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# The genetic component of bicuspid aortic valve and aortic dilation. An exome-wide association study

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ABSTRACT

*Background:* Bicuspid aortic valve is the most common cardiovascular congenital malformation affecting 2% of the general population. The incidence of life-threatening complications, the high heritability, and familial clustering rates support the interest in identifying risk or protective genetic factors. The main objective of the present study was to identify population-based genetic variation associated with bicuspid aortic valve and concomitant ascending aortic dilation.
*Materials and methods:* A cross-sectional exome-wide association study was conducted in 565 Spanish cases and 484 controls. Single-marker and gene-based association analyses enriched for low frequency and rare genetic variants were performed on this discovery stage cohort and for the subsets of cases with and without ascending aortic dilation. Discovery-stage association signals and additional markers indirectly associated with bicuspid aortic valve, were genotyped in a replication cohort that comprised 895 Caucasian cases and 1483 controls.
*Results:* Although none of the association signals were consistent across series, the involvement of *HMCN2* in calcium metabolism and valve degeneration caused by calcium deposit, and a nominal but not genome-wide significant association, supported it as an interesting gene for follow-up studies on the genetic susceptibility to bicuspid aortic valve.
*Conclusions:* The absence of a genome-wide significant association signal shows this valvular malformation may be more genetically complex than previously believed. Exhaustive phenotypic characterization, even larger datasets, and collaborative efforts are needed to detect the combination of rare variants conferring risk which, along with specific environmental factors, could be causing the development of this disease.


## 1. Introduction

Bicuspid aortic valve (BAV) is the most common cardiovascular congenital malformation with a prevalence of 0.5 to 2% in the general population [1,2] and a 2:1 male:female ratio [3]. It is frequently an incidental echocardiographic finding in asymptomatic patients [3];

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**Table 1**
Summary of the more relevant clinical variables collected for all cases included in the discovery stage. M = male, F = female, SD = standard deviation, A = affected, U = unaffected. Thoracic aortic dilation = A, when either the sinuses of Valsalva or the ascending thoracic aorta were dilated. Categories with a number of individuals below the total include undetermined samples.

| | Variable | Discovery stage cohort | Replication cohort |
|---|---|---|---|
| Controls | Gender M/F | 280/203 | 1155/328 |
| | Mean age (SD) | 47 (9) | 65 (10) |
| BAV cases | Gender M/F | 404/161 | 660/235 |
| | Mean age (SD) | 44 (16) | 56 (13) |
| | Mean weight in kg (SD) | 74 (14) | 83 (17) |
| | Mean height in cm (SD) | 170 (10) | 174 (10) |
| | Arterial hypertension A/U | 166/391 | – |
| | Diabetes Mellitus A/U | 24/532 | – |
| | Dyslipidemia A/U | 118/433 | – |
| | Ischemic heart disease A/U | 26/518 | – |
| | Congenital heart disease A/U | 75/418 | 48/815 |
| | BAV morphotype, types 1/2/3 | 413/132/12 | 433/122/9 |
| | BAV raphe A/U | 485/70 | – |
| | BAV calcification A/U | 360/193 | – |
| | BAV sclerosis A/U | 175/378 | – |
| | BAV prolapse A/U | 61/496 | – |
| | BAV insufficiency A/U | 437/123 | – |
| | BAV stenosis A/U | 270/290 | – |
| | Thoracic aortic dilation A/U | 307/232 | 383/512 |
| | Mean sinuses of Valsalva (SD) | 36 (6) | – |
| | Mean ascending thoracic aorta (SD) | 40 (8) | 40 (8) |
| | Mean descending thoracic aorta (SD) | 20 (4) | – |
| | Mean abdominal aorta (SD) | 21 (4) | – |

however, 1/3 of all BAV patients develop valvular or aortic complications in their lifetime, including aortic stenosis, aortic regurgitation, infective endocarditis, and aortic dissection [4–7]. Furthermore, up to 50% suffer from additional congenital cardiovascular defects such as aortic coarctation, patent ductus arteriosus, ventricular septal defects, hypoplastic left ventricle, or Turner and Marfan syndromes [2,3,8–10]. These clinical findings have raised interest in understanding the molecular basis of both the valvular malformation, that seems to no longer refer to a single disease, and associated conditions. Nevertheless, the mechanisms remain unknown [3].

Although BAV can be a sporadic defect, heritability of up to 89% [11], and a high incidence of familial clustering [12] support a genetic origin. Despite the evidence of an autosomal dominant inheritance pattern with variable expression and incomplete penetrance in some families [6], the genetic component does not seem to be as simple as at first assumed. Referring to BAV development, Prakash *et al.* stated in 2014, "Just as BAV phenotypes are highly variable, genetic aetiologies are equally diverse and vary from complex inheritance in families to sporadic cases without any evidence of inheritance" [8]. Despite the repeatedly-proposed complex inheritance of this disease, no genome or exome-wide association studies (GWAS, EWAS) of BAV have been published to date [13], and only one study has investigated the genetic mechanisms of sporadic thoracic aortic aneurysms and dissections in BAV patients [14].

A large cross-sectional EWAS enriched in low frequency and rare genetic variants was conducted. Our main objective was to identify genetic markers associated with BAV. Our secondary objectives were to identify genetic markers associated with ascending aorta dilation (AAD) in BAV patients, and to replicate markers associated with development of sporadic thoracic aortic aneurysms and dissections in BAV patients [14,15].

## 2. Materials and methods

### 2.1. Subjects

#### 2.1.1. Discovery cohort

The discovery cohort was a Spanish population-based series of 565 unrelated cases and 484 controls. We consecutively recruited cases from 8 different hospitals (*Hospital Universitari Vall d'Hebron*, *Hospital Universitario Virgen Macarena*, *Complexo Hospitalario Universitario de Vigo*, *Hospital Universitario Virgen de la Victoria*, *Hospital Clínico Universitario Virgen de la Arrixaca*, *Hospital Universitario 12 de Octubre*, *Hospital General Universitario Gregorio Marañón*, *Hospital Clínico Universitario de Valladolid*) from March 2011 to July 2013. Exclusion criteria were cardiac or ascending aorta surgery, incomplete BAV diagnosis, and any of the main connective tissue syndromes (Marfan, Ehlers Danlos). After institutional approval by the *Hospital Universitari Vall d'Hebron Ethics Committee (Spain)*, and individual patient written informed consent according to the principles outlined in the Declaration of Helsinki, we evaluated all cases by transthoracic echocardiography and collected peripheral blood samples. The *Hospital Universitari Vall d'Hebron* core echocardiography laboratory examined all echocardiographic images. We collected demographic and clinical variables from patient interview and review of medical records, and summarized the distribution of the more relevant in Table 1.

The presence of aortic root or AAD was determined using reference population diameters corrected by body surface area and age based on Devereux *et al.* [16] and Muraru *et al.* [17] recommendations, respectively.

The discovery stage control cohort comprised unrelated individuals attending primary health care centers in Galicia (North-West of Spain) and from *Plataforma en Red Banco Nacional de ADN Carlos III (BNADN)*. All patients were to the best of their knowledge, free of cardiovascular, renal, pulmonary, hepatic, hematologic, and chronic diseases subjected to chronic treatment, as well as AIDS and hepatitis B and C. Moreover, they were subjected to a brief medical examination and questionnaire.

#### 2.1.2. Replication cohort

The replication cohort comprised 2378 unrelated individuals, 895 cases and 1483 Caucasian controls from the International *Bicuspid Aortic Valve Consortium* (*BAVCon*), and was independent of the discovery stage sample. After signature of proper informed consent approved by *Partners Human Research Committee (Massachusetts, USA)*, and following the principles of the Declaration of Helsinki, we reviewed all medical records, evaluated cases by transthoracic echocardiography and collected demographic and clinical variables, and peripheral blood from cases and saliva samples from controls. We finally classified patients with or without AAD based on the dimensions of the tubular ascending aorta, again following Muraru *et al.* recommendations [17].

### 2.2. Genotyping technology and quality control

#### 2.2.1. Discovery cohort

After checking the purity and integrity of the extracted genomic DNA, we performed exome-wide genotyping using the *Axiom Exome Array* and *GeneTitan* technology (*Affymetrix, California, USA*) comprising approximately 319,000 genetic variants (most of them rare) [18], in collaboration with *CEGEN-PRB2-ISCIII* (http://www.prb2.org/cegen) and following manufacturer's instructions (*Axiom Genome-Wide Human Assay Affymetrix* protocol).

We performed variant calling with the *Axiom GT1* algorithm implemented in the *Affymetrix Genotyping Console Software v4.1.4.840*. Using *PLINK v1.07* (http://pngu.mgh.harvard.edu/purcell/plink/) [19] and *GCTA v1.02* (http://cnsgenomics.com/software/gcta/) [20] standard procedures, we performed stringent quality control (QC) of the resulting genotypes following *Affymetrix Best Practices* [21] and published recommendations [22]. We excluded samples according to the following filters: (i) gender discordance; (ii) heterozygosity levels exceeding 3 standard deviations (SD) from the mean; (iii) call rate under 97%; (iv) cryptic relatedness (identity-by-descent > 0.1875 to any other sample in the cohort); and (v) population stratification principal components (PC) 1 or 2 exceeding 6 SD from the mean. We removed genetic variants based on the following criteria: (i) monomorphic in the study population; (ii); genotyping call rate under 99.5%; (iii) significant departure from Hardy-Weinberg equilibrium in the control cohort ($p < 0.001$); and (iv) significantly different genotyping call rate between case and control sample ($p < 0.001$). To confirm reliability, we re-genotyped a single multiplex including 18 of the most significant association signals in the whole discovery stage sample (Table S1). For this technical validation, we used the alternate genotyping technology *IPLEX GOLD MassARRAY* (*Agena Bioscience, California, USA*, formerly *Sequenom*), according to manufacturer's instructions and again in collaboration with *CEGEN-PRB2-ISCIII*. We finally checked consistency between the results obtained with both genotyping technologies.

#### 2.2.2. Replication cohort

We used *IPLEX GOLD MassARRAY* technology (*Agena Bioscience, California, USA*) to genotype the association signals selected for replication: seven of the discovery stage association signals showing $p < 1 \cdot 10^{-4}$ (rs8001733, rs42663, rs17382301, rs4889554, rs13294886, rs10492585, and rs3740526), and the five additional *FBN1* genetic variants previously reported to be associated with the development of sporadic thoracic aortic disease by LeMaire *et al.* in 2011 (rs10519177, rs4774517, rs755251, rs1036477, and rs2118181) [14] in the replication cohort. We excluded samples with mean call rate under 95% and genetic variants with (i) genotyping call rate under 95%; (ii) significant departure from Hardy-Weinberg equilibrium in the control sample ($p < 0.001$); and (iii) significantly different genotyping call rate between cases and controls ($p < 0.001$).

### 2.3. Statistical analysis

We tested each individual variant for association using logistic regression based on both additive (*add*) and dominant (*dom*) inheritance models, and also performed gene-based burden and Sequence Kernel association tests (SKAT) for those variants with minor allele frequency (MAF) < 1% [23]. We considered sex, age, and the first 10 population stratification PCs as covariates in all analyses with the exception of the replication stage, in which ancestry informative markers were not available. We performed all analyses using *PLINK v1.07* [19], and *GenABEL* [24] and *skatMeta R* packages, and created Manhattan plots using the *R* package *qqman* (https://github.com/stephenturner/qqman). We repeated this same analysis procedure for the subset of BAV cases with (n = 307) and without (n = 232) AAD, comparing both subsets to the same 484 discovery stage controls.

We then performed imputation on the whole discovery stage cohort to almost 82 million variants described in the *1000 Genomes Project Phase 3* reference panel [25]. We followed a pre-phasing/imputation stepwise approach using *SHAPEIT v2* and *IMPUTE2 v2.3.2* software [26, 27]. We finally applied the *SNPTEST v2.5* [28] frequentist association test based on both *add* and *dom* inheritance models.

We performed an *add* and *dom* inverse variance weighted meta-analysis using *GWAMA v2.1* software [29]. By default, we chose the fixed-effects model of the Mantel-Haenszel method, but assessed between-series heterogeneity based on Cochran's Q test. Should Q statistics have $p < 0.05$, we selected the random effects model instead. We performed meta-analysis of the imputed association results in the case of the five *FBN1* genetic variants.

Finally, we conducted a post hoc power analysis on the whole discovery stage sample using *Power and Sample Size Calculation (PS) v3.1.2* [30]. We estimated the minimum detectable odds ratio (OR) according to: (i) the number of discovery stage samples and genetic variants that remained after QC; (ii) MAF corresponding to either common or rare variants; (iii) a power of 80%; and (iv) a significance threshold inversely proportional to the number of association tests performed.

## 3. Results

### 3.1. Genotyping, quality control and power calculation

From the 295988 genetic variants that we were able to successfully call in the 565 cases and 484 controls that comprised the whole discovery sample, 163374 were monomorphic in this population and 111039 overcame stringent QC. Therefore, the whole discovery stage final dataset consisted of 31234 polymorphisms and 79805 low frequency/rare variants (MAF > or <5%, respectively), genotyped in 543 cases and 444 controls. A quantile-quantile (Q-Q) plot representing the observed versus expected *p* after QC can be found in Fig. 1.

Regarding the subsets of BAV cases with and without AAD affection, we performed association analysis of 103904 genetic variants in 294 cases versus 450 controls, and 90495 variants in 229 cases versus 448 controls that passed QC, respectively.

The number of individuals and genetic markers based on which we performed association tests represented a significant limitation. Still, assuming a Bonferroni's significance threshold of $4.5 \cdot 10^{-7}$ (0.05/111039), a power of 80%, a MAF of 10%, and allelic association testing, we could detect a minimum OR of 2.180. For a variant with MAF < 1%,

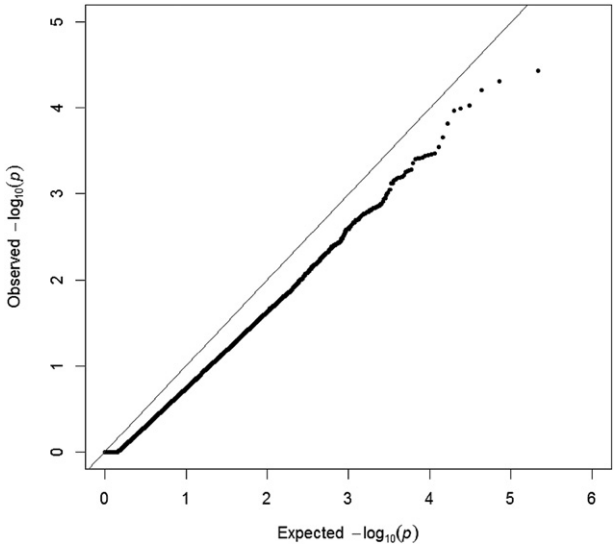


**Fig. 1.** Q-Q plot, illustrating the observed versus expected *p* we obtained from the logistic regression analysis of the whole discovery stage sample after QC based on the *add* inheritance model.

**Table 2**
Best discovery stage, replication, and meta-analysis association results by subgroup. *add* = additive, *dom* = dominant. A1 = tested allele, A2 = alternate allele. Bold print pinpoints the association results published by LeMaire et al. in 2011. 95% confidence interval.

| | ID | Chr | Position (hg19) | Gene | Subset of analysis | Inheritance model | Origin | N (cases/controls) | A1/A2 | MAF | OR (SE) | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Discovery stage | rs8001733 | 13 | 73,824,104 | – | Total | *add* | Discovery stage | 986 (543/443) | A/G | 0.4386 | 1.46 (0.10) | $9.29 \cdot 10^{-5}$ |
| | | | | | | | Replication stage | 2198 (834/1364) | | 0.4290 | 1.08 (0.07) | 0.2458 |
| | | | | | | | Meta-analysis | 3184 (1377/1807) | | 0.4320 | 1.25 (0.16) | 0.1365 |
| | rs42663 | 7 | 89,983,808 | *GTPBP10* | Dilation | *dom* | Discovery stage | 744 (294/450) | T/G | 0.2230 | 2.01 (0.16) | $1.05 \cdot 10^{-5}$ |
| | | | | | | | Replication stage | 1717 (353/1364) | | 0.1983 | 1.10 (0.13) | 0.4864 |
| | | | | | | | Meta-analysis | 2461 (647/1814) | | 0.2058 | 1.48 (0.34) | 0.1998 |
| | rs17382301 | 10 | 90,966,393 | *CH25H* | | | Discovery stage | 744 (294/450) | A/G | 0.0877 | 0.37 (0.23) | $2.27 \cdot 10^{-5}$ |
| | | | | | | | Replication stage | 1717 (353/1364) | | 0.1374 | 1.17 (0.14) | 0.2668 |
| | | | | | | | Meta-analysis | 2461 (647/1814) | | 0.1224 | 0.67 (0.23) | 0.4851 |
| | rs4889554 | 16 | 31,819,166 | – | | *add* | Discovery stage | 744 (294/450) | T/C | 0.2487 | 0.58 (0.14) | $5.53 \cdot 10^{-5}$ |
| | | | | | | | Replication stage | 1717 (353/1364) | | 0.2551 | 1.04 (0.10) | 0.7341 |
| | | | | | | | Meta-analysis | 2461 (647/1814) | | 0.2532 | 0.78 (0.17) | 0.3911 |
| | rs13294886 | 9 | 133,294,244 | *HMCN2* | No dilation | *dom* | Discovery stage | 677 (229/448) | A/G | 0.4911 | 0.37 (0.21) | $2.13 \cdot 10^{-6}$ |
| | | | | | | | Replication stage | 1593 (229/1364) | | 0.4564 | 1.08 (0.17) | 0.6638 |
| | | | | | | | Meta-analysis | 2522 (710/1812) | | 0.4667 | 0.64 (0.21) | 0.3958 |
| | rs10492585 | 13 | 105,386,176 | – | | | Discovery stage | 677 (229/448) | G/A | 0.0820 | 2.71 (0.25) | $7.08 \cdot 10^{-5}$ |
| | | | | | | | Replication stage | 1593 (229/1364) | | 0.0600 | 1.34 (0.22) | 0.1931 |
| | | | | | | | Meta-analysis | 2522 (710/1812) | | 0.0665 | 1.88 (0.48) | 0.0733 |
| Le Maire *et al.* 2011 findings | rs10519177 | 15 | 48,757,195 | *FBN1* | Total | *add* | **LeMaire et al. 2011** | **2813 (1313/1500)** | **G/A** | – | **1.6** | $\mathbf{2.6 \cdot 10^{-11}}$ |
| | | | | | | | Imputed discovery stage | 986 (543/443) | | 0.2596 | 0.97 | 0.6197 |
| | | | | | | | Replication stage | 2198 (834/1364) | | 0.2511 | 1.04 (0.08) | 0.6359 |
| | | | | | | | Meta-analysis | 3184 (1377/1807) | | 0.2537 | 1.01 (0.06) | 0.8574 |
| | rs4774517 | 15 | 48,759,291 | | | | **LeMaire et al. 2011** | **2813 (1313/1500)** | **A/C** | – | **1.5** | $\mathbf{3.8 \cdot 10^{-11}}$ |
| | | | | | | | Imputed discovery stage | 986 (543/443) | | 0.2595 | 0.97 | 0.6138 |
| | | | | | | | Replication stage | 2198 (834/1364) | | 0.2498 | 1.05 (0.08) | 0.5677 |
| | | | | | | | Meta-analysis | 3184 (1377/1807) | | 0.2528 | 1.02 (0.06) | 0.8006 |
| | rs755251 | 15 | 48,812,020 | | | | **LeMaire et al. 2011** | **2813 (1313/1500)** | **G/A** | – | **1.6** | $\mathbf{3.2 \cdot 10^{-11}}$ |
| | | | | | | | Imputed discovery stage | 986 (543/443) | | 0.2511 | 0.97 | 0.6631 |
| | | | | | | | Replication stage | 2198 (834/1364) | | 0.2477 | 1.01 (0.08) | 0.8654 |
| | | | | | | | Meta-analysis | 3184 (1377/1807) | | 0.2487 | 1.00 (0.06) | 0.9595 |
| | rs1036477 | 15 | 48,914,926 | | | | **LeMaire et al. 2011** | **2813 (1313/1500)** | **G/A** | – | **1.8** | $\mathbf{6.5 \cdot 10^{-12}}$ |
| | | | | | | | Imputed discovery stage | 986 (543/443) | | 0.1164 | 0.92 | 0.7091 |
| | | | | | | | Replication stage | 2198 (834/1364) | | 0.1222 | 1.12 (0.10) | 0.2683 |
| | | | | | | | Meta-analysis | 3184 (1377/1807) | | 0.1204 | 1.05 (0.08) | 0.5841 |
| | rs2118181 | 15 | 48,915,884 | | | | **LeMaire et al. 2011** | **2813 (1313/1500)** | **G/A** | – | **1.8** | $\mathbf{5.9 \cdot 10^{-12}}$ |
| | | | | | | | Imputed discovery stage | 986 (543/443) | | 0.1191 | 0.93 | 0.7610 |
| | | | | | | | Replication stage | 2198 (834/1364) | | 0.1219 | 1.12 (0.10) | 0.2836 |
| | | | | | | | Meta-analysis | 3184 (1377/1807) | | 0.1210 | 1.05 (0.08) | 0.5781 |

we could detect a minimum OR = 6.229 based on a significance threshold of $6.3 \cdot 10^{-7}$ (0.05/79805), a power of 80%, a MAF of 1%, and allelic association testing. Nevertheless, we considerably increased power for rare variants collapsing them by burden or SKAT gene-based tests. The minimum OR we could detect according to a significance threshold of $4 \cdot 10^{-6}$ (0.05/12,536), a power of 80%, and an allelic association test was 5.702 if MAF = 1%, and 3.841 if MAF = 2%.

### 3.2. Discovery stage single-marker and gene-based association results

Once we had confirmed the consistency of the original association results through re-genotyping, we summarized the single-marker association signals with a $p < 1 \cdot 10^{-4}$ in Table S2. From these, we specified the genetic variants we considered for replication in Table 2.

rs13294886 in the *HMCN2* gene was the most significant association signal we found, which reached a $p$ of $2.13 \cdot 10^{-6}$ when analyzing the subset of BAV cases without AAD affection (*dom*). We also detected this same association signal from the analysis of the whole discovery stage sample, but with a $p$ of $5.92 \cdot 10^{-5}$ (Table S2). The Manhattan plots we created for the whole discovery stage sample (*add*) and the subset of BAV cases without AAD affection (*dom*) can be found in Fig. 2.

Regarding the association analysis at the gene level, we prioritized those burden or SKAT association results with $p < 1 \cdot 10^{-4}$, considering at least two genetic variants in the same gene. The analysis of the whole discovery stage series and the subsets of cases with and without AAD affection included 12536, 11899, and 10268 genes, respectively. The gene-based association analysis of the whole discovery stage cohort revealed one gene with a $p$ of $3.31 \cdot 10^{-6}$ that met the restrictive Bonferroni's correction significance threshold ($3.99 \cdot 10^{-6}$): *TEKT4*. While this test clustered the single association results from 8 different *TEKT4* genetic variants, the gene-based result was driven by two association signals: rs200887468 and rs111522003, with $p = 1.18 \cdot 10^{-6}$ and $1.65 \cdot 10^{-4}$, respectively. In contrast, in the previous single-marker association analysis (*add*), neither rs200887468 nor rs111522003 reached statistical significance in the discovery cohort ($p$ 0.9969 and 0.001895, respectively).

Gene-based association analysis of either of the two cohorts of BAV cases with and without AAD affection did not identify a significant association. We therefore no longer considered any gene-based association signal as relevant for replication.

### 3.3. Replication stage and meta-analysis association results

From the seven discovery stage genetic markers and the other five previously associated with sporadic thoracic aortic disease selected for replication, we performed logistic regression analysis with eleven passing QC testing (one marker, rs3740526, failed Hardy-Weinberg equilibrium test). Association results for the replication cohort and the inverse variance weighted meta-analysis are in Table 2. We were not able to replicate association for any of the six candidate-variant association results prioritized for replication or the five markers previously associated with thoracic aortic disease after Bonferroni's correction for multiple testing. Similarly, meta-analysis did not identify a significant association.

## 4. Discussion

Despite being a relatively common but complex disease with high heritability in some cohorts, and a high incidence of familial clustering [1,2,11,12], the majority of the hereditable risk for BAV remains unexplained.

Approaches to identify the genetic factors involved have been diverse, and many candidate genes have been proposed. One of those most frequently associated with the development of this valvular malformation is the signaling and transcription regulator *NOTCH1* [31–33]. In fact, some sequencing studies have revealed overrepresentation of non-synonymous missense *NOTCH1* genetic variants among BAV patients [31–37]. Garg *et al.* also demonstrated in 2005 the functional implication of *NOTCH1* in aortic valve development using mice [31]. Even a spontaneous animal model of BAV disease exists, a specific Syrian hamster strain, which will undoubtedly be key for future discoveries, especially involving those BAV familial cases with Mendelian inheritance [38].

Apart from candidate gene sequencing [33], LeMaire *et al.* carried out the only GWAS involving BAV patients, published in 2011 [14]. However, it actually focused on the analysis of the genetic component of sporadic thoracic aortic aneurysm and dissections. Whether dilation of the proximal aorta in BAV patients is a primary manifestation of an underlying genetic disorder or a hemodynamic consequence of the valve's altered morphology, remains controversial [39–41]. On the one hand, aortic dilation can develop even when BAV function is normal, with no evidence of stenosis or insufficiency [42]. These cases in which AAD

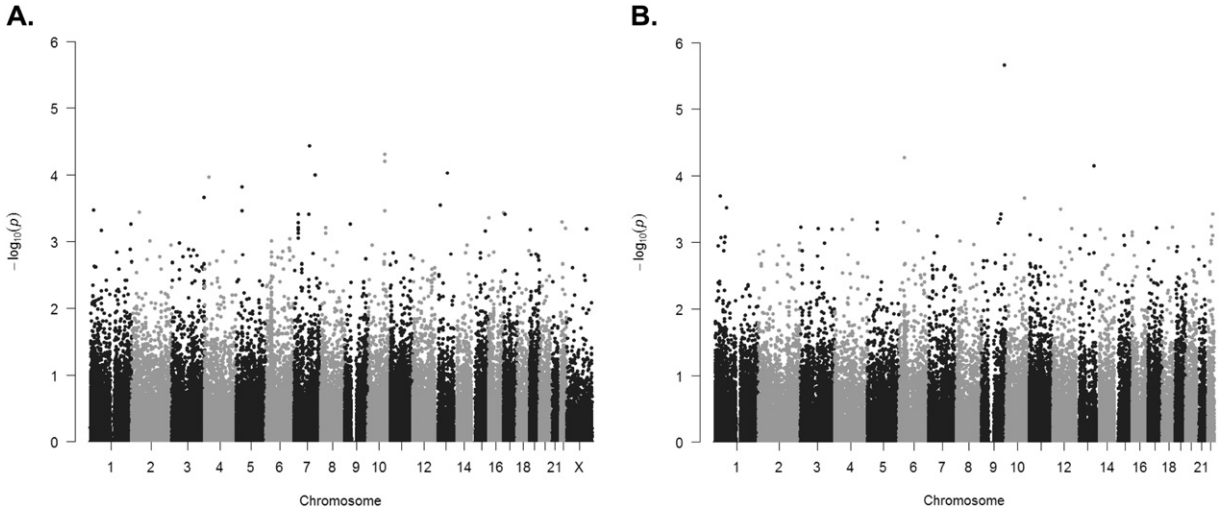


**Fig. 2.** Manhattan plots of the genetic associations with (A) BAV and (B) BAV without AAD affection, by genomic location. In both cases, the *x-axis* represents the chromosomal position for each genetic variant and the *y-axis*, the $-\log_{10}(p)$ from the logistic regression analysis performed based on the *add* (A) and *dom* (B) inheritance models, respectively.

involvement is hemodynamically independent of valve performance [5] suggest that a common valvulo-aortic syndrome may exist [6]. Loscalzo *et al.* concluded that BAV and AAD might be independent phenotypic manifestations of a single gene defect with an autosomal dominant pattern of inheritance with incomplete penetrance [39]. Indeed, a limited number of studies suggested that BAV might be more prevalent in familial cases of thoracic aortic aneurysms and dissections or some hereditary connective tissue disorders associated with an increased risk of aortic aneurysms (for example Marfan syndrome) [10,43,44], further supporting this hypothesis. On the other hand, Martin *et al.* performed bivariate genetic analyses between aortic dimensions and BAV but could not support a shared underlying genetic basis [41]. The high prevalence of BAV in the general population could have a misleading effect in these associations.

Based on this long-lasting debate and our experience, phenotypic variability should be strongly considered when performing genetic analyses of BAV patients, since at least part of the genetic complexity of this disease depends on phenotypic variation and patient misclassification. For instance, variation in valve morphology between different affected family members might result from different etiologies [45]. Trying to best address this issue, we thoroughly examined BAV phenotypes in our study population and, in addition to the association analysis of the whole discovery stage sample, repeated the analysis procedure for the subsets of BAV cases with and without AAD involvement. The clinical utility of a genetic marker able to predict BAV patients at risk of developing AAD dissection would be invaluable.

The present study is the first to attempt to unravel the genetic complexity of BAV and associated AAD from a population-based perspective. With this approach, we sought genetic markers that could help to identify BAV patients at risk of developing AAD dissection, the most feared BAV life-threatening clinical complication. The availability of such a genetic marker would improve BAV clinical diagnosis, better determine the prognosis, and personalize treatment of affected individuals.

Although non-significant, this EWAS revealed several interesting association signals that emerged both from the analyses of the whole discovery stage sample and the subsets of cases with and without AAD involvement. The most significant association result we found was rs13294886 at *HMCN2*. The involvement of this gene in calcium metabolism [46] and valve degeneration caused by calcium deposit [47] together with *NOTCH1* [31–33], supported *HMCN2* as an interesting gene for follow-up studies on the genetic susceptibility to BAV.

Unfortunately, despite the sample size, exhaustive phenotypic characterization, relatively high allele frequencies, and parity among them and those described in *HapMap CEU* [48], none of the association signals selected for replication were consistent across series (Table S3). The lack of significant findings from meta-analysis could be a consequence of either low power, randomness or population singularities, and increased cohort sizes will be needed to further clarify this issue.

Iakoubova *et al.* also tried in 2014 to replicate two of the association signals found by LeMaire *et al.* in 2011, rs2118181 and rs10519177, in 637 thoracic aortic disease cases (497 with aneurysm and 140 with dissection) and 275 controls from the Yale study [14,49]. The only association signal they were able to replicate was rs2118181 and the presence of isolated thoracic aortic dissection; neither this same marker was associated with thoracic aortic aneurysm or thoracic aortic aneurysm and dissection nor rs10519177 was associated with any of the former clinical entities. These, together with our results, suggest the need to further confirm previously described association signals and further detail the phenotype of interest.

### 4.1. Limitations

The main limitation of the present study was the limited statistical power to detect associations at genome-wide significance level, as a result of: (i) the reduced discovery stage sample size; (ii) limitation of the directly genotyped variants to exonic regions; (iii) the elevated number of the genetic variants from the *Axiom Exome Array (Affymetrix)* that were monomorphic in the Spanish population (>50%); and (iv) the possible prevalence of BAV among discovery stage controls.

Furthermore, discovery stage controls were not as well phenotyped as cases and we could not include as covariates some of the variables that might be relevant, such as ascending aortic dimensions. Finally, we could not include the 10 population stratification PCs in the association analysis of the replication cohort since there were no ancestry informative marker genotypes available.

## 5. Conclusions

The failure of this first BAV cross-sectional study to identify any significant association signal supports the concept that the genetic etiology of BAV may be more complex than previously believed. It is probably not dependent on a limited number of high-impact common variants, but a combination of less frequent variants with small effects along with environmental factors. Exhaustive phenotypic characterization of larger cohorts from large collaborative efforts is needed to detect those variants. Finally, further studies should also consider the possibility of overestimated heritability and the potential involvement of genetic factors related to gene expression and regulation.

## Disclosures

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2016.11.012.

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