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IDENTIFICATION AND CHARACTERISATION OF A NOVEL ADRENAL PEPTIDE THAT INHIBITIS OSTEOGENIC TRANSDIFFERENTIATION TO PREVENT VASCULAR CALCIFICATION

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Translational perspective of the manuscript

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The adrenal glands participate in cardiovascular (CV) physiology and in the pathophysiology of CV diseases through their effects on sodium and water metabolism, vascular tone and cardiac function. In this study, the new peptide CBF secreted from the adrenal glands have been identified that modulates vascular calcification processes and its concentration is significantly reduced in patients with increased calcification.

CBF is a highly promising drug candidate for the prevention and/or treatment of vascular calcification (patent application). These findings suggest a novel function of the adrenal medulla in CV regulation: whereas catecholamine secretion of the adrenal glands is important for short-term vasoregulation, CBF may be involved in long-term preservation of heart and vascular health.

ABSTRACT

The adrenal glands participate in cardiovascular (CV) physiology and in the pathophysiology of CV diseases through their effects on sodium and water metabolism, vascular tone and cardiac function. Long-term effects of adrenal glands in relation to processes like vascular calcification are not known.

In the present study, we identified a new adrenal compound with a function in mesenchymal cell differentiation that regulates osteoblastic differentiation in the context of vascular calcification. We we designated this peptide “calcification-blocking factor” (CBF) because of its protective effect against vascular calcification. The substance is a component of plasma and its concentration is significantly reduced in diseases characterized by increased calcification, such as chronic renal insufficiency.

CBF reduced the calcium content of cells and aortic rings under calcifying culture conditions and that of aortas from VDN animals. Pulse pressure, a marker of arterial stiffness, was significantly decreased in VDN animals treated with adrenal CBF. After identification of the enzymes responsible for CBF cleavage from chromogranin A (calpain 1 and kallikrein), the active sequence of CBF, which is secreted into plasma by the adrenal glands, was identified as EGQEEEED. Furthermore, CBF prevented vascular smooth muscle cell (VSMC) transdifferentiation into osteoblast-like cells within the vascular wall via the sodium-dependent phosphate transporter PIT-1 and by inactivation of the BMP2/p-SMAD pathway.

In conclusion, we have identified the new peptide CBF secreted from the adrenal glands that modulates vascular calcification processes and its concentration is significantly reduced in patients with increased calcification. CBF is a highly promising drug candidate for the prevention and/or treatment of vascular calcification (patent application).

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IDENTIFICATION AND CHARACTERISATION OF A NOVEL ADRENAL PEPTIDE THAT INHIBITIS OSTEOGENIC TRANSDIFFERENTIATION TO PREVENT VASCULAR CALCIFICATION

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The adrenal glands participate in cardiovascular (CV) physiology and in the pathophysiology of CV diseases through their effects on sodium and water metabolism, vascular tone and cardiac function. Long-term effects of adrenal glands in relation to processes like vascular calcification are not known.

In the present study, we identified a new adrenal compound with a function in mesenchymal cell differentiation that regulates osteoblastic differentiation in the context of vascular calcification. We have designated this peptide "calcification-blocking factor" (CBF) because of its protective effect against vascular calcification. The substance is a component of plasma and its concentration is significantly reduced in diseases characterized by increased calcification, such as chronic renal insufficiency.

CBF reduced the calcium content of cells and aortic rings under calcifying culture conditions and that of aortas from VDN animals. Pulse pressure, a marker of arterial stiffness, was significantly decreased in VDN animals treated with adrenal CBF. After identification of the enzymes responsible for CBF cleavage from chromogranin A (calpain 1 and kallikrein), the active sequence of CBF, which is secreted into plasma by the adrenal glands, was identified as EGQEEEED. Furthermore, CBF prevented vascular smooth muscle cell (VSMC) transdifferentiation into osteoblast-like cells within the vascular wall via the sodium-dependent phosphate transporter PIT-1 and by inactivation of the BMP2/p-SMAD pathway.

In conclusion, we have identified the new peptide CBF secreted from the adrenal glands that modulates vascular calcification processes and its concentration is significantly reduced in patients with increased calcification. CBF is a highly promising drug candidate for the prevention and/or treatment of vascular calcification (patent application).

These findings suggest a novel function of the adrenal medulla in CV regulation: whereas catecholamine secretion of the adrenal glands is important for short-term vasoregulation, CBF may be involved in long-term preservation of heart and vascular health.

Vascular calcification is an important component of cardiovascular (CV) pathology [1-3]. Increased vascular calcification is highly prevalent in the general population, increases with ageing [4]; and is associated with increased mortality in patients with hypertension, chronic kidney disease (CKD) [5], and atherosclerosis [6-8]. The characteristics of calcified arteries include increased vascular stiffness, reduced stretch, and decreased elasticity. The pathogenesis of vascular calcification is complex and involves imbalance of enhancers and inhibitors of the underlying processes. One essential step in the pathophysiology of vascular calcification is the transdifferentiation of vascular smooth muscle cells (VSMCs) into cells with an osteoblast-like phenotype. Although understanding of arterial calcification has grown rapidly, only a limited number of vascular calcification regulators, such as osteopontin [9], osteoprotegerin [10,11], and bone morphogenetic proteins [12,13], have been identified. Most studies describe local paracrine regulatory mechanisms, such as the roles of bone morphogenetic proteins and Wnt signalling. These studies have mainly focused on the osteoblastic transdifferentiation of VSMCs under pathological conditions resulting from local imbalance between calcifying factors and inhibitors.

In this context, adrenal glands are of particular interest, since adrenal glands are involved in the development and progression of cardiovascular disease through their effects on, among others, salt and water metabolism, vasoregulation and cardiac functions. In particular, the function of catecholamines in short-term vasoregulation is well known. Although specific regulators other than catecholamines have not been identified, considering the proteomic- and peptidomic-base identification of a large number of peptides and proteins produced by the adrenal medulla, it is possible that it contributes to long-term vascular adaptation by a yet undiscovered messenger [14-

[16]. So far only a small number of these peptides, such as adrenomedullin and enkephalins, have been linked to endocrine or paracrine functions [17,18]. While the role of catecholamines in short-term vasoregulation is well known, we hypothesized that the adrenal medulla also contributes to long-term vascular adaptation e.g. osteoblastic transdifferentiation by as yet undiscovered messengers.

In the present study, we aimed to identify an inhibitor of vascular calcification process release by adrenal glands. To this end, instead of traditional proteomic or peptidomic approaches, we used analytic mass spectrometry closely coupled with functional assays to focus specifically on identifying peptides relevant to vascular calcification. We report a new adrenal factor with systemic effects that we named “calcification-blocking factor” (CBF). Using *in vitro, ex vivo* and *in vivo* models we show that CBF modulates VSMC transdifferentiation and thus vascular calcification, thereby participating in the balance between pro- and anti-calcifying mechanisms, and determined the pathways involved in its signal transduction. The level of CBF was found to be decreased in patients with stage 5 CKD (also known as end-stage renal disease (ESRD)), who exhibit a propensity to develop vascular calcification; this correlation, along with the striking capacity of CBF to decrease vascular calcification *in vivo,* makes this peptide a clear candidate to explore a new approach in preventing or treating vascular calcification.

RESULTS

Extraction and identification of the ´ calcification-blocking factor´ from adrenal glands

To identify peptides produced by the adrenal medulla involved in vascular calcification, we screened bovine adrenal glands to identify peptides involved in vascular calcification (Figure 1a). To this end, bovine adrenal gland extracts were desalted, concentrated, and fractionated by preparative reversed-phase chromatography. Each resultant fraction was tested for its inhibitory effect on vascular calcification using the *ex vivo* aortic ring calcification assay, and fractions with inhibitory activity were then further fractionated by anion-exchange chromatography. Inhibitory calcification activity was tested in the resulting fractions and those that showed the activity were

further were further purified by reversed-phase chromatography **(Figure 1b)**. The resulting fractions were again tested for their inhibitory effects on vascular calcification via aortic ring calcification assay. Fractions showing strong inhibitory effects (indicated by the arrow in **Figure 1b**) were analysed by mass spectrometry. The mass spectrum of one of these fractions at a retention time of 34.8 min showed an intense signal peak with a molecular mass (m/z) of 2,297 Da ($[M+H]^+$) (**Figure 1c**). The peptide in this fraction was fragmented and sequenced using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry and identified as LEGEEEEEEDPDRSMRLSF through a Mascot search (**Figure 1d**). This amino acid sequence corresponds to amino acids 300-318 of bovine chromogranin A, which is a precursor to several functional peptides including vasostatin-1, vasostatin-2, pancreastatin, catestatin and parastatin, as discussed recently in detail [19]. (**Figure 1d**; bold)**.** The results of screening via fractionation and calcification assays and sequencing via MS identified the CBF peptide as a component of the adrenal gland with strong inhibitory effects against vascular calcification.

To test whether CBF could also found in humans, we analyzed the plasma since peptides released by the adrenal gland can usually be found in plasma. We followed the purification procedure shown in Figure 1a and isolated a peptide with a m/z of 2,243 ($[M+H]^+$) from human plasma. The amino acid sequence of the peptide was identified as LEGQEEEEDNRDSSMKLSF and corresponds to the amino acid sequence of the human CBF peptide from human chromogranin A (**Figure 1e**; bold). To validate the amino acid sequence, the MS/MS fragment spectrum of the synthetic peptide with the amino acid sequence LEGQEEEEDNRDSSMKLSF and the isolated peptide were compared, confirming the identities of these peptides (data not shown). These data clearly indicate that human plasma contains the CBF peptide and confirmed its identity.

Characterisation of calcification inhibitoric effect of CBF in-vitro and in-vivo

After isolating CBF from human serum, we proceed to characterize its effects on vascular calcification *in vitro* and *ex vivo*. To this end a synthetic human CBF was generated and its effects on the calcification of human aortic smooth muscle cells (hAoSMCs) *in vitro* were investigated. The threshold concentration at which CBF decreased calcification in hAoSMCs was on the order of 1 amol L^{-1}, and the corresponding EC_{50} was on the order of 10 amol L^{-1} (**Figure 2a**). Then, we validated the effects of human CBF on the calcification of rat aortic rings *ex vivo* by culturing aortic rings in calcifying medium (CM) in the presence of CBF at increasing concentrations. Calcification was reduced by CBF in a dose-dependent manner, as shown by quantification of the calcium content (**Figure 2b**). The threshold concentration at which CBF decreased calcification was on the order of 0.01 nmol L^{-1}, and the corresponding EC_{50} value was on the order of 100 pmol L^{-1}. Furthermore, reduced aortic ring calcification in the presence of CBF was confirmed by von Kossa staining, which revealed a 40% reduction in the calcified area upon treatment with CBF at 100 nM compared to calcification of aortic rings cultured in CM alone (**Figure 2c**). CBF decreased calcification in hAoSMCs and rat aortic rings at threshold concentrations in the low picomolar range, and that it showed EC50 values of 10 amol L^{-1} and 100 pmol L^{-1}, respectively.

Next, the VDN animal model was used to assess the impact of CBF on calcification *in vivo*. The calcium content of the vessels was dramatically increased in the VDN animal group compared to the control group (**Figure 3a**). However, continuous CBF treatment via osmotic pump for 4 weeks significantly reduced the calcium content in the vessels of treated VDN rats compared to untreated VDN rats (**Figure 3a**). Aortic calcification was visualized by von Kossa staining of the aortic segments from control, untreated, and CBF-treated male VDN rats. CBF treatment significantly decreased the calcified surface area by 70% (**Figure 3b**). In addition, treatment with CBF *in vivo* decreased arterial pressure in the VDN animals and pulse pressure decreased from 81 ± 6 to 53 ± 3 mmHg ($P \leq 0.001$) (**Figure 3c**). These data confirmed the inhibitory effects of CBF against vascular calcification *in vivo* in the VDN animal model through measurement of the vessel calcium content, von Kossa staining, and measurement of arterial and pulse pressure.

Identification of the active sequence for the inhibitoric effect of CBF

To identify the active sequence of CBF, we synthesized fragments of the full sequence of the CBF peptide (**Figure 4a)** and analysed the inhibitory effect of these peptide fragments on calcification both *in vitro* using hAoSMCs and *ex vivo* using aortic rings incubated in CM in the absence or presence of the CBF fragments for 7 days. Initially, the amino acid sequence of CBF was divided into two peptides consisting of ten amino acids each (CBF 1-10, CBF 6-15) and one peptide consisting of nine amino acids (CBF 11-19) (**Figure 4a**, beige). CBF 01-10 significantly decreased the calcium content of hAoSMCs (**Figure 4b**) and aortic rings (**Figure 4c**) grown in CM in comparison to that in untreated hAoSMCs and aortic rings, while the fragments CBF 6-15 and CBF 11-19 had no significant effect. The inhibitory effects of CBF 1-10 and the complete CBF peptide (CBF 1-19) were not significantly different. Next, the inhibitory effects of the CBF 01-10 sub-fragments CBF 01-05 and CBF 06-10 (**Figure 4a**; dark grey) were analysed. Both sub-fragments significantly reduced calcification in hAoSMCs as well as in aortic rings, although the effect was stronger in cells and aortic rings incubated with the fragment CBF 6-10 (**Figure 4b** and **4c**). To further narrow down the active sequence of CBF, we analysed the fragments CBF 1-4, CBF 2-5, CBF 3-6, CBF 4-7, CBF 5-8, CBF 6-9 and CBF 7-10 (**Figure 4a**; light grey). Each fragment showed an inhibitory effect on vascular calcification in both cells and aortic rings, with the strongest inhibition caused by CBF 4-7, CBF 5-8, and CBF 6-9 (**Figure 4b** and **4c**). The identification of the active side of the CBF peptide has the potential to be the basis for the development of new pharmaceuticals based on this amino acid sequence.

Clarification of the enzymatic cleavage of CBF from chromogranin A

As CBF was identified as a peptide of the protein chromogranin A, we next investigated the enzymes that cleave CBF from chromogranin A by using the Proteasix database. For these experiments, elongated CBF peptides with 10 additional amino acids of chromogranin A at either the N- or the C-terminus were used. First, we examined whether calpain 1 cleaves the N-terminus of CBF from chromogranin A, by incubating LAKELTAEKR-CBF with and without calpain 1 for 48 h. While incubation of LAKELTAEKR-CBF in the absence of enzymes caused no molecular mass signal for CBF (**Figure 5a**), an intense mass signal at 748.66 ($[M+3H]^{3+}$) was detected after incubation in the presence of calpain 1, demonstrating the N-terminal cleavage of CBF from LAKELTAEKR-CBF (**Figure 5a**; lower spectrum). Next, we studied whether kallikrein cleaves the C-terminus of CBF from chromogranin A, by incubating CBF-RARAYGFRGP in the presence and absence of kallikrein for 48 h. While incubation of CBF-RARAYGFRGP in the absence of enzymes caused no molecular mass signal for CBF (**Figure 5b**; upper spectrum), an intense mass signal at 748.66 ($[M+3H]^{3+}$) was detected after incubation in the presence of kallikrein, demonstrating the C-terminal cleavage of CBF from CBF-RARAYGFRGP (**Figure 5b**; lower spectrum). Incubation of chromogranin A in the presence of both calpain 1 and kallikrein, but not in their absence, caused an intense mass signal at 748.66 ($[M+3H]^{3+}$), demonstrating the cleavage of CBF from chromogranin A (**Figure 5c**; lower spectrum).

After identification of the enzymes cleaving CBF from chromogranin A, the stimulus that induces CBF release from the adrenal glands by a physiological stimulus was studied. Carbachol, which binds and activates acetylcholine receptors, significantly increased the release of CBF from adrenal glands (**Figure 5d**), suggesting that CBF release may involve acetylcholine receptors. To examine whether CBF is proteolyzed from intracellular or extracellular chromogranin A, adrenal gland chromaffin granules were isolated by density gradient centrifugation and chromatographic techniques. CBF was detected in one of the fractions from the adrenal gland chromaffin granules, suggesting that CBF is a component of the chromaffin granules of adrenal glands.

Quantification of CBF plasma concentration in chronic kidney diseases

Next we validated the relevance of CBF to human pathophysiology by quantifying the concentration of CBF in plasma in ESRD patients, who suffer from increased vascular calcification and healthy control subjects using mass spectrometry as described above. **Table 2** shows the clinical and biochemical characteristics of ESRD patients and healthy control subjects. The CBF plasma concentration was significantly lower in ESRD patients compared to healthy control subjects (102.4 ± 23.0 nmol L^{-1} and 266.1 ± 56.4 nmol L^{-1}, N = 17 and N = 13, respectively; $P<0.05$) (**Figure 5e**). These results suggest that CBF might have an impact on the increased vascular calcification rate in these patients, most likely caused by modification of the enzyme activity of calpain 1 and/or kallikrein in these patients.

Identification of the pathway mediating the inhibitoric effect of CBF

To understand how CBF inhibits vascular calcification we studied the involvement of CBF in VSMC transdifferentiation into osteoblast-like cells within the vascular wall as this is an essential step in the pathophysiology of vascular calcification. To this end, the expression of α-smooth muscle actin (α-SMA), the predominant actin isoform in VSMCs, was quantified in aortic rings incubated with NCM, as well as under calcifying conditions in the absence or presence of CBF. The α-SMA-stained area was significantly smaller under calcifying conditions compared to non-calcifying conditions (**Figure 6a**). The presence of CBF neutralized this effect of the CM (**Figure 6a**). This effect of CBF on cell transdifferentiation was confirmed *in vivo* using aortas isolated from control rats, VDN rats, and VDN rats treated with CBF (**Figure 6b**). These results suggest that the transdifferentiation of smooth muscle cells to osteoblast-like cells was blocked by CBF.

The type III NaPi cotransporter *PIT-1* has been identified as a pivotal transporter involved in phosphate-induced VSMC transdifferentiation and resulting calcification [20]. Thus, we next analysed the effect of CBF on *PIT-1* expression under calcifying conditions. *PIT-1* expression was significantly higher in VSMCs incubated for 5 days with CM compared to NCM-cultured cells inde-

pendently of the presence or absence of CBF (**Figure 6c**). However, knockdown of PIT-1 in hAoSMCs led to a significant reduction in the inhibitory effect of CBF on calcification compared to that observed in cells transfected with control siRNA, where a 35% inhibition of calcium content by CBF treatment was observed (**Figure 6d**). The results of these expression experiments showed that the inhibitory effect of CBF is mediated via the PIT-1/BMP2/p-SMAD signal transduction pathway.

Since high phosphate uptake via PIT-1 promotes the expression of BMP2 in VSMCs [21] and the BMP2/p-SMAD pathway is strongly involved in vascular calcification, we next analysed whether BMP2 expression *in vitro* and *in vivo* is modified by CBF. Calcifying conditions caused a strong and significant increase in BMP2 mRNA expression compared to that in cells cultured under non-calcifying conditions that was reversed when the cells were incubated in the presence of CBF (**Figure 7a**). This effect was confirmed *in vivo* by analysing aortas isolated from control rats, VDN rats, and VDN rats continuously treated with CBF (**Figure 7b**).

Next, we analysed the phosphorylation levels of the downstream components of the BMP2 pathway SMAD1 and SMAD5 in aortic rings cultured under non-calcifying conditions and in CM in the absence and presence of CBF using immunofluorescence microscopy. We found that the strong and significant increase in the SMAD1 and SMAD5 phosphorylation signals under calcifying conditions was effectively inhibited by CBF (**Figures 7c and 7d**). Similar results were obtained when the phosphorylation of SMAD1 and SMAD5 was quantified in aortas isolated from control rats or from VDN rats with or without treatment via continuous infusion of CBF (**Figure 7e** and **7f**). These results indicate that CBF treatment inhibits SMAD1 and SMAD5 activation under calcifying conditions.

Finally, we assessed the effect of CBF on the relative mRNA expression of runt-related transcription factor 2 (RUNX2), a key transcription factor associated with osteoblast differentiation and

upregulated in response to pro-calcification stimuli, including inflammation, oxidant stress, and BMP2 [22,23]. In addition, we investigated the effect of CBF on the expression of two other genes encoding pro-calcification transcription factors, msh homeobox 2 (MSX2) and osterix (OSX) *in vitro* as well as *in vivo*. Calcifying conditions caused an increase in the mRNA expression of all three transcription factors compared to that under non-calcifying conditions (**Figure 8a**), an effect that was reversed by the presence of CBF although this result was only significant on the mRNA expression of RUNX2 and OSX (**Figure 8a**). Similar results were obtained *in vivo* by analysing aortas isolated from control rats, VDN rats, and VDN rats continuously treated with CBF (**Figure 8b**).

Together, these results clearly demonstrate that osteogenic genes are regulated by BMP-2. In addition to BMP-2/SMAD signalling, MAPK cascades represent an alternative, non-canonical pathway for BMP-2 signal transduction [24,25] to induce osteogenic gene expression and influence calcification [26,27]. In line with the BMP2 expression, kinome analysis revealed that calcifying conditions caused a strong increase in p38 and JNK activity, together with its downstream targets MSK1 and MSK2, respectively (**Figure 8c**; [28]), compared to cell culture in non-calcifying conditions. Importantly, the presence of CBF almost completely abolished this effect of the calcification medium. As p38 and JNK can promote the expression and activation of osteogenic genes, like RUNX2 [29], this further confirms the importance of BMP-2 signalling in the inhibitory role of CBF on calcification.

DISCUSSION

Vascular calcification substantially contributes to CV morbidity and mortality in the general population, particularly in patients suffering from specific diseases, such as chronic renal failure [30]. Imbalances between inhibitory and inducing mediators are known to be involved in vascular calcification [31]. Given the wealth of peptide hormones synthesized by the adrenal medulla and their involvement in vascular calcification [32], we hypothesized that unknown adrenal-derived mediators regulate osteoblastic transdifferentiation, a main feature of vascular calcification. Therefore, the

bovine adrenal gland secretome was screened for novel adrenal-derived peptides that modulate vascular calcification of VSMC by chromatographic and mass spectrometric methods. This approach led to the identification of a novel peptide and specific role for the adrenal glands in vascular calcification in human. We identified a chromogranin A-derived peptide, designated CBF, that strongly inhibits vascular calcification. CBF is a component of human plasma and was identified as LEGQEEEEDNRDSSMKLSF, which corresponds to residues 358-376 of the amino acid sequence of the human CBF peptide from human chromogranin A.

The identified CBF is derived from the protein chromogranin A that is expressed in the secretory cells of different tissues of neuronal, neuro-endocrine, and endocrine origin [33]. This protein, initially identified as a tumour marker [34], is associated with CV (patho)physiology [35]. Chromogranin A is a precursor of several biologically active peptides, e.g., vasostatin-I (human CHGA) [36-38], vasostatin-II (human CHGA 1-113) [38,39], vasoconstriction inhibiting factor (VIF) [40], and catestatin (human CHGA 352-372) [36,41], all of which are essential for the genesis and/or progression of CV disease. For example, in hypertension, the plasma concentration of chromogranin A is increased, while the catestatin plasma concentration is decreased [42]. CBF is thus the first identified, very potent calcification inhibitor secreted by adrenal glands.

The incubation of elongated CBF peptides with calpain 1 and kallikrein, enzymes from the adrenal glands, resulted in enzymatic cleavage between the amino acids arginine and leucine at the N-terminus and the amino acids phenylalanine and arginine at the C-terminus of CBF, demonstrating that CBF can be cleaved from chromogranin A. The enzymatic capacity of these enzymes was validated by incubation of chromogranin A with both enzymes, resulting in the cleavage of CBF. Deregulation of cleavage of CBF might be a mechanism by which CBF plasma levels might be altered, which would ultimately affect the inhibitory function of CBF on vascular calcification. For instance, the CBF-cleavage enzymes could be inhibited by uremic toxins [43] and post-translational modifications [44] in patients with CKD which would reduce the plasma concentration of CBF

in these patients, which offers new prevention and/or treatment options of vascular calcification, since we show that CBF affects calcification processes *in vitro* and *ex vivo*.

The *in vivo* relevance of CBF in vascular calcification was demonstrated using a preclinical animal model of elastocalcinosis caused by deposition of hydroxyapatite on the elastic lamellae of the vessel. Animals in the vehicle-treated group showed more vascular calcification than animals continually treated with CBF via mini-pump, clearly demonstrating the impact of *in vivo* administration of CBF. The effect of CBF was shown by quantification of the vascular calcium content. Interestingly, CBF not only reduced the vascular calcium content but also reduced pulse pressure *in vivo*, an established consequence of arterial stiffness in calcified vessels [45].

For the clinical application of CBF, knowledge of its active sequence is essential. This knowledge might provide the basis to explore new pharmaceutical approaches to prevent and treat vascular calcification in CKD patients and, more widely, vascular calcification in the ageing population, which is known to be caused by the disease. After using a systematic approach to generate CBF fragments of different lengths to optimize the fragment length, we demonstrated that the amino acids between and including the second and ninth amino acids of human CBF (the amino acid sequence EGQEEEED) are mainly responsible for the inhibitory effect of CBF on vascular calcification *in vitro* as well as *ex vivo.* This active sequence of CBF contains one each of the amino acids glutamine, glycine and aspartic acid and five glutamic acid residues. The presence of a carboxylic acid next to a primary amide or two carboxylic acids might be relevant for the inhibitory effect of CBF and/or its fragments. Direct binding of calcium to the amino acid motif might not be the primary effect of CBF, as the calcium concentration clearly exceeded the CBF concentration in the medium. As such, direct binding would not significantly affect the calcium concentration. The identification of active sequence of CBF se findings is highly relevant to the development of peptide mimetics for future therapeutic approaches.

To evaluate the relevance of CBF in humans, the plasma levels of CBF in healthy control subjects and ESRD patients suffering from increased vascular calcification were quantified. Since no appropriate antibody for CBF is available, a mass spectrometry-based approach was used for quantification. In healthy control subjects, plasma concentrations of CBF were in the range at which the peptide exhibited significant inhibitory effects on vascular calcification in our in vitro and ex vivo experiments. While the plasma concentration of CBF is higher than the EC_{50} value of CBF determined by the dose-response curve, the tissue levels of CBF are likely lower than the plasma level owing to several factors, such as the endothelial barrier to CBF, enzymatic degradation, binding, or other effects. The lower serum concentration of CBF observed in patients with ESRD compared to healthy control subjects suggests that the CBF deficit in these patients might account, at least in part, for the increased vascular calcification commonly observed in these patients.

The transdifferentiation of VSMCs to cells with an osteoblast-like phenotype is essential for the pathophysiology of vascular calcification. CBF blocked VSMC transdifferentiation, as demonstrated by the increased expression of the VSMC differentiation marker α-SMA *in vivo* and *ex vivo*. In addition, knockdown in hAoSMCs of the type III NaPi cotransporter PIT-1, involved in phosphate-induced VSMC transdifferentiation [20], led to a reduction in the inhibitory effect of CBF on vascular calcification in these cells. These results demonstrate the essential function of PIT-1 in the inhibition of vascular calcification by CBF.

The BMP2/p-SMAD pathway is involved in VSMC transdifferentiation to cells with an osteoblastic phenotype, which is associated with enhanced vascular calcification [46]. The expression of BMP2 was decreased *in vitro* as well as *in vivo* by chronic administration of CBF. In addition, CBF treatment counteracted increases in the levels of p-SMAD1 and p-SMAD5 in aortic rings in culture as well as in aortas from VDN rats. Phosphorylation of SMAD1 and SMAD5 leads to their association with co-SMAD4. This complex translocates to the nucleus, where it regulates the expression of

osteogenic transdifferentiation genes, including RUNX2, MSX2 and OSX [46]. The presence of CBF limited the effect of the CM in upregulating RUNX2, MSX2, and OSX mRNA expression *in vitro* as well as *in vivo*. Overall, these results show that CBF decreases arterial calcification through inhibition of the PIT-1/BMP2/p-SMAD signal transduction pathway (**Figure 9**).

In addition to identifying a mediator of the complex pathobiology of vascular calcification, these findings have two potential clinical implications. First, we have identified a new mediator that regulates osteoblastic transdifferentiation and describe a new role for the adrenal medulla in the regulation of sympathetic nerve activity and arterial calcification. Second, the decreased plasma CBF concentration in patients suffering from increased vascular calcification may shed further light on the mechanisms of arterial calcification in renal failure. Modification of CBF release from adrenal chromaffin granules by sympathetic nerve stimulation might be an additional attractive approach to prevent or treat arterial calcification in these patients.

In conclusion, this study identifies a mediator of the differentiation of VSMCs and thus vascular calcification. This adrenal gland-derived compound may enhance our understanding of pathophysiological conditions related to calcification, such as calcific arteriopathy. Since CBF is secreted by adrenal chromaffin granules and present at effective concentrations in human plasma, CBF is likely to participate in the regulation of vascular calcification and thus in reducing its harmful consequences. **By the identification and characterisation of the CBF peptide a hitherto unknown function of the adrenal glands in vascular calcification processes was detected. Due to the (patho-)physiological effect of CBF and its active amino acids it is also the basis for the development of a new drug for the prevention and therapy of vascular calcification in CKD patients and beyond that also for other patient groups in the future.**

METHODS

MECHANICAL DISINTEGRATION AND EXTRACTION

Fresh bovine adrenal glands were obtained from a slaughterhouse. The glands were then mechanically pulverized and homogenized.

PREPARATIVE REVERSED-PHASE CHROMATOGRAPHY

The supernatant of the homogenate was loaded onto a reversed-phase chromatography column. A 0.1% trifluoroacetic acid (TFA) solution in water was used as eluent A, and 80% acetonitrile in water was used as eluent B. A stepwise gradient (20, 40, 60, 80, and 100% eluent B) was used to elute the substances retained on the chromatographic.

EFFECT OF CHROMATOGRAPHIC FRACTIONS ON *EX VIVO* AORTIC VASCULAR CALCIFICATION

Aortas from adult male Wistar rats were divided into segments. The aortic rings were incubated with NCM and CM in the presence of adrenal gland chromatographic fractions for 7 days.

ANION-EXCHANGE CHROMATOGRAPHY

The eluate was further fractionated by preparative anion-exchange chromatography. K_2HPO_4 in water was used as eluent A; NaCl in K_2HPO_4 was used as eluent B. The retained substances were eluted from the chromatographic column using a stepwise gradient (20, 40, 60, 80, and 100% eluent B).

REVERSED-PHASE CHROMATOGRAPHY

The eluate from the anion-exchange column was loaded onto a reversed-phase chromatographic column. A 0.1% TFA solution in water (1:1000, v/v; eluent A) was used for equilibration and 80% acetonitrile in water (80:20, v/v; eluent B), and a stepwise gradient of 20, 40, 60, 80, and 100% eluent B was used for elution. Thereafter, the lyophilized fractions were resuspended and fractionated by a reversed-phase chromatographic column. A linear gradient (1% to 100% ACN over

90 min) was used for fractionation. Each fraction from the chromatographic procedure was again screened for its inhibitory effects on calcification by using aortic culture and a calcification assay.

MATRIX-ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY

The lyophilized fractions from reversed-phase chromatography were analysed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and MALDI-TOF/TOF fragment ion analysis. Mass spectrometric measurements were performed on a Bruker Ultraflex-III TOF/TOF instrument. Peptide identification using the obtained fragment ion mass data was performed using the Mascot search engine and the RapiDeNovo 3.0.1 sequencing tool.

ELECTROSPRAY IONIZATION MASS SPECTROMETRY (ESI-MS)

MS/MS fragmentation analysis was also performed by liquid chromatography/electrospray ionization-mass spectrometry (ESI-MS). Two capillary HPLC pumps with a micro-vacuum degasser, microwell-plate autosampler, and diode array multi-wavelength detector were used for chromatography. The mass spectrometric data were collected using the software HyStar 3.2 and analysed using the software Data Analysis 4.0. Glutathione was used as an internal standard to calculate the recovery rate.

PEPTIDE SYNTHESIS

CBF was synthesized automatically by the solid-phase method. Purification of the crude peptide was carried out by preparative HPLC on a PolyEncap A300 column in water with increasing concentrations of ACN as the mobile phase. An eluent gradient of 5-70 (v/v-%) ACN/water (0.1% TFA) was used. The peptide was characterized by MALDI-MS on a Voyager-DE STR BioSpectrometry Workstation MALDI-TOF mass spectrometer.

ISOLATION OF ADRENAL CHROMAFFIN GRANULES AND STIMULATION WITH CARBACHOL

Fresh bovine adrenal glands were obtained from a slaughterhouse. The chromaffin granules were isolated using 0.32 mol L^{-1} sucrose and homogenized with an Ultra-Turrax homogenizer. After several centrifugation steps, the pellet was resuspended in water. To actively release CBF from the chromaffin granules, carbachol was added. PBS was used as a negative control. The supernatants were analysed by ESI-MS.

IDENTIFICATION OF THE ENZYMES THAT CLEAVE CBF FROM CHROMOGRANIN A

The protease prediction tool Proteasix [47] was used to predict the enzymes likely responsible for cleaving CBF from chromogranin A. Enzyme candidates (kallikrein and calpain 1) were identified by entering Protein ID P10645 as the input, with amino acid 358 as the N-terminal amino acid and amino acid 376 as the C-terminal amino acid of CBF. A peptide containing ten additional amino acids from chromogranin A at the N-terminus of CBF (LAKELTAEKR-LEGQEEEEDNRDSSMKLSF) and a peptide containing ten additional amino acids from chromogranin A at the C-terminus (LEGQEEEEDNRDSSMKLSF-RARAYGFRGP) were synthesized and used to identify the enzymes that cleave the N-terminal and C-terminal ends of CBF, respectively, from chromogranin A.

CELL CULTURE AND CALCIFICATION INDUCTION IN HUMAN AORTIC SMOOTH MUSCLE CELLS

hAoSMCs were cultivated in smooth muscle cell medium. Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol L^{-1} glucose supplemented with 2.5% foetal calf serum and 2.8 mmol L^{-1} phosphate was used as CM to induce calcification. The CM was supplemented with CBF or individual CBF fragments. DMEM containing 25 mmol L^{-1} glucose supplemented with 2.5% foetal calf serum and 0.9 mmol L^{-1} phosphate was used as a non-calcifying reference medium. hAoSMCs were incubated for 7 days.

KINASE ACTIVITY PROFILING

Kinase activity profiling using PamChip® Ser/Thr Kinase assay (STK; PamGene International, ´s-Hertogenbosch, The Netherlands) was previously described [Hilhorst, 2013 #3488]. Each STK-PamChip® array contains 144 individual phospho-site(s) that are peptide sequences derived from substrates for Ser/Thr kinases. hAoSMCs were washed once in ice-cold PBS after respective treatments, with three biological replicates per condition, and lysed for 15 min on ice using M-PER Mammalian Extraction Buffer containing Halt Phosphatase Inhibitor and EDTA-free Halt Protease Inhibitor Cocktail (1:100 each; Thermo Fischer Scientific). Lysates were centrifuged for 15 min at 16.000 x g at a temperature of +4°C in a pre-cooled centrifuge. Protein quantification was performed with Pierce™ Coomassie Plus (Bradford) Assay according to the manufacturer's instructions.

For the STK assay, 0.5 µg of protein and 400 µM ATP were applied per array (N=3 per condition) together with an antibody mix to detect the phosphorylated Ser/Thr. After incubation for an hour at a temperature of 30^{o}C where the sample is pumped back and forth through the porous material to maximize binding kinetics and minimize assay time, a second FITC-conjugated antibody was used to detect the phosphorylation signal. Imaging was done using a LED imaging system and the spot intensity at each time point was quantified using the BioNavigator software version 6.3 (PamGene International, 's-Hertogenbosch, The Netherlands). Upstream Kinase Analysis (UKA) [Chirumamilla, 2019 #3490] as the functional scoring method was used to rank kinases based on combined specificity scores based on peptides linked to a kinase derived from 6 databases and sensitivity scores based on treatment-control differences.

EX VIVO CALCIFICATION OF RAT AORTIC RINGS

The thoracic aortae of Wistar rats were divided into segments. The endothelial layer of each aortic ring was manually damaged. The aortic rings were incubated with NCM or CM supplemented with CBF or individual CBF fragments for 7 days.

TRANSFECTION OF HUMAN AORTIC SMOOTH MUSCLE CELLS

For siRNA transfection, hAoSMCs were detached and resuspended in growth medium. Transfection was performed using the Neon Transfection System. PIT-1 siRNA with the sequence 5´-GCCGTAGTTTACAGTATTTAA-3´ was used to knock down PIT-1, and siRNA specific for GFP from Qiagen served as a control. Twenty-four hours after transfection, the growth medium was replaced with CM supplemented with CBF. NCM was used as a reference. The cells were incubated for 7 days.

EFFECT OF CBF IN AN ANIMAL MODEL WITH ENHANCED ARTERIAL CALCIFICATION

An animal model of elastocalcinosis (VDN rats) in which increased arterial calcification has been demonstrated [48] was chosen to assay the effects of CBF *in vivo*. Three groups of 6-week-old Wistar rats were given regular rat chow and spring water *ad libitum* for one week before the experiments. Rats in the two groups received CBF or vehicle infused through an osmotic pump. CBF treatment was initiated 3 or 4 days before the induction of administration of vitamin D3 and nicotine. Untreated rats served as control rats. Four weeks later, the blood pressure and systolic, diastolic, mean arterial, and pulse pressures were determined. Each obtained aorta was cut into segments for further analysis.

HISTOLOGICAL CALCIFICATION STAINING AND IMMUNOFLUORESCENCE STAINING

Formaldehyde-fixed aortic rings were used for histological and immunohistochemical staining. The rings were dehydrated and embedded in paraffin, and 5-µm slices were prepared by a microtome. The slices were deparaffinized and dehydrated before staining. Von Kossa staining was performed to visualize calcified areas of the aortic rings.

For immunohistochemical analyses, sections were blocked and incubated with primary antibodies at 4°C overnight and with secondary antibodies at room temperature for 30 min to 1 h. Samples

were visualized with a LEICA DM5500B microscope. Staining or protein expression was determined as the stained area using ImageJ software, and the corresponding results are expressed as a percentage of the total aortic area per section.

MEASUREMENT OF THE CALCIUM CONTENT

To measure the calcium content, cells or aortic rings were washed with PBS. Aortic rings were dried and weighed. Dried aortic rings and cells were decalcified, and the calcium content in the supernatant after centrifugation was determined with the o-cresolphthalein complexone method according to the manufacturer's protocol. The protein content of the cells was measured by the bicinchoninic acid (BCA) protein assay method. The calcium content was normalized to the total protein content of the cells or dry weight of the aortic rings.

REVERSE TRANSCRIPTION QUANTITATIVE POLYMERASE CHAIN REACTION (RT-qPCR) ANALYSES OF mRNA EXPRESSION IN HUMAN AORTIC SMOOTH MUSCLE CELLS AND VDN RATS

Total RNA was extracted from hAoSMCs and aortic tissue using an RNeasy Mini Kit. Gene expression levels were quantified by real-time PCR using SYBR Green I dye chemistry on a LightCycler 480 system. PCR primers (**Table 1**) were designed using LightCycler Probe Design software 2.0. Expression levels relative to standard curves were determined with LightCycler analysis software (version 3.5).

CLINICAL DETERMINATION OF PLASMA CBF LEVELS IN END-STAGE RENAL DISEASE AND IN HEALTHY CONTROLS

The clinical study included 17 patients who suffered from stage 5 CKD (ESRD) on regular dialysis. Another 13 control individuals had no CKD (controls), with estimated glomerular filtration rates ≥60 mL min^{-1} 1.73^{-1} m^{-2} according to the Modification of Diet in Renal Disease (MDRD) Study formula. Creatinine and haemoglobin were measured using standard autoanalyser techniques.

Intact parathyroid hormone was determined by immunoassay. Plasma samples were immediately separated from blood cells and frozen until all samples were collected, and data were measured side by side within one analytical setup.

STATISTICAL ANALYSES

See Supplementary Methods.

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CONFLICT-OF-INTEREST DISCLOSURE

None

Table 1: The primer sequences for RT-PCR reaction

Target	Species	Sequence (5´-3´)
PIT-for	human	AGCGTGGACTTGAAAGAGGA
PIT-rev	human	TACAGGCCGGAATCCTTATG
BMP2-for	human	GAGGTCCTGAGCGAGTTCGA
BMP2-rev	human	ACCTGAGTGCCTGCGATACA
BMP2-for	rat	ACAGCGGAAGCGTCTTA
BMP2-rev	rat	CACAACCATGTCCTGATAGTTT
RUNX2-for	human	CGCCATGACAGTAACCACAG
RUNX2-rev	human	ACCATGGTGGAGATCATCG
RUNX2-for	rat	CGCATTCCTCATCCCAGTAT
RUNX2-rev	rat	TCTGTAATCTGACTCTGTCCTTGTG
OSX-for	human	GCCAGAAGCTGTGAAACCTC
OSX-rev	human	GCTGCAAGCTCTCCATAACC
OSX-for	rat	CCTACTTACCCGTCTGACTTT
OSX-rev	rat	GCCCACTATTGCCAACTG
MSX2-for	human	AAGAAAACAGGGCTTGGTGCCTC
MSX2-rev	human	GCGCAAGTTCCGTCAGAAACAG
MSX2-for	rat	GGAGGCGGAACTGGAAA
MSX2-rev	rat	ATAGAGTCCCACAGGCG
β-Actin-for	human	CACCAACTGGGACGACAT
β-Actin-rev	human	ACAGCCTGGATAGCAACG
RPLPO-for	rat	CACTGGCTGAAAAGGTCAAGG
RPLPO-rev	rat	GACTTGGTGTGAGGGGCTTA

Table 2: Clinical and biochemical characteristics of ESRD patients and control subjects (values are mean ± SEM. *P<0.05, ***P≤0.001 and ****P≤0.0001 compared with Healthy control subjects based on unpaired t tests)

Parameter	Healthy control subjects (N = 13)	ESRD patients (N = 17)	Significance
age (years)	58.7 ± 3.1	52.8 ± 3.4	n.s.
blood pressure (mmHg)			
systolic	125.9 ± 5.6	125.0 ± 5.9	n.s.
diastolic	70.8 ± 2.0	71.8 ± 3.9	n.s.
weight (kg)	82.6 ± 4.9	77.0 ± 3.6	n.s.
BMI ($kg\ m^{-2}$)	27.9 ± 1.2	26.0 ± 124	n.s.
serum creatinine ($mg\ dl^{-1}$)	1.04 ± 0.03	6.60 ± 0.49	***P≤0.001
eGFR (MDRD ($ml\ min^{-1}\ 1.73^{-1}\ m^{-2}$)	69.7 ± 2.7	9.5 ± 0.6	***P≤0.001
haemoglobin ($g\ L^{-1}$)	219.2 ± 36.8	193.6 ± 8.0	*P<0.05
PTH ($pg\ ml^{-1}$)	39.7 ± 3.7	231.3 ± 37.4	****P≤0.0001
serum calcium ($mg\ dl^{-1}$)	2.2 ± 0.5	2.3 ± 0.7	n.s.
serum phosphate ($mg\ dl^{-1}$)	3.3 ± 0.6	5.4 ± 0.8	***P≤0.001

FIGURE LEGENDS

Figure 1: Identification and characterisation of *Calcium Blocking Factor* (CBF)

(a) Outline of experimental workflow for the identification and characterization of CBF, isolated from bovine adrenal glands.

(b) Reversed-phase chromatogram of the adrenal extract. The pooled fractions were tested in a calcification assay for inhibitory effects on the calcification of aortic rings (AU: arbitrary units).

(c) The fraction shown in **Figure 1b** was analyzed on MALDI-TOF/TOF mass-spectrometry to identify the sequence of the inhibitory factor of calcification. The mass-signal (m/z) at 2,297 Da ($M+H^+$) corresponds to the peptide sequence of LEGEEEEEEDPDRSMRLSF which was named “calcification blocking factor” (CBF).

(d-e) Sequence matching: Peptide sequence of CBF represented in black characters matches the 300-318 amino acid position of (d) bovine and (e) human chromogranin A protein represented in grey characters.

Figure 2: CBF inhibits calcification in aortic rings and vascular smooth muscle cells

(a) Dose-response effect of CBF on Ca^{2+} content of cultivated human aortic smooth muscle cells. Data shown as mean ± SEM. ***P≤0.001, ****P≤0.0001 compared to calcifying condition in the absence of CBF based on one-way ANOVA. Bonferroni’s multiple comparisons were used as a post-test (N=10 per group).

(b) Dose-response effect of CBF on Ca^{2+} content of isolated aortic rings. Data shown as mean ± SEM. *P<0.05 compared to calcifying condition in the absence of CBF based on one-way ANOVA. Bonferroni’s multiple comparisons were used as a post-test (N=3-9 per group).

(c) Quantification of von Kossa stained aortic rings (original magnification x 40, Scale bar 1000 µm), incubated in non-calcifying conditions (non-calcifying medium (NCM)) and under calcifying conditions (calcifying medium (CM)) in the absence or presence of CBF (100nM), respectively. CBF significantly decreased the calcified areas of aortic rings by 40%. Bars represent the mean ± SEM of the percent stained area. ***P≤0.001, ****P≤0.0001 compared to calcifying condition in the absence of CBF based on one-way ANOVA. Bonferroni's multiple comparisons were used as a post-test (N=9 per group).

Figure 3: CBF inhibits calcification and influences pulse pressure *in vivo*

(a) Calcium content was measured in aorta of untreated control Wistar rats and chronically treated or untreated VDN rats with CBF (31µg kg^{-1} per day for 4 weeks). Data shown as mean ± SEM. *P<0.05, ***P≤0.0001 compared with VDN group based on one-way ANOVA. Bonferroni's multiple comparisons were used as a post-test (N=6 per group).

(b) Quantification of von Kossa stained aortic rings (original magnification x 40, Scale bar 1000 µm) of rats from each experimental group are shown. Bars represent the mean ± SEM of the percent stained area. **P≤0.001, ****P≤0.00001 compared with VDN group based on one-way ANOVA. Bonferroni's multiple comparisons were used as a post-test (N=10 per group).

(c) CBF prominently prevented the VDN-induced rise in pulse pressure. Representative graphs of carotid arterial pressure recorded in a rat from each experimental group are shown. The graph represents the mean ± SEM of pulse pressure values. Pulse pressure was increased 2-fold in VDN rats. CBF counteracted the changes in both systolic and diastolic pressure associated with VDN and reduced the pulse pressure by 63%. ***P≤0.0001; ****P≤0.00001 compared with VDN group based on one-way ANOVA. Bonferroni's multiple comparisons were used as a post-test (N=10 per group).

Figure 4: Identification of relevant amino acid sequence of CBF causing the calcification inhibitory effect

(a) Overview of amino acid sequence of CBF fragments used for identification of active site of CBF causing the calcification inhibitory effect. *Black*: Amino acid sequence of CBF. *Beige*: CBF fragments with an amino acid sequence of 9 to 10 amino acids. *Dark grey* CBF fragments with an amino acid sequence of five amino acids. *Light grey*: CBF fragments with an amino acid sequence of four amino acids.

(b) Quantification of Ca^{2+} content of cultivated human aortic smooth muscle cells incubated in non-calcifying conditions (non-calcifying medium (NCM)) and under calcifying conditions (calcifying medium (CM)) in the absence or presence of CBF or CBF fragments as indicated in **Figure 4a** (100 nmol L^{-1}). Data shown as mean ± SEM. **P≤0.001 compared with CM group based on one-way ANOVA. Bonferroni's multiple comparisons were used as a post-test (N=6-10 per group).

(c) Quantification of Ca^{2+} content of cultivated rat aortic rings incubated in non-calcifying conditions (non-calcifying medium (NCM)) and under calcifying conditions (calcifying medium (CM)) in the absence or presence of CBF or CBF fragments as indicated in **Figure 4a** (100 nmol L^{-1}). Data shown as mean ± SEM. **P≤0.001 compared with CM group based on one-way ANOVA. Bonferroni's multiple comparisons were used as a post-test (N=6-9 per group).

Figure 5: Identification of enzymes cleaving CBF from Chromogranin A

(a) LC-qTOF mass-spectrometric analyses of LAKELTAEKR-LEGQEEEEDNRDSSM KLSF incubated in the absence (upper panel) and presence (lower panel) of kallikrein. The molecular mass (m/z) of 748.66 $(M+3H)^{3+}$ of the average spectrum at retention time range 18.8-19.7 corresponds to CBF.

(b) LC-qTOF mass-spectrometric analyses of LEGQEEEEDNRDSSMKLSF-RARAYGFRGP incubated in the absence (upper panel) and presence (lower panel) of calpain 1. The molecular mass (m/z) of 748.66 $(M+3H)^{3+}$ of the average spectrum at retention time range 18.8-19.7 corresponds to CBF.

(c) LC-qTOF mass-spectrometric analyses of chromogranin A incubated in the absence (upper panel) and presence (lower panel) with both kallikrein and calpain 1. The molecular mass (m/z) of 748.66 $(M+3H)^{3+}$ of the average spectrum of retention time range 18.8-19.7 corresponds to CBF.

(d) Quantification of CBF in the supernatant of granules from bovine adrenal glands unstimulated and stimulated with carbachol (0.1 mmol L^{-1}). The release of CBF from granula of adrenal glands into the culture media was doubled after stimulation. Data shown as mean ± SEM. *P<0.05 compared with group without carbachol treatment based on unpaired t tests (N=5 in each group).

(e) Concentration of CBF in plasma of healthy control (N=13) and CKD-5 patients (N=17) were reported in nmol L^{-1}. CBF concentrations were decreased in patients suffering from CKD-5. ** indicates P≤0.001compared to healthy based on unpaired t tests.

Figure 6: CBF inhibits smooth muscle cell transdifferentiation

(a) Quantification of α-smooth muscle actin protein (α-SMA) expression in aortic rings by immunohistochemical detection. Aortic rings analysed after 7 days of culture in non-calcifying medium (NCM) (white bar), and in calcifying media in the absence (grey bar) and presence (back bar) of CBF (100 nmol L^{-1}); representative sections are given (original magnification x 200). The effect of CBF on stained area demonstrates the inhibitory *ex vivo* effect of CBF on differentiation of smooth muscle cells to an osteoblastic phenotype. Data shown as mean ± SEM. *P<0.05 compared with CM group based on

one-way ANOVA. Bonferroni's multiple comparisons were used as a post-test (N=12 per group).

(b) Quantification of α-smooth muscle actin protein (α-SMA) expression in aorta isolated from the control rats (white bar), VDN rats (grey bar) and VDN rats treated with CBF (black bars) by immunohistochemical detection; representative images are shown (original magnification x 200). The reduced α-SMA expression in VDN rats compared to control rats was limited in rats treated with CBF demonstrating inhibitory *in vivo* effect of CBF on differentiation of smooth muscle cells to osteoblast. Data shown as mean ± SEM. *P<0.05 compared with CM group based on one-way ANOVA. Bonferroni's multiple comparisons were used as a post-test (N=7 per group).

(c) Relative quantification of phosphate transporter 1 (PIT-1) gene expression by qRT-PCR analyses. The increased gene expression in human aortic smooth muscle cells cultured for 7 days in calcifying medium ((CM); grey bar) vs. non-calcifying medium ((NCM); white bar) was not affected by CBF (100 nmol L^{-1}) (black bar). Data shown as mean ± SEM. *P<0.05 compared with CM based on one-way ANOVA. Bonferroni's multiple comparisons were used as a post-test (N=3 per group).

(d) Quantification of Ca^{2+} content in transfected human aortic smooth muscle cells with 120 µM control siRNA or 120 µM PIT-1 siRNA after incubation with non-calcifying conditions (non-calcifying medium (NCM)) and under calcifying conditions (calcifying medium (CM)) in the absence or presence of CBF (100 nmol L^{-1}). Data shown as mean ± SEM. ****P≤0.0001 based on two-way ANOVA. Bonferroni's multiple comparisons were used as a post-test (N=3 per group).

Figure 7: CBF inhibits the BMP2 pathway

(a) Relative quantification of bone morphogenetic protein 2 (BMP2) gene expression *in vitro* by qRT-PCR analyses. The increased gene expression in human aortic smooth muscle

cells cultured for 7 days in calcifying medium ((CM); grey bar) vs. non-calcifying medium ((NCM); white bar) was diminished in the presence of CBF (100 nmol L^{-1}) (black bar). Data shown as mean ± SEM. *P<0.05; **P≤0.01 compared with CM based on one-way ANOVA. Bonferroni's multiple comparisons were used as a post-test (N=6 per group).

(b) Relative quantification of bone morphogenetic protein 2 (BMP2) gene expression *in vivo* by qRT-PCR analyses. The increased gene expression in aorta of VDN rats (grey bar) vs. control rats (white bar) was limited in rats treated with CBF (31µg kg^{-1} per day for 4 weeks) (black bar). Data shown as mean ± SEM. *P<0.05; **P≤0.01 compared with VDN group based on one-way ANOVA Bonferroni's multiple comparisons were used as a post-test (N=5 per group).

(c-d) Relative quantification of (C) phospho-SMAD1 and (D) phospho-SMAD5, respectively, protein expression *ex vivo* by immunohistochemical analyses. Phospho-SMAD1 and phospho-SMAD5 proteins are downstream components of BMP2 pathway. The increased protein expression of cultivated rat aortic rings incubated in calcifying conditions ((CM); grey bar) vs. non-calcifying conditions (non-calcifying medium (NCM; white bar) was diminished in the presence of CBF (100 nmol L^{-1}) (black bar). Representative images are shown (original magnification x 200, Scale bar 200 µm). Data shown as mean ± SEM. *P<0.05; **P≤0.01 compared with CM on one-way ANOVA. Bonferroni's multiple comparisons were used as a post-test (N=6-9 in each group).

(e-f) Relative quantification of (E) phospho-SMAD1 and (F) phospho-SMAD5, respectively, protein expression *in vivo* by immunohistochemistry. The increased phospho-SMAD1 protein expression of rat aortic rings from VDN rate (grey bar) was diminished by the treatment with of CBF (31µg kg^{-1} per day for 4 weeks) (black bar), suggesting inhibitory effects of CBF on Smad1 activation on vascular calcification *in vivo*. Representative images are shown (original magnification x 200, Scale bar 200 µm). Data shown as mean ± SEM. *P<0.05; **P≤0.01 compared with VDN group based on one-way ANOVA. Bonferroni's multiple comparisons were used as a post-test (N=7 in each group).

Figure 8: CBF reduces the expression of genes involved in osteoblast differentiation

(a-b) Relative quantification of RUNX2, OSX and MSX2 in (A) *in vitro* and (B) *in vivo*, respectively, gene expression by qRT-PCR analyses. The increased expression of runt-related transcription factor 2 (RUNX2), osterix (OSX) and msh homeobox 2 (MSX2) genes of hAoSMCs incubated in calcifying conditions ((CM); grey bar) vs. non-calcifying conditions (non-calcifying medium ((NCM); white bar) was diminished in presence of CBF (100 nM) (black bars). Data shown as mean ± SEM. **P≤0.01, ****P≤0.0001 compared with CM based on one-way ANOVA. Bonferroni's multiple comparisons were used as a post-test (N=4-6 in each group).

(c) Based on kinase activity profiling, kinase ranking scores (based on their significance and specificity in terms of the set of peptides used for the corresponding kinase) are shown for BMP-2 related kinases (N=3 per condition)

Figure 9: Effect of CBF on vascular calcification processes is mediated by the PIT-1/BMP2/p-SMAD pathway.

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SUPPLEMENTARY METHODS / ONLINE METHODS

CHEMICALS

HPLC water (gradient grade), acetonitrile (ACN), magnesium chloride-hexahydrate and disodium hydrogen phosphate (Na_2HPO_4) were purchased from Merck (Darmstadt, Germany). Sodium dihydrogen phosphate (NaH_2PO_4) and sodium hydroxide (NaOH) were purchased from Roth (Karlsruhe, Germany). All other substances were obtained from Sigma-Aldrich (Munich, Germany).

MECHANICAL DISINTEGRATION AND EXTRACTION

Fresh bovine adrenal glands were obtained on ice from a local slaughterhouse, and the lipid matter was removed. Adrenal glands were then cut into pieces of about 1 g snap-frozen in liquid nitrogen for 30 min and stored at -80°C for 12 h followed by lyophilisation. The freeze-dried adrenal glands were then mechanically pulverized at 4°C. Seventy ml of 0.6 mmol L^{-1} perchloric acid (T=4°C) was added to 700 g of pulverized adrenal glands. The resulting mixture was homogenised with an Ultra-Turrax (9.500 g min^{-1}) for 1 min at 4°C. This procedure was repeated 10 times. The homogenate was centrifuged at 2000 g for 15 min at 4°C. The supernatant's pH was adjusted to 9.0 - 11.0 by adding an aqueous saturated KOH solution. The supernatant was stored at -20°C for at least 12 h to precipitate $KClO_4$.

PREPARATIVE REVERSED-PHASE CHROMATOGRAPHY

The supernatant of the homogenate was adjusted to pH 6.5 by 0.1 mol L^{-1} HCl, and the samples were loaded onto a reversed-phase chromatography column (LiChroprep RP C 18, 310 x 25 mm, Merck, Darmstadt, Germany). The chromatographic step was performed on a BioRad BioLogic DuoFlow HPLC device with UV-detection at 280 nm; 0.1% trifluoroacetic acid (TFA) in water was used as eluent A and 80% acetonitrile in water was used as eluent B with a flow rate of 3 ml min^{-1}.

A stepwise gradient (20, 40, 60, 80, and 100% eluent B) was used to elute the retained substances from the chromatographic column. The fractions were lyophilized and then resuspended in 20 mmol L^{-1} K_2HPO_4 in water.

EFFECT OF CHROMATOGRAPHIC FRACTIONS ON *EX VIVO* AORTIC VASCULAR CALCIFICATION

Aortas from adult male Wistar rats were gently dissected and divided into segments (length: 3-4 mm). The aortic rings were incubated with calcifying medium (Dulbecco's modified Eagle's medium containing 2.8 mmol L^{-1} phosphate) in the presence of chromatographic fractions of the adrenal glands. The aortic rings were cultured for 7 days at 37°C in a humidified atmosphere containing 5% CO_2 and media was changed every two days. Calcium content was determined as described below.

ANION-EXCHANGE CHROMATOGRAPHY

The lyophilized fractions were resuspended in 20 mmol L^{-1} K_2HPO_4 in water, and the pH was adjusted to 8 with 0.1 mol L^{-1} NaOH or 0.1 mol L^{-1} HCl. The eluate was further fractionated by preparative anion exchange chromatography (column: Superformance™ 16, 150 ml, 30 cm x 1.6 cm, gel: Fractogel EMP TMAE™; both Merck, Darmstadt, Germany). Twenty mmol L^{-1} K_2HPO_4 in water was used as eluent A; 1 mol L^{-1} NaCl in K_2HPO_4 (20 mmol L^{-1} in water) was used as eluent B with a flow rate of 1 ml min^{-1}. The retained substances were eluted from the chromatographic column using a stepwise gradient (20, 40, 60, 80, and 100% eluent B), with detection at 280 nm.

REVERSED-PHASE CHROMATOGRAPHY

The eluate from the anion exchange column was adjusted to pH 6 with 0.1 mol L^{-1} NaOH or by 0.1 mol L^{-1} HCl. One hundred µl of 1 mol L^{-1} trifluoroacetic acid (TFA) was added to each fraction of the anion exchange eluate. Thereafter, the samples were loaded onto a reversed-phase chromatographic column (LiChroprep RP C 18, 310 x 25 mm, Merck, Darmstadt, Germany); 0.1%

TFA in water (1:1000, v/v; eluent A) was used for equilibration (flow rate: 3 ml min^{-1}), and 80% acetonitrile in water (80:20, v/v; eluent B) and a stepwise gradient using 20, 40, 60, 80, and 100% eluent B was used for elution (flow rate: 3 ml min^{-1}). The eluate was lyophilized and stored at -20°C until further fractionation. Thereafter, the lyophilized fractions were resuspended in 15 ml H_2O and were fractionated by a reversed-phase chromatographic column (LiChroprep RP C 18e, 100 x 4.6 mm, Merck, Darmstadt, Germany). Once again, 0.1% trifluoroacetic acid (TFA) in water (1:1000, v/v) was used as eluent A and 80% acetonitrile in water (80:20, v/v) as eluent B; a linear gradient (1% to 100% ACN in 90 min; flow rate: 1 ml min^{-1}) was used for fractionation.

The chromatographic purification was performed on a BioLogic DuoFlow HPLC-device (Biorad, Munich, Germany) with a UV-detector at 280 nm. The eluate fractions were lyophilized and were resuspended in 10 µl H_2O. Each fraction of the chromatographic procedure was again screened for inhibitory effects on calcification by using aortic culture and a calcification assay.

MATRIX-ASSISTED LASER DESORPTION/IONIZATION MASS-SPECTROMETRY

The lyophilized fractions from the reversed-phase chromatography were analyzed by matrix-assisted laser desorption/ionization mass-spectrometry (MALDI-MS) and MALDI-TOF/TOF fragment ion analysis. The lyophilized fractions were resuspended in 10 µL H_2O. One µL of each fraction was prepared on a prestructured MALDI sample support (MTP AnchorChip™ 400/384, Bruker Daltonics, Bremen, Germany) using 2,5-dihydroxybenzoic acid (DHB) affinity sample preparation [51]. Mass-spectrometric measurements were performed on a Bruker Ultraflex-III TOF/TOF instrument (Bruker-Daltonics, Bremen, Germany). The instrument was equipped with a Smart beam™ laser operating with a repetition-rate of 100-200 Hz. On average, the presented spectra represent the sum of 200 single-shot spectra for MS mode, and 600 for MS/MS mode. Mass-spectra of positively charged ions were analyzed in the reflector mode using delayed ion extraction. Fragment ion spectra were recorded using the LIFT option of the instrument. The calibration constants were determined using standard peptides prepared on positions adjacent to the sample, resulting in an error of <100 ppm for the recorded mass-spectra. Peptide identification using the obtained fragment ion mass data was performed using the Mascot search engine (Matrix

Science, London, UK) as well as the RapideNovo 3.0.1 sequencing Tool (Bruker-Daltronic, Bremen, Germany).

ELECTROSPRAY-IONIZATION-MASS-SPECTROMETRY (ESI-MS)

The MS/MS fragmentation analysis was also performed by liquid-chromatography/ electrospray-mass-spectrometry (ESI). Two capillary HPLC-pumps (G1376A, Agilent, Böblingen, Germany) with micro vacuum degasser (G1379B, Agilent, Böblingen, Germany), an automatic micro-well-plate autosampler (G1377A, Agilent, Böblingen, Germany), and a diode array multi-wavelength detector (G1365D Agilent, Böblingen, Germany) were used for chromatography. Chromatography was carried out using an Agilent Zorbax SB-C18 column (5 µm, 150 x 0.5 mm, Agilent, Böblingen, Germany), the column temperature was adjusted at 25°C, and 0.01% TFA and 0.1% formic acid (FA) in water was used as eluent A with 98% acetonitrile in water used as eluent B in an injection volume of 5 µL. A flow rate of 10 µL min^{-1} and a linear gradient (0 min: 1% eluent B; 30 min: 60% eluent B) were used for elution of retained substances from the column. The ESI-mass-spectrometry was performed in enhanced positive mode. The range of m/z was 80 to 2800. The scan speed of measured mass-spectra was 26,000 m/z per second. The accumulation time was adjusted to 200 ms. The flow rate of the nitrogen gas was 5 L min^{-1}. The temperature of the transfer-capillary was adjusted to 300°C with the capillary voltage at 1,500 to 4,500 V. The mass-spectrometric data were accumulated by the HyStar software 3.2 (Bruker-Daltonics, Bremen, Germany) and analyzed by Data Analysis 4.0 (Bruker-Daltonics, Bremen, Germany). Glutathione was used as an internal standard for calculation of the recovery rate.

PEPTIDE SYNTHESIS

“Calcification blocking factor” (CBF) was synthesized automatically by the solid-phase method using standard Fmoc chemistry in continuous flow mode TentaGel S Random-Access Memory (RAM) resin 0.21 mmol g^{-1} for peptide amides, TentaGel S p-hydroxybenzoic acid (PHB) resin (Rapp Polymere, Tuebingen, Germany) for the free acid of urocortin, o-benzotriazole-N,N,N’,N’-

tetramethyl- uronium-hexafluoro-phosphate (HBTU), 2 equivalents of N,N-diisopropylethylamine (DIEA), coupling for 20 min, deblocking with 20% piperidine in N,N-dimethyl formamide (DMF) for 15 min, and final cleavage with 95% TFA/5% water for 3 h. Purification of the crude peptide was carried out by preparative HPLC on PolyEncap A300 (10 µm particle size, 250 mm x 20 mm, Bischoff Analysentechnik, Leonberg, Germany) in water with increasing concentrations of ACN as mobile phase. An eluent gradient of 5-70 (v/v-%) ACN/water (0.1% TFA) over 70 min with a flow rate of 10 ml min^{-1} was used. The purified peptide was lyophilized. The peptide was characterized by MALDI mass-spectroscopy on a Voyager-DE STR BioSpectrometry Workstation MALDI-TOF mass-spectrometer (Perseptive Biosystems, Framingham, US) using R-cyano-4- hydroxycinnamic acid and sinapinic acid as matrix, and gave the expected $(M + H)^+$ mass [52].

ISOLATION AND STIMULATION OF ADRENAL GLAND GRANULES BY CARBACHOL

Fresh bovine adrenal glands were obtained on ice from a local slaughterhouse, the lipid matter was removed, and the adrenal glands were dissected. The granules were isolated from the adrenal glands as follows [53,54]. Briefly, 50 ml of 0.32 mol L^{-1} sucrose were added to the adrenal glands, which were then homogenized with an Ultra-Turrax at 4°C (750 rpm) for 4 min, and the homogenate centrifuged at 800 g at 4°C for 10 min. The supernatant was centrifuged again at 10,000 g for 20 min at 4°C. To the supernatant, 50 ml of 1.6 mol L^{-1} sucrose was added, and then the sample was centrifuged at 100,000 g for 45 min at 4°C. The pellet was resuspended in 10 ml of water and divided into two aliquots. For an active release of CBF from the granules, carbachol (10^{-5} mol L^{-1}) was added to one aliquot. Phosphate-buffered saline (PBS, Sigma-Aldrich, Taufkirchen, Germany) was added to the second aliquot as a negative control and vortexed for 5 min at room temperature. The supernatant was analyzed by electrospray-ionization mass-spectrometry as described above.

IDENTIFICATION OF ENZYMES CLEAVING CBF FROM CHROMOGRANIN A

The protease prediction tool Proteasix [49] was used to predict the enzymes likely involved in cleaving CBF from chromogranin A. Enzyme candidates were identified by entering the Protein ID P10645, the N-terminal amino acid 358 and C-terminal amino acid 376. A peptide with ten additional amino acids at the N-terminus of CBF (LAKELTAEKR-LEGQEEEEDNRDSSMKLSF) and ten additional amino acids at the C-terminus (LEGQEEEEDNRDSSMKLSF-RARAYGFRGP) were synthesized for identification of the enzymes cleaving the N-terminal and C-terminal part of CBF, respectively, from chromogranin A.

Human kallikrein (9 µM; Sigma-Aldrich, Taufkirchen, Germany) and the peptide with the amino acid sequence LAKELTAEKR-LEGQEEEEDNRDSSMKLSF (10 µM; Campro, Berlin, Germany) were incubated in 1 ml PBS for 48 h at 37°C. As a control, LAKELTAEKR-LEGQEEEEDNRDSSMKLSF (10 µM) was simultaneously incubated in the absence of enzymes. The reaction mixtures were deproteinized using 70% perchloric acid (Merck, Darmstadt, Germany) at a final concentration of 0.6 mol L^{-1}, centrifuged at 13,000 g for 10 min at 4°C, the pH value was adjusted to 9 by using KOH, and the sample was analyzed by LC-qTOF mass-spectrometry as described above. Calpain 1 (Sigma-Aldrich, Taufkirchen, Germany) was dialyzed using a Pur-A-Lyzer Maxi 12000 dialysis kit (Sigma-Aldrich, Taufkirchen, Germany) for removing EDTA and EGTA from the mixture. Afterwards, calpain 1 (6 µM) and the peptide with the amino acid sequence LEGQEEEEDNRDSSMKLSF-RARAYGFRGP (1.5 µM; Campro, Berlin, Germany) were incubated in 1 ml PBS for 48 h at 37°C. The protein was removed from the reaction mixture using an Amicon Ultra 10K Centrifugal Filter (Merck, Darmstadt, Germany) and the filtrate was analyzed by LC-qTOF mass-spectrometry as described above. Finally, calpain 1 and kallikrein at the same concentrations as mentioned previously and chromogranin A (1 µM; Sigma-Aldrich, Taufkirchen, Germany) were incubated in 1 ml PBS for 48 h at 37°C. The enzymes were removed from the reaction mixture using an Amicon Ultra 10K Centrifugal Filter (Merck, Darmstadt, Germany) and the filtrate was analyzed by LC-qTOF mass-spectrometry as described above.

The reaction mixtures were fractionated by using a nano-HPLC unit (Dionex UltiMate 3000 RSLC-nano system; Thermo Fisher Scientific, Dreieich, Germany) online coupled to a qTOF mass-spectrometer (Impact II, Bruker-Daltonic, Bremen, Germany) equipped with Data Analysis 4.4 (Bruker Daltonic, Bremen, Germany). A Thermo Scientific Acclaim Pepmap RSLC 100 C18-LC-column (75 µm x 15 cm) in combination with a Thermo Scientific Acclaim Pepmap 100 C18-LC-precolumn (100 µm x 2 cm; both Thermo Fisher Scientific, Dreieich, Germany) and gradient elution was used for the chromatographic separation (solvent A. water with 0.1% formic acid; solvent B, 80% acetonitrile and 0.08% formic acid in water: 0-6 min: 2% B; 6–16 min: 0-65% B; 16–19 min: 65% B; 19–21 min: 65-99% B; 21–24 min: 99% B; flow rate of 0.3 µL/min). The mass-spectrometer was operated in positive mode (spray voltage: 4500 V; offset voltage: 500 V; nitrogen dry gas: 4 L min^{-1}; drying temperature: 180°C; nebulizing gas pressure: 0.4 bar). We used an acquisition rate of 2 Hz for MS spectra within the *m/z* range 50-2000. In the case of auto MS/MS analyses, the acquisition rate was set at 2 Hz and 10 Hz for low (<25 000) and for high (>25 000) signal intensities, respectively. Line spectra calculated as a sum of intensities were registered and two technical replicates were accumulated per sample. Raw mass-spectrometric data were recalibrated and the extracted ion chromatograms of $(M+3H)^{3+}$ signal for CBF at m/z 748.66 were calculated using data analysis 4.4.

CELL CULTURE AND CALCIFICATION INDUCTION IN HUMAN AORTIC SMOOTH MUSCLE CELLS

Human aortic smooth muscle cells (hAoSMCs) (Promocell, Heidelberg, Germany) were cultivated in smooth muscle cell medium (Promocell, Heidelberg, Germany). The hAoSMCs were seeded in 48 well plates (5x 10^3 cells/well) at 80% confluence. Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol L^{-1} glucose supplemented with 2.5% fetal calf serum and 2.8 mmol L^{-1} phosphate were used to induce calcification (calcifying medium, CM). The calcifying medium was supplemented with CBF (max. concentration 100 pmol L^{-1}) or CBF fragments (100 nmol L^{-1}). As reference medium, DMEM containing 25 mmol L^{-1} glucose supplemented with 2.5% fetal calf

serum and 0.9 mmol L^{-1} phosphate were used (non-calcifying medium, NCM). The hAoSMCs were incubated for 7 days while the incubation medium was replaced after 48 h.

EX VIVO AND CALCIFICATION OF RAT AORTIC RINGS

The thoracic aorta of 8-13 weeks old male Wistar-rats were gently dissected and were cut in 3-4 mm segments. The endothelial layer of the aortic ring was manually damaged by scratching using a conventional pipette tip. The aortic rings were incubated with DMEM containing 25 mmol L^{-1} glucose supplemented with 2.5% fetal calf serum and 2.8 mmol L^{-1} phosphate to induce calcification. The calcification medium was supplemented with CBF (max. concentration 1 µmol L^{-1}) or CBF fragments (100 nmol L^{-1}). As reference medium, DMEM containing 25 mmol L^{-1} glucose supplemented with 2.5% fetal calf serum and 0.9 mmol L^{-1} phosphate were used (non-calcifying medium, NCM). The aortic rings were incubated for 7 days while the medium was replaced after 48 h.

TRANSFECTION OF HUMAN AORTIC SMOOTH MUSCLE CELLS

For siRNA transfection HAoSMCs were washed once with PBS, detached, collected by centrifugation and resuspended in growth medium. The transfection was performed by the Neon Transfection System (Thermo Fisher Scientific, Germany). The PIT-1 siRNA with sequence 5´-GCCGTAGTTTACAGTATTTAA-3´ was used. A GFP siRNA from Quiagen (Germany) served as a control. 20 µl of cell suspension in Buffer R, containing approximately 650,000 cells and 120 nM siRNA was placed in a sterile electroporation tip from the Neon Transfection System. Cells were subjected to high voltage 1,470V at a setting that had been optimized for HAoSMCs. After electroporation, the cells were immediately plated out using growth medium in 48 well plates 45,000 cells/well. After 24 h the growth medium was replaced with DMEM containing 25 mmol L^{-1} glucose supplemented with 2.5% fetal calf serum and 2.8 mmol L^{-1} phosphate to induce calcification. The calcification medium was supplemented with CBF (100 nmol L^{-1}). As reference medium, DMEM containing 25 mmol L^{-1} glucose supplemented with 2.5% fetal calf serum and 0.9

mmol L^{-1} phosphate were used (non-calcifying medium, NCM). The cells were incubated for 7 days while the medium was replaced after 48 h.

EFFECT OF CBF IN AN ANIMAL MODEL WITH ENHANCED ARTERIAL CALCIFICATION

An animal model of elastocalcinosis with proven increased arterial calcification [50] was chosen to assay the effects of CBF *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 conformed to the Guide for the Care and Use of Laboratory Animals, NIH (National Academies Press US, 8th edition, 2011). Three groups of 6 weeks old Wistar rats (Charles River Laboratories, L'Arbresle, France) were given a regular rat chow (A04, Safe, Villemoisson-sur-Orge, France) and spring water (Mont Roucous®, France; calcium 60 µM) *ad libitum* for one week before experiments. Two groups received CBF (31 µg kg^{-1} per day) or its vehicle (saline) infused through an osmotic pump (Alzet 2004, Charles River, France) implanted under the skin. CBF treatment was initiated 3 or 4 days before induction of elastocalcinosis (vitamin D_3 plus nicotine, or VDN rats) with a single injection of vitamin D_3 (300,000 IU kg^{-1}, i.m.) and two gavages of nicotine (25 mg kg^{-1}, 5 ml kg^{-1}) on the same day, as previously described [55]. Untreated rats served as control rats (sham operation and saline administration). Four weeks later, rats were anaesthetized (ketamine-xylazine), and a catheter was inserted into the right carotid artery for blood pressure measurement. After 10-15 min of equilibration, systolic, diastolic, mean arterial, and pulse (systolic-diastolic) pressures were determined. The aorta was gently dissected, adventitial fat removed, and cut into segments (3-4 mm) for further analysis.

HISTOLOGICAL CALCIFICATION STAINING AND IMMUNOFLUORESCENCE STAINING

Aortic rings were placed in 1 ml of 4% formaldehyde for 1 h to prepare for histological and immunohistochemical staining. The rings were dehydrated and embedded in paraffin and 5 µm slices were prepared by a microtome (Leica RM 2250, Wetzlar, Germany). The slices were placed

on a glass slide, deparaffinized in xylene, and dehydrated in decreasingly concentrated sopropanol solutions before staining. Von Kossa staining was performed to visualize calcified areas of the aortic rings. The aortic ring slides were incubated with 5% silver nitrate in the presence of UV light at 405 nm for 1 h, washed three times with double-distilled water (ddH_2O), incubated in the presence of 5% sodium thiosulfate in water for 1 min, and washed three times with ddH_2O. Next, nuclear fast red (Merck; Darmstadt, Germany) was added to the slides for 5 min. The slides were then rinsed, dehydrated with ethanol, incubated for 5 min in xylol, and covered with Vitro-Clud (R. Langenbrinck; Emmendingen, Germany).

For immunohistochemical analyses, sections were blocked with blocking solution (1% bovine serum albumin (BSA)) for 30 min at room temperature. The sections were incubated with monoclonal mouse anti-α-SMA antibody 1:200 (Dako, Waldbronn, Germany), polyclonal rabbit anti-phospho-SMAD1 antibody 1:100 (Biorbyt, Cambridge, UK), or polyclonal rabbit anti-phospho-SMAD5 antibody 1:200 (Biorbyt, Cambridge, UK) at 4°C overnight. Detection was carried out for α-SMA staining with polyclonal goat anti-mouse-Cy3, 1:300 1 h, at room temperature (Jackson ImmonoResearch, Cambridgeshire, UK) and for p-SMAD1 and p-SMAD5 staining with polyclonal goat anti-rabbit-Biotin (Vector Laboratories, Burlingame, CA, USA), 1:100 for 30 min at room temperature, followed by Strepavidin-FITC (Vector Laboratories, Burlingame, CA, USA), 1:30 for 30 min at room temperature. Samples were visualized with a LEICA DM5500B microscope equipped with a digital imaging system. Staining or protein expression was determined by the area stained and expressed as a percentage of the total aortic area section, using ImageJ software (National Institutes of Health, USA) after RGB split and segmentation of the image.

MEASUREMENT OF CALCIUM CONTENT

For determination of calcium content, cells or aortic rings were washed 3 times with PBS without calcium. Aortic rings were dried and weighed. Dried aortic rings and cells were decalcified with 0.1 M HCl for 24 h, and the calcium content in the supernatant was determined by the o-cresol-

phthalein complexone method (Clinical Chemistry Assay Kit/Calcium, County Antrim, UK) according to the manufacturer's protocol. Cell content was extracted in 0.1 M NaOH 0.1% sodium dodecyl sulfate (SDS), and protein content was measured by the bicinchoninic acid (BCA) protein assay method (Thermo Fisher Scientific, Germany). The calcium content was normalized to total protein for cells or dry weight for aortic rings.

QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR) ANALYSES OF mRNA EXPRESSION IN HUMAN AORTIC SMOOTH MUSCLE CELLS AND VDN RATS

Total RNA was extracted from hAoSMCs and from aortic tissue using RNAeasy mini kit (Qiagen, Hilden, Germany). Reverse transcription was performed using 1 µg total RNA, random hexamers, and Verso reverse transcriptase (Thermo Scientific, France) as per the manufacturer's instructions. For real-time PCR, gene expression levels were quantified using SYBR Green I dye chemistry on a LightCycler 480 system (Roche Applied Sciences, France). PCR primers (**Table 1**) were designed using the LightCycler Probe Design software 2.0. Expression levels were determined with the LightCycler analysis software (version 3.5) relative to standard curves. Data were represented as the mean level of gene expression relative to the expression of the reference gene (β-Actin for cells and RPLPO for rat).

CLINICAL STUDY OF PLASMA CBF LEVELS IN END-STAGE RENAL DISEASE

Study participants having given written informed consent were recruited from University Hospital Essen (Essen, Germany) after approval by the local ethics committee (ethical vote 08–3817). 17 patients suffered from CKD stage 5 (ESRD) and were on regular dialysis. 13 patients had no CKD (controls) with estimated glomerular filtration rates according to the MDRD-formula ≥60 ml min^{-1} 1.73^{-1} m^{-2}. Creatinine and haemoglobin were measured using standard autoanalyzer techniques. Intact parathyroid hormone was determined by immunoassay (Roche Diagnostics, Germany). Plasma samples were immediately separated from blood cells and were frozen until all samples were collected, which were measured side by side within one analysis set-up.

STATISTICS

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, USA), and the data were represented as mean ± SEM. Analysis of variance (ANOVA) for a single factor (one-way) or for multiple factors (two-way) was performed to determine differences between treatment groups and the control group. In both, Bonferroni's multiple comparisons were used as a post-test. Differences at $P<0.05$, *$P<0.05$, **$P\leq0.01$, ***$P\leq0.001$, ****$P\leq0.0001$ were considered to be statistically.

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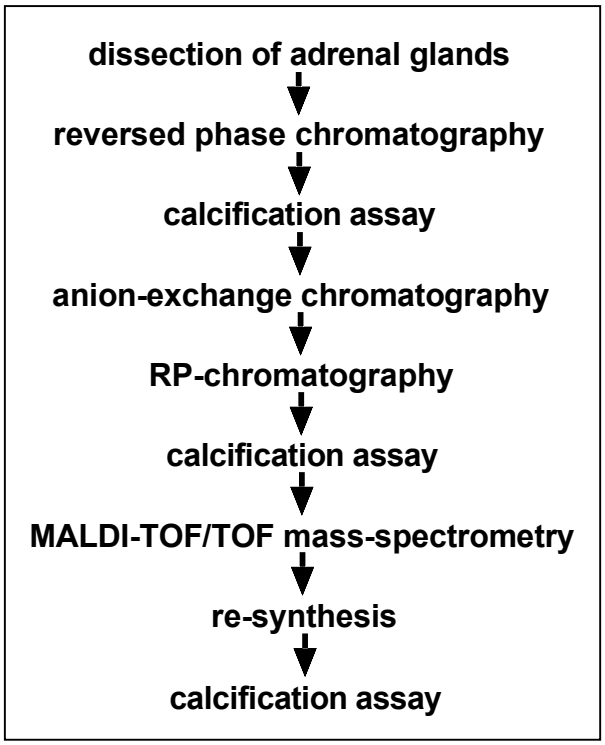
Orth-Alampour *et al.* Figure 1

a



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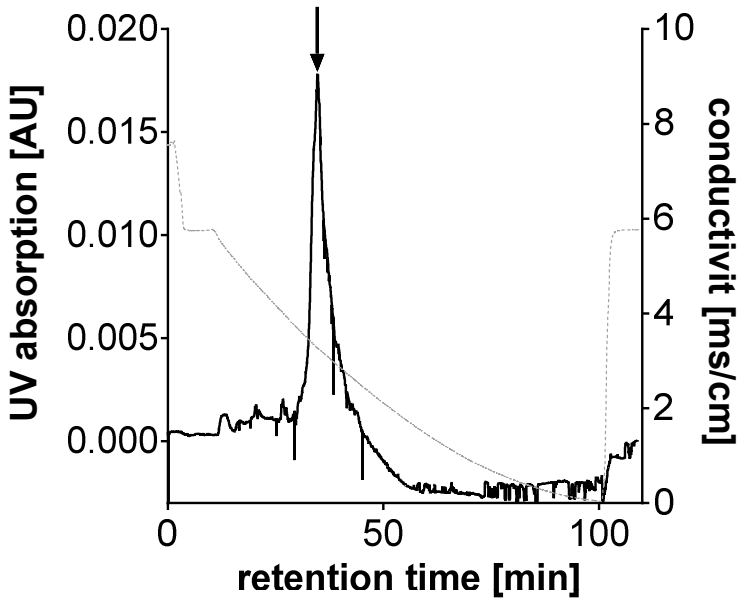

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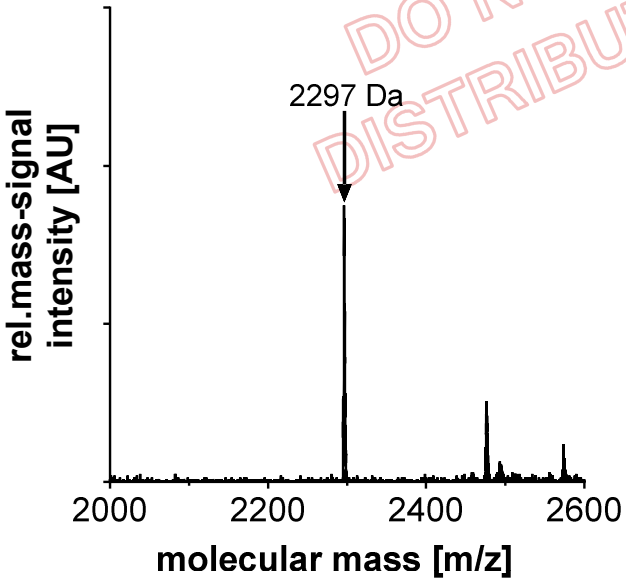

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e

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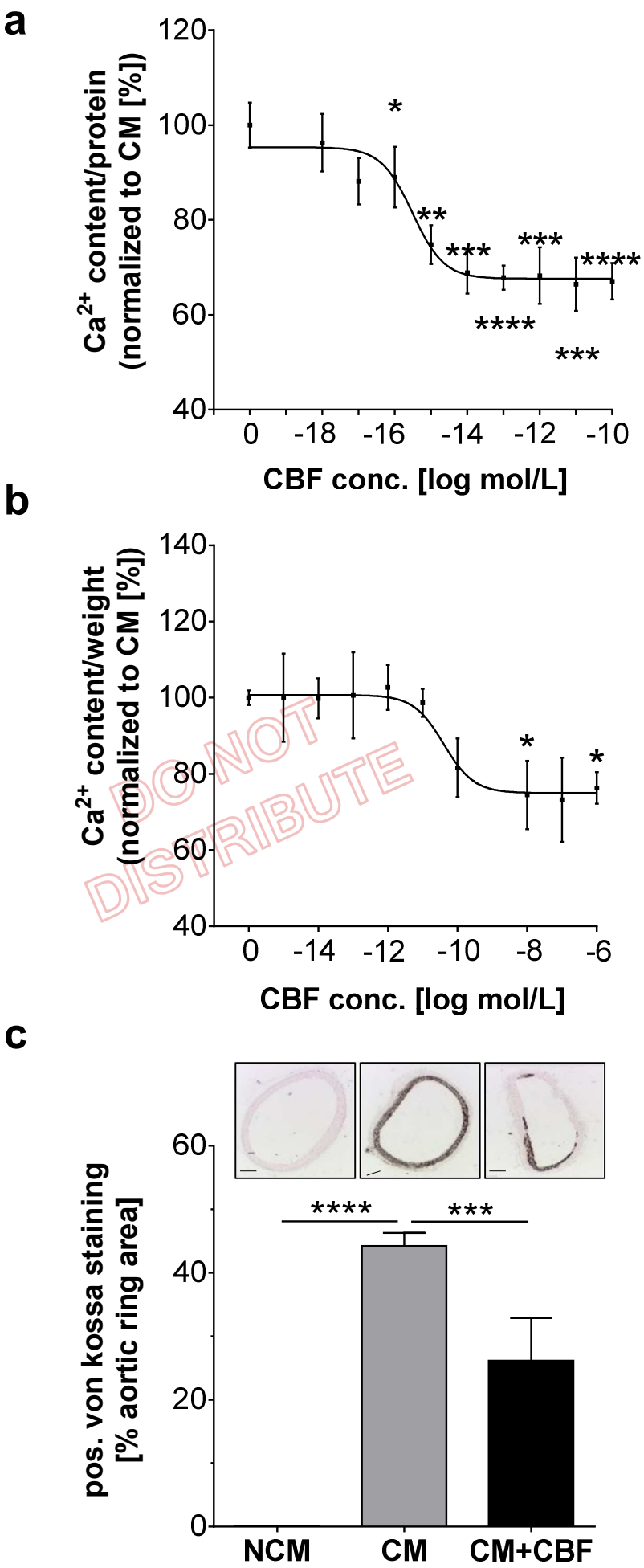
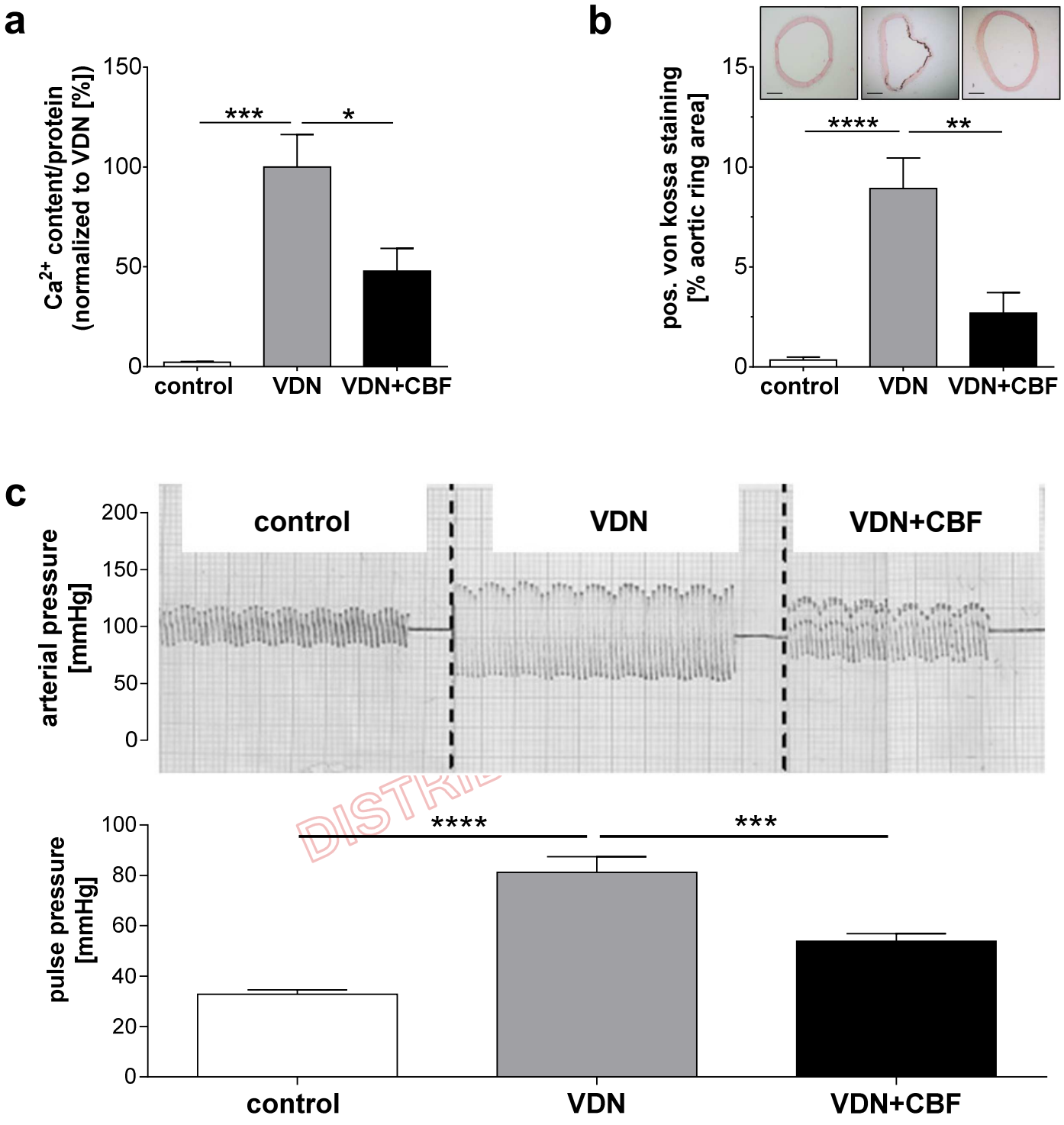
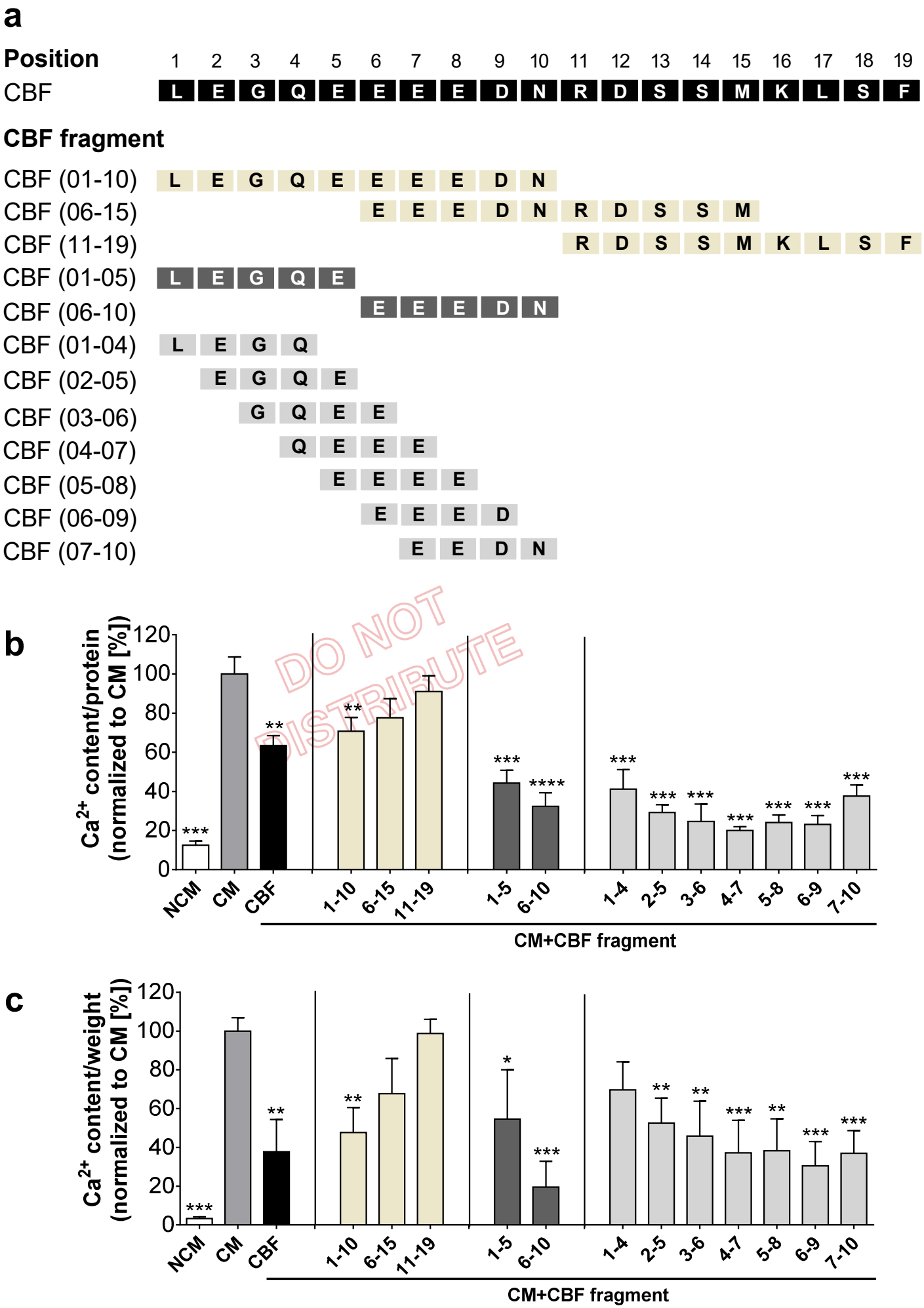
Orth-Alampour *et al.* Figure 2



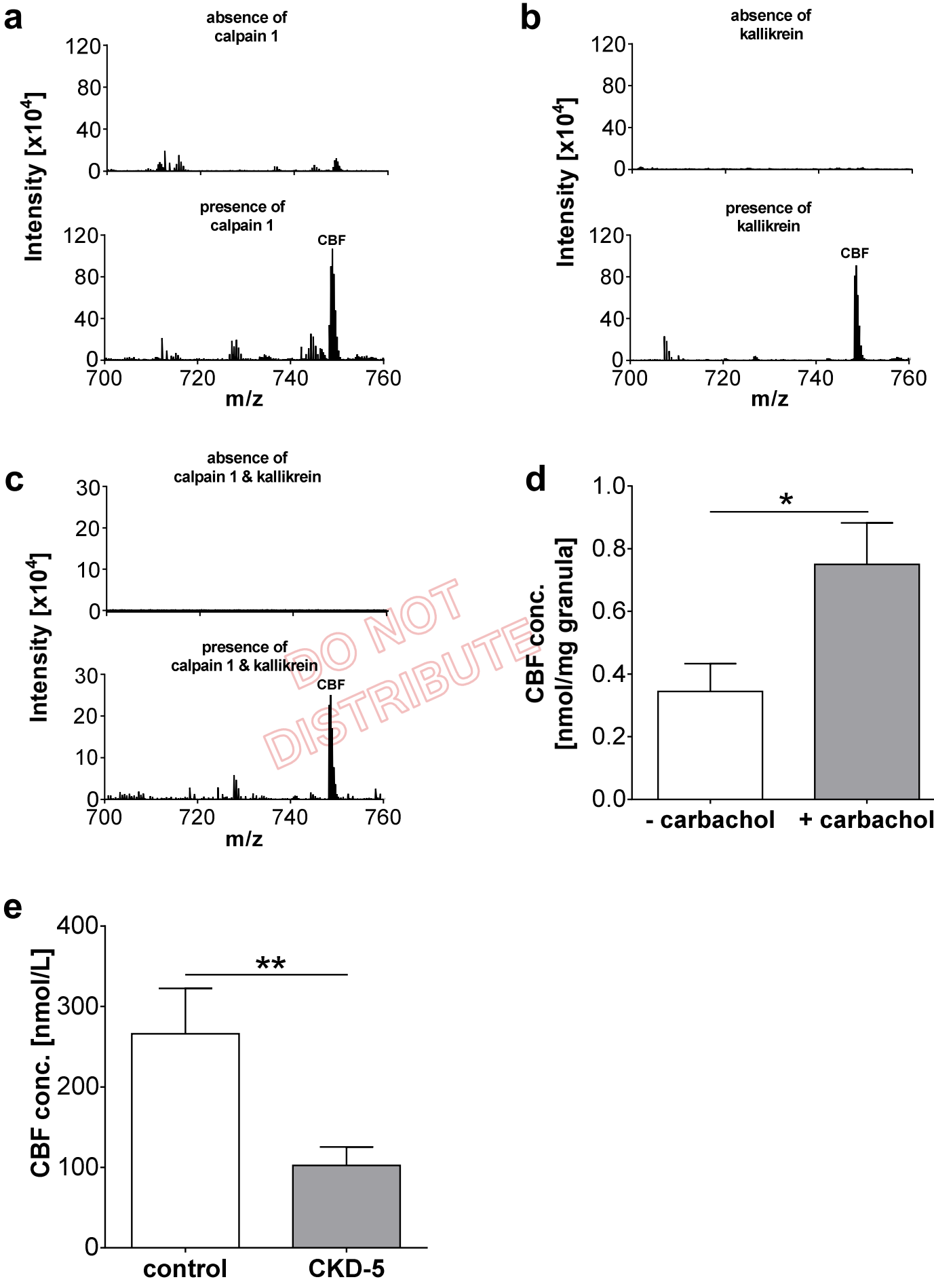
Orth-Alampour *et al.* Figure 3



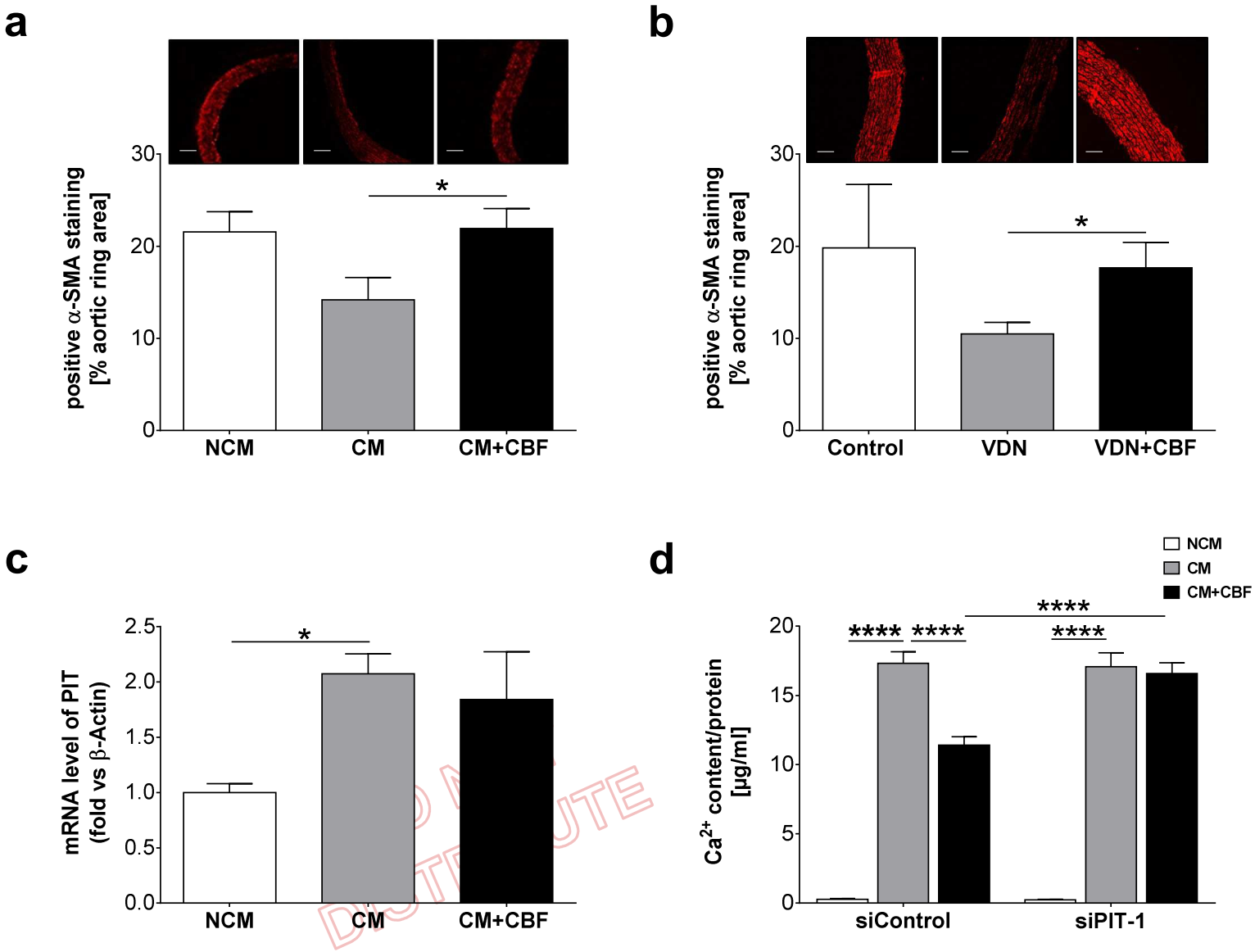
Orth-Alampour *et al.* Figure 4



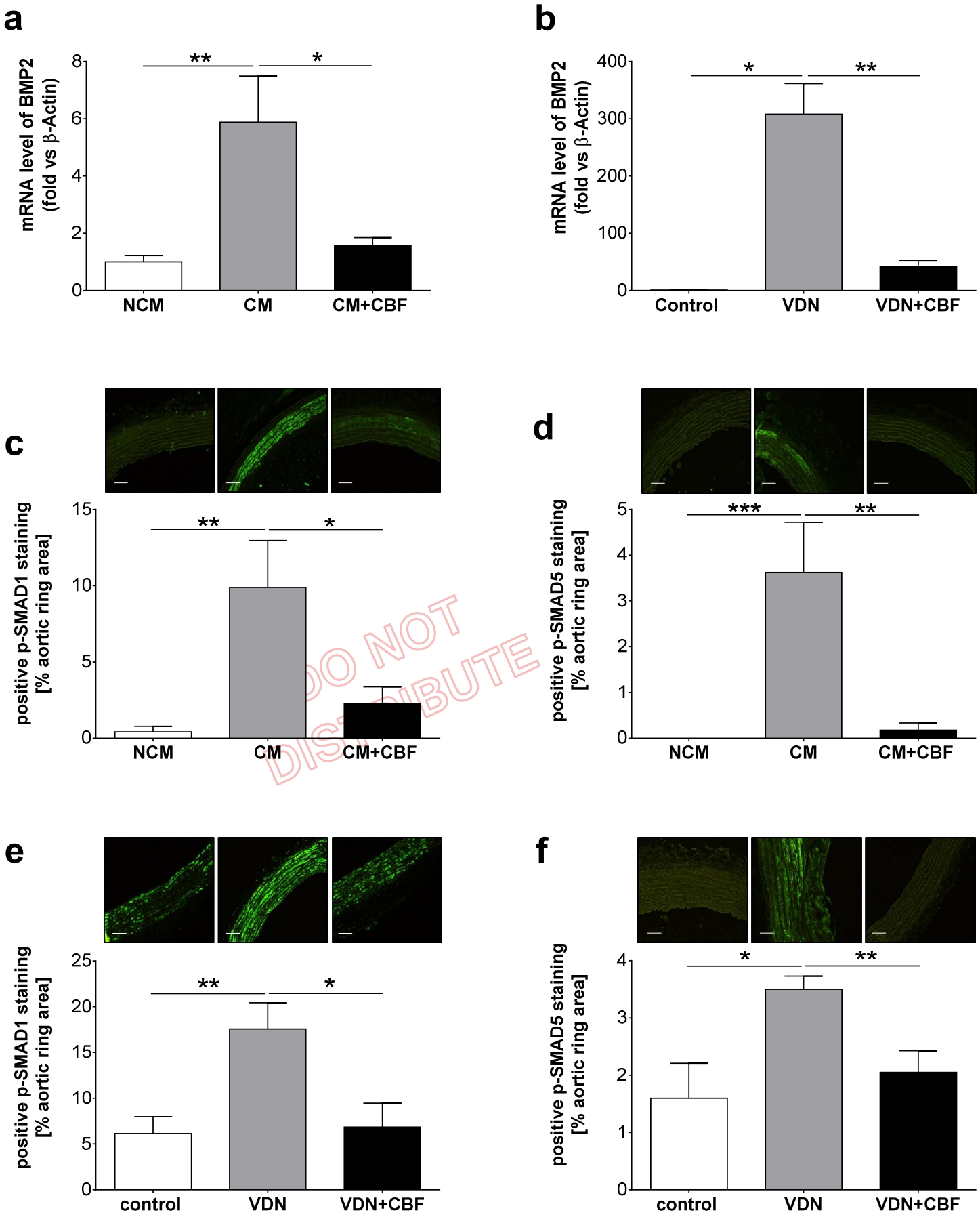
Orth-Alampour *et al.* Figure 5



Orth-Alampour *et al.* Figure 6

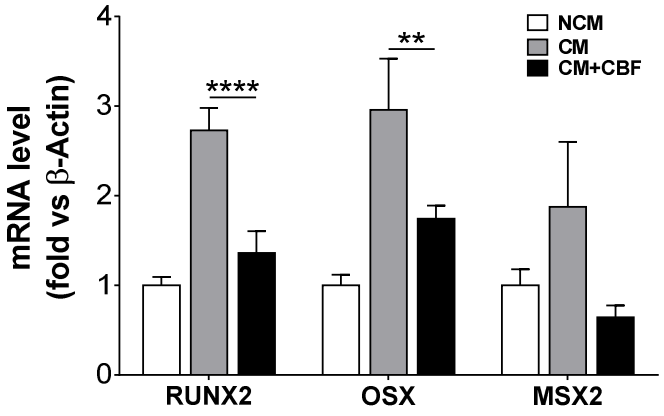


Orth-Alampour *et al.* Figure 7



Orth-Alampour *et al.* Figure 8

a



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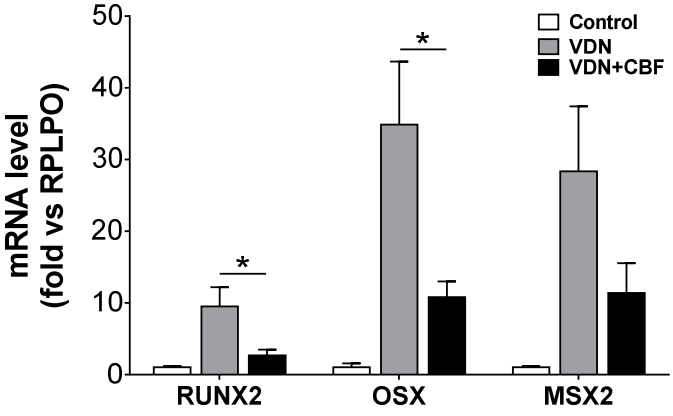
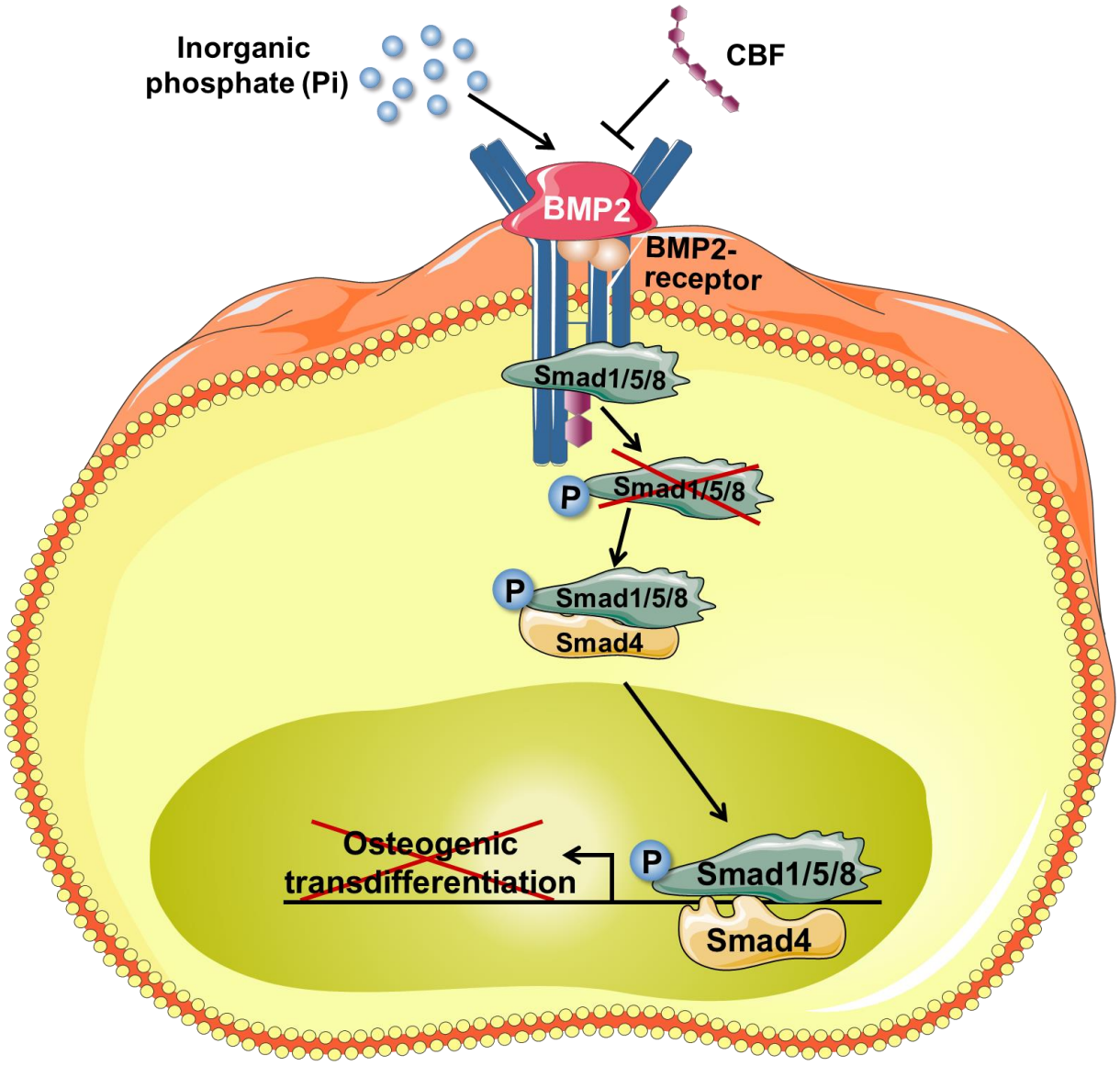


c

Kinase	CM vs. NCM	CM+CBF vs. NCM
MSK1	3,19	1,09
MSK2	2,97	1,10
JNK1	1,86	0,98
JNK2	1,95	0,99
JNK3	1,86	0,98
P38-δ	1,76	1,05

Orth-Alampour *at.al.* Figure 9



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"IDENTIFICATION AND CHARACTERISATION OF A NOVEL ADRENAL PEPTIDE THAT INHIBITIS OSTEOGENIC TRANSDIFFERENTIATION TO PREVENT VASCULAR CALCIFICATION"

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