**Peer Review File**

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**Reviewer A**

**1) Line 67: Please elucidate the rationale behind utilizing neopterin as an indicative marker for inflammation and calcification in the aortic valve. Were other markers of inflammatory response also contemplated?**

**Reply:** We had originally published a study in our group on the involvement of neopterin in atherosclerotic lesions of the coronary arteries. At the same time, we had also reported the involvement of myeloperoxidase in the progression of aortic valve stenosis in hemodialysis patients. Since HOCL is produced from myeloperoxidase of these neutrophils, it is highly possible that neopterin is involved in aortic valve stenosis via the GTP pathway.

**2) Line 82: The control group comprised aortic valves from patients who succumbed to non-cardiovascular diseases. Did you verify whether there were discrepancies in neopterin measurement results between pre- and postmortem instances?**

**Reply:** Thank you for pointing this out. We have not tested whether there is a difference in tissue expression of neopterin before and after death. However, neopterin is a stable molecule.

I will add this to " limitation".

We add reference 34.

**Changes in the text:** *we did not verify the neopterin measurement result between pre- and post mortem instances. However, neopterin is a stable molecule*[34]. (page 14, line 250-252)

*reference 34: Hepokur C, Mısır S, Ali İhsan Hepokur Aİ, Yaylım İ. Association of Neopterin and Wound Healing Process. Experimed 2019; 9(2): 65-68*

(page21 line 367-368)

**3) Line 136: Elaborate on the criteria for choosing factors like medical history and medications for comparative analysis between groups, and cite the relevant references, if available.**

**Reply:** Clinical factors associated with calcific valve disease mirror those associated with coronary atherosclerosis, and coronary artery disease is common among adults with aortic stenosis.（Aortic-Valve Stenosis -- From Patients at Risk to Severe Valve Obstruction N Engl J Med 2014;371:744-756）Therefore, we selected the known coronary risk factors and the associated oral medications. The following text is to be added.

**Change in the text:** *: factors associated with calcific valve disease mirror those associated with coronary atherosclerosis*[8]. (page 9, line 143-144)

*reference 8: Otto CM, Prendergast B. Aortic-Valve Stenosis --From Patients at Risk to Severe Valve Obstruction. N Engl J Med 2014;371:744-756* (page 17 line 295-296)

**4) Line 174: The manuscript posits that elevated neopterin expression is implicated in the early stages of AS due to higher expression levels in bicuspid AS valves compared to tricuspid ones. It is pivotal to establish whether non-AS patients with bicuspid valves exhibit no significant neopterin expression, even at an advanced age, without developing AS.**

**Reply:** Thank you for your valuable remarks. As you say, it is important to evaluate the presence or absence of neopterin expression in the tissue of non-AS patients with bicuspid valves. However, it is ethically difficult to obtain a normal bicuspid valve.

I will add the following statement to “limitation”.

**Changes in the text:** *Furthermore, to verify that neopterin affects bicuspid valve AS, it is also important to show that non-AS patients with bicuspid valves do not develop neopterin at an older age. However, this could not be verified because it is ethically impossible to harvest normal valve tissue from a living human being.*

( page14 , line 246-249 )

**5) Line 189: A prior study by Dr. Naito et al. titled "Increased Serum Neopterin in Patients with Nonrheumatic Aortic Valve Stenosis" discussed the correlation between AS and neopterin. Given the association between neopterin-mediated inflammation and immune responses and the onset of AS, assessing serum neopterin levels is advantageous for AS patients. This manuscript contrasts neopterin levels in**

**AS and AR patients with those in control subjects, featuring a comparative analysis in Fig. 2B between AS and AR patients and controls.**

**Reply:** This paper illustrates the utility of serum neopterin as a biomarker. The current study is about the effect of neopterin on AS. It is modified as follows.

**Changes in the text:** *Naito et al. reported that serum neopterin is a useful marker for AS* [21]. ( page 12, line 196)

*reference 21* :*Naito Y, Tsujino T, Akahori H, Matsumoto M, Ohyanagi M, Mitsuno M, Miyamoto Y, Masuyama T. Increased serum neopterin in patients with nonrheumatic aortic valve stenosis.Int J Cardiol. 2010;19:145(2):360-361* (page 19 line 328-330)

**5) Line 80: The text alludes to limitations; however, the basis for determining the sample size remains unclarified. Could you elaborate?**

**Reply:** The most important reason is that the number of BAV patients is small and only 34 BAV cases were collected during 2012-2015. Since the sample size was small and not normally distributed, nonparametric methods were used to ensure reliability.

**6) Line 89: Specify the guidelines referenced in your description.**

**Reply:** we have modified our text as advised.

**Changes in the text:** *The criteria for hypertension, hyperlipidemia, and diabetes mellitus were in accordance with the guidelines of American heart association and American Diabetes association.* (page 6, line 91-93)

**7) Line 120: While the method to calculate the neopterin-positive macrophage score is clear, the significance of computing CD3-positive T cells needs clarification. Please provide a detailed explanation of the scope of the entire tissue section.**

**Reply:** Macrophages are activated by interferon-γ secreted by T cells; if there are more T cells, we can presume that the macrophages are activated.

Add the following statement.

**Changes in the text:** *Since macrophages are activated by interferon-γ secreted by T cells, we can presume that the macrophages are activated if there are more T cells.*

(page 8, line 127-129)

**8) Line 128: The font for χ should be consistent.**

**Reply:** Thank you for pointing this out. We have corrected it.

**Change in the text:** *χ2 test or Fisher's exact test.* (page 8, line 135)

**9) Line 144: The term "significantly" is employed; could you please elucidate its implication in this context?**

**Reply:** It was used because it looked and felt like a lot. It is not meant in a statistical sense.

This sentence will be erased because it is misleading.

**Changes in the text:** *Calcified areas were observed in all AS lesions in patients with both BAV and TAV. However, the percentage area of calcification was significantly higher in the patients with BAV than in those with TAV patients (Figure 1A and 2A).*

*→Calcified areas were observed in all AS lesions in patients with both BAV and TAV (Figure 1A and 2A).* (page 9, line 151-152)

**10) Line 152: The overlay of red and blue is ambiguous. Could you enhance the magnification to visualize the overlay distinctly?**

**Reply:** Thank you for pointing that out. I did not explain it well enough. It appears purple because the red and blue overlap. Therefore, the overlay is ambiguous. I have explained it in Figure legend and will correct the text.

**Changes in the text:** *The large number of purple cells in neopterin (red) and macrophage (blue) double immunostaining indicated that the majority of neopterin-positive cells were macrophages in AV specimens from BAV patients.* (page10, line 158-161)

**11) Line 154: Incorporate an illustrative image to depict the contrast effectively.**

**Reply:** We added Figure. and the text was also revised.

**Change in the text:** We added new Figure 4.

▪*there were a few macrophages, but no T lymphocytes, Neopterin-positive cells and 4-HNE-positive cells were found (Figure 4).* (page 10 line 161-163)

▪*Figure 4*

*Micrographs of an aortic valve specimen obtained from a control case (A-E). (A) Hematoxylin-eosin stain. The boxed area is enlarged in (B-E). (B) The section stained with anti-CD68 antibody shows a few macrophages in the lesion (red). (C) The adjacent*

*section stained with anti-CD3 antibody reveals that there are no T lymphocytes. (D) The adjacent section stained with anti-neopterin antibody reveals no positivity for neopterin. (E) The anti-4-HNE antibody shows that there are not 4-HNE-positive macrophages. Bar: A; 1000µm, B-F, 100µm.*

(page 22, line 400-407)

**12) Line 200: Cite a study that substantiates the elevation of neopterin levels in conditions like heart failure and unstable angina pectoris.**

**Reply:** References 12, 22, 23, 24 and 25 are applicable. Correct as follows.

**Changes in the text:** *It is important to note that the aforementioned neopterin as a biomarker, which we mentioned above is also elevated in heart failure and unstable angina, refers to plasma neopterin.*[12,22,23,24,25] (page12 , line 207)

**13) Line 203: Elaborate on the mechanism and pathophysiology underlying cardiorenal syndrome.**

**Reply:** The current state of knowledge sees the concept of haemodynamic regulation by the heart and kidneys as a complex and dynamic system in which changes in the function of one organ can lead to a spiral of dysfunction in both through altered balance between nitric oxide and reactive oxygen species, systemic inflammation, activation of both the sympathetic nervous system and the renin-angiotensin-aldosterone system (RAAS), and the influence and interplay of various substances such as endothelin, prostaglandins, vasopressin and natriuretic peptides. We now know that in the setting of kidney dysfunction, the heart experiences varying degrees of accelerated atherosclerosis, left ventricular hypertrophy and remodelling, myocardial micro-angiopathy and vascular calcification, while in the setting of cardiac dysfunction, hypoperfusion and excessive activation of the RAAS contributes to progressive renal insufficiency. Furthermore, individuals who present with the constellation of potentially modifiable risk factors for cardiovascular disease are also at higher risk for renal disease, as many of these factors such as smoking, hypertension, dyslipidaemia, age and diabetes are also factors in the progression of renal disease. Finally, dysfunction of the heart and kidneys can cause similar effects, for example, anaemia, which in turn may contribute to worsening function of both organs.

(Cardiorenal syndrome: refining the definition of a complex symbiosis gone wrong. Intensive Care Medicine. 2008;34:957-962)

**change in the text:** *the current state of knowledge sees the concept of haemodynamic regulation by the heart and kidneys as a complex and dynamic system in which changes in the function of one organ can lead to a spiral of dysfunction in both*[26].

(page 12, line 209-211)

**14) Line 219: Clarify the purpose behind this statement.**

**Reply:** I wanted to mention that the current study evaluated the eventual outcome of neopterin and it is not clear how neopterin was increased; it may tell us whether the cause of accelerated AS progression is congenital or acquired.

**Changes in the text:** *It is unknown why neopterin expression is enhanced in the bicuspid aortic valves. Thus, it may be congenitally expressed in the bicuspid aortic valves.*

→ *We do not know how neopterin is expressed in the bicuspid aortic valve tissue. Neopterin may be congenitally expressed in the bicuspid aortic valve.* (page 13, line 228-229)

**15) Line 225: Could you elucidate why wall shear stress is mentioned and elaborate on its correlation with neopterin expression?**

**Reply:** Blood flow analysis has progressed in recent years, and there are many reports of increased WSS in the ascending aorta in patients with BAV due to disrupted blood flow. It has also been reported that WSS induces oxidative stress on the vascular endothelium. [Autonomous Effects of Shear Stress and Cyclic Circumferential Stretch regarding Endothelial Dysfunction and Oxidative Stress: An ex vivo Arterial Model. *J Vasc Res*;2010:47(4):336–345]

Therefore, if oxidative stress is strong, there is a possibility that neopterin may be produced as an antioxidant reaction.

We added reference 33.

**Changes in the text:**

*and this phenomenon increases wall shear stress in the aortic wall.*[31, 32] *It has also been reported that wall shear stress induces oxidative stress on the vascular endothelium*[33].

(page13 line233- page 14 line 235)

*reference 33: Thacher TN, Silacci P, Stergiopulos N, da Silva RF. Autonomous Effects of Shear Stress and Cyclic Circumferential Stretch regarding Endothelial Dysfunction and Oxidative Stress: An ex vivo Arterial Model. J Vasc Res;2010:47(4):336–345* (page 21, line 364-366)

**16) Lines 355 to 359: For each antibody from (B) to (E), indicate which cell in the photograph is linked to the antibody using arrows.**

**Reply:** Some misleading wording has been corrected. T cells and 4-HNE are not seen in the images posted. Therefore, the description is also corrected and added arrows to neopterin.

**Changes in the text:**

*(B) The section stained with anti-CD68 antibody shows scattered macrophages in the lesion (macrophages: red). (C) The adjacent sections were stained with anti-CD3 antibody, no CD3-positive T lymphocytes were found in this sections (T lymphocyte: red). (D) The adjacent section stained with anti-neopterin antibody reveals that macrophages are scant with positivity for neopterin (neopterin: brown). (E) The anti-4-HNE antibody shows that there are not 4-HNE-positive macrophages (4-HNE: red).* (page 21, line 376-381)

*Figure 1 modified and arrows added.*

**Reviewer B**

**1) Remove BOLD letter ¨b¨**

**What is known and what is new?**

**- It is well known empirically that AS in BAV is associated with a faster rate of AS progression, but the cause is still unknown.**

**Reply:** Thank you for pointing this out. We have corrected it.

**2) Remove BOLD letter ¨ χ ¨**

**128 compared using the χ2 test or Fisher's exact test. Statistical significance was set at…**

**Reply:** Thank you for pointing this out. We have corrected it.

**3) Instead of:**

**91 respective societies. All patients underwent preoperative echocardiography performed by experienced sonographers.**

**It should say:**

**91 respective societies. All patients underwent preoperative echocardiography, which were performed by experienced echocardiographists.**

**Reply:** Thank you for pointing this out. I corrected the same as above. (page 6, line 93-94)

**4) Instead of Helical it should say Helicoidal (Note: both words are correct)**

**blood flow in patients with BAV. The results showed a strong helical flow across the entire**

**Reply:** I change helical to helicoidal as above. (page 13, line 232)

**Reviewer C**

**1) Line 120: The term "neopterin-positive area" should be precisely defined. Additionally, "macrophage-positive" should also be defined explicitly to avoid any ambiguity.**

**Reply:** we have modified our text as advised.

**Changes in the text:** *Immunohistochemical staining would depict neopterin-positive cells as if they were attached to each other, making it impossible to count cells one by one. Therefore, the stained area was measured. This stained area was defined as the neopterin-positive area. The same definition was used for macrophage-positive cells.* (page 8, line 122-126)

**2) Line 194: Please clarify what is meant by "Levels." Specify what levels are being referred to in this context.**

**Reply:** Thank you for pointing this out. We will correct it.

**Changes in the text:** *Neopterin levels were elevated in patients with unstable coronary artery disease compared to control subjects and patients with stable coronary artery disease. (page 12, line 200)*

**3) Line 219: I disagree with the speculation that neopterin expression may be congenitally enhanced in BAV patients. This claim lacks a well-substantiated explanation. Furthermore, it is important to address why not all BAV patients develop AS but some develop aortic regurgitation (AR). Please provide insights or hypotheses on this matter.**

**Reply:** Thank you for your input.

We also believe that the neopterin is not congenitally enhanced expression. However, We wanted to mention that we cannot rule it out because it is impossible to obtain a valve from a BAV patient without lesions.

I believe that some BAV patients develop AR due to some cause of prior aortic annulus enlargement; it has been reported that AR patients with BAV are significantly younger than AS patients with BAV and also have significantly larger aorticannulus diameters. [Comparison of ascending aortic cohesion between patients with bicuspid aortic valve stenosis and regurgitation. *EJCTS*;2014:46(6)e89-e93] My hypothesis is that in AR, the WSS that was originally supposed to hang over the valve is being released into the left ventricle, which further progresses the AR more than the AS.