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Abstract: Background/Objectives: The mechanisms underlying aortic dilation in bicuspid aortic valve (BAV) disease are unknown. Circulating endothelial microparticles (EMPs) have emerged as biomarkers of endothelial damage. We sought to evaluate the relationships among EMPs, BAV disease, and aortic dilation.
Methods: Four evaluations were used. Circulating EMPs (PECAM+, E-selectin+) were compared between BAV patients and tricuspid aortic valve (TAV) control subjects. The variables related to circulating EMPs were investigated in BAV patients. Circulating EMP levels were compared between BAV and TAV patients with a dilated aorta. Finally, circulating EMPs in BAV patients were evaluated over time with respect to aortic valve surgery (AVS) or aortic surgery.
Results: We observed higher levels of circulating PECAM+ EMPs in the BAV patients than in the control subjects (3.98±0.2 vs. 2.39±0.4 per log PECAM+ EMPs/µl, p=0.001). Aortic dilation was the most significant variable that correlated with the PECAM+ EMP levels in the BAV patients (β=0.321, p=0.008). The BAV patients with aortic dilation exhibited higher PECAM+EMP levels than the TAV patients with dilated aortas, and this correlation was independent of aortic valve function. We observed a drastic decrease in the circulating PECAM+ EMPs following AVS and aortic root replacement (4.27±0.6 and 1.75±0.3 per log PECAM+EMPs/µl, p=0.002).
Conclusion: The observed pattern of higher circulating PECAM+ EMP levels links BAV disease to endothelial damage and aortic dilation. Circulating PECAM+ EMPs were identified as a biological variable related to aortic dilation in patients with BAV disease.

Suggested Reviewers:

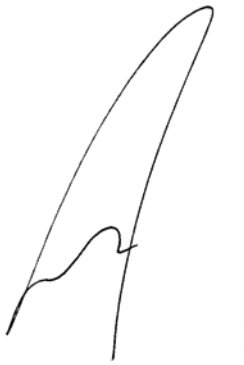
Prof. A.J.S. Coats
Editor-in-Chief of International Journal of Cardiology

Reus, 21th March 2016

Dear Prof. Coats:

In this new version of the manuscript we have included the changes that were required and suggested by the reviewers. We appreciate all these comments, we agree and we think that with these changes the manuscript has been clearly improved. In this new version we have included a new figure showing the flow chart design used and we have also excluded the logistic regression analysis. In addition we have revised the English language. We hope this new version will now be suitable for publication.

Yours sincerely,



Josep M Alegret, MD, PhD.

Response to the Reviewers

Reviewer #1: This is a very interesting paper. Many compliments to the Authors for the brilliant idea, and the appropriate study design.

Thank you to this reviewer for the comments and suggestion. We have modified the manuscript including the proposed changes.

I only have few minor concerns, as follows:

1. Please provide a flow-chart of the study in order to clarify and ease the understanding of group allocation of participants.

In this new version we have included a new figure (Figure 1) with the flow-chart of the study.

2. English language review is needed.

We have review the English language using the American Journal Experts services.

3. The logistic regression analysis (Table 4) is not reliable and should be amended. A role of thumb in regression analysis is 10 variables for every outcome. In your logistic model, AoDil are only 39 (25 Asc + 14 Root), thus you can only carry a logistic regression with 3-4 variables for the entire cohort, or with 1-2 variables for the subgroups, respectively. Given that such a model would be too simple, I strongly recommend to avoid such analysis from the paper because is not fair at all.

As suggested by this reviewer, in this new version of the manuscript, we have excluded the logistic regression analysis and Table 4 in the study, as well as the related comments in the Discussion section.

Reviewer #2: Excellent and highly interesting manuscript. Carefully researched and a very good, substantive discussion. Figures and tables are transparent and supporting the results. From my point no further comments. The use of EPMs as a possible new biomarker of aortic dilation in BAV disease seems to be a very interesting aspect.

Thank you to this reviewer for the comments and support.

Reviewer #3: The article entitled Circulating endothelial microparticles are elevated in bicuspid aortic valve disease and related to aortic dilation proved in a well written, well designed study that bicuspid aortic valve involves important aortic wall endothelial changes.

The authors acknowledge study limitations and they proposed a path through circulating microparticles might be eventually used as markers of disease in clinical practice. Future studies will be needed.

Thank you to this reviewer for the comments. We agree with this reviewer, and we explained in the discussion section, that future studies would be needed to confirm the promising role of circulating microparticles as biomarkers of aortic dilation in BAV disease.

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Circulating endothelial microparticles are elevated in bicuspid aortic valve disease and related to aortic dilation

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Running title: Endothelial microparticles are related to aortic dilation in patients with BAV disease

Abbreviations: AVS = aortic valve surgery; BAV = bicuspid aortic valve; EMPs = endothelial microparticles; LV = left ventricular; TAV = tricuspid aortic valve; WSS = wall systolic stress

Keywords: endothelial microparticles, bicuspid aortic valve, aortic dilation, ascending aorta, aortic root.

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ABSTRACT

Background/Objectives: The mechanisms underlying aortic dilation in bicuspid aortic valve (BAV) disease are unknown. Circulating endothelial microparticles (EMPs) have emerged as biomarkers of endothelial damage. We sought to evaluate the relationships among EMPs, BAV disease, and aortic dilation.

Methods: Four evaluations were used. Circulating EMPs ($PECAM^+$, E-$selectin^+$) were compared between BAV patients and tricuspid aortic valve (TAV) control subjects. The variables related to circulating EMPs were investigated in BAV patients. Circulating EMP levels were compared between BAV and TAV patients with a dilated aorta. Finally, circulating EMPs in BAV patients were evaluated over time with respect to aortic valve surgery (AVS) or aortic surgery.

Results: We observed higher levels of circulating $PECAM^+$ EMPs in the BAV patients than in the control subjects (3.98±0.2 vs. 2.39±0.4 per log $PECAM^+$ EMPs/μl, p=0.001). Aortic dilation was the most significant variable that correlated with the $PECAM^+$ EMP levels in the BAV patients (β=0.321, p=0.008). The BAV patients with aortic dilation exhibited higher $PECAM^+$EMP levels than the TAV patients with dilated aortas, and this correlation was independent of aortic valve function. We observed a drastic decrease in the circulating $PECAM^+$ EMPs following AVS and aortic root replacement (4.27±0.6 and 1.75±0.3 per log $PECAM^+$EMPs/μl, p=0.002).

Conclusion: The observed pattern of higher circulating $PECAM^+$ EMP levels links BAV disease to endothelial damage and aortic dilation. Circulating $PECAM^+$ EMPs were identified as a biological variable related to aortic dilation in patients with BAV disease.

INTRODUCTION

A bicuspid aortic valve (BAV) represents the most common congenital cardiac malformation and is generally associated with the development of aortic valve dysfunction and the progressive dilation of the ascending aorta. The latter is associated with aortic regurgitation [1] and the risk of aortic dissection or rupture and often requires prophylactic aortic surgery.[2–6] The cause of ascending aorta dilation has been debated for several years and may be due to changes in the flow characteristics of the ascending aorta.[7] However, the mechanisms involved have not been fully elucidated. The aortic dilation observed in BAV disease may be related to endothelial dysfunction, as estimated by flow-mediated dilation.[4] Endothelial microparticles (EMPs) are small cell membrane vesicles, less than 1 μm in size, shed by endothelial cells upon activation, injury, or apoptosis.[8] The EMPs are characterized by the presence of endothelial-specific surface antigens, the composition of which is dependent on the cell origin of the microparticles and the generating process.[9] In this way, CD31 (PECAM) and CD62e (E-selectin) are markers of microparticles released from endothelial cells. The $PECAM^+$ EMPs have been related to apoptosis or endothelial damage and the $E\text{-selectin}^+$ EMPs to cellular activation.[9] The EMPs have been linked to inflammation, vascular injury, angiogenesis, and thrombosis and have emerged as markers of endothelial dysfunction.[10] They have also been linked to aortic valve disease.[11] However, the relationships among EMPs, BAV, and aortic dilation have not been extensively studied; thus, the aim of this study was to investigate these associations.

METHODS

Study population

This study included a cohort of patients with BAV and patients with aortic dilation who were prospectively included and followed-up in our facilities. There were 185 patients with BAV and 125 patients with aortic dilation with tricuspid aortic valve (TAV). The participants were prospectively entered into a specific database and provided a blood sample upon enrolment and

written acceptance. The samples were stored until needed in our biological samples bank (Biobanc IISPV - HUSJR). The diagnosis of BAV was made when two aortic leaflets were clearly visualized, with or without a raphe, on the parasternal short-axis view of a transthoracic echocardiogram,[12] on a transesophageal echocardiogram,[12] or by cardiac magnetic resonance.[13] A dilated aortic root or ascending aorta was diagnosed when the aortic diameter was ≥21 mm/m^2.[14] Explorations were performed or supervised by the same observer (JMA). Our database and biobank also included a group of healthy controls. For this study, those individuals who met the characteristics to be included were selected. The participants were divided into different groups depending on the morphology of the aortic valve and the diameter of the aortic root (Figure 1).

The design of the study included four evaluations to determine the possible factors related to the EMP circulation levels and BAV disease. First, the circulating EMP levels of the patients diagnosed with BAV (n=60) were compared with those of the healthy TAV control subjects (n=15), matched by age (ranging from 18-55 years) and sex. We excluded the BAV patients with evidence of significant left ventricular (LV) remodeling (LV end-diastolic diameter>60 mm or LV end-systolic diameter>45 mm) or LV dysfunction (LV ejection fraction<50%) and individuals younger than 18 years or older than 55 years to reduce the possible confounding effects exerted by LV function and remodeling and age. Next, the effects exerted by aortic diameter on other variables potentially related to EMP levels were evaluated. The patients with BAV disease were within a defined age range (18-55 years) and had no restrictions pertaining to LV end-diastolic or end-systolic diameter or LV ejection. Patients were divided based on the presence or absence of aortic dilation, either at the level of the aortic root and/or the ascending aorta ($BAV_{non\text{-}dil}$ n= 32, BAV_{dil}, n= 39) or based on the dilation of the aortic root or the ascending aorta. The type of aortic dilation was categorized into two phenotypes, depending on the dilation pattern, as follows: an ascending phenotype ($BAV_{dil\_ascending,}$ n=25, dilation of the ascending aorta with either a normal

aortic root or a less dilated aortic root) or a root phenotype (BAV_{dil_root}, n=14, dilation in the aortic root with either a normal ascending aorta or a less dilated ascending aorta).[15]

In the third step, the influence of valve morphology on EMP levels was evaluated among the patients with aortic dilation and compared with the previously described control subjects (defined in the first step) and BAV_{dil} (defined in the second step, n=67), as well as a third group composed of TAV patients with a dilated aorta (TAV_{dil}, n=19). Due to the older ages of the TAV patients with a dilated aorta included in our database, no restrictions regarding age range were applied to this step although age was analysed as a possible confounding factor. Patients with Marfan syndrome were excluded. Finally, circulating EMPs were analysed for BAV patients over time with respect to aortic valve surgery (AVS). New blood samples were obtained periodically, and the effect of AVS was evaluated in the BAV patients by measuring the EMP levels from blood samples obtained before and 6 months after the surgery (BAV pre- and post-AVS, respectively, n=10).

Patients diagnosed with aortic coarctation, peripheral arterial disease, stroke, ischemic heart disease, abdominal aortic aneurysm, chronic renal failure (glomerular filtration rate<60 ml/min), or diabetes mellitus were excluded. This study was approved by the Institutional Review Board (the Clinical Ethics Committee) of our institution. Written informed consent was provided by all patients who participated in this study.

Blood sampling and EMP identification

Blood samples in EDTA (K_3) were collected following overnight fasting and were processed within 90 min of collection. Samples were centrifuged at 1500 g for 15 min to obtain plasma, which was further centrifuged at 4000 g for 10 min to obtain platelet-poor plasma. The samples were stored at -80ºC until needed in our biological samples bank (Biobanc IISPV - HUSJR). The concentration of circulating EMPs was determined on an EPICS-XL (Beckman Coulter) flow

cytometer at the low rate setting and 30 s stop time. EMP identification is described in the Supplementary Methods.

Statistical analysis

After undergoing natural logarithmic transformation, the EMP plasma levels were expressed as log-transformed counts per μl (log EMPs/μl). Because of the right-skewed distribution of the values, the quantitative variables are represented as the means ± SDs. The means of two groups were compared using Student's t test. Chi-squared tests or Fisher exact test, when appropriate, were used to compare the frequencies of the categorical variables. The effects of valve morphology and aortic root dilation were assessed using ANOVA. Tukey's test was utilized for pairwise comparisons. Pearson's correlation was used to identify linear relationships between the $PECAM^+$ EMP levels and the factors involved in the development and progression of BAV disease. Backward linear regression models were used to identify independent predictors of the circulating $PECAM^+$ EMP levels. P values<0.05 were considered significant. The statistical analysis was performed using SPSS software, version 21.0 (IBM, Chicago, IL, USA).

RESULTS

BAV patients have higher circulating $PECAM^+$ EMP levels than TAV control subjects

We compared circulating microparticles levels in BAV patients and with age- and sex-matched TAV control subjects. The patients' clinical characteristics and echocardiographic data are presented in Table 1. As expected, the BAV patients exhibited significantly enlarged indexed aortic root and ascending aortic diameters and a high prevalence of aortic valve dysfunction compared with the control group. The circulating $PECAM^+$ EMP levels were higher among the patients diagnosed with BAV disease than in the control group (3.98±0.2 vs. 2.39±0.4 per log $PECAM^+$ EMPs/μl, respectively, p=0.001) (Figure 2). By contrast, the levels of E-$selectin^+$ EMP and platelet-derived microparticles ($CD31^+CD42b^+$) were similar between the BAV patients and healthy controls (25.74±0.3 % vs. 26.09±0.5 %, p=0.619 for E-$selectin^+$ EMPs; p=0.977 for platelet-

derived microparticles). A multivariate analysis that included other possible confounding factors confirmed that valve morphology was an independent predictor of circulating $PECAM^+$ EMP levels (β=0.380, p=0.001) (Table 1 in Ref [30]). In addition, a specific surface marker for cell apoptosis, Annexin V+, was also analysed. There were no changes in BAV patients compared with healthy controls (2.25±0.2 % vs. 2.22±0.2%, p=0.926) (Figure 2).

Circulating $PECAM^+$ EMP levels are elevated in BAV patients with aortic dilation

To identify the factors related to the increases in circulating $PECAM^+$ EMP levels among BAV patients, the BAV-associated clinical and echocardiographic parameters were determined. A significant positive correlation was observed between circulating $PECAM^+$ EMP levels and the indexed aortic root diameter (r=0.342; p=0.004, Table 2). Increased $PECAM^+$ EMPs were noted in the BAV patients when we considered aortic dilation at any level, either the aortic root or ascending aorta ($BAV_{non\text{-}dil}$ 3.29±0.3 and BAV_{dil} 4.34±0.2 per log $PECAM^+$ EMPs/μl, respectively, p=0.008), or at the level of the aortic root ($BAV_{non\text{-}ARdil}$ 3.50±0.2 and BAV_{ARdil} 4.53±0.2 per log $PECAM^+$ EMPs/μl, respectively, p=0.005). Furthermore, we built different multivariate models to find the most important factors predicting circulating $PECAM^+$ EMP levels, and we found aortic dilation at any level, aortic root dilation, and aortic root diameter were independent predictors of $PECAM^+$ EMPs (Table 3). Interestingly, there was no significant effect with aortic stenosis or regurgitation with respect to the $PECAM^+$ EMP levels. The patients with an aortic dilation pattern corresponding to the root phenotype tended to exhibit higher $PECAM^+$ EMP levels than the patients with an ascending aorta phenotype dilation pattern (BAV_{dil_root} 4.73±0.3 and $BAV_{dil\_ascending}$ 4.02±0.2 per log $PECAM^+$ EMPs/μl, respectively, p=0.096).

Dilated aorta: BAV patients have higher circulating $PECAM^+$EMP levels than TAV patients

To better understand the effects exerted by valve morphology and aortic dilation, EMP levels were compared for control subjects vs. patients with aortic dilation, with either BAV or TAV. Although no significant differences were observed in circulating $PECAM^+$ EMP levels between the control

subjects (TAV) and the TAV patients with aortic dilation (TAV_{dil}), the $PECAM^+$ EMP concentration was significantly increased in the patients with BAV_{dil} compared with the patients with TAV_{dil} (Figure 3). The clinical and echocardiographic characteristics of the patients with TAV_{dil} and BAV_{dil} are included in Table 2 in Ref [30]. The multivariate linear analysis confirmed that valve morphology was an independent predictor of circulating EMP levels (β=-0.280, p=0.016) (Table 3 in Ref [30]).

The time course of circulating $PECAM^+$ EMPs: EMP levels decrease following aortic root replacement in BAV patients

After we determined that the circulating $PECAM^+$ EMP levels were significantly increased in the BAV patients and were significantly influenced by aortic dilation, we assessed these levels over time in the BAV patients who underwent AVS. The clinical and echocardiographic characteristics of these patients, as well as the type of surgery, are presented in Table 4 in Ref [30]. We determined that when AVS was performed in BAV patients, the circulating $PECAM^+$ EMP levels were significantly reduced (Figure 4). This effect was observed in patients who underwent aortic valve replacement and in those who underwent aortic root replacement. Interestingly, the two patients who underwent aortic root replacement while preserving the aortic valve also exhibited significantly decreased levels of circulating $PECAM^+$ EMPs following the surgery.

DISCUSSION

Our data indicate that BAV is an independent predictor of increased circulating levels of $PECAM^+$ EMPs, and we identified aortic dilation as an independent predictor of this increased level in BAV patients. On the other hand, the EMP levels of (BAV and TAV) patients with a dilated aorta were strongly influenced by the BAV morphology, suggesting that the dilation of the aorta per se was not sufficient to significantly increase EMP levels. To better understand the relationship between EMP levels and BAV, we assessed the time course of the $PECAM^+$ EMP levels in the BAV patients and evaluated the effects of AVS. We observed that AVS was associated with decreased

PECAM$^+$ EMP production. To our knowledge, this is the first study that after a comprehensive approach, describes a biological variable related to aortic dilation in BAV disease.

Circulating PECAM$^+$ EMP levels are elevated in BAV patients and are related to aortic dilation

Microparticles are small cell membrane vesicles, between 0.1 and 1 μm, shed by different cell types upon activation, injury, or apoptosis. Although platelet-derived microparticles represent the largest population of circulating microparticles, EMPs have gained attention due to their relationship with inflammation, vascular injury, angiogenesis, and thrombosis. Increased levels of circulating EMPs have been observed in the presence of cardiovascular risk factors, such as metabolic syndrome, hypertension,[16,17] and diabetes mellitus,[16,18], and cardiovascular diseases, such as stroke [17] and coronary artery disease.[18] The EMPs have emerged as markers of endothelial damage, and their release has been associated with endothelial dysfunction, assessed by abnormal flow mediated dilatation, in different settings.[19] Accumulating evidence has indicated a pro-coagulant, pro-inflammatory, and pro-angiogenic role for EMPs, possibly as pathogenic agents.[20] Accordingly, persistently elevated levels of circulating EMPs in patients with diabetes [21] or stroke [22] have resulted in poor clinical outcomes.

This study is the first to determine that circulating EMP levels are elevated in patients with BAV disease. We analysed the possible confounding factors and variables related to PECAM$^+$ EMP levels. As expected, BAV patients exhibited more aortic valve dysfunction and aortic dilation than control subjects. Although a previous study determined that aortic stenosis was related to higher levels of circulating EMPs, aortic stenosis and regurgitation did not correlate with PECAM$^+$ EMP levels in our BAV patients. Aortic valve morphology and aortic diameter were not evaluated in the above-mentioned report. In our study, aortic dilation emerged as a predictive factor of higher levels of circulating PECAM$^+$ EMPs in BAV disease. Moreover, to determine whether the increase in PECAM$^+$ EMP levels was related to either aortic dilation or BAV disease itself, we analysed the

$PECAM^+$ EMP levels in BAV and TAV patients with a dilated aorta and similar aortic diameters. We observed that only the patients with BAV exhibited significantly higher $PECAM^+$ levels than the control subjects, which suggested that the combination of BAV and aortic dilation was the main determinant of our findings. In vitro and in vivo experiments have yielded information regarding the role of flow characteristics in EMP release. Wall systolic stress (WSS) is a determinant of endothelial apoptosis, and physiological laminar flow promotes endothelial cell survival and senescence.[23] In vivo, WSS correlates inversely with EMP levels in peripheral arteries.[24,25] However, the characteristics of aortic flow at the aortic root and ascending aorta are much more complex than either of the conditions studied in vitro or the laminar flow observed in the peripheral arteries; in addition, other variables such as the flow angle, rotational component, and eccentricity may account for these findings. Different studies have demonstrated that BAV disease results in anomalous flow in the ascending aorta. A recent study by Bissell et al.[7] using cardiovascular MR with 4D flow analysis demonstrated that an increased systolic flow angle is associated with higher rotational flow among BAV patients. They observed that an asymmetrical jet hits the aortic wall at a more acute angle, resulting in a jet rotating along the aortic wall that increases the in-plane component of the WSS. Their observations suggested that the characteristics of aortic flow were related to aortic dilation. They also observed an asymmetrical and locally increased through-plane WSS and noted that increased rotational WSS was associated with larger ascending aortic diameters. These findings correlated well with the asymmetric pattern of medial degeneration and lesion distribution in BAV-associated aortic dilatations, described by Cotrufo et al.[26] These authors found that patients with BAV and ascending aorta dilation exhibited histological and morphometric differences in the extracellular matrix structure in the aortic convexity, an area of stress concentration and flow turbulence, with respect to the concavity. Recently, Jenkins et al.[27] described experimental in vivo evidence of disturbed blood flow-induced endothelial injury in humans. These authors induced a disturbed blood flow in the forearm that locally increased the release of EMPs.

We hypothesize that the characteristics of the aortic root and ascending aortic flow in patients with BAV disease, particularly the eccentric flow hitting the aortic wall, are related to aortic endothelial damage and may play a role in EMP generation. Moreover, the predisposing effects of eccentric flow with respect to aortic dilation may represent the link between the increased EMP levels observed in our patients and aortic dilation. We found a different expression for specific EMP phenotypes in BAV disease, suggesting potential differences in the origin and the pathophysiological mechanisms involved in the EMP release. We detected higher levels of circulating $PECAM^+$ EMPs in BAV patients; however, we observed similar levels of circulating E-$selectin^+$ EMPs and Annexin V^+ in BAV patients and healthy controls. Previous studies reported that the phenotype of the EMPs released is dependent on the type of the triggering stimulus. In this way, increased levels of E-$selectin^+$ EMPs were identified in situations that lead to cell activation, whereas $PECAM^+$ EMPs were detected predominantly in conditions associated with endothelial apoptosis or destruction.[9,28,29] Amabile et al.[28] observed that $PECAM^+$ EMPs, but not E-$selectin^+$ EMPs, were related to the severity of pulmonary hypertension. Moreover, they did not detect increased levels of Annexin V^+ EMPs in those patients; thus, the higher levels of circulating $PECAM^+$ EMPs were a consequence of the endothelial destruction mediated by haemodynamic causes, rather than apoptosis. Our results showed a similar pattern and the increased circulating $PECAM^+$ EMP levels observed in BAV patients might reflect the endothelial dysfunction mediated by haemodynamic causes due to the anomalous aortic flow associated with BAV. Our study did not elucidate the precise mechanism underlying the release of EMPs or determine why EMP levels correlated more strongly with the aortic root dilation than with the ascending aorta dilation. Future experimental studies analysing the characteristics of aortic flow and the levels of circulating EMPs are necessary to validate this hypothesis.

Limitations

The results presented in this study support the hypothesis that endothelial aortic damage represents the origin of the increased circulating EMP levels observed in patients with BAV disease.

However, there are no markers for identifying the specific territory in which these effects originate. Moreover, the changes in the EMP circulation levels observed following surgery support this hypothesis. We did not observe significant differences in the levels of EMPs between the BAV patients with right-left cusp fusion (the main group) and right-noncoronary cusp fusion. Some differences have been described in previous studies regarding aortic flow with respect to the type of BAV fusion observed. However, we cannot exclude that analysing a larger number of patients with right-noncoronary BAV might have identified differences. Considering our findings, EMPs may emerge as new biomarkers of aortic dilation in BAV disease. However, before use in clinical practice, routine measurement of EMPs is necessary to standardize the sample preparation, the storage protocols, and the techniques for quantification, as well as to consider the conditions that affect the EMP release and the expression of the specific cell surface markers. In conclusion, the pattern of increased circulating $PECAM^+$ EMP levels observed represents the link between BAV disease and endothelial aortic root damage and aortic dilation. These findings are suggestive of the impact of the anomalous flow generated by BAV disease. Therefore, the circulating $PECAM^+$ EMP level is a biological variable related to aortic dilation in patients with BAV disease.

FUNDING

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Figure 1. The participants were divided into different groups based on the morphology of the aortic valve and the diameter of the aortic root for each of the four evaluations used. In the first evaluation, the circulating PECAM$^+$ EMPs were compared between BAV patients and tricuspid aortic valve (TAV) control subjects. In the second evaluation, the variables related to circulating PECAM$^+$ EMPs were investigated in BAV patients. The third evaluation compared the circulating PECAM$^+$ EMP levels in BAV and TAV patients with a dilated aorta. Finally, circulating EMPs in BAV patients were evaluated over time with respect to aortic valve surgery (AVS) or aortic surgery in the fourth evaluation.

Figure 2. BAV patients presented phenotype-dependent higher circulating EMP levels. The BAV patients had increased levels of PECAM$^+$ EMPs (A), while the circulating levels of platelet-derived EMPs (B), E-selectin$^+$ EMPs (C), and Annexin V$^+$ EMPs (D) were unmodified with respect to the control group.

Figure 3. The PECAM$^+$ EMP levels of patients with aortic dilation were influenced by valve morphology. The circulating PECAM$^+$ EMP levels were unmodified with respect to TAV_{dil} compared with the control group; however, the PECAM$^+$ EMP levels were significantly higher in the patients with BAV_{dil} than in the control subjects with TAV_{dil}.

Figure 4. Circulating PECAM$^+$ EMP levels were reduced following AVS in BAV patients.

Table 1. Clinical and echocardiographic characteristics of the patients included in the first evaluation.

	Control	**BAV patients**	**p**
Age (years)	39 ± 2	37 ± 1	0.660
Sex (male/female)	(11/4)	(43/19)	0.803
Body mass index (kg/m^2)	24.17 ± 0.5	24.97 ± 0.6	0.307
Hypertension	1 (6.7%)	12 (20%)	0.222
Hypercholesterolemia	1 (6.7%)	5 (8.3%)	0.831
Smoker	1 (6.7%)	13 (21.6%)	0.611
Treatment:			
Statins	0 (0%)	9 (15%)	0.19
ACE/ARAII	0 (0%)	8 (13.3%)	0.345
β-blockers	0 (0%)	5 (8.3 %)	0.576
Aortic stenosis (mean gradient ≥20 mm Hg)	0 (0%)	9 (15%)	0.110
Aortic regurgitation (≥II)	0 (0%)	28 (47.5%)	<0.001
Aortic valve gradient (mean, mm Hg)	3.67 ± 0.2	12.27 ± 1.9**	<0.001
Left ventricle diastolic diameter (mm)	49.73 ± 1.1	51.67 ± 0.6	0.170
Left ventricle systolic diameter (mm)	30.40 ± 0.7	31.98 ± 0.5	0.157
Left ventricular ejection fraction (%)	75.54 ± 1.6	72.37 ± 0.7	0.072
Indexed aortic root diameter (mm/m^2)	16.47 ± 0.6	19.72 ± 0.5**	<0.001
Indexed ascending aorta diameter (mm/m^2)	15.69 ± 0.5	20.87 ± 0.6**	<0.001
Aortic dilation (aortic root or ascending aorta)	0 (0%)	32 (52.5%)	<0.001

** Significant values (p<0.01); BAV, bicuspid aortic valve; [#]determined based on 59 patients

Table 2. Clinical and echocardiographic factors related to the circulating $PECAM^+$ EMP levels in the BAV patients.

	$PECAM^+$ EMP levels (log EMPs/µl)	
Quantitative variables	**R**	**p**
Age (years)	-0.003	0.982
Weight (kg)	-0.038	0.753
Body surface area (m^2)	-0.104	0.393
Body mass index (kg/m^2)	0.101	0.406
Left ventricle diastolic diameter (mm)	0.026	0.831
Left ventricle systolic diameter (mm)	0.081	0.513
Left ventricular ejection fraction (%)	-0.122	0.321
Mean transvalvular aortic gradient (mean, mm Hg)	-0.191	0.119
Indexed aortic root diameter (mm/m^2)	0.342**	0.004
Indexed ascending aorta diameter (mm/m^2)	0.138	0.259

	$PECAM^+$ EMP levels (log EMPs/µl)		
Qualitative variables	**Absence (n)**	**Presence (n)**	**p**
Male gender	3.83 ± 0.2 (53)	3.97 ± 0.3 (18)	0.759
Hypertension	3.72 ± 0.2 (57)	4.48 ± 0.4 (14)	0.115
Hypercholesterolemia	3.84 ± 0.2 (66)	4.29 ± 0.9 (5)	0.548
Aortic stenosis (mean gradient ≥ 20 mm Hg)	3.96 ± 0.2 (59)	3.43 ± 0.5 (12)	0.309
Aortic regurgitation (≥2/4)	3.70 ± 0.3 (34)	4.05 ± 0.2 (36)	0.373
Aortic dilation (aortic root or ascending aorta)	3.29 ± 0.3 (32)	4.34 ± 0.2 (39)**	0.008
Dilated aortic root (≥21 mm/m^2)	3.50 ± 0.3 (44)	4.53 ± 0.2 (26)**	0.005
Dilated ascending aorta (≥21 mm/m^2)	3.58 ± 0.3 (38)	4.17 ± 0.2 (31)	0.125
Root dilation phenotype (vs. ascending dilation)	4.02 ± 0.2 (25)	4.73 ± 0.3 (14)	0.096
Valve morphology (right-left cusp fusion vs. right-noncoronary)[#]	4.19 ± 0.3 (17)	3.92 ± 0.3 (42)	0.546

** Significant values ($p<0.01$); EMP, endothelial microparticles; [#]determined based on 59 patients

Table 3. Summary of the multivariate linear analysis of the PECAM$^+$ EMP levels in patients with BAV disease.

	PECAM$^+$ EMP levels (log EMPs/uL)								
	β	p	95% CI	β	p	95% CI	β	p	95% CI
Age (years)	-0.057	0.656	-	-0.051	0.691	-	-0.066	0.611	-
Hypertension	0.089	0.475	-	0.114	0.350	-	0.126	0.295	-
Aortic stenosis (mean gradient ≥ 20 mm Hg)	-0.188	0.122	-	-	-	-	-	-	-
Aortic regurgitation	0.047	0.699	-	0.073	0.541	-	0.039	0.751	-
Aortic dilation (root/ascending)	0.321**	0.008	0.27-1.74	-	-	-	-	-	-
Aortic root dilation	-	-	-	0.323**	0.008	0.29-1.81	-	-	-
Indexed aortic root diameter (x mm/m^2)	-	-	-	-	-	-	0.321**	0.008	0.04-0.23
Indexed ascending aorta diameter (x mm/m^2)	-	-	-	-	-	-	-0.183	0.279	-

** Significant values (p<0.01); EMP, endothelial microparticles

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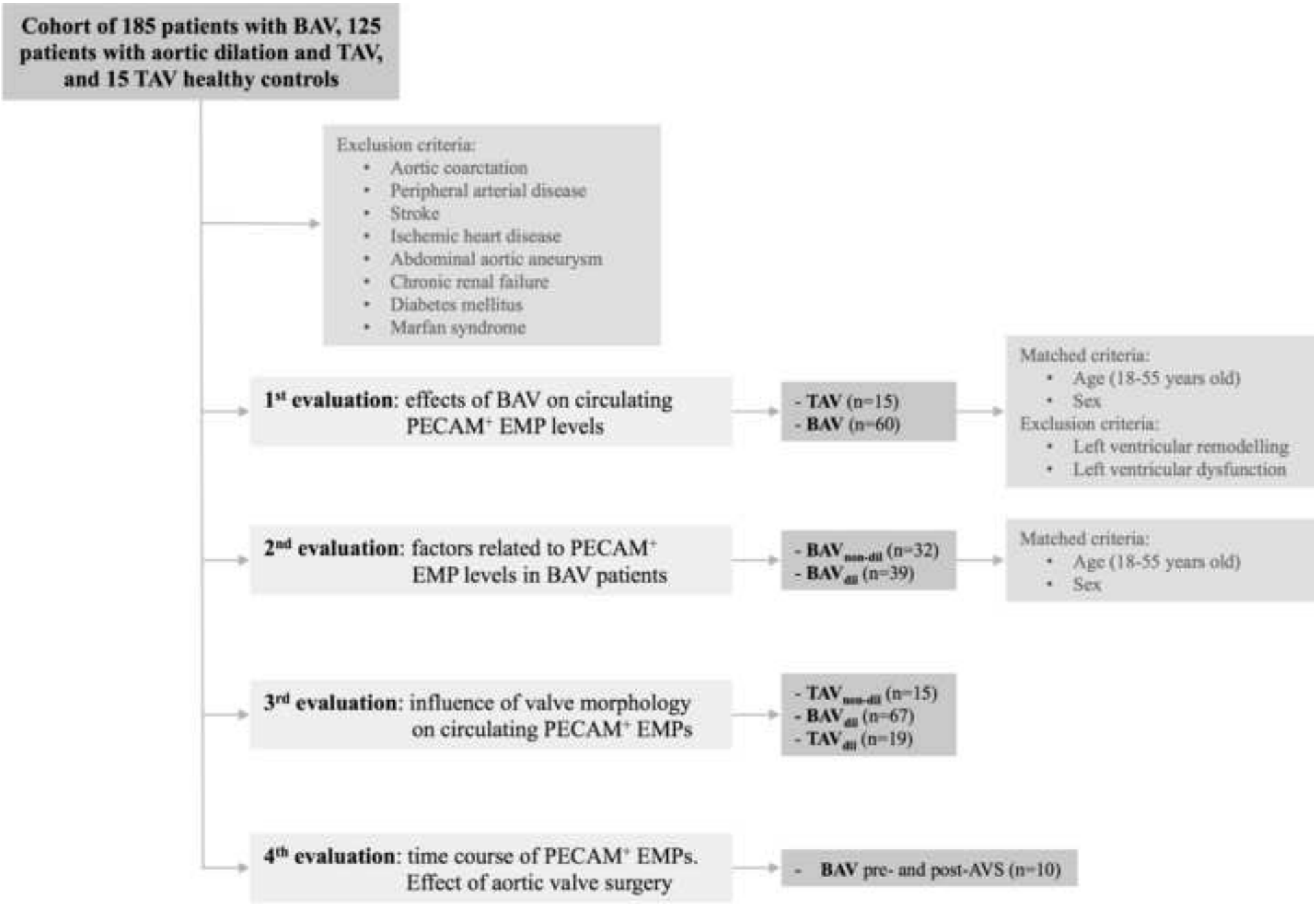

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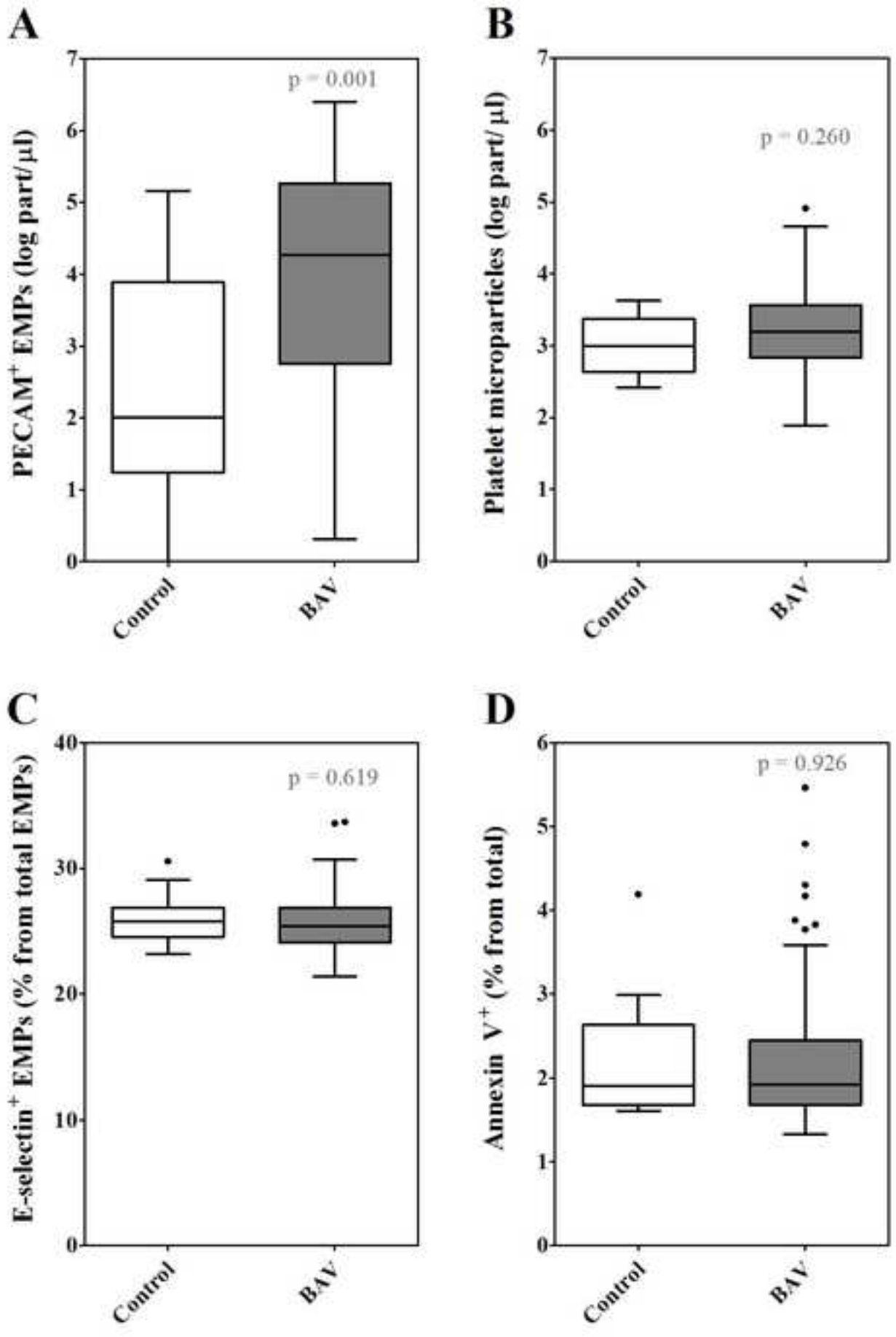

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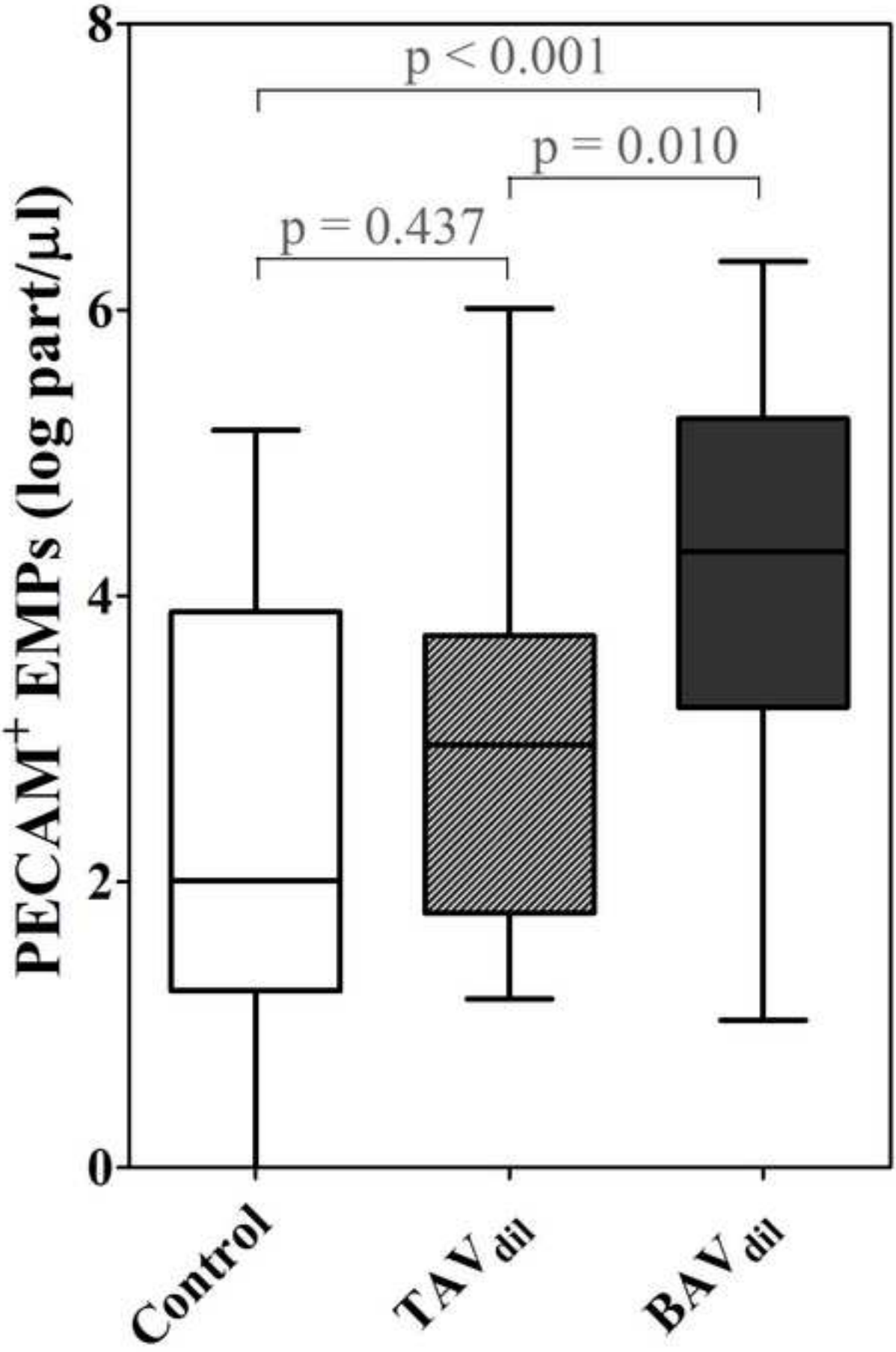

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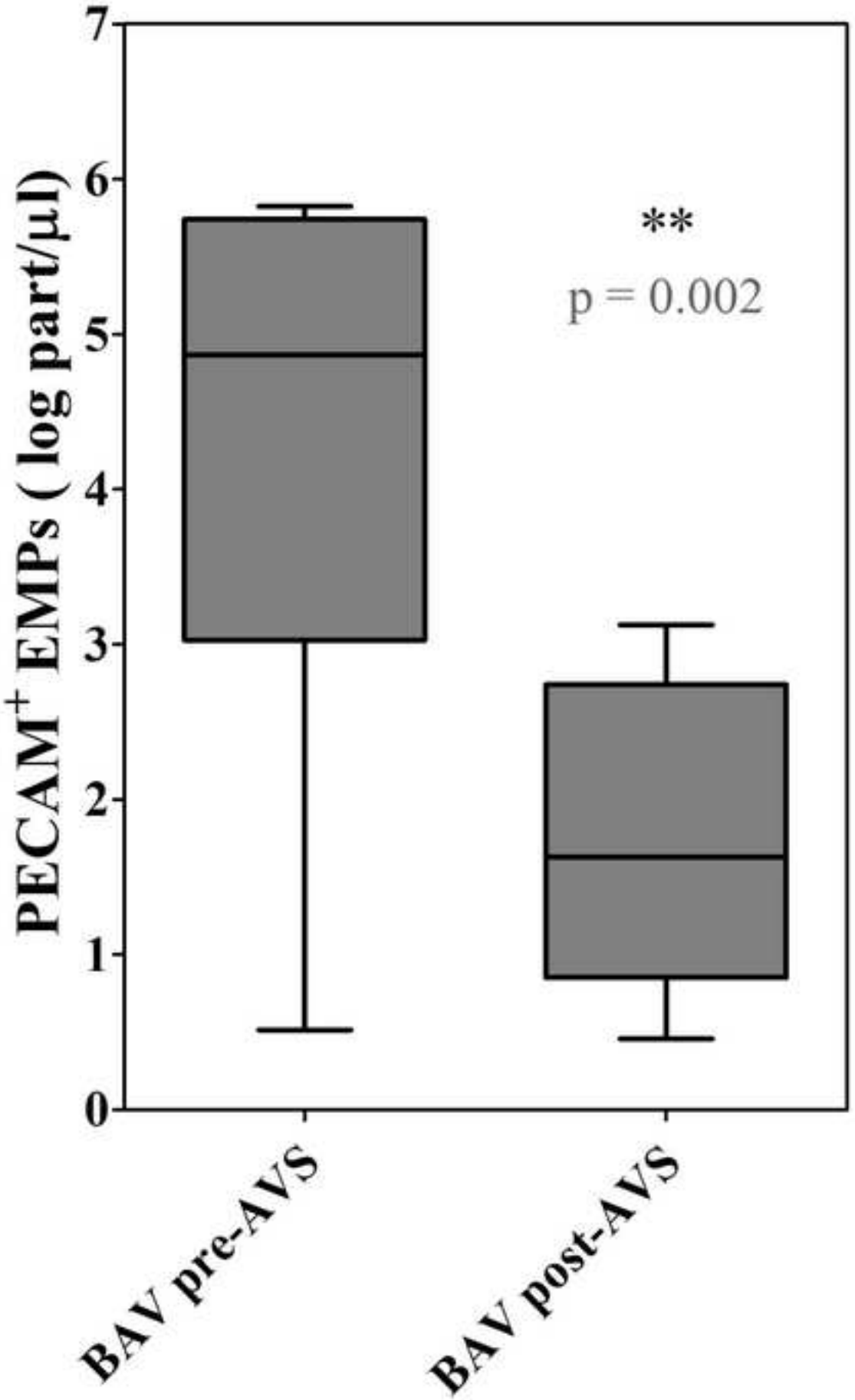

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