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Vasoconstriction-inhibiting factor: an endogenous inhibitor of vascular calcification as a calcimimetic of calcium-sensing receptor

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Aims

Patients with chronic kidney disease (CKD) show a high risk of cardiovascular diseases, predominantly caused by accelerated vascular calcification. Vascular calcification is a highly regulated process with no current treatment. The vasoconstriction-inhibiting factor (VIF) peptide was recently discovered with vasoregulatory properties, but no information regarding calcification has been described.

Methods and results

In the present work, the inhibitory calcification effect of the VIF peptide was analysed *in vitro* in vascular smooth muscle cells (VSMCs), *ex vivo* in rat aortic rings, as well as *in vivo* in rats treated with vitamin D and nicotine (VDN). The VIF peptide inhibits vascular calcification by acting as a calcimimetic for the calcium-sensing receptor, increasing carboxylated matrix Gla protein production and blocking the activation of calcification pathways. The VIF peptide decreased calcium influx, the production of reactive oxygen species, and the activation of multiple kinases in VSMCs. Furthermore, calcium deposition in the aortas of patients with CKD negatively correlates with the VIF peptide concentration. Moreover, we show the cleavage of the VIF peptide from chromogranin-A by 'proprotein convertase subtilisin/kexin type 2' and 'carboxypeptidase E' enzymes. In addition, 'cathepsin K' degrades the VIF peptide. The active site of the native 35 amino acid-sequence long VIF peptide was identified with seven amino acids, constituting a promising drug candidate with promise for clinical translation.

Conclusion

The elucidation of the underlying mechanism by which the VIF peptide inhibits vascular calcification, as well as the active sequence and the cleavage and degradation enzymes, forms the basis for developing preventive and therapeutic measures to counteract vascular calcification.

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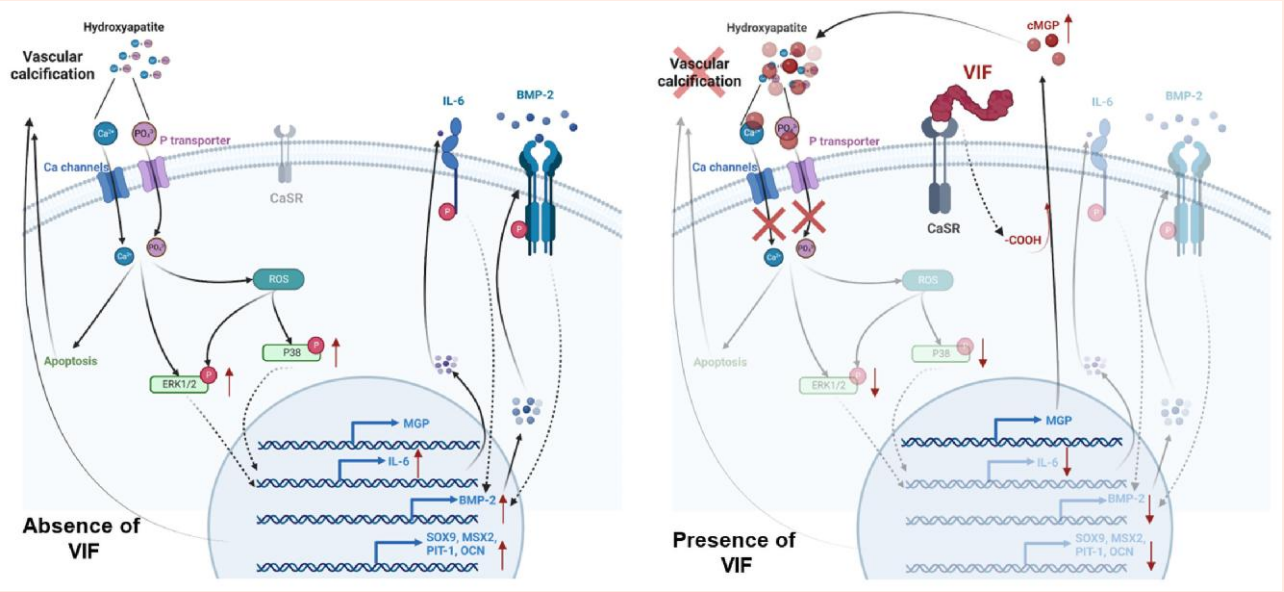
Graphical Abstract



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Keywords CaSR • Vascular Calcification • smooth muscle cells • inhibitor • CKD

1. Introduction

Vascular calcification is the pathological deposition of calcium phosphate crystals in the vessel wall.[1] It is associated with vascular aging,[2] atherosclerosis,[3] and chronic kidney disease (CKD).[4] Vascular calcification leads to a significant change in the mechanical properties of the vessels and elevated blood pressure[1,2] and contributes to morbidity and mortality as it significantly increases the risk of cardiovascular disease (CVD).[5,6] The clinic has two different forms of vascular calcification: intimal and medial. Both have different clinical manifestations, resulting in different treatments, prognoses, and clinical consequences.[7,8] While intimal calcification is associated with atherosclerosis, medial calcification is prevalent in patients with diabetes or CKD.[9] Accumulating evidence indicates that vascular calcification is an active, highly regulated process.[10–12] In this context, mediators regulate the underlying calcium phosphate deposition and transdifferentiation of vascular smooth muscle cells (VSMCs) into an osteoblast-like phenotype.[13] Although the involvement of calcium, phosphate, and uraemic toxins as enhancers, and fetuin-A, matrix Gla protein, and vitamin K as inhibitors in these processes is known,[14] our knowledge of the calcification processes is still incomplete, and intervention options remain limited.

The chromogranin A (CgA)-derived peptide vasoconstriction-inhibiting factor (VIF) was recently isolated from both bovine adrenal glands and human plasma and identified as an inhibitor of angiotensin II (Ang II)-induced vasoconstriction.[15] It has also been described that Ang II induces calcification in VSMCs under osteogenic conditions[16–18] but inhibits calcium deposition under high phosphate.[19] Therefore, we investigated which effect the VIF peptide has on vascular calcification.

2. Methods

2.1 Cell culture

Primary human aortic smooth muscle cells (hAoSMCs) were obtained from PromoCell for calcification assays. The human embryonic kidney-293 (HEK) cells stably expressing the full-length human calcium-sensing receptor (CaSR-HEK) were used for CaSR-mediated intracellular Ca^{2+}-ion mobilization. For details, see the Supplementary Material.

2.2 Calcification induction in hAoSMCS cells and rat aortic rings

The regional ethics committee approved the animal experiments and conformed to the Guide for the Care and Use of Laboratory Animals (permit number 80056A4). The experiments were performed according to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Eight to ten weeks, male Wistar rats (Janvier, France) were anaesthetised with 4–5% isoflurane (Abbvie, USA), the thoracic aortas of Wistar rats were dissected, and the animals were euthanized with an overdose of 5% isoflurane. The aortas were then dissected and cut into 3–4 mm segments, and the endothelial layer of the thoracic aortic ring was manually damaged. The aortic rings and hAoSMCs were cultivated in Dulbecco's modified Eagle's medium (DMEM), supplemented with penicillin, streptomycin, and 2.5% fetal calf serum (FCS). Then, 2.5 mmol L^{-1} phosphate was used to induce calcification [calcifying medium (CM)]. The CM was supplemented with corresponding peptides. As a reference medium, DMEM supplemented with 2.5% FCS was used [non-CM (NCM)]. The hAoSMCs and rings were cultured for 1–7 days, replacing the medium after 48 h. For details, see the Supplementary Material.

2.3 Peptide synthesis

Using the solid-phase, the VIF peptide and fragments used were synthesised by Campro (Scientific GmbH, Germany). VIF: HSGFEDE LSEVLENQSSQAELKEAVEEPSSKDVME; VIF1–7: HSGFEDE; VIF8–14: LSEVLEN; VIF15–21: QSSQAEL; VIF22–28: KEAVEEP; VIF29–35: SSKDVME.

2.4 Effect of VIF peptide in an animal model of elastocalcinosis

An animal model of elastocalcinosis with proven increased arterial calcification[20] was chosen to assay the effects of the VIF peptide *in vivo*. Animal experiments were approved by the regional ethics committee 'Comité d'éthique pour l'expérimentation animale languedoc Roussillon N°36', with the agreement number (APAFIS 2018072715539434#17270v4) and were performed according to the guidelines from Directive 2010/63/EU. Two groups of four and one group of six rats received VIF (31 µg kg^{-1} per day; $n = 4$) or a vehicle (saline; $n = 10$), respectively, infused through an osmotic pump. The osmotic pump was implanted under the skin under general gas anaesthesia with gas inhalation of 2% isoflurane and subcutaneous injection of 0.5% xylocaine. Four days later, the induction of elastocalcinosis (VDN rats) was done as previously described, with an intramuscular injection of vitamin D3 (300 000 IU kg^{-1}) and two gavages of nicotine (25 mg kg^{-1}, 5 mL kg^{-1}) 9 h apart on the same day ($n = 6$ for VDN group, and $n = 4$ for VIF group). A higher number of animals for the VDN compared with the other groups was used because it is the most harmful treatment, and to avoid a low *N* number due to possible death of the animals. Untreated rats were control rats (sham operation and saline administration, $n = 4$).[21] Four weeks later, the rats were anaesthetised and a catheter into the right carotid artery to measure the blood pressure. All animals were killed by a lethal intraperitoneal injection of pentobarbital (200 mg kg^{-1}), after the last blood pressure measurement was carried out under gas sedation with 2% isoflurane without the animal waking up. The aortas were gently dissected and cut into segments (3–4 mm) for further analysis. For details, see the Supplementary Material.

2.5 Quantification and visualization of the calcium deposition

Calcium content was assessed using the o-cresolphthalein method. Alizarin red and von Kossa staining were performed to visualize calcified areas, and hydroxyapatite crystals in the isolated rat aortic rings were quantified by MALDI mass imaging as described in the Supplementary Material.

2.6 Real-time quantitative polymerase chain reaction analyses of human aortic smooth muscle cells

For real-time quantitative polymerase chain reaction (RT-qPCR) analyses, hAoSMCs were incubated for 2 and 5 days, respectively. The total mRNA was extracted from hAoSMCs, and the gene expression levels were quantified using SYBR Green I dye chemistry on a QuantStudio3 Real-Time PCR System I. For details, see the Supplementary Material.

2.7 Identification of synthesizing and degrading enzymes of the VIF peptide

The protease prediction tool Proteasix[22] was used to predict the enzymes likely involved in cleaving the VIF peptide from chromogranin A and for the identification of degrading enzymes. The VIF peptide and the VIF peptide degraded fragments were quantified by a capillary-HPLC system interfaced with an electrospray ioniser mass spectrometer. For details, see the Supplementary Material.

2.8 Intracellular calcium and ROS measurements using fluorescence spectrophotometry

For the intracellular calcium measurement, imaging was performed on human coronary artery smooth muscle cells. Cells were loaded with 1 µmol L^{-1} of the calcium-sensitive, cell-permeable, intracellular fluorescence dye for 1 h and 5 µmol L^{-1} ionomycin calcium salt was added. Fluorescence was measured at 425 nm excitation and 535 nm emission. The reactive oxygen species (ROS) production was quantified using the 'Detection Assay Kit' according to the manufacturer's protocol (Biovision, USA). For details, see the Supplementary Material.

2.9 Western blotting

Western blotting was used for the quantitative analysis of proteins in hAoSMCs lysates. For details, see the Supplementary Material.

2.10 Interleukin-6 determination by enzyme-linked immunosorbent assay

The secretion of interleukin-6 (IL-6) by hAoSMCs was quantified following the enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Invitrogen, USA). The hAoSMCs were incubated in the corresponding condition for 48 h. For details, see the Supplementary Material.

2.11 Quantification of VIF peptide concentration from plasma samples of patients under dialysis

The VIF peptide concentration was measured in patients from the VitaVasK cohort[23] using a competitive ELISA (BMA Biomedicals, Switzerland). The VitaVasK study is registered on ClinicalTrials.gov (NCT01742273) and EudraCT (2010-021264-14). For details, see the Supplementary Material.

2.12 Coronary artery calcium volume of patients with CKD in the VitaVasK cohort

Unenhanced, electrocardiogram-synchronized MSCT of the chest, extending from the aortic arch to the oesophageal hiatus of the diaphragm, was performed to assess cardiovascular calcifications. For details, see the Supplementary Material.

2.13 Statistical analyses

Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, USA), and the data were represented as mean ± SEM for the *in vitro* and *ex vivo* experiments and mean ± SD for the *in vivo*. Analysis of variance for a single factor (one-way) or multiple factors (two-way) was performed to determine differences between treatment groups and the reference group. In both, Bonferroni's multiple comparisons were used as a post-test. Simple linear regression was used to study the correlation of non-parametric data with a confidence interval of 95%. Differences with $*P < 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$ were considered statistically significant.

3. Results

3.1 Calcification inhibitory effect of the VIF peptide *in vitro* and *ex vivo*

First, we investigated whether the VIF peptide affects the inhibitory effect of Ang II on vascular calcification. *In vitro*, calcification was induced in hAoSMCs using the calcification medium (CM) conditions, as recently described.[24] As expected, incubation of hAoSMCs in the presence of Ang II (100 nmol L^{-1}) showed a 70% reduction in calcium precipitation. However, a co-incubation of cells with Ang II and the VIF peptide showed no significant reduction or increase in this inhibitory effect of Ang II. Surprisingly, incubation of the cells in a CM with the VIF peptide alone yielded a 58% reduction in the calcium content of the cells compared with CM, while no effect was observed when VIF was added in NCM (*Figure 1A*). The VIF peptide inhibited the calcification process in hAoSMCs in a dose-dependent manner with an EC_{50} value in the 1 fmol L^{-1} range (*Figure 1B*). The inhibitory effect was validated *ex vivo* by quantification of the calcium content of rat aortic rings incubated in

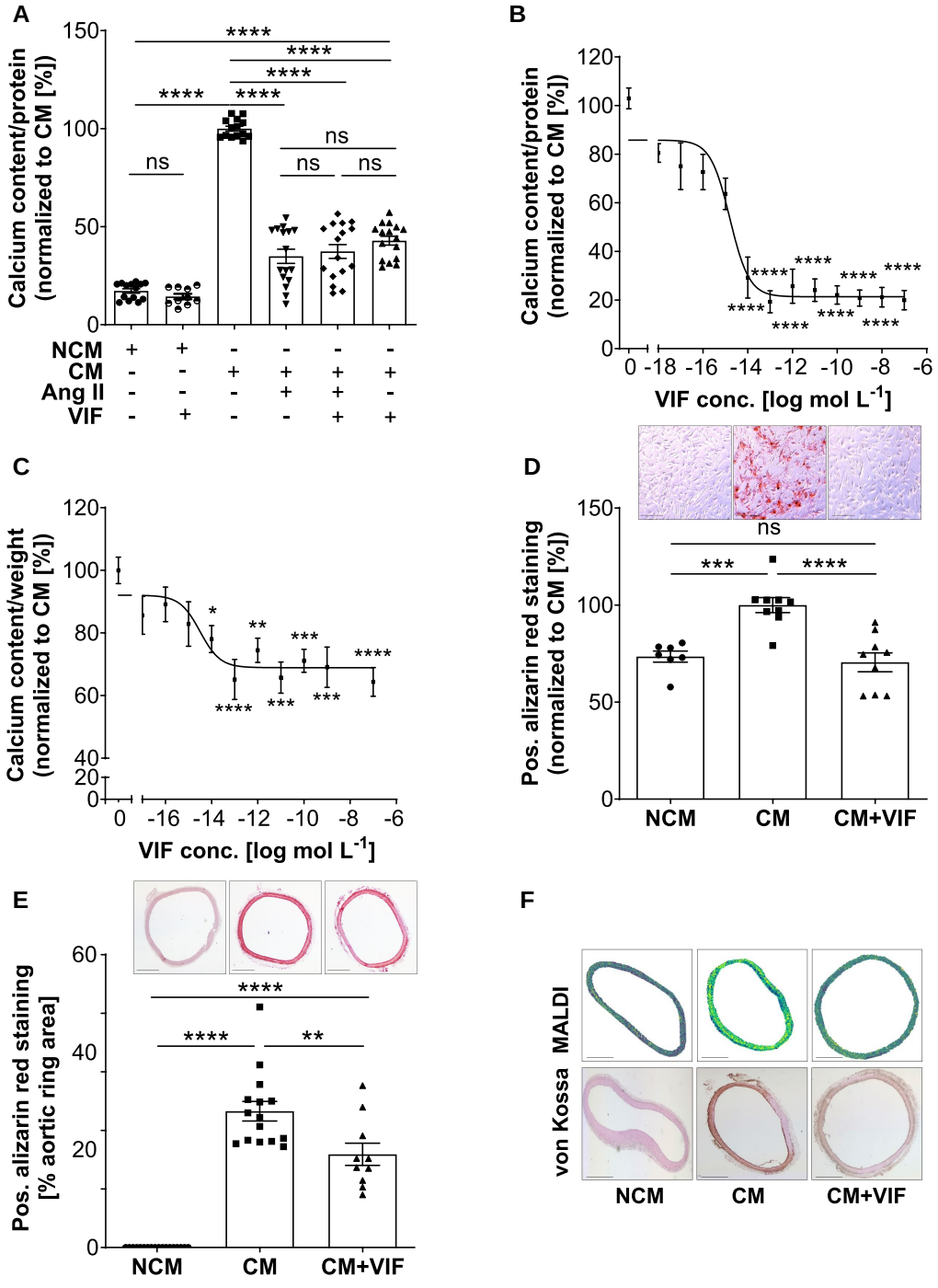

Figure 1 The vasoconstriction-inhibiting factor (VIF) peptide does not affect the angiotensin II inhibitory calcification effect in human aortic smooth muscle cells (hAoSMCs) and inhibits vascular calcification in *in vitro* and *ex vivo*. (*A*) Relative quantification of Ca^{2+} in hAoSMCs incubated in non-calcifying medium (NCM; circle dots) and calcifying medium (CM) in the absence (square dots) or presence of Ang II (100 nmol L^{-1}; CM + Ang II; inverse triangle dots); Ang II and the VIF peptide (each 100 nmol L^{-1}; CM + Ang II + VIF; diamond dots); or the VIF peptide (100 nmol L^{-1}; NM + VIF; black and white circles dots and CM + VIF; triangle dots). Data are compared with NM, CM, or CM + Ang II ($N = 4$ independent experiments with 3–4 pseudoreplicates per group). Dose–response effect of the VIF peptide on Ca^{2+} content of (*B*) cultivated human aortic smooth muscle cells ($N = 6$ independent experiments with 3 pseudoreplicates per group) and (*C*) isolated thoracic rat aortic rings ($N = 3$ independent experiments with 3 pseudoreplicates per group). Representative images of alizarin red staining in (*D*) hAoSMCs and (*E*) thoracic aortic rat rings incubated in NCM (circle dots) and in a CM in the absence (square dots) or presence of VIF (100 nmol L^{-1}; CM + VIF; triangle dots; magnification: 4×; scale bar: 1000 μm for hAoSMCs; and magnification: 2.5×; scale bar: 200 μm for the rings) and the corresponding quantification ($N = 4$ independent experiments with 3 pseudoreplicates per group). (*F*) Representative MALDI images of calcium phosphate crystals in the thoracic aortic rings incubated in a NCM and calcifying medium in the absence (CM) or presence of the VIF peptide (100 nmol L^{-1}; CM + VIF) and the corresponding von Kossa staining (magnification: 2.5×; scale bar: 200 μm; $N = 3$).

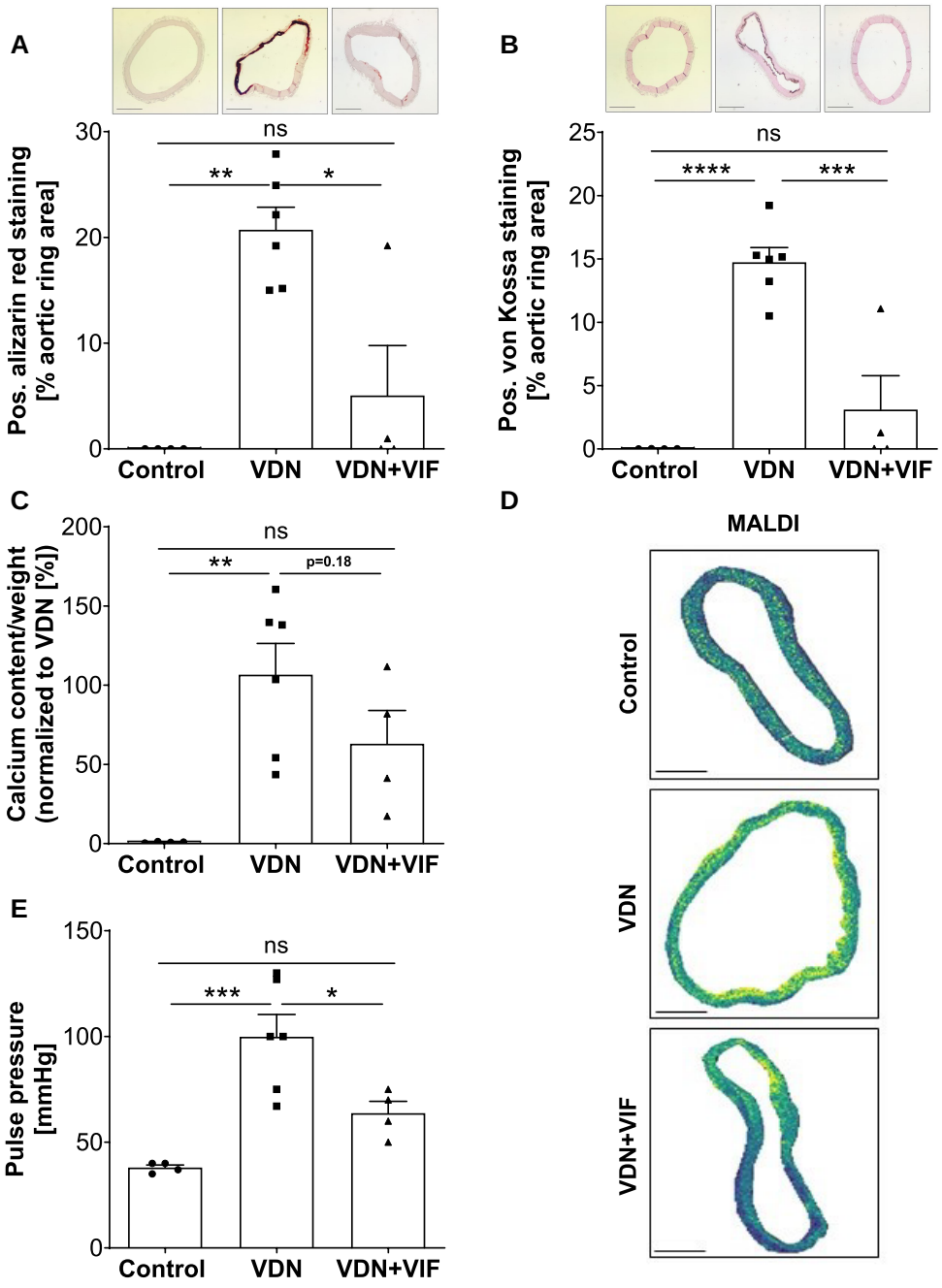

Figure 2 The vasoconstriction-inhibiting factor (VIF) peptide inhibits vascular calcification and influences pulse pressure *in vivo*. Representative images of (*A*) alizarin red-stained and (*B*) von Kossa-stained thoracic aortic rings of Wistar rats (control; circle dots) and VDN Wistar rats treated with a vehicle (VDN; square dots) or VIF peptide infusion (31 μg kg^{-1} per day for 4 weeks; VDN + VIF; triangle dots) via an osmotic pump (magnification: 2.5×; scale bar: 200 μm) and the corresponding quantification of the stained area (percentage of total aortic area section; $N = 4–6$ per group). (*C*) Calcium content of thoracic aorta of Wistar rats (control; circle dots) and VDN Wistar rats treated with a vehicle (VDN; square dots) or VIF peptide infusion (31 μg kg^{-1} per day for 4 weeks; VDN + VIF; triangle dots; $N = 4–6$ per group). (*D*) Representative images of calcium phosphate crystals in the thoracic aortic rings from Wistar rats (control) and VDN Wistar rats treated with a vehicle (VDN) or VIF peptide infusion (31 μg kg^{-1} per day for 4 weeks; VDN + VIF; magnification: 2.5×; scale bar: 200 μm). (*E*) Quantification of carotid arterial pulse pressure of Wistar rats (control; circle dots) and VDN Wistar rats treated with a vehicle (VDN; square dots) or VIF infusion (31 μg kg^{-1} per day for 4 weeks; VDN + VIF; triangle dots; $N = 4–6$ per group).

the CM in the absence and presence of increasing VIF peptide concentration. The EC_{50} value *ex vivo* remains comparable to the corresponding values *in vitro* (EC_{50} value 1 fmol L^{-1} range; *Figure 1C*). To visualise the inhibitory effect of the VIF peptide on calcification, alizarin red staining of hAoSMCs incubated in NCM and CM in the absence (CM) and presence of the VIF peptide (CM + VIF) was performed. *Figure 1D* shows an increase in alizarin red-positive hAoSMCs in CM compared with cells incubated in NCM. The presence of the VIF peptide in CM significantly reduced the

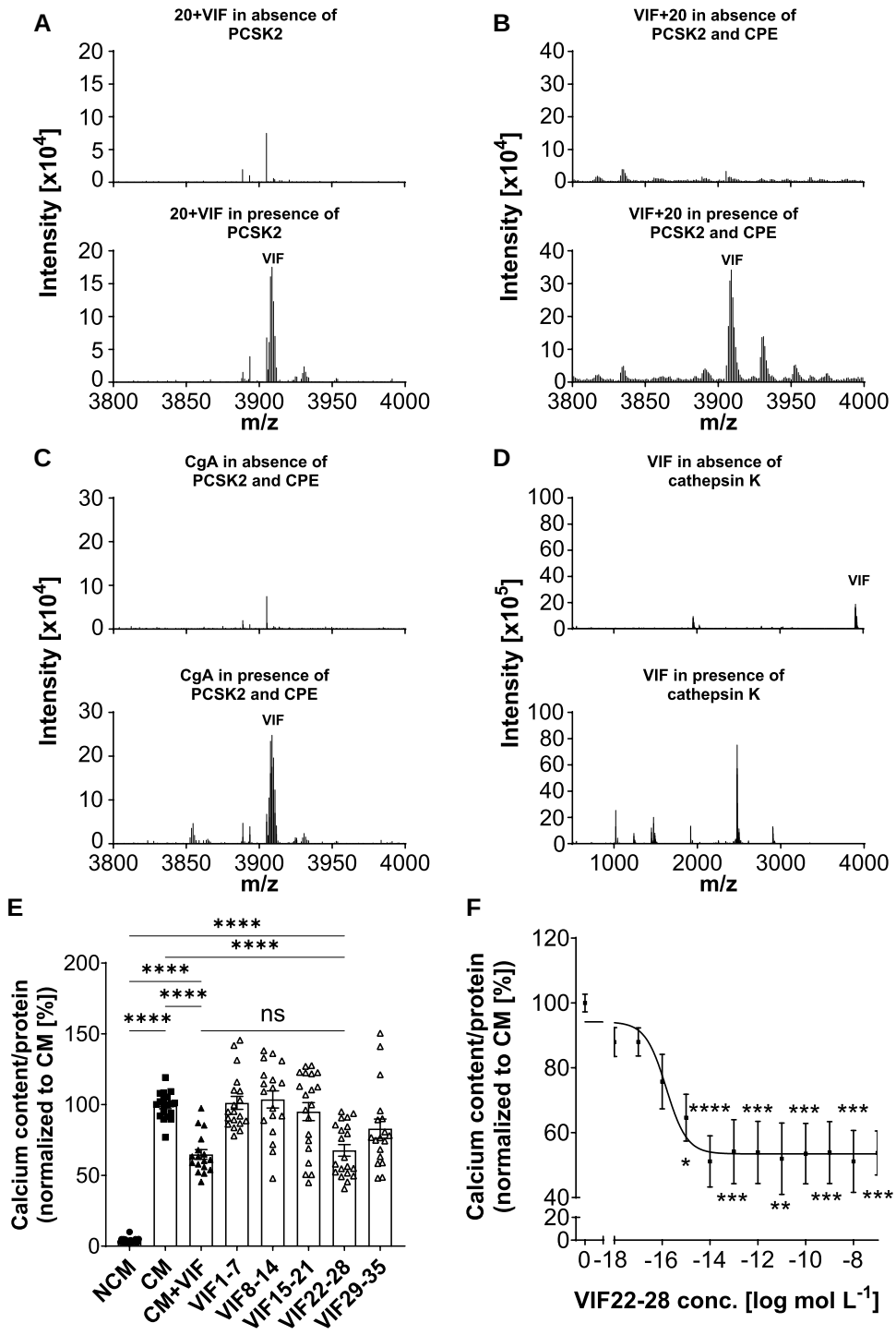

Figure 3 Identification of synthesizing and degrading enzymes and active amino acid sequence causing the inhibitory effect of vasoconstriction-inhibiting factor (VIF) peptide. (*A*) MALDI mass-spectrometric analyses of '20 + VIF' peptide incubated in the absence (upper panel) and presence (lower panel) of 'pro-protein convertase subtilisin/kexin type 2' (PCSK2). The molecular mass (m/z) of 3908.7 $[M + H]^+$ corresponds to VIF. (*B*) MALDI mass-spectrometric analyses of 'VIF + 20' peptide incubated in the absence (upper panel) and presence (lower panel) of PCSK2 and 'carboxypeptidase E' (CPE). The molecular mass (m/z) of 3908.7 $[M + H]^+$ corresponds to the VIF peptide. (*C*) MALDI mass-spectrometric analyses of CgA incubated in the absence (upper panel) and presence (lower panel) of PCSK2 and CPE. The molecular mass (m/z) of 3908.7 $[M + H]^+$ corresponds to the VIF peptide. (*D*) MALDI mass-spectrometric analyses of the VIF peptide incubated in the absence (upper panel) and presence (lower panel) of cathepsin K. The molecular mass (m/z) of 3908.7 $[M + H]^+$ corresponds to the VIF peptide. (*E*) Quantification of Ca^{2+} content of cultivated human aortic smooth muscle cell (hAoSMC) incubated in non-calcifying medium (NCM; circle dots) or calcifying medium in the absence (CM; square dots) or presence of VIF (CM + VIF; triangle dots) or VIF fragments (white triangle dots; see Supplementary material online, *Figure S2*; each 100 nmol L^{-1}; $N = 7$ independent experiments with 3 pseudoreplicates per group). (*F*) Dose–response effect of VIF peptide fragment VIF22–28 on Ca^{2+} content of cultivated hAoSMC ($N = 10$ per group independent experiments with 3 pseudoreplicates per group).

calcification of hAoSMCs compared with the absence of the VIF peptide in the medium. In addition, the inhibitory effect of the VIF peptide on vascular calcification was validated by alizarin red staining in rat thoracic aortic rings, which revealed a 30% reduction of the calcified area on the medial layer of the aortas upon treatment with the VIF peptide at 100 nmol L^{-1} compared with aortic rings cultured in CM alone (*Figure 1E*). As VIF is a chromogranin A-derived peptide, hAoSMCs were incubated with CgA, and the calcium deposition was analysed. No inhibitory effect, but an increase in calcification by CgA could be observed (100 ± 7.3% vs. 140 ± 15.5%).

For further validation of the effect of the VIF peptide, hydroxyapatite crystals of isolated rat aortic rings were quantified by MALDI mass-spectrometric imaging. The hydroxyapatite characteristic mass signal intensities at 94 (m/z) were increased in the aortic rings incubated in CM compared with NCM. Moreover, the VIF peptide significantly attenuated the increase in hydroxyapatite crystals in aortic rings in CM (*Figure 1F*; upper image). Staining in the thoracic aortic rings using von Kossa staining confirmed the result of MALDI mass imaging (*Figure 1F*; lower image).

3.2 Calcification inhibitory effect of the VIF peptide *in vivo*

Next, the VDN rat model was used to assess the impact of the VIF peptide on vascular calcification *in vivo*. Calcium-phosphate deposits on the medial layer of the thoracic aortic segments from sham-untreated (control), vehicle (VDN), or VIF peptide-treated (VND + VIF) rats were stained with alizarin red and von Kossa staining. The treatment with the VIF peptide significantly decreased the calcified surface area compared with VDN animals, as shown by alizarin red and von Kossa staining (*Figure 2A* and *B*). The calcium content of the aorta significantly increased in the VDN group receiving the vehicle compared with the control group without any treatment. Although treatment with the VIF peptide in VDN rats for 4 weeks showed only a trend towards reducing aortic calcium content (*Figure 2C*), this finding is consisted with the results shown in *Figure 2A* and *B*. In addition, hydroxyapatite crystals [mass signal intensities at 94 (m/z)] of isolated aortic rings were quantified by MALDI mass imaging. The hydroxyapatite crystal density in isolated aortic rings was also reduced by the VIF peptide treatment of VDN rats compared with vehicle-treated VDN rats (*Figure 2D*).

Further, the treatment with the VIF peptide significantly reduced the pulse pressure compared with VDN rats (*Figure 2E*). Systolic, diastolic, and mean arterial pressure measurements can be seen in Supplementary material online, *Figure S1A–C*. Endogenous concentration of the VIF peptide was measured in the rats, with no difference found between control and VDN rats. However, as expected, there was a significant increased concentration of the VIF peptide in the VDN rats pumped with the peptide animals (see Supplementary material online, *Figure S1D*).

3.3 Identification of synthesizing and degrading enzymes of the VIF peptide

The VIF peptide was identified as a peptide derived from chromogranin A protein.[15] However, the enzymes involved in this cleavage process are unknown, and thus, we aimed to identify the enzymes cleaving the VIF peptide from CgA. For this, elongated VIF peptides were used, with 20 additional amino acids of CgA at the N-terminus (named 20 + VIF) or at the C-terminus of the VIF peptide (VIF + 20; see Supplementary material online, *Figure S2A*). Based on 'Proteasix database'[22] searches, 'proprotein convertase subtilisin/kexin type 2' (PCSK2) was identified as a potential candidate for the cleavage of the N-terminus of the VIF peptide from CgA. Incubation of 20 + VIF in the presence of PCSK2 (*Figure 3A*; lower spectrum) but not in the absence of the enzyme (*Figure 3A*; upper spectrum) caused a mass signal at 3908.78 ($[M + H]^+$), which corresponds to the molecular mass (m/z) of the VIF peptide, demonstrating the N-terminal cleavage of the VIF peptide from 20 + VIF (*Figure 3A*). For the C-terminus, the 'Proteasix database'[22] searches suggested PCSK2 combined with 'carboxypeptidase E' (CPE) for cleaving the VIF peptide from CgA. Both proteases would be required since first PCSK2 would cleave VIF two amino acids after its C-terminus, and CPE, as exopeptidase, would be able to cleave the remaining two amino acids. Incubation of VIF + 20 in the presence of PCSK2 and CPE (*Figure 3B*; lower spectrum), but not in the absence of both enzymes (*Figure 3B*; upper spectrum), caused a mass signal at 3908.7 ($[M + H]^+$), reflecting the molecular mass (m/z) of the VIF peptide and demonstrating the C-terminal cleavage of the VIF peptide from VIF + 20 by PCSK2 and CPE. Incubation of CgA in the presence of both enzymes, PCSK2 and CPE (*Figure 3C*; lower spectrum), but not in their absence (*Figure 3C*; upper spectrum), caused an intense mass signal at 3908.7 ($[M + H]^+$), demonstrating the cleavage of the VIF peptide from the full CgA protein.

In addition to identifying the enzymes cleaving the VIF peptide from CgA, enzymes involved in the degradation of the VIF peptide were investigated. Incubation of the VIF peptide with cathepsin K, a well-known protease,[25] yielded small peptide fragments of the VIF peptide (*Figure 3D*; lower spectrum), while the absence of the enzyme (*Figure 3D*; upper spectrum) caused a mass signal at 3908.7 ($[M + H]^+$), reflecting only the molecular mass (m/z) of the VIF peptide.

3.4 Identification of the active amino acid sequence causing the inhibitory effect of the VIF peptide

To identify the active calcification-inhibitory site of the VIF peptide, 7-amino acid peptides of the entire amino acid sequence of the VIF peptide were synthesized (see Supplementary material online, *Figure S2B*), and their inhibitory effect was analysed *in vitro* using hAoSMCs. The fragment of the VIF peptide with the amino acids 22–28 significantly decreased the calcium deposition on hAoSMCs, while the remaining fragments did not cause significant effects on the calcification processes (*Figure 3E*). Moreover, the dose dependency of this effect was analysed. *Figure 3F* shows an EC_{50} value for this fragment in the femto molar range, similar to that of the intact VIF peptide (*Figure 1C*).

3.5 Identification of the pathway mediating the inhibitory effect of the VIF peptide

High phosphate concentrations increase calcium influx in hAoSMCs, which is essential for vascular calcification.[26] We thus investigated whether the VIF peptide affects phosphate-induced calcium influx in hAoSMCs using the fluorescent dye technique. *Figure 4A* shows an increased calcium influx in hAoSMCs incubated with CM compared with NCM. Adding the VIF peptide to the CM in hAoSMCs significantly reduced the calcium influx. After the addition of the calcium ionophore ionomycin (Iono), as a positive control of increased calcium influx,[27] no difference in calcium influx could be observed between NCM and CM in the presence of the VIF peptide, both reaching high calcium influx (*Figure 4A*). Phosphate-induced calcium influx elicits cell oxidative stress, resulting in phenotypic alterations and subsequent calcification of VSMCs.[26] Therefore, the effect of the VIF peptide on ROS production in the CM was investigated using the probe H_2DCFDA. Increased ROS production was found in hAoSMCs incubated in CM vs. NCM after 24 h while the presence of the VIF peptide caused a significant reduction (*Figure 4B*). *In vivo* staining with 8-OdGH (marker of oxidation in the DNA) showed increased positive staining in the VDN compared with the animals treated with VIF and the control rats (*Figure 6D*).

Next, using kinase activity profiling of hAoSMCs, molecular signalling cascades triggered by a phosphate concentration of 2.5 mmol L^{-1}[28] were investigated. Exposure to phosphate increased the activity of mitogen-activated protein kinases (MAPK) P38 as well as extracellular signal-regulated kinases ERK1/2 activity compared with cells cultivated in the non-CM. The VIF peptide reduced this effect of the calcification medium (see Supplementary material online, *Table S1*). By Western blot, we confirmed that kinase activation induced by CM was reduced in the presence of the VIF peptide (*Figure 4C* and *D*).

Inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) are well-known inducers

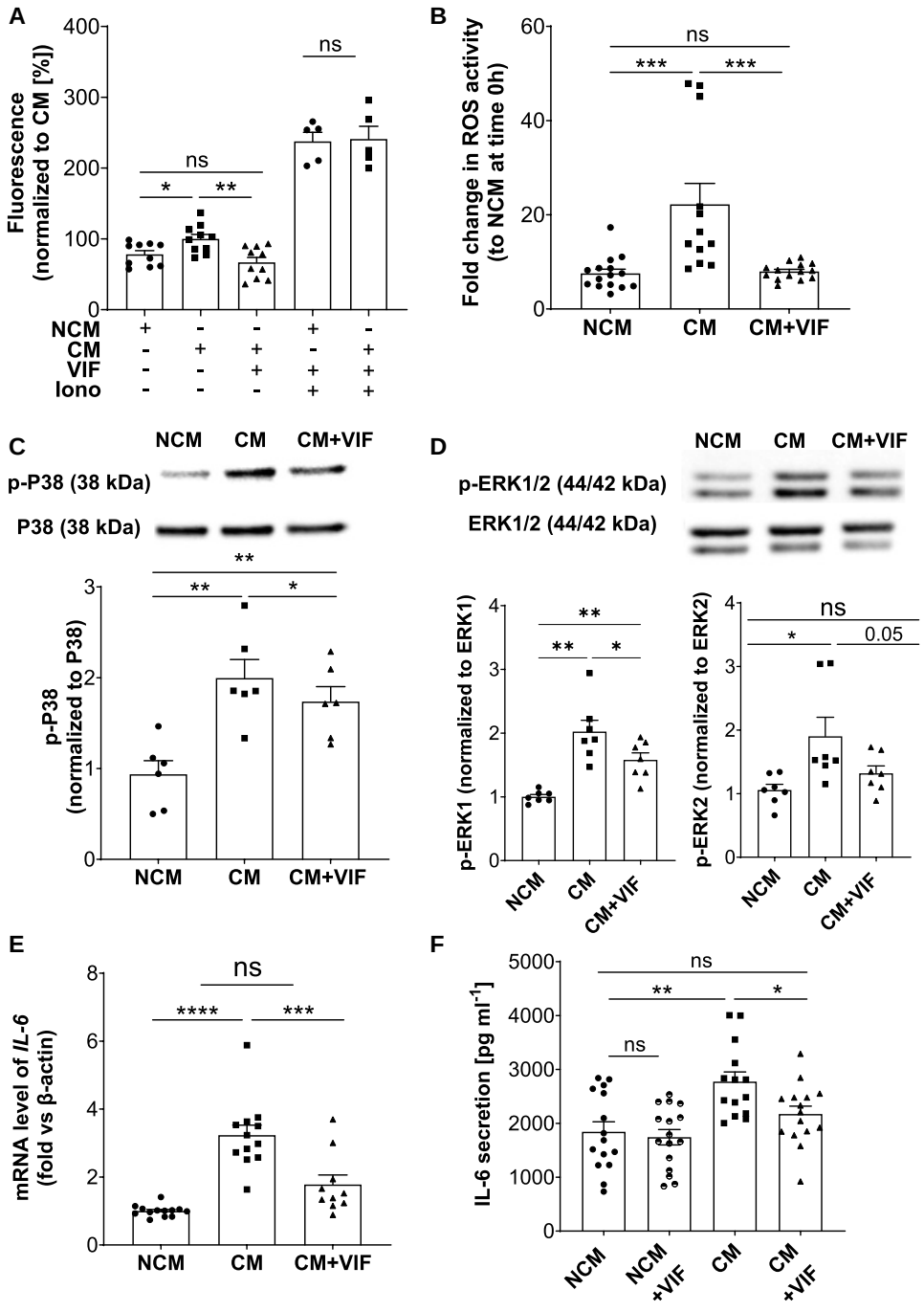

Figure 4 The vasoconstriction-inhibiting factor (VIF) peptide inhibits calcium influx and reactive oxygen species (ROS) production in human aortic smooth muscle cells (hAoSMCs) and reduces the phosphate-induced activity and phosphorylation of P38 and ERK1/2, and expression and secretion of IL-6. (*A*) Relative quantification of calcium influx in cultivated hAoSMCs incubated in non-calcifying medium (NCM; circle dots) or in calcifying medium (CM) in the absence (square dots) or presence of the VIF peptide (100 nmol L^{-1}; CM + VIF; triangle dots), and NCM in the presence of the VIF peptide and ionomycin (Iono; NCM + VIF + ionomycin; white circle dots) and CM in the presence of the VIF peptide and ionomycin (CM + VIF + ionomycin; white triangle dots; $N = 3–4$ independent experiments with 3 pseudoreplicates per group). (*B*) Relative quantification of ROS activity in cultivated hAoSMCs incubated in NCM (circle dots) or in calcifying medium in the absence (CM; square dots) or presence of the VIF peptide (100 nmol L^{-1}; CM + VIF; triangle dots) for 24 h. Fold change compared with NCM at time point zero (N = 7 per group). (*C–D*) Western blot and quantitative analysis of P38– (*C*) and ERK1/2- (*D*) phosphorylation in hAoSMCs incubated in a NCM (circle dots) or calcifying medium in the absence (CM; square dots) or presence of the VIF peptide (100 nmol L^{-1}; CM + VIF; triangle dots) after 72 h ($N = 3$ independent experiments with 2–3 pseudoreplicates per group). (*E–F*) Relative quantification of interleukin-6 (IL-6) gene expression (*E*) or secretion (*F*) in hAoSMCs incubated in a NCM (circle dots) or CM in the absence (square dots) or presence of the VIF peptide (100 nmol L^{-1}; NM + VIF; black and white circle dots and CM + VIF; triangle dots) after 48 h using RT-qPCR analyses and ELISA ($N = 3–4$ independent experiments with 3 pseudoreplicates per group).

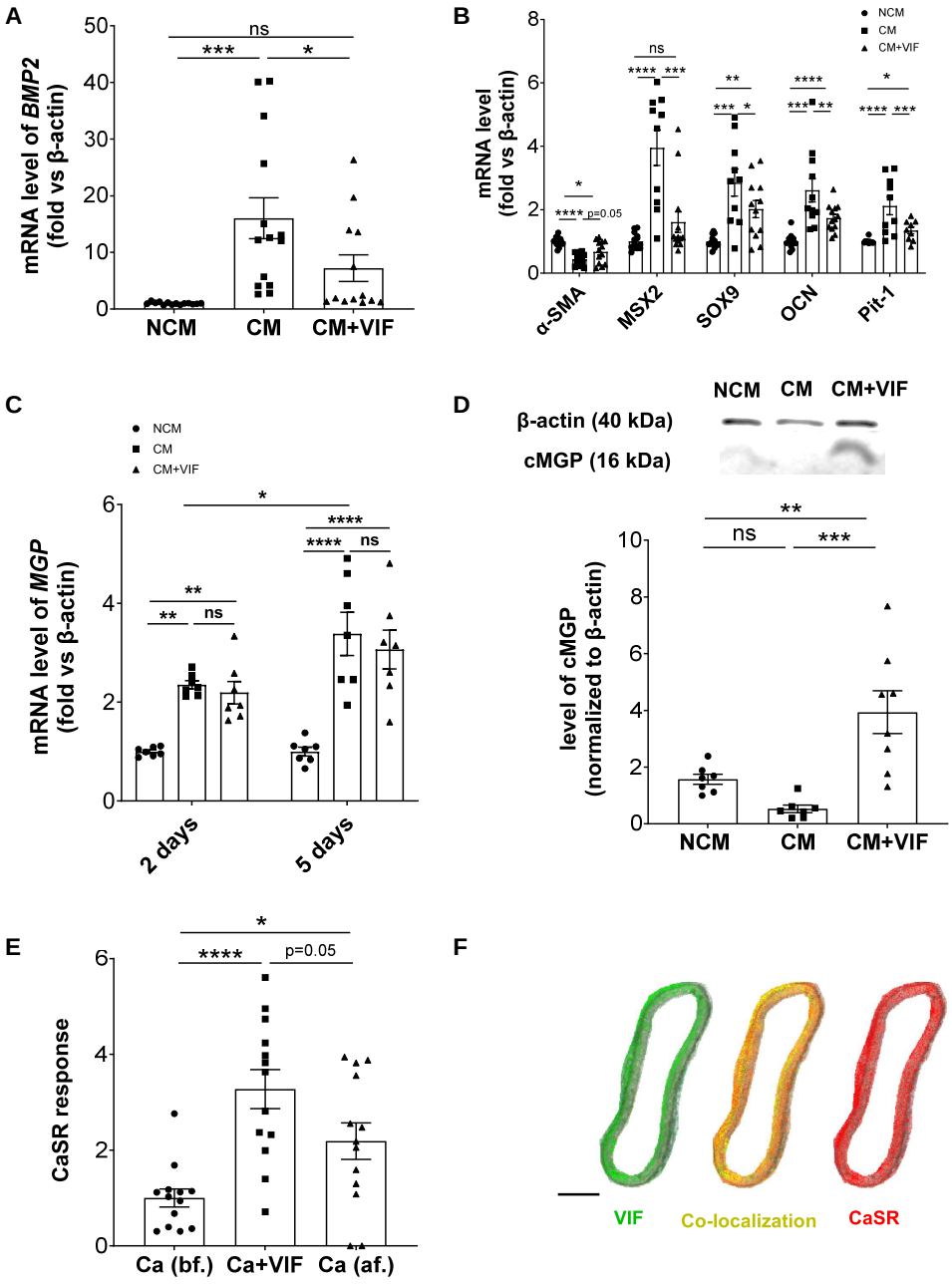

Figure 5 The vasoconstriction-inhibiting factor (VIF) peptide reduces the expression of genes involved in the development of vascular calcification and increases cMGP production after binding to CaSR. (*A*) Relative quantification of 'bone morphogenetic protein 2' (*BMP2*) gene expression in human aortic smooth muscle cell (hAoSMC) incubated for 5 days in a non-calcifying medium (NCM; circle dots) or calcifying medium (CM) in the absence (square dots) or presence of the VIF peptide (100 nmol L^{-1}; CM + VIF; triangle dots) using RT-qPCR analyses (N = 4–5 independent experiments with 3 pseudoreplicates per group). (*B*) Relative quantification of 'α-smooth muscle actin' (*α-SMA*), 'msh homeobox 2' (*MSX2*) and 'SRY-box transcription factor 9' (*SOX9*), 'osteocalcin' (*OCN*) and 'sodium-dependent phosphate cotransporter-1' (*PIT-1*) gene expression *in vitro* by RT-qPCR analyses in hAoSMCs incubated in a NCM (circle dots) or calcifying medium (CM) in the absence (square dots) or presence of the VIF peptide (100 nmol L^{-1}; CM + VIF; triangle dots; *N* = 4–5 independent experiments with 3 pseudoreplicates per group). (*C*) Relative quantification of 'matrix Gla protein' (*MGP*) gene expression *in vitro* by RT-qPCR analyses in hAoSMC incubated 2 or 5 days in a NCM (circle dots) or calcifying medium in the absence (CM; square dots) or presence of the VIF peptide (100 nmol L^{-1}; CM + VIF; triangle dots; *N* = 3 independent experiments with 2–3 pseudoreplicates per group). (*D*) Western blot and quantitative analysis of cMGP production in hAoSMCs incubated 5 days in a NCM (circle dots) or calcifying medium in the absence (CM; square dots) or presence of the VIF peptide (100 nmol L^{-1}; CM + VIF; triangle dots; N = 3 independent experiments with 2–3 pseudoreplicates per group). (*E*) Effect of the VIF peptide (300 nmol L^{-1}; Ca + VIF; square dots) on CaSR-mediated $Ca^{2+}{}_i$ mobilisation upon stimulation with 2.5 mmol l^{-1} calcium (Ca) (bf: before addition of VIF; circle dots and af: after addition of VIF peptide, triangle dots). (*F*) Representative MALDI image of the co-localization (yellow) of calcium-sensing receptor (red) and VIF (green) in thoracic aortic rings from VDN Wistar rats treated with VIF peptide (31 μg kg^{-1} per day for 4 weeks; 2.5x; scale bar: 200 μm; N = 4).

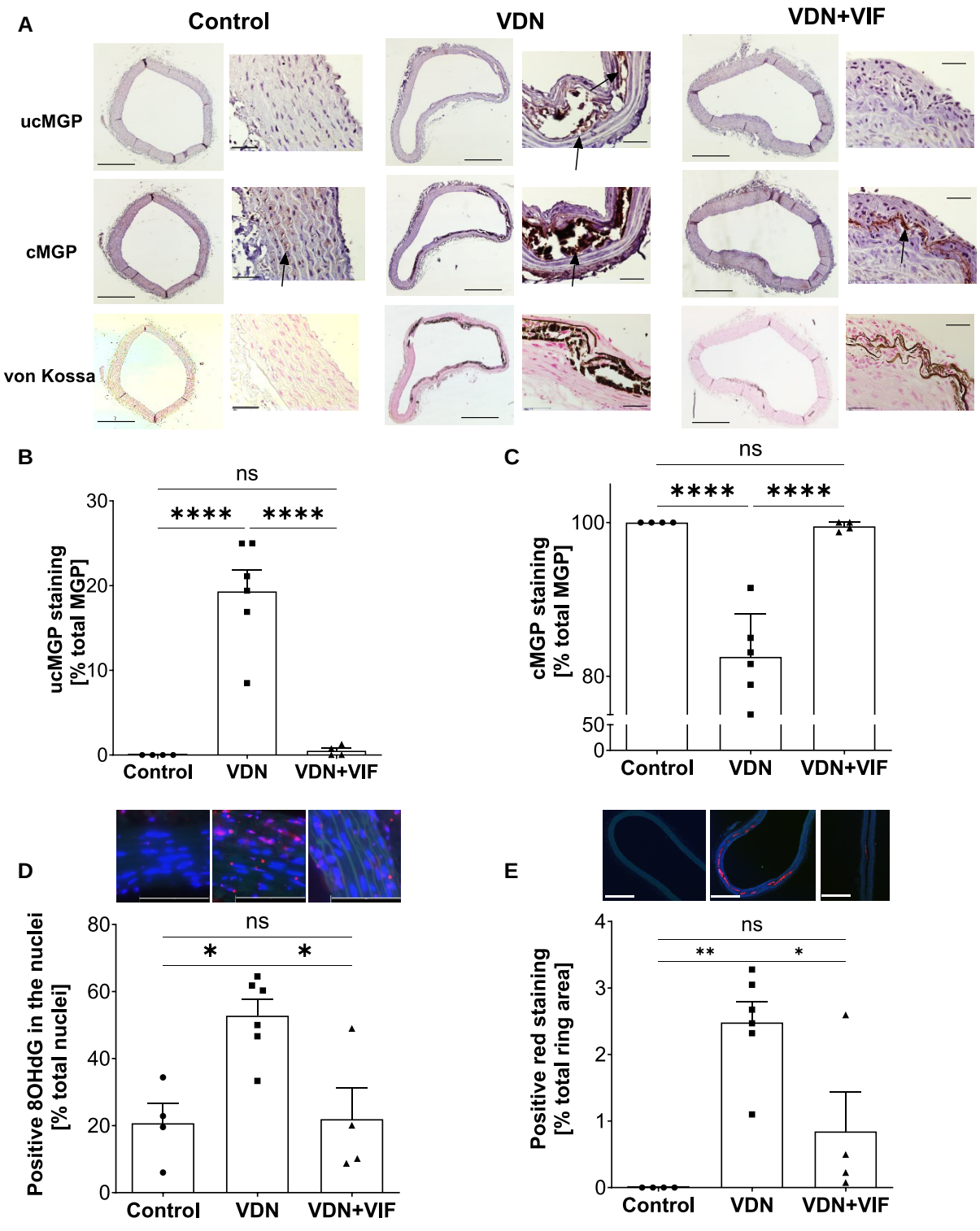

Figure 6 The vasoconstriction-inhibiting factor (VIF) peptide increases cMGP production and reduces reactive oxygen species (ROS) and apoptosis in the VDN rat model. Representative images of (*A*) cMGP, ucMGP, and von Kossa staining in the thoracic aortic rings from control, VDN, or VDN + VIF rats (magnification: 2.5×; scale bar: 200 μm and 40×; scale bar 50 μm) (*B–C*) and the corresponding ratio of cMGP/ucMGP and vice versa ($N = 4–6$ per group; arrows pointing to positive staining). (*D*) Percentage of positive 8OdGH staining (red) in the nuclei of SMCs (blue) in rat aortic rings, elastin fibers are shown in green (autoflurescence; magnification: 100×; scale bar: 100 μm) and its representative pictures. (*E*) Percentage of positive TUNEL staining (red) in rat aortic rings (percentage of total aortic area section), nuclei of SMCs (blue), elastin fibers are shown in green (autofluorescence; magnification: 5×; scale bar: 500 μm) and its representative pictures of Wistar rats (control; circle dots) and VDN Wistar rats treated with a vehicle (VDN; square dots) or VIF peptide infusion (31 μg kg^{-1} per day for 4 weeks; VDN + VIF; triangle dots) via an osmotic pump ($N = 4–6$ per group).

of mineralization in VSMC,[29–31] and a relationship between inflammation, ROS production, and increased vascular calcification in CKD has been shown.[32] Calcifying conditions did not affect TNF-α and IL-1β expression and secretion in hAoSMCs (data not shown), but a 3-fold increase in the mRNA expression of IL-6 was detected using CM, which was reduced by 65% in the presence of the VIF peptide (*Figure 4E*). Incubation of cells with the VIF peptide showed a decrease in CM-induced IL-6 secretion of hAoSMCs, while no effect was observed when VIF was added to NCM (*Figure 4F*).

Since the expression of 'bone morphogenetic protein 2' (BMP2) as a regulator of vascular calcification[33,34] is regulated by IL-6,[35] the effect of VIF on BMP2 in the CM was investigated. Increased BMP2 gene expression induced by CM was reduced by up to 60% in the presence of VIF (*Figure 5A*). BMP2 was detected as preform at protein level in the cell lysates at 44 kDa under calcifying conditions, and as the active form in the medial layer of aortic rings from VDN rats. There was no significant expression in non-calcifying media and control rats, and expression was reduced in presence of VIF (see Supplementary material online, *Figure S3A* and *B*). Analysis of BMP2-dependent osteogenic factors in hAoSMCs showed an increased expression of the transcription factors 'msh homeobox 2' (MSX2) and 'SRY-box transcription factor 9' (SOX9), and osteocalcin (OCN) and the 'sodium-dependent phosphate cotransporter-1' (Pit-1) in CM compared with NCM, this was significantly decreased in the presence of the VIF peptide. Increased expression of 'α-smooth muscle actin' (α-SMA) in the CM was observed, with no difference after VIF addition (*Figure 5B*). Incubation of hAoSMCs in the CM in the presence and absence of the VIF peptide showed no difference in gene expression of MGP at any time point (*Figure 5C*). However, after five days, a significant increase in the production and secretion of carboxylated MGP (cMGP), the form that inhibits calcium deposition, in the CM in the presence of the VIF peptide (CM + VIF) was detected (*Figure 5D*). Detection of cMGP after two days was not possible via western blot (data not shown). Staining of cMGP and ucMGP *in vivo* in thoracic rings from the rats showed cMGP in all the conditions (control, VDN and VDN + VIF), while ucMGP was only visualised in the VDN rats (*Figure 6A–C*, arrows pointing to positive staining).

3.6 VIF-binding partner

Loss of CaSR expression on VSMCs leads to vascular calcification,[36] and calcimimetics that stimulate this receptor decrease mineralisation in VSMCs.[37] Co-immunoprecipitation was performed to identify the interaction between the VIF peptide and CaSR. Therefore, hAoSMC lysates were incubated with the VIF peptide, followed by immunoprecipitation of CaSR using a CaSR antibody. In addition, an IgG isotype antibody was used as a control. The samples were analysed by using a mass spectrometric approach. In the samples incubated with the CaSR antibody, both the VIF peptide (see Supplementary material online, *Figure S3C*) and the CaSR (see Supplementary material online, *Figure S3D*) were detected. In contrast, the samples incubated with the corresponding control isotype antibody did not show a matching spectrum for the CaSR or the VIF peptide (see Supplementary material online, *Figure S3E*). To validate the stimulatory action of the VIF peptide on CaSR, intracellular Ca^{2+}-ion ($Ca^{2+}{}_i$) mobilization was measured in HEK cells expressing the CaSR. Representative $Ca^{2+}{}_i$ mobilization time-course traces at different calcium concentrations are shown in Supplementary material online, *Figures S4A, B, D,* and *E*. The HEK cells were treated with 2.5 mmol L^{-1} calcium [Ca (bf); bf: before addition of the VIF peptide] to induce submaximal $Ca^{2+}{}_i$ mobilization followed by the addition of the VIF peptide (Ca + VIF) before returning to 2.5 mmol L^{-1} calcium [Ca (af); af: after addition of the VIF peptide]. *Figure 5E* demonstrates that adding the VIF peptide significantly increased the CaSR-mediated $Ca^{2+}{}_i$ mobilization, which declined after removing the VIF peptide [Ca (af.)], though not immediately to previous levels. Submaximal concentration was used to potentiate the ligand's effect at submaximal levels. VIF was 30% more efficient in activating the calcium-sensing receptor than Ca^{2+} alone (see Supplementary material online, *Figure S4C*). Further, the VIF peptide effect was ablated using NPS-2143 (see Supplementary material online, *Figure S4E* and *F*), a known CaSR-selective negative allosteric modulator.[38] The VIF peptide had no stimulatory effect at low (0.5 mmol L^{-1}) calcium concentration, increased the $Ca^{2+}{}_i$ mobilization after the addition of the VIF peptide with 2.5 mmol L^{-1} calcium and lost its effect when the negative allosteric modulator NPS-2143 was added (see Supplementary material online, *Figure S4E* and *F*), indicating that VIF acts as a positive allosteric modulator at higher calcium concentrations. *In vitro* and *ex vivo* experiments on SMCs and rat aortic rings under high phosphate conditions, in the presence of VIF and/or NPS-2143, showed that the addition of NPS-2143 neither negates the effect of the VIF peptide nor has any synergic additive or inhibitory effect. (see Supplementary material online, *Figure S5A* and *B*). Furthermore, the binding of VIF to CaSR could be confirmed *in vivo,* after performing co-localization assay in aortic rings from VDN rats treated with VIF. The signals from both VIF (shown in green) and CaSR (red) m/z were detected in the medial layer of SMCs in the animals' aortas via MALDI imaging. The overlay of both signals provided a yellow colour, showing the co-localization of VIF and calcium-sensing receptors (*Figure 5F*).

3.7 Inhibitory effect of the VIF peptide on apoptosis

Since high phosphate levels induce apoptosis in VSMCs[39] and apoptosis has been suggested as an underlying mechanism of vascular calcification,[12,40] next, the effect of the VIF peptide on apoptosis was investigated. Apoptotic cells were analysed via flow cytometry by counting the annexin V-positive cells in hAoSMCs incubated in NCM and CM in the absence or presence of the VIF peptide. The incubation of hAoSMCs in the CM had a population of annexin V-positive cells three times higher compared with NCM. The presence of the VIF peptide in the CM reduced annexin V-positive cells to values like NCM (*Figure 7A* and *B*). Tunel staining in the *in vivo* model corroborated the *in vitro* data, showing an increased population of apoptotic cells in the VDN rats, which decreased when the rats were treated with VIF, reaching similar levels to the control rats (*Figure 6E*).

3.8 VIF effect on osteoblast mineralization *in vitro*

In order to assess the VIF effect on bone remodelling, *in vitro* studies using osteoblast cultivated with high phosphate media (2.5 mmol L^{-1}; CM) as well as osteogenic media (ascorbic acid 0.3 mmol L^{-1}, dexamethasone 10–5 mmol L^{-1}, and β-glycerolphosphate 3 mmol L^{-1}; OM) in the absence or presence of the VIF peptide have been performed. Calcium deposition and osteoblast transdifferentiation have been analysed, but no significant difference was found between the presence or absence of the VIF peptide in osteoblast (see Supplementary material online, *Figure S5C–E*).

3.9 The VIF peptide concentration in patients with CKD with artery calcification

Previous studies have found an increased VIF peptide concentration in patients with CKD compared with healthy individuals.[15] The present study measured the VIF peptide in plasma from patients with CKD with artery calcification (see Supplementary material online, *Table S2*). Patients with extensive coronary artery calcium (CAC) volume exhibited lower levels of the VIF peptide in plasma, showing a negative correlation between them ($r = -0.5025$; P-value = 0.0398; *Figure 7C*), suggesting an inhibitory effect of the VIF peptide on vascular calcification in patients with CKD. No effect of statins was observed on the correlation between VIF and CAC score (*Figure 7D*).

4. Discussion

The chromogranin A-derived peptide, 'vasoconstriction inhibiting factor' (VIF), was recently identified as an endogenous inhibitor of the Ang II-mediated vasoconstriction.[15] Since Ang II has been described as an inhibitor of vascular calcification under high phosphate conditions,[19] we investigated the impact of the VIF peptide on this process. While Ang II inhibits calcification, the VIF peptide does not affect the Ang II-mediated process.

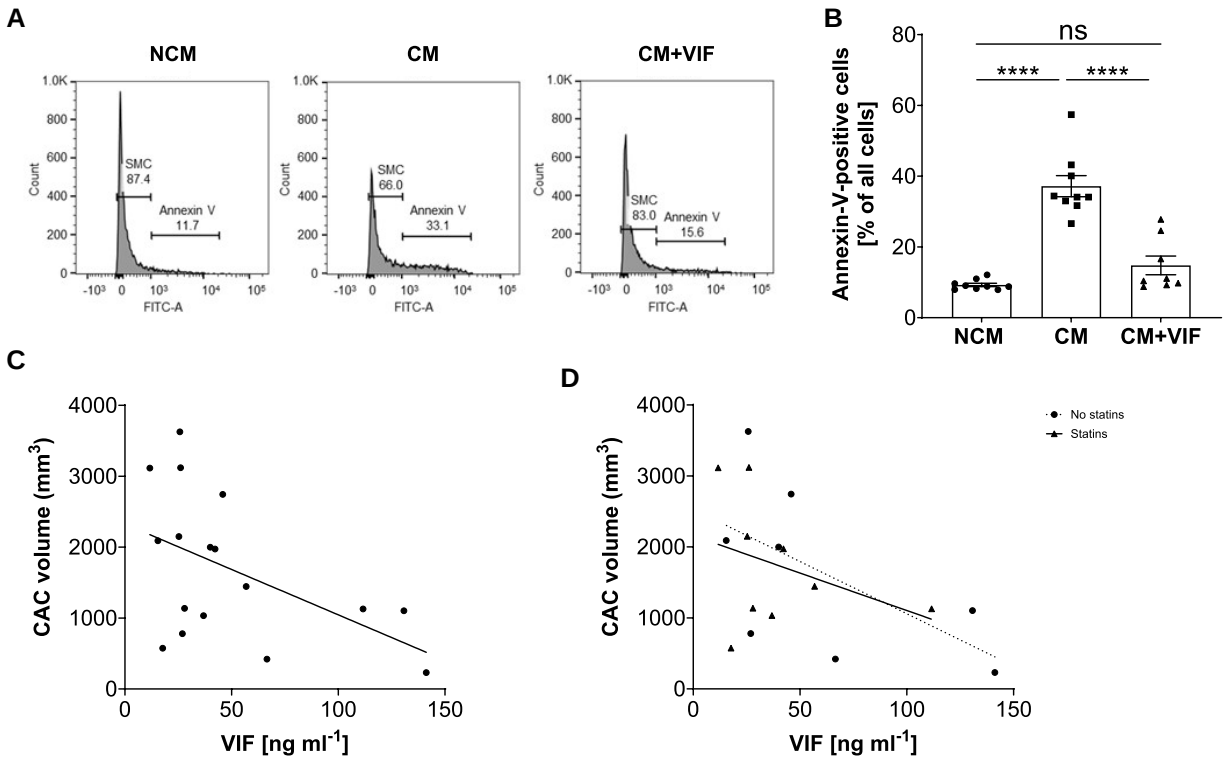

Figure 7 The vasoconstriction-inhibiting factor (VIF) peptide decreases apoptosis of human aortic smooth muscle cells (hAoSMC) culture in calcifying medium (CM) and correlates negatively with CAC volume in patients with chronic kidney disease (CKD). (*A*) Representative flow cytometry histograms of hAoSMCs with the population of FITC-annexin V-positive cells. (*B*) Relative quantification of FITC-annexin V-positive cells in hAoSMC incubated in a non-calcifying medium (NCM; circle dots) or calcifying medium in the absence (CM; square dots) or presence of the VIF peptide (100 nmol L^{-1}; CM + VIF; triangle dots). (*C*) Correlation between the VIF peptide concentration analysed by ELISA in plasma and the coronary artery calcium of patients with CKD under haemodialysis ($r = -0.5025$; *P*-value = 0.0398; $N = 17$). (*D*) Correlation between the VIF peptide concentration analysed by ELISA in plasma and the coronary artery calcium of patients with CKD under haemodialysis with (triangle dots; $r = -0.3480$; *P*-value = 0.3588; $N = 9$) or without (circle dots; $r = -0.5978$; *P*-value = 0.1175; $N = 8$) statin treatment.

However, it strongly reduced calcium deposition on hAoSMCs in a dose-dependent manner both *in vitro* and *ex vivo* with an EC_{50} value of 10^{-15} mol L^{-1}, showing its strong potency. A limitation of our culture model is that it relies solely on SMCs, which are directly responsible for the development of vascular calcification[41]. As a result, the model lacks a multicellular environment and various factors and mediators that other cells might contribute to this pathology. To address this, we performed experiments in aortic rings, which better mimic the conditions in the organism by involving different cell types. However, this model also has limitations, such as the absence of organ crosstalk interactions. For *in vivo* validation, a preclinical animal model of elastocalcinosis was used (VDN animals), in which medial calcification was induced, mimicking calcification in patients with CKD. Only male rats were used to avoid hormone influence. The inhibitory effect of the VIF peptide on the deposition of calcium phosphate on the medial layer was confirmed in VDN animals by different calcification measurements. In line with vascular calcification contributing to arterial stiffness, the treatment with the VIF peptide caused a reduction of pulse pressure in the VDN animals treated with the peptide, which is a reliable parameter for the elasticity of the arteries but never reaching significant values below the control rats. Moreover, Salem *et al.*[15] described the VIF peptide as a vasoconstriction inhibitor factor with a $EC_{50} = 10^{-11}$ mol L^{-1}, but in the current study, the EC_{50} regarding calcification inhibition is much lower, $EC_{50} = 10^{-15}$ mol L^{-1}. These facts point to the VIF effect in the VDN rats is mainly related to changes in calcification-induced arterial stiffness with a minor influence on the vascular tone.[21,42] However, the possible effect of the VIF peptide on the vascular tone should not be disregarded, as a decrease in blood pressure could help to prevent the remodelling of the extracellular matrix that leads to calcium and phosphate deposition.[43] In both our models (*ex vivo* and *in vivo*) VIF inhibited medial calcification in the aortic rings. A limitation of the present study is the low number of animals in the *in vivo* experiment. Another limitation is using only male rats, which has been addressed by using male and female donors in the *in vitro* studies.

Furthermore, the amino acids at positions 22–28 of the VIF peptide are essential for the inhibitory effect. For clinical application, a biologically active peptide consisting of seven amino acids is highly relevant in terms of synthesis costs compared with the full-length peptide. The shorter potent VIF fragment allows the hypothesis that the VIF peptide may be a precursor mediator, which activate intracellular yet unknown processes.

The incubation of both elongated VIF peptides or the entire CgA with PCSK2 and CPE enzymes results in the release of the VIF peptide. Since CgA is not able to inhibit calcium deposition, the VIF peptide is cleaved from CgA to be activated. Moreover, the enzyme cathepsin K metabolises the VIF peptide. Other authors have observed a correlation between cathepsin K levels, CAC score and increased mineralization of the vascular matrix.[44,45]

The influx of calcium into the cells, triggered by high ambient phosphate, enhances vascular calcification.[46,47] Since calcium and phosphate concentrations are orders of magnitude higher than the VIF peptide concentration, the inhibitory effect of the VIF peptide is unlikely to involve direct

interactions between the VIF peptide and calcium/phosphate crystal formation. This was also proved in our calcium influx experiments with ionomycin. In addition, increased calcium influx and resulting vascular calcification are associated with increased ROS production[26,48] as we have also observed both *in vitro* and *in vivo*, with decreased oxidative species in presence of VIF.

Furthermore, hyperphosphatemia and the resulting increase in ROS production lead to the activation of signalling pathways, contributing to vascular calcification.[12,49–52] Consistent with this, the activity of the P38 and ERK1/ERK2 MAPKs was increased in the calcification medium, which agrees with prior findings,[32] and in the presence of the VIF peptide, their activity was reduced. Increased ROS production, as well as activation of P38 and ERK1/2, leads to increased expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6[32,53,54] inducing osteogenic differentiation and mineralisation of VSMCs.[29–31] The preform of BMP2, a key regulator of vascular calcification,[34] was detected in calcifying media, while the active protein was found in the calcified layer of aortic rings from VDN rats. In both models, VIF reduced the expression of BMP2. As expected, an increased inflammatory environment, as well as an increase in the expression of calcification-related genes (MSX2, OCN, SOX9, and Pit-1),[33,35,54,55] was observed when the cells were cultured in CM while the addition of VIF decreased all these processes. Moreover, the expression of α-SMA was reduced by incubating the cells in a CM. However, the presence of the VIF peptide did not significantly alter this phenomenon, suggesting that the VIF peptide is not involved in the phenotypic conversion of smooth muscle cells.[56] In addition, increased apoptosis of VSMCs is a relevant mechanism of vascular calcification.[39,41] In the current study, an increase in apoptotic cell population was observed in the CM and VDN rats, but the presence of the VIF peptide reduced this population. The apoptotic cells in the *in vivo* model overlapped with the areas of calcification staining with von Kossa, showing the relationship between apoptosis and vascular calcification.

Furthermore, the calcium-sensing receptor (CaSR) was identified as the VIF peptide-binding partner. Due to the substantial difference in the molecular weight of VIF (3907 Da) and CaSR (120 000 Da), detection of co-immunoprecipitation by Western blot could not be performed. Therefore, the samples were analysed by using a mass spectrometric approach. Mass spectrometry analyses also showed co-localization of VIF and the receptor on the aortas of VDN rats treated with the VIF peptide. The paradox observed between VIF reducing calcium influx (*Figure 4A*), and increasing CaSR efficacy (*Figure 5E*) by increasing mobilization of Ca^{2+}, could be explained by the nature of the different cells and conditions used: in SMCs, which are by nature excitable cells,[47] VIF reduced calcium influx under high phosphate concentrations; while in HEK-293, which are non-excitable cells and where no calcifying media was used, VIF increased calcium mobilization and thus increasing the efficacy of the receptor. The VIF peptide acts as a calcimimetic of CaSR, leading to increased cMGP production, as already shown before for calcimimetics like R-568 and AMG641.[37,57,58] The addition of NPS-2143 to the calcifying conditions in the presence of VIF did not alter VIF´s effectiveness. Although NPS-2143 itself inhibited calcium deposition *in vitro*, this effect is likely due to its anti-inflammatory properties[59] and its ability to inhibit VGCC channels,[60] which in turn reduces the entry of calcium into cells, lowers the production of ROS and prevents activation of calcification pathways.[26] mRNA levels of MGP include cMGP, but also uncarboxyMGP, phosphoMGP and unphosphoMGP.[61,62] No difference was observed at the gene level for this protein. Carboxylation of MGP is needed to be active and thus inhibit calcium deposition by binding hydroxyapatite crystals.[61,62] As observed *in vivo*, only the VDN rats have ucMGP present, with the calcified areas of these rats being positive for both cMGP and ucMGP. On the other hand, the few calcified areas from VDN + VIF rats are positive only for the active cMGP. These results show that the administration of VIF increases the production of cMGP; therefore, they bind to the hydroxyapatite crystals, inhibiting the development of vascular calcification. The regulation of MGP by CaSR activation has been previously reported,[63] although the mechanism has not been fully elucidated, and further research has to be done in this field.

To consider the VIF peptide as a potential therapeutic agent against calcification, its effect on osteoblasts mineralization, and thus bone formation, was investigated. No effect was observed in this regard.

An increased plasma concentration for the VIF peptide in patients with CKD compared with control patients with normal renal function, as well as in patients with heart failure compared with control subjects without heart failure, are described.[15] The increased VIF peptide plasma concentration might be a compensatory response to the increased blood pressure.[15] In our study, patients with CKD with a lower VIF plasma concentration have a higher degree of calcification independent of statin treatment. The number of patients included in the present study is limited due to restricted access to appropriate patients with CT data. Patients with CKD have an increased concentration of uraemic toxins, leading to post-translational modifications (PTMs) of enzymes,[64,65] altering their enzymatic activity. This could explain the difference in VIF concentration in patients with CKD. The next step would be to further research on this area, looking for PTMs in VIF-related enzymes.

In conclusion, the VIF peptide is characterized as a novel and highly potent inhibitor of vascular calcification by acting as an endogenous calcimimetic and positive allosteric modulator of CaSR. Furthermore, it was shown that the VIF peptide increases the production of cMGP, the active form of the MGP, which binds strongly to hydroxyapatite crystals and thus inhibits crystal growth. The scavenge of hydroxyapatite crystals by cMGP leads to reduced calcium influx, ROS production, and IL-6 expression and secretion. The activation of various kinases cascades in the cells, involved in the expression of calcification related genes, is prevented and, thus vascular calcification is inhibited.

Translational perspective

Identification of vasoconstriction-inhibiting factor (VIF) peptide as an endogenous inhibitor of vascular calcification through calcium-sensing receptor modulation presents opportunities for novel preventive and therapeutic strategies against vascular calcification, thereby enhancing the therapeutic potential of VIF peptide. Furthermore, the development and availability of an ELISA assay to detect the peptide in plasma makes the VIF peptide a promising candidate as a biomarker in the clinic.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Authors' contributions

S.d.l.P.-S. acquired, analysed, interpreted, and wrote the manuscript. D.M., A.M., N.G., K.A., and D.W. acquired, analysed, and interpreted the data. V.J., J.H., T.S., M.T., and E.P.C.v.d.V. acquired and analysed data. S.B. acquired data. S.O. interpreted, wrote, and designed the manuscript. A.A., Z.W., H.N, and J.J. designed the work. H.N., J.F., L.S., W.Z., M.W., and J.J. interpreted the data. All authors have read and approved the manuscript.

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Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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