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Differential expression patterns of Toll Like Receptors and Interleukin-37 between calcific aortic and mitral valve cusps in humans

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ABSTRACT

Background: Significant differences are mentioned in the progress of calcification between aortic and mitral valve. Evidence of inflammation in calcific aortic and mitral valve disease suggests that pathways of Toll Like Receptors (TLR) and Interleukin (IL)-37 expression may contribute to this process. We sought to investigate the role of TLR-mediated inflammatory response and IL-37 pathway expression on aortic and mitral valve calcification.

Material and methods: One-hundred twenty stenotic valve cusps/leaflets (60 aortic, 60 mitral) were excised during surgery and were collected for histological, immunohistochemistry and morphometric analysis at our department. After total RNA isolation from a second part of valve cusps/leaflets, cDNA synthesis and quantitative reverse transcription polymerase chain reaction (qRT-PCR) protocols were performed and relative mRNA levels of target genes were assessed.

Results: By histological analysis, the anti-inflammatory IL-37 levels were increased in mitral valve leaflets (MVL) compared to aortic valve cusps (AVCu) while all other biomarkers, including TLR, presented a reverse pattern with decreased levels as compared to AVCu. In terms of calcification biomarkers, only osteopontin differed between AVCu and MVL. mRNA analysis confirmed increased expression of IL-37 and decreased levels of TLR in MVL compared to AVCu.

Conclusions: Stenotic cusps of aortic valves express lower IL-37 and increased TLRs levels than stenotic mitral valve leaflets, suggesting a differential pro-calcification and pro-inflammatory profile between the two valves. This may explain the higher incidence of calcification of AVCu than MVL and offer therapeutic considerations.

1. Introduction

Valvular heart disease (VHD) and specifically calcific aortic valve disease (CAVD) and calcific mitral valve disease (CMVD) is an important cause of morbidity and mortality worldwide [1,2]. CAVD is becoming quite common with increasing age, with a prevalence of 2.8% in individuals older than 75 years, rising to 9.8% in octogenarians [3]. Currently, histopathologic and clinical data suggest that calcific valve disease is, in fact, not a simple degenerative process, in which calcium passively accumulates on the valve cusps, but an active and multifaceted condition involving chronic inflammation, and active leaflet calcification [4,5].

Evidence of inflammation in valve calcification suggests that the Toll Like Receptor (TLR) pathway may contribute to this process [6]. TLRs are pattern recognition receptors for ligands [7] from fungi, viruses and microbes or host cells [8]. Interleukin (IL)-37, is an anti-inflammatory member of the IL-1 family, which broadly inhibits innate and acquired immune responses in vitro and in vivo [9,10]. The role of TLRs and IL-37 as promoters or suppressors of both aortic (AVC) and mitral valve calcification (MVC) and pathobiology associated with CAVD and CMDV has not been adequately studied. Moreover, additional effectors molecules, including CD14 circulating monocytes and

Abbreviations: AVC, aortic valve calcification; CAVD, calcific aortic valve disease; CMVD, calcific mitral valve disease; CNP, C-type natriuretic peptide; LDL, low-density lipoprotein; MVC, mitral valve calcification; MVL, mitral valve leaflets; PLTP, phospholipid transfer protein; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RHD, rheumatic heart disease; TLR, Toll Like Receptor; VHD, valvular heart disease; AVCu, aortic valve cusps

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BNP, might selectively regulate the pro- and anti-calcification effects of TLRs and IL-37 on aortic and mitral valve.

Importantly, differences exist between aortic and mitral valve disease which have a clinical consequence. Mitral stenosis is usually due to rheumatic heart disease (RHD), but heavy calcification of the mitral annulus with extension into the leaflets might also cause obstruction to left ventricular inflow, particularly in the elderly population [11]. While aortic calcification is quite frequent, if mitral annulus calcification is excluded, mitral valve leaflets (MVL) do not frequently calcify in patients without RHD which has become practically extinct in western societies. Thus, we hypothesized that intrinsic differences may exist between these two valves that may help to explain why aortic valves calcify more and mitral valves less frequently. In particular, in this study, we (a) investigated whether TLR mediated inflammatory response may play an important role in the osteogenic process and whether AVC and/or MVC are associated with the TLR and IL-37 pathway expression, (b) examined if TLR expression is associated with osteogenetic markers, such as osteocalcin, osteopontin, osteoprotegerin and CD14+ monocytes which have already been studied in CAVD, (c) assessed the relationship of calcification factors response with the expression of TLR1,2,3,4,5,6,7,9 and their receptor ligands IRAK4, MYD88, NFkB, TIRAP as well as IL-37 in both calcified aortic and mitral valves, (d) assessed if TLR mRNA expression is related to IL37 expression and (e) evaluated BNP expression in both aortic and mitral valve cusps, as well as its possible correlation with other osteogenic markers.

2. Material and methods

This study is a continuous project. Patient selection criteria for aortic valve samples are described in our previous study [12]. We examined in total 120 cusps: 60 aortic valve cusps (AVCu) and 60 MVL collected during surgery. As regards CAVD, cusps of aortic valves removed at the time of valve replacement were examined. We also included 60 patients who were scheduled to undergo surgery for severe mitral valve stenosis. MVL were studied but not the annulus, which obviously could not be excised. Mitral valves with regurgitation grade $> +2\,cm^2$ by preoperative echocardiography were excluded.

All patients underwent examination by standard at the Onassis Cardiac Surgery Center (OCSC) echocardiographic techniques. Most were treated with diuretics, b-blockers, anticoagulants and occasionally antiarrhythmic drugs during the last two years of their course. The patients included in this study had dyspnea as clinical symptom, characterized as NYHA III-IV.

The study was conducted in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the OCSC according to clinical valvular heart disease guidelines. All patients signed informed consent for the use of their clinical and laboratory results for scientific purposes under condition of anonymity.

2.1. Valve cusp histology, immunohistochemistry and quantitative morphometrical analysis

Calcified aortic valve cusps or MVL were excised at the time of surgery; one part of each valve cusp/leaflet was placed in a container for histological, immunohistochemistry and morphometric analysis at the pathology department of the OCSC and the Biomedical Research Foundation of Academy of Athens.

Hematoxylin and eosin stain were performed in both AVCu and MVL to study disorganization of cellular valve matrix; and Alizarin Red stain was performed for the presence of calcification.

Ten serial sections per tissue, at equal intervals (50 μm) over a distance of about 500 μm were taken and stained with H&E, Alizarin Red and with the following antibodies in their concentrations were used in order to perform immunohistochemistry technique. The procedure has been validated in our lab [13]. IRAK-4 (Abcam, UK; ab32511; 1 μg/ml); MYD88 (Santa Cruz Biotechnology, INC, USA; sc-11356; 5 μg/ml); NFkB (Santa Cruz Biotechnology, INC, USA; sc-372; 5 μg/ml); TIRAP (Abcam, UK; ab17218; 2 μg/ml); TLR1 (Santa Cruz Biotechnology, INC, USA; sc-130896; 5 μg/ml); TLR2 (Thermoscientific, USA; PA1-41045; 5 μg/ml); TLR3 (Santa Cruz Biotechnology, INC, USA; sc-12509; 5 μg/ml); TLR4 (Abcam, UK; ab47093; 5 μg/ml); TLR5 (Imgenex, USA; IMG-580; 10 μg/ml); TLR6 (Abcam, UK; ab71429; 5 μg/ml); TLR7 (Imgenex, USA; IMG-581A; 10 μg/ml); TLR9 (Imgenex, USA; IMG-305A; 10 μg/ml); IL37 (Abcam, UK; ab57187; 3 μg/ml); BNP (Thermoscientific, USA; MA5-15362; 5 μg/m); CD14 (Abcam, UK; ab181470; 2 μg/ml); osteocalcin (R&D systems, Minneapolis, MN, USA; MAB1419; 25 μg/ml); osteopontin (R&D systems, Minneapolis, MN, USA; AF1433; 10 μg/ml); osteoprotegerin (R&D systems, Minneapolis, MN, USA; AF805; 15 μg/ml). Immunohistochemistry methods performed according to the manufacturer's protocol by using the development kit (Zytochem Plus; Zytomed system, Germany). Appropriate isotype controls (negative control) were performed at the same concentrations as the primary antibodies.

The immunohistochemistry and staining sections were investigated microscopically with stereology-upright Leica DMRA2 camera and were analyzed by stereo-investigator 10 program (version 10.1, MBF Bioscience, Microbrightfield. Inc, Willinston, Vermont, USA) and quantitate the extent of the tissue covered by each staining and antibody.

2.2. RNA isolation and qRT-PCR analysis

Total RNA was isolated from a second part of valve cusps/leaflets by using the Tri Reagent (Sigma, USA) according to the manufacturer's protocol [14]. Then the quantity and purity of RNA was evaluated by absorbance on a Spectrometer (Molecular devices, California, USA) at 260 nm and 280 nm. cDNA synthesis and quantitative reverse transcription polymerase chain reaction (qRT-PCR) protocols were performed as previously described [13]. PCR products were evaluated by melting curve analysis.

Relative mRNA levels of target genes were calculated by the $2^{-\Delta\Delta Ct}$ method [15]. All human primers for b-actin, IRAK4, MYD88, NFkB, TIRAP, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR9, IL-37, BNP, CD14, OCN (Osteocalcin), Osteopontin (OPN), osteoprotegerin (OPG) were designed by Eurofins Genomics (German) (supplementary Table 1).

2.3. Statistical analysis

Data are presented as mean ± standard deviation (mean ± SD). Normal distribution of continuous variables was assessed by histograms and distributional plots (i.e. quantile-quantile plots). Correlation between measured variables was assessed by Pearson analysis. Student's *t*-test was used to compare variables between AVCu and MVL groups. Subsequently, we implement linear regression analysis for dependent variables of interest (i.e. TLRs and IL-37 expression levels). All statistical calculations were performed using GraphPad Prism version 4.03 (Graphpad Inc., CA, USA). Level of statistical significance was set at $p < 0.05$. All tests are two-tailed.

3. Results

3.1. Patient's characteristics and differences in tissue staining biomarkers between AVCu and MVL group

Among patients' characteristics we found that mean age in the two groups was (a) AVCu: 68.9 ± 7.9 years; (b) MVL: 69.55 ± 7.1 years; prevalence of male gender was 50% for both valves. Biomarker tissue data from staining and immunohistochemistry shows: a. Significant higher tissue area of the following biomarkers IRAK4, MYD88, NFkB, TIRAP, TLR1, TLR2, TLR3, TLR4, TLR6, CD14 and OPN was found in AVCu compared to MVL ($p < 0.05$; Fig. 1A, B, C). TLR5, TLR7, TLR9,

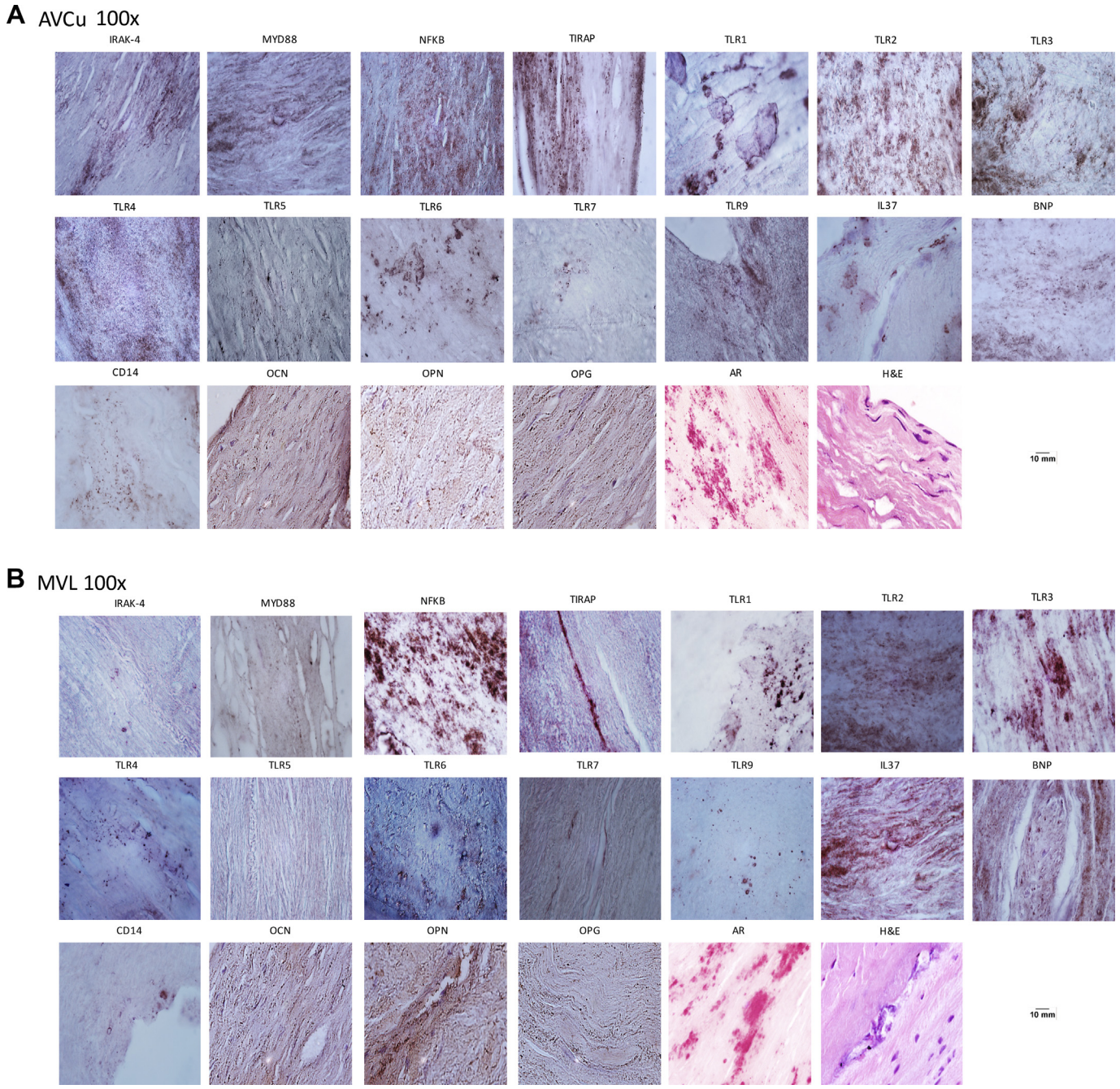

Fig. 1. (A) Representative photos of toll like receptor (TLR) pathway, inflammation and calcification biomarkers from aortic valve cusp (AVCu). Nuclei stained with celestian blue are shown in blue, expression of biomarkers shown in brown. 100× magnification. (B) Representative photos of TLR pathway, inflammation and calcification biomarkers from mitral valve leaflets (MVL). Nuclei stained with celestian blue are shown in blue, expression of biomarkers shown in brown. 100× magnification. (C) Quantification of histology and immunohistochemistry staining in AVCu (black) and MVL (grey bars). TLR pathway, IL-37, brain-type natriuretic peptide (BNP), CD14 and calcification biomarkers are compared between AVCu (n = 60) and MVL (n = 60). Asterisks denote significant difference ($p < 0.05$) by Student's T test. Bars represent mean values and spikes standard errors. (D) Linear regression analysis between IL-37 and variables in tissue AVCu (n = 60) and MVL (n = 60). Statistical significance ($p < 0.05$) and r value presented in graph. NS means no significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

BNP, OCN, OPG and AR tissue area biomarkers were higher in AVCu compared to MVL but not significantly ($p > 0.05$). b. IL-37 tissue area in MVL was significant higher compared to AVCu ($p < 0.05$; Fig. 1C). All the differences between the groups are summarized in Table 1.

3.2. Correlation of IL-37 staining tissue area with inflammation and calcification process

We observed that IL-37 tissue area was a. positively correlated with TLR2, BNP and OPG in AVCu ($p < 0.05$; Fig. 1D); b. IL-37 tissue area was inversely correlated with CD14 in AVCu (Fig. 1D); c. In MVL, only IL-37 tissue area was significantly correlated with TLR7 (Fig. 1D).

3.3. Correlation between calcification and inflammation process in both AVCu and MVL

Calcification (AR staining) significantly correlated a. with the up-regulation of TIRAP and the downregulation of OPG in AVCu; and b.

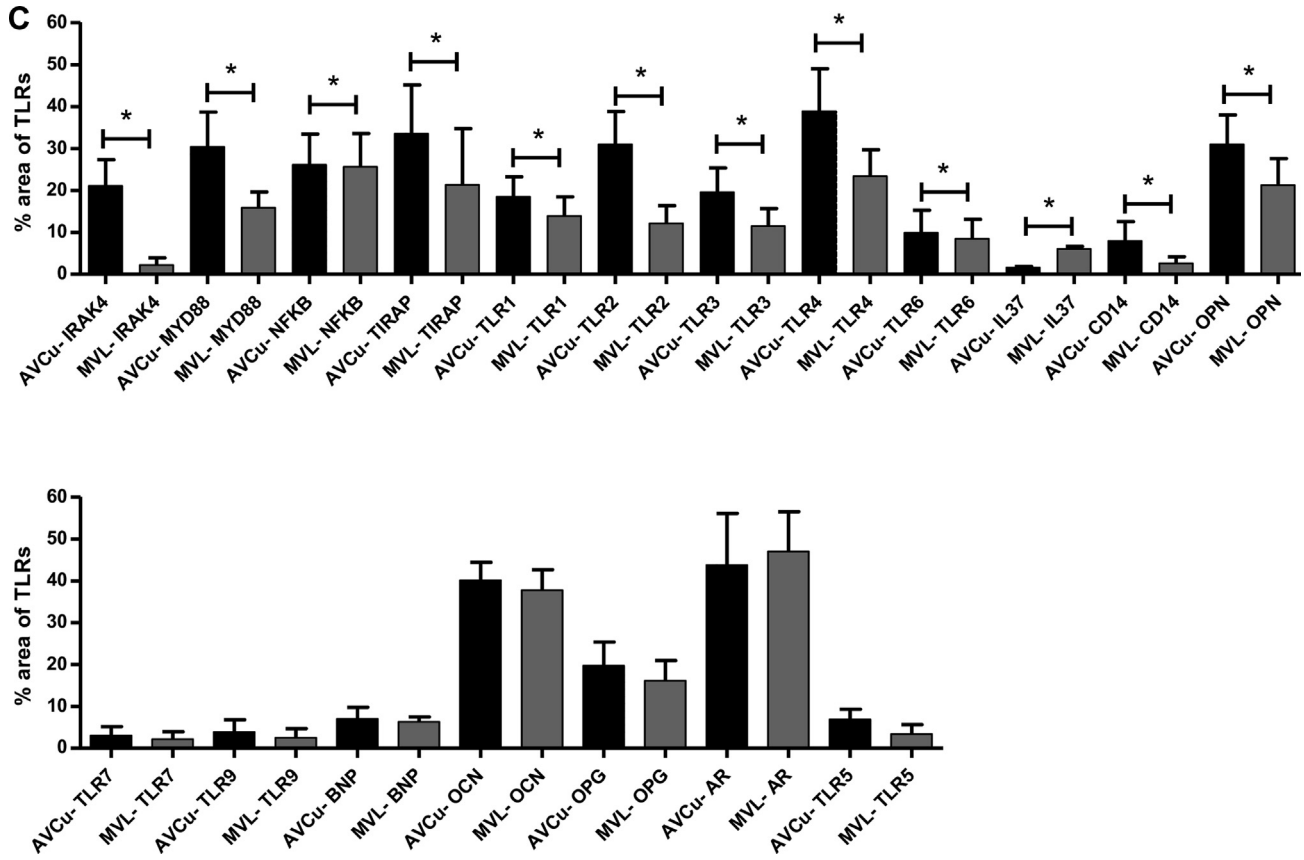

Fig. 1. (*continued*)

with the upregulation of OPG, TLR9, and TLR6 and downregulation of TLR5, TLR2 and MYD88 pathway in MVL ($p < 0.05$; Table 2). Osteocalcin production correlated a. positively with MYD88, TLR4, TLR5 and CD14; and b. negatively with IRAK4, NFkB, TLR2, and TLR6 in AVCu, while it was a. positively with the TIRAP and TLR3 in MVL and b. inversely correlated with IRAK4 and ($p < 0.05$; Table 2). In addition, correlation was found among osteoprotegerin and osteocalcin, and TLR6 in MVL. Osteopontin correlated a. positively with the IRAK4 and NFkB and b. negatively with MYD88, TLR2 and TLR3 in MVL. No correlation was found between OPN and all the other biomarkers in AVCu. Various significant correlations between inflammatory TLRs and their pathways were established in both AVCu and MVL, suggesting a significant presence of TLR in the process of valve inflammation and calcification ($p < 0.05$; Table 2). Another tissue biomarker, BNP was associated with TLR6 and IL-37; and negatively with the MYD88 in both AVCu and MVL and TIRAP and TLR7 in AVCu.

Finally, CD14+ monocytes positively correlated with MYD88, TLR3 and TLR9; and inversely with IRAK4, TLR2, TLR6 and IL-37 in AVCu. In contrast, CD14+ monocytes correlated positively with TIRAP and negatively with TLR1 in MVL ($p < 0.05$; Table 2).

3.4. Differences in mRNA expression of inflammation and calcification biomarkers between AVCu and MVL

To determine mRNA expression differences of biomarkers between AVCu and MVL, we applied qRT-PCR analysis (Table 3). a. Calcific aortic valve mRNA expression was found significantly higher ($p < 0.05$) in AVCu compared to MVL regarding IRAK4, MYD88, TIRAP, TLR1, TLR2, TLR3, TLR4, TLR6, BNP, CD14, OCN, OPN and OPG. b. IL-37 mRNA expression was higher ($p < 0.05$) in MVL compared to AVCu (Fig. 2A).

3.5. Correlation between age and mRNA expression of calcification and inflammation biomarkers in both AVCu and MVL

In order to assess whether age is responsible for the progression of calcification and inflammation, we performed a mean value analysis of all biomarkers and age. In detail, we found that age is not correlated with the mRNA expression of MYD88, TLR7, IL37, BNP and OPG in AVCu; and IRAK4, TLR1, TLR3, TLR4, TLR7, TLR9, BNP, CD14 and OPN in MVL. All other variables significantly correlated to age (Table 4).

3.6. IL37 mRNA expression correlated with the inflammation process expressed by TLRs

The following correlations were found as shown in Fig. 2B and C, IL-37 was associated with TIRAP and TLR1 signaling (upregulation) in AVCu; while IL-37 was associated with downregulated TIRAP and NFkB signaling in MVL.

3.7. TLR mediated inflammatory response correlated with calcification-associated factors

To determine the relative role of the TLR-mediated signaling pathways in the inflammatory response and calcification process, we evaluated correlations among representative biomarkers. (A) AVCu group: Table 5 shows that (i) mRNA expression of osteoprotegerin is positively correlated with TLR7 mRNA expression, while is negatively correlated with IRAK4, TLR1, BNP and CD14 mRNA expression. (ii) Osteopontin is positively correlated with TLR1 and negatively with TLR5. (iii) osteocalcin mRNA production is positively correlated with TLR1, TLR3 and CD14 mRNA expression. (iv) Calcification (AR) is not related to MYD88, NFkB and TIRAP pathway. (B) MVL group: (i) Osteopontin mRNA expression significantly correlated with the MYD88 pathway

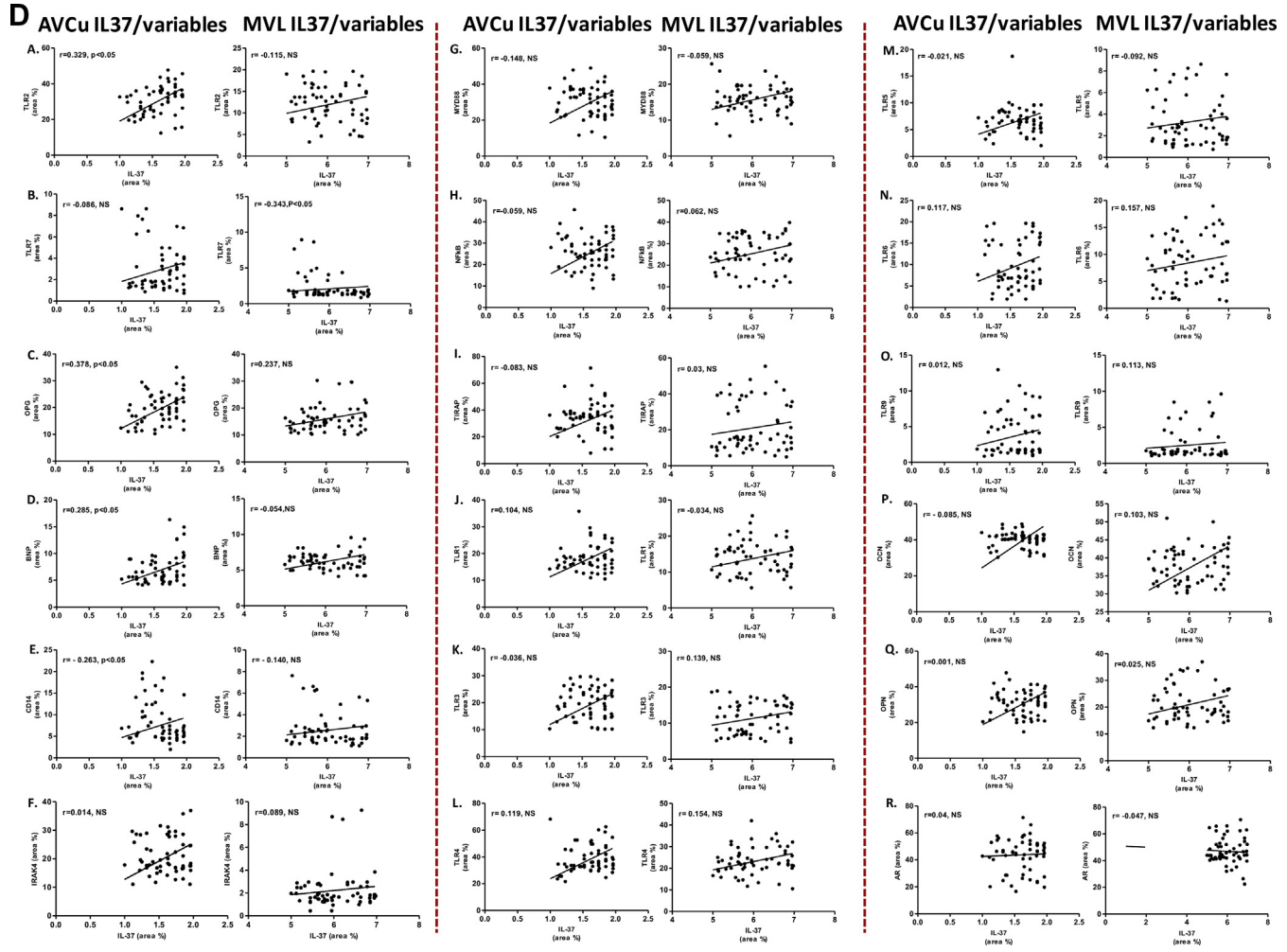

Fig. 1. (*continued*)

Table 1
Tissue biomarkers in both AVC and MVC groups.

Tissue Biomarkers	Groups		P < 0.05
	AVCu	MVL	
IRAK4	21.03 ± 6.3	2.23 ± 1.67	*
MYD88	30.38 ± 8.31	15.87 ± 3.78	*
NFkB	26.09 ± 7.36	25.59 ± 8.01	*
TIRAP	33.48 ± 11.7	21.30 ± 13.5	*
TLR1	18.44 ± 4.79	13.94 ± 4.5	*
TLR2	30.98 ± 7.85	12.13 ± 4.24	*
TLR3	19.57 ± 5.75	11.43 ± 4.28	*
TLR4	38.89 ± 10.1	23.39 ± 6.29	*
TLR5	6.81 ± 2.48	3.33 ± 2.24	NS
TLR6	9.89 ± 5.37	8.45 ± 4.61	*
TLR7	3.04 ± 0.26	2.17 ± 0.22	NS
TLR9	3.83 ± 0.37	2.51 ± 0.27	NS
IL37	1.6 ± 0.26	6.03 ± 0.6	*
BNP	6.97 ± 2.76	6.28 ± 1.17	NS
CD14	7.95 ± 4.61	2.62 ± 1.54	*
OCN	40.08 ± 4.29	37.71 ± 4.9	NS
OPN	30.98 ± 6.98	21.26 ± 6.31	*
OPG	19.66 ± 5.68	16.1 ± 4.81	NS
AR	43.73 ± 12.4	46.94 ± 9.56	NS

Data presented as mean ± Standard Deviation.
Statistical analysis between AVC and MVL (P < 0.05) *Significant; NS: no significant.
Abbreviations: AVCu, aortic valve cusp; MVL, mitral valve leaflet; NFkB, nuclear factor-kappa B; TLR, toll like receptor; IL, interleukin; BNP, brain-type natriuretic peptide; OCN, osteocalcin; OPN, Osteopontin; OPG, osteoprotegerin.

TLR4, BNP and CD14 and negatively with IRAK4 pathway, TLR1 and TLR3; (ii) osteocalcin is positively correlated with BNP and was inversely related to TLR4; (iii) osteoprotegerin is inversely correlated with TLR2 and OPN but is positively associated with TLR5.

3.8. CD14 and BNP mRNA expression correlated with inflammation process

(A) CD14 correlated (Table 5) (i) with suppressed mRNA expression of TLR5, TLR7 and BNP in AVCu; and IRAK4, NFkB, TLR1, TLR2, TLR3 and TLR9 in MVL; (ii) with upregulated TLR1 in AVCu; and (B) BNP (Table 5) is correlated (i) with lower mRNA expression of TLR3,4,6,9 in AVCu; and IRAK4, NFkB, TIRAP, TLR1, TLR3, TLR5, TLR9 in MVL; and (ii) with the upregulation of TLR1 in AVCu; and upregulation of IL37 in MVL.

4. Discussion

The main finding of our study is that the calcific aortic valve overall expresses increased pro-inflammatory TLRs and lower IL-37 levels than stenotic MVL. Several correlations shown in this study at histological and gene expression level among inflammatory and calcific molecules strongly support a differential pro-calcification and pro-inflammatory profile for the aortic valve.

Importantly, CAVD is becoming more frequent with advancing age. Conversely, although mitral valve annulus calcification is quite frequent in older patients [16,17], together with degenerative mitral valve

Table 2
Correlation between tissue biomarkers in AVCu and MVL.

	IRAK4	MYD88	NFKB	TIRAP	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR9	IL37	BNP	CD14	OCN	OPN	OPG	AR
IRAK4							–									–			
MYD88	–													–			–		–
NFKB		–					–												
TIRAP					–														
TLR1		–										–			–				
TLR2		–										–					–		–
TLR3																	–		
TLR4	–		–				–												
TLR5										–									
TLR6				–			–	–	–		–								
TLR7							–						–						
TLR9			–								–								
IL37																			
BNP		–		–							–								
CD14	–					–				–			–						
OCN	–		–			–				–									
OPN																			
OPG																			
AR																		–	

Downleft: AVCu group; upright: MVL group.
Red boxes denote no correlation; green boxes denote significant correlation (upregulation); symbol "–" denotes significant correlation (downregulation).
Abbreviations: AVCu, aortic valve cusp; MVL, mitral valve leaflet; NFkB, nuclear factor-kappa B; TLR, toll like receptor; IL, interleukin; BNP, brain-type natriuretic peptide; OCN, osteocalcin; OPN, Osteopontin; OPG, osteoprotegerin.

Table 3
mRNA expression of tissue biomarkers in both AVCu and MVL.

Tissue Biomarkers	Groups		P < 0.05
	AVCu	MVL	
IRAK4	3.08 ± 0.48	1.52 ± 0.26	*
MYD88	3.09 ± 0.56	1.78 ± 0.36	*
NFkB	4.93 ± 0.69	4.71 ± 1.22	NS
TIRAP	3.15 ± 0.56	1.54 ± 0.25	*
TLR1	5.07 ± 1.34	2.71 ± 0.76	*
TLR2	5.9 ± 1.19	4.36 ± 1.08	*
TLR3	2.77 ± 0.5	1.5 ± 0.27	*
TLR4	7.66 ± 1.11	1.72 ± 0.39	*
TLR5	1.89 ± 0.59	1.37 ± 0.28	NS
TLR6	4.72 ± 0.68	2.05 ± 0.64	*
TLR7	1.64 ± 0.45	1.63 ± 0.43	NS
TLR9	1.54 ± 0.34	1.37 ± 0.29	NS
IL37	1.58 ± 0.26	3.07 ± 0.53	*
BNP	41.48 ± 5.27	25.37 ± 4.31	*
CD14	14.7 ± 2.9	8.06 ± 1.09	*
OCN	15.49 ± 2	9.89 ± 1.84	*
OPN	5.96 ± 0.61	2.53 ± 0.87	*
OPG	2.53 ± 1.09	1.07 ± 0.4	*

Data presented as mean ± Standard Deviation. Statistical analysis between AVCu and MVL ($P < 0.05$) *Significant; NS: no significant.
Abbreviations: AVCu, aortic valve cusp; MVL, mitral valve leaflet; NFkB, nuclear factor-kappa B; TLR, toll like receptor; IL, interleukin; BNP, brain-type natriuretic peptide; OCN, osteocalcin; OPN, Osteopontin; OPG, osteoprotegerin.

regurgitation [18], MVL calcification and stenosis are becoming quite infrequent with the virtual extinction of RHD. Progress in imaging techniques has helped to support this assumption. Willmann et al. [19] using electrocardiogram-gated multi-detector row computed tomography found an excellent agreement with operative findings. Accordingly, Mahnken et al. [20] using the same technique found a 8.2% incidence of MVL calcification in 32/390 patients, of whom 15 (3.8% overall) had mitral stenosis. There was an overlap with MVL calcification in that 13 patients showed only leaflet calcification, 17 only annular, and 19 calcification of both. Age range of these patients was 33–91 (mean 62.4 ± 12.2) years. Patients with calcification were older; as expected, no gender differences were found in contrast to data in CVAD. Although, typically RHD produces mitral stenosis at an early age, delays up to three decades have been described [21] especially with early penicillin treatment.

In recapitulation, MVLs seem to have a lower calcific potential than AVCu. We have previously studied various biomarkers in CAVD and have tried correlations among calcification, fibrotic and inflammatory pathways [12]. In the current study, we additionally assessed a central inflammatory pathway, that of TLR expression and the associated antinflammatory cytokine, IL-37. Recently, a new dimension in the description of the spectrum of CAVD has emerged [22]. In this context, progression from aortic sclerosis to severe stenosis occurs only 10–15% of patients over 2–5 years. However, when even mild stenosis occurs [23], progression to severe obstruction is observed in nearly all patients [24]. This reminds of the old adage that '' mitral regulation begets mitral regulation '', but evokes a different mechanism, indicating that aortic stenosis begets stenosis. Thus, in partial support to our findings, once obstruction to flow occurs, flow abnormalities may provoke lipid infiltration, increased oxidative stress and procalcific stimuli and inflammation. Because serial tissue studies are impossible in humans, the course of these processes is difficult to study accurately. Under the same prism, is not yet delineated whether in AS a systemic perturbation exists leading to valve calcification or if the sclerotic valve itself produces proinflammatory and pro-calcific factor and creates a self-propagating process. To this end, in the MESA study [25], CAVD was associated with 50% increased risk of cardiovascular events. Similar findings emerged from LIFE study [26], the Cardiovascular Health Study [27] and the Heinz Nixdorf Recall study [28].

Toll Like Receptors expression was a main aspect of our study. In regard to TLRs which are expressed by cells of the innate immunity system [29], Yang et al. showed that human aortic valve interstitial cells (AVICs) express TLR2 and TLR4 and that stimulation of their receptors induces pro osteogenic factor expression [30]. Meng et al. [31] found that aortic valves express more TLR2 and TLR4 than pulmonary valves in both total tissue and isolated interstitial cells. Significantly, after stimulating with agonists such as peptidoglycan for TLR2 and LPS for TLR4, AVICs expressed higher levels of various pro-inflammatory and pro-osteogenic factors such as bone morphogenic protein-2 (BMP-2), runt-related transcription factor 2 and osteogenic changes such as alkaline phosphatase compared to pulmonary valve interstitial cells (PVICs). Also, when they silenced TLRs in AVICs, the expression of the already mentioned factors was reduced to the level of PVICs. To our knowledge, TLRs have not been studied in MVLs. We cannot postulate from our findings if a genetic difference exists between the two valves or if underlying hemodynamic factors induce differential inflammatory patterns. To this direction, Lopez et al. [32] found that in aortic valves

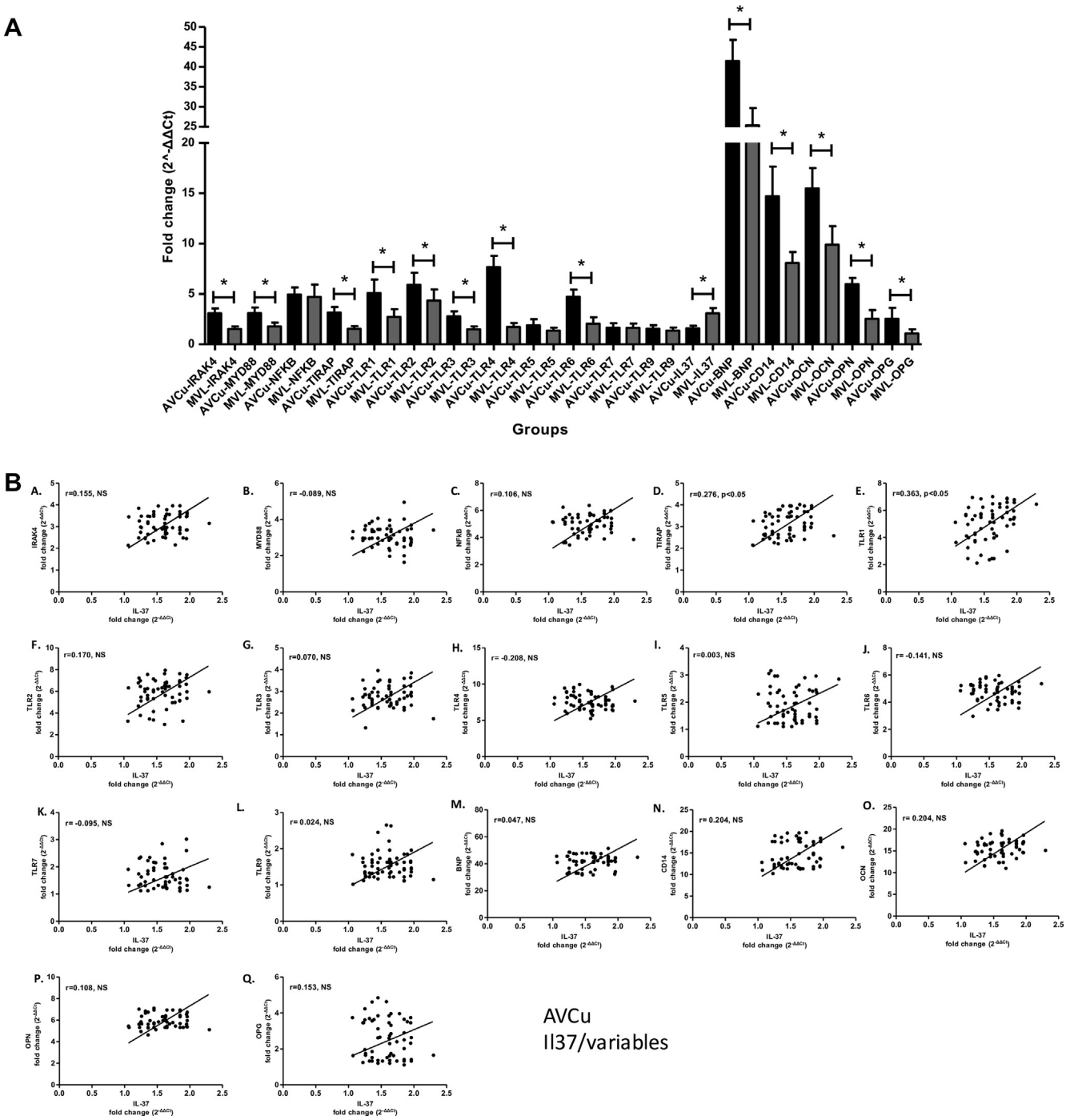

Fig. 2. (A) Quantification of mRNA expression of inflammation and calcification biomarkers in aortic valve cusp (AVCu) and mitral valve leaflet (MVL). Statistical significance presented in graph as * ($p < 0.05$) between AVCu (n = 60) and MVL (n = 60) group by Student's T test. (B) Linear regression analysis between IL-37 and variables in tissue AVCu (n = 60). Statistical significance ($p < 0.05$) and r value presented in graph. NS denotes no significant. (C) Linear regression analysis between IL37 and variables in tissue MVL (n = 60). Statistical significance ($p < 0.05$) and r value presented in graph. NS means no significant.

from transplanted patients without valve disease, all TLRs except TLR8 were expressed, with TLR4 being the most abundantly represented. In AVICs from stenotic valves, a comparative increase of TLR4 and TLR2 was observed; no differences in TLR1 and TLR6 were shown, while TLR5 and TLR9 levels were lower. Importantly, most of TLRs were not expressed to the cell surface, but in cellular content [33] in both stenotic and non-stenotic aortic valves.

Moreover, biglycan which is associated with LDL deposition [34] and the phospholipid transfer protein (PLTP) were over-expressed in stenotic valves and colocalized with TLR2. In more detail, biglycan induced the production of PLTP via stimulation of TLR2. Thus, biglycan colocalizes with oxidized low-density lipoprotein (LDL) inflammatory pathways [35] a main determinant of aortic calcification. It should be noted that PLTP has been found in human coronary atherosclerotic plaques, where it modifies lipoproteins such as Apo AI [36]. Our group has shown in rabbit aortas [13] that after an atherogenic diet for 3 months, together with an increase of the atherosclerotic burden, mRNA of TLR2,3,4 and 8 also increased significantly. The association of increased lipid levels and inflammatory expression has already been described [37,38]. We cannot, from our data, postulate which is the

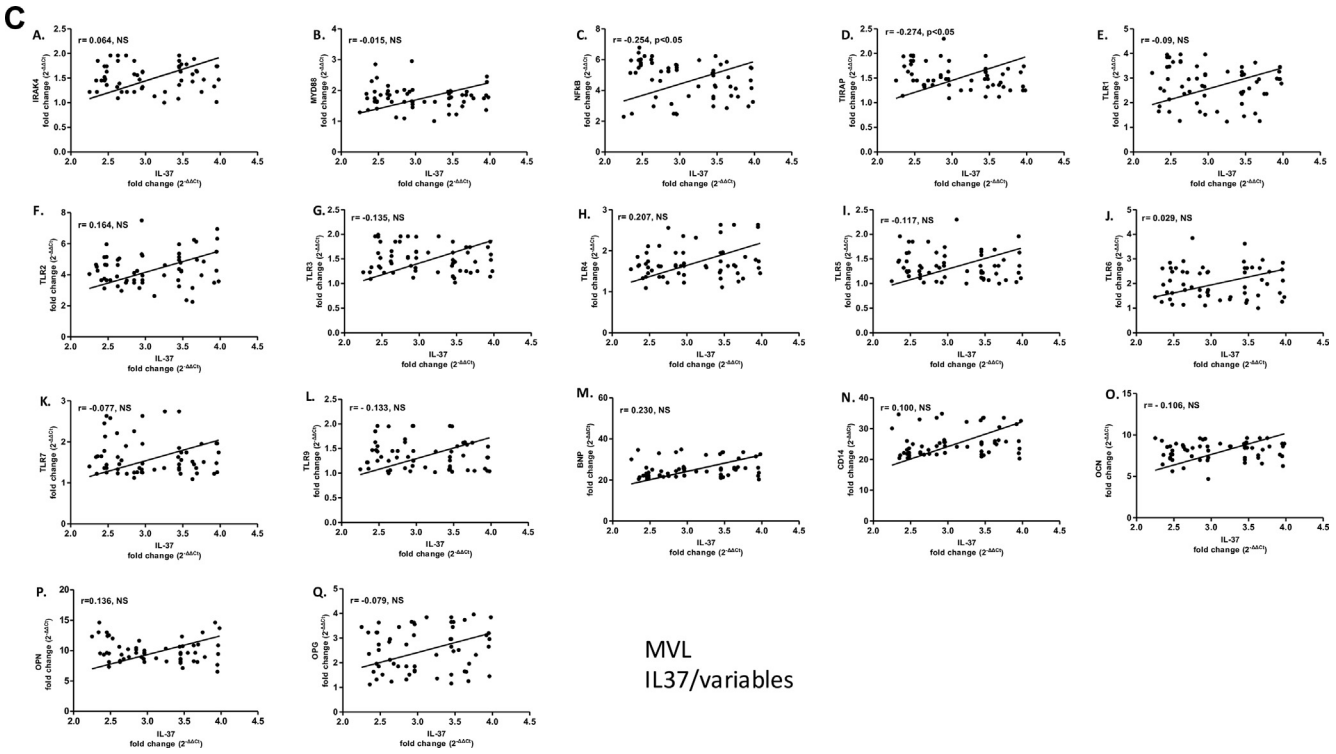

Fig. 2. (*continued*)

Table 4
Correlation between age and mRNA biomarkers expression of tissue AVCu and MVL.

Variables	AVCu-AGE			MVL-AGE		
	R2	P	Significant	R2	P	Significant
IRAK4	0.304	0.018	Yes	−0.234	0.073	No
MYD88	0.115	0.38	No	0.496	< 0.001	Yes
NFKB	0.865	< 0.001	Yes	0.296	0.002	Yes
TIRAP	0.408	< 0.001	Yes	−0.467	< 0.001	Yes
TLR1	−0.530	< 0.001	Yes	0.107	0.457	No
TLR2	−0.684	< 0.001	Yes	0.432	0.001	Yes
TLR3	−0.883	< 0.001	Yes	0.102	0.440	No
TLR4	0,454	< 0.001	Yes	0.180	0.170	No
TLR5	0,498	< 0.001	Yes	−0.775	< 0.001	Yes
TLR6	0,697	< 0.001	Yes	0.788	< 0.001	Yes
TLR7	−0.2	0.124	No	0.071	0.588	No
TLR9	0,403	0.001	Yes	−0.054	0.684	No
IL37	−0.219	0.092	No	0.517	< 0.001	Yes
BNP	−0.016	0.905	No	0.030	0.819	No
CD14	−0.357	0.005	Yes	0.066	0.615	No
OCN	−0.728	< 0.001	Yes	−0.579	< 0.001	Yes
OPN	−0.285	0.027	Yes	0.100	0.449	No
OPG	−0.009	0.948	No	−0.701	< 0.001	Yes

Results are given as mean values; R^2 value and $P < 0.05$ were calculated.
Abbreviations: AVCu, aortic valve cusp; MVL, mitral valve leaflet; NFkB, nuclear factor-kappa B; TLR, toll like receptor; IL, interleukin; BNP, brain-type natriuretic peptide; OCN, osteocalcin; OPN, Osteopontin; OPG, osteoprotegerin.

first initiator of TLR overexpression; inflammation alone or hemodynamic disturbances, of both, acting in a feedback mechanism. The TLRs may play additional roles in CAVD [39]. Human AVICs were treated with LPS only (a TLR2 activator) or LPS plus oxidized LDL. The combination produced higher levels of BMP-2 and ALP than the single exposure to LPS alone, as well as greater NFkB activation, and increased Notch 1 activation. These findings suggest that the combination of noxious factors results into greater osteogenetic actions. Another action of TLR2 is to mediate the effects of endogenous high mobility group box 1 (HMGB1) protein [40]. Release of this factor elicits or augments inflammatory reactions [41,42], and has also been found to be involved in the action of osteopontin on endothelial and valve interstitial cells behavior in aortic stenosis [43]. As regards TIRAP and MYD88, they are involved in the action of TLR4 and CD14 and the production of proinflammatory cytokines. IRAK-4 is activated by MYD88 in the MYD88 dependent pathway with subsequent downstream activation of NFkB [44].

We also found BNP to be expressed in a higher level in the stenotic aortic than the mitral valve. In this context, Peltonen et al. [45] demonstrated atrial natriuretic peptide (ANP) and BNP genes to be expressed in a similar degree in the regurgitant and stenotic human aortic valves. They also studied C-type natriuretic peptide (CNP) which was found to be lower in the stenotic valves; it was localized to valvular endothelial cells and myofibroblasts, which we also postulate that produce these peptides. Yip et al. [46] studied CNP in normal and sclerotic porcine valves; they also observed a reduction of this peptide in the sclerotic cusps. Tsuruda et al. [47] found that cultured adult canine cardiac fibroblasts produced BNP as detected by a specific radioimmunoassay. The secretion of BNP was augmented by tumor necrosis factor-a (TNFa), and inhibited de novo collagen synthesis, as well as that of MMP-1,-2 and -3. In cultured human fibroblasts, BNP inhibited transforming growth factor-b (TGFb) effects: profibrotic (collagen 1, fibronectin, TGFb, PAI-1, TIMP3); pro-proliferative (IGF1, FGF18) and pro-inflammatory (COX2, IL6, TNFa induced protein b and TNF superfamily member-4) [48]. Therefore, BNP has an antifibrotic and anti-inflammatory effect. ANP and BNP have also been found to inhibit the sympathetic and renin-angiotensin-aldosterone system [49,50] which have also been implicated in the process of CAVD.

We found many interesting correlations among osteogenetic mechanisms in both the aortic and mitral valve. There were some discrepancies in that the same factors showed differences between AVCu and MVL. Obviously, not all correlations are completely clear. Importantly, osteocalcin, osteoprotegerin and osteopontin had many positive correlations. Also, BNP had many inverse correlations in both valves, further advocating for its antifibrotic/anticalcifying action.

Another important finding of the study is the correlation between

Table 5
Correlation between mRNA expression of inflammation and calcification biomarkers in AVCu and MVL.

	IRAK4	MYD88	NFKB	TIRAP	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR9	IL37	BNP	CD14	OCN	OPN	OPG
IRAK4														–	–		–	
MYD88																		
NFKB													–	–	–			
TIRAP													–	–				
TLR1														–	–		–	
TLR2															–			–
TLR3														–	–		–	
TLR4																–		
TLR5														–				
TLR6														–				
TLR7					–	–												
TLR9						–								–	–			
IL37																		
BNP							–	–		–	–	–						
CD14									–		–			–				
OCN																		
OPN									–									–
OPG	–				–									–	–			

Downleft: AVCu group; upright: MVL group.
Red boxes denote no correlation; green boxes denote significant correlation (upregulation); symbol "–" denotes significant correlation (downregulation).
Abbreviations: AVCu, aortic valve cusp; MVL, mitral valve leaflet; NFkB, nuclear factor-kappa B; TLR, toll like receptor; IL, interleukin; BNP, brain-type natriuretic peptide; OCN, osteocalcin; OPN, Osteopontin; OPG, osteoprotegerin.

serum NT-proBNP and density of staining of this peptide in the stenotic valves. Research on the role of serum NT-proBNP in the context of increased endomyocardial tension and left ventricular end-diastolic pressure in aortic stenosis has already been performed [51,52]. To our knowledge, this is the first localization of BNP in the mitral valve. We also established correlation between BNP and calcifying processes (negative with osteoprotegerin and positive with osteopontin), indicating that this peptide as well as the already mentioned CNP may have a role on calcification which should be further investigated. We cannot explain the higher expression of BNP in AVCu than MVL; further experiments are required in order to understand the internal mechanism.

Osteoprotegerin was inversely correlated to CD14 mRNA expression in the AVCu while osteocalcin was positively associated with CD14 in the mitral leaflets. These data suggest an association of monocyte heterogeneity with the calcification process. Increased levels of circulating intermediate monocytes have been found in severe aortic stenosis [53] and actually diminish after transcatheter aortic valve replacement [54]. Monocyte subsets are shifted towards the intermediate sub-type (CD14++, CD16+) in inflammation [55,56]. Similarly, Shimoni et al. [57] who found higher CD14+ monocytes in patients with aortic stenosis attributed their increase to inflammation. As Hewing et al. [58] point out intermediate monocytes are the most potent inflammatory monocytes producing more cytokines. Neuser et al. [59] did not find a correlation of their levels with C-reactive protein levels. Obviously, many other factors may be involved in this association; Neuser et al. also postulated the role of hemodynamic associations.

Finally, we found for the first time that the anti-inflammatory IL-37 is higher in MVL. This may partially explain why MVLs are protected from calcification at more advanced ages compared to the AVCu. This interleukin also negatively regulates signaling mediated by TLR agonists [60]. IL-37 has been studied in the foam cell of atherosclerotic plaques [61]. Experimentally, it suppresses the osteogenic responses of human AVICs, but is also lower in AVIC of stenotic valves [62]. The same group also found that it suppresses human AVICs response to TLR agonists [63]. According to our results, aortic valve shows a higher expression of IRAk4 and MYD88 which are activated by TLR and TIRAP, that interact with CD14 in the LPS/TLR pathway [64].

To date, preventive therapy for CAVD, especially statin and anti-renin-angiotensin system treatment has not yielded satisfactory results. In this context, our findings indicating increased TLRs and decreased levels of anti-inflammatory IL-37 in stenotic aortic cusps may have clinical implications. In specific, it is tempting to postulate that early detection of calcified or stenotic aortic cusps by echocardiographic or incidental CT studies may call for initiation of anti-inflammatory treatment along with intensification of the statin therapy that could delay disease progression. To the same direction, we have previously shown that IL-2 is increased in the serum of patients with aortic stenosis [12]. Interestingly, the CANTOS study found that anti-inflammatory treatment against IL-1β in high-risk CAD patients reduces a composite endpoint of non-fatal myocardial infarction, stroke and cardiovascular death [65]. Given that severe aortic stenosis contributes to cardiovascular mortality, these results provide further support to anti-inflammatory treatment in stenotic aortic cusps. Whether anti-inflammatory therapy in patients with mitral annulus calcification could provide favorable clinical outcomes in accordance to stenotic aortic cusps, remains to be elucidated.

5. Conclusions

In conclusion, stenotic cusps of the aortic valves express more, on the whole, pro-inflammatory TLRs and lower IL-37 levels than stenotic MVL. Additional, correlations among intermediate molecules in the inflammation cascade point out toward a higher pro-calcification and pro-inflammatory profile of the aortic; than that of the mitral valve; these findings explain the greater prevalence of aortic than non-rheumatic mitral stenosis in advanced age. Also, they suggest that if anti-inflammatory treatments are to be adopted in atherosclerotic vascular disease, according to the CANTOS study, a preventive effect on CAVD occurring in later ages may be hoped for.

Conflict of 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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