role in the differentiation of osteoclasts, which originate from hematopoietic stem cells.⁴⁵ Loss of CaSR expression in monocyte lineage might therefore also contribute to the abnormal bone turnover associated with CKD. This hypothesis deserves further study.

The present study had limitations and strengths. The main limitations are the absence of scans quantifying vascular calcifications in patients, an inflammatory exploration limited to C-reactive protein, the cross-sectional design of the study, and the lack of precise identification of the mechanism explaining the variation of monocyte CaSR expression in CKD. The strengths include the translational nature of the work, the quantification of both membrane and total CaSR expression in monocytes, the independence of the association between membrane CaSR and eGFR, and the striking correlation between monocyte CaSR and monocyte anticalcifying capacity.

In conclusion, we demonstrate a decrease in CaSR expression in CD14⁺ monocytes of patients with CKD. Exposure of monocytes to serum of CKD patients similarly reduces CaSR expression *in vitro*. We further demonstrate that monocytes are able to reduce and even resorb carotid artery mineral deposits *in vitro*, and that this capacity depends on normal CaSR expression and/or CaSR activation. The CKD-induced reduction of monocyte CaSR expression therefore appears to enhance vascular calcification *in vitro*. Whether abnormal monocyte CaSR expression and function contribute to the progression of vascular calcification in patients with CKD deserves further investigation.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

[Supplementary File \(PDF\)](#)

Supplementary Methods. Routine biochemistry laboratory parameters; uremic toxin measurements; quantification of CaSR expression in monocytes (RT-qPCR and WB); CaSR agonist or antagonist pretreatment; quantification of calcium deposition.

Figure S1. Western blot shows decrease of CaSR expression in PBMCs from CKD patients.

Figure S2. Flow cytometry patterns of CaSR expression.

Figure S3. CaSR mRNA expression between healthy volunteers and CKD patients.

Figure S4. Alizarin-red staining of the carotid calcification model (day 5).

Figure S5. Physical contact of monocytes and PBMCs with calcium deposits needed for anticalcifying action.

Figure S6. Decrease by CaSR allosteric inhibitor NPS-2143 of monocyte capacity to reduce carotid calcium content.

Figure S7. Increase by CaSR allosteric activator NPS-R568 of monocyte and PBMC capacity to reduce carotid calcium content.

Figure S8. Impact of the CKD-associated decrease in monocyte CaSR expression on PBMCs' ability to reduce carotid calcification after calcimimetic pretreatment.

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