Author’s response to reviews

Title: Changes of liver enzymes and bilirubin during ischemic stroke. Mechanisms and possible significance

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Author’s response to reviews:

Reply to first Reviewer

1. “All of your data are based on simple correlations and regression models. I would therefore hesitate to conclude figure 1 is valid – it is very speculative at best. I believe it would be better to delete this and drop most of the speculation as to pathways – the main conclusion being that GOT is independent of CRP, and not a lot of other conclusions can be drawn”.

Simple correlations are shown only in Table 3, while the new Tables 4 and 5, and the Figure, show multivariate associations. Figure 1 is not speculative. In fact it is closely adherent to the data, and is exactly what the legend and the text say: a graphical representation of the independent associations reported in Tables 4 and 5. To avoid any doubt, the legend has been made even more explicit: “Schematic representation of the independent associations with infarct volume reported in Table 4 and of the independent associations among the study variables reported in Table 5”.

We have eliminated the previous Table 5, as requested by the Reviewer, but we would keep at least the Figure, which in our opinion is useful in helping the reader to understand the complex interrelations among the variables (which are instead more difficult to appreciate looking at the numbers reported in the Tables). However, since the second Reviewer criticized the fact that only the correlations with # >=0.3 were represented, we have modified the Figure, which now shows all the associations (strong and weak) among the variables. We agree that he main conclusion of this study is that GOT is the only liver enzyme
directly associated with the ischemic cerebral lesion independently from inflammation: these are the exact words reported in the last paragraph of the Discussion. A sentence in the Results that proposed a pathway too explicitly (“In addition, inflammation favours the progressive decrease in red blood cells and haemoglobin, with consequent decrease in indirect bilirubin”) has been modified as follows (end of page 6): “In addition, CRP is inversely associated with hemoglobin, which progressively decreases together with red blood cells and unconjugated bilirubin (see also Table 2”).

2. “You have chosen by and large to present “all data available” rather than to exclude all subjects with missing data, which would be the more accepted epidemiological practice. Given that this would lead to large amounts of missing data, I would suggest that you perform a sensitivity analysis to test whether you can state ‘when analyses were restricted to those with complete data, the overall conclusions were unchanged’ or similar.”

We used all data available when we had to analyze one variable at a time (temporal course, Table 2, and simple correlations with infarct volume, Table 3). However, in the more relevant multivariate analyses we have excluded all patients with missing data (Independent associations with infarct volume and among variables, Tables 4 and 5). Since also the second Reviewer suggested verifying whether there were differences between patients with all data and patients with missing data, we compared these two subgroups in relation to the general characteristics reported in Table 1 (page 6). We found no significant differences for most of the variables considered, including age, infarct volume and NIHSS score. The two groups differed only for sex (more males among the patients with missing data) and for the prevalence of previous TIAAs (no TIAAs in the patients with missing data).

3. “Table 3, rather than being based on simple correlations, would be better presented using linear regression, and also including a more complete adjustment model (as a separate model) adjusting for patient demographics and clinical characteristics, as well as all the biomarkers adjusted for each other. This would tell us whether the associations you observe are truly independent. For instance on page 6 you have not included WBC in the multivariable model. Using a systematic approach avoids the question as to what should be included”.

On previous page 6 the variables included in the multivariate model of the associations with infarct volume were “the log-transformed values of GOT, GPT, #GT, leukocytes and CRP”. Thus, WBC (leukocytes) were actually included in the multivariate model. However, the new Table 4 concerns a more complete adjustment model, including age, sex and NIHSS score, in addition to all the 9 variables listed in Table 3. The table shows the final result of the multiple linear regression, after backward elimination of non-significant associations, together with beta coefficients, standard errors and intercept.

4. “I struggle to believe that table 5 really tells us much, as the cutoffs used to determine rising or non-rising GOT is really completely arbitrary, and excludes an arbitrary number of patients from the analysis. I would delete this, or at the very
least make it more systematic, e.g. compare the upper half with the lower half of changes in GOT”.

Previous Table 5 has been deleted (together with 13 lines of text in the Results), as requested.

5. “Please state the power calculation used in study design, and how the figure of 180 patients was arrived at. Was the study designed specifically to address the present question?”

The figure of 180 patients was the result of multiple exclusion criteria, starting from 313 patients, as described in detail in the “Patients” subheading. Power calculation is now reported under the same subheading (page 4): “In the ADIS Study [11], in 31 patients with ischemic stroke a mean increment of 5 ± 8 U of both GOT and #GT was obtained after 7 days from admission (P=0.001). Hypothesizing, for the present study, a mean increment of only 3 ± 8 U with a P value of 0.01, 110 patients would be needed to get a power of 90% (a size well below the number of our patients, even considering missing data)”.

6. “Table 4 needs all data included, not just data you feel warrants presentation”.

Previous Table 4 (present Table 5) does not report the data that we feel warrants presentation. It is a matrix of significant multivariate associations, and all non-significant associations cannot be shown. As reported in both text and footnote, non-significant associations were progressively removed from the models with a backward elimination procedure. The numbers shown (standardized beta coefficients) are the final result of these procedures: there is no way to fill the empty cells.

7. “Table 2 has some data presented as non-parametric, and in table 3 it is presented as parametric, please check normality assumptions and make presentation consistent”.

Now Table 2 reports erythrocyte, haemoglobin and platelet values as mean and SD, in accordance with their presentation in Table 3.

8. “In the abstract ‘The correlation of IV with GOT increased progressively from admission to day 14’. You don’t show this. Please delete”.

Probably the Reviewer did not see this paragraph in the Results (now on page 7): “The correlation between the logarithm of infarct volume and the logarithm of GOT was not present on admission to the Emergency Department (median delay from onset of symptoms 1.7 hours; r = 0.13; P = 0.11; N = 158), it was first detectable on admission to Stroke Unit (median delay 16.8 hours; r = 0.24; P = 0.002; N = 172), it increased on the 7th day (r = 0.35; P < 0.001; N = 137), and was maximum on the 14th day (r = 0.50; P < 0.001; N = 95).”

9. “In the abstract conclusion – I don’t think the ‘(toxic glutamate?)’ adds anything”
The phrase “(toxic glutamate?)” has been removed.

10. “Please state the software used for stats”

The software used for the statistical analyses is now specified at the end of the Methods section: “The analyses were performed using SYSTAT 10 (SPSS Inc, Chicago, IL, USA)”.

11. “In the limitations section discuss the use of IV as an outcome rather than a more clinical endpoint such as Rankin score, or death/disability”.

Our goal was not to predict stroke prognosis from hepatic markers, but to find possible explanations for their changes during acute stroke. This is clearly stated in the last paragraph of the Introduction. There was no reason to associate liver enzymes and bilirubin with the subsequent clinical outcome, since this could not be the cause of their changes. From this point of view the use of IV is certainly more reasonable, and we do not think it is a limitation.

Reply to second Reviewer

1. “One major problem would be the clinical relevance of all the changes described. Although most of the markers are significantly increased/decreased, changes are very mild and probably for most of them, concentrations remain within normal lab ranges, so the biological effect is not very clear to me. Probably the info provided would be much more clear to the reader if it was represented in graphs and the values for the normal range or data from controls were provided”.

We agree that the changes found, although quite significant, were rather small. This was even anticipated in the first sentence of the Abstract and in the Introduction, reporting the results of the ADIS study. For most hepatic markers the cause of these changes seems to be the acute phase, which in turn is proportional to infarct volume. The biological significance of such changes (i.e. their function in the acute phase) is largely unknown. In addition, the association of GOT levels to infarct volume seems to be independent from inflammation (although also CRP, like infarct volume, may influence GOT levels). In this case, not only the function of GOT elevation, but also the mechanism connecting infarct volume with GOT levels, is unknown. This is clearly stated at the beginning of the Discussion, in the Conclusion and all over the text.

We have added normal values to Table 2, as suggested by the Reviewer. In this way we could notice that some variables were high (in more than a quarter of cases) already on admission to Stroke Unit. A comment to this fact has been added to the Results (end of page 5), and in the Limitations section.

2. “Although both CRP and GOT were associated with infarct volume, this multivariate analysis was not controlled for other clinical determinants of outcome such as age or baseline stroke severity (NIHSS scores). I would strongly suggest to include them in the predictive model of infarct volume.”

The new Table 4 now shows the multivariate analysis of the factors associated
with infarct volume, including age, sex and baseline NIHSS score as adjustment factors.

3. “Table 4 shows how CRP and GOT are indeed related to each other (beta coefficient 0.18), so probably their interaction should also be considered in the multivariate analysis.”

We found that GOT levels are influenced by infarct volume independently of CRP. However, GOT levels may also be affected by CRP, as the previous Table 4 (now Table 5) showed. To solve the problem of a possible interaction of CRP in the relationship of infarct volume with GOT levels, we have analyzed another model, including an interaction term of infarct volume X CRP. The following text has been added to the Results (end of page 6): “GOT levels are independently influenced by cerebral infarct volume, but are also associated with CRP levels (#=0.18). It is therefore possible that the effect of a variable on GOT levels may vary in relation to the variations of the other variable. To test this hypothesis we constructed a linear regression model including log (GOT) as dependent variable, and log(infarct volume), log(CRP) and log(infarct volume) X log (CRP) (interaction term) as independent variables. In this analysis only log(infarct volume) remained associated with log(GOT) (#=0.26, P=0.007), while both log(CRP) (#=-0.011, P=0.92) and the interaction term (#=0.112, P=0.25) were not significant, so that the hypothesis of an interaction between the 2 variables (infarct volume and CRP) on GOT levels was rejected.

Thus, the association between GOT and infarct volume seems to be independent from inflammation, and appears of particular interest.”

4. “Other major problem would be that missing data was frequent for liver enzymes, bilirubin, blood counts and CRP determinations on admission or seventh day (about 40 cases lost out of 180). Authors should clarify whether baseline characteristics between those patients with available data and those with missing data were similar. One could think that only more severe strokes (except those who died before the 7th day) remained at the hospital by that time (and therefore they had a blood sample available) whereas mild strokes were discharged earlier and did not”.

We have compared the general characteristics of the patients with missing data with those of the patients without missing data, and found only 2 differences. In particular, stroke severity (NIHSS score and OCSP classification) did not differ. We have added this new text to the Results, on page 6: “Seventy patients were not included in this analysis as they were lacking of some of the above 9 variables. Thus, we compared the general characteristics (see Table 1) of these 70 patients with those of the 110 patients that had all the data. There were no significant differences for most of the variables considered, including, in particular, age, infarct volume and NIHSS score. The two groups differed only for sex (57.1% males among the patients with missing data, vs. 40.9% among the other patients, P=0.03), and for the prevalence of previous TIA (respectively, 0 vs. 8.2%, P=0.01).”
5. “Figure 1. It is not clear to me why only associations with a beta coefficient higher than 0.3 were chosen to hypothesize the relationship between variables.”

The Figure has been modified, and now it shows all the associations (strong and weak) reported in Tables 4 and 5.

6. “Main results are based on data on admission and the 7th day, whereas levels on the emergency department, 3rd day and 14th day are only mentioned on specific parts of the text or just omitted.”

The main results are based on data obtained on admission and on the 7th day for the reasons reported at the beginning of page 6: “The mean or median values on the 3rd and 14th day are not reported since none of them differed significantly from the mean or median values obtained, respectively, on admission and on the 7th day. Thus, during the second week all variables displayed a “plateau” course. Moreover, due to the random presence of missing values, the addition of these data to the Table would have caused a further reduction in sample size.” On admission to the Emergency Department only the transaminases (GOT and GPT) were measured (see Methods, page 4).

7. “Also it is not clear why the third day determinations were not used, since it would make more sense to correlate liver changes with infarct volume at the same time, too.”

The correlations of hepatic markers with infarct volume were much stronger on the 7th day than on admission (see Table 3), and the values on the 3rd day were similar to those obtained on admission (see previous point). Thus, we chose to use the 7th day values in the multivariate analysis of the associations with infarct volume.

8. “The description of the study variables, in particular the timing for liver enzymes, etc. determinations and their delay relative to the stroke onset is confusing. Please clarify.”

As stated at the beginning of the Methods section of the Abstract, and at the beginning of the Study variables subsection of the text, serial determinations were performed on days 0, 3, 7 and 14 after the admission to Stroke Unit. The delays of admission to Stroke Unit and to the Emergency Dept. relative to the stroke onset are reported in the 4th and 5th line of Table 1. To make this more clear, we have added these words to the footnote of Table 1 “Delays are referred to stroke onset”. Moreover, the first sentence of the Study variables subsection has been so modified: “On admission to Stroke Unit (median delay from stroke onset 16.8 hours, see Table 1), and after 7 days ...”. And also the second sentence of the same subsection has been modified: “Finally, the transaminases (GOT and GPT) were also measured on admission to the Emergency Department (median delay from stroke onset 1.7 hours, see Table 1).”

9. “Also, it is not clear to me which infarct volume was chosen (baseline?, third day?) to calculate correlations with admission and 7th days markers.”
We did not measure the baseline infarct volume. Infarct volume was calculated only on the third day, as stated both in the Abstract (last line of Methods subsection: “… and on day 3 cerebral infarct volume was calculated from CT scan slices”), and in the text at the end of the Study variables subsection (“…while on day 3 a second brain CT scan … was performed to precisely define the site and volume of cerebral infarct.”). However, to make the concept more clear, we have added these words to the last paragraph of the Study variables subsection: “For each patient the infarct volume, expressed in ml, was obtained by one of the authors (A.C.) from the 3rd day scan measuring the lesion area…”

10. “Conclusions on other signals different from inflammation leading to the increase in GOT levels might be too speculative, since Table 4 shows indeed that GOT was partly explained by CRP (beta=0.18). I strongly recommend to revise conclusions in the abstract and main text.”

GOT levels are influenced by infarct volume independently from CRP (see new Table 4, and point 3 of this reply), although GOT levels may also be influenced by CRP. Since, according to our data, this is certainly true, we believe that some other mechanism, different from inflammation, should explain the association of infarct volume with GOT levels. This conclusion seems to be reasonable, as also the first and third reviewer believe.

Third Reviewer’s report

“The authors sought to investigate the significance of changes of liver enzymes and bilirubin often observed in the acute phase after ischemic stroke. … The authors postulate inflammation as a cause of elevated CRP correlative with IV and attribute increased GOT to an inflammation-independent cause, possibly in response to neurotoxic levels of glutamate seen after ischemic infarction. … I am now happy that this is worthy of publication and would contribute significantly to the understanding of ischemic stroke sequelae.”