Cardiovascular Pathology 25 (2016) 362–370

Contents lists available at ScienceDirect

Cardiovascular Pathology

Original Article

Associations of rs3918242 and rs2285053 MMP-9 and MMP-2 polymorphisms with the risk, severity, and short- and long-term complications of degenerative mitral valve diseases: a 4.8-year prospective cohort study

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ARTICLE INFO

Article history:
Received 9 August 2015
Received in revised form 4 May 2016
Accepted 17 May 2016
Available online xxxx

Keywords:
Degenerative forms of mitral valve diseases
Metalloproteinases
rs3918242, rs243865, rs2285053 *MMP-2* and *MMP-9* gene SNPs
Management and outcome

ABSTRACT

Background: Degenerative forms of mitral valve diseases (MVDs) are very complex pathologies. Thus, it is difficult to make generalizations about the disease pathways or genetic risk factors contributing to these diseases. However, a key role of metalloproteinases (MMPs) in their pathophysiology is emerging. Thus, we performed for the first time a perspective study to assess eventual associations of some functional single nucleotide polymorphisms (SNPs) in *MMP-2* and *MMP-9* genes with the MVD risk, symptom severity, and short- and long-term (4.8 years) complications.
Materials and methods: For this purpose, 90 patients and two control groups were genotyped for rs3918242, rs243865, and rs2285053 *MMP-2* and *MMP-9* gene SNPs, and systemic levels of pro-atrial natriuretic peptide (pro-ANP) and two enzymes were quantified and correlated to genotypes of *MMP-2* and *MMP-9* SNPs studied. In addition, associations between these SNPs and symptom severity and short- and long-term (4.8 years) complications were evaluated.
Results: Interestingly, rs3918242 *MMP-9* and rs2285053 *MMP-2* SNPs were significantly represented in cases than two control groups and were associated with a higher MVD risk, as demonstrated using dominant/recessive models. Cases stratified for NYHA symptoms and particularly those NYHA III + IV with rs3918242 CT + TT MMP-9 and rs2285053CT + TT genotypes also showed higher severity related to significant higher systemic levels of MMP enzymes and pro-ANP at enrolment and 4.8 follow-up times. In addition, cases with these genotypes and particularly those NYHA III + IV had a very significant percentage of complications, particularly at the 4.8 follow-up. Surprisingly, 20% of patient controls developed MVD at 4.8-year follow-up and were carriers of these genotypes.
Conclusion: Thus, the associations observed seem to suggest that the two SNPs might represent useful biomarkers and targets for preventing and monitoring MVDs and developing personalized treatments, consenting a more appropriate management and outcome.

1. Introduction

Degenerative mitral valve diseases (MVDs) are a group of heart valve pathologies, very common in European population and with a significant increase in old people [1,2]. They, indeed, manifest after the fifth decade of life and show a difficult early diagnosis and a complex management, which represent the real challenge for clinicians [3]. Until now, there is not a panel of diagnostic and prognostic biomarkers for sporadic forms, and their physiopathology remains unclear. Echocardiography evaluation represents until now unique "gold standard diagnostic" tool, even if it always reveals no appropriate for diagnosing moderate forms [3–5]. This underlines the necessity of innovative biomarker tools to identify in molecular pathways associated with mechanisms related to MVD pathogenesis. Recently, it is emerging that

Authors Contributions: Dr. Balistreri was involved in conception and study design. Drs. Allegra and Pisano were involved in enrolling study's controls/patients and in collecting their demographic and clinical data. Dr. Triolo was involved in determining echocardiographic parameters. Drs. Balistreri and Crapanzano performed experimental assays. Dr. Balistreri acquired the results obtained and performed their analysis. Dr. Balistreri was involved in the data interpretation and their translation in clinical suggestions. Dr. Balistreri was involved in drafting the paper and in its critical revision. Dr. Balistreri gave the final approval of the version to be published.

Funding: This work was supported by grants from the Italian Ministry of Education, University and Research to Dr. Balistreri and Professor Ruvolo.

Conflict of Interests: The authors declare that there is no conflict of interests regarding the publication of this paper.

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http://dx.doi.org/10.1016/j.carpath.2016.05.004
1054-8807/

degenerative MVDs are the result of *modified adult valve biology* [4,5]. Particularly, Levine and colleagues have recently underlined that MVDs are the result of a 'living valve', which with advancing age shows active changes mediated both by valvular endothelial and interstitial cells and alterations in composition and turnover of elements of extracellular matrix [6]. Accordingly, in surgically excised human myxomatous tissues, an overexpression of transforming growth factor-β cytokine (TGF-β) pathway, an increased release of metalloproteinases (MMPs), and down-expression of related inhibitors (TIMPs), responsible for degeneration of collagen and elastin structures, have been observed [7–15]. Increased systemic and tissue levels of MMPs in cases affected by degenerative sporadic MVDs and with stenosis or regurgitation complications have been also assessed [7–15]. In contrast, no exhaustive data about eventual associations between single nucleotide polymorphisms (SNPs) in *MMP* genes and the risk of degenerative sporadic MVDs are reported until now in literature, since they are a very limited number [16,17]. However, genetic association studies have the potential to identify molecular pathways as early diagnostic biomarkers and targets. Ulterior evidence might also derive performing clinical studies and correlations between polymorphisms and severity and complications of these diseases.

Here, we performed for the first time a 4.8-year prospective case–control study in order to identify eventual associations of some functional *MMP-2* and *MMP-9* gene SNPs (known to determine an increased release of these enzymes and higher susceptibility for several cardiovascular diseases [18]; see Table 1S online supplementary materials) with the MVD risk, severity, and short- and long-term follow-up complications. Thus, we enrolled a homogenous group of patients ($N=90$), all affected by myxomatous degeneration and at late stage of disease, when they were referred for mitral valve surgery (repair or replacement), and a control group of patients ($N=80$), showing no degenerative MVD at the time of its enrollment, but with a manifested myxomatous mitral degeneration at the 4.8 follow-up time in 20% of its components with NYHA I symptoms. Genotyping of the selected SNPs and quantification of systemic levels of MMP-2 and MMP-9 were assessed for evaluating associations between the genotypes and MVD risk and with the clinical disease phenotypes (i.e., circulating amount of two enzymes, severity, and complications). For this last objective, systemic levels of pro-atrial natriuretic peptide (pro-ANP) were also quantified due to the emerging evidence suggesting a significant association between high circulating pro-ANP levels and severity and complication of degenerative MVDs [19–22]. These investigations might permit to evidence whether the genetic variants selected and the circulating blood levels of their molecules and the pro-ANP may be potential predictive, diagnostic, and prognostic biomarkers. As a result, the management strategies for the medical and surgical treatment of degenerative MVD forms might become gene personalized. In addition, these insights might suggest new treatment therapeutic options, such as use of agonist and/or antagonists of TGF-β and ACE pathways and monoclonal antibodies against MMP-2 and MMP-9.

2. Materials and methods

2.1. Enrolled patients: Clinical and demographic characteristics

Our study included 90 patients, all affected by myxomatous degeneration, 46 women and 44 men, mean age 66 ± 10.8 years, and with demographic and clinical characteristics shown in Table 1 (data collected at the time of enrolment). They were randomly selected from patients who underwent to mitral valve plastic surgery or replacement in the Unit of Cardiac Surgery (Department of Surgery and Oncology, University of Palermo). Demographic, clinical, and echocardiographic data (as described in detail in the next paragraph) related to the preoperative and postoperative periods of hospitalization were also collected. All 90 patients were symptomatic before surgery and divided into two subgroups according to the degree of symptom's severity [3]. Precisely, the subgroup NYHA I + II was composed by 52 patients (58%) and NYHA III + IV consisted of 38 patients (42%) (see Tables 3a and 3b). All patients were evaluated preoperatively by echocardiography, angiography and underwent surgery (see paragraph 2.3). Mitral valve replacement with mechanical or biological prosthesis was the principal surgery approach used than mitral valve plastic surgery applied only in few patients. Furthermore, they were also subjected to a 4.8-year follow-up, in order to prospectively assess the echocardiographic data (see Tables 3a and 3b), complications and to evaluate the weight of specific MMP-9 and MMP-2 gene polymorphisms in the MVD outcome. To

Table 1
Patient's clinical and demographic characteristics

	$N=90$
Variables	**Mean ± SD**
Age	66.01 ± 10.8
Weight (kg)	68.7 ± 12.1
Height (cm)	164.1 ± 7.1
BSA	1.78 ± 0.65
Ejection fraction (%)	57.4 ± 8.34
Left atrium (cm^2)	32.1 ± 10.6
Left ventricular end-diastolic diameter (mm)	55.3 ± 17.5
Left ventricular end-diastolic volume (ml)	112.8 ± 35.4
Left ventricular end-systolic diameter (mm)	37.1 ± 6.2
Left ventricular end-systolic volume (ml)	43.1 ± 9.8
Pulmonary arterial systolic pressure (mmHg)	43.4 ± 8.5

	Patients	Percentage (%)
Male	44	49
Female	46	51
Mortality	0	0
Emergencies	6	6.6
Dilatative cardiomyopathy	5	5.5
NYHA		
Grade I-II	42	47
Grade III-IV	48	53
Smokers		
Ex	30	33.4
Yes	20	22.2
No	40	44.4
Hypertension	56	62.2
Diabetes	12	13.3
Renal failure	7	7.7
Chronic obstructive pulmonary disease	3	3.3
Atrial fibrillation	39	43.3
Heart failure	3	3.3
Cerebrovascular disease	12	13.3
Mitral lesions		
Regurgitation	56	62.2
Stenosis	14	15.5
Mixed	20	22.3
Mitral dysfunction		
Chordae tendineae rupture	4	4.4
Calcific degeneration	8	9
Prolapse	12	13.3
Tricuspid regurgitation grades		
Absent	12	36
Faint	32	39
Moderate	26	29
Severe	10	11.1
Pulmonary hypertension	65	72.2
Pharmacological treatment	***N* (%) at preoperative time treatment**	***N* (%) at follow-up time**
Beta-blockers	50	69
Alpha-agonists	53	65
Sartans	51	68
Calcium antagonists	61	79
ACE inhibitors	40	45
Oral hypoglycemic agents	42	49
Antiaggregants	90	90
Antidyslipidemics	90	90
Diuretics	46	53

Data collected at the time of enrolment.

Table 2
Clinical and demographic characteristics of the two control groups

	Control patients	Healthy controls
	N = 80 (%)	*N* = 168 (%)
Age (SD)	65.6 ± 10.7	61 ± 5.8
Men (%)	35 (44%)	81 (48%)
Women (%)	45 (56%)	87 (52%)
Body mass index (SD)	27.8 ± 8.6	25.8 ± 8.7
Comorbidity	***N* (%)**	***N* (%)**
Familiarity for coronaropathy	24 (30%)	40 (24%)
Smokers	46 (57.5%)	63 (38%)
Hypertension	28 (35%)	0 (0%)
Dyslipidemia	14 (17.5%)	0 (0%)
Diabetes mellitus	12 (15%)	0 (0%)
Renal failure	0 (0%)	0 (0%)
Ongoing cardiac diseases	0 (0%)	0 (0%)
Coronary atherosclerosis	0 (0%)	0 (0%)
Therapy	***N* (%)**	***N* (%)**
Beta-blockers	0 (0%)	0 (0%)
Alpha-agonists	0 (0%)	0 (0%)
Sartans	0 (0%)	0 (0%)
Calcium antagonists	0 (0%)	0 (0%)
ACE inhibitors	14 (17.5%)	0 (0%)
Oral hypoglycemic agents	12 (15%)	0 (0%)
Antiaggregants	28 (35%)	0 (0%)
Antidyslipidemics	0 (0%)	0 (0%)
Diuretics	28 (35%)	0 (0%)

this aim, peripheral blood samples were also collected from patients in order to assess the genetic background and to perform ELISA assays.

2.2. Enrolled controls: Clinical and demographic characteristics

Two control groups were also enrolled. The first consisted of 80 patients (mean age 65.6 ± 10.7 years; 45 women and 35 man) selected among individuals who arrived in the outpatient department for routine health screening for employment and without a manifested degenerative MVD, as evidenced after meticulous physical and echocardiography imaging examinations. Demographic and clinical features, eventual comorbidities, and pharmacological treatments were collected (see Table 2; data at the enrolment time). They were subjected to the same follow-up (4.8 years) of the 90 patients. Of them, the 25% (*N* = 20) at the 4.8 follow-up time showed onset of myxomatous degeneration with NYHA I symptoms. Their clinical features were reported in Table 3b.

The second control group included 168 healthy subjects, belonging to the same ethnic group of patients, in order to include in the study a very homogenous population. Ethnicity was confirmed, since parents and grandparents of both patients and controls were born in Western Sicily, at least two generations. Controls were in good health, evidenced by their medical history and laboratory data (blood count, erythrocyte sedimentation rate, blood glucose, blood urea nitrogen, trim electrolyte, creatinine, CRP, and liver function tests) (see Table 2). Demographic and clinical features were collected, as reported in Table 2. The absence of alterations in cardiac structures was confirmed by echocardiography imaging examinations. Peripheral blood samples were also collected in order to assess the genetic background and to perform ELISA assays.

2.3. Ethical study approval

Our study received approval from local ethic committees and all participants gave their informed consent. Data were encoded to ensure patient and control protection. All measurements were performed without knowledge about nature of material.

2.4. Preoperative and follow-up hemodynamic and anatomical modifications: Echocardiographic measurements

Precise echocardiographic parameters were collected in the several steps (preoperative and follow-up times), including left and right ventricular functions and left atrial and ventricular dimensions, mitral gradient, presence and degree of pulmonary hypertension, and tricuspid insufficiency. In addition, other parameters were as follows: ejection fraction percentage, ventricular size, pulmonary artery systolic pressure, and right atrial pressure. We defined pulmonary hypertension as a systolic pulmonary pressure >30 mmHg. Tricuspid insufficiency was quantified through color Doppler method, measuring the area of the regurgitant volume. Their values were reported in tables as mean ± standard deviation (SD).

2.5. DNA samples and genotyping

DNA case samples were extracted from peripheral blood samples of all individuals enrolled by using a commercial kit, Gen-Elute Blood Genomic DNA kit (Sigma, Saint Louis, USA). They were genotyped for the three SNPs in *MMP-2* and *MMP-9* genes selected in our study. Information about these SNPs was acquired from dbSNP NCBI, the ENSEMBL database (http://www.ensembl.org/index.html), and the UCSC Genome

Table 3a
Clinical characteristics of 90 patients stratified for NYHA class at preoperative and follow-up (4.8 years) times

Variables	Preoperative		Follow-up (4.8 years)			
	NYHA I + II	NYHA III + IV	NYHA I + II	NYHA III + IV	*P*1^	*P*2^
	(N = 52)	(N = 38)	*	*		
Ejection fraction (SD)	57.9 ± 8.97	57.5 ± 8.2	53.9 ± 8.6	55.1 ± 9.67	NS	NS
Left atrium, cm^2 (SD)	30.2 ± 9.87	39.4 ± 10.3	48 ± 9.7	51.1 ± 13.1	<.001	<.0001
Left ventricular end-diastolic diameter, mm (SD)	55.8 ± 15.8	56 ± 13.3	52 ± 4.2	52 ± 8.6	NS	NS
Left ventricular end-diastolic volume, ml (SD)	116 ± 18.5	118 ± 21.1	97 ± 15.6	99 ± 26.5	NS	NS
Left ventricular end-systolic diameter, mm (SD)	38 ± 5.5	36 ± 5.6	40.4 ± 5.2	39 ± 6.5	NS	NS
Left ventricular end-systolic volume, ml (SD)	44 ± 10.4	43 ± 6.4	49 ± 12.8	45 ± 14.7	NS	NS
Pulmonary artery systolic pressure, mmHg (SD)	42 ± 8.3	47 ± 6.8	32 ± 5.6	31 ± 4.5	.005	.005
Renal failure	3 (6%)	4 (12.5%)	0 (0%)	3 (8%)		
Heart failure	0 (0%)	3 (8%)	0 (0%)	3 (8%)		
Chronic obstructive pulmonary disease	0 (0%)	3 (8%)	2 (4%)	3 (8%)		
Cerebrovascular disease	4 (8%)	8 (25%)	2 (4%)	3 (8%)		
Atrial fibrillation	20 (38%)	19 (50%)	16 (31%)	12 (31.5%)		
Tricuspid regurgitation grades						
Absent	12 (23%)	11 (29%)	0 (0%)	0%		
Faint	32 (61.5%)	0 (0%)	48 (92%)	10 (26%)		
Moderate	6 (11.5%)	20 (53%)	4 (8%)	28 (74%)		
Severe	2 (4%)	7 (18%)	0%	0%		
Pulmonary hypertension	40 (77%)	25 (66%)	18 (35%)	22 (58%)		

Table 3b
Clinical characteristics of 20 individuals with MVD at the 4.8-year follow-up time with NYHA I symptoms

Variables	Values
Ejection fraction (SD)	51 ± 5.4
Left atrium, cm^2 (SD)	28.5 ± 3.8
Left ventricular end-diastolic diameter, mm (SD)	52 ± 14.6
Left ventricular end-diastolic volume, ml (SD)	115.3 ± 7.3
Left ventricular end-systolic diameter, mm (SD)	32.6 ± 4.8
Left ventricular end-systolic volume, ml (SD)	36.8 ± 6.1
Pulmonary artery systolic pressure, mmHg (SD)	39.1 ± 8.1
Renal failure	0 (0%)
Heart failure	0 (0%)
Chronic obstructive pulmonary disease	0 (0%)
Cerebrovascular disease	0 (0%)
Atrial fibrillation	5 (25%)
Tricuspid regurgitation grades	
Absent	5 (25%)
Faint	15 (75%)
Moderate	0 (0%)
Severe	0 (0%)
Pulmonary hypertension	7 (35%)

^, *t* test; *NYHA class refers to preoperative time.

Browser website (http://genome.ucsc.edu) and was reported in Table 1S and 2S (*online Supplementary Materials*; *in Table 1S, their biological effects are reported*; *in Table 2S, their frequencies in European population are reported*). Genotyping was performed using procedures described in our previous study [23].

2.6. MMPs and pro-ANP systemic evaluations

Systemic levels of pro-ANP, MMP-9, and MMP-2 were detected through ELISA [Pro Human Atrial Natriuretic Peptide kit (Cusabio, China); Quantikine ELISA Human MMP-9 Immunoassay kit (RD, Minneapolis, USA); Elisa Quantikine Total MMP-2 kit (RD, Minneapolis, USA), respectively], according to the manufacturer's instructions. To standardize our results, reference preparations of pro-ANP, MMP-9, and MMP-2 were tested in all assays. Results were expressed as picograms per milliliter (pg/ml) for the quantitative values of pro-ANP and as nanograms per milliliter (ng/ml) for MMP-9 and MMP-2. Detection limits in our laboratory were 15.6 pg/ml for pro-ANP, 0.014 ng/ml for MMP-2, and 0.156 ng/ml for MMP-9.

Table 4b
Genotype distribution and allele frequencies of rs3918242(−1562C/T) MMP-9, rs2285053(−735C/T), and rs243865(−1306C/T) MMP-2 polymorphisms in two groups of 90 MVD patients identified in according to NYHA classification

Candidate genes	Reference SNP number	Candidate SNPs	NYHA I+II patients (*N*=52)	NYHA III+IV patients (*N*=38)	*P* (cases vs. cases) (3×2 table) (2×2 table)
MMP-9	**rs3918242**	−1562C/C	45 (86%)	25 (66%)	.03
		−1562C/T	5 (10%)	6 (16%)	
		−1562T/T	2 (4%)	7 (18%)	
		−1562C	95 (91%)	56 (74%)	.002
		−1562T	9 (9%)	20 (26%)	
MMP-2	**rs2285053**	−735C/C	45 (86%)	25 (66%)	.002
		−735C/T	6 (12%)	7 (18%)	
		−735 T/T	1 (2%)	6 (16%)	
		−735C	96 (92%)	57 (75%)	.002
		−735T	8 (8%)	19 (25%)	

2.7. Statistical analysis

All analyses were performed with R and Microsoft Excel software. Quantitative variables were expressed as mean ± SD. *t* and the Wilcoxon tests were used to analyze the relationship between quantitative variables. The alleles and genotypes frequency was assessed by gene count. Data were tested for goodness of fit between observed and expected genotype frequencies according to Hardy–Weinberg equilibrium, by χ^2 tests. Significant differences in frequencies, among the groups, were calculated by χ^2 test (3×2, 2×2 tables, where appropriate). In addition, significant differences among qualitative variables were calculated by using χ^2 and appropriated tables (3×2; 2×2). The *P* value <.05 was considered statistically significant. Multiple logistic regression analyses of dominant (major allele homozygotes plus heterozygotes vs. minor allele homozygotes) and recessive (major allele homozygotes vs. heterozygotes plus minor allele homozygotes) models were applied to MVD patient group compared with control groups and between the two patient NYHA groups. Odds ratios (OR), 95% confidence intervals (95% CI), and *P* values were determined using SPSS (SPSS Inc., Chicago, IL, USA). A *P*<.05 was considered statistically significant. The significant differences of the MVD complications between the individual carriers of rs3918242 CC+TT and rs2285053 CC+TT genotypes and no carriers were detected using Fisher's Exact Test and Pearson's chi-square test. *t* and the Wilcoxon tests were also used for calculating significant differences of circulating MMP and pro-ANP levels between the individual carriers of rs3918242 CC+TT and rs2285053 CC+TT genotypes and no carriers.

Table 4a
Genotype distributions and allele frequencies of rs3918242(−1562C/T) MMP-9, rs2285053(−735C/T), and rs243865(−1306C/T) MMP-2 polymorphisms in 90 MVD patients, 80 patient controls, and 168 healthy controls

Candidate genes	Reference SNP number	Candidate SNPs	Patients (*N*=90)	Healthy controls (*N*=168)	*P*1 (cases vs. healthy controls) (3×2 table) (2×2 table)	Patient controls (*N*=80)	*P*2 (cases vs. patient controls) (3×2 table) (2×2 table)
MMP9	**rs3918242**	−1562C/C	70 (78%)	156 (93%)	**.001**	74 (93%)	**.001**
		−1562C/T	11 (12%)	9 (5%)		5 (6%)	
		−1562T/T	9 (10%)	3 (2%)		1 (1%)	
		−1562C	151 (84%)	321 (95%)	**.00001**	153 (96%)	**.0008**
		−1562T	29 (16%)	15 (5%)		7 (4%)	
MMP2	**rs2285053**	−735C/C	70 (78%)	161 (96%)	**.00001**	73 (91%)	**.05**
		−735T/C	13 (14%)	6 (3.5%)		5 (6%)	
		−735T/T	7 (8%)	1 (0.5%)		2 (2%)	
		−735C	153 (85%)	328 (98%)	**1.5e-7**	151 (94%)	**.008**
		−735T	27 (15%)	8 (2%)		9 (6%)	
MMP2	**rs243865**	−1306C/C	82 (91%)	160 (95%)	NS	78 (97%)	NS
		−1306C/T	6 (7%)	7 (4.5%)		2 (3%)	
		−1306T/T	2 (2%)	1 (0.5%)		0 (0%)	
		−1306C	170 (94%)	327 (97%)	NS	158 (99%)	NS
		−1306T	10 (10%)	9 (3%)		2 (1%)	

Table 5a
Multiple logistic regression analyses of dominant (major allele homozygotes plus heterozygotes vs. minor allele homozygotes) and recessive (major allele homozygotes vs. heterozygotes plus minor allele homozygotes) models applied to MVD patient group compared with control groups

MVD cases vs. healthy controls					MVD cases vs. patient controls		
SNPs	Model		OR (95% CI)	*P*		OR (95% CI)	*P*
rs3918242	Dominant	**CC + C/T/TT**			**CC + CT/TT**		
		Cases: 81/9			Cases 81/9		
		Controls:165/3	0.163 (0.043–0.621)	.0004	Patient Controls:79/1	0.113 (0.014–0.920)	.01
	Recessive	**CC/C/T + TT**			**CC/CT + TT**		
		Cases: 20/70			Cases: 20/70		
		Controls:12/156	**3.71 (1.72–8.017)**	**.0006**	Patient Controls:6/74	**3.52 (1.337–9.289)**	**.006**
rs2285053	Dominant	**CC + CT/TT**			**CC + CT/TT**		
		Cases: 83/7			Cases: 83/7		
		Controls:167/1	0.071 (0.008–0.58)	.003	Patient Controls:78/2	0.662 (0.453–0.969)	.115
	Recessive	**CC/CT + TT**			**CC/CT + TT**		
		Cases: 20/70			Cases: 20/70		
		Controls:7/161	**2.444 (1.817–3.289)**	**<.0001**	Patient Controls:7/73	**1.513 (1.145–2.000)**	**.01**

3. Results

3.1. Patients characteristics according to NYHA score

The 90 patients had different symptoms severity in according to NYHA score (see Tables 3a and 3b). Precisely, 52 patients were classified as NYHA I + II, and 38 patients were classified as NYHA III + IV. The comparative analysis of their echocardiographic data at preoperative and 4.8-year follow-up times showed a general clinical improvement of hemodynamic profile of patients. No significant difference was observed in the left ventricular diastolic diameter in two groups at two different times. The same result was obtained comparing the end-systolic left ventricular diameter in two groups at two different times (see Tables 3a and 3b). Furthermore, pulmonary artery pressures were reduced in both classes. However, a slight disease progression, mainly due to the physiological aging process, was observed. For example, in both the two groups, we observed a significant increase in left atrium medium enlargement at the 4.8 follow-up (see Tables 3a and 3b).

3.2. Genotype distribution and allele frequencies of rs3918242(−1562C/T) MMP-9, rs2285053(−735C/T) MMP-2, and rs243865(−1306C/T) MMP-2 SNPs in 90 patients, 80 and 168 controls

By comparing genotype distributions and allele frequencies of three SNPs selected in our study between cases and the two control groups, significant differences were detected, as reported in Table 4a. In particular, the genotypes of rs3918242(−1562C/T) *MMP-9* were significantly distributed between cases and healthy controls and cases vs. patient controls ($P = .001$ and $P = .001$, respectively, by χ^2 test and 3×2 tables). Accordingly, the −1562T allele was overrepresented in cases vs. the two control groups (Yates corrected $P = .00001$ and $P = .0008$, respectively, by χ^2 test and 2×2 tables). Likewise, the rs2285053(−735C/T) *MMP-2* SNP showed very significant differences in genotype and allele frequencies, by comparing its values between cases vs. healthy controls ($P = .00001$ and Yates corrected $P = 1.5e\text{-}7$, by χ^2 test and appropriate tables) and cases vs. patient controls ($P = .05$ and Yates corrected $P = .008$, by χ^2 test and appropriate tables). In contrast, no significant differences were observed in genotype distributions and allele frequencies of rs243865(−1306C/T) *MMP-2* SNP between cases vs. healthy controls and cases vs. patient controls.

These results were confirmed by logistic regression analyses of dominant and recessive models performed between patient and control groups (Table 5a). Interestingly, the data obtained through comparisons between cases and patient and healthy controls for dominant model and recessive models evidenced that patients carrying of the minor alleles (−1562T and −735T) of rs3918242(−1562C/T) *MMP-9* and rs2285053(−735C/T) *MMP-2* SNPs were significantly susceptible for MVD disease in recessive models (see data reported Table 5a).

3.3. Genotype distribution and allele frequencies of rs3918242(−1562C/T) MMP-9 and rs2285053(−735C/T) MMP-2 SNPs in two NYHA patient groups and 20 NYHA I subjects

The data's analysis of genotype distributions and allele frequencies of two SNPs in *MMP-9* and *MMP-2* genes between 52 NYHA I + II and 38 NYHA III + IV patients evidenced significant differences (see Table 4b). The genotypes of rs3918242(−1562C/T) *MMP-9* were significantly distributed between 52 NYHA I + II and 38 NYHA III + IV patients ($P = .03$, by χ^2 test and 3×2 tables). Accordingly, the −1562T allele was overrepresented in 38 NYHA III + IV patients vs. the 52 NYHA I + II patients (20 vs. 9, Yates corrected $P = .002$ by χ^2 test and 2×2 tables). In addition, the rs2285053(−735C/T) *MMP-2* SNP showed very significant differences in genotype and allele frequencies, by comparing its values between 52 NYHA I + II and 38 NYHA III + IV patients. In particular,

Table 5b
Multiple logistic regression analyses of dominant (major allele homozygotes plus heterozygotes vs. minor allele homozygotes) and recessive (major allele homozygotes vs. heterozygotes plus minor allele homozygotes) models applied between the two patients groups

MVD NYHA I + II cases vs. MVD NYHA III + IV cases				
SNPs	Model		OR (95% CI)	*P*
rs3918242	Dominant	**CC + CT/TT**		
		MVD NYHA I + II cases: 50/2		
		MVD NYHA III + IV cases: 31/7	**2.778 (0.808–9.547)**	**.02**
	Recessive	**CC/CT + TT**		
		MVD NYHA I + II cases: 7/45		
		MVD NYHA III + IV cases: 13/25	**1.82 (1.161–2.854)**	.01
rs2285053	Dominant	**CC + CT/TT**		
		MVD NYHA I + II cases: 51/1		
		MVD NYHA III + IV cases: 32/6	**9.563 (1.099–83.183)**	**.02**
	Recessive	**CC/CT + T**		
		MVD NYHA I + II cases: 6/46		
		MVD NYHA III + IV cases: 13/25	**3.98 (1.349–11.778)**	**.009**

Table 6a
Circulating levels of MMP-2 and MMP-9 and pro-ANP at enrolment and 4.8-year follow-up in NYHA patients, 168 healthy controls, and 20 individuals with MVD at 4.8-year follow-up stratified for the genotypes of rs3918242C/T MMP-9 SNP

	rs3918242CT + TT NYHA I + II cases	rs3918242CC NYHA I + II cases	*P*	rs3918242CT + TT NYHA III + IV cases	rs3918242CC NYHA III + IV cases	*P*	rs3918242CT + TT 6 follow-up patients	rs3918242CC 14 follow-up patients	*P*	rs3918242CT + TT healthy controls	rs3918242CC healthy controls	*P*
	N = 7	*N* = 45		*N* = 13	*N* = 25		*N* = 6	*N* = 14		*N* = 12	*N* = 156	
ANP at 1 time	498 ± 51	415 ± 82	.002	525 ± 87	459 ± 69	.01	160 ± 59	130 ± 47	NS	16 ± 8.6	15.2 ± 7.4	NS
ANP at follow-up	547 ± 32	456 ± 64	<.0001	593 ± 59	486 ± 45	<.0001	296 ± 69	198 ± 32	.01	19 ± 13.2	17 ± 13	NS
MMP-9 at 1 time	58 ± 18	41 ± 12	.02	59 ± 11	43 ± 12	.0002	38 ± 19	22 ± 15	.05	30 ± 11	18 ± 13	.001
MMP-9 at follow-up	66 ± 15	52 ± 9	.02	64 ± 8	56 ± 7	.003	56 ± 18	41 ± 13	.05	39 ± 12	23 ± 10	<.0001

Table 6b
Circulating levels of MMP-2 and MMP-9 and pro-ANP at enrolment and 4.8-year follow-up in NYHA patients, 168 healthy controls, and 20 individuals with MVD at 4.8-year follow-up stratified for the genotypes of rs2285053C/T MMP-2 SNP

	rs2285053CT + TT NYHA I + II cases	rs2285053CC NYHA I + II cases	*P*	rs2285053CT + TT NYHA III + IV cases	rs2285053CC NYHA III + IV cases	*P*	rs2285053CT + TT 6 follow-up patients	rs2285053CC 14 follow-up patients	*P*	rs2285053CT + TT healthy controls	rs2285053CC healthy controls	*P*
	N = 7	*N* = 45		*N* = 13	*N* = 25		*N* = 6	*N* = 14		*N* = 7	*N* = 161	
ANP at 1 time	501 ± 47	380 ± 61	<.0001	565 ± 54	460 ± 61	.008	158 ± 57	128 ± 44	NS	17 ± 6	14 ± 8	NS
ANP at follow-up	575 ± 28	472 ± 71	<.0001	601 ± 63	479 ± 39	<.0001	287 ± 63	195 ± 31	.007	18 ± 10	16 ± 11	NS
MMP-2 at 1 time	59 ± 18	40 ± 15	.01	60 ± 10	50 ± 9	.003	39 ± 20	25 ± 16	.01	27 ± 10	19 ± 9	.01
MMP-2 at follow-up	69 ± 15	50 ± 11	.007	71 ± 14	58 ± 11	.004	55 ± 20	33 ± 12	.02	40 ± 11	22 ± 13	.0002

Table 7a
Prevalence expressed in percentages of MVD complications at enrolment and 4.8-year follow-up in NYHA patients and 20 individuals with MVD at 4.8-year follow-up stratified for the genotypes of rs3918242C/T MMP-9 SNP

	rs3918242CT+TT NYHA I+II cases	rs3918242CC NYHA I+II cases	*P*	rs3918242 CT+TT NYHA III+IV cases	rs3918242CC NYHA III+IV cases	*P*	rs3918242CT+TT 6 follow-up patients	rs3918242CC 14 follow-up patients	*P*
	N=7	*N*=45		*N*=13	*N*=25		*N*=6	*N*=14	
Pulmonary hypertension at 1time	4 (57%)	36 (80%)	.007	6 (46%)	19 (76%)	NS	0 (0%)	0 (0%)	NS
Pulmonary hypertension at follow-up	7 (100%)	11 (24%)	.0002	13 (100%)	9 (36%)	.00009	6 (100%)	1 (7%)	.001
Tricuspid regurgitation at 1 time	7 (100%)	33 (73%)	NS	13 (100%)	14 (56%)	.00004	0 (0%)	0 (0%)	NS
Tricuspid regurgitation at follow-up	7 (100%)	45 (100%)	1e-8	13 (100%)	25 (100%)	.0003	6 (100%)	14 (100%)	.03
Cerebrovascular disease at 1 time	2 (28%)	0 (0%)	.01	7 (100%)	1 (4%)	.000002	0 (0%)	0 (0%)	
Cerebrovascular disease at follow-up	0 (0%)	0 (0%)	NS	3 (23%)	0 (0%)	.007	0 (0%)	0 (0%)	NS
Heart failure at 1tme	2 (28%)	0 (0%)	.01	3 (23%)	0 (0%)	.007	0 (0%)	0 (0%)	NS
Heart failure at follow-up	3 (43%)	0 (0%)	.001	3 (23%)	0 (0%)	.007	0 (0%)	0 (0%)	NS

the allele −735T was overrepresented in 38 NYHA III+IV patients vs. the 52 NYHA I+II patients (19 vs. 8, Yates corrected $P=.002$, by χ^2 test and 2×2 tables).

Furthermore, we observed that rs3918242(−1562C/T) *MMP-9* and rs2285053(−735C/T) *MMP-2 SNPs* in two NYHA patient groups conferred a higher risk for MVD in both dominant and recessive models (see Table 5b).

Concerning the 20 individuals with NYHA I symptoms, we interestingly observed that 6 (30%) were carriers of both −1562C/T+−1562TT and −735C/T+−735T/T genotypes of rs3918242(−1562C/T) *MMP-9* and rs2285053(−735C/T) *MMP-2* SNPs (data not shown).

3.4. Associations of rs3918242(−1562C/T) MMP-9 and rs2285053(−735C/T) MMP-2 SNPs with the MVD severity, prevalence of its complications at short and long times, and outcome

As described in the previous paragraphs, the two rs3918242(−1562C/T) *MMP-9* and rs2285053(−735C/T) *MMP-2* SNPs were significantly overrepresented in the patients and associated with high MVD risk. In particular, in the two NYHA patient's groups, these SNPs conferred a very significant high risk in both dominant and recessive models (see Table 5b). In the light of these results, we also detected eventual associations of these SNPs with severity and prevalence of disease complications at short and long times and consequently with its outcome. To this aim, circulating levels of pro-ANP and the two MMP enzymes were firstly quantified at the two (enrolment and 4.8 follow-up) times in the plasma samples from the three groups studied, and subsequently, their mean values were stratified for the rs3918242(−1562C/T) *MMP-9* and rs2285053(−735C/T) *MMP-2* SNP genotypes (see Tables 6a and 6b). The data obtained confirmed that the patient carriers of rs3918242 CC+TT and rs2285053 CC+TT genotypes had significant high levels of circulating pro-ANP and the two enzymes at two times, but particularly at 4.8 follow-up, than patient carriers of rs3918242 CC and rs2285053 CC genotypes (see Tables 6a and 6b, which report levels of these molecules in mean values ± DS and *P* values detected by using *t* test). In particular, higher levels of these molecules were assessed in patient carriers bearing the rs3918242 CC+TT and rs2285053 CC+TT genotypes and belonging at NYHA III and IV classes. Similar results were obtained by stratifying for these genotypes the 20 individuals, who at 4.8 follow-up showed MVD onset with NYHA I symptoms, even if their number was very small (see Tables 6a and 6b).

Furthermore, in the patient carriers of rs3918242 CC+TT and rs2285053 CC+TT genotypes, we observed a significant prevalence (expressed in percentage, %; see Tables 7a and 7b, which show the percentage values of each complications at the two times and *P* values) at both enrolment and 4.8 follow-up times of MVD complications, including pulmonary hypertension, tricuspid regurgitation, cerebrovascular disease, and heart failure. Precisely, we assessed that, at 4.8 follow-up time, all patients of both NYHA classes carriers of rs3918242 CC+TT and rs2285053 CC+TT genotypes [7 (100%) and 13 (100%); see Table 4b] showed pulmonary hypertension and tricuspid regurgitation (even if of faint and moderate grades). Differently, few cases of both NYHA classes and with rs3918242 CC and rs2285053 CC genotypes had these MVD complications. Furthermore, the 28% of NYHA I+II patients at enrolment time with rs3918242 CC+TT and rs2285053 CC+TT genotypes developed cerebrovascular disease, but not at 4.8 follow-up. The 28% and 43% of NYHA I+II cases with rs3918242 CC+TT and rs2285053 CC+TT genotypes also had heart failure. No cases of cerebrovascular disease and heart failure were observed at the two times in group of NYHA I+II patients with rs3918242 CC and rs2285053 CC genotypes. Similar data were detected in NYHA III+IV patients at two times with rs3918242 CC+TT and rs2285053 CC+TT genotypes (23%) (see Tables 7a and 7b). In addition, the 23% of patients of NYHA III+IV class with rs3918242 CC+TT and rs2285053 CC+TT genotypes displayed a severe clinical status with heart failure at the two times. Likewise, the 6 individuals with rs3918242 CC+TT and rs2285053 CC+TT genotypes of the 20 subjects, who at 4.8 follow-up showed MVD onset with NYHA I symptoms, showed all pulmonary hypertension and tricuspid regurgitation of faint grades (see Tables 7a and 7b).

Table 7b
Prevalence expressed in percentages of MVD complications at enrolment and 4.8-year follow-up in NYHA patients and 20 individuals with MVD at 4.8-year follow-up stratified for the genotypes of rs2285053C/T MMP-2 SNPs

	rs2285053CT+TT NYHA I+II cases	rs2285053CC NYHA I+II cases	*P*	rs2285053CT+TT NYHA III+IV cases	rs2285053CC NYHA III+IV cases	*P*	rs228505CT+TT 6 follow-up patients	rs2285053CC 14 follow-up patients	*P*
	N=7	*N*=45		*N*=13	*N*=25		*N*=6	*N*=14	
Pulmonary hypertension at 1time	4 (57%)	36 (80%)	.007	6 (46%)	19 (76%)	NS	0 (0%)	0 (0%)	NS
Pulmonary hypertension at follow-up	7 (100%)	11 (24%)	.0002	13 (100%)	9 (36%)	.00009	6 (100%)	1 (7%)	.001
Tricuspid regurgitation at 1 time	7 (100%)	33 (73%)	NS	13 (100%)	14 (56%)	.00004	0 (0%)	0 (0%)	NS
Tricuspid regurgitation at follow-up	7 (100%)	45 (100%)	1e-8	13 (100%)	25 (100%)	.0003	6 (100%)	14 (100%)	.03
Cerebrovascular disease at 1 time	2 (28%)	0 (0%)	.01	7 (100%)	1 (4%)	.000002	0 (0%)	0 (0%)	
Cerebrovascular disease at follow-up	0 (0%)	0 (0%)	NS	3 (23%)	0 (0%)	.007	0 (0%)	0 (0%)	NS
Heart failure at 1tme	2 (28%)	0 (0%)	.01	3 (23%)	0 (0%)	.007	0 (0%)	0 (0%)	NS
Heart failure at follow-up	3 (43%)	0 (0%)	.001	3 (23%)	0 (0%)	.007	0 (0%)	0 (0%)	NS

3.5. Levels of systemic MMP-2 and MMP-9 and pro-ANP molecules in the healthy control group stratified for rs3918242 CC + TT and rs2285053 CC + TT genotypes

We also compared the systemic levels of MMP-2 and MMP-9 and pro-ANP molecules in control carriers versus no control carriers, as reported in Tables 6a and 6b. As shown in these tables, these comparisons demonstrated significant differences of two enzymes at two times, but not certainly for pro-ANP. In particular, carriers of rs3918242 CC + TT and rs2285053 CC + TT genotypes showed significant levels of systemic MMP-2 and MMP-9 than no control carriers.

4. Discussion

In this study, we focused our attention in evaluating the potential associations of rs3918242(−1562C/T *MMP*-9), rs243865(−1306C/T *MMP-2*), and rs2285053(−735C/T *MMP-2*) *MMP-9* and *MMP-2* functional gene SNPs with susceptibility, symptom severity, and short- and long-term (at 4.8 years) complications of degenerative MVD forms. Our interest is supported by experimental studies on animal models and humans, which have suggested a key role of MMPs in their onset and progression [7–15]. However, in literature, there are few data [16,17] showing associations of SNPs in *MMP* genes with MVD susceptibility. Thus, our study represents the first report that analyzed, in a perspective manner, associations of functional genetic variants in the *MMP-2* and *MMP-9* genes not only with MVD risk but also with its severity and complications, as well as with long-term outcome after surgical approaches (i.e., mitral valve replacement or plastic surgery). We also used a particular study's strategy characterized by selecting a homogenous group of patients ($N = 90$), all affected by myxomatous degeneration and at late stage of disease, when they were referred for mitral valve surgery (repair or replacement), and a control group of patients ($N = 80$), showing no degenerative MVD at the time of its enrollment but with a manifested myxomatous mitral degeneration at the 4.8 follow-up time in 20% of its components with NYHA I symptoms.

By analyzing the data of genotyping, we observed that the rs3918242(−1562C/T *MMP*-9) and rs2285053(−735C/T *MMP-2*) SNPs, determining an increased expression of both gelatinases, were significantly represented in cases respect to controls (see Table 4a). We also assessed that cases with rs3918242 CT + TT MMP-9 and rs2285053CT + TT genotypes had significant high risk of MVD in recessive model (see Table 5a). In addition, the 90 cases divided in two groups (52 and 38, respectively) with NYHA I + II and NYHA III + IV symptom grades also showed significant differences in genotype and allele frequencies of two SNPs and a very significant high risk for MVD both in dominant and recessive models (see Table 5b). Concerning the 20 individuals of patient control group with MVD and NYHA I symptoms at 4.8-year follow-up, we interestingly observed that the 6 (30%) were carriers of both −1562C/T + −1562TT and −735C/T + −735T/T genotypes of rs3918242(−1562C/T) *MMP-9* and rs2285053(−735C/T) *MMP-2* SNPs (data not shown).

In the light of these relevant results obtained, we also assessed the associations of these SNPs with the severity and prevalence of short- and long-time complications (at enrolment and at 4.8-year follow-up) and consequently with MVD outcome. To this aim, we firstly quantified the systemic levels of two gelatinases in the plasma samples from cases and two control groups and subsequently we stratified them for the genotypes. By comparing the systemic amounts, we found that cases of the two NYHA symptoms classes carrying rs3918242 CT + TT MMP-9 and rs2285053CT + TT genotypes showed higher circulating levels of MMP-9 and MMP-2 enzymes at the two times (enrolment and 4.8 follow-up) than cases of the same NYHA classes and the healthy controls carrying rs3918242CC MMP-9 and rs2285053CC genotypes (see Tables 6a and 6b). Thus, the interesting results obtained also led us to suggest that the SNPs studied can modulate risk, onset, and severity of symptoms shown by patient's population and detected in accordance to NYHA degrees as established by guidelines. Accordingly, cases of the two NYHA symptom classes carrying rs3918242 CT + TT MMP-9 and rs2285053CT + TT genotypes also had very significant circulating pro-ANP levels at the two times compared to cases of the same NYHA classes and the healthy controls carrying rs3918242CC MMP-9 and rs2285053CC genotypes (see Tables 6a and 6b). Surprisingly, significant differences in the circulating levels of two enzymes were also assessed in 6 individuals with MVD at 4.8-year follow-up carrying rs3918242 CT + TT MMP-9 and rs2285053CT + TT genotypes than the 14 subjects with MVD at 4.8-year follow-up carrying rs3918242CC MMP-9 and rs2285053CC genotypes (see Tables 6a and 6b).

Furthermore, in all patient (52 and 32 NYHA patients) carriers of rs3918242 CC + TT and rs2285053 CC + TT genotypes, we observed a significant prevalence at both enrolment and 4.8 follow-up times of MVD complications, including pulmonary hypertension, tricuspid regurgitation, cerebrovascular disease, and heart failure. In particular, the cases of NYHA III + IV class with rs3918242 CC + TT and rs2285053 CC + TT genotypes displayed a more severe clinical status at the two times. Interestingly, all 6 individuals with MVD at 4.8-year follow-up carrying rs3918242 CT + TT MMP-9 and rs2285053CT + TT genotypes also showed pulmonary hypertension and tricuspid regurgitation (of faint grades) than the 14 subjects with MVD at 4.8-year follow-up carrying rs3918242CC MMP-9 and rs2285053CC genotypes, even if these last had all tricuspid regurgitation, but pulmonary hypertension was found only in 1 of them.

In accordance of the data assessed in cases, we compared systemic levels of MMP-2 and MMP-9 and pro-ANP in healthy group, stratified for the genotypes of MMP-9 and MMP-2 SNPs. Controls bearing rs3918242 CT + TT MMP-9 and rs2285053CT + TT genotypes showed higher levels of two enzymes than control carriers of rs3918242CC MMP-9 and rs2285053CC genotypes. These results are in agreement with the data of a previous study performed on another cardiovascular disease, such as sporadic thoracic aneurysm [23,24].

5. Limitations and conclusions

In complex, the results obtained suggest, for the first time, significant associations of *MMP-9* and *MMP-2* genetic variants with the susceptibility of degenerative MVD forms, their severity, and their short- and long-term complications. Furthermore, cases bearing the rs3918242 CT + TT MMP-9 and rs2285053CT + TT genotypes, even if underwent to mitral valve replacement, also showed a worse outcome at long term (4.8 years).

Certainly, future and additional more large studies are needed to validate our results, since our sample size was relatively small. This might permit to open new perspectives in the management and outcome of degenerative MVD forms, using these genetic variants and MMP-9 and MMP-2 as innovative biomarkers and targets for developing personalized treatments. Revealing the role of MMP-9 and MMP-2 in degenerative MVD forms may serve as a starting point for future studies leading to a better understanding of the pathophysiological basis and perhaps effective treatment of these human diseases. As result, the management and outcome strategies for the medical and surgical treatment of degenerative MVD forms might become gene tailored. In addition, these insights might lead to propose new treatment options such as the use of agonist and/or antagonists of TGF-β pathways and monoclonal antibodies against MMP-2 and MMP-9. These treatments might likely have more advantage effects with respect to those mediated by diuretics, calcium-channel blocker, angiotensin receptor blockers, ACE inhibitors, statins, and antidiabetic and antiplatelet agents, which seem to influence, in different ways, the MMP pattern with beneficial effects on cardiovascular outcome, as recently reported by Hopps and Caimi [25]. As regards, we assessed in this study whether these drugs were able to limit severity and complications at long term (4.8 years) in cases bearing high responder genotype. No significant data were obtained (data not shown). This led us to suppose that a more specific therapy might

be necessary to reduce the effects of genetic propensity, and probably a better understanding of MVD pathophysiology might be useful. As consequence, future studies and additional efforts are imperative as well as a combination of analysis based on genetic, transcriptomic, proteomic, and epigenomic evaluations. Epigenomic, transcriptomic, and proteomic approaches might particularly provide valuable insights about disease pathobiology, although it is very difficult obtaining human tissue valve samples and appropriate controls.

Acknowledgments

The authors gratefully acknowledge Dr. Vincenzo Argano who ulteriorly contributed in the support of study's materials. This work was supported by grants from the Italian Ministry of Education, University, and Research to Prof Ruvolo, and Dr. Balistreri.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.carpath.2016.05.004.

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