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PROJECT COMPLETION REPORT

1. **Project Number** : **6115**

2. **Title of the Project** : **The Role of Biomarkers in Predicting the Risk of Hemorrhagic Transformation in Acute Ischemic Stroke**

3. **Funding Agency Name** : **Intramural-TDF scheme**

4. **Project Reference Number provided by the Funding Agency**:None

5. **Principal Investigator (Name & Address) :**

   Dr Sapna Erat Sreedharan,
   Professor of Neurology,SCTIMST,TVM-11

6. **Co-Investigators (Name & Address):**
   - **i.** Dr Sylaja P N,
     Professor and HOD,Neurology,SCTIMST,TVM-11
   - **ii.** Dr Soumya Krishnamoorthy,
     PhD Scholar,Department of Neurology,SCTIMST,TVM-11
   - **iii.** Dr Lissy Krishnan,Scientist G,SCTIMST,TVM-11

7. **Implementing Institution** : SCTIMST

8. **Collaborating Institutions** : NONE

9. **Date of Commencement** : 27.09.2018

10. **Duration** : 3 years 6 months

11. **Date of Completion** : 22.04.2021

12. **Objectives as approved :**
    To determine the impact of 5 biomarkers namely, MMP-9, s100B, soluble ST2, Occludin (OCLN), and Claudin-5 (CLDN5) in predicting the risk of Hemorrhagic Transformation (HT) in acute ischemic stroke (AIS).
    To plan out a diagnostic panel of biomarkers which will be most useful in predicting HT and its severity in AIS patients.

13. Deviation made from original objectives if any, while implementing the project and reasons there of :
None

14. Field/Experimental work giving full details of summary of methods adopted, data collected supported by necessary tables, charts, diagrams and photographs :

Recruitment of the acute ischemic stroke patients was carried out at the comprehensive stroke unit of SCTIMST. The inclusion criteria were as follows: (i) patients of 18 – 80 years of age were enrolled, (ii) with first-ever ischemic stroke, (iii) within 24 hours of stroke onset (iv) initiation of thrombolysis within 4.5 hours of symptom onset and, (v) the absence of hemorrhagic transformation on initial brain CT or MRI. Patients were excluded if they had (i) previous intracerebral hemorrhage (ii) stroke within the past 3 months (iii) other central nervous system diseases (iv) serum creatinine level >2 mg/dL and, (v) any malignant diseases. All reports were collected by the study research coordinator at SCTIMST and was uploaded to the study database. Enrolled patients' demographics, vascular risk factors, medical history, diagnostic workup (including all standard imaging and blood tests) was collected through the course of hospital admission. HT was documented by means of CT\MRI imaging and was assessed using the European Cooperative Acute Stroke Study (ECASS) III criteria. A TOAST classification was used for etiological classification of stroke subtypes. The severity of symptoms at admission and at discharge were documented according to National Institutes of Health Stroke Scale (NIHSS) & modified Rankin Scale (mRS) scores. Standard 3-month post-stroke functional outcome data was assessed using mRS scores and was documented in the study database.

BIOCHEMICAL ANALYSIS
Blood samples from each patient were collected in K2 EDTA BD vacutainers at three timepoints – (i) at baseline i.e., at the time of admission or before thrombolytic intervention and, (ii) after 12 hours and (ii) after 24 hours from stroke onset. For the group of patients who have undergone thrombolytic therapy, blood samples were collected at baseline before thrombolysis. The serum was separated from the blood by centrifugation at 2,500 rpm for 15 minutes and stored in aliquots at -80˚C until further use. Each marker was separately assessed to find out their threshold levels and cut-off values which were evaluated by enzyme linked immunosorbent assay (ELISA). After analyzing the biomarkers and its values, its sensitivity and specificity was computed, it can be combined to form a panel to predict the risk of HT in AIS. A logistics model was created which will provide the most discriminative layout in predicting HT.

15. Detailed analysis of results :
The study included 112 consecutive AIS patients: 76 patients underwent intervention and 36 non-intervention patients not undergoing thrombolytic therapy and were willing to give a written informed consent. The TOAST classification of the index ischemic strokes were 32 cases of large-artery atherosclerosis, 2) 28 cases of cardioembolism, 3) 8 cases with small-vessel occlusion, 4) 3 strokes of other determined etiology, and 5) 41 patients with stroke of undetermined etiology. Of these, 23 patients developed HT. We found that significantly increased levels of baseline MMP-9 predicted HT in AIS patients. Temporal profile showed a peak within 12 hours from onset consistent to previous studies that MMP-9 levels was the highest between 8 and 12 hours from stroke onset. An increased level of MMP-9 was observed in the baseline serum of HT patients as compared to non-HT patients. However, baseline levels of soluble ST2 and S100B were not shown to be significant in patients with HT.
Multiple logistic regression model including MMP-9, sST2, and Claudin-5 levels at 12 h from onset and the
covariates that were statistically significant in the univariate analysis .Claudin-5 did not show statistically significant association for prediction of HT in the univariate analysis. However,after adjusting for the risk factors such as age, atrial fibrillation, baseline plasma glucose and systolic blood pressure, ASPECTS, NIHSS scores, IV rt-PA, EVT and cardioembolic etiology, a statistically significant predictive performance was found (OR 9.46; 95% CI:1.97_64.6;
P = 0.01) and low ASPECTS scores at baseline (OR 20.3; 95% CI:3.46_193;P = 0.003).

We examined the correlation of these biomarkers with baseline stroke severity . Of these, 11 patients (22%) with baseline NIHSS scores(5_15) and 18 patients(44%) with severe stroke(NIHSS_16) were detected with HT (P = 0.006). Patients with severe strokes with NIHSS_16 had median MMP-9 levels significantly elevated at 12 h (62.0 ng/Ml [IQR:48.1_162.7] vs 116.8 ng/mL [IQR: 60.1,233.6] vs 160.0 ng/mL [IQR: 107.0,285.9];P = 0.04).

16. Summary sheet of not more than 2 pages under following heads :
(Title, Introduction, Rationale, Objectives, Methodology, Results, Translational Potential)
Attached

17. Contributions made towards increasing the state of knowledge in the subject :

The study has increased our understanding on the role of biomarkers and their limitations in predicting hemorrhagic transformation risk in AIS.

18. Conclusions summarising the achievements and indication of scope for future work :

Claudin-5 levels, combined with low ASPECTS score at baseline, independently predicted the risk of HT in AIS.
MMP-9 was positively correlated with stroke severity in AIS.
We could not identify a panel of biomarkers which may help predict hemorrhagic transformation at baseline,from the available results.

19. Science and Technology benefits accrued :

a. List of research publications with complete details :

1. Soumya Krishnamoorthy,P.N. Sylaja,Sapna Erat Sreedharan,Gurpreet Singh,Deepa Damayanthi, Srinivas Gopala,UK Madhusoodanan,and Harikrishnan Ramachandran Biomarkers predict hemorrhagic transformation and stroke severity after acute ischemic stroke Journal of Stroke and Cerebrovascular Diseases, Vol. 32, No. 1 (January), 2023: 106875
2. Soumya Krishnamoorthy,Gurpreet Singh,Sapna Erat Sreedharan,Deepa Damayanthi,Srinivas Gopala,U.K. Madhusoodanan,P.N. Sylaja Soluble ST2 Predicts Poor Functional Outcome in Acute Ischemic Stroke Patients Cerebrovasc Dis Extra 2023;13:33–40

b. Manpower trained on the project :

i. Research Scientists or Research Fellows	**:**	**1**
ii. No. of PhD's produced	**:**	**1-under guideship of Prof Sylaja P N**
iii. Other Technical Personnel trained	**:**	**Nil**
c. Patents taken, if any		**nil**
d. Products developed, if any	**:**	**Nil**

20. Abstract: (In 300 words for possible publication in ..... Bulletin)

a. Background:
Hemorrhagic transformation (HT) is a complication occurring in patients with acute ischemic stroke (AIS) either spontaneously or post-thrombolysis leading to significant morbidity and mortality. We assessed circulating matrix metalloproteinase-9 (MMP-9), Claudin-5, and soluble serum stimulation-2 (sST2) in HT and

stroke severity in AIS based on their temporal distribution.

b. Materials:

We prospectively enrolled 111 AIS patients within 12 h from onset. Patient demographic, clinical, and imaging details were documented. Follow-up imaging was conducted 24_48 h after admission. Blood samples were taken at three timepoints from stroke onset. HT was classified according to the European Co-operative Acute Stroke Study-III(ECASS-III). Stroke severity was assessed using the National Institutes of Health Stroke Scale (NIHSS). Multiple logistic regression and receiver operating characteristic curve were conducted to determine the discriminative capacity.

c. Results:

Mean age was 62.3 § 11.7 years and median baseline NIHSS was 12[IQR 8.0_18.0]. HT was detected in 30(27%) patients. Biomarker levels at 12 h were elevated with median MMP-9 concentration of 153.9 ng/mL[IQR 110.6_309 ng/mL] indicating a trend toward significant positive correlation with HT(P = 0.05). Claudin-5 levels at 12 h was elevated but was not statistically significant (43.1 pg/mL[IQR:26.7_72.6 pg/mL] vs 59.4 pg/mL[IQR:24.5_100.8 pg/mL]; P = 0.4). Multiple logistic regression indicated Claudin-5 levels at 12 h (OR 9.46;95% CI:1.97_64.6;P = 0.010) and baseline low ASPECTS score(OR 20.3;95% CI:3.46_193; P = 0.003) independently predicted HT. MMP-9 at 12 h was significantly elevated in patients with moderate to severe strokes (P = 0.04).

d. Conclusion:

Claudin-5 and low
ASPECTS independently predicted HT. MMP-9 was positively correlated with baseline stroke severity.

21. Procurement/Usage of Equipment:none

a. Details of Equipment:

Sl. No.	Name of Equipment	Make/ Model	Cost (Rs.)	Date of Installation	Utilisation	Remarks regarding maintenance breakdown

b. Suggestions for disposal of equipment(s):NA

25.11.2023

(Name and Signature of PIs with date)

Routing: *Signed copy of "Project completion Report" by PI →* root@sctimst.ac.in, rpc@sctimst.ac.in

Summary sheet of not more than 2 pages under following heads :

Title: The Role of Biomarkers in Predicting the Risk of Hemorrhagic Transformation in Acute Ischemic Stroke

Introduction:

Despite successful recanalization by intravenous thrombolysis (IV rt-PA) or endovascular thrombectomy (EVT),some patients may not achieve favorable outcomes due to the occurrence of hemorrhagic transformation (HT).This makes the use of thrombolytic intervention a prime concern in AIS, especially in individuals with severe strokes. HT is a post-stroke complication occurring in 10_40% of patients and is characterized by bleeding in the ischemic zone either spontaneously or post-recanalization,leading to poorer outcomes with significant morbidity and mortality. Severity of neurological deficits, large ischemic core, advanced age, hyperglycemia, higher baseline systolic blood pressure, and anticoagulant use are recognized as some of the risk factors of HT.
Although the pathophysiology is not well established, it is speculated that disruption of the blood-brain barrier (BBB) within 4_5 h post-ischemia, leads to HT. Various components of the barrier contribute to its selective and protective function which, during disintegration, may leak out and be expressed as circulating biomarkers.Plasma biomarkers may act as adjuncts to clinical decision- making. Potential biomarkers such as Claudin-5, matrix metalloproteinase-9(MMP-9), and soluble serum stimulation-2(sST2) have also been correlated with HT but there is not enough data to establish their predictive roles. However, heterogeneity of data and variability in the methodology across studies have made validation of these markers uncertain in a clinical setting.

Rationale and Objectives

To determine the impact of 5 biomarkers namely, MMP-9, s100B, soluble ST2, Occludin (OCLN), and Claudin-5 (CLDN5) in predicting the risk of Hemorrhagic Transformation (HT) in acute ischemic stroke (AIS).

To plan out a diagnostic panel of biomarkers which will be most useful in predicting HT and its severity in AIS patients.

Methodology

The study protocol was approved by the institute ethics committee and each participant had given their written informed consent. Consecutive patients with AIS were recruited during their admission at the comprehensive stroke unit of our institute. Patients included in the study were: (i) 18_85 years of age, (ii) first-ever ischemic stroke,(iii) admitted within 12_14 h of symptom onset (iv) thrombolysis initiated if the patient was within 4.5 h of onset (v) absence of HT on baseline brain CT or MRI and, (vi) no prior anticoagulant use. Patients were excluded if they had (i) history of intracerebral hemorrhage, (ii) serum creatinine levels >2 mg/dL at the time of admission, (iii) absence of other central nervous system diseases, (iv) signs of concomitant infection or, (v) any malignant diseases.
Baseline clinical data Patient demographics, vascular risk factors, medical history, diagnostic workup were collected through the course of hospital admission. Etiological classification of stroke subtypes was done using trial of ORG 10172 in acute stroke treatment (TOAST). The stroke severity at admission and discharge were documented using the National Institutes of Health Stroke Scale (NIHSS). The details of the reperfusion therapies received were noted in detail. All values of systolic blood pressure (SBP) above and below 180 mmHg were dichotomized for the subjects in order to further demonstrate the effect of SBP on the outcome.Hemorrhagic transformation was classified as per ECASS II criteria .
Biochemical analysis :Blood samples were collected at three time points: (i) at the time of admission, (ii)12 h, and (ii)24 h from stroke onset. Patients who underwent revascularization at the time of admission had their baseline blood samples collected before intervention. Plasma was separated by centrifugation at 2,500 rpm for 15 minutes and stored as aliquots at _80 8C until analysis. Each marker was assessed to determine their cut-off values in each group and was evaluated by commercially available enzymelinked immunosorbent

assays(ELISA). MMP-9 was assessed using Quantikine ELISA Human Immunoassay kit(DM900;R&D Systems,MN,USA). Soluble ST2 levels were determined using Duoset ELISA kits(DY523B-05; R&D Systems,MN,USA), and Claudin-5 was assessed using Cusabio ELISA kits(Cusabio Technology LLC, Houston,TX,USA). Absorbance of the analytes was measured at 450 nm using a microplate reader(Biotek ELX
800).

Results

Between December 2018 and April 2021, 111 patients were enrolled in the study. Mean age of the population was 62.3 § 11.7 years and 70% were males. NIHSS score was significantly higher (12.0[IQR:8.0_18.0]) indicating stroke severity at admission.
The mean time of arrival to the hospital was 4.2 h. Mean ASPECTS score was 6. Intravenous thrombolysis with rt-PA was administered to 43(39%) patients, whereas 35(32%) patients underwent EVT, and 6(5.4%) patients were given bridging therapy. Thirty patients(27%) were detected with HT with a significantly higher number of patients with atrial fibrillation, low ASPECTS scores, reperfusion therapies, and delayed time of arrival had HT as compared to those without HT. Although 47% of the patients who underwent EVT were detected with HT, this included both the HI and PH subtypes. A maximum elevation of all three biomarkers at the 12-hour timepoint. The median 12-hour MMP-9 level of 153.9 ng/mL [IQR:110.6_309 ng/mL] showed a trend towards statistical significance in HT(P = 0.05). Claudin-5 levels were elevated at 12 h as compared to the other two time-points but was not found to be statistically significant (43.1 pg/ mL[IQR:26.7_72.6 pg/mL] vs 59.4 pg/mL [IQR:24.5_100.8 pg/mL];P = 0.4). There was no correlation between any biomarkers at baseline or at the 24 h timepoint with HT. No significant correlation was reported between sST2 levels and HT.
The ROC curves generated for the biomarker levels at all timepoints, showed baseline MMP-9 levels showed maximum sensitivity of 85.7% for a cut-off value of 56.06 ng/mL which was maintained at 12 h (80%) for a threshold of 96.3ng/mL(AUC = 0.63) and declined by the 24 hour timepoint. Similarly, Claudin-5 had the maximum sensitivity of 64% and specificity of 62.3% at the 12 h time point for a cut-off of 50 pg/mL(AUC = 0.553). sST2 thresholds progressively increased from baseline to 24 h and yielded a maximum sensitivity of 95% and low specificity of 21.6% at baseline albeit for a lower cut-off value of 8.865 ng/mL.

Multiple logistic regression model showed that Claudin-5 did not show statistically significant association for prediction of HT in the univariate analysis. However,after adjusting for the risk factors such as age, atrial fibrillation, baseline plasma glucose and systolic bloodpressure, ASPECTS, NIHSS scores, IV rt-PA, EVT and cardioembolic etiology, a statistically significant predictive performance was found (OR 9.46; 95% CI:1.97_64.6;P = 0.01) and low ASPECTS scores at baseline (OR 20.3; 95% CI:3.46_193;P = 0.003).
Of these, 11 patients (22%) with baseline NIHSS scores(5_15) and 18 patients(44%) with severe stroke(NIHSS_16) were detected with HT (P = 0.006) .Patients with severe strokes with NIHSS_16 had median MMP-9 levels significantly elevated at 12 h (62.0 ng/mL [IQR:48.1_162.7] vs 116.8 ng/mL [IQR: 60.1,233.6] vs 160.0 ng/mL [IQR: 107.0,285.9];P = 0.04).

Translational Potential

Utility of panel of biomarkers including Claudin -5 and MMP-9 needs to be studied further for predicting hemorrhagic transformation risk.