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Mitral Valve Endothelial Cells Secrete Osteoprotegerin During Endothelial Mesenchymal Transition

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Keywords

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Introduction

Mitral valve prolapse (MVP) is a common valve pathology and has an estimated prevalence of 2% to 3% in the general population, affecting more than 176 million people worldwide[1]. Despite the pathology was first described in the late 1800s[1], no major risk factor has been identified[2] and the initiating mechanisms driving MVP are not fully understood. To date, the diagnostic gold standard[1] is two dimensional (2D) echocardiography, whereas surgical intervention is the therapeutic choice when the prolapse causes severe regurgitation and symptoms[3].

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Disclosures
None.

Myxomatous mitral valve degeneration is the primary cause of MVP. The leaflets lose their well-organized structure showing a characteristic thickening, due to proteoglycans accumulation, structural alteration of collagen and thinning or elongation of the chordae tendinae[4]. Valve interstitial cells (VIC), within the valve layers, maintain the normal architecture of the leaflets by regulating extracellular matrix production and degradation[1]. The disruption of this tight regulation and the constitutive activation of VICs have been linked to MVP, as well as the endothelial to mesenchymal transition (EndMT)[5, 6]. Diseased mitral valve display prominent VIC activation with overexpression of smooth muscle actin (SMA), bone morphogenetic proteins (BMP2 and 4), metalloproteinases (MMPs)[6], biglycan (BGN) and versican (VCAN)[7]. In addition, human isolated valve endothelial cells (VEC)[5] exhibited evidence of EndMT, as well as ovine and diseased human[8] mitral valves. Recently, Shapero et al[9] showed that the cross talk between endothelial and interstitial cells is essential for normal cell homeostasis and that inhibits VEC EndMT and VIC activation. In this study, we focus our attention on osteoprotegerin (OPG), a soluble glycoprotein belonging to the tumour necrosis factor (TNF) family[10], and its receptors, the syndecan family (SDC1, 2, 3, 4) that are heparan sulphate proteoglycans involved in cell proliferation, differentiation, adhesion and migration[11].

In this study we have investigated the involvement of OPG in EndMT and its effect(s) on the cell population within the heart valve leaflets. We also provide the proof of concept that plasma OPG may represent a new circulating marker to identify MVP patients. To achieve these goals we implemented an *in vitro* system forcing EndMT of isolated human VECs.

Materials and Methods

Patient population

The study was approved by Institutional Review Board of Centro Cardiologico Monzino, IRCCS. Written informed consent to participate to this observational study was obtained from patients undergoing posterior mitral valve repair (MVR) and control subjects. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Preoperative inclusion criteria were the need for elective, isolated surgical procedure, over 18 year of age, ejection fraction of > 30%, normal sinus rhythm and no history of atrial fibrillation. Exclusion criteria were: presence of bicuspid aortic valve, premature menopause and/or osteoporosis, prior aortic or mitral valve surgery, rheumatic heart disease, endocarditis, active malignancy, chronic liver failure, calcium regulation disorders (hyperparathyroidism, hyperthyroidism, and hypothyroidism), and chronic or acute inflammatory states (sepsis, autoimmune disease, and inflammatory bowel disease).

Fifty-three patients, who were candidates for mitral valve replacement (MVR) due to posterior mitral valve prolapse (MVP) with severe regurgitation, were enrolled in the study. Patients characteristic are listed in Supplemental Table 1 (n = 28, blood collection) and Supplemental Table 2 (n = 25, mitral valve cell isolation). Blood samples were obtained the day before surgery and mitral valve specimens were collected in saline solution immediately after mitral valve resection. Blood from control subjects (over 18 years of age) with normal sinus rhythm, no electrocardiographic alterations and no history of atrial fibrillation

(Controls, n = 29) were screened from those attending the Clinic for Global control of Cardiovascular Risk at Centro Cardiologico Monzino, IRCCS. Three healthy mitral valve leaflets were obtained from the Penn Cardiac Bioregistry at University of Pennsylvania. Control subjects had prevalence of cardiovascular risk factors similar to that of MVP patients, but they were clinically free from cardiovascular disease (Supplemental Table 1).

Isolation and treatments of valve endothelial and interstitial cells

Isolation of both mitral endothelial (VEC) and interstitial cells (VIC) was performed using a modified method described by Poggio et al.[12]. Briefly, mitral leaflets were placed in 2 mg/mL type II collagenase (Worthington Biochemical Corp.) in Advanced Dulbecco's modified Eagle's medium (DMEM – Life Technologies) containing 10% fetal bovine serum (FBS) 1% Penicillin, 1% Streptomycin solution and incubated for 20 min at 37°C. Loosened endothelial layer was removed by wiping the leaflet surfaces with sterile cotton swabs. The resulting cells were washed and isolated using Dynabeads® conjugated with platelet endothelial cell adhesion molecule (CD31 – Life Technologies), an endothelial marker, and cultured in supplemented M200 media (Life Technologies) on 0.1% gelatine (Sigma-Aldrich) coated tissue culture plate. Tissues were then finely minced and dissociated with type II collagenase (1 mg/mL) and hyaluronidase (100 U/mL) overnight at 37°C. The resulting VICs were seeded in tissue culture plates in supplemented advanced DMEM media (Life Technologies). All the experiments were performed on cultured cells between their second and fifth passages. Cells were left untreated or treated with 10 mM β -glycerophosphate and 50 mg/mL ascorbic acid (β GAA) for 6 and 12 days or 50 ng/mL osteoprotegerin (OPG) for 6 and 12 days unless specified elsewhere.

Reverse transcription, Real-Time PCR and digital PCR

Extraction of RNA was performed from VECs and VICs using the Total RNA Purification Plus Kit (Norgen Biotek Corp.). RNA was quantified using Nanodrop and used for two steps PCR amplification with TaqMan Reverse Transcription Reagent kit (Life Technologies). Total RNA (1 μ g) was converted into cDNA. Real Time PCR (qPCR) was performed on ABI Prism 7900 HT (Applied Biosystems), according to the manufacturer's instructions and analysis were performed using software SDS2.4 (Life Technologies). Digital PCR (dPCR) was performed on a QuantStudio™ 3D Digital PCR System platform composed by the QuantStudio™ 3D Instrument, the Dual Flat Block GeneAmp® PCR System 9700 and the QuantStudio™ 3D Digital PCR Chip Loader (Life Technologies). dPCR was performed according to the manufactures' instructions and analysis were executed with QuantStudio® 3D AnalysisSuite™ (Life Technologies). Primers labelled with FAM® dyes were used to evaluate the expression of target genes, while for housekeeping genes primers labelled with VIC® dye were implemented. For normalization we used glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein large P0 (RPLP0) gene expression levels and the primers used are listed in Supplemental Table 3

Western Blot Analysis

Protein expression was evaluated by western blot using specific antibodies against pSDC4 (Santa Cruz Biotechnology, Inc - cat. sc-16852 - observed band molecular weight (MW) 24 kDa), SDC4 (Santa Cruz Biotechnology, Inc - cat. sc-12766 - observed band MW 24 kDa),

pERK (Cell Signaling Technology - cat. #4370 - observed band MW 42/44 kDa), ERK (Santa Cruz Biotechnology, Inc - cat. sc-135900 - observed band MW 44 kDa), MMP9 (Santa Cruz Biotechnology, Inc - cat. sc-10737 - observed band MW 89/92 kDa) and GAPDH (Cell Signaling Technology - cat. #5174 - observed band MW 37 kDa) following standard protocols.

Collagen measurements

Total soluble collagen in cell culture supernatants was quantified by Sircol Soluble Collagen Assay Kit according to the manufacturer's instructions (Biocolor).

Matrix Metalloproteinase Zymography

Matrix metalloproteinase 9 activity was detected by gelatin zymography using cell culture supernatant collected after OPG treatment, following manufacturer's instructions (Life Technologies).

Staining and flow cytometry analysis

Flow cytometry analysis was performed on 1% paraformaldehyde fixed cells. For intracellular staining cells were permeabilized with cold methanol for 30 minutes at 4°C followed by incubation with specific antibody TRITC-conjugated. A total of 10,000 events per sample were acquired on a BD FACSCalibur. Cytometer performances were checked by daily running BD Cytometer Setup and Tracking Beads. Data are reported as Mean Fluorescence Intensities (arbitrary units), calculated from fluorescence histograms for population or as percentage of cells positive for the analysed antigens. All the data were analysed with FlowJo vX.0.7 Software.

Osteoprotegerin quantification

Blood was collected prior to surgery from the patients and while fasting from the control subjects. Blood was processed to obtain plasma then stored at -80°C until the assay was performed. Plasma analysis for OPG levels was conducted using enzyme-linked immunosorbent assay (ELISA; DuoSet, R&D Systems - cat. DY805) following manufacturer instructions.

Reactive oxygen species evaluation

Cells were grown to sub-confluency in complete medium and incubated with or without OPG for the mentioned times. At the end of the treatments CellROX® Detection Reagent (Life Technologies), at a final concentration of 500 nM, were added for 60 minutes and incubated at 37°C, 5% CO₂, protected from light. The samples were analysed on a BD FACSCalibur following manufacturer's instructions.

Migration assay

The directional cell migration assay was performed using 24-well plate with culture inserts (IBIDI). Briefly, VECs and VICs were seeded in the inserts of the 24-well system and allow adhering overnight. Inserts were removed to create a cell-free gap of approximately 500 µm, and cells were allowed to migrate for 24 h at 37°C and 5% CO₂ in the presence of OPG or

in medium alone. Every 3 hours pictures of the gaps were taken with ZEISS ApoTome and ImageJ (Version 1.49m – National Institute of Health) was used to analyse the area free of cells.

Statistical analysis

Data were analysed using IBM SPSS statistic software (version 22) and Graph Pad Prism software (version 6). Continuous variables were expressed as mean $\pm$ standard error (SEM). For comparisons of continuous and categorical data, we used the parametric T-test and the Pearson Chi-square test, respectively. To determine the specificity and sensitivity of OPG quantification, area under the receiver operating characteristic curve (AUC of ROC curve) was calculated using Graph Pad Prism software (version 6). A value of $p < 0.05$ was considered to be statistically significant.

Results

Human mitral valve endothelial and interstitial cell characterization

Endothelial cells (VECs) and interstitial cells (VICs) were isolated using a brief collagenase procedure (see method section) from the valve leaflets of 25 patients affected by posterior mitral valve prolapse (MVP) and undergoing surgical repair. VECs showed classical cobblestone morphology at phase contrast microscopy (Figure 1A). In addition, immunofluorescence staining revealed expression of platelet endothelial cell adhesion molecule (CD31) at the cell-cell borders, vimentin (VIM) throughout the cytoplasm but not α -smooth muscle actin (SMA) (Figure 1A). VICs had a marked spindle-shaped morphology and immunofluorescence staining showed the presence of SMA (representing activated interstitial cells), VIM expression and no sign of endothelial markers (Figure 1B).

Human mitral valve endothelial cells undergo endothelial to mesenchymal transition

To evaluate the role of endothelial to mesenchymal transition (EndMT) on isolated MVP cells, we implemented an *in vitro* system forcing VECs to undergo EndMT. Cells were treated with β -glycerophosphate and ascorbic acid (β GAA) for 6 and 12 days. EndMT was confirmed by quantitative PCR (qPCR), flow cytometry and morphology analysis (Figure 2). After 6 days of β GAA treatment, RNA analysis showed a significant upregulation of SMA and collagen III (Col3A1) by 4.5 ± 0.9 and 3.0 ± 0.6 fold ($p < 0.05$), respectively (Figure 2A). We also noticed a significant upregulation of bone morphogenetic protein 4 (BMP4 – a member of the transforming growth factor beta superfamily) and collagen I (Col1A1) by 3.0 ± 0.7 and 10.8 ± 3.2 fold ($p < 0.05$), respectively after 12 days of treatment (Figure 2B). Flow cytometry analysis showed a significantly overexpression of SMA on VECs treated with β GAA (Figure 2C). In addition, phase contrast microscopy confirmed morphological changes from cobblestone to spindle-shape (Figure 2D).

Endothelial to mesenchymal transition induces osteoprotegerin expression and secretion

Western blot analysis of OPG was performed using whole extract of three control and three MVP leaflets and revealed a significant OPG overexpression ($p < 0.05$) in prolapsed tissue when compared to healthy tissue (Figure 3A and B).

To determine VEC ability to express and secrete osteoprotegerin (OPG) during EndMT, VECs were exposed to β GAA. At both time points (6 and 12 days) VECs showed a significant upregulation of OPG mRNA levels by 1.9 ± 0.4 and 4.1 ± 1.3 fold ($p < 0.05$), respectively (Figure 3C). In addition, during the first 6 days of treatment VECs undergoing EndMT secreted more OPG compared to untreated cells (2369 ± 564.7 pg/ μ g of total protein vs. 923.5 ± 261.1 pg/ μ g of total protein, respectively; $p < 0.05$ – Figure 3D). OPG involvement has never been described during EndMT before. We therefore tested whether OPG could have a direct effect on endothelial cells, by triggering an autocrine response in VECs. First, we analysed the presence of OPG receptors, syndecan-1, -2, -3 and -4 (SDC1, SDC2, SDC3 and SDC4) on nine different VEC preparations. We evaluate SDC levels by digital PCR (dPCR) in relation to RPLP0. As shown in Figure 3E all the four SDCs were expressed in VECs. The expression levels were: SDC1 $2.07 \pm 0.62\%$ with a precision of 2.97%; SDC2 $0.41 \pm 0.05\%$ with a precision of 5.49%; SDC3 $2.42 \pm 0.50\%$ with a precision of 3.50%; and SDC4 1.03 ± 0.23 with a precision of 4.85%. The data used for the absolute quantification are listed in Supplemental Table 4.

Osteoprotegerin exacerbate mitral valve prolapse phenotype

Considering the presence of syndecan receptors, we investigated if OPG treatments could affect proteins already known to be involved in mitral valve degeneration[6]. First of all, we checked expression of known OPG downstream partners, such as integrin alpha V and beta 3[13]. OPG treatments increased significantly both integrins by 4.4 ± 0.9 and 20.9 ± 7.5 , respectively (Figure 4A; $p < 0.05$). Secondly, the incubation of cells with OPG, for 12 days, significantly increased mRNA levels of Col1A1 ($+4.0 \pm 0.6$; $p < 0.001$), Col3A1 ($+3.0 \pm 0.6$; $p < 0.01$), BMP4 ($+2.4 \pm 0.7$; $p < 0.05$), SMA ($+1.6 \pm 0.2$; $p < 0.05$), fibroblastic specific protein 1 (FSP1, $+1.9 \pm 0.3$; $p < 0.05$), and extracellular matrix proteoglycan versican (VCAN, $+3.8 \pm 0.2$; $p < 0.001$) (Figure 4B). In addition, we evaluated total collagen production by colorimetric assay. After 48 hours of OPG, treated cells secreted more collagen compared to untreated cells (13.4 ± 0.7 vs. 11.3 ± 0.4 ng of collagen/ μ g of total protein, respectively; $p < 0.05$) (Figure 4C). After all, we also detected higher metalloproteinases 9 (MMP9) activity and expression after OPG treatment (Figure 4D and E).

To evaluate if OPG acts through syndecan receptors, we analysed SDC4 phosphorylation (serine 179 - pSDC4) upon OPG treatment. This residue is highly conserved among the family[14] and once it is phosphorylated the signalling cascade is shutdown[15]. Upon OPG treatment, there was a decrement in pSDC4 at 5 and 15 minutes (Figure 4F).

To assess further the OPG implications, we inhibited all the syndecans with Heparinase I (Hep I). Hep I is known to inactivate the syndecans by removing heparan sulphates present on the extracellular portion of these receptors. Hep I pre-treatment completely inhibited the dephosphorylation of SDC4 (Figure 4G - top panels). The apparent loss of SDC4 could be due to protein destabilization after Hep I pre-treatment or the specific antibody used may require heparan sulphate to completely bind SDC4. Finally, since extracellular signal-regulated kinase 1 and 2 (ERK) phosphorylation was previously documented upon OPG treatment[13, 16], we analysed if Hep I could also block this phosphorylation. As shown in Figure 4G - bottom panels, Hep I pre-treatment were able to block ERK activation. In

addition, we evaluated Hep I specificity on SDCs. TGF β -1 treatment, a known ERK activator, in the presence of Hep I pre-treatment did not affect ERK phosphorylation (Supplemental Figure 1).

Osteoprotegerin induces ROS and cell migration in endothelial cells

VEC behaviour after incubation with OPG was assessed in terms of reactive oxygen species (ROS) generation, percentage of cell death and cell migratory ability. Flow cytometry analysis showed that OPG incubated with VECs for 24 hours induced an increased ROS production (+39% – Figure 5A) together with a significant increment in cell death ($p < 0.05$ – Figure 5B). Since it has been shown that mitral valve degeneration might start with EndMT and that transforming cells acquire migratory properties[9], we performed a migration assay using isolated VECs. The percentage of the area closed at 24 hours by the treated cells was $43.3 \pm 3.9\%$, compared to $21.8 \pm 2.6\%$ for the untreated cells ($p < 0.001$ – Figure 5C and D). These results indicate that VECs exposed to OPG are able to close the wound faster than the untreated cells. Notably, VECs incubated with OPG started to migrate faster than the controls at 6 hours ($p < 0.05$ – Figure 5C and D).

The percentage of the area closed at 24 hours by cells pre-treated with Hep I and OPG was $16.5 \pm 3.6\%$, compared to $43.0 \pm 3.1\%$ for cells treated only with OPG ($p = 0.001$ – Figure 5E and F). The loss of migratory properties by VECs incubated with heparinase I indicated that cell migration induced by OPG occurred throughout the activation of the syndecan receptors.

Osteoprotegerin induces proliferation and proteoglycan overexpression in interstitial cells

Valvular interstitial cells represent another cell population involved in the mitral valve degeneration[6]. Therefore, we analysed the presence of OPG receptors (SDC1, SDC2, SDC3 and SDC4) on three different VIC preparations. We evaluate SDC levels by digital PCR (dPCR) in relation to RPLP0. As shown in Figure 6A all the four SDCs were expressed in VICs. The expression levels were: SDC1 $0.64 \pm 0.34\%$ with a precision of 11.74%; SDC2 $11.43 \pm 1.53\%$ with a precision of 2.56%; SDC3 $7.31 \pm 1.28\%$ with a precision of 3.61%; and SDC4 4.08 ± 1.04 with a precision of 3.80%. The data used for the absolute quantification are listed in Supplemental Table 5.

It has been shown by Candido et al.[17] that OPG increases smooth muscle cell proliferation but there is no evidence of its effects on interstitial cells isolated from mitral valves. OPG, incubated with cells for 24 hours, significantly increased proliferation of VICs (22.4% increment; p value < 0.05 – Figure 6D), whereas OPG treatment did not increase the migratory ability of the cells (Supplemental Figure 2). Finally, the incubation of cells with OPG, for 12 days, significantly increased mRNA levels of biglycan (BGN, $+1.9 \pm 0.3$; $p < 0.001$), VCAN ($+1.5 \pm 0.2$; $p < 0.05$), BMP4 ($+1.6 \pm 0.1$; $p < 0.05$), metalloproteinase 2 (MMP2, $+1.6 \pm 0.1$; $p < 0.01$), SMA ($+1.6 \pm 0.2$; $p < 0.05$) and OPG ($+1.5 \pm 0.2$; $p < 0.05$ – Figure 6B). The upregulation of all these proteins corroborated the hypothesis that OPG is involved in extracellular matrix changes during the progression of mitral valve degeneration. In addition, we evaluated total collagen production by colorimetric assay. After 48 hours of OPG, treated cells secreted more collagen compared to untreated cells (18.5 ± 0.4 vs. 16.4 ± 0.3 ng of collagen/ μ g of total protein, respectively; $p < 0.05$) (Figure 6C).

Circulating plasma levels of osteoprotegerin identify patients with mitral valve prolapse in a surgical patient population independently of age, sex, and common cardiovascular comorbidities

Since OPG has a direct effect on endothelial cells, we evaluated its plasma levels. Moreover, we assess if OPG could be a potential circulating marker for posterior mitral valve prolapse. To achieve this goal we measured OPG plasma levels in 57 subjects (29 control subjects and 28 MVP patients – Supplemental Table 1) matching the inclusion criteria reported in the methods section. Control subjects were selected with similar cardiovascular risk factor profile of MVP patients. In addition, because OPG plasma levels directly increase with age (Supplemental Figure 3) our analysis was performed using a model adjusted for age.

Patients with posterior MVP had significantly higher OPG levels compared to control subjects (1953 ± 127.5 vs. 1109 ± 45.3 pg/mL, respectively; $p < 0.0001$ – Figure 7B), with a cut-off equal to 1246 pg/mL of OPG plasma level the maximized area under the curve (AUC) being 0.92, with sensitivity of 89.3%, specificity of 72.4% and likelihood ratio of 3.24 (Figure 7B).

Discussion

Patients with mitral valve prolapse (MVP) and severe regurgitation have no pharmacological treatment choice since the only successful treatment of their disease is surgical repair or replacement of the mitral valve. It is known that a tight cross talk between valve endothelial (VEC) and interstitial cells (VIC) regulates cellular homeostasis by inhibiting VEC endothelial to mesenchymal transition (EndMT) and VIC activation[9].

Data reported in this study corroborate that EndMT is involved in the pathogenesis of mitral valve degeneration and show that osteoprotegerin (OPG) might represent a novel player in the progression of this disease. OPG regulates bone metabolism throughout paracrine signalling between osteoblast and osteoclast[13]. OPG interacts mainly with receptor activator of nuclear factor kappa-B ligand (RANKL) and tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)[13]. Several studies already described the relationship of OPG with coronary artery syndromes[18–20], aortic valve stenosis[21–23], myocardial infarction[24] and cardiovascular postoperative outcome[25] focusing only on the TNF superfamily. In addition, a recent review article consolidate the overall knowledge regarding OPG/RANK/TRAIL in cardiometabolic disorders[26]. This comprehensive review analysed the possibility of OPG to be considered as a novel biomarker of complications and severity, but extensive research are needed to fully assess OPG usefulness[26].

Interestingly, the OPG heparin-binding domain has the potential to interact with heparan sulphate proteoglycans, such as the syndecan family[10], regulating cellular apoptosis[10], migration and proliferation[17], intracellular levels of reactive oxygen species (ROS)[10] and metalloproteinases secretion[27]. We explored the interaction between OPG and syndecans in isolated human cells from MVP patients in order to give new insights in the progression of this common disease. The impossibility of obtaining healthy mitral valve specimens for cell isolation forced us to use only cells isolated from the posterior portion of

the diseased mitral valves for the *in vitro* experiments, while we did not have any problem with blood collection from control subjects.

Roberts et al.[28] showed that the formation of new fibrous tissue is the cause of the posterior leaflet thickening in MVP. The newly formed tissue was consequent to a deregulation in the extracellular matrix (ECM) homeostasis. In addition, it is well known that metalloproteinases (MMPs)[29, 30], bone morphogenetic protein 4 (BMP4) and VIC over proliferation[6] are involved in the progression of MVP inducing the thickening of the mitral leaflets. Besides MMPs, growing evidence suggests lysosomal cysteine proteases, generally known as cathepsins, play an important role in cardiac wall and valve diseases[31]. These enzymes have been discovered to be secreted into the extracellular space and to be overexpressed in valve tissues[30]. In addition, there is evidence that pharmacological cathepsin inhibition have a protective role in cardiovascular disease animal models[32]. Finally, cathepsin K (CatK) has been linked to endothelial cell invasion, proliferation and tube formation[33]. In this context, OPG and CatK have been described to be involved in vascular remodelling[34], leaving this intriguing possible association to be investigated in future studies addressing the MVP issue.

Here we show for the first time that MVP leaflets have high levels of OPG and endothelial cells increase, during EndMT, production and secretion of OPG. As a result, OPG autocrine effects strengthen EndMT induction, collagen production, MMP9 overexpression and its paracrine effects increase VICs proliferation with enhanced production of ECM components. In summary, OPG interferes with the correct valve endothelial function, increases collagen deposition, overexpresses BMP4, proteoglycan and MMPs. All these effects suggest a direct involvement of OPG in MVP pathogenesis. Additionally, higher concentrations of OPG are found in plasma of MPV patients compared to control subjects, indicating a possible role as a novel circulating marker. Unfortunately, the lack of OPG specificity undermines the purpose to use only this molecule to identify MVP patients. However, the combination with other circulating markers combined in multivariate regression model could improve OPG specificity. An example could be represented by oxidative stress, already documented to have the highest value in patients with MVP compared to coronary bypass and aortic stenosis patients[35].

Finally, considering the prevalence of this disease (over 6 % 65 years old[36]), the aging population and that the gold standard to diagnose MVP is based on echocardiographic evaluation[1] after medical referral due to symptom occurrence, the health system costs may rise if no pharmacological treatment or cheaper diagnosis will be identified. Thus, the study of new circulating markers and new molecular players involved in MVP progression is essential. Although larger group size and further prospective studies are needed, our data provide evidence of OPG involvement in mitral valve degeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- MVP leaflets showed higher levels of OPG than control subjects.
- Endothelial-to-mesenchymal transition increase OPG production and secretion.
- OPG induced endothelial cell migration and reactive oxygen species production.
- OPG induced VIC proliferation and collagen production.
- OPG plasma levels were highly increased in MVP patients than control subjects.

Figure 1. Human mitral valve endothelial and interstitial cell characterization

(A–B) Phase contrast imaging and immunofluorescence staining for platelet endothelial cell adhesion molecule (CD31 – Red), smooth muscle actin (SMA – Green), vimentin (VIM – Green) and 4',6-diamidino-2-phenylindole (DAPI, for nuclei detection – Blue), in human isolated valve endothelial (VEC) and interstitial cells (VIC) from mitral valve prolapse patients. Scale bar, 20 μ m.

Figure 2. Endothelial to mesenchymal transition of human mitral valve endothelial cells (A–B) Quantitative PCR (qPCR) of smooth muscle actin (SMA), bone morphogenetic protein 4 (BMP4), collagen I (Col1A1) and collagen III (Col3A1) of valve endothelial cells (VEC) in absence or in presence of β -glycerophosphate and ascorbic acid (β GAA) for 6 or 12 days (n = 3). (C) Flow cytometry analysis (FACS) of SMA for isotype control, untreated VEC, VEC treated with β GAA for 6 days and activated valve interstitial cells (VIC) as a positive control (n=3). (D) Phase contrast imaging of untreated VECs or treated for 12 days with β GAA. Magnification: 20 $\times$. * p < 0.05.

Figure 3. Osteoprotegerin expression and secretion during endothelial to mesenchymal transition (A) Western blot of osteoprotegerin (OPG) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as endogenous control, of mitral valve prolapse (MVP) patients and controls subjects (CTRL). (B) Western blot quantification using ImageJ (n=3). * $p < 0.05$. (C) Quantitative PCR (qPCR) of osteoprotegerin (OPG) of valve endothelial cells (VEC) in absence or in presence of β -glycerophosphate and ascorbic acid (β GAA) for 6 or 12 days (n = 3). (D) OPG enzyme-linked immunosorbent assay (ELISA) on media of untreated and

β GAA treated VEC for 6 days ($n = 3$). * $p < 0.05$. (E) Digital PCR analysis of the syndecan family (SDC1, 2, 3 and 4) on nine different VEC populations.

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Figure 4. Osteoprotegerin treatment on valve endothelial cells

(A) Quantitative PCR (qPCR) of integrins alpha 5 ($\alpha 5$) and beta 3 ($\beta 3$), (B) collagen I (Col1A1), collagen III (Col3A1), bone morphogenetic protein 4 (BMP4), smooth muscle actin (SMA), fibroblast specific protein 1 (FSP1) and versican (VCAN) of valve endothelial cells (VEC) untreated or treated for 12 days with 50 ng/ml of osteoprotegerin (OPG) (n=5). * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$ (C) Total soluble collagen produced by untreated or osteoprotegerin (OPG - 50 ng/ml) treated VECs for 24 and 48 hours (n = 5); * $p < 0.05$. (D) Gelatin zymography gel and (E) western blot of matrix metalloproteinase 9 (MMP9) in

VECs untreated or treated with OPG (50 ng/ml) (n = 3). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as endogenous control. **(F)** Western blot of phosphorylated syndecan 4 (pSDC4) and syndecan 4 (SDC4) in VECs after OPG treatment for 5 and 15 minutes. (n = 3). **(G)** Western blot of pSDC4, SDC4, pERK and ERK, in absence or presence of OPG or Hepriase I (Hep I) pre-treatment (5 U/ml) and OPG (n = 3).

Figure 5. Osteoprotegerin induces ROS and cell migration in endothelial cells

(A) Flow cytometry analysis (FACS) of reactive oxygen species (ROS) for untreated or osteoprotegerin (OPG - 50 ng/ml) treated valve endothelial cells (VEC) for 24 hours (n = 3). (B) Bar graph depicting positive cells to SYTOX® (dead cells) after 24 hours treatment with 50 ng/mL of OPG (n = 3). (C) Representative images of wound healing assay for VECs in presence of OPG (50 ng/mL) or 10 % fetal bovine serum (FBS - positive control). (D) Time course representing the percentage of the area without migrating VECs in presence of OPG (50 ng/mL) or 10 % FBS (n = 5). * p < 0.05; ** p < 0.01. (E) Representative images of

wound healing assay for VECs in presence of OPG (50 ng/mL) with or without heparinase I pre-treatment (5 U/mL). (F) Bar graph depicting the percentage of migrating VECs in absence or presence of OPG (50 ng/ml) or heparinase I pre-treatment (5 U/mL) after 24 hours in presence of OPG (50 ng/mL) (n = 3). * $p < 0.05$ vs. untreated; # $p < 0.05$ vs. OPG treated VECs.

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Figure 6. Osteoprotegerin induces proliferation and proteoglycan expression in interstitial cells (A) Digital PCR (dPCR) analysis of the syndecan family (SDC1, 2, 3 and 4) on three different valve interstitial cells (VIC) populations. (B) Quantitative PCR (qPCR) of bone morphogenetic protein 4 (BMP4), biglycan (BGN), versican (VCAN), metalloproteinase 2 (MMP2), smooth muscle actin (SMA), osteoprotegerin (OPG), collagen I (Col1A1) and collagen III (Col3A1) of VICs left untreated or OPG treated (50 ng/ml) for 6 days (n = 3). * p < 0.05; ** p < 0.01. (C) Total soluble collagen produced by untreated or osteoprotegerin (OPG - 50 ng/ml) treated VICs for 24 and 48 hours (n = 5); * p < 0.05. (D) Proliferation

assay of VICs in absence or presence of osteoprotegerin (OPG - 50 ng/mL) for 6 days (n = 3).

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Figure 7. Osteoprotegerin plasma levels identify patients with mitral valve prolapse in a surgical patient population independently of age, sex, and common cardiovascular comorbidities
(A) Osteoprotegerin (OPG) enzyme-linked immunosorbent assay (ELISA) on plasma samples from control subjects (Control) or mitral valve prolapse (MVP) patients. **(B)** Receiver operating characteristic (ROC) curve and area under the curve (AUC) of OPG. (Control subjects n = 29 and Mitral Valve Patients n = 28) *** p < 0.0001.