PEER REVIEW HISTORY

BMJ Open publishes all reviews undertaken for accepted manuscripts. Reviewers are asked to complete a checklist review form (see an example) and are provided with free text boxes to elaborate on their assessment. These free text comments are reproduced below.

This paper was submitted to the JNNP but declined for publication following peer review. The authors addressed the reviewers' comments and submitted the revised paper to BMJ Open. The paper was subsequently accepted for publication at BMJ Open.

ARTICLE DETAILS

TITLE (PROVISIONAL)	Predictive role of C-reactive protein in stroke recurrence after cardioembolic stroke: the Fukuoka Stroke Registry
AUTHORS	Kuwashiro, Takahiro; Sugimori, Hiroshi; Ago, Tetsuro; Kuroda, Junya; Kamouchi, Masahiro; Kitazono, Takanari

VERSION 1 - REVIEW

REVIEWER	Di Napoli , Mario San Camillo de Lellis General Hospital, Rieti, Italy, Neurological Service
REVIEW RETURNED	25-Apr-2013

GENERAL COMMENTS	I have the pleasure to read this interesting manuscript on the role of CRP in predicting recurrence after cardioembolic stroke. It is a confirmatory study in Japanese population. The study is well planned, and the results are well and clearly reported. However, there are several points that deserve attention by the Authors. 1. The patients were enrolled within 7 days of onset. This is a quite large window to assess the CRP values considering its time course after an acute insult. From previous studies the prognostic value of CRP after ischemic stroke is time dependent, the readers should know when the CRP was assayed after stroke onset. Furthermore, CRP is an acute phase reactant that is fairly non-specific, in that, it is modulated in acute and chronic inflammatory conditions (bacterial, viral, or fungal infections) as well as rheumatologic and malignancies. Therefore, there are a number of confounders that have not been accounted for in the study and determining CRP values considering a so long time window for assaying. For example, infections such as pneumonia that occur frequently in this subset of patients (aspiration at the time of ictus) could account for elevated CRP in many cases. Other confounders include urinary tract infections and deep venous thrombosis. Thus could elevations in CRP simply be an epiphenomenon and have little to do stroke recurrence? Please clarify. 2. CRP concentration is reported using mg/dl, the international units are mg/l. At the same time, it is reported as mean value. CRP concentration is usually non normally distributed. Did the Authors tested for normality in reporting the values of their continue variables? 3. The multivariable association between CRP concentration is quite weak. Did the Authors tested the goodness of their model (calibration, discrimination, overfitting, etc)? 4. Because the time in range during anticoagulant therapy is the major determinant of recurrence after embolic stroke, unfortunately,

	this variable is not considered in their model, making it problematic. It could be an important selection bias. I think without knowing this aspect any conclusion of this study is misleading. At the same time, it is important to know if there is an interaction between CRP and AOT. This limitation should be added in discussion together with the other included by the Authors. 4. Interventions against elevated CRP have not been shown to be beneficial in acute stroke, but the present paper suggested that CRP is a predictor of recurrence after cardioembolic stroke. Therefore, it is very important for the authors to show whether CRP can improve predictivity of recurrence using c-statistics, NRI and/or RIDI.

REVIEWER	Tuttolomondo, Bruno
REVIEW RETURNED	30-Apr-2013

GENERAL COMMENTS	The article by Kuwashiro et al. is an interesting article and altough not original has the value of a large sample I believe that is article is important in its field but I have some minor concerns: 1) Lip G et al demonstred that a subclinical inflammation is present in atrial fibrillation patients and owing to the fact that patients with cardioembolic stroke are mostly suffering from atrial fibrillation I should appreciate that this issue will be underlyned on discussion section 2) Please add on Introduction a reference about the higher degree of immunoinflammatory activation of the acute phase of stroke in patients with cardioembolic stroke and about this issue I should like that authors add these two references (Licata G, Tuttolomondo A, Di Raimondo D, Corrao S, Di Sciacca R, Pinto A. ; Immuno-inflammatory activation in acute cardio-embolic strokes in comparison with other subtypes of ischaemic strokeThromb Haemost. 2009 May;101(5):929-37; Tuttolomondo A, Di Sciacca R, Di Raimondo D, Serio A, D'Aguanno G, La Placa S, Pecoraro R, Arnao V, Marino L, Monaco S, Natalè E, Licata G, Pinto A Plasma levels of inflammatory and thrombotic/fibrinolytic markers in acute ischemic strokes: relationship with TOAST subtype, outcome and infarct site, Neuroimmunol. 2009 Oct 30;215(1-2):84-9)Methods: We enrolled 2084 consecutive ischemic stroke patients who 3) some previous articles reported that immunoinflammatory activation has a different degree in relation of TOAST subtype of stroke, on this basis it appears clearly interesting to add some information about subtype of stroke prevalence in incident strokes at follow-up- Are incident strokes all of cardioembolic subtype? or not. Please add this information and a brief comment about on discussion section

VERSION 1 – AUTHOR RESPONSE

Reply to the Reviewer 1

Thank you for your comments. We have tried to respond to your comments as much as possible. We have highlighted the points of correction using bold color letters in the revised text. We hope that our response meets your requests.

Comment 1
The patients were enrolled within 7 days of onset. This is a quite large window to assess the CRP values considering its time course after an acute insult. From previous studies the prognostic value of CRP after ischemic stroke is time dependent, the readers should know when the CRP was assayed after stroke onset. Furthermore, CRP is an acute phase reactant that is fairly non-specific, in that, it is modulated in acute and chronic inflammatory conditions (bacterial, viral, or fungal infections) as well as rheumatologic and malignancies. Therefore, there are a number of confounders that have not been accounted for in the study and determining CRP values considering a so long time window for assaying. For example, infections such as pneumonia that occur frequently in this subset of patients (aspiration at the time of ictus) could account for elevated CRP in many cases. Other confounders include urinary tract infections and deep venous thrombosis. Thus could elevations in CRP simply be an epiphenomenon and have little to do stroke recurrence? Please clarify.

Our response
As reviewer pointed out, the timing of blood sampling was important for CRP. In the present study, we obtained a CRP level within 24 hours after admission in all enrolled patients. When we investigated the interval time from stroke onset to admission, it was found the time of 0.59 ± 1.08 days. We think that we could collect blood samples during an acute phase. We have added the following sentence in the third paragraph of METHODS (page 12); “We collected blood samples within 24 hours after admission.” Further, we assessed the inflammatory conditions, pneumonia and urinary tract infections. We have added the following sentence in the third paragraph of METHODS (page 13); “Moreover, we investigated the frequency of infections such as pneumonia and urinary tract infections in acute phase.” Therefore, we conducted the univariate and multivariate Cox proportional regression analysis using those confounders. Then, we found the same results with adjustments for pneumonia and urinary tract infections. We have added the results of univariate Cox regression analysis for pneumonia and urinary tract infections on Table 1 (page 34), and the following sentence below Table 3 (page 37); “*: $p < 0.05$ by multivariate Cox regression analysis using sex, age, pneumonia and urinary tract infections as well as the clinical characteristics which showed a significant ($p < 0.05$) or marginally significant ($0.05 \leq p < 0.1$) correlation with stroke recurrence in the univariate analyses.” However, we could not exclude the possibility that other factors (e.g., rheumatologic, malignancies and deep venous thrombosis) influenced the value of CRP. Further, we have added the following sentence in the seventh paragraph of DISCUSSION (page 21); “Then, we cannot completely exclude the possibility that CRP values are affected by several factors (e.g., rheumatologic, malignancies and deep venous thrombosis) even if it was collected blood samples during an acute phase.”

Comment 2
CRP concentration is reported using mg/dl, the international units are mg/l. At the same time, it is reported as mean value. CRP concentration is usually non normally distributed. Did the Authors tested for normality in reporting the values of their continue variables?

Our response
Thank you for your valuable comments. We have changed the methods of the presentation for CRP concentration. We expressed the CRP value with international units, mg/l, and median and

interquartile range in the present study. In the text, we have revised all appropriate points for CRP indication. Then, analyzing it again, we have shown the results.

Comment 3
The multivariable association between CRP concentration is quite weak. Did the Authors tested the goodness of their model (calibration, discrimination, overfitting, etc)?

Our response
Thank you for your valuable comments. We performed multivariate Cox regression analyses for stroke recurrence by other models, and found the corresponding results.
Adjustment with 1) age and gender, 2) age, gender, hypertension, dyslipidemia, diabetes mellitus, and atrial fibrillation, 3) age, gender, hypertension, dyslipidemia, diabetes mellitus, atrial fibrillation, smoking habit, and alcohol consumption, 4) age, gender, hypertension, dyslipidemia, diabetes mellitus, atrial fibrillation, smoking habit, alcohol consumption, pneumonia, and urinary tract infections, were conducted. In every model, we have shown that age and C-reactive protein were independent risk factors for recurrence in the first year after onset

Comment 4
Because the time in range during anticoagulant therapy is the major determinant of recurrence after embolic stroke, unfortunately, this variable is not considered in their model, making it problematic. It could be an important selection bias. I think without knowing this aspect any conclusion of this study is misleading. At the same time, it is important to know if there is an interaction between CRP and AOT. This limitation should be added in discussion together with the other included by the Authors.

Our response
We are sorry, we did not have those data about the international normalized ratio (INR) of prothrombin time at the time of stroke recurrence. Because our study was derived from the multicenter prospective data base, we were not able to collect additional information for the INR at the recurrence. In the present study, we investigated the predictor about the stroke recurrence, using factors available at the first onset. As reviewer mentioned comments, INR at the time of recurrence was important factor for stroke onset. Then we will add INR at the recurrence into analysis in a future study, and reexamine the risk factors. We have added the following sentence in the seventh paragraph of DISCUSSION (page 21); “In particular, effectiveness of the anticoagulant treatment was not examined at the time of recurrence.”

Comment 5
Interventions against elevated CRP have not been shown to be beneficial in acute stroke, but the present paper suggested that CRP is a predictor of recurrence after cardioembolic stroke. Therefore, it is very important for the authors to show whether CRP can improve predictivity of recurrence using c-statistics, NRI and/or RIDI.

Our response
Thank you for your suggestion and valuable comments. We analyzed it using c-statistics, net reclassification improvement (NRI) and integrated discrimination improvement (IDI).
We calculated the area under the curve (AUC) of the recurrent factor of age, and found those results; AUC 0.6154 (95%CI 0.5343 - 0.6966), sensitivity 0.6938776, specificity 0.5597826, positive predictive value 0.1734694, negative predictive value 0.9321267. Then, we calculated the AUC of the recurrent factors of age and CRP, and found those results; AUC 0.6390 (95%CI 0.5584 - 0.7195), sensitivity 0.6530612, specificity 0.5733696, positive predictive value 0.1693122, negative predictive value

0.9254386. Although there was not the statistical different, the model of age and CRP had higher AUC than it of age only. It seems that the combination of age and CRP had better predictive role than age only.
Further, we reexamined the results using the methods of NRI and IDI. These results were shown in reclassification table.

Reclassification table

Outcome: absent
Updated Model
Initial Model [0,0.0908) [0.0908,0.116) [0.116,0.143) [0.143,1] % reclassified
[0,0.0908) 86 1 0 3 4
[0.0908,0.116) 35 57 7 3 44
[0.116,0.143) 0 35 38 11 55
[0.143,1] 0 0 24 68 26

Outcome: present
Updated Model
Initial Model [0,0.0908) [0.0908,0.116) [0.116,0.143) [0.143,1] % reclassified
[0,0.0908) 6 0 0 1 14
[0.0908,0.116) 1 4 0 1 33
[0.116,0.143) 0 7 10 2 47
[0.143,1] 0 0 3 14 18

Combined Data
Updated Model
Initial Model [0,0.0908) [0.0908,0.116) [0.116,0.143) [0.143,1] % reclassified
[0,0.0908) 92 1 0 4 5
[0.0908,0.116) 36 61 7 4 44
[0.116,0.143) 0 42 48 13 53
[0.143,1] 0 0 27 82 25

NRI (Continuous) [95% CI]: 0.1719 [-0.0875 - 0.4313] ; p-value: 0.1939
IDI [95% CI]: 0.0122 [-0.0049 - 0.0293] ; p-value: 0.16349

Then, we have added the following sentence in the seventh paragraph of DISCUSSION (page 21); “In the present study, the sample size was relatively small and the statistical power may be insufficient to draw conclusions.”

Reply to the Reviewer 2

Thank you for your comments. We have tried to respond to your comments as much as possible. We have highlighted the points of correction using bold color letters in the revised text. We hope that our response meets your requests.

Comment 1
Lip G et al demonstred that a subclinical inflammation is present in atrial fibrillation patients and owing to the fact that patients with cardioembolic stroke are mostly suffering from atrial fibrillation I should appreciate that this issue will be underlyned on discussion section.

Our response
Thank you for your valuable comments. According to your suggestion, we have cited the paper as reference [32] and discussed it in the fourth paragraph of DISCUSSION (page 19); “In the study of 880 subjects with atrial fibrillation, CRP was positively correlated to stroke risk and related to stroke prognosis.[32]”

Comment 2
Please add on Introduction a reference about the higher degree of immunoinflammatory activation of the acute phase of stroke in patients with cardioembolic stroke and about this issue I should like that authors add these two references (Licata G, Tuttolomondo A, Di Raimondo D, Corrao S, Di Sciacca R, Pinto A.; Immuno-inflammatory activation in acute cardio-embolic strokes in comparison with other subtypes of ischaemic strokeThromb Haemost. 2009 May;101(5):929-37; Tuttolomondo A, Di Sciacca R, Di Raimondo D, Serio A, D'Aguanno G, La Placa S, Pecoraro R, Arnao V, Marino L, Monaco S, Natalè E, Licata G, Pinto A Plasma levels of inflammatory and thrombotic/fibrinolytic markers in acute ischemic strokes: relationship with TOAST subtype, outcome and infarct site, Neuroimmunol. 2009 Oct 30;215(1-2):84-9)

Our response
Thank you for your valuable comments. According to your advice, we have cited the papers as reference [16, 17] and added the following sentence in the paragraph of INTRODUCTION (page 9); “Indeed, several studies have shown the different plasma levels of inflammatory activation according to stroke subtypes.[16, 17]”

Comment 3
some previous articles reported that immunoinflammatory activation has a different degree in relation of TOAST subtype of stroke, on this basis it appears clearly interesting to add some information about subtype of stroke prevalence in incident strokes at follow-up- Are incident strokes all of cardioembolic subtype? or not. Please add this information and a brief comment about on discussion section.

Our response
Unfortunately, we did not have those data concerning the classification of recurrent stroke. Because our study was derived from the multicenter prospective data base, we were not able to collect the additional information about the recurrence of stroke. We have added the following sentence in the seventh paragraph of DISCUSSION (page 21); “Furthermore, as we did not investigate the classification of the recurrent stroke, the explanation about the relationship between CRP and stroke recurrence may be insufficient.” As reviewer mentioned comments, stroke subtype at the time of recurrence was important factor for the study. We will register detailed information for the recurrence in future.

VERSION 2 – REVIEW

REVIEWER	Mario Di Napoli Neurological Service, San Camillo de' Lellis General Hospital, Rieti, Italy
REVIEW RETURNED	15-Oct-2013

- The reviewer completed the checklist but made no further comments.