Gla-Rich Protein Acts as a Calcification Inhibitor in the Human Cardiovascular System

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Objective—Vascular and valvular calcifications are pathological processes regulated by resident cells, and depending on a complex, they interplay between calcification promoters and inhibitors, resembling skeletal metabolism. Here, we study the role of the vitamin K–dependent Gla-rich protein (GRP) in vascular and valvular calcification processes.

Approach and Results—Immunohistochemistry and quantitative polymerase chain reaction showed that GRP expression and accumulation are upregulated with calcification simultaneously with osteocalcin and matrix Gla protein (MGP). Using conformation-specific antibodies, both γ-carboxylated GRP and undercarboxylated GRP species were found accumulated at the sites of mineral deposits, whereas undercarboxylated GRP was predominant in calcified aortic valve disease valvular interstitial cells. Mineral-bound GRP, MGP, and fetuin-A were identified by mass spectrometry. Using an ex vivo model of vascular calcification, γ-carboxylated GRP but not undercarboxylated GRP was shown to inhibit calcification and osteochondrogenic differentiation through α-smooth muscle actin upregulation and osteopontin downregulation. Immunoprecipitation assays showed that GRP is part of an MGP–fetuin-A complex at the sites of valvular calcification. Moreover, extracellular vesicles released from normal vascular smooth muscle cells are loaded with GRP, MGP, and fetuin-A, whereas under calcifying conditions, released extracellular vesicles show increased calcium loading and GRP and MGP depletion.

Conclusions—GRP is an inhibitor of vascular and valvular calcification involved in calcium homeostasis. Its function might be associated with prevention of calcium-induced signaling pathways and direct mineral binding to inhibit crystal formation/maturation. Our data show that GRP is a new player in mineralization competence of extracellular vesicles possibly associated with the fetuin-A–MGP calcification inhibitory system. GRP activity was found to be dependent on its γ-carboxylation status, with potential clinical relevance. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.114.304823.)

Key Words: aortic valve, calcification of gene expression vascular calcification

Cardiovascular calcification is a life-threatening complication of cardiovascular disease, affecting tissues such as arteries, heart valves, and cardiac muscle. Although many aspects on the pathogenesis of calcified aortic valve disease (CAVD) are still unclear, it has been shown to share many features with the mostly studied vascular calcification (VC), such as chronic inflammation, increased extracellular matrix (ECM) remodeling, proliferation and differentiation of resident cells, and the development of calcific lesions. Both VC and CAVD are active, naturally occurring, controlled cell-mediated processes of osteochondrogenic differentiation of vascular smooth muscle cells (VSMCs) and valvular interstitial cells (VICs), resembling developmental skeletal formation. Several growth factors, matrix inhibitory proteins, and other bone-related proteins, including osteocalcin, alkaline phosphatase, runt-related transcription factor 2, bone morphogenetic protein 2, collagen type I, and osteopontin, were found to be associated with VC and CAVD. Importantly, VC is a process that must be actively inhibited, relying on the presence of functional calcification inhibitors, including matrix Gla protein (MGP) and fetuin-A. MGP, and fetuin-A is a liver-derived blood protein acting as a potent inhibitor of ectopic calcification. An association between both proteins has been observed at tissue and systemic levels.
circulation levels, forming a potentiocalcification inhibitory system, whereas decreased levels of functional γ-carboxylated MGP (cMGP) and fetuin-A have been linked to an enhanced calcification environment.11–15 A key event in the initiation of VSMC calcification is the release of mineralization-competent extracellular vesicles (EVs), capable of efficiently nucleating hydroxyapatite in the absence of calcification inhibitors, such as MGP.3,5,15 However, the existence of additional calcification inhibitors has been repeatedly suggested.16,17 Gla-rich protein (GRP) is a vitamin K–dependent protein18 whose function and molecular mechanisms of action remain unknown, albeit zebrafish knockdown19 and knockout mouse20 studies have been performed. Also, different alternatively spliced transcripts, the function of which remains to be clarified, were shown to exist in mouse/zebrafish19,21 and human,22 highlighting the existence of distinct gene regulation in animal models and humans. However, GRP has been suggested to act as a negative regulator of osteogenic differentiation,23 a modulator of calcium availability in the ECM,18,24 and as a potential inhibitor of soft tissue calcification in connective tissues.24 In concordance, its potential calcium-binding properties and association with calcification processes have been demonstrated through immunohistochemical and in vitro studies, showing (1) high levels of protein accumulation at the sites of pathological calcification22,24,25 and (2) its capacity to directly bind basic calcium phosphate crystals.25 γ-Carboxylation of human GRP has been recently evidenced using a conformation-specific antibody for the γ-carboxylated GRP (cGRP) conformation,25 whereas an impaired γ-carboxylation status has been associated with pathological calcification–related diseases, such as osteoarthritis,22 and certain cancers.23 In this work, we aimed to study the relationship of GRP at gene and protein levels, with valvular calcification and VC processes, highlighting GRP function and molecular mechanisms of action.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.
patterns were observed in the regions of massive calcification in nondecalcified tissue sections, colocalizing with mineralized materials (Figure IV in the online-only Data Supplement). Foam cells, found in the regions of lipid accumulation mostly present in atheromas surrounding areas, were shown to accumulate tGRP (Figure 2G). The lower staining observed for cGRP (Figure 2H), when compared with ucGRP (Figure 2I), and the colocalization of ucGRP with tGRP indicate ucGRP as the predominant protein form in foam cells. Both cGRP and ucGRP were detected in VICs in the regions of high and dispersed cellularity although with different patterns of accumulation; whereas ucGRP is mostly found intracellularly in most VICs (Figure 2L and 2O), cGRP is predominantly localized...
in the ECM on the vicinity of cells while a lower percentage of cells are also stained intracellularly (Figure 2K and 2N). Of notice, some of the fibroblast-like cells were GRP negative (white arrow head), a pattern confirmed with all antibodies (Figure 2J–2O). Cells accumulating GRP were mostly assumed as VICs, and although at this stage we cannot rule out the possibility of GRP colocalization with T lymphocytes, the majority of cells accumulating GRP were found to be cluster of differentiation 68 and cluster of differentiation 3 negative results not shown). Overall, these results indicate that higher GRP accumulation in CAVD is associated with calcification together with VIC disarray and specifically associated with particular VIC populations. Although ucGRP is apparently predominant in CAVD, cGRP seems to be mostly targeted to the sites of calcification. Negative controls showed the absence of signal (Figure V in the online-only Data Supplement).

GRP, MGP, and Osteocalcin Are Concomitantly Upregulated in CAVD

The GRP gene expression profile in CAVD was evaluated to correlate it with the degree of cellular differentiation and calcification. Gene expression of known osteoblastic, differentiation, and inflammation markers—MGP, osteocalcin, osteopontin, $\alpha$-smooth muscle actin ($\alpha$SMA), and tumor necrosis factor-$\alpha$—is determined in CAVD and non-CAVD groups (Figure 3). Although no significant differences in GRP, MGP, and osteocalcin levels were found between samples in each sample group, GRP was clearly upregulated in the majority of CAVD samples with an expression pattern highly similar to that of MGP and osteocalcin. In concordance with the immunohistochemistry results showing specific GRP accumulation in certain VICs, gene expression is higher in the particular set of samples presenting higher levels of MGP and osteocalcin. In contrast, a significant upregulation of osteopontin, $\alpha$SMA, and tumor necrosis factor-$\alpha$ is shown between all CAVD and non-CAVD samples, indicative of typical stenotic processes occurring in CAVD, characterized by VIC phenotypic transitions. These results suggest that GRP is upregulated in specific differentiation stages of VIC populations, concomitantly expressing MGP and osteocalcin, probably in a later stage of VIC osteochondrogenic differentiation. Gene expression of GRP-F5 and F6 was also analyzed, and the results showed barely detectable expression of both transcripts in the majority of CAVD samples (Figure VI in the online-only Data Supplement).

GRP, MGP, and Fetuin-A Are Associated With the Mineral Phase of CAVD, Resembling Bone and VC

To characterize GRP protein forms associated with the mineral phase of calcified aortic valves and to further identify additional proteins involved in aortic valve calcification, the mineral-associated (M-P) and 2 extractions of organic matrix (E1 and E2) protein contents, extracted from calcified aortic valves, were analyzed by SDS-PAGE and Western blot (WB;
of relevant molecular mass markers (kDa) is indicated on the side of Figure 4A). SDS-PAGE analysis revealed distinct protein profiles, showing an efficient protein extraction of the organic matrixes (E1 and E2, Figure 4A) and a clear preponderance of low-molecular weight proteins associated with the mineral phase (M-P, Figure 4A). Both GRP and MGP were confirmed to be present mainly in the M-P (Figure 4B). Four protein bands were detected with CTerm-GRP and ucGRP antibodies (tGRP and ucGRP in Figure 4B), corresponding to the protein profile marked as 1, 2, 3, and 4 on SDS-PAGE gel (Figure 4A), whereas cGRP antibodies preferably detect band 2 (cGRP in Figure 4B). In E1 and M-P extracts, a 25-kDa protein band was also detected, particularly with CTerm-GRP and cGRP antibodies, which may either represent intracellular nonprocessed protein or aggregated forms of GRP (Figure 4B). Both undercarboxylated MGP and cMGP protein forms were detected in M-P, where 2 main protein bands named 2 and 5 were detected with both specific antibodies (Figure 4B). Fetuin-A was found to be present mostly in the M-P and E1 extracts, whereas E2 showed only residual detection (Figure 4B). Further identification of proteins present in SDS-PAGE gel slices marked as 1, 2, 3, 4, 5, and 6 was performed by nano-liquid chromatography–mass spectrometry (MS)/MS analysis (Figure 4C; Tables IV and V in the online-only Data Supplement). Protein identities were confirmed in bands 1 and 2 for GRP, in bands 1 to 5 for MGP, and in band 6 for fetuin-A (Figure 4C; Table IV in the online-only Data Supplement), in concordance with WB detections. These results indicate that GRP species present in bands 3 and 4 are predominantly undercarboxylated, although identification by MS was not successful, most possibly because of lower abundance. Identification of additional known osteogenic markers, such as osteopontin, calcium-binding proteins, such as protein S100-A9 (S10A9), and lipid-associated proteins, such as apolipoprotein E, were also identified in the M-P extract (Table V in the online-only Data Supplement).

Identification of Gla residues could not be achieved by liquid chromatography–MS/MS neither for GRP nor for MGP, most possibly because Gla residues may undergo neutral loss of CO₂ from the γ-carboxy carbon, either during ionization by matrix-assisted laser desorption/ionization or after collision-induced dissociation tandem MS (MS/MS). Overall, our results showed that GRP is associated with the mineral phase of calcified stenotic valves together with other calcification inhibitors known to accumulate at the sites of calcification, both in bone and vascular tissues.

\( \gamma \)-Carboxylated GRP Inhibits Calcification in an Ex Vivo Aortic Calcification Model by Upregulating \( \alpha \)SMA and Downregulating Osteopontin Expression

To determine whether GRP directly affects VC and to examine the relevance of its \( \gamma \)-carboxylation status, we adapted an ex vivo model of aortic rings by culturing small human aortic fragments both under control or mineralizing conditions; these cultures were supplemented with either ucGRP or cGRP.

Calcification obtained under Ca+P supplementation was characterized by substantial accumulation of calcium deposits along the media, whereas aortic fragments cultured under control conditions had generally no detectable calcification (results not shown); only in a few cases, the control tissues contained some minor disperse calcium spots (Figure 5A). After 14 days of in vitro culture, hematoxylin–eosin staining showed an intact structure of the vessels and an apparently normal VSMC.

Figure 4. Gla-rich protein (GRP), matrix Gla protein (MGP), and fetuin-A are associated with the mineral phase of calcified aortic valves. A, SDS-PAGE analysis of protein extracts corresponding to 2 sequential extractions of the aortic matrix components (E1 and E2) and the demineralized fraction containing mineral-bound proteins (M-P), stained with Coomassie Brilliant Blue. Numbers 1 to 6 indicate the relative positions of protein bands that were further analyzed and identified by nano-high-performance liquid chromatography (HPLC)-matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF)/TOF (C and Tables IV and V in the online-only Data Supplement). B, Western blot detection of total GRP (tGRP), \( \gamma \)-carboxylated GRP (cGRP), undercarboxylated GRP (ucGRP), \( \gamma \)-carboxylated MGP (cMGP), undercarboxylated MGP (ucMGP), and fetuin-A in E1, E2, and M-P extracts. Both forms of GRP and MGP are mainly detected in the M-P extract, whereas fetuin-A is preferentially detected in E1 and M-P extracts (corresponding to band 6 in A). Four protein bands are detected with both CTerm-GRP (tGRP) and ucGRP antibodies corresponding to bands 1 to 4 marked in A, whereas the cGRP antibody majorly detects band 2. C, Identification of target proteins present in gel band slices 1 to 6 by nano-HPLC-MALDI-TOF/TOF. Additional information of identified proteins is given in Tables IV and V in the online-only Data Supplement. The position of relevant molecular mass markers (kDa) is indicated on the side of A and C.
Figure 6. A Gla-rich protein (GRP)–matrix Gla protein (MGP)–fetuin-A (Fet-A) complex exists at calcification sites, and vascular smooth muscle cell-derived extracellular vesicles (EVs) are differentially loaded with GRP, MGP, and Fet-A under control and calcifying conditions. A, Glycol methacrylate calcified aortic valve disease tissue samples show massive mineralized areas (von Kossa) with colocalization of γ-carboxylated GRP (cGRP), γ-carboxylated MGP (cMGP), and Fet-A, as determined by double immunohistochemistry staining using the following combination of antibodies: cGRP/cMGP (cGRP in red and cMGP in blue), cGRP/Fet-A (Santa Cruz; cGRP in red and Fet-A in blue), and cMGP/Fet-A (cMGP in red and Fet-A in blue). Scale bar, 100 μm. B, Western blot (WB) with CTerm-GRP, cMGP, and Fet-A (Santa Cruz) antibodies (indicated on the left side) of eluted proteins obtained after immunoprecipitation (IP) reactions with CTerm-GRP, cMGP, and IgG antibodies (IP-GRP, IP-MGP, and IP-IgG, respectively), using mineral phase extract (described in Figure 4 legend). GRP, cMGP, and Fet-A are detected in both IP-GRP and IP-MGP immunoprecipitants and not in the negative IgG-IP reaction. C, WB analysis of EVs isolated from cultured aortic segments for 3 and 6 days by differential centrifugation at 100,000g and 100,000g, using CTerm-GRP, cMGP, and Fet-A (BioVendor) antibodies. GRP and MGP are only detected in 100,000g isolated EVs and are similarly present under tested conditions at day 3, whereas a strong subsequent reduction is observed after long-term calcifying treatment. D, EVs isolated at 100,000g...
phenotype under all conditions (Figure VIIA in the online-only Data Supplement); at this stage, no outgrowth or proliferation of VSMCs was observed (results not shown). A significant increase in Ca accumulation was determined in Ca+P relative to control, whereas addition of ucGRP did not significantly affect Ca levels relative to Ca+P (Figure 5B; Figure VIIIB and VIIC in the online-only Data Supplement). However, Ca+P supplemented with 500 ng/mL of cGRP results in a significant decrease in Ca, whereas 250 ng/mL showed a nonsignificant tendency for calcification inhibition (Figure 5B; Figure VIIIB and VIIC in the online-only Data Supplement). Reduction of calcium deposits in vessels treated with 500 ng/mL of cGRP was confirmed by VK staining (Figure 5A). Different inhibition degrees were obtained in the 3 experiments performed with 500 ng/mL of sturgeon GRP (28.2% [Figure VIIIB in the online-only Data Supplement], 42.6% [Figure 5B], and 50% [Figure VIIC in the online-only Data Supplement]) but were found similar to those obtained with 500 ng/mL of both bovine MGP and bovine fetuin-A (Figure 5B; Figure VIIIB in the online-only Data Supplement). Mineralizing conditions induced a decrease of α-SMA and an increase in osteopontin expression, indicative of an osteochondrogenic differentiation occurring in VSMCs, whereas exogenous cGRP is able to induce an increased α-SMA and decreased osteopontin expression relative to Ca+P (Figure 5C). Nonsignificant changes were observed with ucGRP treatment (Figure 5C). These results clearly show that (1) calcification inhibition is dependent on GRP dosage and γ-carboxylation status, indicating a preponderant role of Gla residues in the calcification inhibitory function of GRP, (2) carboxylated GRP has similar capacity of preventing calcification as the 2 widely accepted VC inhibitors, MGP and fetuin-A, and (3) GRP interferes with the osteochondrogenic differentiation process by retaining VSMCs in a contractile phenotype.

**GRP–MGP–Fetuin-A Complex Is Associated With Calcification Sites, and Increased Calcium Loading of VSMC-Derived EVs Is Related to GRP and MGP Depletion**

Immunohistochemistry of double-staining experiments, using the respective antibodies, showed the colocalization of (1) cGRP and cMGP, (2) cGRP and fetuin-A, and (3) cMGP and fetuin-A, all in association with the mineral phase (VK, Figure 6A). These data point to a possible association of the 3 proteins at the sites of calcification. To further test this hypothesis, immunoprecipitation of an M-P extract resuspended in 10 mmol/L of Tris–HCl pH 7.5 was carried out using either CTerm-GRP or cMGP as capture antibodies. Before immunoprecipitation assays, the presence of both GRP and MGP in the Tris–HCl buffer was confirmed by WB because both proteins are known to have poor solubility in neutral buffers.19,27 Only part of the initially resuspended proteins was recovered in the soluble fraction, whereas the predominant GRP soluble protein form was detected as a 15-kDa band with the cGRP antibody (Figure VIII in the online-only Data Supplement). WB analysis of the resulting eluted proteins after GRP and MGP immunoprecipitation assays showed positive detection of the 15-kDa GRP band in the anti-GRP and anti-MGP immunoprecipitates. Control IgG immunoprecipitation gave negative results (Figure 6B). In concordance, MGP was also detected in both immunoprecipitation assays (Figure 6B) and was further identified by liquid chromatography–MS/MS in the eluted GRP-immunoprecipitation proteins (results not shown). Additionally, fetuin-A was detected in both GRP- and MGP-immunoprecipitation eluted proteins, and a residual signal was observed in the IgG-immunoprecipitation reaction, most certainly corresponding to the heavy IgG chain of antibodies (Figure 6B). To discard a possible cross-reactivity of cMGP antibody with GRP, immunoprecipitation assays were reproduced as described for M-P extract, using conditioned media of transiently transfected HEK293T cells with GRP-F1–mkate2 fusion protein and the control mkate2-N plasmid. Protein immunodetection was achieved with the CTerm-GRP antibody showing positive detection of GRP-F1–mkate2 fusion protein only in the GRP-immunoprecipitation eluted proteins (Figure IX in the online-only Data Supplement), further confirming the specificity of the immunoprecipitation assays. Overall, these results provide strong evidence for the existence of a GRP–MGP–fetuin-A containing complex at the sites of VC.

Because VC is a process highly related to the mineralization capacity of EVs and to the presence of cMGP and fetuin-A,5,9,15 the association of GRP with EVs in VSMC mineralization was investigated using the developed human vessel ex vivo culture system. EVs were isolated from the media of aortic segments cultured under C and Ca+P conditions, by differential centrifugation after 3 and 6 days in culture, and the presence of GRP, MGP, and fetuin-A was analyzed by WB. Overall, GRP and MGP were only detected in EVs obtained by ultracentrifugation at 100000g, whereas high levels of fetuin-A were found in Ca+P EVs obtained at both 10000g and 100000g (Figure 6C). Importantly, although at day 3, levels of GRP and MGP are similar between C and Ca+P, a strong reduction is observed for both proteins under mineralizing conditions at day 6 (Figure 6C). This reduction of MGP and GRP loading in EVs is also related to increased EV calcium loading (Figure 6D). Further characterization of isolated EVs containing MGP and GRP was performed by scanning electron microscopy, showing a highly homogenous population of small vesicles with an average size range of 65±12 nm that were found positive for cluster of differentiation 9 (Figure 6E).

**Discussion**

In this study, we demonstrated that GRP has a function in calcium homeostasis in the cardiovascular system, most likely by acting as a calcification inhibitor through its calcium chelator and mineral-binding capacity, and involvement in the mineralization competence of VSMC-derived EVs.

VSMCs and VICs synthesize low basal levels of GRP in normal histopathologic human aortas and aortic valves, but its levels of protein accumulation and gene expression highly
increase with calcification. Immunohistochemistry results evidence the colocalization of GRP with the mineral phase, whereas gene expression analysis reveals a GRP upregulation concomitant with the osteoblastic marker osteocalcin. The parallel upregulation of GRP with osteocalcin and MGP, and not necessarily with an increase in osteopontin, αSMA, or tumor necrosis factor-α, further suggests an association of GRP with osteoblast-like VICs after myofibroblast-VIC differentiation. VICs are a highly plastic population of valvular resident cells with the ability to differentiate into a variety of other cell types, such as myofibroblasts and osteoblast-like cells with calcifying potential.1,2 Under endothelial damage and lipid deposition, inflammation is triggered with consequent macrophage differentiation and accumulation, expressing tumor necrosis factor-α and osteopontin.1,3,8,29 Inflammation activity initiates a subpopulation of VICs to differentiate into myofibroblasts expressing αSMA and matrix metalloproteinases. Consequently, disease progression from sclerotic to stenotic states is thought to be driven by the differentiation of myofibroblasts into osteoblast-like cells, with local production of factors, such as osteocalcin, commonly associated with bone and VC.1–3,7 Although abnormal circulating MGP levels have been associated with CAVD,30 studies of MGP at tissue levels in CAVD are lacking. However, MGP expression is known to increase with osteoblast development/maturation and has been considered a late marker of osteoblast-like differentiation in calcifying vascular cells. MGP upregulation in areas of extra cellular VC both in vivo1,3,22 and in vitro31 precludes a mechanism to limit further the extent of calcification through cell differentiation modulation and control of matrix calcification.

We have recently reported that although both cGRP and ucGRP forms possess Ca/P mineral–binding affinity and are present in healthy connective tissues, the increased accumulation of ucGRP is associated with a pathological condition.1,2 In concordance, both cGRP and ucGRP were detected in VSMCs and VICs in normal tissues, and similarly, they are highly accumulated at the sites of mineral deposition. Also, a predominance of ucGRP relative to cGRP was confirmed in CAVD by immunohistochemistry. The pattern of cGRP accumulation further suggests that the reduced amount of cGRP produced may be directed to the sites of calcification where it accumulates probably as a mechanism to control or limit the levels of calcification. In concordance, both GRP species were detected in extracts of mineral-bound proteins derived from calcified valvular materials. The pattern of GRP in WB was shown to be complex with multiple positive bands consistently detected with all available anti-GRP antibodies. The presence of the alternatively spliced variants F5 and F6 in this protein profile can be excluded because their expression was nearly undetectable in both normal and calcified valves. Moreover, the conformation-specific antibodies were raised against synthetic peptides comprising partial Gla/Glu domains, so multiple patterning may represent different degrees of γ-carboxylation, with a predominant γ-carboxylated protein form migrating at 15 kDa. In fact, Gla proteins with different degrees of γ-carboxylation have been shown to alter their migration behavior on SDS-PAGE gels.34 In addition, the fact that we used a pool of individual aortic valves may have contributed to the high Gla content heterogeneity. GRP identification was confirmed by MS analysis of 2 immunopositive bands. With this approach, we have also identified MGP and fetuin-A as mineral-bound proteins associated with CAVD. Other proteins involved in vascular and bone mineralization (eg, osteopontin) were also identified, providing additional evidence that common mechanisms exist in valvular, VC, and physiological bone mineralization.

The similar patterns of GRP and MGP upregulation, and mineral colocalization shown in CAVD, further support the previously proposed calcification inhibitory function of GRP. More importantly, using an ex vivo model of human vessel culture, we showed that cGRP is able to inhibit the calcification to the same extent as cMGP and fetuin-A. Although a reduction in calcification was observed to some extent with noncarboxylated GRP supplementation, only with cGRP, a clear dose-dependent calcification inhibition was obtained, suggesting that γ-carboxylation is essential for GRP’s calcification inhibitory function. γ-Carboxylation has been shown to be required for biological activity of vitamin K–dependent proteins whose function is known, and under- or uncarboxylated species are generally regarded as proteins with low or no functional activity.17,32,35,36 This has been extensively shown for MGP, the inhibitory function of which is highly dependent on its γ-carboxylation status, in particular, in VSMCs.1,28 In our model, vessel calcification was characterized mostly by a medial localization, decreased α-SMA, and simultaneously increased osteopontin expression. These events are counteracted by cGRP through the upregulation of αSMA and down-regulation of osteopontin, indicating a protective effect on the Ca/P-induced effect on VSMCs, shifting from a contractile to a synthetic osteochondrogenic phenotype. High extracellular calcium has been shown to act as a key trigger for VSMC calcification by activation of intracellular signaling pathways that culminate in the secretion of mineralization–competent EVs.3 Inhibition of calcium or cytosolic calcium chelation efficiently prevents VSMC calcification.15 It is conceivable to propose that addition of exogenous cGRP to VSMCs could act as a calcium chelator agent, decreasing extracellular calcium levels and reducing calcification. In addition, we have previously shown that GRP can directly bind basic calcium phosphate crystals in vitro, and in this way, it may interfere with crystal growth or maturation through a similar mechanism previously proposed for the calcification inhibitory activity of MGP.25 Altogether, our in vitro and ex vivo results show that although both cGRP and ucGRP have calcium mineral affinity, cGRP is a more efficient VC inhibitor.

To uncover additional mechanisms involving GRP in cardiovascular calcification, we further explored the relationship between GRP and MGP. Our results from immunoprecipitation assays strongly suggest that GRP is part of a larger complex containing MGP and fetuin-A, acting at the sites of calcification. A relationship between MGP and fetuin-A has been documented previously, at both circulation13 and tissue levels.12 It is known that VC is a process initiated with the deposition in the ECM of basic calcium phosphate containing EVs derived from VSMCs, forming a nidus of calcification in a process similar to bone mineralization.3,5,9,11,15 It has
been reported that under normal conditions, VSMC-derived EVs do not calcify because of their loading with mineralization inhibitors, such as cMGP and fetuin-A, that act to block mineral nucleation.\textsuperscript{3,5,9,11,15} In this context, it was important to further understand whether GRP was also involved in EV biogenesis or mineralization competence associated with ECM calcification. The presence of GRP was confirmed in EVs released by aortic fragments, with <100-nm size and expressing cluster of differentiation 9, consistent with exosome profiling.\textsuperscript{37,38} Importantly, these EVs were also loaded with MGP and fetuin-A under control conditions, whereas MGP and GRP EV contents dramatically decrease after long-term calcifying conditions, concomitantly with an increase in calcium loading. These results are in agreement with the knowledge that loss of mineralization inhibitors is crucial for promoting EV mineralization.\textsuperscript{5,15} Like previously shown for MGP, GRP is loaded in noncalcifying EVs and absent in high calcium–loaded vesicles, suggesting GRP as an important mineralization inhibitor highly related to the EV mineralization process. Furthermore, it is possible that inhibition of EV calcification capacity might occur constitutively via a GRP–MGP–fetuin-A containing complex, forming a potent inhibitory system. In concordance, an MGP–fetuin-A complex was described in chondrocyte-derived EVs,\textsuperscript{15} and it was proposed that the calcification inhibitory function of the MGP–fetuin-A complex in circulation results from the strong calcium–phosphate binding capacity of these proteins.\textsuperscript{15} This GRP–MGP–fetuin-A complex was detected at the sites of calcification and associated with known EV components, such as annexin A2 and S100A9,\textsuperscript{15,39} and ECM calcification is well known to be initiated with the deposition of basic calcium–phosphate-containing vesicles.\textsuperscript{3,5,15} Considering the characterization of VSMC-derived vesicles loaded with GRP and MGP, it could be suggested that those were mainly exosomes, as previously suggested for MGP-loaded EVs because of identification of proteins from endosomal origin.\textsuperscript{15} Additional functional studies altering endogenous GRP, both by knockdown or gain of function experiments, will be crucial to definitively establish the GRP mechanism of action in VC and will bring new knowledge on the characterization of EV biogenesis, mechanisms of protein loading, and mineralization-related regulation.

From a research point of view, our study was pioneer in establishing GRP as a key factor involved in cardiovascular calcification, providing new data in the field of CAVD. Although it provides new insights into the GRP function as a calcification inhibitor, probably through mechanisms involving the MGP–fetuin-A calcification inhibitory system, acting on the inhibition of EV mineralization capacity, it raises new and important questions requiring further investigation.

From a clinical perspective, our work reinforces the notion that special care should be given to the widely used anticoagulant agents, such as warfarin, acting as vitamin K antagonists\textsuperscript{40} and interfering with MGP and GRP functionalities. Further studies aiming to correlate circulating levels and \(\gamma\)-carboxylation status of GRP with the degree of calcification and disease progression are currently in progress and will enable us to evaluate the potential use of GRP as an additional marker for ectopic calcification.

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\section*{References}
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SUPPLEMENTAL MATERIAL

Detailed methods

Materials

Antibodies against tGRP and cGRP, CTerm-GRP and cGRP pAb, respectively, were provided by GenoGla Diagnostics (Faro, Portugal); anti-ucGRP (ucGRP mAb) was provided by VitaK BV (Maastricht, The Netherlands), anti-cMGP and ucMGP by IDS (Boldon, UK); antibodies against CD68, CD3, CD9, GAPDH and monoclonal fetuin-A were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Polyclonal antibody against fetuin-A was purchased by BioVendor (Karasek, Czech Republic). Secondary goat anti-rabbit-HRP, goat anti-chicken-HRP, rabbit anti-mouse-HRP, and goat anti-mouse-ALP antibodies, primers, fetal bovine serum (FBS) and all other chemical/reagents were purchased from Sigma-Aldrich (St. Luis, MO, USA), unless otherwise stated.

Biological material

Fetal samples were collected from 37-40 gestational weeks' stillbirths at HPP, Cascais Hospital. Five aortas, six aortic valves (further designated as non-CAVD group) and six pulmonary valves were obtained at autopsy from six individuals with a mean age of 52 +/- 10 years (3 females and 3 males). Five of those individuals had no medical history of cardiovascular associated disease and one died with stroke (Supplemental Table I). Calcified aortic valve disease tissues (CAVD) were obtained from patients undergoing aortic valve replacement: eleven tissue samples were used in IHC and gene expression studies, from 6 males and 5 females (age 72 +/- 11 years, Supplemental Table II); and a pool of 12 samples, from 4 males and 8 females (age 71 +/- 12 years) was used for protein extraction. All patients were diagnosed with aortic valve stenosis and two from the group used in IHC and gene expression were additionally diagnosed with coronary artery disease (Supplemental Table II); none of the patients was on coumarin therapy. Fragments of human ascending thoracic aorta were obtained at autopsy and from patients at the time of aortic valve replacement. This study complies with the guidelines for good clinical practice and was performed in accordance with the Declaration of Helsinki and was approved by the ethics committees of all hospitals and institutions involved. Written informed consent was obtained from all participants. Further details of sample processing are provided in the online-only Data Supplement methods.

Sample processing

For gene expression and IHC studies, samples were collected into RNAlater or sterile 4% (w/v) PFA solution, respectively, while for protein extraction, samples were frozen at -20°C until processing. Tissue samples were embedded either in paraffin or glycol methacrylate as described [1] and histologically classified by pathologists. Physiological structures were identified by regular haematoxylin-eosin (HE) staining and mineral deposits were detected with silver nitrate by the von Kossa method, and counterstained with haematoxylin.

RNA extraction and gene expression

Total RNA was extracted from fetal, non-CAVD, CAVD and cultured aortic fragment tissues as described by Chomczynski and Sacchi [2]. RNA integrity was evaluated by
agarose-formaldehyde gel electrophoresis and concentration determined by spectrophotometric analysis at 260 nm using a Nanodrop spectrophotometer (Thermo Scientific).

One microgram of total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) and reverse-transcribed at 37°C with MMLV-RT (Invitrogen, Carlsbad, CA, USA) using a dT-adapter. PCR amplifications for GRP-F1, F5 and F6 splice variants were performed as previously described [3, 4] with SsoFast EvaGreen Supermix (Bio-Rad, Richmond, CA, USA) for 50 cycles and specific primer sets GRP_F1Fw/GRP_F1Rv, GRP_F5_F6Fw/GRP_F5Rv and GRP_F5_F6Fw/GRP_F6Rv, respectively. Ribosomal 18S was used as loading control. A list of all PCR primer sequences is presented in Supplemental Table III.

Quantitative PCR was performed with an iCycler iQ apparatus (Bio-Rad) using 25 ng cDNA and the conditions described above. GRP-F1, F5, F6 and 18S, MGP, OC (osteocalcin), OPN (osteopontin), alpha smooth muscle actin (αSMA), TNFα (tumor necrosis factor alpha), and GAPDH were amplified using primer sets as described in Supplemental Table III. Fluorescence was measured at the end of each extension cycle in the FAM-490 channel and melting profiles of each reaction were performed to check for unspecific product amplification. Levels of gene expression were calculated using the comparative method (ddCt) and normalized using gene expression levels of both GAPDH and 18S housekeeping genes, with the iQ5 software (Bio-Rad); qPCR was performed in duplicates (minimum) and a normalized SD was calculated.

Immunolocalization

Single immunohistochemical staining was performed on paraffin-embedded tissue sections as described elsewhere [1, 4]. Briefly, endogenous peroxidase activity was blocked with 3% (v/v) H2O2 in TBST buffer (TBST: 0.1 mol/L Tris, 0.15 mol/L NaCl, 0.1% (v/v) Triton X-100) for 15 min. Nonspecific antibody binding was blocked with TBT (0.5% (w/v) BSA in TBST) for 1 h at 37°C. Incubations with CTerm-GRP, cGRP pAb and ucGRP mAb were performed as previously described [4]. Primary antibodies were detected using species specific HRP-conjugated secondary antibodies, and 0.025% (w/v) 3,3-diaminobenzidine. Double staining was performed on glycol methacrylate using a combination of cGRP/cMGP (1:200), cGRP/fetuin-A (1:200) and cMGP/fetuin-A primary antibodies. First and second antibodies were detected with ImmPACT NovaRED substrate kit and Vector Blue-ALP substrate (both from Vector laboratories, Burlingame, CA, USA) originating a red and blue colors, respectively, using species specific HRP-conjugated secondary antibodies. Negative controls consisted in the substitution of primary antibody with TBT. Single staining experiments were counterstained with HE. Microphotographs were acquired in a Zeiss AXIOIMAGER Z2 microscope, with an AxioCam ICc3 camera and AxioVision software version 4.8 (Carl Zeiss), at the Light Microscopy Facility, Department of Biomedical Sciences and Medicine, University of Algarve (Faro, Portugal).

Protein extraction

A pool of 12 individual CAV tissue samples was ground to powder in a mortar, and the organic matrix associated proteins were extracted twice (E1 and E2) with RIPA buffer (50mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) for 2 h each extraction at 4°C with rotation. After separation of the remaining mineral phase by centrifugation at 16000 xg for 20 min at 4°C, mineral-bound proteins (M-P) were extracted using a 10-fold excess of 10% (v/v) formic acid for 4 h at 4°C as previously described [5]. Extracted proteins were separated from the insoluble material by centrifugation and then dialyzed at 4°C against 50 mM HCl using 3,500 molecular
weight tubing (SpectraPor 3; Spectrum, Gardena, CA, USA) over 2 days. Aliquots of the dialyzed extracts were freeze-dried until further use.

Sturgeon GRP further referred as cGRP was extracted and purified as previously described [5]. Identification of purified protein, obtained after RP-HPLC purification, was confirmed by N-terminal amino acid sequencing. Bovine MGP (bMGP) was extracted from bovine calcified costal cartilage, obtained from local slaughterhouse, as described [4]. Briefly, the formic acid demineralized fraction containing mineral-bound proteins was dialyzed against 50 mM HCl using 3,500 molecular weight tubing, over 2 days, then freeze-dried and dissolved in 6 M guanidine-HCl, 0.1 M Tris, pH 9.0. Precipitated bMGP, obtained after dialysis against 5 mM ammonium bicarbonate, was dissolved in 6 M guanidine-HCl, 0.1 M Tris, pH 9.0, and purified through RP-HPLC using the conditions described for cGRP purification [5]. Recombinant human GRP further referred as ucGRP was produced and purified through affinity chromatography followed by RP-HPLC, as previously reported [4].

Electrophoresis and western blot

Aliquots of total protein extracts were size-separated on a 4-12% (w/v) gradient polyacrylamide precast gel containing 0.1% (w/v) SDS (NuPage, Invitrogen) and either stained with G-250 coomassie brilliant blue or transferred onto a nitrocellulose membrane (Bio-Rad) as previously described [1]. Detection of tGRP, cGRP, ucGRP, cMGP, ucMGP, CD9, GAPDH and fetuin-A proteins was performed through overnight (O/N) incubation with CTerm-GRP (5 µg/ml), cGRP (1 µg/ml), ucGRP (1 µg/ml), cMGP (1:1000), ucMGP (1:1000), CD9 (1:200), GAPDH (1:200) and fetuin-A, either from Santa Cruz (1:200) or BioVendor (1:500) antibodies, respectively. Immunodetection was achieved using species-specific secondary horseradish peroxidase-conjugated antibodies and Western Lightning Plus-ECL (PerkinElmer Inc., Waltham, MA, USA).

Protein identification by nano-HPLC-MALDI-TOF/TOF

Protein bands were excised manually from SDS-PAGE gel, destained with 25 mM ammonium bicarbonate/50 % (v/v) acetonitrile and dried under vacuum (SpeedVac, Thermo Savant). The dried gel pieces were rehydrated with 25 µL of 10 µg/mL trypsin (Promega V5111) in 50 mM ammonium bicarbonate and digested O/N at 37 °C. Tryptic peptides were extracted from the gel with 10 % formic acid/ 50 % acetonitrile (v/v), dried in a vacuum concentrator, and re-suspended in 10 µL of a 50 % acetonitrile/0.1 % formic acid solution (v/v). Separation of tryptic peptides by nano-HPLC was performed on the module separation Ultimate 3000 (Dionex) using a capillary column (Pepmap100 C18; 3 µm particle size, 0.75 µm internal diameter, 15 cm in length). A gradient of solvent A, [water/acetonitrile/trifluoroacetic acid (98:2:0.05, v/v/v)] to solvent B [water/acetonitrile/trifluoroacetic acid (10:90:0.045, v/v/v)] was used. The separation of 2 µg/µL sample was performed using a linear gradient (5-50 % B for 30 min, 50-70 % B for 10 min and 70-5 % A for 5 min) with a flow rate of 0.3 µL/min. The eluted peptides were mixed with a continuous flow of α-CHCA matrix solution (270 nL/min, 2 mg/mL in 70% ACN/0.1% TFA and internal standard Glu-Fib at 15 fmol) and applied directly on a MALDI plate in 7 seconds fractions using an automatic fraction collector Probot (Dionex).

Mass spectra were obtained on a matrix-assisted laser desorption/ionization–time-of-flight MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems) in the positive ion reflector mode and obtained in the mass range from 700-4500 Da with 900 laser shots. Glu-Fib was used for internal calibration. A data-dependent acquisition method was created to select the 16 most intense peaks in each sample spot (considering 2 spots per fraction) for subsequent tandem mass
spectrometry (MS/MS) data acquisition, excluding those from the matrix, due to trypsin autolysis or acrylamide peaks. A fragmentation voltage of 2kV was used throughout the automated runs. The spectra were processed and analyzed by the T2S (v1.0, Matrix Science) and submitted in Mascot software (v.2.1.0.4, Matrix Science) for protein/peptide identification based on MS/MS data using the following criteria: trypsin as enzyme; a maximum of two missed cleavages; mass tolerances of 30 ppm for peptide precursors, mass tolerance of 0.3 Da was set for fragment ions. Protein identifications based on MS/MS data were considered as reliable when the Mascot ion score confidence level for each individual peptide was higher than 32. The local FDR was calculated by searching the spectra against SwissProt (*Homo sapiens*, release date 01052013) decoy (random) database.

**Aortic segments ex vivo assay**

Previously described models of aortic rings [6-8] were adapted. After adventitia removal by careful dissection, the aortas were cut into small fragments of 2-to 3 mm squares and cultured for 14 days at 37°C and 5% CO₂ atmosphere, with media changes every 2 days. Aortic segments were cultured either in control conditions (C: DMEM/ 10% FBS/ 1% PS/ 1% L-Gln) or in high P and Ca conditions (Ca + P: C supplemented with 3.6 mM Ca and 1.6 mM P). Testing proteins were added to Ca + P media at the concentrations of 250 ng/ml and 500 ng/ml for ucGRP and cGRP, and 500 ng/ml for bMGP and bFetuin-A. ucGRP, cGRP and bMGP were dialyzed against milliQ water prior experiments, and protein quantification estimated through measurement of absorbance at 280 nm, based on the assumption that $E_{0.1\%1cm} = 1.0$. Fetuin-A was dissolved in milliQ water as manufacturer’s recommendations. Solubility and stability of ucGRP, cGRP and bMGP in water was determined by SDS-PAGE analysis immediately after dialysis and after 24 h at RT. After 14 days the aortic segments were washed 3× in PBS and 3× in water, dried and weighted. Dried tissues were completely digested with nitric acid and Ca content was measured by O-cresolphthalein complexone chemistry using a colorimetric assay (Randox Laboratories, UK) according to manufacturer’s recommendations, and normalized to aortic segments dry weight. ucGRP and cGRP proteins were tested in three independent experiments and bMGP and bFetuin-A were tested in two experiments. Each of the three experiments was performed at least in duplicates with a minimum of two fragments per duplicate.

For gene expression and histology, experiments were performed as described and fragments were either immediately used for RNA extraction or collected into 4% (w/v) PFA solution, respectively, after washing. Gene expression and von Kossa staining were performed as described above.

**Extracellular vesicles isolation and protein analysis**

Aortic fragments were cultured under C and Ca + P conditions for 6 days as described above, with the exception that FBS was depleted of EVs through ultracentrifugation at 100,000 xg for 4 h at 4°C. Media were collected at days 3 and 6, and EVs size fractionation was performed by differential centrifugation at 2000 xg for 15 min, 10,000 xg for 1 h, and 100,000 xg for 2 h, at 4°C, after filtration through 0.2 μm. Sedimented EVs were washed with PBS, pelleted, and resuspended in PBS. Half of those vesicles were used for protein extraction with RIPA buffer while the other half was used for scanning electron microscopy (SEM) as described below. Aortic segments total protein extracts (tExt) were obtained by extraction with RIPA buffer. Protein quantification was determined with MicroBCA protein assay kit (Pierce), and 10 μg of protein were analyzed by WB as described above. Calcium quantification was obtained using O-
cresolphthalein complexone colorimetric assay and normalized to total protein content. Four independent experiments were performed using aortic tissues from 4 individuals. Samples from each time point per condition were pooled from the 4 experiments and analyzed at least in duplicates.

**Scanning Electron Microscopy (SEM)**

EVs obtained after centrifugation at 100,000 xg and resuspended in PBS were fixed in 3.7% glutaraldehyde, washed twice with PBS and dehydrated with an ascending sequence of ethanol (40%, 60%, 80% and 98%). Dehydrated EVs were applied into a silica substrate and dried. Samples were examined in a scanning electron microscope (Hitachi S-2400) with elemental analysis (Bruker) after chromium sputtering.

**Immunoprecipitation**

Immunoprecipitation was performed according to a method previously used to detect MGP from blood [9], using protein A sepharose with cross-linked CTerm-GRP, cMGP and rabbit IgG antibodies. Briefly, 100 µg of each antibody were incubated for 1 h at RT with 500 mg of protein A-sepharose (GE Healthcare, Waukesha, WI, USA). After incubation, antibodies were cross-linked with dimethyl pimelimidate and the remaining groups were quenched with ethanolamine. Aliquots of M-P extract were resuspended for 4 h in 10 mM Tris-HCl pH 7.5 and then centrifuged for 20 min at 16000 xg and 4°C, to remove eventual non-resuspended proteins. 48 h conditioned media of HEK293T cells transfected with GRP-F1-mKate2 fusion protein and pmKate2-N vector expressing mkate2-only, obtained as previously described [3], were dialyzed against 10 mM Tris-HCl pH 7.5. M-P extract and conditioned media in 10 mM Tris HCl pH 7.5 were added to the protein A-antibody complexes and incubated with rotation O/N for binding of target proteins. After extensive washes, elution was performed with 100 mM glycine pH 2.5 and eluted proteins were either analyzed by staining with CBB for protein identification or WB for specific protein detection.

**Statistical analysis**

Data are presented as mean ± standard error (SE). Student’s t-test was used for comparison between two groups. The non-parametric Mann-Whitney U test was performed to confirm the difference of two group comparison. For more than two groups significance was determined using one-way analysis of variance (ANOVA) with comparison between groups by Dunnett test. Statistical significance was defined as \( P\leq0.05 \) (*), \( P\leq0.005 \) (**), and \( P\leq0.0005 \) (***)

**Supplemental References**


Supplemental Figure I: GRP-F1 is the main transcript expressed in adult cardiovascular tissues. Qualitative gene expression analysis of GRP splice variants F1, F5 and F6 in fetal (heart (Ht); aortic valve (AV); aorta (Ao); umbilical artery (UA); umbilical vein (UV)) and adult (heart (Ht); aorta (Ao); aortic valve (AV); pulmonary valve (PV)) tissues, showing the predominance of GRP-F1 transcript in all tissues analyzed. 18S was used as loading control for sample integrity.
Supplemental Figure II: A, c/ucGRP accumulation patterns are similar to tGRP in control non-calcified and calcified aorta and aortic valve tissues. A, Representative immunodetection of c and ucGRP protein forms by IHC using the conformation-specific c/ucGRP antibodies. VK, von Kossa staining of consecutive tissue sections. B, Representative negative controls performed by omitting primary antibodies in consecutive tissue sections. Positive signal in brown and counterstaining with HE. Scale bar represents 100 μm.
Supplemental Figure III: Representative histopathological features of CAVD samples by staining with haematoxylin-eosin, showing heterogeneous phenotype. The degree of the normal trilaminar stratification was variable, ranging from normal structure (A) to highly disorganized ECM (B). Leaflets thickness and cellularity were variable in affected tissue, with either VICs disarray forming clusters (C) or dense regions of almost acellular ECM (D). Detected calcifications ranged from early mineralization characterized by disperse spots (E) to localize and dense areas of mineral in the fibrosa layer (F), or massive areas of calcification occupying almost all leaflet (G), or within atheromas (H). Scale bar represents 100 μm.
Supplemental Figure IV: GRP is highly accumulated at regions of massive mineral accumulation with similar c/ucGRP patterns. IHC staining was performed as described in Figure 2 and Supplemental Figure 2 legends and shows high accumulation of cGRP (B) and ucGRP (C) at regions of massive mineral deposition (von Kossa), with similar patterns. Positive signal in brown and counterstaining with HE. Scale bar represents 100 μm.
Supplemental Figure V: Representative negative controls performed by omitting primary antibodies, in consecutive tissue sections presented in Figure 2. A, negative control of tissues presented in panels A-C of Figure 2. B, negative control of tissues in panels D-F of Figure 2. C, negative control of tissues in panels G-I of Figure 2. D, negative control of tissues in panels J-O of Figure 2. Tissues were counterstaining with HE. Scale bar represents 100 μm.
Supplemental Figure VI: GRP-F5 and F6 are barely detected in the majority of the eleven CAVD samples analyzed. 18S was used as loading control for sample integrity.
Supplemental Figure VII: Experimental duplicates of assay described in Figure 5B legend, showing the effect of carboxylated (cGRP) and noncarboxylated GRP (ucGRP) in an *ex vivo* model of aortic tissue calcification. A, HE histological characterization of aortic segments cultured for 14 days in control, Ca + P, and Ca + P supplemented with cGRP and ucGRP conditions, showing an intact structure of the vessel with apparent VSMCs phenotype. Darker purple spots in Ca + P and ucGRP indicate mineral deposition. Scale bar represents 100 μm. B, Ca + P calcifying media induce significant increase in Ca relative to control (P<0.01), and treatments with 500 ng/ml of cGRP, bMGP and bFet-A result in similar 28.2% reduction in Ca relative to Ca + P condition (P<0.05). No significant effects are observed with ucGRP neither with 250 ng/ml cGRP. C, Ca + P calcifying media induce significant increase in Ca relative to control (P<0.01), and treatments with 500 ng/ml of cGRP result in 50% reduction in Ca relative to Ca + P condition (P<0.05). No significant effects are observed with ucGRP neither with 250 ng/ml cGRP. SD was calculated (n=2) and ANOVA with comparison between groups by Dunnett test was performed.
**Supplemental Figure VIII:** Patterning of GRP and MGP prior to IP assays. M-P was resuspended in 10 mM Tris-HCl, pH 7.5 buffer, and centrifuged to remove eventual insoluble components (Ins). Both Ins and soluble (Sol) fractions were analyzed by WB using CTerm-GRP, cGRP and cMGP antibodies, and indicate that only part of the initially resuspended proteins (Figure 4B) are in the soluble fraction, and that the predominant GRP soluble protein form is detected with the cGRP antibody as a 15 kDa band.
Supplemental Figure IX: Cross-reactivity test of cMGP antibody to GRP detection. WB analysis with CTerm-GRP antibody of IP assays performed as described in Figure 6B legend, using conditioned media of transiently transfected HEK293T cells with GRP-F1-mkate2 fusion protein and respective control, the empty plasmid pmkate2-N. GRP is detected in IP-GRP assay from GRP-F1-mkate2 conditioned media but not in the IP-MGP assay from GRP-F1-mkate2 media, indicating that cMGP antibody cannot pull-down GRP-F1-mkate2 fusion protein. Arrow indicates positive GRP detection with CTerm-GRP antibody at expected size of 43 kDa. Relevant molecular weight markers are indicated (kDa) on the right side of the panel.
### Supplemental Table I. Post mortem cardiovascular samples used for gene expression and IHC analysis

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*Samples were collected at the Histopathology department, Algarve Medical Centre and National Institute of Legal Medicine and Forensic Sciences, Public Institute: At, aorta, AV, aortic valve, PV, pulmonary valve.
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*Samples collected at Hospital Santa Cruz, Lisbon*
**Supplemental Table III: Primers used for PCR analysis**

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Supplemental Table IV. GRP (UCMA), MGP and fetuin-A proteins identified by LC-MALDI-MS/MS in calcified aortic valves M-P extract

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*Bands 1, 2, 3, 4, 5 and 6 according to Figure 4A.
### Supplemental Table V: Additional proteins identified by LC-MALDI-MS/MS in calcified aortic valves M-P extract

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<th>ppm</th>
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* Bands 1, 2, 3, 4, 5 and 6 according to Figure 4A.