Author’s response to reviews

Title: An ultrasound model to calculate the brain blood outflow through collateral vessels. A pilot study

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Version: 8 Date: 3 July 2013

Author’s response to reviews: see over
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Version: 5 Date: 30 April 2013

Author's response to reviews: see over
Dear Editor,

please find attached our revised manuscript which has been amended according to the suggestions of the Reviewers. We addressed all the points raised by reviewers indicating with Q the reviewers points and with A our reply. We thank the reviewers for comments that have made a major contribution to the worth of this paper.

Thank you for your time and consideration.

Sincerely,
the authors

Reply to 1st reviewer's comments

1. Q: The authors should report the duration of the Doppler spectrum on which it was carried out the measurement of the TAV. In the artery the duration of Doppler spectrum should be at least three complete cardiac cycles, in the case of the veins over a period of at least one complete respiratory cycle.
A: This issue has been addressed in the text at the end of the paragraph “Evaluation of Doppler venous haemodynamics”, thank you.

2. Q: To perform venous flow measurements with an uniform insonation technique could be better to use a sample volume of magnitude of at least half the diameter of the vessel and positioned in the center of the vessel in order to avoid the vessel wall artifacts.
A: Thank you for the suggestion, in order to avoid vessel wall artifacts we used “assumed profile techniques” for the veins. This has been specified in paragraph “Evaluation of Doppler venous haemodynamics” as point 3), line 128.

3. Q: it is unclear which measurement of CSA was used for the calculation of venous flow, the minimum or maximum? I think that the most useful measure may be the mean value of CSA during a complete respiratory cycle. In addition, the CSA vein was measured with manual tracing or by other method? Please specify in the text.
A: We have now specified that in the text paragraph “Refinement of Doppler haemodynamics assessment”.

4. Q: Is not very clear the formula used to evaluate the uncertainty of measurement of TAV. The authors delta TAV means the difference between the measured velocity and actual one. However, in this case, the reported formula seems to be wrong, (as, in the limit of vanishing epsilon, according to the formula, delta TAV = TAV instead of 0, as expected). Instead, what can be said is that TAV_actual = TAV_measured * cos(theta)/cos(theta+epsilon).

A: We missed a -1 in equation 1. It has been now corrected. Thank you.

5. Q: The authors in the text and in the figures insert the resistances that are not calculated. This make more difficult the interpretation of the figures and that does not add anything to the results of the work. In the lumped model that authors used I suggest to remove the resistance and the corresponding boxes. The figures thus becomes more simple and easy to understand.

A: We followed the suggestion to provide a simplified version of the model which is now easy to understand (see Figure 1 and 2).

6. Q: In the section Calculated indexes the DJDI should be renamed Distal Cerebral Venous Outflow as it includes the blood flow of the vertebral veins.

A: We have modified the mentioned index by specifying the vertebral vein (see equation 10). The new index name is Distal Jugular and Vertebral Draining Index (DJVDI).

7. Q: For the calculation of the inflow would be better to use the velocity integrated in time of cardiac cycle and the velocity integrated in the respiratory cycle time for the calculation of the venous outflow.

A: This is exactly what we have done and it is now specified in the text “Off-line assessment of Doppler inflow and outflow haemodynamics” line 146.

8. Q: The calculation of cerebral collateral draining index could be better described by the formula...

A: The mentioned index (CCDI) has been redefined in terms of the collateral flows. See eq. 12.

9. Q: The references 14-15 do not correspond to the text.

A: Corrected, thank you.

10. Q: The neck veins lumped model section “and in collateral segments
C2-C3” add C1-2. Change: “and in collateral segments C1-C2 and C2-C3”
A: It has been corrected.

11. Q: In formulas the definition of parameters the subscript “s” is not define.
   A: It has been now defined at line 251.

12. Q: The total flow measured at the level of the common carotid artery both in patients than in controls, does not correspond to the sum of the flow in the internal carotid artery and external carotid artery. In addition the authors also included the flow of the vertebral arteries that should be considered separately. Can the authors discuss this topic?
   A: The carotids have been measured in the CCA segment, in the ICA segment and in the ECA segment. The flow in the CCA corresponds to the one measured directly in the CCA segment or to the sum of ICA+ECA. In order to minimize the experimental error we assumed HBinF=1/2*(CCA measured+ICAs+ECAs) +VAs. This is now explained in the text (See equation 2). Concerning the VAs we have corrected the wrong sentence.

13. Q: “In our sample the rate of HBinF drained by the IJVs is 37% in J3, 55% in J2 and more than 90% in J1, respectively, and thus suggesting a re-entry of significant volume of blood along the course of the jugular vein through the collaterals. “ This is different from the values showed in the table where in the normal subjects the trend looks completely different and the blood flow is reduced from J3 to J1 (167ml/min J1 293 ml/min J2 607 ml/mi J3).
   A: Unfortunately, the label J1 and J3 have been erroneously swapped in the text and thus leading to confusion. It has been corrected. Thank you.

14. Q: in Figures 2a and 2b the right part of the writing circuit is incorrect: QJ3 200, QJ3 410, QJ3 310 must be modified: QJ3 200, QJ2 410, QJ1 310; in the same way fig. 2b Moreover it would be better to place the Q at level of the segments and not at level of the joints of circuit.
   A: Such figure has been completely redrawn by taking into account the comments of the reviewer.
Reply to 2nd reviewer’s comments

Reviewer’s report:

1. Q: The methods applied are clearly described and the data provided could permit to replicate this work but the method applied is not so easy to understand and has to be widely validated before considering it for clinical trial.

A: We have now largely improved the description of our model and we hope it is now easier to understand. Limitations of the present study have been discussed in the text, see line 370. In addition we simplified Fig 1 to be more readable.

2. Q: The use of a constant volume sample size ensures the repeatability of the measure and so far so good but a small sample volume (adjusted for artery) does not seem ideal in a vein where the trend of the flow may not be parallel to the direction of the vessel (helical, etc.).

A: The mean velocities of the blood can be calculated according to Womersley by sampling the TAVmax with a small sample volume. The operator assessed the position in the vein where the TAV was max and then sampled the velocities. Normally the velocities was max in the center of the lumen. This has been now clarified in the text, see line 122.

3. Q: We read the letter to the editor in which the bibliographic reference but we can not understand how one can, in the proposed manner, to minimize the parameter epsilon (angle error induced by the operator).

A: The use of correct angle is crucial to assess the correct velocity of the blood as reported by several authors in our reference list. For such reason we put particular attention both to the on-line acquisition (two operator verify the angle) and to the off-line refinement. All studies have been exported as DICOM study in order to cross check the CSA and the Doppler angle.

4. Q: Furthermore, the authors provide tables and diagrams to try to help with the interpretation. However they aren’t entirely clear and the scheme which shows this new model can be improved by specifying more clarity the entity of collateral and Jugular flow, the flow direction in the distal portion of the Jugular in CCSVI patients, which collateral vessels are involved and finally why the right Jugular drains more than the left Jugular.

A: As already stated, the description of the model has been largely improved. Furthermore a new table has been added (see Table 2) and all the figures have been completely redrawn to better explain the model.
5. Q: Considering the population studied, controls were screened for CCSVI absence by means of established ECD criteria whereas cases are patients affected by CCSVI, screened by the same ECD criteria among patients affected by multiple sclerosis. The results are not totally conclusive and leave room for new interpretations. The authors provide references to other work to confirm some but not all the data obtained. However, some references are overly cited throughout the text. For example, reference number 5 is cited six times.

A: We admit that the ECD methodology adopted for subset CCSVI from non CCSVI subjects might lead to heterogeneous results. Accordingly, as suggested by this reviewer we have now updated the reference list and changed the text at the top of the discussion.

6. Q: The text is for the most part clearly written but could use some revision by someone with better English. For example:

1. "... which could potentially compress the neck veins and consequently affecting measurements."
2. "...blood can flow throw all such pathways."
3. "This quota is dramatically increased because does not include only the..."

"As an example, we apply the proposed model to compare HC subjects with a CCSVI ones having same age and gender."

In addition, abbreviations are not kept consistent throughout the manuscript. For example: CSA is defined as “cross sectional area” as well as “cross section area” EC/ECA are both used to define “external carotid artery”

A: All suggestions of the reviewer have been taken into account and text has been amended accordingly.

7. Q: The title and abstract sum up adequately the subject matter below, but the term “lumped” is not straightforward to understand.

A: The word “lumped” has been removed from both text and abstract.

8. Q: Off-line assessment of Doppler inflow and outflow haemodynamics "The acquisition phase was performed by two operators (FS and EM)” What was the inter and intra rater reproducibility?

A: This has been our mistake because only EM performed the investigation. At the time of the acquisition EM and FS agreed on the Doppler technique regarding angle, position of the SV etc. Therefore we cannot calculate inter and intra rater variance and we have now changed the text accordingly, see line 141.

9. Q: While the formulae are given, besides CFI, what these values actually represent are not clearly described in the text. The manuscript needs to be updated to better explain them.

A: As suggested, explanation of all 4 indexes has been added after the
indexes definition.

10. Q: "As shown in Table 1, this is a consistent amount of blood never measured before: up to 350 ml/min for C2-3 and more than 500 ml/min for C1-2" I am unable to follow this statement this based on the data presented in the table.

A: Unfortunately, the label J1 and J3 have been erroneously swapped in the text and thus leading to confusion. It has been corrected. Thank you.

11. Q: For DJDI/CCDI/DCVO data presented, are these the mean values for all ten controls/patients? This should be more clearly described, as well as presenting the standard deviations.

A: These are mean values for all ten controls/patients. This has been clarified in the text and SD values have been added. Also, Table 2, which contains all the index values, has been added.

12. Q: "Moreover, we estimated an uncertainty of about 5 degrees in theta when the operator places the sample volume into the J1 lumen." How was the degree of uncertainty estimated?

A: An uncertainty of 5 degrees has been estimated by asking the operator to assess recursively the correct Doppler angle and to evaluating the standard deviation of the mean. It has been now clarified in the text at line 399.

13. Q: Several of the proposed indicies and subsequent results aren’t even mentioned in the discussion! (CCDI, DJDI, DCVO). Why is this?

A: Two new paragraphs have been added in the discussion (Comparison in supine position and Comparison in upright position) and the indexes have been discussed.

14. Q: Table. "right" / "left" should be used in favor of "dx" / "sx" "supine" / "upright" should be used in favor of "clino" / "orto"

A: Ok, corrected.

15. Q: The term “mean” should be used in favor of “values”

A: The term “mean values” has been used in the table caption.

16. Q: It is not clear why the resistance boxes are not labeled in the “IJV-sx” and VV-sx” columns in Figure 1a. In Figure 1b, why are there no flow arrows in the “IJV-sx” and VV-sx” columns? Neither of these questions are explained in the manuscript nor in the text of the figures themselves.

A: The figures have been changed and corrected following the suggestion.
Reply to 3rd reviewer's comments

1. **Q:** Title: “A lumped model to calculate non invasively in clinical practice the brain outflow through collateral vessels”. “An ultrasound model to calculate the brain blood outflow through collateral vessels” would be a more adequate title.

   **A:** We changed the title according to this suggestion.

2. **Q:** “Such evaluation is linked to a condition known as chronic cerebro-spinal venous insufficiency (CCSVI), characterized by some blockages in the IJV which are bypassed by collateral circulation, in turn activated to transport blood into the caval system”. This paragraph should be deleted, because: 1. Not relevant. 2. CCSVI is not a condition recognized by the medical community. “We developed a novel lumped model to calculate the cerebral venous return, normalized to the arterial inflow, in the different segments of the IJV”. Drop the word “lumped”. “Our preliminary application of the novel lumped model in the clinical setting suggests the pivotal role of the collateral network in draining the blood into the superior vena cava under CCSVI condition”. Drop the word “lumped”.

   **A:** We deleted both the sentence about the condition known as CCSVI and the word lumped throughout the abstract. For benefit of the readership we now define CCSVI patients in the section “Methods” of the abstract.

3. **Q:** There is general agreement in considering the internal jugular veins (IJVs) as the major route of cerebral outflow in the supine position, and the vertebral veins (VVs) as the major route of brain drainage in upright [1-4]. This true for most subjects but not for everybody. So your model should not disregard that small percentage of subjects who are exceptions to this rule.

   **A:** This sentence is simply introductory and not related to the model subsequently proposed.

4. **Q:** “Total of eleven healthy volunteers were screened for CCSVI absence by means of established ECD criteria [5]. Ten out of eleven were enrolled in this phase (age ranging from 23 to 42 yo, male female ratio 6:4)”. Are you really sure that 10 subjects will suffice to build a universal model? I believe not. This is a major limitation of this study and the review process should end right here. Moreover, how did you select those 10 subjects: please detail all the inclusion and exclusion criteria. Was CCSVI the only exclusion criterium? Why was the eleventh healthy subject excluded?

   **A:** This is a preliminary study which aims to test in clinical practice the
feasibility of the model. Of course, it needs further validation and this is admitted in the discussion section (See line 510). Finally we add “pilot study” in the title to avoid any misunderstanding from the beginning. The patients underwent a CCSVI screening by US as recommended in a consensus document [5]. We admit that the ECD methodology adopted for subset CCSVI from non CCSVI subjects might led to heterogeneous results. As suggested by this reviewer we now updated the reference list and change the text at the top of the discussion as follows: “In the first part of the study we tested the model on a HC cohort based on medical history and a controversial US CCSVI screening [5] [19] [22-29]. However, a recent meta-analysis clearly shows that the majority of HC are not affected by CCSVI [30].” Finally, one out of eleven subjects who underwent US Doppler screening for CCSVI showed >2 criteria and was excluded from the controls. Now this is specified at line 80.

5. Q: “Measurements were all performed in the morning hours following recommendation to drink 500 ml after the wake, in order to have comparable conditions of hydration”. Please explain where you found that 500ml of any liquid will produce the same baseline condition of hydration. Please cite your references.

A: In order to establish the level of baseline condition of hydration, actually there are no studies which non invasively measure the central venous pressure in subjects who undergo vascular US Doppler investigation. We simply have been compliant with the recommendations published in the consensus document on CCSVI investigation by US [5]. The recommendation was intended to avoid the investigation of dehydrated subjects. Such reference has been added into the text (line 88).

6. Q: “The vertebral artery (VA) was evaluated at V2 level”. Please cite your references and explain why at this level and not at V3.

A: We chose the V2 level because it is the level easier to investigate and so more reproducible. It was recommended in the consensus document [5]. This issue has been addressed in the text at line 110.

7. Q: “in the veins a smaller sample volume of 0.5 mm has been adopted”. Sometimes, especially in the supine position, the blood rushes along the far wall of the vein. If a small sample volume is used and applied in the center of the vessel, the collected velocity data will be underestimated. Please explain how you managed to avoid this error. Also explain how you managed to collect accurate data from J1, where physiologically there is always turbulence.

A: The mean velocities of the blood can be calculated according to the Womersley theory by sampling the TAVmax. The operator assessed the position in the vein where the TAV was max and then sampled the velocity. Typically, the velocities were max in the center of the lumen. This has been now clarified in the text at line 122. Concerning the collection of accurate data from J1 we have now added a sentence in the text at line 129.
to address the turbulence issue through a detailed description of the adopted technique.

8. Q: “We carefully acquired the images and traces as above described, trying to improve as much as we could the reliability of the Doppler assessment and of the variables...”. Please explain how you improved the data collected and who made the corrections: a third author? Was he blinded?

A: This is now explained in the text at line 138

9. “The acquisition phase was performed by two operators (FS and EM)”. What was the inter-observer agreement?

A: This has been our mistake because only EM performed the investigation. At the time of the acquisition EM and FS agreed on the Doppler technique regarding angle, position of the SV etc. Therefore we cannot calculate inter and intra rate variance and we have now changed the text accordingly.

10. Q: “We tested our model on a second population represented by ten patients (age ranging from 37 to 45 yo, male to female ratio 5:5) affected by CCSVI...”. Only 10 patients were recruited to test your model? This is another major limitation of this study. How did you select those 10 patients? Please detail all the inclusion and exclusion criteria. It is obviously not enough to say they all had CCSVI.

A: As already here admitted, this is a preliminary study to test the feasibility and the clinical applicability of the proposed model. The CCSVI subjects were recruited according to the CCSVI criteria, recommended by experts from seven international scientific societies [5]. Finally the selection criteria were already discussed above (see our reply to “first phase of the study”).

11. Q: “The control subjects were successfully investigated. Measured CCAs flow was 836 167 ml/min, subdivided in 464 74 ml/min in the ICs, 226 59 ml/min in the ECs...” CCAs flow is greater than ICAs+ECAs flow: explain.

A: The carotids have been measured in the CCA segment, in the ICA segment and in the ECA segment respectively. The flow in the CCA corresponds to the one measured directly in the CCA segment or to the sum of ICA+ECA. In order to minimize the experimental error we assumed HBinF=1/2*(CCA measured+ICAs+ECAs) +VAs. This is now explained in the text (see Equation 2). Concerning the VAs we have corrected the wrong sentence.

12. Q: “In Table 1 we report CSA, major axis, TAV, and Q respectively for left and right IJV, measured in sitting; TAV increases from J3 to J1, whereas CSA and major axis are apparently constant. In our sample the rate of HBinF drained by the IJVs is 26% in J3, 33% in J2 and more than 90% in J1, respectively, and thus suggesting, also in upright, a re-entry of significant volume of blood along the course of the jugular vein
through the collaterals". Explain how it is possible that more than 90% of HBinF is drained by the IJVs in upright posture? Aren’t the VVs the main draining route in this position?

A: This issue has been addressed in the text by adding a sentence after the cited sentence (Line 285).

13. Q: By turning the subjects in sitting posture, we did not find out significant differences between patients and controls. ? Explain these results.

A: Our statement is particularly referred to the controls’ flow where we did not find significant differences by comparing upright with supine values; the major limitation is linked with the small sample and the big sd. We need more cases to draw definitive conclusions. On the contrary, by turning the CCSVI patients from supine to upright there is a significant difference, as previously assessed by Doepp [19] and Monti [23] with results comparable to ours. Now this is better specified at line 365.
1. Q: It is not clear how healthy subjects and patients were selected to limit biases
   A: The patients underwent a CCSVI screening by US as recommended in a consensus document [5]. We admit that the ECD methodology adopted for subset CCSVI from non CCSVI subjects might lead to heterogeneous results. To deal with this issue we updated the reference list and changed the text at the top of the discussion as follows: “In the first part of the study we tested the model on a HC cohort based on medical history and a controversial US CCSVI screening [5] [19] [22-29]. However, a recent meta-analysis clearly shows that the majority of HC are not affected by CCSVI [30].”

2. Q: I think that the small sample size should not allow to make any valuable conclusion nor for healthy subjects and for patients, i.e. it is not possible to extend the findings from ten subjects to the entire burden of healthy people or diseased one. It is likely that healthy people can show a significant activation of the collateral outflow routes if the sample number increases and also diseased people could become a more heterogeneous group concerning the brain venous outflow
   A: Please, see the answer above for the first part of the question. Furthermore, this is a preliminary study which aims to test in clinical practice the feasibility of the model. Of course, it needs further validation and this is admitted in the discussion section (line 454). Finally, we added “pilot study” in the title in order to clarify from the beginning our aims.

3. Q: The p values of some measured and calculated parameters are very impressive (p < 0.000002) and it is hard to accept it in a such small cohort of subjects; moreover figures and tables show only CSA, TAV and in-outflow values, and therefore it is not easy to follow the reasoning of the authors step by step without reproducible analysis
   A: The mentioned p-value corresponds to the CFI index that can be calculated following the text (see equation 11). You are right that we just reported the mean values and not the measured values for each healthy control and patient but you can understand our results by verifying in Table 2 that CFI for health controls is separated by two standard deviations from the CFI of the MS patients. To make this point clear we have added a pertinent sentence at line 361.

4. Q: Maybe a validation of the proposed model in a greater cohort of healthy subject would be desirable before to test the model in other situations and the use of another technique besides ultrasound technique to compare data and show the effective collateral activation
would add methodological value to this work, whose founding idea is
good and interesting.

A: As stated above, the aim of the present paper is only to test
clinical applicability of the model. Besides, in the discussion section
we admit the general limitation of our study and propose further
perspectives, for example, that our model should be tested in a large
multicentric blinded trial (see line 454).

5. Q: IJV branches are always reported in the paper as re-entry channels,
but they drains face and neck and also they may represent a component
of a complex collateral network for brain drainage, therefore this last
role is only one of the possibilities.

A: Our model permits to calculate both the re-entry of collateral
channels and veins independently, which both drain into the superior
caval system. In particular, our manuscript has been largely improved
thanks to the many comments of the reviewers, by discussing in detail
the network of the neck and relative flows (see paragraph “Model of
neck veins”).

6. Q: Re-entry may be a misleading term, because it implies that the blood
not only skips an IJV segment, but it can also flows more than one time
in a local re-flow circuit, making very hard to assess the real flow
volume, according to this hypothesis

A: We used the term re-entry to indicate when a collateral channel is
tributary of the major truncal pathway. Now this is clarified in the
text at line (63) in order to avoid any misunderstanding.

7. Q: The use of a fixed sample volume for TAV measurements on the venous
side (for veins of different size) is associated to a consistent risk
of missing low flow velocities, that more likely occur at the edge of
the central flow lamina, and sometimes single flow laminae with
inverted flow direction, making not assessable the reliability of TAV
measurement in this context (Major Compulsory Revisions)

A: The mean velocities of the blood can be calculated according to the
Womersley theory by sampling the TAVmax. The operator assessed the
position in the vein where the TAV was max and then sampled the
velocity. Typically, the velocities were max in the center of the
lumen. This has been now clarified in the text at line 122.

8. Q: It is not clear if IJV CSA measurement has been performed by
manually tracking the boundaries of the lumen or by using an automatic
adjustable elliptic shape

A: CSA measurement has been performed by manually tracking the
boundaries of the lumen. It is now clarified in the text at line 119.

9. Q: Where VV outflow was calculated? V2 or V1 segment, referring to the
correspondent artery segment?
We chose the V2 level because it is the level easier to investigate and so more reproducible. It was recommended in the consensus document [5]. This issue has been addressed in the text at line 110.

10. Q: It is not clear if and how much the CSA postprocessing refinement affect the final measurement: in all subjects? Only in subjects with a great breath variability?

A: This issue has been now better addressed and details are shown at line 153.

11. Q: The off line calculation of the insonation angle is not possible in the ultrasound platform, therefore it can be supposed that DICOM images were analyzed and measured with a DICOM viewer and TAV measurements without angle correction were used for the application of the formula. Is it correct?

A: Please, see answer below.

12. Q: The paper does not report the measure of angle correction and of the epsilon factor, although a great attention was paid to the relevant question of angle (Major Compulsory Revisions)

A: The use of the correct angle is crucial to assess the proper velocity of the blood as reported by several authors in our reference list. For such reason we put particular attention both to the on-line acquisition (two operators verify the angle) and to the off-line refinement. All studies have been exported as DICOM study in order to cross check the CSA and the Doppler angle. The text has been changed at line 157 to emphasize the need to verify the Doppler angle.

13. Q: Measurements in J1 IJV segment are not easy and probably are not reliable (as the authors wrote), therefore it is not possible to build on them the interpretation of the findings (Major Compulsory Revisions)

A: In J1 there are more turbulences and it is more difficult to properly place Doppler sample into the lumen with the correct angle. Specifically, we have now added one more sentence in the text to make this issue clearer (line 389).
Reply to 5th reviewer’s comments

1. Q: There is currently much controversy regarding the ultrasound criteria used for diagnosis of CCSVI and its potential association with Multiple Sclerosis. Therefore the authors should have described collateral outflow of cervical veins in patients with angiographically confirmed IJV thrombosis or stenosis. There have been previous reports indicating that ultrasound diagnosis of CCSVI does not correspond to abnormal cervical vein anatomy on DSA.

   A: The patients underwent a CCSVI screening by US as recommended in a consensus document [5]. Anyway, we admit that the ECD methodology adopted for subset CCSVI from non CCSVI subjects might lead to heterogeneous results. Accordingly, as suggested by this reviewer we have now updated the reference list and changed the text at the top of the discussion.

2. Q: The authors do not report the inter-rater and intra-rater reliability of outflow measurements using their model

   A: This has been our mistake because only EM performed the investigation. At the time of the acquisition EM and FS agreed on the Doppler technique regarding angle, position of the SV etc. Therefore we can not calculate inter and intra rate variance and we have now changed the text accordingly.

3. Q: Sample size was limited to ten healthy controls and ten patients with CCSVI

   A: This is a preliminary study which aims to test in clinical practice the feasibility of the model. Of course, it needs further validation and this is admitted in the conclusion section (line 454). Finally, we added “pilot study” in the title in order to clarify from the beginning our aims.

4. Q: Statistical comparisons were not adjusted for demographics and vascular risk factors

   A: This analysis is beyond the aim of the present study where we tested the feasibility of the proposed model in a limit sample size. This is now clarified at lines 404.

5. Q: The Discussion is speculative and does not highlight the potential clinical implications of this study.

   A: In the conclusion section we now highlight the potential clinical implications of the present pilot study.

6. Q: The authors do not cite recent studies rejecting CCSVI hypothesis in their Discussion and in particular MRI studies indicating that there is no
impaired cerebral venous outflow in patients with MS

**A:** Now we have updated our reference list with studies rejecting CCSVI condition by means of US screening. As correctly suggested by this reviewer we also add MRI studies confirming and rejecting CCSVI hypothesis (line 370).