THE ROLE OF BIOMARKERS IN PREDICTING THE RISK OF HEMORRHAGIC TRANSFORMATION IN ACUTE ISCHEMIC STROKE

SOUMYA KRISHNAMOORTHY

PhD THESIS

2022

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM

An Institution of National Importance established by an Act of the Indian Parliament
(Act No.52 of 1980)

Dept. of Science and Technology, Govt. of India

www.sctimst.ac.in

THE ROLE OF BIOMARKERS IN PREDICTING THE RISK OF HEMORRHAGIC TRANSFORMATION IN ACUTE ISCHEMIC STROKE

A THESIS SUBMITTED BY

SOUMYA KRISHNAMOORTHY

TO

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM.

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

2022

DECLARATION BY THE STUDENT

CERTIFICATE

I, **Soumya Krishnamoorthy** hereby certify that I had personally carried out the work depicted in the thesis titled, "**The Role of Biomarkers in Predicting the Risk of Hemorrhagic Transformation in Acute Ischemic Stroke**", except where due acknowledgment has been made in the text. No part of this thesis has been submitted for the award of any other degree or diploma prior to this date.

Date: 14 October 2022 *Soumya Krishnamoorthy*

श्री चित्रा तिरुनाल आयुर्विज्ञान और प्रौद्योगिकी संस्थान, त्रिवेन्द्रम
तिरुवनन्तपुरम - ६९५०११, केरल, इंडिया
SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM
Thiruvananthapuram - 695 011, Kerala, India
(An Institute of National Importance under Govt. of India)
Grams : Chitramet, Phone : +91-471-2443152, Fax : +91-471-2550728 / 2446433, E-mail : sct@sctimst.ac.in, Website : www.sctimst.ac.in

CERTIFICATE BY THE RESEARCH GUIDE

Dr. P.N. Sylaja MD, DM, FRCP, FESO
Professor and Head, Department of Neurology
In-charge, Comprehensive Stroke Care Program
SCTIMST, Trivandrum

This is to certify that **Soumya Krishnamoorthy**, Department of Neurology of this institute has fulfilled the requirements prescribed for the Ph.D. degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum.

The thesis entitled, "**The Role of Biomarkers in Predicting the Risk of Hemorrhagic Transformation in Acute Ischemic Stroke**" was carried out under my direct supervision. No part of the thesis was submitted for the award of any degree or diploma prior to this date.

*Clearance was obtained from the Institutional Ethics Committee for carrying out the study.

Date: 14 October 2022

Sylaja PN

Dr. P. N. Sylaja

श्री चित्रा तिरुनाल आयुर्विज्ञान और प्रौद्योगिकी संस्थान, त्रिवेन्द्रम
तिरुवनन्तपुरम - ६९५०११, केरल, इंडिया
SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM
Thiruvananthapuram - 695 011, Kerala, India
(An Institute of National Importance under Govt. of India)
Grams : Chitramet, Phone : +91-471-2443152, Fax : +91-471-2550728 / 2446433, E-mail : sct@sctimst.ac.in, Website : www.sctimst.ac.in

CERTIFICATE BY THE RESEARCH CO-GUIDE

Dr. Srinivas Gopala, PhD, MAMS
Scientist 'G' and Head
Department of Biochemistry
SCTIMST, Trivandrum

This is to certify that **Soumya Krishnamoorthy**, Department of Neurology of this institute has fulfilled the requirements prescribed for the Ph.D. degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum.

The biomarker analysis under the thesis entitled, "**The Role of Biomarkers in Predicting the Risk of Hemorrhagic Transformation in Acute Ischemic Stroke**" was carried out under my direct supervision. No part of the thesis was submitted for the award of any degree or diploma prior to this date.

*Clearance was obtained from the Institutional Ethics Committee for carrying out the study.

Date: 14 October 2022

Dr. Srinivas Gopala

श्री चित्रा तिरुनाल आयुर्विज्ञान और प्रौद्योगिकी संस्थान, त्रिवेन्द्रम
तिरुवनन्तपुरम - ६९५०११, केरल, इंडिया
SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM
Thiruvananthapuram - 695 011, Kerala, India
(An Institute of National Importance under Govt. of India)
Grams : Chitramet, Phone : +91-471-2443152, Fax : +91-471-2550728 / 2446433, E-mail : sct@sctimst.ac.in, Website : www.sctimst.ac.in

APPROVAL OF THE THESIS

The thesis entitled

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

Submitted by

Soumya Krishnamoorthy

for the degree of

Doctor of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND

is evaluated and approved by

Dr. P.N. Sylaja (PhD Guide) 17/2/23

Thesis Examiner 17/2/23

Dr. JEYARAJ D. PANDIAN
PRINCIPAL / DEAN
CHRISTIAN MEDICAL C[illegible] LUDHIANA
PUNJAB STATE, INDIA-[illegible]

Whatever I have, wherever I am, I can make it through anything in the One Who makes me who I am.

ACKNOWLEDGEMENTS

First and foremost, I thank God Almighty for His grace and the ability He has bestowed upon me to complete my doctoral dissertation. My deepest gratitude to my beloved parents for their incessant support through thick and thin. I owe my beloved family everything; their supplication, love, toil, and support have made my work possible. I especially dedicate my thesis to my dearest mother whose perpetual prayers and unwavering belief in me have taught me to scale new heights.

The pride of place goes to my dissertation committee who have guided me throughout the years. At the very top of this prodigious list is my Ph.D. supervisor. I wish to place on record my deep sense of gratitude to Dr. P.N. Sylaja, Professor and Head of the Department of Neurology, and in charge, of the Comprehensive Stroke Care Program, who has academically and emotionally supported me through the path to finish this thesis by guiding my progress with a keen eye for detail. She is a role model to me, and I highly regard her for her tenacity and incredible work ethic. Being her student has been a privilege.

I express my immense gratitude to my co-guide, Dr. Srinivas Gopala, Scientist 'G' and Head, Department of Biochemistry, for his expert advice and his active guidance throughout my thesis work by providing me exceptional support and the opportunity to work at his department without any restrictions. I am extremely indebted to my DAC members, Dr. Harikrishnan S, Associate

Dean and Professor of Cardiology, Dr. C Kesavadas, Deputy Director and Professor (Senior grade) of Radiology and Dr. Madhusoodanan UK, Associate Professor, Department of Biochemistry, for their constructive suggestions at every stage of my thesis work. I sincerely thank Dr. Madhusoodanan for his support and for allowing me to work at his lab. My heartfelt gratitude to Dr. Sapna Erat Sreedharan, Professor of Neurology, who was willing to help me with the application of funding for the study.

I have been granted the honor of doing my Ph.D. studies at Sree Chitra, one of the finest institutes in the nation and, I thank our eminent former Directors, Prof. Asha Kishore, Prof. K. Jayakumar, Prof. Ajith Kumar, and our present Director, Prof. Sanjay Behari, for allowing me to be part of this institute. I thank the institute for providing me with the scholarship to pursue my doctoral studies. I would also like to thank our esteemed Deans, our Registrar, Dr. Santhosh Kumar B., and Deputy Registrar, Mrs. Radha M. for the constant encouragement and support throughout my Ph.D. course.

My deepest appreciation to the nurses at the stroke ICU, under the supervision of our current head nurse, Mrs. Rajalakshmi, and previous head nurse Mrs. Laisa Kumar, who have helped to coordinate the blood sample collection at the specified timepoints for the study. I extend my sincere gratitude to Dr. Deepa Damayanthi, Technical assistant, at the department of biochemistry, for her guidance and support for the lab work and to all my fellow lab mates who have all been so warm and helpful. I am truly grateful to Prof. Biju Soman, Head, AMCHSS for his expertise and guidance for

conducting the meta-analysis and Dr. Gurpreet Singh, Ph.D. scholar, AMCHSS, for helping me with the statistical analysis. I also extend my gratitude to my dear colleagues at the Comprehensive Stroke Care Program for their companionship and encouragement. My sincere thanks to our librarian, Ms. Sudha T, and the other staff at our library for their assistance.

I hereby also take the opportunity to express gratitude to my dear friends and colleagues, Dr. Tiju I Varghese, Scientist B, National Centre for Earth Science Studies, Dr. Aneesh Dhasan, Research Associate and National Coordinator, Indian Stroke Clinical Trial Network and Dr. Vishnu Renjith, Faculty of the Royal College of Surgeons, Ireland, who have extended their moral and intellectual support during my Ph.D. tenure.

Last, but not least, I would like to thank the Technology Development Fund (TDF) intramural scheme of SCTIMST for funding my work.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACA	Anterior Cerebral Artery
ADP	Adenosine diphosphate
AEC	Absolute Eosinophil Count
AF	Atrial Fibrillation
aHT	Asymptomatic Hemorrhagic Transformation
AIS	Acute Ischemic Stroke
AJ	Adherens junctions
ASPECTS	Alberta Stroke Program Early CT Score
ATLANTIS	Alteplase Thrombolysis for Acute Noninterventional Therapy in Ischemic Stroke
ATP	Adenosine triphosphate
AUC	Area under the curve
BA	Basilar Artery
BBB	Blood-brain barrier
BEST	Biomarkers, Endpoints, And Other Tools
BMI	Basal Metabolic Rate
BNP	B-type Natriuretic Peptide
Ca^{2+}	Calcium ion
CBF	Cerebral Blood Flow
CCCK-18	Caspase-cleaved Cytokeratin-18
CE	Cardioembolism

c-FN	Cellular-Fibronectin
CLDN5	Claudin-5
CMB	Cerebral microbleeds
CNS	Central Nervous System
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CT	Computed tomography
CTA	Computed tomography angiography
DALYs	Disability-adjusted Life Years
DAMPs	Damage-associated molecular patterns
DOR	Diagnostic Odds Ratio
DWI	Diffusion-weighted Imaging
ECAD	Extracranial atherosclerotic disease
ECASS	European Cooperative Acute Stroke Study
ECM	Extracellular matrix
EDTA	Ethylenediamine Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assays
EVT	Endovascular Therapy
FBS	Fasting blood glucose
FDP	Fibrin and fibrinogen degradation product
FGF	Fibroblast growth factor
g	grams
GBD	Global Burden of Disease

GFAP	Glial Fibrillary Acid Protein
h	hours
HB-EGF	Heparin Binding Epidermal Growth Factor-like Growth Factor
HGF	Hepatocyte growth factor
HI	Hemorrhagic infarction
HIC	High-income countries
HMGB1	High-mobility group box 1
Hs-CRP	High-sensitivity C-reactive protein
HSROC	Hierarchical summary receiver operator characteristic
HT	Hemorrhagic Transformation
IA-tPA	Intra-arterial tissue Plasminogen Activator
ICA	Internal Carotid Artery
ICAD	Intracranial Atherosclerotic Disease
ICAM	Intercellular adhesion molecule
ICH	Intracerebral Hemorrhage
IL-6	Interleukin-6
IQR	Interquartile range
IR	Ischemia-reperfusion
IST-3	The third International Stroke Trial
IV rtPA	Intravenous recombinant tissue plasminogen activator
JAM	Junctional Adhesion Molecule
kDa	Kilo Dalton

KGF	Keratinocyte Growth Factor
LMIC	Low- and middle-income country
Lp (a)	Lipoprotein (a)
Lp-PLA2	Lipoprotein phospholipase A2
LRP	Lipoprotein receptor protein
LVD	Large Vessel Disease
MAST	Multicenter Acute Stroke Trial
MCA	Middle cerebral artery
mg/dL	Miligram per deciliter
Mg^{2+}	Magnesium ion
min	Minute
MMP	Matrix metalloproteinase
MRI	Magnetic Resonance Imaging
mRS	modified Rankin Score
MT	Mechanical thrombectomy
Na^{+}	Sodium ion
NF-κB	Nuclear factor-kappa B
ng/mL	Nanogram per milliliter
NIHSS	National Institutes of Health Stroke Scale
NINDS	National Institute of Neurological Disorders and Stroke
NLR	Neutrophil-lymphocyte ratio
NMDA	N-Methyl-D-Aspartate

NR2	NMDA receptor 2
NSE	Neuron-specific Enolase
NVU	Neurovascular unit
O_2	Oxygen
OCLN	Occludin
PAI-1	Plasminogen Activator Inhibitor-1
PARP-1	Poly (ADP-ribose) polymerase 1
PCA	Posterior Cerebral Artery
PDGF-BB	Platelet Derived Growth Factor BB
pg/mL	Picogram per milliliter
PH	Parenchymal Hematoma
pH	Potential of Hydrogen
PNS	Peripheral Nervous System
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PROSPERO	International Prospective Register of Systematic Reviews
PVS	Perivascular Spaces
PWI	Perfusion-Weighted Imaging
QUADAS-2	Quality Assessment of Diagnostic Accuracy Score-2
REML	Restricted Maximum Likelihood
RNS	Reactive Nitrogen Species
ROC	Receiver Operator Characteristic

ROS	Reactive Oxygen Species
S100B	S100 Calcium-binding Protein B
SAH	Subarachnoid Hemorrhage
SD	Standard deviation
sHT	Symptomatic Hemorrhagic Transformation
sICH	Symptomatic Intracranial Hemorrhage
SITS-MOST	Safe Implementation of Thrombolysis in Stroke-Monitoring Study
sST2	Soluble Serum Stimulation-2
STROBE	Statement: Guidelines for Reporting Observational Studies
SVD	Small Vessel Disease
TAFI	Thrombin activatable fibrinolysis inhibitor
T_H	Helper T cells
TJ	Tight junctions
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α
TOAST	Trial of Org 10172 in Acute Stroke Treatment
tPA	Tissue Plasminogen Activator
TPO	Thrombopoietin
TSP-1	Thrombospondin-1
TSP-2	Thrombospondin-2

VAP-1-SSAO	Vascular Adhesion Protein-1-Semicarbazide-Sensitive Amine Oxidase
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand factor
WHO	World Health Organization
WMH	White Matter Hyperintensities
ZO	Zonula Occludens
µg/mL	Microgram per mililiter

SYNOPSIS

Stroke is a public health emergency worldwide. 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 post-stroke complications such as hemorrhagic transformation (HT). HT occurs in 10-40% of patients and is characterized by bleeding in the ischemic area either spontaneously or post-recanalization, leading to poorer outcomes with significant morbidity and mortality. The severity of neurological deficits, large ischemic core, advanced age, hyperglycemia, higher baseline systolic blood pressure, and anticoagulant use are some of the known risk factors of HT. Although the pathophysiology is not well established, it is speculated that disruption of the blood-brain barrier leads to HT. Various components of the barrier contribute to its selective and protective function which, during disintegration, may be expressed as circulating biomarkers.

Plasma biomarkers may act as adjuncts for clinical decision-making, and few have been correlated with HT. However, there is not enough evidence to establish their predictive roles and their clinical applicability owing to the heterogeneity in the data and the variability in the methodology across studies that have made their validation uncertain in a clinical setting.

Based on this hypothesis, the objectives of the thesis were:

1. To identify the biomarkers that predict hemorrhagic transformation in a South Indian cohort of patients with acute ischemic stroke: (1a) to conduct a systematic review and meta-analysis of the biomarkers that predict hemorrhagic transformation, (1b) to investigate the temporal relationship of the biomarkers in HT.

2. To examine the relationship of the biomarkers with short-term functional outcome in acute ischemic stroke.

For the first sub-objective, a systematic review and meta-analysis were conducted to determine the biomarkers with greater diagnostic accuracies. Based on a search of publications available between 1 October 2000 and 30 November 2020 in the CENTRAL, MEDLINE, and Web of Science databases, relevant studies were screened and data extraction was done. These studies were full-text publications in English, that enrolled AIS patients above 18 years with HT confirmed by follow-up CT or MRI and biomarkers measured in pre-treatment or baseline blood samples collected within 24 h from stroke onset.

Thirty quality-appraised articles were selected for the systematic review with most studies employing Trial of ORG 10172 in acute stroke treatment (TOAST) classification for diagnosing the ischemic stroke etiology and European Cooperative Acute Stroke Study (ECASS) criteria for assessing HT subtypes. The systematic review found that Caveolin-1, Thrombin Activatable Fibrinolysis Inhibitor, Plasminogen Activator Inhibitor-1, and soluble Serum Stimulation - 2 (sST2) were found to be potential biomarkers for HT risk prediction.

Among the articles selected for the review, sixteen studies were meta-analyzed that investigated the biomarkers namely, Matrix Metalloproteinase-9 (MMP-9),

Ferritin, cellular-Fibronectin, S100 Calcium-binding protein B (S100B), and Neutrophil-lymphocyte ratio (NLR). The proteolytic enzyme, MMP-9 with serum levels greater than 140 ng/mL had 29.5 times greater risk of developing symptomatic HT after stroke with a high pooled sensitivity of 84.9% and a low false positivity rate, signifying that MMP-9 had a higher diagnostic accuracy for predicting risk of HT. Cellular-Fibronectin showed the highest sensitivity which when combined with MMP-9 increased the positive predictive value and specificity for predicting severe subtypes of HT. The marker had the highest discriminative power with an area under the curve (AUC) of 0.972, however, it yielded a wide confidence interval in the univariate analysis thereby affecting its reliability as a marker. Similarly, a wide confidence interval was also reported for Ferritin levels despite the high sensitivity of 80.2%, whereas, both S100B and NLR yielded lower pooled sensitivity of 78.2% and 67.2%, respectively, with S100B having the highest false-positive rate. Baseline levels of MMP-9, Ferritin, and NLR also predicted poor functional outcome at 90 days from stroke onset.

Based on these findings MMP-9, S100B, sST2, and the tight junction proteins namely, Claudin-5 and Occludin were selected for the second sub-objective, which was to analyze the temporal profile of the biomarkers in patients with HT.

Consecutive AIS patients enrolled in the study were above 18 years of age, with first-ever ischemic stroke admitted within 24 hours of symptom onset. Patients with the absence of hemorrhage on baseline brain CT or MRI, serum creatinine levels less than 2 mg/dL at the time of admission, no history of anticoagulant use, with the absence of central nervous system diseases, infections, systemic inflammations, or

malignant diseases were considered eligible for the study. The demographics, vascular risk factors, medical history, and diagnostic workup were documented. Ischemic stroke was classified using the TOAST classification. Baseline stroke severity was documented using the National Institutes of Health Stroke Scale (NIHSS) and functional outcome at 90 days was assessed using the modified Rankin Scale (mRS) with an unfavorable outcome defined as a score greater than 2. CT or MRI was performed on all patients upon admission and at 24-48 h after admission. HT, if detected, was documented and defined according to the ECASS criteria. Plasma samples were quantified at three time-points – at admission or before the intervention, at 12 h, and 24 h from stroke onset using commercially available immunoassays.

One hundred and eleven consecutive AIS patients were enrolled in the study after receiving informed consent with a mean age of 62.3±11.7 years and 70% being male. The mean time of arrival at the hospital was 4.2 h with a median baseline NIHSS score of 12. Among these, IV tPA was administered to forty-three patients, and thirty-five patients underwent EVT.

Thirty patients (27%) were detected with HT which was significantly associated with Atrial fibrillation, low baseline ASPECTS (Alberta Stroke Program Early CT Score), high baseline NIHSS scores, Cardioembolism etiology, reperfusion therapies and delayed time of arrival.

The temporal profile indicated a maximum elevation of MMP-9, and Claudin-5 at the 12-hour time-point in HT. The median 12-hour MMP-9 level of 153.9 ng/mL [IQR:110.6 - 309 ng/mL] showed a trend toward statistical significance in HT (P = 0.05). MMP-9 showed a good sensitivity of discrimination at baseline (85.7%) which

was maintained at 12 hours and declined by the 24 h time-point signifying that an earlier measurement of the marker would predict the risk of HT. Claudin-5 levels were elevated at 12 h as compared to the other two time-points but were not found to be statistically significant. Similarly, Claudin-5 had an optimum sensitivity of 62% and specificity of 62.3% at the 12 h time-point for a cut-off of 50 pg/mL (AUC = 0.552). Although this elevation was not statistically significant, Claudin-5 (OR 9.46; 95% CI:1.97-64.6; P = 0.01) and low baseline ASPECTS score (OR 20.3; 95% CI:3.46-193; P = 0.003) emerged as an independent predictor after adjusting for covariates in the multiple logistic regression model. These findings supported the presence of Claudin-5 in blood after the ischemia-reperfusion injury as an important indicator of HT and are influenced by the activation of MMP-9.

There was no correlation between any biomarkers at baseline or at the 24 h time-point with HT possibly owing to the variability in the baseline samples collected from patients admitted at different times from onset. There was no significant difference in the sST2, S100B, and Occludin levels between both groups and did not yield any association with HT. The temporal distribution of sST2 showed a delayed increase from onset indicating its role in the inflammatory phase of the ischemic cascade. Although the highest sensitivity was observed at baseline (95.2%), the low cut-off value of 8.8 ng/mL may affect its discriminative capacity.

Furthermore, baseline stroke severity significantly correlated with the median MMP-9 levels at 12 h (160 ng/mL [IQR: 107.0-285.9]; P = 0.04) in patients with moderate to severe baseline NIHSS scores thereby establishing a relevant relationship

between stroke severity and increased expression of MMP-9. No other markers were found to correlate with stroke severity.

The second objective was to investigate the relationship of these biomarkers with short-term functional outcome in AIS. Following the same methodology, the temporal profiles of MMP-9, Claudin-5, and sST2 presented an overall increase in the plasma levels at 12 h in patients with unfavorable outcomes at 90 days. Notably, sST2 levels showed a gradual increase in the concentration across the three time-points, and the maximum levels were obtained at the 24 h time-point. Poor outcome was significantly associated with sST2 levels at 12 h and 24 h with mean levels of 50.4±51.0 ng/mL (P = 0.047) and 81.8±101.3 ng/mL (P = 0.001), respectively. This temporal profile was consistent with previous findings that showed an increase in its activity beyond 24 hours from onset associated with its role in the inflammatory phase of ischemia. Both S100B, and Occludin were not associated with functional outcome.

Although MMP-9 and Claudin-5 levels were elevated at 12 h, there was no significant correlation of either marker with the outcome. The diagnostic accuracy of plasma sST2 levels at 24 h was 67% for a cut-off value of 71.8 ng/mL with 96.9% specificity. MMP-9 levels showed higher sensitivity at all three time-points with an optimum cut-off value of 77.212 ng/mL at the 12 h (sensitivity: 86.1%, specificity: 44.2%). The highest sensitivity (92.3%) was attained at 24 h for a cut-off value of 50.663 ng/mL but the diagnostic accuracy was only 56.6%. Claudin-5 had the lowest AUC values with the lowest sensitivity at all three time-points.

sST2 levels assessed at 24 h from onset emerged as an independent predictor of poor functional outcome at 3 months with the probability of an unfavorable outcome

being six times higher when the cut-off value at 24 h was applied to the model generating better discriminative capacity and high specificity.

The significance of the findings of the study were that the tight junction protein, Claudin-5 measured at 12 h from stroke onset was an independent predictor of the risk of HT in AIS. Although MMP-9 was not associated with HT, it was positively correlated with baseline stroke severity in AIS when assessed within 12 h. MMP-9 and Claudin-5 followed similar time courses of expression indicating the direct role of MMP-9 in the degradation of Claudin-5 leading to blood-brain barrier disruption causing an early occurrence of HT. The association of poor functional outcome and increased expression of sST2 in a proinflammatory context at 12 h from stroke onset indicated sST2 may be considered a prognostic marker for predicting short-term outcomes in AIS. Temporal distribution is an essential factor of biomarker expression relative to the pathophysiological mechanism, hence, conducting sequential analyses of the markers from stroke onset, rather than at the time of admission or at a single time-point, as adopted in earlier studies. This helped understand the dynamic changes in the biomarker expression over time. Some limitations of the study are the monocentric design and the small sample size, making it difficult to appropriately assess the differences in biomarker levels between the groups, leading to wide confidence intervals in the regression analyses. A prospective study with a larger sample size and evaluation of these proteins at specific time intervals from the onset may help establish their predictive roles in HT.

1 INTRODUCTION

The chapter begins with an overview of stroke. The epidemiology, etiology, pathophysiology, and interventional treatments for acute ischemic stroke are explored. This is followed by a brief account of hemorrhagic transformation as a major complication in acute ischemic stroke, its classification, known predictors, and its mechanism. The role of biomarkers in stroke and its implications as a possible adjunctive therapy in stroke and hemorrhagic transformation is explored. The rationale of looking into biomarkers as predictors of hemorrhagic transformation is finally justified.

1.1 Global burden of stroke

Stroke is the second leading cause of death and the third leading cause of disability worldwide and is considered one of the most devastating neurological conditions (Feigin et al., 2021; Owolabi et al., 2021). The Global Burden of Disease (GBD) reported 12.2 million incident strokes, a whopping estimate of 101 million prevalent strokes, and 6.5 million deaths in 2019 (Feigin et al., 2021). A stroke is an abrupt focused lesion to the brain, retina, or spinal cord that has a vascular origin and results in neurological disability (Sacco et al., 2013). In clinical practice, it refers to the disease condition arising from either an occlusion, attributable to arterial blockage or due to hemorrhage of the blood vessels supplying to the brain caused due to the arterial rupture in the cerebral parenchyma or the subarachnoid region (Campbell and Khatri, 2020). Based on regional epidemiology, the worldwide incidence of stroke

accounts for around 70–80% of ischemic strokes and the remaining 20%–30% of hemorrhagic strokes (Feigin et al., 2021; Johnson et al., 2019).

Although stroke continues to affect developed and developing nations alike, mortality and disability resulting from stroke are higher in low- and middle-income countries (LMICs) as shown in Fig. 1.1, indicating a higher incidence particularly in the Asian and African countries (Krishnamurthi et al., 2013).

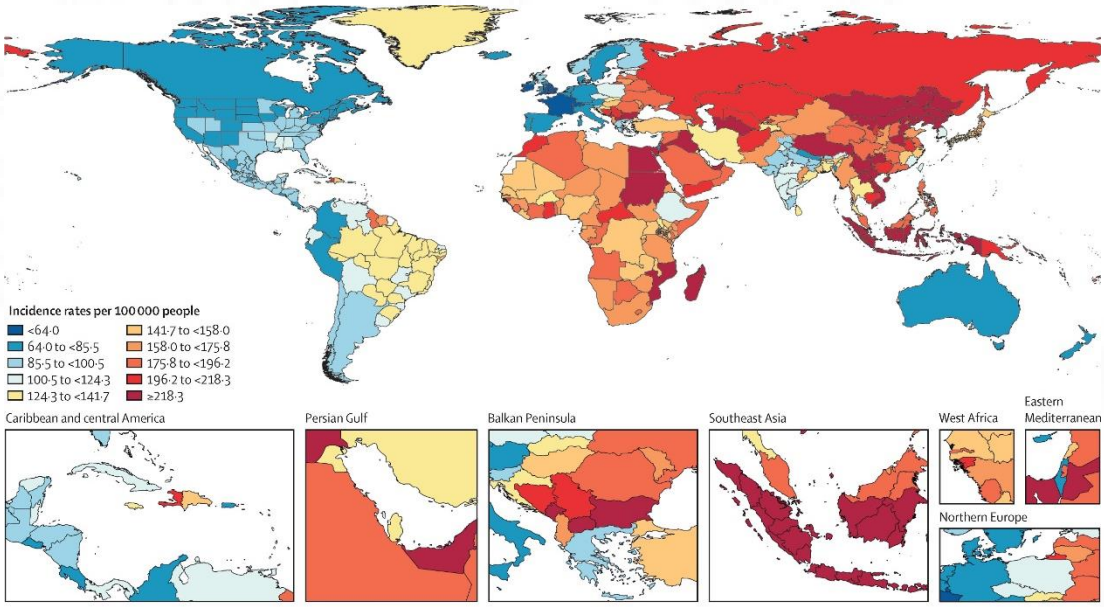


Fig. 1.1 The global distribution of stroke incidence as reported by the GBD in 2019. (Source: Feigin et al., 2021)

With the impact of urbanization, changing lifestyles, and aging populations, the burden of stroke in the LMICs is expected to rise over the next decade (Thrift et al., 2017). Noteworthy, the mean age of patients with stroke in the LMICs was 15 years younger than that of the high-income countries (HICs).

1.2 The burden of stroke in India

Stroke is a public health emergency in India and is currently the fourth leading cause of death and the fifth leading cause of disability (Jones et al., 2022). The Indo-USA National Stroke Registry revealed that stroke incidence had a lower mean age when compared to Western nations (Sylaja et al., 2018). In India, the yearly incidence of stroke varied from 108 to 172 per 100,000 people (Jones et al., 2022). The reported case fatality rates were likewise higher than those for HICs; several community-based studies found substantial variation across the subcontinent, ranging from 18% to 42% (Jones et al., 2022). As more stroke units and imaging facilities become available to people living in cities, stroke patients in rural locales may be impacted as a result of their limited access to specialized stroke care services (Pandian and Sudhan, 2013).

The age-adjusted prevalence rate ranged from 84 to 262 per 100,000 persons and 334 to 424 per 100,000 persons in rural and urban populations, respectively (Jones et al., 2022). Although intravenous thrombolysis was the major therapy for acute stroke in India, only 11% of patients received thrombolytic treatment (Sylaja et al., 2018). Most ischemic strokes in the subcontinent were reported to be caused by large-artery atherosclerosis (29.9%), particularly the intracranial atherosclerosis subtype (Sylaja et al., 2018; Kaul et al., 2018). Obesity, hypertension, diabetes mellitus, alcoholism, and sedentary lifestyles are snowballing the illness burden in India and might be attributed to the rapid socioeconomic changes causing lifestyle changes, job-related stress, and adjusting dietary habits (Sylaja et al., 2018).

1.3 Acute ischemic stroke

Acute ischemic stroke (AIS) involves the abrupt depletion of blood supply in the region of the brain due to arterial occlusion blocking blood flow either by an *in situ* thrombus or embolus (Campbell et al., 2019; Caplan, 2016). The extent of metabolic and functional impairments during ischemia may depend on the artery that is occluded. (Jayaraj et al., 2019). A decline in glucose and oxygen levels results from the occlusion triggering a series of molecular events leading to cell death (Lo et al., 2003). Energy levels plummet in the affected tissue thereby impairing the energy-dependent ion transport system followed by the disruption of the cell volume, cerebral hypoxia, apoptosis, and necrosis of the brain tissue (Xing et al., 2012; Lo et al., 2003). A stroke patient may exhibit typical symptoms such as impairments in vision and motor movement, acute facial paresis, speech deficits, coordination challenges, disorientation, and unconsciousness that range from moderate to severe depending on the cortical area affected (Goldstein and Simel, 2005).

The Trial of Org 10172 in acute stroke treatment (TOAST) classifies ischemic stroke into five subtypes namely, large vessel disease (LVD), cardioembolism (CE), small vessel disease (SVD), stroke of other determined causes, and stroke of undetermined etiology, based on the cause of the occlusion (Adams et al., 1993). Treatment of AIS may be directed according to the time of onset and severity of symptoms. The gold standard for diagnosing acute stroke is neuroimaging i.e., non-contrast head computed tomography (CT), magnetic resonance imaging (MRI), and more recently, diffusion-weighted imaging (DWI). Intravenous thrombolysis is one of the major treatments opted for AIS patients brought within 4.5 hours of symptom onset

(Xiong et al., 2022; Bivard et al., 2013). For patients who do not qualify for thrombolysis, endovascular therapy (EVT) may be conducted to mechanically retrieve the clot following imaging evaluation (Campbell and Khatri, 2020). Medical intervention includes dual antiplatelet therapy administered within 24 hours thereby benefitting patients with mild strokes and those that do not qualify for either thrombolysis or EVT (Xiong et al., 2022).

1.4 Hemorrhagic transformation

Hemorrhagic transformation (HT) is a frequent complication reported in 10% to 40% of AIS patients and accounts for adverse clinical outcomes in patients with a mortality rate of 13.4% and significant morbidity in the survivors (Spronk et al., 2021). HT may occur spontaneously as a part of the natural history of ischemic stroke (Khatri et al., 2007; Zhang et al., 2014). However, a 10-fold increase was reported in patients after receiving thrombolytic treatment which offsets potential worsening (Wardlaw et al., 2014; Alberts, 2012; Thomalla et al., 2006). Apart from thrombolysis, anticoagulation medication, and EVT may also exacerbate HT (Zhang et al., 2014). Symptomatic HT involves clinical deterioration and is associated with a higher risk of clinical complications, prolonged hospital stays, mortality, and poor clinical outcomes (Andrade et al., 2020). HT may also develop asymptomatically after AIS, which is not as benign as once believed, making it challenging to diagnose based on only clinical manifestations.

Consequently, the widely used classification system developed by the European Cooperative Acute Stroke Study (ECASS) makes use of the radiographic characteristics to distinguish petechial hemorrhagic infarctions (HI) from the far more

severe forms of parenchymal hematomas (PH) and symptomatic intracranial hemorrhage (sICH) (Hacke et al., 1998; Hacke et al., 2008). The PH subtypes occur rarely, in about 4% of the patients, but are closely associated with poor outcomes often resulting in midline shift and mass effect in the cerebral hemisphere (Álvarez-Sabín et al., 2013).

Some of the known clinical, imaging, and biochemical predictors of HT are older age, hyperglycemia, higher systolic blood pressure, renal impairment, and cardiac risk factors such as atrial fibrillation and congestive heart disease (van Kranendonk et al., 2019; Kablau et al., 2011; Spronk et al., 2021; Thanvi et al., 2008). Medium- and large-sized cardioembolic infarcts and large vessel occlusion strokes frequently exhibit HT (Hornig et al., 1993; Iwamoto et al., 2021). Cerebral microbleeds and leukoaraiosis have been identified as imaging biomarkers that are linked to severe forms of HT (D'Anna et al., 2021).

Due to its complex nature, the pathophysiology of HT is still up for debate. The principal cause of HT is the blood-brain barrier disruption, which results in the loss of integrity of the neurovascular unit, comprising the endothelial cells, tight junction proteins, the pericytes, extracellular matrix (ECM), astrocyte endfeet, and microglia (Iadecola, 2017; Hawkins and Davis, 2005). The ischemia-reperfusion damage and an increase in oxidative stress in the ischemic zone are the major causes of barrier disruption (Kalogeris et al., 2016). In addition, coagulation, angiogenesis, and inflammation equally play important roles in the pathophysiology of HT (Thanvi et al., 2008). An overview of the mechanism of the pathogenesis of HT based on the current knowledge is illustrated in Fig. 1.2.

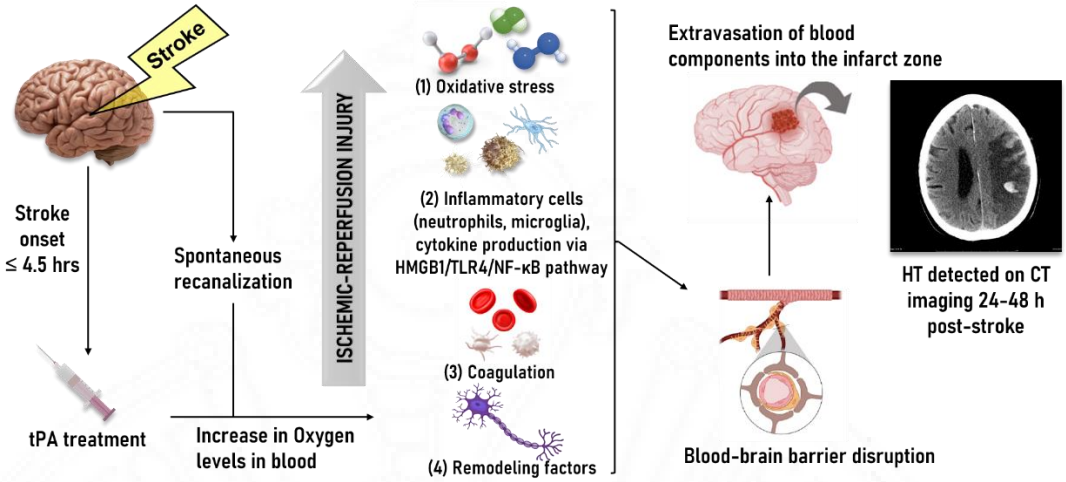


Fig. 1.2 Overview of the mechanisms involved in hemorrhagic transformation.

It has been discovered that several proteins associated with these pathways affect the timing and severity of the onset of HT (Jickling et al., 2014). This is extremely important in locating molecular markers relevant to predicting HT. There are many uncertainties regarding the pathophysiology of HT, and more research is needed to define the underlying biochemical mechanisms.

1.5 Implications of biomarkers in stroke

In its simplicity, the term 'biomarker' denotes the measurement of a biological function of a healthy or a disease condition (Atkinson et al., 2001). Biomarkers are necessary for the rational development of medical interventions, and their applicability underpins their use in clinical practice and research, particularly in the context of chronic diseases. For instance, in the assessment of myocardial infarction, Troponin and Creatine Kinase MB levels are monitored in an acute clinical setting (Laskowitz et al., 2009). But unlike Creatine Kinase-MB, the Troponin test has virtually hundred

percent specificity for the cardiac tissue and high sensitivity, which has in turn contributed to its widespread use in clinical therapy (Maas and Furie, 2009). Other biomarkers have also been incorporated into routine clinical practice for the assessment of other major diseases like pulmonary embolism and congestive heart failure (Laskowitz et al., 2009). These include circulating D-dimer - a fibrin degradation product that indicates clot formation - and the cardiac neurohormone biomarker, B-type natriuretic peptide, which is secreted from the heart ventricles during increased stress (Bounameaux et al., 1991; Gibler et al., 2003; de Lemos et al., 2003).

Approaches to accurately identify patients at risk for stroke and post-stroke complications are necessary, in addition to improving patient diagnosis and prognosis following a stroke. Clinicians are adept at assessing stroke and its causes, and biomarkers may be used adjunctively for risk stratification, clinical decision-making, therapeutic response assessment, and for better prognosis (Jickling and Sharp, 2011; Whiteley et al., 2012a; Maas and Furie, 2009). Acute stroke diagnostic approaches, mostly rely on neuroimaging techniques and may benefit from the supplementary evaluation of circulating blood-based biomarkers (Kim et al., 2013; Jickling and Sharp, 2011).

Several blood-borne biomarkers have been examined for their ability to predict long-term outcomes, aid in the diagnosis, and to be associated with prospective risk for stroke. These include tissue injury-specific markers such as S100 calcium-binding protein B (S100B), glial fibrillary acid protein (GFAP), Neuron-specific enolase (NSE), inflammatory markers such as C-reactive protein (CRP), interleukin-6 (IL-6),

Tumor necrosis factor (TNF-α) and coagulation pathway markers such as Fibrin and fibrinogen degradation product (FDP), von Willebrand factor (vWF), and D-dimer based on their pathophysiological role in stroke (Foerch et al., 2006; Whiteley et al., 2008; Montaner et al., 2011; Jickling and Sharp, 2011). However, the brain presents several difficulties despite the vast literature examining the significance of these biomarkers in cerebral ischemia. These comprise the existence of the blood-brain barrier, various cell types, and their locations throughout the central nervous system, as well as their ischemic tolerance and ischemic cascade complexity (Laskowitz et al., 2009).

1.6 Biomarkers of hemorrhagic transformation

Currently, assessing the risk of HT largely relies on clinical conditions and neuroimaging. The development of adjunct therapeutic approaches, such as the identification of individuals at risk of developing HT either post-intervention or spontaneously by using highly discriminative biomarkers, is crucial to accurately predict HT (Jickling and Manolescu, 2012). Increased blood-brain barrier permeability primarily accounts for post-stroke HT. Hence, degradation and subsequent leakage of protein components conducive to the integrity of the blood-brain barrier may affect the overall stability of the neurovascular unit (NVU) (Steliga et al., 2020). Proteins activated during dysregulated extracellular proteolysis post-thrombolysis involve the matrix metalloproteinases (MMP) specifically MMP-2, MMP-3, and MMP-9 (Yang and Rosenberg, 2015; Lakhan et al., 2013). MMPs play a direct role in the blood-brain barrier breakdown through the degradation of the extracellular protein components (Planas et al., 2001; Kurzepa et al., 2014). Moreover, the tight junction proteins restrict

the paracellular transport of particles such as Claudin-5, Occludin, and Zonula occludens-2. There are currently no clinically used HT-related biomarkers. Potential blood biomarkers may be utilized to identify high-risk groups for HT in both acute ischemic stroke patients who had undergone thrombolytic intervention and those who had not.

1.7 Statement of the problem

Several clinical and imaging markers are available for predicting HT. Biomarkers may be useful in providing adjunctive information for medical decision-making. However, despite extensive research conducted, their clinical predictability has not been well represented. Although various circulating biomarkers have been reported, the data have been conflicting possibly owing to heterogeneity in the clinical, methodological, or statistical approaches. Additionally, potential biomarkers may be useful in further identifying HT severity among patients, screening for high-risk patients, and offering a better understanding of the pathophysiological process of HT and outcome in ischemic stroke.

1.8 Hypothesis

1. Elevated baseline circulating levels of matrix metalloproteinase-9 (MMP-9), S100 calcium-binding protein B (S100B), Claudin-5, Occludin, and soluble Serum stimulation-2 (sST2) may be associated with HT in AIS, independent of known stroke risk factors.
2. HT severity can be distinguished by the heterogeneity of circulating levels of each of the above markers.

1.9 Broad objectives and sub-objectives of the study

1. To identify the biomarkers that predict hemorrhagic transformation in a South Indian cohort of patients with acute ischemic stroke: (1a) to conduct a systematic review and meta-analysis of the biomarkers that predict hemorrhagic transformation, and (1b) to investigate the temporal relationship of the biomarkers in HT.
2. To examine the relationship of the biomarkers with short-term functional outcome in acute ischemic stroke.

2 REVIEW OF LITERATURE

The chapter provides an elaborate account of the epidemiology, classification, and pathophysiology of acute ischemic stroke and hemorrhagic transformation - as a major complication of acute ischemic stroke. The role of biomarkers in stroke and hemorrhagic transformation as provided in the literature is explored by discussing the gaps and controversies in the research.

2.1 Epidemiology of stroke

Globally, stroke is the second leading cause of death, accounting for mortality of 6.5 million each year, and the third leading cause of disability and mortality combined, with at least 12.2 million new strokes reported each year (Feigin et al., 2021; Feigin et al., 2022). Over the past three decades, the number of stroke survivors has doubled with an estimated 67% of the global population surviving a stroke under the age of 70 years (Kim et al., 2020; Feigin et al., 2022). By 2019, it was reported by the Global Burden of Disease (GBD) study that the absolute stroke incidence had increased by 70% within the past two decades, prevalent strokes increased by 85%, stroke fatalities climbed by 43%, and the disability-adjusted life-years (DALYs) attributable to stroke increased by 32% (Feigin et al., 2021). According to estimates, one in four people will experience a recurrent stroke during their lifetime (Feigin et al., 2022).

Ischemic strokes account for 7.6 million cases every year, with an incidence of at least 61% in those under the age of 70 years and a mortality rate of 19% (Feigin et al., 2022). Stroke rates have undoubtedly increased with increased exposure to several

key risk factors, such as high fasting plasma glucose, high systolic blood pressure, alcohol intake, high basal metabolic rate (BMI), ambient particulate matter pollution, lack of physical activity, kidney dysfunction, and high temperature (Feigin et al., 2021). Despite a decline in mortality and incidence over time in high-income countries (HICs), no similar shift was observed in the low- and middle-income countries (LMICs), which account for more than 85% of the global burden (Thrift et al., 2017).

In India, stroke is the fourth leading cause of death and the fifth leading cause of disability (Jones et al., 2022). According to a comprehensive analysis of the epidemiology of stroke in the urban and rural regions of India, the annual incidence of stroke ranged from 108 to 172 per 100,000 persons (Jones et al., 2022). The Trivandrum Stroke Registry had documented an overall incidence rate of stroke as 135 per 100,000 persons in the state of Kerala alone (Sridharan et al., 2009). Several community-based studies found substantial regional variation, with rural regions reporting higher case fatality rates than urban areas (18% to 42%) (Jones et al., 2022).

Predominant comorbidities of ischemic stroke prevalent in India consisted of hypertension (60.8%), diabetes mellitus (35.7%), tobacco use (32.2%), and alcohol intake (34.2%) followed by cardiac risk factors such as coronary artery disease (16.9%), hypercholesterolemia (14.4%), nonvalvular atrial fibrillation (4.0%), rheumatic heart disease (5.6%), previous transient ischemic attack (7.7%) and familial history of stroke and ischemic heart disease (Sylaja et al., 2018). An overview of the incidence prevalence and case fatality rates reported in the subcontinent are given in Fig. 2.1.

Study and publication year	No. of cases of stroke identified	Imaging not available *N* (%)	Stroke type *N* (%)	Mean age (years) *N* (SD)	Female *N* (%)	Crude annual incidence/ 100,000 *N* (95% CI)	Sex-disaggregated incidence rate male/100,00 *N* (95% CI)	Sex-disaggregated incidence rate female/ 100,000 *N* (95% CI)	Age-adjusted incidence/ 100,000 *N* (95% CI)	Crude prevalence/ 100,000 *N* (95% CI)	Age-adjusted prevalence/ 100,000 *N* (95% CI)	One-month case fatality % (95% CI)
Bhattacharya et al.[22]	128	NS	NS	61 (NS)	60 (47)	124 (NS)	124 (NS)	123 (NS)	108 (88–130)[a]	618 (509–1707)[a]	NS	18 (NS)
Das et al.[23]	247	81 (33)	IS 108 (65) ICH 58 (35)	NS	110 (45)	123 (103–233)	100 (75–130)	149 (117–166)	145 (120–175)	472 (41–534)	545 (479–617)	41 (31–54)
Dalal et al.[29]	456	0 (0)	IS 366 (80) ICH 81 (18) US 9 (2)	66 (14)	218 (48)	145 (120–170)	149 (120–170)	141 (120–160)	92 (74–113)[a]	291 (264–318)[a]	NS	30 (NS)
Sridharan et al.[28]	541	169 (31)	IS 311 (84) ICH 43 (12) SAH 18 (4)	67 (NS)	279 (52)	117 (NS)	115 (NS)	119 (NS)	135 (123–146)	58 (53–63)[a]	135 (123–146)	27 (NS)
Ray et al.[27]	763	NS	NS	NS	341 (45)	108 (88–131)	113 (86–146)	102 (75–136)	141 (114–171)	757 (702–817)[a]	NS	42 (38–46)[a]
Pandian et al.[25]	493	NS	NS	58 (15)	185 (38)	NS	NS	NS	NS	53 (48–57)[a]	NS	NS
Pandian et al.[26]	3441	2122 (62)	IS 976 (74) ICH 290 (22) SAH 53 (4)	NS	NS	140 (133–147)	151 (141–161)	106 (97–115)	130 (123–137)	323 (312–334)[a]	NS	22 (21–23)[a]
Kaur et al.[24]	4989	26 (0)	IS 3260 (66) ICH 1656 (33) CVT 47 (1)	59 (15)	1865 (37)	NS	NS	NS	NS	26 (25–27)[a]	NS	NS
Singh et al.[30]	596	NS	NS	62 (15)	286 (48)	172 (NS)	170 (NS)	173 (NS)	209 (NS)	229 (211–248)[a]	NS	NS

NS: not stated; IS: ischemic stroke; ICH: intracerebral hemorrhage; CVT: cerebral venous thrombosis; NA: no scan available; US: unspecified stroke type; SD: standard deviation; CI: confidence interval.
[a]Calculated manually based on information in article.

Fig. 2.1 An overview of the study characteristics appraising the incidence, prevalence, and one-month case fatality rates of stroke based on the regional registries and community-based surveys in India. (Source: Jones et al., 2022).

In comparison to the global mean age of people with stroke, at least 20% of the people in India with first-ever strokes were younger (Pandian and Sudhan, 2013; Sylaja et al., 2018). These data emphasize the problems of fewer in-hospital and outpatient rehabilitation facilities, as well as the lack of public awareness (Owolabi et al., 2021; Pandian and Sudhan, 2013). According to the Indo-USA National Stroke Registry, there was a significant risk of postischemic bleeding (10.9%) and high stroke severity, with a considerable percentage having big infarcts, especially after thrombolysis (Sylaja et al., 2018). The functional outcome in patients after 28 days from stroke onset did not vary among gender, age groups, or regions, and reported that moderate disability was present in 43% of patients (Sylaja et al., 2018).

2.2 Acute ischemic stroke

The World Health Organization (WHO) defined stroke as a syndrome of "rapidly developing clinical symptoms and/or signs of focal (or global) disturbance of cerebral function lasting more than 24 hours (unless interrupted by surgery or death), with no apparent cause other than of vascular origin" (Aho et al., 1980). Acute ischemic stroke involves the occlusion of major arteries of the brain pertaining to a sudden focal neurological deficit of the central nervous system (Sacco et al., 2013).

2.1.1 Clinical presentation

Clinical presentation of ischemic stroke primarily depends on the localization of the affected vascular region (Goldstein and Simel, 2005). The middle cerebral artery (MCA) is the most affected territory in almost 80% of ischemic strokes and is characterized by contralateral hemiparesis, contralateral hemisensory loss, hemianopia, aphasia – if the dominant hemisphere is involved – and neglect if the non-

dominant hemisphere is involved (Chandra et al., 2017a). On the other hand, anterior cerebral artery (ACA) territory infarcts are rare (~2%) and these patients present with dysarthria, aphasia, unilateral, contralateral motor weakness, minimal sensory changes (two-point discrimination), left limb apraxia and urinary incontinence (Goldstein and Simel, 2005). In the case of lacunar infarcts, it is generally known to be an occlusion in the small perforating artery resulting from an embolism or intrinsic vessel occlusion although these mechanisms are being debated (Caplan, 2016; Chandra et al., 2017b). Clinical presentation of this territory includes a pure motor or sensory loss, sensorimotor deficit, or ataxia with hemiparesis (Chandra et al., 2017b; Goldstein and Simel, 2005).

When the posterior cerebral artery (PCA) is involved, the patients may present with ocular findings, hypersomnolence, cognitive deficits, ataxia, and to a lesser extent homonymous hemianopsia (Chandra et al., 2017a). Basilar artery (BA) infarcts are rare (~1%) but may have a devastating effect on the patient (Caplan, 2016). They are characterized by sudden death or loss of consciousness, top of the basilar syndrome, visual and oculomotor deficits, behavioral abnormalities, somnolence, and hallucinations (Goldstein and Simel, 2005). Motor dysfunctions are often absent. If the proximal and mid portions of BA are damaged, it may lead to one of the most complex medical conditions known as the 'locked-in syndrome' characterized by quadriplegia, bulbar palsy, and whole-body sensory loss (Caplan, 2016). However, these individuals may typically still have normal hearing, vertical eye movement, blinking, and cognition (Caplan, 2016). Clinical symptoms of cerebellar infarctions might include ataxia, vomiting, headaches, nausea, dysarthria, and vertigo (Goldstein and Simel,

2005). These symptoms may also be exacerbated by the presence of edema followed by rapid clinical deterioration (Goldstein and Simel, 2005).

2.1.2 Risk factors

Determinants of outcome in stroke include non-modifiable and modifiable risk factors. Non-modifiable factors comprise age, gender, race, and genetics (Boehme et al., 2017). Of these, age is the prime risk factor whereby advanced age is a predictor of stroke (Singhal et al., 2013; Soriano-Tárraga et al., 2018). Modifiable risk factors are hypertension, diabetes mellitus, heart disease, hypercholesterolemia, tobacco use, alcohol intake, apolipoproteins B/A1 ratio, diet quality, and obesity (Feigin et al., 2021; Johnson et al., 2019; O'Donnell et al., 2016). The major risk factor for all types of strokes is high blood pressure or hypertension, particularly in middle-aged individuals (Boehme et al., 2017). By maintaining optimal levels of arterial pressure, stroke risk can be reduced by about 40% in these patients (Kannel, 2000). Diabetes mellitus is another major risk factor responsible for the enhancing stroke rates in the LMICs and may be in conjunction with metabolic syndrome, high cholesterol levels, and obesity (Feigin et al., 2021). High blood sugar levels can damage the blood vessels, which increases the chance of having a stroke by 1.5 to 3 fold (Ergul et al., 2012). Atherosclerosis involves atheromatous plaque build-up in the major arteries and may predispose an individual to carotid stenosis and large vessel disease (Campbell et al., 2019). Increased levels of low-density lipoprotein cholesterol (LDL-C) may also hasten the development of stenosis by forming plaque in the artery walls that is prone to thrombus formation and obstructing blood flow (Chandra et al., 2017b).

A proven risk factor for blood clot development from a potential intracardiac cause of embolism includes coronary heart disease, valve disorders, cardiomyopathies, arrhythmias including atrial fibrillation, and other conditions may be among them (Chandra et al., 2017b; Campbell and Khatri, 2020). Dietary intake has a major influence on the incidence of stroke as well, indicating that increased daily consumption of fruits and vegetables may considerably reduce the risk of stroke (Feigin et al., 2021). Other risk factors include peripheral artery disease, angiopathies, congenital heart diseases, thrombophilia with stroke, and other causes specific to certain age groups (Boehme et al., 2017).

2.1.3 Pathophysiology of stroke

Ischemic stroke has multifactorial pathophysiology encompassing the functions of several cellular and protein components thereby challenging the subsequent development of targeted therapy (Albers et al., 2011). Hence, therapeutic options accessible for stroke patients are relatively limited due to the incomplete information that exists even after decades of study dedicated to unraveling the pathophysiological mechanisms of stroke (Xing et al., 2012).

The cerebral ischemia pathophysiology encompasses the cerebral blood flow (CBF), the ischemic core and penumbra, collateral circulation, and the ischemic cascade (Campbell and Khatri, 2020). Although the brain constitutes only 2% of the total body weight, it consumes 20% of the oxygen and glucose intake of the body, therefore, requiring its continuous supply for neurons to function (Lo et al., 2003). The critical and undisrupted supply of energy derived from oxygen and glucose to the brain is reflected in the extensive vascularization of the brain (Sarvari et al., 2020). When a

stroke occurs, the supply is affected and death of neural cells ensues almost as rapidly as within minutes after the occlusion has taken place (Lo et al., 2003).

2.1.3.1 Core and penumbra

Cerebral autoregulation is a regulatory mechanism that helps maintain constant brain perfusion over a relatively wide range of cerebral perfusion pressures (CPPs) (Caplan, 2016). During a stroke, disruption of the blood flow alters neurologic metabolism in less than a minute leading to cell death (Deb et al., 2010; Jovin et al., 2008). As a result, the center of ischemia is known as the ischemic core where irreversible neuronal damage ensues due to low ATP (Adenosine triphosphate) levels, low energy storage, severe ionic and metabolic failure, and other factors (Campbell et al., 2019). The abrupt cessation of blood supply within the territory engenders the ischemic core comprising dead tissue with minimal perfusion (<10 mL/100 mg/min) which is the most affected region characterized by CBF less than 20% of the normal blood flow to the brain (Chandra et al., 2017b).

The core is immediately surrounded by a hyperperfused region called the penumbra harboring partially functioning cells with a critical supply of energy enough for its survival, making this region salvageable and a target for reperfusion therapies (Campbell et al., 2019). Autoregulation of blood flow in the penumbra is disturbed (CBF 18-20 mL/100g /min) with carbon dioxide reactivity of blood vessels partially preserved, a slight decrease of tissue glucose content, however, ATP levels are almost normal providing cells with enough energy to survive (Campbell et al., 2019). With a CBF range of 22–35 mL/100g/min, the penumbra's periphery designates the area of oligemia, which contains hypoperfused tissue but is not in danger under typical

conditions (Campbell et al., 2019). Fig 2.2 shows the infarct core surrounded by the penumbra and oligemia and their respective CBF.

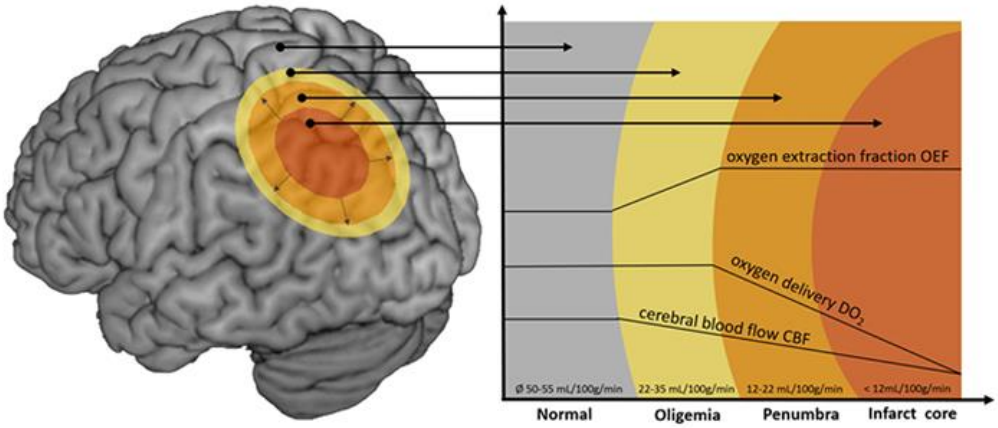


Fig. 2.2 Depiction of the infarct core (red), penumbra (orange) and oligemia (yellow). (Source: Jung et al., 2017)

2.1.3.2 Collateral circulation

When a major intracranial artery is occluded, cerebral collateral circulation or a network of specialized endogenous bypass vessels that include the leptomeningeal anastomoses allows alternative blood flow pathways to sustain the blood flow to the penumbra for some time (Liebeskind, 2003). The extent of the collateral blood flow, however, is variable across individuals based on genetic and environmental factors (Campbell et al., 2019). The ischemic core would be lesser than the territory supplied by the occluded artery for an individual with a good collateral system whereas, in the case of poor collaterals, the ischemic area is equal to the territory supplied by the occluded artery (Jung et al., 2017). Fig. 2.3 illustrates the collateral circulation of the intracranial arteries.

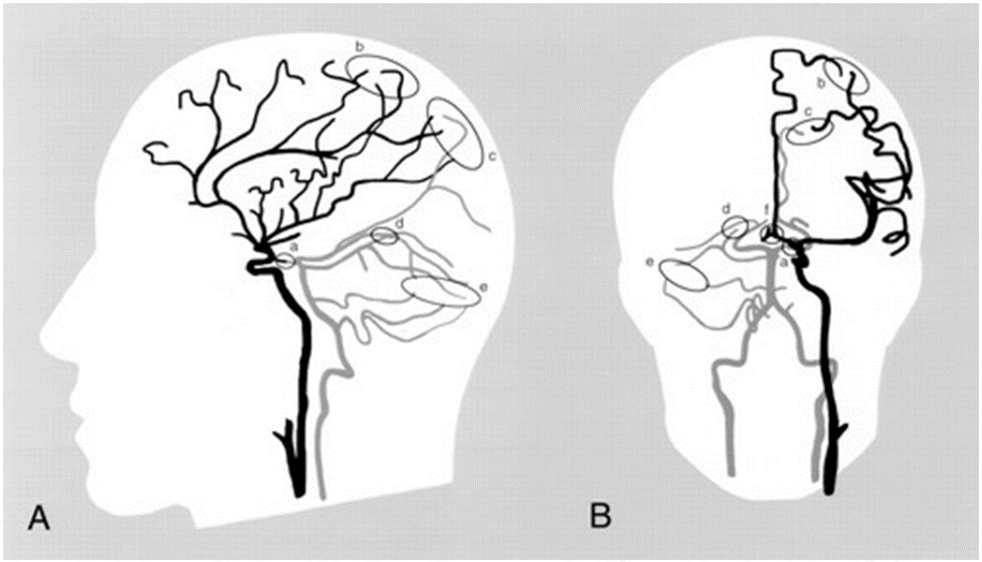


Fig. 2.3 Collateral circulation of intracranial arteries shown in lateral (A) and frontal (B) views depicting the (a) posterior communicating artery, (b) leptomeningeal anastomoses between anterior and middle cerebral arteries, and (c) between posterior and middle cerebral arteries, (d) the tectal plexus between posterior cerebral and superior cerebellar arteries, (e) anastomoses of distal cerebellar arteries, and (f) anterior communicating artery. (Source: Liebeskind, 2003)

2.1.3.3 Ischemic cascade

The pathophysiological mechanisms in response to ischemia occur temporally and spatially spanning from hours to weeks (Brouns and De Deyn, 2009). Temporally, ischemia may be observed in three distinct phases: acute, subacute, and chronic phase (Qin et al., 2022). The basic mechanisms of the ischemic cascade involve energy failure, neurotransmitter-mediated excitotoxicity, oxidative stress, hemostatic activation, cell death, and post-ischemic inflammation (Caplan, 2016). The temporal distribution of the major pathways involved in the ischemic cascade is given in Fig. 2.4.

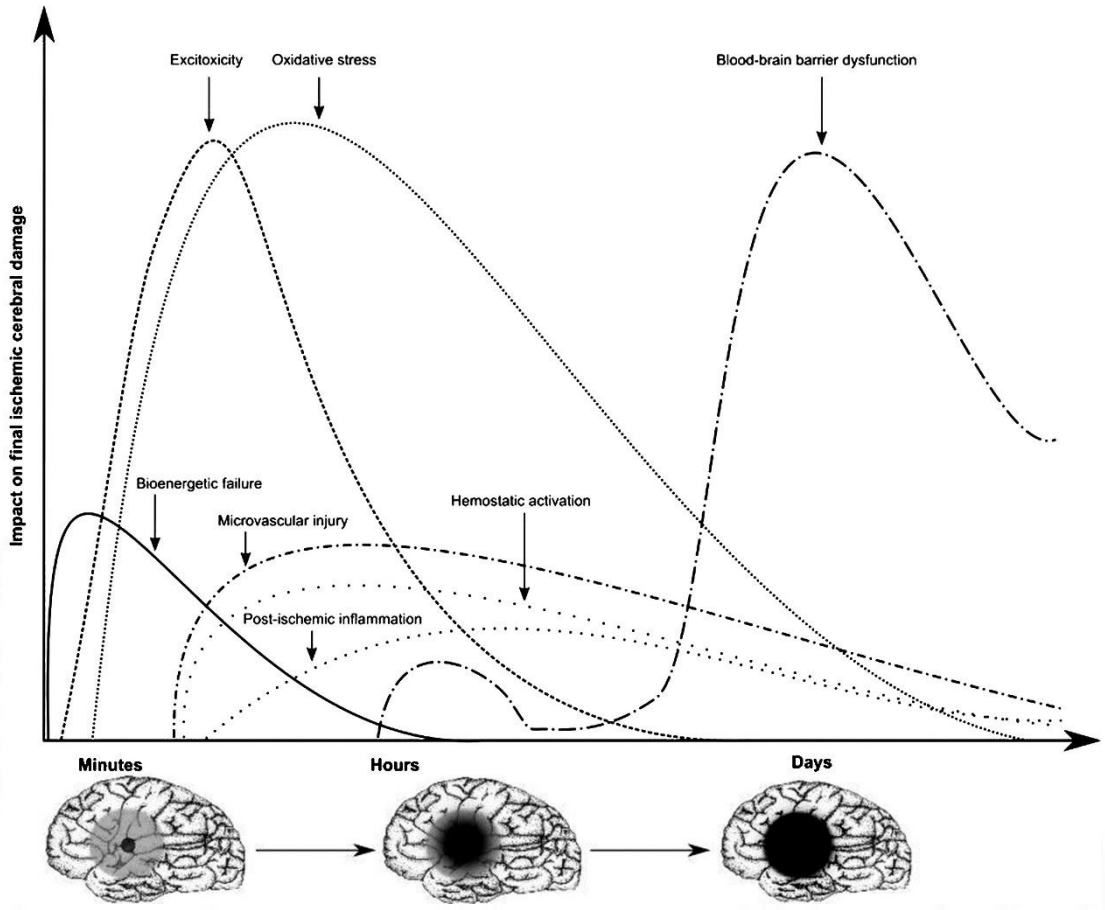


Fig. 2.4 The timeline of the key pathophysiological processes of the ischemic cascade and their effect on the subsequent ischemic damage. (Source: Brouns and De Deyn, 2009)

The extent to which these phases affect the region is a frequent cause of debilitating disease and death (Jovin et al., 2008). Animal studies have shown that oxidative stress may cause secondary damage involving Wallerian degeneration of the corticospinal tracts associated with persisting impairment of motor functions and indicate pyramidal tract damage (Campbell et al., 2019).

i. Energy failure

Ischemic stroke alters the cellular and molecular functions resulting in a dereliction in the cellular function subsequently leading to deficits in oxidative phosphorylation and ATP synthesis during the acute phase of stroke (Campbell et al., 2019; Jovin et al., 2008). With severely depleted levels of ATP, the sodium-potassium (Na^+-K^+ ATPase) pump becomes dysfunctional, and neurons can no longer maintain their transmembrane gradient as it leads to the influx of Na^+ and the release of K^+ ions resulting in the depolarization of the neuronal plasma membrane (Deb et al., 2010). Intracellular pH thereby decreases with the insufficient synthesis of ATP and the increase in anaerobic metabolism and lactate accumulation (Campbell et al., 2019).

ii. Glutamate excitotoxicity

A sudden and progressive anoxic depolarization at the presynaptic terminals resulting from inadequate blood supply leads to untimely neurotransmitter release (Campbell et al., 2019). The dearth of clearing of the excitatory neurotransmitter molecules from the synaptic cleft leads to its accumulation (Deb et al., 2010). This depolarization may often spread to the surrounding regions of the penumbral tissue causing a rise in the metabolic demand and can transform this region into infarction (Brouns and De Deyn, 2009). The glutamate receptor, N-methyl-d-aspartate (NMDA) present on the surface of neurons and glial cells remains open due to the removal of the Magnesium ion by membrane depolarization (Jovin et al., 2008). This causes an influx of calcium ions (Ca^{2+}) (Campbell et al., 2019).

As Calcium pumps malfunction, there is an abrupt rise in intracellular Ca^{2+} levels. This causes the activation of several Ca^{2+}-dependent processes, including the

production of free radicals by neuronal nitric oxide synthase and the induction of apoptosis, necrosis, necroptosis, and autophagy in the cells (Belov Kirdajova et al., 2020). This further depletes intracellular ATP levels by releasing proteases, pro-apoptotic proteins, free radicals, and Ca^{2+} ions into the mitochondrial cisternae (Brouns and De Deyn, 2009). As a result, the cells' reliance on anaerobic glycolysis as their only fuel source increases (Campbell et al., 2019). For the surrounding cells to preserve ion homeostasis and carry out other crucial actions, this is only momentarily accessible which causes the ischemic milieu to accumulate lactate - a by-product of glycolysis - lowering intracellular pH (Kalogeris et al., 2012). The adult brain only has a finite quantity of anaerobic glycolysis energy and time to sustain ion homeostasis and other crucial functions before various events take place that results in brain cell damage and death (Campbell et al., 2019).

iii. Oxidative stress

An unwarranted accumulation of Ca^{2+}, Na^{+}, and adenosine diphosphate (ADP) in cells of the ischemic region cause reactive oxygen species (ROS) formation that target destroying the lipid, protein, nucleic acid, and carbohydrate components of these cells (Qin et al., 2022). A steady state of increase in ROS and reactive nitrogen species (RNS) which are highly reactive formed either enzymatically or non-enzymatically within the cells when the anti-oxidative defense mechanism of the cell becomes overwhelmed (Ramon and Julio César, 2016). ROS is produced within the mitochondrial cisternae, during prostaglandin synthesis, and the degradation of hypoxanthine (Campbell et al., 2019). Oxidative stress is a unified process that occurs during the dysfunction of the mitochondria whereby free radicle formation and NO-mediated oxidation, triggered by the oxidation of essential macromolecules, leads to

cell damage in the early phase of ischemia i.e., within 24 hours from onset (Belov Kirdajova et al., 2020).

iv. Hemostatic activation

Hemostatic activation occurs during the acute phase of stroke, where critical elements exposed to tissue factors cause endothelial damage, resulting in platelet activation, the coagulation cascade, the generation of fibrin, and the suppression of fibrinolytic activity by the carboxypeptidase U enzyme (Brouns and De Deyn, 2009). During ischemia, shear stress causes the platelets to become activated, which is followed by the activation of various biochemical mediators that interact with the coagulation factors (Campbell et al., 2019). Within 72 hours after stroke onset, Factor VIIIa in the blood interacts with membrane phospholipids to synthesize fibrin by activating thrombin, which then polymerizes the fibrin monomers and triggers carboxypeptidase U (Brouns and De Deyn, 2009). Plasmin is subdued by the activity of carboxypeptidase U and platelet activity. The inflammation in the ischemic zone may be made worse by a variety of secondary hemostatic causes. Leukocyte migration across the endothelium may be aided by thromboxane A2 and free radical generation produced by platelets resulting in the "no reflow mechanism," which may help explain why cell damage in the ischemic zone is irreversible (Brouns and De Deyn, 2009). In a study, it was shown that elevated blood levels of the fibrinolysis markers β-thromboglobulin and von Willebrand factor might predict mortality following an ischemic stroke (Carter et al., 2007). Fig. 2.5 illustrates the hemostatic mechanism during stroke.

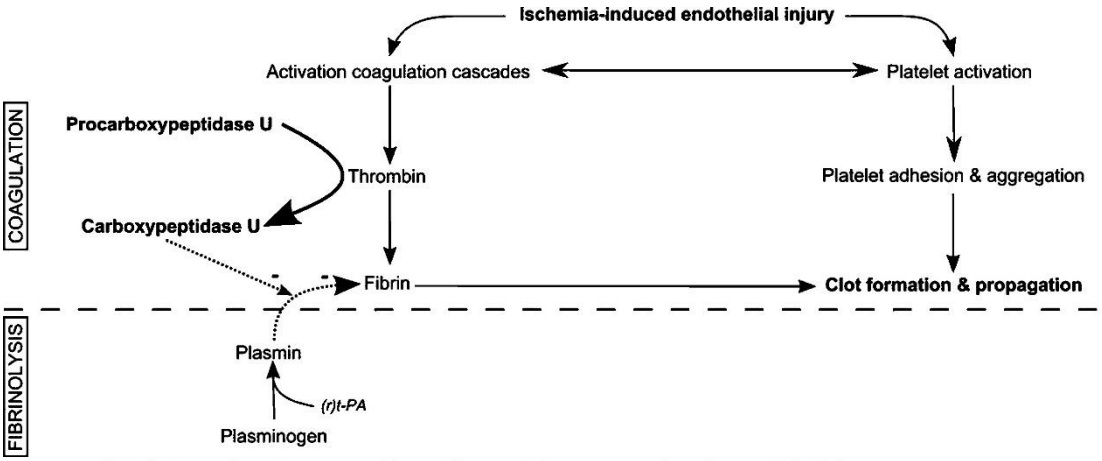


Fig. 2.5 Hemostatic mechanism leads to clot formation. (Source: Brouns and De Deyn, 2009)

v. Cell death

The destabilization of the cell membrane and malfunctioning of organelles collectively lead to four different types of cell death—apoptosis, necrosis, autophagy, and recently emerging data identifying the phenomenon of necroptosis (Belov Kirdajova et al., 2020). Apoptosis may be triggered by the activation of caspase-dependent and caspase-independent pathways primarily in the ischemic penumbra as functional activity of cells may persist due to the presence of a certain level of ATP in the milieu (Ramon and Julio César, 2016). Apoptotic cell death may be brought on by ionic imbalance, the production of ROS, the ligation of death receptors, the activation of lysosomal proteins, and DNA damage (Belov Kirdajova et al., 2020). During the first stage of reperfusion, the executioner caspase, Caspase-3, is cleaved within damaged neurons; this is followed by the release of cytochrome c, which may promote the formation of apoptosomes, boost the synthesis of gelsolin, and release Poly(ADP-ribose) polymerase 1 (PARP1), which increases nucleosomal endonuclease activity and DNA fragmentation (Xing et al., 2012; Deb et al., 2010).

In addition to apoptosis, ischemic cells may experience necrosis, an uncoordinated death in response to extreme stress that often entails swelling of the cells and their accompanying organelles before it ruptures and intracellular debris leaks out (Kalogeris et al., 2012). Necrosis may usually be predominant in the ischemic core due to the extreme deficiency of glucose and oxygen (Ramon and Julio César, 2016). Under certain circumstances, necrosis may sometimes involve coordinated specific signaling mechanisms which have been termed 'programmed necrosis' or necroptosis (Qin et al., 2022). The fourth type of cell death seen during ischemia is autophagy, which is often a cell survival strategy that is usually activated under hunger, hypoxia, or mitochondrial failure, but may also be another tightly controlled event leading to cell death (Kalogeris et al., 2012; Wang et al., 2018).

vi. Post-ischemic inflammation

Apart from neurons, cells of the immune system are activated during ischemia (Campbell et al., 2019). Neuroinflammation plays an important role throughout ischemia, by recruiting a wide range of cells such as neutrophils, monocytes, T cells, and macrophages which infiltrate into the ischemic region (Jayaraj et al., 2019). Recent findings also suggested that innate and adaptive immunity may be involved in stroke pathophysiology (Anrather and Iadecola, 2016). The brain parenchyma is an immune-privileged site involved in a complex interplay between the central nervous system (CNS) and the immune system after an acute stroke (Enzmann et al., 2013). When pericytes are associated with the blood-brain barrier, there is a complex interplay between the CNS and immune system as acute stroke triggers the inflammatory cascade in the ischemic zone of the brain parenchyma (Jin et al., 2010; Jayaraj et al., 2019). The presence of the blood-brain barrier, the lack of cerebral lymphatic vessels,

and the inefficiency of microglia and astrocytes for antigen presentation to T cells may challenge a complete immune response in the brain parenchyma (Chamorro et al., 2012).

The activation of neuroinflammation may exert either beneficial or detrimental effects during ischemia (Shi et al., 2019). The recruitment of neutrophils and their extravasation through the endothelial wall is a multistep process that involves the activation of endothelium by cytokines such as tumor necrosis factor-α (TNF-α) and Interleukin-1β (IL-1β) (Enzmann et al., 2013). Microglia are resident macrophages of the cerebral cortex which when activated, produce cytokines, MMPs, and phagocytose cellular debris. They may play a dual role by also causing secondary cell death in the penumbral region (Jayaraj et al., 2019; Chamorro et al., 2012). On the other hand, its depletion may exacerbate ischemia and neuronal deficits as they essentially promote neurogenesis after ischemia increasing the expression of neurotrophic factors (Jayaraj et al., 2019; Jin et al., 2010). Damage-associated molecular patterns (DAMPs) such as chromatin-associated protein, high mobility group protein B1 (HMGB1), heat shock proteins, and cytokines are highly expressed during this phase (Shi et al., 2019). These mediators use putative pattern recognition receptors which are released from necrotized cells (Enzmann et al., 2013). S100 proteins are activated when neural cells are damaged during ischemia thereby initiating the innate and adaptive immune responses (Rothermundt et al., 2003). The innate immune response begins with the release of circulating neutrophils and monocytes to the site of ischemia whereby they play a major role in modulating tissue repair and inflammation (Anrather and Iadecola, 2016). Fig. 2.6 provides a summary of the pathways involved in the ischemic cascade of stroke.

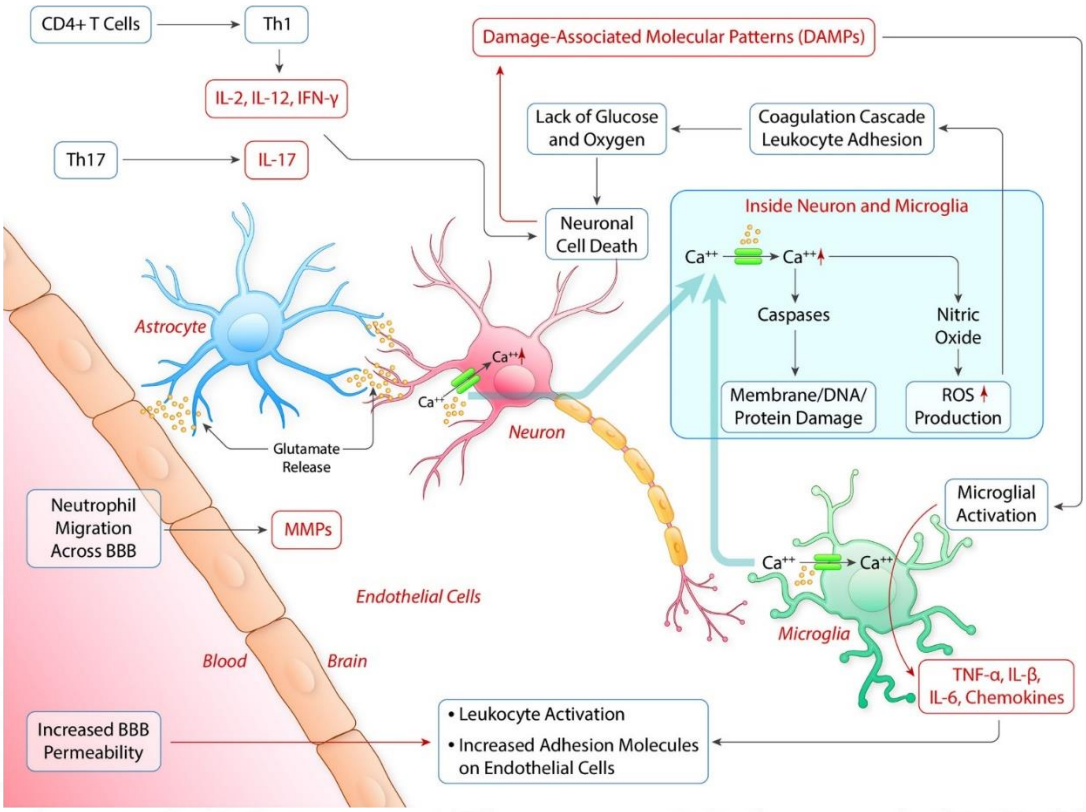


Fig. 2.6 An illustration of the various molecular pathways of the ischemic cascade leading to cell death in the infarct core. (Source: Xiong et al., 2022)

2.1.4 Etiology

A widely adopted system for classifying ischemic strokes is the Trial of Org 10172 in acute stroke treatment (TOAST) classification based on causal subtyping (Adams et al., 1993). The subtypes are diagnosed based on the patient's clinical features, baseline neuroimaging, cerebral angiography, neurosonography, and echocardiography (Caplan, 2016). TOAST classifies stroke into five subtypes: large vessel disease (LVD), cardioembolism (CE), small vessel disease (SVD), stroke of other determined causes, and stroke of unknown causes (Adams et al., 1993). These stroke subtypes vary among each other with the medical management employed, the prognosis, and the risk of recurrence (Chandra et al., 2017b).

i. LVD results from a blockage due to atherosclerotic plaques formed in the major arteries of the brain and is one of the major subtypes of ischemic strokes prevalent in India (Amarenco et al., 2009; Sylaja et al., 2018).

ii. Cardioembolism denotes the presence of a potential intracardiac source of embolism including atrial fibrillation, myocardial infarction, rheumatic heart disease, and other underlying cardiac risk factors (Adams et al., 1993; Campbell et al., 2019).

iii. Small vessel disease is thought to result from pathologies in perforating cerebral arterioles, capillaries, and venules, and the imaging changes including the small subcortical infarcts, lacunes, white matter hyperintensities (WMHs), prominent perivascular spaces (PVS), cerebral microbleeds (CMBs) and atrophy (Caplan, 2015).

iv. Stroke of other determined etiology includes patients with an uncommon cause of strokes such as arteritis, dissection, Moyamoya disease, and vasculitis (Adams et al., 1993; Campbell et al., 2019).

v. Ischemic strokes that occur without a well-defined etiology are categorized under the stroke of undetermined etiology (Adams et al., 1993).

2.1.5 Diagnosis and evaluation

Neuroimaging methods are the mainstay of most diagnostic strategies for the assessment of acute stroke, relying on neuroimaging modalities such as computed tomography (CT) of the brain and magnetic resonance imaging (MRI) (Caplan, 2016; Kim et al., 2014). This is followed by a CT or MR angiography to create high-resolution images of the arteries of the brain that can be used for diagnosis, based on

careful neurologic examination, the severity of the neurologic deficits is quantified by using various scales that have been developed, mostly for use in research investigations (Campbell and Khatri, 2020).

2.1.5.1 Alberta stroke program early CT score

A 10-point quantitative topographic CT scan score called the Alberta stroke program early CT score (ASPECTS) is used for assessing stroke patients in the MCA territory (Pexman et al., 2001; Barber et al., 2000). One point is deducted from the initial score of 10 for every MCA region involved which includes the Caudate, Insular ribbon, Internal capsule, Lentiform nucleus, M1 or the anterior MCA cortex, M2 or the MCA cortex lateral to the insular ribbon, M3 or the posterior MCA cortex, and the anterior (M4), the lateral (M5) and the posterior MCA (M6) territories immediately superior to M1, M2 and M3, rostral to basal ganglia (Barber et al., 2000).

2.1.5.2 National Institutes of Health Stroke Scale

The National Institutes of Health Stroke Scale (NIHSS) score is used for evaluating stroke severity and comprises a 42-point scale for 15 individually appraised components, which has become the “gold standard scale” in clinical trials (Brott et al., 1989; Goldstein et al., 1989). Baseline NIHSS scores are indicators of the clinical outcomes for patients with acute stroke (Goldstein et al., 1989). Patients with minor strokes usually have a score of less than 5 and patients with moderate or severe strokes greater than 5 as shown in Table 2.1.

Table 2.1 The NIHSS scoring range.

NIHSS score range	Deficits
0	No stroke or no deficits
1–4	Minor stroke
5–15	Moderate stroke
15–20	Moderate to severe stroke
21–42	Severe stroke

Note. (Adopted from Hage, 2011)

2.1.5.3 Modified Rankin Score

The Modified Rankin Score (mRS) is a 6-point scale to assess the outcomes of stroke patients (Rankin, 1957; Sulter et al., 1999). It is also a frequently used endpoint in randomized clinical trials and a clinician-reported measure of global impairment and consists of a six-point scoring system (Banks and Marotta, 2007). The scoring system of mRS is given in Table 2.2

Table 2.2 The modified Rankin scale scoring system.

mRS score	Deficits
0	The patient has no residual symptoms
1	The patient has no significant disability; able to carry out all pre-stroke activities.
2	The patient has a slight disability; unable to carry out all pre-stroke activities but can look after themself without daily help.

Table 2.2 The modified Rankin scale scoring system *(contd.)*

mRS score	Deficits
3	The patient has a moderate disability; requiring some external help but can walk without the assistance of another individual.
4	The patient has a moderately severe disability; unable to walk or attend to bodily functions without the assistance of another individual.
5	The patient has a severe disability; is bedridden, incontinent, and requires continuous care.
6	The patient has expired (during the hospital stay or after discharge from the hospital).

Note. (Adopted from Banks and Marotta, 2007).

2.1.6 Treatment and outcome

The treatment strategies for AIS have transformed in recent decades with the introduction of effective recanalization therapies such as intravenous recombinant tissue plasminogen activator (IV rtPA) and EVT or mechanical clot retrieval therapy (Xiong et al., 2022). Apart from these, three other interventions which may greatly improve the clinical outcome in patients with AIS are, (i) Aspirin given within the first 48 hours from onset (ii) the decompressive craniectomy procedure for the treatment of elevated intracranial pressure and (iii) a dedicated stroke unit that manages AIS patients (Dagonnier et al., 2021).

2.1.6.1 Thrombolytic therapy

Reperfusion is pharmacologically administered using rt-PA also known as chemical reperfusion (Bivard et al., 2013). It involves the use of a genetically modified tissue plasminogen activator (tPA) administered within the first 4.5 hours of the onset of stroke symptoms (Hacke et al., 2008). Early recanalization remains the keystone of the treatment of AIS as an improved clinical outcome is associated with accelerated reperfusion (Wardlaw et al., 2014). This treatment showed a better functional outcome at 3 months from onset even among older patients above 70 years and was the first FDA-approved treatment for AIS (Sandercock et al., 2008).

Various thrombolytic drugs have been developed in recent years to improve reperfusion in acute stroke, some of which have shown greater efficacy in clot dissolution (Bivard et al., 2013). In a meta-analysis of nine trials that investigated the efficacy of alteplase, 31% of the patients achieved good outcomes when alteplase was administered within 4.5 hours of onset with substantial improvement in disability scores (Emberson et al., 2014). Recently, Tenecteplase, a genetically modified version of alteplase, has gained popularity for its benefits over alteplase in terms of clot specificity and extended half-life (Campbell et al., 2018).

Despite the efficacy of reperfusion therapies in improving functional outcomes in AIS, a major complication arises in the form of intracranial hemorrhages leading to increased morbidity and mortality (Thomalla et al., 2006). Patients with minor strokes do not benefit from alteplase but have an increased risk of ICH after intervention (Powers 2018). Another major factor that may limit its applicability in AIS is the resistance of the thrombus to the t-PA (Bambauer et al. 2006). Other limitations

include low recanalization rates for large vessel occlusion and the requirement for continuous infusion. Moreover, due to the narrow time window required for the efficacy of tPA, only 10% of patients admitted with AIS may be found eligible for receiving the thrombolytic intervention. Reperfusion therapies will remain the mainstay for AIS and so in the coming years, the main goal would be to improve reperfusion rates and functional outcomes while at the same time reducing the risk of hemorrhage.

2.1.6.2 Endovascular therapy

An endovascular intervention involves the removal of a thrombus under image guidance. Originally established at 6 hours, it has recently been increased to 24 hours from the known onset of symptoms following the criteria of the ischemic penumbra having increased oxygen extraction fraction, a cerebral blood flow of approximately 18–20 ml/100 g/min, and a diffusion (DWI) and perfusion-weighted imaging (PWI) mismatch (Albers, 2018; Camara et al., 2021). Patients brought in after the tPA time window period may be eligible for EVT, which is one of this intervention's main benefits for patients with large vessel occlusion.

According to the HERMES meta-analysis of major trials, early treatment with EVT combined with medical therapy as opposed to medical therapy alone was related to reduced degrees of impairment at 3 months in patients with large vessel occlusions (Goyal et al., 2016). However, a major limitation is that it requires expertise and infrastructure that are not evenly distributed across centers. Furthermore, like thrombolysis, there is a risk of bleeding during the insertion of catheters and guidewires into arteries (Spronk et al., 2021).

2.1.6.3 Medical intervention

Secondary stroke prevention accounts for at least an 80% reduction in the risk of recurrent strokes when initiated in the acute phase (Phipps and Cronin, 2020). Patients who do not qualify for either IV rtPA or EVT are administered dual antiplatelet therapy including Aspirin and Clopidogrel within 24 hours of onset and may be continued on the medication for 21 days to lower their risk of recurrence (Phipps and Cronin, 2020). Lipid-lowering drugs known as statins are 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase inhibitors effective in the primary, and secondary prevention of ischemic stroke even with minimal elevation of blood lipid values when the treatment is initiated (Heart Protection Study Collaborative Group, 2002).

2.2 Hemorrhagic transformation

The early clinical course and functional outcome of a patient are significantly impacted by post-stroke complications such as hemorrhagic transformation (HT), which is reported in 10% to 40% of AIS patients and increases morbidity and mortality (Spronk et al., 2021). HT ensues after an ischemic event and usually develops in the grey matter of the cerebral cortex characteristically resulting from the loss of vascular integrity with clinical manifestations as in the case of a symptomatic HT (Jickling et al., 2014). It is challenging to identify HT in infarcted tissues without neuroimaging, such as CT or MRI, as HT can also develop asymptomatically after AIS, which is not as innocuous as previously believed (Dzialowski et al., 2007). Although HT typically is exacerbated with thrombolytic reperfusion (Hacke et al., 2004), anticoagulant medication (Paciaroni et al., 2018), or EVT (Goyal et al., 2016), it can also occur

spontaneously as part of the natural history of ischemic stroke (Khatri et al., 2007). The duration of occlusion, reperfusion, and vascular risk factors are correlated with an elevated risk of HT caused by thrombolysis (Spronk et al., 2021).

There is much uncertainty regarding the incidence, risk factors, and effect of HT on the outcome of ischemic stroke patients (Paciaroni et al., 2008). Based on the studies conducted so far, it is known that the post-ischemic blood-brain barrier becomes abnormally friable due to the ischemic-reperfusion injury in the infarct region resulting in the extravasation of blood components from the capillary endothelium (Abdullahi et al., 2018; Bernardo-Castro et al., 2020; Kalogeris et al., 2016).

2.2.1 Classification systems

HT is classified depending on the radiographic appearance of the hemorrhage and the existence of concomitant neurological impairment clinical and imaging characteristics (Yaghi et al., 2017). The major clinical trials based on reperfusion therapies classified HT into subtypes based on radiological and clinical criteria, both of which, are widely adopted in clinical studies for assessing HT.

2.2.1.1 Radiological assessment

The European Cooperative Acute Stroke Study (ECASS) is the most widely accepted classification of HT based on radiological standards (Hacke et al., 1998; Hacke et al., 2008). Petechial hemorrhages are classified as hemorrhagic infarction (HI-1 and HI-2) if they appeared as heterogeneous hyperdensity occupying the ischemic infarct zone and parenchymal hematoma (PH-1 and PH-2), which is characterized by a more homogeneous, dense hematoma with mass effect (Hacke et

al., 1998; Fiorelli et al., 1999). HI-1 denotes small hyperdense petechiae along the margins of the infarcts and HI-2 denoted confluent petechiae throughout the infarct regions without any mass effect (Thomalla et al., 2006). Whereas, in PH-1, blood clots are present within 30% of the infarcted area with a certain amount of mass effect; and PH2 denotes more than 30% of blood clots in the infarcted area with a substantial amount of mass effect (Thomalla et al., 2006). The incidence of HI is higher (~9%) than that of PH (~3%) (Pascual-Figal et al., 2016; Zhang et al., 2014). Fig. 2.7 shows the four subtypes of HT as reported by CT imaging.

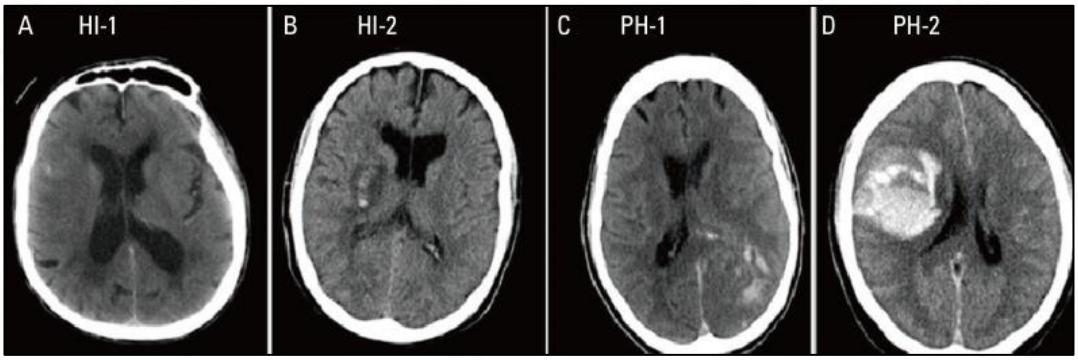


Fig. 2.7 The four grades of hemorrhagic transformation are classified according to the ECASS criteria. (Source: Kim et al., 2014)

Though HI-1 and HI-2 are mostly asymptomatic, PH-1 and PH-2 usually appear to be symptomatic and are associated with adverse outcomes at 3 months, early neurological deterioration, and mortality after ischemic stroke. Whereas HI-1 and HI-2 were not usually associated with the poor outcome although conflicting reports exist (Honig et al., 2022; van Kranendonk et al., 2019). Moreover, HI-2 and PH-1 have been reported to occur in patients who underwent thrombolytic treatment (Berger et al., 2001). A meta-analysis conducted on twenty-seven trials noted an increased rate

(6.1%) of parenchymal hemorrhage type-2 (PH-2) in the alteplase group as compared to 1.3% in the placebo arm (Wardlaw et al., 2014).

2.2.1.2 Clinical classification

HT has been clinically classified as symptomatic and asymptomatic in seminal clinical trials (NINDS rt-PA Stroke Study Group, 1995; MAST Group, 1996). The NINDS study defined symptomatic HT as "any CT-documented hemorrhage that was temporally related to deterioration in the patient's condition in the judgment of the clinical investigator" (NINDS rt-PA Stroke Study Group, 1995). An asymptomatic HT, on the other hand, is characterized by no visible clinical changes observed as a result of the necrotic staining only to be detected at the time of CT imaging (Álvarez-Sabín et al., 2013). A higher incidence of asymptomatic HT in the treatment arm was reported in major IV rtPA clinical trials as given in Table 2.3.

Table 2.3 Rate of HT in major IV tPA clinical trials.

Clinical trial	Sample size	Duration of radiographic follow-up	aHT rate %(N)	sHT rate %(N)	PH-2 rate %(N)	Time to treatment (median); in hours
NINDS	312	7–10 days	4.5 (14/312)	6.4 (20/312)	N/A	1.5
ECASS-II	409	7 days	39.6 (161/407)	8.8 (36/407)	8.1 (33/407)	N/A
ATLANTIS	272	18–30 h	11.4 (31/272)	7.0 (19/272)	N/A	4.36
SITS-MOST	6483	22–36 h	9.6 (617/6438)	7.3 (468/6483)	2.9 (184/6352)	2.3

Table 2.3 Rate of HT in major IV tPA clinical trials *(contd.)*

Clinical trial	Sample size	Duration of radiographic follow-up	aHT rate %(N)	sHT rate %(N)	PH-2 rate %(N)	Time to treatment (median); in hours
ECASS-III	418	36 h	27 (113/418)	2.4 (10/418)	1.9 (8/418)	3.98
IST-III	1515	7 days	N/A	6.9 (104/1515)	N/A	4.2

Note. NINDS, National Institute of Neurological Disorders and Stroke; ECASS, European Cooperative Acute Stroke Study; ATLANTIS, Anti-Thrombotic Strategy After Trans-Aortic Valve Implantation for Aortic Stenosis; SITS-MOST, Safe Implementation of Thrombolysis in Stroke-Monitoring Study; IST, International Stroke Trial; aHT, asymptomatic HT; sHT, symptomatic HT (Reprinted from Sussman and Connolly Jr., 2013).

2.2.1.3 Heidelberg bleeding classification

The Heidelberg bleeding classification was proposed recently during the XII Thrombolysis Symposium on Thrombolysis, Thrombectomy, and Ischemic stroke treatment (Yaghi et al., 2017). The ECASS classification has some limitations, such as the absence of a precise definition for subarachnoid, subdural, or intraventricular hemorrhages, as well as the inability to distinguish between parenchymal hematomas located near or far from the site of infarction (Yaghi et al., 2017). This classification altered the pre-existing subgroups in order to harmonize clinical practice and RCT approaches. For instance, by placing symptomatic and asymptomatic HTs, localization of the infarct and the intraparenchymal, subdural, and subarachnoid ICH topographies were under one roof (Kummer et al., 2015). Whereas the PH2 subtype is categorized as a separate entity due to its effects on the functional outcome and mortality (Kummer et al., 2015). A summary of the classification systems used for HT is given in Table 2.4.

Table 2.4. Classification systems of hemorrhagic transformation.

HT Classification System	Type	Criteria
ECASS	Hemorrhagic Infarction 1 (HI1)	Small petechial hemorrhagic infarction
	Hemorrhagic Infarction 2 (HI2)	Confluent petechial hemorrhagic infarction
	Parenchymal Hematoma 1 (PH1)	Small parenchymal hemorrhage (<30% of infarct, mild mass effect)
	Parenchymal Hematoma 2 (PH2)	Large parenchymal hemorrhage (>30% of infarct, marked mass effect)
NINDS	Symptomatic ICH (sICH)	Increase in the NIHSS by >4 points within the first 36 h of stroke onset
	Asymptomatic ICH (aICH)	Increase in the NIHSS by ≤4 points within the first 36 h of stroke onset
Heidelberg Bleeding Classification	1aHI1	Scattered small petechiae, no mass effect
	1bHI2	Confluent petechiae, no mass effect
	1cPH1	Hematoma within infarcted tissue, occupying <30%, no substantive mass effect
	2PH2	Hematoma occupies 30% or more of the infarcted tissue, with obvious mass effect
	3	Intracerebral hemorrhage outside the infarcted brain tissue or intracranial-extracerebral hemorrhage
	3a	Parenchymal hematoma remote from infarcted brain tissue
	3b	Intraventricular hemorrhage
	3c	Subarachnoid hemorrhage

Note. (Reprinted from Spronk et al., 2021)

2.2.2 Risk factors

Recent research has shown various clinical, imaging, and biochemical factors linked to the inflammatory mechanism and activation of the immune system, such as advanced age, the severity of stroke, hypertension, higher NIHSS scores, atrial fibrillation, admission level hyperglycemia, and cardioembolic etiology, to increase the risk of HT (Yang et al., 2016; Álvarez-Sabín et al., 2013; Thomas et al., 2021).

2.2.2.1 Clinical markers

Demographic factors such as age, gender, and race have been indicated as risk factors for HT but with many discrepancies among studies (Thomas et al., 2021). Advanced age was found to specifically predict PH-2 in many studies. A meta-analysis consisting of 55 studies showed age as a determinant among older patients who had undergone IV r-tPA (Whiteley et al., 2012b). However, the NINDS rt-PA trial there reported no correlation between age and the occurrence of sICH (NINDS rt-PA Stroke Study Group, 1995). This is contrary to the findings in the IST-3 trial that reported elderly patients have a better outcome and fewer occurrences of HT post IV rt-PA as compared to the younger group (Sandercock et al., 2008). Most studies have shown that HT occurs in both men and women in equal proportion although some studies have reported men to be at a greater risk than women (Wen et al., 2020). A recent meta-analysis of 65 studies observed increased rates of the PH subtype of HT in East-Asian patients who had undergone IV r-tPA in comparison to non-East Asian patients (Honig et al., 2022).

Hyperglycemia has a major association with severe forms of HT and was found to predict an unfavorable outcome 3 months from stroke onset (Paciaroni et al., 2009).

The mechanism of hyperglycemia is said to involve hypoxia and necrosis of the arterial wall predisposing to the incidence of HT and as evidenced in animal models, exacerbates thrombo-inflammation of the microvasculature leading to increased blood-brain barrier permeability and infarct volume (Zhang et al., 2014; Desilles et al., 2017)

Stroke severity was an independent predictor of HT in several studies (Whiteley et al., 2012b; Thomas et al., 2021). Paciaroni *et al.* reported that patients with a higher NIHSS score at presentation were at a greater risk of having HT (Paciaroni et al., 2008). The risk of HT was remarkably higher in patients with large vessel occlusions; the larger the size of the infarction the greater incidence of HT often accompanied by cerebral edema leading to vascular compression (Spronk et al., 2021; Zhang et al., 2014). Castellanos et al. found that large infarct volume predicted early HT in AIS (Castellanos et al., 2003).

The risk of sICH is increased by other variables, such as renal impairment, which is indicated by a rise in creatinine levels (>1.0 mg/dL) or estimated glomerular filtration rate after thrombolysis (Marsh et al., 2013). Over 60% of AIS patients have hypertension, and HT may develop as a result of the dysfunction of the cerebral vasculature and endothelium (Spronk et al., 2021).

Hacke et al. reported that in a pooled study of six randomized trials, stroke patients receiving rt-PA treatment had a 5.9% incidence of symptomatic HT compared to a 1% risk in the control groups (Hacke et al., 2004). Tissue plasminogen is responsible for the coactivation of matrix metalloproteinase (MMP) enzyme that is involved in the breakdown of the blood-brain barrier. Kablau and colleagues found

tPA treatment to be the main predictor of HT in their cohort (Kablau et al., 2011). Higher rates of occurrence of severe subtypes of HT namely, PH-1 and PH-2 were reported in thrombolysed patients (Honig et al., 2022). The frequency of PH as reported from the pooled analysis of ATLANTIS, ECASS, and NINDS rt-PA stroke trials given in Table 2.5 indicated that earlier thrombolysis dramatically reduced the rates of PH in patients.

Table 2.5 Frequency of parenchymal hematoma within 6 hours after thrombolytic intervention.

	Placebo		rt-PA	
Onset to treatment (minutes)	N	Patients with parenchymal hematoma (90%, 95% CI)	N	Patients with parenchymal hematoma (90%, 95% CI)
0–90	150	0 (0, ··)	161	5 (3.1, 1.6–5.6)
91–180	315	3 (1.0, 0.4–2.0)	302	17 (5.6, 3.9–7.9)
181–270	411	7 (1.7, 1.0–2.9)	390	23 (5.9, 4.3–8.0)
271–360	508	5 (1.0, 0.5–1.8)	538	37 (6.9, 5.3–8.7)

Note. CI, confidence interval; N, sample size; rt-PA, recombinant tissue plasminogen activator. This data is taken from the review, "Association of outcome with early stroke treatment: a pooled analysis of ATLANTIS, ECASS, and NINDS rt-PA stroke trials" (Reprinted from Hacke et al., 2004).

A history of oral anticoagulant medication prior to having a stroke and EVT has also been linked to HT (Zhang et al., 2014). Atrial fibrillation, which is the major risk factor for cardioembolic strokes, was identified as an independent predictor of spontaneous HT (Tan et al., 2014). Atrial Fibrillation was associated with the occurrence of PH-2 in the ECASS trial (Kablau et al., 2011). Cardioembolic etiology is said to develop higher rates of HT, especially in post-thrombolytic patients. On the

other hand, the Lacunar etiology was least associated with HT (Honig et al., 2022). Patients with a history of congestive heart failure were at a greater risk of developing HT after stroke (Paciaroni et al., 2018). Cardioembolic etiology was found to have a significant propensity towards HT in especially non-thrombolysed patients with cardioembolic strokes who were found to have a greater risk of developing spontaneous HT (Alexandrov et al., 1997).

2.2.2.2 Radiological markers

Leukoaraiosis or cerebral white matter lesions (WMLs) are associated with sICH as intracerebral hemorrhage is one of the manifestations of small vessel stroke, and WMLs are a surrogate sign for this condition (Bivard et al., 2016; Curtze et al., 2015). WML in the chronic state is thought to result from incomplete infarction due to hypoxic hypoperfusion, as the deep white matter is a watershed area, making it susceptible to fluctuations in tissue oxygen levels (Yang and Rosenberg, 2015). Additionally, it is thought that cerebral white matter abnormalities are a relative contraindication to thrombolysis (Curtze et al., 2015).

MMPs produced in blood vessels may play a role in the secondary damage to the white matter, disrupting the blood-brain barrier and causing vasogenic edema (Yang and Rosenberg, 2015). Individuals detected with PH displayed WMLs with Fazekas grade 3 (D'Anna et al., 2021). Brain parenchyma damage from cerebral microbleeds (CMBs), a kind of subclinical bleeding, is caused by cerebral microvascular dysfunction (Wang et al., 2021).

2.2.2.3 Biochemical markers

Biochemical markers include high plasma glucose, thrombocytopenia, low levels of plasminogen activator inhibitor (PAI-1), and low-density lipoprotein cholesterol (Thanvi et al., 2008). A risk factor for HT after thrombolysis for ischemic stroke is thrombocytopenia, therefore, thrombolysis treatment is contraindicated for platelet counts below 100,000/mL (Fugate and Rabinstein, 2015). Lower baseline platelet counts have been identified as independent risk factors for early HT (Zhang et al., 2014). The baseline procoagulant or fibrinolytic states in individuals who are not on any anticoagulants may have an impact on the risk of HI with thrombolysis (Zhang et al., 2014). In one study but not in another, low levels of PAI-I are discovered to be a risk factor for HT (Wang et al., 2015). Low levels of low-density lipoprotein (LDL) were also associated with higher rates of early HT (Kim et al., 2009).

2.2.3 Pathophysiological mechanisms

The pathophysiology of HT is still a matter of discussion due to its complex and multifactorial process (Jickling et al., 2014). HT primarily occurs within the ischemic zone either in the deep grey nuclei or the cerebral cortex due to the presence of dense capillary networks in these regions (Álvarez-Sabín et al., 2013). Nevertheless, it has been proposed that two of the major conditions leading to HT are either preserved collateral perfusion of adjacent vessels or the reperfusion of infarcted tissues which have vessels with increased blood-brain barrier permeability resulting from extravasation of blood components in the ischemic zone (Spronk et al., 2021). In the development of HT, the ischemic cascade which involves metabolic derangements disrupts the blood-brain barrier which is exacerbated by reperfusion of the ischemic

zone as the presence of oxygen in blood leads to the increase in ROS further compromising the vessel permeability (Sussman and Connolly Jr., 2013).

Several perspectives can be used to understand HT-related mechanisms as shown in the schema of the pathogenesis of HT in Fig. 2.8.

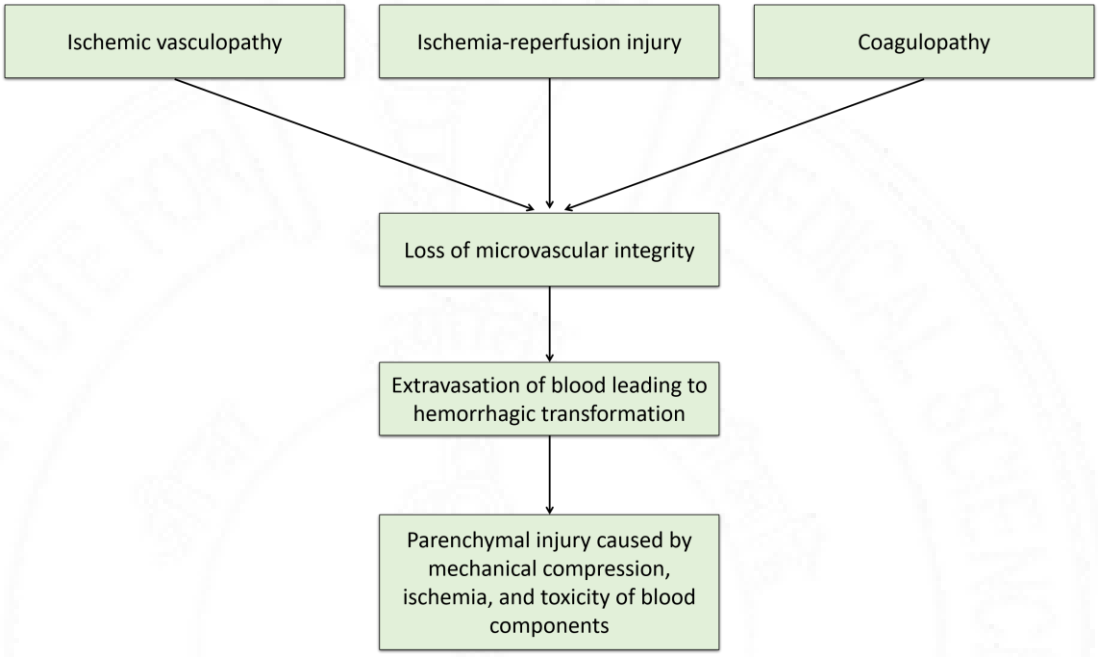


Fig. 2.8 The possible mechanisms leading to hemorrhagic transformation. Adapted from the original figure from Thanvi BR et al. Haemorrhagic transformation in acute ischaemic stroke following thrombolysis therapy: classification, pathogenesis and risk factors. *Postgrad Med J*. 2008 Jul 1;84(993):361-7.

An abnormal permeability of the blood-brain barrier is caused by the dysfunctional endothelium of the capillaries. This may be led by the fragmentation and distal migration of the embolus that cause reperfusion of the ischemia-reperfusion near the ischemic zone. Or it may result in HT occurring at the distal portion of an ischemic

arterial territory by reperfusion via the Leptomeningeal collaterals (Álvarez-Sabín et al., 2013). Whereas studies have also reported HT to be increased from the activation of the collateral circulation into the ischemic territory due to the presence of hypertension (Honig et al., 2022).

Most importantly, the pre-existence of risk factors may also influence the expression of the molecular factors leading to HT (Spronk et al., 2021). Asymptomatic HT occurs within the ischemic zone which is already necrotic, and no visible clinical changes may be observed in the region (Hong et al., 2021). Hence, it requires a high-precision microenvironment essential for the seamless electrical conduction of neurons within the CNS to the spinal cord and its peripheries (Abbott et al., 2010; Reinhold and Rittner, 2017).

2.2.3.1 Blood-Brain Barrier

The impermeability of the blood vessels in the brain is attributed to the unique properties of the brain microvascular endothelial cells, the pericytes, and the astrocyte end-feet that envelope the vessels forming the blood-brain barrier (Haorah et al., 2007; Liddelow, 2011; Liebner et al., 2018; Obermeier et al., 2013). It represents a physical as well as a metabolic barrier, at the capillary level, between the CNS and the endothelium from the peripheral blood circulation protecting the brain from pathogens and macromolecules such as albumin, plasminogen, and pro-thrombin present in the plasma that are damaging to neuronal signaling (Alberts, 2012; Lochhead et al., 2010). It is present at the interface of the cerebral circulation and neuronal tissue by forming a specialized structure around the blood vessels, synapses, and axons critical for the regulation of cerebral blood flow and reliable transmission (Abbott et al., 2010). Other

functions of the blood-brain barrier include the regulated transport of molecules in and out of the CNS, restricted paracellular, and an extremely low rate of transcytotic vesicle diffusion (Wolburg and Lippoldt, 2002).

The structure of the blood-brain barrier is unique that it is composed of specific junctional proteins, basement lamina, transporters, and receptor components that are not only morphologically distinct but functionally different (Sweeney et al., 2018). This is achieved by regulating the trafficking of ions, molecules, and leukocytes across the barrier (Obermeier et al., 2013). Moreover, as the CNS and PNS use the same neurotransmitters, the blood-brain barrier helps keep the central and peripheral transmitter pools separate by minimizing 'cross talk' (Kalogeris et al., 2012). Fig. 2.9 illustrates the cross-section of the blood-brain barrier.

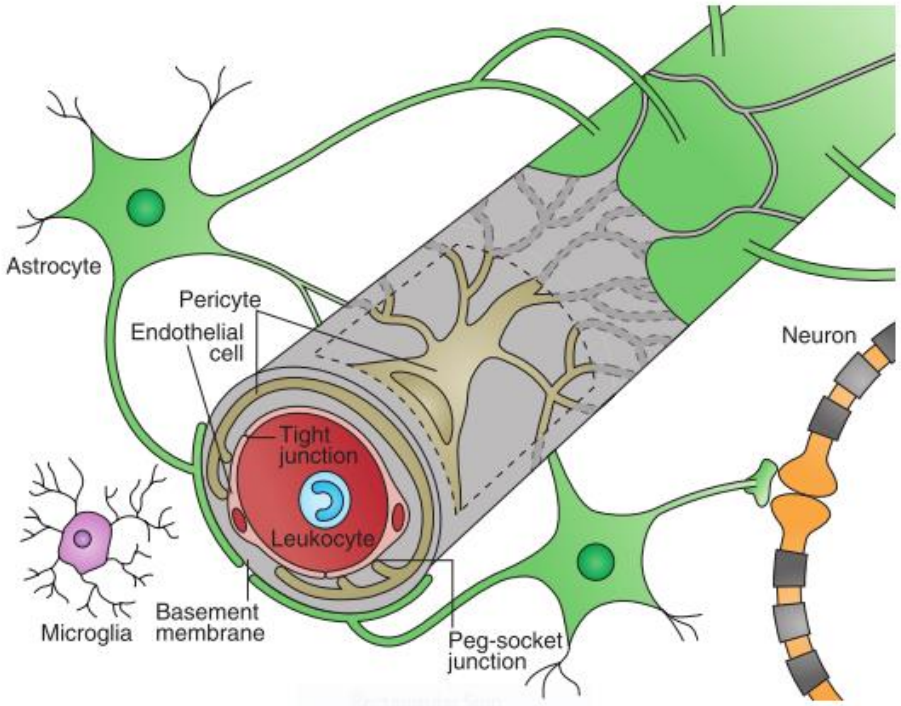


Fig. 2.9 The blood-brain barrier. (Source: Obermeier et al., 2013)

It is crucial in maintaining brain homeostasis and optimizes ionic concentrations by employing specific ion channels and transporters thereby protecting the brain from the dynamic milieu of the blood (Obermeier et al., 2013). The vessel's distinctive lack of fenestrations to limit solute paracellular permeability is due to the presence of specialized proteins at the junctional complex in the paracellular region of the endothelial cells, which includes Tight junctions (TJs), Junctional adhesion molecules (JAMs), and Adherens Junctions (AJs) (Iadecola, 2017). These proteins support the blood-brain barrier's structural integrity by having a strongly associated with the endothelial cells of the barrier (Haorah et al., 2007). The major components of the blood-brain barrier are described below.

i. Basal Lamina

The extracellular matrix (ECM) components including proteoglycans, collagen, laminin, and heparin sulfate are found in the specialized, acellular layer known as basal lamina or basement membrane (Bernardo-Castro et al., 2020). By serving as a framework, anchoring the cellular components, and preserving the cellular cross-talk among them, the basal lamina stabilizes the blood-brain barrier (Bernardo-Castro et al., 2020).

ii. Pericytes

Pericytes, which are found in the basal lamina, are crucial for preserving the integrity of the blood-brain barrier (Bernardo-Castro et al., 2020). By creating a peg-and-socket interface with endothelial cells, they stop leukocyte infiltration into the CNS (Bernardo-Castro et al., 2020). Additionally, they produce certain ECM proteins and use contractile proteins to control capillary width. After the blood-brain barrier

has leaked, they are also known to have phagocytic capabilities comparable to macrophages and to be able to degrade cell debris and erythrocytes (Bernardo-Castro et al., 2020).

iii. Astrocytes

The end-feet of astrocytes completely cover the cerebral capillaries which are already sheathed by the pericytes and functioning as the major glial cells of the blood-brain barrier forming a bridge between the neural system and the cerebral vasculature (Abbott et al., 2006). Astrocytic end-feet show a high expression of aquaporin IV, a protein channel that is critical in maintaining water homeostasis in the CNS (Hawkins and Davis, 2005). Similar to pericytes, the astrocytes' end feet respond to local neurons by regulating the contractility of the cerebral capillaries thereby modulating the CBF (Abbott et al., 2006). Other major functions of astrocytes include the provision of energy substrate, reuptake of neurotransmitters, and synaptic plasticity (Bernardo-Castro et al., 2020).

iv. Microglia

These cells are resident macrophages of the CNS and can detect the primary signs of tissue damage and mediate inflammatory responses and come in contact with the cerebral microvessels (Bernardo-Castro et al., 2020). Microglia are an important source of MMPs, particularly MMP-9, and its activation during HT is directly linked to elevation in the circulating levels of MMP-9 leading to the substantial loss of the ECM components (del Zoppo et al., 2012; Liu et al., 2021).

v. Tight Junctions

The principal tight junction components are involved in cell adhesion thereby providing integrity to the blood-brain barrier comprising the transmembrane adhesion proteins, cytoplasmic scaffolding proteins, and actin cytoskeleton that restrict the passage of polar solutes between the apical and basolateral surfaces of the endothelial cells (Pandit et al., 2020). These include Claudin-5, Occludin, and Zonula occludens-1 (ZO-1) which are associated with the blood-brain barrier. and ZO-1 belongs to the class of membrane-associated guanylate kinase localized within the cytoplasm and is associated with the polymerization of Claudins (Wolburg and Lippoldt, 2002).

a) **Claudin-5:** These tight junction proteins are integral membrane proteins which are a group of proteins that work as primary sealants and are found primarily in the endothelium and are crucial for the maintenance of the blood-brain barrier by forming strong barrier sealing (Weiss et al., 2009). Knockout mice that lacked genes encoding Claudin-5 showed barrier permeation (Nitta et al., 2003). Recently, it was found that Claudin-5 has a promoter function in the activation of pro- metalloproteinase-2 by membrane-type MMP (Miyamori et al., 2001). This may be indicated in angiogenesis and disease pathology affecting permeability (Liebner et al., 2018).

b) **Occludin:** Occludin is also specific for the endothelium that comprises the Brain-nerve barrier (BNB) and regulates and provides a platform for signaling processes Occludin is another tight junction protein with 60-65 kDa with its carboxyl-terminal domain capable of binding with Zonula Occludens-2 (Obermeier et al., 2013). It was the first tight junction molecule to be discovered which is localized in the endothelial tight junctions and functions

in regulating tight junctions (Abbott et al., 2006). Tight junction permeability is determined by the phosphorylation of Occludin domains in a G-protein-dependent or -independent manner (Weiss et al., 2009). Occludin-deficient mice did not show any changes but showed the presence of inflammation and hyperplasia which shows that Occludin plays an important role in the regulation of paracellular permeability rather than contributing to barrier properties (Wolburg and Lippoldt, 2002).

c) **Zonula Occludens**: Scaffolding proteins link Claudins with the actin cytoskeleton employing intracellular proteins called Zonula occludens (ZO) which promote stability to the junctional complex (Bernardo-Castro et al., 2020). Fig. 2.10 depicts the components of junctional proteins in the paracellular region of the blood-brain barrier.

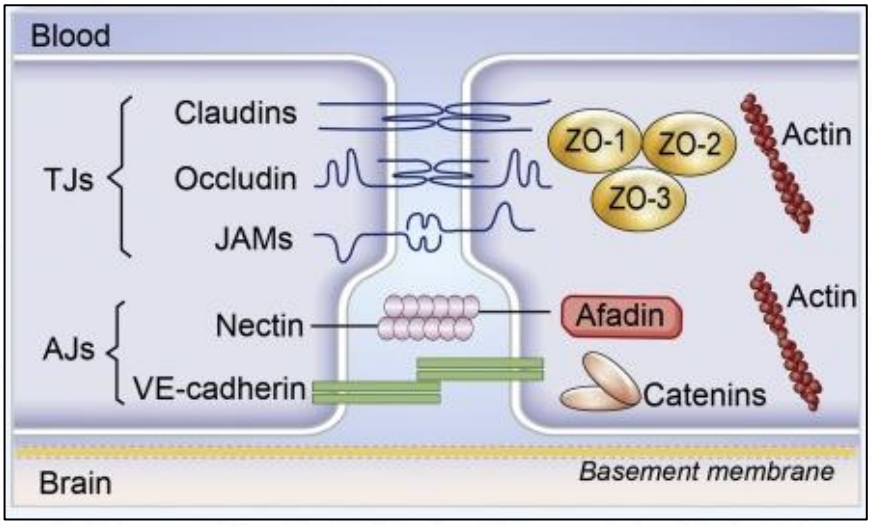


Fig. 2.10 The junctional components promoting paracellular restriction in the blood-brain barrier. Adapted from the original figure from Lv J et al. Focusing on Claudin-5: A promising candidate in the regulation of BBB to treat ischemic stroke. Prog. Neurobiol. 2018 Feb 1; 161:79-96.

d) **Junction Adhesion Molecules:** The junctional adhesion molecules (JAMs) are members of the immunoglobulin subfamily that are present within tight junctions and are involved in tubule formation, leukocyte adhesion, and its transmigration (Bernardo-Castro et al., 2020).

e) **Adherens Junctions:** Adherens junctions promote structural stability to the endothelial cells and tissues and are composed of transmembrane proteins called Cadherins that help in cell adhesion and cytoplasmic proteins called Catenins that are associated with cadherins (Wolburg and Lippoldt, 2002).

f) **Gap Junctions:** Gap junctions are necessary for intercellular communication and consist of paracellular proteins called connexins which form a channel to maintain crosstalk between cells (Bernardo-Castro et al., 2020).

This close association between neuronal and vascular components and their functioning is often termed the neurovascular unit (NVU) as shown in Fig. 2.11.

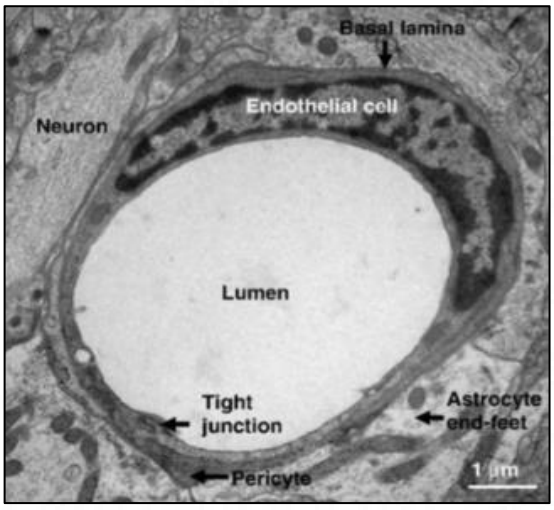


Fig. 2.11 An electron microscope image of the cross-section of the neurovascular unit. (Source: Weiss et al., 2009)

The endothelium, astrocytes, pericytes, neural system, basal lamina, and the ECM collectively form a well-structured, modular organization of the blood-brain barrier which is known as the neurovascular unit (NVU) which helps the brain regulate its blood supply and this functional interdependence is frequently underscored in the pathophysiology of stroke (Hawkins and Davis, 2005; Iadecola, 2017).

2.2.3.2 Ischemia-Reperfusion Injury

The major goal of recanalization is the dissolution of the thrombus leading to the restoration of blood flow. This will help salvage the penumbral region that has not yet progressed to irreversible injury (Enzmann et al., 2013). However, the phenomenon of re-establishing blood supply in hypoperfused areas has a paradoxical effect and may further exacerbate tissue injury in the ischemic zone known as the ischemia-reperfusion (IR) injury (Kalogeris et al., 2012).

This multifactorial phenomenon converts the ischemic brain tissue into hemorrhagic lesions as a result of blood vessel leakage owing to either damage or remodeling of the blood vessels constituting the blood-brain barrier occurs within the region of the infarct and very rarely outside its perimeter (Enzmann et al., 2013). Oxidative stress, leukocyte infiltration, vascular activation, and dysregulated extracellular proteolysis are the primary mechanisms involved in causing IR injury (Wang and Lo, 2003).

Neutrophils may act as precursors by exacerbating tissue injury by infiltrating the blood-brain barrier and releasing proteases leading to oxidative stress in the penumbral region in turn increasing the barrier permeability (Enzmann et al., 2013). Free radical formation plays a major role in cerebral ischemia-reperfusion injury

(Haorah et al., 2007). Although the blood-brain barrier disruption posits one of the major hallmarks of ischemia, hemorrhage in this region is attributed to the increased buildup of ROS and RNS species in the infarct zone (Abdullahi et al., 2018). ROS was shown to induce the activity of MMPs - a family of zinc-dependent endopeptidases that specialize in degrading ECM components leading to the loss of stability of the endothelium (Haorah et al., 2007). This is coupled with the phosphorylation of TJ proteins as part of redox-associated signal transduction and the structural alteration of Occludin which is one of the key components of TJ proteins (Haorah et al., 2007; Lochhead et al., 2010).

MMP-2 and MMP-9, two of the several metalloproteinases in the superfamily, are crucial regulators of blood-brain barrier permeability (Kurzepa et al., 2014; Sarvari et al., 2020). MMP-2 activity is considered the key event in oxidative stress-related injury (Planas et al., 2001). Although both enzymes have diverse substrates, MMP-9 is involved in the proteolytic opening of the blood-brain barrier and the post-ischemic increase in capillaries permeability (Lakhan et al., 2013). The activation of MMP-9 after ischemia was confirmed by animal studies (Planas et al., 2001; Justicia et al., 2003).

MMP-9 is usually present in the inactive, zymogenic form to prevent unwanted proteolysis which is activated during ischemia/reperfusion (Kurzepa et al., 2014). MMP activities are usually inhibited by general protease inhibitors such as α_2-macroglobulin and a family of natural MMP inhibitors, tissue inhibitors of metalloproteinases, or TIMPs (Yang and Rosenberg, 2015).

2.2.3.3 Pleiotropic effects of tissue plasminogen activator

In AIS, thrombolytic treatment with alteplase makes for 14–25% of HT and can also be triggered by other than fibrinolytic causes (Spronk et al., 2021). This includes activation of the immune system by activating neutrophils and PDGF-alpha both of which increase MMP-9 expression. MMP-9 in turn acts on protease-activated receptor 1 (PAR-1) which leads to blood-brain barrier disruption (Spronk et al., 2021). Emerging data suggests that tPA not only functions as a thrombolytic agent but is directly involved in endothelial damage by modulating the immune system and dysregulated extracellular proteolysis leading to HT (Shi et al., 2021). MMP-9 has been produced by microglia in the brain and neutrophils in the blood can both be activated by tPA (Jickling et al., 2014). Degranulation of neutrophils is induced by exogenous tPA which in turn increases MMP-9 and MMP-2 levels while endogenous tPA can operate on endothelial cells to enhance MMP-2 release from astrocytes (Lakhan et al., 2013). MMP-9 is also released from microglia or brain-derived MMP-9, whereby the brain tissue is a primary source of MMP-9 within the first 18–24 hours post-stroke in probable pathways for delayed HT (Ma et al., 2021; Jickling et al., 2014; Hong et al., 2021). In tPA knockout mice, there was a significant decrease in the expression of MMP-9 further proving that MMP-9 is upregulated by t-PA in the brain (Wang and Lo, 2003).

Exogenous t-PA can also act on neutrophils to increase the secretion of MMP-9 which may explain why clinical trials have shown a higher frequency of HT in patients who have undergone thrombolytic treatment (Jickling et al., 2014). Fig. 2.12 shows the ischemia-reperfusion and tPA leading to the activation of MMPs and subsequently leading to the barrier disruption.

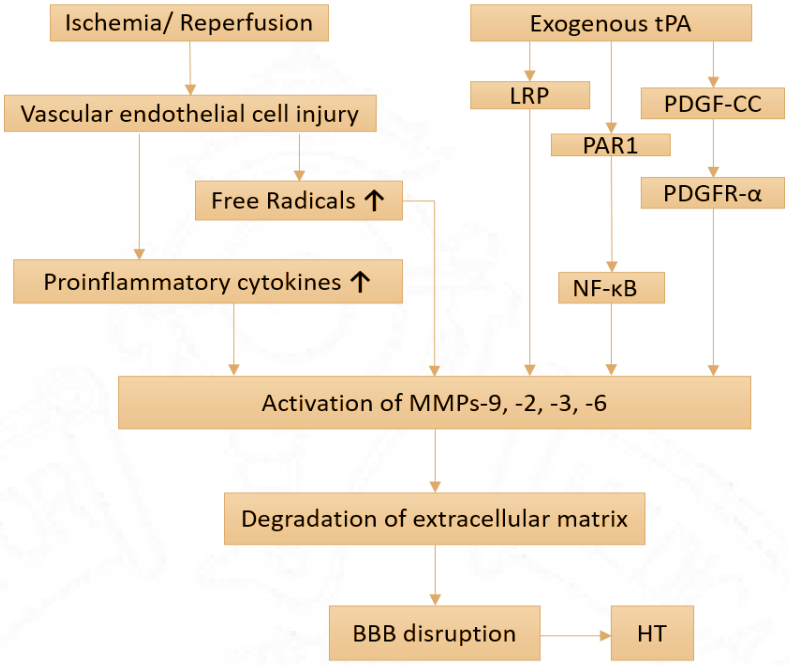


Fig. 2.12 The roles of exogenous tPA and ischemia-reperfusion injury in the blood-brain barrier disruption by activating MMPs. Adapted from the original figure from Lakhan SE et al. MMPs and blood-brain barrier disruption in acute ischemic stroke. Front. Neurol. 2013 Apr 3; 4:32.

Following thrombolysis with rt-PA for acute ischemic stroke, it has been demonstrated that an early rise in fibrin degradation products (FDP) increases the likelihood of developing PH indicating its relationship with tPA (Trouillas et al., 2004).

2.2.3.4 Neuroinflammation

Inflammation is established as a contributor to cerebral infarctions (Ma et al., 2021). Blood-brain barrier disruption and inflammation share a common ground as inflammatory cells when activated lead to the production of ROS and RNS which exacerbate the barrier disruption (Spronk et al., 2021). During reperfusion, more cells

express ROS thereby exacerbating the barrier disruption, especially after thrombolysis. Certain inflammatory components such as nuclear factor-kappa B (NF-κB), MMP-9, Vascular adhesion protein 1 (VAP-1), Neutrophil-lymphocyte ratio (NLR), soluble intercellular adhesion molecule-1 (sICAM-1), high mobility group box 1 (HMGB1), and Toll-like receptor 4 (TLR4) are known to directly cause HT (Shi et al., 2019). Dying cells and necrotic tissue trigger infiltration of peripheral inflammatory mediators by their activation coupled with glial activation in the brain (Campbell et al., 2019).

Neutrophils release ROS and superoxide free radicals that in turn mediate intracerebral vascular damage leading to blood-brain barrier disruption (Ma et al., 2021). The neutrophil-lymphocyte ratio is elevated in patients with HT and with poor outcomes (Guo et al., 2016). Another inflammatory protein known as high-sensitivity C-reactive protein (Hs-CRP) was found to be elevated in patients detected with HT, however, there is heterogeneity in the data owing to the different study populations (Ma et al., 2021).

The interaction of leukocyte-derived MMP-9 with endothelial cell lipoprotein receptor protein (LRP) to raise MMP-3 levels after being stimulated by exogenous tPA and brain-derived MMP-2 activated by platelet-derived growth factor-CC was implicated in the early onset of HT within the first 18 to 24 hours following a stroke (Lakhan et al., 2013). Conversely, ROS, microglia-derived MMP-3, and MMP-9, vascular remodeling factors such as VEGF and HMGB1, and neuroinflammatory mechanisms were primarily involved in delayed HT occurring beyond 24 hours (Hong

et al., 2021; Jickling et al., 2014). Mechanisms of early and late HT are illustrated in Fig. 2.13.

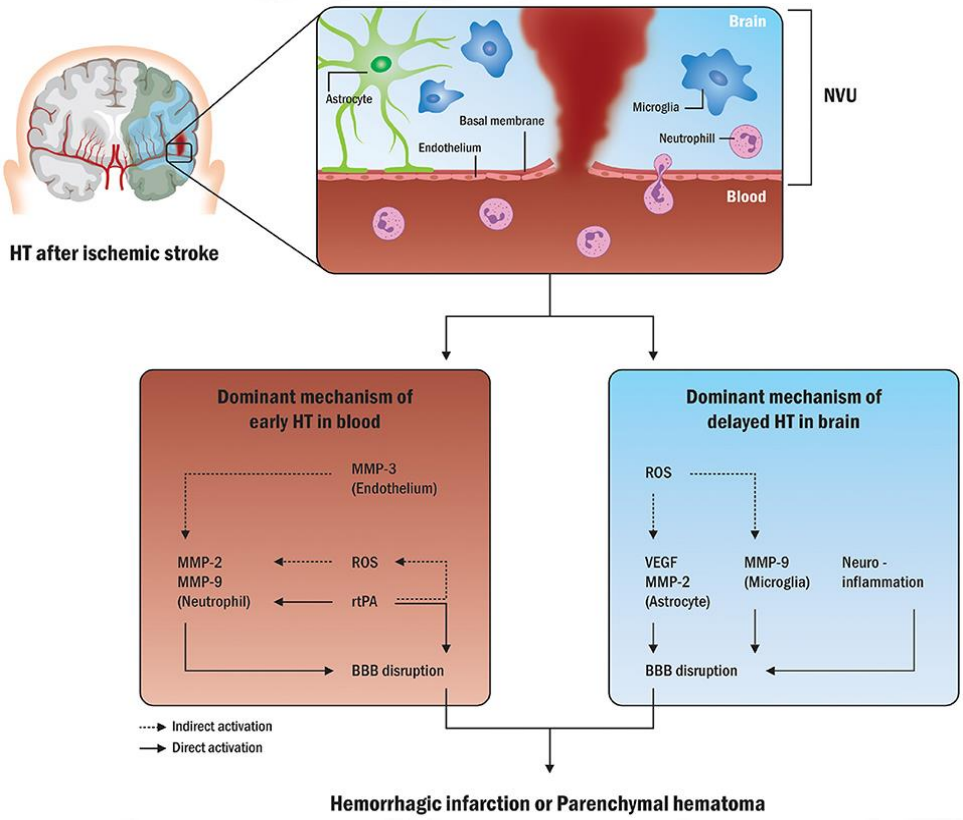


Fig. 2.13 The mechanisms that may be involved in early and delayed HT. (Source: Hong et al., 2021)

2.3 Clinical utility of biomarkers

Biomarker, an acronym for 'biological marker', refers to a biological parameter or an indicator that is accurately quantifiable to determine the nature or the course of a disease condition (Strimbu and Tavel, 2010). The latest definition was given by the U.S. Food and Drug Administration and the National Institutes of Health as part of their joint Biomarkers, EndpointS, and other Tools (BEST) resource as, "a defining characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including

therapeutic interventions" (Atkinson et al., 2001). Thus, biomarkers are precise clinical measurement tools and technologies that typically encompass physiological, molecular, genetic, histologic, and imaging characteristics that can aid in the prediction, diagnosis, progression, and outcome of disease (Selleck et al., 2017). An ideal biomarker yields high reproducibility and may have widespread applications in diagnostics, and drug discovery (Kim et al., 2013). They can also be used in clinical trials for selecting suitable patients and substituting clinical endpoints as surrogate endpoints to ensure the safety of treatments and to guide clinical decisions (Atkinson et al., 2001; Mayeux, 2004).

Proteomics has the potential for the development of biomarkers since the protein domain is most likely the most widely affected in stroke (Campbell et al., 2019). Using biological media, such as blood or cerebrospinal fluid, circulating protein biomarkers are frequently investigated qualitatively and quantitatively using techniques like Western blotting, enzyme-linked immunosorbent assays (ELISA), immunohistochemical labeling, 2D gel electrophoresis, mass spectrometry, which includes the ionization technique matrix aided laser desorption/ionization analysis. (Jickling and Sharp, 2011).

2.3.1 Development of a biomarker

Six crucial process elements include candidate identification, qualification, verification, research assay optimization, biomarker validation, and commercialization of the biomarker (Rifai et al., 2006). The first phase is to identify a potential candidate marker through biomarker discovery, which frequently comprises a binary, basic comparison between samples from patients and healthy controls; this stage may

involve weak credentialing and a high risk of false discovery (Rifai et al., 2006). It is possible to employ a variety of human biological resources, including blood, cerebrospinal fluid (CSF), tissue samples, and model systems like cell lines or animal models (Selleck et al., 2017). During the qualification of the biomarker, it is proven that the divergent candidate expression seen during discovery is also detectable utilizing additional, focused techniques and expression of candidate biomarkers in streamlined comparisons of sick and healthy human plasma samples (Gromova et al., 2020). Both stages give more importance to the marker's sensitivity or the chance that a sample with the disease would test positive than its specificity or the likelihood that an unaffected sample will test negative (Rifai et al., 2006). Sensitivity and specificity are statistical evaluations used for analyzing the diagnostic accuracy of a biomarker and its capacity to discriminate across groups (Califf, 2018). Sensitivity is the capacity to identify a disease in individuals who are suffering from it i.e., a true positive, while specificity is the capacity to exclude the condition in those who are free of it i.e., a true negative (Selleck et al., 2017). Verification of the biomarker involves the expansion of the sample size and the inclusion of cases and controls to test the generalizability of the results to test for assay optimization and that the immunoassay is refined to meet the rigorous standards required for clinical tests (Rifai et al., 2006). The validation process involves confirming the analytical assays by assessing their precision, robustness, accuracy, and detection limit (Mayeux, 2004).

2.3.2 Role of circulating biomarkers in stroke

The molecular mechanisms of the ischemic cascade, reperfusion injury, and blood-brain barrier disruption are the primary focus of circulating biomarkers for

stroke, as the variable expressions of these molecules may serve as significant indicators in the clinical practice of prediction, diagnosis, and prognosis (Maas and Furie, 2009; Dagonnier et al., 2021). This may aid in clinical decisions and urgent treatments may be addressed as deregulated molecules involved in the stroke pathophysiology can be used as therapeutic agents (Montaner et al., 2020). In recent years, several biomarkers have been studied extensively and some of them have found potential applicability in diagnosis, prediction, prognostication, and risk stratification yet, to date, none of them are in clinical use (Gromova et al., 2020).

Various biomarkers in the form of proteins, nucleic acids, and lipids are among the markers that have been discovered and have been reported in stroke. Circulating biomarkers such as the B-type natriuretic peptide (BNP), a vasoactive peptide hormone of cardiac and cerebral origin with natriuretic, diuretic, and vasodilator activity, showed that its elevation in blood above the threshold of 76 pg/mL predicts post-stroke mortality and identified cardioembolic etiology (Montaner et al., 2008). The glial fibrillary acidic protein (GFAP), an intermediate filament protein present in the cytoskeleton of astrocytes, had remarkable specificity and sensitivity for a cutoff value of 2.9 ng/L for the identification of ICH in acute stroke (Foerch et al., 2006) and for differentiating between ischemic stroke and ICH (Bustamante et al., 2021). The biomarkers associated with various pathways during ischemia that are currently relevant to stroke are listed in Fig. 2.14.

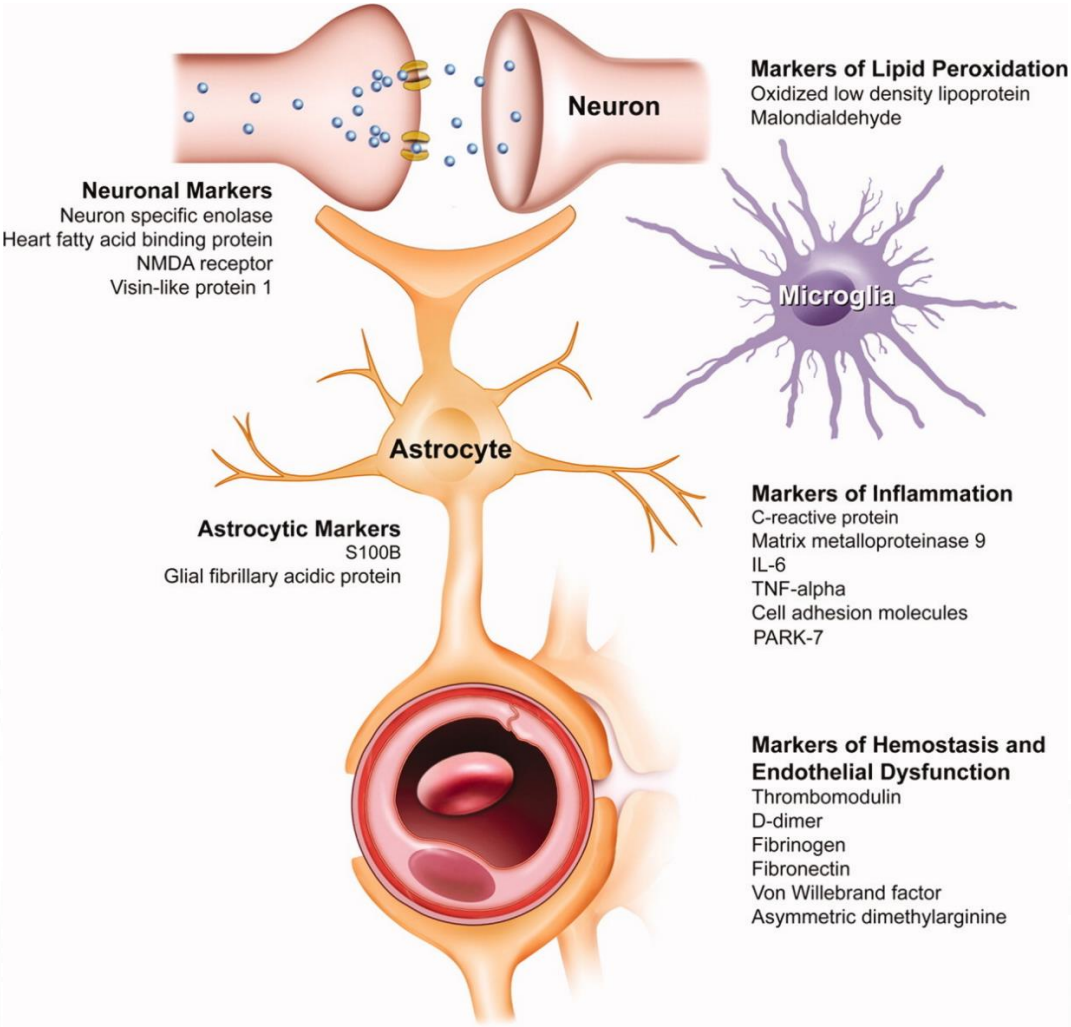


Fig. 2.14 Biomarkers of stroke. (Source: Kernagis and Laskowitz, 2012)

Biomarkers belonging to the inflammatory phase of ischemia such as interleukin-6 (IL-6) have been implicated in plaque severity (Kamtchum-Tatuene et al., 2022). High sensitivity C-reactive protein (hs-CRP), and the vascular inflammatory marker, lipoprotein phospholipase A_2 (Lp-PLA_2), which are involved in the early pro-inflammatory phase during ischemia, have been associated with prognosis (Elkind et al., 2006), recurrence (Elkind et al., 2009; Krishnamoorthy et al., 2021), stroke severity and long-term outcome (Elkind et al., 2009), respectively.

Synaptic NR2 peptide proteolytic degradation caused by NMDA-r autoantibodies NR2A/NR2B subunit Ab (97% sensitivity and 97% specificity) by thrombin-activated serine proteases NMDA-R diagnosis of ischemic stroke (Dambinova et al., 2012). Among the panel of biomarkers evaluated, S100B, MMP-9 and D-dimer were able to differentiate between stroke subtypes and strokes from stroke mimics (Lynch et al., 2004; Laskowitz et al., 2009). Increased serum level of D-dimer was also implicated in fibrin degradation products (FDP) in AIS patients with large artery occlusion within 6 hours of onset independent of the time of recanalization (Skoloudík et al. 2010).

Despite the substantial amount of research, there are presently no recommendations for the use of biomarkers in treatment planning for stroke patients (Jickling and Sharp, 2011). The development of a suitable biomarker panel entails simultaneously assessing many biomarkers, which would increase the diagnostic precision and time-sensitive diagnostic information for the treatment of stroke (Montaner et al., 2020). Biomarkers, however, can only be used to predict events in groups where they are useful, and the diverse results of putative biomarkers across studies frequently restrict their application in clinical practice (Jickling and Sharp, 2011). Despite these drawbacks, biomarkers are an essential part of personalized therapy, hence investigating stroke biomarkers should continue to remain a top priority.

2.3.3 Biomarkers related to hemorrhagic transformation

Proteins associated with causal pathways leading to HT including the blood-brain barrier disruption, neuro-inflammation, oxidative stress, angiogenesis, and coagulation pathway, have been studied as potential biomarkers for risk prediction

(Spronk et al., 2021). One of the most extensively researched biomarkers is MMP-9, whose elevated blood levels were strongly linked to the PH subtype and HT caused by thrombolytic therapy (Montaner et al., 2001; Montaner et al., 2003; Castellanos et al., 2003). When MMP-9, a neutrophil, and microglia-derived protein, is activated during neuroinflammation and in the presence of tPA, it greatly affects HT and contributes to the breakdown of the blood-brain barrier (Vafadari et al., 2016; Jickling et al., 2014). Rosell et al. showed an increased expression of neutrophil-borne MMP-9 within the area of HT in infarct tissue samples and microvessels of fatal ischemic strokes with hemorrhagic complications (Rosell et al., 2008).

Baseline serum levels of S100 Calcium-binding protein B (S100B) secreted by astrocytes, is a classic blood-brain barrier breakdown protein that was found to be an independent predictor of HT in AIS (Foerch et al., 2007). Kazmierski *et al.* investigated tight junction proteins and found increased levels of Occludin and the Claudin-5/ZO-1 ratio associated with spontaneous HT (Kazmierski et al., 2012). Degradation of these tight junction proteins is frequently observed in ischemic stroke and their presence in the blood can be correlated to the degree of blood-brain barrier damage.

Proteins involved in the coagulation pathway, endothelial dysfunction, and inflammatory mechanism have also been found to be positively correlated with HT. Fibrin degradation product (FDP) and thrombin-activated fibrinolysis inhibitor (TAFI) are involved in the coagulation pathway and have shown increased levels in the serum of patients with HT (Ribo et al., 2004). FDPs are components of the blood produced by clot degeneration and were found elevated in patients who developed PH post-

thrombolysis (Trouillas et al., 2004). At baseline, increased levels of the protein Thrombin activatable fibrinolysis inhibitor (TAFI), which is thought to represent the molecular connection between coagulation and fibrinolysis, were associated with the development of sICH in AIS post-thrombolysis (Ribo et al., 2004). C-Fibronectin is indicated in endothelial dysfunction and accumulates in the region of cerebral hemorrhage and it has been positively correlated with parenchymal hemorrhage after stroke (Castellanos et al., 2004). Recently, Interleukin-33 and its decoy receptor, soluble serum stimulation-2 (ST2) have been implicated in HT and as prognostic markers (Chen et al., 2021). Soluble ST2 is an inflammatory protein that has been indicated as a marker primarily for cardiovascular diseases and was found to predict HT, outcome, and mortality after stroke, independent of cardiovascular risk factors (Wolcott et al., 2017).

Although biomarkers offer many benefits, the disparity in study findings due to heterogeneity of data, inadequate sampling frame, and variability in the methodologies across studies have made validation of these markers uncertain in a clinical setting (Jickling and Manolescu, 2012). For instance, even though many studies have consistently reported the predictive value of a specific marker, some studies find no association between the same marker and HT. Variability in sampling also contributes to this disparity as these molecules exhibit a temporal pattern of expression during HT . Taking into account the causes of variation in the measurement of a biomarker, the possibility that the exposure will be misclassified may be reduced.

3 MATERIALS AND METHODS

The chapter outlines the methodology in two parts. Part one begins with the methodology used to conduct the systematic review and meta-analysis which included a description of the inclusion and exclusion criteria, the search strategy employed followed by the procedure for data extraction, and statistical analysis.

Part two follows the second sub-objective by evaluating the biomarkers for the risk of HT and the second objective which investigates the role of the biomarkers in predicting short-term functional outcomes in AIS. A prospective, observational study was carried out to investigate the association of circulating MMP-9, Claudin-5, soluble ST2, Occludin, and S100B levels with HT and stroke severity in AIS. The eligibility criteria of the participants enrolled, intervention, study design, and short-term functional outcome in AIS are given.

3.1 PART 1: Systematic review and meta-analysis

The study protocol followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement and was filed in the PROSPERO database with the registration number CRD42020201334.

3.1.1 Inclusion and exclusion criteria

3.1.1.1 Participants

The articles that were selected included patients with a first-ever ischemic stroke and who were 18 years of age or older.

3.1.1.2 Intervention

Patients who underwent IV-tPA, intra-arterial thrombolysis (IA-tPA), EVT, or bridging therapy were included in the review.

3.1.1.3 Study design

Studies that were qualified included observational, cross-sectional, and case-control studies with full-text publications available in the English language. Conference proceedings and abstracts were excluded. Studies that did not offer sufficient information during the screening of the full-text publications, regarding the diagnostic information of its recruited subjects, the detection of HT, and the evaluation of biomarkers were excluded.

The blood samples taken at baseline within 24 h of the stroke's occurrence were used for the biomarker evaluation. Articles were considered if they were either single or serial assessments of the marker. Research on biomarkers based on gene expression, microRNA, metabolomics, tissue biopsies, cerebrospinal fluid, or any other bodily fluid outside blood was not included in this study.

3.1.1.4 Outcome

The following outcome measures were used to evaluate the biomarkers: (i) baseline biomarker comparison between HT and non-HT patients; (ii) estimation of biomarker levels among HT subtypes; (iii) association of biomarker levels in HT post-intervention; and (iv) functional outcome at 90 days after onset using the mRS score. HT was verified by a brain CT or MRI imaging, and the publications contained information about the baseline and follow-up CT or MRI results.

3.1.2 Operational Definitions

Symptomatic HT was defined as any patient with clinical worsening within 48 hours, HI, or PH on follow-up neuroimaging, and a rise of more than 4 points on the NIHSS score (Hacke et al., 1998). Asymptomatic HT as neuroimaging-documented bleeding without neurological deterioration with an increase of <4 points in the NIHSS score (Berger et al., 2001). Symptomatic ICH was defined the as per the NINDS definition, indicating the presence of clinical deterioration in NIHSS ≥4 points or death within 7 days along with any type of intracerebral hemorrhage on post-intervention imaging (NINDS rt-PA Stroke Study Group, 1995).

3.1.3 Systematic Search Strategy

The search criteria involved screening for eligible studies using the Cochrane Library (CENTRAL), MEDLINE using PubMed, and Web of Science databases with keywords and search strings for articles published in English between 01 October 2000 and 30 November 2020. The title and abstract screening of all eligible articles were conducted to ensure that only studies that were found to be germane were included after the data extraction. The full-text screening was carried out for the final articles included in the review.

3.1.4 Risk of Bias in Individual Studies

The Quality Assessment of Diagnostic Accuracy Score-2 (QUADAS-2) instrument, which has four domains based on patient selection, index test, reference standard, and flow and time, was used to determine the risk of bias and applicability

issues (Whiting et al., 2006). The selected studies were evaluated using the questionnaire available under this tool (Bristol Medical School, 2002).

We assessed the methodology's suitability using the Statement: Guidelines for Reporting Observational Studies (STROBE) criteria (von Elm et al., 2008).

3.1.5 Statistical Analysis

Statistical analysis was conducted for the meta-analysis of the selected studies. These descriptive statistics for the research comprised sensitivity, specificity, diagnostic odds ratio (DOR), false-positive rates, and likelihood ratios of individual investigations. Chi-square tests were used to determine whether sensitivities and specificities were equivalent. The DerSimonian-Laird estimator was used in the univariate analysis for the pooled diagnostic odds ratio estimations. The variance parameters were estimated using Cochran's Q statistic. Hierarchical Summary Receiver Operator Characteristic (HSROC) curves were created using a hierarchical bivariate model, which is a linear mixed model with known variances of the random effects. Restricted maximum likelihood was used to estimate the variance components (REML). Deeks' funnel plots were created to estimate any publication bias. The funnel plot asymmetry was quantified statistically using weighted regression with a multiplicative dispersion model utilizing standard error and sample size as variables. All analyses were carried out using RevMan 5.0.3 and R version 4.0.2 (*mada* and *metafor* packages) software.

3.2 PART 2: Assessment of Biomarkers in HT and Outcome

3.2.1 Study Design

The study had received ethical approval to be conducted at Sree Chitra Tirunal Institute of Medical Science and Technology, a tertiary care and academic medical center in Trivandrum, Kerala, India, and the study protocol was approved by the institute's ethics committee on human research. Each patient had provided their written informed consent before being enrolled in the study.

3.2.2 Eligibility Criteria

Consecutive patients included in the study were: (i) 18 years of age or older, (ii) first-ever ischemic stroke, (iii) admitted within 24 hours of symptom onset, and (iv) absence of HT on baseline brain CT or MRI. Patients were excluded if (i) serum creatinine levels >2 mg/dL at the time of admission, (ii) were on anticoagulants (iii) stroke or TIA within the past 3 months, (iv) absence of central nervous system diseases, (v) signs of concomitant infection and systemic inflammation or, (vi) any malignant diseases. Patients who underwent intravenous thrombolysis, endovascular treatment, or bridging therapy as well as those who were not thrombolysed were recruited following admission at the comprehensive stroke unit of our institute.

3.2.3 Data Collection

Patient demographics, vascular risk factors, medical history, and diagnostic workup were collected through the course of hospital admission. Etiological classification of stroke subtypes was done using the TOAST classification (Adams et

al., 1993). The stroke severity at the time of admission and discharge was documented using the NIHSS score. The details of the patients who underwent reperfusion therapies were also recorded.

3.2.4 Imaging Modalities

CT or MRI was performed on all patients upon admission and at 24-48 h after admission. HT was documented using CT or MRI imaging and was defined according to the ECASS III criteria (Hacke et al., 2008). HT detected within 48 h was graded as either HI or PH based on the follow-up neuroimaging diagnosis. Symptomatic ICH was defined as the clinical and neurological deterioration of greater or equal to 4 points on the NIHSS from baseline or leading to death within 24 h.

3.2.5 Sample Collection

Plasma samples of patients admitted to the stroke unit were taken after overnight fasting for analysis of routing blood tests which included fasting blood glucose (FBS), and lipid profile estimation.

Plasma samples were evaluated at three time points: (i) at the time of admission or before revascularization for patients who underwent intervention, (ii) 12 hours, and (ii) 24 hours from stroke onset. Six patients who were admitted 12-14 hours from the onset had their baseline samples taken at the 12 hours timepoint. EDTA Plasma was separated by centrifugation at 2,500 rpm for 15 minutes and stored as aliquots at -80˚C until analysis for the biomarkers was conducted.

3.2.6 Biochemical Estimation of Risk Factors

3.2.6.1 Fasting Blood Glucose (FBS)

The FBS of patients admitted to the stroke unit was measured following the manufacturer's protocol using the GLU FLEX® reagent cartridge (Siemens, USA) in the Dimension® Clinical Chemistry auto-analyzer with 70-110 mg/dL as the standardized reference range.

3.2.6.2 Estimation of Lipid Profile

The lipid profile estimation comprised the total cholesterol, Triglyceride, High-Density Lipoprotein-Cholesterol (HDL-C), Low-Density Lipoprotein-Cholesterol (LDL-C), and total cholesterol was estimated in serum by the cholesterol esterase-cholesterol oxidase-peroxidase method using CHOL FLEX® reagent cartridge (Siemens, USA) in Dimension® Clinical Chemistry autoanalyzer according to manufacturer's protocol. Less than 200 mg/dl of blood is considered the normal range for total serum cholesterol.

Triglyceride in serum was quantitated by the enzymatic method using TGL FLEX® reagent cartridge (Siemens, USA) in Dimension® Clinical Chemistry auto analyzer according to the manufacturer's protocol. Normal range of serum Triglycerides: 30-150 mg/dL. HDL-C in serum was determined by the enzymatic method using the AHDL FLEX® reagent cartridge in Dimension® Clinical Chemistry autoanalyzer according to the manufacturer's protocol. For males, the typical HDL-C range was >40 mg/dl, and for females, >50 mg/dl.

LDL-C in serum was derived indirectly using the Friedewald equation (Friedewald et.al., 1972) LDL-C= Total Cholesterol- (VLDL-C+HDL-C, (where

VLDL-C =Triglycerides/5). The normal range of LDL-C was considered less than 100 mg/dL.

3.2.7 Analysis of Blood Biomarkers

Each marker was assessed to determine its cut-off values in each group and was evaluated by commercially available enzyme-linked immunosorbent assays (ELISA). MMP-9 was assessed using the Quantikine ELISA Human Immunoassay kit (DM900; R&D Systems, MN, USA). Soluble ST2 levels were determined using the Duoset ELISA kits (DY523B-05; R&D Systems, MN, USA), and Claudin-5 was assessed using the Cusabio ELISA kits (Cusabio Technology LLC, Houston, TX, USA). The absorbance of the analytes was measured at 450 nm with the correction wavelength set at 540 nm using a microplate reader (Biotek ELX 800).

3.2.8 Statistical Analysis

The sample size was calculated based on the assumptions for one of the biomarkers namely, MMP-9 as 75% and 24.6% above the cut-off value as reported in previous literature. To achieve a power of 90% with an alpha error of 5%, the minimum sample size required was approximately 25 each. By adjusting for four to five confounding variables, another 50 samples, 25 in each group, were added to get a minimum of 100 patients. Continuous variables were expressed as means and standard deviation (SD) or medians and interquartile range (IQR) based on their distribution, and categorical variables were given in percentages. The predictive discrimination of each marker was first analyzed by univariate analysis. Pearson's Chi-squared test, Wilcoxon rank sum test, and Fisher's exact test were conducted for bivariate analyses.

Pearson Product–Moment correlation was conducted to determine the relationship between the variables and biomarkers levels associated with poor outcomes. Based on the intercorrelation of these markers and statistical significance, a multiple logistic regression model was created using covariates identified as statistically significant in bivariate analyses. Separate models were developed for the three time points of marker sampling. Receiver operator characteristic (ROC) curves and area under the curve (AUC), in which sensitivity was plotted as a function of (1-specificity), were used to identify optimal cut-off levels and compare predictive accuracies of the markers. The logistic regression model including the biomarkers that provided good discriminative capacity to predict HT and functional outcome was finally included. A probability value less than 0.05 was considered to be statistically significant.

4 RESULTS

The chapter begins with a detailed account of the outcome of the literature search including a PRISMA diagram outlining the results of the systematic review and meta-analysis of Part 1 of the thesis work. A description of the eligible studies and the study quality is also given. The findings from the meta-analysis and assessment of publication bias are also reported. Part 2 of the chapter presents the findings of the evaluation of the biomarkers at three specific time intervals with the aim of answering the primary research question regarding its association with HT. The findings of the biomarkers related to the functional outcome of AIS are outlined as part of the secondary research questions.

4.1 PART 1: Systematic Review and Meta-analysis

4.1.1 Study Selection

Based on the keywords, the literature search, which was completed on August 20, 2020, had preliminarily identified a database of 2230 articles. During the screening for titles and abstracts, 2175 articles were removed from the list as duplicates and studies that were not relevant to the review. Of these, 30 quality-appraised articles were chosen for full-text review and were determined to be admissible. The PRISMA flow diagram of the screening of articles were shown in Fig. 4.1.

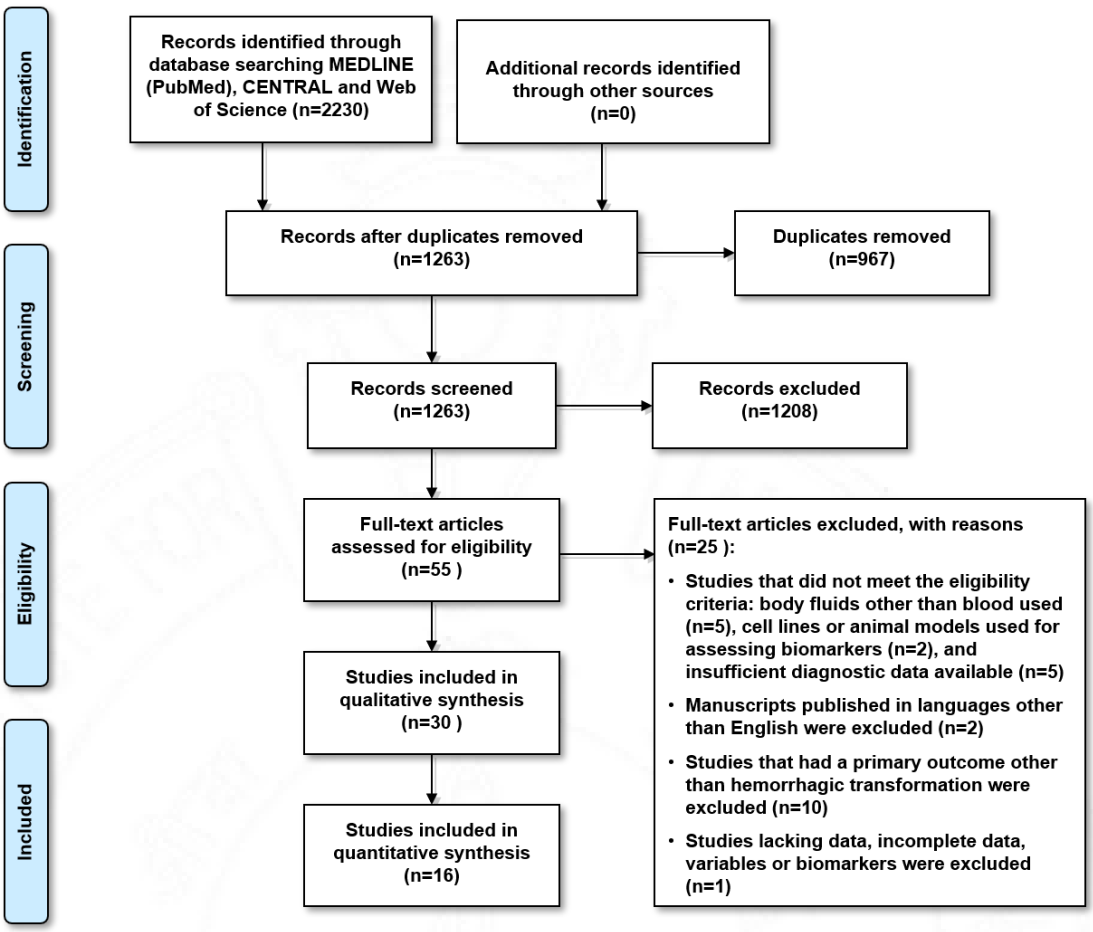


Fig. 4.1 The PRISMA flow diagram indicating the process of study screening through to the final selection of articles.

4.1.2 Study Characteristics

The studies that were deemed suitable had a total sample size of 9751. The baseline characteristics of these studies are given in Table 4.1.

Table 4.1. Basic characteristics of included studies.

Author of the study	Place	Study design	Sample size (n)	Mean age±SD (in years)	Males (n)	Interventio n(s)	First CT/MRI (in hours)	Follow-up CT/MRI (in hours)	Groups (n)	Biomarker
Montaner et al. 2003	Spain	P	41	70±10.6	16	IV tPA	≤ 3	< 36-48	sHT = 15; non-sHT = 26	MMP-9
Eman El-Banhawy et al. 2014	Egypt	P, case-control	70	60.9±9.37	30	IV tPA	≤ 24	> 48	sHT = 8; non-sHT = 62	MMP-9
Kazmierski et al. 2012	Poland	P	458	68±12.5	247	none	≤ 3	< 24	cdHT = 33; non-cdHT = 425	MMP-9, s100B, OCLN, CLDN5, ZO-1, VEGF, NSE
Yuan et al. 2018	China	P, case-control	208	66.7±13.1	90	none	≤ 24	< 24-36	sHT = 29; non-sHT = 139	MMP-9
Molnar et al. 2018	Hungary	P	54	HT=71±7.8; non-HT= 66±10	32	IV tPA, EVT	≤ 6	< 24-36	sHT = 9 ; non-sHT = 45	CCCK-18

Table 4.1. Basic characteristics of included studies *(contd.)*

Author of the study	Place	Study design	Sample size (n)	Mean age±SD (in years)	Males (n)	Interventio n(s)	First CT/MR (in hours)	Follow-up CT/MRI (in hours)	Sample groups (n)	Biomarker
Castellanos et al. 2007	Spain	P	134	67±12	88	IV tPA	≤ 3	< 24-36	sHT = 27 ; non-sHT = 107	MMP-9, c-Fibronectin
Castellanos et al. 2003	Spain	P	250	72.3±9.2	134	none	≤ 24	> 48	sHT = 38 ; non-sHT = 212	MMP-9
Castellanos et al. 2004	Spain	P	87	67±12	48	IV tPA	≤ 6	< 24-36	sHT = 26 ; non-sHT = 61	MMP-9, c-Fn
Castellanos et al. 2018	Spain	P	133	72±13	67	IV tPA	≤ 4.5	< 24-36	sHT = 26 ; non-sHT = 107	Caveolin-1
Ribo et al. 2004	Spain	P	77	70±12	47	IV tPA	≤ 3	< 36-48	sHT = 17 ; non-sHT = 60	Fibrinogen, PAI-1, TAFI, Lp(a), homocysteine

Table 4.1. Basic characteristics of included studies *(contd.)*

Author of the study	Place	Study design	Sample size (n)	Mean age±SD (in years)	Males (n)	Intervention (s)	First CT/MR (in hours)	Follow-up CT/MRI (in hours)	Groups (n)	Biomarker
Foerch et al. 2007	Spain	R	275	69±13	126	IV tPA	≤ 6	< 24	sHT = 80 ; non-sHT = 195	s100B
Choi et al. 2012	Korea	R	752	NA	455	IV tPA	≤ 6	> 48	HT = 90 ; non-HT = 662	Ferritin
Montaner et al. 2001	Spain	P	39	74±15	20	none	≤12	<36-48	sHT = 15 ; non-sHT = 24	MMP-9
Ruiwen Che et al. 2017	China	P	428	NA	316	IV tPA	≤4.5	>48	sHT = 24 ; non-sHT = 404	Serum albumin
Millan et al. 2008	Spain	R	134	67±11	88	IV tPA	≤ 3	< 24-36	sHT = 27 ; non-sHT = 107	Ferritin, MMP-9, c-Fn, IL-6, glutamate

Table 4.1. Basic characteristics of included studies *(contd.)*

Author of the study	Place	Study design	Sample size (n)	Mean age±SD (in years)	Males (n)	Intervention (s)	First CT/MR (in hours)	Follow-up CT/MRI (in hours)	Groups (n)	Biomarker
Zhenhui Duan et al. 2018	China	R	616	NA	368	EVT, Bridging therapy	≤ 4.5	< 24	sICH = 292 ; non-sICH = 324	NLR
Malhotra et al. 2018	USA	R	657	64.3±14.4	333	IV tPA, EVT, bridging therapy	≤ 4.5	< 24	sICH = 21 ; non-sICH = 635	NLR
Guo et al. 2016	China	P	189	NA	123	IV tPA, EVT, Bridging therapy	≤ 4.5	< 36-48	PH = 28 ; non-PH = 161	NLR
Pikija et al. 2018	Austria	R	187	NA	86	IV tPA, EVT, Bridging therapy	≤ 6	< 24	sHT = 31 ; non-sHT = 156	NLR
Navarro-Sobrino et al. 2011	Spain	P	109	71±12.4	59	IV tPA	≤3	<24-48	sHT = 8 ; non-sHT = 101	Angiogenic factors*
Xing et al. 2014	China	P	216	NA	154	IA tPA, EVT	≤6	<24	sHT = 41 ; non-sHT = 175	Globulin

Table 4.1. Basic characteristics of included studies *(contd.)*

Author of the study	Place	Study design	Sample size (n)	Mean age±SD (in years)	Males (n)	Intervention (s)	First CT/MR (in hours)	Follow-up CT/MRI (in hours)	Groups (n)	Biomarker
Hernandez-Guillamon et al. 2010	Spain	P	140	71.9±10.5	76	IV tPA	≤3	<36-48	sHT = 48 ; non-sHT = 92	VAP-SSAO activity
Matosevic et al. 2013	Austria	P	547	68±15.1	316	IV tPA	≤6	<24-36	sICH = 33 ;non-sICH=514	Fibrinogen
Wolcott et al. 2017	USA	P	646	69±15	364	IV tPA	≤9	>48	sHT = 42 ; non-sHT = 604	Soluble ST2
Mechtouff et al. 2020	France	P	148	69±15	89	IV tPA, EVT, Bridging therapy	≤4.5	<24	sHT = 40 ; non-sHT = 108	MMP-9
Switonska et al. 2020	Poland	R	51	65±16	22	IV tPA, EVT, Bridging therapy	≤24	>24	sHT = 10 ; non-sHT = 41	NLR
Goyal et al. 2018	USA	P	293	62±14	147	EVT	≤6	<24-36	sHT = 21 ; non-sHT = 272	NLR

Table 4.1. Basic characteristics of included studies *(contd.)*

Author of the study	Place	Study design	Sample size (n)	Mean age±SD (in years)	Males (n)	Intervention (s)	First CT/MR (in hours)	Follow-up CT/MRI (in hours)	Groups (n)	Biomarker
Maestrini et al. 2015	France and Finland	R	846	NA	430	IV tPA	≤3	<24-36	sHT = 54 ; non-sHT = 792	NLR
Sheng-Feng Lin et al. 2019	Taiwan	P	1840	NA	1174	IV tPA, EVT	≤3	<24-36	sHT = 38 ; non-sHT = 1802	Serum cholesterol
Neringa Jucevičiūtė et al. 2019	Lithuania	R	201	NA	81	IV tPA	≤4.5	<24-36	sHT = 23 ; non-sHT = 178	NLR, AEC

Note. SD, Standard deviation; NA, data not available; P, prospective; R, retrospective; HT, hemorrhagic transformation; sHT, symptomatic hemorrhagic transformation; sICH, symptomatic intracranial hemorrhage; cdHT, hemorrhagic transformation with clinical deterioration; PH, parenchymal hematoma; HI, hemorrhagic infarction; IV tPA, intravenous tissue plasminogen activator; IA tPA, intra-arterial tissue plasminogen activator; EVT, Endovascular treatment; CT, Computed tomography; MRI, Magnetic resonance imaging; h, hours; MMP-9, matrix metalloproteinase-9; NLR, neutrophil-lymphocyte ratio; c-Fibronectin, cellular-fibronectin; IL-6, interleulin-6; PAI-1, plasminogen activator inhibitor-1; TAFI, thrombin-activated fibrinolysis inhibitor; Lp (a), Lipoprotein (a); AEC, Absolute eosinophil count; VAP-1-SSAO, Vascular adhesion protein-1-semicarbazide-sensitive amine oxidase; *Angiogenic factors include PDGF-BB, platelet derived growth factor BB; HGF, hepatocyte growth factor; FGF, fibroblast growth factor; VEGF; vascular endothelial growth factor; HB-EGF, heparin binding epidermal growth factor like growth factor; KGF, keratinocyte growth factor; PDGF-AA, platelet derived growth factor AA; TPO, thrombopoietin;VEGFR-1, vascular endothelial growth factor receptor 1; VEGFR-2, vascular endothelial growth factor receptor-2; TSP-1, thrombospondin-1; TSP-2, thrombospondin-2; CCCK-18, Caspase-cleaved cytokeratin-18; s100B, S100 Calcium-binding protein B; OCLN, Occludin; CLDN5, Claudin5, ZO-1, Zonula occludens-1, NSE, Neuron-specific enolase protein B.

The patients were all enrolled in the study within 24 hours from stroke onset. Nineteen studies used the TOAST classification to determine the cause of stroke (Adams et al., 1993). Two studies used the Oxfordshire Community for Stroke patients (OCSP) classification (Che et al., 2017; Świtońska et al., 2020). The etiology of stroke in five studies was not categorized using any specific classification (Castellanos et al., 2018; El-Banhawy E et al., 2014; Lin et al., 2019; Malhotra et al., 2018; Molnar et al., 2019; Maestrini et al., 2015; Xing et al., 2014). In the majority of the included studies, stroke severity was assessed using the NIHSS score, whereas one study (Castellanos et al., 2003) employed the Canadian Stroke Scale (CSS).

Except for four studies (Castellanos et al., 2003; Yuan et al., 2018; Montaner et al., 2001; Kazmierski et al., 2012), all patients underwent interventions such as IV tPA, EVT, or bridging therapy. One study included patients who received IA tPA (Xing et al., 2014).

Assessment of HT varied across studies. Twenty-three studies (Che et al., 2017; Castellanos et al., 2003; Castellanos et al., 2007; Castellanos et al., 2004; Castellanos et al., 2018; Choi et al., 2012; Guo et al., 2016; Hernandez-Guillamon et al., 2010; Jucevičiūtė et al., 2019; Kazmierski et al., 2012; Lin et al., 2019; Maestrini et al., 2015; Mechtouff et al., 2020; Millán et al., 2008; Molnar et al., 2019; Montaner et al., 2001; Montaner et al., 2003; Ribo et al., 2004; Świtońska et al., 2020; Navarro-Sobrino et al., 2011; Wolcott et al., 2017; Xing et al., 2014; Yuan et al., 2018) defined HT according to the ECASS criteria, whereas two studies (Duan et al., 2018; Pikija et al., 2018) used the Heidelberg bleeding classification. Two studies (Lin et al., 2019; Matosevic et al., 2013) used the NINDS criteria. One study defined sICH according to

the Safe Implementation of Thrombolysis in Stroke-Monitoring Study (SITS-MOST) criteria (Malhotra et al., 2018). In one study, early HT was defined as intracranial bleeding occurring within 48 hours of the stroke onset and was compared to late HT, which occurred around 7-14 days after stroke (Montaner et al., 2001). Using ROC curve analysis, cut-off values for the biomarkers were established in 16 studies. Twenty studies looked at the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of each marker. The remaining studies used predetermined thresholds as reference values for the markers.

4.1.3 Risk of bias

The risk of bias and applicability of the studies' graph for the included studies and the summary is shown in Fig. 4.2. and Fig. 4.3, respectively.

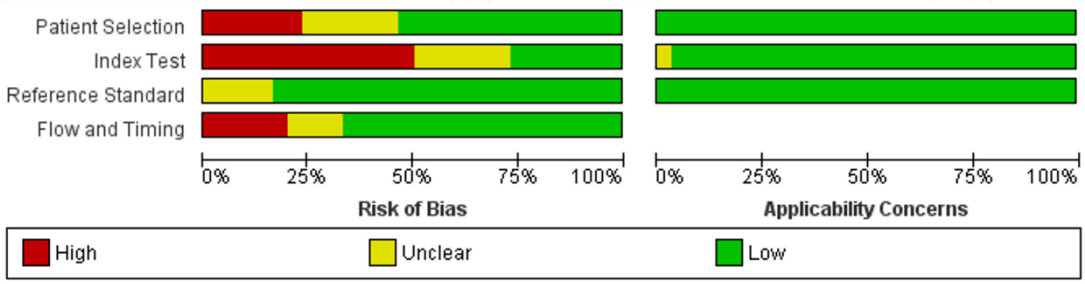


Fig. 4.2 The risk of bias graph for all studies is presented as percentages using the QUADAS-2 tool.

Fig. 4.3 The summary of the risk of biases based on the QUADAS-2 tool presenting the risk of bias items for each included study.

Sixteen studies accurately defined their study population, including information on missing data and patients lost during follow-up, seventeen studies addressed the possibility of bias, two studies provided flow diagrams, and twenty studies released funding information. The methodological quality of studies following the STROBE recommendations is given in Fig. 4.4.

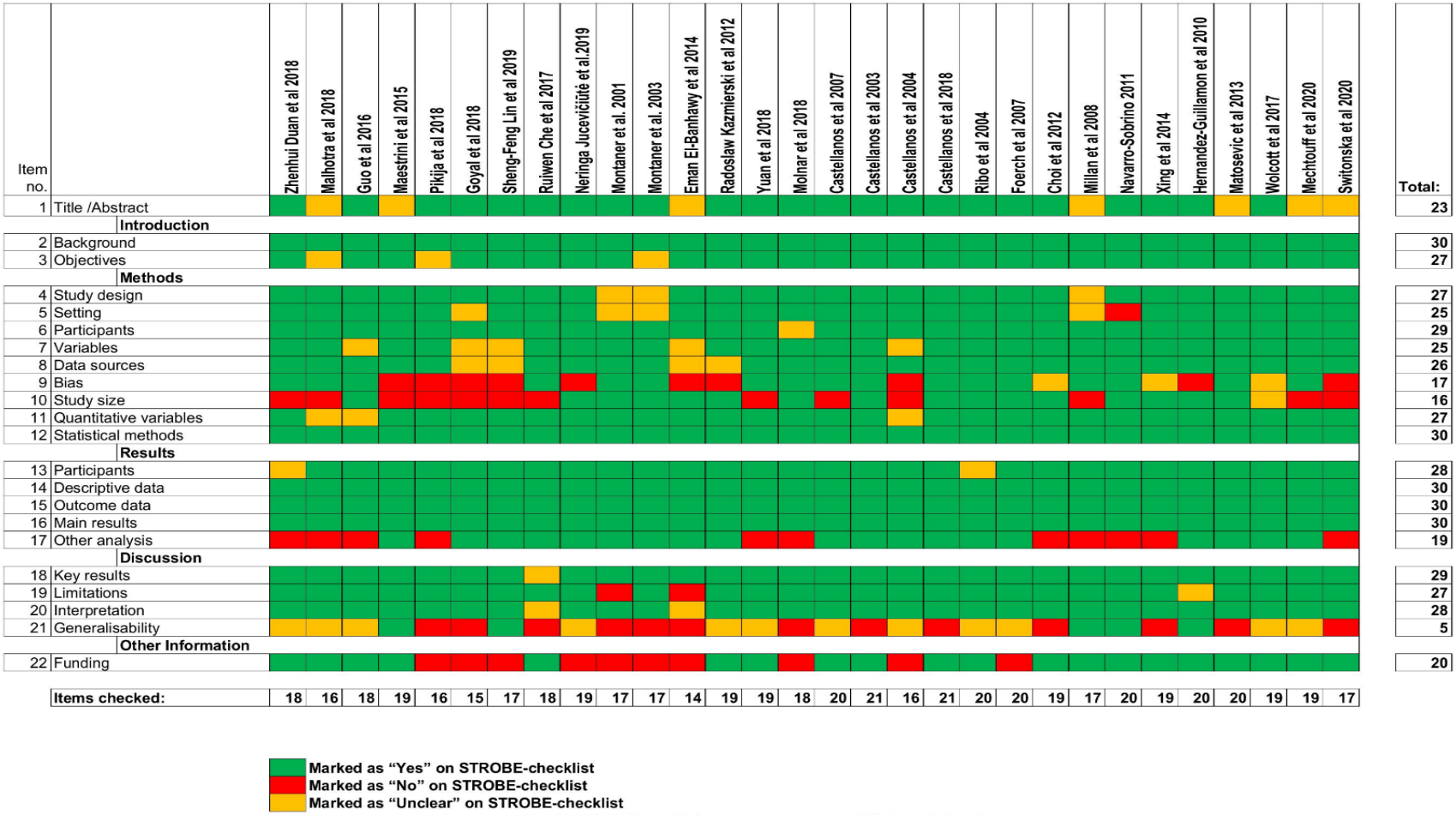


Fig. 4.4 The methodological quality of included studies following the STROBE guidelines.

4.1.4 Biomarkers Included in the Meta-analysis

Sixteen studies were found eligible to be included in the meta-analysis with seven studies analyzing MMP-9 (Castellanos et al., 2003; Castellanos et al., 2007; Millán et al., 2008; Montaner et al., 2001; Montaner et al., 2003; Castellanos et al., 2004; Yuan et al., 2018; El-Banhawy et al., 2014), two studies investigating c-Fibronectin (Castellanos et al., 2004; Castellanos et al., 2007), two studies Ferritin (Choi et al., 2012; Millán et al., 2008), two studies S100B levels in symptomatic HT (Foerch et al., 2007; Kazmierski et al., 2012), and five studies (Maestrini et al., 2015; Goyal et al., 2018; Duan et al., 2018; Pikija et al., 2018; Guo et al., 2016) assessing Neutrophil-lymphocyte ratio (NLR) in sICH. Table 4.2 provides the summary statistics for diagnostic accuracy parameters of the included studies.

Table 4.2 The pooled Diagnostic odds ratio, sensitivity, and false positivity rates of the meta-analyzed biomarkers.

Marker	DOR (univariate)	Sensitivity (bivariate)	FPR (bivariate)	AUC
MMP-9 for sHT	29.571 (17.750, 49.267)	0.849	0.180	0.881
c-Fn for sHT	299.253 (20.508, 4366.709)	0.982	0.153	0.972
NLR for sICH	5.036 (2.898, 8.749)	0.672	0.284	0.751
Ferritin for sHT	24.032 (2.557, 225.871)	0.802	0.140	0.87
S100B for sHT	6.286 (1.861, 21.230)	0.782	0.329	0.766

Note. sHT, symptomatic hemorrhagic transformation; sICH, symptomatic intracranial hemorrhage; DOR, Diagnostic Odds ratio; FPR, False positivity ratio; AUC, Area under the SROC curve MMP-9, matrix metalloproteinase-9; c-Fn, cellular-fibronectin; NLR, neutrophil-lymphocyte ratio; s100B, S100 Calcium-binding protein B.

Fig. 4.5 depicted the forest plots for the DOR-based univariate analysis, and Fig. 4.6 showed the forest plots for the sensitivity and specificity of all the biomarkers, respectively.

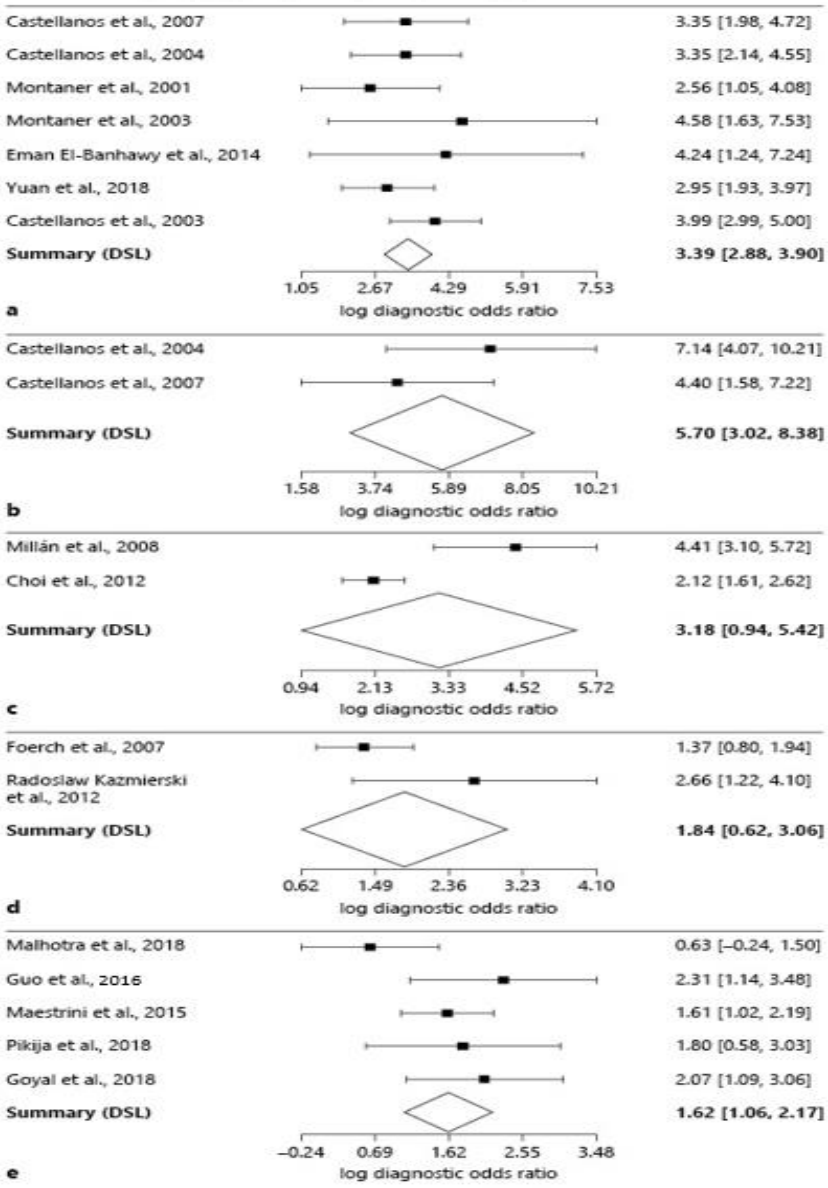


Fig. 4.5 Forest plots of univariate analysis using diagnostic odds ratios (DOR) of meta-analyzed studies: pooled DOR for (a) MMP-9, (b) c-Fibronectin, (c) Ferritin, (d) S100B, and (e) NLR.

MMP-9 for HT

Study	TP	FP	FN	TN	cut off value	Sensitivity (95% CI)	Specificity (95% CI)
Castellanos et al 2003	33	21	5	191	140.0	0.87 [0.72, 0.96]	0.90 [0.85, 0.94]
Castellanos et al 2004	21	7	5	54	140.0	0.81 [0.61, 0.93]	0.89 [0.78, 0.95]
Castellanos et al 2007	25	28	2	79	140.0	0.93 [0.76, 0.99]	0.74 [0.64, 0.82]
Eman El-Banhawy et al 2014	8	5	0	22	900.0	1.00 [0.63, 1.00]	0.81 [0.62, 0.94]
Montaner et al. 2001	13	5	3	18	144.81	0.81 [0.54, 0.96]	0.78 [0.56, 0.93]
Montaner et al. 2003	15	6	0	20	191.3	1.00 [0.78, 1.00]	0.77 [0.56, 0.91]
Yuan et al 2018	24	26	5	113	181.7	0.83 [0.64, 0.94]	0.81 [0.74, 0.87]

c-FN for HT

Study	TP	FP	FN	TN	cut off value	Sensitivity (95% CI)	Specificity (95% CI)
Castellanos et al 2004	26	2	0	59	3.6	1.00 [0.87, 1.00]	0.97 [0.89, 1.00]
Castellanos et al 2007	27	43	0	64	3.6	1.00 [0.87, 1.00]	0.60 [0.50, 0.69]

Ferritin for HT

Study	TP	FP	FN	TN	cut off value	Sensitivity (95% CI)	Specificity (95% CI)
Choi et al 2012	67	172	23	490	144.8	0.74 [0.64, 0.83]	0.74 [0.70, 0.77]
Millan et al 2008	23	7	4	100	79.0	0.85 [0.66, 0.96]	0.93 [0.87, 0.97]

S100B for HT

Study	TP	FP	FN	TN	cut off value	Sensitivity (95% CI)	Specificity (95% CI)
Foerch et al 2007	37	35	43	160	0.23	0.46 [0.35, 0.58]	0.82 [0.76, 0.87]
Radoslaw Kazmierski et al 2012	31	221	2	204	0.01189	0.94 [0.80, 0.99]	0.48 [0.43, 0.53]

NLR for sICH

Study	TP	FP	FN	TN	cut off value	Sensitivity (95% CI)	Specificity (95% CI)
Goyal et al 2018	15	65	6	207	6.62	0.71 [0.48, 0.89]	0.76 [0.71, 0.81]
Guo et al 2016	13	42	4	130	10.59	0.76 [0.50, 0.93]	0.76 [0.68, 0.82]
Maestrini et al 2015	36	227	18	565	4.8	0.67 [0.53, 0.79]	0.71 [0.68, 0.74]
Malhotra et al 2018	11	235	10	401	2.2	0.52 [0.30, 0.74]	0.63 [0.59, 0.67]
Pikija et al 2018	9	47	4	127	3.89	0.69 [0.39, 0.91]	0.73 [0.66, 0.79]

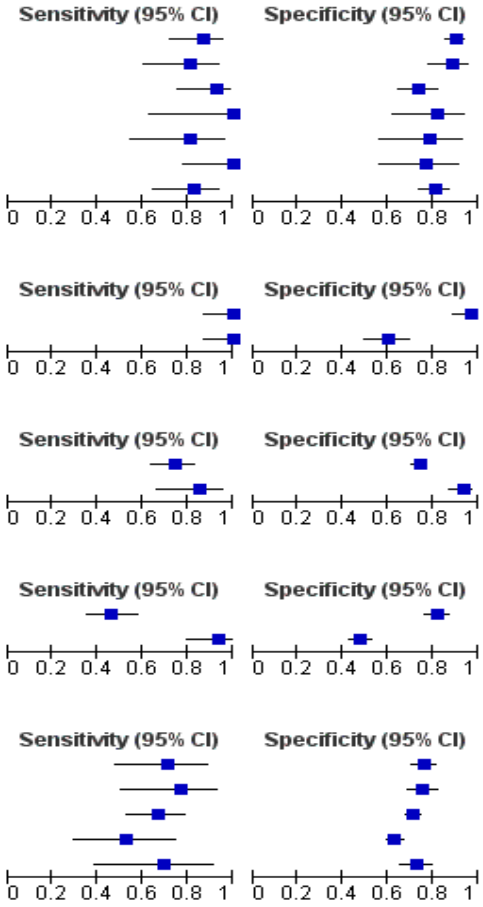


Fig. 4.6 Forest plots for sensitivities and specificities corresponding to MMP-9, c-Fibronectin, Ferritin, S100B, and NLR.

Using the estimated HSROC parameters as shown in Fig. 4.7, an SROC plot was created for five biomarkers and Table 4.3 provides the HSROC parameters that were applied to the graph.

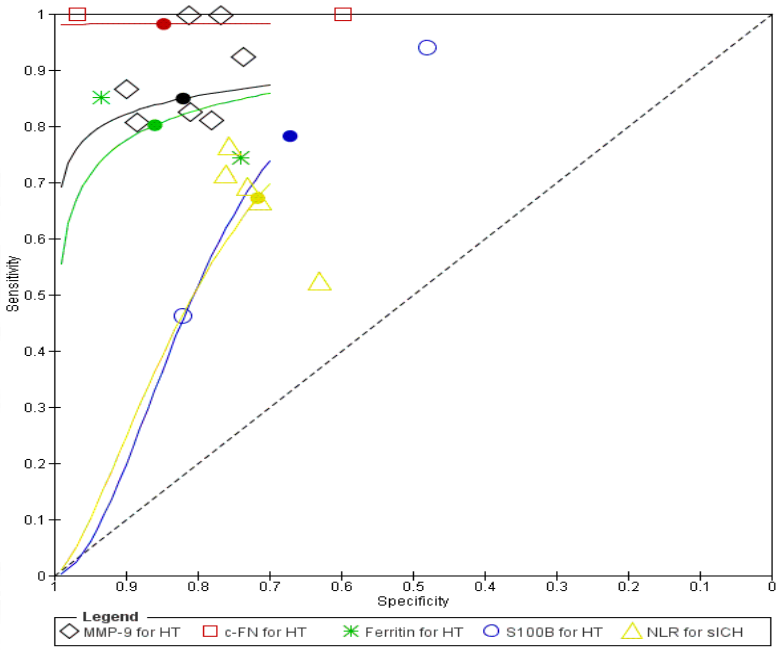


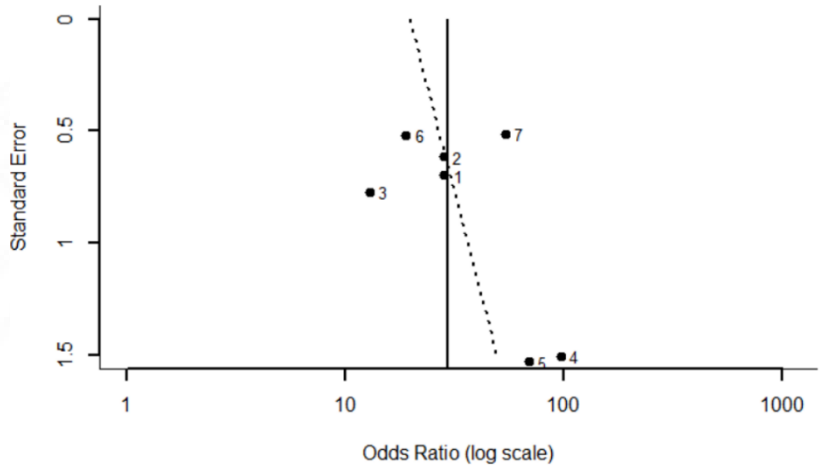
Fig. 4.7 SROC plot for the meta-analyzed biomarkers using calculated HSROC parameters.

Table 4.3 HSROC parameters for the bivariate analysis of each biomarker.

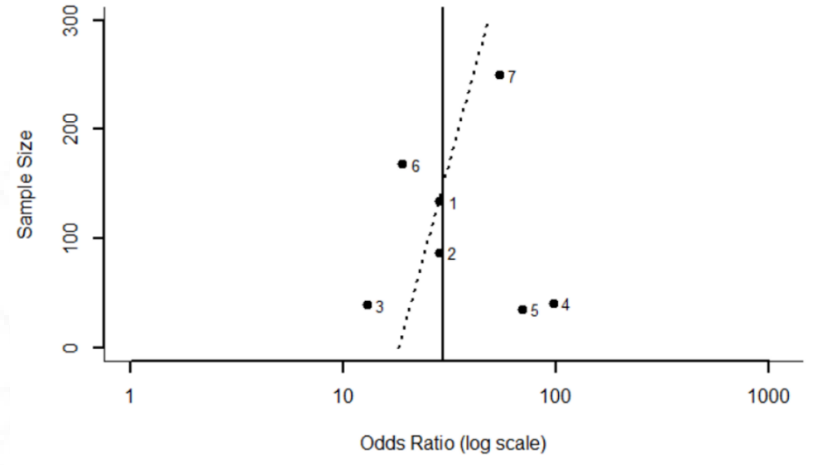
Marker	Lambda	Theta	Beta	Sigma2alpha	Sigma2theta	Symbol
MMP-9 for sHT	3.985	1.162	1.205	0.000	0.043	Diamond
c-FN for sHT	34.708	17.156	4.316	0.000	0.048	Square
NLR for sICH	1.709	-0.258	-0.367	0.329	0.000	Triangle
Ferritin for sHT	3.326	0.487	0.863	2.067	0.000	Star
S100B for sHT	1.910	-0.004	-0.592	0.000	2.281	Ellipse

Note. HSROC, Hierarchical Summary Receiver Operator Characteristic; MMP-9: Matrix metalloproteinase-9; c-Fn: cellular-Fibronectin; NLR: Neutrophil-lymphocyte ratio; s100B: S100 Calcium-binding protein B.

Fig. 4.8 represents the funnel plots used to calculate publication bias. Standard error and sample size were used as variables in a regression test for funnel plot asymmetry, which revealed no evidence of publication bias (p-value of 0.5574 and 0.3119, respectively).



(a) standard error



(b) sample size as predictors

Fig. 4.8 Funnel plots for estimating the publication bias in the included studies with standard error and sample size as predictors.

4.1.4.1 Matrix Metalloproteinase-9

High baseline MMP-9 in symptomatic HT as confirmed by commercially available enzyme-linked immunosorbent assay (ELISA) kits was reported in ten investigations including 1494 patients. Two studies serially measured MMP-9 levels at four time points (Montaner et al., 2001; Montaner et al., 2003).

Four studies (Castellanos et al., 2003; Castellanos et al., 2007; Castellanos et al., 2004; Montaner et al., 2001) reported a baseline cut-off >140 ng/mL yielding high sensitivity and specificity to predict symptomatic HT. There was no significant variance between non-thrombolysed individuals with early HI and early PH, according to two trials (Montaner et al., 2001; Castellanos et al., 2003). However, individuals detected with PH had a higher median baseline MMP-9 while receiving IV tPA. Pretreatment values of 191.3 ng/mL were observed in two studies to predict PH (Yuan et al., 2018; Montaner et al., 2003). One study revealed that patients with poor functional results had significantly elevated MMP-9 levels (Millán et al., 2008).

The meta-analysis comprised 754 patients from seven studies. Increased circulating MMP-9 levels were shown to accurately predict PH (DOR_{pooled}, 29.571 [95% CI 17.750-49.267]). The analysis revealed that the pooled sensitivity was 0.849 and the pooled false positive rate was 0.180.

4.1.4.2 Cellular-Fibronectin

The biomarker, c-Fibronectin was correlated with symptomatic HT in three studies consisting of a total of 355 participants, using commercial ELISA (Castellanos et al., 2007; Castellanos et al., 2004; Millán et al., 2008). All studies have assessed the concentrations of c-Fibronectin in patients who received thrombolysis.

All studies reported that post-thrombolysis individuals with PH had a significant elevation of c-Fibronectin (Castellanos et al., 2004). When combined with MMP-9 (140 ng/mL), a cut-off of 3.6 g/mL produced good sensitivity and NPV, which boosted specificity and PPV for predicting PH (Castellanos et al., 2004).

Functional outcome positively correlated with c-Fibronectin in one study (P=0.010) (Millán et al., 2008).

Two studies (Castellanos et al., 2004; Castellanos et al., 2007) with 221 patients were meta-analyzed. The highest pooled DOR was reported for c-Fibronectin (299.253[95% CI,20.508-4366.709]), and the pooled sensitivity and false positivity rates were 0.982 and 0.153, respectively.

4.1.4.3 Ferritin

Using immunoassay methods, two studies including 886 patients assessed Ferritin levels in symptomatic HT (Millán et al., 2008; Choi et al., 2012). Choi *et al.* evaluated Ferritin levels within 48 h from onset whereas Millán *et al.* serially measured the marker using three time points.

Baseline Ferritin levels were increased in patients with PH and symptomatic HT (cut-off ≥144.8 ng/mL). At 24 hours after intervention, individuals with PH had the highest ferritin levels (Millán et al., 2008). Similarly, Choi *et al.* found thresholds with good sensitivity and specificity ≥164.1 ng/mL to predict PH and ≥171.8 ng/mL to predict symptomatic HT (Choi et al., 2012).

Baseline Ferritin levels were significantly elevated in patients who had poor outcomes (P<0.001) (Millán et al., 2008).

Both studies were included in the meta-analysis. Pooled DOR was 24.032 (95% CI 2.557 - 225.871), and pooled sensitivity and false positivity rate were 0.802, and 0.140, respectively.

4.1.4.4 S100 Calcium-binding protein B

In two studies consisting of 733 individuals in total, baseline S100B in symptomatic HT was assessed using commercial immunoassays (Kazmierski et al., 2012; Foerch et al., 2007). Median S100B levels were elevated in patients with symptomatic HT with a threshold ≥0.23 µg/L predicting PH-2 (Foerch et al., 2007). S100B was significantly elevated in spontaneous HT than in non-HT (0.1002 µg/L versus 0.01415 µg/L; P=0.003) (Kazmierski et al., 2012).

Meta-analysis revealed pooled DOR to be 6.286 (95% CI 1.861 - 21.230), pooled sensitivity value of 0.782, and false positivity rate of 0.32.

4.1.4.5 Neutrophil-lymphocyte ratio

Eight studies comprised a total of 3040 patients, who received thrombolytic intervention within six hours of stroke onset and had their NLR values estimated using complete blood count. Additionally, three time points were used in one investigation for NLR calculation (Guo et al., 2016).

In four studies, baseline NLR levels were significantly correlated with sICH (Maestrini et al., 2015; Goyal et al., 2018; Duan et al., 2018; Pikija et al., 2018) and in one study with symptomatic HT post-revascularization (Świtońska et al., 2020). The thresholds varied between studies, with 2.2 serving as the lowest cut-off value and 10.59 serving as the highest (Malhotra et al., 2018; Jucevičiūtė et al., 2019; Guo et al., 2016). Guo et al. reported that there was no association between baseline NLR and

sICH and that the peak was at its highest between 12 and 18 h post-revascularization (Guo et al., 2016).

Baseline NLR was shown to be significantly associated with poor outcomes in four studies (Duan et al., 2018; Pikija et al., 2018; Goyal et al., 2018; Maestrini et al., 2015) with a higher mortality rate in patients with baseline NLR ≥7 (Duan et al., 2018).

For the meta-analysis, five studies including 2172 patients were evaluated. Pooled DOR for baseline NLR was 5.036 (95% CI 2.898 - 8.749) predicting sICH in patients who underwent revascularization (pooled sensitivity - 0.672, pooled false positivity rate - 0.284).

4.1.5 Biomarkers not Included in the Meta-analysis

A forest plot could not be synthesized for biomarkers in fourteen studies due to insufficient data or reporting in a single study. Among the single studies, Caveolin-1 (99% NPV and 86% sensitivity) (Castellanos et al., 2018), Plasminogen activator inhibitor-1 (PAI-1), and thrombin-activated fibrinolysis inhibitor (TAFI) (97.6% specificity, 97% NPV) (Ribo et al., 2004), and soluble ST2 (AUC = 0.747) (Wolcott et al., 2017) were found to be independent predictors of symptomatic HT.

4.2 PART 2: Assessment of Biomarkers in HT and Outcome

Between December 2018 and April 2021, 111 patients were enrolled in the prospective observational study. A summary of the screening of patients, the timeline of the blood sample collection, and follow-up at 3 months is provided in Fig. 4.9.

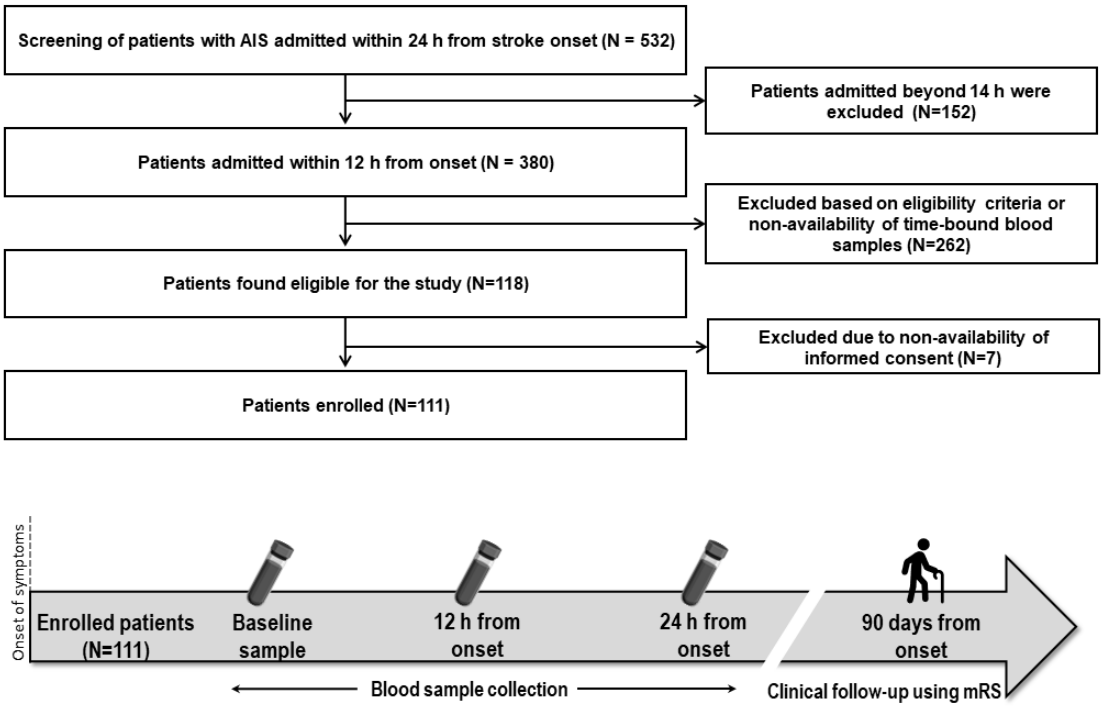


Fig. 4.9 Flow diagram of the study participants, and timeline of the sample collection for biomarker analysis and clinical follow-up at 3 months.

4.2.1 Biomarkers Associated with HT in AIS

The baseline clinical and imaging characteristics of the patients enrolled in the study are given in Table 4.4.

Table 4.4 Baseline characteristics of the sample population.

Baseline characteristics	Total (N= 111)	Non-HT (N= 81)	HT (N= 30)	P value[1†]
Male, n (%)	78 (70)	55 (68)	23 (77)	0.4
Age, in years, mean (SD)	62.3 (11.7)	63.1 (11.7)	60.2 (11.5)	0.3
Mean time of arrival to hospital, in minutes, mean (SD)	257 (220)	226 (185.8)	359 (269.8)	0.004**
Hypertension, n (%)	74 (67)	52 (64)	22 (73)	0.4

Table 4.4 Baseline characteristics of the sample population *(contd.)*

Baseline characteristics	Total (N= 111)	Non-HT (N= 81)	HT (N= 30)	P value[1†]
Diabetes mellitus, n (%)	54 (49)	40 (49)	14 (47)	0.8
Coronary artery disease, n (%)	22 (20)	16 (20)	6 (20)	>0.9
Hypercholesterolemia, n (%)	33 (30)	24 (30)	9 (30)	>0.9
Atrial fibrillation, n (%)	20 (18)	11 (14)	9 (30)	0.04*
Rheumatic heart disease, n (%)	7 (6.3)	4 (4.9)	3 (10)	0.4
Tobacco use, n (%)	39 (35)	30 (37)	9 (30)	0.5
Alcohol intake, n (%)	31 (28)	24 (30)	7 (23)	0.5
Baseline plasma glucose, mg/dL, mean (SD)	173.3 (74.5)	174.6 (71.2)	169.9 (84.0)	0.8
Plasma glucose >140 mg/dL,mean (SD)	67 (61)	49 (61)	18 (60)	>0.9
HbA1c, (%), mean (SD)	6.6 (5.7, 8.4)	6.4 (5.6, 8.7)	6.8 (5.8, 8.1)	0.9
Lipid profile				
Total cholesterol, mg/dL, mean (SD)	189.8 (55.6)	194.9 (55.6)	176.3 (54.2)	0.2
High-density lipoproteins, mg/dL, mean (SD)	47.2 (13.1)	47.1 (13.7)	47.4 (11.7)	>0.9
Low-density lipoproteins, mg/dL, mean (SD)	123.9 (47.1)	126.7 (48.7)	116.3 (42.3)	0.4
Triglycerides, mg/dL, mean (SD)	97.1 (48.8)	98.3 (48.3)	93.8 (50.7)	0.7
Systolic BP, mmHg, mean (SD)	155.1 (26.6)	155.2 (26.0)	154.9 (28.6)	>0.9
Systolic BP ≥180 mmHg, n (%)	24 (22)	14 (18)	10 (33)	0.073
Diastolic BP, mmHg, mean (SD)	88.1 (14.7)	88.2 (15.1)	88.0 (13.8)	>0.9
Baseline ASPECTS score (≥6), n (%)	56 (60)	47 (72)	9 (32)	<0.001**

Table 4.4 Baseline characteristics of the sample population *(contd.)*

Baseline characteristics	Total (N= 111)	Non-HT (N= 81)	HT (N= 30)	P value[1†]
TOAST classification, n (%)				0.02*
Cardioembolism	30 (27)	16 (20)	14 (47)	
Large vessel disease	35 (32)	29 (36)	6 (20)	
Small vessel disease	8 (7.2)	8 (9.9)	0 (0)	
Stroke of other determined etiology	3 (2.7)	3 (3.7)	0 (0)	
Stroke of undetermined etiology	35 (32)	25 (31)	10 (33)	
Intravenous thrombolysis, n (%)	43 (39)	38 (47)	5 (17)	0.004**
Endovascular therapy, n (%)	35 (32)	21 (26)	14 (47)	0.03*
Bridging Therapy, n (%)	6 (5.4)	4 (4.9)	2 (6.7)	0.7
Baseline NIHSS score, Median (IQR)	12.0 (8.0, 18.0)	10.0 (6.0, 17.0)	17.0 12.2, 21.0)	<0.001**
modified Rankin scale 3 – 6 at 3 months, n (%)	39 (38)	24 (31)	15 (58)	0.01*
Mortality at 3 months, n (%)	11 (9.9)	6 (7.4)	5 (17)	0.08

Note. [1]Pearson's Chi-squared test; Wilcoxon rank sum test; Fisher's exact test; [†]P-value computed for comparison of HT and non-HT; *P < 0.05, **P < 0.01 and ***P < 0.001; NIHSS, National Institutes of Health Stroke Scale; ASPECTS, Alberta stroke program early CT score; HT, hemorrhagic transformation; mg/dL, milligram/deciliter; SD, standard deviation; IQR, interquartile range; TOAST, Trial of Org 10172 in Acute Stroke Treatment; BP, blood pressure.

The 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 at the hospital was 4.2 h. The mean ASPECTS score was 6. IV rtPA was administered to 43 (39%) patients, whereas 35 (32%) patients underwent EVT, and 6 (5.4%) patients were given bridging therapy. HT was detected in 30 patients (27%) with a significantly higher number of patients with AF, low ASPECTS scores, reperfusion therapies, and delayed time of arrival having HT as

compared to non-HT patients. Of the patients with HT, we 22 patients were diagnosed with HI and 8 patients with PH.

Although 47% of the patients who underwent EVT were detected with HT, this included both the HI and PH subtypes. According to the functional outcome data recorded using mRS scores as shown in Fig. 4.10, HT revealed a larger proportion of individuals with moderate to severe disability at 3 months after stroke onset.

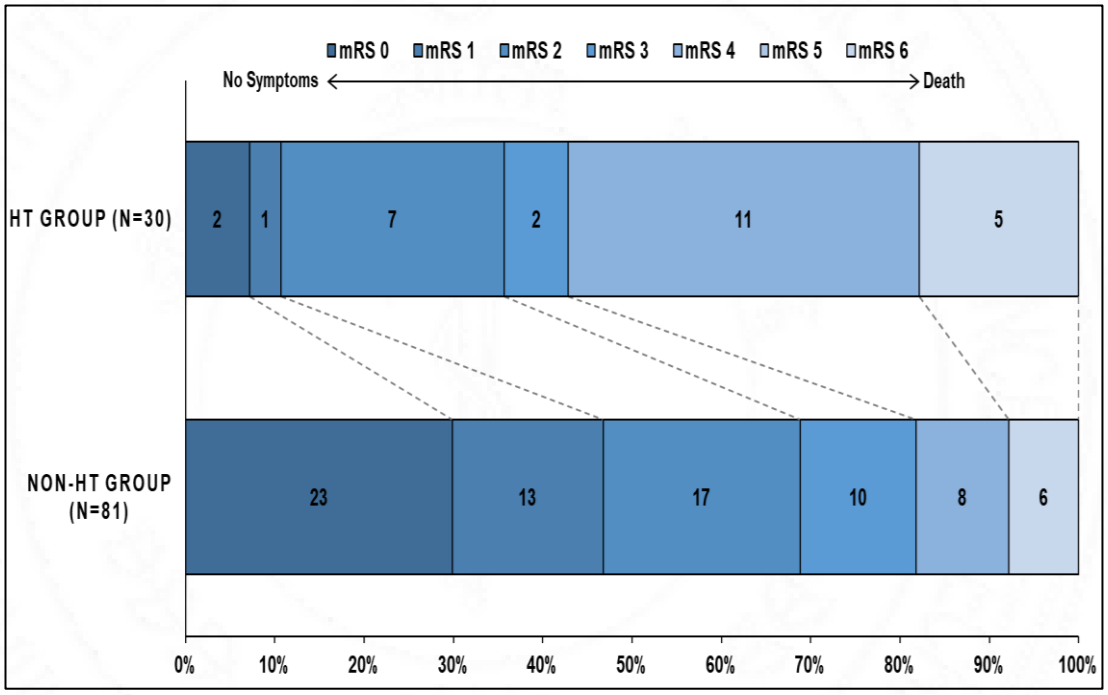


Fig. 4.10 The distribution of modified Rankin scores at 3 months among HT and non-HT patients.

The temporal profile is shown in Fig. 4.11, indicating a maximum elevation of MMP-9, Claudin-5, and sST2 at the 12-hour timepoint.

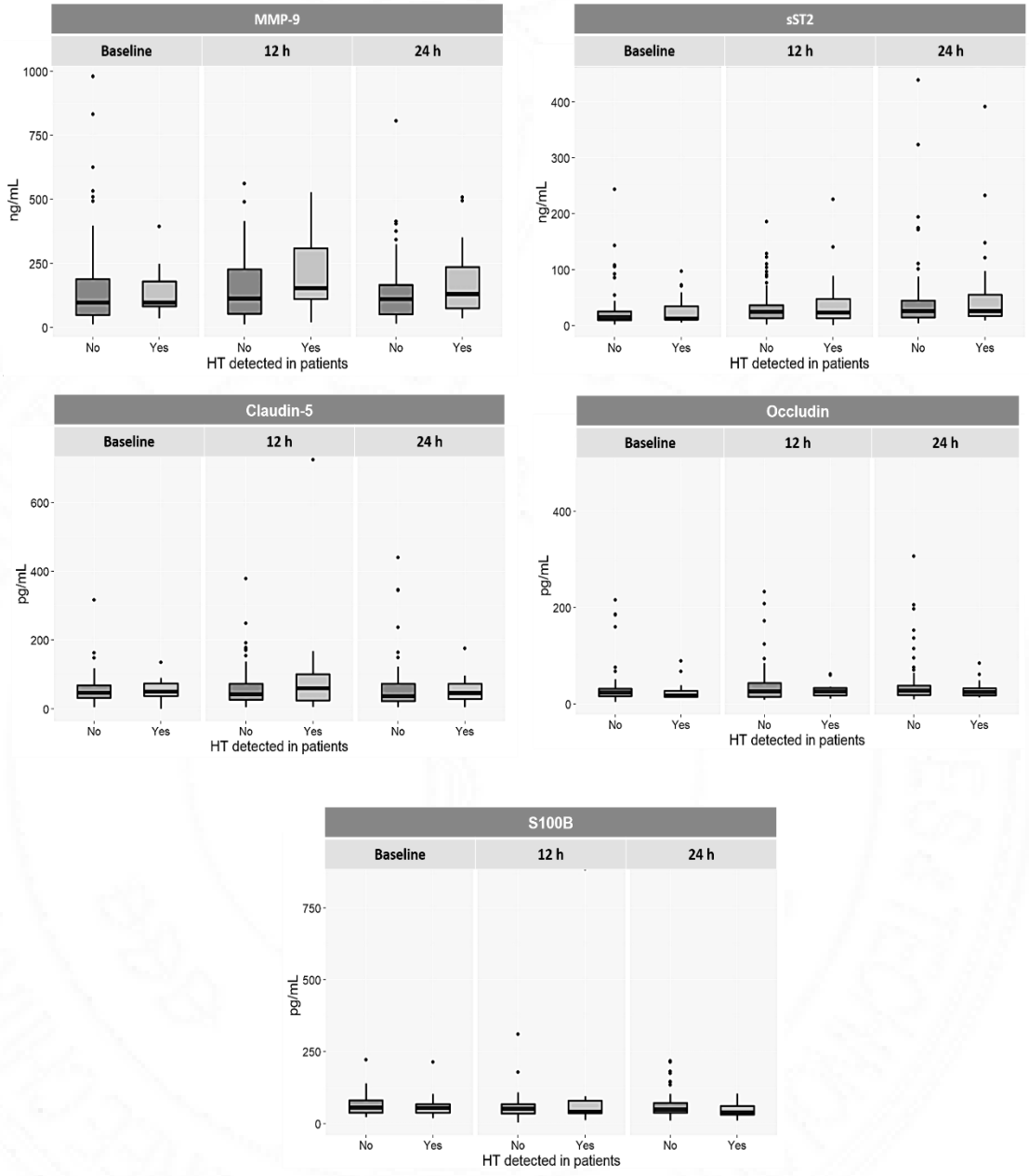


Fig. 4.11 Box plot graphs showing median biomarker levels at each time-points between patients who had HT and non-HT patients.

The median 12-hour MMP-9 level of 153.9 ng/mL [IQR: 110.6 – 309 ng/mL] showed a trend toward statistical significance in HT(P=0.05). Claudin-5 levels were elevated at 12 h as compared to the other two time-points but were not found to be statistically significant (43.1 pg/mL [IQR: 26.7-72.6 pg/mL] versus 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. Both Occludin and S100B yielded very low values and did not show significant variation in their median levels between the groups.

The ROC curves generated for the biomarker levels at all time points given in Table 4.5 showed baseline MMP-9 levels with a maximum sensitivity of 85.7% for a cut-off value of 56.06 ng/mL which was maintained at 12 hours (80%) for a threshold of 96.3 ng/mL (AUC = 0.63) and declined by the 24 hour-timepoint.

Table 4.5 ROC curve analysis discriminating HT and non-HT patients.

Biomarker levels		AUC	Cut-off value	Sensitivity, %	Specificity, %	PPV, %	NPV, %
MMP-9	Baseline	0.563	56.06 ng/mL	85.7	33.8	10.7	73.1
	12 h	0.630	96.34 ng/mL	80.0	46.4	13.5	64.9
	24 h	0.583	214.17 ng/mL	33.3	84.0	22.7	56.5
Claudin5	Baseline	0.551	65.17 pg/mL	47.6	71.6	17.2	67.7
	12 h	0.553	50.02 pg/mL	64.0	62.3	17.3	61.9
	24 h	0.515	40.30 pg/mL	60.0	54.3	21.4	67.3
sST2	Baseline	0.547	8.865 ng/mL	95.2	21.6	5.9	74.4
	12 h	0.522	34.58 ng/mL	40.0	73.9	22.7	64.3

Table 4.5 ROC curve analysis discriminating HT and non-HT patients *(contd.)*

Biomarker levels		AUC	Cut-off value	Sensitivity, %	Specificity, %	PPV, %	NPV, %
Occludin	24 h	0.558	87.56 ng/mL	23.3	91.4	23.7	50.0
	Baseline	0.455	36.73 pg/mL	81.0	23.0	19.0	77.0
	12 h	0.509	70.03 pg/mL	40.0	78.3	21.7	60.0
	24 h	0.375	18.64 pg/mL	96.6	61.7	16.6	72.3

Note. AUC, Area under the curve; PPV, Positive predictive value; NPV, Negative predictive value; MMP-9, matrix metalloproteinase-9; sST2, soluble serum stimulation-2; h, hours; pg/mL, picogram/milliliter; ng/mL, nanogram/milliliter.

Similarly, Claudin-5 had a maximum sensitivity of 62% and specificity of 62.3% at the 12 h time point for a cut-off of 50 pg/mL (AUC = 0.552). 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 8.65 ng/mL. Occludin yielded the lowest performance of the model at distinguishing between HT and non-HT patients. Due to the low levels, S100B displayed erroneous AUC values.

The multiple logistic regression model included MMP-9, sST2, and Claudin-5 levels at 12 h from the onset and the covariates that were statistically significant in the univariate analysis as shown in Table 4.6.

Table 4.6 Multiple logistic model for predicting HT after adjusting for biomarker levels at 12 hours.

Characteristic	OR[1]	95% CI[1]	p-value
Age	0.96	0.90, 1.02	0.2
Atrial fibrillation	1.35	0.06, 28.5	0.8
Plasma glucose ≥180 mg/dL	0.80	0.20, 3.33	0.8
Admission Systolic blood pressure>140 mmHg	5.19	0.77, 39.2	0.092
Baseline ASPECTS score <6	20.3	3.46, 193	0.003*
Intravenous thrombolysis	0.52	0.09, 2.65	0.4
Mechanical thrombectomy	3.91	0.72, 26.4	0.13
Baseline severe stroke NIHSS ≥16	0.75	0.16, 3.24	0.7
Cardioembolism etiology	0.22	0.02, 2.09	0.2
MMP-9 at 12 h (ng/mL)	1.39	0.26, 8.43	0.7
Claudin-5 at 12 h (pg/mL)	9.46	1.97, 64.6	0.010*
sST2 at 12 h (ng/mL)	1.29	0.23, 6.79	0.8

Note. [1]OR, odds ratio, CI, confidence interval; *P value <0.05; NIHSS, National Institutes of Health Stroke Scale; MMP-9, matrix metalloproteinase-9; sST2, soluble serum stimulation-2; h, hours; pg/mL, picogram/milliliter; ng/mL, nanogram/milliliter; mg/dL, milligram/deciliter; IQR, Interquartile range.

Claudin-5 did not show a statistically significant association for the prediction of HT in the univariate analysis. However, after adjusting for the risk factors such as age, AF, baseline plasma glucose and systolic blood pressure, ASPECTS, NIHSS scores, IV tPA, EVT, and cardioembolism 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).

The correlation of these biomarkers with baseline stroke severity is given in Fig. 4.12.

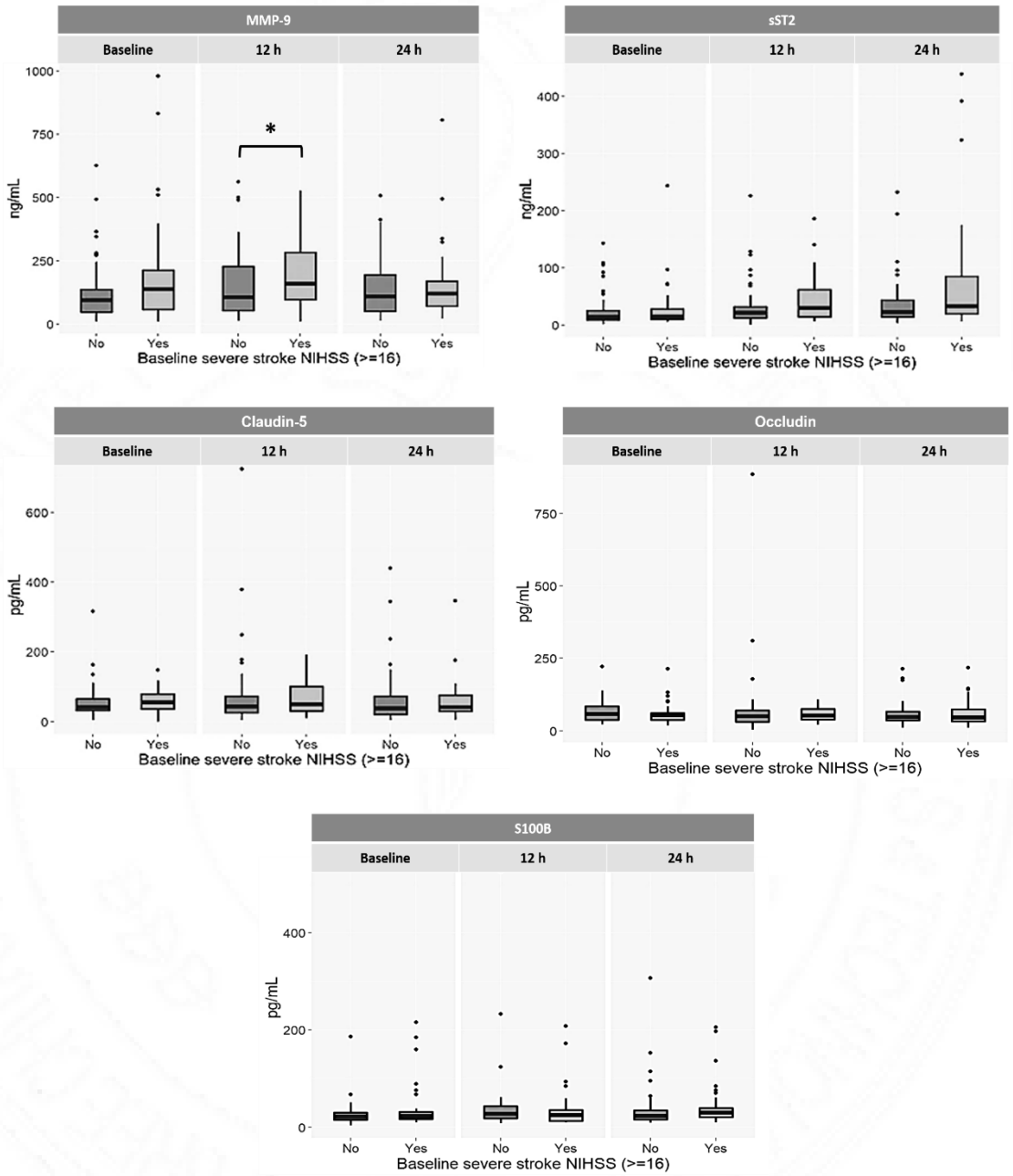


Fig. 4.12 Comparison of baseline NIHSS scores and biomarker levels at three time points (*P < 0.05).

Of these, 11 patients (22%) with baseline NIHSS scores 5-15 and 18 patients (44%) with severe strokes (≥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). An elevation was observed for Claudin-5 at 12 h and sST2 at 24 h, but these were not statistically significant between the two groups. No significant association was reported for Occludin and S100B levels and stroke severity.

4.2.2 Biomarkers Associated with Functional Outcome in AIS

The baseline characteristics of the patient population based on their functional outcome at 90 days are given in Table 4.7.

Table 4.7 Comparison of patients with good outcomes and poor outcomes at 3 months from stroke onset.

Characteristic	Good outcome at 90 days, N = 65	Poor outcome at 90 days, N = 43	p-value[1]
Male, n (%)	45 (69%)	30 (70%)	>0.9
Age, in years, Mean (SD)	62.0 (11.2)	62.2 (12.5)	0.9
Hypertension, n (%)	38 (58%)	33 (77%)	0.050
Diabetes Mellitus, n (%)	27 (42%)	26 (60%)	0.054
Coronary Artery Disease, n (%)	11 (17%)	10 (23%)	0.4
Hypercholesterolemia, n (%)	19 (29%)	12 (28%)	0.9
Atrial Fibrillation, n (%)	8 (12%)	11 (26%)	0.076
Rheumatic Heart Disease, n (%)	7 (11%)	0 (0%)	0.040*
Tobacco use, n (%)	26 (40%)	13 (30%)	0.3
Alcohol use, n (%)	20 (31%)	10 (23%)	0.4
Single antiplatelets, n (%)	5 (7.7%)	3 (7.0%)	>0.9
Dual antiplatelets, n (%)	2 (3.1%)	4 (9.3%)	0.2

Table 4.7 Comparison of patients with good outcomes and poor outcomes at 3 months from stroke onset *(contd.)*

Characteristic	Good outcome at 90 days, N = 65	Poor outcome at 90 days, N = 43	p-value[1]
Plasma glucose at admission, mg/dL Mean (SD)	158.0 (58.6)	196.7 (91.0)	0.016*
Plasma glucose >140 mg/dL	34 (52%)	31 (74%)	0.026*
HbA1c, n (%), Median (IQR)	6.3 (5.6, 8.0)	7.6 (6.2, 9.8)	0.008**
Total Cholesterol, mg/dL Mean (SD)	193.5 (48.3)	189.2 (64.4)	0.2
HDL, mg/dL, Mean (SD)	48.6 (14.7)	45.6 (10.7)	0.5
LDL, mg/dL, Mean (SD)	127.4 (39.7)	122.2 (56.3)	0.2
Triglycerides, mg/dL, Mean (SD)	98.1 (53.2)	98.6 (42.2)	0.6
ESR, mm/hr, Mean (SD)	22.7 (22.2)	39.1 (36.0)	0.038*
Systolic blood pressure, mm Hg, Mean (SD)	151.3 (23.6)	160.8 (30.6)	0.2
Systolic Blood Pressure (≥180 mmHg), Mean (SD)	10 (15%)	14 (33%)	0.030*
Diastolic blood pressure, mm Hg, Mean (SD)	85.9 (13.7)	91.1 (15.3)	0.13
Baseline ASPECTS score <6, n (%)	15 (27%)	20 (56%)	0.007**
TOAST classification, n (%)			0.039*
Cardioembolism	16 (25%)	12 (28%)	
Large Vessel Disease	17 (26%)	18 (42%)	
Small Vessel Disease	8 (12%)	0 (0%)	
Stroke of other determined etiology	1 (1.5%)	2 (4.7%)	
Stroke of undetermined etiology	23 (35%)	11 (26%)	

Table 4.7 Comparison of patients with good outcomes and poor outcomes at 3 months from stroke onset *(contd.)*

Characteristic	Good outcome at 90 days, N = 65	Poor outcome at 90 days, N = 43	p-value[1]
Intravenous Thrombolysis, n (%)	34 (52%)	9 (21%)	0.001**
Mechanical Thrombectomy, n (%)	17 (26%)	18 (42%)	0.088
Bridging Therapy, n (%)	5 (7.7%)	1 (2.3%)	0.4
HT detected in patients, n (%)	11 (17%)	17 (40%)	0.009**
Baseline NIHSS score, Median (IQR)	10.0 (7.0, 15.0)	17.0 (11.0, 20.0)	0.003**
NIHSS score at discharge, Median (IQR)	2.0 (0.0, 6.2)	12.0 (7.5, 15.5)	<0.001***
mRS at discharge (0-2), n (%)	34 (52%)	1 (2.3%)	<0.001***
mRS at discharge (3-6), n (%)	31 (48%)	41 (95%)	<0.001***
Mortality, n (%)	1 (1.5%)	10 (23%)	<0.001***

Note. [1]Pearson's Chi-squared test; Wilcoxon rank sum test; Fisher's exact test (*P < 0.05, **P < 0.01 and ***P < 0.001); NIHSS: National Institutes of Health Stroke Scale; ASPECTS: Alberta stroke program early CT score; HT: hemorrhagic transformation; mg/dL: milligram/deciliter; SD: Standard deviation; IQR: Interquartile range; mRS: Modified Rankin scale; IV-rt-PA: Intravenous recombinant tissue plasminogen activator; EVT: Endovascular therapy; TOAST: Trial of Org 10172 in Acute Stroke Treatment; ESR: LDL: Low density lipoproteins; HDL: high density lipoproteins.

The mean age was 62.3±11.7 years and 70% were men. At 90 days, 65 (60.1%) patients had a good functional outcome. The median baseline NIHSS score was 12 IQR [8 - 18]. Among the etiological subtypes, large vessel atherosclerotic disease (P=0.039) was most common. The presence of elevated plasma glucose (196.7±91.0 mg/dL; P = 0.016), stroke severity 17.0 IQR [11.0 - 20.0]; (P = 0.003) and HT of the infarct (P = 0.009) predicted poor functional outcome.

The temporal profile of all the biomarkers given in Fig. 4.13 showed an overall increase in the plasma levels at 12 h.

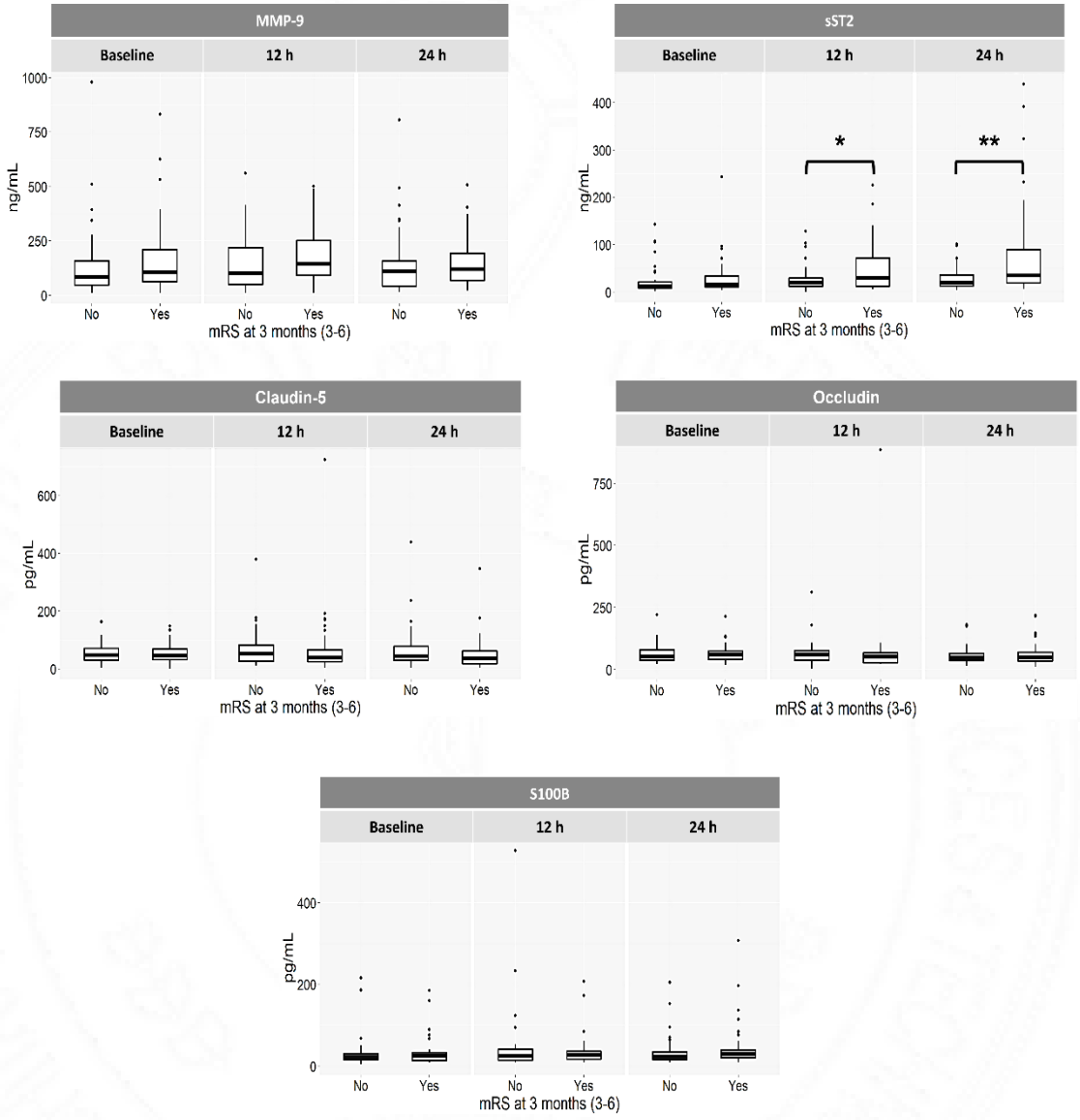


Fig. 4.13 Box plot graphs showing MMP-9, sST2, Claudin-5, Occludin, and S100B levels at each time-points between patients who had favorable and unfavorable outcomes at 3 months from onset (*P < 0.05, **P < 0.01).

In addition, sST2 showed a significant elevation at the 12 h and 24 h timepoints, and poor outcome was correlated at 12 h and 24 h with mean levels of 50.4±51.0 ng/mL (P = 0.047) and 81.8±101.3 ng/mL (P = 0.001), respectively. There was no correlation of either MMP-9 or Claudin-5 at 12 h with the 90-day functional outcome. Pearson product moment correlation showed significant associations between mRS at discharge, and 3 months, and the ST2 levels measured at 12 h and 24 h as given in Table 4.8.

Table 4.8 Pearson Product–Moment Correlation Coefficients for sST2 levels at three time points and mRS scores.

Correlation of mRS		Pearson's r	95% CI	P value
mRS at discharge	sST2 at baseline	0.15	-0.04, 0.34	0.13
	sST2 at 12 h	0.16	-0.03, 0.35	0.1
	sST2 at 24 h	0.25	0.07, 0.42	0.006*
mRS at 3 months	sST2 at baseline	0.17	-0.02, 0.36	0.08
	sST2 at 12 h	0.31	0.11, 0.48	0.002*
	sST2 at 24 h	0.28	0.10, 0.45	0.002*

Note. *P<0.01; CI, Confidence Interval; Pearson's r, Pearson's correlation coefficient; sST2, soluble serum stimulation-2; ng/mL, nanogram per milliliter; mRS, Modified Rankin scale; h, hours.

The ROC curve in Table 4.9 showed that the diagnostic accuracy of plasma sST2 levels at 24 h was 0.670 for a cut-off value of 71.8 ng/mL (specificity: 96.9%, sensitivity: 38.5%).

Table 4.9 ROC curve Analysis discriminating favorable outcomes and unfavorable outcomes in acute ischemic stroke.

Biomarker levels		AUC	Cut-off value	Sensitivity, %	Specificity, %	PPV, %	NPV, %
MMP-9	Baseline	0.585	56.06 ng/mL	81.8	39.3	21.4	55.7
	12 h	0.610	77.21 ng/mL	86.1	44.2	17.9	48.3
	24 h	0.566	50.66 ng/mL	92.3	30.8	13.0	55.6
sST2	Baseline	0.593	11.09 ng/mL	75.8	44.6	24.2	55.4
	12 h	0.619	36.67 ng/mL	44.4	90.4	29.9	23.8
	24 h	0.670	71.81 ng/mL	38.5	96.9	27.6	11.8
Claudin5	Baseline	0.474	116.77 pg/mL	9.1	98.2	35.2	25.0
	12 h	0.417	168.10 pg/mL	11.1	96.2	39.0	33.3
	24 h	0.409	164.38 pg/mL	5.1	96.9	37.0	50.0
Occludin	Baseline	0.500	48.91 pg/mL	66.7	46.4	29.7	59.7
	12 h	0.423	15.23 pg/mL	100.0	38.4	0.0	58.1
	24 h	0.508	103.36 pg/mL	15.4	96.9	34.4	25.0

Note. AUC, Area under the curve; PPV, Positive predictive value; NPV, Negative predictive value; MMP-9, matrix metalloproteinase-9; sST2, soluble serum stimulation-2; h, hours; pg/mL, picogram/milliliter; ng/mL, nanogram/milliliter.

MMP-9 levels showed a higher sensitivity for all three time points with an optimum cut-off value at 12 h timepoint (77.212 ng/mL; sensitivity: 86.1% and specificity: 44.2%). The highest sensitivity (92.3%) was attained at 24 h for a cut-off value of 50.663 ng/mL but the AUC was only 56.6%. Based on their discriminative values Claudin-5 and Occludin yielded the lowest discriminative capacity between the outcome groups. The ROC analysis of S100B was not available due to the low concentrations yielding erroneous results.

Multiple logistic regression analysis is given in Table 4.10 and showed that elevated sST2 levels assessed at 24 h from onset emerged as an independent predictor of poor functional outcome at 3 months (OR: 6.44, 95% CI: 1.40 - 46.3, P=0.029) by applying cut-off value derived from the ROC curve analysis to the model.

Table 4.10 Multiple logistic model for predicting functional outcome after adjusting for risk factors.

Variable	OR[1]	95% CI[1]	p-value
Plasma glucose (>140 mg/dL)	2.06	0.73, 6.20	0.2
Systolic blood pressure (≥180 mmHg)	1.17	0.32, 4.14	0.8
Baseline ASPECTS score (<6)	2.10	0.68, 6.54	0.2
Hemorrhagic transformation	0.42	0.13, 1.30	0.13
Baseline NIHSS score (>16)	1.87	0.62, 5.53	0.3
Large vessel disease etiology	0.45	0.15, 1.27	0.13
sST2 levels at 24 h (ng/mL)	6.44	1.40, 46.3	0.029*

Note. [1]OR: Odds Ratio, CI: Confidence Interval; *P<0.05; sST2: soluble serum stimulation-2; ng/mL: nanogram per milliliter; mg/dL: milligram/deciliter; mRS: Modified Rankin scale; h: hours; NIHSS: National Institutes of Health Stroke Scale; ASPECTS: Alberta Stroke Program Early CT Score.

5 DISCUSSION

The chapter begins with a summary of the results from the systematic review and meta-analysis. The plasma levels of biomarkers that are associated with HT, stroke severity, and functional outcome are reviewed in connection to the research hypotheses in the findings of the observational study. The theoretical and clinical implications of the research findings are considered. The methodological strengths and limitations of the study are reflected with suggestions for future research and concluding remarks.

5.1 MMP-9 had the highest discriminative capacity

The meta-analysis of studies examining the association of four biomarkers, specifically, MMP-9, c-Fibronectin, Ferritin, and S100B, with symptomatic HT and NLR with sICH, revealed that MMP-9 levels were elevated 6 to 8 h post-ischemia and were significantly higher in individuals who developed symptomatic HT, particularly the PH subtype (Montaner et al., 2001; Montaner et al., 2003). Patients with increased levels of MMP-9, which were often assessed in baseline samples prior to thrombolysis, had a 29.5-fold higher likelihood of developing symptomatic HT following a stroke. In the SROC plot evaluating the performance of all five biomarkers, MMP-9 demonstrated a high pooled sensitivity and low false positive rate, validating previous studies (Castellanos et al., 2003; Montaner et al., 2003), and indicating that MMP-9 had a better discriminating capacity for predicting the risk of HT.

Fibronectin was the molecule with the highest sensitivity. Studies have shown that c-Fibronectin and MMP-9 combined, improved the specificity and predictive capacity for determining the risk of PH (Castellanos et al., 2007). High sensitivity was

also obtained from Ferritin levels, which were shown to be related to both c-Fibronectin and MMP-9 at 24 h from the onset but not at baseline (Millán et al., 2008). However, the wide confidence intervals that both c-Fibronectin and Ferritin generated in the meta-analysis had an impact on the accuracy of their prediction. The meta-analysis reported low pooled sensitivities for both S100B and NLR and S100B was reported to have the highest false-positive rate. The difference in the HT subtypes investigated in the two studies may have contributed to the poor sensitivity of S100B (Foerch et al., 2007; Kazmierski et al., 2012).

Functional outcome is closely related to HT, and its correlation with biomarkers was also examined. Baseline concentrations of MMP-9, Ferritin, and NLR were linked to unfavorable outcomes. NLR's weak correlation with sICH suggests that it was less discriminative than the other markers. Although Caveolin-1, TAFI, PAI-1, and sST2, reported in single studies, were identified as potential biomarkers with the ability to predict HT, further research may be necessary to establish these findings.

5.2 Claudin-5 was an independent predictor of HT

The prospective, observational study investigated the temporal relationship of circulating MMP-9, Claudin-5, soluble ST2, Occludin, and S100B levels with HT in AIS. The study also looked at how these indicators affected baseline stroke severity and the functional outcome within the first three months after stroke onset as the secondary objective.

On admission, patients enrolled for the study who were detected with HT showed an increased correlation with AF, EVT, baseline ASPECTS score, and cardioembolic etiology. There was a significant delay in the hospital arrival time of

patients who developed HT as compared to those that did not after the stroke. Most patients who underwent IV rtPA were not detected with HT. The majority of studies have found that early reperfusion during the initial three hours of stroke results in a smaller infarct volume, which lowers the risk of HT (Wardlaw et al., 2014). Noteworthy, in this study, the median period admission from stroke onset for most patients who had undergone reperfusion was within three hours. Secondly, there may be a larger risk of spontaneous HT in patients who were hospitalized after the thrombolytic window period. Molina et al. found that spontaneous recanalization occurred beyond six hours from stroke onset and was associated with HT (Molina et al., 2001). According to experimental data, petechial hemorrhages that occur within 24 hours after an MCA occlusion may be brought about by the loss of the basal lamina of the blood-brain barrier (Molina et al., 2001).

Among the biomarkers, Claudin-5 in combination with low ASPECTS score independently predicted HT in AIS. Although temporal profiles of MMP-9, Claudin-5, and sST2 indicated an overall increase within the first 12 hours after onset, MMP-9 at 12 hours showed a trend toward a significant positive correlation with HT. Pathological data suggested that MMP-9 released by degranulation of neutrophils during ischemia are highly expressed in blood 6-8 hours after stroke onset in patients with HT, subsequently plummeting around 24-26 hours (Jickling et al., 2014; Kelly et al., 2008). However, studies have also shown that temporal distribution between HT subtypes tends to vary due to slightly different pathophysiologies (Montaner et al., 2003; Hong et al., 2021). The findings showed a differential time of release of MMP-9 reaching the pinnacle at around 12 hours that gradually declined, which was consistent with the preceding findings (Jickling et al., 2014).

The ROC curve of MMP-9 showed a high sensitivity of discrimination for a cut-off value of 96.3 ng/mL, which was maintained from baseline to 12 hours signifying that an earlier measurement of the marker would predict the risk of HT. This represented a progressive increase in the threshold levels in both MMP-9 and sST2 with a maximum sensitivity obtained for MMP-9 and Claudin-5 at 12 hours. Claudin-5 did not show a statistically significant association for the prediction of HT in the bivariate analysis. However, when all biomarkers which were elevated at the 12-hour timepoint were included in the multiple logistic regression model after adjusting for covariates, a statistically significant predictive performance was reported. Claudin-5 is a principal TJ protein restricting the paracellular permeation of polar solutes and transcytotic vesicles across the barrier (Greene et al., 2019). The presence of Claudin-5 in the blood after the ischemia-reperfusion injury is an important indicator of HT, given its direct involvement in the blood-brain barrier disruption. Experimental data in animals have shown a biphasic elevation of Claudin-5 – the first spike at 3 to 6 hours from stroke onset and the second, at 120 hours, indicating its role in barrier leakage and redistribution (Jiao et al., 2011). Our findings supported the elevation of the marker within the first 12 hours from the onset in HT patients thereby proving to be an important marker for screening for high-risk patients.

The temporal distribution of soluble ST2 showed a gradual increase across the baseline to 24 hours indicating its role in the inflammatory phase of the ischemic cascade, however, no significant association was observed contrary to previous findings (Wolcott et al., 2017; Mechtouff et al., 2021). Although the highest sensitivity was observed at baseline for sST2, the low threshold value may affect its discriminative capacity in HT. Our study did not show any baseline level association

between all five markers and the risk of HT presumably owing to the variability in the time of sample collection during admission. Occludin and S100B did not show any difference between HT and non-HT groups at any of the time points.

5.3 MMP-9 was correlated with stroke severity

Furthermore, MMP-9 correlated with baseline stroke severity in the study population demonstrating a relevant relationship between severe strokes and the increased expression of MMP-9. Patients with moderate to severe NIHSS scores at the time of admission had significantly elevated levels of MMP-9 when assessed at 12 hours and twenty-nine of these patients were detected with HT. This finding is similar to a previous study that showed the association of MMP-9 with stroke severity whereby an NIHSS score over 8 correlated with neurological deterioration within 48 hours from stroke onset (Sotgiu et al., 2006).

However, MMP-9 was not significantly correlated with HT contrary to previous findings (Castellanos et al., 2003; Castellanos et al., 2007; Montaner et al., 2003; Mechtouff et al., 2020). MMP-9 is the most studied biomarker in the prediction of HT with data indicating the efficacy of the marker in predicting HT and outcome (Mechtouff et al., 2020; Castellanos et al., 2003; Montaner et al., 2003). On the other hand, some studies have reported the lack of correlation of MMP-9 with either HT or stroke severity, possibly owing to its heterogeneity in the eligibility criteria, and time of blood collection which usually ranges between 6 to 20 hours from the onset in the studies (Maestrini et al., 2020; Iwamoto et al., 2021). Nevertheless, the trend of elevation in the HT group indicates that MMP-9 plays a major role in HT by infiltrating the endothelium of the blood-brain barrier via neutrophil transmigration (Enzmann et al., 2013).

5.4 Soluble ST2 predicted Poor Functional Outcome

Our findings demonstrated the prognostic impact of circulating sST2 levels measured within 24 hours from stroke onset predicting short-term functional outcomes in AIS. Of the five markers, sST2 showed a gradual increase in its concentration across the three time points in the poor outcome group with significantly elevated levels at 12 h and 24 h from the onset. This temporal profile was consistent with the previous findings of its elevation beyond 24 hours from onset indicating its expression during the inflammatory phase of ischemia (Sastre et al., 2021).

After adjusting for covariates, sST2 emerged as an independent predictor of poor functional outcomes after stroke. The probability of an unfavorable outcome was six times higher when the cut-off value of sST2 levels assessed at 24 hours was applied to the model yielding better discriminative capacity and high specificity.

We found that there was no significant association of both MMP-9 and Claudin-5 with the outcome, although a trend towards elevation was observed at the 12-hour time point in patients with poor outcomes. Furthermore, the discriminatory capacity of Claudin-5 was low, thereby affecting the reliability of the marker. Experimental studies have indicated the role of neuroinflammatory markers in the disruption of the blood-brain barrier affecting outcomes after stroke (Yang et al., 2019; Anrather and Iadecola, 2016; Korhonen et al., 2015). Elevated levels of sST2 have been previously reported as a prognostic indicator of AIS in some studies (Wolcott et al., 2017; Mechtouff et al., 2021; Tian et al., 2020). One study reported the highest tertile of sST2 levels above a cut-off level of 22.96 ng/mL, which was measured at a median time of 19 hours from stroke onset, to predict poor outcome and all-cause

mortality at 90 days (Tian et al., 2020). A similar correlation was reported for an earlier timepoint by Wolcott and colleagues, whereby sST2 >44.6 ng/mL was independently associated with outcome and mortality in AIS (Wolcott et al., 2017). Although these baseline assessments were consistent with the results of the current study, no significant correlation between the baseline levels and the functional outcome was found in our cohort. Another recent study that looked into the relationship of sST2 levels in AIS and cardiac patients reported a significant correlation between the levels of sST2 evaluated at 24 hours from admission with all-cause mortality at three months, but relatively lower levels at baseline and in the subsequent time points were observed among AIS group (Mechtouff et al., 2021). Contrary to these findings, in a multi-marker study conducted by Dieplinger and colleagues, sST2 showed no correlation with the functional outcome (Dieplinger et al., 2017). Nevertheless, the heterogeneity in these results may be attributed to the sampling done at different time points of admission rather than at a specific time course from the onset of stroke. This was evident in the dynamics observed in the temporal distribution of each marker in our study. The plasma levels of sST2 were more pronounced during the late phase of acute stroke when inflammatory mechanisms are activated (Altara et al., 2018) but both MMP-9 and Claudin-5 were found to be expressed earlier during the blood-brain barrier dysfunction owing to their response to oxidative stress (Rosell et al., 2008). sST2 is a receptor of the immunomodulatory cytokine, IL-33, and is a known prognostic marker for cardiac diseases as the IL-33/sST2 system was found to respond to cardiac stress (Lotierzo et al., 2020). However, recent studies have also implicated its key role in various central nervous diseases (De la Fuente et al., 2015; Yang et al., 2017). Whereas IL-33 function as neuroprotective, increased levels of circulating

sST2 inhibit IL-33 and exacerbate the pro-inflammatory processes (Pascual-Figal et al., 2016; Yang et al., 2017). The opposing functions of IL-33 and sST2 is necessary for modulating the neuroprotective effects post-ischemia and remodeling of the ECM that may influence the outcome after stroke (Sastre et al., 2021; Yang et al., 2017). This relationship is evident in a study that showed the presence of lower IL-33 levels in patients with poor outcome in ischemic stroke (Chen et al., 2021).

5.5 Strengths and Limitations of the Study

The strengths of the systematic review and the meta-analysis conducted were the availability of individual studies with at least two well-studied biomarkers such as MMP-9 and NLR. However, the variable levels of MMP-9 and NLR among studies might be explained by heterogeneity in the patient group, timing of blood samples, whether plasma or serum was utilized, the use of different commercial assay kits, and the reporting of HT subtypes. The pooled analysis employed in the current investigation was based on a random effects model due to the large variety of cut-off values and the use of different methodologies. Even though heterogeneity is a well-known trait of research on diagnostic test accuracy, large sample studies are advised to assess the diagnostic test accuracies of different biomarkers. Additionally, only two studies were available for c-Fibronectin and Ferritin among the included studies, which limited the pooled analysis. Additionally, several studies failed to exclude individuals who had previously used anticoagulants, antithrombotic medications, or both, all of which may have an impact on the severity and incidence of HT and its subtypes. Much of the included research used a monocentric design, which may restrict how broadly we may generalize our findings.

As for the observational study ensuing the systematic review and meta-analysis, the thorough sample collection at predetermined intervals following the onset of stroke was one of the major merits. The biomarkers were evaluated using serial measurements from onset to observe the dynamic changes in the plasma from baseline to 24 hours after onset. Few studies that investigated the association of sST2 with AIS assessed the plasma levels at baseline alone. Additionally, the study had limitations due to its monocentric design and its small sample size, which may restrict the generalizability of its results to other ethnic groups. Due to the qualifying requirements, a sizable number of patients had to be excluded, and since this information was not gathered, the difference between the included and excluded patients was not reported. This may have led to a selection bias in the findings. Furthermore, despite the small numbers, the analysis was conducted to see whether there may be a trend between the five biomarkers and the HT subtypes, but yielded no correlation. Previous studies have shown an association between the PH subtype and the baseline levels of MMP-9 and S100B (Montaner et al., 2003; Castellanos et al., 2007). But our analysis did not show any correlation with the same. Hence, we did not include these findings in the thesis. To confirm the therapeutic application of these biomarkers, a larger sample set may need to be used to further study the association of these proteins with HT and outcome in AIS.

6 SUMMARY AND CONCLUSIONS

Hemorrhagic transformation (HT) is a frequent and potentially serious complication of acute ischemic stroke (AIS). Plasma biomarkers may be useful in predicting HT with accuracy and operability and they may be helpful adjuncts for clinical decision-making. However, due to the heterogeneity in the data reported, the disparities in the timing of sampling, and the techniques used among research studies, their validation in a clinical setting is uncertain. Hence there is insufficient evidence to support their therapeutic application. This study sought to identify the biomarkers that may predict HT in a South Indian cohort of patients diagnosed with AIS with the foremost objective of conducting a systematic review and meta-analysis of the biomarkers that have been indicated in previous literature to predict HT, and the subsequent objective of investigating the temporal relationship of the biomarkers in HT. Additionally, the relationship of these biomarkers with short-term functional outcome was examined in AIS.

6.1 Summary of results

For the systematic review, the databases' screening of 2230 research studies yielded 30 papers that were quality appraised and eligible for the review. Of these, 16 studies investigated five biomarkers namely, Matrix metalloproteinase-9 (MMP-9), cellular-Fibronectin (c-Fibronectin), Ferritin, S100B levels in symptomatic HT, and Neutrophil-lymphocyte ratio (NLR) in symptomatic intracranial hemorrhage (sICH), were included in the meta-analysis. The meta-analysis revealed that MMP-9, which is an enzyme specialized in the blood-brain barrier disintegration, had a proclivity to lead

to symptomatic HT after stroke with a 29.5-fold increased risk. MMP-9 was found to be a highly sensitive and specific marker for predicting symptomatic HT, as previous literature pointed out, with excellent diagnostic accuracy, particularly for predicting severe forms of HT. The systematic review reported baseline levels of MMP-9, Ferritin, and NLR to predict poor short-term functional outcomes in AIS. Biomarkers that had a high predictive value but could not be included in the meta-analysis due to the availability of a single study were Caveolin-1, thrombin-activated fibrinolysis inhibitor (TAFI), plasminogen activator inhibitor-1 (PAI-1) and soluble serum stimulation (sST2) protein.

In the prospective observational study, the multiple logistic regression analysis revealed that plasma Claudin-5 levels measured within 12 hours from stroke onset, combined with low ASPECTS scores at baseline, independently predicted the risk of HT in AIS. MMP-9 was positively correlated with baseline stroke severity in AIS. Patients with higher NIHSS scores at baseline were found to have elevated plasma levels of MMP-9 at 12 hours from stroke onset. MMP-9 and Claudin-5 followed a similar time course of expression demonstrating the direct role of MMP-9 in the degradation of Claudin-5 tight junction proteins of the blood-brain barrier thereby playing a major role in the disruption of the blood-brain barrier. SST2 was an independent predictor of poor outcomes and mortality in AIS. The plasma levels were significantly elevated in the samples assessed at 12 hours and 24 hours from stroke onset. The temporal profile of sST2 showed a gradual elevation which was associated with the inflammatory mechanism during ischemia. Both Occludin and S100B yielded the lowest results and were not found to be associated with HT and outcome.

6.2 Future Scope of the Work

- A prospective study with a large sample size consisting of plasma samples drawn at specific time intervals from stroke onset may help further establish the predictive roles of these biomarkers in HT
- Investigating the correlation between infarct volume and biomarker levels in HT and poor outcome.
- Blood-brain barrier disruption measured with quantitative model studies.
- Cerebral autoregulation correlation with biomarker levels.

7 REFERENCES

Abbott NJ, Patabendige AAK, Dolman DEM, et al. (2010) Structure and function of the blood–brain barrier. *Neurobiology of Disease* 37(1): 13-25.

Abbott NJ, Rönnbäck L and Hansson E. (2006) Astrocyte–endothelial interactions at the blood–brain barrier. *Nature Reviews Neuroscience* 7(1): 41-53.

Abdullahi W, Tripathi D and Ronaldson PT. (2018) Blood-brain barrier dysfunction in ischemic stroke: targeting tight junctions and transporters for vascular protection. *American Journal of Physiology-Cell Physiology* 315(3): C343-C356.

Adams HP, Bendixen BH, Kappelle LJ, et al. (1993) Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. *Stroke* 24(1): 35-41.

Aho K, Harmsen P, Hatano S, et al. (1980) Cerebrovascular disease in the community: results of a WHO collaborative study. *Bulletin of the World Health Organization* 58(1): 113-130.

Albers GW. (2018) Late Window Paradox. *Stroke* 49(3): 768-771.

Albers GW, Goldstein LB, Hess DC, et al. (2011) Stroke Treatment Academic Industry Roundtable (STAIR) Recommendations for Maximizing the Use of Intravenous Thrombolytics and Expanding Treatment Options With Intra-arterial and Neuroprotective Therapies. *Stroke* 42(9): 2645-2650.

Alberts MJ. (2012) Cerebral Hemorrhage, Warfarin, and Intravenous tPA: The Real Risk Is Not Treating. *JAMA* 307(24): 2637-2639.

Alexandrov AV, Black SE, Ehrlich LE, et al. (1997) Predictors of Hemorrhagic Transformation Occurring Spontaneously and on Anticoagulants in Patients With Acute Ischemic Stroke. *Stroke* 28(6): 1198-1202.

Altara R, Ghali R, Mallat Z, et al. (2018) Conflicting vascular and metabolic impact of the IL-33/sST2 axis. *Cardiovascular Research* 114(12): 1578-1594.

Álvarez-Sabín J, Maisterra O, Santamarina E, et al. (2013) Factors influencing haemorrhagic transformation in ischaemic stroke. *The Lancet Neurology* 12(7): 689-705.

Amarenco P, Bogousslavsky J, Caplan LR, et al. (2009) Classification of Stroke Subtypes. *Cerebrovascular Diseases* 27(5): 493-501.

Andrade JBC, Mohr JP, Lima FO, et al. (2020) The Role of Hemorrhagic Transformation in Acute Ischemic Stroke Upon Clinical Complications and Outcomes. *Journal of Stroke and Cerebrovascular Diseases* 29(8): 104898.

Anrather J and Iadecola C. (2016) Inflammation and Stroke: An Overview. *Neurotherapeutics* 13(4): 661-670.

Atkinson AJ, Colburn WA, DeGruttola VG, et al. (2001) Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. *Clinical Pharmacology & Therapeutics* 69(3): 89-95.

Banks JL and Marotta CA. (2007) Outcomes Validity and Reliability of the Modified Rankin Scale: Implications for Stroke Clinical Trials. *Stroke* 38(3): 1091-1096.

Barber PA, Demchuk AM, Zhang J, et al. (2000) Validity and reliability of a quantitative computed tomography score in predicting outcome of hyperacute stroke before thrombolytic therapy. *The Lancet* 355(9216): 1670-1674.

Belov Kirdajova D, Kriska J, Tureckova J, et al. (2020) Ischemia-Triggered Glutamate Excitotoxicity From the Perspective of Glial Cells. *Frontiers in Cellular Neuroscience* 14: 51.

Berger C, Fiorelli M, Steiner T, et al. (2001) Hemorrhagic transformation of ischemic brain tissue: asymptomatic or symptomatic? *Stroke* 32(6): 1330-1335.

Bernardo-Castro S, Sousa JA, Brás A, et al. (2020) Pathophysiology of Blood–Brain Barrier Permeability Throughout the Different Stages of Ischemic Stroke and Its Implication on Hemorrhagic Transformation and Recovery. *Frontiers in Neurology* 11: 1605.

Bivard A, Cheng X, Lin L-T, et al. (2016) Global White Matter Hypoperfusion on CT Predicts Larger Infarcts and Hemorrhagic Transformation after Acute Ischemia. *CNS Neuroscience & Therapeutics* 22(3): 238-243.

Bivard A, Lin L and Parsons MW. (2013) Review of stroke thrombolytics. *Journal of stroke* 15(2): 90-98.

Boehme AK, Esenwa C and Elkind MSV. (2017) Stroke Risk Factors, Genetics, and Prevention. *Circulation research* 120(3): 472-495.

Bounameaux H, Cirafici P, de Moerloose P, et al. (1991) Measurement of D-dimer in plasma as diagnostic aid in suspected pulmonary embolism. *The Lancet* 337(8735): 196-200.

Brott T, Adams HP, Olinger CP, et al. (1989) Measurements of acute cerebral infarction: a clinical examination scale. 20(7): 864-870.

Brouns R and De Deyn PP. (2009) The complexity of neurobiological processes in acute ischemic stroke. *Clinical Neurology and Neurosurgery* 111(6): 483-495.

Bustamante A, Penalba A, Orset C, et al. (2021) Blood Biomarkers to Differentiate Ischemic and Hemorrhagic Strokes. *Neurology* 96(15): e1928-e1939.

Califf RM. (2018) Biomarker definitions and their applications. *Experimental Biology and Medicine* 243(3): 213-221.

Camara R, Matei N and Zhang JH. (2021) Evolution of the stroke paradigm: A review of delayed recanalization. *Journal of Cerebral Blood Flow & Metabolism* 41(5): 945-957.

Campbell BCV, De Silva DA, Macleod MR, et al. (2019) Ischaemic stroke. *Nature Reviews Disease Primers* 5(1): 70.

Campbell BCV and Khatri P. (2020) Stroke. *The Lancet* 396(10244): 129-142.

Campbell BCV, Mitchell PJ, Churilov L, et al. (2018) Tenecteplase versus Alteplase before Thrombectomy for Ischemic Stroke. *New England Journal of Medicine* 378(17): 1573-1582.

Caplan LR. (2015) Lacunar infarction and small vessel disease: pathology and pathophysiology. *Journal of stroke* 17(1): 2-6.

Caplan LR. (2016) *Caplan's stroke*: Cambridge University Press.

Carter AM, Catto AJ, Mansfield MW, et al. (2007) Predictive Variables for Mortality After Acute Ischemic Stroke. *Stroke* 38(6): 1873-1880.

Castellanos M, Eendenburg Cv, Gubern C, et al. (2018) Low Levels of Caveolin-1 Predict Symptomatic Bleeding After Thrombolytic Therapy in Patients With Acute Ischemic Stroke. *Stroke* 49(6): 1525-1527.

Castellanos M, Leira R, Serena J, et al. (2004) Plasma Cellular-Fibronectin Concentration Predicts Hemorrhagic Transformation After Thrombolytic Therapy in Acute Ischemic Stroke. *Stroke* 35(7): 1671-1676.

Castellanos M, Leira R, Serena J, et al. (2003) Plasma Metalloproteinase-9 Concentration Predicts Hemorrhagic Transformation in Acute Ischemic Stroke. *Stroke* 34(1): 40-46.

Castellanos M, Sobrino T, Millán M, et al. (2007) Serum Cellular Fibronectin and Matrix Metalloproteinase-9 as Screening Biomarkers for the Prediction of Parenchymal Hematoma After Thrombolytic Therapy in Acute Ischemic Stroke. *Stroke* 38(6): 1855-1859.

Chamorro Á, Meisel A, Planas AM, et al. (2012) The immunology of acute stroke. *Nature Reviews Neurology* 8(7): 401-410.

Chandra A, Li WA, Stone CR, et al. (2017a) The cerebral circulation and cerebrovascular disease I: Anatomy. *Brain circulation* 3(2): 45-56.

Chandra A, Stone C, Li W, et al. (2017b) The cerebral circulation and cerebrovascular disease II: Pathogenesis of cerebrovascular disease. *Brain Circulation* 3(2): 57-65.

Che R, Huang X, Zhao W, et al. (2017) Low Serum Albumin level as a Predictor of Hemorrhage Transformation after Intravenous Thrombolysis in Ischemic Stroke Patients. *Scientific Reports* 7(1): 7776.

Chen Z, Hu Q, Huo Y, et al. (2021) Serum Interleukin-33 is a Novel Predictive Biomarker of Hemorrhage Transformation and Outcome in Acute Ischemic Stroke. *Journal of Stroke and Cerebrovascular Diseases* 30(2): 105506.

Choi K-H, Park M-S, Kim J-T, et al. (2012) The serum ferritin level is an important predictor of hemorrhagic transformation in acute ischaemic stroke. *European Journal of Neurology* 19(4): 570-577.

Curtze S, Haapaniemi E, Melkas S, et al. (2015) White Matter Lesions Double the Risk of Post-Thrombolytic Intracerebral Hemorrhage. *Stroke* 46(8): 2149-2155.

D'Anna L, Filippidis FT, Harvey K, et al. (2021) Extent of white matter lesion is associated with early hemorrhagic transformation in acute ischemic stroke related to atrial fibrillation. *Brain and Behavior* 11(8): e2250.

Dagonnier M, Donnan GA, Davis SM, et al. (2021) Acute Stroke Biomarkers: Are We There Yet? *Frontiers in Neurology* 12: 619721.

Dambinova SA, Bettermann K, Glynn T, et al. (2012) Diagnostic Potential of the NMDA Receptor Peptide Assay for Acute Ischemic Stroke. *PLOS ONE* 7(7): e42362.

De la Fuente M, MacDonald TT and Hermoso MA. (2015) The IL-33/ST2 axis: Role in health and disease. *Cytokine & Growth Factor Reviews* 26(6): 615-623.

de Lemos JA, McGuire DK and Drazner MH. (2003) B-type natriuretic peptide in cardiovascular disease. *The Lancet* 362(9380): 316-322.

Deb P, Sharma S and Hassan KM. (2010) Pathophysiologic mechanisms of acute ischemic stroke: An overview with emphasis on therapeutic significance beyond thrombolysis. *Pathophysiology* 17(3): 197-218.

del Zoppo GJ, Frankowski H, Gu Y-H, et al. (2012) Microglial cell activation is a source of metalloproteinase generation during hemorrhagic transformation. *Journal of Cerebral Blood Flow and Metabolism* 32(5): 919-932.

Desilles J-P, Syvannarath V, Ollivier V, et al. (2017) Exacerbation of Thromboinflammation by Hyperglycemia Precipitates Cerebral Infarct Growth and Hemorrhagic Transformation. *Stroke* 48(7): 1932-1940.

Dieplinger B, Bocksrucker C, Egger M, et al. (2017) Prognostic Value of Inflammatory and Cardiovascular Biomarkers for Prediction of 90-Day All-Cause Mortality after Acute Ischemic Stroke—Results from the Linz Stroke Unit Study. *Clinical Chemistry* 63(6): 1101-1109.

Duan Z, Wang H, Wang Z, et al. (2018) Neutrophil-Lymphocyte Ratio Predicts Functional and Safety Outcomes after Endovascular Treatment for Acute Ischemic Stroke. *Cerebrovascular Diseases* 45(5-6): 221-227.

Dzialowski I, Pexman JHW, Barber PA, et al. (2007) Asymptomatic Hemorrhage After Thrombolysis May Not Be Benign. *Stroke* 38(1): 75-79.

El-Banhawy E, Amer H, Younes K, et al. (2014) Plasma matrix metalloproteinase-9 (MMP-9) and hemorrhagic transformation in acute ischemic stroke. *The Egyptian Journal of Neurology, Psychiatry and Neurosurgery* 51: 159-166.

El-Banhawy E, Amer H, Younes K, et al. (2014) Plasma matrix metalloproteinase-9 (MMP-9) and hemorrhagic transformation in acute ischemic stroke. *The Egyptian Journal of Neurology, Psychiatry and Neurosurgery* 51: 159-166.

Elkind MSV, Tai W, Coates K, et al. (2006) High-Sensitivity C-Reactive Protein, Lipoprotein-Associated Phospholipase A2, and Outcome After Ischemic Stroke. *Archives of Internal Medicine* 166(19): 2073-2080.

Elkind MSV, Tai W, Coates K, et al. (2009) Lipoprotein-Associated Phospholipase A_2 Activity and Risk of Recurrent Stroke. *Cerebrovascular Diseases* 27(1): 42-50.

Emberson J, Lees KR, Lyden P, et al. (2014) Effect of treatment delay, age, and stroke severity on the effects of intravenous thrombolysis with alteplase for acute

ischaemic stroke: a meta-analysis of individual patient data from randomised trials. *The Lancet* 384(9958): 1929-1935.

Enzmann G, Mysiorek C, Gorina R, et al. (2013) The neurovascular unit as a selective barrier to polymorphonuclear granulocyte (PMN) infiltration into the brain after ischemic injury. *Acta Neuropathologica* 125(3): 395-412.

Ergul A, Kelly-Cobbs A, Abdalla M, et al. (2012) Cerebrovascular Complications of Diabetes: Focus on Stroke. *Endocrine, Metabolic & Immune Disorders - Drug Targets* 12(2): 148-158.

Feigin VL, Brainin M, Norrving B, et al. (2022) World Stroke Organization (WSO): Global Stroke Fact Sheet 2022. *International Journal of Stroke* 17(1): 18-29.

Feigin VL, Stark BA, Johnson CO, et al. (2021) Global, regional, and national burden of stroke and its risk factors, 1990 - 2019: a systematic analysis for the Global Burden of Disease Study 2019. *The Lancet Neurology* 20(10): 795-820.

Fiorelli M, Bastianello S, Kummer Rv, et al. (1999) Hemorrhagic Transformation Within 36 Hours of a Cerebral Infarct. *Stroke* 30(11): 2280-2284.

Foerch C, Curdt I, Yan B, et al. (2006) Serum glial fibrillary acidic protein as a biomarker for intracerebral haemorrhage in patients with acute stroke. *Journal of Neurology, Neurosurgery and Psychiatry* 77(2): 181-184.

Foerch C, Wunderlich MT, Dvorak F, et al. (2007) Elevated Serum S100B Levels Indicate a Higher Risk of Hemorrhagic Transformation After Thrombolytic Therapy in Acute Stroke. *Stroke* 38(9): 2491-2495.

Fugate JE and Rabinstein AA. (2015) Absolute and Relative Contraindications to IV rt-PA for Acute Ischemic Stroke. *The Neurohospitalist* 5(3): 110-121.

Gibler WB, Blomkalns AL and Collins SP. (2003) Evaluation of chest pain and heart failure in the emergency department: impact of multimarker strategies and B-type natriuretic peptide. *Reviews in Cardiovascular Medicine* 4 Suppl 4: S47-55.

Goldstein LB, Bertels C and Davis JN. (1989) Interrater Reliability of the NIH Stroke Scale. *Archives of Neurology* 46(6): 660-662.

Goldstein LB and Simel DL. (2005) Is This Patient Having a Stroke? *JAMA* 293(19): 2391-2402.

Goyal M, Menon BK, van Zwam WH, et al. (2016) Endovascular thrombectomy after large-vessel ischaemic stroke: a meta-analysis of individual patient data from five randomised trials. *The Lancet* 387(10029): 1723-1731.

Goyal N, Tsivgoulis G, Chang JJ, et al. (2018) Admission Neutrophil-to-Lymphocyte Ratio as a Prognostic Biomarker of Outcomes in Large Vessel Occlusion Strokes. 49(8): 1985-1987.

Greene C, Hanley N and Campbell M. (2019) Claudin-5: gatekeeper of neurological function. *Fluids and Barriers of the CNS* 16(1): 3.

Gromova M, Vaggelas A, Dallmann G, et al. (2020) Biomarkers: Opportunities and Challenges for Drug Development in the Current Regulatory Landscape. *Biomarker insights* 15: 1177271920974652.

Group HPSC. (2002) MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20 536 high-risk individuals: a randomised placebocontrolled trial. *The Lancet* 360(9326): 7-22.

Group Nr-PSS. (1995) Tissue Plasminogen Activator for Acute Ischemic Stroke. *New England Journal of Medicine* 333(24): 1581-1588.

Group TMAST-ES. (1996) Thrombolytic Therapy with Streptokinase in Acute Ischemic Stroke. *New England Journal of Medicine* 335(3): 145-150.

Guo Z, Yu S, Xiao L, et al. (2016) Dynamic change of neutrophil to lymphocyte ratio and hemorrhagic transformation after thrombolysis in stroke. *Journal of Neuroinflammation* 13(1): 199.

Hacke W, Donnan G, Fieschi C, et al. (2004) Association of outcome with early stroke treatment: pooled analysis of ATLANTIS, ECASS, and NINDS rt-PA stroke trials. *The Lancet* 363(9411): 768-774.

Hacke W, Kaste M, Bluhmki E, et al. (2008) Thrombolysis with Alteplase 3 to 4.5 Hours after Acute Ischemic Stroke. *New England Journal of Medicine* 359(13): 1317-1329.

Hacke W, Kaste M, Fieschi C, et al. (1998) Randomised double-blind placebo-controlled trial of thrombolytic therapy with intravenous alteplase in acute ischaemic stroke (ECASS II). *The Lancet* 352(9136): 1245-1251.

Hage V. (2011) The NIH stroke scale: a window into neurological status. *J Nursing Spectrum* 24(15): 44-49.

Haorah J, Ramirez SH, Schall K, et al. (2007) Oxidative stress activates protein tyrosine kinase and matrix metalloproteinases leading to blood–brain barrier dysfunction. *Journal of neurochemistry* 101(2): 566-576.

Hawkins BT and Davis TP. (2005) The Blood-Brain Barrier/Neurovascular Unit in Health and Disease. *Pharmacological reviews* 57(2): 173-185.

Hernandez-Guillamon M, Garcia-Bonilla L, Solé M, et al. (2010) Plasma VAP-1/SSAO activity predicts intracranial hemorrhages and adverse neurological

outcome after tissue plasminogen activator treatment in stroke. *Stroke* 41(7): 1528-1535.

Hong JM, Kim DS and Kim M. (2021) Hemorrhagic Transformation After Ischemic Stroke: Mechanisms and Management. *Frontiers in Neurology* 12.

Honig A, Percy J, Sepehry AA, et al. (2022) Hemorrhagic Transformation in Acute Ischemic Stroke: A Quantitative Systematic Review. *Journal of clinical medicine* 11(5): 1162.

Hornig CR, Bauer T, Simon C, et al. (1993) Hemorrhagic transformation in cardioembolic cerebral infarction. *Stroke* 24(3): 465-468.

Iadecola C. (2017) The Neurovascular Unit Coming of Age: A Journey through Neurovascular Coupling in Health and Disease. *Neuron* 96(1): 17-42.

Iwamoto T, Kitano T, Oyama N, et al. (2021) Predicting hemorrhagic transformation after large vessel occlusion stroke in the era of mechanical thrombectomy. *PLOS ONE* 16(8): e0256170.

Jayaraj RL, Azimullah S, Beiram R, et al. (2019) Neuroinflammation: friend and foe for ischemic stroke. *Journal of Neuroinflammation* 16(1): 142.

Jiao H, Wang Z, Liu Y, et al. (2011) Specific Role of Tight Junction Proteins Claudin-5, Occludin, and ZO-1 of the Blood–Brain Barrier in a Focal Cerebral Ischemic Insult. *Journal of Molecular Neuroscience* 44(2): 130-139.

Jickling GC, Liu D, Stamova B, et al. (2014) Hemorrhagic Transformation after Ischemic Stroke in Animals and Humans. *Journal of Cerebral Blood Flow and Metabolism* 34(2): 185-199.

Jickling GC and Manolescu BN. (2012) Breaking down barriers to identify hemorrhagic transformation in ischemic stroke. 79(16): 1632-1633.

Jickling GC and Sharp FR. (2011) Blood Biomarkers of Ischemic Stroke. *Neurotherapeutics* 8(3): 349-360.

Jin R, Yang G and Li G. (2010) Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. *Journal of leukocyte biology* 87(5): 779-789.

Johnson CO, Nguyen M, Roth GA, et al. (2019) Global, regional, and national burden of stroke, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet Neurology* 18(5): 439-458.

Jones SP, Baqai K, Clegg A, et al. (2022) Stroke in India: A systematic review of the incidence, prevalence, and case fatality. *International Journal of Stroke* 17(2): 132-140.

Jovin TG, Demchuk AM and Gupta R. (2008) Pathophysiology of Acute Ichemic Stroke. *CONTINUUM: Lifelong Learning in Neurology* 14(6): 28-45.

Jucevičiūtė N, Mikužis P and Balnytė R. (2019) Absolute blood eosinophil count could be a potential biomarker for predicting haemorrhagic transformation after intravenous thrombolysis for acute ischaemic stroke. *BMC Neurology* 19(1): 127.

Jung S, Wiest R, Gralla J, et al. (2017) Relevance of the cerebral collateral circulation in ischaemic stroke: time is brain, but collaterals set the pace. *Swiss medical weekly* 147(w14538): w14538.

Justicia C, Panés J, Solé S, et al. (2003) Neutrophil Infiltration Increases Matrix Metalloproteinase-9 in the Ischemic Brain after Occlusion/Reperfusion of the Middle Cerebral Artery in Rats. *Journal of Cerebral Blood Flow & Metabolism* 23(12): 1430-1440.

Kablau M, Kreisel SH, Sauer T, et al. (2011) Predictors and Early Outcome of Hemorrhagic Transformation after Acute Ischemic Stroke. *Cerebrovascular Diseases* 32(4): 334-341.

Kalogeris T, Baines CP, Krenz M, et al. (2012) Cell Biology of Ischemia/Reperfusion Injury. In: Jeon KW (ed) *International Review of Cell and Molecular Biology.* Academic Press, 229-317.

Kalogeris T, Baines CP, Krenz M, et al. (2016) Ischemia/Reperfusion. *Comprehensive Physiology* 7(1): 113-170.

Kamtchum-Tatuene J, Saba L, Heldner MR, et al. (2022) Interleukin-6 Predicts Carotid Plaque Severity, Vulnerability, and Progression. *Circulation Research* 131(2): e22-e33.

Kannel WB. (2000) Risk stratification in hypertension: new insights from the Framingham study*. *American Journal of Hypertension* 13(S1): 3S-10S.

Kaul S, Alladi S, Jabeen SA, et al. (2018) Intracranial Atherosclerosis is the Most Common Stroke Subtype: Ten-year Data from Hyderabad Stroke Registry (India). *Ann Indian Acad Neurol* 21(3): 209-213.

Kazmierski R, Michalak S, Wencel-Warot A, et al. (2012) Serum tight-junction proteins predict hemorrhagic transformation in ischemic stroke patients. *Neurology* 79(16): 1677-1685.

Kelly PJ, Morrow JD, Ning M, et al. (2008) Oxidative Stress and Matrix Metalloproteinase-9 in Acute Ischemic Stroke. *Stroke* 39(1): 100-104.

Kernagis DN and Laskowitz DT. (2012) Evolving role of biomarkers in acute cerebrovascular disease. 71(3): 289-303.

Khatri P, Wechsler LR and Broderick JP. (2007) Intracranial Hemorrhage Associated With Revascularization Therapies. *Stroke* 38(2): 431-440.

Kim BJ, Kang HG, Kim H-J, et al. (2014) Magnetic resonance imaging in acute ischemic stroke treatment. *Journal of stroke* 16(3): 131-145.

Kim BJ, Lee S-H, Ryu W-S, et al. (2009) Low Level of Low-Density Lipoprotein Cholesterol Increases Hemorrhagic Transformation in Large Artery Atherothrombosis but Not in Cardioembolism. *Stroke* 40(5): 1627-1632.

Kim J, Thayabaranathan T, Donnan GA, et al. (2020) Global Stroke Statistics 2019. *International Journal of Stroke* 15(8): 819-838.

Kim SJ, Moon GJ and Bang OY. (2013) Biomarkers for stroke. *Journal of Stroke* 15(1): 27-37.

Korhonen P, Kanninen KM, Lehtonen Š, et al. (2015) Immunomodulation by interleukin-33 is protective in stroke through modulation of inflammation. *Brain, Behavior, and Immunity* 49: 322-336.

Krishnamoorthy S, Damayanthi D, Gopala S, et al. (2021) High-Sensitivity C-Reactive Protein and Lipoprotein-Associated Phospholipase A2 in Predicting Recurrence and Severity of Stenosis in Symptomatic Intracranial Atherosclerotic Disease. *Current Proteomics* 18(2): 231-236.

Krishnamurthi RV, Feigin VL, Forouzanfar MH, et al. (2013) Global and regional burden of first-ever ischaemic and haemorrhagic stroke during 1990–2010: findings from the Global Burden of Disease Study 2010. *The Lancet Global Health* 1(5): e259-e281.

Kummer Rv, Broderick JP, Campbell BCV, et al. (2015) The Heidelberg Bleeding Classification. 46(10): 2981-2986.

Kurzepa J, Kurzepa J, Golab P, et al. (2014) The significance of matrix metalloproteinase (MMP)-2 and MMP-9 in the ischemic stroke. *International Journal of Neuroscience* 124(10): 707-716.

Lakhan SE, Kirchgessner A, Tepper D, et al. (2013) Matrix metalloproteinases and blood-brain barrier disruption in acute ischemic stroke. *Frontiers in neurology* 4: 32-32.

Laskowitz DT, Kasner SE, Saver J, et al. (2009) Clinical Usefulness of a Biomarker-Based Diagnostic Test for Acute Stroke. *Stroke* 40(1): 77-85.

Liddelow SA. (2011) Fluids and barriers of the CNS: a historical viewpoint. *Fluids and Barriers of the CNS* 8(1): 2.

Liebeskind DS. (2003) Collateral Circulation. *Stroke* 34(9): 2279-2284.

Liebner S, Dijkhuizen RM, Reiss Y, et al. (2018) Functional morphology of the blood–brain barrier in health and disease. *Acta Neuropathologica* 135(3): 311-336.

Lin S-F, Chao AC, Hu H-H, et al. (2019) Low Cholesterol Levels Increase Symptomatic Intracranial Hemorrhage Rates After Intravenous Thrombolysis: A Multicenter Cohort Validation Study. *Journal of Atherosclerosis and Thrombosis* 26(6): 513-527.

Liu J, Liu L, Wang X, et al. (2021) Microglia: A Double-Edged Sword in Intracerebral Hemorrhage From Basic Mechanisms to Clinical Research. 12.

Lo EH, Dalkara T and Moskowitz MA. (2003) Mechanisms, challenges and opportunities in stroke. *Nature Reviews Neuroscience* 4(5): 399-414.

Lochhead JJ, McCaffrey G, Quigley CE, et al. (2010) Oxidative Stress Increases Blood–Brain Barrier Permeability and Induces Alterations in Occludin during

Hypoxia–Reoxygenation. *Journal of Cerebral Blood Flow and Metabolism* 30(9): 1625-1636.

Lotierzo M, Dupuy AM, Kalmanovich E, et al. (2020) sST2 as a value-added biomarker in heart failure. *Clinica Chimica Acta* 501: 120-130.

Lynch JR, Blessing R, White WD, et al. (2004) Novel Diagnostic Test for Acute Stroke. *Stroke* 35(1): 57-63.

Ma G, Pan Z, Kong L, et al. (2021) Neuroinflammation in hemorrhagic transformation after tissue plasminogen activator thrombolysis: Potential mechanisms, targets, therapeutic drugs and biomarkers. *International Immunopharmacology* 90: 107216.

Maas MB and Furie KL. (2009) Molecular biomarkers in stroke diagnosis and prognosis. *Biomarkers in medicine* 3(4): 363-383.

Maestrini I, Strbian D, Gautier S, et al. (2015) Higher neutrophil counts before thrombolysis for cerebral ischemia predict worse outcomes. 85(16): 1408-1416.

Maestrini I, Tagzirt M, Gautier S, et al. (2020) Analysis of the association of MPO and MMP-9 with stroke severity and outcome. *Neurology* 95(1): e97-e108.

Malhotra K, Goyal N, Chang JJ, et al. (2018) Differential leukocyte counts on admission predict outcomes in patients with acute ischaemic stroke treated with intravenous thrombolysis. 25(12): 1417-1424.

Marsh EB, Llinas RH, Hillis AE, et al. (2013) Hemorrhagic transformation in patients with acute ischaemic stroke and an indication for anticoagulation. *European Journal of Neurology* 20(6): 962-967.

Matosevic B, Knoflach M, Werner P, et al. (2013) Fibrinogen degradation coagulopathy and bleeding complications after stroke thrombolysis. 80(13): 1216-1224.

Mayeux R. (2004) Biomarkers: potential uses and limitations. *NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics* 1(2): 182-188.

Mechtouff L, Bochaton T, Paccalet A, et al. (2020) Matrix Metalloproteinase-9 Relationship With Infarct Growth and Hemorrhagic Transformation in the Era of Thrombectomy. *Frontiers in Neurology* 11(473).

Mechtouff L, Paccalet A, Crola Da Silva C, et al. (2021) Prognosis value of serum soluble ST2 level in acute ischemic stroke and STEMI patients in the era of mechanical reperfusion therapy. *Journal of Neurology* 269(5): 2641-2648.

Millán M, Sobrino T, Arenillas JF, et al. (2008) Biological Signatures of Brain Damage Associated with High Serum Ferritin Levels in Patients with Acute Ischemic Stroke and Thrombolytic Treatment. *Disease Markers* 25: 380356.

Miyamori H, Takino T, Kobayashi Y, et al. (2001) Claudin Promotes Activation of Pro-matrix Metalloproteinase-2 Mediated by Membrane-type Matrix Metalloproteinases *. *Journal of Biological Chemistry* 276(30): 28204-28211.

Molina CA, Montaner J, Abilleira S, et al. (2001) Timing of Spontaneous Recanalization and Risk of Hemorrhagic Transformation in Acute Cardioembolic Stroke. 32(5): 1079-1084.

Molnar T, Borocz K, Berki T, et al. (2019) Subacute Elevation of Plasma Level of Caspase-Cleaved Cytokeratin-18 is Associated with Hemorrhagic Transformation and Functional Outcome in Ischemic Stroke. *Journal of Stroke and Cerebrovascular Diseases* 28(3): 719-727.

Montaner J, Alvarez-Sabín J, Molina C, et al. (2001) Matrix Metalloproteinase Expression After Human Cardioembolic Stroke. *Stroke* 32(8): 1759-1766.

Montaner J, Mendioroz M, Ribó M, et al. (2011) A panel of biomarkers including caspase-3 and D-dimer may differentiate acute stroke from stroke-mimicking conditions in the emergency department. *Journal of internal medicine* 270(2): 166-174.

Montaner J, Molina CA, Monasterio J, et al. (2003) Matrix Metalloproteinase-9 Pretreatment Level Predicts Intracranial Hemorrhagic Complications After Thrombolysis in Human Stroke. *Stroke* 107(4): 598-603.

Montaner J, Perea-Gainza M, Delgado P, et al. (2008) Etiologic Diagnosis of Ischemic Stroke Subtypes With Plasma Biomarkers. *Stroke* 39(8): 2280-2287.

Montaner J, Ramiro L, Simats A, et al. (2020) Multilevel omics for the discovery of biomarkers and therapeutic targets for stroke. *Nature Reviews Neurology* 16(5): 247-264.

Navarro-Sobrino M, Rosell A, Hernández-Guillamon M, et al. (2011) A large screening of angiogenesis biomarkers and their association with neurological outcome after ischemic stroke. *Atherosclerosis* 216(1): 205-211.

Nitta T, Hata M, Gotoh S, et al. (2003) Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *The Journal of cell biology* 161(3): 653-660.

O'Donnell MJ, Chin SL, Rangarajan S, et al. (2016) Global and regional effects of potentially modifiable risk factors associated with acute stroke in 32 countries (INTERSTROKE): a case-control study. *The Lancet* 388(10046): 761-775.

Obermeier B, Daneman R and Ransohoff RM. (2013) Development, maintenance and disruption of the blood-brain barrier. *Nature Medicine* 19(12): 1584-1596.

Owolabi MO, Thrift AG, Martins S, et al. (2021) The state of stroke services across the globe: Report of World Stroke Organization–World Health Organization surveys. *International Journal of Stroke* 16(8): 889-901.

Paciaroni M, Agnelli G, Caso V, et al. (2009) Acute Hyperglycemia and Early Hemorrhagic Transformation in Ischemic Stroke. *Cerebrovascular Diseases* 28(2): 119-123.

Paciaroni M, Agnelli G, Corea F, et al. (2008) Early Hemorrhagic Transformation of Brain Infarction: Rate, Predictive Factors, and Influence on Clinical Outcome. *Stroke* 39(8): 2249-2256.

Paciaroni M, Bandini F, Agnelli G, et al. (2018) Hemorrhagic Transformation in Patients With Acute Ischemic Stroke and Atrial Fibrillation: Time to Initiation of Oral Anticoagulant Therapy and Outcomes. *Journal of the American Heart Association* 7(22): e010133.

Pandian JD and Sudhan P. (2013) Stroke epidemiology and stroke care services in India. *Journal of Stroke* 15(3): 128-134.

Pandit R, Chen L and Götz J. (2020) The blood-brain barrier: Physiology and strategies for drug delivery. *Advanced Drug Delivery Reviews* 165-166: 1-14.

Pascual-Figal DA, Lax A, Perez-Martinez MT, et al. (2016) Clinical relevance of sST2 in cardiac diseases. *J Clinical Chemistry and Laboratory Medicine* 54(1): 29-35.

Pexman JHW, Barber PA, Hill MD, et al. (2001) Use of the Alberta Stroke Program Early CT Score (ASPECTS) for Assessing CT Scans in Patients with Acute Stroke. 22(8): 1534-1542.

Phipps MS and Cronin CA. (2020) Management of acute ischemic stroke. *The BMJ* 368: l6983.

Pikija S, Sztriha LK, Killer-Oberpfalzer M, et al. (2018) Neutrophil to lymphocyte ratio predicts intracranial hemorrhage after endovascular thrombectomy in acute ischemic stroke. *Journal of Neuroinflammation* 15(1): 319.

Planas AM, Solé S and Justicia C. (2001) Expression and Activation of Matrix Metalloproteinase-2 and -9 in Rat Brain after Transient Focal Cerebral Ischemia. *Neurobiology of Disease* 8(5): 834-846.

Qin C, Yang S, Chu Y-H, et al. (2022) Signaling pathways involved in ischemic stroke: molecular mechanisms and therapeutic interventions. *Signal Transduction and Targeted Therapy* 7(1): 215.

Ramon R and Julio César G. (2016) Excitotoxicity and Oxidative Stress in Acute Stroke. In: Bernhard S (ed) *Ischemic Stroke.* Rijeka: IntechOpen, Ch. 2.

Rankin J. (1957) Cerebral Vascular Accidents in Patients over the Age of 60: II. Prognosis. *Scottish Medical Journal* 2(5): 200-215.

Reinhold AK and Rittner HL. (2017) Barrier function in the peripheral and central nervous system—a review. *Pflügers Archiv - European Journal of Physiology* 469(1): 123-134.

Ribo M, Montaner J, Molina CA, et al. (2004) Admission Fibrinolytic Profile Is Associated With Symptomatic Hemorrhagic Transformation in Stroke Patients Treated With Tissue Plasminogen Activator. *Stroke* 35(9): 2123-2127.

Rifai N, Gillette MA and Carr SA. (2006) Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nature Biotechnology* 24(8): 971-983.

Rosell A, Cuadrado E, Ortega-Aznar A, et al. (2008) MMP-9-positive neutrophil infiltration is associated to blood-brain barrier breakdown and basal lamina type IV collagen degradation during hemorrhagic transformation after human ischemic stroke. *Stroke* 39(4): 1121-1126.

Rothermundt M, Peters M, Prehn JHM, et al. (2003) S100B in brain damage and neurodegeneration. *Microscopy Research and Technique* 60(6): 614-632.

Sacco RL, Kasner SE, Broderick JP, et al. (2013) An Updated Definition of Stroke for the 21st Century. *Stroke* 44(7): 2064-2089.

Sandercock P, Lindley R, Wardlaw J, et al. (2008) Third international stroke trial (IST-3) of thrombolysis for acute ischaemic stroke. *Trials* 9: 37-37.

Sarvari S, Moakedi F, Hone E, et al. (2020) Mechanisms in blood-brain barrier opening and metabolism-challenged cerebrovascular ischemia with emphasis on ischemic stroke. *Metabolic Brain Disease* 35(6): 851-868.

Sastre C, Bevers MB and Kimberly WT. (2021) Role of Interleukin-1 Receptor-Like 1 (ST2) in Cerebrovascular Disease. *Neurocritical Care* 35(3): 887-893.

School UoB-BM. (2002) *QUADAS-2*. Available at: https://www.bristol.ac.uk/population-health-sciences/projects/quadas/quadas-2/.

Selleck MJ, Senthil M and Wall NR. (2017) Making Meaningful Clinical Use of Biomarkers. *Biomarker insights* 12: 1177271917715236.

Shi K, Tian D-C, Li Z-G, et al. (2019) Global brain inflammation in stroke. *The Lancet Neurology* 18(11): 1058-1066.

Shi K, Zou M, Jia DM, et al. (2021) tPA Mobilizes Immune Cells That Exacerbate Hemorrhagic Transformation in Stroke. *Circ Res* 128(1): 62-75.

Singhal AB, Biller J, Elkind MS, et al. (2013) Recognition and management of stroke in young adults and adolescents. *Neurology* 81(12): 1089-1097.

Soriano-Tárraga C, Giralt-Steinhauer E, Mola-Caminal M, et al. (2018) Biological Age is a predictor of mortality in Ischemic Stroke. *Scientific Reports* 8(1): 4148.

Sotgiu S, Zanda B, Marchetti B, et al. (2006) Inflammatory biomarkers in blood of patients with acute brain ischemia. *European Journal of Neurology* 13(5): 505-513.

Spronk E, Sykes G, Falcione S, et al. (2021) Hemorrhagic Transformation in Ischemic Stroke and the Role of Inflammation. *Frontiers in neurology* 12.

Sridharan SE, Unnikrishnan JP, Sukumaran S, et al. (2009) Incidence, Types, Risk Factors, and Outcome of Stroke in a Developing Country. *Stroke* 40(4): 1212-1218.

Steliga A, Kowiański P, Czuba E, et al. (2020) Neurovascular Unit as a Source of Ischemic Stroke Biomarkers—Limitations of Experimental Studies and Perspectives for Clinical Application. *Translational Stroke Research* 11(4): 553-579.

Strimbu K and Tavel JA. (2010) What are biomarkers? *Current opinion in HIV and AIDS* 5(6): 463-466.

Sulter G, Steen C and Keyser JD. (1999) Use of the Barthel Index and Modified Rankin Scale in Acute Stroke Trials. 30(8): 1538-1541.

Sussman E and Connolly Jr. E. (2013) Hemorrhagic Transformation: A Review of the Rate of Hemorrhage in the Major Clinical Trials of Acute Ischemic Stroke. *Frontiers in neurology* 4: 69.

Sweeney MD, Sagare AP and Zlokovic BV. (2018) Blood–brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders. *Nature Reviews Neurology* 14(3): 133-150.

Świtońska M, Piekuś-Słomka N, Słomka A, et al. (2020) Neutrophil-to-Lymphocyte Ratio and Symptomatic Hemorrhagic Transformation in Ischemic Stroke Patients Undergoing Revascularization. 10(11): 771.

Sylaja PN, Pandian JD, Kaul S, et al. (2018) Ischemic Stroke Profile, Risk Factors, and Outcomes in India. *Stroke* 49(1): 219-222.

Tan S, Wang D, Liu M, et al. (2014) Frequency and predictors of spontaneous hemorrhagic transformation in ischemic stroke and its association with prognosis. *Journal of Neurology* 261(5): 905-912.

Thanvi BR, Treadwell S and Robinson T. (2008) Haemorrhagic transformation in acute ischaemic stroke following thrombolysis therapy: classification, pathogenesis and risk factors. *Postgraduate Medical Journal* 84(993): 361-367.

Thomalla G, Schwark C, Sobesky J, et al. (2006) Outcome and Symptomatic Bleeding Complications of Intravenous Thrombolysis Within 6 Hours in MRI-Selected Stroke Patients. *Stroke* 37(3): 852-858.

Thomas SE, Plumber N, Venkatapathappa P, et al. (2021) A Review of Risk Factors and Predictors for Hemorrhagic Transformation in Patients with Acute Ischemic Stroke. *International Journal of Vascular Medicine* 2021: 4244267.

Thrift AG, Thayabaranathan T, Howard G, et al. (2017) Global stroke statistics. *International Journal of Stroke* 12(1): 13-32.

Tian X, Guo Y, Wang X, et al. (2020) Serum soluble ST2 is a potential long-term prognostic biomarker for transient ischaemic attack and ischaemic stroke. *European Journal of Neurology* 27(11): 2202-2208.

Trouillas P, Derex L, Philippeau F, et al. (2004) Early Fibrinogen Degradation Coagulopathy Is Predictive of Parenchymal Hematomas in Cerebral rt-PA Thrombolysis. *Stroke* 35(6): 1323-1328.

Vafadari B, Salamian A and Kaczmarek L. (2016) MMP-9 in translation: from molecule to brain physiology, pathology, and therapy. *Journal of neurochemistry* 139(S2): 91-114.

van Kranendonk KR, Treurniet KM, Boers AMM, et al. (2019) Hemorrhagic transformation is associated with poor functional outcome in patients with acute ischemic stroke due to a large vessel occlusion. *Journal of neurointerventional surgery* 11(5): 464-468.

von Elm E, Altman DG, Egger M, et al. (2008) The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. *Journal of Clinical Epidemiology* 61(4): 344-349.

Wang H-L, Zhang C-L, Qiu Y-M, et al. (2021) Dysfunction of the Blood-brain Barrier in Cerebral Microbleeds: from Bedside to Bench. *Aging and disease* 12(8): 1898-1919.

Wang P, Shao B-Z, Deng Z, et al. (2018) Autophagy in ischemic stroke. *Progress in Neurobiology* 163-164: 98-117.

Wang W, Li M, Chen Q, et al. (2015) Hemorrhagic Transformation after Tissue Plasminogen Activator Reperfusion Therapy for Ischemic Stroke: Mechanisms, Models, and Biomarkers. *Molecular neurobiology* 52(3): 1572-1579.

Wang X and Lo EH. (2003) Triggers and mediators of hemorrhagic transformation in cerebral ischemia. *Molecular Neurobiology* 28(3): 229-244.

Wardlaw JM, Murray V, Berge E, et al. (2014) Thrombolysis for acute ischaemic stroke. *Cochrane Database of Systematic Reviews* (7).

Weiss N, Miller F, Cazaubon S, et al. (2009) The blood-brain barrier in brain homeostasis and neurological diseases. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1788(4): 842-857.

Wen L, Zhang S, Wan K, et al. (2020) Risk factors of haemorrhagic transformation for acute ischaemic stroke in Chinese patients receiving intravenous thrombolysis: A meta-analysis. *Medicine* 99(7): e18995-e18995.

Whiteley W, Tseng M-C and Sandercock P. (2008) Blood Biomarkers in the Diagnosis of Ischemic Stroke. *Stroke* 39(10): 2902-2909.

Whiteley W, Wardlaw J, Dennis M, et al. (2012a) The Use of Blood Biomarkers to Predict Poor Outcome After Acute Transient Ischemic Attack or Ischemic Stroke. *Stroke* 43(1): 86-91.

Whiteley WN, Slot KB, Fernandes P, et al. (2012b) Risk Factors for Intracranial Hemorrhage in Acute Ischemic Stroke Patients Treated With Recombinant Tissue Plasminogen Activator. *Stroke* 43(11): 2904-2909.

Whiting PF, Weswood ME, Rutjes AWS, et al. (2006) Evaluation of QUADAS, a tool for the quality assessment of diagnostic accuracy studies. *BMC Medical Research Methodology* 6(1): 9.

Wolburg H and Lippoldt A. (2002) Tight junctions of the blood–brain barrier: development, composition and regulation. *Vascular Pharmacology* 38(6): 323-337.

Wolcott Z, Batra A, Bevers MB, et al. (2017) Soluble ST2 predicts outcome and hemorrhagic transformation after acute stroke. *Annals of Clinical and Translational Neurology* 4(8): 553-563.

Xing C, Arai K, Lo EH, et al. (2012) Pathophysiologic Cascades in Ischemic Stroke. *International Journal of Stroke* 7(5): 378-385.

Xing Y, Guo Z-N, Yan S, et al. (2014) Increased globulin and its association with hemorrhagic transformation in patients receiving intra-arterial thrombolysis therapy. *Neuroscience Bulletin* 30(3): 469-476.

Xiong Y, Wakhloo AK and Fisher M. (2022) Advances in Acute Ischemic Stroke Therapy. *Stroke* 130(8): 1230-1251.

Yaghi S, Willey JZ, Cucchiara B, et al. (2017) Treatment and Outcome of Hemorrhagic Transformation After Intravenous Alteplase in Acute Ischemic Stroke: A Scientific Statement for Healthcare Professionals From the American Heart Association/American Stroke Association. *Stroke* 48(12): e343-e361.

Yang C, Hawkins KE, Doré S, et al. (2019) Neuroinflammatory mechanisms of blood-brain barrier damage in ischemic stroke. *Am J Physiol Cell Physiol* 316(2): C135-C153.

Yang N, Lin M, Wang BG, et al. (2016) Low level of low-density lipoprotein cholesterol is related with increased hemorrhagic transformation after acute ischemic cerebral infarction. *European Review for Medical and Pharmacological Sciences* 20(4): 673-678.

Yang Y, Liu H, Zhang H, et al. (2017) ST2/IL-33-Dependent Microglial Response Limits Acute Ischemic Brain Injury. *The Journal of Neuroscience* 37(18): 4692-4704.

Yang Y and Rosenberg GA. (2015) Matrix metalloproteinases as therapeutic targets for stroke. *Brain Research* 1623: 30-38.

Yuan R, Tan S, Wang D, et al. (2018) Predictive value of plasma matrix metalloproteinase-9 concentrations for spontaneous haemorrhagic transformation in patients with acute ischaemic stroke: A cohort study in Chinese patients. *Journal of Clinical Neuroscience* 58: 108-112.

Zhang J, Yang Y, Sun H, et al. (2014) Hemorrhagic transformation after cerebral infarction: current concepts and challenges. *Annals of translational medicine* 2(8): 81.

ANNEXURES

List of publications from thesis

1. Krishnamoorthy S, Singh G, Sreedharan SE, Damayanthi D, Gopala S, Uk M, **Sylaja PN**. Soluble ST2 Predicts Poor Functional Outcome in Acute Ischemic Stroke Patients. Cerebrovasc Dis Extra. 2023 Feb 8;13(1):33–40.
2. Krishnamoorthy S, **Sylaja PN**, Sreedharan SE, Singh G, Damayanthi D, Gopala S, Madhusoodanan UK, Ramachandran H. Biomarkers predict hemorrhagic transformation and stroke severity after acute ischemic stroke. J Stroke Cerebrovasc Dis. 2023 Jan;32(1):106875.
3. Krishnamoorthy S, Singh G, Jose K J, Soman B, Foerch C, Kimberly WT, Millán M, Świtońska M, Maestrini I, Bordet R, Malhotra K, Mechtouff L, **Sylaja PN**. Biomarkers in the Prediction of Hemorrhagic Transformation in Acute Stroke: A Systematic Review and Meta-Analysis. Cerebrovasc Dis. 2022;51(2):235-247.

Curriculum Vitae

Soumya Krishnamoorthy

Permanent Postal Address:	112, Prasanth Nagar, Ulloor, Medical College P.O., Trivandrum, Kerala, India, PIN - 695011
Phone:	+91 956 7038 239
Email:	soumyamoorthy.k@gmail.com
Date of Birth:	27 July 1985
Gender:	Female
Nationality:	Indian

Education

Degree	Institute/ University	Year of Graduation
Doctor of Philosophy	Comprehensive Stroke Care Program, Department of Neurology, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Government of India, Trivandrum, Kerala, India	2023
Advanced Masters in Biotechnology	School of Biological Sciences, University of Queensland, St Lucia Campus, Brisbane, Australia	July 2009
Bachelors in Science (triple	University of Bangalore, Karnataka, India	April 2006

major: Biotechnology, Chemistry, Botany)		

Previous Work Experience

1. Junior Research Fellow, Comprehensive Stroke Care Program, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Trivandrum India. **Project title:** Apolipoprotein B/ A1 ratio in stroke subtypes	October 2015 – December 2017
2. Research Assistant, Comprehensive Care Center for Movement Disorder, SCTIMST, Trivandrum India. **Project title:** Association of Dopamine receptor (DRD3), glutamate receptor (GRIN2B) and serotonin transporter (5HTR2A) gene polymorphisms in Parkinson's disease patients with impulse control disorders while on dopamine agonist therapy	September 2013 – December 2014
3. Scientific Assistant, Thrombosis Research Laboratory, Trivandrum, India. **Project title:** Development of a suitable anti-venom treatment by means of avian antibodies (Technology transfer project)	September 2012 – August 2013

Internship and Master's thesis

1. Short project titled ‘Construction of dengue Replicons’ - Dr. E. Sreekumar at the Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum, Kerala.	January 2011-March 2011
2. Final year Master’s thesis, ‘Subgenomic Flaviviral RNA (sfRNA) tags in Kunjin strain of West Nile Virus,’ - Associate Professor Alexander Khromykh at Sir Albert Sakzewski Virus Research Centre (SASVRC), Queensland, Australia.	November 2008-June 2009
3. Master’s Project: ‘Role of Hepcidin in the treatment of Iron-related disorders,’ - Dr. Richard Clark at the Institute of Molecular Bioscience (IMB), University of Queensland, Australia.	July 2008-September 2008

Publications

1. **Krishnamoorthy S**, Singh G, Sreedharan SE, Damayanthi D, Gopala S, Uk M, Sylaja PN. Soluble ST2 Predicts Poor Functional Outcome in Acute Ischemic Stroke Patients. Cerebrovasc Dis Extra. 2023 Feb 8;13(1):33–40. doi: 10.1159/000529512. Epub ahead of print. PMID: 36754033.
2. **Krishnamoorthy S**, Sylaja PN, Sreedharan SE, Singh G, Damayanthi D, Gopala S, Madhusoodanan UK, Ramachandran H. Biomarkers predict hemorrhagic transformation and stroke severity after acute ischemic stroke. J Stroke Cerebrovasc Dis. 2023 Jan;32(1):106875. doi:

10.1016/j.jstrokecerebrovasdis.2022.106875. Epub 2022 Nov 14. PMID: 36395663.

3. Damayanthi D, **Krishnamoorthy S**, Sylaja PN, Gopala S. Increased high-density lipoprotein-oxidant index in ischemic stroke patients. Biomed Rep. 2022 Sep 14;17(5):87. doi: 10.3892/br.2022.1570. PMID: 36237288; PMCID: PMC9500476.
4. **Krishnamoorthy S**, Singh G, Jose K J, Soman B, Foerch C, Kimberly WT, Millán M, Świtońska M, Maestrini I, Bordet R, Malhotra K, Mechtouff L, Sylaja PN. Biomarkers in the Prediction of Hemorrhagic Transformation in Acute Stroke: A Systematic Review and Meta-Analysis. Cerebrovasc Dis. 2022;51(2):235-247. doi: 10.1159/000518570. Epub 2021 Sep 22. PMID: 34569521.
5. **Krishnamoorthy S**, Damayanthi D, Gopala S, Paul R, Sylaja PN. High-sensitivity C-reactive protein and lipoprotein-associated phospholipase A2 in predicting recurrence and severity of stenosis in symptomatic intracranial atherosclerotic disease. Current Proteomics. 2021 Apr 1;18(2):231-6.
6. Kalani R, **Krishnamoorthy S**, Deepa D, Gopala S, Prabhakaran D, Tirschwell D, Sylaja PN. Apolipoproteins B and A1 in Ischemic Stroke Subtypes. J Stroke Cerebrovasc Dis. 2020 Apr;29(4):104670. doi: 10.1016/j.jstrokecerebrovasdis.2020.104670. Epub 2020 Feb 10. PMID: 32057650; PMCID: PMC7085346.
7. **Krishnamoorthy S**, Rajan R, Banerjee M, Kumar H, Sarma G, Krishnan S, Sarma S, Kishore A. Dopamine D3 receptor Ser9Gly variant is

associated with impulse control disorders in Parkinson's disease patients. Parkinsonism Relat Disord. 2016 Sep;30:13-7. doi: 10.1016/j.parkreldis.2016.06.005. Epub 2016 Jun 15. PMID: 27325396.

Patent applied

Title: "Production of pure IgY against a mixture of two Neurotoxins (anti-neurotoxins) and two hemotoxins (anti-hemotoxins) for neutralizing effects of envenomation in snake bite patients" Co-inventors: Dr. Lissy K Krishnan, Soumya Krishnamoorthy, Institute: Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Government of India, Trivandrum, Kerala, India

Awards received

1. SCTIMST Certificate of Excellence 2023 (PhD students' category) awarded on 28 February 2023
2. Neurologists Association of Tiruchirapally Annual Award for Laboratory Research in Neuroscience -18th Asian Oceanian Congress of Neurology and 29th Annual Conference of the Indian Academy of Neurology (IANCON-AOCN) 2022.
3. Young investigator award - 14th World Stroke Congress (WSC) on October 26-29, 2022, Singapore.
4. Travel grant award - 8th European Stroke Organization Conference (ESOC) 2022, Lyon, France.
5. Young investigator award - 13th World Stroke Congress (Virtual)
6. Top 10 Young Investigator Award - 11th World Stroke Congress, Montreal, Canada

Conferences

1. Award presentation of the abstract titled, “Biomarkers Predict Hemorrhagic Transformation and Stroke Severity in Acute Ischemic Stroke Patients” at the IANCON-AOCN 2022, November 2-6, 2022 in New Delhi, India.
2. Oral Presentation of the abstract titled, “Soluble ST2 is a Predictor of Poor Functional Outcome in Acute Ischemic Stroke” during the Free Communication Sessions in the 14th World Stroke Congress (WSC) on October 26-29, 2022 in Singapore.
3. E-poster presentation of the abstract titled, “Biomarkers predict stroke severity and hemorrhagic transformation after acute ischemic stroke” at the 8th ESOC 2022, Lyon, France.
4. E-poster presentation of the abstract titled, “Biomarkers in the prediction of hemorrhagic transformation in acute ischemic stroke: a systematic review and meta-analysis,” at the 13th WSC 2021 (Virtual).
5. E-poster presentation titled, “Hs-CRP and Lp-PLA2 in predicting recurrence and severity of stenosis in symptomatic intracranial atherosclerotic disease” at the ESO-WSO 2020 Stroke Conference (virtual).
6. Poster presentation - International Seminar on Recent Biochemical Approaches in Therapeutics (RBAT-VI) 2019, University of Kerala.
7. Oral presentation for the abstract titled, “Increased Levels of Serum Apolipoprotein B in Symptomatic Intracranial Atherosclerotic Disease” at the 11th World Stroke Congress, Montreal, October 17-20, 2018.
8. Rajan R, Krishnamoorthy S, Krishnan S, Sarma G, Kishore A. A Gene for Risk Taking: Effect of Genotypic Variants on Decision Making, Response Inhibition

and Impulsivity in Parkinson's Disease (P4. 325). Neurology. 2016 Apr 5;86(16 Supplement): P4-325

Software and programing Skills

PC and MAC operating systems, Microsoft office, Excel, Adobe Photoshop, knowledge of R, RevMan

Professional Memberships

World Stroke Organization (WSO)

Indian Academy of Neurology (IAN)

Manuscript Reviewer

For the following journals: Stroke, Cerebrovascular Diseases and World Journal of Surgical Oncology.

Web of Science Researcher ID - AAZ-7344-2021

ORCID ID - 0000-0003-4233-8811

Other activities and interests

Blogging, reading, calligraphy

APPENDIX A – ETHICS COMMITTEE APPROVAL

श्री चित्रा तिरुनाल आयुर्विज्ञान और प्रौद्योगिकी संस्थान, त्रिवेन्द्रम
तिरुवनन्तपुरम - ६९५०११, केरल, इंडिया
SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM
Thiruvananthapuram - 695 011, Kerala, India
(An Institute of National Importance under Govt. of India)
Grams : Chitramet, Phone : +91-471-2443152, Fax : +91-471-2550728 / 2446433, E-mail : sct@sctimst.ac.in, Website : www.sctimst.ac.in

Institutional Ethics Committee
(IEC Regn No. ECR/189/Inst/KL/2013/RR-16)

SCT/IEC/1295/OCTOBER-2018 12.11.2018

Dr. Sapna Erat Sreedharan
Associate Professor
Department of Neurology
SCTIMST, Thiruvananthapuram

Dear Dr. Sapna Erat Sreedharan,

The Institutional Ethics Committee reviewed and discussed your application to conduct the study entitled "THE ROLE OF BIOMARKERS IN PREDICTING THE RISK OF HEMORRHAGIC TRANSFORMATION IN ACUTE ISCHEMIC STROKE (IEC/1295)" on 26th October, 2018.

The following documents were reviewed:

Original submission

1. *Covering letter addressed to the Chairman, IEC, SCTIMST dated 27.09.2018 with check list*
2. *TAC Approval Letter*
3. *IEC Application Form*
4. *Project Proposal*
5. *Proforma*
6. *Patient Information Sheet and Informed Consent Form in English and Malayalam*
7. *CV of Principal Investigator and Co-Principal Investigators*

Revised submission

1. *Covering letter addressed to the Chairman, IEC, SCTIMST dated 08.11.2018 with check list*
2. *TAC Approval Letter*
3. *IEC Application Form*
4. *IEC comments and reply*
5. *Project Proposal*
6. *Proforma*
7. *Patient Information Sheet and Informed Consent Form in English and Malayalam*
8. *CV of Principal Investigator and Co-Principal Investigators*

Page 1 of 2

The following members of the Ethics Committee were present at the meeting held on 26th October, 2018 at G. Parthasarathi Board Room, AMCHSS, SCTIMST

SL. No.	Member Name	Highest Degree	Gender	Scientific /Non Scientific	Affiliation with Institution(s)
1.	Dr. R V G Menon	M Tech, PhD	Male	Lay Person (Chairman)	No
2.	Dr. Rema M. N	MD	Female	Basic Medical Scientist	No
3.	Smt. Sathi Nair	MA (English Literature)	Female	Lay Person	No
4.	Dr. Kala Kesavan. P	MBBS, MD	Female	Basic Medical Scientist	No
5.	Dr. Harikrishna Varma PR	Ph.D(Materials Science)	Male	Medical Technology	Yes
6.	Dr. Christina George	MD Psychiatry	Female	Clinician	No
7.	Dr. S S Giri Sankar	LL.M. Ph.D.	Male	Legal Expert	No
8.	Dr. Aneesh V Pillai	BA. LLB (Hons.), LLM, Ph. D, SET (Law)	Male	Legal Expert	No
9.	Mr. Satheesh Chandran	MSW, PGDPM	Male	Lay person/ NGO/ Social Scientist	No
10.	Dr. Harikrishnan S	MD, DM (Cardiology) DNB (Cardiology)	Male	Clinician	Yes
11.	Dr. Anand Kumar A	MD, DM	Male	Clinician	No
12.	Dr. V. Raman Kutty	M D, M Phil, M P H	Male	Health Sciences Expert/Clinician	Yes
13.	Dr. Mala Ramanathan	PhD	Female	Social Scientist (Member Secretary)	Yes

IEC Decision

The IEC approved the conduct of the study in the present form.

Remarks:
The Institutional Ethics Committee expects to be informed about the progress of the study, any SAE occurring in the course of the study, any changes in the protocol and patient information/informed consent and asks to be provided a copy of the final report.

There was no member of the study team who participated in voting / decision making process. The ethics committee is organized and operated according to the requirements of Good Clinical Practice and the requirements of the Indian Council of Medical Research (ICMR).

Sincerely,

Mala Ramanathan
Member Secretary, IEC

APPENDIX B - PUBLICATIONS

Publications relevant to the thesis

1. Krishnamoorthy S, Singh G, Sreedharan SE, Damayanthi D, Gopala S, Uk M, Sylaja PN. Soluble ST2 Predicts Poor Functional Outcome in Acute Ischemic Stroke Patients. Cerebrovasc Dis Extra. 2023 Feb 8;13(1):33–40.
2. Krishnamoorthy S, Sylaja PN, Sreedharan SE, Singh G, Damayanthi D, Gopala S, Madhusoodanan UK, Ramachandran H. Biomarkers predict hemorrhagic transformation and stroke severity after acute ischemic stroke. J Stroke Cerebrovasc Dis. 2023 Jan;32(1):106875.
3. Krishnamoorthy S, Singh G, Jose K J, Soman B, Foerch C, Kimberly WT, Millán M, Świtońska M, Maestrini I, Bordet R, Malhotra K, Mechtouff L, Sylaja PN. Biomarkers in the Prediction of Hemorrhagic Transformation in Acute Stroke: A Systematic Review and Meta-Analysis. Cerebrovasc Dis. 2022;51(2):235-247.

Other Publications during the PhD tenure

1. Damayanthi D, Krishnamoorthy S, Sylaja PN, Gopala S. Increased high-density lipoprotein-oxidant index in ischemic stroke patients. *Biomedical Reports*. 2022 Nov 1;17(5):1-6.
2. Krishnamoorthy S, Damayanthi D, Gopala S, Paul R, Sylaja PN. High-Sensitivity C-Reactive Protein and Lipoprotein-Associated Phospholipase A2 in Predicting Recurrence and Severity of Stenosis in Symptomatic Intracranial Atherosclerotic Disease. *Current Proteomics.* 2021 Apr 1;18(2):231-6.
3. Kalani R, Krishnamoorthy S, Deepa D, Gopala S, Prabhakaran D, Tirschwell D, Sylaja PN. Apolipoproteins B and A1 in ischemic stroke subtypes. *Journal of Stroke and Cerebrovascular Diseases.* 2020 Apr 1;29(4):104670.

APPENDIX C – PLAGIARISM CHECK REPORT

Ouriginal
by Turnitin

Document Information

Analyzed document	Thesis_14th_for Plagiarism check.docx (D146396680)
Submitted	10/14/2022 10:09:00 AM
Submitted by	PN Sylaja
Submitter email	sylajapn@sctimst.ac.in
Similarity	5%
Analysis address	sylajapn.sctims@analysis.urkund.com

Sources included in the report

SA	**del Valle_Vargas_Cristina.pdf** Document del Valle_Vargas_Cristina.pdf (D138132718)	4
W	URL: https://www.frontiersin.org/articles/10.3389/fnagi.2016.00142/full Fetched: 11/15/2021 3:57:21 AM	2
SA	**Karolinska Institutet / seminarieversion.matusevicius.marius.docx** Document seminarieversion.matusevicius.marius.docx (D14368530) Submitted by: marius.matusevicius@stud.ki.se Receiver: pingpong-urkund.ki@analys.urkund.se	3
SA	**Karolinska Institutet / rättad.slutversion.almqvist.niklas.docx** Document rättad.slutversion.almqvist.niklas.docx (D39815488) Submitted by: niklas.almqvist@stud.ki.se Receiver: pingpong-urkund.ki@analys.urkund.se	2
W	URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8160112/ Fetched: 6/7/2021 4:27:07 AM	5
SA	**Avhandling Kajsa hela.pdf** Document Avhandling Kajsa hela.pdf (D138284097)	5
W	URL: https://www.frontiersin.org/articles/10.3389/fnint.2021.638114/full Fetched: 10/3/2021 12:28:40 PM	1
W	URL: https://www.karger.com/Article/FullText/518570 Fetched: 11/11/2021 6:52:50 AM	37
W	URL: https://www.oatext.com/hemorrhagic-transformation-of-ischemic-stroke.php Fetched: 1/19/2020 1:01:58 PM	2

SA	**Karolinska Institutet / slutversion.l%C3%B6fberg.harald.docx** Document slutversion.l%C3%B6fberg.harald.docx (D62630118) Submitted by: harald.lofberg@stud.ki.se Receiver: kristina.leif.ki@analys.urkund.se	2
W	URL: https://www.frontiersin.org/articles/10.3389/fncel.2021.716825/full Fetched: 9/15/2021 3:28:35 PM	1
W	URL: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0059420 Fetched: 11/2/2019 7:53:38 AM	1
W	URL: https://thejns.org/focus/view/journals/neurosurg-focus/36/1/article-pE3.xml Fetched: 6/8/2020 3:09:42 AM	1
W	URL: https://www.spandidos-publications.com/10.3892/etm.2015.2495 Fetched: 1/24/2022 5:47:54 AM	1
W	URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7410632/ Fetched: 4/10/2021 10:32:25 AM	1
W	URL: https://www.newcastle.edu.au/profile/christopher-levi Fetched: 5/7/2021 12:26:33 AM	1
W	URL: https://link.springer.com/article/10.1007/s11239-021-02523-2 Fetched: 2/12/2022 5:47:50 PM	1
SA	**University of Glasgow / LiteratureReview.docx** Document LiteratureReview.docx (D44020309) Submitted by: 2238162A@student.gla.ac.uk Receiver: moodle.glas@analysis.urkund.com	1
W	URL: https://link.springer.com/chapter/10.1007/978-2-287-99031-1_3 Fetched: 6/27/2021 4:37:59 PM	1
W	URL: https://n.neurology.org/content/95/1/e97 Fetched: 10/13/2022 12:32:38 PM	1
SA	**CNRS - Labs Recherche - France / marc-ferrigno-19mai2016.pdf** Document marc-ferrigno-19mai2016.pdf (D19550859) Submitted by: marc_ferrigno@yahoo.fr Receiver: rech-med-lille2.cnrs@analyse.urkund.com	1

Entire Document