

Peer Review File

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Reviewer A

The authors present the results of a single-center retrospective observational and comparative clinical study aimed at exploring the correlation between different right-to-left shunt (RLS) indices and the occurrence of cryptogenic stroke (CS) and its clinical diagnostic value. A total of 117 patients with CS undergoing agitated saline contrast echocardiography (ASCE) were collected. As a control group, 93 patients presenting only with headache and dizziness were collected. The authors observed that the proportion of RLS in the CS group (77.7%) was significantly higher than that in the control group (35.4%). RLS grade and type correlated significantly with the occurrence of CS. The study is potentially interesting, but can be improved if the following considerations are addressed:

1. Please, clearly indicate in the Abstract the proportion of RLS in the CS group and in the control group.

Reply: Thanks for your comment. We have added the proportion of RLS in the CS group and in the control group to the Abstract section (line 44-46, “The proportion of RLS (77.78%) in the CS group was significantly higher than that in the control group (35.48%) (P<0.001).”).

2. In the Introduction section, authors should clearly reflect that a classic cardioembolic presentation includes the onset of symptoms after a Valsalva provoking activity (coughing, bending, etc.) suggesting paradoxical embolism facilitated by a transient rise in right atrial pressure and the co-occurrence of cerebral and systemic emboli (Int J Cardiol 2004; 95; 129-134)

Reply: Thanks for your comment. We have added this detailed description to the introduction section (line 73-76, “Furthermore, a classic cardioembolic presentation includes the onset of symptoms after a Valsalva provoking activity (coughing, bending, etc.), suggesting paradoxical embolism facilitated by a transient rise in right atrial pressure and the co-occurrence of cerebral and systemic emboli [7].”).

3. In the Results, it would be interesting to add the positive predictive value and negative predictive value in the multivariate analysis performed.

Reply: Thanks for your comment. We have added the positive predictive value and negative predictive value in the revised Table 5.

Table 5 Comparison of different RLS indexes in the diagnosis of cryptogenic stroke.

| | AUC (95% CI) | Youden index | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | P value |
|-----------|----------------------------|-----------------|--------------------|--------------------|------------|------------|---------|
| RLS Type | 0.700 (0.626, 0.773) | 0.423 | 77.8 | 64.5 | 77.8 | 63.4 | <0.001 |
| RLS Grade | 0.807 | 0.555 | 68.4 | 87.1 | 68.4 | 87.1 | <0.001 |

| | | | | | | |
|----------|---------|-------|------|------|------|--------|
| | (0.748, | | | | | |
| | 0.866) | | | | | |
| RLS | 0.707 | 0.423 | 77.8 | 64.5 | 77.8 | 64.5 |
| Duration | (0.634, | | | | | <0.001 |
| Time | 0.780) | | | | | |

Note: PPV= positive predictive value; NPV= negative predictive value

4. Did the authors find differences with respect to sex? A recently published study on the impact of female sex on the distribution of risk factors, stroke subtype, stroke severity, and outcome in acute cardioembolic ischemic stroke should also be commented on the Discussion (Inogés M et al. Gender Predicts Differences in Acute Ischemic Cardioembolic Stroke Profile: Emphasis on Woman-Specific Clinical Data and Early Outcome-The Experience of Sagrat Cor Hospital of Barcelona Stroke Registry. *Medicina* (Kaunas). 2024 Jan 5;60(1):101. doi: 10.3390/medicina60010101. PMID: 38256361; PMCID: PMC10819324).

Reply: Thanks for your comment. We have added the relevant description in the Discussion section (line 321-325, “In addition, recent studies have shown that female CS patients have different clinical characteristics and poorer early prognosis compared with male CS patients [27]. Our study has not found similar results, which may be related to the small sample size we included, and subsequent studies still need to expand the sample size.”).

5. Authors will be advised to begin the Discussion by evaluating the most relevant data of their study.

Reply: Thanks for your comment. The data included in this study are all closely related to the study objectives. Therefore, we believe that discussion in the order in which the results are presented makes it easier for readers to understand the objectives and conclusions of our study.

6. A brief concluding comment on other possible lines of future research on the presented topic would be appreciated.

Reply: Thanks for your comment. We have added the comment on other possible lines of future research on the presented topic in Discussion section (line 386-389, “In addition, previous studies have shown that CS populations have different clinical characteristics and prognosis in different genders [27]. The sample size was subsequently expanded to explore the value of RLS type, RLS grade, or duration of RLS in CS patients of different genders.”).

Reviewer B

In this study, the authors investigated the role of patent foramen ovale (PFO) in cryptogenic stroke. They studied 117 patients with cryptogenic stroke and used a control group of 93 patients who underwent a saline contrast study for neurological symptoms such as dizziness and headaches. They found a higher incidence of intracardiac shunting in patients with cryptogenic stroke compared to the control group, with rates of 78% versus 35%, respectively. Additionally, the grade of shunting was higher in the cryptogenic stroke patients compared to controls but not the duration of shunting and this was used to demonstrate a high ROC value.

There are several comments related to this paper:

****Strengths of the Paper:****

- The use of a control group is a significant strength.
- The authors counted the number of bubbles to determine the grade of shunting, as well as the duration during which bubbles were present on the left side of the heart after opacification of the right side. While the grade of shunting was higher in patients with cryptogenic stroke, the duration of shunting was not different. This primarily differentiated intrapulmonary shunting from PFO, where the duration of bubbles in the left side lasted longer than in the PFO group, both in controls and in cryptogenic stroke patients.

****Specific Comments:****

1. Multiple edits are needed to improve the readability of the manuscript, and these have been highlighted in the attached version of the paper.

Reply: Thanks for your comment. We have revised the manuscript according to the attached version of the paper, and the modifications have been highlighted in red.

2. Besides visualizing bubbles directly arriving from the PFO versus the pulmonary vein, the authors need to elaborate on how they distinguished intracardiac versus intrapulmonary shunts. This is important because shunting from the pulmonary vein is not easy to detect on transthoracic echocardiography (TTE) and for that matter it may be difficult even during TEE. If the differentiation was based solely on the number of beats it took for the bubbles to arrive on the left side of the heart, that too has its own issues. While bubbles arrive before 5th beat of RA opacification are considered to arise from PFO and those after from intrapulmonary shunting, that rule is broken almost on a daily basis in our echo lab.

Reply: Thanks for your comment. As you said, how to more accurately distinguish between intracardiac and intrapulmonary shunts needs to be further explored. Unfortunately, echocardiographic visualization is currently used in our department for the determination and quantification of intracardiac and intrapulmonary shunts. This is also the mainstream approach for the vast majority of current research (Stickland MK et al. Intra-pulmonary shunt and pulmonary gas exchange during exercise in humans. J Physiol. 2004;561:321–329; Tedjasaputra V et al. Dopamine receptor blockade improves pulmonary gas exchange but decreases exercise performance in healthy humans. J Physiol. 2015 Jul 15;593(14):3147-57.). How to distinguish intracardiac from intrapulmonary shunts is described in detail in the Methods section. Thank you again for your advice.

3. I assume that most patients underwent TTE only for this study. Please clarify how many patients needed additional transesophageal echocardiography (TEE) and in how many patients the diagnosis was ultimately confirmed by TEE.

Reply: Thanks for your comment. We have added this detailed information in Method section as you suggested (line 153-154, “All patients included in this study completed TTE. Of these, 32 patients required further TEE.”).

4. The stroke group had patients who were slightly older, although not statistically significant, and had more cardiovascular risk factors. Even though these factors were not individually significant, there may be significance if one combines drinking history, hypertension, and diabetes. It would be interesting to discuss whether this group might have atheromatous embolic disease or atrial

fibrillation, and whether there were any such findings on TEE. It may also be possible that cardiovascular risk factors also increase the risk of deep vein thrombosis (DVT) and hence an increased incidence of paradoxical embolism.

Reply: Thanks for your comment. We agree strongly with you. However, the patients included in this study did not contain clinical data such as atherosclerosis or DVT. In addition, we believe that serum biological parameters are also risk factors for atherosclerosis and DVT, reflecting to some extent the occurrence of cardiovascular disease in patients.

6. The incidence of intrapulmonary shunting is rather high in this young cohort in both groups. This raises concern that the diagnosis of intrapulmonary shunting may not always have been from bubbles coming from the pulmonary veins, as these are hard to distinguish on a transthoracic echo. If the authors used this method to differentiate intracardiac versus intrapulmonary shunts, it should be noted that this method has issues. Often, bubbles appear in the right side five beats after opacification due to the pressure gradient timing. On page 10, table 4, please indicate the unit of time in the second column.

Reply: Thanks for your comment. We judged whether it was P-RLS by observing the pattern of microbubbles entering the left atrium in combination with ASCE. When microbubbles continued from the pulmonary veins into the left atrium, they were judged as P-RLS. In addition, Feng et al showed that 14 RLS were detected in 21 CS patients. Specifically, two of them had both PFO-RLS and P-RSL, nine had PFO-RL, and three had P-RLS, similar to our findings (Ref: Feng C, Luo T, Luo Y, Zhao N, Huang K, Xiao C. Contrast-enhanced transthoracic echocardiography applied in evaluation of pulmonary right-to-left shunt: A preliminary study. *Comput Med Imaging Graph.* 2018 Sep;68:55-60. doi: 10.1016/j.compmedimag.2018.04.007). In addition, we have added the unit of RLS duration time in revised **Table 4**.

Table 4. Correlation analysis of RLS duration with RLS type and grade.

| Contents | | RLS Duration Time (cardiac cycles) | r | P value |
|----------------------------------|---------------|---------------------------------------|--------|---------|
| RLS Type | PFO-RLS | 8.0 (7.0, 9.0) | 0.902 | <0.001 |
| | P-RLS | 19.0 (17.0, 20.0) | | |
| | PFO-RLS+P-RLS | 20.0 (18.0, 22.0) | | |
| RLSRLS semi-quantitative grading | 1 | 10.0 (8.0, 20.0) | -0.154 | 0.088 |
| | 2 | 9.0 (8.0, 17.0) | | |
| | 3 | 9.0 (8.0, 10.0) | | |

****Minor Comments:****

- Page 4, line 110 should be deleted as it is a repetition.

Reply: Thanks for your comment. We have deleted this duplicate description as you suggested.

- Page 7, line 180: Provide percentages of patients next to the numbers as the groups are not equal.

Reply: Thanks for your comment. We have provided the percentages of patients next to the numbers as the groups are not equal (**line 193, 194, 202, and 203**).

- Figure 1, panel B: Bubbles coming from the right superior pulmonary vein are hard to see in this still image.

Reply: Thanks for your comment. We have provided the clear one.

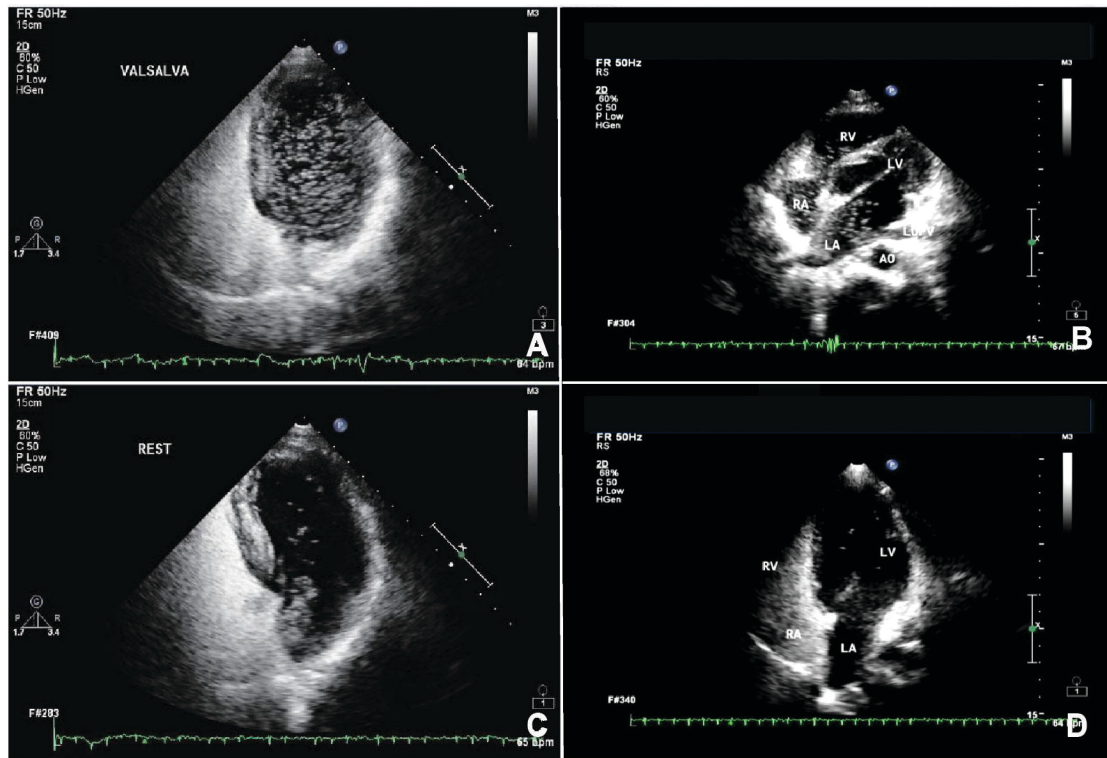


Figure 1. Representative images of RLS signal diagnosed by TTE combined with ASCE. (A) A large number of RLS signals under Valsalva action; (B) left atrial RLS signal from left superior pulmonary vein; (C) RLS signal from interatrial septum in resting state; (D) A small amount of RLS signals at rest.

Additional comments of reviewer B

1. Provide percentages next to numbers here as the two groups are not equal.

Reply: it has been revised (**line 193, 194, 202, and 203**).

2. Bubbles coming from right superior pulmonary vein are hard to see.

Reply: Thanks for your comment. We have provided the clear one.

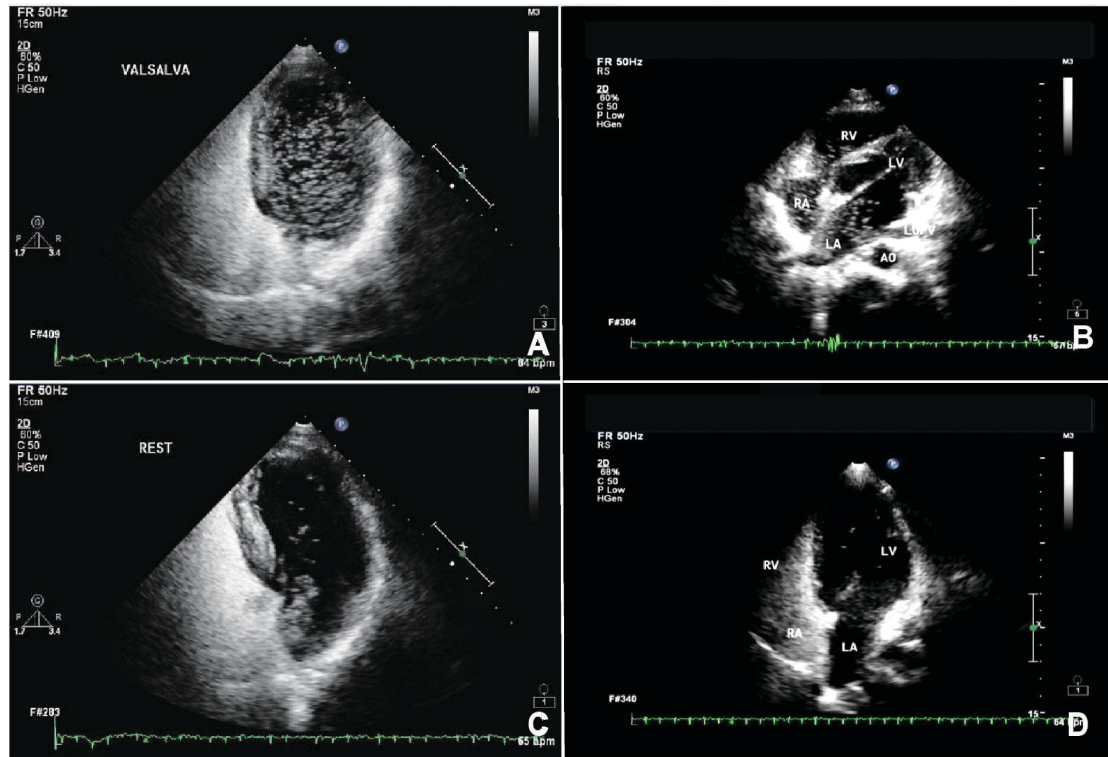


Figure 1. Representative images of RLS signal diagnosed by TTE combined with ASCE. (A) A large number of RLS signals under Valsalva action; (B) left atrial RLS signal from left superior pulmonary vein; (C) RLS signal from interatrial septum in resting state; (D) A small amount of RLS signals at rest.

3. Stroke gps have more CV risk factors suggesting alternate etiology such as atheromatous embolism also possible. If gps were larger, this might reach statistical significance. Or CV risk factors may increase the risk of DVT

Reply: We agree strongly with you. However, the patients included in this study did not contain clinical data such as atherosclerosis or DVT. In addition, we believe that serum biological parameters are also risk factors for atherosclerosis and DVT, reflecting to some extent the occurrence of cardiovascular disease in patients.

4. Almost 28 patients had pulmonary shunting. This number is rather high in this young population. This begs the question of what was the criterion for distinguishing between intracardiac or intrapulmonary shunt. Number of beats after RA opacification is not a very sensitive method.

Reply: We judged whether it was P-RLS by observing the pattern of microbubbles entering the left atrium in combination with ASCE. When microbubbles continued from the pulmonary veins into the left atrium, they were judged as P-RLS. In addition, Feng et al showed that 14 RLS were detected in 21 CS patients. Specifically, two of them had both PFO-RLS and P-RSL, nine had PFO-RL, and three had P-RLS, similar to our findings (Ref: Feng C, Luo T, Luo Y, Zhao N, Huang K, Xiao C. Contrast-enhanced transthoracic echocardiography applied in evaluation of pulmonary right-to-left shunt: A preliminary study. *Comput Med Imaging Graph.* 2018 Sep;68:55-60. doi: 10.1016/j.compmedimag.2018.04.007).

5. Again prevalence of P-RLS high in the control group as well.

Reply: Feng et al showed that 7 cases of RLS were detected in 20 healthy adult volunteers, including 3 cases with both PFO-RLS and P-RLS, 2 cases with PFO-RL, and 2 cases with P-RLS. In this study, the detection rate of P-RLS was also relatively high (Ref: Feng C, Luo T, Luo Y, Zhao N, Huang K, Xiao C. Contrast-enhanced transthoracic echocardiography applied in evaluation of pulmonary right-to-left shunt: A preliminary study. Comput Med Imaging Graph. 2018 Sep;68:55-60. doi: 10.1016/j.compmedimag.2018.04.007).

6. What is the unit of time, secs?

Reply: The unit of RLS duration time is cardiac cycles.

7. how many patients were diagnosed on TTE and how many needed a TEE?

Reply: All patients included in this study completed TTE. Of these, 32 patients required further TEE.