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Research paper

Sequencing of NOTCH1 gene in an Italian population with bicuspid aortic valve: Preliminary results from the GISSI OUTLIERS VAR study

Silvana Pileggi[a,*], Benedetta De Chiara[b,c], Michela Magnoli[a], Maria Grazia Franzosi[a], Bruno Merlanti[d], Francesca Bianchini[e], Antonella Moreo[b], Gabriella Romeo[f], Claudio Francesco Russo[d], Stefania Rizzo[g], Cristina Basso[g], Luigi Martinelli[h], Attilio Maseri[i], , on behalf of the VAR Study Group[1]

[a] Department of Cardiovascular Research, IRCCS Istituto di Ricerche Farmacologiche "Mario Negri", Milan, Italy
[b] Cardiology IV, "A.De Gasperis" Department, ASST GOM Niguarda, Milan, Italy
[c] School of Medicine and Surgery, University of Milano-Bicocca, Milan, Italy
[d] Cardiac Surgery, "A.De Gasperis" Department, ASST GOM Niguarda, Milan, Italy
[e] ANMCO Research Center, Florence
[f] Department of Cardiac, Vascular and Thoracic Sciences, University of Padua, Padua
[g] Cardiovascular Pathology, Department of Cardiac, Thoracic and Vascular Sciences, University of Padua, Padua
[h] Cardiothoracic Surgery, ICLAS-Istituto Clinico Ligure Alta Specialità, Rapallo
[i] Heart Care Foundation, Florence

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ABSTRACT

Background: Bicuspid aortic valve (BAV) formation is genetically determined, with reduced penetrance and variable expressivity. NOTCH1 is a proven candidate gene and its mutations have been found in familial and sporadic cases of BAV.
Methods: 66 BAV patients from the GISSI VAR study were genotyped for the NOTCH1 gene.
Results: We identified 63 variants, in heterozygous and homozygous states. Fifty-two are common polymorphisms present in almost all patients. Eleven variants are new and never yet reported: two are non-synonymous substitutions, Gly540Asp in exon 10 and Glu851Gln in exon 16; one is in the 3'UTR region and seven in introns, one corresponds to a T allele insertion in intron 27. We selected four statistically noteworthy and seven new variants identified in six BAV patients and correlated them with clinical and demographic variables and with imaging and histological parameters. Preliminary data show that four were BAV patients with isolated stenosis in patients over 60 aged. These variants may correlate with a later need for surgery for the presence of stenosis and not aortic valve regurgitation or ascending aortic aneurysm.
Conclusions: Completing the genotyping of 62 BAV patients we found 11 new variants in the NOTCH1 gene never yet reported. These findings confirm that the identification of new, clinically remarkable biomarkers for BAV requires a deeper genetic understanding of the NOTCH1 gene variants, which could be targeted by future diagnostic and therapeutic strategies.

1. Introduction

During heart development, cusp fusion or failure of the cusps to separate can result in a valve with two cusps (bicuspid) or one cusp (unicuspid) (Fedak et al., 2002). Bicuspid aortic valve (BAV) results from abnormal aortic cusp formation during valvulogenesis with adjacent cusps fused into a bigger single cusp that operates with the normal cusp as the valve (Tadros et al., 2009). BAV is a congenital heart

Abbreviations: Bicuspid aortic valve, BAV; epithelial-to mesenchyme transition, EMT; GISSI Group, Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto; trans-thoracic echocardiography, TTE; trans-esophageal echocardiography, TOE; Biobanking and BioMolecular resources Research Infrastructure, BBMRI; polymerase chain reaction, PCR; Analysis of Variance, ANOVA

* Corresponding author at: Department of Cardiovascular Research, IRCCS - Istituto di Ricerche Farmacologiche "Mario Negri", Via Giuseppe La Masa, 19 - 20156 Milano, Italy.
E-mail address: silvana.pileggi@marionegri.it (S. Pileggi).

[1] See Appendix A for a complete list of participating Centers and Investigators.

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defect with a prevalence estimated at 1% (Hoffman and Kaplan, 2002) and is frequently (10–35% of cases) associated with enlargement of the ascending aorta (Svensson, 2008) with consequent severe complications such as aortic rupture or dissection (Cripe et al., 2004). BAV can present significant morphological variability. Most commonly, the right and left coronary cusps comprise the larger fused cusp and the non-coronary cusp is separate, with true commissures. The coronary arteries usually arise in front of the cusp with a raphe.

Only 20% of patients with a congenital BAV will maintain a normally functioning valve throughout life and > 30% suffer serious morbidity throughout their life (Michelena et al., 2008; Della Corte et al., n.d.). Patients may develop progressive calcification and stenosis. BAV is the main cause of pure aortic stenosis in most series. Patients may develop pure regurgitation with or without infection (40–60% of severe aortic regurgitation in the BAV population is secondary to infective endocarditis). Valvular and aortic complications usually occur in BAV patients earlier in life than in those with tricuspid aortic valves (Michelena et al., 2008); 30–50% of all patients who underwent surgical aortic valve replacement for stenosis have BAV (Roberts and Ko, 2005). All these aspects indicate that BAV is an important medical and economic problem that needs to be tackled.

BAV appears to be sporadically transmitted through families with an autosomal dominant inheritance, the heritability estimates range from 0.75 to 0.89.BAV is highly hereditable, and mutations in single genes have been reported in a few human cases (Cripe et al., 2004). The NOTCH1 gene (on chromosome 9q34-35) is a proven candidate and its mutations have been found both in familial and sporadic cases of BAV. However, their role in the pathophysiology is still debated (Garg et al., 2005). The genetic background of BAV, the real influence of NOTCH1 genetic variants and whether other genes are involved in the impaired cardiogenesis leading to BAV are still not known. The importance of considering NOTCH1 as a potential candidate gene for BAV formation is stressed in several studies in human and animal models summarized in the recent review of Giusti et al. (Giusti et al., 2017). Targeted inactivation of Notch in genetically modified mice impairs endocardial epithelial-to mesenchyme transition (EMT) leading Notch1 null mice to develop serious cardiac alterations (von Gise and Pu, 2012). Moreover, Notch1 signaling has also been described as affecting molecular processes involved in aortic valve calcification: Notch1 +/− mice have been shown to undergo a higher aortic valve calcification level (> 5-fold) with respect to the wild-type counterparts (Giusti et al., 2017).

Other studies based on targeted mutational approaches underlined a possible real role of NOTCH1 in aortic valve diseases and allowed the identification of new variants in the NOTCH1 gene related to specific forms of cardiac alteration, such us left-sided congenital heart disease (LS-CHD) (Mohamed et al., 2006; Kerstjens-Frederikse et al., 2016).

The GISSI Group (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto) focused on small groups of patients with homogeneous outlier phenotypes at opposite extremes of their overall distribution, starting the “GISSI OUTLIERS studies” that includes the GISSI OUTLIERS VAR study. Based on the hypothesis of an underlying genetic contribution in the pathogenesis of BAV, we planned a study enrolling patients with BAV, with the main aim of studying small homogeneous groups of BAV in terms of age, valve morphology and/or dysfunction, type of aortic enlargement-associated. We employed the clinical data and genetic material of the GISSI OUTLIERS VAR database. Integration with data on the presence of BAV disease in family groups permitted better definition of the genetic aspect, particularly for the NOTCH1 gene. Some recent studies suggested that mutations in this gene, identified in a small number of families, might be associated with syndromic forms of cardiac alteration, such as hypoplastic left heart syndrome, in some patients (Garg et al., 2005). To our knowledge, this is the first study that enrolled a quite large number of well-classified BAV patients and considered a known condition of connective disease in patients or in family groups as exclusion criteria of enrolment. A clinical evaluation at the time of enrolment has confirmed the absence of features related to connective disease. This approach could result of particular interest from a diagnostic point of view.

2. Material and methods

2.1. Study population

The GISSI OUTLIERS VAR protocol was designed as a prospective longitudinal study; ten of the 13 leading cardiac centers in Italy recruited patients. The full protocol, published by Merlanti et al. (Merlanti et al., 2015), had the aim to identify markers/predictors of favorable–unfavorable aortic wall evolution selecting homogeneous small groups of surgical patients with the same subtype of BAV and same aortic behaviour in order to evaluate if there is a BAV phenotype more likely to be at high risk for aortic degeneration. Briefly, each center enrolled at least one BAV patient eligible for cardiac surgery for each of these conditions (Fig. 1): BAV with isolated regurgitation (group A); BAV with normal valvular function but associated aorta

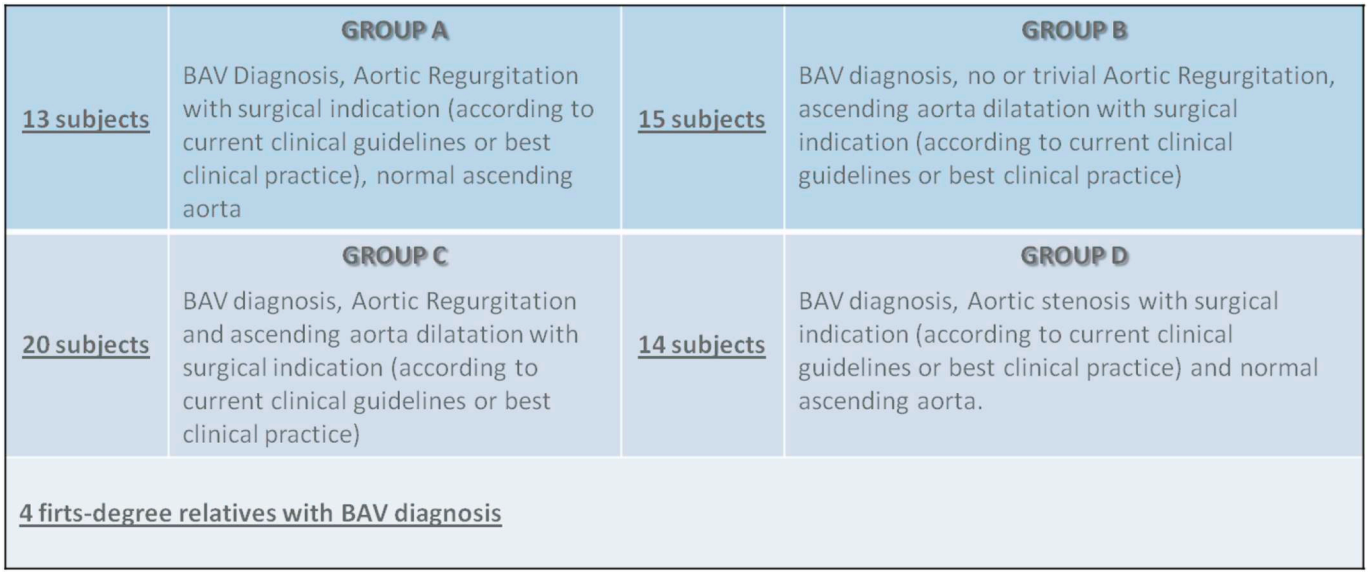

Fig. 1. Basic details of the GISSI OUTLIERS VAR population.

dilatation (group B); BAV with both valve regurgitation and aortic dilatation (group C); BAV with isolated stenosis in patients over 60 years old (group D).

BAV was diagnosed for individuals whose aortic valves had two clearly defined cusps or the characteristic systolic fish mouth appearance of the cusps and two of three supporting features of BAV, including systolic doming or diastolic prolapse of the aortic valve cusps and eccentric valve leaflet closure. Bicuspid valve morphology was confirmed by independent review of each echocardiogram by two observers who assessed and reported the morphology of the cusps and commissure positions, morphology and dimension of the aortic annulus, aortic root, ascending aorta and aortic arch and the grade of valve disease. If transthoracic echocardiography (TTE) gave an uncertain result, a transesophageal echocardiography (TOE) and/or 3D imaging was suggested, whenever possible. The GISSI VAR study is still in progress, but enrolment was closed in September 2014, reaching the planned number of subjects in each group. The protocol also considered the first-degree relatives of enrolled patient, who were asked to take a screening TTE in order to detect- in view of the familial etiology of BAV – the presence of BAV and/or associated disease (aortic root or ascending aorta enlargement or coronary ostia displacement).

Clinical and echo follow-up after 6 months and at 1, 2, and 3 years from surgery have been performed in order to evaluate changes/evolution of the disease in the valve/aorta not surgically treated.

2.2. Methods

EDTA-blood was available for all subjects. Biological samples are stored at the certified biobank of IRCCS Istituto di Ricerche Farmacologiche “Mario Negri”, which operates under ISO 9001:2015 rules and is part of the Italian BBMRI (Biobanking and BioMolecular resources Research Infrastructure) network. The 34 coding exons and their flanking regions of NOTCH1 gene were amplified by polymerase chain reaction (PCR) as previously described (Merlanti et al., 2015). PCR products were purified by solid-phase extraction (QIAquick PCR purification Kit; QIAGEN, Hilden, Germany) and sequenced according to standard protocols on the ABI 3130Xl platform and analyzed by the Sequence Analysis software version 5.1. The different contigs are assembled with the SeqScape software (applied Biosystems). Histological sections of aortic specimens were stained with Hematoxylin–Eosin, Alcian PAS and Weigert Van Gieson to evaluate the aortic wall degenerative changes. In detail, a semi-quantitative analysis was performed on the tunica media adopting grades from 0 to 4 according to Larson and Edwards concerning the following parameters: medial necrosis, cystic degeneration, and elastic fragmentation (Merlanti et al., 2015). Moreover, overall medial degeneration was evaluated using a mild/moderate/severe scale for severity according to the consensus-grading scheme from the Society for Cardiovascular Pathology and the Association For European Cardiovascular Pathology (Halushka et al., 2016). Grading was performed at the most severe region from each aortic segment.

2.3. In silico analysis of missense variants

A computational analysis of novel missense variants of NOTCH1 gene was performed with three different programs – PolyPhen, PROVEAN (Protein Variation Effect Analyzer) and SIFT – that are *in silico* tools predicting the possible impact of an amino acid substitution on the structure and function of a human protein (Anon, n.d.-a; Anon, n.d.-b; Anon, n.d.-c). The hg19 Genome Reference has been considered.

2.4. Statistical analysis

Categorical variables are presented as frequency and proportions, and continuous variables as means and standard deviations. Differences in baseline characteristics of the four groups (A,B,C,D) were compared by the Chi-square test for categorical variables, and for continuous variables we adopted Analysis of Variance (ANOVA).We used Fisher's Exact Test to detect any genotypic-phenotypic association and identify genetic variants with different distribution within the four groups.

A two-sided p-value of 5% was deemed statistically significant. All statistical analyses were done with SAS software, version 9.2 (SAS Institute, Inc., Cary, NC).

3. Results

3.1. BAV population

In all 62 unrelated patients were enrolled, 53 male (85.5%), with mean age 48.8 years (± SD 14.32). Patients were divided into four small groups according to the echographic classification of BAV and aortic disease (Fig. 1). Four more subjects appertaining to the ‘relatives with BAV group’ were enrolled, all male with a mean age of 46.2 years.

As expected, group A patients were younger (mean age 34.2 years) than groups B, C and D patients (respectively 46.1, 46.5 and 68.2, $p < .0001$); in group A only 23.1% were over 40 years old. Patients within the group D seem to have a benign presentation of the BAV condition and a later need for surgery presumably due to the old age.

3.2. NOTCH1 genotyping

We identified 63 variants, in heterozygous and homozygous states (Table S1); 52 were polymorphisms already reported in literature, 23 were in exon sequences or in untranslated regions and three caused aminoacidic changes in the mature protein sequence: rs199654211 Asn104Ser in exon 3; rs61751543 Arg1279His in exon 23 and rs61751489 Val2285Ile in exon 34. Twenty-nine polymorphisms fall in the intronic sequences.

Eleven variants are new and never yet reported in BAV literature. Three were identified in exons and two of these were non-synonymous substitutions: Gly540Asp in exon 10 and Glu851Gln in exon 16. One was in the 3′UTR region and 7 in the intron: one corresponded to a T allele insertion in intron 27 (Table 1).

3.3. Genotypic-phenotypic association

Once the genotyping was complete, we examined the genotype and allele frequencies of the variants identified in the whole BAV population ($n = 62$) and in the four groups, in order to establish their possible specific distribution. Among the 52 known polymorphisms in the NOTCH1 gene, we found two variants that had a significantly different genotype distribution among the four groups, at $p < .05$: rs9411208 in intron 8 ($p = .029$) and rs11574889 in exon 10 ($p = .017$). Another interesting variant was IVS13 + 15 in intron 13, which appeared only in group D.

Considering all the ‘rare and new’ variants emerging from a preliminary genetic analysis, we identified seven subjects of particular interest: six BAV patients and one first-degree relative. Table 2 reports the main clinical, echo and histologic features of these subjects and Fig. 2 shows surgical pathologic features and histology of the aortic tunica media of the same patients. Four out of six patients belong to group D (BAV with isolated stenosis in patients over 60 years old).

4. Discussion

Heart valve disease with a surgical indication, observed in a specific cardiac disorder with different clinical presentations, can offer many opportunities for investigation, from imaging to real view, from genetic blood tests to analysis of surgical tissue samples. Patients with BAV provide an ideal setting to put into practice this way of dealing with a disease and its multiple possible evolutions: from pure regurgitation to pure dilatation of the ascending aorta to aortic stenosis with various

Table 1
New NOTCH1 genetic variants identified for the first time in the GISSI VAR study. Each variant was found in a single subject. The number of variants found in each group is shown with their position, in exons or intron (IVS), the aminoacidic change when present and the predictive consideration with the three different tools used (Polyphen, SIFT and PROVEAN).

Group	Number of variants	Position	Nucleotidic change	Aminoacid change	Polyphen	SIFT	PROVEAN
A (n = 13)	1	ex10	c. G1619A	Gly540Asp	Probably damaging	Damaging	Deleterious
B (n = 15)	3	IVS22 + 48		–			
		IVS28 + 17		–			
		ex34 (3' UTR)		–			
D (n = 14)	7	IVS13 + 10		–			
		IVS13 + 15		–			
		ex16	c.G6255C	Glu851Gln	Probably damaging	Tolerated	Neutral
		IVS16–92		–			
		IVS23–4		–			
		IVS27 + 42insT		–			
		ex 29	c. C5415T	Leu1805Leu			

intermediate phenotypes (Merlanti et al., 2015).

Recently the BAVCon (Bicuspid Aortic Valve Consortium) discussed current knowledge about the pathophysiology and genetic etiology of BAV and indicated the possible clinical implications and translational application of the genetic discoveries (Prakash et al., n.d.). The authors noted that genetic studies of cohorts or family with predisposition to BAV offer an important opportunity for gene discovery. There are very few papers about the genetics of BAV formation and all comprise only quite small populations of patients. The limited numbers of genes investigated in these studies underline the great uncertainty in current knowledge of the genetic basis of BAV formation.

Given this complexity, a more recent review by Giusti et al.

Table 2
Clinical, echographic and histologic features of the six BAV patients and onerelative with 'rare and new' variants emerging from a preliminary genetic analysis.

	Genetic variants	Group	Characteristics and type of BAV	Echo features	Histologic features Grading* and overall medial degeneration severity^
Patient 1	Gly540Asp#	A	Man 21 yrs. No risk factors. Fusion of right coronary and non-coronary cusps; prolapsed, no calcifications, a raphe, normal coronary anatomy	Mild annulus dilatation, normal ascending aorta	Elastic fragmentation 1 Medial necrosis 2 Cystic degeneration 1 Mild medial degeneration
Patient 2	rs148331061; rs61751541	C	Man 55 yrs. Hypertension. Fusion of left and right coronary cusps; prolapsed, no calcifications, a raphe, normal coronary anatomy	Annulus not dilated, ascending aorta dilatation	Elastic fragmentation 3 Medial necrosis 2 Cystic degeneration 2 Moderate medial degeneration
Relative 1	rs148331061; rs61751541		Man, 64 yrs. Diabetes, hypertension. Fusion of right coronary and non-coronary cusps; not prolapsed, calcifications, a raphe.	Annulus not dilated, mild ascending aorta dilatation	n.a. (not surgery)
Patient 3	rs11574889; IVS27 + 42insT#	D	Woman, 63 yrs. Diabetes, dyslipidemia. Fusion of left and right coronary cusps; not prolapsed, calcifications, a raphe, normal coronary anatomy	Small aortic annulus, normal ascending aorta	Elastic fragmentation 1 Medial necrosis 2 Cystic degeneration 1 Mild medial degeneration
Patient 4	rs11574889; IVS13 + 10#; IVS23–4#	D	Man 68 yrs. Hypertension. Fusion of right coronary and non-coronary cusps; not prolapsed, calcifications, a raphe, normal coronary anatomy	Annulus not dilated, normal ascending aorta	Elastic fragmentation 2 Medial necrosis 2 Cystic degeneration 1 Moderate medial degeneration
Patient 5	rs11574889; rs61751489	D	Woman, 70 yrs. No risk factors. Fusion of left and right coronary cusps; not prolapsed, presence of calcifications, presence of a raphe, normal coronary anatomy	Small aortic annulus, normal ascending aorta	Elastic fragmentation 1 Medial necrosis 3 Cystic degeneration 1 Moderate medial degeneration
Patient 6	IVS13 + 15# Glu851Gln# Leu1805Leu#	D	Man, 68 y.o. Hypertension. Fusion of left and right coronary cusps; not prolapsed, calcifications, a raphe, normal coronary anatomy	Annulus not dilated, normal ascending aorta	n.a.

^According to the consensus grading scheme of the Society for Cardiovascular Pathology and the Association For European Cardiovascular Pathology (von Gise and Pu, 2012).

New variants.

* According to the grading scale of Larson and Edwards (Giusti et al., 2017).

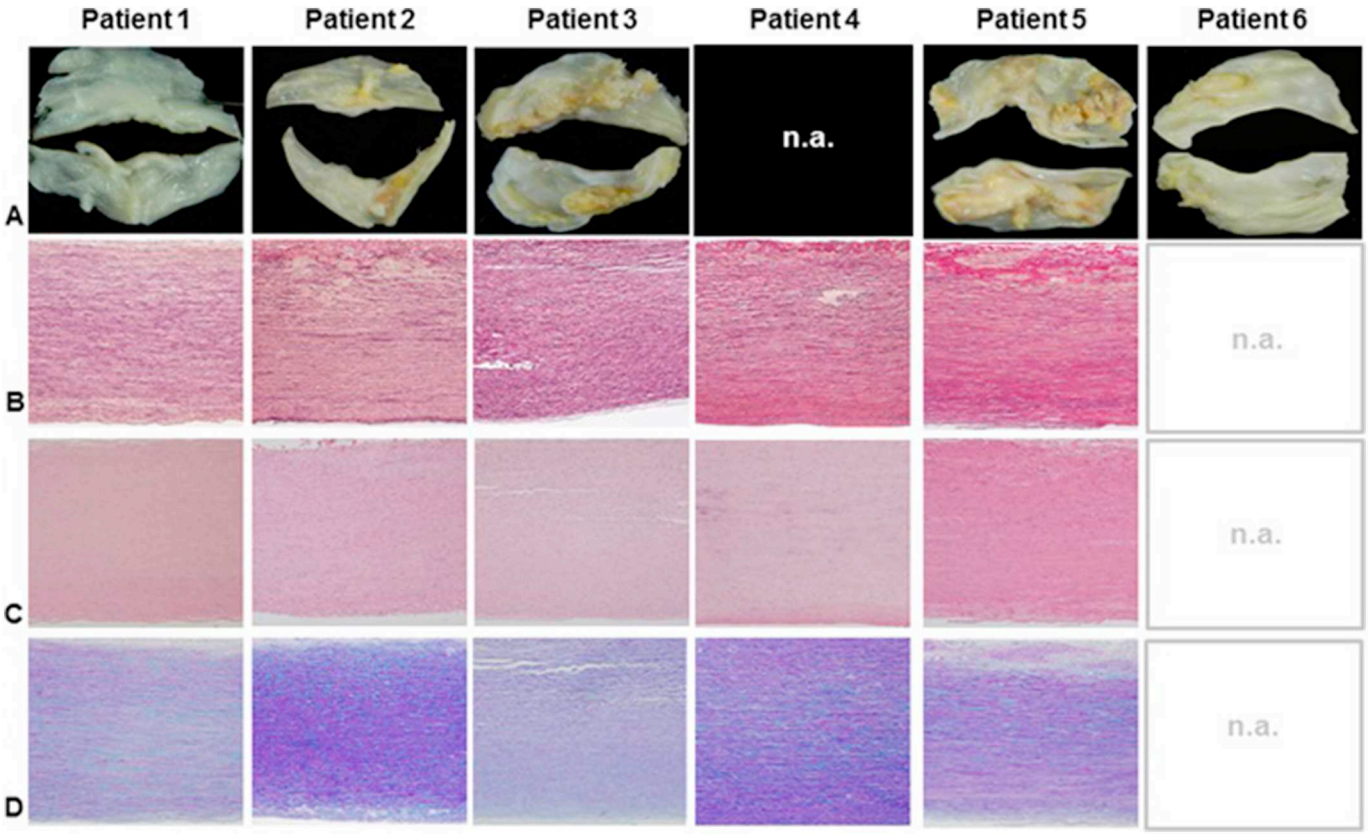

Fig. 2. Surgical pathologic features of the bicuspid aortic valve (A) and aorta specimens (B-D) in the 6 patients with 'rare and new' variants. A. Gross view of surgically resected aortic bicuspid valves; patient 1 show thickening of the free edge; note coarse nodular calcium macro aggregates and the raphe in patients 2, 3, 5 and 6 with aortic stenosis owing to dystrophic calcification. B-D Histology of the aortic tunica media showing different grades of elastic fragmentation (B, elastic Weigert Van-Gieson stain), medial necrosis (C,Hematoxylin-Eosinstain) and cystic degeneration (D, Alcian–PAS stain) of the lamellar units.

underlined the necessity of consider animal model studies, targeted mutational approaches and High-Throughput Sequencing (HTS) technologies to give a deeper and complete view of the genetic background of aortic alterations (Giusti et al., 2017).

Several family-based studies have shown that BAV, either alone or in combination with other malformations, can recur in families, with an autosomal dominant inheritance. Only 20% of patients will maintain a normally functioning valve and > 30% will develop serious morbidity (Michelena et al., 2008; Della Corte et al., n.d.).

BAVs have a heterogeneous presentation that is age-related and the nature of the valve lesion differs among patients. The severity of the aortic disease is depends on the type of valve lesion, but valve dysfunction alone does not account for the degree of aortic dilatation (Michelena et al., 2008).

All these aspects underline the need for new genetic markers to indicate a better approach for prevention or improvement of disability due to the disease. For this reason, we planned the GISSI OUTLIERS VAR protocol, designed as an Italian prospective longitudinal study enrolling BAV patients eligible for cardiac surgery as described by Merlanti et al. (Merlanti et al., 2015). Part of this study focused on the genetic bases of BAV formation and progression. Since the entire protocol considered also echo and histological aspects, a complete analysis will be the issue of a separate paper: here we report the preliminary genetic characterization of the GISSI-VAR population. Integration with data about the presence of BAV disease in relatives enable us to define the genetic aspects better. We studied the 34 coding exons of the NOTCH1 gene.

The sample size of our BAV biobank (62 unrelated BAV patients and 4 relatives) means that these results could add important information about the variability of BAV phenotypes.

This study describes eleven variants in the NOTCH1 gene (Table 1) never described in association with the BAV condition. Since we could not study the relatives of the two subjects with the non-synonymous substitutions, we do not know whether these variants segregated with the disease; however we analyzed them with three different *in silico* tools predicting the possible impact of an amino acid substitution on the structure and function of a human protein (Anon, n.d.-a; Anon, n.d.-b; Anon, n.d.-c). As reported in Table 1, the Gly540Asp variant in exon 10 has been described as 'probably damaging and damaging' by Polyphen and SIFT and as 'deleterious' by PROVEAN: it causes substitution of an aliphatic amino acid, glycine, with an amino acid with an electrically charged side chain, aspartic acid. In our cohort, the subject carrying this mutation was a young man 21 years old, who underwent cardiac surgery for BAV. He was classified in group A (patient eligible for cardiac surgery due to BAV with isolated regurgitation) and presented a fusion of the right coronary and non-coronary cusps, significant prolapse, no calcification, a raphe and normal coronary anatomy. The ExAC database describes p.Gly540Asp as a singleton variant without any specific annotation in the ClinVar database confirming that it could be a rare variant of particular interest to study. The second variant, Glu851Gln, causes substitution of a negatively charged glutamic acid with glutamine that has an amide side chain instead of the hydroxyl one of glutamic acid, with an amine functional group. It was considered 'probably benign' by Polyphen, 'tolerated' and 'neutral' by SIFT and PROVEAN, respectively. This variant was found in a subject with two other novel variants: the intronic IVS13 + 15 and the synonymous point substitution Leu1805Leu in exon 29. He belonged to group D (BAV with isolated stenosis in patients over 60) and presented hypertension, fusion of the left and right coronary cusps, not prolapsed, calcification, raphe and normal coronary anatomy. The echo analysis showed no hypertrophic

ventricle, even with aortic stenosis. Glu851Gln is not present in any public database of known variants (HGMD, ExAC for example); conversely, Leu1805Leu is present in EXAC and defined as singleton.

Some of the novel variants are still under review, looking for their functional and genetic characterization, and we found 52 known polymorphisms in the NOTCH1 gene. Two variants had a different genotype distribution in the four groups, statistically significant at $p < .05$, rs9411208 in intron 8, rs11574889 in exon 10, and one that was statistically borderline, IVS13 + 15 in intron 13.

Starting from these 'rare and new' variants for the BAV condition, we performed a preliminary association analysis and selected six BAV patients and one first-degree relative of particular interest. Four of the six patients were in group D, that assembled subjects with a benign presentation of the BAV condition and a later need for surgery presumably due to the old age (Table 2). Although none of the known or new variants identified in our study seemed to be associated with any specific clinical feature, further investigations are needed to clarify their role. We hypothesize that these variants may correlate with a later need for surgery, driven by the presence of stenosis, not cardiac failure. Our results confirm a role of NOTCH1 gene in susceptibility to dystrophic calcification and aortic stenosis in BAV, corroborating previous studies which reported frameshift mutations and variants of NOTCH1 in patients with calcific BAV (Anon, n.d.-c; Mohamed et al., 2006; Nigam and Srivastava, 2009). Association analyses for the four first-degree relatives are now in progress and the findings should be helpful to understand the inheritance of BAV. A recent paper by Girdauskas et al. (Girdauskas et al., 2017) shows the results of a next generation sequencing of 63 patients with BAV root phenotype. The custom-made panel used for this analysis included also the NOTCH1 gene. None of the six different rare variants in NOTCH1 gene identified in this study corresponds with those found in our population. Even if the sample sizes of the two studies are quite similar, we speculate that the observed differences are due to the phenotypic heterogeneity of the two study populations. The group of Girdaukas enrolled young men with BAV insufficiency and aortic root dilatation. Our population consists in 62 unrelated patients divided into four small groups according to the echographic classification of BAV and aortic disease and eligible for cardiac surgery for the conditions shown in Fig. 1. The differences in the planning of the populations to recruit also correspond to the different rationales of the two studies. Our study focused on the identification of markers associated to the aortic wall evolution selecting homogeneous small groups of surgical patients with the same subtype of BAV and same aortic behaviour. The one of Girdauskas had as primary end-point to study the prevalence of genetic defects in patients with the BAV root phenotype. Even though we could count on a quite big genetic biobank of BAV patients of Italian origin ($n = 62$), the main limitation was the lack of a control group of healthy subjects that allowed us to better define and validate the identified variants so we can just speculate on their possible pathogenicity. However, so far this is the first study that describes the correlation of genetics with clinical and demographic variables and with imaging and histological parameters in a BAV population.

BAV disease has the characteristic of heredity with variable genetic penetrance and many studies had shed light upon the genetic and morphologic biomarkers for the diagnosis and prognosis of this condition. A very recent review defined NOTCH1 as one of the main implicated gene in BAV (23) and most of its highly penetrant mutations (75%) determining BAV. We also believe in the role of the NOTCH1 gene mutations affecting the normal aortic valve formation as suggested by several previous studies both in humans and in animal models (Giusti et al., 2017). For this purpose, the present work has the intent to describe the NOTCH1 gene background in a quite big BAV population in the Italian GISSI VAR study. Many other association studies – part of the GISSI VAR protocol - are ongoing and consider the clinical data obtained at follow-up visits. The integration of all these findings could support the role of new genetic markers in finding a better approach for prevention or improvement of disability due to the disease.

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Authorship

The authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation. All authors have read and approved the manuscript.

The study was registered as a clinical trial on the Clinical Trial **Registry,** www.clinicaltrials.gov (Identifier: NCT02283970).

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CRediT authorship contribution statement

Silvana Pileggi: Supervision**Benedetta De Chiara**Supervision**Maria Grazia Franzosi:**Supervision**Bruno Merlanti**Supervision

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A

Steering committee

A. Maseri (Chairman), L. Martinelli (Co-Chairman), L.P. Badano, M.G. Franzosi, A.P. Maggioni, B. Merlanti, A. Moreo, C.F. Russo, G. Thiene

Coordinating Center (ANMCO Research Center)

A.P. Maggioni (Director), M. Gorini, A. Lorimer, I. Cangioli, F. Bianchini, M. Tricoli, C. Alongi

Genetic Core Laboratory (Lab. Valutazione Clinica Farmaci Istituto Mario Negri Milano)

M.G. Franzosi, S. Pileggi

Echo Core Laboratory (Padova and Milano)

L.P. Badano, G. Kocabay, G. Romeo (Clinica Cardiologica Università di Padova), A. Moreo, B. De Chiara (Cardiologia 4 Osp. Niguarda Milano).

Istological Core Laboratory (Istituto di Anatomia Patologica Università di Padova)

G. Thiene, C. Basso, S. Rizzo

Centralized Biobank (Istituto Mario Negri Milano)

S. Masson

Participating centers

Catanzaro, Casa di Cura Villa Sant'Anna; Firenze, AOU Careggi;

Mestre, Ospedale dell'Angelo; Milano, Centro Cardiologico Monzino; Milano, Ospedale Niguarda; Milano, Ospedale San Raffaele; Roma, Ospedale San Camillo; San Donato Milanese, IRCCS Policlinico San Donato; Torino, Ospedale Molinette; Udine, AOU Santa Maria della Misericordia.

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