**Author’s response to reviews**

**Title:** An Association of Spleen Volume and Aortic Diameter in Patients and in Mice with Abdominal Aortic Aneurysm

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**Version:** 1 Date: 29 Aug 2017

**Author’s response to reviews:**

Amine Mazine (Reviewer 1): The authors are to be congratulated on an interesting and well-conducted study.

The manuscript is well-written and easy to follow.

My only reservation has to do with the control group in the clinical arm of the study. As the authors point out, patients in the control group were significantly younger than in the study group. Furthermore, no data were collected regarding height/weight, and the spleen volumes were not indexed. Could the authors comment on how they selected the 25 controls for this study? Why did they only choose 25 patients? The 50-80 age frame seems quite wide and certainly insufficient to consider the two groups comparable.

Answer: Thank you for your kind suggestions. Actually, we selected 432 cases at the beginning, supposing to find more cases to make the two groups more comparable. But to exclude the potential influence of several conditions on the spleen volume, we firstly excluded 165 cases with basic lesions about spleen, including tumors, cysts, infection, hematopoietic system diseases, the lymphatic system diseases, connective tissue disease, metabolic disease, autoimmune diseases and parasitic diseases, unexplained fever for a long time, etc. The study by Meier et al (Meier et al. Assessment of age-related changes in abdominal organ structure and function with computed tomography and positron emission tomography. Semin Nucl Med. 2007 May;37(3):154-72.) has found that pediatric, but not adult, spleen volume to be positively associated with age (Data shown in the table below). In their study, they divided the adult group into two subgroups, and the spleen volume revealed a trend of shrinkage. Also, considering the higher incidence of abdominal aortic aneurysm among older populations, to make the AAA group and the control group more comparable, we excluded the cases younger than 50 year-old-
age. Besides, accompanying with the increase of age, the severity of atherosclerosis reveals more severe. And previous studies have proved the atherosclerosis might be associated with the spleen size because the inflammation responses also play important role in its pathological process. As a result, to exclude the influence of the contaminant atherosclerosis, as well as unknown influence of older age, on the spleen volume, we further excluded the cases older than 80 year-old-age and selected 25 cases in the control group at last.

Aly Ghoneim (Reviewer 2):

I would like to congratulate the authors for their well conducted and interesting study (An Association of Spleen Volume and Aortic Diameter in Patients and in Mice with Abdominal Aortic Aneurysm.)

I do agree with the authors about the importance of identifying other morphological indices that serve as predictors of the risk of rupture in AAA. The manuscript is well-written, easy to follow and exploring an interesting question about the presence of an association between the increase in size of spleen coexisting with AAA. Indeed, it arises the questions; if this can be useful in the future and if it can be used as a predictor of an increase in the inflammatory reactions co-existing with an increased risk of AAA rupture.

However, I would like to address the following important points:

1. Why is the control group in the study significantly younger than the study group?
Answer: Thank you for your kind responses. Actually, to exclude the potential influences of several conditions on the spleen size, we firstly excluded 165 cases with basic lesions about spleen, including tumors, cysts, infection, autoimmune diseases, etc. During the study, we found that accompanying with the increase of age, aortic atherosclerosis reveals more severe. And previous studies have proved the atherosclerosis might be associated with the spleen size because the pathological process shares some similar characteristics with autoimmune inflammation responses. To exclude the influence of the contaminant atherosclerosis on the spleen volume, we further excluded the cases with aortic atherosclerosis, resulting in a younger control group because atherosclerosis is quite common among the population older than 50 year-old-age.

2. What was the selection criteria of the control group?
Answer: Thank you for your kind responses. The selection criteria of the control group include: (1) patients without abdominal aortic lesions including abdominal aortic aneurysm, aortic dissection, or intramural aortic hematoma; (2) patients without abdomen operation history; (3) patients without abdominal aortic atherosclerosis; (4) patients’ age not younger than 50 years or greater than 80 years; (5) patients without diseases proved to lead to spleen size and/or shape changes such as tumors, cysts, infection, hematopoietic system diseases, the lymphatic system diseases, connective tissue disease, metabolic disease, autoimmune diseases and parasitic diseases, unexplained fever for a long time, etc.

3. Why the splenic volumes were not indexed?
Answer: Thank you for your kind response, which is quite critical. Actually, we have considered the influence of height and weight on the spleen size. But it is a pity that, limited to the study design, we could not collect the data of height, weight to adjust the spleen volume. To make the results more credible, we have review the previous studies on the radiological analysis of the spleen volume but to find some studies (Konus et al. Normal liver, spleen, and kidney dimensions in neonates, infants, and children: Evaluation with sonography. AJR Am J Roentgenol. 1998 Dec;171(6):1693-8; Megremis et al. Spleen length in childhood with US: Normal values based on age, sex, and somatometric parameters. Radiology. 2004 Apr;231(1):129-34.) have proved that the relationship between age and spleen size was independent of body surface area, weight, and height. Thus, the splenic volumes were not indexed in our study at last.

4. Why the wide range of age (50-80)? A range of 10 years' difference would have made both groups more comparable.

Answer: Thank you for your kind response, which is quite critical. Actually, it was quite ambivalent to choose such a wide range of age (50-80). Though it has been proved that the adult spleen volume did not change significantly, the older patients almost accompanied with abdominal aortic atherosclerosis. However, the incidence of abdominal aortic aneurysm increases with the old age, as well as the incidence of acute events of abdominal aortic aneurysm. In 2010, the global age-specific prevalence rate per 100,000 ranged from 7.88 (95% CI: 6.54 to 9.59) in the 40 to 44 years age group to 2,274.82 (95% CI: 2,149.77 to 2,410.17) in the 75 to 79 years age group (Sampson et al. Estimation of global and regional incidence and prevalence of abdominal aortic aneurysms 1990 to 2010. Glob Heart. 2014 Mar;9(1):159-70.). The incidence of acute AAA events per 100,000 population per year was 55 in men aged 65-74 years, but increased to 112 at age 75-84 years and to 298 at age 85 years or above (Howard et al. Age-specific incidence, risk factors and outcome of acute abdominal aortic aneurysms in a defined population. Br J Surg. 2015 Jul;102(8):907-15.). Study of older populations seemed more clinically meaningful for abdominal aortic aneurysm and could get more cases in a relative small series. As a result, we chose the wide range of age (50-80) also to get more cases.

5. How can a 28 days' laboratory experiment with inflammatory induced changes mimic the natural inflammatory process causing the main pathology in human?

Answer: Thank you for your kind response, which is quite critical. The development of AAAs involves a series of complex pathologies in the abdominal aorta. There is accumulating evidence that the renin angiotensin system plays an important role in the development of human AAAs. This is complemented by the demonstration that subcutaneous infusion of angiotensin II (Ang II) into mice leads to the development of AAAs. According to previous studies (Daugherty et al. Complex pathologies of angiotensin II-induced abdominal aortic aneurysms. J Zhejiang Univ Sci B. 2011 Aug;12(8):624-8;), the animal model shares several common features of the complex pathologies with human AAAs, including: (1) medial and adventitial accumulation of macrophages as early as 48 h after the beginning of Ang II infusion and being persistent during the progression of AAAs; (2) early stage of elastin fiber disruption associated with profound macrophage infiltration that rapidly progresses across the entire aortic media; (3) progressive
luminal expansion that manifests rapidly in the first 5–14 d and continuous and gradual expansion beyond the early stage of AngII infusion; (4) thrombus formation, propagation, and resolution; (5) disarrayed extracellular matrix deposition; (6) neovascularization; and (7) atherosclerotic lesions in hypercholesterolemic mice. Since this initial description of this 28 days’ laboratory experiment over a decade ago, this model has been regarded as the most similar animal model to mimic the natural inflammatory process causing the main pathology in human and used by many laboratories to define the mechanisms of AAAs. So we chose this model to conduct our laboratory experiment.

6. What are the chances that the splenic enlargement is a temporary reaction to the induced inflammation?

Answer: Thank you for your kind response, which is quite critical. Actually, we have considered the chances that the splenic enlargement is a temporary reaction to the induced inflammation. After reviewing the relative article, we have excluded the conditions that could influence the spleen volume according to the descriptions from the clinicians, including hematological disorders (hemoglobinopathies, hereditary spherocytosis, primary neutropenia, thalassemia, myelofibrosis, polycythemia vera, thrombotic thrombocytopenic purpura, osteopetrosis, myelofibrosis), congestive diseases (cirrhosis, portal hypertension, splenic vein and portal vein thromboses, right sided heart failure, cystic fibrosis, acute splenic sequestration secondary to sickle cell anemia crisis), storage diseases (Gaucher disease, Niemann-Pick disease, amyloidosis, hemosiderosis, hemachromatosis, diabetes mellitus, histiocytosis), collagen vascular diseases (systemic lupus erythematosus, rheumatoid arthritis, Felty syndrome), neoplastic diseases (cysts, hemangioma, lymphangioma, leiomyosarcoma, fibrosarcoma, lymphoma, leukemia, myeloma, metastases) and inflammatory diseases (hepatitis, septicemia, bacterial endocarditis, infective mononucleosis, tuberculosis, syphilis, histoplasmosis, brucellosis, malaria, leishmaniasis, kala-azar). However, with just listing the main diagnosis, those descriptions were inaccuracy to some extend. Thus we could not definitely assure that we have excluded all the possible causes of splenic enlargement. Because our study is just retrospective cross-sectional design, limitations do exist. To solve the problem, under the guidance of the results of present study, we would conduct prospective cohort study in the future to learn more about the details.

7. Were there any splenic volumes followed on a longer duration in the study group? If yes, were there any decrease in volumes later on?

Answer: Thank you for your kind response. It is a pity that our study was just retrospective cross-sectional design to learn the potential association of splenic volume and abdominal aortic aneurysm, inspired by our preliminary laboratory experiments which is quite controllable. As a result, we did not get any data about the changes of splenic volumes followed on duration. However, I do agree that the splenic volumes have the potential to decrease with the increase of age, but the previous studies have proved that the shrinkage was not significant among the whole populations at least among those at the age of 18–81. Nowadays, more and more evidences have proved that abdominal aortic aneurysm was provoked by lasting chronic inflammatory responses induced by the spleen mobilized T lymphocytes and macrophages. On the basis of the
pathological studies, we suggest that the changes of spleen might be long lasting, which needs future studies to clarify.

8. Is there any data about an associated thrombocytopenia concomitant with the increase in splenic volumes?

Answer: Thank you for your kind response. It is a pity that we might miss some important information limited to the retrospective cross-sectional design. As our clinical study was just focused on the radiological parameters, we could not get any data about the blood platelet. Follow your suggestion, to clarify the problem we will collect the results of blood routine test in our future prospective cohort study to learn more details.

Robert Doonan (Reviewer 3): I read this manuscript with great interest. The authors have identified an important avenue of investigation. Although I believe the topic to be of interest for the vascular surgery community, this manuscript suffers from methodological flaws, which will require extensive revision.

General comments:

1. I would recommend an outside party review this manuscript for spelling and grammatical errors.

Answer: Thank you for your kind suggestions. Because time limited after we have collected the relative material to answer all of the reviewers’ questions and revised the manuscript, we hope to ask the Nature Research Editing Service for help if our answer could meet your requirements.

2. The figures contain some incorrectly labelled information. Some of these are noted in specific comments below. I recommend reviewing this manuscript thoroughly for these errors.

Specific comments:

Methods

1. It is not clear how the authors chose their final 30 male cases to be included in the study. Please provide a detailed description of how the number of screened patients went from 432 to the 30 included in the study. This can be in methods or results. However, the description in the results section currently is incomplete. Were there eventually more than 30 patients who did not meet exclusion criteria and 30 were selected at random? This is not clear.

Answer: Thank you for your kind suggestions. We feel sorry for not making it clear how the 30 patients were selected. The enrollment procedures were as follows:
1. Step one: We retrospectively analyzed 432 male patients’ imaging (mean age 70 years, range from 44 to 88 years) from January 1, 2012 to December 31, 2014.

2. Step two: We ruled out 104 cases with age less than 50-year-old and greater than 80-year-old.

3. Step three: We ruled out 55 cases with abdominal aortic dissection, intramural aortic hematoma, postoperation, or previous aneurysm surgery. Also to excluded the influences of abdominal aortic atherosclerosis, we ruled out 53 cases with abdominal aortic atherosclerosis.

4. Step four: We ruled out the 165 cases with diseases leading to spleen size and/or shape changes such as tumors, cysts, infection, hematopoietic system diseases, the lymphatic system diseases, connective tissue disease, metabolic disease, autoimmune diseases and parasitic diseases, unexplained fever for a long time, etc.

5. Step five: For the remaining 55 cases, we enrolled 30 patients in the AAA group and 25 patients in the control to analyze the correlation of spleen volume with Dmax. However, when analyze the correlation of spleen volume and age, we included the 53 cases because atherosclerosis is quite common and the previous study about the normal spleen volume did not exclude this factor either.

Results

1. Animal experiments - the authors state that 'the number and size of follicles in the medullary region were increased…' Was this determined simply by gross observation or was there a quantification method to determine this? I suggest quantifying these analyses and showing this data.

   Answer: Thank you for your kind suggestions. Referring to the study by Gopal et al. (Gopal et al. High-fat diet- and angiotensin II-induced aneurysm concurrently elicits splenic hypertrophy. Eur J Clin Invest. 2014 Dec;44(12):1169-76.), we analyzed the number and size of follicles simply by gross observation. And our results focused on the changes of spleen volume in animals with abdominal aortic aneurysm, so we did not quantify the pathologic changes of spleen.

2. Clinical cohort - the presentation of demographic and clinical data is confusing. If you are presenting data of 108 patients aged 50-80, this data should be in a table.

   Answer: Thank you for your kind suggestions. As described in the answer to your first question about the description in the method, we have explained how we enrolled 30 cases in the patient group and 25 cases in the control group. Besides for the 108 patients aged 50-80 to analyze the correlation of spleen volume and age, we added the 53 patients with abdominal aortic atherosclerosis. The reason that we did not include the data of the 53 patients in table one is to show the statistics of the patient group and the control group clearly. If you think it necessary, we will add this data.
3. Table 1 - this table is sparse. Typically a Table 1 contains significant information with respect to the patient characteristics. The authors should include CV risk factors, smoking history, medications etc… For example, perhaps the groups differ with respect to smoking history, anti-hypertensive treatments, statin treatments etc… These may not only affect AAA size, but perhaps spleen volume? In addition, this data should be shown for the large and small AAA sizes since this is a key group differentiation in your analyses.

Answer: Thank you for your kind suggestions. It is a pity that our study was just retrospective cross-sectional design to learn the potential association of spleen volume and abdominal aortic aneurysm, inspired by our preliminary laboratory experiments, which is quite controllable. Because our study is just retrospective cross-sectional design, limitations do exist. One of the limitations is that we could not get any data about the CV risk factors, smoking history, medications etc., because the clinicians only recorded the main diagnosis to apply CT scan. To solve the problem, under the guidance of the results of present study, we would conduct prospective cohort study in the future to learn more about the details. Moreover, the data about statistic analysis of spleen volume between large and small AAA sizes has been shown in the quantitative analysis of spleen volume section of the method part, though we did not show the data in a table. If you think it necessary, we will modify the graphs to add the data in a table.

4. It appears as though all the analyses in this manuscript are unadjusted. The clinical characteristics suggested above to be included in Table 1 should be entered into the author's models to determine if there is a significant association between these covariates and pertinent variables (spleen size, AAA size).

Answer: Thank you for your kind suggestions. It is a pity that our study was just retrospective cross-sectional design, and limitations do exist. One of the limitations is that we could not get any data about the characteristics you mentioned. Though it is necessary to include those characteristics and adjust the analysis to make it more precise, we hope to solve the problem by conducting prospective cohort study in the future.

5. It is unclear to me the clinical significance of a correlation of 0.36. Please comment.

Answer: Thank you for your kind response. According to the results of our study, the clinical significance of a correlation of 0.3611 (P=0.0423) means the spleen volume do significantly increase with Dmax of AAA. However, the conclusion needs further proof by further clinical study with large sample size, and a prospective cohort study may be more helpful for exclude the unknown factors influencing the results.

6. Figure 1c - typo 'volumn'

Answer: Thank you for your kind suggestions. We have modified the 'volumn’ into ‘volume’. Sorry for the mistake.

7. Figure 1 d - the text states that this figure shows spleen to body weight ratio. It appears that the figure actually shows absolute weight of spleen and not a ratio.
Answer: Thank you for your kind suggestions. Actually, the result shows spleen to body weight ratio because we have normalized the spleen volume according to the weight of the mice. The formula for the normalization is Spleen Volume=Measured Spleen Volume/(Weight of mice/30g). The 30g is the mean of the weight of mice.

8. Figure 1 e and f - I see no arrows on the figure as is described in the figure legends.

Answer: Thank you for your kind suggestions. We have added the arrows as is described in the figure legends. Sorry for the mistakes.

Discussion

1. 'we hypothesized that in patients… cause hypertrophy of the spleen'. I do not believe you can make the link between inflammation, inflammatory cells and hypertrophy of the spleen in your data set, at least not in humans. This statement should be clarified please.

Answer: Thank you for your kind suggestions. Follow your suggestions, we have modified the statement into ‘We hypothesized that in patients with large AAA and high risk of rupture, more inflammatory cells may be mobilized in the spleen and infiltrate the aorta to promote the local inflammation and the degradation of the aortic wall. And the lasting mobilization of the inflammatory cells causes the gradual increase of the spleen volume. However, the precise pathological mechanisms still remain elusive and need further investigations.’ Hope it more helpful for the explanation of the correlation of spleen volume and Dmax of abdominal aortic aneurysm.

2. Conclusion - first line. Your study does not demonstrate an association of spleen morphological changes in humans and AAA size, only spleen size (not morphology per se). Morphology typically refers to many features beyond size including heterogeneity, homogeneity, tissue types etc… Please fix this here and throughout the manuscript.

Answer: Thank you for your kind suggestions. We have modified the ‘spleen morphological changes’ into ‘spleen size changes’ to make the conclusion more precise.