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**POINT-OF-CARE OPTICAL BIOSENSOR PLATFORMS FOR
STROKE DIAGNOSTICS WITH BLOOD-BASED BIOMARKERS**

HARPAZ DORIN

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2020

POINT-OF-CARE OPTICAL BIOSENSOR PLATFORMS FOR STROKE DIAGNOSTICS WITH BLOOD-BASED BIOMARKERS

HARPAZ DORIN

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2020

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

01 January 2020

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Date

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Harpaz Dorin

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

01 January 2020

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Date

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Alfred Ling Yong Tok

Authorship Attribution Statement

This thesis contains materials from 6 papers and 1 book chapter published in the following peer-reviewed journals, where I was the first author.

Chapter 2 is published as Dorin Harpaz, Evgeni Eltzov, Raymond Chee Seong Seet, Robert S. Marks* and Alfred Iing Yoong Tok*, ‘Point-of-Care-Testing in Acute Stroke Management: An Unmet Need Ripe for Technological Harvest’, Aug 2017 Biosensors 7, no. 3: 30.

The contributions of the co-authors are as follows:

- Prof. Robert S. Marks, Prof. Alfred Iing Yoong Tok and Prof. Raymond Chee Seong Seet proposed the initial literature research direction and guidance.
- I conducted the literature review research and drafted the manuscript.
- Dr. Evgeni Eltzov revised the manuscript.

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The contributions of the co-authors are as follows:

- Prof. Robert S. Marks and Prof. Alfred Iing Yoong Tok proposed the initial research direction and guidance.
- I designed the research experiments, preformed the 3D printing of the biosensor models, conducted the dissolution characterization, tested the control of the sample flow and drafted the manuscript.
- Alicia Lu Yitian helped to conduct the research experiments.
- Tim Axelrod helped to design the 3D models.
- Dr. Evgeni Eltzov revised the manuscript.

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- I preformed the membrane structure characterization using FE-SEM, membrane parameters testing and drafted the manuscript.
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- Prof. Robert S. Marks and Prof. Alfred Iing Yoong Tok proposed the initial research direction and guidance.
- Dr. Evgeni Eltzov and I designed the research experiments.
- Timothy S.E. Ng and I conducted the study initial investigation, which included the colorimetric signal comparison in solution.
- I performed the colorimetric signal comparison within the sensor, and drafted the manuscript.

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The contributions of the co-authors are as follows:

- Prof. Ibrahim Abdulhalim, Prof. Alfred Iing Yoong Tok and Prof. Robert S. Marks proposed the initial research direction and guidance.
- I designed the research experiments.
- I and Brescia Koh performed the functionalization of the plasmonic chips, conducted the plasmonic characterization, tested the performance of the biosensor and drafted the manuscript.
- Raymond Chee Seong Seet revised the manuscript.

Chapter 10 is published as Dorin Harpaz, Brescia Koh, Raymond Chee Seong Seet, Ibrahim Abdulhalim* and Alfred Iing Yoong Tok*, 'Functionalized silicon dioxide self-referenced plasmonic chip as point-of-care biosensor for stroke biomarkers NT-proBNP and S100 β ', Talanta 212 (2020): 120792.

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- I designed the research experiments.
- I performed the functionalization of the plasmonic chips, conducted the plasmonic characterization, tested the performance of the biosensor and drafted the manuscript.
- Brescia Koh contributed to the initial investigation of the study experiments and functionalization of the plasmonic chips.
- Raymond Chee Seong Seet revised the manuscript.

01 January 2020

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Date

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Harpaz Dorin

Abstract

Stroke, a top leading cause of death, occurs as a result of an acute interruption in the brain blood flow. After stroke onset, brain cells die rapidly. Therefore, therapeutic treatment must be administered early. The ‘time is brain’ rationale is a major limitation in therapeutic approaches, due to the association of time delays with worsened outcome in patients. Stroke diagnosis is mainly based on utilizing imaging technologies (magnetic resonance imaging (MRI) or computed tomography (CT)) in order to identify the stroke event in the brain. Stroke is classified to ischemic stroke (blockage) in 87% of cases, or to haemorrhagic stroke (bleeding) in the remaining 13% of cases. Then, ischemic stroke is further classified into different etiologies, based on the stroke mechanism: large artery atherosclerosis (LAA); cardioembolic (CEI); lacunar (LAC); others and undetermined. Stroke etiology classification main value is for therapeutic decision-making process, to allow the admission of time-limited window life-saving therapeutics (IV-tPA), within 4.5 hours from stroke onset. However, ischemic stroke etiologies classification schemes are complexed, time consuming (between hours to days), and require professional personnel.

Incorporating biomarkers measurement holds big potential as a new stroke etiology classification. Stroke is associated with a variety of pathophysiological changes, which leads to triggering different bio-chemical processes. This results in a big variety of stroke related biomarkers, for which their clinical practice value is yet to be fully determined. An ideal brain biomarker is usually a protein which can be measured from bio-fluids in a safe method, in order to provide additional data on specific organs, mainly the brain and spinal cord. In this thesis, candidate biomarkers were reviewed, and only biomarkers that were available in hospital laboratory analytical platforms were included. The levels of the following multiple biomarkers were investigated: NT-proBNP, D-dimer, S100 β , NSE, vitamin D, cortisol, IL-6, insulin, uric acid and albumin. After, the performance of these biomarkers to identify stroke mechanisms was examined, with the use of statistical and data-mining tools. The methodology of using receiver operating characteristic (ROC) analysis was followed in order to identify optimal cut-off values. Multivariable regression was employed using backward stepwise logistic regression model to identify useful biomarkers.

Biomarkers measured by a rapid point-of-care biosensor will enable an improved diagnosis approach. A point-of-care test is rapid (results within minutes), user-friendly, robust and can be used on-site. Successful examples are the glucometer and lateral flow pregnancy test. The challenge is to reach the required sensitivity sufficient to make a clinical decision. Several biomarkers were identified as useful to elucidate stroke mechanisms, as well as showed value for additional stroke management needs, such as diagnosis, severity, mortality and therapeutic admission. The use of a multi biomarker panel strategy, instead of the measurement of a single biomarker, is more useful but it is still not used in the clinical practice. After identifying selected and specific stroke biomarkers as potential target analytes, several optical quantitative biosensor platforms technologies were considered and compared. This thesis focuses on developing a point-of-care biosensor platform, for the multiplex and quantitative detection of specific stroke related biomarkers panel, directed specifically to ischemic stroke etiology classification.

Four optical biosensor platforms were considered and examined. Two paper-based sensing platforms were examined, based on colorimetric and chemiluminescent signals detection, because of their main advantages for multiplex detection. Moreover, two chip-based (gold-silver and silicon dioxide chips) sensing platforms were examined, based on surface plasmon resonance (SPR) sensing technology, because of their main advantage for sensitive detection. However, each of these four biosensor platforms also exhibit a problematic disadvantage, which must be overcome. First, in order to overcome the disadvantage for sensitive detection of the chemiluminescent and colorimetric paper-based biosensor platform, three different approaches were examined: dissolvable polyvinyl-alcohol film, membrane type comparison and signal amplification. Moreover, in order to overcome the disadvantage for ease of use as point-of-care platform of the SPR biosensor platform, specific functionalized chips from gold, silver and silicon dioxide were developed for use in a miniaturized SPR system. To conclude, the SPR chip-based biosensor platform demonstrated clinically relevant limit-of-detection lower than 1 ng/mL, for both biomarkers tested (NT-proBNP and S100 β). While, the paper-based biosensor platforms did not reach the required sensitivity and specificity.

Lay Summary

Stroke is a major cause of death worldwide. A stroke event is defined as an interference in the blood flow into the brain. After stroke occurs, the brain cells die rapidly, which cause irreversible damage to the patient brain. Treatment must be given rapidly in order to reduce the damage to the brain and to improve the patient outcome. Currently, stroke diagnosis mainly relies on imaging technologies, such as magnetic resonance imaging (MRI) or computed tomography (CT), in order to identify the stroke event in the brain. Stroke can be caused due to blockage of a brain blood vessel (termed ischemic stroke) or due to bleeding from a brain blood vessel (termed haemorrhagic stroke). In addition, the type of blockage is also further investigated to identify the exact ischemic stroke mechanism. This is important in order to make clinical decisions and to allow the admission of life saving treatment. The treatment must be given to the patient within 4.5 hours from stroke onset. However, the current diagnostic tests are complexed, time consuming (between hours to days), and require professional personal.

There is a big potential in the use of blood-based biomarkers measurement, in order to improve stroke diagnosis. Biomarkers are small molecules, usually proteins, which are secreted into the blood in different amounts in different medical conditions. Each disease is associated with different biomarkers in different concentrations in the blood. The stroke event triggers several bio-chemical processes in the patient brain, which leads to the secretion of different biomarkers into the blood. These biomarkers can then be safely measured from a blood sample of the patient, in order to provide additional information on the patient medical status. In this thesis, several potential stroke biomarkers were reviewed. Then, the amounts of the following biomarkers were examined in the blood samples of stroke patients: NT-proBNP, D-dimer, S100 β , NSE, vitamin D, cortisol, IL-6, insulin, uric acid and albumin. After, analysis was conducted in order to identify which of these biomarkers show high diagnostic accuracy for the ischemic stroke mechanisms. Analysis of individual and multiple biomarkers was conducted in order to identify a biomarkers panel. Several biomarkers were identified as useful to classify the stroke mechanisms.

The medical device, that enables the measurement of the selected biomarkers, was then developed. A device that measures blood-based biomarkers is termed a biosensor. A sensing device that measures the quantity of biological molecules. A point-of-care biosensor was developed, which can be used on the site of the event, with rapid results within minutes and is user friendly. Examples of successful point-of-care devices are the glucometer (for diabetes) and the lateral flow pregnancy test. Several point-of-care optical biosensor platforms were examined and compared. The optical biosensor platforms included an optical signal detection such as color, luminescent, and refractive index. And the platforms examined were either paper-based or chip-based biosensor platforms. These platforms were selected because of their main advantage for multiplex, sensitive and quantitative detection. However, each of these four biosensor platforms also exhibit a problematic disadvantage, which must be overcome.

First, in order to overcome the disadvantage of lack of sensitivity in the paper-based biosensor platform (with a color and luminescent signal detection), different engineering approaches were examined. They included the development of a dissolvable film, comparison of different paper types and the increase of the color signal. Moreover, in order to overcome the disadvantages of ease of use as a point-of-care device in the chip-based biosensor platform (with a refractive index signal detection), specific functionalized chips were developed from gold, silver and silicon. These functionalized chips were then incorporated and used in a miniaturized biosensor platform for the detection of the refractive index signal. To conclude, the chip-based biosensor platform demonstrated clinically relevant limit-of-detection lower than 1 ng/mL, for both biomarkers tested (NT-proBNP and S100 β). While, the paper-based biosensor platforms did not reach the required sensitivity and specificity.

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Abbreviations

AB	Antibody
AC	Alternating Current
ACT	Activated Clotting Time
AF	Atrial Fibrillation
ALT	Alanine Aminotransferase
APTT	Activated Partial Thromboplastin Time
ARU	Aspirin Reaction Units
AST	Aspartate Aminotransferase
ATP	Adenosine Triphosphate
AUC	Area under Curve
AVA	Agri-Food & Veterinary Authority
AVM	Arterio Venous Malformation
BBB	Blood Brain Barrier
BNP	Brain Natriuretic Peptide
CCS	Causative Classification Systems
CEI	Cardioembolic
CFN	Cellular Fibronectin
CI	Cerebral Infarction
CK	Creatine Kinase
CKMB	Creatine Kinase-Muscle/Brain
CNS	Central Nervous System
CRP	C Reactive Protein
CT	Computed Tomography
CV	Cyclic Voltammetry
DA	Dalton
DI	Deionized
EA	Ethanolamine
ECL	Electro-Chemiluminescence
ED	Emergency Department

EDC	1-Ethyl-3-(3-Dimethylaminopropyl) Carbodiimide
EDTA	Ethylene Diamine Tetra Acetic Acid
EIS	Electrochemical Impedance Spectroscopy
ELISA	Enzyme Linked Immuno Sorbent Assay
EMS	Emergency Medical Services
ER	Emergency Room
ESP	Extended Surface Plasmon
FDP	Fibrin Degradation Products
FFA	Free Fatty Acid
FMC	Fibrin Monomer Complex
GFAP	Glial Fibrillary Acidic Protein
HDL	High Density Lipoprotein
HF	Heart Failure
HFABP	Heart Fatty Acid Binding Protein
HRP	Horse Radish Peroxidase
ICH	Intra Cerebral Haemorrhage
IL	Interleukin
IMI	Insulator Metal Insulator
INR	International Normalized Ratio
IQR	Interquartile Range
IV-tPA	Intervenors Tissue Plasminogen Activator
LAA	Large Artery Atherosclerosis
LAC	Lacunar
LAD	Large Artery Disease
LD	Lactate Dehydrogenase
LDL	Low Density Lipoprotein
LMB	Langmuir Blodgett
LOD	Limit Of Detection
LSP	Localized Surface Plasmon
LTC	Long Term Care
LVEF	Left Ventricular Ejection Fraction

MAP	Microtubule-Associated Protein
MBP	Myelin Basic Protein
MES	2-(N-Morpholino) Ethanesulfonic Acid
MMP	Matrix Metallo Proteinase
MRI	Magnetic Resonance Imaging
MSU	Mobile Stroke Unit
MSTU	Mobile Stroke Treatment Unit
MUA	11-Mercaptoundecanoic Acid
MW	Molecular Weight
NF	Neurofilaments
NHS	N-Hydroxysuccinimide
NIHSS	National Institutes of Health Stroke Scale
NINDS	National Institute of Neurological Disorders and Stroke
NLR	Neutrophil Lymphocyte Ratios
NP	Nanoparticle
NPV	Negative Predictive Value
NSE	Neuron-Specific Enolase
NUH	National University Hospital
OCSP	Oxford Community Stroke Project
OTH	Others
PBS	Phosphate Buffered Saline
PE	Plasma Equivalent
PK	Pyruvate Kinase
PMT	Photomultiplier Tube
POCT	Point-of-Care-Test
PPV	Positive Predictive Value
PT	Prothrombin Time
PTT	Partial Thromboplastin Time
PVA	Polyvinyl Alcohol
PWR	Plasmon Waveguide Resonance
RAGE	Receptor for Advanced Glycation End products

RI	Refractive Index
RIU	Refractive Index Units
ROC	Receiver Operating Characteristic
RSD	Relative Standard Deviation
SAH	Subarachnoid Haemorrhage
SBDP	Spectrin Break-Down Products
SD	Standard Deviation
SE	Standard Echocardiography
SF	Soluble Fibrin
SP	Surface Plasmon
SPE	Screen Printed Electrode
SPGE	Screen Printed Gold Electrode
SPR	Surface Plasmon Resonance
SRPWR	Self Referenced Plasmon Waveguide Resonance
STEMO	Stroke Emergency Mobile
TC	Total Cholesterol
TG	Thromboglobulin
TIA	Transient Ischemic Attack
TNF	Tumor Necrosis Factor
TOAST	Trial of Org 10172 in Acute Stroke Treatment
UCH	Ubiquitin Carboxyl-Terminal Hydrolase
UND	Undetermined
UFC	Ultra-Fast Ceramics
VWF	Von Willebrand Factor

Chapter 1.   **Introduction**

This chapter gives a brief introduction of this thesis. Firstly, the hypotheses concerning the optical biosensor platforms for stroke diagnostics with blood-based biomarkers are introduced. Then, the objectives and scopes are described, which includes both sections of the identification of biomarkers and the biosensor platforms technologies. Later, the dissertation overview for each chapter is presented. Finally, the findings and outcomes are summarized.

1.1 Hypothesis/Problem Statement

Stroke, a top leading cause of death, occurs as a result of an acute interruption in the brain blood flow ^[1]. This interference is either due to ischemic stroke (blockage) in 87% of cases, or due to haemorrhagic stroke (bleeding) in the remaining 13% of cases ^[2]. After stroke onset, brain cells die rapidly, therefore, therapeutic treatment need to be administered early. The ‘time – outcome’ relationship is a major limitation in therapeutic approaches, due to the association of time delays with patients worsen outcome. Stroke diagnostics main focus is a rapid identification of stroke patients in the ED for the admission of time-limited life-saving therapeutics (IV-tPA) ^[3-5]. In the ED, MRI or CT screenings are usually used in order to classify between haemorrhagic and ischemic stroke ^[6-8]. Followed by the second important classification, which is between ischemic stroke etiologies, which includes: LAA, CEI, LAC, OTH and UND ^[9]. The most commonly used classification scheme is the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification ^[10-13]. Stroke etiology classification main value is for therapeutic decision-making process, in order to reduce the time to IV-tPA admission ^[9]. The current average time is of 3 hours from stroke symptoms, it must be administered within 4.5 hours. However, ischemic stroke etiologies classification schemes are complexed, time consuming (between hours to days), and require professional personal.

Incorporating biomarkers measurement holds big potential as a new stroke etiology classification ^[14]. Stroke is associated with a variety of pathophysiological changes, which leads to triggering different bio-chemical processes ^[15]. This results in a big variety of stroke related biomarkers, for which their clinical practice value is yet to be fully determined. An ideal brain biomarker is usually a protein which can be measured from bio-fluids in a safe method, in order to provide additional data on specific organs, mainly the brain and spinal cord. This biomarker should be specific, uniquely present in the CNS and reflect the extent of brain damage; sensitive, abundant and easily detected; and selective, for example, reflect therapeutic efficacy ^[16]. Stroke biomarkers are categorized based on their different origins, which includes glial cells origin, neuronal cells origin, heart muscle cells (cardiomyocytes) origin, blood vessels cells (myocytes) origin, general inflammatory cytokines, cytoskeleton

proteins, haemostatic proteins, lipids, metabolic proteins and others. In previous studies, several biomarkers have been associated to the different ischemic stroke etiologies. BNP [17-27], D-dimer [28-34], FMC, SF, FDP [35], FFA [36], TNF- α , IL-6, IL-1 β [34, 37-39] and CRP [40, 41] increased serum/plasma levels were found to be associated with CEI stroke. Whereas albumin [42] decreased serum/plasma levels were associated with CEI. In addition, CRP, NLR [43-52], Apo-lipoprotein (a) [53, 54], fibrinogen, vWF, Beta-TG [55], triglycerides, and LDL [56-59] increased serum/plasma levels were associated with LAA. Whereas HDL and decorin [33, 60] decreased serum/plasma levels were associated with LAA. Also, Apo-lipoprotein (E) [61] increased serum/plasma levels were associated with LAC stroke. Whereas, TNF- α , IL-6 and IL-1 β decreased serum/plasma levels were associated with LAC stroke. Biomarkers measured by a fast POCT on-site will enable an improved care approach for stroke patients at their most critical state.

A POCT technology is a rapid test (results within minutes), which is user-friendly, robust and can be used on-site [62]. Successful POCTs are the glucometer [63] and lateral flow pregnancy test. POCTs advantages includes: mobility, fast data processing, simple measurement and small volume of sample [64]. ELISA is the most common technology for immunoassay [65], with high sensitivity, but complicated with multiple steps. However, sensitivity is not always needed, in some cases fast results are more important. A POCT biosensor can have sufficient sensitivity while enabling a more practical approach [66]. However, not all POCTs contain the functions needed for a diagnostic test. As POCT is defined by its function and usage, it does not hold restrictions to a particular structure or method [67]. However, a POCT based biosensor will be more suitable as a stroke POCT. A biosensor is an integrated biorecognition-transducer device, able of giving quantitative results [68-70]. It is based on: (1) biorecognition – allow the detection of target biological molecule; (2) interface – the system main structure and function and (3) transducer – enable signal measurement and results processing. The choice of biorecognition element (e.g., antibodies) is done according to the target analyte. Towards the creation of POCTs for commercial applications in healthcare diagnostics, there is a constant focus on minimizing the device size, while still obtaining highly sensitive and accurate results, and in addition, to simplify the fabrication technologies [71-73].

The use of a multi biomarker panel strategy, instead of the measurement of a single biomarker, is a more useful approach, but it is still mostly research based and of unproven value. While the results for certain biomarkers answer on certain clinical requirements, there is no currently available biomarker to be recommended for use in the clinical settings. Multi biomarker studies will make effective panels in clinical settings ^[74], by increasing the diagnostic accuracy ^[16]. In addition, there is a need for a quantitative biomarker measurement and a specific cut-off value, which will allow an improved decision-making process in the clinical practice. In order to develop such multiplex and quantitative POCT for stroke biomarker measurement panel, there is a need to engineer novel POCT platforms.

This thesis focuses on developing a biosensor based POCT platform, for the multiplex and quantitative detection of specific stroke related biomarkers panel, directed specifically to ischemic stroke etiology classification. A POCT for this targeted clinical gap, has the potential to be integrated into the stroke management scheme and potentially shorten time-to-treatment and improve stroke patient outcome.

Considering the information discussed, the following hypotheses are proposed in this thesis:

- 1) Specific biomarkers can triage stroke patients for ischemic stroke etiology classification.
- 2) A point-of-care optical biosensor platform can quantitatively measure selected specific stroke biomarkers in clinically relevant limit-of-detection.
- 3) A point-of-care optical biosensor platform can rapidly measure selected specific stroke biomarkers in order to expedite ischemic stroke etiology classification.

1.2 Objectives and Scope

The main objective of this thesis is optical biosensor platform development for stroke diagnostics, for the multiplex and quantitative detection of selected specific stroke biomarkers at point-of-care, as a new ischemic stroke etiology classification device (Figure 1.1).

The specific objectives of this thesis are as follow:

- 1) To measure a potential panel of selected biomarkers in plasma samples of ischemic stroke patients, focusing on the biomarkers associated to ischemic stroke etiologies.
- 2) To analyze the stroke biomarkers levels using statistical and data mining tools, in order to select specific biomarkers which best classify the different ischemic stroke etiologies.
- 3) To develop a rapid point-of-care optical biosensor platform for the quantitative and multiplex detection of the chosen biomarkers, at the required clinically relevant limit-of-detection.

Figure 1.1. Thesis Scope and Objectives.

Detailed objectives of the thesis, with specific targets on how to achieve them.

1.3 Dissertation Overview

This thesis focuses on optical biosensor platform development for stroke diagnostics, for the multiplex and quantitative detection of selected specific stroke biomarkers at point-of-care, as a new ischemic stroke etiology classification device

Chapter 1 present the thesis hypotheses and describes the scope and objectives.

Chapter 2 review the literature concerning the current stroke diagnostics, the point-of-care devices clinically tested for stroke care and stroke related biomarkers.

Chapter 3 describe the rationale for the selected experimental methods, the characterization techniques and biosensor platforms technologies.

Chapter 4 elaborates on the selected biomarkers measurement in plasma samples of stroke patients, and the results of the statistical and data mining analysis to elucidate stroke mechanisms.

Chapter 5 elaborates on the selected biomarkers measurement in plasma samples of stroke patients, and the results of the statistical and data mining analysis with additional value in stroke management.

Chapter 6 elaborates on the development of a paper based optical biosensor platform, through the examination of increased sensitivity via dissolvable film to control sample flow.

Chapter 7 elaborates on the development of a paper based optical biosensor platform, through the examination of increased sensitivity via membrane type comparison and their modifications.

Chapter 8 elaborates on the development of a paper based optical biosensor platform, through the examination of increased sensitivity via colorimetric signal amplification.

Chapter 9 elaborates on the development of a surface plasmon resonance biosensor platform, for NT-proBNP and S100 β stroke biomarkers detection using gold-silver plasmonic chips in both water and plasma samples.

Chapter 10 elaborates on the development of a surface plasmon resonance biosensor platform, for NT-proBNP and S100 β stroke biomarkers detection using silicon dioxide plasmonic chips in both water and plasma samples.

Chapter 11 concludes the dissertation and elaborates on future studies.

Chapter 12 contains the appendix that supports the findings in this dissertation.

1.4 Findings and Outcomes/Originality

The use of POCTs in stroke care is only now starting to be integrated into the established clinical practice. However, those most commonly used POCTs are mainly well-known devices for the general management of ED patients, such as coagulation system parameters and blood chemistry measurements, however, they are not specifically customized to the care of stroke patients. There are only a few innovative POCTs that are still in the research scale and have not been tested in a clinical study, and which show a direction for the development of tailored-made stroke POCT device. As stroke diagnosis and outcome improvement mostly rely on time, POCT incorporation in stroke prognosis will save the lives of patients. There is a clear need for new stroke POCT devices, specifically directed to stroke clinical practice gaps. It is within the current scientific knowledge to develop such POCT devices directly to improve stroke care, with the use of novel on-site immunoassays to detect raising stroke related biomarkers. Stroke related biomarkers research is a growing field, with new biomarkers being discovered regularly. In order to fully make use of biomarkers measurement for the integration of POCT devices in stroke care there is a need to make use of modern analytical tools as well.

The original outcomes of this thesis are as follow:

- 1) The target of the point-of-care biosensor platform: towards the clinical need of ischemic stroke etiologies classification. This gap was identified after a broad study which included 10 interviews with professionals (companies, physicians and researchers) in the stroke research field (the interviews summaries can be found in appendix L).
- 2) The stroke biomarkers panel: the choice of the best combination of stroke biomarkers for stroke etiology classification.
- 3) The point-of-care optical biosensor platform: the development of a multiplex and quantitative platform, specifically for selected stroke biomarkers detection.

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Chapter 2. Literature Review

This chapter presents the literature review on the roles of a portable and rapid point-of-care-test (POCT) for stroke. Stroke, a leading cause of death, is caused by an abrupt interruption of blood flow to the brain. The supply of blood needs to be promptly restored to salvage brain tissues from irreversible neuronal death. Existing assessment of stroke patients is based largely on detailed clinical evaluation that is complemented by neuroimaging methods. However, emerging data point to the potential use of blood-derived biomarkers in aiding clinical decision-making, especially in the diagnosis of ischemic stroke, triaging patients for acute reperfusion therapies, and in informing stroke mechanisms and prognosis. The demand for newer techniques to deliver individualized information on-site for incorporation into a time-sensitive workflow has become greater. This literature review examines the roles of a portable and easy to use point-of-care-test (POCT) in shortening the time-to-treatment, classifying stroke subtypes and improving outcome of patients. The conventional stroke management workflow is first examined, then situations where a bedside biomarker assessment might aid clinical decision-making are highlighted. A novel stroke POCT approach is also presented, which combines the use of quantitative and multiplex POCTs platforms for the detection of specific stroke biomarkers, as well as data-mining tools to drive analytical processes. Further work is needed in the development of POCTs to fulfill an unmet need in acute stroke management.

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2.1 Stroke

2.1.1 Top Killer

Stroke is a top killer, with 6.7 million deaths globally in 2015 ^[3]. Each year, 15 million people suffer a stroke event, 30% die, while another 30% are left with permanent disabilities that affect their daily activities ^[4, 5]. Projections for 2030 show that stroke deaths are estimated to rise to 7.8 million ^[4]. Stroke, e.g. ‘brain attack’, is caused due to an acute interference in the brain blood flow ^[6]. This interference can be either due to a burst or blocked blood vessel. 87% of stroke cases are acute ischemic stroke (blockage), while the remaining 13% are hemorrhage stroke (burst, e.g. bleeding) ^[7]. Stroke is estimated to have around 150 causes ^[8]. Atherothrombotic disease is the most common cause for stroke, both diseases also share the following risk factors: smoking, alcohol consumption, inactive lifestyle, dietary factors, hypertension, dyslipidaemia, diabetes and obesity ^[9, 10]. Individuals of any age can suffer from stroke, however it mostly common in the elderly population (> 65 years) ^[8, 11, 12]. Brain changes related to aging have been linked to stroke, moreover stroke mechanisms differ between young and elderly patients ^[13]. Together with the aging population growth, stroke prevalence and costs will significantly rise in the following years ^[14-16]. A stroke victim is also considered to be in an increased health risk for heart function, breathing abnormalities, body temperature control, and paralysis ^[8]. Stroke patients recovery ranges in months at best but can last even years, which increase the burden and costs on the healthcare system in developed countries worldwide ^[14-16].

2.1.2 Symptoms & Hospital Admission

Stroke diagnostics main focus is rapid identification of stroke patients in the ED for the admission of time-limited life-saving therapeutics, mostly IV-tPA ^[17-20]. Stroke symptoms usually start suddenly (seconds to minutes). Identified by the American Stroke Association, stroke major symptoms are: limbs or face weakness (one side), difficulty speaking or understanding, dizziness, loss of balance, vision trouble confusion and severe headaches ^[7]. Once a stroke suspect is recognize, there is a need to rapidly contact the emergency medical services (EMS) dispatch for additional assessment ^[21]. Then, an urgent transportation to a

stroke unit or hospital with a pre-notification on a stroke case ^[22]. 70% of stroke cases receive medical treatment from the EMS ^[23, 24]. EMS miss stroke symptoms in more than 50% of cases ^[23, 25]. In this case, the patient arriving to the hospital will go through a regular ED admission and only then be identified as a stroke patient, while losing the patient critical survival ^[26]. NIHSS, a stroke scoring system ^[27, 28], is not successful in differentiating between stroke and its mimics (metabolic disorders, migraine and mass brain lesions) ^[29-31]. Additional technologies are: electrocardiogram, electrocardiogram, blood tests, carotid angiography, carotid ultrasound, echocardiography and more (Table 12.1) ^[32]. Poor stroke diagnostics results in critical time delays which worsen the patient outcome, mainly due to a lack of fast and accurate testing ^[8, 33].

2.1.3 Ischemic & Haemorrhagic Strokes

The initial classification in stroke prognostics is between ischemic and haemorrhagic stroke ^[34]. Ischemic stroke is the cause of stroke in 87% of cases. It is usually as a result of fat accumulation and the formation of a clot in vessel walls. If the clot is created where the accumulation of fat is, it is termed cerebral thrombosis, or if in the circulatory system, it is termed cerebral embolism. In cerebral embolism, the clot is in the heart or in the chest/neck large arteries, once the clot breaks down it is released into the blood stream, which then results in a small vessels blockage. Atrial Fibrillation (AF), e.g. irregular heartbeat, is a medical condition in which clots are formed in the heart, this is also a common cause of embolism. The remaining 13% of cases are as a result of haemorrhagic stroke, which occurs due to the burst of a weakened blood vessel ^[7]. Haemorrhagic stroke is usually caused by either: hypertension (high blood pressure), aneurysms (weakened blood vessel swelling) or arteriovenous malformations, AVMs, (abnormal blood vessels cluster) ^[8]. 9% of haemorrhagic strokes are as a result of intra-cerebral haemorrhage (ICH), and 3% are as a result of subarachnoid haemorrhage (SAH) ^[35]. Haemorrhagic stroke is with a higher risk for young individuals and its death rate is higher in compare to ischemic stroke. Haemorrhagic stroke symptoms appear suddenly: loss of consciousness, nausea, vomiting and severe headaches ^[8]. The classification between ischemic vs. haemorrhagic stroke is critical for patient prognostics and therapeutic treatment (Figure 2.2). In the ED, MRI or CT

screening (Table 12.1) are usually used to exclude haemorrhagic stroke, and thereby to determine if the patient suffer from stroke and whether it is haemorrhagic or ischemic stroke. However, CT and MRI technologies are not best for detecting ischemia, their availability is limited, and with high cost ^[36].

2.1.4 Ischemic Stroke Etiologies

As mentioned previously, ischemic stroke accounts for 87% of stroke cases, making ischemic stroke etiologies (subtypes) classification the second most important classification in stroke care. There are 4 main ischemic stroke etiologies: 20% are LAA, 20% are CEI, 25% are LAC, and 5% are other causes ^[37]. In addition, 30% are due to ‘cryptogenic strokes’, which includes stroke from unknown causes ^[38]. The main value in subtyping ischemic stroke is for a better targeted therapeutic decision-making process in the clinical practice and to minimize time-to-thrombolysis with treatment of IV-tPA admission. In addition, stroke classification serves other purposes, such as describing the characteristics of patients and phenotyping patients in a clinical study ^[37]. From previous studies ^[39-41], it was found out that: (1) CEI is common in the elderly (above 70 years), show smaller rate of secondary stroke, higher use of IV-tPA treatment and the highest stroke severity; (2) LAA is the most frequent stroke etiology in middle-aged patients (45-70 years), show the highest rate of secondary stroke, the highest percentage of smoking, previous TIA and alcohol consumption; (3) LAC show the highest rate of hypercholesterolemia, obesity, hypertension, diabetes mellitus and was associated with the lowest mortality and stroke severity. Because the pathologic pathway is very different among different ischemic stroke etiologies (atherosclerosis versus cardiac embolism), the hypothesis that different biomarkers will be associated to different stroke etiologies is a raising concept in the last decade. There are 3 common ischemic stroke subtypes classification schemes: (1) TOAST, (2) NINDS and (3) OCSP. Table 2.1 presents the comparison between the 3 common ischemic stroke etiologies classification methods. In addition, Figure 2.1 presents the complexed TOAST classification scheme. The current ischemic stroke etiologies classification methods are complexed, time consuming (between hours to days), and require professional personal. The improvement of the existing stroke etiology classification using

specific biomarkers measurement has a promising potential. Stroke etiology classification main value is therapeutic decision-making and to reduce time to IV-tPA treatment ^[37]. The average time is 3 hours from stroke symptoms, it was found useful only when administered within 4.5 hours ^[42, 43].

Table 2.1. Classification Schemes of Stroke Etiologies.

Description	Stroke Subtypes	Strengths	Weaknesses	Ref.
Trial of Org 10172 in Acute Stroke Treatment (TOAST) Classification				
Since 1993, the most clinically used method for ischemic stroke subtyping is TOAST, which is mainly based on clinical symptoms	LAA - Thrombosis or embolism from atherosclerosis of a large artery. CEI - Embolism from a cardiac origin. LAC - Occlusion of a small blood vessel. OTH and UND - includes either of the following cases: (1) more than one possible cause; (2) no cause is identified; (3) incomplete investigation	Reliability has been improved by the use of a computerized algorithm	Stroke from UND cause is the most heterogeneous group in the TOAST system, as well as in the Stroke Data Bank. Once a patient matches more than one possible cause he is equally grouped as a patient with a no cause identified or an incomplete investigation. This weakness could flaw the medical decision-making process	[44-47]
National Institute of Neurological Disorders and Stroke (NINDS) Classification				
Derived from the Harvard Stroke Registry classification	Brain Hemorrhages Brain Infarctions, which includes: LAA CEI LAC UND	The best option in the search of new causes of stroke among patients with no known cause or with another disease not causally related to the stroke event	Most of the currently used diagnostic tools were not available at that time, such as modern MRI with diffusion-weighted imaging, transesophageal echocardiography, TEE, magnetic resonance angiography, MRA, duplex ultrasound examination, and transcranial Doppler	[48]
Oxford Community Stroke Project (OCSF, Bamford/Oxford) Classification				
Relies on the initial symptoms and based on their extent	Total anterior circulation stroke (TAC) Partial anterior circulation stroke (PAC) LAC Posterior circulation stroke (POC) The type of stroke is then coded by adding a final letter to the above: I – for infarct (e.g. TACI) H – for hemorrhage (e.g. TACH) S – for syndrome (e.g. TACS)	Patients are easily classified into groups based on clinical grounds and CT scanning, which is usually done in all stroke patients; the outcome of the stroke event is driven strongly by the severity of the stroke, which is well reflected in this classification, without addressing the cause of the stroke	The extent and site of the brain infarct is unlikely to be specific to a particular stroke etiology, patients classified as having a LAC infarct may have a missed cardiac source of embolism. In addition, this classification should no longer be used to investigate potential risk factors or causes of stroke	[49, 50]

Figure 2.1. TOAST Classification Scheme.

Trial of Org 10172 in Acute Stroke Treatment ^[44].

2.1.5 Recovery & Prevention of Stroke Reoccurrence

Once the patient receives treatment and his condition is stable, he is transferred to a long-term care (LTC) facility for recovery and rehabilitation process. The recovery procedure depends on each individual and can last between days to months. The main importance in stroke recovery is to improve patient outcome and to prevent secondary stroke ^[51]. Stroke survivors accounts for 30 million people, which are one-quarter of the LTC facilities residents. The U.S. National Stroke Association reports that 10% of stroke survivors fully recover, 25% with minor disabilities, 40% with moderate-severe disabilities, 10% require full care in LTC, and 15% die ^[8]. Mapping the patient condition, using neuroimaging and neurophysiological assessments (Table 12.1), followed by predicting his motor functions recovery, improve the recovery process planning. For example, for predicting the recovery of upper-limb functions, shoulder abduction and finger extension testing within five days of stroke is needed. Also, in order to test the effects on caudal motor pathways and on motor disabilities, diffusion-weighted imaging testing within seven days of stroke is needed. Moreover, for motor and language recovery, fMRI activation pattern is needed. In addition, for motor outcome prediction, MRI DTI sequence is needed ^[52]. Stroke recovery is highly important for improving the patient outcome, there is potential in adding value to mapping the patient medical condition, directed for the recovery process needs.

2.1.6 ‘Time is Brain’

After stroke event, brain cells die fast, so treatment must start early. ‘Time to outcome’ relationship is a major limitation in therapeutic approaches. After suffering stroke, the damage to the brain tissue in the ischemic lesion core is irreversible, however the perilesional penumbra temporarily lost its function but still maintains metabolic and structural integrity, meaning it can still be saved ^[53]. Stroke can be treated, IV-tPA admission significantly improves patients outcome ^[18, 42, 43, 54]. However, the therapeutic treatments effectiveness is time dependent, with a small percentage of stroke patients receiving treatment ^[53, 55]. Time delays are associated with patients worsen outcome ^[56, 57]. Recent studies examined additional biological values, like genetic testing and blood

biomarkers for the prediction of improved outcome. In addition, targeted strategies are identified and applied [56, 58, 59]. Proposed pre-hospital strategies includes: public education, EMS stroke priority, better medical records access and mobile CT [60, 61]. In-hospital strategies includes: stroke code system, increased availability of stroke physicians, POCTs, CT co-location with ED and access to neuroimaging experts [62]. Post-hospital strategies includes: IV-tPA increased availability and consent bureaucracy simplification [56]. In addition, several biomarkers became valuable in guiding clinical decision-making [63, 64]. A fast POCT at the bedside can enable the admission of time-sensitive treatment, preferably already at the first patient medical care by physicians and paramedics. A portable and an easy to use POCT will enable an improved care approach for stroke patients.

Figure 2.2. POCTs vs. Conventional Time-to-Treatment in Stroke.

(1) Upon stroke symptoms onset, EMS dispatch is contacted ($t = 0$). (2) EMS follows 'case entry protocol' and evaluate the medical status of the patient by specific parameters. (3) The patient is then transported to the hospital ($t \leq 15\text{min}$). (4) Conventional prognosis: at best, stroke diagnosis has already been confirmed by the EMS, so the patient is immediately transported to a stroke specialized unit. Otherwise, the patient will go through the ED, like any other patient suffering from a range of medical problems ($t \leq 30\text{min}$). The suspected stroke patient will then only be evaluated by a neurologist ($t \leq 60\text{min}$). The most common neurologic examination used is NIHSS, and followed by a CT/MRI scan for initial stroke diagnosis ($t \leq 90\text{min}$). Subsequently, stroke subtype classification ($t \leq 120\text{min}$) is conducted by either TOAST, NINDS or OCSF classification schemes. Admission of IV-tPA is usually within 3 hours from stroke-symptoms-onset ($t \leq 180\text{min}$). (5) Improved prognosis using POCTs: shorten the time from stroke-symptoms-onset to neurologist examination ($t \leq 20\text{min}$). In addition, the time to CT/MRI scan can be reduced ($t \leq 45\text{min}$) and, most importantly, the time to IV-tPA admission can be reduced as well ($60\text{min} \leq t \leq 90\text{min}$)^[1].

2.2 Biosensors

2.2.1 Point-of-Care-Test

A POCT will enable a faster and better approach to caring for stroke patients in their most critical state, as it provides relevant information on-site to expedite specific diagnostic delays^[65]. A POCT platform is a mobile laboratory device that is located at the site where the patient is treated, which can be used to perform the needed tests by the same personnel who is treating the patient, thus potentially reducing interface times and examination times^[66]. POCT holds many advantages, such as simple measurement procedures, use of low sample volumes, no sample processing, automated data processing and off-course mobility (Figure 2.3)^[67]. The potential for POCT use in stroke care is therefore enormous. Conventional technologies are usually found in an analytical lab due to their more complicated set-up and operation procedure. Current conventional technologies do provide sufficient sensitivity and reliable results; however, they are not always considered practical. An important factor of POCT development is that the test will be considered effective only if action is taken based on its results^[68]. Only when the POCT outcome is utilized on-site as a basis for patient treatment can POCT successfully and efficiently reduce hospital stay,

improve adherence to treatment, and reduce complications ^[69]. The current POCT development and design research focuses on minimizing the device size, while still obtaining highly sensitive and accurate results ^[70, 71]. POCT successes include the glucose biosensor strips ^[72] and lateral flow immunoassay strips with the most well-known being the pregnancy test. The conventional clinical technology is ELISA (enzyme-linked immunosorbent assay) ^[73] which can reach higher sensitivities than most POCTs available, but which require multiple steps and a complicated testing procedure. It is important to note that sensitivity is not always the most critical factor, especially in cases where there needs to be faster and more accessible treatments. POCTs may result in sufficient sensitivity and accuracy standards being obtained.

Figure 2.3. POCT vs. Conventional Technologies.

POCTs advantages includes mobility, fast results, robust set up and being user-friendly. However, have lower sensitivity. Conventional technologies have more sensitive and accurate results. However, they have limitations that does not allow their usage as an on-site tool. Their disadvantages includes lab based equipment, long results processing (hours to days) and complicated method that requires expertise ^[1].

2.2.2 Biosensors Structure & Composition

Not all POCT devices demonstrate the full features required of a diagnostic device. A POCT is not defined by any particular technology or method of use, in that it does not require reagent-free operation, battery-powered operation, or a specific degree of operator training^[74]. However, a biosensor as a POCT will be a more accurate approach for a novel-stroke POCT^[75]. Biosensors are an important components of POCT devices because they are directly responsible for the assay bioanalytical performance^[76]. There is a variety of POCT-based sensor analytical formats, such as microfluidics^[77], microarrays^[78], paper-based immunoassays^[79-81] and optical-based sensors^[82-84]. A biosensor is usually described as a self-contained device, capable of providing selective and quantitative or semi-quantitative analytical information and which uses a biological recognition element and a transducer placed in intimate contact via some form of chemical immobilization^[85-87]. A typical biosensor is designed based on three different aspects: the bio-specific capture entity - biological detection of the target molecule; the chemical interface – controls the main function of the system; and transducer – signal detection and measurement (Figure 2.4). The bio-specific capture entity (e.g., whole cells, enzymes, DNA or RNA strands, antibodies, antigens or biomimetic molecules) is chosen according to the target analyte, while the interfacial chemistry ensures that the bio-specific capture entity molecule is immobilized upon the relevant transducer. A successful POCT sensor must fulfil at least three critical conditions after the immobilization steps: (1) maintain stability and activity of the biological part during and after functionalization; (2) maximize proximity of the biological layer to the transducer; (3) maintain sensitivity and specificity of the biological components to a target analyte^[88]. Adsorption, cross-linking, covalent binding, entrapment, and less useful Langmuir–Blodgett (LMB) deposition, self-assembled monolayers and bulk modifications are the most reported methods used in biosensor functionalization applications^[88, 89]. The transducer, which converts the molecular recognition event to a measurable signal, may be electrochemical, acoustic or of optical origin etc.^[90]. An ongoing trend in biosensors research is the development of healthcare diagnostic tools^[91, 92].

Figure 2.4. POCT Biosensor Structure.

A POCT biosensor consists of three components: bio-specific capture entity, chemical interface and transducer. A variety of bio-specific capture entities are used for the bio-detection of the target analyte in biosensors, such as antibodies, enzymes, DNA and whole cells. The biosensor chemical interface can be based on either a covalent, entrapment, absorption, encapsulation or cross-linking binding. In addition, the transducer, which is used for the signal transmission and measurement, can be based on electrochemical signal (potentiometric or amperometric), optical signal (absorbance, luminescence or fluorescence) or acoustic signal (quartz crystal microbalance, surface acoustic wave or surface transverse wave) ^[1].

2.2.3 POCT Biosensors in Stroke

Prior to the administration of IV-tPA treatment, laboratory results such as coagulation profile (international normalized ratio (INR) and activated partial thromboplastin time (APTT)), blood-count (platelet, leukocyte and erythrocyte count) and blood-chemistry (hemoglobin, glucose, c-glutamyltransferase and p-amylase test) were found as highly useful. However, in clinical practices, these valuable tests are not fully completed due to the time consuming diagnostic procedures ^[19, 93]. Most guidelines of stroke prognosis

recommend that thrombolytic therapy should not be delayed to wait for these test results unless there is clinical suspicion of: bleeding abnormality, thrombocytopenia, and cases where either the patient has received anticoagulants (heparin or warfarin) or if the use of anticoagulants is not known ^[19]. This might lead to an increased risk of overlooking stroke mimics or patients with contraindications for thrombolysis treatment administration ^[93]. In the ED, 50% of non-strokes are misdiagnosed as stroke ^[94]. The current clinical standard is based solely on the assessment of symptoms and rudimentary stroke scale tools. There is no simple, immediate, and unbiased way to diagnose stroke ^[95]. For example, studies have showed that serum glucose could be safely obtained by paramedics and that INR POCT can reduce door-to-needle times ^[56, 96, 97]. Part of these tests can be completed even before arriving to the hospital. Furthermore, a recent study showed that the use of POCT instead of the central hospital laboratory could reduce the time-to-therapy from 84 ± 26 to 40 ± 24 min ($p < 0.001$) and that the results of most laboratory tests (except APTT and INR) revealed close agreement with those from a standard centralized hospital laboratory ^[93]. Different POCTs have been tested for their usefulness in stroke monitoring (Table 2.2) ^[2]. However, their accuracy and effectiveness still have not been fully clinically tested ^[98].

Table 2.2. POCT in Stroke.

Category	POCT Device	Description	Stroke Application	Ref.
Mobile Stroke Unit	Mobile Stroke Unit	Ambulance containing imaging tools (CT scanners), POCTs (coagulation profile, blood-count, and blood-chemistry), telemedicine connection to hospital and therapeutic treatments (thrombolysis for ischemic stroke and anticoagulant reversal for haemorrhagic stroke)	Diagnosis	[99-112]
Imaging POCTs	CereTom® (Samsung/Neurologica)	Portable CT scanner that produce images with sufficient quality, however, does not allow the assessment of the neck vessels or the aortic arch	Ischemic vs. haemorrhagic stroke diagnosis	[113, 114]
	SOMATOM Scope (Siemens)	Portable CT scanner that allows higher resolution and assessment of neck vessels and the aortic arch, however it is bigger in size and require on-board power generation	Ischemic vs. haemorrhagic stroke diagnosis	[113, 115]
	Vivid q® (GE Healthcare)	Portable cardiac ultrasound device, show the condition of the carotid	Ischemic vs. haemorrhagic	[116, 117]

		arteries in the neck and intracranial vessels	stroke diagnosis	
Electrochemical POCTs Assays	CoaguChek® (Roche)	Amperometric determination of the PT time after activation of the coagulation with human recombinant thromboplastin, can determine the INR value from a drop of capillary whole blood, results within 1 minute	Diagnosis	[98, 104, 118-122]
	SMARTChip (Sarissa Biomedical)	3-layered micro-electrode consists of a biolayer, mediator and electrode, which measures purines from a drop of whole blood, results within 3-5 minutes	Diagnosis	[123-126]
	i-STAT® (Abbott)	Specific antibodies are located on an electrochemical sensor fabricated on a silicon chip, includes tests such as blood gases, electrolytes, metabolites, coagulation and cardiovascular biomarkers, results within 2 minutes	Diagnosis	[104, 127-131]
	pocH-100i (Sysmex)	Micro-fluidics. WBCs, RBCs and PLTs are counted using the direct current detection method with hydrodynamic focusing technology. Hemoglobin analysis is conducted using a non-cyanide method. Provides a full blood count and a 3-part differential leukocyte count	Diagnosis	[93, 104, 132, 133]
Optical POCTs Assays	Hemochron® Junior (ITC)	Whole blood micro-coagulation system, LED optical detectors detects the reduced sample flow once a clot is formed within the test channel	Diagnosis	[93, 134, 135]
	Reflotron® plus analyzer (Roche)	Blood-chemistry test (c-glutamyltransferase, p-amylase, and glucose), based on reflectance photometry, using LED as a light source and photodiodes as light detectors, results within 2-3 minutes	Diagnosis	[93, 136, 137]
	Cobas® h 232 (Roche)	Cardiac biomarkers (troponin T, NT-proBNP, D-dimer, myoglobin and CK-MB) immunoassay, based on gold-labelled monoclonal and biotinylated polyclonal antibodies, optical system converts the intensity of the signal line into a quantitative result	Prognosis (correlation to CEI) and stroke recovery (indication on second stroke reoccurrence)	[103, 138-166]
	Triage® BNP Test (Alere)	Fluorescence immunoassay for quantitative BNP measurement, the specimen reacts with fluorescent antibody conjugates, the concentration of BNP in the specimen is proportional to the fluorescence detected, results within 15 minutes	Prognosis (correlation to CEI) and stroke recovery (indication on second stroke reoccurrence)	[103, 138-165, 167]
	Cornell University	Luminescent assay of a biomimicry approach, based on enzymes tethered	Diagnosis	[168-174]

		to nanoparticles, for the detection of NSE. Immobilization of pyruvate kinase (PK) and luciferase on silica NPs, results within 10 minutes		
	VerifyNow® (Accumetrics/Accriva Diagnostics)	Qualitative turbidimetric based optical detection system which measures platelet induced aggregation as an increase in light transmittance. As aggregation occurs, the system converts luminosity transmittance results into 'Aspirin Reaction Units', for the identification of aspirin non-responsive patients	Prevention of second stroke recurrence	[175-192]
Other POCTs	PFA-100®, (Dade/Siemens)	Simulates under high shear stress, the interaction of platelets with an injured blood vessel, measures the time required for platelets to adhere and aggregate, used for identification of aspirin non-responsive patients	Prevention of second stroke recurrence	[175-191, 193, 194]
	Prediction Sciences LLC	Lateral flow-based test for the measurement of the proteomic marker cellular fibronectin (c-Fn), elevated serum levels in stroke patients at IV-tPA admission can identify if the patient is at high or low risk for a subsequent hemorrhage, results within 10 minutes	Diagnosis	[195]
	ReST™ (Valtari Bio™ Inc.)	Measurement of blood brain-specific biomarkers associated with immune responses, the degree and direction of the immune system activation allow the accurate identification of acute stroke from non-stroke, results within 10 minutes	Diagnosis	[94, 95, 196]

2.2.4 Mobile Stroke Unit

The mobile stroke unit (MSU) enables administration of treatment at the emergency site, which saves critical time and helps to expedite treatment with the time-dependent therapeutics [106-110]. It was developed in 2003 and clinically tested in 2008. The MSU contains imaging tools, POCTs, telemedicine connection to hospital and therapeutic treatments (thrombolysis for ischemic stroke and anticoagulant reversal for haemorrhagic stroke). The size of the devices used has been minimized, in order to fit into the standard ambulance. Since then, non-contrast CT, CT angiography, and CT perfusion (excluding MRI) have been integrated into MSUs [113]. The integration of mobile CT scanner and POCTs in ambulances was first clinically tested in the MSU project of the University of

Homburg, Saarland, Germany. These ambulances allow IV-tPA treatment on-site to test for ischemic stroke. After excluding the possibility of haemorrhagic stroke, the patient would then be transported to the hospital in a normal ambulance. The results of the controlled study showed a remarkable reduction of time from alarm to therapy decision (median of 35 minutes compared to 76 minutes in regular care) ^[99, 100]. However, scanning failures (mainly technical) were reported in a number of patients (12 of 53) ^[103]. Later, The Stroke Emergency Mobile (STEMO) project of the Charité in Berlin integrated new features into a fully equipped ambulance, enabling hyper-acute treatment and transport in the same vehicle ^[101, 105]. This pilot study showed encouraging results for the safety of treatment and in the number of prehospital IV-tPA applications (23 treatments within 52 days) with a mean call-to-needle time of 62 minutes compared to 98 minutes. The data suggests that prehospital stroke care in STEMO is feasible, without any safety concerns. A third clinical study of the mobile stroke treatment unit (MSTU) in the Cleveland Pre-Hospital Acute Stroke Treatment Study Group, also incorporated the presence of a registered nurse, paramedic, emergency medical technician, and a CT technologist. A cerebrovascular specialist evaluates the patient via telemedicine, and a neuroradiologist remotely evaluates images obtained by the portable CT scanner. In addition, a variety of POCTs were used, such as coagulation profile, complete blood-count, and blood-chemistry ^[104].

2.2.5 Imaging POCTS in Stroke

2.2.5.1 CereTom® (Samsung/Neurologica) & SOMATOM Scope (Siemens)

Most of the mobile brain imaging tools used in ambulances are portable, accumulator driven and radiation shielded, as they were originally designed for intensive care units. Two commonly used CT scanners were shown to be significantly useful when used in the MSUs: CereTom® (Samsung/Neurologica) and SOMATOM Scope (Siemens) ^[113]. CereTom® produces images with sufficient quality but does not allow assessment of the neck vessels or the aortic arch. It is an 8-slice CT scanner that delivers non-contrast, angiography and contrast perfusion scans. It uses a radiolucent universal scan board made from carbon fiber material designed to fit any bed ^[114]. SOMATOM Scope, on the other hand, allows higher

resolution and assessment of neck vessels and the aortic arch. However, it is bigger in size and requires on-board power supply. It uses an ultra-fast ceramics (UFC) detector with adaptive signal boost that reduces image noise by up to 50% and has a posterior fossa optimization filter that delivers reduced beam hardening artifacts in the base of the skull. Using Siemens proprietary UFC, SOMATOM Scope provides excellent image detail even at low radiation doses. This industry-leading device achieves exceptional efficiency thanks to high X-ray absorption combined with extremely effective conversion of X-ray energy into visible light, reducing noise in the images ^[115].

2.2.5.2 Vivid q® (GE Healthcare)

Standard echocardiography (SE) is also used in ischemic stroke recovery, though it is less commonly utilized. In a recent study in the stroke unit of the University Hospital Würzburg, Germany, the feasibility and accuracy of the POCT echocardiography was compared with SE using Vivid q® (GE Healthcare), a portable cardiac ultrasound device ^[116]. Results showed that amongst the patients tested, there was good agreement for cardiac parameters, interrater agreement for left ventricular ejection fraction (LVEF), as well as good detection of systolic dysfunction. They concluded that the use of POCT echocardiography in a stroke unit setting is feasible, enabling reliable preliminary assessment of selected cardiac parameters. However, its potential clinical utility in triaging stroke patients needs to be further investigated in larger diagnostic studies. Vivid q® is a comprehensive, compact and portable ultrasound system, which produces raw data image quality with matrix array imaging which is one of the most advanced probe technologies available. The matrix probe technology allows electronic beam focusing in three dimensions, enabling higher image quality. With its 16 probes and compact size, the Vivid q® allows clinicians to share exceptional ultrasound imaging in any clinical environment ^[117].

2.2.6 Electrochemical POCTs Assays in Stroke

2.2.6.1 CoaguChek® (Roche)

By using a coagumeter POCT, INR values can be measured immediately at the bedside^[98]. Roche company commercialized CoaguChek®, an electrochemical POCT, a convenient, portable and user-friendly instrument for monitoring oral anticoagulation therapy and determining the INR value from a drop of capillary whole blood. This POCT was used for both pre-hospital stroke care^[104, 118] and in-hospital stroke care^[98, 119]. CoaguChek® test principle is based on amperometric (electrochemical) detection of the coagulation response of the platelet after activation with the human recombinant thromboplastin. After placing a blood drop on the test strip (8 µL of fresh capillary whole blood), results are obtained within 1 minute. The INR values can be measured from 0.8 to 8. The POCT can be directly connected using Bluetooth or USB to send the test results to a healthcare professional quickly and with ease^[120]. The INR POCT results correlates well with laboratory values and can be used to shorten door-to-needle time^[118, 121, 122].

2.2.6.2 SMARTChip (Sarissa Biomedical)

A second electrochemical POCT is developed by Sarissa Biomedical, a spin-off company from Coventry (UK). SMARTChip^[123] is a POCT device that monitor stroke biomarkers. It can be used for stroke diagnosis by a paramedic, which will allow faster identification of stroke victims at the point of injury and facilitate rapid coordination of the clinical treatment pathway to maximize the chances of the best possible patient outcomes. The assay uses a hand-held reader and disposable biosensor. The SMARTChip can measure purines from just a drop of whole unprocessed blood and provide the results within minutes. Purines (e.g. adenosine, inosine and hypoxanthine) are neurochemicals that influence the function of the nervous system and can be used as stroke biomarkers as they are released after stroke onset. This SMARTChip POCT consists of a 3-layer structure, that converts a "dumb" microelectrode into a "smart" device capable of measuring specific analytes in real-time. The analyte sensitivity depends upon the nature of the enzymes included in the sensing

layer. The upper sensing layer is termed ‘biolayer’ and contains the bioreporter (enzyme) and sample, with or without the target analyte. The layer underneath the ‘biolayer’ is termed ‘mediator’ layer, and the bottom layer is the electrode which transmits the signal. The current SMARTChip platforms available are used in the detection of the following analytes: adenosine triphosphate (ATP), adenosine, inosine, hypoxanthine, acetylcholine, choline, glutamate, glucose, lactate and D-serine. The user initially calibrates a SMARTChip by applying a drop of calibration fluid. Then, the fluid is removed, and a finger-prick drop of blood from the patient is added. The reader is started, and the purine level displayed as a numerical value on the device. The process from calibration to the result takes approximately 3-5 minutes. SMARTChip was tested in one completed and two ongoing clinical studies: (1) a field deployable blood test for stroke, capable of detecting brain ischemia ^[124]; (2) validation of a purine biosensor in detecting acute cerebral ischemia: carotid endarterectomy model in SMARTChip ^[125]; (3) SMARTChip: a field deployable blood test for stroke, capable of detecting brain ischemia from the earliest stages of pathology ^[126].

2.2.6.3 i-STAT® (Abbott)

The i-STAT® is a portable, electrochemical clinical analyzer designed to be used at the patient bedside for critical care tests for blood gases, electrolytes, metabolites, coagulation and cardiovascular biomarkers ^[129]. It was tested for use in stroke monitoring ^[104, 127, 128]. A clinical study, supervised by Abbott and conducted in an anonymous hospital, aimed to revitalize the ED system by including new emergent protocols that integrated bedside POCTs. As part of this new protocol, i-STAT® System CHEM8+ (basic metabolic panel), cTnI (troponin I), CG4+ (blood gas with lactate), and PT/INR were implemented to improve diagnostic efficiency and patient flow. Incorporating i-STAT CHEM8+, cTnI, CG4+, and PT/INR into nurse-driven emergent protocols made measurable advancements in the diagnostic-process efficiency. The ED staff was empowered to accelerate diagnosis, treatment and disposition of patients. The full parameters include sodium, potassium, chloride, ionized calcium, BUN, glucose, creatinine, lactate, pH, PCO₂, PO₂, HCO₃, TCO₃, hematocrit, ACT celite, PT/INR, ACT kaolin, cTnI, CK-MB, BNP. Most of the test

principles are different. For the cardiovascular biomarkers, cTnI, CK-MB, BNP, the test is determined amperometrically using a two-site ELISA method. Specific antibodies are located on an electrochemical sensor fabricated on a silicon chip. An antibody/alkaline phosphatase enzyme conjugate specific to a separate portion of the analyte molecule is also deposited in another location on the sensor silicon chip. The whole blood or plasma sample is brought into contact with the sensor, allowing the enzyme conjugate to dissolve into the sample. The analyte within the sample becomes labelled with alkaline phosphatase and is captured onto the surface of the electrochemical sensor during an incubation period of approximately seven minutes. The sample, as well as excess enzyme conjugate, is washed off the sensor. Within the wash fluid is a substrate for the alkaline phosphatase enzyme. The enzyme bound to the antibody/antigen/antibody sandwich cleaves the substrate releasing an electrochemically detectable product. The electrochemical (amperometric) sensor measures this enzyme product which is proportional to the concentration of analyte within the sample. Each test has a different measurement range, for example the i-STAT BNP test range is 15 to 5000 pg/mL. Results for BNP and cTnI are obtained within 10 minutes, while that for CK-MB are obtained within 5 minutes. Blood-gas, blood-chemistry, electrolytes and hematology results are obtained within only 2 minutes ^[130].

2.2.6.4 PocH-100iTM (Sysmex)

Blood-count tests are also needed in stroke patient prognosis. A POCT specific for blood-count is the PocH-100iTM hematology analyzer (Sysmex), which is a compact device designed specifically for a POCT environment. The technology is based on hydrodynamic focused impedance cell counting. The analyzer provides a full blood count and a 3-part differential leukocyte count ^[132]. This POCT is used for both pre-hospital stroke care ^[104] and in-hospital stroke care ^[93]. PocH-100iTM is designed for laboratory testing of up to 25 samples per day and can detect red blood cells and platelets count. White blood cells, red blood cells and platelets are counted using the direct current detection method with hydrodynamic focusing technology to minimize coincidence or recirculation. Hemoglobin analysis is conducted using a non-cyanide method. Hematocrit is directly determined based on the red cell count and volume detection of each individual red blood cell. The instrument

requires only 15 μL of EDTA whole blood for reporting of 17 clinical parameters including a 3-part WBC differential ^[133].

2.2.7 Optical POCTs Assays in Stroke

2.2.7.1 Hemochron® (ITC/Accriva Diagnostics)

The optical POCT, Hemochron® (ITC/Accriva Diagnostics), is a whole blood micro-coagulation system, which was tested as an improved strategy for in-hospital stroke care ^[93, 134]. Being a micro-coagulation system, it offers point-of-care monitoring of: (1) ACT-LR: Low Range Activated Clotting Time, (2) ACT+: Activated Clotting Time Plus, (3) PT, (4) Citrate PT, (5) APTT and (6) Citrate APTT. The test technology is cuvette-based and uses finger-stick or venous samples with results received within minutes. The ACT+ assay accurately measures heparin (anticoagulant) effects from 1-6 μmL of blood. Results are dependent on actual fibrin clot formation, and not thrombin generation. It uses a mixture of silica, kaolin, and phospholipids as an activator to create a rapid and highly sensitive alternative to existing ACT tests. The ACT-LR assay is sensitive to low doses of heparin (0-2.5 μmL of blood) and it uses a celite activator due to its excellent heparin sensitivity. This POCT also reports INR, plasma-equivalent seconds and whole blood seconds, with an effective INR range from 0.8 to 10.0. As mentioned, the system measures whole blood clotting times using disposable single-use cuvettes. Each cuvette contains all the reagents necessary for a specified test. First, the operator inserts a cuvette for the test into the instrument and then once the cuvette has warmed to $37^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$, the instrument will signal that a blood sample can be added to the cuvette to start the test. The instrument measures the required volume of blood and automatically moves it into the cuvette test channel, where it is mixed with reagents. The sample is then moved back and forth at a predetermined rate within the test channel and monitored for clot formation. The test channel is maintained at $37^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$ during the test. The rate of movement of the sample is monitored by a series of LED optical detectors that are aligned with the test channel. When the blood clots, the flow of the blood sample within the test channel is impeded, reducing its rate of flow between the optical detectors. A reduction in flow below a

predetermined value signals to the instrument that a clot has formed. An internal timer measures the elapsed time between the start of the test and the clot formation. During the test, the whole blood clotting time (in seconds) is displayed. APTT and APTT Citrate results are displayed as plasma equivalent (PE) values, PT and PT Citrate results are displayed as the International Normalized Ratios (INR) and PE values, and ACT+ and ACT-LR results are displayed as Celite ACT equivalent time ^[135].

2.2.7.2 Reflotron® plus analyzer (Roche)

Another optical POCT is the Reflotron® plus analyzer (Roche, Cobas series), which is also used for blood-chemistry measurements of c-glutamyltransferase, p-amylase, and glucose. It was tested as an improved strategy for in-hospital stroke care ^[93, 136]. This is a single-test clinical chemistry system which can measure whole blood, plasma or serum for liver and pancreas enzymes, metabolites and blood lipids. The test gives on-site and reliable test results within 2-3 minutes, without sample or reagent preparation. It uses capillary or venous whole blood, plasma, serum, EDTA or heparin. The measuring principle is reflectance photometry, using LED as a light source (wavelengths 567 nm; 642 nm; 951 nm) and photodiodes as light detectors. A reflectance measurement is recorded based on the color change of the test strip. First, the operator applies 30 µL of the sample material to the test strip, without any need for reagent preparation or calibration of the instrument. Then, the operator inserts the test strip into the measuring chamber. On the test strip, the sample passes through the separation pad which separates erythrocytes and other cellular components. Then, the pre-reaction layer helps to eliminate any interfering substances and helps the sample to be pre-incubated with special activators or auxiliary reagents. After, the plasma or serum that is obtained passes to the transport pad (glass fiber) and underneath the reaction zone. In this zone, the photometer starts the analyte determination reaction at a defined time by pushing the reaction zone onto the transport pad. The reaction layers absorb the quantity of plasma or serum required for the reaction from the transport pad by capillary action. The reagents that are required for the analyte determination reaction are present on carriers. Reagents that would normally be unstable together on a single carrier can be placed on separate carriers. The reaction starts when the reaction zone is pushed onto

the filled plasma reservoir. The color intensity of the reaction product is measured at various times (kinetic measurement) or on completion of the reaction (end-point measurement). The depth of color is determined by the concentration of analyte in the sample being investigated. The instrument, a microprocessor-controlled reflectance photometer, takes full charge of the test and automatically measures reflectance values as well as performs calculations. It receives the test- and lot-specific information from the magnetic stripe affixed to the underside of each Reflotron® reagent strip. In this way, the microprocessor system can control and monitor all functions such as testing procedure, heating, automatic calibration and evaluation of reflectance values and calculation of results, specific to each test. At the heart of the photometer is the Ulbricht's sphere where the measurements take place. The full 17 parameters include (range): bilirubin (8.5-205 µmol/L), cholesterol (2.59-12.9 mmol/L), HDL (0.26-2.59 mmol/L), creatinine (44.5-884 µmol/L), glucose (0.56-33.3 mmol/L), hemoglobin (5-20 g/dL), potassium (2-12 mmol/L), triglycerides (0.8-6.86 mmol/L), uric acid (120-1190 µmol/L), urea (3.33-50 mmol/L), alkaline phosphates (20-1250 U/L), amylase (29-860 U/L), pancreatic amylase (14-850 U/L), creatine kinase (CK) (37°C: 24.4-1400 IU/L, 30°C: 15.4-900 IU/L, 25°C: 10-600 IU/L), gamma glutamyl transpeptidase (γ-GT) (37°C: 5-3500 U/L, 30°C: 3.85-2700 U/L, 25°C: 2.8-2000 U/L), aspartate aminotransferase (AST) (37°C: 5-500 U/L, 30°C: 3.25-325 U/L, 25°C: 2.25-225 U/L) and alanine aminotransferase (ALT) (37°C: 5-2000 U/L, 30°C: 3.8-1520 U/L, 25°C: 2.66-1060 U/L) ^[137].

2.2.7.3 Cobas® h 232 (Roche)

The Cobas h 232 point-of-care testing (POCT) system manufactured by Roche Diagnostics has been introduced as a near-patient instrument for measurement of several cardiac biomarkers, including NT-proBNP. Recent studies demonstrated the usefulness of using the B-Type Natriuretic Peptide (BNP) POCT platform on suspected ischemic stroke patients in the ED in order to complete stroke prognosis ^[138-142]. BNP, a well-known biomarker for heart failure (HF), has now been identified as potentially useful for stroke patient prognostic and recovery management. BNP elevated serum levels in stroke patients show correlation with CEI stroke ^[103, 143-155], increased mortality ^[155-162] and indication of

second stroke recurrence ^[163-165]. Cobas h 232 technology is based upon an immunological method and uses 150 µL heparinized venous whole blood as sample material. The test strips (CARDIAC proBNP+) contain monoclonal and polyclonal antibodies against epitopes of the NT-proBNP molecule of which one is gold-labelled and the other biotinylated. The test strip detection zone for NT-proBNP has two lines: A signal line and a control line. The signal line indicates if NT-proBNP is present in the blood sample. The control line indicates that the test is valid. The antibodies form a sandwich complex with the analyte in the blood. Following removal of erythrocytes from the sample, plasma passes through the detection zone where the gold-labelled sandwich complexes accumulate, and the positive signal is displayed as a reddish line (the signal line). Excess gold-labelled antibodies accumulate along the control line, signaling that the test is valid. An optical system converts the intensity of the signal line into a quantitative result which is shown on the display. Calibration is done automatically when a test strip is inserted. Each lot of the Roche CARDIAC proBNP+ test strip is calibrated against the Elecsys proBNP test. The measuring range is 7 – 1062 pmol/L. The full 5 parameters includes: troponin T, NT-proBNP, D-dimer, myoglobin and CK-MB ^[166].

2.2.7.4 Triage® BNP Test (Alere)

Another optical POCT for BNP monitoring is the Alere Triage® BNP Test. It is a rapid fluorescence immunoassay used with Alere Triage® Meters for the quantitative measurement of BNP in EDTA anticoagulated whole blood or plasma specimens. The test is intended to be used as an aid in the diagnosis and assessment of severity of congestive heart failure. The test procedure involves the addition of several drops of an EDTA anticoagulated whole blood or plasma specimen to the sample port on the test device. After addition of the specimen, the whole blood cells are separated from the plasma using a filter contained in the test device. The specimen reacts with fluorescent antibody conjugates and flows through the test device by capillary action. Complexes of each fluorescent antibody conjugate are captured on discrete zones specific to the analyte. The test device is inserted into the Alere Triage® Meter (hereafter referred to as Meter), which is programmed to perform the BNP analysis after the specimen has reacted with the reagents within the test

device. The concentration of BNP in the specimen is directly proportional to the fluorescence detected within a measurement zone on the test device. The results are displayed on the Meter screen in approximately 15 minutes from the addition of specimen. A larger amount of fluorescence detected by the Meter indicates a higher BNP concentration in the specimen. All results are stored in the Meter memory to display or print when needed. If connected, the Meter can transmit results to the lab or hospital information system. The Alere Triage® BNP Test contains all reagents necessary for the quantification of BNP in EDTA anticoagulated whole blood or plasma specimens. The test device contains murine monoclonal antibodies and polyclonal antibodies against BNP, fluorescent dye and stabilizers. The test devices need to be stored in a refrigerator at 2-8°C or stabilized for up to 14 days at room temperature. The lowest detectable concentration determined, with 95% confidence limit of the analytical sensitivity, was less than 5 pg/mL ^[167].

2.2.7.5 Cornell University

Additional optical POCT directed against stroke biomarker measurement, was developed by researchers from Cornell University, State University of New York and the New York Presbyterian Hospital. This POCT is based on biomimicry-based approach to demonstrate a new diagnostic platform, based on enzymes tethered to nanoparticles (NPs), for the detection of neuron-specific enolase (NSE) ^[168]. Immobilization of pyruvate kinase (PK) and luciferase on silica NPs was used to achieve rapid and sensitive detection of NSE, a well-known and clinically relevant biomarker for stroke. Their approach couple production of ATP by PK with activity of firefly luciferase (Luc) to generate a highly rapid and sensitive bioluminescent readout for the detection of NSE. The researchers showed that their data matched well ($r = 0.815$) with the current gold standard for biomarker detection, ELISA. Moreover, they have a great advantage over ELISA, as they can achieve detection in 10 minutes as opposed to the several hours required for traditional ELISA. Although no single biomarker will likely provide a definitive diagnosis of any disease, the glycolytic enzyme, NSE, is released from damaged neurons and has been suggested to be valuable for the diagnosis of various brain injuries. NSE has been suggested to be useful in distinguishing stroke from mimics, an important first step in expediting the diagnostic

process ^[169-171]. As an alternative to antibody capture, POCTs based on fluid phase enzymatic activities ^[172, 173] or semi-solid phase bioluminescence ^[174] are used for plasma NSE monitoring.

2.2.7.6 VerifyNow® (Accumetrics/Accriva Diagnostics)

A potentially useful POCT for stroke recovery is to screen for aspirin responsiveness after stroke ^[175]. Aspirin, the most commonly used antiplatelet agent, reduces the relative risk of major vascular events and vascular death by 20% after ischemic stroke ^[176]. However, the antiplatelet properties of aspirin are not uniform between individuals and recurrent events, which may be caused by ‘aspirin non-responsiveness’ ^[177-184]. There is evidence that aspirin non-responsive individuals may be at increased risk of ischemic vascular events ^[185, 186]. The response to aspirin should be monitored in post-hospital care of stroke patients for the prevention of second stroke recurrence ^[188, 191]. However, the platelet function tests for aspirin monitoring are time-consuming and difficult to follow as a routine practice. There are simpler platelet function POCTs, such as the PFA-100® Platelet Function Analyzer (Dade/Siemens) and the VerifyNow® Aspirin test (Accumetrics/Accriva Diagnostics) ^[187, 189, 193] available to screen stroke patients for aspirin non-responsiveness. Harrison *et al.* compared the use of both POCTs in 100 patients with transient ischemic attack or stroke patients receiving daily low-dose aspirin treatment ^[175]. These POCTs offer the possibility of a rapid and reliable identification of aspirin non-responsive patients without the requirement of a specialized laboratory ^[190]. However, aspirin non-responsiveness is highly test-specific and large prospective studies should determine the prognostic value for each POCT used. The VerifyNow® aspirin test system is a qualitative turbidimetric based optical detection system which measures platelet induced aggregation as an increase in light transmittance. The system consists of a stand-alone instrument and disposable assay device with reagents based on microbead agglutination technology. The quality control system includes an electronic quality control, an assay device internal control, and two levels of external, wet quality controls. The instrument controls assay sequencing, temperature, reagent-sample mixing, determines the degree of platelet function, and displays the results and status information, as well as self-diagnostics. The assay device contains a lyophilized

preparation of human fibrinogen coated beads, platelet agonist, a peptide, bovine serum albumin, buffer and stabilizer. The patient sample is citrated whole blood, which is automatically dispensed from the blood collection tube into the assay device, with no pre-treatment. Fibrinogen-coated microparticles are used in the assay device to bind activated platelet GP IIb/IIIa receptors. When the activated platelets are exposed to the fibrinogen-coated microparticles, aggregation occurs proportionally to the number of activated platelet receptors. To ensure consistent and uniform activation of the platelets, the agonist arachidonic acid is incorporated into the assay device. The results are reported in aspirin reaction units (ARU). Results ≤ 549 shows evidence of platelet dysfunction due to aspirin; > 550 : no evidence of aspirin-induced platelet dysfunction ^[192]. The details for PFA-100® are presented under the following section.

2.2.7 Other POCTs in Stroke

2.2.7.1 PFA-100® (Dade/Siemens)

As previously mentioned, screening for aspirin non-responsiveness is useful for secondary stroke prevention. There are POCTs available to screen stroke patients for aspirin non-responsiveness, such as the PFA-100® system, which provides a tool for clinicians to use in the detection of platelet dysfunction. The PFA-100® system simulates, under high shear stress, the interaction of platelets with an injured blood vessel. These conditions allow the PFA-100® system to measure in vitro platelet function. The PFA-100® system measures the time required for platelets to adhere and aggregate and result in occlusion of an aperture under high shear conditions. The single use test cartridge unit consists of a capillary, a sample reservoir and a biologically active membrane with a central aperture coated with collagen and epinephrine. By applying constant vacuum, anticoagulated whole blood is aspirated from the sample reservoir through the capillary and the aperture under standardized rheological conditions that expose platelets to high shear stress. First, a trigger solution is dispensed to wet the membrane. Then, similar to an in vivo mechanism, platelets adhere and aggregate at the aperture, thereby gradually diminishing and finally arresting the blood flow. The instrument then determines the time from the start of the test until the

platelet plug occludes the aperture and reports the time interval as the closure time. The platelet plug formation in the PFA-100® is dependent on adequate platelet activity and adequate von willebrand factor status. Therefore, the closure time is an indicator of the platelet function in the analyzed whole blood sample ^[194].

2.2.7.2 Prediction Sciences LLC

Another POCT directed against a stroke biomarker was developed by Prediction Sciences LLC (California, USA). This POCT is directed for the measurement of proteomic marker cellular fibronectin (c-Fn) ^[195], which has been shown in recent studies to predict hemorrhagic transformation in IV-tPA-treated patients with a sensitivity of 100%. As mentioned previously, administration of IV-tPA is limited to a critical time window of 4.5 hours and the amount of c-Fn in the blood of stroke patients at administration can identify if the patient is at high or low risk for a subsequent hemorrhage. This POCT platform is based on lateral flow technology detection of c-Fn, with the ability to obtain results within 10 minutes.

2.2.7.3 ReST™ (Valtari Bio™ Inc.)

Valtari Bio™ Inc., a company from West-Virginia (USA), is also developing a POCT for the detection of stroke related biomarkers. ReST™, is a rapid evaluation stroke triage test ^[196], which is aimed at improving the initial stroke versus no stroke determination in 10 minutes or less. This approach is based on measurement of blood brain-specific biomarkers associated with immune responses, for better stroke identification. The degree and direction of the immune system activation, following stroke and brain injury, allow the accurate identification of acute stroke from non-stroke. In addition, they employ machine learning and pattern recognition tools in order to identify different immune response patterns in the peripheral blood following various types of neuro-related brain injury. Their method is optimized due to their use of pattern recognition and ratios of biomarkers, rather than the absolute measurement of specific biomarkers. The company conducted clinical studies on over 500 real-world patients and their preliminary clinical trial data suggests that the

sensitivity and specificity for diagnosing stroke, using a pattern of expression of associated immune related biomarkers, are much higher than current clinical practice.

2.3 Biomarkers

2.3.1 Ideal Brain Biomarker

As a priority, a novel stroke POCT device should show the detection of novel biomarkers. An ideal brain biomarker is usually a protein which can be measured from bio-fluids in a safe method, in order to provide additional data on specific organs, mainly the brain and spinal cord. This biomarker should be specific, uniquely present in the CNS (central nervous system) and reflect the extent of brain damage; sensitive, abundant and easily detected; and selective, for example, reflect therapeutic efficacy ^[197]. Brain biomarkers identification can be used for suspected stroke patient diagnosis, and also to monitor their treatment and recovery ^[198]. The use of a multi biomarker panel strategy, instead of the measurement of a single biomarker, is probably the more useful approach, but it is still mostly research based and of unproven value ^[199]. While the results for certain biomarkers answer on certain clinical requirements, there is no currently available biomarker to be recommended for use in the clinical settings. Multi biomarker studies will make effective panels in clinical settings ^[200], increasing diagnostic accuracy, by minimizing the cross-effect of any individual biomarker ^[197]. The majority of the brain biomarkers tested so far for stroke show characteristic differentiation due to other medical conditions ^[17, 201]. In addition, there is a need for a quantitative biomarker measurement and a specific cut-off value, which will allow an improved decision-making process in the clinical practice. In order to develop such a multiplex and quantitative POCT for stroke biomarker measurement panel, there is a need to engineer novel POCT platforms.

2.3.2 Stroke Biomarkers

Stroke is associated with a variety of pathophysiological changes, which leads to triggering different bio-chemical processes ^[63]. This results in a big variety of stroke related

biomarkers, for which their clinical practice value is yet to be fully determined ^[202-213]. As presented in Table 2.3, a convenient way to categorize stroke biomarkers is by their origin, which includes: glial cells origin, neuronal cells origin, heart muscle cells (cardiomyocytes) origin, blood vessels cells (myocytes) origin, general inflammatory cytokines, cytoskeleton proteins, haemostatic proteins, lipids, metabolic proteins and others ^[214].

Table 2.3. Stroke Related Biomarkers.

Family	Biomarker	Expression Association	Stroke Clinical Value	Ref.
Glial cells origin	S100-Beta	Blood–brain barrier (BBB) dysfunction	Diagnosis	[197, 198, 215-220]
	Glial Fibrillary Acidic Protein (GFAP)	Size of brain lesions, neurological status and short-term functional outcome	Prognosis: outcome prediction	[197, 200, 215, 216, 221-226]
	Myelin Basic Protein (MBP)	Worsen outcome	Prognosis: outcome prediction	[227-230]
Neuronal cells origin	Neuron-Specific Enolase (NSE)	Neurological outcomes and infarct volume	Prognosis: outcome prediction	[171, 197, 198, 200, 215-217, 231, 232]
	Ubiquitin Carboxyl-terminal Hydrolase L1 (UCH-L1)	Extent of the neuronal injury	Prognosis: outcome prediction	[197, 198, 233-236]
	Creatinine Kinase-BB (CKBB)	Extent and severity of brain damage and recovery potential	Prognosis and recovery	[237, 238]
Heart muscle cells (cardiomyocytes) origin	B-Type Natriuretic Peptide (BNP)	CEI ischemic stroke, increased mortality and second stroke indication	Prognosis: subtype classification and recovery: second stroke prevention	[103, 143-165, 239-250]
Blood vessels cells (myocytes) origin	Matrix Metallo-Proteinase (MMP) 7, 8, 9 and 26	Blood–brain barrier (BBB) dysfunction and second stroke indication	Diagnosis and recovery: second stroke prevention	[201, 251-255]
General inflammatory cytokines and proteins	Interleukin (IL) – 1b, 6 and 8, tumour necrosis factor- α (TNF- α)	CEI ischemic stroke subtype	Prognosis: subtype classification	[200, 256-266]
	Neutrophil Lymphocyte Ratios (NLR)	Increased mortality and LAA ischemic stroke subtype	Prognosis: subtype classification and recovery	[263, 267-275]
Cytoskeleton proteins	Neurofilaments (NF)	Disconnected axons	Prognosis	[276-278]
	Cleaved-tau (C-tau)	Neuronal degeneration and disease progression	Prognosis	[197, 198, 227, 279-282]

	Microtubule-associated protein 2 (MAP2)	Dendritic injury	Prognosis	[283-286]
	Alpha-II spectrin break-down products (SBDPs)	Apoptosis and necrotic neuronal death	Prognosis	[197, 287-290]
Haemostatic proteins	D-dimer (DD)	CEI ischemic stroke subtype and recurrence strokes	Prognosis: subtype classification	[17, 291-313]
	C-reactive protein (CRP)	Acute ischemic stroke diagnosis, stroke severity and LAA ischemic stroke subtype	Prognosis: subtype classification	[17, 314-325]
	Fibrin monomer complex (FMC)	Stroke early recognition and CEI ischemic stroke subtype	Diagnosis and prognosis: subtype classification	[326]
	Soluble fibrin (SF)	Stroke early recognition and CEI ischemic stroke subtype	Diagnosis and prognosis: subtype classification	[326]
	Fibrinogen	Stroke early recognition	Diagnosis	[326, 327]
	Fibrin/fibrinogen degradation products (FDPs)	Stroke early recognition	Diagnosis	[326]
	Von willebrand factor (vWF)	Stroke early recognition and LAC ischemic stroke subtype	Diagnosis and prognosis: subtype classification	[327]
Lipids origin	Triglycerides	LAA ischemic stroke subtype	Prognosis: subtype classification	[328-331]
	Low density lipoprotein (LDL)/High density lipoprotein (HDL)	LAA ischemic stroke subtype	Prognosis: subtype classification	[328-330]
	Heart fatty acid binding protein (H-FABP)	Early diagnosis of stroke	Diagnosis	[332, 333]
	Free fatty acid (FFA)	CEI ischemic stroke subtype	Prognosis: subtype classification	[334]
	ApoA	LAA ischemic stroke subtype, and stroke severity	Prognosis: subtype classification and outcome prediction	[335-337]
	ApoE4	Ischemic stroke diagnosis (vs. haemorrhagic stroke) and with LAC and LAA ischemic stroke subtypes	Diagnosis and prognosis: subtype classification	[329, 338]
Metabolic proteins	lactate dehydrogenase (LD)	Extent and severity of the brain damage and the recovery potential	Prognosis and recovery	[237, 339]
	Albumin	CEI ischemic stroke subtype	Prognosis: subtype classification	[321, 340]
	Uric Acid	Mortality and functional outcomes	Prognosis: outcome prediction	[341]
Others	Decorin	LAA ischemic stroke subtype	Prognosis: subtype classification	[342]

2.3.3 Ischemic Stroke Etiologies Biomarkers

Several biomarkers have been associated to the different ischemic stroke etiologies (CEI, LAA and LAC), as presented in Table 2.4 [343]. NT-proBNP, D-dimer, FMC, SF, FDPs, FFA, TNF- α , IL-6, IL-1 β and CRP increased serum/plasma levels were found to be associated with CEI stroke. Whereas albumin decreased serum/plasma levels were associated with CEI. In addition, CRP, NLR, Apo-a, fibrinogen, vWF, beta-TG, triglycerides, and LDL increased serum/plasma levels were associated with LAA. Whereas HDL and decorin decreased serum/plasma levels were associated with LAA. Also, Apo-E4 increased serum/plasma levels were associated with LAC stroke. Whereas, TNF- α , IL-6 and IL-1 β decreased serum/plasma levels were associated with LAC stroke.

Table 2.4. Stroke Etiologies Biomarkers.

Arrows indicate on elevated ($\uparrow$) or decreased ($\downarrow$) levels in serum or plasma.

Biomarker	Cardioembolic	Large Artery Atherosclerosis	Lacunar	Ref.
NT-proBNP	$\uparrow$			[140, 143, 144, 146, 148-150, 152-154, 240, 344]
D-dimer	$\uparrow$			[295, 297, 300, 302, 304, 308, 309, 344, 345]
FMC	$\uparrow$			[326]
SF	$\uparrow$			[326]
FDP	$\uparrow$			[326]
FFA	$\uparrow$			[334]
TNF- α	$\uparrow$		$\downarrow$	[258, 259, 295, 346]
IL-6	$\uparrow$		$\downarrow$	[258, 259, 295, 346]
IL-1 β	$\uparrow$		$\downarrow$	[258, 259, 295, 346]
Albumin	$\downarrow$			[340]
CRP	$\uparrow$	$\uparrow$		[317, 318, 323]
NLR		$\uparrow$		[263, 267-273, 347, 348]
Apo (a)		$\uparrow$		[335, 336]
Fibrinogen		$\uparrow$		[327]
vWF		$\uparrow$		[327]
Beta-TG		$\uparrow$		[327]
Triglycerides		$\uparrow$		[328-330, 349]
LDL		$\uparrow$		[328-330, 349]
HDL		$\downarrow$		[328-330, 349]
Decorin		$\downarrow$		[297, 342]
Apo (E) 4			$\uparrow$	[338]

2.3.4 Data-Mining for Clinical Integration

As modern research is developing, the use of big-data studies became increasingly common. Incorporating data engineering tools in scientific research helps to achieve better results' analysis and conclusions, and has become mandatory in clinical research, with a powerful impact on the research directions and choices. Big data analytics in healthcare have evolved significantly as an innovative approach ^[350]. Data mining and knowledge discovery as an approach to examining medical data can limit some of the inherent bias in the hypothesis assumptions that can be found in traditional clinical data analysis ^[351]. Big data has four characteristics—Volume, Variety, Velocity and Value (the 4 Vs)—which traditional systems are incapable of processing. Medical data sets are continuously becoming larger, thus making it increasingly difficult for traditional systems to process them ^[352]. Data mining is one of the technologies called to improve the quality of service in clinical medicine through the intelligent analysis of biomedical information. From the enunciation of evidence-based medicine in early 1990s ^[353], the need for creating evidence that could be quickly transferred to physician daily practice is one of the most important challenges in medicine. The use of statistics to prove the validity of the treatment over discrete populations; the creation of predictive models for diagnosis, prognosis, and treatment; and the inference of clinical guidelines as decision trees or workflows from instances of healthcare protocols are examples of how data mining can help in the application of evidence based medicine. For example, in a recent study ^[304], N. Dougu *et al.* developed an algorithm combining AF and D-dimer level in order to classify the diagnosis of cerebral infarction (CI). CI patients were classified into LAA, CEI, LAC infarction or others. If the D-dimer level exceeds a certain cutoff point, the patient would be suspected of having cardioembolic disease. However, despite the great penetration of these techniques in literature, their application in real clinical practice is far from complete ^[354]. In order to make use of the strong clinical potential of a POCT for stroke biomarkers measurement, there is a need to develop a multi biomarker panel, combined with a strong analytical tool, to create a customized useful POCT for improved stroke prognosis care.

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Chapter 3.   **Experimental Methodology**

This chapter describe the rationale for the selected experimental methods, which includes the stroke biomarkers measurement, the biosensor platforms technologies and the characterization techniques.

3.1 Rationale for Selection

The main objective of this thesis is to develop an optical biosensor platform for stroke diagnostics, for the multiplex and quantitative detection of selected specific stroke biomarkers at point-of-care, as a new ischemic stroke etiology classification device ^[1, 2]. The development of a biosensor platform is firstly focused on the selection of the target analyte (biomarker). As ischemic stroke involves different biomarkers from tissues in the brain, cardiac and vasculature ^[3], multiple biomarkers that target different aspects of stroke pathogenesis were examined for their performance to classify stroke mechanisms. Candidate biomarkers that interrogate different aspects of stroke pathogenesis (e.g. glial/neuronal death, inflammation, cardiac function, hemostasis, oxidative damage and neuroendocrine responses) were reviewed ^[2]. Then, only biomarkers that were available in hospital laboratory analytical platforms were included.

The levels of the following multiple biomarkers were investigated: NT-proBNP, D-dimer, S100 β , NSE, vitamin D, cortisol, IL-6, insulin, uric acid and albumin. After, the performance of these biomarkers to identify stroke mechanisms was examined with the use of statistical and data-mining tools. The combination and cut-off values of the biomarkers to elucidate stroke mechanisms were examined in this analysis. The methodology of using receiver operating characteristic (ROC) analysis was followed in order to identify optimal cut-off values for continuous markers that were subsequently reconstructed as dichotomous variables and used in the logistic regression analysis ^[4]. Multivariable regression was employed using backward stepwise logistic regression model to identify useful biomarkers ^[5]. Several biomarkers were identified as useful to elucidate stroke mechanisms, as well as showed value for additional stroke management needs, such as diagnosis, severity, mortality and therapeutic admission. After identifying selected and specific stroke biomarkers as potential target analytes for the biosensor platforms development, several optical biosensor platforms technologies were considered and compared.

As previously discussed, the second important focus of this thesis is to develop a multiplex and quantitative point-of-care biosensor platform for stroke. In this thesis, four optical biosensor platforms were considered and examined to answer this need (Figure 3.1). Two paper-based sensing platforms were examined, based on colorimetric and chemiluminescent signals detection, because of their main advantages for multiplex detection. Moreover, two chip-based (gold-silver and silicon dioxide chips) sensing platforms were examined, based on surface plasmon resonance sensing technology, because of their main advantage for sensitive detection. These four biosensors platforms were selected due to their strong advantages for multiplex, quantitative and sensitive detection. All these three parameters are needed in order to answer the need for a point-of-care biosensor platform for stroke. Sensitive biosensor platform with low limit-of-detection is required in order to detect the biomarkers in the low cut-offs needs for the stroke biomarkers detection. However, each of these four biosensor platforms also exhibit a problematic disadvantage, which must be overcome. Chemiluminescent and colorimetric paper-based biosensor platforms usually exhibit very low detection sensitivity. And, surface plasmon resonance biosensor platforms are often bulky and not user friendly, as well as require challenging data acquisition, which previously made this technology less attractive as point-of-care biosensor platform.

Figure 3.1. Multiplex & Quantitative Point-of-Care Biosensors Platforms.

In this thesis, several experimental methodologies were examined in order to overcome the disadvantages of each tested biosensor platform. First, in order to overcome the disadvantage for sensitive detection of the chemiluminescent and colorimetric paper-based biosensor platform, three different approaches were examined. Which included novel engineering of different aspects of the paper-based biosensor platform. Firstly, a dissolvable film made from PVA was examined for its use in controlling the sample flow, both in time as well as in the critical areas of the biosensor platform. The film was integrated into the paper-based biosensor platform after the conjugation pad and blocking pad, and before the signal pad for detection. Thus, to increase the exposure time between immunoreagents in order to increase detection sensitivity and reduce background noise (increase specificity by reducing false-positive signal). Moreover, different membranes were screened and compared in terms of their material, structure, and performance in paper-based biosensor platform. Selection of a specific membrane type over another allow the control of sample flow and the direction of antibodies flow within the paper-based biosensor platform. This control can increase the detection sensitivity by directing the antibodies into the area of choice for signal acquisition. Lastly, colorimetric signal amplification was also examined by enhancing the colorimetric signal using a specific stopping solution to the chromogenic substrate used in the paper-based biosensor platform. Moreover, in order to overcome the disadvantage for ease of use as point-of-care platform of the surface-plasmon-resonance biosensor platform, specific functionalized chips from gold, silver and silicon dioxide were developed for use in a miniaturized SPR system. The miniaturized SPR system allows for rapid on-site detection, with user-friendly signal acquisition, while still making use of the great advantages of SPR technology for highly sensitive detection of the stroke biomarkers.

3.2 Biomarkers

3.2.1 Study Design and Participants

Between January and December 2017, consecutive patients with ischemic stroke and TIA who presented within 3-days from onset of stroke symptoms from the Stroke Unit at the National University Hospital (NUH), Singapore, were recruited (Figure 3.2). Diagnosis of ischemic stroke was made based on a corroborative history-taking, neurological assessment, and brain neuroimaging (CT and/or MRI). Stroke severity was determined using NIHSS. Risk factors, including diabetes mellitus, hypertension, hyperlipidemia, prior stroke, coronary artery disease, atrial fibrillation, peripheral vascular disease, chronic renal disease and cigarette smoking, were obtained from each patient. Angiography (ultrasonography, CT or MRI), echocardiogram and 24-hour electrocardiogram investigations were performed to investigate stroke mechanisms and, on the basis of these results, patients were classified using the TOAST criteria into cardioembolism, atherosclerosis, lacunar and other determined and undetermined causes ^[6]. In the “cardioembolism subtype”, the presence of a high- or medium-risk cardiac source of embolism is necessary while, in the “atherosclerosis subtype”, a stenosis > 50% of an appropriate intracranial or extracranial artery should be demonstrated. “Lacunar subtype” requires a traditional clinical lacunar syndrome and infarction with a diameter < 1.5cm, while “strokes of undetermined etiology” include patients with 2 or more causes identified, those patients with a negative evaluation and patients with incomplete evaluation. The TOAST criteria was used instead of the ASCO and CCS to allow comparisons of our findings with previous studies; noteworthy, both ASCO and CCS have good-to-excellent agreement with TOAST ^[7]. Patients < 21 years old, with pregnancy, intracranial hemorrhage, active cancer, autoimmune diseases and not willing to provide blood samples were excluded. As part of the stroke protocol, patients were routinely screened for new-onset AF by inpatient telemetry, Holter and implantable loop recorder; the extent of search is not standardized and vary according to clinical suspicion and patient suitability for anticoagulation. The study protocol was approved by the Domain-Specific Review Board, National Healthcare Group, and all subjects provided written informed consent prior to their study participation.

Figure 3.2. Participant Recruitment.

Patients were recruited between January and December 2017, consecutive patients with ischemic stroke and TIA who presented within 3-days from onset of stroke symptoms from the Stroke Unit at the National University Hospital (NUH), Singapore.

3.2.2 Biomarkers Measurement in Clinical Samples

Blood was drawn into EDTA tubes and centrifuged at 1800 rpm for 15 minutes. Plasma was stored at -80°C . Plasma levels of NT-proBNP, IL-6, S100 β , NSE, cortisol, vitamin D and insulin were measured using Roche Cobas e-411 analyzer, whereas D-dimer, uric acid and albumin levels were measured using a Roche c-111 analyzer. All reagents used for the measurement were purchased from Roche: NT-proBNP (proBNP II, Cat. 04842464), IL-6 (IL-6, Cat. 05109442), S100 β (S100, Cat. 03175243), NSE (NSE, Cat. 12133113), D-dimer (D-D12 Gen.2, Cat. 05077753), albumin (ALB2 Gen.2, Cat. 04657357), cortisol (cortisol, Cat.11875116), vitamin D (Vitamin D total, Cat. 05894913), uric acid (UA2 Gen.2, Cat. 04657608) and insulin (insulin, Cat. 12017547). Results of clinical investigations were extracted from their records: white blood cells, haemoglobin, haematocrit, platelets, MPV, neutrophils, lymphocytes, PT, INR, PTT, total cholesterol, triglycerides, LDL, HDL, AST and ALT.

3.2.3 Roche Cobas e-411 Analyzer

Plasma levels of NT-proBNP, IL-6, S100 β , NSE, cortisol, vitamin D and insulin were measured using Roche e-411 analyzer (Figure 3.3). The cobas e-411 analyzer is based on an electro-chemiluminescence (ECL) technology, which provides superior analytical performance. The detection is based on sandwich principle, and each test takes 18 minutes. A bionylated monoclonal antibody and a ruthenium complex (Tris (2, 2' -bipyridy) ruthenium (II)-complex ($\text{Ru}(\text{bpy})_3^{2+}$) labelled monoclonal antibody, form a sandwich complex. Then streptavidin-coated microparticles binds the complex to the solid phase via biotin-streptavidin interaction. Later, the microparticles are magnetically captured onto the electrode surface. Voltage application induce chemiluminescent emission that is then measured by a photomultiplier [8].

3.2.4 Roche Cobas c-111 Analyzer

Plasma levels of D-dimer, uric acid and albumin were measured using Roche c-111 analyzer (Figure 3.3). The cobas c-111 analyzer is based on photometric testing for clinical chemistry and homogeneous immunoassays. The D-dimer immunoassay is based on particle enhanced immunoturbidimetric assay. Latex particles are coated with monoclonal D-dimer antibody, the change of absorbance is tested with time. The albumin immunoassay is based on colorimetric assay. At pH=4.1, albumin binds to bromcresol green (BCG) forming a blue-green complex, the change in color intensity is tested. The uric acid immunoassay is based on an enzymatic colorimetric test. Uricase cleaves uric acid to form allantoin and hydrogen peroxide. In the presence of peroxidase, 4-aminophenazone is oxidized by hydrogen peroxide to a quinone-diimine dye. The red color intensity of the quinone-diimine formed is directly proportional to the uric acid concentration and is determined by measuring the increase in absorbance [9].

Figure 3.3. Roche Cobas e-411 and c-111 Analyzers.

3.2.5 Statistical and Data-Mining Analysis

Continuous data are reported as mean (with standard deviation) or median (with interquartile range) and categorical data are summarized as proportions. Normality of continuous variables was tested by Shapiro-Wilk test and probability-probability (P-P) plots. Demographic, clinical, laboratory and biomarker parameters were compared across the different stroke mechanisms (cardioembolism, atherosclerosis, lacunar and undetermined causes). Differences between categorical variables were assessed by chi-squared test (if cell frequency < 5 , Fisher's exact test was used) and Kruskal-Wallis test for continuous variables. The methodology by Barrio *et al.* was followed in order to identify optimal cut-off values for continuous markers using receiver operating characteristic (ROC) analysis that were subsequently used in the logistic regression analysis ^[4]. Then, the univariable and multivariable logistic regression models were fitted in order to identify variables associated with cardioembolism. Predictors from the univariate analysis with $p < 0.1$ were included in multivariable regression using backward stepwise logistic regression model to identify useful markers ^[5]. The odds ratios (OR) were calculated and reported with their corresponding 95% confidence intervals (CI). The assessment for possible interactions between study variables and outcome was also conducted and reported in the regression table if its p-value was found to be less than 0.1. The goodness-of-fit for the multivariable model was assessed by overall fit, discrimination (by C-statistic), calibration (by Hosmer-Lemeshow goodness-of-fit test) and classification (by observed cell frequencies). All statistical analysis was performed using SPSS version 22 (IBM Corp, Armonk, NY, USA)

and Stata version 13 (StataCorp LLC, TX, USA). A two-sided p-value < 0.05 was considered as statistically significant.

3.2.6 Data Type Definition and Analysis Determination

Firstly, the clinical biomarkers data was defined. There are four different data types: nominal, ordinal, interval and ratio. Nominal data refers to categorically discrete data, such as names. Ordinal data refers to quantities that have a natural ordering, such as order of people place in a line, without the certainty whether the intervals between each value are equal. Interval data refers to ordinal data with equal intervals. Ratio data refers to interval data with a natural zero point, such as time. These terms are used to describe types of data and by some to dictate the appropriate statistical test to use. The clinical biomarkers data was defined as ordinal data type. Median and mode (vs. mean) values are preferred in order to report this data type. In addition, an important analysis determination is to distinguish between parametric vs. non-parametric analysis. Parametric test assume that the data is normally distributed. Whereas, non-parametric test does not assume that the data follow a specific distribution. The clinical biomarkers data is not normally distributed, therefore non-parametric analysis was used. The non-parametric tests includes the following: Wilcoxon rank-sum test (compare means between two independent groups); Wilcoxon signed-rank test (compare two measurement from the same group); Kruskal-Wallis test (compare means between three or more independent groups) and spearman's rank correlation (estimate the degree of association between two groups) ^[10].

3.2.7 Receiver Operating Characteristic (ROC) Analysis

The ROC analysis is a fundamental tool for the evaluation of a diagnostic test. It results in a ROC curve and a complete sensitivity/specificity report. In a ROC curve the true positive rate (sensitivity) is plotted as a function of the false positive rate (100-specificity) for different variable cut-offs. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. The area under the ROC curve (AUC) is a measure of how well a parameter can distinguish between two diagnostic groups. The

significance level (p-value) is the probability that the observed sample area under the ROC curve is found, when in fact the true (population) area under the ROC curve is 0.5 (null hypothesis: area = 0.5). If p-value is small ($p\text{-value} < 0.05$) then it can be concluded that the area under the ROC curve is significantly different from 0.5 and that therefore there is evidence that the laboratory test does have an ability to distinguish between the two groups. The ROC analysis was used in order to identify and characterize the biomarkers ability to classify the mechanism of stroke patients ^[11].

3.2.8 Logistic Regression Analysis

Logistic regression is a widely used multivariable modeling method of dichotomous outcomes. It is used in order to analyze the effect of several predictor variables on an outcome variable. Adjustments for confounding factors can also be made. The standard logistic regression model is applicable only to binary (yes/no) outcome variables ^[12]. The model results in a predicted value, which represents the outcome variable through a sum of values that are the multiplication of each variable with its coefficient. The model can be used in order to predict an outcome variable for a panel of predictor variables. Moreover, it can be used to understand further the analyzed data through identifying the relative contribution and influence of each predictor variable to the outcome variable ^[13]. Several parameters must be carefully examined in the process of logistic regression modelling. Firstly, there must be sufficient observations per predictor variable. The higher events per variable, the greater reliability of the regression coefficients. In addition, there is a need to test for correlation between the predictor variables. If two predictor variables are highly correlated with each other, there might be a problem in their estimated contributions as reported by their regression coefficients. It might even influence the contribution of other predictor variables, and therefore on the entire regression model. Moreover, a fitting process must be conducted (forward, backward or best-fit) in order to include only the predictor variables with the most significant contribution to the outcome variable. Lastly, statistical tests of significance and goodness-of-fit measures must be calculated, in order to optimize the model fit to the tested data. These measures give indication to how well the model matches to the observed data values ^[14].

3.3 Colorimetric & Chemiluminescent Paper-Based Biosensors

3.3.1 PolyJet 3D Printing

The different biosensor platforms prototypes were 3D printed using a PolyJet 3D printer. PolyJet 3D printing is well known for smooth surfaces, fine precision and diverse material properties. The principle is similar to inkjet document printing only that instead of jetting drops of ink onto paper, the print head jets microscopic layers of liquid photopolymer onto a build tray and instantly cures them with UV light ^[15]. The fine layers build up to create a prototype or production part. Along with the selected model material, the 3D printer also jets a gel-like support material designed to uphold overhangs. When printing is done, the nontoxic support material is easily removed with a water jet. Models can be handled and used immediately, without additional post-curing. With its astonishingly realistic aesthetics and ability to deliver special properties such as transparency, flexibility and even biocompatibility, PolyJet 3D printing offers a competitive edge in products prototyping, precision tooling and specialized production parts. The PolyJet 3D printer used was Stratasys Eden260V (Figure 3.4), which can 3D print 15 different types of materials (rigid and flexible), producing models up to 255 x 252 x 200 mm ^[16].

Figure 3.4. PolyJet 3D Printing.

The PolyJet 3D printer Stratasys Eden260V, which can 3D print 15 different types of materials (rigid and flexible), producing models up to 255 x 252 x 200 mm ^[16]

3.3.3 Spectrophotometer

The colorimetric signal in the development of the paper-based biosensor platform was detected by a spectrophotometer. A spectrophotometer detects the solution absorbance by passing a light in a specific wavelength. The sequence of events in a spectrophotometer firstly starts with the shone of the light source into a monochromator, diffracted into a rainbow, and split into two beams. It is then scanned through the sample and the reference solutions. Fractions of the incident wavelengths are transmitted through, or reflected from, the sample and the reference. The resultant light strikes the photodetector device, which compares the relative intensity of the two beams. Electronic circuits convert the relative currents into linear transmission percentages and/or absorbance/concentration values. The spectrophotometer used was TECAN Infinite M200 PRO (Tecan Trading AG, Seestrasse 103, 8708 Männedorf, Switzerland) ^[17].

Figure 3.5. Spectrophotometer.

The spectrophotometer TECAN Infinite M200 PRO ^[17] and general mechanism illustration.

3.3.4 Luminometer

The chemiluminescent signal in the development of the paper-based biosensor platform was detected by a Luminometer. The chemiluminescent signal is a form of light (form of energy). To create light, another form of energy must be supplied. There are two common ways for this to occur, incandescence and luminescence. Incandescence refers to light from heat energy. While, luminescence, "cold light", can be emitted at normal and lower temperatures. In luminescence, an energy source kicks an electron of an atom out of its lowest energy "ground" state into a higher energy "excited" state; then the electron returns the energy in the form of light so it can fall back to its "ground" state. With few exceptions, the excitation energy is always greater than the energy (wavelength, color) of the emitted light. Chemiluminescence is a form of luminescence where the energy is supplied by chemical reactions. In the Luminometer, the chemiluminescent signal is detected with a photomultiplier tube (PMT). PMT is useful for light detection of weak signals, it is a photoemissive device in which the absorption of a photon results in the emission of an electron. The detectors work by amplifying the electrons generated by a photocathode exposed to a photon flux. A photocathode is a negatively charged electrode that is coated with a photosensitive compound. When it is struck by a quantum of light (photon), the absorbed energy causes electron emission due to the photoelectric effect. The Luminometer used was Luminoskan Ascent Luminometer (Thermo Fisher Scientific, Waltham, MA, USA) ^[18].

Figure 3.6. Luminometer.

Luminoskan Ascent Luminometer (Thermo Fisher Scientific, Waltham, MA, USA) ^[18]. 1) Light source is a quartz-halogen lamp. 2) Excitation filter. 3) Lamp reference detector. 4) Excitation light beam (normal 3mm diameter vs. small 1.5 mm). 5) Light shield to avoid cross measurement. 6) Emission filter. 7) Photomultiplier tube.

3.4 Surface Plasmon Resonance Chip-Based Biosensor

3.4.1 Equipment

PhotonicSys SPR H5 (Figure 3.7) was purchased from PhotonicSys (Neveh Shalom-Wahat Alsalam, Israel; www.photonicsys.com). Peristaltic pump Masterflex L/S was purchased from Cole-Parmer (625 East Bunker Ct Vernon Hills, IL 60061 United States). Ultrasonic Cleaner SONICA model MH was purchased from Soltec (Soltec S.r.l. via Castelbarco, 17 - 20136 Milano, Italy). Field Emission Scanning Electron Microscope (FE-SEM), model: Supra 55, was purchased from Carl Zeiss (Oberkochen, Germany). Balance was purchased from Mettler Toledo (Columbus, Ohio, USA). Binder oven was purchased from Gaia Science Pte Ltd (102E Pasir Panjang Rd, Singapore 118529).

3.4.2 Materials

3-aminopropyltriethoxysilane, 99% (APTES) (SKU 440140), glutaraldehyde, 25% in H₂O (SKU G5882), phosphate buffer saline (PBS, pH 7.4) (SKU P4417), Tween 20 (SKU P7949), bovine serum albumin (BSA) (SKU 7030), 2-(N-Morpholino) ethanesulfonic acid (MES) sodium salt (SKU M5057), ethanolamine (EA) ($\geq 99.0\%$) (SKU 398136), 11-Mercaptoundecanoic (MUA) acid, 95% (SKU 450561), absolute ethanol (SKU 02856), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (SKU E1769), N-Hydroxysuccinimide, 98% (NHS) (SKU 130672) were purchased from Sigma-Aldrich. Immunoreagents: NT-proBNP peptide (CAT#orb81959), Anti-NT-proBNP 24E11 Antibody (CAT#orb79568) and Anti-NT-proBNP 15F11 Antibody (CAT#orb79568) were purchased from Biorbyt. S100 β protein (CAT#8S9b), Anti-S100 β 6G1 Antibody (CAT#4S37) and Anti-S100 β 8B10 Antibody (CAT#4S37) were purchased from HyTest.

SPR Substrate: SPR silver-gold substrates which we refer to them simply as “gold-chips” were purchased from PhotonicSys.

Figure 3.7. Point-of-care Surface Plasmon Resonance Biosensor.

The system is 5 cm tall and weighs 1 kg. It currently handles two flow micro-channels, but the number of channels can be expanded to enable the monitoring of several analytes simultaneously.

3.4.3 Porcine Plasma

Porcine plasma was used as a medium to mimic the sample environment of human plasma, which is the preferred sample type for diagnostic testing of NT-proBNP and S100 β . Porcine whole blood was purchased from Primary Industries Pte. Ltd. (Singapore), with an official permit from Singapore Agri-Food & Veterinary Authority (AVA). To prevent blood coagulation before plasma extraction, heparin solution was prepared by dissolving 100 mg heparin sodium salt in 20 mL cold 0.1 M Krebs-Ringer Phosphate buffer (pH 7.3) and then mixed with 1 L of the blood. Once collected, the blood was aliquoted to 50-mL falcon tubes and centrifuged for 30 min at 400 x g. Subsequently, the resulting plasma was purified once

again with the same separation condition so as to remove any remains of red blood cells. For storage, the aliquoted porcine plasma stocks were frozen at -80°C , and whenever needed, one aliquot was thawed in a 37°C -incubator for at least 30 min. Thereafter, various concentrations of NT-proBNP and S100 β were spiked into the porcine plasma for biosensor validation.

3.4.4 Biomarker Measurement

The surface plasmon resonance biosensor setup is shown in Figure 3.8. Firstly, the SPR chip were cleaned and then functionalized with the bio-specific capture entity (antibody). Thereafter, specific biomarker detection was done by passing different concentration of analyte (biomarkers NT-proBNP or S100 β) over the SPR chip. The biomarkers were either diluted in DI water or in spiked plasma samples. Lastly, the changes in RI value were recorded and analysed.

Figure 3.8. Surface Plasmon Resonance Biosensor System Setup.

(1) PhotonicSys SPR H5; (2) peristaltic pump; (3) source; (4) drain; (5) detection software. Illustration of SPR System Setup. The SPR system is connected to an LD power source. In addition, a camera was connected to the computer for signal acquisition through a customized software, for signal translation. The system is also connected to a peristaltic pump, via a first tube to the source solution and a second tube to the drain solution.

3.4.5 Data Analysis

Data was collected in Microsoft Excel and then analyzed using SPSS 23.0 (IBM SPSS Statistics, version 23). Differences in the pixel and RI value were analyzed by descriptive analysis. Results are presented as the median value 50% percentile with the corresponding interquartile range (IQR) of 25%-75% percentiles. The limit-of-detection (LOD) for each measurement was determined based on the sensitivity and the average standard-deviation (SD), according to the following equation:

$$\text{Limit - Of - Detection (LOD)} = \frac{(k \times SD)}{m}$$

Where k is the confidence level (k = 3; 98.3% confidence level), SD (refractive index units (RIU)) is the average SD for each specific measurement and m (RIU / (ng/mL)) is the calibration sensitivity (the slope of the linear plot for each specific measurement).

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Chapter 4. Biomarkers to Elucidate Stroke Mechanisms

Accumulating data suggest a role of blood-based biomarkers in stroke management. The potential applications of multiple blood-based biomarkers were investigated, implicated in stroke pathogenesis to etiologically classify stroke patients and direct the search for cardioembolic risk factors. In this study, consecutive patients with acute ischemic stroke admitted at the National University Hospital, Singapore, were recruited. Levels of NT-proBNP, D-dimer, S100 β , NSE, vitamin D, cortisol, interleukin-6, insulin, uric acid and albumin were compared between stroke mechanisms (using the TOAST criteria) and their ability to identify patients at high-risk of new-onset atrial fibrillation and to distinguish between cardioembolism and large artery disease (LAD) mechanisms. 515 patients with ischemic stroke (mean age, 61 years; 71% men) were enrolled. The combination of two laboratory parameters (total cholesterol $\leq$ 169 mg/dl and triglycerides $\leq$ 44.5 mg/dl) and two biomarkers (NT-proBNP $\geq$ 507 pg/ml, S100 β $\geq$ 65 pg/ml) correctly identified cardioembolism from LAD in ischemic stroke patients with NPV 86%, PPV 78%, sensitivity 73%, specificity 89% and AUC 87%. A panel of blood-based biomarkers centering on NT-proBNP may expedite and direct the search for cardioembolic risk factors.

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*This section submitted substantially as Dorin Harpaz, Ram Bajpai, Geelyn J. L. Ng, Michael Soljak, Robert S. Marks, Christine Cheung, Thiruma Valavan Arumugam, Amy M.L. Quek, Alfred Iing Yoong Tok and Raymond Chee Seong Seet, 'Blood Biomarkers Predict New Onset Atrial Fibrillation and Cardioembolism in Ischemic Stroke'.

4.1 Introduction

Epidemiology data estimate that 1 in 3 ischemic stroke patients will develop another stroke in their lifetime ^[1]. The ability to mitigate the risk of a stroke recurrence hinges considerably on whether the correct mechanism of stroke could be ascertained and whether patients are treated with the correct antithrombotic agent; patients with a cardioembolic stroke etiology derive a net benefit from anticoagulation while patients with large artery disease and lacunar stroke mechanisms benefit from antiplatelet treatment ^[1-3]. The task of elucidating a stroke etiology often entails a careful interrogation of risk factors through an extensive battery of investigations that are performed hours or days after a stroke ^[1]. A lack of thoroughness in organizing these investigations could inadvertently result in an incomplete set of evidence to inform stroke etiology, rendering the etiology to be undetermined ^[1, 2]. On the contrary, concerns have been raised on the risk of over-investigation, especially given a wider availability of investigational modalities such as cardiac rhythm monitoring, arterial-wall/plaque imaging, transesophageal echocardiogram and MRI-based cardiac morphology studies ^[1]. As these investigations are costly and are not without risks ^[4-6], there are emergent calls to rationalize the number of investigations an ischemic stroke patient undergoes in the pursuit of etiological risk factors.

Existing methods of patient selection for further investigations center around the use of clinical predictors ^[7]. In a systematic review, the yield of prolonged cardiac rhythm investigations increases substantially when additional patient selection criteria are applied, doubling from 5.3% to 10.9% ^[7]. Despite this increase, the yield of these investigations remains suboptimal as the etiology of stroke continues to be undetermined in close to a third of stroke patients ^[1, 2]. It is unclear whether patients excluded by these clinical criteria harboured undiagnosed occult risk factors and whether a longer duration of cardiac rhythm monitoring could further increase the detection yield. Establishing an objective set of criteria is important to ensure only suitable patients are subjected to an extended set of investigations. Increasingly, researchers have turned to blood markers to pursue biological signatures of cardioembolic stroke that could serve as gate-keepers to these investigational resources ^[8-15]. By interrogating protein substrates of the heart, brain and vasculature,

accumulating evidence suggest that several of these blood markers could be incorporated in routine clinical workflow aimed at triaging patients according to their risk of cardioembolism ^[13]. More recent studies revealed unique gene and microRNA signatures of cardioembolic stroke, adding to the growing conviction that blood biomarkers could indeed provide important circumstantial evidence of cardioembolism ^[16-18].

A pre-requisite for clinical use, however, is external validation; previous biomarker studies are mostly confined to small patient cohorts, incorporating single biomarker measurements and use of biomarkers that are accessible only in research laboratories. Few studies have simultaneously measured biomarkers reported in the literature in a single cohort or incorporated existing parameters (clinical and laboratory) when verifying the performance of these biomarkers to inform stroke etiology. By examining key targets of stroke pathogenesis, a panel was identified of previously reported biomarkers involved in glial/neuronal death, inflammation, cardiac function, haemostasis, oxidative damage and neuroendocrine responses, and selected only biomarkers that are accessible in most clinical laboratories for clinical applications ^[13, 14]. Selected biomarkers, which include NT-pro-brain derived peptides (NT-proBNP), D-dimer, S100 β , neuron-specific enolase (NSE), vitamin D, cortisol, interleukin-6, insulin, uric acid and albumin, were examined for their ability to identify ischemic stroke patients at high-risk of new-onset AF and cardioembolism.

4.2 Results and Discussion

4.2.1 Clinical and Biomarker Characteristics

515 ischemic stroke patients were recruited, forming the study cohort. Median age of the study cohort was 61 (IQR 53-71) years, 363 were men (71%) and 379 were Chinese (74%). The distribution of risk factors is summarized in Table 4.1; 32 (6%) patients had pre-existing atrial fibrillation. Following ECG monitoring, a further 44 patients were diagnosed with new-onset paroxysmal AF using inpatient telemetry (n = 30), Holter (n = 8) and implantable loop recorder (n = 6); their stroke mechanisms were subsequently reclassified from

undetermined to cardioembolism. Overall, 14.5% patients had cardioembolism, 25.4% with LAD, 47.9% with lacunar stroke and 12.0% with undetermined stroke etiology (Table 4.1). Compared with LAD and lacunar stroke subtypes, patients with cardioembolism were older, had a higher frequency of ischemic heart disease and pre-existing AF, elevated levels of INR and AST, but lower levels of lymphocytes, total cholesterol, triglycerides and low-density lipoprotein. Median time of blood sampling from stroke onset was 2 days (range, 0.5 - 3 days). Levels of NT-proBNP, S100 β , cortisol and interleukin-6 were significantly higher in patients with cardioembolism compared with the other subtypes. Noteworthy, median age in our cohort of 61 years is younger and the prevalence of diabetes mellitus is higher than in previous studies involving Caucasians, which may influence the amount of thrombogenic substrates and protein expressions in these cohorts. By contrast, this study that included a larger sample size did not observe differences in levels of D-dimer, neuron-specific enolase, vitamin D, insulin and uric acid between different stroke subtypes despite employing comparable criteria for patient selection.

4.2.2 Identifying Ischemic Stroke Patients for New-Onset Atrial Fibrillation

To examine the feasibility of biomarkers to distinguish stroke mechanisms, individual clinical and biomarker variables were compared between stroke patients with new-onset AF and a control group comprising patients with a defined stroke mechanism (LAD and lacunar stroke) (Table 4.2). Compared with controls, stroke patients with new-onset AF were older (median, 67 vs 60 years), had a greater burden of ischemic heart disease (32% vs 15%), and higher levels of INR (1.03 vs 0.97), NT-proBNP (966 vs 80 pg/mL), S100 β (87 vs 50 pg/mL), cortisol (601 vs 403 nmol/L) and interleukin-6 levels (8.10 vs 3.57 pg/mL). Contrary to previous reports, only several biomarkers (NT-proBNP, S100 β , cortisol and interleukin-6) were significantly elevated in patients with cardioembolic stroke; these biomarkers were similarly found to be elevated in patients with a high-risk of developing new-onset AF following their stroke hospitalization. Stepwise logistic regression analysis identified age $\geq$ 63 years, ischemic heart disease, total cholesterol $\leq$ 169 mg/dL, triglycerides $\leq$ 44.5 mg/dL, NT-proBNP $\geq$ 294 pg/mL, S100 β $\geq$ 64 pg/mL and cortisol $\geq$ 471 nmol/L as significant predictors of new-onset AF (Table 4.3). The combination of two

laboratory parameters (total cholesterol ≤ 169 mg/dL and triglycerides ≤ 44.5 mg/dL) and three biomarkers (NT-proBNP ≥ 294 pg/mL, S100 β ≥ 64 pg/mL and cortisol ≥ 471 nmol/L) correctly identified new-onset AF with negative predictive value (NPV) 90%, positive predictive values (PPV) 73%, sensitivity 24%, specificity 95% and area under curve (AUC) 85% (Table 4.4). In this study, marked increase in circulatory cortisol is associated with cardioembolism. However, it is unclear whether the increase in cortisol is triggered by the release of glial proteins into the circulation or part of a systemic response to an acute illness. For the first time, optimal cut-offs of S100 β (≥ 64 ng/mL) and cortisol (≥ 471 nmol/L) to identify patients at high-risk of new-onset AF are proposed.

The clinical application of this biomarker-based solution was repurposed to identify a higher NT-proBNP cut-off of 294 pg/mL to identify ischemic stroke patients at high-risk of new-onset AF. Patients with pre-existing AF were excluded from this analysis since the presence of AF would imply cardioembolism and obviate the need for a biomarker solution to diagnose cardioembolism. The findings are consistent with those of a previous study by Rodriguez-Yanez *et al.* that employed a cut-off of 360 pg/mL to diagnose new-onset AF^[19]; both these studies observed NT-proBNP cut-offs that were substantially higher compared to when diagnosing cardioembolism by measuring NT-proBNP levels (60 - 140 pg/mL)^[8-11]. Despite sharing common risk factors, it is possible that ischemic stroke patients at risk of new-onset AF harbor high expressions of thrombogenic cardiac substrates prior to AF detection. Previous studies have shown P-wave terminal force in ECG lead V1 (which indicates left atrial abnormalities)^[20] and echocardiographic measures of left atrial size and contractile function have been associated with incident cardioembolic stroke independent of AF^[21]. When this combination of biomarkers is applied to patients with undetermined stroke mechanisms, 25% patients harbored high-risk signatures while the remaining 75% were classified as having low-risk of new-onset AF who may not benefit from expensive and cumbersome long-term AF monitoring.

Table 4.1. Descriptive Analysis According to Stroke Mechanisms.

	All patients (n=515, 100%)	Cardio- embolism (n=75, 14.5%)	Large artery disease (n=131, 25.4%)	Lacunar (n=247, 47.9%)	Undetermined (n=62, 12.0%)	P- value
Demographic						
Age (years)	61 (53-71)	71 (61-76) a, b	60 (52-71) ^c	59 (52-65) ^c	61 (50-74)	<0.001
Men (%)	363 (71%)	45 (60%)	92 (70%)	186 (75%)	40 (65%)	0.051
Chinese (%)	379 (74%)	54 (72%)	93 (71%)	191 (77%)	41 (66%)	0.250
Risk factors						
Hypertension (%)	345 (67%)	60 (80%)	86 (66%)	159 (64%)	40 (65%)	0.079
Hyperlipidemia (%)	242 (47%)	39 (52%)	73 (56%) ^b	106 (43%) ^a	24 (39%)	0.044
Diabetes mellitus (%)	221 (43%)	32 (43%)	65 (50%)	100 (41%)	24 (39%)	0.328
Cigarette smoker (%)	241 (47%)	25 (33%)	65 (60%)	120 (49%)	31 (50%)	0.092
Prior ischemic stroke/TIA (%)	81 (16%)	15 (20%)	25 (19%)	33 (13%)	8 (13%)	0.313
Ischemic heart disease (%)	90 (18%)	25 (33%) ^b	29 (22%) ^c	26 (11%) ^{a, c}	10 (16%)	<0.001
Pre-existing atrial fibrillation (%)	32 (6%)	31 (41%) ^{a, b}	0 ^c	0 ^c	1 (2%)	<0.001
Laboratory parameters						
Full blood count						
White cell count (x10 ⁹ /L)	8.17 (6.69-9.89)	7.71 (6.36-10.6)	8.55 (7.04-10.2) ^b	7.86 (6.54-9.26) ^a	8.67 (7.29-9.98)	0.008
Neutrophils (x 10 ⁹ /L)	5.14 (3.87-6.61)	5.26 (3.98-7.77) ^b	5.47 (4.27-7.08) ^b	4.73 (3.68-6.00) ^{a, c}	5.53 (4.21-7.54)	0.001
Lymphocytes (x 10 ⁹ /L)	1.95 (1.44-2.57)	1.73 (1.29-2.27) ^{a, b}	1.97 (1.44-2.51) ^c	2.06 (1.50-2.69) ^c	1.89 (1.43-2.48)	0.013
Haemoglobin (g/dL)	14.2 (13.0-15.4)	13.6 (12.4-15.2) ^b	14.1 (13.0-15.3)	14.5 (13.3-15.5) ^c	14.1 (12.8-15.7)	0.036
Platelets (x 10 ⁹ /L)	237 (199-281)	210 (176-259) ^{a, b}	248 (202-303) ^c	237 (201-282) ^c	252 (205-298)	0.001
Coagulation profile						
International normalized ratio	0.98 (0.95-1.04)	1.03 (0.97-1.18) ^{a, b}	0.98 (0.95-1.03) ^c	0.97 (0.93-1.01) ^c	0.97 (0.93-1.04)	<0.001
Partial thromboplastin time (sec)	28.7 (27.0-30.8)	28.7 (27.5-31.1) ^a	27.8 (25.8-30.8) ^{b, c}	29.0 (27.7-30.5) ^a	29.7 (27.3-31.4)	0.023
Lipid panel						
Total cholesterol (mg/dL)	186 (156-221)	160 (133-189) ^{a, b}	195 (157-230) ^c	188 (163-215) ^c	197 (169-239)	<0.001
Triglycerides (mg/dL)	51.2 (38.2-75.9)	34.0 (27.5-58.0) ^{a, b}	52.6 (40.2-82.6) ^c	52.6 (40.6-81.2) ^c	52.9 (38.3-76.6)	<0.001
High-density lipoprotein (mg/dL)	40.6 (34.8-49.5)	43.7 (33.9-61.2)	40.9 (35.2-47.9)	39.8 (35.2-49.1)	40.6 (32.9-48.3)	0.102
Low-density lipoprotein (mg/dL)	115 (88.3-144)	87.2 (71.5-115) ^{a, b}	121 (90.9-150) ^c	117 (93.0-140) ^c	126 (93.7-157)	<0.001
Liver panel						

Aspartate aminotransferase (U/L)	22.0 (18.0-28.0)	24.0 (20.8-31.0) ^{a, b}	22.0 (17.0-28.0) ^c	21.0 (18.0-27.8) ^c	20.0 (17.3-28.5)	0.045
Alanine transaminase (U/L)	20.0 (14.0-29.0)	17.0 (12.0-26.0) ^b	19.0 (14.0-28.0) ^b	23.0 (15.0-31.0) ^{a, c}	16.5 (13.3-21.0)	0.007
Biomarker levels						
NT-proBNP (pg/mL)	120 (44-503)	1110 (443-2893) ^{a, b}	155 (55-498) ^{b, c}	65 (27-177) ^{a, c}	147 (50-925)	<0.001
D-dimer (ng/mL)	350 (210-685)	350 (227-660)	350 (200-880)	375 (195-755)	310 (200-600)	0.970
S100 β (pg/mL)	52.0 (38.0-86.0)	87.0 (50.8-165) ^{a, b}	55.0 (40.0-88.0) ^{b, c}	47.0 (35.0-67.0) ^{a, c}	58.0 (39.0-98.0)	<0.001
Neuron-specific enolase (ng/mL)	15.1 (11.6-21.4)	15.1 (11.4-23.1)	14.8 (11.7-20.6)	14.7 (11.3-19.9)	19.1 (13.9-30.8)	0.532
Vitamin D (mg/dL)	21.5 (14.9-29.2)	20.6 (15.5-29.3)	21.3 (14.7-28.8)	21.6 (15.2-29.5)	22.3 (11.4-30.2)	0.901
Cortisol (nmol/L)	419 (268-576)	562 (341-713) ^b	433 (305-602) ^b	383 (235-495) ^{a, c}	491 (315-703)	<0.001
Interleukin-6 (pg/mL)	4.31 (2.08-10.3)	8.38 (4.21-22.4) ^{a, b}	4.70 (2.50-9.20) ^{b, c}	3.14 (1.80-6.48) ^{a, c}	5.98 (2.56-13.5)	<0.001
Insulin (pmol/L)	54.6 (32.8-98.6)	48.1 (21.4-83.5)	55.3 (34.7-100)	54.3 (35.8-99.1)	62.9 (28.1-110)	0.108
Uric acid (μ mol/L)	358 (304-437)	371 (270-448)	376 (321-455)	349 (304-430)	358 (297-406)	0.474
Albumin (g/L)	40.4 (37.6-43.4)	40.4 (37.4-43.3)	40.1 (37.5-43.0)	40.4 (38.1-43.3)	42.3 (39.5-43.9)	0.873

* All values are described as median (interquartile range) and percentages (%). P-values are assessed by chi-squared test for categorical variables and Kruskal-Wallis test for continuous variables. Superscripts “a” denotes $p < 0.05$ compared with LAD, “b” denotes $p < 0.05$ compared with lacunar, “c” denotes $p < 0.05$ compared with cardioembolism and “d” denotes $p < 0.05$ compared with undetermined group. Abbreviations: TIA = transient ischemic attack, NT-proBNP = N-terminal pro-brain natriuretic peptide.

Table 4.2. Descriptive Analysis According to New-Onset Atrial Fibrillation.

	All patients (n=422, 100%)	New-onset Atrial Fibrillation with cardioembolism (n=44, 10.4%)	LAD and Lacunar Stroke (n=378, 89.6%)	P-value
Demographic				
Age (years)	61 (53-71)	67 (57-75)	60 (52-66)	0.001
Men (%)	307 (73%)	29 (66%)	278 (74%)	0.282
Chinese (%)	315 (75%)	31 (71%)	284 (75%)	0.500
Risk factors				
Hypertension (%)	278 (66%)	33 (75%)	245 (65%)	0.177
Hyperlipidemia (%)	198 (47%)	19 (43%)	179 (47%)	0.600

Diabetes mellitus (%)	186 (44%)	21 (48%)	165 (44%)	0.606
Cigarette smoker (%)	199 (47%)	14 (32%)	185 (49%)	0.031
Prior ischemic stroke/TIA (%)	66 (16%)	8 (18%)	58 (15%)	0.624
Ischemic heart disease (%)	69 (16%)	14 (32%)	55 (15%)	0.003
Laboratory parameters				
Full Blood Count				
White cell count (x 10 ⁹ /L)	8.17 (6.69-9.89)	7.52 (6.66-10.1)	8.095 (6.68-9.78)	0.913
Neutrophils (x 10 ⁹ /L)	5.14 (3.87-6.61)	5.22 (3.74-8.75)	5.03 (3.81-6.33)	0.269
Lymphocytes (x 10 ⁹ /L)	1.95 (1.44-2.57)	1.71 (1.28-2.05)	2.05 (1.48-2.61)	0.006
Hemoglobin (g/dL)	14.2 (13.0-15.4)	13.7 (12.7-15.5)	14.4 (13.1-15.4)	0.142
Platelets (x 10 ⁹ /L)	237 (199-281)	231 (179-265)	239 (202-289)	0.106
Coagulation profile				
International normalized ratio	0.98 (0.95-1.04)	1.03 (0.97-1.11)	0.97 (0.94-1.01)	<0.001
Partial thromboplastin time (sec)	28.7 (27.0-30.8)	28.9 (27.4-31.2)	28.6 (26.9-30.5)	0.389
Lipid panel				
Total cholesterol (mg/dL)	186 (156-221)	155 (129-182)	189 (159-221)	<0.001
Triglycerides (mg/dL)	51.2 (38.2-75.9)	33.3 (28.4-56.6)	52.6 (40.22-81.2)	<0.001
High-density lipoprotein (mg/dL)	40.6 (34.8-49.5)	42.2 (32.9-60.7)	40.22 (35.2-48.3)	0.479
Low-density lipoprotein (mg/dL)	115 (88.3-144)	87.0 (71.5-114)	118 (92.0-144)	<0.001
Liver panel				
Aspartate aminotransferase (U/L)	22.0 (18.0-28.0)	23.0 (19.0-28.0)	21.0 (18.0-28.0)	0.367
Alanine transaminase (U/L)	20.0 (14.0-29.0)	18.0 (12.3-27.5)	21.0 (14.0-29.0)	0.279
Biomarkers levels				
NT-proBNP (pg/mL)	120 (43.6-503)	966 (278-2474)	80.3 (34.7-267)	<0.001
D-dimer (ng/mL)	350 (210-685)	480 (280-1060)	370 (200-760)	0.224
S100 β (pg/mL)	52.0 (38.0-86.0)	87.0 (50.3-186)	50.0 (36.0-73.0)	<0.001
Neuron specific enolase (ng/mL)	15.1 (11.6-21.4)	17.8 (11.7-23.7)	14.7 (11.4-20.4)	0.186
Vitamin D (mg/dL)	21.5 (14.9-29.2)	22.9 (16.4-30.1)	21.5 (15.1-29.3)	0.351
Cortisol (nmol/L)	419 (268-576)	601 (409-755)	403 (251-538)	<0.001
Interleukin-6 (pg/mL)	4.31 (2.07-10.33)	8.10 (3.56-21.3)	3.57 (1.92-7.47)	0.001
Insulin (pmol/L)	54.6 (32.8-98.6)	58.7 (28.7-96.7)	55.1 (35.8-99.4)	0.736
Uric acid (μ mol/L)	358 (304-437)	371 (262-456)	352 (312-440)	0.960
Albumin (g/L)	40.39 (37.6-43.4)	39.7 (37.1-43.4)	40.3 (37.7-43.2)	0.652

* All values are described as median (interquartile range) and percentages (%). P-values are assessed by chi-squared test for categorical variables and Kruskal-Wallis test for continuous variables (if cell frequency was lower than 5, Fisher's exact test was used). Abbreviations: TIA = transient ischemic attack, NT-proBNP = N-terminal pro-brain natriuretic peptide.

Table 4.3. Multivariable Logistic Regression for New-Onset Atrial Fibrillation.

Variables	Cut-off values & conditions	Univariate Predictors		Multivariable Predictors	
		Odds Ratios (95% CI)	P-values	Odds Ratios (95%CI)	P-values
Clinical					
Age (years)	≥ 63	2.49 (1.31-4.74)	0.005	2.06 (1.06-3.99)	0.032
Cigarette smoker (%)	Yes	0.49 (0.25-0.95)	0.034	0.51 (0.26-1.01)	0.054

Ischemic heart disease (%)	Yes	2.74 (1.37-5.49)	0.005	2.43 (1.18-4.99)	0.016
Laboratory					
Lymphocytes (x 10 ⁹ /L)	≤ 1.86	2.39 (1.19-4.80)	0.014	-	-
International normalized ratio	≥ 1.02	5.37 (2.55-11.33)	<0.001	-	-
Total cholesterol (mg/dL)	≤ 169	5.15 (2.52-10.49)	<0.001	3.86 (1.84-8.08)	<0.001
Triglycerides (mg/dL)	≤ 44.5	4.19 (2.09-8.41)	<0.001	2.93 (1.41-6.07)	0.004
Low-density lipoprotein (mg/dL)	≤ 106	3.49 (1.74-7.04)	<0.001	-	-
Biomarkers					
NT-proBNP (pg/mL)	≥ 294	8.60 (3.98-18.6)	<0.001	5.85 (2.62-13.1)	<0.001
S100β (pg/mL)	≥ 64	4.06 (1.95-8.44)	<0.001	2.52 (1.15-5.54)	0.021
Cortisol (nmol/L)	≥ 471	4.23 (2.04-8.75)	<0.001	3.01 (1.36-6.68)	0.007
Interleukin-6 (pg/mL)	≥ 5.39	3.05 (1.51-6.16)	0.002	-	-

* Optimal cut-off values were derived using receiver operating characteristic (ROC) analyses, and variables were subsequently reconstructed as dichotomous. All univariate predictors with p-values < 0.05 were analyzed using a stepwise backward logistic regression model and only significant predictors are presented. Data are summarized using odds ratios and 95% confidence intervals.

Table 4.4. Multivariable Models Performance for New-Onset Atrial Fibrillation.

Categories of Predictors	Chi-squared (P-values)	Probability Cut-off	Sensitivity (%)	Specificity (%)	Negative predictive values (%)	Positive predictive values (%)	Accuracy (%)	Area under curve (%)
Clinical	2.01 (0.73)	0.30	15.9	95.2	90.7	28.0	87.0	67.7
Laboratory	0 (0.97)	0.30	52.5	84.1	94.1	26.9	81.0	74.6
Biomarkers	3.03 (0.55)	0.40	38.9	94.2	93.0	43.8	88.5	82.2
Clinical + laboratory	30.3 (0.17)	0.40	10.0	98.3	90.8	40.0	89.5	78.6
Clinical + biomarkers	37.2 (0.89)	0.50	8.3	98.7	90.3	42.9	89.4	83.2
Laboratory + biomarkers	21.2 (0.73)	0.50	24.2	99.0	92.3	72.7	91.6	85.2
Clinical + laboratory + biomarkers	161 (<0.01)	0.45	33.3	98.7	93.1	73.3	92.2	85.8

* Multivariable predictors with p < 0.05 were categorized into clinical (age > 63 years, smoking and ischemic heart disease), laboratory (total cholesterol ≤ 169 mg/dL and triglycerides ≤ 44.5 mg/dL) and biomarkers (NT-proBNP ≥ 294 pg/mL, S100β ≥ 64 pg/mL and cortisol ≥ 471 nmol/L).

Table 4.5. Undetermined Stroke Reclassification.

Applying the model conditions to identify new-onset atrial fibrillation				
Condition	Yes	Total	Percentage	Chi-Squared (p-value)
Clinical Parameters				
Age ≥ 63 (Years)	28	62	45.2%	0.0138 (0.906)
Smoking (Yes)	31	62	50.0%	0.2906 (0.590)
Ischemic Heart Disease (Yes)	10	62	16.1%	0.0886 (0.766)
Laboratory Parameters				
Total Cholesterol ≤ 169 (mg/dL)	15	59	25.4%	2.9585 (0.085)
Triglycerides ≤ 44.5 (mg/dL)	22	59	37.3%	0.0209 (0.885)
Biomarkers Levels				
NT-proBNP ≥ 294 (pg/mL)	20	55	36.4%	0.2114 (0.638)
S100 ≥ 64 (pg/mL)	24	54	44.4%	0.6986 (0.403)
Cortisol ≥ 471 (nmol/L)	29	55	52.7%	4.9672 (0.026)

* Given p-value of 0.000 is less than alpha of 0.05, we reject the null hypothesis that the variables are independent. We conclude that they are dependent, that there is an association between the two variables.

4.2.3 Distinguishing Cardioembolism from Large Artery Disease

Compared with LAD, patients with cardioembolism were older (median, 71 vs 60 years), had a greater burden of hypertension (80% vs 66%) and pre-existing AF (41% vs 0%), INR (1.03 vs 0.98), AST (24 vs 22 U/L), NT-proBNP (1110 vs 155 pg/mL), S100 β (87 vs 55 pg/mL) and interleukin-6 (8.38 vs 4.70 pg/mL), but lower frequency of cigarette smoking (33% vs 60%), lymphocyte count (1.73 vs 1.97×10^9 /L), platelet count (210 vs 248×10^9 /L), total cholesterol (160 vs 195 mg/dL), triglycerides (34 vs 53 mg/dL) and low-density lipoprotein (87 vs 121 mg/dL) (Table 4.6). Stepwise logistic regression analysis identified age ≥ 67 years, total cholesterol ≥ 169 mg/dL, triglycerides ≤ 44.5 mg/dL, AST ≥ 23.0 U/L, NT-proBNP ≥ 507 pg/mL and S100 β ≥ 65.0 pg/mL as significant predictors (Table 4.7). The combination of two laboratory parameters (total cholesterol ≤ 169 mg/dL and triglycerides ≤ 44.5 mg/dL) and two biomarkers (NT-proBNP ≥ 507 pg/mL, S100 β ≥ 65 pg/mL) correctly identified cardioembolism from LAD in ischemic stroke patients with NPV 86%, PPV 78%, sensitivity 73%, specificity 89% and AUC 87% (Table 4.8).

The combination of biomarkers (NT-proBNP and S100 β) was found useful to distinguish cardioembolism from LAD. Consistent with previous studies [8, 10, 11, 22], a significant increases was observed in levels of NT-proBNP, S100 β , cortisol and interleukin-6 in patients with cardioembolic stroke. Interestingly, in a systematic review, two of the identified biomarkers (NT-proBNP and S100 β) identified through this study were found to be reliable biomarkers to aid in the diagnosis of ischemic stroke [22]. Distinguishing cardioembolism from intracranial LAD can be challenging especially in communities with a high prevalence of intracranial stenosis. Data from this study support the potential use of blood-based biomarkers (NT-proBNP ≥ 507 pg/mL and S100 $\beta \geq 65$ pg/mL) to direct patients at high-risk of cardioembolism for long-term AF monitoring and anticoagulation and those at high-risk of LAD for vessel-wall imaging and catheter angiography studies to select suitable patients for endarterectomy and carotid/intracranial stenting.

Table 4.6. Descriptive Analysis According to Cardioembolism and Large Artery Disease.

	All patients (n=206, 100%)	Cardioembolism (n=75, 15%)	Large artery disease (n = 131, 64%)	P-value
Demographic				
Age (years)	61 (53-71)	71 (61-76)	60 (52-71)	<0.001
Men (%)	137 (66.5%)	45 (60.0%)	92 (70.2%)	0.134
Chinese (%)	147 (71.4%)	54 (72.0%)	93 (71.0%)	0.878
Risk factors				
Hypertension (%)	146 (70.9%)	60 (80.0%)	86 (65.6%)	0.029
Hyperlipidemia (%)	112 (54.4%)	39 (52.0%)	73 (55.7%)	0.606
Diabetes mellitus (%)	97 (47.1%)	32 (42.7%)	65 (49.6%)	0.336
Cigarette smoker (%)	90 (43.7%)	25 (33.3%)	65 (59.6%)	0.023
Prior ischemic stroke/TIA (%)	40 (19.4%)	15 (20.0%)	25 (19.1%)	0.873
Ischemic heart disease (%)	54 (26.2%)	25 (33.3%)	29 (22.1%)	0.079
Atrial fibrillation (%)	31 (15.0%)	31 (41.3%)	0	<0.001
Laboratory parameters				
Full Blood Count				
White cell count (x 10 ⁹ /L)	8.17 (6.69-9.89)	7.71 (6.36-10.6)	8.55 (7.04-10.2)	0.366
Neutrophils (x 10 ⁹ /L)	5.14 (3.87-6.61)	5.26 (3.98-7.77)	5.47 (4.27-7.08)	0.949
Lymphocytes (x 10 ⁹ /L)	1.95 (1.44-2.57)	1.73 (1.29-2.27)	1.97 (1.44-2.51)	0.030
Hemoglobin (g/dL)	14.2 (13.0-15.4)	13.6 (12.4-15.2)	14.1 (13.0-15.3)	0.154
Platelets (x 10 ⁹ /L)	237 (199-281)	210 (176-259)	248 (202-303)	<0.001
Coagulation profile				
International normalized ratio	0.98 (0.95-1.04)	1.03 (0.97-1.18)	0.98 (0.95-1.03)	<0.001
Partial thromboplastin time (sec)	28.7 (27.0-30.8)	28.7 (27.5-31.1)	27.8 (25.8-30.8)	0.025
Lipid panel				
Total cholesterol (mg/dL)	186 (156-221)	160 (133-189)	195 (157-230)	<0.001
Triglycerides (mg/dL)	51.2 (38.2-75.9)	34.0 (27.5-58.0)	52.6 (40.2-82.6)	<0.001
High-density lipoprotein (mg/dL)	40.6 (34.8-49.5)	43.7 (33.9-61.2)	40.9 (35.2-47.9)	0.083

Low-density lipoprotein (mg/dL)	114.7 (88.3-144)	87.2 (71.5-115)	121 (90.9-150)	<0.001
Liver panel				
Aspartate aminotransferase (U/L)	22.0 (18.0-28.0)	24.0 (20.8-31.0)	22.0 (17.0-28.0)	0.030
Alanine transaminase (U/L)	20.0 (14.0-29.0)	17.0 (12.0-26.0)	19.0 (14.0-28.0)	0.241
Biomarkers levels				
NT-proBNP (pg/mL)	120 (43.6-503)	1110 (443-2893)	155 (54.9-498)	<0.001
D-dimer (ng/mL)	350 (210-685)	350 (227-660)	350 (200-880)	0.884
S100 β (pg/mL)	52.0 (38.0-86.0)	87.0 (50.8-165)	55.0 (40.0-88.0)	<0.001
Neuron specific enolase (ng/mL)	15.1 (11.6-21.4)	15.1 (11.4-23.1)	14.8 (11.7-20.6)	0.895
Vitamin D (mg/dL)	21.5 (14.9-29.2)	20.6 (15.5-29.3)	21.3 (14.7-28.8)	0.841
Cortisol (nmol/L)	419 (268-576)	562 (341-713)	433 (305-602)	0.068
Interleukin-6 (pg/mL)	4.31 (2.08-10.3)	8.38 (4.21-22.4)	4.70 (2.50-9.20)	0.003
Insulin (pmol/L)	54.6 (32.8-98.6)	48.1 (21.4-83.5)	55.3 (34.7-100)	0.054
Uric acid (μ mol/L)	358 (304-437)	371 (270-448)	376 (321-455)	0.477
Albumin (g/L)	40.4 (37.6-43.4)	40.4 (37.4-43.3)	40.1 (37.5-43.0)	0.889

* All values are described as median (interquartile range) and percentages (%). P-values are assessed by chi-squared test for categorical variables and Kruskal-Wallis test for continuous variables (if cell frequency was lower than 5, Fisher's exact test was used). Abbreviations: TIA = transient ischemic attack, NT-proBNP = N-terminal pro-brain natriuretic peptide.

Table 4.7. Multivariable Logistic Regression for Cardioembolism.

Variables	Cut-off values & conditions	Univariate Predictors			Multivariable Model	
		Odds (95% CI)	Ratios	P-values	Odds (95%CI)	Ratios
Clinical						
Age (years)	≥ 67	4.79 (2.59-8.84)	<0.001	4.79 (2.59-8.84)	<0.001	
Hypertension (%)	Yes	2.09 (1.07-4.09)	0.031	-	-	
Cigarette smoker (%)	Yes	0.51 (0.28-0.92)	0.024	-	-	
Laboratory						
Lymphocytes (x 10 ⁹ /L)	≤ 1.86	1.79 (0.97-3.27)	0.061	-	-	
Platelets (x 10 ⁹ /L)	≤ 225	2.22 (1.24-3.98)	0.007	-	-	
International normalized ratio	≥ 1.00	2.95 (1.51-5.75)	0.002	-	-	
Partial thromboplastin time (sec)	≥ 28.5	1.76 (0.90-3.45)	0.097	-	-	
Total cholesterol (mg/dL)	≤ 169	4.09 (2.17-7.72)	<0.001	3.44 (1.58-7.48)	0.002	
Triglycerides (mg/dL)	≤ 44.5	3.69 (1.96-6.92)	<0.001	3.14 (1.44-6.84)	0.004	
Low-density lipoprotein (mg/dL)	≤ 106	3.06 (1.63-5.74)	0.001	-	-	
Aspartate aminotransferase (U/L)	≥ 23.0	1.99 (1.04-3.82)	0.037	2.36 (1.12-4.95)	0.023	
Biomarkers						
NT-proBNP (pg/mL)	≥ 507	8.43 (4.14-17.2)	<0.001	8.38 (4.02-17.5)	<0.001	
S100β (pg/mL)	≥ 65.0	2.62 (1.38-5.00)	0.003	2.71 (1.30-5.65)	0.008	
Interleukin-6 (pg/mL)	≥ 6.28	2.55 (1.34-4.82)	0.004	-	-	

* Optimal cut-off values were derived using receiver operating characteristic (ROC) analyses to distinguish between groups of patients with cardioembolism and large artery disease. All univariate predictors with p-values <0.05 were analyzed using a stepwise backward logistic regression model and only significant predictors are presented. Cut-off values were determined from receiver operating characteristics curves which were subsequently reconstructed as dichotomous variables. Data are summarized using odds ratios and 95% confidence intervals.

Table 4.8. Multivariable Models Performance for Cardioembolism.

Categories of Predictors	Chi-squared (P-values)	Probability Cut-off	Sensitivity (%)	Specificity (%)	Negative predictive values (%)	Positive predictive values (%)	Accuracy (%)	Area under curve (%)
Clinical	0	0.5	69.3	67.9	79.5	55.3	68.5	68.6
Laboratory	0.50 (0.97)	0.5	38.2	92.6	74.6	72.4	74.2	76.8
Biomarkers	2.52 (0.11)	0.5	72.6	75.7	82.7	63.4	74.6	79.3
Clinical + laboratory	19.23 (0.06)	0.6	49.1	90.7	77.8	72.9	76.7	82.0
Clinical + biomarkers	4.93 (0.29)	0.5	64.5	84.1	80.4	70.2	76.9	80.8
Laboratory + biomarkers	30.59 (0.20)	0.5	72.9	89.0	86.2	77.8	83.5	86.6
Clinical + laboratory + biomarkers	57.55 (0.05)	0.5	68.8	90.1	84.5	78.6	82.7	87.8

* Multivariable predictors with p<0.05 were categorized into clinical (age > 67 years), laboratory (total cholesterol ≤ 169 mg/dL, triglycerides ≤ 44.5 mg/dL and AST ≥ 23 U/L) and biomarkers (NT-proBNP ≥ 507 pg/mL and S100β ≥ 65 pg/mL).

4.3 Conclusions

A wider availability of safer and more efficacious anticoagulants has driven the search of occult AF in ischemic stroke patients who might be at risk of cardioembolism ^[1]. In this study, multiple blood biomarkers were explored and identified a panel of biomarkers capable of triaging patients according to their risk of new-onset AF and cardioembolism. Importantly, the blood biomarkers were superior to clinical methods of selecting patients,

suggesting the presence of a biological signature of cardioembolism that is previously elusive to existing methods of ascertaining stroke etiology. This study is the first to put together a panel of biomarkers selected through a meticulous search of the literature aimed at providing important data to translate advances in biomarker research to clinical practice.

Contrary to findings of previous studies ^[8-12], not all of the previously reported biomarkers were found to be differentially expressed in patients with cardioembolic stroke. For example, biomarkers such as D-dimer, neuron-specific enolase, vitamin D, insulin, uric acid and albumin were unchanged, while the extent of rise in NT-proBNP was highest compared with other biomarkers such as S100 β , cortisol and interleukin-6. Brain-type natriuretic peptide (BNP) is synthesized mainly in the ventricular myocardial as a prehormone that is subsequently cleaved into C- and N-terminal fragments upon release into the circulation following myocardial wall stress ^[23]. NT-proBNP has a half-life of 120 mins, which is 6 times longer than BNP, making it a reliable biomarker for commercial development using fully automated platforms with proven excellent test precision (AxSYM BNP, Abbot; ADVIA centaur, Bayer; Elecsys, Roche Diagnostics) ^[23]. In this study, measurement of NT-proBNP was found to signal an increased risk of new-onset AF and capable of deciphering the origins of a thrombus, either from cardioembolism or an intracranial large artery disease, where a cut-off value of ≥ 294 pg/mL predicted new-onset AF while a higher cut-off ≥ 507 pg/mL identified cardioembolism. Differences in NT-proBNP cut-off values could indicate relate to the chronicity of AF; patients with new-onset AF have lesser myocardial stress compared to those with cardioembolism who harboured a greater burden of AF. The cut-off values of NT-proBNP observed in the current study to identify new-onset AF are comparable with another Caucasian cohort which reported a cut-off value of 360 pg/mL ^[19], suggesting generalizability of the findings across different populations.

Glial proteins (e.g. S100 β) are typically confined to brain tissues and their release into the circulation occurs following breakdown in the blood-brain barrier after a stroke; their levels therefore correlate closely with the extent of stroke severity and infarct size ^[13]. In this study, there were no observed differences in S100 β cut-offs to distinguish patients with new-onset

AF and cardioembolism (both approximately ≥ 65 pg/mL), suggesting that clots associated with AF result in tissue damage that are extensive yet comparable, regardless whether the AF is new-onset or of the chronic persistent type. The rise in cortisol levels in ischemic stroke patients with new-onset AF suggest a profound amount of circulatory stress during the early stages of AF pathogenesis; it remains unclear, however, whether the rise in cortisol level is a cause or consequence of new-onset AF and whether excessive amounts of circulatory stress could indeed trigger the onset of AF in high-risk individuals. Elucidating the etiology of an acute thrombus poses a conundrum to clinicians especially when the appearance of a thrombus arising from a cardiac or intracranial LAD etiology may appear indistinguishable; thrombus from these different etiologies may both present as a hyperdense artery on plain CT brain or as a filling defect on angiographic studies ^[1, 13]. Intracranial stenosis is prevalent in Asians where close to 50% of patients with ischemic stroke harbour various degrees of intracranial stenosis, compared to 8% in Northern America ^[24]. Ascertaining the likelihood of a cardioembolic stroke etiology is important as this could guide critical decisions on the timing and duration of cardiac rhythm monitoring and to direct patients to anticoagulation for secondary stroke prevention. By contrast, patients with intracranial LAD could be subjected to more appropriate investigations to elucidate the extent of arterial narrowing, arterial wall/plaque morphologies and adequacy of collateral blood supply ^[25].

Several limitations merit mention. First, only blood biomarkers that are accessible to most clinicians through their hospital laboratories were included in order to triage patients into risk categories, this may inadvertently exclude promising research biomarkers that have yet been included in most commercial analytical platforms. Second, the standardize of the duration of cardiac monitoring was not conducted in each patient and relied on clinical reasoning of attending neurologists to decide on the intensity of AF search. Although this closely mirrors clinical practice, there is a possibility of missed detection of AF in a proportion of patients had the duration of monitoring been extended beyond what is routinely performed ^[7]. Third, as the study focus was to validate the performance of blood biomarkers reported in other studies, the findings are also subjected to external validation in independent cohorts of stroke patients. To conclude, blood biomarkers could provide

information on stroke etiology and direct the search of occult risk factors that could significantly impact on treatment for secondary stroke prevention. Data from this study could have immediate clinical implications to provide an objective method to increase the detection yield of investigations aimed at elucidating stroke etiology using a panel of blood biomarkers that are accessible in most clinical laboratories in an effort to beat the risk of cardiovascular recurrence in stroke patients.

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Chapter 5.   **Biomarkers with Additional Value in Stroke Management**

Accumulating data suggest a role of blood-based biomarkers in stroke management. The potential applications of multiple blood-based biomarkers were investigated, to examine additional value in stroke management. Different aspects in stroke management were examined, which included stroke diagnosis, severity, mortality, tPA admission and ischemic stroke vs. TIA classification. Consecutive patients with acute ischemic stroke admitted at the National University Hospital, Singapore, were recruited. Levels of NT-pro-brain derived peptides (NT-proBNP), D-dimer, S100 β , neuron-specific enolase (NSE), vitamin D, cortisol, interleukin-6, insulin, uric acid and albumin were compared. 516 patients with ischemic stroke were enrolled. A panel of blood-based biomarkers centering on NT-proBNP may expedite and direct the search for improved stroke management. Further studies are needed to externally validate these findings.

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*This section is under preparation for submission.

5.1 Introduction

Recent studies have showed great value in blood biomarkers for stroke care, in order to identify biological pathways and signatures ^[1-8]. By interrogating protein substrates of the heart, brain and vasculature, accumulating evidence suggest that several of these blood biomarkers could be incorporated in routine clinical workflow aimed at triaging patients ^[6]. Moreover, additional studies revealed unique gene and microRNA signatures of cardioembolic stroke, adding to the growing conviction that blood biomarkers could indeed provide important circumstantial evidence of cardioembolism ^[9-11]. A prerequisite for clinical use, however, is external validation; previous biomarker studies are mostly confined to small patient cohorts, incorporating single biomarker measurements and use of biomarkers that are accessible only in research laboratories. Few studies have simultaneously measured biomarkers reported in the literature in a single cohort or incorporated existing parameters (clinical and laboratory) when verifying the performance of these biomarkers. By examining key targets of stroke pathogenesis, a panel was identified of previously reported biomarkers involved in glial/neuronal death, inflammation, cardiac function, haemostasis, oxidative damage and neuroendocrine responses, and selected only biomarkers that are accessible in most clinical laboratories for clinical applications ^[6, 7]. Selected biomarkers, which include NT-pro-brain derived peptides (NT-proBNP), D-dimer, S100 β , neuron-specific enolase (NSE), vitamin D, cortisol, interleukin-6, insulin, uric acid and albumin, were examined for their ability to identify additional value in stroke management. Different aspects in stroke management were examined, which included stroke diagnosis, severity, mortality, tPA admission and ischemic stroke vs. TIA classification.

5.2. Results and Discussion

5.2.1 Stroke Diagnosis

The study cohort included of 577 patients. 516 (89.4%) stroke patients were recruited, while comparison was made with 61 (10.6) healthy control patients as reference. The

descriptive analysis is presented in Table 5.1. The distribution of the clinical parameters was compared between the two groups, stroke patients vs. healthy control. Most of the study cohort were males, both in the stroke patients (70.5%) and in the control group (57.4%). The stroke patients group presented statistically significant (p -value=0.050) higher percentage of hypertension (68.2%) in comparison with the healthy control group (55.7%). In addition, NT-proBNP plasma levels (ng/mL), were found to be statistically significant (p -value < 0.001) different (median (IQR)) between the stroke patients (188 (51.4 – 1098)) and the healthy control groups (48.9 (17.5 - 102)).

Table 5.1. Descriptive analysis according to stroke patients and healthy control.

Clinical Feature	Total	Stroke	Control	p-Value
Number (%)	577 (100%)	516 (89.4%)	61 (10.6%)	-
Males (%)	399 (69.2%)	364 (70.5%)	35 (57.4%)	0.035
Age (years), median (IQR)	61 (52 - 70)	61 (52.3 - 72)	60 (47 - 71)	0.006
Race (%) -				0.150
Chinese	419 (72.6%)	380 (73.6%)	39 (63.9%)	-
Malay	109 (18.9%)	92 (17.8%)	17 (27.9%)	-
Indian	40 (6.9%)	37 (7.2%)	3 (4.9%)	-
Others	9 (1.6%)	7 (1.4%)	2 (3.3%)	-
Stroke severity, median NIHSS score (IQR)	2 (1 - 6)	2.5 (0.250 - 7.75)	1 (0 - 3)	<0.001
Vascular Clinical Features (%) -				
Vascular	130 (22.5%)	125 (24.2%)	5 (8.2%)	0.005
Hypertension	386 (66.9%)	352 (68.2%)	34 (55.7%)	0.050
Diabetes Mellitus	188 (32.6%)	174 (33.7%)	14 (23.0%)	0.090
Hyperlipidaemia	299 (51.8%)	268 (51.9%)	31 (50.8%)	0.869
Chronic Renal Failure	13 (2.3%)	10 (1.9%)	3 (4.9%)	0.138
Previous Stroke	107 (18.5%)	99 (19.2%)	8 (13.1%)	0.249
Atrial Fibrillation	34 (5.9%)	32 (6.2%)	2 (3.3%)	0.360
Smoking	147 (25.5%)	134 (26.0%)	13 (21.3%)	0.430
Modified Ranking Scale -				0.927
No symptoms	510 (88.4%)	456 (88.4%)	54 (88.5%)	-
No significant disability	30 (5.2%)	28 (5.4%)	2 (3.3%)	-
Slight disability	19 (3.3%)	16 (3.1%)	3 (4.9%)	-
Moderate disability	9 (1.6%)	8 (1.6%)	1 (1.6%)	-
Moderately severe disability	7 (1.2%)	6 (1.2%)	1 (1.6%)	-
Severe disability	2 (0.3%)	2 (0.4%)	0.000	-
Dead	0.000	0.000	0.000	-
Biomarkers				
Cortisol (ng/mL)	149 (93.9 - 206)	168 (112 - 217)	162 (86.2 -213)	0.059
NT-proBNP (pg/mL)	105 (38.4 - 446)	188 (51.4 - 1098)	48.9 (17.5 - 102)	<0.001
IL-6 (pg/mL)	4.31 (2.12 - 9.80)	4.61 (2.33 - 10.8)	7.16 (2.17 - 13.2)	0.898
Insulin (pg/mL)	20.08 (12.0 - 36.5)	20.2 (12.6 - 34.2)	23.4 (17.5 - 58.2)	0.075

NSE (ng/mL)	15.0 (11.5 - 21.3)	14.9 (11.9 - 20.6)	13.9 (11.6 - 21.3)	0.408
S100 (pg/mL)	52.0 (38.0 - 83.0)	55.5 (40.0 - 94.8)	43.0 (35.0 - 75.0)	0.058
Albumin (g/L)	40.5 (37.6 - 43.6)	40.5 (37.5 - 43.4)	42.9 (38.0 - 44.3)	0.262
D-dimer (ng/mL)	370 (210 - 755)	375 (213 - 873)	320 (160 - 500)	0.221

* Interquartile Range (IQR): Q1 – Q3. Kruskal Wallis H-Test was used to calculate p-value for continues variables, while chi-square test was used for categorical variables (if cell frequency was lower than 5%, fisher's exact test was used). NIHSS: National Institutes of Health Stroke Scale. Vascular: either stroke (ischemic / haemorrhage), transient ischemic attack (TIA), Acute myocardial infarction (AMI) or Angina Pectoris.

Univariable logistic regression analysis was conducted in order to identify the biomarkers classification performance of the stroke patients vs. the healthy control group. First, the biomarkers diagnostic performance was examined with ROC analysis (Table 12.11), in order to determine the biomarkers optimal cut-off and reconstruct the biomarkers into dichotomous variables according to stroke diagnosis classification. In the univariable logistic regression analysis (Table 5.2), the plasma levels of three biomarkers were found to be individually statistically significant associated to stroke diagnosis classification: cortisol ≥ 138 (nmol/L) (p-value = 0.025; OR = 1.927), NT-proBNP ≥ 0.064 (ng/mL) (p-value < 0.001; OR = 2.905) and insulin ≤ 23.4 (pmol/L) (p-value = 0.025; OR = 1.923). Moreover, multivariable logistic regression modelling (Table 5.3), identified that the combination of the two biomarkers panel: NT-proBNP ≥ 0.064 (ng/mL) (p-value < 0.001; OR = 2.905) and insulin ≤ 23.4 (pmol/L) (p-value = 0.026; OR = 1.934), results in 85.2% sensitivity, 90.9% PPV, 79.2% correctly classified and 67.3% AUC (Figure 12.4).

Table 5.2. Univariable Logistic Regression between Stroke and Healthy Control.

Variable	n	Stroke Diagnosis						
		Logistic Regression			Classification Table			
		Condition	OR (95% CI)	p-value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Cortisol (nmol/L)	489	≥ 138	1.927 (1.088-3.416)	0.025	58.85	57.41	91.76	14.76

NT-proBNP (pg/mL)	487	≥ 0.064	2.905 (1.624-5.197)	<0.001	64.90	61.11	93.05	17.84
IL-6 (pg/mL)	487	≥ 3.99	1.221 (0.693-2.149)	0.491	53.12	51.85	89.84	12.12
Insulin (pmol/L)	486	≤ 23.4	1.923 (1.085-3.409)	0.025	58.80	57.41	91.70	14.83
NSE (ng/mL)	485	≥ 14.5	1.485 (0.842-2.620)	0.172	56.15	53.70	90.64	13.30
S100 (pg/mL)	483	≥ 0.05	1.558 (0.881-2.753)	0.127	55.48	55.56	90.84	13.57
Albumin (g/L)	180	≤ 40.5	1.185 (0.411-3.418)	0.753	50.91	53.33	92.31	8.99
D-dimer (ng/mL)	180	≥ 0.33	1.513 (0.524-4.368)	0.444	56.97	53.33	93.07	10.13

Table 5.3. Multivariable Logistic Regression for Stroke Diagnosis.

Stroke Vs. Control			
Model II (n = 486)			
Biomarkers	Condition	Adjusted OR (95% CI)	p-value
NT-proBNP (pg/mL)	≥ 0.064	2.905 (1.618-5.214)	<0.001
Insulin (pmol/L)	≤ 23.4	1.934 (1.082-3.457)	0.026
Goodness-of-fit test		Chi-squared = 1.27	0.259
AUC	67.3%		
Classification Table Probability Cut-Off 0.8			
classified	No	Yes	Total
positive	368	37	405
negative	64	17	81
Total	432	54	486
sensitivity	85.2%	false positives rate	68.5%
specificity	31.5%	false negatives rate	14.8%
positive predictive value	90.9%	false positives rate	9.1%
negative predictive value	20.9%	false negatives rate	79.0%
correctly classified			79.2%

5.2.2 Stroke Severity

The study cohort included 516 stroke patients. The stroke patients were grouped according to the different levels of stroke severity. Firstly, the descriptive analysis (both baseline characteristics (Table 12.9) and biomarkers (Table 12.10)) was conducted between 5 groups of stroke severity level: no stroke symptoms (NIHSS = 0); minor stroke (NIHSS = 1-4); moderate stroke (NIHSS = 5-15); moderate to severe stroke (NIHSS = 16-20) and severe stroke (NIHSS = 21-42). Secondly, the descriptive analysis (both baseline characteristics (Table 5.4) and biomarkers (Table 5.5)) was conducted between 2 groups of stroke severity level: minor to moderate stroke (NIHSS = 0-15) and moderate to severe stroke (NIHSS = 16-42).

In the first case, in the baseline characteristics distribution analysis (Table 12.9) between the 5 groups of stroke severity levels, 25% did not show stroke symptoms, 46.9% suffered a minor stroke, 20.4% suffered a moderate stroke, 2.52% suffered moderate to severe stroke and 5.23% suffered a severe stroke. Several parameters were found to be statistically significant different between the 5 stroke severity groups. Males were the majority in the lower stroke severity levels of NIHSS = 0-15 (~70%), while women were the majority in the higher stroke severity levels of NIHSS = 16-42 (~60%). In addition, older patients (≥ 70 years) suffered a more severe stroke of NIHSS = 16-42, while patients that were 10 years younger (≤ 60 years) suffered a less severe stroke of NIHSS = 0-15. Moreover, tPA was administered more frequently in the higher stroke severity levels (moderate to severe stroke 84.6% and severe stroke 66.7%) in comparison to the lower stroke severity levels (minor stroke 3.3% and moderate stroke 19%). Regarding the biomarkers distribution analysis (Table 12.10), several biomarkers levels were found to be statistically significant different between the 5 groups: haemoglobin, haematocrit, lymphocytes, prothrombin time; INR, AST, cortisol, NT-proBNP and IL-6.

In the second case, in the baseline characteristics distribution analysis (Table 5.4) between the 2 groups of stroke severity levels, 92.25% suffered a minor to moderate stroke while only 7.75% suffered a moderate to severe stroke. Several parameters were found to be statistically significant different between the 2 stroke severity groups. Males were the majority in the lower stroke severity levels of NIHSS = 0-15 (73.1%), while women were the majority in the higher stroke severity levels of NIHSS = 16-42 (60%). In addition, older patients (≥ 70 years) suffered a more severe stroke of NIHSS = 16-42, while patients that were 10 years younger (≤ 60 years) suffered a less severe stroke of NIHSS = 0-15. Moreover, tPA was administered more frequently in the higher stroke severity levels (72.5%) in comparison to the lower stroke severity levels (5.9%). Regarding the biomarkers distribution analysis (Table 5.5), several biomarkers levels were found to be statistically significant different between the 2 groups: haemoglobin, haematocrit, platelets, triglycerides, HDL, AST, cortisol, NT-proBNP, IL-6, NSE and S100.

Table 5.4. Baseline Characteristics Distribution between 2 Different Levels of Stroke Severity.

Clinical Feature	Stroke Severity (NIHSS) †			p-Value**
	Total	Minor - Moderate Stroke	Moderate - Severe Stroke	
	-	NIHSS = 0 - 15	NIHSS = 16 - 42	
Number (%)	516 (100%)	476 (92.25%)	40 (7.75%)	-
Stroke (%)	433 (83.9%)	393 (82.6%)	40 (100%)	0.004
Transient Ischemic Attack (%)	83 (16.1%)	83 (17.4%)	0.000	0.004
Males (%)	364 (70.5%)	348 (73.1%)	16 (40%)	<0.001
Age (years), median (IQR*)	61 (53-71)	60 (52 - 69)	71.5 (61.25 - 75.75)	<0.001
Abdominal Circumference (cm)	91 (84-97)	91 (84 - 97)	90 (84 - 96)	0.993
Height (cm)	163 (157 - 169)	164 (157 - 170)	156 (150 - 164.75)	<0.001
Weight (kg)	66 (59.1 - 74.3)	66.4 (59.6 - 74.9)	60.9 (55.3 - 70.9)	0.029
Body Mass Index (BMI)	24.8 (22.3-27.7)	24.8 (22.3 - 27.7)	24.9 (21.7 - 27.7)	0.889
Race (%) -				0.229
Chinese	380 (73.6%)	349 (73.3%)	31 (77.5%)	-
Malay	92 (17.9%)	83 (17.4%)	9 (22.5%)	-
Indian	37 (7.2%)	37 (7.8%)	0.000	-
Others	7 (1.4%)	7 (1.5%)	0.000	-
Duration of Symptoms (days), median (IQR*)	12 (3-29)	13 (4 - 31.3)	4 (0.49 - 15.5)	0.032
Duration of Observed Event (days), median (IQR*)	50 (25-59)	50.5 (26 - 59.8)	32.5 (4 - 56.8)	0.008
Stroke Severity, NIHSS Score, median (IQR*)	2 (0.25 - 6)	2 (0 - 4)	22 (20 - 24)	<0.001
Tissue Plasminogen Activator (%)	57 (11.1%)	28 (5.9%)	29 (72.5%)	<0.001
Vascular Clinical Features (%) -				
Vascular #	125 (24.2%)	110 (23.1%)	15 (37.5%)	0.041
Hypertension	346 (67.1%)	319 (67%)	27 (67.5%)	0.95
Diabetes Mellitus	221 (42.9%)	204 (42.9%)	17 (42.5%)	0.965
Hyperlipidaemia	243 (47.1%)	226 (47.5%)	17 (42.5%)	0.545
Chronic Renal Failure	4 (0.8%)	1 (0.2%)	3 (7.5%)	<0.001
Previous Stroke	81 (15.7%)	71 (14.9%)	10 (25%)	0.092
Ischemic Heart Disease	90 (17.4%)	78 (16.4%)	12 (30%)	0.029
Peripheral Vascular Disease	4 (0.8%)	3 (0.6%)	1 (2.5%)	0.277
Atrial Fibrillation	32 (6.2%)	24 (5%)	8 (20%)	<0.001
Smoking	242 (46.9%)	231 (48.5%)	11 (27.5%)	0.010
Modified Ranking Scale -				0.001
No symptoms	471 (91.5%)	440 (92.4%)	32 (80%)	-
No significant disability	15 (2.9%)	15 (3.2%)	0.000	-
Slight disability	11 (2.1%)	8 (1.7%)	3 (7.5%)	-
Moderate disability	14 (2.7%)	10 (2.1%)	4 (10%)	-
Moderately severe disability	4 (0.8%)	3 (0.6%)	1 (2.5%)	-
Severe disability	0.000	0.000	0.000	-
Dead	0.000	0.000	0.000	-
Location (%) -				0.054
Left Hemisphere	186 (41.1%)	162 (39.1%)	24 (60%)	-
Right Hemisphere	180 (39.7%)	166 (40.1%)	14 (35%)	-

Brainstem	14 (3.1%)	13 (3.1%)	1 (2.5%)	-
Other	5 (1.1%)	5 (1.2%)	0.000	-
Unknown	69 (15.2%)	68 (16.4%)	1 (2.5%)	-
Stroke Syndrome † (%) -				<0.001
TACS	57 (11.2%)	35 (7.5%)	22 (55%)	-
PACS	101 (19.9%)	88 (18.8%)	13 (32.5%)	-
LACS	292 (57.6%)	290 (62.1%)	2 (5%)	-
POCS	55 (10.8%)	53 (11.3%)	2 (5%)	-
Undetermined	2 (0.4%)	1 (0.2%)	1 (2.5%)	-

* Interquartile Range (IQR): Q1 – Q3. ** Kruskal Wallis H-Test was used to calculate p-value for continues variables, while chi-square test was used for categorical variables (if cell frequency was lower than 5%, fisher's exact test was used). † NIHSS: National Institutes of Health Stroke Scale. 0 - 15 minor to moderate stroke, 16 - 42 = moderate to severe stroke. # Vascular: either stroke (ischemic / haemorrhage), transient ischemic attack (TIA), Acute myocardial infarction (AMI) or Angina Pectoris. ‡ Stroke syndromes: TACS, total anterior circulation syndrome; LACS, lacunar syndrome; PACS, partial anterior circulation syndrome; POCS, posterior circulation syndrome. *** Stroke Etiology: LAA, large artery atherosclerosis, CEI, cardioembolic, LAC, lacunar.

Table 5.5. Biomarkers Distribution between 2 Different Levels of Stroke Severity.

Biomarker	N	Stroke Severity (NIHSS) ‡			
		Total	Minor - Moderate Stroke	Moderate - Severe Stroke	p-Value**
		-	NIHSS = 0 - 15	NIHSS = 16 - 42	
White Blood Cells (10 ⁹ /L)	509	8.17 (6.69-9.89)	8.1 (6.67 - 9.88)	8.95 (7.07 - 11.9)	0.059
Haemoglobin (g/dL)	509	14.2 (13-15.4)	14.3 (13.1 - 15.5)	13.2 (11.7 - 14.9)	0.001
Haematocrit (%)	509	41.8 (38.9-45.1)	42.1 (39 - 45.2)	40.1 (35.1 - 43.6)	0.003
Platelets (10 ⁹ /L)	509	237 (199-281)	239 (201 - 288)	219 (185 - 251)	0.010
Mean Platelet Volume (fL)	505	9.6 (8.28-10.3)	9.6 (8.4 - 10.3)	9.4 (7.2 - 10.6)	0.925
Neutrophils (%)	509	64.1 (56.9-72.2)	63.8 (56.9 - 72)	68.2 (59.7 - 75.4)	0.137
Neutrophils (10 ⁹ /L)	508	5.14 (3.87-6.61)	5.09 (3.84 - 6.52)	5.56 (4.04 - 8.55)	0.054
Lymphocytes (%)	464	25.6 (18.9-31.9)	25.8 (19 - 31.9)	22.9 (17.1 - 30.8)	0.395
Lymphocytes (10 ⁹ /L)	464	1.95 (1.44-2.57)	1.95 (1.44 - 2.56)	1.87 (1.37 - 2.89)	0.808
Prothrombin Time (sec)	318	12.8 (12.4-13.2)	12.7 (12.4 - 13.2)	13 (12.4 - 13.8)	0.066
International Normalized Ratio (INR)	318	0.98 (0.95-1.04)	0.98 (0.95 - 1.03)	1.03 (0.96 - 1.06)	0.076

Partial Thromboplastin Time (sec)	299	28.7 (27-30.8)	28.9 (27 - 30.8)	28.2 (26.75 - 30.4)	0.224
Total Cholesterol (mg/dL)	485	186 (156-221)	188 (157 - 221)	167 (151 - 218)	0.295
Triglycerides (mg/dL)	485	51.2 (38.2-75.9)	52.2 (38.9 - 78.3)	40.6 (29.8 - 65.7)	0.015
HDL (mg/dL)	485	40.6 (34.8-49.5)	40.2 (34.8 - 49.1)	43.3 (39.3 - 55.1)	0.034
LDL (mg/dL)	471	115 (88.3-144)	116 (89.3 - 144)	101 (86.0 - 139)	0.243
Aspartate Aminotransferase (U/L)	425	22 (18-28)	21.5 (18 - 27)	29.5 (23.3 - 37.8)	<0.001
Alanine Transaminase (U/L)	424	20 (14-29)	20 (14 - 29)	19.5 (13 - 30.5)	0.802
Fasting Blood Glucose (mg/dL)	329	95.5 (84.7-114)	93.7 (84.7 - 110)	106 (88.3 - 150)	0.069
Glycohaemoglobin, HbA1c (%)	407	6.3 (5.7-7.8)	6.3 (5.7 - 7.8)	6.1 (5.7 - 7.4)	0.405
Vitamin D (mg/dL)	486	21.5 (14.9-29.2)	21.6 (14.9 - 29.7)	20.3 (15.2 - 25.1)	0.354
Cortisol (nmol/L)	433	419 (268-576)	408 (259 - 557)	660 (433 - 988)	<0.001
NT-proBNP (pg/mL)	432	120 (43.6-503.3)	105 (39.7 - 416)	1112 (205 - 2421)	<0.001
IL-6 (pg/mL)	432	4.31 (2.08-10.3)	3.99 (1.98 - 8.29)	14.5 (6.56 - 26.5)	<0.001
Insulin (pmol/L)	431	54.6 (32.8-98.6)	55.0 (32.9 - 97.9)	51.3 (29.6 - 161)	0.927
NSE (ng/mL)	430	15.1 (11.6-21.4)	14.9 (11.5 - 20.7)	18.2 (12.8 - 29.4)	0.029
S100 (pg/mL)	428	52 (38-86)	51 (37.5 - 79)	98.5 (70.25 - 193.25)	<0.001
Urate (μ mol/L)	228	358 (304-437)	357 (304 - 431)	397 (292 - 460)	0.512
Albumin (g/L)	156	40.4 (37.6-43.4)	40.4 (37.6 - 43.6)	40.3 (37.6 - 42.6)	0.640
D-dimer (ng/mL)	156	350 (210-685)	370 (210 - 670)	300 (185 - 1065)	0.651

* Interquartile Range (IQR): Q1 – Q3. ** Kruskal Wallis H-Test was used to calculate p-value.

† NIHSS: National Institutes of Health Stroke Scale. 0 - 15 minor to moderate stroke, 16 - 42 = moderate to severe stroke.

Univariable logistic regression analysis was conducted in order to identify the biomarkers classification performance of the stroke severity level. First, the biomarkers diagnostic performance was examined with ROC analysis (Table 12.12), in order to determine the biomarkers optimal cut-off and reconstruct the biomarkers into dichotomous variables according to stroke severity level classification. In the univariable logistic regression analysis (Table 5.6), the plasma levels of 14 biomarkers were found to be individually statistically significant associated to stroke severity level classification: haemoglobin $\leq$ 13.9 (g/dL), haematocrit $\leq$ 41.1 (%), prothrombin time $\geq$ 13 (sec), INR $\geq$ 1, total

cholesterol ≤ 175 (mg/dL), triglycerides ≤ 46.4 (mg/dL), LDL ≤ 109 (mg/dL), AST ≥ 25 (U/L), fasting blood glucose ≥ 103 (mg/dL), cortisol ≥ 529 (nmol/L), NT-proBNP ≥ 275 (pg/mL), IL-6 ≥ 7.7 (pg/mL), NSE ≥ 17.1 (ng/mL) and S100 ≥ 71 (pg/mL). Moreover, multivariable logistic regression modelling (Table 5.7), identified that the combination of the five biomarkers panel: HDL ≥ 43.3 (mg/dL) (p-value = 0.046, OR = 2.53), AST ≥ 25 (U/L) (p-value < 0.001, OR = 6.25), cortisol ≥ 529 (nmol/L) (p-value = 0.034, OR = 2.72), IL-6 ≥ 7.7 (pg/mL) (p-value = 0.001, OR = 4.72), and S100 ≥ 71 (pg/mL) (p-value = 0.005, OR = 3.96) results in 96.9% specificity, 94.7% NPV, 92.2% correctly classified and 88.5% AUC (Figure 12.5).

Table 5.6. Univariable Logistic Regression between 2 Different Levels of Stroke Severity.

Stroke Severity (NIHSS)								
Variable	n	Logistic Regression			Classification Table			
		Condition	OR (95% CI)	p-value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Biomarkers								
White Blood Cells (10 ⁹ /L)	510	≥8.6	1.83 (0.95 - 3.54)	0.072	56.4	58.6	10.1	94.2
Haemoglobin (g/dL)	510	≤13.9	2.44 (1.24 - 4.81)	0.010	64.1	57.8	11.2	95.1
Haematocrit (%)	510	≤41.1	2.19 (1.12 - 4.28)	0.022	61.5	57.8	10.8	94.8
Platelets (10 ⁹ /L)	510	≤220	1.79 (0.93 - 3.45)	0.083	53.9	60.5	10.1	94.1
Mean Platelet Volume (fL)	506	≤9.4	1.52 (0.79 - 2.93)	0.215	56.4	53.9	9.28	93.7
Neutrophils (%)	510	≥66	1.71 (0.88 - 3.30)	0.111	56.4	56.9	9.78	94.0
Neutrophils (10 ⁹ /L)	509	≥5.3	1.72 (0.89 - 3.34)	0.109	58.9	54.5	9.70	94.1
Lymphocytes (%)	465	≤23.5	1.64 (0.81 - 3.31)	0.169	55.9	56.4	9.18	94.2
Lymphocytes (10 ⁹ /L)	465	≥1.9	0.89 (0.44 - 1.79)	0.744	50	47.1	6.94	92.3
Prothrombin Time (sec)	319	≥13	2.94 (1.36 - 6.37)	0.006	64.5	61.8	15.4	94.2
International Normalized Ratio (INR)	319	≥1	2.35 (1.09 - 5.02)	0.028	61.3	59.7	14.1	93.5
Partial Thromboplastin Time (sec)	300	≤28.6	2.19 (0.98 - 4.88)	0.056	65.5	53.5	13.1	93.6
Total Cholesterol (mg/dL)	486	≤175	2.28 (1.15 - 4.50)	0.018	59.5	60.8	11.1	94.8
Triglycerides (mg/dL)	486	≤46.4	2.19 (1.11 - 4.34)	0.024	59.5	59.9	10.9	94.7
HDL (mg/dL)	486	≥43.3	1.91 (0.97 - 3.75)	0.062	56.8	59.2	10.3	94.3
LDL (mg/dL)	472	≤109	2.16 (1.09 - 4.28)	0.027	59.5	59.5	11.1	94.5
Aspartate Aminotransferase (U/L)	426	≥25	5.08 (2.38 - 10.85)	0.000	72.2	66.2	16.5	96.3
Alanine Transaminase (U/L)	425	≤20	1.08 (0.54 - 2.14)	0.829	52.8	49.1	8.76	91.8
Fasting Blood Glucose (mg/dL)	330	≥103	2.66 (1.19 - 5.95)	0.017	59.3	64.7	13.0	94.7
Glycohaemoglobin, HbA1c (%)	408	≤6.2	1.34 (0.64 - 2.80)	0.432	54.8	52.5	8.67	93.4
Vitamin D (mg/dL)	487	≤20.4	1.41 (0.73 - 2.73)	0.312	52.6	55.9	9.17	93.3

Cortisol (nmol/L)	434	≥529	5.93 (2.67 - 13.20)	0.000	71.9	69.9	15.9	96.9
NT-proBNP (pg/mL)	433	≥275	4.75 (2.18 - 10.32)	0.000	68.8	68.3	14.8	96.5
IL-6 (pg/mL)	433	≥7.7	5.97 (2.74 - 13.01)	0.000	68.8	73.1	16.9	96.7
Insulin (pmol/L)	432	≥52.3	0.83 (0.40 - 1.71)	0.615	46.9	48.5	6.79	91.9
NSE (ng/mL)	431	≥17.1	2.33 (1.12 - 4.84)	0.024	59.4	61.4	10.9	94.9
S100 (pg/mL)	429	≥71	7.01 (3.06 - 16.05)	0.000	75	70.0	16.8	97.2
Urate (μmol/L)	228	≥384	2.04 (0.73 - 5.68)	0.174	56.3	61.3	9.89	94.9
Albumin (g/L)	156	≤40.3	0.95 (0.30 - 2.95)	0.923	46.2	52.5	8.11	91.5
D-dimer (ng/mL)	156	≤320	2.09 (0.65 - 6.70)	0.215	61.5	56.6	11.4	94.2

Table 5.7. Multivariable Logistic Regression between 2 Different Levels of Stroke Severity.

Stroke Severity (NIHSS)			
Model II (n = 357)			
Biomarkers	Condition	Adjusted OR (95% CI)	p-value
HDL (mg/dL)	≥43.3	2.53 (1.02 - 6.31)	0.046
Aspartate Aminotransferase (U/L)	≥25	6.25 (2.35 - 16.66)	<0.001
Cortisol (nmol/L)	≥529	2.72 (1.08 - 6.88)	0.034
IL-6 (pg/mL)	≥7.7	4.72 (1.85 - 12.01)	0.001
S100 (pg/mL)	≥71	3.96 (1.51 - 10.33)	0.005
Goodness-of-fit test		Chi-squared = 13.73	0.976
AUC		0.8846	
Classification Table Probability Cut-Off 0.4			
classified	Minor-Moderate Stroke	Moderate-Severe Stroke	Total
positive	10	10	20
negative	319	18	337
Total	329	28	357
sensitivity	35.71%	false positives rate	3.04%
specificity	96.96%	false negatives rate	64.29%
positive predictive value	50.00%	false positives rate	50.00%
negative predictive value	94.66%	false negatives rate	5.34%
correctly classified			92.16%

5.2.3 Stroke Mortality

The study cohort included 516 stroke patients. The stroke patients were grouped according to mortality. Firstly, the descriptive analysis, both baseline characteristics (Table 5.8) and biomarkers (Table 5.9) was conducted. 82.2% of the stroke patients survived, while 17.8% died. There was no statistically significant difference in the mortality frequency in both cases of stroke (83.3% alive vs. 87% dead) and transient ischemic attack (16.7% alive vs. 13% dead). There was a statistically significant difference (p-value < 0.001) in the gender percentage, even though that males were the majority in both groups, also in the stroke patients group that survived (74.1%), and in the stroke patients group that died (54.3%). The stroke patients that died (72.5 (63.3 - 80))

were statistically significant (p -value < 0.001) older than the stroke patients that survived (59 (52 - 67)). Moreover, the stroke severity levels were statistically significant (p -value $= 0.001$) higher in the stroke patients group that died (3 (1 - 12.3)), in comparison to the stroke patients group that survived (2 (0 - 5)).

Several vascular parameters were statistically significant more frequent among the stroke patients group that died in comparison to the stroke patients group that survived, which included: hypertension (82.6% vs. 63.7%); diabetes mellitus (53.3% vs. 40.6%); previous stroke (27.2% vs. 13.2%); ischemic heart disease (28.3% vs. 15.1%) and atrial fibrillation (15.2% vs. 4.2%). The modified ranking scale upon admission also showed statistically significant (p -value < 0.001) difference between the two groups. Most stroke patients that did not show symptoms were in the group that survived (95.8%) in comparison to the group that died (71.7%). In the rest of the scale indications, the stroke patients group that died shows increased frequencies in comparison to the group that survived, accordingly: no significant disability 4.3% vs. 2.6%; slight disability 10.9% vs. 0.2%; moderate disability 8.7% vs. 1.4% and moderate to severe disability 4.3% vs. 0. Moreover, the stroke syndrome and etiology classification revealed that lacunar etiology was more frequent among the stroke patients group that survived (62.4% vs. 34.8%; 53.2% vs. 23.9%), while the rest of the etiologies (atherosclerosis, cardioembolic and undetermined) were more frequent in the stroke patients group that died.

Table 5.8. Baseline Characteristics Distribution for Mortality Analysis.

Clinical Feature	Mortality			p-Value**
	Total	Alive	Dead	
Number (%)	516 (100%)	424 (82.2%)	92 (17.8%)	-
Stroke (%)	433 (83.9%)	353 (83.3%)	80 (87%)	0.381
Transient Ischemic Attack (%)	83 (16.1%)	71 (16.7%)	12 (13%)	0.381
Males (%)	364 (70.5%)	314 (74.1%)	50 (54.3%)	<0.001
Age (years), median (IQR*)	61 (53-71)	59 (52 - 67)	72.5 (63.3 - 80)	<0.001
Abdominal Circumference (cm)	90.5 (84-97)	91 (84 - 97)	89 (82.3 - 96)	0.116
Height (cm)	163 (157 - 169)	164 (158 - 170)	161 (150 - 167)	0.002
Weight (kg)	66 (59.1 - 74.3)	67 (60 - 75)	61.1 (50 - 70)	<0.001
Body Mass Index (BMI)	24.8 (22.3-27.7)	25.0 (22.6 - 27.9)	24.0 (21.2 - 26.3)	0.004
Race (%) -				0.333
Chinese	380 (73.6%)	312 (73.6%)	68 (73.9%)	-

Malay	92 (17.9%)	72 (17%)	20 (21.7%)	-
Indian	37 (7.2%)	34 (8%)	3 (3.3%)	-
Others	7 (1.4%)	6 (1.4%)	1 (1.1%)	-
Duration of Symptoms (days), median (IQR*)	12 (3-29)	15 (5 - 36)	9 (1.29 - 16.8)	0.008
Duration of Observed Event (days), median (IQR*)	50 (25-59)	53 (43 - 61)	13 (2.68 - 29)	<0.001
Stroke Severity, NIHSS Score, median (IQR*)	2 (0.25 - 6)	2 (0 - 5)	3 (1 - 12.3)	0.001
Tissue Plasminogen Activator (%)	57 (11.1%)	44 (10.4%)	13 (14.1%)	0.298
Vascular Clinical Features (%) -				
Vascular #	125 (24.2%)	66 (15.6%)	59 (64.1%)	<0.001
Hypertension	346 (67.1%)	270 (63.7%)	76 (82.6%)	<0.001
Diabetes Mellitus	221 (42.9%)	172 (40.6%)	49 (53.3%)	0.026
Hyperlipidaemia	243 (47.1%)	194 (45.8%)	49 (53.3%)	0.191
Chronic Renal Failure	4 (0.8%)	1 (0.2%)	3 (3.3%)	0.019
Previous Stroke	81 (15.7%)	56 (13.2%)	25 (27.2%)	0.001
Ischemic Heart Disease	90 (17.4%)	64 (15.1%)	26 (28.3%)	0.003
Peripheral Vascular Disease	4 (0.8%)	2 (0.5%)	2 (2.2%)	0.148
Atrial Fibrillation	32 (6.2%)	18 (4.2%)	14 (15.2%)	<0.001
Smoking	242 (46.9%)	203 (47.9%)	39 (42.4%)	0.339
Vascular Death	52 (10.1%)	0.000	52 (56.5%)	<0.001
Stroke as Cause of Vascular Death	29 (5.6%)	0.000	29 (31.5%)	<0.001
Modified Ranking Scale -				<0.001
No symptoms	471 (91.5%)	406 (95.8%)	66 (71.7%)	-
No significant disability	15 (2.9%)	11 (2.6%)	4 (4.3%)	-
Slight disability	11 (2.1%)	1 (0.2%)	10 (10.9%)	-
Moderate disability	14 (2.7%)	6 (1.4%)	8 (8.7%)	-
Moderately severe disability	4 (0.8%)	0.000	4 (4.3%)	-
Severe disability	0.000	0.000	0.000	-
Dead	0.000	0.000	0.000	-
Location (%) -				0.792
Left Hemisphere	186 (41.1%)	149 (40.2%)	37 (44.6%)	-
Right Hemisphere	180 (39.7%)	147 (39.6%)	33 (39.8%)	-
Brainstem	14 (3.1%)	11 (3%)	3 (3.6%)	-
Other	5 (1.1%)	4 (1.1%)	1 (1.2%)	-
Unknown	69 (15.2%)	60 (16.2%)	9 (10.8%)	-
Stroke Syndrome [†] (%) -				<0.001
TACS	57 (11.2%)	39 (9.3%)	18 (20.2%)	-
PACS	101 (19.9%)	75 (17.9%)	26 (29.2%)	-
LACS	292 (57.6%)	261 (62.4%)	31 (34.8%)	-
POCS	55 (10.8%)	42 (10%)	13 (14.6%)	-
Undetermined	2 (0.4%)	1 (0.2%)	1 (1.1%)	-
Stroke Etiology*** (%) -				<0.001
LAA	131 (25.4%)	103 (24.3%)	28 (30.4%)	-
CEI	75 (14.6%)	50 (11.8%)	25 (27.2%)	-
LAC	247 (48%)	225 (53.2%)	22 (23.9%)	-
Undetermined	62 (12%)	45 (10.6%)	17 (18.5%)	-

* Interquartile Range (IQR): Q1 – Q3. ** Kruskal Wallis H-Test was used to calculate p-value for continues variables, while chi-square test was used for categorical variables (if cell frequency was lower than 5%, fisher's exact test was used). † NIHSS: National Institutes of Health Stroke Scale. # Vascular: either stroke (ischemic / haemorrhage), transient ischemic attack (TIA), Acute myocardial infarction (AMI) or Angina Pectoris. ‡ Stroke syndromes: TACS, total anterior circulation syndrome; LACS, lacunar syndrome; PACS, partial anterior circulation syndrome; POCS, posterior circulation syndrome. *** Stroke Etiology: LAA, large artery atherosclerosis, CEI, cardioembolic, LAC, lacunar.

The biomarkers distribution analysis (Table 5.9) identified several statistically significant differences between the two groups. The plasma levels of several biomarkers were found to be statistically significant different between the stroke patients group that died in comparison to those that survived, which included the following: haemoglobin, haematocrit, platelets, neutrophils, lymphocytes, prothrombin time, INR, partial thromboplastin time, triglycerides, ALT, cortisol, NT-proBNP, IL-6 and S100.

Table 5.9. Biomarkers Distribution for Mortality Analysis.

Biomarker	N	Mortality			P-Value**
		Total	Alive	Dead	
White Blood Cells (10 ⁹ /L)	509	8.17 (6.69-9.89)	8.06 (6.71 - 9.79)	8.6 (6.54 - 10.24)	0.233
Haemoglobin (g/dL)	509	14.2 (13-15.4)	14.4 (13.2 - 15.5)	13.2 (11.8 - 15.1)	<0.001
Haematocrit (%)	509	41.8 (38.9-45.1)	42.3 (39.68 - 45.2)	39.6 (35.4 - 44.3)	<0.001
Platelets (10 ⁹ /L)	509	237 (199-281.25)	239 (202 - 283.75)	214 (186 - 281)	0.032
Mean Platelet Volume (fL)	505	9.6 (8.28-10.3)	9.6 (8.48 - 10.3)	9.6 (7.43 - 10.5)	0.713
Neutrophils (%)	509	64.05 (56.98-72.2)	63.3 (56.18 - 71.5)	66.9 (59.4 - 75.2)	0.007
Neutrophils (10 ⁹ /L)	508	5.14 (3.87-6.61)	5.03 (3.84 - 6.52)	5.56 (4.14 - 7.13)	0.068
Lymphocytes (%)	464	25.6 (18.85-31.9)	26.2 (19.5 - 32.4)	22.9 (15.7 - 30.0)	0.003
Lymphocytes (10 ⁹ /L)	464	1.95 (1.44-2.57)	2 (1.48 - 2.6)	1.82 (1.3 - 2.43)	0.030
Prothrombin Time (sec)	318	12.8 (12.4-13.2)	12.7 (12.3 - 13.2)	13 (12.63 - 13.9)	0.001
International Normalized Ratio (INR)	318	0.98 (0.95-1.04)	0.97 (0.94 - 1.03)	1.01 (0.96 - 1.11)	<0.001
Partial Thromboplastin Time (sec)	299	28.7 (27-30.8)	28.7 (16.9 - 30.4)	30.2 (27.4 - 31.8)	0.033

Total Cholesterol (mg/dL)	485	186 (156-221)	188 (158 - 219)	176 (149 - 232)	0.286
Triglycerides (mg/dL)	485	51.2 (38.2-75.9)	52.0 (38.8 - 80.1)	46.9 (32.9 - 62.6)	0.060
HDL (mg/dL)	485	40.6 (34.8-49.5)	40.2 (34.8 - 49.1)	42.9 (35.6 - 53.8)	0.356
LDL (mg/dL)	471	115 (88.3-144)	116 (89.8 - 143.3)	109 (81.1 - 151)	0.310
Aspartate Aminotransferase (U/L)	425	22 (18-28)	22 (18 - 28)	22.5 (19 - 29.8)	0.296
Alanine Transaminase (U/L)	424	20 (14-29)	21 (14.5 - 29)	15 (12.25 - 22.8)	0.001
Fasting Blood Glucose (mg/dL)	329	95.5 (84.7-114)	93.7 (85.1 - 108)	102 (85 - 137)	0.077
Glycohaemoglobin, HbA1c (%)	407	6.3 (5.7-7.8)	6.3 (5.7 - 7.8)	6.4 (5.7 - 8.15)	0.289
Vitamin D (mg/dL)	486	21.5 (14.9-29.2)	21.9 (15.3 - 29.6)	20.4 (12.1 - 28.3)	0.120
Cortisol (nmol/L)	433	419 (268-576)	406 (245 - 567)	468 (364 - 671)	<0.001
NT-proBNP (pg/mL)	432	120 (43.6-503)	86.3 (35.3 - 300)	1021 (202 - 3007)	<0.001
IL-6 (pg/mL)	432	4.31 (2.08-10.3)	3.76 (1.91 - 7.89)	8.34 (3.64 - 23.2)	<0.001
Insulin (pmol/L)	431	54.6 (32.8-98.6)	56.5 (33.8 - 99.2)	48.8 (21.7 - 85.5)	0.077
NSE (ng/mL)	430	15.1 (11.6-21.4)	14.9 (11.5 - 21.3)	15.6 (11.7 - 24.2)	0.261
S100 (pg/mL)	428	52 (38-86)	51 (37 - 75)	79.5 (47 - 144)	<0.001
Urate (μ mol/L)	228	358 (304-437)	353 (305 - 437)	380 (300 - 439)	0.563
Albumin (g/L)	156	40.4 (37.6-43.4)	40.3 (37.6 - 43.4)	41.6 (37.7 - 43.6)	0.591
D-dimer (ng/mL)	156	350 (210-685)	350 (210 - 700)	400 (200 - 630)	0.940

* Interquartile Range (IQR): Q1 – Q3. ** Kruskal Wallis H-Test was used to calculate p-value.

Table 5.10. Univariable Logistic Regression for Mortality.

Mortality								
Variable	n	Logistic Regression			Classification Table			
		Condition	OR (95% CI)	p-value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Biomarkers								
White Blood Cells (10 ⁹ /L)	510	≥8.36	1.38 (0.88 - 2.17)	0.162	53.3	54.8	20.6	84.2
Haemoglobin (g/dL)	510	≤14	2.04 (1.28 - 3.23)	0.003	60.9	56.7	23.6	86.8
Haematocrit (%)	510	≤41.3	1.76 (1.12 - 2.78)	0.015	57.6	56.5	22.6	85.8
Platelets (10 ⁹ /L)	510	≤231	1.75 (1.11 - 2.75)	0.017	57.6	56.2	22.5	85.8
Mean Platelet Volume (fL)	506	≥9.6	0.99 (0.63 - 1.56)	0.983	51.1	48.8	18.2	81.8
Neutrophils (%)	510	≥65.5	1.59 (1.01 - 2.52)	0.043	55.4	56.2	21.8	85.1
Neutrophils (10 ⁹ /L)	509	≥5.34	1.54 (0.98 - 2.43)	0.061	55.4	55.4	21.5	84.9
Lymphocytes (%)	465	≤24	1.77 (1.09 - 2.87)	0.020	57.3	56.9	22.2	86.2
Lymphocytes (10 ⁹ /L)	465	<1.9	1.47 (0.91 - 2.38)	0.115	56.1	53.5	20.5	85.1

Prothrombin Time (sec)	319	≥ 12.9	1.83 (1.00 - 3.34)	0.049	57.7	57.3	20.8	87.4
International Normalized Ratio (INR)	319	≥ 1	2.55 (1.38 - 4.69)	0.003	61.5	61.4	23.7	89.1
Partial Thromboplastin Time (sec)	300	≥ 28.9	1.34 (0.72 - 2.48)	0.351	55.1	52.2	18.4	85.6
Total Cholesterol (mg/dL)	486	≤ 183	1.41 (0.88 - 2.24)	0.153	53.5	55.0	20.4	84.6
Triglycerides (mg/dL)	486	≤ 51	1.29 (0.81 - 2.07)	0.275	53.5	53.0	19.7	84.1
HDL (mg/dL)	486	≥ 41.4	1.34 (0.84 - 2.13)	0.220	52.9	54.4	20.2	84.1
LDL (mg/dL)	472	≤ 113	1.46 (0.91 - 2.34)	0.119	54.8	54.6	20.7	84.8
Aspartate Aminotransferase (U/L)	426	≥ 23	1.19 (0.72 - 1.95)	0.497	50.0	54.3	19.2	83.3
Alanine Transaminase (U/L)	425	≤ 19	2.01 (1.21 - 3.33)	0.007	61.8	55.3	23.2	86.9
Fasting Blood Glucose (mg/dL)	330	≥ 101	2.33 (1.29 - 4.23)	0.005	59.3	61.6	23.2	88.5
Glycohaemoglobin, HbA1c (%)	408	≥ 6.4	1.28 (0.78 - 2.10)	0.334	53.3	52.9	20.8	82.9
Vitamin D (mg/dL)	487	≤ 21.1	1.42 (0.89 - 2.26)	0.133	55.1	53.8	21.0	84.3
Cortisol (nmol/L)	434	≥ 441	1.92 (1.17 - 3.15)	0.010	57.7	58.4	23.3	86.3
NT-proBNP (pg/mL)	433	≥ 271	7.54 (4.33 - 13.10)	0.000	73.1	73.5	37.8	92.6
IL-6 (pg/mL)	433	≥ 5.65	3.37 (2.02 - 5.62)	0.000	64.1	65.4	28.9	89.2
Insulin (pmol/L)	432	≤ 50.7	1.47 (0.89 - 2.41)	0.124	55.1	54.5	21.1	84.7
NSE (ng/mL)	431	≥ 15.4	1.33 (0.81 - 2.17)	0.257	53.9	53.3	20.3	83.9
S100 (pg/mL)	429	≥ 63	3.13 (1.88 - 5.21)	0.000	62.8	64.9	28.5	88.7
Urate (μ mol/L)	228	≥ 359	1.41 (0.71 - 2.78)	0.325	56.1	52.4	20.5	84.5
Albumin (g/L)	156	≥ 40.5	1.40 (0.64 - 3.09)	0.401	54.8	53.6	22.7	82.7
D-dimer (ng/mL)	156	≤ 400	0.71 (0.32 - 1.57)	0.398	51.6	40.0	17.6	76.9

Univariable logistic regression analysis was conducted in order to identify the biomarkers classification performance of stroke mortality. First, the biomarkers diagnostic performance was examined with ROC analysis (Table 12.13), in order to determine the biomarkers optimal cut-off and reconstruct the biomarkers into dichotomous variables according to stroke mortality classification. In the univariable logistic regression analysis (Table 5.10), the plasma levels of 13 biomarkers were found to be individually statistically significant associated to stroke severity level classification: haemoglobin ≤ 14 (g/dL), haematocrit ≤ 41.3 (%), platelets ≤ 231 (10^9 /L), neutrophils ≥ 65.5 (%), lymphocytes ≤ 24 (%), prothrombin time ≥ 12.9 (sec), INR ≥ 1 , ALT ≤ 19 (U/L), fasting blood glucose ≥ 101 (mg/dL), cortisol ≥ 441 (nmol/L), NT-proBNP ≥ 271 (pg/mL), IL-6 ≥ 5.65 (pg/mL) and S100 ≥ 63 (pg/mL). Moreover, multivariable logistic regression modelling (Table 5.11), identified that the combination of the two biomarkers panel: NT-proBNP ≥ 271 (pg/mL) (p-value < 0.001 , OR = 6.62), and S100 ≥ 63 (pg/mL) (p-value = 0.005, OR = 2.18) results in 86.9% specificity, 88.4% NPV, 79.9% correctly classified and 77.4% AUC (Figure 12.6).

Table 5.11. Multivariable Logistic Regression for Mortality.

Mortality			
Model II (n = 429)			
Biomarkers	Condition	Adjusted OR (95% CI)	p-value
NT-proBNP (pg/mL)	≥ 271	6.62 (3.76 - 11.66)	<0.001
S100 (pg/mL)	≥ 63	2.18 (1.26 - 3.77)	0.005
Goodness-of-fit test		Chi-squared = 0.25	0.6149
AUC		0.7737	
Classification Table Probability Cut-Off 0.45			
classified	Alive	Dead	Total
positive	46	38	84
negative	305	40	345
Total	351	78	429
sensitivity	48.72%	false positives rate	13.11%
specificity	86.89%	false negatives rate	51.28%
positive predictive value	45.24%	false positives rate	54.76%
negative predictive value	88.41%	false negatives rate	11.59%
correctly classified			79.95%

5.2.4 tPA Admission

The study cohort included 516 stroke patients. The stroke patients were grouped according to tPA admission. Firstly, the descriptive analysis, both baseline characteristics (Table 5.12) and biomarkers (Table 5.13) was conducted. 88.9% of the stroke patients were not administered with tPA, while 11.1% received tPA. There was a statistically significant (p-value < 0.001) difference in the tPA admission frequency between the cases of stroke (100%) and transient ischemic attack (0). All the patients that received tPA suffered from stroke and not from transient ischemic attack. There was a statistically significant difference (p-value = 0.002) in the gender percentage, even though that males were the majority in both groups, also in the stroke patients group that did not receive tPA (72.8%), and in the stroke patients group that did receive tPA (52.6%). Moreover, the stroke severity levels were statistically significant (p-value < 0.001) higher in the stroke patients group that received tPA (16 (8 - 21)), in comparison to the stroke patients group that did not receive tPA (2 (0 - 4)).

Most of the vascular parameters were not statistically significant different between the two groups. Only the smoking frequency was found to be statistically significant (p-value = 0.006) different between the stroke patients that received tPA (29.8%), in comparison

to the stroke patients that did not receive tPA (49%). Interestingly, the modified ranking scale upon admission did not show a statistically significant difference between the two groups. In addition, the stroke location was found to be statistically significant (p -value = 0.028) different between the two groups. The stroke patients that received tPA, suffered more frequently from a stroke event located in the left (51.8%) or right (44.6%) hemisphere. While, the stroke patients that did not receive tPA suffered significantly more frequently from a stroke event in an unknown location (17.1%). Moreover, the stroke syndrome and etiology classification revealed that lacunar etiology was more frequent among the stroke patients group that did not receive tPA (63.6% vs. 10.5%; 53.3% vs. 5.3%), while the other two etiologies (atherosclerosis and cardioembolic) were more frequent in the stroke patients group that received tPA.

Table 5.12. Baseline Characteristics Distribution for tPA Admission Analysis.

Clinical Feature	Tissue Plasminogen Activator			p-Value**
	Total	No	Yes	
Number (%)	516 (100%)	459 (88.9%)	57 (11.1%)	-
Stroke (%)	433 (83.9%)	376 (81.9%)	57 (100%)	<0.001
Transient Ischemic Attack (%)	83 (16.1%)	83 (18.1%)	0.000	<0.001
Males (%)	364 (70.5%)	334 (72.8%)	30 (52.6%)	0.002
Age (years), median (IQR*)	61 (53-71)	61 (53 - 69)	66 (54.5 - 74.5)	0.070
Abdominal Circumference (cm)	90.5 (84-9)	90 (84 - 97)	91 (84 - 96)	0.915
Height (cm)	163 (157 - 169)	163 (157 - 170)	160 (151 - 168)	0.016
Weight (kg)	66 (59.1 - 74.3)	66 (59.5 - 75)	61.8 (57.7 - 71.5)	0.197
Body Mass Index (BMI)	24.8 (22.3-27.7)	24.8 (22.2 - 27.7)	25.4 (23.2 - 27.3)	0.577
Race (%) -				0.159
Chinese	380 (73.6%)	338 (73.6)	42 (73.7%)	-
Malay	92 (17.9%)	80 (17.4%)	12 (21.1%)	-
Indian	37 (7.2%)	36 (7.8%)	1 (1.8%)	-
Others	7 (1.4%)	5 (1.1%)	2 (3.5%)	-
Duration of Symptoms (days), median (IQR*)	12 (3-29)	13 (3 - 31)	8.5 (1.03 - 26.8)	0.287
Duration of Observed Event (days), median (IQR*)	50 (25-59)	50 (25 - 59)	51 (19.5 - 59)	0.839
Stroke Severity, NIHSS Score, median (IQR*)	2 (0.25 - 6)	2 (0 - 4)	16 (8 - 21)	<0.001
Vascular Clinical Features (%) -				
Vascular [#]	125 (24.2%)	114 (24.8%)	11 (19.3%)	0.357
Hypertension	346 (67.1%)	310 (67.5%)	36 (63.2%)	0.507
Diabetes Mellitus	221 (42.9%)	200 (43.6%)	21 (36.8%)	0.333
Hyperlipidaemia	243 (47.1%)	217 (47.3%)	26 (45.6%)	0.813
Chronic Renal Failure	4 (0.8%)	3 (0.7%)	1 (1.8%)	0.371
Previous Stroke	81 (15.7%)	73 (15.9%)	8 (14%)	0.714

Ischemic Heart Disease	90 (17.4%)	75 (16.3%)	15 (26.3%)	0.061
Peripheral Vascular Disease	4 (0.8%)	4 (0.9%)	0.000	0.479
Atrial Fibrillation	32 (6.2%)	26 (5.7%)	6 (10.5%)	0.151
Smoking	242 (46.9%)	225 (49%)	17 (29.8%)	0.006
Modified Ranking Scale -				0.236
No symptoms	471 (91.5%)	419 (91.3%)	53 (93%)	-
No significant disability	15 (2.9%)	15 (3.3%)	0.000	-
Slight disability	11 (2.1%)	8 (1.7%)	3 (5.3%)	-
Moderate disability	14 (2.7%)	13 (2.8%)	1 (1.8%)	-
Moderately severe disability	4 (0.8%)	4 (0.9%)	0.000	-
Severe disability	0.000	0.000	0.000	-
Dead	0.000	0.000	0.000	-
Location (%) -				0.028
Left Hemisphere	186 (41.1%)	157 (39.4%)	29 (51.8%)	-
Right Hemisphere	180 (39.7%)	155 (38.9%)	25 (44.6%)	-
Brainstem	14 (3.1%)	13 (3.3%)	1 (1.8%)	-
Other	5 (1.1%)	5 (1.3%)	0.000	-
Unknown	69 (15.2%)	68 (17.1%)	1 (1.8%)	-
Stroke Syndrome [†] (%) -				<0.001
TACS	57 (11.2%)	28 (6.2%)	29 (50.9%)	-
PACS	101 (19.9%)	82 (18.2%)	19 (33.3%)	-
LACS	292 (57.6%)	286 (63.6%)	6 (10.5%)	-
POCS	55 (10.8%)	52 (11.6%)	3 (5.3%)	-
Undetermined	2 (0.4%)	2 (0.4%)	0.000	-
Stroke Etiology*** (%) -				<0.001
LAA	131 (25.4%)	102 (22.3%)	29 (50.9%)	-
CEI	75 (14.6%)	58 (12.7%)	17 (29.8%)	-
LAC	247 (48%)	244 (53.3%)	3 (5.3%)	-
Undetermined	62 (12%)	54 (11.8%)	8 (14%)	-

* Interquartile Range (IQR): Q1 – Q3. ** Kruskal Wallis H-Test was used to calculate p-value for continues variables, while chi-square test was used for categorical variables (if cell frequency was lower than 5%, fisher's exact test was used). † NIHSS: National Institutes of Health Stroke Scale. # Vascular: either stroke (ischemic / haemorrhage), transient ischemic attack (TIA), Acute myocardial infarction (AMI) or Angina Pectoris. ‡ Stroke syndromes: TACS, total anterior circulation syndrome; LACS, lacunar syndrome; PACS, partial anterior circulation syndrome; POCS, posterior circulation syndrome. *** Stroke Etiology: LAA, large artery atherosclerosis, CEI, cardioembolic, LAC, lacunar.

The biomarkers distribution analysis (Table 5.13) identified several statistically significant differences between the two groups. The plasma levels of several biomarkers were found to be statistically significant different between the stroke patients group that did not receive tPA in comparison to those that did, which included the following: haemoglobin, haematocrit, partial thromboplastin time, triglycerides, HDL, HbA1c, cortisol, NT-proBNP, IL-6, NSE and S100.

Table 5.13. Biomarkers Distribution for tPA Admission Analysis.

Biomarker	N	Tissue Plasminogen Activator			
		Total	No	Yes	P-Value**
White Blood Cells (10 ⁹ /L)	509	8.17 (6.69-9.89)	8.11 (6.67 - 9.88)	8.75 (7.13 - 10.0)	0.193
Haemoglobin (g/dL)	509	14.2 (13-15.4)	14.3 (13 - 15.5)	13.8 (12.9 - 14.9)	0.034
Haematocrit (%)	509	41.8 (38.9-45.1)	42.25 (38.9 - 45.2)	40.8 (38 - 43.78)	0.022
Platelets (10 ⁹ /L)	509	237 (199-281)	239 (201 - 286)	232 (187 - 260)	0.117
Mean Platelet Volume (fL)	505	9.6 (8.28-10.3)	9.6 (8.2 - 10.3)	9.85 (8.38 - 10.5)	0.295
Neutrophils (%)	509	64.1 (56.9-72.2)	64.1 (57.2 - 72.2)	63.9 (55.9 - 74.7)	0.937
Neutrophils (10 ⁹ /L)	508	5.14 (3.87-6.61)	5.12 (3.86 - 6.58)	5.33 (3.9 - 7.52)	0.524
Lymphocytes (%)	464	25.6 (18.9-31.9)	25.8 (19.1 - 31.9)	23.7 (16.7 - 31.8)	0.487
Lymphocytes (10 ⁹ /L)	464	1.95 (1.44-2.57)	1.94 (1.45 - 2.56)	1.95 (1.42 - 2.87)	0.801
Prothrombin Time (sec)	318	12.8 (12.4-13.2)	12.7 (12.4 - 13.2)	12.9 (12.3 - 13.5)	0.670
International Normalized Ratio (INR)	318	0.98 (0.95-1.04)	0.98 (0.95 - 1.03)	0.99 (0.95 - 1.04)	0.459
Partial Thromboplastin Time (sec)	299	28.7 (27-30.8)	29 (27.3 - 31.1)	27.8 (25.6 - 28.8)	<0.001
Total Cholesterol (mg/dL)	485	186 (156-221)	186 (155 - 218)	185 (164 - 228)	0.493
Triglycerides (mg/dL)	485	51.2 (38.2-75.9)	52.6 (39.1 - 80.1)	42.2 (30.6 - 60.5)	0.002
HDL (mg/dL)	485	40.6 (34.8-49.5)	40.2 (34.5 - 48.6)	46.0 (36.7 - 60.3)	0.002
LDL (mg/dL)	471	115 (88.3-144)	115 (88.2 - 144)	113 (88.2 - 150)	0.662
Aspartate Aminotransferase (U/L)	425	22 (18-28)	22 (18 - 28)	23.5 (15.8 - 31)	0.566
Alanine Transaminase (U/L)	424	20 (14-29)	20 (14 - 29)	17 (12 - 26.8)	0.165
Fasting Blood Glucose (mg/dL)	329	95.5 (84.7-114)	93.7 (84.7 - 114)	99.9 (85.6 - 123)	0.515
Glycohaemoglobin, HbA1c (%)	407	6.3 (5.7-7.8)	6.4 (5.7 - 7.9)	6 (5.5 - 6.9)	0.008
Vitamin D (mg/dL)	486	21.5 (14.9-29.2)	21.5 (14.3 - 29.5)	21.9 (16.5 - 26.8)	0.822
Cortisol (nmol/L)	433	419 (268-576)	406 (256 - 553)	620 (437 - 828)	<0.001
NT-proBNP (pg/mL)	432	120 (43.6-503)	113 (38.4 - 443)	286 (65.1 - 1567)	0.002
IL-6 (pg/mL)	432	4.31 (2.08-10.3)	4.21 (2.01 - 8.76)	7.9 (2.98 - 22.18)	0.015
Insulin (pmol/L)	431	54.6 (32.8-98.6)	55.3 (33.6 - 98.1)	49.9 (20.4 - 150)	0.357

NSE (ng/mL)	430	15.1 (11.6-21.4)	14.9 (11.5 - 20.5)	20.3 (14.2 - 44.7)	<0.001
S100 (pg/mL)	428	52 (38-86)	51 (37 - 80)	80.5 (60.3 - 102)	<0.001
Urate (μmol/L)	228	358 (304-437)	358 (304 - 430)	333 (275 - 462)	0.875
Albumin (g/L)	156	40.4 (37.6-43.4)	40.4 (37.8 - 43.5)	39.3 (37.5 - 42.8)	0.730
D-dimer (ng/mL)	156	350 (210-685)	350 (210 - 650)	600 (230 - 1420)	0.274

* Interquartile Range (IQR): Q1 – Q3. ** Kruskal Wallis H-Test was used to calculate p-value.

Table 5.14. Univariable Logistic Regression for tPA Admission.

Tissue Plasminogen Activator								
Variable	n	Logistic Regression			Classification Table			
		Condition	OR (95% CI)	p-value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Biomarkers								
White Blood Cells (10 ⁹ /L)	510	≥8.6	1.52 (0.87 - 2.65)	0.140	51.8	58.6	13.4	90.8
Haemoglobin (g/dL)	510	≤14.1	2.26 (1.26 - 4.05)	0.006	66.1	53.7	14.9	92.8
Haematocrit (%)	510	≤41.3	1.95 (1.1 - 3.43)	0.022	60.7	55.7	14.5	92.0
Platelets (10 ⁹ /L)	510	≤233	1.49 (0.85 - 2.62)	0.159	57.1	52.9	13.0	90.9
Mean Platelet Volume (fL)	506	≥9.8	1.4 (0.8 - 2.45)	0.233	53.6	54.9	12.9	90.5
Neutrophils (%)	510	≤64.2	1.04 (0.59 - 1.81)	0.898	51.8	49.1	11.2	89.2
Neutrophils (10 ⁹ /L)	509	≥5.3	1.27 (0.73 - 2.21)	0.407	51.8	54.1	12.2	90.1
Lymphocytes (%)	465	≤26.4	1.01 (0.57 - 1.81)	0.964	53.9	46.5	11.2	88.9
Lymphocytes (10 ⁹ /L)	465	≥1.9	1.05 (0.59 - 1.88)	0.859	53.9	47.5	11.4	89.1
Prothrombin Time (sec)	319	≥12.8	1.27 (0.69 - 2.31)	0.433	55.8	50.2	17.9	85.4
International Normalized Ratio (INR)	319	≥0.9	1.1 (0.4 - 2.99)	0.850	90.4	10.5	16.4	84.9
Partial Thromboplastin Time (sec)	300	≤28.3	3.33 (1.74 - 6.37)	0.000	67.4	61.8	25.6	90.6
Total Cholesterol (mg/dL)	486	≥185	0.94 (0.53 - 1.65)	0.822	50.0	48.4	10.8	88.6
Triglycerides (mg/dL)	486	≤49.1	2.19 (1.22 - 3.92)	0.009	62.9	56.3	15.3	92.4
HDL (mg/dL)	486	≥43.3	2.19 (1.24 - 3.91)	0.007	59.3	60.2	15.7	92.2
LDL (mg/dL)	472	≥114	0.91 (0.51 - 1.6)	0.732	49.1	48.5	10.7	88.3
Aspartate Aminotransferase (U/L)	426	≥23	1.41 (0.78 - 2.54)	0.258	54.0	54.5	13.6	89.9
Alanine Transaminase (U/L)	425	≤20	1.37 (0.76 - 2.49)	0.297	58.0	49.9	13.4	89.9
Fasting Blood Glucose (mg/dL)	330	≥99.1	1.46 (0.76 - 2.79)	0.252	50.0	59.4	15.2	89.1
Glycohaemoglobin, HbA1c (%)	408	≤6.2	1.98 (1.08 - 3.63)	0.027	62.8	54.1	16.3	91.0
Vitamin D (mg/dL)	487	≥21.9	1.16 (0.66 - 2.05)	0.610	50.9	52.8	11.6	89.8

Cortisol (nmol/L)	434	≥529	5.63 (2.89 - 10.96)	0.000	69.6	71.1	22.2	95.2
NT-proBNP (pg/mL)	433	≥195	2.42 (1.3 - 4.52)	0.005	58.7	63.1	15.9	92.8
IL-6 (pg/mL)	433	≥5.4	1.97 (1.06 - 3.65)	0.032	56.5	60.2	14.4	92.1
Insulin (pmol/L)	432	≤50.7	1.38 (0.75 - 2.54)	0.307	54.4	53.6	12.3	90.8
NSE (ng/mL)	431	≥16.8	2.7 (1.43 - 5.09)	0.002	63.0	61.3	16.3	93.3
S100 (pg/mL)	429	≥65	4.45 (2.29 - 8.63)	0.000	69.6	66.1	19.8	94.8
Urate (μmol/L)	228	≤333	1.85 (0.8 - 4.26)	0.149	52.0	63.1	14.8	91.4
Albumin (g/L)	156	≤39.4	2.08 (0.712 - 6.07)	0.180	53.3	64.5	13.8	92.9
D-dimer (ng/mL)	156	≥370	1.61 (0.54 - 4.76)	0.389	60.0	51.8	11.7	92.4

Univariable logistic regression analysis was conducted in order to identify the biomarkers classification performance of tPA admission. First, the biomarkers diagnostic performance was examined with ROC analysis (Table 12.14), in order to determine the biomarkers optimal cut-off and reconstruct the biomarkers into dichotomous variables according to tPA admission classification. In the univariable logistic regression analysis (Table 5.14), the plasma levels of 11 biomarkers were found to be individually statistically significant associated to tPA admission classification: haemoglobin ≤ 14.1 (g/dL), haematocrit ≤ 41.3 (%), partial thromboplastin time ≤ 28.3 (sec), triglycerides ≤ 49.1 (mg/dL), HDL ≥ 43.3 (mg/dL), HbA1c ≤ 6.2 (%), cortisol ≥ 529 (nmol/L), NT-proBNP ≥ 195 (pg/mL), IL-6 ≥ 5.4 (pg/mL), NSE ≥ 16.8 (ng/mL) and S100 ≥ 65 (pg/mL). Moreover, multivariable logistic regression modelling (Table 5.15), identified that the combination of the three biomarkers panel: HDL ≥ 43.3 (mg/dL) (p-value = 0.050, OR = 1.95), cortisol ≥ 529 (nmol/L) (p-value < 0.001, OR = 4.09) and S100 ≥ 65 (pg/mL) (p-value = 0.001, OR = 3.18) results in 95.1% specificity, 92.3% NPV, 88.6% correctly classified and 76.7% AUC (Figure 12.7).

Table 5.15. Multivariable Logistic Regression for tPA Admission.

Tissue Plasminogen Activator			
Model II (n = 411)			
Biomarkers	Condition	Adjusted OR (95% CI)	p-value
HDL (mg/dL)	≥43.3	1.95 (1.00 - 3.80)	0.050
Cortisol (nmol/L)	≥529.1	4.09 (2.04 - 8.23)	<0.001
S100 (pg/mL)	≥65	3.18 (1.58 - 6.41)	0.001
Goodness-of-fit test		Chi-squared = 2.56	0.6338
AUC		0.7668	
Classification Table Probability Cut-Off 0.35			
classified	No	Yes	Total
positive	18	15	33
negative	349	29	378
Total	367	44	411
sensitivity	34.09%	false positives rate	4.90%

specificity	95.10%	false negatives rate	65.91%
positive predictive value	45.45%	false positives rate	54.55%
negative predictive value	92.33%	false negatives rate	7.67%
correctly classified			88.56%

5.2.5 Ischemic Stroke vs. TIA

The study cohort included 516 stroke patients. The stroke patients were grouped according to ischemic stroke and transient ischemic attack patients groups. Firstly, the descriptive analysis, both baseline characteristics (Table 5.16) and biomarkers (Table 5.17) was conducted. 83.9% of the patients suffered from ischemic stroke, while 16.1% suffered from transient ischemic attack. There were no statistically significant differences between the two groups in the demographic parameters, such as gender, age and race. A statistically significant differences were identified between the two groups in the three main lifestyle parameters: abdominal circumference (p-value = 0.026), height (p-value = 0.037) and weight (p-value = 0.021). Moreover, the stroke severity levels were statistically significant (p-value < 0.001) higher in the ischemic stroke patients group (3 (1 - 6)), in comparison to the transient ischemic attack patients group (0 (0 - 2)).

Most of the vascular parameters were not statistically significant different between the two groups. Only the diabetes mellitus frequency was found to be statistically significant (p-value < 0.001) different between the ischemic stroke patients (46.2%), in comparison to the transient ischemic attack patients (25.3%). Interestingly, the modified ranking scale upon admission did not show a statistically significant difference between the two groups. In addition, the stroke location was found to be statistically significant (p-value < 0.001) different between the two groups. As expected, the ischemic stroke patients, suffered more frequently from a stroke event located in the left (45.6%) or right (43.8%) hemisphere. While, the transient ischemic attack patients suffered significantly more frequently from a stroke event in an unknown location (74.6%).

Table 5.16. Baseline Characteristics Distribution between IS vs. TIA.

Clinical Feature	Ischemic Stroke (IS) Vs. Transient Ischemic Attack (TIA)			p-Value**
	Total	Transient Ischemic Attack	Ischemic Stroke	
Number (%)	516 (100%)	83 (16.1%)	433 (83.9%)	-
Males (%)	364 (70.5%)	62 (74.7%)	302 (69.7%)	0.365
Age (years), median (IQR*)	61 (53-71)	58 (51 - 66)	61 (53 - 71)	0.090
Abdominal Circumference (cm)	90.50 (84-97)	92 (87 - 99)	90 (84 - 97)	0.026
Height (cm)	163 (157 - 169)	164 (160 - 170)	163 (156 - 169)	0.037
Weight (kg)	66 (59.1 - 74.3)	68.7 (61.1 - 78.5)	65.2 (58.4 - 73.1)	0.021
Body Mass Index (BMI)	24.8 (22.3-27.7)	25.1 (22.9 - 29.1)	24.8 (22.2 - 27.4)	0.129
Race (%) -				0.081
Chinese	380 (73.6%)	58 (69.9%)	322 (74.4%)	-
Malay	92 (17.9%)	12 (14.5%)	80 (18.5%)	-
Indian	37 (7.2%)	11 (13.3%)	26 (6%)	-
Others	7 (1.4%)	2 (2.4%)	5 (1.2%)	-
Duration of Symptoms (days), median (IQR*)	12 (3-29)	12 (8 - 36)	12.5 (3 - 28)	0.511
Duration of Observed Event (days), median (IQR*)	50 (25-59)	51 (33 - 59)	50 (23 - 59.5)	0.681
Stroke Severity, NIHSS Score, median (IQR*)	2 (0.25 - 6)	0 (0 - 2)	3 (1 - 6)	<0.001
Tissue Plasminogen Activator (%)	57 (11.1%)	0	57 (13.2%)	0.000
Mortality (%)	92 (17.8%)	12 (14.5%)	80 (18.5%)	0.381
Vascular Clinical Features (%) -				
Vascular [#]	125 (24.2%)	16 (19.3%)	109 (25.2%)	0.251
Hypertension	346 (67.1%)	60 (72.3%)	286 (66.1%)	0.268
Diabetes Mellitus	221 (42.9%)	21 (25.3%)	200 (46.2%)	<0.001
Hyperlipidaemia	243 (47.1%)	37 (44.6%)	206 (47.6%)	0.616
Chronic Renal Failure	4 (0.8%)	0	4 (0.9%)	0.379
Previous Stroke	81 (15.7%)	10 (12%)	71 (16.4%)	0.318
Ischemic Heart Disease	90 (17.4%)	14 (16.9%)	76 (17.6%)	0.880
Peripheral Vascular Disease	4 (0.8%)	1 (1.2%)	3 (0.7%)	0.626
Atrial Fibrillation	32 (6.2%)	4 (4.8%)	28 (6.5%)	0.569
Smoking	242 (46.9%)	43 (51.8%)	199 (46%)	0.328
Modified Ranking Scale -				0.492
No symptoms	471 (91.5%)	79 (95.2%)	393 (90.8%)	-
No significant disability	15 (2.9%)	2 (2.4%)	13 (3%)	-
Slight disability	11 (2.1%)	0.000	11 (2.5%)	-
Moderate disability	14 (2.7%)	1 (1.2%)	13 (3%)	-
Moderately severe disability	4 (0.8%)	1 (1.2%)	3 (0.7%)	-
Severe disability	0.000	0.000	0.000	-
Dead	0.000	0.000	0.000	-
Location (%) -				<0.001
Left Hemisphere	186 (41.1%)	6 (10.2%)	180 (45.6%)	-
Right Hemisphere	180 (39.7%)	7 (11.9%)	173 (43.8%)	-
Brainstem	14 (3.1%)	1 (1.7%)	13 (3.3%)	-

Other	5 (1.1%)	1 (1.7%)	4 (1%)	-
Unknown	69 (15.2%)	44 (74.6%)	25 (6.3%)	-

* Interquartile Range (IQR): Q1 – Q3. ** Kruskal Wallis H-Test was used to calculate p-value for continues variables, while chi-square test was used for categorical variables (if cell frequency was lower than 5%, fisher's exact test was used). † NIHSS: National Institutes of Health Stroke Scale. # Vascular: either stroke (ischemic / haemorrhage), transient ischemic attack (TIA), Acute myocardial infarction (AMI) or Angina Pectoris.

The biomarkers distribution analysis (Table 5.17) identified several statistically significant differences between the two groups. The plasma levels of several biomarkers were found to be statistically significant different between the ischemic stroke patients group in comparison to those that suffered a transient ischemic attack, which included the following: neutrophils, lymphocytes, prothrombin time, INR, triglycerides, fasting blood glucose, HbA1c, cortisol, NT-proBNP, S100 and urate.

Table 5.17. Biomarkers Distribution between IS vs. TIA.

Biomarker	N	Ischemic Stroke (IS) Vs. Transient Ischemic Attack (TIA)			
		Total	Transient Ischemic Attack	Ischemic Stroke	p-Value**
White Blood Cells (10 ⁹ /L)	509	8.17 (6.69-9.89)	7.79 (6.59 - 8.93)	8.24 (6.71 - 9.93)	0.064
Haemoglobin (g/dL)	509	14.2 (13-15.4)	14.6 (13.5 - 15.5)	14.1 (13 - 15.4)	0.216
Haematocrit (%)	509	41.8 (38.9-45.1)	42.4 (39.9 - 44.9)	41.8 (38.4 - 45.2)	0.300
Platelets (10 ⁹ /L)	509	237 (199-281)	223 (201 - 277)	237 (199 - 286)	0.514
Mean Platelet Volume (fL)	505	9.6 (8.28-10.3)	9.80 (8.90 - 10.3)	9.6 (8.00 - 10.3)	0.194
Neutrophils (%)	509	64.1 (56.9-72.2)	62.2 (52.1 - 68.3)	64.8 (57.3 - 73.3)	0.004
Neutrophils (10 ⁹ /L)	508	5.14 (3.87-6.61)	4.68 (3.73 - 5.79)	5.23 (3.98 - 6.91)	0.006
Lymphocytes (%)	464	25.6 (18.9-31.9)	27.8 (22.0 - 36.1)	24.7 (18.4 - 31.2)	0.003
Lymphocytes (10 ⁹ /L)	464	1.95 (1.44-2.57)	2.11 (1.60 - 2.77)	1.90 (1.41 - 2.47)	0.023
Prothrombin Time (sec)	318	12.8 (12.4-13.2)	12.6 (12.1 - 12.8)	12.8 (12.4 - 13.3)	0.002
International Normalized Ratio (INR)	318	0.98 (0.95-1.04)	0.96 (0.93 - 1.01)	0.99 (0.95 - 1.04)	0.020
Partial Thromboplastin Time (sec)	299	28.7 (27-30.8)	28.9 (28.2 - 30.4)	28.7 (26.9 - 30.9)	0.362
Total Cholesterol (mg/dL)	485	186 (156-221)	196 (162 - 222)	185 (154 - 220)	0.169
Triglycerides (mg/dL)	485	51.2 (38.2-75.9)	59.6 (46.0 - 86.4)	49.5 (36.5 - 73.7)	0.004
HDL (mg/dL)	485	40.6 (34.8-49.5)	39.4 (34.6 - 48.3)	40.9 (34.8 - 49.7)	0.432

LDL (mg/dL)	471	115 (88.3-144)	119 (95.9 - 145)	114 (87 - 143)	0.277
Aspartate Aminotransferase (U/L)	425	22 (18-28)	23 (19 - 27)	22 (18 - 28)	0.457
Alanine Transaminase (U/L)	424	20 (14-29)	22 (15 - 30)	20 (14 - 28)	0.083
Fasting Blood Glucose (mg/dL)	329	95.5 (84.7-114)	91.9 (83.8 - 101)	97.3 (86.5 - 121)	0.028
Glycohaemoglobin, HbA1c (%)	407	6.3 (5.7-7.8)	6 (5.63 - 6.98)	6.4 (5.7 - 8.08)	0.033
Vitamin D (mg/dL)	486	21.5 (14.9-29.2)	23.0 (16.0 - 30.6)	21.2 (14.5 - 28.9)	0.185
Cortisol (nmol/L)	433	419 (268-576)	373 (205 - 481)	425 (281 - 589)	0.002
NT-proBNP (pg/mL)	432	120 (43.6-503)	66.7 (22.2 - 263)	125 (48.8 - 624)	0.002
IL-6 (pg/mL)	432	4.31 (2.08-10.33)	4.28 (1.85 - 7.89)	4.44 (2.14 - 11.37)	0.313
Insulin (pmol/L)	431	54.6 (32.8-98.6)	60.6 (38.3 - 107)	52.2 (31.9 - 98.3)	0.165
NSE (ng/mL)	430	15.1 (11.6-21.4)	15.7 (12.2 - 22.9)	14.9 (11.5 - 21.2)	0.325
S100 (pg/mL)	428	52 (38-86)	42 (34 - 57)	57 (40 - 90)	<0.001
Urate (μ mol/L)	228	358 (304-437)	381 (337 - 490)	352 (301 - 429)	0.022
Albumin (g/L)	156	40.4 (37.6-43.4)	41.5 (37.2 - 44.1)	40.4 (37.7 - 43.2)	0.466
D-dimer (ng/mL)	156	350 (210-685)	340 (223 - 645)	370 (200 - 705)	0.776

* Interquartile Range (IQR): Q1 – Q3. ** Kruskal Wallis H-Test was used to calculate p-value.

Univariable logistic regression analysis was conducted in order to identify the biomarkers classification performance of ischemic stroke vs. transient ischemic attack. First, the biomarkers diagnostic performance was examined with ROC analysis (Table 12.15), in order to determine the biomarkers optimal cut-off and reconstruct the biomarkers into dichotomous variables according to ischemic stroke vs. transient ischemic attack classification. In the univariable logistic regression analysis (Table 5.18), the plasma levels of 8 biomarkers were found to be individually statistically significant associated to ischemic stroke vs. transient ischemic attack classification: prothrombin time ≥ 12.7 (sec), INR ≥ 0.9 , total cholesterol ≤ 192 (mg/dL), triglycerides ≤ 54.9 (mg/dL), fasting blood glucose ≥ 93.7 (mg/dL), HbA1c ≥ 6.2 (%), cortisol ≥ 387 (nmol/L) and S100 ≥ 49 (pg/mL). Moreover, multivariable logistic regression modelling (Table 5.19), identified that the combination of the four biomarkers panel: platelets ≥ 224 ($10^9/L$) (p-value = 0.005, OR = 2.22), triglycerides ≤ 54.9 (mg/dL) (p-value = 0.003, OR = 2.35), cortisol ≥ 387 (nmol/L) (p-value = 0.006, OR = 2.19) and S100 ≥ 49 (pg/mL) (p-value = 0.018, OR = 1.97) results in 98.5% sensitivity, 85.5% PPV, 84.7% correctly classified and 68.8% AUC (Figure 12.8).

Table 5.18. Univariable Logistic Regression between IS vs. TIA.

Ischemic Stroke (IS) Vs. Transient Ischemic Attack (TIA)								
Variable	n	Logistic Regression			Classification Table			
		Condition	OR (95% CI)	p-value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Biomarkers								
White Blood Cells (10 ⁹ /L)	510	≥7.9	1.3 (0.81 - 2.09)	0.273	54.8	51.9	85.8	17.8
Haemoglobin (g/dL)	510	≤14.5	1.34 (0.83 - 2.15)	0.229	56.6	50.6	85.9	18.1
Haematocrit (%)	510	≤42.3	1.22 (0.76 - 1.96)	0.415	54.3	50.6	85.4	17.3
Platelets (10 ⁹ /L)	510	≥224	1.52 (0.94 - 2.44)	0.087	59.7	50.6	86.45	19.2
Mean Platelet Volume (fL)	506	≤9.8	1.13 (0.69 - 1.84)	0.619	59.9	43.0	85.1	16.6
Neutrophils (%)	510	≥63.1	1.48 (0.92 - 2.39)	0.106	55.5	54.3	86.6	18.7
Neutrophils (10 ⁹ /L)	509	≥5	1.57 (0.97 - 2.53)	0.066	55.6	55.6	86.9	19.2
Lymphocytes (%)	465	≤26.6	1.58 (0.96 - 2.62)	0.073	56.6	54.8	87.1	19.1
Lymphocytes (10 ⁹ /L)	465	≤2	1.58 (0.95 - 2.62)	0.076	53.8	57.5	87.2	18.8
Prothrombin Time (sec)	319	≥12.7	2.45 (1.26 - 4.74)	0.008	62.5	59.5	91.1	19.4
International Normalized Ratio (INR)	319	≥0.9	2.88 (1.23 - 6.71)	0.015	91.3	21.4	88.5	27.3
Partial Thromboplastin Time (sec)	300	≤28.9	0.99 (0.5 - 1.99)	0.994	53.9	45.9	87.7	12.3
Total Cholesterol (mg/dL)	486	≤192	1.66 (1.02 - 2.71)	0.043	56.7	55.8	87.2	19.6
Triglycerides (mg/dL)	486	≤54.9	1.99 (1.22 - 3.27)	0.006	58.7	58.4	88.2	21.0
HDL (mg/dL)	486	≥40.2	1.42 (0.87 - 2.32)	0.156	54.3	54.6	86.4	18.3
LDL (mg/dL)	472	≤117	1.22 (0.74 - 2.01)	0.438	52.9	52.1	85.8	16.8
Aspartate Aminotransferase (U/L)	426	≤23	1.03 (0.59 - 1.79)	0.917	59.7	40.9	85.8	14.5
Alanine Transaminase (U/L)	425	≤22	1.4 (0.81 - 2.41)	0.225	60.7	47.5	87.4	16.9
Fasting Blood Glucose (mg/dL)	330	≥93.7	1.88 (1.06 - 3.32)	0.031	53.2	62.3	86.1	23.2
Glycohaemoglobin, HbA1c (%)	408	≥6.2	1.75 (1 - 3.04)	0.048	57.2	56.7	88.4	18.6
Vitamin D (mg/dL)	487	≤22.6	1.49 (0.92 - 2.43)	0.102	56.9	53.2	86.3	19.3
Cortisol (nmol/L)	434	≥387	1.87 (1.12 - 3.13)	0.016	59.2	56.3	87.4	21.3
NT-proBNP (pg/mL)	433	≥98.1	1.54 (0.92 - 2.57)	0.099	55.8	54.9	86.3	19.6
IL-6 (pg/mL)	433	≥4.3	1.12 (0.68 - 1.87)	0.650	50.8	52.1	84.4	17.2
Insulin (pmol/L)	432	≤58.8	1.38 (0.83 - 2.3)	0.213	54.6	53.5	85.7	18.8
NSE (ng/mL)	431	≤15.6	1.2 (0.72 - 2)	0.479	53.9	50.7	84.7	17.8
S100 (pg/mL)	429	≥49	2.29 (1.36 - 3.87)	0.002	60.5	60.0	88.6	22.8
Urate (μmol/L)	228	≤379	1.53 (0.74 - 3.15)	0.250	59.1	51.4	87.0	18.6
Albumin (g/L)	156	≤41.2	1.71 (0.71 - 4.09)	0.231	59.1	54.2	87.6	19.4
D-dimer (ng/mL)	156	≥350	1.29 (0.54 - 3.09)	0.562	52.3	54.2	86.3	17.1

Table 5.19. Multivariable Logistic Regression between IS vs. TIA.

Ischemic Stroke (IS) Vs. Transient Ischemic Attack (TIA)			
Model II (n = 406)			
Biomarkers	Condition	Adjusted OR (95% CI)	p-value
Platelets (10 ⁹ /L)	≥224	2.22 (1.27 - 3.89)	0.005
Triglycerides (mg/dL)	≤54.9	2.35 (1.34 - 4.12)	0.003

Cortisol (nmol/L)	≥ 387	2.19 (1.25 - 3.84)	0.006
S100 (pg/mL)	≥ 49	1.97 (1.13 - 3.46)	0.018
Goodness-of-fit test		Chi-squared = 10.60	0.477
AUC		0.6879	
Classification Table Probability Cut-Off 0.55			
classified	No	Yes	Total
positive	335	57	392
negative	5	9	14
Total	340	66	406
sensitivity	98.53%	false positives rate	86.36%
specificity	13.64%	false negatives rate	1.47%
positive predictive value	85.46%	false positives rate	14.54%
negative predictive value	64.29%	false negatives rate	35.71%
correctly classified			84.73%

5.3 Conclusions

Different biomarkers were examined for their classification contribution with additional value in stroke management. Stroke diagnosis multivariable logistic regression modelling identified that the combination of the two biomarkers panel: NT-proBNP ≥ 0.064 (ng/mL) (p-value < 0.001; OR = 2.905) and insulin ≤ 23.4 (pmol/L) (p-value = 0.026; OR = 1.934), results in 85.2% sensitivity, 90.9% PPV, 79.2% correctly classified and 67.3% AUC. Moreover, stroke severity multivariable logistic regression modelling identified that the combination of the five biomarkers panel: HDL ≥ 43.3 (mg/dL) (p-value = 0.046, OR = 2.53), AST ≥ 25 (U/L) (p-value < 0.001, OR = 6.25), cortisol ≥ 529 (nmol/L) (p-value = 0.034, OR = 2.72), IL-6 ≥ 7.7 (pg/mL) (p-value = 0.001, OR = 4.72), and S100 ≥ 71 (pg/mL) (p-value = 0.005, OR = 3.96) results in 96.9% specificity, 94.7% NPV, 92.2% correctly classified and 88.5% AUC. In addition, stroke mortality multivariable logistic regression modelling identified that the combination of the two biomarkers panel: NT-proBNP ≥ 271 (pg/mL) (p-value < 0.001, OR = 6.62), and S100 ≥ 63 (pg/mL) (p-value = 0.005, OR = 2.18) results in 86.9% specificity, 88.4% NPV, 79.9% correctly classified and 77.4% AUC. Also, tPA admission multivariable logistic regression modelling identified that the combination of the three biomarkers panel: HDL ≥ 43.3 (mg/dL) (p-value = 0.050, OR = 1.95), cortisol ≥ 529 (nmol/L) (p-value < 0.001, OR = 4.09) and S100 ≥ 65 (pg/mL) (p-value = 0.001, OR = 3.18) results in 95.1% specificity, 92.3% NPV, 88.6% correctly classified and 76.7% AUC. Additionally, ischemic stroke vs. TIA multivariable logistic regression modelling identified that the combination of the four

biomarkers panel: platelets ≥ 224 ($10^9/L$) (p-value = 0.005, OR = 2.22), triglycerides ≤ 54.9 (mg/dL) (p-value = 0.003, OR = 2.35), cortisol ≥ 387 (nmol/L) (p-value = 0.006, OR = 2.19) and S100 ≥ 49 (pg/mL) (p-value = 0.018, OR = 1.97) results in 98.5% sensitivity, 85.5% PPV, 84.7% correctly classified and 68.8% AUC. To conclude, four biomarkers, NT-proBNP, HDL, cortisol and S100, appeared more than once for additional value in stroke management (Table 5.20). HDL and cortisol are common biomarkers for different diseases, while NT-proBNP and S100 are more specific to cardiovascular and neuronal diseases respectively.

Table 5.20. Summary Biomarkers with Additional Value in Stroke Management.

Stroke Application	Diagnosis	Severity	Mortality	tPA Admission	Ischemic Stroke vs. TIA
Multivariable Model	NT-proBNP ≥ 0.064 (ng/mL) OR=2.905 Insulin ≤ 23.4 (pmol/L) OR=1.934	HDL ≥ 43.3 (mg/dL) OR=2.53 AST ≥ 25 (U/L) OR=6.25 Cortisol ≥ 529 (nmol/L) OR=2.72 IL-6 ≥ 7.7 (pg/mL) OR=4.72 S100 ≥ 71 (pg/mL) (p-value=0.005, OR=3.96)	NT-proBNP ≥ 271 (pg/mL) OR=6.62 S100 ≥ 63 (pg/mL) OR=2.18	HDL ≥ 43.3 (mg/dL) OR=1.95 cortisol ≥ 529 (nmol/L) OR=4.09 S100 ≥ 65 (pg/mL) OR=3.18	Platelets ≥ 224 ($10^9/L$) OR=2.22 Triglycerides ≤ 54.9 (mg/dL) OR=2.35 Cortisol ≥ 387 (nmol/L) OR=2.19 S100 ≥ 49 (pg/mL) OR=1.97
Classification Results	85.2% sensitivity 90.9% PPV 79.2% correctly classified 67.3% AUC	96.9% specificity 94.7% NPV 92.2% correctly classified 88.5% AUC	86.9% specificity 88.4% NPV 79.9% correctly classified 77.4% AUC	95.1% specificity 92.3% NPV 88.6% correctly classified 76.7% AUC	98.5% sensitivity 85.5% PPV 84.7% correctly classified 68.8% AUC

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Chapter 6. Dissolvable Polyvinyl-Alcohol Film

Integrating a dissolvable membrane into a sensor allows the control of sample flow in critical areas. These time-barrier films stop the flow of samples until the membrane has dissolved, thus, for example, allow increased exposure time between immunoreagents for the formation of greater numbers of immuno-complexes, ensuring higher sensitivity, reactivity and helping to reduce false-positive signals. In this study, dissolvable polyvinyl alcohol (PVA) films are used in a 3D-printed sensor holder, which enables film integration without the use of glue. PVA is a synthetic hydrophilic linear polymer, its solubility is dependent on its molecular weight and degree of hydrolysis. Three types of PVAs films were tested herein: (1) PVA 1 - Mw: 30-70K, 87-90% hydrolyzed; (2) PVA 2 - Mw: 31-50K, 98-99% hydrolyzed and (3) PVA 3 - Mw: 89-98K, >99% hydrolyzed. The films were exposed to water in (1) the novel 3D-printed holder and (2) directly immersed into a water droplet. After comparing the time taken to dissolve PVA 1-3 films, PVA 1 films of 5% - 20% (w/v) are found to be most suitable as time barrier films, due to their optimal dissolution times and physical properties for integration into the customized 3D-printed holder.

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6.1 Introduction

Integrating a dissolvable membrane into a sensor allows the control of sample flow in critical areas. These time-barrier films stem the flow of samples until the membrane has dissolved, thus allowing increased exposure time between immunoreagents for the formation of more immuno-complexes. This helps to ensure higher sensitivity, reactivity and in some cases reduces false-positive signals ^[2-5]. The ideal material to function as a time-barrier film should be stable in dry form but still dissolves easily upon wetting to allow the flow of the sample. A recent study demonstrated the use of a polyacrylamide-based dissolvable membrane in a microfluidic chemical and biological sensor. In the presence of the sample, the membrane dissolves and permits the sample flow into interdigitated electrodes. This results in a resistance change between the electrodes and transduces the chemical event into an electrical signal ^[6]. However, for such dissolvable membranes to be employed in high-speed sensing technologies in the medical field, the total operation time of the device should be approximately 30 minutes or less. This should include the time taken for the time-barrier films to dissolve, subsequent protein-antibody reaction and signal testing. As a result, the time taken for the time-barrier films to dissolve should ideally be within the range of 10-15 minutes ^[7].

Dissolvable films contain a water-soluble polymer, such as hydroxyl-propyl-methyl-cellulose, polyvinyl-pyrrolidone, polyvinyl-alcohol, carboxy-methyl-cellulose, polyethylene-oxide, hydroxyl-propyl-cellulose, hydroxyl-ethyl-cellulose, methyl-cellulose, pullulan, gelatin, pectin, sodium alginate, maltodextrin, polymerized rosin and xanthan ^[8]. The polymer of choice is usually selected based on its physical characterization as well as its rate of dissolution, which is inversely related to its molecular weight. Moreover, plasticizers, such as glycerol, propylene glycol, poly (ethylene glycol), glycerin, dimethyl; dicetyl; dibutyle phthalate, triacetin, castor oil, citrate ether and tryethyle citrate are added for the control of the film mechanical properties ^[9]. In addition, there are two main methods for dissolvable films preparation: solvent-casting and hot-melt extrusion. In the solvent-casting method, the polymer is dissolved together with the plasticizer in a selected solvent (usually water or ethanol), which is later spread out using casting technique and drying.

This method is ideal for heat-sensitive materials, however, can contain trace amounts of the solvent used. In the hot-melt extrusion method, the dry form of the ingredients is heated and extruded until they are mixed. Then, the melted material is forced through an extrusion die to form the desired shape. In this method there is no addition of solvent material, however the material can go through thermal degradation due to the high temperature used [10, 11].

In this study, a PVA film inside a 3D-printed sensor holder was employed to control sample flow and the time taken for dissolution of the film was measured. Polyvinyl alcohol (PVA) is a synthetic hydrophilic linear polymer which forms copolymers of vinyl alcohol and vinyl acetate. PVA was first synthesized by Hermann and Haehnel in 1924 by hydrolysing polyvinyl acetate in ethanol with potassium hydroxide [12]. Since vinyl alcohol is unstable and rapidly tautomerizes into acetaldehyde, PVA is commercially produced via the hydrolysis of Polyvinyl Acetate. As such, the physical and structural properties of PVA are dependent on the extent of polymerization and hydrolysis. Esterification, etherification, crystallization, ion-polymer complexation in the polymer can be caused by changes in these factors [13]. The unique properties of these modified PVA structures, such as their adhesiveness, strength, film forming, biocompatibility, swelling, safety, and non-carcinogenicity, are beneficial in different industries including textile, paper, adhesives, food, biomedical and pharmaceutical in particular [14].

PVA is widely used as a non-protein blocking agent in immunoassays to saturate the unoccupied sites on the solid phase. This prevents non-specific binding of the immune-reactants, since it does not get contaminated by interfering proteins like immunoglobulins or endogenous peroxidases [15-19]. Moreover, PVA is widely used as a humidity-sensing material. The hydrophilic functional groups in PVA give the polymer its high affinity to water. They absorb the moisture in the air, which changes the water content in the PVA film [20-23]. In addition, since PVA is a water-soluble polymer, it is also widely used in the creation of biodegradable packaging. Plasticizers, such as glycerol, are added for increased flexibility and reduced brittleness, whereas surfactants, such as tween 20, are added to enhance the plasticizer function [24]. Together with reagents such as chitosan, cellulose and

cross linking reagents, a biodegradable film which is highly dissolvable in water is created [25-31]. PVA is highly soluble in water as it comprises a large amount of hydroxyl groups, which interact with the water molecules through hydrogen bonds. The PVA solubility depends on its degree of hydrolysis and molecular weight [32]. The degree of hydrolysis of the PVA is inversely proportional to its solubility. PVA with a high degree of hydrolysis will be less soluble in water due to inter- and intra- molecular H-bonds that form between the hydroxyl groups of the PVA molecules. With higher degree of hydrolysis, there will be greater interactions amongst the PVA molecules than with the water molecules, thus PVA solubility is reduced. Conversely, with a lower degree of hydrolysis, inter- and intra-molecular H-bonds are reduced due to steric hindrance from a large quantity of hydrophobic acetate groups. This helps to increase interactions between PVA molecules and water molecules, thus boosting solubility. Similarly, PVA with lower molecular weights will dissolve faster in water over higher molecular weights as they exhibit lesser intramolecular interaction [33]. Moreover, with the recent emergence of 3D printing technology, it has become possible to rapidly and easily produce customized objects. 3D printing has allowed for constant design-build-test cycles for objects ranging from microfluidic chips to smartphone interfaces, which are complements to sensors applications [34]. This technology has allowed the creation of a prototype that can securely hold the PVA film for it to function as a time barrier film inside a paper-based sensor casing, without the use of glue.

6.2 Materials and Methods

6.2.1 Materials

PVA 1, Mw: 30-70K, 87-90% hydrolyzed (Cat. 8136), PVA 2, Mw: 31-50K, 98-99% hydrolyzed (Cat. 363138), PVA 3, Mw: 89-98K, > 99% hydrolyzed (Cat.341584), Glycerol (Cat. G6279) and Tween20 (Cat. P7949) were purchased from Sigma-Aldrich. Sample (Cat. GFB-R4), absorbent (Cat. AP-080) and conjugate release matrix (Cat. PT-R5) pads were purchased from Advanced Microdevices Pvt. Ltd (India). Nitrocellulose membrane 0.45 μm (Cat. No. 1620115) was purchased from Bio-Rad. Parafilm “M” was purchased from Bemis.

6.2.2 Equipment

Balance was purchased from Mettler Toledo. Hotplate (WH220PLUS) and Binder oven were purchased from Gaia Science Pte Ltd. Film cutter (PAT/RE38, 219 & D613, 795) was purchased from EK. Digital Vernier Caliper was purchased from Kincrome. PowerBlast was purchased from Balco UK for objet Geometries Limited.

6.2.3 Preparation of Films

Using casting evaporated technique, the PVA films were made from aqueous polymer solutions. PVA was first weighed, and then added into deionized water according to the required concentration. For the preparation of 1% PVA, 0.1g PVA were added slowly into 10 mL of deionized (DI) water. Once added, the solution was mixed on a hotplate at 250 rpm and 80°C for 1 hour to obtain a homogenous solution. The PVA solution was heated up to 80°C in order to break up the crystal structure and thus allow it to dissolve. Then, 10 mL of the homogenous PVA solution was placed in a disposable plastic petri dish and placed into the oven at 37°C for 22 hours. The PVA solution volume was also varied and the following were tested: 10 mL, 15 mL and 20 mL.

6.2.4 3D-Printing of Customized Holder

The customized 3D-printed holder was modelled using Autodesk Fusion 360 software and printed using Stratasys Eden 260 Inkjet 3D Printer, using VeroClear and Sup705 as supporting material. Once printed, the prototypes were washed using PowerBlast to remove the excess support material.

6.2.5 Determination of Dissolution Time in Water

In order to eliminate factors such as, breaking or tearing of film and poor exposure of film to sample within the 3D-printed holder, the dissolution time of the tested PVA 1-3 films was first determined under standard conditions. Each tested PVA film was cut into small circles of 6 mm diameter then immersed into a 100 μ L DI-water droplet on a parafilm. The films were visually and physically examined to determine the time for dissolution.

6.2.6 Determination of Dissolution Time in 3D-Printed Holder

PVA 1-3 films were also integrated into customized 3D-printed holders and the time taken for the films to dissolve inside the holder was determined. First, an absorption pad was cut into 5 mm x 20 mm and placed in the designated rectangle at the bottom of the holder. Then, each tested PVA film was cut into a 15 mm x 15 mm square which is placed between the top and bottom components of the 3D-printed holder, exactly on the ring of the bottom of the holder (Figure 6.1). After the tested PVA film was integrated into the holder an ink sample was then added through the top entrance of the holder. The dissolution time of each tested film was measured after observing the visible flow of the ink sample into the absorption pad (Figure 6.2). Since reagent-containing membranes are a common component of many sensors, the effect of such membranes on the dissolution time was also tested by adding sample pads above the PVA film before addition of the ink sample.

6.2.7 Characterization of Film Weight and Thickness

The thickness of the tested PVA films was measured using a Digital Vernier Caliper. The weight of the tested PVA films was measured after the films were cut into 15 mm x 15 mm squares.

Figure 6.1. Schematic Description of Customized 3D-Printed PVA Film Holder.

A) Top Part: outer circle with 13 mm diameter and inner circle with 8 mm diameter. B) Bottom Part: ring thickness of 1 mm. C) PVA film (15 mm x 15 mm) integration and sample addition.

Figure 6.2. Control of Sample Flow.

A) The PVA film placed inside the customized 3D-printed holder, enabled a visible delay of flow. B) Dissolved PVA film.

6.3 Results & Discussion

6.3.1 Comparison of PVA 1-3 Films Dissolving Time in Water

Three types of PVA were tested for their use as time barrier films. The three types of PVA used were as follow: (1) PVA 1 - Mw: 30-70K, 87-90% hydrolyzed; (2) PVA 2 - Mw: 31-50K, 98-99% hydrolyzed and (3) PVA 3 - Mw: 89-98K, > 99% hydrolyzed. The three types of PVAs were chosen based on the differences in their Mw and level of hydrolysis. Theoretically, PVA degree of hydrolysis is inversely proportional while the PVA Mw is directly proportional to its solubility ^[32, 33]. As such, it was hypothesized that PVA 1 will dissolve most quickly because of its lower Mw and lower level of hydrolysis. This is followed by PVA 2 which has a higher level of hydrolysis, then PVA 3 due to its high Mw and high level of hydrolysis. The dissolution time of the tested PVA 1-3 films were first determined under standard conditions. Each tested PVA film was cut into small circles of 6 mm diameter and immersed into 100 μ L of DI-water droplet on a parafilm. The films were visually and physically examined to determine the time for dissolution and the results are presented in Figure 6.3. A range of PVA concentrations were used to obtain films with different physical properties for testing since their ease of integration into the 3D-printed holder is an essential factor. The dissolution time of PVA 1-3 films was determined for the following concentrations: 1% to 25% (w/v), 1% to 5% and 0.5% - 1% respectively. PVA 1 1% and 2% (w/v) films dissolved rapidly after less than 1 minute, while the PVA 1 25% (w/v) film dissolved too slowly, taking up to ~18 minutes. These films were unsuitable as a time-barrier film as they either did not provide sufficient time for the formation of the immuno-complexes or dissolved too slowly to be effective in a sensing device. PVA 1 5% - 20% (w/v) films, however, dissolved after 4-10 minutes, which is within the time range of interest for a time barrier film for paper-based sensors. The PVA 2 films were tested with a narrower range of concentrations: 1% to 5% (w/v), as the films did not dissolve even after 2 hours for 10% (w/v) concentration. The results presented in Figure 6.3B shows that even a 5% (w/v) concentration gave a very long dissolution time of ~ 40 minutes. Although PVA 2 1% - 2.5% (w/v) films dissolved after 5-13 minutes, which is within the time range of interest, it was ultimately deemed unsuitable due to its brittleness which would be a problem

during film-holder integration. The PVA 3 films dissolving time was determined in the concentrations: 0.5% to 1% (w/v), since 2%, 5% and 10% (w/v) films did not dissolve even after 2 hours. The results presented in Figure 6.3C shows that PVA 3 0.5% (w/v) film dissolved only after ~2 minutes while the PVA 3 0.8% and 1% (w/v) films dissolved after 12-16 minutes. Similarly, PVA 3 0.8% and 1% films were determined to be too brittle and thus unsuitable for use as a time-barrier film inside paper-based sensors. To conclude, after the comparison of PVA 1-3 films, PVA 1 5% - 20% (w/v) films were found to be the most suitable for use as time barrier films, due to their optimal dissolution times and physical properties for integration into the 3D-printed holder.

Figure 6.3. Comparison of PVAs 1-3 Films Dissolving Time in DI-Water.

The dissolution time was determined after immersing the PVA films into 100 μ L of DI-water droplet on parafilm. Dissolution time was compared between three types of PVA: A) PVA 1 (Mw:

30-70K, 89-90% Hydrolysed); B) PVA 2 (Mw: 31-50K, 98-99% Hydrolysed) and C) PVA 3 (Mw: 89-98K, > 99% Hydrolysed). Different concentrations were selected for each PVA type, according to the Mw and hydrolysis level.

6.3.2 PVA 1 Film Dissolving Time in 3D-Printed Holder

PVA 1 5% - 17.5% films were selected for further testing in the customized 3D-printed holder since they were determined to have optimal dissolution times and physical properties than the other tested films. After they were integrated into the holder, as described in section 6.2.6, the dissolution time of each tested film was determined after the visible flow of the ink sample into the absorption pad (Figure 6.2). Moreover, the effect of three other factors, (1) sample volume, (2) presence of membranes and (3) plasticizers and surfactants, on the dissolution time were also tested and the results are presented in Figure 6.4. As presented in Figure 6.4A, the effect of sample volume on dissolution time were tested for two volumes of sample: 100 μ L and 500 μ L. The resultant dissolution time is similar between the 100 μ L and 500 μ L samples: 10% (w/v): ~11-12 minutes; 15% (w/v): ~14 minutes and 17.5% (w/v): ~15-17 minutes, except for a small difference in the case of PVA 1 5% (w/v) films (100 μ L: ~10 minutes, 500 μ L: ~6 minutes). Moreover, the PVA 1 films have a longer dissolution time in the 3D-printed holder than in the water drop: 5% (w/v) - drop ~4 minutes vs. holder ~6 minutes; 10% (w/v) - drop ~5 minutes vs. holder ~12 minutes and 15% (w/v) - drop ~7 minutes vs. holder ~14 minutes. The time taken for dissolution was longer in the 3D-printed holder as the exposure area of the PVA film to water in the 3D printed holder is limited to only 6 mm $\varnothing$ surface, whereas the PVA film was fully immersed inside the water droplet, which increased the exposure area. The results suggest that the 3D-printed holder can be employed in sensors to effectively tune the dissolution time of PVA films by adapting the area of film-sample exposure based on the needs of the sensor. As previously mentioned, reagent-containing membranes are a common component of many sensors. Therefore, the effect of the presence of membranes was also tested in the 3D-printed holder and the results are presented in Figure 6.4B. Five sample pads were placed on top of the PVA 1 films before the addition of the ink sample. The addition of membranes reduced the dissolution time of the PVA 1 films: 5% (w/v) without ~6 minutes vs. with ~3 minutes and

10% (w/v) without ~11 minutes vs. with ~6 minutes. Furthermore, the effect of adding plasticizer (0.5% (v/v) Glycerol) and surfactant (0.05% (v/v) Tween20) to the PVA film was also tested in the 3D-printed holder and the results are presented in Figure 6.4C. The plasticizer and surfactant were added to the PVA film with the intention of improving the film flexibility for better integration into the 3d-printed holder. However, from the resultant dissolution time, the plasticizer and surfactant addition into the PVA 1 film content reduced the dissolution time significantly from ~4 minutes to ~1 minute.

Figure 6.4. PVA 1 Film Dissolving Time in Customized 3D-Printed Holder.

A) Effect of sample volume, comparing dissolution time with 100 μL and 500 μL of DI-water. B) Effect of Membranes on 7.5% (w/v) PVA 1 film dissolution time, using 500 μL DI-water. C) Effect of plasticizer & surfactant on 7.5% (w/v) PVA 1 film dissolution time, using 500 μL DI-water.

6.3.3 PVA 1 Film Weight and Thickness Characterization

The characterization of the weight and thickness of the PVA 1 films was also conducted. Results are presented in Figure 6.5. PVA 1 7.5% (w/v) films were prepared and then cut into 6 mm diameter circles. The PVA circles were first weighed then tested for their dissolution time when immersed in a DI-water droplet on parafilm. From the results presented in Figure 6.5A, a linear connection between the weight of the PVA circles to its dissolution time was observed. After standardization per area, the linear connection between dissolution time (minutes) to weight/area (mg/cm^2) can be described with the following equation: $y = 0.1934x - 1.278$, $R^2 = 0.9626$. Moreover, the characterization of the weight and thickness of the PVA 1 7.5% (w/v) films based on three different PVA solution volumes was also conducted and the results are presented in Figure 6.5B. After the preparation of the PVA 1 7.5% (w/v) films, the films were cut into 15 mm x 15 mm squares. From the results presented in Figure 6.5B, a linear connection between the weight of the films to the solution volume can be observed. After standardization per area, the linear connection between weight/area (mg/cm^2) to solution volume (mL) can be described with the following equation: $y = 7.8759x + 3.468$, $R^2 = 0.98$. The linear connection between thickness (μm) to solution volume (mL) can be described with the following equation: $y = 61.667x + 57.778$, $R^2 = 0.9343$.

Figure 6.5. PVA 1 Film Weight and Thickness Characterization.

A) Effect of the weight of 7.5% (w/v) PVA 1 film on dissolution time when immersed in 100 μ L of DI-water droplet on parafilm. B) Weight and thickness as a function of PVA 1 solution volume.

6.4 Conclusions

In this study, dissolvable PVA films are used in a novel, customized 3D-printed sensor holder for the control of sample flow. Integrating a dissolvable membrane inside a paper-based sensor allow the control of the sample flow in selected critical areas. With the use of PVA as a time barrier film, the sample will not flow until the required time. This allows increased exposure time between proteins and antibodies in order to allow more immunocomplexes to be formed and to ensure higher sensitivity, reactivity and reduced false-positive signal [2, 3, 6]. As previously discussed, the PVA solubility depends on its molecular weight and degree of hydrolysis. Three types of PVAs films were tested: (1) PVA 1 - Mw: 30-70K, 87-90% hydrolyzed; (2) PVA 2 - Mw: 31-50K, 98-99% hydrolyzed and (3) PVA 3 - Mw: 89-98K, > 99% hydrolyzed. PVA 1 5% - 20% (w/v) films were found to be the most suitable for use as time barrier films, due to ideal dissolution times and physical properties for integration into the 3D-printed holder. As expected, the dissolution times of PVA 1 films were slower in the 3D-printed holder than in the DI-water droplet: 5% (w/v) drop ~4 minutes holder ~6 minutes; 10% (w/v) drop ~5 minutes holder ~12 minutes and 15% (w/v) drop ~7 minutes holder ~14 minutes. Moreover, a linear relation can be observed between the PVA weight/area and the dissolution time. The intended use of the dissolvable PVA films is inside a paper-based sensor. A potential sensor platform is Stack-pad [35, 36]. Stack-pad is based on vertically stacked functional membranes. The sample is added from the bottom-up, and as each layer is wetted, the analyte is pushed upwards. Through the immunocomplex (analyte-antibody conjugated with HRP) migration, a measurable signal is produced. In order to prevent a false positive result, a proprietary blocking layer membrane is added to stop unbounded antibodies from reaching to the top membrane. Thus, only the analyte/antibody-HRP complex will generate a signal. The dissolvable PVA film has the potential to improve the sensitivity and specificity of such sensors by allowing increased exposure time, which will potentially increase immunocomplex formation.

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Chapter 7. Membrane Type Comparison and Modification

Paper-based diagnostics are rapid, robust, affordable and user-friendly, thus have potential for point-of-care. Modulation of sample flow in these technologies have the advantage of increased exposure between immunoreagents, which ensures higher sensitivity and specificity. In this study, common membrane types were compared and modified to modulate sample flow. Five different membranes were compared: glassfiber, polyester, nitrocellulose, polyvinylidene-difluoride (PVDF) and cellulose. Several parameters were tested for their effect on the sample flow: membranes absorption capacity, immobilization of buffer solution, different membrane type as separation layer and sample volume. The effect of separation membrane on the sample flow was tested using the 'Stack-Pad' sensor layout. Higher antibody levels could reach the three absorption pads with the use of polyester separation membrane (340, 440 and 408 RLU) as compared to the glassfiber separation membrane (208, 198 and 107 RLU). Lastly, the effect of the membrane type and its modifications on the detection sensitivity was also compared. The modified setup (862 RLU) demonstrated higher detection sensitivity vs. the initial setup (290 RLU). To conclude, the selection of membrane types and their modifications can modulate sample flow and increase the sensitivity of detection in paper diagnostics.

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7.1 Introduction

Widely used diagnostic technologies, such as Enzyme-linked Immune Sorbent Assay (ELISA), are expensive and not always accessible. On the other hand, paper-based diagnostic technologies are usually cheaper, rapid, robust and user friendly, thus have potential for the delivery of point-of care (POC) diagnostics ^[2, 3]. Paper-based diagnostics are divided to three main categories: dipstick assays, lateral flow assays (LFAs), and microfluidic paper-based analytical devices (μ PADs) ^[4, 5]. Dipstick assays are based on a functionalized paper, such as a pH strip. However, their simplicity prevents them from having a more sophisticated mechanism which is needed for advanced diagnostics assays ^[6]. Although the LFAs are also based on functionalized paper, they also help to integrate sample flow, allowing a gradual flow through different areas of the assay. The most famous LFA is the pregnancy test. This method has the advantage of allowing sample flow in sandwich, competitive or multi-detection formats. However, its main drawback is in obtaining quantitative results ^[7, 8]. This can be overcome with the μ PADs since they are based on the integration of paper diagnostics with microfluidics detection. They require low amount of sample and are based on the creation of hydrophilic channels integrated inside a paper-based hydrophobic surface, through applying techniques such as photolithography, inkjet etching, poly-dimethyl-siloxane (PDMS) plotting, cutting, plasma etching and wax printing ^[9, 10].

The modulation of sample flow may allow increased exposure time between immunoreagents. As a result, the sensor sensitivity and reactivity may increase and it may also reduce false-positive signals ^[11-14]. Previous studies had examined different mechanical strategies to modulate sample flow. Hossain *et al.* had introduced the tested sample from one direction of the lateral flow and water from the other direction. By doing so, the sample was forced into the sensing area, which allowed the initiation of the enzyme-catalyzed hydrolysis of the substrate ^[15]. In addition, Elain *et al.* had examined the effect of a two-dimensional format on enhancing sensitivity. While the sample was introduced from one direction, the buffer and the signal amplification solutions were introduced from a diagonal direction, in order to enhance the test sensitivity ^[16]. Moreover, Andres *et al.* had

examined the combination of a three-dimensional format with an integrated adhesive tape for controlling the initial sample flow by compressing, as well as the sample flow direction by holes in the integrated adhesive tape ^[17]. In addition, researchers have also examined the use of dissolvable films in order to modulate sample flow. Dissolvable films is usually based on a polymer that is water-soluble, common examples include polyvinyl-alcohol, polyethylene-oxide, polyvinylpyrrolidone, hydroxylpropyl-methylcellulose, hydroxylpropyl-cellulose, hydroxyethyl-cellulose, carboxymethyl-cellulose, methyl-cellulose, gelatin, sodium alginate, maltodextrin, polymerized rosin, pectin, pullulan and xanthan ^[18, 19]. For example, Harpaz *et al.* examined the use of polyvinyl-alcohol dissolvable films in order to modulate sample flow in capillary flow paper diagnostics ^[20]. In addition, Barry *et al.* had tested the use of a paper with dissolvable sugar in order to modulate sample flow through introducing localized time delays. The tested format used these time delays to create an automated fluidic protocol ^[21]. Also, Sudheer *et al.* examined the performance of a microfluidic sensor with an integrated dissolvable membrane that is based on polyacrylamide. The membrane dissolves after introducing the sample, which then allowed its flow into the inter-digitated electrodes. After the sample flow, the electrodes exhibited a resistance change. This converted a chemical reaction into an electric measurable signal ^[22].

Sample flow can be also modulated by the type of membrane and its modifications. Generally, paper diagnostics contain the following four parts: sample, conjugation, detection and absorption layers. The sample layer filters impurities and is immobilized with the assay buffer. The conjugation layer is immobilized with a labelled agent, usually an antibody, which detects the assay target. The detection layer contains the capture reagent, e.g. antibodies, recorded for the signal measurement. Lastly, the absorption layer wicks the rest of the sample ^[4]. Each of the four parts of the paper diagnostics has a different function and thus requires the use of different types of membrane. Membranes are a semi-permeable interfaces that are capable of blocking unwanted molecules while simultaneously transporting selected ones ^[23]. There are a variety of membranes available that can be selected based on the required fabrication procedure and the specific application. Important parameters that differentiate each membrane are polymer type, porosity, particle retention,

capillary flow rate, thickness and surface characteristics ^[24]. Membranes are categorized as porous or dense, symmetrical or asymmetrical and organic or inorganic ^[23]. The membrane porosity is defined as the pores volume divided by the membrane total volume. ^[25]. As previously discussed, a membrane is chosen based on the required function. For example, a sample layer is usually made of hydrophilic cellulose membrane, after introducing the solvent, the cellulose fibres swell, which prevent the liquid penetration because the pores are restricted. A second example is the glossy paper, a flexible membrane made from cellulose and an inorganic filler. Because of its smooth surface, it is mainly used as a filter paper, when it is necessary to modify reagents on a surface rather than within the membrane ^[26]. The use of modified cellulose membranes is common, such modifications include acid treatments in order to add different mechanical and chemical properties ^[24]. Commercially available examples include ion-exchange cellulose papers and composite papers of cellulose and polyester, which are attractive as a conjugation layer. The detection layer, on the other hand, utilizes membranes such as nitrocellulose which prevents the binding of non-specific biomolecules and are also attractive for the immobilization of proteins, DNA and enzymes ^[26]. Polyvinylidene difluoride (PVDF) membrane is also attractive as a detection layer, it is hydrophobic and exhibit a dense, porous and asymmetric structure. The membrane pore size is an important factor associated with the membrane wetting properties ^[27].

In this study, the effect of different membrane types and membrane modifications on the sample flow were examined. Five different membrane types (e.g., glassfiber, polyester, nitrocellulose, polyvinylidene difluoride (PVDF) and cellulose) were compared and modified using buffers in order to modulate sample flow in paper diagnostic. The membranes effect was tested using ‘Stack-Pad’ sensor layout ^[28, 29]. ‘Stack-Pad’ is based on functional membranes that are stacked vertically one upon the other. The sample is then introduced from the bottom of the stack, and as the layers are wetted, the sample moves up towards the top-most layer. There, a chemiluminescent signal is generated based on the amount of horseradish peroxidase (HRP) – conjugated antibody. Moreover, in order to reduce background signal, there is use of a blocking layer that is added for the filtering of the remaining free antibodies. Firstly, the membrane structures were characterized using

Field Emission Scanning Electron Microscope (FE-SEM). In addition, the absorption capacities of the membranes were determined. The effect of immobilization buffer solution content, separation layer and sample volume on the antibody release and sample flow were also tested. Lastly, the effect of the membrane type and its modifications was examined by comparing two 'Stack-Pad' setups, initial setup vs. modified setup. As a measure of the test sensitivity, the positive vs. negative signal was compared.

7.2 Materials and Methods

7.2.1 Materials

Glassfiber (GFB-R4), polyester (PT-R5) and cellulose (AP080) were purchased from MDI membrane technologies. Nitrocellulose (1620115), PVDF (1620177) and Clarity western ECL solutions (Luminol – H₂O₂) (Cat. 1705061) were purchased from Bio-Rad. Phosphate buffered saline (PBS) tablets (Cat. No. P4417), polyoxyethylene sorbitan monolaurate (Tween20) (Cat. P7949) and Skim Milk powder (Cat. 70166) were purchased from Sigma-Aldrich. HRP Conjugated Goat anti Human IgG (Cat. ab97175) was purchased from Abcam. Recombinant protein G (Cat. 21193) was purchased from Thermo. Milli-Q ultrafiltered (UF) H₂O (with a resistivity of 18.2 MΩ cm at 25°C) were used in the preparation of all solutions.

7.2.2 Equipment

Field Emission Scanning Electron Microscope (FE-SEM), model: Supra 55, was purchased from Carl Zeiss. Sputter coater, model: SPIN 150i, was purchased from POLOS. Balance was purchased from Mettler Toledo. Membrane cutter (PAT/RE38, 219 & D613, 795) was purchased from EK. Luminoskan Ascent Luminometer was purchased from Thermo Fisher Scientific, Waltham, MA, USA.

7.2.3 Membranes Structure Characterization

FE-SEM was used to characterize the structure of the different tested membranes. SEM consists of an electron gun which generates an electron beam that scans the sample surface, producing an image of the surface structure of the sample. The electrons interaction with the sample surface generates signals that are detected by the various detectors (X-ray, backscattered electrons (BSE) and secondary electrons (SE)). Different detected electrons hold different information on the sample topography and content. The membranes were coated with conductive platinum for 30 seconds on 40 mA before SEM scanning.

7.2.4 Membranes Absorption Capacity

The different membranes were cut into 2 cm X 2 cm squares. The cut membrane squares were weighed before and after incubation in water. Two incubation times were tested namely, 10 seconds and 30 minutes. The absorption capacities of the membranes were then calculated and standardized per area (cm²). The density of water is 1 g/mL, which means 1 mL of water volume is equal to 1 gram of mass. Based on that, 1 mg of water equals 1 μ L of water.

7.2.5 Effect of Immobilization Buffer Solution on Sample Flow

The effect of buffer solution content on antibody release and flow was tested. Different buffer solutions immobilize antibodies differently on the membrane, resulting in different membrane modification. Polyester membrane was used as 'conjugate and release' pad and was cut into circles of 6 mm $\varnothing$ and then immobilized with 40 μ L of 100 ng/mL HRP - conjugated antibody diluted in different buffer solutions. The five different tested buffer solutions were: DI water, PBS, 0.05% (v/v) PBSTween20 (PBST), 5% (w/v) skim milk (SM) and 5% (w/v) skim milk – 0.05% (v/v) PBSTween20 (SM-PBST). The pads were then dried for 1 hour in RT. Three absorption pads were placed above each tested conjugation pad before a 60 μ L DI water sample was added. Following sample flow, antibodies are released from the conjugation pads. These pads were then placed inside a

96-well plate for signal measurement. 100 μL of Luminol – H_2O_2 substrate was added to each pad in each well and a kinetic measurement was conducted. The results are presented in relative light units (RLU), as a measure of chemiluminescent enzymatic reaction, which corresponds to the antibody amount.

7.2.6 Effect of Separation Membrane Type and Sample Volume on Sample Flow

The effect of separation membrane type and sample volume on the antibody flow were tested using ‘Stack-Pad’ sensor layout [28, 29]. ‘Stack-Pad’ is based on functional membranes that are stacked vertically one upon the other. The sample is then introduced from the bottom of the stack, and as the layers are wetted, the sample moves up towards the top-most layer. There, a chemiluminescent signal is generated based on the amount of horseradish peroxidase (HRP) – conjugated antibody. Moreover, in order to reduce background signal, there is use of a blocking layer that is added for the filtering of the remaining free antibodies. Unmodified glassfiber membrane was used as the sample pad. Conjugation and release pads (polyester) were immobilized with 100 ng/mL antibody diluted in 0.05% PBST buffer, then dried for 1 hour in RT. Nitrocellulose membrane was used as a blocking pad and was immobilized with 800 ng/mL protein G diluted in PBS buffer for 1 hour in RT. Then non-specific blocking was conducted by incubation in 5% (w/v) skim milk – 0.05% (v/v) PBST for 1 hour in RT. PBS buffer was used to wash away the excess non-bonded reagents. Two different types of membranes (glassfiber vs. polyester) were compared based on their functionality as separation membranes. In order to test the effect of sample volume, four different sample volumes were tested (20, 40, 60 and 80 μL), with polyester membrane used as the separation layer. The release and flow of the HRP – conjugated antibody was tested by separating the ‘Stack-Pad’ layers after sample flow. In the case of the effect of sample volume, the measurement was done on the conjugation layer after the sample flow. Each layer was placed inside a 96-well plate for signal measurement. 100 μL of 1:1 (v/v) Luminol – H_2O_2 substrate was added to each pad in each well before a kinetic measurement was conducted. The results are presented in relative light units (RLU).

7.2.7 Effect of Membrane Type and Modification on Detection Sensitivity

The effect of the membrane type and its modification on the detection sensitivity was tested using ‘Stack-Pad’ sensor layout (see section 7.2.6.). Two ‘Stack-Pad’ setups were compared, namely the initial setup and the modified setup. Both setups comprised of unmodified glassfiber membrane as the sample pad, modified polyester as the conjugation pad and modified nitrocellulose as the blocking pad (see details of the membrane modification at section 7.2.6.). Cellulose membrane, which was used as the absorption pad, was immobilized with 60 μL of 1:1 (v/v) Luminol – H_2O_2 substrate, then dried in the dark for 1 hour at RT. The initial setup made use of glassfiber membrane as the separation layer while the modified setup utilized polyester. Moreover, the conjugation pad in the initial setup was immobilized with antibody diluted in water only while the antibody was diluted in 5% (w/v) skim milk – 0.05% (v/v) PBS-Tween20 (SM-PBST) in the case of the modified setup. After the construction of both layouts, a 120 μL sample was added to each tested setup. The negative sample comprised of DI-water while the positive sample comprised of 2500 ng/mL protein G diluted in DI-water. The relative light signal (RLU) of the negative and positive, samples and the two setups was measured and compared. In addition, the ratio of positive to negative signals was calculated as a measure of the detection sensitivity.

7.3 Results and Discussion

7.3.1 Membranes Structure Characterization

In paper diagnostics, there is a wide variety of membranes to choose from. The membranes are commonly selected based on their properties such as porosity, particle retention, capillary flow rate, thickness and surface characteristics ^[24]. Membranes with different properties are used for different functions inside the sensor, for example as sample, conjugation, detection and absorption layers. Firstly, the structures of the different membranes were characterized using FE-SEM (

Figure 7.1) and a summary of their physical properties and advantages is recorded in Table 7.1. The unmodified membranes (glassfiber, polyester, nitrocellulose, polyvinylidene

difluoride (PVDF) and cellulose) were screened using FE-SEM with SE2 detector. As shown in

Figure 7.1A, the glassfiber membrane was observed to be a less organized, highly porous structure with thin fibres. The glassfiber membrane is a hydrophilic membrane with low pressure drop, making it attractive as a pre-filter membrane for fluids that have high viscosity and/or a high level of particulates. It is known to be cheap and convenient to work with and commonly functions as a ‘sample layer’ for the direction of the sample flow ^[30]. Based on

Figure 7.1B, the polyester membrane showed a more organized structure with thick fibres. Polyester membrane is a hydrophilic membrane as well and it can prevent damage and/or aggregation of dried conjugates. Moreover, it ensures a quick and clean release of conjugates when the sample is applied. Another advantage of the polyester membrane is its high mechanical strength which allows for ease of handling. Therefore, it is commonly used as a ‘conjugation and release’ layer ^[31]. As shown in

Figure 7.1C, the nitrocellulose membrane had a very dense and thick structure. Nitrocellulose membrane is a hydrophilic membrane which is usually used for the transfer of proteins and nucleic acids ^[32]. The PVDF membrane, as observed in

Figure 7.1D, showed a very similar structure to that of nitrocellulose, only much denser. Contrary to the nitrocellulose membrane, the PVDF membrane is hydrophobic and retains proteins very strongly during protein transfer ^[33]. The cellulose membrane, as shown in

Figure 7.1E, demonstrated a tangled structure with thicker fibres. It is known as a membrane with high absorption capacity and therefore functions well as a good absorption layer for absorption of the sample ^[34]. Moreover, the surface area to volume ratio is much higher in glassfiber membrane, compare to polyester and cellulose membranes. This parameter can further influence the differences in the membranes absorption capacity ^[35]. To conclude, the observed membranes possess different structures, which may potentially influence important functions that are related to the sensor performance such as the absorption capacities of the membranes and their immobilization efficiencies.

Table 7.1. Membranes Properties and Description.

Membrane	Function	Description	Advantages	Ref.
Glassfiber	Sample	Hydrophilic membrane. Low pressure drop, making it attractive as a pre-filter membrane for fluids that have high viscosity and/or a high level of particulates	Attractive for the following applications: salt, sugar, sera, tissue lysates, protein solutions, and environmental samples, where there is an elevated level of suspended or undissolved particles	[30]
Polyester	Conjugation	Hydrophilic membrane. Prevents damage or aggregation of dried conjugate, ensures quick and clean release of conjugate when the sample is applied	High mechanical strength for ease of handling, and high absorption capacity	[31]
Nitrocellulose	Blocking	Hydrophilic membrane. Pore size 0.45 μm . Commonly used for the transfer of proteins and nucleic acids	Easy to use and to work with water-based solutions	[32]
PVDF	Blocking	Hydrophobic membrane. Pore size 0.2 μm . Strongly retains the target protein for transfer	Reduces non-specific binding of proteins, which increases the detection sensitivity	[33]
Cellulose	Absorption	Hydrophilic membrane. Excellent absorption capacity	Drive the flow of test fluids	[34]

Figure 7.1. Membranes Structure Characterization.

FE-SEM imaging (SE2 detector) of unmodified membranes: A) glassfiber, B) polyester, C) nitrocellulose, D) polyvinylidene difluoride (PVDF), and E) cellulose.

7.3.2 Membrane Absorption Capacity

The membrane type used in paper diagnostics influence the sensor performance, mainly through sample flow. As previously discussed, membranes differ in the polymer type and their structures which in turn affect the membrane absorption capacity ^[4]. Absorption occurs when a liquid (such as the sample) is absorbed into the membrane. As a result, the membrane either grow in size or swell. Different polymers can absorb different amount of liquid ^[36]. The absorption capacities of the tested membranes were also determined, as shown in Figure 7.2A. The membranes were cut and incubated for 10 seconds or 30 minutes in water. From the results, it can be determined that PVDF (10 seconds: 26.3 $\mu\text{L}/\text{cm}^2$ and 30 minutes: 19.3 $\mu\text{L}/\text{cm}^2$) and nitrocellulose (10 seconds: 32.7 $\mu\text{L}/\text{cm}^2$ and 30 minutes: 22.2 $\mu\text{L}/\text{cm}^2$) demonstrated lower absorption capacities. Polyester (10 seconds: 50.9 $\mu\text{L}/\text{cm}^2$ and 30 minutes: 44.3 $\mu\text{L}/\text{cm}^2$) and glassfiber (10 seconds: 86.9 $\mu\text{L}/\text{cm}^2$ and 30 minutes: 90.4 $\mu\text{L}/\text{cm}^2$) demonstrated medium range absorption capacities. Cellulose (10 seconds: 138.3 $\mu\text{L}/\text{cm}^2$ and 30 minutes: 137.9 $\mu\text{L}/\text{cm}^2$), on the other hand, demonstrated the highest absorption capacity. As previously discussed, the cellulose membrane absorption mechanism is different, after introducing the solvent, the cellulose fibres swell ^[26]. This findings agrees with the characterization of cellulose, which suggested that it has an excellent absorption capacity and is usually used as an absorption layer in order to drive the flow of the tested fluid ^[34]. Surprisingly, glassfiber membrane also demonstrated a high absorption capacity, which is a parameter that needs to be considered, since it is usually used as a ‘sample layer’ in the beginning of the test, but also sometimes used as a ‘separation layer’ in certain tests ^[30]. Polyester membrane demonstrated an average level of absorption capacity. This parameter should be taken into account when trying to immobilize conjugates on this membrane, when it is used as a ‘conjugate and release layer’. Moreover, since PVDF and nitrocellulose demonstrated low absorption capacities, they are attractive as isolating layers between functionalized membranes since they do not absorb

the sample and will therefore prevent any flow through. Comparing the results of the two different incubation times (10 seconds and 30 minutes), most of the membranes, except for glassfiber, demonstrated slightly lower absorption capacities in the case of 30 minutes incubation. This may be due to a slight degradation of the membranes when they were incubated for a longer period inside the water solution. This finding can also serve as an insight into the physical properties of the membranes. It is suggested that glassfiber membranes can be stable inside a water solution for a longer incubation time as demonstrated by its higher absorption capacity. The weight per area of the membranes was also determined (Figure 7.2B). Glassfiber (7.8 mg/cm^2) and polyester (8.8 mg/cm^2) demonstrated a similar weight per area, while cellulose (15.9 mg/cm^2) demonstrated a higher weight per area. To conclude, the different membranes not only demonstrated different structures, but also demonstrated significantly different absorption capacities. This must be considered when developing a paper diagnostic sensor since their effect on sample flow will significantly influence the sensor performance.

Figure 7.2. Membranes Absorption Capacity.

A) All membranes were scaled, then dipped in water for 10 seconds or 30 minutes and scaled again. Five different membranes (PVDF, nitrocellulose, polyester, glassfiber and cellulose) were

compared for their absorption capacity. PVDF and nitrocellulose membranes demonstrated the lowest absorption capacity ($20 \mu\text{L}/\text{cm}^2$). While polyester membrane ($50 \mu\text{L}/\text{cm}^2$) and glassfiber ($90 \mu\text{L}/\text{cm}^2$) demonstrated medium absorption capacity. Cellulose membrane showed the highest absorption capacity ($140 \mu\text{L}/\text{cm}^2$), the cellulose membrane absorption mechanism is different, after introducing the solvent, the cellulose fibres swell. B) Membranes weight per area.

7.3.3 Effect of Immobilization Buffer Solution on Sample Flow

Modulation of sample flow can give rise to several advantages in sensor technologies [15-19, 21, 22]. An important parameter in sensor technologies is the reaction between the analyte and the bioreceptor, which is usually an antibody. Firstly, the analyte in the sample is recognized by the bioreceptor (antibody). Therefore, there is a great importance of the antibodies release during sample flow. Different immobilization buffer solutions immobilize antibodies differently on the membrane. In order to examine the effect of buffer solution content on sample flow, the amount of antibody release from the conjugation layer was measured. The conjugation layer is immobilized with a labelled agent, usually antibody, which is the capture reagent for the signal measurement [4]. The results are presented as relative light units (RLU) as a measure of luminescent enzymatic reaction, which corresponds to the antibody amount. Polyester membrane was used as the conjugation layer in this test due to its hydrophilic nature that prevents damage and/or aggregation of dried conjugates, and its ability to ensure quick and clean release of conjugates when the sample is applied [31]. The effect of buffer solution content on antibody release and flow was tested (Figure 7.3). Polyester membrane was immobilized with HRP - conjugated antibody diluted in different buffer solutions, e.g. DI water, PBS, 0.05% (v/v) PBSTween20 (PBST), 5% (w/v) skim milk (SM) and 5% (w/v) skim milk – 0.05% (v/v) PBSTween20 (SM-PBST). As shown in Figure 7.3A, the least amount of antibody was released when water was used as the buffer. 3218 RLU of antibodies remained on the polyester membrane. On the other hand, the use of SM-PBST buffer solution released the most antibody, with only 459 RLU of antibodies remaining on the membrane. Since the purpose of the ‘conjugate and release’ membrane layer is to hold the conjugates during immobilization and then to release them once wetted during sample flow, SM-PBST is the most preferred buffer solution for antibody immobilization. The other buffer solutions demonstrated increasing antibody

release function in this order: PBST (1779 RLU), PBS (1535 RLU) and SM (1033 RLU). These results support the theory. The non-ionic detergent Tween 20 (polysorbate 20) is widely used in different biochemical applications. It prevents antibody aggregation and therefore allows greater ease of antibody release. The presence of Tween 20 significantly increased the release of the immobilized HRP - antibodies from the conjugation layer. It was shown in a previous study that Tween 20 may disassociate proteins immobilized on nitrocellulose membrane ^[37]. Furthermore, the addition of skim milk (SM-PBST) in the buffer solution also significantly increased the antibody release from the conjugation layer. The skim milk may have prevented the absorption of antibodies onto the membrane surface and therefore enabled an increased release ^[38]. This observation is strengthened by the FE-SEM structure characterization that was performed for polyester membrane immobilized with HRP – conjugated antibody diluted in the different immobilization buffer solutions. As shown in Figure 7.3B, the SM-PBST buffer solution immobilization on the polyester membrane is clearly visible as compared to the unmodified polyester membrane structure (Figure 7.3B). The coating of the polyester membrane fibres with the SM-PBST solution is also clearly visible. To conclude, the addition of Tween 20 and skim milk significantly improved the antibody release from the conjugation layer after sample flow. This effect of membrane modification by buffer solution can be further considered for enhanced control of the antibody release during sample flow.

Figure 7.3. Effect of Immobilization Buffer on Sample Flow.

A) Polyester membranes (conjugation and release pads) were immobilized with HRP-antibody (100 ng/mL) diluted in different buffer solutions: water, 0.05% PBSTween20 (PBST), PBS, skim milk (SM) and skim milk-0.05%-PBSTween20 (SM-PBST). After immobilization (40 μ L solution followed with drying in RT), three absorption pads were placed above each tested conjugation pad, then a 60 μ L DI water sample was added. After sample flow, the conjugation pads were then placed inside a 96-well plate for signal measurement, then light signal (RLU) was measured. In the case of SM-PBST as buffer, more antibodies were released from the membrane, making it preferred as a buffer for antibody immobilization on conjugation membrane. B) Modified membranes SEM structure characterization. FE-SEM imaging (SE2 detector) of the unmodified vs. modified polyester membrane with SM-PBST immobilization buffer solution.

7.3.4 Effect of Separation Membrane Type and Sample Volume on Sample Flow

Sample flow can be modulated not only by the membrane type and its modification, but also by the construction of a specific layout ^[15-19, 21, 22]. As previously mentioned, paper diagnostics usually contain the following four parts: sample, conjugation, detection and

absorption layers ^[4]. Separation layer is often incorporated in the sensor layout in order to further control and direct the sample flow in the sensor critical areas as well as to serve as a ‘buffer layer’ between functional layers. The effect of different types of separation membrane on the antibody flow was tested using a ‘Stack-Pad’ sensor layout ^[28, 29] (Figure 7.4A). ‘Stack-Pad’ is based on functional membranes that are stacked vertically one upon the other. The sample is then introduced from the bottom of the stack, and as the layers are wetted, the sample moves up towards the top-most layer. There, a chemiluminescent signal is generated based on the amount of horseradish peroxidase (HRP) – conjugated antibody. Moreover, in order to reduce background signal, there is use of a blocking layer that is added for the filtering of the remaining free antibodies. Conjugation and release pads (polyester) were immobilized with antibody diluted in 0.05% PBST buffer. Tween 20 prevents antibody aggregation and therefore also increases antibody release ^[37]. Two different types of membranes (glassfiber vs. polyester) were compared for their function as separation membranes.

The release and flow of the HRP – conjugated antibody was examined by separating the ‘Stack-Pad’ layers after sample flow and measuring the RLU in the luminometer. The antibody levels that were measured from the three absorption pads were two times higher in the case of polyester as separation membrane (340, 440 and 408 RLU), than glassfiber (208, 198 and 107 RLU). This suggests that when polyester was used as the separation membrane, the antibodies could flow through the ‘Stack-Pad’ layers with greater ease and with less trapping in the membrane. This means that higher levels of HRP - conjugated antibodies would eventually reach the absorption pads, and thus potentially increase sensor sensitivity. From Figure 7.4A, it can be observed that the glassfiber separation membranes absorbed and trapped higher levels of antibodies during sample flow (862, 459, 518 and 520 RLU) as compared to polyester as separation membrane (573, 235, 238 and 146 RLU). These conclusions are supported by the results obtained from both the structure characterization of the membranes (

Figure 7.1) and their absorption capacities (Figure 7.2). The polyester membrane was observed to be more porous (

Figure 7.1A), while the glassfiber membrane was observed to have a tangled structure (

Figure 7.1B). The porosity of the polyester separation membrane allowed more antibodies to flow through the ‘Stack-Pad’. In addition, as shown in Figure 7.2A, the polyester membrane demonstrated half the absorption capacity ($44.3 \mu\text{L}/\text{cm}^2$) of the glassfiber membrane ($90.4 \mu\text{L}/\text{cm}^2$). This further supports the results that more antibodies were absorbed and trapped in the glassfiber separation layers in the ‘Stack-Pad’. In addition, the effect of sample volume was also tested (Figure 7.4B). Four different sample volumes were compared: 20, 40, 60 and 80 μL . From the results, the amount of antibodies released from the ‘conjugation and release pad’ was doubled when the sample volumes were 60 - 80 μL (1477 and 1493 RLU). At lower sample volumes of 20 – 40 μL , twice as much antibodies (2915 and 3119 RLU) remained on the ‘conjugation and release pad’. This is because in a higher sample volume, more antibodies are ‘pushed out’ of the ‘conjugation and release pad’. This may be explained by the capillary flow of the sample through the membrane, which resulted in the release of more antibodies from the membrane surface. To conclude, the use of different types of separation layer membranes as well as different sample volumes can significantly influence sample flow inside paper diagnostics sensors, and thus affect the amount of antibodies that reach the detection area for signal measurement.

Figure 7.4. Effect of Separation Membrane and Sample Volume on Sample Flow.

Conjugation and release pads were immobilized with 100 ng/mL antibody diluted in 0.05% PBS-Tween20 buffer. A) Two different types of membranes (glassfiber vs. polyester) were compared for their function as separation membrane. Higher antibody levels reached to the absorption pads in the case of polyester as separation membrane. In the case of glassfiber as separation membrane, higher antibody levels were absorbed throughout the sample flow. Abbreviations: S.P. = separation pad (glassfiber vs. polyester), C.R. = conjugation & release (polyester), N.C. = nitrocellulose, A.P. = absorption pad (cellulose). B) Four different sample volumes were compared: 20, 40, 60 and 80 µL. from the results, it is possible to determine that the amount of antibody released from the ‘conjugation and release pad’ was double in the cases of 60 - 80 µL sample (1477 and 1493 RLU).

7.3.5 Effect of Membrane Type and Modification on Detection Sensitivity

Membrane type and its modification by buffer solution are important factors that may affect the sample flow inside paper diagnostics, and therefore may also influence the sensor performance. The last part of this study examined the effect of the cumulative conclusions, gathered from the different conditions that were previously discussed, on sample flow. The effect of the membrane type and its modification on detection sensitivity was tested using ‘Stack-Pad’ sensor layout (Figure 7.5). Two ‘Stack-Pad’ setups were compared. The ‘conjugation pad’ was immobilized with only water in the case of the initial setup, vs. 5% (w/v) SM-PBST in the case of the modified setup. In addition, the initial setup contained glassfiber membrane as separation layer, vs. polyester membrane as separation layer in the modified setup. The same sample volume as well as the same sensor construction were

tested in both setups. The light signal (RLU) was compared between negative vs. positive detection, and between the two setups. As concluded from the previous sections, it is expected that the modified setup will demonstrate higher detection sensitivity due to the selected parameters. Firstly, the ‘conjugation layer’ membrane in the modified setup was modified with the SM-PBST buffer solution for antibody immobilization, which was previously determined to release the most antibodies during the wetting phase. Secondly, the type of separation membrane in the modified setup was selected to be polyester, which was previously determined to allow higher levels of antibodies to reach the absorption pads for detection. The combination of parameters in the modified setup is thus expected to result in higher detection sensitivities. Indeed, as shown in Figure 7.5A, it can be determined that the modified setup demonstrated a higher detection sensitivity (862 RLU) vs. the initial setup (290 RLU). Moreover, the difference between the positive and the negative sample signal was also significantly higher in the modified setup (862 vs. 281 RLU) compared to the initial setup (290 vs. 162 RLU). The ratio between the positive to negative signals was calculated as a measure of the detection sensitivity. From this, the modified setup was also found to demonstrate a higher positive/negative signal ratio (3.10) vs. the initial setup (1.80). To conclude, the membrane type and its modification not only help to control sample flow inside the paper diagnostics sensor, but also significantly increase the sensor detection sensitivity.

Figure 7.5. Effect of Membrane Type and Modification on Detection Sensitivity.

Two ‘Stack-Pad’ setups were compared. The ‘conjugation pad’ was immobilized with water only in the case of initial setup, vs. 5% (w/v) SM-PBST in the case of modified setup. The initial setup contained glassfiber membrane as separation layer, vs. polyester membrane as separation layer in the modified setup. The negative sample comprised of DI-water, vs. the positive sample which comprised of 2500 ng/mL protein G diluted in DI – Water. (A) It is visible to determine that the modified setup (862 RLU) demonstrated a higher detection sensitivity vs. the initial setup (290 RLU). (B) The modified setup demonstrated a higher positive/negative signal ratio (3.10) vs. the initial setup (1.80).

7.4 Conclusions

Sample flow allows several advantages in sensor technologies^[15-19, 21, 22]. Two main factors that influence the sample flow inside a sensor are membrane type and membrane modification with different buffers. Paper diagnostics show the use of different types of membranes available. The membranes are commonly selected based on their properties such as porosity, particle retention, capillary flow rate, thickness and surface characteristics

[24]. Membranes with different properties are used for different functions inside the sensor, for example as sample, conjugation, detection and absorption layers. In this study, different membrane types were compared and modified in order to modulate sample flow in paper diagnostic. Five different membrane types were compared: glassfiber, polyester, nitrocellulose, polyvinylidene difluoride (PVDF) and cellulose. Firstly, the membranes structures were characterized using FE-SEM. The glassfiber membrane (functions as the 'sample layer') showed a less organized structure with thin fibres, while the cellulose membrane (functions as the 'absorption layer') had a tangled structure with thicker fibres. The polyester membrane (functions as the 'conjugate and release layer') showed a more organized structure with thick fibres. Both nitrocellulose and PVDF membranes (function as the 'blocking layer'), showed very dense and thick structures. However, the PVDF membrane is hydrophobic and was much denser than the nitrocellulose membrane. The absorption capacities of the membranes were also determined. PVDF ($26.3 \mu\text{L}/\text{cm}^2$) and nitrocellulose ($32.7 \mu\text{L}/\text{cm}^2$) demonstrated lower absorption capacities, while polyester ($50.9 \mu\text{L}/\text{cm}^2$) and glassfiber ($86.9 \mu\text{L}/\text{cm}^2$) demonstrated medium range absorption capacities. Cellulose ($138.3 \mu\text{L}/\text{cm}^2$) demonstrated the highest absorption capacity. As different buffer solutions immobilize antibodies differently on the membrane, it results in different membrane modifications. In order to examine the effect of buffer solution content on sample flow, the amount of antibody release from the conjugation layer was measured. Five different buffer solutions were tested: DI water, PBS, 0.05% (v/v) PBSTween20 (PBST), 5% (w/v) skim milk (SM) and 5% (w/v) skim milk – 0.05% (v/v) PBSTween20 (SM-PBST). The use of SM-PBST as buffer solution for antibody immobilization on the polyester 'conjugate and release layer' was preferred, since most of the antibody (459 RLU) was released from the polyester membrane during the wetting phase. The effect of separation membrane type on the antibody flow was tested using 'Stack-Pad' sensor layout [28, 29]. Two different types of membranes (glassfiber vs. polyester) were compared for their function as separation membrane. Higher antibody levels could reach the three absorption pads with the use of polyester separation membrane (340, 440 and 408 RLU) as compared to the glassfiber separation membrane (208, 198 and 107 RLU), thus potentially increasing the sensor sensitivity. The effect of four different sample volumes on sample flow were also compared: 20, 40, 60 and 80 μL . From the results, it is possible to determine that the

amount of antibody released from the ‘conjugation and release pad’ was double in the cases of 60 - 80 μ L sample (1477 and 1493 RLU). Lastly, the effect of the membrane type and its modification on the detection sensitivity was tested using ‘Stack-Pad’ sensor layout. Two ‘Stack-Pad’ setups were compared, initial vs. modified setup. The combination of parameters in the modified setup (i.e. use of SM-PBST buffer and polyester separation layer) was expected to demonstrate higher detection sensitivity. It was then determined that the modified setup demonstrated a higher detection sensitivity (862 RLU) vs. the initial setup (290 RLU). In addition, the ratio between the positive to negative signal was calculated as a measure of the detection sensitivity. The modified setup demonstrated a higher positive/negative signal ratio (3.10) vs. the initial setup (1.80). To conclude, the membrane type and its modification not only help to control sample flow inside the paper diagnostics sensor, but also significantly increased the sensor detection sensitivity.

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Chapter 8.   **Colorimetric Signal Amplification**

Paper-based colorimetric biosensors combine the use of paper with colorimetric signal detection. However, they usually demonstrate lower sensitivities because a signal amplification procedure was not used. Stopping the reaction of colorimetric signal is often used in lab-based assays in order to amplify and stabilize the colorimetric signal for detection. In this study, the generation of a stopped colorimetric signal was examined for accurate and enhanced signal detection in paper-based biosensors. The colorimetric reaction in biosensors is usually based on the interaction between the enzyme horseradish peroxidase (HRP) and a selected chromogenic substrate. The two most commonly used HRP substrates, 3, 3', 5, 5'-tetramethylbenzidine (TMB) and 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic-acid) (ABTS), were compared in terms of their ability to generate a stopped colorimetric signal on membrane. The stopped colorimetric signal was visible for TMB but not for ABTS. Moreover, the generation of stopped colorimetric signal was dependent on the presence of polyvinylidene-difluoride (PVDF) membrane as the separation layer. With PVDF the signal color intensity was higher (TMB: 126 ± 6 and ABTS: 121 ± 9) in comparison to without PVDF (TMB: 110 ± 2 and ABTS: 102 ± 4). The TMB stopped signal demonstrated a stable signal with lower SD. To conclude, a stopped colorimetric signal can be generated in paper-based biosensors for enhanced and accurate signal detection.

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8.1 Introduction

The main advantages of biosensors include simplicity, cost-effectiveness and rapid results. Colorimetric detection puts to best use these important biosensor advantages. Current technologies based on colorimetric detection are mainly focused on point-of-care, miniaturization of size and cost, without the incorporation of additional instruments ^[2-4]. A colorimetric sensor is based on the detection of analytes via a change in color that can be observed visually. Colorimetric sensors are categorized according to their different molecular interaction. Chemical or biomolecular-based interactions are categorized as chemical-sensors or biosensors respectively. Biosensors allow the detection of proteins, amino acids, nucleic acids, bacteria and pathogens whereas chemical-sensors mainly detect organic compounds, heavy-metals, toxic gases and explosives ^[5-7]. Paper-based colorimetric biosensors combine the use of paper diagnostics with colorimetric signal detection. They are attractive due to their simple fabrication, accessibility, and low-cost ^[8]. The use of paper for biosensors technologies show two main advantages, which are sample capillary flow and compatibility with biomolecules. Although, they still exhibit lower sensitivity and accuracy ^[9, 10]. Paper-based colorimetric biosensors often exhibit low sensitivities because a signal amplification procedure was not used. Therefore, the current research is focused on signal amplification procedures for enzyme-mediated reactions ^[11].

Colorimetric biosensing main challenge is to transform the biomolecule detection event into a reaction of a visible change in color. The colorimetric reaction in paper-based biosensors is mainly based on the interaction between the labelled antibody – protein immunocomplex and a selected chemical substrate. Most commercially available antibodies are labelled with the enzyme horseradish peroxidase (HRP), and are used in immunoassay developments ^[12-14]. The traditional enzyme-linked immunosorbent assay (ELISA) show the use of an HRP - labelled secondary antibody that is used in order to quantify the binding reaction between the target analyte and the specific primary antibody. This specific binding interaction is then detected by measuring the oxidizing reaction of HRP enzyme with a chromogenic substrate ^[15]. This reaction occurs in the presence of hydrogen peroxide, which is the natural substrate. The HRP enzyme breaks two hydrogen

peroxide molecules into water and oxygen. However, the specificity of the HRP enzyme for the second molecule of hydrogen peroxide is low and therefore other electron donors may be considered. This low specificity increased the development of additional chromogenic substrates for HRP enzyme. These hydrogen donors substrates are oxidized and form a colored product which can be spectrophotometrically monitored ^[12]. There are several well studied HRP chromogenic substrates, such as 3, 3', 5, 5'-tetramethylbenzidine (TMB); 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS); o-phenylenediamine (OPD); 5-aminosalicylic acid (5-AS); 3-amino-9-ethylcarbazole (AEC); 3-methyl-2-benzothiazolinone hydrazone (MBTH); 3,3'-diaminobenzidine (DAB) and 4-chloro-1-naphthol (4-CN) ^[16-18]. In addition, the detection of colorimetric signals can be further enhanced for a more accurate signal measurement using a selected stopping solution.

In this study, the generation of a stopped colorimetric signal was examined for accurate and enhanced signal detection in paper-based biosensors. Stopping the reaction of colorimetric signal generation not only enhances the signal detection, but also stabilizes the detected signal, allowing a more accurate signal to be detected in comparison to unstopped colorimetric signals. Two most commonly used HRP substrates, TMB and ABTS, were compared in terms of their ability to generate a stopped colorimetric signal on membrane. First, the stopped colorimetric signals were compared in solution. Three different concentrations of stopping solution were tested for each substrate. For more accurate comparison between unstopped and stopped colorimetric signal detections in paper-based biosensors, the unstopped colorimetric signal on membrane was also examined. Subsequently, the stopped colorimetric signals were detected on membrane for paper-based biosensors, using 'Stack-Pad' sensor layout ^[19-21], which consists of vertically stacked functional membranes (Figure 8.1). The unstopped colorimetric signal was detected with and without the use of polyvinylidene difluoride (PVDF) ^[22] as a separation layer between the two functionalized layers of cellulose absorption pads containing substrate and stop solution ^[23].

Biosensor Schematic Layout

Figure 8.1. Biosensor Schematic Description.

A) The detection of stopped colorimetric signal on membrane was examined using ‘Stack-Pad’ sensor layout ^[19, 20]. B) The colorimetric signal was generated based on the reaction of the enzyme horseradish peroxidase (HRP) with the chromogenic substrates 3, 3', 5, 5'-tetramethylbenzidine (TMB) and 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Sulfuric acid and oxalic acid were used as the stopping solutions for TMB and ABTS respectively.

8.2 Materials and Methods

8.2.1 Materials

Phosphate buffered saline (PBS) tablets (Cat. No. P4417), polyoxyethylene sorbitan monolaurate (Tween20) (Cat. P7949), skim Milk powder (Cat. 70166), TMB liquid substrate for ELISA (Cat. No. T0440), sulfuric acid (Cat. No. 84716) and oxalic acid (Cat. No. 241172) were purchased from Sigma-Aldrich. Glassfiber (GFB-R4), polyester (PT-R5) and cellulose (AP080) were purchased from MDI membrane technologies. Nitrocellulose pore size 0.45 μm (1620115) and PVDF pore size 0.2 μm (1620177) were purchased from Bio-Rad. HRP Conjugated Goat anti Human IgG (Cat. ab97175) was purchased from Abcam. Recombinant protein G (Cat. 21193) and ABTS single solution ready for use (Cat. No. 00-2024) were purchased from Thermo Fisher. Milli-Q ultrafiltered (UF) H_2O (with a resistivity of 18.2 $\text{M}\Omega\text{ cm}$ at 25°C) were used in the preparation of all solutions.

8.2.2 Equipment

Weighing balance was purchased from Mettler Toledo. The hotplate (WH220PLUS) and the Binder oven were purchased from Gaia Science Pte Ltd. Membrane cutter (PAT/RE38, 219 & D613, 795) was purchased from EK Tools, Vaessen Creative (Vaessen B.V. Thermiekstraat 25 6361HB Nuth, The Netherlands). Digital Vernier Caliper was purchased from Kincrome. Luminoskan Ascent Luminometer was purchased from Thermo Fisher Scientific, Waltham, MA, USA. TECAN Infinite M200 PRO was purchased from Tecan Trading AG, Switzerland (Seestrasse 103, 8708 Männedorf, Switzerland).

Figure 8.2. Colorimetric Reaction of HRP with Chromogenic Substrates TMB and ABTS.

The colorimetric signal was generated based on the reaction of the enzyme horseradish peroxidase (HRP) with the chromogenic substrates 3, 3', 5, 5'-tetramethylbenzidine (TMB) and 2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Sulfuric acid and oxalic acid were used as the stopping solutions for TMB and ABTS respectively.

8.2.3 Stopped Colorimetric Signal in Solution

The stopped colorimetric signal was first tested in solution inside a 96-well plate. Two substrates were tested, TMB and ABTS, for the stopped colorimetric signal with HRP enzyme (Figure 8.2). In both cases, 50 ng/mL of HRP – antibody concentration was used. In the case of TMB, three sulfuric acid concentrations (0.5, 1 and 2 M) were tested as stopping solutions, and the stopped colorimetric signal was measured at the wavelength of 450 nm. In the case of ABTS, three oxalic acid concentrations (0.3, 0.6 and 1 M) were tested as stopping solutions, and the stopped colorimetric signal was measured at 405 nm. Firstly, 100 μ L of 20 ng/mL HRP – antibody, diluted in PBS buffer, was added to each well together with 100 μ L of substrate (either TMB or ABTS). After 8 minutes of reaction at RT and in dark conditions, 100 μ L of stopping solution was added accordingly. The absorbance signal was then immediately measured using a TECAN reader. The stopped colorimetric signal was further tested with a range of HRP – antibody concentrations 0 – 75 ng/mL (0, 0.5, 1, 2.5, 10, 25, 50 and 75 ng/mL) for both substrates. In this case, the stopping solutions used for TMB and ABTS were kept at 0.5 M sulfuric acid and 0.6 M oxalic acid respectively.

8.2.4 Unstopped Colorimetric Signal on Membrane

Colorimetric signal detection was then carried out on membrane in order to compare unstopped and stopped colorimetric signal detections in paper diagnostics. Firstly, the unstopped colorimetric signal on membrane was examined for two HRP substrates, TMB and ABTS, and examined over a range of HRP – antibody dilutions. The 0.5 mg/mL HRP – antibody stock concentration was diluted in 0.05% (v/v) PBS - Tween20 buffer into the following dilutions: 1:4K; 1:6K; 1:8K; 1:10K; 1:12K; 1:14K; 1:16K and 1:18K. The substrate, TMB or ABTS, was first immobilized on a 6 mm $\varnothing$ cut cellulose absorption pad. Four different substrate volumes (10, 20, 30 and 40 μ L) were then tested for each substrate. After placing the substrates on the absorption pads, the pads were dried at RT for 2 hours. In order to test for unstopped colorimetric signal reaction on the membrane, the pads were

then exposed to a 50 μL drop of diluted HRP – antibody accordingly. After 8 minutes of reaction, the pads were visually inspected, and a picture was taken for further examination.

8.2.5 Stopped Colorimetric Signal on Membrane

Subsequently, stopped colorimetric signal detection on membrane for paper diagnostics was also examined. The stopped colorimetric signal on membrane was tested for the two substrates, TMB and ABTS, with 100 ng/mL HRP enzyme, and with or without a polyvinylidene difluoride (PVDF) separation layer. The detection of stopped colorimetric signal on membrane was examined using ‘Stack-Pad’ sensor layout, which consists of vertically stacked functional membranes (Figure 8.1) ^[19, 20]. The three layers of PVDF membrane serve as a separation layer between the functionalized substrate and the stopping layers in the ‘Stack-Pad’. The sample is added from the bottom-up and as the layers are wetted, the sample moves upwards. In the top-most layer, a colorimetric signal is generated from the reaction between the substrate and the HRP – conjugated antibody. The ‘Stack-Pad’ test was constructed as follows: (From bottom up) 0.05% PBS-Tween20 immobilized on ‘sample layer’ (glassfiber membrane), 50 μL of 100 ng/mL HRP – antibody (diluted in 0.05% PBS-Tween20) immobilized on the ‘conjugation layer’ (polyester membrane), 1% (w/v) polyvinyl alcohol (PVA) immobilized on ‘buffer layer’ (polyester membrane), 50 μL substrate (TMB or ABTS) immobilized on the ‘substrate layer’ (polyester membrane), unmodified ‘separation layer’ (PVDF membrane) and 50 μL stopping solution (0.5M sulfuric acid for TMB and 0.6M oxalic acid for ABTS) immobilized on the ‘stopping layer’ (cellulose membrane). After immobilization of the chemicals, all pads were dried for 1 – 2 hours at RT. After 8 minutes of reaction, the pads were visually inspected, and a picture was taken for further examination. The experiment was also conducted for the unstopped reaction, as a measure of control for the generation of an unstopped colorimetric signal with and without the use of the PVDF separation layer. In addition, in order to examine the colorimetric signal stability over time, the signals were further examined in several time points.

8.2.6 Color Intensity Analysis

The images of the pads color intensity were firstly recorded in JPEG files. Then, they were analyzed using Image J software (US National Institutes of Health). The colored images were firstly transformed to a 32-bit format and converted into grey contrast. Thereafter, the images were inverted for further analysis. Lastly, the average color intensities were recorded for each tested pad.

8.3 Results and Discussion

8.3.1 Stopped Colorimetric Signal in Solution

Firstly, the stopped colorimetric signal was compared in solution, and the results are presented in Figure 8.3. The two substrates, TMB and ABTS, were both exposed to the same HRP - antibody concentration of 50 ng/mL. For each substrate, three different concentrations of stopping solution were tested ^[24]. In the case of TMB, sulfuric acid solution was used as the stopping solution (0.5, 1 and 2 M) ^[25, 26]. As shown in Figure 8.3A, in all three tested concentrations of stopping solution, the stopped signal measured was approximately the same (3.49, 3.55 and 3.50 absorbance). Similarly for ABTS, as shown in Figure 8.3B, the three tested concentrations of oxalic acid stopping solution (0.3, 0.6 and 1 M) ^[27, 28] showed similar stopped signal values (2.76, 2.81 and 2.82 absorbance) as well. The stopped colorimetric signal was further tested with a range of HRP – antibody concentrations from 0 – 75 ng/mL (0, 0.5, 1, 2.5, 10, 25, 50 and 75 ng/mL) for both substrates (Figure 8.3C). The stopped signals for TMB and ABTS were tested with 0.5 M sulfuric acid and 0.6 M oxalic acid stopping solutions respectively. As observed in Figure 8.3C, TMB showed increased sensitivity in the stopped colorimetric signal in all the tested HRP – antibody concentrations (0 ng/mL: 0.077 vs. 0.066; 0.5 ng/mL: 0.138 vs. 0.098; 1 ng/mL: 0.229 vs. 0.119; 2.5 ng/mL: 0.519 vs. 0.195; 10 ng/mL: 1.68 vs. 0.540; 25 ng/mL: 3.51 vs. 1.61; 50 ng/mL: 3.56 vs. 3.04 and 75 ng/mL: 3.58 vs. 3.43). The increased TMB sensitivity in the stopped colorimetric signal was mainly detected between 1 – 25 ng/mL of HRP – antibody concentrations.

The color gradient in the stopped colorimetric signal is also clearly visible for the range of HRP – antibody concentrations tested, as shown in Figure 8.3D. To conclude, a stopped colorimetric signal was detected in solution for both tested substrates, TMB and ABTS, with a clear color gradient observed for the range of HRP – antibody concentrations. It was also observed that measurements obtained from stopping the reaction were stable over the next several hours. Stopping the reaction of colorimetric signal generation thus not only enhances the signal detection, but also stabilizes the detected signal, allowing a more accurate signal to be detected in comparison to unstopped colorimetric signals.

Figure 8.3. Stopped Colorimetric Signal in Solution.

A) Stopped colorimetric signal for TMB (450 nm) with three sulfuric acid concentrations (0.5, 1 and 2 M) as a stopping solution, using 50 ng/mL HRP – antibody concentrations. B) Stopped colorimetric signal measurement for ABTS (405 nm) with three oxalic acid concentrations (0.3, 0.6 and 1 M) as a stopping solution, using 50 ng/mL HRP – antibody concentrations. C) Stopped colorimetric signal of TMB (450 nm; 0.5 M sulfuric acid) vs. ABTS (405 nm; 0.6 M oxalic acid) in a range of HRP – antibody concentrations. TMB showed increased sensitivity, with higher absorbance signals for the same HRP – antibody concentrations. D) Visible stopped colorimetric signal for TMB (450 nm) and ABTS (405 nm) with a range of HRP – antibody concentrations.

8.3.2 Unstopped Colorimetric Signal on Membrane

Colorimetric signal detection was then carried out on membrane in order to compare unstopped and stopped colorimetric signal detections in paper diagnostics (Figure 8.4, Figure 8.5 and Table 8.1). The unstopped colorimetric signal on membrane was compared for the two HRP substrates, TMB and ABTS, and also examined with a range of HRP – antibody dilutions in 0.05% (v/v) PBS - Tween20 buffer (1:4K; 1:6K; 1:8K; 1:10K; 1:12K; 1:14K; 1:16K and 1:18K). Four different substrate volumes (10, 20, 30 and 40 μ L) were also tested for each substrate. TMB (Figure 8.4) showed overall higher sensitivity for the diluted HRP – antibody samples compared to ABTS (Figure 8.5). In the lowest substrate volume tested of 10 μ L, ABTS did not show any unstopped colorimetric signal generated for the case of 1:18K HRP – antibody dilution. However, the unstopped colorimetric signal is clearly visible for 10 μ L TMB with 1:18K HRP - antibody. In the color intensity analysis (Figure 8.4B and Figure 8.5B), the results showed a clear gradient for the HRP – antibody dilutions (40 μ L substrate: TMB: 1:4K (195) vs. 1:18K (153) and ABTS: 1:4K (192) vs. 1:18K (122)). As well as, a clear gradient for the substrate volume (1:4K HRP – antibody: TMB: 10 μ L (146) vs. 40 μ L (195) and ABTS: 10 μ L (115) vs. 40 μ L (192)). In general, a nice and clear gradient is visible for both substrates tested, over the range of different substrate volumes and HRP – antibody dilutions tested. However, as the unstopped colorimetric signal in TMB was generally stronger and produced more intense coloration in all the tested HRP – antibody dilutions, the unstopped colorimetric signal gradient was thus more easily observed in the case of ABTS. To conclude, the unstopped colorimetric signal on membrane can be clearly observed for both TMB and ABTS, for the tested HRP – antibody

concentrations. A colorimetric signal can be generated on membrane using both TMB and ABTS substrates with HRP conjugated antibodies.

Figure 8.4. TMB Unstopped Colorimetric Signal on Membrane.

Colorimetric signal on membrane with a range of HRP – antibody concentrations (dilutions in 0.05% (v/v) PBS - Tween20 buffer (1:4K; 1:6K; 1:8K; 1:10K; 1:12K; 1:14K; 1:16K and 1:18K)) and a range of TMB substrate volumes (10, 20, 30 and 40 µL), for: A) picture and B) color intensity analysis.

Figure 8.5. ABTS Unstopped Colorimetric Signal on Membrane.

Colorimetric signal on membrane with a range of HRP – antibody concentrations (dilutions in 0.05% (v/v) PBS - Tween20 buffer (1:4K; 1:6K; 1:8K; 1:10K; 1:12K; 1:14K; 1:16K and 1:18K)) and a range of ABTS substrate volumes (10, 20, 30 and 40 μ L), for: A) picture and B) color intensity analysis.

Table 8.1. Color Intensity Analysis for TMB and ABTS Unstopped Colorimetric Signal on Membrane.

Color Intensity									
TMB									
	HRP – Antibody Dilution								
		1:4K	1:6K	1:8K	1:10K	1:12K	1:14K	1:16K	1:18K
TMB Volume	10μl	146 ± 17	151 ± 14	145 ± 17	153 ± 10	137 ± 17	129 ± 15	139 ± 11	128 ± 12
	20μl	176 ± 13	175 ± 7	172 ± 11	168 ± 13	168 ± 16	162 ± 7	161 ± 7	146 ± 8
	30μl	190 ± 13	183 ± 19	175 ± 16	178 ± 14	178 ± 8	163 ± 15	161 ± 12	148 ± 12
	40μl	195 ± 10	184 ± 13	184 ± 12	186 ± 19	178 ± 17	173 ± 7	170 ± 19	153 ± 20
ABTS									
	HRP – Antibody Dilution								
		1:4K	1:6K	1:8K	1:10K	1:12K	1:14K	1:16K	1:18K
ABTS Volume	10μl	115 ± 13	95 ± 4	85 ± 6	101 ± 8	92 ± 5	75 ± 14	81 ± 15	62 ± 16
	20μl	169 ± 9	143 ± 6	127 ± 13	129 ± 11	130 ± 13	99 ± 5	119 ± 6	97 ± 13
	30μl	180 ± 5	159 ± 13	149 ± 10	157 ± 17	154 ± 16	127 ± 9	138 ± 15	109 ± 13
	40μl	192 ± 8	172 ± 10	163 ± 9	171 ± 10	166 ± 14	147 ± 9	148 ± 15	122 ± 16

8.3.3 Stopped Colorimetric Signal on Membrane

Stopped colorimetric signal was detected on membrane, using ‘Stack-Pad’ sensor layout [19, 20] (Figure 8.1), which consists of vertically stacked functional membranes (Figure 8.6). The stopped colorimetric signal on membrane was tested for the two substrates, TMB and ABTS, with 100 ng/mL HRP enzyme, and with or without a PVDF separation layer. The comparison between the unstopped vs. stopped colorimetric signal was also conducted. Firstly, the unstopped colorimetric signal was detected for both cases of TMB and ABTS, with and without the use of PVDF [22] as a separation layer. The unstopped reaction was conducted as a measure of control for the generation of an unstopped colorimetric signal with and without the use of PVDF as a separation layer. The unstopped signals obtained for both substrates, TMB and ABTS, are visibly similar in the cases of with and without PVDF membrane (Figure 8.6). This suggests that the use of PVDF membrane as a separation layer does not interfere with the generation of a colorimetric signal. For the generation of stopped colorimetric signal on membrane, a clear, yellow stopped colorimetric signal was

visible on the membrane when TMB substrate was used in the presence of a PVDF layer. However, in the case of ABTS substrate, the colorimetric signal was less visible. When the stopped colorimetric signals obtained with PVDF separation layer was compared to the signals obtained without PVDF, it was observed that the stopped colorimetric signals were not visible without PVDF. These results strengthen the importance of using PVDF membrane as a separation layer between the substrate and the stopping solution layers, for the generation of a stopped colorimetric signal. This can be explained by the hydrophobic nature of the PVDF membrane, which slows the flow of sample between the substrate and the stopping solution layers, giving rise to a buffer layer for the generation of a colorimetric signal ^[22]. The results of the color intensity analysis also support these findings (Figure 8.6). The blank control with PVDF showed similar signal level (TMB: 108 and ABTS: 96) as the stopped test without PVDF (TMB: 110 and ABTS: 102). Meaning, that without PVDF membrane as separation layer, the stopped signal was not visually and quantitatively detected. Moreover, when comparing the stopped signal with and without PVDF membrane, the stopped signal detected with PVDF showed an increased value (TMB: 126 and ABTS: 121). In addition, the unstopped signal for both with (TMB: 176 and ABTS: 161) and without (TMB: 186 and ABTS: 157) PVDF membrane did show an elevated value, this is expected since the colorimetric reaction is not stopped. However, looking closer at the standard deviation values, the stopped signal is more accurate with lower standard deviation values compare to the unstopped signal. These quantitative signal value also provide evidence to support the claim that the addition of PVDF membrane as separation layer does not interfere to the generation of the colorimetric signal (TMB with: 176 vs. without: 186 and ABTS with: 161 vs. without: 157). To conclude, the stopped colorimetric signal was clearly observed for TMB but less observed for ABTS. Moreover, the generation of stopped colorimetric signal was dependent on the presence of PVDF membrane as separation layer between the two functionalized layers of substrate (TMB) and stop solution (sulfuric acid).

Figure 8.6. Stopped Colorimetric Signal on Membrane.

A) The stopped colorimetric signal was examined with and without three layers of polyvinylidene difluoride (PVDF) membrane as a separation layer between the functionalized substrate and the stopping layers. B) Two substrates were compared: TMB vs. ABTS. For unstopped vs. stopped colorimetric signal comparison on membrane, using 100 ng/mL HRP – antibody.

8.3.4 Colorimetric Signal Stability over Time

The colorimetric signal stability was also examined further over several time points (Table 8.2 and Figure 8.7). The stability of both stopped and unstopped colorimetric signals on membrane were compared, with three layers of PVDF membrane as a separation layer between the substrate and the stopping solution layers. In the case of TMB (Figure 8.7A), the unstopped signal values (171-192) were higher than the stopped signal values (67-111). Looking closer at the standard deviation values, it is possible to determine that the stopped colorimetric signal demonstrated a more stable and accurate signal detection with lower standard deviation values. On the contrary, in the case of ABTS (Figure 8.7C), both unstopped (66-149) and stopped (77-152) signals were in a similar range. This might indicate that the ABTS colorimetric reaction was not stopped. However, from both the image (Figure 8.7B) and color intensity analysis (Figure 8.7C), it is possible to still determine that there was a slight difference between the unstopped and stopped colorimetric signal. A difference is already visible after the 20 minutes time point (stopped: 81 ± 11 vs. unstopped: 96 ± 12) and can be identified until the 100 minutes time point (stopped: 130 ± 20 vs. unstopped: 141 ± 15). The ABTS stopped colorimetric reaction developed slightly slower than the unstopped reaction. To conclude, the stopped colorimetric signal was more stable and accurate over time for TMB but less observed for ABTS.

Figure 8.7. Stopped vs. Unstopped Colorimetric Signal Stability Over Time.

Two substrates were compared: TMB vs. ABTS. for unstopped vs. stopped colorimetric signal comparison on membrane. A) TMB; B) picture; C) ABTS.

Table 8.2. Color Intensity Analysis for Stopped vs. Unstopped Colorimetric Signal Stability Over Time.

Time (min)	Color Intensity			
	TMB		ABTS	
	Stopped	Unstopped	Stopped	Unstopped
0	38 ± 3	44 ± 6	35 ± 2	36 ± 2
8	67 ± 6	171 ± 14	77 ± 5	66 ± 7
20	79 ± 7	192 ± 18	81 ± 11	96 ± 12
30	80 ± 8	188 ± 22	89 ± 18	109 ± 14
40	77 ± 7	189 ± 18	96 ± 24	121 ± 12
50	77 ± 8	187 ± 17	102 ± 26	117 ± 15
60	81 ± 7	190 ± 15	115 ± 26	129 ± 14
90	83 ± 8	188 ± 13	122 ± 20	131 ± 13
100	86 ± 8	184 ± 20	130 ± 20	141 ± 15
110	88 ± 9	184 ± 18	117 ± 20	136 ± 13
120	92 ± 6	187 ± 14	126 ± 16	143 ± 13
140	89 ± 7	184 ± 16	129 ± 18	146 ± 14
150	92 ± 6	182 ± 17	123 ± 16	137 ± 12
180	96 ± 5	184 ± 15	134 ± 16	148 ± 11
210	103 ± 3	191 ± 12	136 ± 15	149 ± 9
240	99 ± 2	190 ± 10	135 ± 12	142 ± 8
300	111 ± 3	192 ± 11	152 ± 10	147 ± 9

8.4 Conclusion

In this study, the generation of a stopped colorimetric signal was examined for accurate and enhanced signal detection in paper-based biosensors. Firstly, the stopped colorimetric signal was compared in solution. The two substrates, TMB vs. ABTS, were exposed to the HRP - antibody. For each substrate, three different concentrations of stopping solution were tested. In the case of TMB, sulfuric acid solution was used as the stopping solution (0.5, 1 and 2 M), and the stopped signal obtained in each case was approximately the same (3.49, 3.55 and 3.50 absorbance). Similarly, in the case of ABTS, oxalic acid (0.3, 0.6 and 1 M) was used as the stopping solution and the stopped signals obtained were approximately the same (2.76, 2.81 and 2.82 absorbance). The stopped colorimetric signal

was then further tested with a range of HRP – antibody concentrations. TMB showed increased sensitivity in the stopped colorimetric signal in all the tested HRP – antibody concentrations. In order to compare unstopped and stopped colorimetric signal detection in paper diagnostics, the unstopped colorimetric signal on membrane was also examined, with a range of HRP – antibody dilutions. TMB showed increased sensitivity for the diluted HRP – antibody samples as compared to ABTS. In general, a clear gradient was visible for both substrates tested. Lastly, stopped colorimetric signal was detected on membrane, using ‘Stack-Pad’ sensor layout ^[19, 20], which consists of vertically stacked functional membranes. The unstopped reaction was also conducted, as a measure of control for the generation of an unstopped colorimetric signal with and without the use of the PVDF separation layer. In the case of TMB substrate, a clear, yellow stopped colorimetric signal was visible on the membrane. However, in the case of ABTS substrate, colorimetric signal was not visible. Moreover, when comparing between the stopped colorimetric signals obtained with PVDF as separation layer and those without, it was concluded that the stopped colorimetric signal was not visible without PVDF. To conclude, the stopped colorimetric signal was clearly observed for TMB but not for ABTS. Moreover, the generation of stopped colorimetric signal was dependent on the presence of PVDF membrane as separation layer between the two functionalized layers of substrate (TMB) and stop solution (sulfuric acid). In addition, the stopped colorimetric signal was more stable and accurate (lower standard deviation values) over time for TMB but less observed for ABTS. This study concludes that stopped colorimetric signals can be generated in paper-based biosensors for enhanced and accurate signal detection.

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Chapter 9.   **Gold Plasmonic Chip**

Surface-plasmon-resonance (SPR) is a quantum-electromagnetic phenomenon arising from the interaction of light with free electrons at a metal-dielectric interface. At a specific angle or wavelength of light, the energy of the photons is transferred to excite the free electrons oscillation on the surface. A change in the refractive index (RI) may occur, which is influenced by the analyte concentration in the medium in close contact with the metal surface. SPR has been widely used for the detection of gaseous, liquid, or solid samples. In this study, a functionalized specific SPR chip was designed and used in a point-of-care SPR module (PhotonicSys SPR H5) for the detection of the stroke biomarkers NT-proBNP and S100 β . These biomarkers have proven to be good for stroke diagnosis, with sensitivity and specificity > 85%. Specific detection was done by binding a biomolecular-recognizing antibody onto the Au SPR-chip. Detection was tested in water and plasma samples. NT-proBNP and S100 β were detected in a range of concentrations for stroke, from 0.1 ng/mL-10 ng/mL. The RI of blank plasma samples was 1.362412, and the lowest concentration tested for both biomarkers showed a prominent shift in the RI signal (0.25 ng/mL NT-proBNP (1.364215) and S100 β (1.364024)). The sensor demonstrated clinically relevant limit-of-detection less than 1 ng/mL.

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9.1 Introduction

Surface plasmon resonance (SPR) biosensors are often used in the detection of gaseous, liquid, or solid samples ^[2], since their origin in 1983 ^[3]. Sensing is conducted directly, without labeling, thus allowing real – time measurements. They require small sample sizes, and exhibit highly sensitive, quantitative and reproducible results. These advantages have led to applications in diagnostics, pharmaceuticals and military defense ^[4]. SPR is advantageous over other various optical biosensors, such as fluorescence, interferometry, spectroscopy and optical waveguides, as they often require the use of labels and multi-step detection protocols ^[5]. Moreover, SPR biosensors are characterized as zero background techniques because the analyte is the only signal source, therefore they enable highly sensitive measurements. In addition, transmitting conduits are not needed for the signal measurement in SPR biosensors, because optical signals travel in an open path ^[6]. SPR biosensors are categorized as angle, intensity, wavelength, polarization modulation or phase sensors.

They are based on a quantum electromagnetic phenomenon that arises when a metal-dielectric interface interacts with light ^[7]. At a certain angle or wavelength of the light, the energy of the photons excites the free electrons (surface plasmons (SPs)) on the metal interface ^[8], which results in a dip in the reflectance ^[9]. The excited extended SPs are considered as compressive electromagnetic surface waves on the metal interface. The SPs are strongly localized across the interface and may be considered as electromagnetic surface waves that propagate along the interface between a dielectric and a metal, and evanescent along the direction perpendicular to the interface ^[7, 8]. These surface waves are sensitive to refractive index (RI) shifts in the medium near the metal interface, which may quantitatively shift the resonant wavelength or the incident light angle ^[10]. Localized SPs (LSPs), which are usually excited from free space on the surface of metal nanoparticles, are differentiated from extended SPs (ESPs) which have longer propagation length and stronger sensitivity to the refractive index of the environment. The interaction between LSPs and ESPs is of interest for biosensing and other applications due to the ultra-high local field enhancement ^[11].

The extended SPR phenomenon causes a dip in reflectance at that specific wavelength or angle due to the absorption of the photon energy in the metal. In the case of exciting ESPs using nano-perforated metals such as periodic nanoslit a transmission peak is observed in what is called extraordinary or enhanced optical transmission ^[12]. The wave vector of SPs at a metal-dielectric interface is larger than that of the light wave in the dielectric ^[8] and this prevents SPs from exciting when a direct light wave is incident on the interface. To allow optical excitation of SPs, the wave vector of the incident light must be increased by passing the light wave through a diffracting structure or an optically denser medium via attenuated total reflection in a prism configuration at an angle larger than the critical angle. In order to excite the SPs, the incident light must be transverse magnetic (TM) – polarized and have enough momentum along the interface. This can be achieved by using refraction and diffraction elements such as prism, waveguide and grating coupling. In a SPR sensor, prism coupling is favorable for the development of simple miniaturized multichannel sensing devices on a single chip, in which the metal surface is placed directly on top of the prism surface ^[13]. The angle of incidence needs to be higher than the critical angle for total internal reflection (TIR). The wave on the interface is exceptionally sensitive to changes in the RI of the medium adjacent to the metal surface within the range of the SP field ^[9]. These changes may result in a shifting of the incident light resonant wavelength, a change in reflected light intensity ^[14], a change in incident light resonant angle ^[15] or a change in the phase of the reflected wave ^[16]. The metal interface on the SPR chip used for the generation of plasmon waves, is critical for the SPR biosensor performance ^[17]. The overall performance of the SPR biosensor depends on both the characteristics of the surface functionalization as well as the optical performance of the SPR sensor. The fact that the optical field is evanescent, i.e. penetrates only to a small distance (usually few hundreds of nm) inside the analyte medium, helps to make the sensor specific when a specific binder is used. SPR-based biosensors are able to detect biomolecular interactions directly with no need for labelling ^[9]. This characteristic allows real-time measurements of the reaction kinetics, analyte concentration, and binding parameters. Detection of large analyte (> 1 kDa) is usually performed using direct assay format ^[2]. In direct detection method, the bio-specific capture entity is attached to the sensor chip surface, thereby inducing a sensor response.

Several metal interfaces were tested for their use in SPR biosensors, such as gold, silver, aluminium, copper, titanium, sodium, indium and chromium. Different metals exhibit different limitations in SPR biosensing, in terms of the metal atmospheric inertness, resonance sharpness, compatibility with the chemicals needed for the assay functionalization and cost. Gold is the most well studied and practically used metal in SPR biosensors, because of several important advantages that include having a strong SPR dip, its inertness and ease of functionalization for biomolecules immobilization ^[18]. Other metals showed significant limitations. For example, silver is sensitive to oxidation ^[19], copper and aluminum exhibit low sensitivities, sodium is violently reactive and indium is expensive ^[9]. A bimetallic layer of silver and gold has also been explored, which enable the combination of the advantages of both metals, while compensating on their disadvantages. The silver layer maintains the surface plasmon wave while the gold layer ensures a chemically stable surface for the biomolecule immobilization ^[20]. In order to excite the SP, an incorporation of a second material layer is also required, either above or below the metal interface. Both of the materials are selected carefully so as to allow for the resonance condition ^[9].

In this study, a functionalized SPR chips were developed for the specific detection of biomarkers using an SPR POC device. This is the first time SPR technology is utilized as a POC device for the detection of blood-based biomarkers. SPR sensing technology is well known for its high detection sensitivity, which is required for the measurement of biomarkers. However, due to its bulky setup and complicated signal acquisition, SPR has never been utilized as a POC device. This study shows the use of a SPR module (PhotonicSys SPR H5). This miniature SPR module is portable and can be easily integrated with other instruments, making it attractive as a POC sensor. The novelty of this SPR technology arises from its ability to measure sub-ng/mL concentrations of blood-based biomarkers for point-of-care tests. The shift in RI is measured by a camera and line detection algorithm integrated within the SPR device that identifies a change in pixels, which is then translated into a shift in RI, based on the device pre-calibration. The SPR biosensor mechanism is based on the detection of biomarkers using a functionalized SPR chip.

The SPR chip is functionalized with a bio-specific capture entity (antibody) which acts as the biomolecular recognition element [8]. Immobilizing the recognition molecule onto the SPR chip surface is an essential step that is highly important and can affect the biosensor performance. The immobilization can be conducted via different methods, including physical absorption, hydrophobic electrostatic interactions and covalent coupling (carboxylic, thiol or aldehyde groups) [4]. When a concentration of biomarker is inserted onto the biosensor, the biomolecular interaction of specific binding of analyte is detected as a RI change on the sensor surface. The SPR biosensor chips were developed for the detection of two candidate biomarkers that have been extensively studied and have shown great diagnostic accuracy, the brain natriuretic peptide (NT-proBNP) and S100 β . Our data mining analysis showed that plasma levels of NT-proBNP ≥ 0.5 ng/mL and S100 β ≥ 0.1 ng/mL are associated with cardioembolic stroke, with a sensitivity and a specificity higher than 85%. These low cut-off values make it a necessity to have a highly sensitive and rapid point-of-care biosensor capable of measuring these biomarkers below 1 ng/mL concentrations. In addition, the type of the metal film on the SPR chip where plasmon waves are generated is critical for sensitive SPR sensing [17]. In order to achieve high-sensitivity in SPR sensing, silver substrate is a good option as plasmon coupling on silver substrates exhibits a sharper angular resonance, thus yielding a higher sensitivity [21]. However, silver has poor chemical stability and is susceptible to thermal desorption and oxidation [19] which precludes its wide use for SPR sensing. Therefore, gold is mostly used in the SPR chip as they possess stable chemical and optical properties [18]. Most SPR chips utilizes a gold nano-layer for sensing [9, 22, 23]. In this study, a bi-metallic layer is used consisting of silver and gold chip, utilized to detect sub nano concentrations of the stroke biomarkers [8, 24-26].

9.2 Results and Discussion

9.2.1 Biosensor Calibration to the Gold Plasmonic Chip

The detection system is factory-calibrated and does not require user intervention. However, there could be cases where calibration is lost. The device can then be recalibrated by

measuring the pixel value of different concentrations of glycerol (5% v/v - 25% v/v) diluted in DI-water (Figure 9.1). Then, the pixel was analyzed according to the solution known RI value. DI water is used in the reference channel.

Figure 9.1. Biosensor Calibration to the Gold Plasmonic Chip.

9.2.2 Gold Plasmonic Chip Functionalization

The gold SPR chip is functionalized as presented in Figure 9.2. The gold chip is first cleaned in an ultrasonic bath and incubated with deionized (DI) water for 20 minutes at room temperature (RT) to remove inorganic and organic contaminants on the surface. Then, the chip is incubated in 1 mM MUA-ethanolic solution for 18 hours at 4°C in the dark. This is to enable the formation of a self-assembled monolayer with carboxyl functional group on the surface [8, 24-26]. Thereafter, the chip is soaked in activation reagent solution (0.1 mM NHS - 0.4 mM EDC - 10 mM MES buffer pH 5) for 60 minutes at RT to activate the terminal carboxyl acid group [24-26]. After the activation of carboxylic acid group, specific antibody (Anti-NT-proBNP or Anti-S100β) is immobilized on the chip surface by soaking the chip in 20 µg/mL antibody solution diluted in 0.05% (v/v) PBS-Tween20 for 18 hours at 4°C. Lastly, the chip is soaked in 50 mM EA-PBS solution for 30 minutes at RT for blocking of non-specific binding sites.

Figure 9.2. Gold Plasmonic Chip Functionalization.

Gold chip is functionalized with a specific antibody, based on MUA-EDC/NHS reaction (MUA: 11-Mercaptoundecanoic acid; EDC: N-(3-Di-methylaminopropyl)-N'-ethyl carbodiimide hydrochloride; NHS: N-Hydroxysuccinimide).

The SPR biosensor mechanism is based on the detection of biomarkers using a functionalized SPR chip. The SPR chip is functionalized with a bio-specific capture entity (antibody) acting as the biomolecular recognition element [8]. The functionalization of the gold SPR chip includes the removal of inorganic and organic contaminants with the ultrasonic bath, cleaning and activation using MUA-ethanolic solution to allow the formation of a self-assembled monolayer with carboxyl functional group on the surface, coupling of antibody onto substrate surface with EDC/NHS solution and blocking with ethanolamine. In order to validate the surface modification of the gold SPR chip, the pixel value of DI Water is monitored after each functionalization step (Figure 9.3). Adsorptions on the gold surface changes the SPR resonance angle θ_{SPR} , and consequently the RI of the analyte near the gold layer [27], which results in a change in the pixel value measured in the SPR detection system. The pixel value decreases over each functionalization step with the linear equation $Y = -6.1494X + 593.14$ ($R^2 = 0.9735$). The decrease in pixel value indicates

that the SPR dip shifts up and that the SPR resonance angle θ_{SPR} increases. This positive shift in the SPR dip occurs when adsorptive structure is applied on the chip ^[28]. Therefore, the change in surface chemistry on the chip is validated with the result shown in Figure 9.3. The results presented are in agreement with the findings from Asta Kausaite *et al.* ^[29], who found an increase in SPR resonance angle through the surface functionalization procedure with MUA, EDC/NHS, and Ethanolamine, and Omar *et al.* ^[30], who found an increase in SPR resonance angle with the introduction of EDC/NHS onto the chip layer during immobilization procedure. In addition, these results are also in agreement with Karabchevsky *et al.* ^[27], who found an increase in SPR resonance angle following 11-MUA incubation and antibody immobilization on silver SPR chips. To conclude, the pixel negative shift, which is equivalent to a SPR dip positive shift, validates the change of surface chemistry on the gold SPR chip.

Figure 9.3. Characterization of the Gold Plasmonic Chip Functionalization.

9.2.3 Biomarkers Measurement with Gold Plasmonic Chip

NT-proBNP and S100 β were chosen as the two stroke biomarkers candidates as they have been extensively studied and have shown great diagnostic accuracy. Firstly, the gold SPR chips were functionalized with a specific antibody via a MUA-EDC/NHS-based reaction. After specific antibody functionalization, the chips were exposed to increasing biomarker concentrations between 0.25 ng/mL to 10 ng/mL diluted in DI water. NT-proBNP detection showed consistent results with a linear trend of increasing RI signal with increasing NT-proBNP concentrations. The increase in RI with increasing NT-proBNP concentrations is characterized with the linear equation $Y = 0.0005X + 1.3309$ ($R^2 = 0.6753$) (Figure 9.4A). The LOD of 0.12 ng/mL NT-proBNP was determined based on the sensitivity (0.0005 (RIU / (ng/mL))) and the average SD (0.00002 (RIU)). NT-proBNP sensing was previously explored by Binbin Luo *et al.* [31], with a label-free immunosensor platform based on excessively tilted fiber gratings (Ex-TFGs). The lowest detectable concentration of 0.5 ng/mL for NT-proBNP was obtained. Yuji Teramura *et al.* [32] explored the detection of BNP (NT-proBNP is the derivative of BNP) using SPR biosensor. The SPR signal was amplified by using a sandwich biosensor layout with a secondary antibody conjugated with streptavidin nanobeads. By this method, the SPR signals were intensified and sub-nano (0.25 ng/mL) concentrations of BNP could thus be detected. Hye Ri Jang *et al.* [33] also investigated the detection of BNP using SPR sandwich biosensor layout, but with the use of aptamer as the bio-specific capture entity. Kurita *et al.* [34] also explored BNP detection using a microfluidic device combined with a portable SPR sensor system. The sensor achieved a detectable concentration range of 5 pg/mL - 100 ng/mL by monitoring the SPR angle shift.

The functionalized gold SPR chips were also functionalized with specific anti-S100 β antibody, and then exposed to increasing S100 β concentrations. S100 β detection showed consistent results with a linear trend of increased RI signal with increasing S100 β concentrations. The increase in RI with increasing S100 β concentrations is characterized by the linear equation $Y = 0.0002X + 1.3322$ ($R^2 = 0.9411$) (Figure 9.4B). The LOD of 0.75 ng/mL S100 β in water was determined based on the sensitivity (0.0002 (RIU / (ng/mL)))

and the average SD (0.00005 (RIU)). Previously, SPR technology was utilized for the testing of protein-protein interactions of S100 β . Harald Seitz *et al.* [35] showed that by using SPR technology and functional protein microarrays, specific calcium-dependent interactions between Arg-Gly-Ser-(RGS)-His6 tagged proteins and GST-tagged S100 β can be detected. In a different study by Estelle Leclerc *et al.* [36], SPR technology was utilized to examine the interaction between S100 proteins and isolated domains of the receptor for advanced glycation endproducts (RAGE), in particular, the measurement of the binding of S100 β to RAGE V domain. Our results demonstrate for the first time a sensitive and label free SPR biosensor for the detection of NT-proBNP and S100 β .

9.2.4 Validation in Spiked Porcine Plasma Samples with Gold Plasmonic Chip

Porcine plasma was used as a medium to mimic the sample environment of human plasma, which is the preferred sample type for diagnostic testing of NT-proBNP and S100 β . NT-proBNP detection in plasma showed consistent results with a linear trend of increased RI signal with increasing NT-proBNP concentrations. The increase in RI with increasing NT-proBNP concentrations is characterized by the linear equation $Y = 0.0016X + 1.3613$ ($R^2 = 0.8873$) (Figure 9.5A). The LOD of 0.075 ng/mL NT-proBNP in plasma was determined based on the sensitivity (0.0016 (RIU / (ng/mL))) and the average SD (0.00004 (RIU)). S100 β detection in plasma also showed consistent results with a linear trend of increased RI signal with increasing S100 β concentrations. The increase in RI with increasing S100 β concentrations is characterized by the linear equation $Y = 0.0011X + 1.362$ ($R^2 = 0.9406$) (Figure 9.5B). The LOD of 0.136 ng/mL S100 β in plasma was determined based on the sensitivity (0.0011 (RIU / (ng/mL))) and the average SD (0.00005 (RIU)).

Figure 9.4. Stroke Biomarkers Measurement using Gold Plasmonic Chip.

A) NT-proBNP; B) S100 β .

Figure 9.5. Validation in Spiked Plasma Samples with Gold Plasmonic Chip.

A) NT-proBNP; B) S100 β .

9.3 Conclusions

This is the first time SPR technology is utilized as a POC device for the detection of blood-based biomarkers. NT-proBNP and S100 β were chosen as the two stroke biomarkers candidates. The SPR biosensor mechanism is based on a functionalized SPR chip with bio-specific capture entity (antibody) [8]. The biomolecular interaction of specific binding is detected as a RI shift. Gold chip was chosen as it possess stable chemical and optical properties [9, 18, 22, 23]. Firstly, the gold SPR chips were functionalized with a specific antibody via a MUA-EDC/NHS-based reaction. After specific antibody functionalization, the chips were exposed to increasing biomarker concentrations. NT-proBNP detection in water showed consistent results with a linear trend of increased RI signal with increasing NT-proBNP concentrations. The increase in RI with increasing NT-proBNP concentrations is characterized by the linear equation $Y = 0.0005X + 1.3309$ ($R^2 = 0.6753$). The LOD of 0.12 ng/mL NT-proBNP was determined based on the sensitivity (0.0005 (RIU / (ng/mL))) and the average SD (0.00002 (RIU)). Also, NT-proBNP detection in plasma showed consistent results with a linear trend of increased RI signal with increasing NT-proBNP concentrations, $Y = 0.0016X + 1.3613$ ($R^2 = 0.8873$). The LOD of 0.075 ng/mL NT-proBNP in plasma was determined based on the sensitivity (0.0016 (RIU / (ng/mL))) and the average SD (0.00004 (RIU)). Moreover, S100 β detection showed consistent results with a linear trend of increased RI signal with increasing S100 β concentrations. The increase in RI with increasing S100 β concentrations is characterized by the linear equation $Y = 0.0002X + 1.3322$ ($R^2 = 0.9411$). The LOD of 0.75 ng/mL S100 β in water was determined based on the sensitivity (0.0002 (RIU / (ng/mL))) and the average SD (0.00005 (RIU)). Also, S100 β detection in plasma showed consistent results with a linear trend of increased RI signal with increasing S100 β concentrations, $Y = 0.0011X + 1.362$ ($R^2 = 0.9406$). The LOD of 0.136 ng/mL S100 β in plasma was determined based on the sensitivity (0.0011 (RIU / (ng/mL))) and the average SD (0.00005 (RIU)). To conclude, NT-proBNP and S100 β were detected in a range of concentrations for stroke, from 0.1 ng/mL-10 ng/mL. The biosensor demonstrated a clinically relevant limit-of-detection less than 1 ng/mL.

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Chapter 10.   **Silicon Dioxide Plasmonic Chip**

Plasmon waveguide resonance (PWR) mode is obtained when a dielectric waveguide layer is added to the metal film. In this study, a self-referenced PWR (SRPWR) silicon dioxide (SiO₂) chip was examined. The self-referenced measurement is important to compensate for temperature fluctuations and other instabilities and allows to conduct RI signal measurement without an additional reference sample, thus minimizing the sample volume needed. The chip was fabricated with a multi-layer of metals and dielectrics, consisting of a 420 nm SiO₂ layer, a 40 nm Ag layer and another a 480 nm SiO₂ layer. This chip was shown to give one internal plasmon excited on the bottom interface SiO₂/Ag which is used as self-reference in the detection. The top layer acts as a waveguide layer and can be designed to give modes with ultrahigh penetration depth. A direct assay format was developed, where the recognition molecule (antibody) was immobilized onto the SiO₂ plasmonic chip surface, via a covalent coupling protocol based on 3-aminopropyltriethoxysilane and glutaraldehyde. The SRPWR biosensor was developed for sensing two chosen stroke biomarkers: NT-proBNP and S100 β , which are sensitive and specific for stroke diagnostics. For both biomarkers, a linear decreasing pattern in the RI signal was recognized with the increasing biomarkers concentrations. The SiO₂ based plasmonic chip demonstrates a limit-of-detection of less than 1 ng/mL that is clinically relevant for both stroke biomarkers.

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10.1 Introduction

In recent years, silicon dioxide (SiO_2) chips have been increasingly used in various biosensor applications ^[2-4]. SiO_2 is a good SPR chip cover candidate because of its higher thermal stability and low cost ^[2]. The thermal stability of the chip surface is important for the immobilization of the bio-specific capture entity, which may involve high temperature condition. Furthermore, the ease and cost effectiveness of SiO_2 processing makes it a good candidate as an SPR chip ^[3]. Studies have also shown that the sensitivity of bare gold chip has no significant difference as compared to the SiO_2 covered gold chip ^[4]. Plasmon waveguide resonance (PWR) mode is obtained when a dielectric waveguide layer is added to the metal film and exhibit narrow dips. A novel self-referenced PWR (SRPWR) silicon dioxide (SiO_2) chip was examined. The chip was fabricated with a multi-layer of insulator-metal-insulator (IMI), consisting of a 420 nm SiO_2 layer, a 40 nm Ag layer and another a 480 nm SiO_2 layer. This chip was shown to give one internal plasmon excited on the bottom interface SiO_2/Ag which is used as self-reference in the detection. The top layer acts as a waveguide layer and can be designed to give modes with ultrahigh penetration depth. This chip was shown to give one plasmon excited at the internal SiO_2/Ag interface which is used as self-reference, while the other mode behaving like long range SPR has a high penetration depth controllable from few hundred nm till few microns ^[5]. This later mode is in fact a guided mode. The plasmon waveguide resonance (PWR) is a mode obtained when a dielectric waveguide layer is deposited on top of the metal film. Both TE and TM polarizations can be used to excite guided modes exhibiting narrow resonances, thus improved figure of merit. Isaacs *et al.* ^[6] had recently used this PWR sensor for determining the orientation of molecules on the surface. The addition of the bottom SiO_2 layer to the PWR sensor generates a SRPWR, benefiting both from the higher figure of merit and the accurate measurement due to the self-reference. The self-referenced measurement (Figure 10.1) is important to compensate for temperature fluctuations and other instabilities. This also allows to conduct accurate measurements of the RI signal or analyte concentration without the need for an additional reference sample, thus minimizing the sample volume needed.

Figure 10.1. Self-Referenced Silicon Dioxide Plasmonic Chip.

The SPRWR SiO₂ chip allows for a stable self-reference measurement due to its multi-layered structure of 420 nm SiO₂ – 40 nm Ag – 480 nm SiO₂, thus minimising the sample volume required. The top dark line is the self-reference SPR line while the bottom dark line corresponds to the PWR mode.

The top dark line corresponds to the self-reference SPR while the bottom dark line corresponds to the PWR mode. Before sample addition, the bottom dark lines correspond to air. After DI water sample addition, into both reference and analyte channels, two dark lines are seen. The software red color marker covers the thin dark line, which is prove of how thin it is, while it is clearly seen in the reference channel. The thinner dark line means much narrower resonance and therefore higher figure of merit. Moreover, the sensor has a wide dynamic range, as it can detect gases and liquids using the same chip. The IMI chip is designed to be compatible with the PhotonicSys SPR system and the software, which enables the monitoring of both the reference line as well as the two sample channels ^[7]. A direct assay format was developed, where the recognition molecule was immobilized onto the SiO₂ plasmonic chip surface. A covalent coupling immobilization protocol based on 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde was utilized in order to covalently bind the recognition molecule (antibody) onto the SiO₂ surface. The SRPWR biosensor was developed with the purpose of detecting two chosen stroke biomarkers, NT-proBNP and S100β.

10.2 Results and Discussion

10.2.1 Biosensor Calibration to the Silicone Dioxide Plasmonic Chip

Though the SPR detection system was previously factory-calibrated, it can be further calibrated to adjust to specific work setups, in order to accommodate any temperature and humidity changes. This can be easily achieved by monitoring the pixel value of diluted glycerol (5% v/v–25% v/v) solutions in deionized water (Figure 10.2). Subsequently, the known RI value of the solution was used in order to analyze the pixel value accordingly. The PhotonicSys SPR H5 detects the location of the dark line in pixels through a camera connection. The shift in RI signal is then monitored with a line detection algorithm that first identifies the change in pixels, before translating it into a shift in RI.

Figure 10.2. Biosensor Calibration to the Silicone Dioxide Plasmonic Chip.

The PhotonicSys SPR H5 detects the location of the dark line in pixels through a camera connection. The shift in RI signal is then monitored with a line detection algorithm that firstly identifies the change in pixels, before translating it into a shift in RI.

10.2.2 Silicon Dioxide Plasmonic Chip Functionalization

Figure 10.3. Functionalization of the Silicon Dioxide Plasmonic Chip.

Immobilization protocol based on the utilization of 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde, for the covalent binding of the recognition molecule onto the silicon dioxide surface.

Multiple steps were conducted for the functionalization of the silicon dioxide chip surface using APTES and glutaraldehyde, as presented in the schematic in Figure 10.3. Firstly, the cleaning and activation of the surface of the silicon dioxide chips has a significant effect on the subsequent surface functionalization steps. The silicon dioxide chip was initially cleaned by washing with de-ionized (DI) water in ultrasonic bath for 20 minutes at room temperature (RT), in order to remove inorganic and organic surface contaminants. Subsequently, the chip was soaked in piranha solution ($\text{H}_2\text{SO}_4:\text{H}_2\text{O}_2 = 7:3$) for 1 minute at RT, for further removal of the remaining organic contaminants and to improve the surface hydrophilicity by hydroxylating the silicon dioxide surface^[8]. The chip surface is activated when it is hydroxylated and is then ready for silanization by APTES. In the silanization step, the chip was soaked in 2% APTES solution in preheated ethanol with a drop of acetic

acid for 60 minutes at RT for the formation of monolayer silane (~ 2 nm) ^[8] and to promote protein adhesion. Acetic acid was also added to the diluted APTES solution to orientate the amino groups outward from the interdigitated surface ^[9]. After 60 minutes incubation in the oven at 60°C, the dried chip was soaked in 2.5% (v/v) glutaraldehyde in PBS solution for 60 minutes at RT, to convert the amine-terminated surface into an aldehyde-terminated surface. After the generation of aldehyde group, a specific antibody (Anti-S100 β or Anti-NT-proBNP) was immobilized on the chip surface by soaking the chip in a 20 μ g/mL antibody solution diluted in 0.05% (v/v) PBS-Tween20 for 18 hours at 4°C. Lastly, the chip was soaked in a 0.2% (w/v) BSA-PBS-0.05% (v/v) Tween20 solution, in order to block and to maintain a constant pH on the surface, as well as to minimize non-specific binding and background signal ^[10].

As previously discussed, SiO₂ chips have been increasingly used in various biosensor applications ^[2-4]. SiO₂ is a good candidate as a SPR chip because of its higher thermal stability ^[2], the ease and cost effectiveness of SiO₂ processing ^[3]. Studies have also shown that the sensitivity of bare gold chips has no significant difference as compared to the SiO₂ covered gold chip ^[4]. Moreover, higher sensitivity can be obtained in angular mode when a thin dielectric layer with high RI is deposited (below the cutoff for guided mode excitation), while the figure of merit remains the same ^[11, 12]. On the other hand, the sensitivity and the figure of merit can be both enhanced in the spectral mode ^[13]. The chip examined in this study is novel because it is constructed with thick dielectric layers below and above the metal film, in order to excite both the self-reference plasmon and the higher figure of merit guided mode. The chip was fabricated with a multi-layer of insulator-metal-insulator, consisting of a 420 nm SiO₂ layer, a 40 nm silver layer then another layer of 480 nm SiO₂. A covalent coupling immobilization protocol was examined in order to immobilize the recognition molecule on the SPR chip surface and to develop a direct assay format. The protocol is based on the utilization of 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde, in order to covalently bind the recognition molecule onto the silicon dioxide surface.

Previous studies, had examined the covalent coupling immobilization protocol on SiO₂ surface, based on APTES and glutaraldehyde [8-10]. However, it was never tested for use in SPR sensing. The functionalization of the SPR SiO₂ chip required the use of an ultrasonic bath to remove any inorganic and organic contaminants. Then, the chips were cleaned and activated with piranha solution to improve the surface hydrophilicity via hydroxylating the silicon surface. Silanization with APTES was then conducted for the formation of monolayer silane (~2 nm) [8] and promotion of protein adhesion. The chips were subsequently soaked in glutaraldehyde solution in order to convert the amine-terminated surface into an aldehyde-terminated surface, before the antibody coupling onto the substrate surface and blocking with BSA. The surface modifications of the SiO₂ SPR chip after each functionalization step were validated by monitoring the changes in the pixel value of deionized Water (Figure 10.4).

Adsorptions on the SiO₂ surface changes the SPR resonance angle θ_{SPR} , and consequently the RI of the analyte near the SiO₂ layer, which results in a change in the pixel value measured by the SPR biosensor. As shown in Figure 10.4, the pixel value decreased over the functionalization steps. The decrease in pixel value indicated a shift in the SPR dip upwards meaning the SPR resonance angle θ_{SPR} increased as expected. A significant change is visible following the two functionalization steps of antibody (pixel 5A: 722.3 and 5B: 721.0) and BSA protein (pixel 5A: 718.8 and 5B: 716.9) immobilizations. Moreover, after introducing increasing levels of the biomarkers (NT-proBNP and S100 β) concentrations, an increase in pixel value is shown. For NT-proBNP, a steeper increase is visible (Figure 10.4A), with the pixel value increasing from 718.3 to 724.1 in the highest concentration tested. For S100 β , a milder pattern is visible (Figure 10.4B), with the pixel value increasing from 714.7 to 718.9 in the highest concentration tested. To conclude, the changes on the SiO₂ SPR chip surface were confirmed by the observation of a negative shift in the pixel values, which is equivalent to a positive shift in the SPR dip.

Figure 10.4. Characterization of the Silicon Dioxide Plasmonic Chip Functionalization

Monitoring the pixel value after each of the functionalization steps, and detection of different biomarkers concentrations of: A) NT-proBNP; B) S100 β . Signal is given pixels representing the resonance angle.

10.2.3 NT-proBNP Measurement

N-terminal pro brain natriuretic peptide (NT-proBNP) was chosen as a stroke biomarker candidate as it is both well-studied and has also shown excellent diagnostic accuracies. NT-proBNP, 76-amino acid peptide, is brain natriuretic peptide (BNP) biologically inactive derivative^[14, 15]. The neurohormone BNP is secreted after excessive stretching of heart muscle cells^[16] from the heart ventricles^[17]. Most assays prioritize the detection of NT-proBNP over BNP because of its longer half-life and higher molecular weight (NT-proBNP - Mw: 8.5kDa; half-life: 120 minutes vs. BNP - Mw: 3.5kDa; half-life: 20 minutes)^[18]. NT-proBNP elevated plasma levels (≥ 0.5 ng/mL) were found predictive of stroke mechanisms^[19-33]. The low cut-off value of less than 1 ng/mL set a need for a sensitive analytical device. Combined with the additional requirements for stroke diagnostics, there is a need for a highly sensitive and rapid point-of-care biosensor that can measure NT-proBNP in sub-nano concentrations. Therefore, a sensitive biosensor based on SPR detection was developed. The SiO₂ plasmonic chips were functionalized with specific anti-NT-proBNP antibody, based on an APTES and glutaraldehyde reaction. Subsequently, the different samples containing increasing NT-proBNP concentrations between 0.5 ng/mL and 10 ng/mL diluted in deionized water, were introduced to the plasmonic chips. NT-proBNP detection showed an inverse, linear relationship between the RI signal and NT-proBNP concentrations. This decreasing pattern in the RI signal with increasing NT-proBNP concentrations in water was quantified by the following linear equation: $Y = -0.0008X + 1.3337$ ($R^2 = 0.9542$) (Figure 10.5A). In addition, the LOD in water was calculated and identified as 0.1275 ng/mL NT-proBNP. The calculation (assuming $k = 3$ confidence level) was based on the following two specific parameters: sensitivity (0.0008 (RIU/(ng/mL))) and the average SD (0.000034 (RIU)). The sensing of NT-proBNP with SPR technology was previously examined by Binbin Luo *et al.*^[34], with excessively tilted fiber gratings (Ex-TFGs) (LOD of 0.5 ng/mL), and Harpaz *et al.*^[7] with a gold-silver plasmonic chip (LOD of 0.12 ng/mL). In addition, the sensing of BNP with SPR technology was previously examined only with gold as the metal interface in the plasmonic chip. Yuji Teramura *et al.*^[35] explored BNP detection with a sandwich biosensor layout, Jang *et al.*^[36] also examined a sandwich biosensor layout while utilizing aptamer as the bio-specific capture entity, and

Kurita *et al.* [37] developed BNP detection using a portable SPR sensor system combined with a microfluidic device. Our results demonstrate a novel, sensitive and self-referenced SiO₂ plasmonic chip utilized as an SPR biosensor for NT-proBNP sensing in clinically relevant limit-of-detection for stroke diagnosis. The system used is also miniature and cost effective so it can be used for point-of-care diagnostics.

10.2.4 S100 β Measurement

S100 β was also chosen as a stroke biomarker candidate as it has demonstrated great diagnostic accuracies. S100 β is part of the non-ubiquitous Ca²⁺-modulated protein family. It is an intracellular 10 kDa glial protein, secreted mainly from astrocytes [38]. This biomarker has a significant contribution to the differentiation, growth and repair of nerves [39-41]. It is mainly found in high levels in the cerebral astroglial compartment, peripheral Schwann cells, extraneuronally in melanocytes, adipocytes and chondrocytes [42]. Previous studies found that S100 β elevated plasma levels (≥ 0.1 ng/mL) are predictive of stroke mechanisms [43-49]. Again, this low cut-off value of less than 1 ng/mL makes it necessary to develop a highly sensitive and rapid point-of-care biosensor that can measure S100 β below ng/mL concentrations. The SiO₂ SPR chips were functionalized with a specific anti-S100 β antibody based on an APTES and glutaraldehyde reaction. Subsequently, the different samples containing increasing S100 β concentrations between 0.5 ng/mL and 10 ng/mL diluted in deionized water, were introduced to the plasmonic chips. S100 β detection showed a similar inverse, linear relationship between the increasing S100 β concentrations and the decreasing RI signal. This decreasing pattern in the RI signal with increasing S100 β concentrations in water was quantified by the following linear equation: $Y = -0.0001X + 1.3327$ ($R^2 = 0.9519$) (Figure 10.5B). In addition, the LOD in water was calculated and identified as 0.18 ng/mL S100 β . The calculation (assuming $k = 3$ confidence level) was based on the following two specific parameters: sensitivity (0.0001 (RIU/(ng/mL))) and the average SD (0.000006 (RIU)). S100 β sensing based on SPR technology was previously examined only by Harpaz *et al.* [7] with a gold-silver plasmonic chip (LOD of 0.75 ng/mL). Previous studies only examined the use of SPR technology for the testing of protein – protein interactions of S100 β , by Seitz *et al.* [50] and Leclerc [51]. Our results demonstrate a

novel, sensitive and self-referenced SiO₂ plasmonic chip utilized as an SPR biosensor for S100 β sensing in clinically relevant limit-of-detection for stroke diagnosis.

Figure 10.5. Biomarkers Measurement with Silicone Dioxide Plasmonic Chip.

RI value of various clinically relevant concentrations of A) NT-proBNP and B) S100 β .

10.2.5 Validation in Plasma

The sensing of the stroke biomarkers, NT-proBNP and S100 β , with the SiO₂ plasmonic chip, was later also validated in plasma samples. The biomarkers were spiked into porcine plasma, which was used as a substitute to human plasma, because it is the sample type that is mostly used for the diagnostic testing of NT-proBNP and S100 β . In addition, 90 nm silicon dioxide layer was added to the plasmonic chip in order to optimize the sensing range for the RI range of the plasma samples. The detection of increasing NT-proBNP concentrations in the spiked plasma samples showed consistent results with an inverse linear pattern of decreasing RI signal. This decreasing pattern in the RI signal with increasing NT-proBNP concentrations in plasma was quantified by the following linear equation: $Y = -0.00003X + 1.3624$ ($R^2 = 0.9802$) (Figure 10.6A). In addition, the LOD in plasma was calculated and identified as 1.3 ng/mL NT-proBNP. The calculation (assuming $k = 3$ confidence level) was based on the following two specific parameters: sensitivity (0.00003 (RIU/(ng/mL))) and the average SD (0.000013 (RIU)). Moreover, the detection of increasing S100 β concentrations in the spiked plasma samples also showed consistent results with an inverse linear pattern of decreasing RI signal. This decreasing pattern in the RI signal with increasing S100 β concentrations in plasma was quantified by the following linear equation: $Y = -0.0005X + 1.3631$ ($R^2 = 0.9168$) (Figure 10.6B).). In addition, the LOD in plasma was calculated and identified as 0.096 ng/mL S100 β . The calculation (assuming $k = 3$ confidence level) was based on the following two specific parameters: sensitivity (0.0005 (RIU/(ng/mL))) and the average SD (0.000016 (RIU)).

Figure 10.6. Validation in Plasma.

RI value of various clinically relevant concentrations of A) NT-proBNP and B) S100 β .

10.3 Conclusions

In this study, a functionalized, novel silicon dioxide (SiO₂) SPR chip was developed for application in an SPR point-of-care device for the detection of specific stroke biomarkers. This is the first known study whereby SiO₂ SPR chip was used in an SPR-based POC device to detect blood-based biomarkers. The stroke biomarkers, NT-proBNP and S100 β , were chosen as extensive research has been conducted on them, with great diagnostic accuracy demonstrated. The novel SiO₂ SPR chip was shown to give one internal plasmon which is used as self-reference, while the other mode behaving like long range SPR has a high penetration depth controllable from few hundred of nm till few microns ^[5]. In recent years, SiO₂ chips have been increasingly used in various biosensor applications ^[2-4] due to a higher thermal stability and low cost of SiO₂ material ^[2]. Thermal stability of the surface is a critical factor for the immobilization of bio-specific capture entity which may involve high temperature condition. Furthermore, the ease and cost effectiveness of SiO₂ processing makes it a good candidate as a SPR chip ^[3]. It is well understood that silver, gold and SiO₂ are suitable materials for the fabrication of SPR chips. Studies have also shown that the sensitivity of bare gold chip has no significant difference as compared to the SiO₂ covered gold chip ^[4]. The SPR biosensor is capable of detecting biomarkers using a functionalized SPR chip with a bio-specific capture entity (antibody) serving as the biomolecular recognition element ^[52]. The specific binding of the analyte is detected as a shift in RI. The SiO₂ SPR chips were first functionalized with a specific antibody via an APTES-glutaraldehyde-based reaction. Subsequently, the chips were exposed to increasing biomarker concentrations between 0.5 ng/mL and 10 ng/mL. Pixel change was first detected by a camera, before being translated into a shift in RI using a customized software. NT-proBNP detection in water showed consistent results with a linear trend of decreasing RI signal with increasing NT-proBNP concentrations. The decrease in RI with increasing NT-proBNP concentrations was characterized with the linear equation $Y = -0.0008X + 1.3337$ ($R^2 = 0.9542$). The LOD of 0.1275 ng/mL NT-proBNP was determined based on the sensitivity (0.0008 (RIU/(ng/mL))) and the average SD (0.000034 (RIU)). Moreover, S100 β detection in water showed consistent results with a linear trend of decreasing RI signal with increasing S100 β concentrations. The decrease in RI with increasing S100 β

concentrations was characterized with the linear equation $Y = -0.0001X + 1.3327$ ($R^2 = 0.9519$). The LOD of 0.18 ng/mL S100 β was determined based on the sensitivity (0.0001 (RIU/(ng/mL))) and the average SD (0.000006 (RIU)). To conclude, NT-proBNP and S100 β were detected in a range of concentrations for stroke, from 0.5 ng/mL to 10 ng/mL. The SiO₂ plasmonic chip demonstrated a clinically relevant limit-of-detection for stroke for both biomarkers, NT-proBNP (0.1275 ng/mL) and S100 β (0.18 ng/mL).

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Chapter 11.   **Implications & Impact**

This chapter presents the discussion on the thesis impact, concerning the development of the four optical biosensor platforms for stroke diagnostics, which includes two paper-based (colorimetric and chemiluminescent) and two plasmonic chip-based (gold-silver and silicone dioxide). Lastly, the outlook on different aspects that can be conducted in the future is also presented.

11.1 Conclusions

11.1.1 Stroke Biomarkers Identification

This thesis main objective is biosensor platform development for stroke diagnostics, for the multiplex and quantitative detection of selected specific stroke biomarkers at point-of-care, as a new ischemic stroke etiology classification device ^[1, 2]. The development of the optical biosensor platforms was firstly focused on the selection of the target analytes, the stroke biomarkers. As ischemic stroke involves different biomarkers from tissues in the brain, cardiac and vasculature ^[3], multiple biomarkers that target different aspects of stroke pathogenesis were examined for their performance to classify stroke mechanisms. Candidate biomarkers that interrogate different aspects of stroke pathogenesis (e.g. glial/neuronal death, inflammation, cardiac function, hemostasis, oxidative damage and neuroendocrine responses) were reviewed ^[2]. Then, only biomarkers that were available in hospital laboratory analytical platforms were included.

Consecutive patients with acute ischemic stroke admitted at the National University Hospital, Singapore, were recruited. Levels of NT-proBNP, D-dimer, S100 β , NSE, vitamin D, cortisol, interleukin-6, insulin, uric acid and albumin were compared between stroke mechanisms (using the TOAST criteria) and their ability to identify patients at high-risk of new-onset atrial fibrillation and to distinguish between cardioembolism and large artery disease mechanisms. The performance of these biomarkers to identify stroke mechanisms was examined with the use of statistical and data-mining tools. The combination and cut-off values of the biomarkers to elucidate stroke mechanisms were examined in this analysis. The methodology of using receiver operating characteristic (ROC) analysis was followed in order to identify optimal cut-off values for continuous markers that were subsequently reconstructed as dichotomous variables and used in the logistic regression analysis ^[4]. Multivariable regression was employed using backward stepwise logistic regression model to identify useful biomarkers ^[5]. Several biomarkers were identified as useful to elucidate stroke mechanisms (chapter 4), as well as showed value for additional stroke management needs, such as stroke diagnosis, severity, mortality and therapeutic admission (chapter 5).

From 515 ischemic stroke patients (mean age, 61 years; 71% men), 44 (8.5%) patients were diagnosed with new-onset AF and 75 (14.5%) patients had cardioembolism. The combination of two laboratory parameters (total cholesterol ≤ 169 mg/dL and triglycerides ≤ 44.5 mg/dL) and three biomarkers (NT-proBNP ≥ 294 pg/mL, S100 β ≥ 64 pg/mL and cortisol ≥ 471 nmol/L) identified patients with new-onset AF (negative predictive value (NPV) 90%, positive predictive values (PPV) 73% and area under curve (AUC) 85%). Whereas, the combination of two laboratory parameters (total cholesterol ≤ 169 mg/dL and triglycerides ≤ 44.5 mg/dL) and two biomarkers (NT-proBNP ≥ 507 pg/mL and S100 β ≥ 65 pg/mL) identified those with cardioembolism (NPV 86%, PPV 78% and AUC 87%). Adding clinical predictors, however, did not improve the performance of these models. To conclude, blood biomarkers that are accessible in most hospital laboratories could provide important hints on stroke etiology and direct the search for occult risk factors.

In addition, the different biomarkers were examined for their classification contribution with additional value in stroke management. Stroke diagnosis multivariable logistic regression modelling identified that the combination of the two biomarkers panel: NT-proBNP ≥ 0.064 ng/mL (p-value < 0.001 ; OR = 2.905) and insulin ≤ 23.4 pmol/L (p-value = 0.026; OR = 1.934), results in 85.2% sensitivity, 90.9% PPV, 79.2% correctly classified and 67.3% AUC. Moreover, stroke severity multivariable logistic regression modelling identified that the combination of the five biomarkers panel: HDL ≥ 43.3 mg/dL (p-value = 0.046, OR = 2.53), AST ≥ 25 U/L (p-value < 0.001 , OR = 6.25), cortisol ≥ 529 nmol/L (p-value = 0.034, OR = 2.72), IL-6 ≥ 7.7 pg/mL (p-value = 0.001, OR = 4.72), and S100 ≥ 71 pg/mL (p-value = 0.005, OR = 3.96) results in 96.9% specificity, 94.7% NPV, 92.2% correctly classified and 88.5% AUC. In addition, stroke mortality multivariable logistic regression modelling identified that the combination of the two biomarkers panel: NT-proBNP ≥ 271 pg/mL (p-value < 0.001 , OR = 6.62), and S100 ≥ 63 pg/mL (p-value = 0.005, OR = 2.18) results in 86.9% specificity, 88.4% NPV, 79.9% correctly classified and 77.4% AUC. Also, tPA admission multivariable logistic regression modelling identified that the combination of the three biomarkers panel: HDL ≥ 43.3 mg/dL (p-value = 0.050, OR = 1.95), cortisol ≥ 529 nmol/L (p-value < 0.001 , OR = 4.09) and S100 ≥ 65 pg/mL (p-value

= 0.001, OR = 3.18) results in 95.1% specificity, 92.3% NPV, 88.6% correctly classified and 76.7% AUC. Additionally, ischemic stroke vs. TIA multivariable logistic regression modelling identified that the combination of the four biomarkers panel: platelets $\geq 224 \times 10^9/L$ (p-value = 0.005, OR = 2.22), triglycerides $\leq 54.9 \text{ mg/dL}$ (p-value = 0.003, OR = 2.35), cortisol $\geq 387 \text{ nmol/L}$ (p-value = 0.006, OR = 2.19) and S100 $\geq 49 \text{ pg/mL}$ (p-value = 0.018, OR = 1.97) results in 98.5% sensitivity, 85.5% PPV, 84.7% correctly classified and 68.8% AUC. To conclude, four biomarkers, NT-proBNP, HDL, cortisol and S100, appeared more than once for additional value in stroke management. HDL and cortisol are common biomarkers for different diseases, while NT-proBNP and S100 are more specific to cardiovascular and neuronal diseases respectively.

11.1.2 Optical Biosensor Platforms

After identifying selected and specific stroke biomarkers as potential target analytes for the biosensor platforms development, several optical biosensor platforms technologies were considered and compared. As previously discussed, the second important focus of this thesis is to develop a multiplex and quantitative optical point-of-care biosensor platform for stroke. Four optical biosensor platforms were considered and examined to answer this need. Two paper-based sensing platforms were examined, based on colorimetric and chemiluminescent signals detection, because of their main advantages for multiplex detection. Moreover, two chip-based (gold-silver and silicon dioxide chips) sensing platforms were examined, based on surface plasmon resonance sensing technology, because of their main advantage for sensitive detection. These four biosensors platforms were selected due to their strong advantages for multiplex, quantitative and sensitive detection. All these three parameters are needed in order to answer the need for a point-of-care biosensor platform for stroke. Sensitive biosensor platform with low limit-of-detection is required in order to detect the biomarkers in the low cut-offs needs for the stroke biomarkers detection.

However, each of these four biosensor platforms also exhibit a problematic disadvantage, which must be overcome. Chemiluminescent and colorimetric paper-based biosensor platforms usually exhibit very low detection sensitivity. And, surface plasmon resonance biosensor platforms are often bulky and not user friendly, as well as require challenging data acquisition, which previously made this technology less attractive as point-of-care biosensor platform. In this thesis, several experimental methodologies were examined in order to overcome the disadvantages of each tested biosensor platform. First, in order to overcome the disadvantage for sensitive detection of the chemiluminescent and colorimetric paper-based biosensor platform, three different approaches were examined. Which included novel engineering of different aspects of the paper-based biosensor platform.

A dissolvable film made from polyvinyl alcohol (PVA) was examined for its use in controlling the sample flow, both in time as well as in the critical areas of the biosensor platform. Integrating a dissolvable membrane into a sensor allows the control of sample flow in critical areas. These time-barrier films stop the flow of samples until the membrane has dissolved, thus, for example, allow increased exposure time between immunoreagents for the formation of greater numbers of immuno-complexes, ensuring higher sensitivity, reactivity and helping to reduce false-positive signals. In this thesis, dissolvable PVA films were used in a 3D-printed sensor holder, which enables film integration without the use of glue (chapter 6). PVA is a synthetic hydrophilic linear polymer, its solubility is dependent on its molecular weight and degree of hydrolysis. Three types of PVAs films were tested herein: (1) PVA 1 - Mw: 30-70K, 87-90% hydrolyzed; (2) PVA 2 - Mw: 31-50K, 98-99% hydrolyzed and (3) PVA 3 - Mw: 89-98K, > 99% hydrolyzed. The films were exposed to water in (1) the novel 3D-printed holder and (2) directly immersed into a water droplet. After comparing the time taken to dissolve PVA 1-3 films, PVA 1 films of 5% - 20% (w/v) are found to be most suitable as time barrier films, due to their optimal dissolution times and physical properties for integration into the customized 3D-printed holder.

Moreover, different membranes were screened and compared in terms of their material, structure, and performance in paper-based biosensor platform. Selection of a specific membrane type over another allow the control of sample flow and the direction of antibodies flow within the paper-based biosensor platform. This control can increase the detection sensitivity by directing the antibodies into the area of choice for signal acquisition. Paper-based diagnostics are rapid, robust, affordable and user-friendly, thus have potential for point-of-care. Modulation of sample flow in these technologies have the advantage of increased exposure between immunoreagents, which ensures higher sensitivity and specificity. In this thesis, common membrane types were compared and modified to modulate sample flow (chapter 7). Five different membranes were compared: glassfiber, polyester, nitrocellulose, polyvinylidene-difluoride (PVDF) and cellulose. Several parameters were tested for their effect on the sample flow: membranes absorption capacity, immobilization of buffer solution, different membrane type as separation layer and sample volume. The absorption capacities of the membranes were determined. PVDF ($26.3 \mu\text{L}/\text{cm}^2$) and nitrocellulose ($32.7 \mu\text{L}/\text{cm}^2$) demonstrated lower absorption capacities, while polyester ($50.9 \mu\text{L}/\text{cm}^2$) and glassfiber ($86.9 \mu\text{L}/\text{cm}^2$) demonstrated medium range absorption capacities. Cellulose ($138.3 \mu\text{L}/\text{cm}^2$) demonstrated the highest absorption capacity. In addition, the effect of immobilization buffer solution on antibody release and flow was also tested. The use of SM-PBST was preferred, since most of the antibody (459 RLU) was released from the polyester membrane during the wetting phase. The effect of separation membrane on the sample flow was tested using a 'Stack-Pad' sensor layout. Higher antibody levels could reach the three absorption pads with the use of polyester separation membrane (340, 440 and 408 RLU) as compared to the glassfiber separation membrane (208, 198 and 107 RLU), thus potentially increasing the sensor sensitivity. The effect of sample volume was also tested. Four different sample volumes were compared: 20, 40, 60 and 80 μL . The amount of antibodies released was double in the cases of 60 - 80 μL sample (1477 and 1493 RLU). Lastly, the effect of the membrane type and its modifications on the detection sensitivity was also compared. The modified setup (862 RLU) demonstrated a higher detection sensitivity vs. the initial setup (290 RLU). To conclude, the selection of membrane types and their modifications can modulate sample flow and increase the sensitivity of detection in paper diagnostics.

Colorimetric signal amplification was also examined by enhancing the colorimetric signal using a specific stopping solution to the chromogenic substrate used in the paper-based biosensor platform. Paper-based colorimetric biosensors combine the use of paper with colorimetric signal detection, which exhibit a change in color that can be observed visually. However, they usually demonstrate lower sensitivities because a signal amplification procedure was not used. In this thesis, the generation of a stopped colorimetric signal was examined for accurate and enhanced signal detection in paper-based biosensors (chapter 8). Stopping the reaction of colorimetric signal generation is often used in lab-based assays in order to amplify and stabilize the colorimetric signal for detection. The colorimetric reaction in biosensors is usually based on the interaction between the enzyme horseradish peroxidase (HRP) and a selected chromogenic substrate. The two most commonly used HRP substrates, 3, 3', 5, 5'-tetramethylbenzidine (TMB) and 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic-acid) (ABTS), were compared in terms of their ability to generate a stopped colorimetric signal on membrane. The stopped colorimetric signal was visible for TMB but not for ABTS. Moreover, the generation of stopped colorimetric signal was dependent on the presence of polyvinylidene-difluoride (PVDF) membrane as the separation layer. To conclude, a stopped colorimetric signal can be generated in paper-based biosensors for enhanced and accurate signal detection.

These three different approaches (chapters 6-8) were examined in order to overcome the disadvantage for sensitive detection of the chemiluminescent and colorimetric paper-based biosensor platform. They all included novel engineering of different aspects of the paper-based biosensor platform. The results and conclusions that were obtained from these studies will hopefully lead to new developments in the field of paper-based optical biosensor platforms. Unfortunately, the sensitivity that was reached after these developments was not sufficient for the detection required for the specific stroke biomarkers.

Moreover, in order to overcome the disadvantage for ease of use as point-of-care platform of the surface-plasmon-resonance biosensor platform, specific functionalized chips from gold, silver and silicon dioxide were developed for use in a miniaturized SPR system. The miniaturized SPR system allows for rapid on-site detection, with user-friendly signal acquisition, while still making use of the great advantages of SPR detection for highly sensitive detection of the stroke biomarkers.

SPR is a quantum-electromagnetic phenomenon arising from the interaction of light with free electrons at a metal-dielectric interface. At a specific angle or wavelength of light, the energy of the photons is transferred to excite the free electrons oscillation on the surface. A change in the refractive index may occur, which is influenced by the analyte concentration in the medium in close contact with the metal surface. SPR has been widely used for the detection of gaseous, liquid, or solid samples. In this thesis, a functionalized specific gold-silver plasmonic chip was designed and used in a point-of-care SPR module (PhotonicSys SPR H5) for the detection of the stroke biomarkers NT-proBNP and S100 β (chapter 9). These biomarkers have proven to be good for stroke diagnosis, with sensitivity and specificity > 85%. Specific detection was done by binding a biomolecular-recognizing antibody onto the gold-silver plasmonic chip. Detection was tested in water and plasma samples. NT-proBNP and S100 β were detected in a range of concentrations for stroke, from 0.1 ng/mL - 10 ng/mL. The RI of blank plasma samples was 1.362412, and the lowest concentration tested for both biomarkers showed a prominent shift in the RI signal (0.25 ng/mL NT-proBNP (1.364215) and S100 β (1.364024)). The sensor demonstrated clinically relevant limit-of-detection less than 1 ng/mL.

In addition, plasmon waveguide resonance (PWR) mode is obtained when a dielectric waveguide layer is added to the metal film. In this thesis, a self-referenced PWR (SRPWR) silicon dioxide (SiO₂) chip was also examined (chapter 10). The self-referenced measurement is important to compensate for temperature fluctuations and other instabilities and allows to RI signal measurement without an additional reference sample, thus minimizing the sample volume needed. The chip was fabricated with a multi-layer of metals and dielectrics, consisting of a 420 nm SiO₂ layer, a 40 nm Ag layer and another a 480 nm

SiO₂ layer. This chip was shown to give one internal plasmon excited on the bottom interface SiO₂/Ag which is used as self-reference in the detection. The top layer acts as a waveguide layer and can be designed to give modes with ultrahigh penetration depth. A direct assay format was developed, where the recognition molecule (antibody) was immobilized onto the SiO₂ plasmonic chip surface, via a covalent coupling protocol based on 3-aminopropyltriethoxysilane and glutaraldehyde. The SRPWR biosensor was developed for sensing two chosen stroke biomarkers: NT-proBNP and S100 β , which are sensitive and specific for stroke diagnostics. For both biomarkers, a linear decreasing pattern in the RI signal was recognized with the increasing biomarkers concentrations. The SiO₂ based plasmonic chip demonstrates a limit-of-detection of less than 1 ng/mL that is clinically relevant for both stroke biomarkers.

To conclude, several approaches were examined in order to fulfill this thesis main objective, which is optical biosensor platform development for stroke diagnostics, for the multiplex and quantitative detection of selected specific stroke biomarkers at point-of-care, as a new ischemic stroke etiology classification device. Firstly, several biomarkers were identified as useful to elucidate stroke mechanisms (chapter 4), as well as showed value for additional stroke management needs, such as stroke diagnosis, severity, mortality and therapeutic admission (chapter 5). Then, four optical biosensor platforms were considered and examined for the detection of the selected stroke biomarkers. Two paper-based sensing platforms, based on colorimetric and chemiluminescent signals detection, and two chip-based (gold-silver and silicon dioxide chips) sensing platforms, based on surface plasmon resonance sensing technology. Three different approaches (chapters 6-8) were examined in order to overcome the disadvantage for sensitive detection of the chemiluminescent and colorimetric paper-based biosensor platform. Unfortunately, the sensitivity that was reached after these developments was not sufficient for the detection required for the specific stroke biomarkers. The plasmonic chip-based biosensor platform, both gold-silver and silicon dioxide chips, demonstrated clinically relevant limit-of-detection lower than 1 ng/mL, for both biomarkers tested (NT-proBNP and S100 β).

11.2 Outlook

This thesis discussed two major sections that concerned the optical biosensor platform development for stroke diagnostics, which includes stroke biomarkers identification and the optical biosensor platform itself. Based on previous studies and this thesis findings, this section presents the future directions regarding both stroke biomarkers identification and the development of the optical biosensor platform.

11.2.1 Stroke Biomarkers Identification

11.2.1.1 Stroke Etiology Classification Schemes, Origin & Biomarkers Assays

In this thesis, consecutive patients with acute ischemic stroke admitted at the National University Hospital, Singapore, were recruited. Levels of NT-proBNP, D-dimer, S100 β , NSE, vitamin D, cortisol, interleukin-6, insulin, uric acid and albumin were compared between stroke mechanisms. This study was based on stroke etiology classification determined according to the TOAST criteria ^[6-9]. However, similarly to other classification systems, the weakness of the study is the lack of an absolute 100% reliable ‘gold standard’. Due to the lack of criterion standard, bias may be present in the determination of the TOAST criteria. Additional validation can be added to the biomarkers identification analysis, by comparing the information obtained from several stroke etiology classification schemes. For example, there are 3 common ischemic stroke subtypes classification schemes: (1) TOAST, (2) NINDS ^[10] and (3) OCSF ^[11, 12]. In addition, the biomarkers data that is presented and analysed in this thesis is limited to patients in south-east Asia, which are mostly from Chinese race. Additional validation is needed by comparing the results found with a cohort from different continents, for example, USA and Europe. Regarding the biomarkers measurement, variability does exist among the different assay used (Roche Cobas analysers c-111 ^[13] and e-411 ^[14]). In the future, if those assays are to be used in clinical practice in stroke, assays validation needs to be conducted.

11.2.1.2 Missing Data & Additional Biomarkers

In addition, this study encountered the issue of missing data. The biomarkers were measured in a total cohort of 515 stroke patients. However, the biomarkers were measured in different samples among the cohort, which lead to the issue of missing data in the logistic regression model building. Due to small sample size, selected biomarkers needed to be removed from the model building. Imputing the missing data was also tested, however, because of the sensitive character of clinical data, this method was not found useful in this case. Moreover, the expansion of the study presented in this thesis can also be through the examination of additional stroke biomarkers. In this thesis, a panel of biomarkers was selected based on previous knowledge, as well as accessibility to the analytical devices for their measurement in the clinical samples. Testing additional types of stroke biomarkers can add value to stroke diagnostics, in the hospital setting as well as to promote point-of-care biosensor platforms development.

11.2.1.3 Decision Tree Classifier Algorithm

In addition, a decision tree classifier (DTC) algorithm can be developed in order to promote the use of stroke biomarkers analysis into real clinical practice. Decision tree classifier algorithm will be constructed for the multi biomarkers panel measurement, incorporating useful clinical parameters and risk factors, similar to the one used in TOAST classification. DTC is an essential tool for classification. It is possible to construct the DTC automatically from the data or to customize it. A DTC is based on a ‘training set’ that contain objects. To create a tree from a data set is termed ‘induction’. DTCs can be used for ‘discrimination’, which is to conclude the classification parameters from a sample of already classified objects, or for ‘classification’, which is to apply the parameters to objects without a pre-known classification. For the biomarkers measurement in the biosensor platform, the focus will be on ‘classification’. As the rules of classification will be determined by the statistical analysis (logistic regression model ^[15, 16] and ROC analysis ^[17]) conducted previously. In addition, it is possible that the use of ‘discrimination’ function can also be utilized.

As presented in Figure 11.1, the DTC starts from the ‘root node’, then splits into ‘internal nodes’ and ‘leaf nodes’, each connected to a different ‘arc’. Each ‘internal node’ further splits into ‘child nodes’. Each ‘arc’ to the ‘child node’ has a different outcome, and each ‘leaf node’ has a classification label. An object is classified from the ‘root node’, down through the DTC. It is tested in each ‘internal node’ in order to continue to the designated ‘arc’ accordingly. The label of the ‘leaf node’ where the object ends up is its classification. If the object classification output is not the same as its original classification, then it means the object was misclassified by the tree. The tree ‘accuracy’ refers to the proportion of correctly classified objects. The tree ‘error’ refers to the proportion of misclassified objects [18-21].

Figure 11.1. Decision Tree Classifier Algorithm.

Decision tree classifier example from a study conducted by Dougu *et al.* [21]

11.2.2 Optical Biosensor Platform

In this thesis, four optical biosensor platforms were considered and examined for stroke diagnostics. Two paper-based sensing platforms, based on colorimetric and chemiluminescent signals detection, and two plasmonic chip-based (gold-silver and silicon dioxide chips) sensing platforms based on surface plasmon resonance sensing technology. These four biosensors platforms were selected due to their strong advantages for multiplex (paper-based platforms), quantitative and sensitive detection (plasmonic chip-based platforms). All these three parameters are needed in order to answer the need for a point-of-care biosensor platform for stroke. Sensitive biosensor platform with low limit-of-detection is required in order to detect the biomarkers in the low cut-offs needs for the stroke biomarkers detection. Three different approaches (chapters 6-8) were examined in order to overcome the disadvantage for sensitive detection of the chemiluminescent and colorimetric paper-based biosensor platform. They all included novel engineering of different aspects of the paper-based biosensor platform: dissolvable polyvinyl-alcohol film, membrane type comparison and colorimetric signal amplification. The results and conclusions that were obtained from these studies will hopefully lead to new developments in the field of paper-based optical biosensor platforms. Unfortunately, the sensitivity that was reached after these developments was not sufficient for the detection required for the specific stroke biomarkers.

Additional examination is still needed for the development of optical paper-based biosensor platforms for stroke diagnostics. In this thesis, the efforts made were centered on the biosensor platform itself, for sensitivity improvement through layout modifications and functionalization. An additional important aspect that was not tested in this thesis is sample pre-processing for the analyte pre-concentration. Several studies showed the value in sample pre-processing especially in order to improve the sensitivity in paper-based diagnostics through pre-concentration. Example of such sample pre-treatment includes the conjugation of the bio-recognition entity to magnetic beads, for improved separation (Figure 11.2). The analyte separation from the sample medium would significantly improve the biosensor detection sensitivity ^[22].

Figure 11.2. Magnetic Beads for Improved Separation and Sensitivity.

(a) Antibodies conjugated to magnetic beads; (b) Activation of magnetic field for analyte isolation; (c) Removal of magnetic field to allow flow to test zone ^[22].

Moreover, in this thesis (chapters 9-10) two plasmonic chip-based (gold-silver and silicon dioxide) were functionalized for the specific detection of two stroke biomarkers (NT-proBNP and S100 β). The plasmonic chip-based biosensor platform, both gold-silver and silicon dioxide chips, demonstrated clinically relevant limit-of-detection lower than 1 ng/mL, for both biomarkers tested (NT-proBNP and S100 β). Detection was demonstrated with the biomarkers samples diluted in deionized water. As well as, validated using spiked porcine plasma samples. Additional examination is needed in real clinical samples. This will allow real value demonstration of the developed point-of-care optical biosensor platform. Moreover, it is required to conduct such examination in the same clinical samples in parallel to validation with hospital based assays (Roche Cobas analysers c-111 ^[13] and e-411 ^[14]).

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Publications List

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- **Dorin Harpaz**, Brescia Koh, Raymond Chee Seong Seet, Ibrahim Abdulhalim* and Alfred Iing Yoong Tok*, 'Functionalized silicon dioxide self-referenced plasmonic chip as point-of-care biosensor for stroke biomarkers NT-proBNP and S100 β ', *Talanta* 212 (2020): 120792.
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- **Dorin Harpaz**, Evgeni Eltzov, Raymond C.S. Seet, Robert S. Marks and Alfred I.Y. Tok, ‘Point-of-Care-Testing in Acute Stroke Management: An Unmet Need Ripe for Technological Harvest’, Biosensors 2017
- Prima Dewi Sinawang, **Dorin Harpaz**, Luka Fajs, Raymond C.S. Seet, Alfred I.Y. Tok and Robert S. Marks, ‘Electrochemical impedimetric detection of stroke biomarker NT-proBNP using disposable screen-printed gold electrodes’, The Euro-Biotech Journal 2017
- **Dorin Harpaz**, Raymond C.S. Seet, Robert S. Marks and Alfred I.Y. Tok, ‘Stroke Biomarkers Panel for Subtype Classification’, conference paper in the 2nd International Conference in Sports Science and Technology (ICSST 2016)

Awards

- **Finalist** in the Falling Walls Lab Singapore competition, ‘Breaking the Wall of Diagnostic Delays’ (2019).
- **Best Group Presentation Award**, the 12th Asia-Oceania Top University League on Engineering (AOTULE), Vietnam (2017).
- **Finalist** in the 3-Minute Thesis (3MT) NTU competition, ‘Stack-Pad, a Novel Point-Of-Care-Test’ (2017).
- **Semi-finalist** in the 3-Minute Thesis (3MT) NTU competition, ‘Future Point-Of-Care-Testing in Stroke Care’ (2016)
- **3rd place** in the Young person’s lecture competition Singapore (YPLCS), ‘Future Point-Of-Care in Stroke Care’ (2016).
- **Scholarship**, Singapore international graduate award (SINGA) for 4-years PhD program (2016).

Conferences

- **Oral presentation**, ‘Point-of-Care Surface Plasmon Resonance Biosensor for Stroke Biomarkers NT-proBNP&S100 β ’, 10th International Conference on Materials for Advanced Technologies (ICMAT), Singapore (23rd-28th Jun 2019)

- **Oral presentation**, ‘Point-of-Care Surface Plasmon Resonance Biosensor for Stroke Biomarkers NT-proBNP&S100 β ’, Materials Research Society Spring Meeting 2019 (MRS2019), USA, Arizona, Phoenix (22nd – 26th Apr 2019)
- **Poster presentation**, ‘Dissolvable Polyvinyl-Alcohol Film, Time-Barrier for Sample Flow Using a 3D-Printed Sensor Holder’, 10th Singapore international chemistry conference (SICC10), Singapore (16th–19th Dec 2018)
- **Poster presentation**, ‘Point-of-Care-Testing in Acute Stroke Management: An Unmet Need Ripe for Technological Harvest’, ACS on Campus (NTU), Singapore (2nd Aug 2018)
- **Oral and poster presentation**, ‘Electrochemical Impedimetric Detection of Stroke Biomarker NT- proBNP using Disposable Screen-Printed Gold Electrodes’, BIO-X, Singapore (21st-24th Feb 2018)
- **Oral presentation**, ‘Electrochemical Impedimetric Detection of Stroke Biomarker NT-proBNP’, 12th Asia-Oceania Top University League on Engineering, Vietnam (22nd-24th Nov 2017)
- **Two posters presentation**, ‘Stroke Biomarkers Panel for Etiology Classification’ and ‘Electrochemical Impedimetric Detection of Stroke Biomarker NT-proBNP using Disposable Screen-Printed Gold Electrodes’, 2nd International Meeting on Comprehensive Approaches in Stroke Treatment (CAST), Israel (22nd-23rd Oct 2017)
- **Poster presentation**, ‘Stack-Pad, a Novel Quantitative & Multiplex Point-of-Care-Test (POCT) Biosensor’, Symposium on Advances in Molecular Sensory Engineering (SAMSE), Singapore (27th-28th Jul 2017)
- **Oral presentation**, ‘Stack-Pad, a Novel Quantitative & Multiplex Point-of-Care-Test (POCT) Biosensor’, 9th International Conference on Materials for Advanced Technologies (ICMAT), Singapore (18th-23rd Jun 2017)
- **Oral and poster presentation**, ‘Stroke Biomarkers Panel for Subtype Classification’, BIO-X, Israel (3rd-8th Jun 2017)
- **Oral and poster presentation**, ‘Stroke Biomarkers Panel for Subtype Classification’, 2nd International Conference in Sports Science and Technology (ICSST), Singapore (12th-13th Dec 2016)

Undergraduate Students Supervision – Final Year Project

- **Brescia Koh Rui Wen**, Nanyang Technological University, School of Materials Science and Engineering, ‘Portable Surface Plasmon Resonance Biosensor for Stroke Triaging’ (2019).
- **Suk Ping Lai**, Singapore University of Social Sciences, ‘Portable Surface Plasmon Resonance Biosensor for Stroke Triaging’ (2019).
- **Alicia Lu Yitian**, Nanyang Technological University, School of Materials Science and Engineering, ‘Dissolvable Polyvinyl-Alcohol Film, to Modulate Sample Flow in Paper Diagnostics’ (2018).
- **Timothy Ng Si En**, Nanyang Technological University, School of Materials Science and Engineering, ‘Stopped Colorimetric Signal Detection in Paper Diagnostics’ (2017).
- **Soo Bin Choo**, Singapore Institute of Management, School of Bio-Medical Engineering, ‘Modified Membranes Characterization in Paper Diagnostic Sensor’ (2017).
- **Edmund Lau Wei Zhi**, Singapore Institute of Management, School of Bio-Medical Engineering, ‘Stack-Pad, a Novel Quantitative & Multiplex Point-of-Care Test (POCT) Biosensor’ (2017).
- **Trish Hui Ping Koon**, Singapore Institute of Management, School of Bio-Medical Engineering, ‘Biosensing of Artificial Sweeteners Toxicity Effects using Bioluminescent Bacteria’ (2016).

Chapter 12. APPENDIX

Appendix A. Stroke Diagnostics Conventional Technologies

Table 12.1. Conventional Technologies for Stroke Diagnosis.

Technology	Name	Application	Clinical Value	Ref .
Imaging Technique which uses computerized x-ray imaging	Computed Tomography (CT)	3D scan of body tissues. Shows evidence of early ischemia and rules out haemorrhage. Can be performed with contrast agent for better visualization	Ischemic stroke diagnosis and admission of thrombolytic therapy	[1-3]
	Multi-detector Computed Tomography (MDCT)	2D array of detector elements which enables multiple slices simultaneously, and faster image acquisition. High resolution and long range scans	Ischemic stroke diagnosis and admission of thrombolytic therapy	[1]
	SPECT Computed Tomography (SPECT-CT)	This technology uses radioisotopes. Shows cross-sectional image of the target organ. The patient either swallows or is injected with a radioisotope, which travels to a target organ. The radioisotope emits radiation, which is detected. Does not reliably distinguish between hemorrhage and infarction	Determine if a specific area of the body is receiving adequate blood flow	[4]
	XENON-Contrast Computed Tomography (XENON-CT)	This technology uses the inert gas xenon to measure cerebral blood flow (CBF) in various brain regions. The patient inhales a mixture of xenon and oxygen over a period of a few minutes, allowing measurement of an increase in their density caused by their presence in the brain tissue	Determine local cerebral blood flow in small area	[4]
	Positron Emission Tomography (PET)	Measures related changes in cortical function by tracking the chemical changes which occur in tissues. Detect biochemical changes in an organ or tissue that can identify the stroke onset before anatomical changes can be seen with other imaging processes	Guide decision making for brain surgical planning	[5]

	Carotid Angiography (CA)	This technology uses dye to show the inside of human carotid arteries. A small tube (catheter) is put into an artery, usually in the groin (upper thigh), then moved upwards into one of the carotid arteries	Identification of extracranial vessel disease	[1]
Imaging Technique which uses magnetic fields and radio waves	Magnetic Resonance Imaging (MRI)	Show the slowing of water movement through the injured brain tissue, which is caused by ischemia and ruling out haemorrhage. Can detect a variety of changes in the brain and blood vessels and visualize blockages in the arteries	Ischemic stroke diagnosis and admission of thrombolytic therapy	[1, 6-9]
	Magnetic Resonance Arteriogram (MRA)	Permits the visualization of blood flow in vessels and allows rapid characterization of the cervical and cephalic large vessels. Detects and grades cervical internal carotid stenosis with an accuracy of 85% to 96% compared to digital subtraction angiography	Identification of extracranial vessel disease	[6, 7, 10]
	Diffusion-Weighted Imaging (DWI-MRI)	Can render ischemic fields visible within minutes of ischemia onset and detects the effects of stroke on caudal motor pathways in the recovery process. Can also predict lasting motor impairment in stroke recovery	Early visualization of site and extent of ischemia	[7, 10, 11]
	Perfusion-Weighted Imaging (PWI-MRI)	Used to assess cerebral blood flow and blood volume in various brain regions. Usually performed by injecting a contrast agent and then obtaining a rapid series of MRIs using an ultrafast technique	Early visualization of site and extent of ischemia	[7]
	Magnetic Resonance Spectroscopy (MRS)	Spectroscopy measurement, enabling measurement of ATP, lactate levels, and pH at discrete locations within the brain. Can distinguish areas that have no viable neurons from areas that may be salvageable	Identify areas in the brain that may be salvageable	[4]
	Functional Magnetic Resonance Imaging (f-MRI)	Measurement of brain activity by detecting the changes in blood oxygenation and flow. Can measure differences in cognitive reserve	Monitoring patient recovery	[1, 5, 12-15]
	Magnetic Resonance Imaging Diffusion Tensor (MRI-DTI)	Provide information on white matter damage. Correlate better with cognition than conventional MRI measures	Monitoring damage progression	[12, 16-18]

Imaging Technique which uses sound waves	Carotid Ultrasound (CU)	Show the inside of human carotid arteries and detect whether plaque has narrowed or blocked carotid arteries. Can include Doppler to show the speed and direction of blood flow through the blood vessels	Show the condition of the carotid arteries in the neck and/or intracranial vessels	[1]
	B-Mode Carotid Ultrasound (B-CU)	Provides images of various levels, or planes, enabling the creation of a three-dimensional image of the carotid artery wall and surrounding structures. Provides information on the type and extent of arterial damage, though blood clots sometimes do not appear and the method cannot distinguish a narrowed from a completely occluded artery	Show the condition of the carotid arteries wall and structure	[1]
	Duplex-Carotid Ultrasound (D-CU)	Show the human carotid arteries and detect their condition. Combines B-mode imaging and pulsed Doppler ultrasound to provide more detail on the condition of arteries	Show the condition of the carotid arteries	[19]
	Transcranial Doppler (TCD)	Probe is placed over areas on the head to detect blood velocity and pressure in certain arteries at various depths in the brain. Allows the assessment of the location and extent of occlusions or atheromatous plaques in extracranial carotid and large intracranial vessels	Show the condition of the carotid arteries and location of occlusions	[20]
	Echo-Cardiography (ECHO)	Show images of the human heart, gives information on the size, shape and function of the organ. Can also detect possible blood clots inside the heart, and problems with the aorta	Heart pathologies diagnosis	[1]
Other	Electro-Cardiogram (ECG)	Records the heart's electrical activity, showing how fast the heart is beating, and its rhythm (steady or irregular). Can detect heart problems that may lead to a stroke and can also record the strength and timing of electrical signals which pass through the heart	Atrial fibrillation or previous heart attack diagnosis	[1]
	Blood Tests (BT)	Includes: Glucose test, Platelets count and PT/PTT. Low glucose levels can cause symptoms similar to stroke and abnormal platelet levels can be a sign of bleeding/thrombotic disorder. Can also test whether the blood is clotting normally	Bleeding/Thrombotic disorders diagnosis	[1]

Appendix B. Stroke POCT Biosensors

Figure 12.1. Mobile Stroke Unit and Imaging Tools.

A) Mobile Stroke Unit ^[21]. B) CereTom® (Samsung/ Neurologica). C) SOMATOM® (Siemens). D) Vivid q® (GE Healthcare) ^[22].

Figure 12.2. Electrochemical POCTs for Stroke.

A) CoaguChek® (Roche) ^[23]. B) SMARTChip (Sarissa Biomedical Ltd.) ^[24]. C) i-STAT® BNP (Abbott) ^[25]. D) pocH-100i™ (Sysmex).

Figure 12.3. Optical POCTs for Stroke.

A) Hemochron® (ITC/Accriva Diagnostics) ^[26]. B) Reflotron® plus analyzer (Roche, Cobas series) ^[27]. C) Cobas® h 232 (Roche) ^[28]. D) Triage® BNP Test (Alere) ^[29]. E) VerifyNow® (Accumetrics, Inc.) ^[30].

Appendix C. Biomarkers Correlation Analysis

Table 12.2.Spearman rho Correlation Coefficient.

Spearman rho Correlation Coefficient (Sig. (2-tailed))						
	Age (years)	Abdominal Circumference	BMI	NIHSS	Duration of Event (days)	Duration Observed Event (days)
Laboratory parameters						
Full Blood Count						
White cell count (x 10 ⁹ /l)	-0.001696	0.193** (0.000)	0.221** (0.000)	0.097* (0.029)	-0.051 (0.512)	-0.007 (0.867)
Neutrophils (x 10 ⁹ /l)	-0.031 (0.479)	0.102* (0.022)	0.141** (0.001)	0.117** (0.009)	-0.094 (0.223)	-0.001 (0.983)
Lymphocytes (x 10 ⁹ /l)	-0.230** (0.000)	0.255** (0.000)	0.214** (0.000)	-0.070 (0.131)	0.048 (0.554)	0.016 (0.726)
Hemoglobin (g/dl)	-0.322** (0.000)	0.149** (0.001)	0.171** (0.000)	-0.126** (0.004)	0.066 (0.397)	0.038 (0.396)
Platelets (x 10 ⁹ /l)	-0.238** (0.000)	0.011 (0.808)	0.083 (0.060)	-0.043 (0.330)	-0.099 (0.199)	0.071 (0.112)
Coagulation profile						
International normalized ratio	0.275** (0.000)	-0.009 (0.870)	-0.005217	0.192** (0.001)	-0.047 (0.624)	-0.168** (0.003)
Partial thromboplastin time (sec)	0.107 (0.064)	-0.018 (0.751)	-0.035 (0.550)	-0.057 (0.323)	0.078 (0.433)	-0.065 (0.264)
Lipid panel						
Total cholesterol (mg/dL)	-0.261** (0.000)	0.031 (0.490)	0.027 (0.549)	-0.034 (0.455)	-0.112 (0.158)	0.001 (0.981)
Triglycerides (mg/dL)	-0.260** (0.000)	0.228** (0.000)	0.205** (0.000)	-0.003104	0.053 (0.504)	-0.048 (0.288)
High-density lipoprotein (mg/dL)	0.122** (0.007)	-0.212** (0.000)	-0.194** (0.000)	0.000 (0.993)	-0.021 (0.795)	0.004 (0.936)
Low-density lipoprotein (mg/dL)	-0.241** (0.000)	0.019 (0.686)	0.017 (0.710)	-0.040 (0.385)	-0.118 (0.143)	0.037 (0.420)
Liver panel						
Aspartate aminotransferase (U/l)	0.038 (0.430)	0.060 (0.219)	0.021 (0.663)	0.109* (0.024)	0.028 (0.735)	-0.053 (0.276)
Alanine transaminase (U/l)	-0.265** (0.000)	0.197** (0.000)	0.226** (0.000)	-0.060 (0.220)	0.077 (0.354)	0.043 (0.381)
Biomarkers levels						
NT-proBNP (pg/ml)	0.445** (0.000)	-0.003045	-0.187** (0.000)	0.213** (0.000)	-0.080 (0.334)	-0.220** (0.000)
D-dimer (ng/ml)	0.032 (0.691)	-0.103 (0.201)	-0.056 (0.487)	-0.011 (0.889)	0.016 (0.908)	0.168* (0.036)
S100 β (pg/ml)	0.206** (0.000)	-0.034 (0.479)	-0.046 (0.345)	0.193** (0.000)	-0.003564	-0.002052
Neuron specific enolase (ng/ml)	0.001 (0.986)	0.106* (0.028)	0.050 (0.303)	0.070 (0.147)	-0.102 (0.221)	0.000 (0.994)

Vitamin D (mg/dl)	0.110* (0.015)	-0.079 (0.081)	-0.088 (0.051)	-0.020 (0.653)	-0.051 (0.518)	0.024 (0.594)
Cortisol (nmol/l)	0.108* (0.024)	-0.051 (0.286)	0.003 (0.949)	0.218** (0.000)	-0.220** (0.008)	-0.033 (0.497)
Interleukin-6 (pg/ml)	0.380** (0.000)	0.049 (0.314)	-0.039 (0.414)	0.217** (0.000)	-0.013 (0.872)	-0.003193
Insulin (pmol/l)	-0.003296	0.324** (0.000)	0.338** (0.000)	-0.048 (0.319)	0.060 (0.470)	-0.031 (0.518)
Uric acid (μmol/l)	-0.101 (0.130)	0.235** (0.000)	0.210** (0.001)	0.048 (0.472)	0.101 (0.362)	-0.048 (0.467)
Albumin (g/l)	0.078 (0.332)	0.003 (0.967)	0.012 (0.886)	0.011 (0.888)	-0.01132	-0.134 (0.095)

* Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

Appendix D. ROC Biomarkers to Elucidate Stroke Mechanisms

Table 12.3. ROC Cardioembolism without AF (Reference: Lacunar & Atherosclerosis Stroke).

[CEI WITHOUT AF] VS. [LAA AND LAC]										
Biomarker	Cut-Off	Sensitivity (%)	Specificity (%)	Accuracy (%)	LR+	LR-	AUC (%)	SE	95% CI (%)	
Demographic										
Age (years)	≥63	61.4	61.1	61.1	1.578	0.632	64.9	0.047	55.8	74.2
Laboratory parameters										
Full Blood Count										
White cell count (x 10 ⁹ /l)	≥7.76	46.5	44.4	44.6	0.836	1.205	50.5	0.052	40.4	60.6
Neutrophils (x 10 ⁹ /l)	≥5.22	51.2	54.2	53.9	1.116	0.902	55.2	0.053	44.8	65.5
Lymphocytes (x 10 ⁹ /l)	≥1.86	40.5	40.2	40.2	0.678	1.48	36.2	0.044	27.5	44.9
Hemoglobin (g/dl)	≥14.0	44.2	40.6	41.0	0.744	1.373	43.2	0.049	33.6	52.8
Platelets (x 10 ⁹ /l)	≥233	46.5	45.9	46.0	0.861	1.163	42.5	0.048	33.2	51.8
Coagulation profile										
International normalized ratio	≥1.02	63.9	75.2	73.6	2.579	0.48	70.1	0.053	59.8	80.4
Partial thromboplastin time (sec)	≥29.0	50	56.1	55.2	1.139	0.891	54.6	0.057	43.5	65.7
Lipid panel										
Total cholesterol (mg/dL)	≥169	30	30.6	30.6	0.433	2.285	30.3	0.048	20.9	39.7
Triglycerides (mg/dL)	≥44.5	32.5	32.6	32.6	0.482	2.071	28.9	0.049	19.3	38.4
High-density lipoprotein (mg/dL)	≥41.4	53.9	54.7	54.6	1.189	0.843	53.5	0.059	41.8	65.1
Low-density lipoprotein (mg/dL)	≥106	35.9	35.2	35.3	0.554	1.819	31.2	0.048	21.7	40.7
Liver panel										
Aspartate aminotransferase (U/l)	≥23	53.1	55.8	55.6	1.202	0.84	54.8	0.054	44.2	65.5
Alanine transaminase (U/l)	≥20	46.9	43.1	43.4	0.824	1.233	44.2	0.058	32.9	55.5
Biomarkers levels										
NT-proBNP (pg/ml)	≥294	75.7	76.1	76.1	3.168	0.32	81.3	0.039	73.5	88.9
D-dimer (ng/ml)	≥480	52.9	61.2	60.0	1.363	0.769	59.2	0.064	46.6	71.8
S100β (pg/ml)	≥64.0	66.7	66.9	66.9	2.019	0.498	72.8	0.047	63.6	81.9
NSE (ng/ml)	≥15.9	55.6	59.1	58.7	1.359	0.752	56.7	0.052	46.5	66.9
Vitamin D (mg/dl)	≥22.4	55	54.3	54.4	1.205	0.828	54.5	0.049	44.8	64.2
Cortisol (nmol/l)	≥471	67.6	66.9	67.1	2.047	0.484	71.3	0.048	61.9	80.7
Interleukin-6 (pg/ml)	≥5.39	62.2	64.9	64.7	1.774	0.582	67.3	0.048	57.9	76.6
Insulin (pmol/l)	≥62.0	50	56.7	56.0	1.154	0.882	48.3	0.054	37.8	58.8
Uric acid (μmol/l)	≥371	57.9	55.8	56.0	1.31	0.754	50.4	0.08	34.6	66.1
Albumin (g/l)	≥40.4	47.1	52.4	51.7	0.989	1.01	46.6	0.082	30.5	62.7

Table 12.4. ROC Cardioembolism (Reference: Atherosclerosis Stroke).

[CEI] VS. [LAA]										
Biomarker	Cut-Off	Sensitivity (%)	Specificity (%)	Accuracy (%)	LR+	LR-	AUC (%)	SE	95% CI (%)	
Demographic										
Age (years)	≥67	69.3	67.9	68.5	2.16	0.45	68.6	0.04	61.0	76.2
Laboratory parameters										
Full Blood Count										
White cell count (x 10 ⁹ /l)	≥8.52	43.8	46.9	45.8	0.83	1.20	46.2	0.04	37.4	54.9
Neutrophils (x 10 ⁹ /l)	≥5.39	46.6	47.7	47.3	0.89	1.12	50.3	0.05	41.5	59.1
Lymphocytes (x 10 ⁹ /l)	≥1.86	43.9	43.9	43.9	0.78	1.28	40.4	0.04	31.9	48.9
Hemoglobin (g/dl)	≥41.2	41.1	42.3	41.9	0.71	1.39	43.5	0.04	35.1	51.9
Platelets (x 10 ⁹ /l)	≥225	41.1	36.9	38.4	0.65	1.60	33.9	0.04	26.2	41.7
Coagulation profile										
International normalized ratio	≥1.00	65.6	60.7	62.8	1.67	0.57	68.5	0.04	59.8	77.2
Partial thromboplastin time (sec)	≥28.5	57.6	56.4	56.9	1.32	0.75	60.9	0.05	51.7	70.2
Lipid panel										
Total cholesterol (mg/dL)	≥169	32.8	32.5	32.6	0.49	2.07	31.6	0.04	23.5	39.7
Triglycerides (mg/dL)	≥44.5	34.3	33.3	33.7	0.51	1.97	27.5	0.04	19.6	35.4
High-density lipoprotein (mg/dL)	≥42.9	57.6	54.9	55.9	1.28	0.77	57.7	0.05	48.3	67.1
Low-density lipoprotein (mg/dL)	≥106	37.9	37.1	37.4	0.60	1.68	30.7	0.04	22.6	38.8
Liver panel										
Aspartate aminotransferase (U/l)	≥23.0	62.1	54.9	57.4	1.38	0.69	60.2	0.05	51.3	69.1
Alanine transaminase (U/l)	≥18.0	44.8	43.2	43.8	0.79	1.28	44.5	0.05	35.1	53.9
Biomarkers levels										
NT-proBNP (pg/ml)	≥507	74.6	75.7	75.3	3.07	0.34	79.7	0.04	72.7	86.7
D-dimer (ng/ml)	≥350	53.3	48.7	50.7	1.04	0.96	51.0	0.07	37.1	64.9
S100β (pg/ml)	≥65.0	62.9	60.8	61.5	1.60	0.61	66.3	0.04	57.8	74.8
Neuron specific enolase (ng/ml)	≥15.1	51.6	54.2	53.3	1.13	0.89	50.6	0.05	41.4	59.8
Vitamin D (mg/dl)	≥20.8	49.3	46.7	47.6	0.92	1.09	50.9	0.04	42.2	59.5
Cortisol (nmol/l)	≥471	57.1	56.1	56.5	1.30	0.76	58.4	0.05	49.2	67.6
Interleukin-6 (pg/ml)	≥6.28	60.3	62.6	61.8	1.61	0.63	63.6	0.05	54.8	72.4
Insulin (pmol/l)	≥50.0	46.8	46.7	46.8	0.88	1.14	41.1	0.05	31.9	50.3
Uric acid (μmol/l)	≥371	51.6	49.2	50.0	1.02	0.98	45.5	0.07	32.4	58.5
Albumin (g/l)	≥40.3	53.3	53.9	53.6	1.16	0.87	50.9	0.07	36.9	65.0

Table 12.5. ROC Cardioembolism (Reference: Non-Cardioembolism).

Cardioembolic Stroke										
Biomarker	Cut-Off	Sensitivity (%)	Specificity (%)	Accuracy (%)	LR+	LR-	AUC (%)	SE	95% CI (%)	
White Blood Cells (109/L)	≥7.84	0.48	0.44	0.45	0.86	1.18	0.51	0.04	0.42	0.59
Haemoglobin (g/dL)	≥14	0.41	0.42	0.41	0.7	1.42	0.42	0.04	0.35	0.49
Haematocrit (%)	≥41.2	0.41	0.41	0.41	0.7	1.43	0.42	0.04	0.35	0.5
Platelets (109/L)	≥229	0.41	0.42	0.42	0.71	1.4	0.37	0.04	0.3	0.44
Mean Platelet Volume (fL)	≥9.7	0.53	0.52	0.52	1.11	0.89	0.54	0.04	0.46	0.62
Neutrophils (%)	≥67.3	0.6	0.64	0.63	1.65	0.63	0.63	0.04	0.56	0.7
Neutrophils (109/L)	≥5.23	0.52	0.53	0.53	1.1	0.91	0.55	0.04	0.47	0.63
Lymphocytes (%)	≥23.4	0.42	0.41	0.41	0.72	1.4	0.41	0.04	0.33	0.48
Lymphocytes (109/L)	≥1.86	0.44	0.41	0.41	0.74	1.37	0.39	0.04	0.32	0.47
Prothrombin Time (sec)	≥13	0.64	0.65	0.65	1.83	0.55	0.74	0.04	0.66	0.81
International Normalized Ratio	≥1	0.66	0.63	0.64	1.79	0.54	0.72	0.04	0.64	0.79
Partial Thromboplastin Time (sec)	≥28.8	0.49	0.5	0.5	0.99	1.01	0.55	0.04	0.46	0.63
Total Cholesterol (mg/dL)	≥172	0.33	0.34	0.34	0.5	1.95	0.32	0.04	0.24	0.39
Triglycerides (mg/dL)	≥45.2	0.34	0.34	0.34	0.52	1.91	0.29	0.04	0.22	0.36
HDL (mg/dL)	≥42.9	0.58	0.58	0.58	1.38	0.73	0.58	0.04	0.5	0.67
LDL (mg/dL)	≥105	0.38	0.35	0.35	0.58	1.77	0.32	0.04	0.24	0.39
Aspartate Aminotransferase (U/L)	≥23	0.62	0.56	0.57	1.41	0.68	0.6	0.04	0.52	0.68
Alanine Transaminase (U/L)	≥19	0.43	0.42	0.42	0.75	1.34	0.42	0.04	0.34	0.5
Fasting Blood Glucose (mg/dL)	≥97.3	0.47	0.52	0.51	0.97	1.02	0.46	0.05	0.37	0.56
Glycohaemoglobin, HbA1c (%)	≥6.3	0.5	0.48	0.48	0.95	1.05	0.44	0.04	0.36	0.51
Vitamin D (mg/dL)	≥21.1	0.49	0.47	0.48	0.93	1.07	0.5	0.04	0.43	0.57
Cortisol (nmol/L)	≥444	0.6	0.59	0.59	1.47	0.67	0.63	0.04	0.55	0.72
NT-proBNP (pg/mL)	≥377	0.79	0.79	0.79	3.75	0.26	0.84	0.03	0.79	0.89
IL-6 (pg/mL)	≥5.54	0.65	0.63	0.63	1.77	0.55	0.67	0.04	0.59	0.74
Insulin (pmol/L)	≥50.4	0.47	0.46	0.46	0.86	1.17	0.42	0.04	0.34	0.5
NSE (ng/mL)	≥15.1	0.52	0.5	0.5	1.04	0.96	0.5	0.04	0.42	0.58
S100 (pg/mL)	≥64	0.66	0.65	0.65	1.91	0.52	0.72	0.04	0.65	0.79
Urate (μmol/L)	≥370	0.55	0.56	0.56	1.26	0.8	0.49	0.06	0.37	0.61
Albumin (g/L)	≥40.4	0.5	0.49	0.49	0.98	1.02	0.48	0.06	0.36	0.59
D-dimer (ng/mL)	≥350	0.53	0.49	0.5	1.05	0.95	0.52	0.06	0.41	0.63

Table 12.6. ROC Atherosclerosis (Reference: Non-Atherosclerosis).

Large Artery Atherosclerosis Stroke										
Biomarker	Cut-Off	Sensitivity (%)	Specificity (%)	Accuracy (%)	LR+	LR-	AUC (%)	SE	95% CI (%)	
White Blood Cells (109/L)	≥8.34	0.55	0.56	0.56	1.25	0.8	0.57	0.03	0.51	0.63
Haemoglobin (g/dL)	≥14.2	0.49	0.48	0.48	0.94	1.06	0.49	0.03	0.43	0.55
Haematocrit (%)	≥41.9	0.51	0.51	0.51	1.03	0.97	0.5	0.03	0.44	0.56
Platelets (109/L)	≥239	0.53	0.53	0.53	1.12	0.89	0.56	0.03	0.5	0.62
Mean Platelet Volume (fL)	≥9.6	0.53	0.49	0.5	1.04	0.96	0.51	0.03	0.46	0.57
Neutrophils (%)	≥63.7	0.52	0.5	0.5	1.04	0.96	0.51	0.03	0.45	0.57
Neutrophils (109/L)	≥5.21	0.56	0.54	0.55	1.22	0.81	0.56	0.03	0.51	0.62
Lymphocytes (%)	≥25.5	0.49	0.48	0.48	0.95	1.06	0.46	0.03	0.4	0.52
Lymphocytes (109/L)	≥1.96	0.5	0.51	0.51	1.02	0.98	0.51	0.03	0.45	0.57
Prothrombin Time (sec)	≥12.8	0.46	0.47	0.47	0.87	1.14	0.45	0.03	0.38	0.52
International Normalized Ratio	≥0.98	0.54	0.47	0.49	1.02	0.98	0.49	0.03	0.42	0.56
Partial Thromboplastin Time (sec)	≥28.6	0.41	0.41	0.41	0.69	1.45	0.4	0.04	0.32	0.47
Total Cholesterol (mg/dL)	≥188	0.55	0.53	0.54	1.18	0.84	0.54	0.03	0.48	0.6
Triglycerides (mg/dL)	≥51.8	0.52	0.52	0.52	1.07	0.94	0.55	0.03	0.49	0.6
HDL (mg/dL)	≥40.6	0.54	0.5	0.51	1.09	0.92	0.49	0.03	0.44	0.55
LDL (mg/dL)	≥117	0.55	0.55	0.55	1.23	0.81	0.56	0.03	0.49	0.62
Aspartate Aminotransferase (U/L)	≥22	0.52	0.48	0.49	1	1	0.47	0.03	0.41	0.54
Alanine Transaminase (U/L)	≥20	0.49	0.47	0.47	0.91	1.1	0.47	0.03	0.41	0.53
Fasting Blood Glucose (mg/dL)	≥95.5	0.56	0.51	0.52	1.14	0.87	0.55	0.04	0.48	0.62
Glycohaemoglobin, HbA1c (%)	≥6.4	0.59	0.55	0.56	1.31	0.75	0.59	0.03	0.52	0.65
Vitamin D (mg/dL)	≥21.5	0.49	0.49	0.49	0.96	1.04	0.49	0.03	0.43	0.55
Cortisol (nmol/L)	≥424	0.53	0.54	0.54	1.15	0.87	0.55	0.03	0.48	0.61
NT-proBNP (pg/mL)	≥130	0.55	0.55	0.55	1.23	0.81	0.54	0.03	0.48	0.6
IL-6 (pg/mL)	≥4.53	0.53	0.52	0.53	1.12	0.89	0.52	0.03	0.46	0.58
Insulin (pmol/L)	≥55.2	0.51	0.51	0.51	1.05	0.95	0.53	0.03	0.47	0.59
NSE (ng/mL)	≥14.9	0.49	0.48	0.48	0.93	1.08	0.5	0.03	0.43	0.56
S100 (pg/mL)	≥54	0.54	0.53	0.53	1.14	0.87	0.53	0.03	0.47	0.6
Urate (μmol/L)	≥361	0.56	0.55	0.55	1.24	0.8	0.56	0.04	0.47	0.64
Albumin (g/L)	≥40.3	0.46	0.46	0.46	0.86	1.17	0.46	0.05	0.35	0.57
D-dimer (ng/mL)	≥350	0.51	0.49	0.49	1	1	0.51	0.05	0.4	0.61

Table 12.7. ROC Lacunar (Reference: Non-Lacunar).

Biomarker	Lacunar Stroke									
	Cut-Off	Sensitivity (%)	Specificity (%)	Accuracy (%)	LR+	LR-	AUC (%)	SE	95% CI (%)	
White Blood Cells (109/L)	≥8.12	0.45	0.45	0.45	0.83	1.2	0.41	0.03	0.36	0.46
Haemoglobin (g/dL)	≥14.3	0.55	0.55	0.55	1.22	0.82	0.55	0.03	0.5	0.6
Haematocrit (%)	≥41.9	0.55	0.55	0.55	1.23	0.81	0.55	0.03	0.5	0.6
Platelets (109/L)	≥237	0.5	0.49	0.5	0.98	1.02	0.49	0.03	0.44	0.54
Mean Platelet Volume (fL)	≥9.7	0.52	0.55	0.53	1.15	0.87	0.53	0.03	0.48	0.58
Neutrophils (%)	≥64.1	0.44	0.45	0.45	0.8	1.24	0.4	0.03	0.35	0.45
Neutrophils (109/L)	≥5.1	0.44	0.43	0.43	0.77	1.31	0.39	0.02	0.34	0.44
Lymphocytes (%)	≥25.6	0.57	0.56	0.57	1.3	0.76	0.6	0.03	0.55	0.65
Lymphocytes (109/L)	≥1.95	0.56	0.55	0.55	1.24	0.8	0.55	0.03	0.5	0.61
Prothrombin Time (sec)	≥12.8	0.45	0.45	0.45	0.82	1.22	0.4	0.03	0.33	0.46
International Normalized Ratio	≥0.98	0.45	0.41	0.43	0.77	1.33	0.38	0.03	0.32	0.45
Partial Thromboplastin Time (sec)	≥28.9	0.55	0.55	0.55	1.23	0.81	0.54	0.03	0.47	0.6
Total Cholesterol (mg/dL)	≥185	0.54	0.52	0.53	1.12	0.89	0.53	0.03	0.48	0.58
Triglycerides (mg/dL)	≥51.4	0.53	0.53	0.53	1.14	0.88	0.56	0.03	0.51	0.62
HDL (mg/dL)	≥40.6	0.45	0.44	0.45	0.81	1.25	0.48	0.03	0.42	0.53
LDL (mg/dL)	≥115	0.51	0.51	0.51	1.05	0.95	0.52	0.03	0.47	0.57
Aspartate Aminotransferase (U/L)	≥22	0.49	0.44	0.46	0.87	1.16	0.48	0.03	0.43	0.54
Alanine Transaminase (U/L)	≥21	0.6	0.62	0.61	1.57	0.65	0.6	0.03	0.55	0.66
Fasting Blood Glucose (mg/dL)	≥95.5	0.47	0.46	0.47	0.88	1.14	0.46	0.03	0.4	0.52
Glycohaemoglobin, HbA1c (%)	≥6.4	0.47	0.5	0.48	0.93	1.07	0.47	0.03	0.41	0.53
Vitamin D (mg/dL)	≥21.5	0.51	0.51	0.51	1.04	0.96	0.51	0.03	0.46	0.56
Cortisol (nmol/L)	≥419	0.4	0.4	0.4	0.67	1.5	0.36	0.03	0.31	0.41
NT-proBNP (pg/mL)	≥114	0.34	0.33	0.34	0.51	1.97	0.29	0.02	0.24	0.34
IL-6 (pg/mL)	≥4.28	0.38	0.37	0.38	0.6	1.67	0.37	0.03	0.32	0.42
Insulin (pmol/L)	≥52.8	0.52	0.5	0.51	1.04	0.96	0.52	0.03	0.46	0.57
NSE (ng/mL)	≥15.0	0.46	0.46	0.46	0.84	1.19	0.43	0.03	0.38	0.49
S100 (pg/mL)	≥52	0.42	0.39	0.4	0.68	1.51	0.35	0.03	0.3	0.4
Urate (μmol/L)	≥353	0.45	0.43	0.44	0.79	1.29	0.47	0.04	0.39	0.55
Albumin (g/L)	≥40.4	0.5	0.5	0.5	1	1	0.5	0.05	0.41	0.59
D-dimer (ng/mL)	≥370	0.52	0.52	0.52	1.08	0.93	0.5	0.05	0.41	0.6

Table 12.8. Biomarkers cut-offs according to stroke mechanism.

Biomarker	Cardioembolic	Large Artery Atherosclerosis	Lacunar
White Blood Cells (10 ⁹ /L)	>= 7.84; < 7.84 [Ref]	>= 8.34; < 8.34 [Ref]	<= 8.12; > 8.12 [Ref]
Haemoglobin (g/dL)	<= 14; > 14 [Ref]	<= 14.2; > 14.2 [Ref]	>= 14.3; < 14.3 [Ref]
Haematocrit (%)	<= 41.2; > 41.2 [Ref]	>= 41.9; < 41.9 [Ref]	>= 41.9; < 41.9 [Ref]
Platelets (10 ⁹ /L)	<= 229; > 229 [Ref]	>= 239; < 239 [Ref]	<= 237; > 237 [Ref]
Mean Platelet Volume (fL)	>= 9.7; < 9.7 [Ref]	>= 9.6; < 9.6 [Ref]	>= 9.7; < 9.7 [Ref]
Neutrophils (%)	>= 67.3; < 67.3 [Ref]	>= 63.7; < 63.7 [Ref]	<= 64.1; > 64.1 [Ref]
Neutrophils (10 ⁹ /L)	>= 5.23; < 5.23 [Ref]	>= 5.21; < 5.21 [Ref]	<= 5.1; > 5.1 [Ref]
Lymphocytes (%)	<= 23.4; > 23.4 [Ref]	<= 25.5; > 25.5 [Ref]	>= 25.6; < 25.6 [Ref]
Lymphocytes (10 ⁹ /L)	<= 1.86; > 1.86 [Ref]	>= 1.96; < 1.96 [Ref]	>= 1.95; < 1.95 [Ref]
Prothrombin Time (sec)	>= 13; < 13 [Ref]	<= 12.8; > 12.8 [Ref]	<= 12.8; > 12.8 [Ref]
International Normalized Ratio	>= 1; < 1 [Ref]	<= 0.98; > 0.98 [Ref]	<= 0.98; > 0.98 [Ref]
Partial Thromboplastin Time (sec)	>= 28.8; < 28.8 [Ref]	<= 28.6; > 28.6 [Ref]	>= 28.9; < 28.9 [Ref]
Total Cholesterol (mg/dL)	<= 172.4; > 172.4 [Ref]	>= 188.3; < 188.3 [Ref]	>= 185.2; < 185.2 [Ref]
Triglycerides (mg/dL)	<= 45.24; > 45.24 [Ref]	>= 51.82; < 51.82 [Ref]	>= 51.43; < 51.43 [Ref]
HDL (mg/dL)	>= 42.92; < 42.92 [Ref]	<= 40.61; > 40.61 [Ref]	<= 40.6; > 40.6 [Ref]
LDL (mg/dL)	<= 105.9; > 105.9 [Ref]	>= 117.5; < 117.5 [Ref]	>= 114.8; < 114.8 [Ref]
Aspartate Aminotransferase (U/L)	>= 23; < 23 [Ref]	<= 22; > 22 [Ref]	<= 22; > 22 [Ref]
Alanine Transaminase (U/L)	<= 19; > 19 [Ref]	<= 20; > 20 [Ref]	>= 21; < 21 [Ref]
Fasting Blood Glucose (mg/dL)	<= 97.29; > 97.29 [Ref]	>= 95.49; < 95.49 [Ref]	<= 95.49; < 95.49 [Ref]
Glycohaemoglobin, HbA1c (%)	<= 6.3; > 6.3 [Ref]	>= 6.4; < 6.4 [Ref]	<= 6.4; > 6.4 [Ref]
Vitamin D (mg/dL)	>= 21.14; < 21.14 [Ref]	<= 21.46; > 21.46 [Ref]	>= 21.49; < 21.49 [Ref]
Cortisol (nmol/L)	>= 444.3; < 444.3 [Ref]	>= 424.2; < 424.2 [Ref]	<= 418.9; > 418.9 [Ref]
NT-proBNP (pg/mL)	>= 377.2; < 377.2 [Ref]	>= 130.1; < 130.1 [Ref]	<= 113.7; > 113.7 [Ref]
IL-6 (pg/mL)	>= 5.54; < 5.54 [Ref]	>= 4.53; < 4.53 [Ref]	<= 4.28; > 4.28 [Ref]
Insulin (pmol/L)	<= 50.35; > 50.35 [Ref]	>= 55.21; < 55.21 [Ref]	>= 52.78; < 52.78 [Ref]
NSE (ng/mL)	>= 15.07; < 15.07 [Ref]	>= 14.97; < 14.97 [Ref]	<= 15.03; > 15.03 [Ref]
S100 (pg/mL)	>= 64; < 64 [Ref]	>= 54; < 54 [Ref]	<= 52; > 52 [Ref]
Urate (μmol/L)	<= 370.8; > 370.8 [Ref]	>= 361.4; < 361.4 [Ref]	<= 352.6; > 352.6 [Ref]
Albumin (g/L)	<= 40.37; > 40.37 [Ref]	<= 40.33; > 40.33 [Ref]	>= 40.4; < 40.4 [Ref]
D-dimer (ng/mL)	>= 350; < 350 [Ref]	>= 350; < 350 [Ref]	>= 370; < 370 [Ref]

Appendix E. Distribution between 5 Different Levels of Stroke Severity.

Table 12.9. Baseline Characteristics Distribution between 5 Different Levels of Stroke Severity.

Clinical Feature	Stroke Severity (NIHSS) [†]						p-Value**
	Total	No Stroke Symptoms	Minor Stroke	Moderate Stroke	Moderate to Severe Stroke	Severe Stroke	
	-	NIHSS = 0	NIHSS = 1 - 4	NIHSS = 5 - 15	NIHSS = 16 - 20	NIHSS = 21 - 42	
Number (%)	516 (100%)	129 (25%)	242 (46.9%)	105 (20.4%)	13 (2.52%)	27 (5.23%)	-
Stroke (%)	433 (83.9%)	83 (64.3%)	209 (86.4%)	101 (96.2%)	13 (100%)	27 (100%)	<0.001
Transient Ischemic Attack (%)	83 (16.1%)	46 (35.7%)	33 (13.6%)	4 (3.8%)	0.000	0.000	<0.001
Males (%)	364 (70.5%)	91 (70.5%)	180 (74.4%)	77 (73.3%)	6 (46.2%)	10 (37%)	<0.001
Age (years), median (IQR*)	61 (53-71)	59 (50 - 65)	61 (53 - 70)	61 (54 - 71)	71 (58.5 - 75)	73 (67 - 77)	<0.001
Abdominal Circumference (cm)	90.5 (84-97)	92 (84 - 99.3)	90 (84 - 97)	91 (84 - 97)	88 (82 - 95.5)	90 (84 - 97)	0.629
Height (cm)	163 (157 - 169)	163 (157 - 170)	164 (157 - 170)	164 (158 - 169)	160 (149 - 162)	155 (150 - 165)	0.003
Weight (kg)	66 (59.1 - 74.3)	66.8 (57.6 - 78.5)	66.6 (59.6 - 74.4)	66 (60 - 73)	60 (51.6 - 65.6)	61 (57.8 - 72)	0.146
Body Mass Index (BMI)	24.8 (22.3-27.7)	25.0 (22.8 - 28.7)	24.6 (22.3 - 27.7)	25 (21.9 - 26.9)	23.6 (21.6 - 27.4)	25.4 (22.0 - 28.3)	0.733
Race (%) -							0.402
Chinese	380 (73.6%)	98 (76%)	174 (71.9%)	77 (73.3%)	10 (76.9%)	21 (77.8%)	-
Malay	92 (17.9%)	17 (13.2%)	48 (19.8%)	18 (17.1%)	3 (23.1%)	6 (22.2%)	-
Indian	37 (7.2%)	14 (10.9%)	16 (6.6%)	7 (6.7%)	0.000	0.000	-
Others	7 (1.4%)	0.000	4 (1.7%)	3 (2.9%)	0.000	0.000	-
Duration of Symptoms (days), median (IQR*)	12 (3-29)	14 (3.75 - 26)	11.5 (3 - 31.5)	19.5 (6.25 - 33.8)	5 (0.5 - 51.5)	4 (0.37 - 15)	0.209
Duration of Observed Event (days), median (IQR*)	50 (25-59)	48 (21.5 - 61)	52 (28.8 - 60)	49 (28 - 56)	52 (5 - 61)	15 (2.03 - 55)	0.010
Stroke Severity, NIHSS Score, median (IQR*)	2 (0.25 - 6)	0.000	2 (1 - 3)	7 (5.5 - 9)	19 (17 - 20)	23 (22 - 24)	<0.001

Tissue Plasminogen Activator (%)	57 (11.1%)	0.000	8 (3.3%)	20 (19%)	11 (84.6%)	18 (66.7%)	<0.001
Vascular Clinical Features (%) -							
Vascular #	125 (24.2%)	30 (23.3%)	58 (24%)	22 (21%)	4 (30.8%)	11 (40.7%)	0.287
Hypertension	346 (67.1%)	77 (59.7%)	179 (74%)	63 (60%)	10 (76.9%)	17 (63%)	0.021
Diabetes Mellitus	221 (42.9%)	48 (37.2%)	100 (41.3%)	56 (53.3%)	8 (61.5%)	9 (33.3%)	0.050
Hyperlipidaemia	243 (47.1%)	76 (58.9%)	110 (45.5%)	40 (38.1%)	7 (53.8%)	10 (37%)	0.016
Chronic Renal Failure	4 (0.8%)	1 (0.8%)	0.000	0.000	1 (7.7%)	2 (7.4%)	<0.001
Previous Stroke	81 (15.7%)	14 (10.9%)	40 (16.5%)	17 (16.2%)	1 (7.7%)	9 (33.3%)	0.052
Ischemic Heart Disease	90 (17.4%)	23 (17.8%)	38 (15.7%)	17 (16.2%)	5 (38.5%)	7 (25.9%)	0.201
Peripheral Vascular Disease	4 (0.8%)	2 (1.6%)	0.000	1 (1%)	1 (7.7%)	0.000	0.024
Atrial Fibrillation	32 (6.2%)	3 (2.3%)	17 (7%)	4 (3.8%)	3 (23.1%)	5 (18.5%)	0.001
Smoking	242 (46.9%)	64 (49.6%)	108 (44.6%)	59 (56.2%)	4 (30.8%)	7 (25.9%)	0.031
Modified Ranking Scale -							<0.001
No symptoms	471 (91.5%)	128 (99.2%)	224 (92.6%)	88 (83.8%)	12 (92.3%)	20 (74.1%)	-
No significant disability	15 (2.9%)	0.000	6 (2.5%)	9 (8.6%)	0.000	0.000	-
Slight disability	11 (2.1%)	0.000	3 (1.2%)	5 (4.8%)	0.000	3 (11.1%)	-
Moderate disability	14 (2.7%)	0.000	8 (3.3%)	2 (1.9%)	0.000	4 (14.8%)	-
Moderately severe disability	4 (0.8%)	1 (0.8%)	1 (0.4%)	1 (1%)	1 (7.7%)	0.000	-
Severe disability	0.000	0.000	0.000	0.000	0.000	0.000	-
Dead	0.000	0.000	0.000	0.000	0.000	0.000	-
Location (%) -							<0.001
Left Hemisphere	186 (41.1%)	23 (24.5%)	93 (41.5%)	46 (47.9%)	7 (53.8%)	17 (63%)	-
Right Hemisphere	180 (39.7%)	34 (36.2%)	88 (39.3%)	44 (45.8%)	6 (46.2%)	8 (29.6%)	-
Brainstem	14 (3.1%)	2 (2.1%)	8 (3.6%)	3 (3.1%)	0.000	1 (3.7%)	-
Other	5 (1.1%)	1 (1.1%)	3 (1.3%)	1 (1%)	0.000	0.000	-
Unknown	69 (15.2%)	34 (36.2%)	32 (14.3%)	2 (2.1%)	0.000	1 (3.7%)	-
Stroke Syndrome ¹ (%)							<0.001
TACS	57 (11.2%)	6 (4.7%)	11 (4.6%)	18 (17.6%)	8 (61.5%)	14 (51.9%)	-
PACS	101 (19.9%)	21 (16.5%)	47 (19.7%)	20 (19.6%)	5 (38.5%)	8 (29.6%)	-
LACS	292 (57.6%)	83 (65.4%)	152 (63.9%)	55 (53.9%)	0.000	2 (7.4%)	-

POCS	55 (10.8%)	16 (12.6%)	28 (11.8%)	9 (8.8%)	0.000	2 (7.4%)	-
Undetermined	2 (0.4%)	1 (0.8%)	0.000	0.000	0.000	1 (3.7%)	-

* Interquartile Range (IQR): Q1 – Q3.

** Kruskal Wallis H-Test was used to calculate p-value for continues variables, while chi-square test was used for categorical variables (if cell frequency was lower than 5%, fisher's exact test was used).

‡ NIHSS: National Institutes of Health Stroke Scale. 0 = no stroke symptoms, 1-4 = minor stroke, 5-15 = moderate stroke, 16-20 = moderate to severe stroke, 21-42 = severe stroke.

Vascular: either stroke (ischemic / haemorrhage), transient ischemic attack (TIA), Acute myocardial infarction (AMI) or Angina Pectoris.

‡ Stroke syndromes: TACS, total anterior circulation syndrome; LACS, lacunar syndrome; PACS, partial anterior circulation syndrome; POCS, posterior circulation syndrome.

Table 12.10. Biomarkers Distribution between 5 Different Levels of Stroke Severity.

Biomarker	N	Stroke Severity (NIHSS) †						p-Value**
		Total	No Stroke Symptoms	Minor Stroke	Moderate Stroke	Moderate to Severe Stroke	Severe Stroke	
		-	NIHSS = 0	NIHSS = 1 - 4	NIHSS = 5 - 15	NIHSS = 16 - 20	NIHSS = 21 - 42	
White Blood Cells (10 ⁹ /L)	509	8.17 (6.69-9.89)	8.01 (6.78 - 9.13)	8.19 (6.61 - 9.93)	8.24 (6.65 - 9.92)	9.01 (7.24 - 12.6)	8.8 (6.83 - 11.3)	0.297
Haemoglobin (g/dL)	509	14.2 (13-15.4)	14.6 (13.3 - 15.4)	14.3 (12.9 - 15.5)	14.1 (13.1 - 15.4)	13.7 (12.4 - 15.1)	13.2 (11.5 - 14.3)	0.025
Haematocrit (%)	509	41.8 (38.9-45.1)	42.4 (39.9 - 45.2)	41.9 (38.4 - 45.3)	41.8 (39.4 - 44.5)	41.2 (36.1 - 44.6)	39.1 (35.1 - 42.2)	0.022
Platelets (10 ⁹ /L)	509	237 (199-281)	238 (202 - 285)	238 (201 - 281)	239 (198 - 297)	191 (172 - 240)	226 (189 - 267)	0.064
Mean Platelet Volume (fL)	505	9.6 (8.28-10.3)	9.7 (8.7 - 10.4)	9.6 (8.33 - 10.2)	9.55 (7.05 - 10.3)	9.2 (7.55 - 10.4)	9.4 (7.03 - 10.6)	0.748
Neutrophils (%)	509	64.1 (56.9-72.2)	62.9 (54.6 - 70.3)	64.5 (57.6 - 71.9)	63.5 (57.4 - 74.1)	70.3 (66.7 - 80.3)	63.9 (56.7 - 73.3)	0.073
Neutrophils (10 ⁹ /L)	508	5.14 (3.87-6.61)	5.04 (3.65 - 5.95)	5.22 (4.12 - 6.61)	5.07 (3.86 - 6.81)	6.85 (4.82 - 10.1)	5.44 (3.76 - 7.38)	0.056
Lymphocytes (%)	464	25.6 (18.9-31.9)	26.8 (20.7 - 35.7)	25.4 (19.1 - 30.8)	24 (16.8 - 32.5)	18 (10.3 - 24.2)	27.2 (19.2 - 33.4)	0.018
Lymphocytes (10 ⁹ /L)	464	1.95 (1.44-2.57)	2.13 (1.51 - 2.63)	1.92 (1.44 - 2.47)	1.88 (1.4 - 2.35)	1.52 (1.16 - 2.26)	2.19 (1.58 - 3.02)	0.086
Prothrombin Time (sec)	318	12.8 (12.4-13.2)	12.7 (12.2 - 12.9)	12.7 (12.3 - 13.3)	13 (12.6 - 13.6)	13 (12.3 - 13.9)	13.1 (12.5 - 13.7)	0.002
International Normalized Ratio (INR)	318	0.98 (0.95-1.04)	0.97 (0.93 - 1)	0.97 (0.94 - 1.04)	1 (0.96 - 1.05)	1.02 (0.95 - 1.08)	1.03 (0.96 - 1.05)	0.007
Partial Thromboplastin Time (sec)	299	28.7 (27-30.8)	28.7 (26.9 - 30.1)	29.1 (27 - 31.1)	28.7 (27.2 - 30.9)	27.9 (25.8 - 30.9)	28.2 (26.6 - 30.8)	0.689
Total Cholesterol (mg/dL)	485	186 (155-220)	194 (158 - 219)	186 (154 - 218)	188 (157 - 234)	183 (155 - 268)	165 (151 - 208)	0.500
Triglycerides (mg/dL)	485	51.2 (38.2-75.9)	52.6 (40.2 - 81.0)	52.6 (38.7 - 80.4)	50.3 (37.7 - 69.2)	41.6 (27.9 - 57.8)	40.2 (29.8 - 70.6)	0.159
HDL (mg/dL)	485	40.6 (34.8-49.5)	42.3 (35.6 - 49.3)	39.8 (34.4 - 49.7)	40.2 (33.6 - 47.9)	46.2 (39.4 - 67.1)	43.3 (38.7 - 52.2)	0.147

LDL (mg/dL)	471	114 (88.3- 144)	121.0 (92.2 - 144)	114 (86.6 - 141)	119 (86.6 - 150)	121 (86.5 - 170)	98.9 (83.9 - 116)	0.436
Aspartate Aminotransferase (U/L)	425	22 (18- 28)	21 (18 - 27)	22 (18 - 27)	22 (17 - 28.5)	30.5 (27.3 - 38.8)	27.5 (21.5 - 34.8)	0.001
Alanine Transaminase (U/L)	424	20 (14- 29)	22 (14 - 30)	20 (14.5 - 29)	19 (12.5 - 27)	29 (14.8 - 43)	17 (12.3 - 23)	0.115
Fasting Blood Glucose (mg/dL)	329	95.5 (84.7- 113)	90.9 (82.9 - 105)	93.7 (84.7 - 114)	101 (86.9 - 118)	103 (89.2 - 153)	106 (87.8 - 141)	0.072
Glycohaemoglobin, HbA1c (%)	407	6.3 (5.7- 7.8)	6.3 (5.7 - 7.75)	6.2 (5.7 - 7.6)	6.75 (5.8 - 8.5)	6.4 (5.9 - 9.3)	6.05 (5.6 - 7.25)	0.132
Vitamin D (mg/dL)	486	21.5 (14.9- 29.2)	21.1 (14.9 - 28.5)	22.3 (15.4 - 29.9)	20.5 (13.6 - 29.9)	19.9 (16.7 - 25.8)	20.9 (11.7 - 25.1)	0.627
Cortisol (nmol/L)	433	419 (267- 575)	403 (232 - 539)	405 (253 - 548)	448 (294 - 588)	720 (602 - 978)	616 (412 - 1007)	<0.001
NT-proBNP (pg/mL)	432	119 (43.6- 503)	79.9 (26.9 - 295)	104 (36.4 - 461)	120 (58.6 - 721)	2217 (969 - 5747)	503 (193 - 2155)	<0.001
IL-6 (pg/mL)	432	4.31 (2.08- 10.3)	2.92 (1.5 - 6.15)	4.67 (2.24 -9.69)	4.1 (2.15 - 11.3)	20.9 (12.6 - 35.3)	12.5 (5.06 - 24.1)	<0.001
Insulin (pmol/L)	431	54.6 (32.8- 98.6)	59.8 (34.7 - 107)	52.2 (33.8 - 91.9)	52.2 (26.2 - 103)	60.4 (30.2 - 206)	46.4 (28.3 - 150)	0.771
NSE (ng/mL)	430	15.1 (11.6- 21.4)	14.8 (11.3 - 20.4)	15 (11.4 - 21.2)	15.6 (11.7 - 21.2)	17.5 (12.2 - 27.2)	20.6 (12.7 - 34.1)	0.180
S100 (pg/mL)	428	52 (38- 86)	50.5 (33.5 - 79.3)	51 (37 - 82.3)	52 (39 - 74.5)	93.5 (65 - 196)	99 (70.8 - 213)	<0.001
Urate (μmol/L)	228	357 (303- 436)	361 (297 - 429)	350 (303 - 417)	374 (325 - 456)	507 (440 - .)	358 (290 - 452)	0.305
Albumin (g/L)	156	40.4 (37.6- 43.4)	40.3 (37.1 - 42.9)	40.4 (37.9 - 43.9)	41 (38.8 - 43.4)	39.5 (37.7 - 42.1)	41.9 (37.5 - 42.8)	0.817
D-dimer (ng/mL)	156	350 (210- 685)	395 (220 - 715)	360 (200 - 650)	340 (210 - 890)	250 (158 - 850)	310 (230 - 1420)	0.877

* Interquartile Range (IQR): Q1 – Q3.

** Kruskal Wallis H-Test was used to calculate p-value.

‡ NIHSS: National Institutes of Health Stroke Scale. 0 = no stroke symptoms, 1-4 = minor stroke, 5-15 = moderate stroke, 16-20 = moderate to severe stroke, 21-42 = severe stroke.

Appendix F. ROC Biomarkers with Additional Value in Stroke

Table 12.11. Biomarkers Diagnostic Performance for Stroke Diagnosis.

Stroke Diagnosis										
Biomarker	Cut-Off	Sensitivity (%)	Specificity (%)	Accuracy (%)	LR+	LR-	AUC (%)	SE	95% CI (%)	
Cortisol (nmol/L)	≥ 137	58.9	57.4	58.7	1.3817	0.7168	57.9	0.0413	49.8	65.9
NT-proBNP (pg/mL)	≥ 0.064	64.9	61.1	64.5	1.6688	0.5744	64.8	0.0384	57.3	72.4
IL-6 (pg/mL)	≥ 3.99	53.1	51.9	52.9	1.1032	0.9042	50.5	0.0411	42.5	58.6
Insulin (pmol/L)	≥ 23.4	41.2	42.6	41.4	0.7177	1.3804	42.6	0.0439	33.9	51.2
NSE (ng/mL)	≥ 14.5	56.2	53.7	55.9	1.2128	0.8165	53.5	0.0408	45.5	61.4
S100 (pg/mL)	≥ 0.05	55.5	55.6	55.5	1.2483	0.8014	57.9	0.04	50.1	65.8
Albumin (g/L)	≥ 40.5	49.1	40.0	48.3	0.8182	1.2727	41.3	0.0806	25.5	57.0
D-dimer (ng/mL)	≥ 0.33	56.9	53.3	56.7	1.2208	0.8068	59.6	0.0685	46.1	72.9

Table 12.12. Biomarkers Diagnostic Performance between 2 Different Levels of Stroke Severity.

Biomarker	Stroke Severity (NIHSS) ‡									
	Cut-Off	Sensitivity (%)	Specificity (%)	Accuracy (%)	LR+	LR-	AUC	SE	95% CI (%)	
White Blood Cells (10 ⁹ /L)	≥8.57	58.9	58.2	58.2	1.41	0.7052	59.1	0.0511	49.1	69.1
Haemoglobin (g/dL)	≥13.9	41.0	38.0	38.2	0.6617	1.5518	34.6	0.0464	25.5	43.7
Haematocrit (%)	≥41.1	41.0	41.8	41.8	0.7052	1.41	35.6	0.0472	26.4	44.9
Platelets (10 ⁹ /L)	≥220	46.2	39.1	39.6	0.7574	1.3783	37.7	0.0447	28.9	46.4
Mean Platelet Volume (fL)	≥9.4	51.3	42.8	43.5	0.897	1.1376	49.6	0.0546	38.8	60.3
Neutrophils (%)	≥66	56.4	56.9	56.9	1.3088	0.7661	57.2	0.502	47.3	67.0
Neutrophils (10 ⁹ /L)	≥5.34	56.4	55.3	55.4	1.2625	0.788	59.3	0.0517	49.2	69.4
Lymphocytes (%)	≥23.5	44.1	43.2	43.2	0.7761	1.2949	45.6	0.0539	35.1	56.2
Lymphocytes (10 ⁹ /L)	≥1.95	50	49.9	49.9	0.9977	1.0023	51.3	0.0551	40.5	62.1
Prothrombin Time (sec)	≥13	64.5	61.8	62.1	1.6891	0.5741	60.0	0.0589	48.5	71.6
International Normalized Ratio	≥1	61.3	59.7	59.9	1.5217	0.6482	59.7	0.058	48.3	71.1
Partial Thromboplastin Time (sec)	≥28.6	37.9	43.9	43.3	0.6763	1.4135	43.1	0.0572	31.9	54.4
Total Cholesterol (mg/dL)	≥175	40.5	39.2	39.3	0.6668	1.5169	44.8	0.0519	34.7	55.0
Triglycerides (mg/dL)	≥46.4	40.5	40.1	40.1	0.6767	1.4832	37.9	0.0505	28.1	47.9
HDL (mg/dL)	≥43.3	56.8	59.2	59.1	1.3926	0.7299	60.5	0.0481	51.1	69.9
LDL (mg/dL)	≥109	40.5	40.5	40.5	0.6809	1.4696	44.2	0.0513	34.2	54.3
Aspartate Aminotransferase (U/L)	≥25	72.2	66.2	66.7	2.1338	0.4199	70.9	0.0488	61.4	80.5
Alanine Transaminase (U/L)	≥20	50	47.6	47.8	0.9534	1.0514	48.7	0.0543	38.1	59.4
Fasting Blood Glucose (mg/dL)	≥103	59.3	64.7	64.2	1.6781	0.6298	60.5	0.0624	48.3	72.8
Glycohaemoglobin, HbA1c (%)	≥6.2	45.2	44.0	44.1	0.8069	1.2454	45.5	0.0535	35.0	55.9
Vitamin D (mg/dL)	≥20.4	47.4	43.7	43.9	0.8406	1.2057	45.5	0.0429	37.1	53.9
Cortisol (nmol/L)	≥529	71.9	69.9	70.1	2.3879	0.4024	76.6	0.049	67.0	86.2
NT-proBNP (pg/mL)	≥275	68.8	68.3	68.4	2.1708	0.4573	77.7	0.0364	70.5	84.8
IL-6 (pg/mL)	≥7.68	71.9	73.1	72.9	2.6687	0.3849	75.6	0.0441	66.9	84.3
Insulin (pmol/L)	≥52.3	50	48.5	48.6	0.9709	1.0309	50.5	0.0597	38.8	62.2
NSE (ng/mL)	≥17.1	59.4	61.4	61.3	1.5384	0.6616	61.6	0.054	50.9	72.2
S100 (pg/mL)	≥71	75	70.0	70.4	2.5021	0.357	79.8	0.0353	72.9	86.7
Urate (μmol/L)	≥384	56.3	61.3	60.9	1.4543	0.7135	54.9	0.0869	37.9	71.9
Albumin (g/L)	≥40.3	53.9	47.6	48.1	1.0267	0.9706	46.1	0.0749	31.4	60.8
D-dimer (ng/mL)	≥320	38.5	41.9	41.7	0.6627	1.4667	46.2	0.0909	28.4	64.0

‡ NIHSS: National Institutes of Health Stroke Scale. 0 - 15 minor to moderate stroke, 16 - 42 = moderate to severe stroke.

Table 12.13. Biomarkers Diagnostic Performance for Mortality.

Biomarker	Mortality									
	Cut-Off	Sensitivity (%)	Specificity (%)	Accuracy (%)	LR+	LR-	AUC (%)	SE	95% CI (%)	
White Blood Cells (10 ⁹ /L)	≥8.36	54.4	54.8	54.7	1.202	0.8333	53.9	0.036	46.9	60.9
Haemoglobin (g/dL)	≥14	41.3	40.7	40.8	0.6962	1.4432	35.8	0.035	28.9	42.6
Haematocrit (%)	≥41.3	42.4	42.1	42.2	0.7322	1.3682	36.3	0.036	29.3	43.4
Platelets (10 ⁹ /L)	≥231	42.4	42.8	42.8	0.7414	1.3453	42.9	0.034	36.2	49.6
Mean Platelet Volume (fL)	≥9.6	51.1	48.8	49.2	0.9976	1.0025	51.2	0.037	43.9	58.5
Neutrophils (%)	≥65.5	55.4	56.2	56.1	1.2662	0.7927	58.9	0.034	52.4	65.6
Neutrophils (10 ⁹ /L)	≥5.34	55.4	56.6	56.4	1.2771	0.7874	56.1	0.034	49.3	62.8
Lymphocytes (%)	≥24	42.7	42.8	42.8	0.7465	1.3386	39.7	0.036	32.6	46.7
Lymphocytes (10 ⁹ /L)	≥1.9	45.0	45.7	45.6	0.8309	1.201	42.4	0.037	35.2	49.6
Prothrombin Time (sec)	≥12.9	57.7	57.3	57.4	1.3512	0.7383	64.2	0.044	55.5	72.8
International Normalized Ratio	≥1	61.5	61.4	61.4	1.5952	0.6262	65.6	0.044	57.1	74.2
Partial Thromboplastin Time (sec)	≥28.9	55.1	52.2	52.7	1.1526	0.8603	59.7	0.048	50.2	69.1
Total Cholesterol (mg/dL)	≥183	46.5	45.0	45.3	0.8457	1.1886	46.3	0.038	38.9	53.8
Triglycerides (mg/dL)	≥51.0	46.5	47.0	46.9	0.8776	1.1381	43.5	0.034	36.8	50.2
HDL (mg/dL)	≥41.4	54.0	53.4	53.5	1.1589	0.8613	53.2	0.036	46.2	60.1
LDL (mg/dL)	≥113	45.2	45.4	45.3	0.8279	1.2073	46.5	0.038	39.1	53.8
Aspartate Aminotransferase (U/L)	≥23	50.0	54.3	53.5	1.0938	0.9211	53.8	0.038	46.4	61.2
Alanine Transaminase (U/L)	≥19	40.8	40.9	40.9	0.691	1.4451	37.9	0.036	30.9	44.8
Fasting Blood Glucose (mg/dL)	≥101	59.3	61.6	61.2	1.543	0.6614	57.6	0.047	48.4	66.8
Glycohaemoglobin, HbA1c (%)	≥6.4	53.3	52.9	52.9	1.1298	0.8843	53.9	0.036	46.8	60.9
Vitamin D (mg/dL)	≥21.1	46.1	46.2	46.2	0.8568	1.1666	44.7	0.035	37.9	51.6
Cortisol (nmol/L)	≥441	57.7	58.4	58.3	1.3877	0.7241	63.0	0.033	56.5	69.6
NT-proBNP (pg/mL)	≥271	73.1	73.5	73.4	2.7598	0.3662	80.6	0.027	75.4	85.9
IL-6 (pg/mL)	≥5.65	65.4	65.4	65.4	1.8871	0.5297	69.1	0.033	62.7	75.6
Insulin (pmol/L)	≥50.7	44.9	44.9	44.9	0.8146	1.2274	43.6	0.038	36.1	51.1
NSE (ng/mL)	≥15.4	53.9	53.3	53.4	1.152	0.8666	54.1	0.037	46.8	61.3
S100 (pg/mL)	≥63	63.0	64.9	64.6	1.7927	0.5724	67.0	0.036	59.9	74.0
Urate (μmol/L)	≥359	56.1	52.4	53.1	1.1787	0.8377	52.9	0.053	42.5	63.3
Albumin (g/L)	≥40.5	58.1	53.6	54.5	1.2514	0.7824	53.1	0.059	41.6	64.6
D-dimer (ng/mL)	≥400	51.6	55.2	54.5	1.1521	0.8766	49.6	0.059	38.0	61.1

Table 12.14. Biomarkers Diagnostic Performance for tPA Admission.

Biomarker	Tissue Plasminogen Activator									
	Cut-Off	Sensitivity (%)	Specificity (%)	Accuracy (%)	LR+	LR-	AUC (%)	SE	95% CI (%)	
White Blood Cells (10 ⁹ /L)	≥8.57	53.6	58.2	57.7	1.2801	0.7984	55.3	0.042	47.1	63.5
Haemoglobin (g/dL)	≥14.1	37.5	44.5	43.7	0.6756	1.4047	41.3	0.038	33.8	48.9
Haematocrit (%)	≥41.3	42.9	43.4	43.3	0.7571	1.3169	40.7	0.038	33.2	48.1
Platelets (10 ⁹ /L)	≥233	42.9	46.5	46.1	0.8007	1.2295	43.6	0.042	35.5	51.7
Mean Platelet Volume (fL)	≥9.8	53.6	54.9	54.7	1.1875	0.8459	54.3	0.041	46.3	62.3
Neutrophils (%)	≥64.2	50.0	50.4	50.4	1.0089	0.9913	49.7	0.044	41.0	58.3
Neutrophils (10 ⁹ /L)	≥5.32	51.8	54.3	54.0	1.1333	0.8878	52.6	0.044	44.0	61.2
Lymphocytes (%)	≥26.4	48.1	52.8	52.3	1.0182	0.9837	47.0	0.045	38.2	55.9
Lymphocytes (10 ⁹ /L)	≥1.95	52.0	50.1	50.3	1.041	0.9592	51.1	0.045	42.2	59.9
Prothrombin Time (sec)	≥12.8	55.8	50.2	51.1	1.1196	0.8813	51.9	0.046	42.9	60.8
International Normalized Ratio	≥0.99	50.0	52.4	52.0	1.0512	0.9536	53.2	0.045	44.5	61.9
Partial Thromboplastin Time (sec)	≥28.3	32.7	36.3	35.7	0.5122	1.8576	33.9	0.045	25.2	42.8
Total Cholesterol (mg/dL)	≥185	50.0	48.4	48.6	0.9686	1.0335	52.9	0.041	44.9	60.8
Triglycerides (mg/dL)	≥49.1	37.0	43.8	43.0	0.6584	1.4392	37.2	0.041	29.2	45.2
HDL (mg/dL)	≥43.3	59.3	60.2	60.1	1.4884	0.6769	63.1	0.04	55.2	70.9
LDL (mg/dL)	≥114	49.1	48.5	48.5	0.9516	1.0515	51.8	0.042	43.5	60.2
Aspartate Aminotransferase (U/L)	≥23	54.0	54.5	54.5	1.1874	0.8437	52.5	0.05	42.6	62.4
Alanine Transaminase (U/L)	≥20	46.0	46.9	46.8	0.8668	1.1506	43.9	0.049	34.4	53.5
Fasting Blood Glucose (mg/dL)	≥99.1	52.4	56.3	55.8	1.1973	0.8466	53.1	0.048	43.7	62.6
Glycohaemoglobin, HbA1c (%)	≥6.2	39.2	42.6	42.2	0.6829	1.4276	38.5	0.041	30.4	46.6
Vitamin D (mg/dL)	≥21.9	50.9	52.8	52.6	1.0785	0.9297	50.9	0.038	43.6	58.3
Cortisol (nmol/L)	≥529	69.6	71.1	70.9	2.4099	0.4279	73.8	0.043	65.5	82.2
NT-proBNP (pg/mL)	≥195	58.7	63.1	62.6	1.5885	0.6551	63.7	0.042	55.6	71.9
IL-6 (pg/mL)	≥5.39	58.7	59.7	59.6	1.4561	0.692	60.9	0.046	51.9	69.9
Insulin (pmol/L)	≥50.7	45.7	45.9	45.8	0.8431	1.1852	45.8	0.053	35.5	56.2
NSE (ng/mL)	≥16.8	63.0	61.6	61.7	1.64	0.6003	66.7	0.046	57.7	75.8
S100 (pg/mL)	≥65	70.0	66.1	66.4	2.0495	0.4607	71.3	0.033	64.9	77.7
Urate (μmol/L)	≥333	48.0	36.5	37.7	0.7553	1.4265	49.0	0.073	34.8	63.3
Albumin (g/L)	≥39.4	46.7	34.8	35.9	0.7152	1.5347	47.3	0.078	32.1	62.5
D-dimer (ng/mL)	≥370	60.0	51.8	52.6	1.2441	0.7726	58.6	0.08	42.9	74.3

Table 12.15. Biomarkers Diagnostic Performance between IS vs. TIA.

Ischemic Stroke (IS) Vs. Transient Ischemic Attack (TIA)										
Biomarker	Cut-Off	Sensitivity (%)	Specificity (%)	Accuracy (%)	LR+	LR-	AUC (%)	SE	95% CI (%)	
White Blood Cells (10 ⁹ /L)	≥7.96	54.1	53.1	53.9	1.1527	0.865	56.5	0.03	50.5	62.4
Haemoglobin (g/dL)	≥14.5	45.2	46.9	45.5	0.8518	1.1676	45.7	0.032	39.3	51.9
Haematocrit (%)	≥42.3	46.9	48.2	47.1	0.9036	1.1038	46.4	0.032	40.1	52.7
Platelets (10 ⁹ /L)	≥224	59.7	50.6	58.2	1.2084	0.7967	52.3	0.034	45.6	59.0
Mean Platelet Volume (fL)	≥9.8	44.9	48.1	45.5	0.8664	1.1442	45.4	0.034	38.7	52.1
Neutrophils (%)	≥63.1	55.5	54.3	55.3	1.2145	0.8196	60.1	0.032	53.7	66.4
Neutrophils (10 ⁹ /L)	≥5	55.6	55.6	55.6	1.2512	0.7991	59.7	0.031	53.6	65.7
Lymphocytes (%)	≥26.6	44.1	43.8	44.1	0.7858	1.2745	38.9	0.034	32.3	45.7
Lymphocytes (10 ⁹ /L)	≥2.04	45.0	43.8	45.2	0.8085	1.2454	41.6	0.035	34.8	48.5
Prothrombin Time (sec)	≥12.7	62.5	59.5	62.1	1.543	0.6308	64.9	0.043	56.7	73.3
International Normalized Ratio	≥0.97	63.2	52.4	61.8	1.3267	0.703	61.2	0.045	52.3	69.9
Partial Thromboplastin Time (sec)	≥28.9	48.7	48.7	48.7	0.9478	1.0551	45.4	0.043	36.9	53.8
Total Cholesterol (mg/dL)	≥192	43.3	44.2	43.4	0.7749	1.2846	45.1	0.034	38.3	51.8
Triglycerides (mg/dL)	≥54.9	41.3	41.6	41.4	0.707	1.412	39.6	0.033	33.1	46.1
HDL (mg/dL)	≥40.2	54.3	54.6	54.3	1.1941	0.8382	52.8	0.035	45.9	59.7
LDL (mg/dL)	≥117	47.1	47.9	47.3	0.9052	1.103	46.0	0.035	39.1	52.9
Aspartate Aminotransferase (U/L)	≥23	45.5	47.5	45.8	0.867	1.1468	47.0	0.036	39.9	54.2
Alanine Transaminase (U/L)	≥22	43.1	44.3	43.3	0.7738	1.2848	43.1	0.041	35.1	51.0
Fasting Blood Glucose (mg/dL)	≥93.7	56.9	55.7	56.7	1.285	0.7737	59.0	0.036	51.9	66.1
Glycohaemoglobin, HbA1c (%)	≥6.2	57.2	56.7	57.1	1.3196	0.7556	58.6	0.038	51.2	66.0
Vitamin D (mg/dL)	≥22.6	43.1	46.8	43.7	0.8114	1.2141	45.3	0.036	38.3	52.3
Cortisol (nmol/L)	≥387	59.2	56.3	58.8	1.3565	0.7237	61.8	0.034	55.1	68.5
NT-proBNP (pg/mL)	≥98.1	56.1	54.9	55.9	1.2442	0.7996	61.9	0.037	54.6	69.1
IL-6 (pg/mL)	≥4.25	51.1	49.3	50.8	1.0079	0.9919	53.8	0.037	46.6	60.9
Insulin (pmol/L)	≥58.8	45.7	45.1	45.6	0.8321	1.2046	44.8	0.036	37.8	51.8
NSE (ng/mL)	≥15.6	46.7	47.9	46.9	0.8955	1.1137	46.3	0.037	39.0	53.6
S100 (pg/mL)	≥49	60.0	60.0	60.4	1.5111	0.6592	64.7	0.033	58.2	71.2
Urate (μmol/L)	≥379	40.9	45.7	41.7	0.754	1.2921	37.8	0.054	27.3	48.3
Albumin (g/L)	≥41.2	40.9	45.8	41.7	0.7552	1.2893	45.3	0.07	31.7	58.9
D-dimer (ng/mL)	≥350	52.3	54.2	52.6	1.1405	0.8811	51.8	0.062	39.7	63.9

Appendix G. ROC Multivariable Model Performance of Biomarkers with Additional Value in Stroke.

Figure 12.4. Multivariable Logistic Regression for Stroke Diagnosis Determination ROC Curve.

Figure 12.5. Multivariable Logistic Regression for Stroke Severity Determination ROC Curve.

Figure 12.6. Multivariable Logistic Regression for Stroke Mortality Determination ROC Curve.

Figure 12.7. Multivariable Logistic Regression for tPA Admission Determination ROC Curve.

Figure 12.8. Multivariable Logistic Regression between IS vs. TIA ROC Curve.

Appendix H. ROC Biomarkers STATA Code for Cardioembolic Stroke as an Analysis Example.

```
roctab TOAST_CEI WBC, summary detail specificity graph
roctab TOAST_CEI Hb, summary detail specificity graph
roctab TOAST_CEI Hct, summary detail specificity graph
roctab TOAST_CEI Platelets, summary detail specificity graph
roctab TOAST_CEI MPV, summary detail specificity graph
roctab TOAST_CEI Neutro_Precantage, summary detail specificity graph
roctab TOAST_CEI Neutrophils, summary detail specificity graph
roctab TOAST_CEI Lympho_Precantage, summary detail specificity graph
roctab TOAST_CEI Lymphocytes, summary detail specificity graph
roctab TOAST_CEI PT, summary detail specificity graph
roctab TOAST_CEI INR, summary detail specificity graph
roctab TOAST_CEI PTT, summary detail specificity graph
roctab TOAST_CEI TC, summary detail specificity graph
roctab TOAST_CEI TG, summary detail specificity graph
roctab TOAST_CEI HDL, summary detail specificity graph
roctab TOAST_CEI LDL, summary detail specificity graph
roctab TOAST_CEI AST, summary detail specificity graph
roctab TOAST_CEI ALT, summary detail specificity graph
roctab TOAST_CEI FB_GLC, summary detail specificity graph
roctab TOAST_CEI HbA1c, summary detail specificity graph
roctab TOAST_CEI VitaminD, summary detail specificity graph
roctab TOAST_CEI Cortisol, summary detail specificity graph
roctab TOAST_CEI NTProBNP, summary detail specificity graph
roctab TOAST_CEI IL6, summary detail specificity graph
roctab TOAST_CEI Insulin, summary detail specificity graph
roctab TOAST_CEI NSE, summary detail specificity graph
roctab TOAST_CEI S100, summary detail specificity graph
roctab TOAST_CEI Urate, summary detail specificity graph
roctab TOAST_CEI Albumin, summary detail specificity graph
roctab TOAST_CEI Ddimer, summary detail specificity graph
```

Appendix I. Reconstruct Biomarkers as Binary STATA Code for Cardioembolic Stroke as an Analysis Example.

```
gen WBC_CEI = 0
replace WBC_CEI = 1 if (WBC >= 7.84)
replace WBC_CEI = . if (WBC == .)
```

```
gen Hb_CEI = 0
replace Hb_CEI = 1 if (Hb <= 14)
replace Hb_CEI = . if (Hb == .)
```

```
gen Hct_CEI = 0
replace Hct_CEI = 1 if (Hct <= 41.2)
replace Hct_CEI = . if (Hct == .)
```

```
gen Platelets_CEI = 0
replace Platelets_CEI = 1 if (Platelets <= 229)
replace Platelets_CEI = . if (Platelets == .)
```

```
gen MPV_CEI = 0
replace MPV_CEI = 1 if (MPV >= 9.7)
replace MPV_CEI = . if (MPV == .)
```

```
gen NeutroP_CEI = 0
replace NeutroP_CEI = 1 if (Neutro_Precantage >= 67.3)
replace NeutroP_CEI = . if (Neutro_Precantage == .)
```

```
gen Neutro_CEI = 0
replace Neutro_CEI = 1 if (Neutrophils >= 5.23)
replace Neutro_CEI = . if (Neutrophils == .)
```

```
gen LymphoP_CEI = 0
replace LymphoP_CEI = 1 if (Lympho_Precentage <= 23.4)
replace LymphoP_CEI = . if (Lympho_Precentage == .)
```

```
gen Lympho_CEI = 0
replace Lympho_CEI = 1 if (Lymphocytes <= 1.86)
replace Lympho_CEI = . if (Lymphocytes == .)
```

```
gen PT_CEI = 0
replace PT_CEI = 1 if (PT >= 13)
replace PT_CEI = . if (PT == .)
```

```
gen INR_CEI = 0
replace INR_CEI = 1 if (INR >= 1)
replace INR_CEI = . if (INR == .)
```

```
gen PTT_CEI = 0
replace PTT_CEI = 1 if (PTT >= 28.8)
replace PTT_CEI = . if (PTT == .)
```

```
gen TC_CEI = 0
replace TC_CEI = 1 if (TC <= 172.4)
replace TC_CEI = . if (TC == .)
```

```
gen TG_CEI = 0
replace TG_CEI = 1 if (TG <= 45.24)
replace TG_CEI = . if (TG == .)
```

```
gen HDL_CEI = 0
replace HDL_CEI = 1 if (HDL >= 42.92)
replace HDL_CEI = . if (HDL == .)
```

```
gen LDL_CEI = 0
replace LDL_CEI = 1 if (LDL <= 105.9)
replace LDL_CEI = . if (LDL == .)
```

```
gen AST_CEI = 0
```

```
replace AST_CEI = 1 if (AST >= 23)
replace AST_CEI = . if (AST == .)
```

```
gen ALT_CEI = 0
replace ALT_CEI = 1 if (ALT <= 19)
replace ALT_CEI = . if (ALT == .)
```

```
gen GLC_CEI = 0
replace GLC_CEI = 1 if (FB_GLC >= 97.29)
replace GLC_CEI = . if (FB_GLC == .)
```

```
gen HbA1c_CEI = 0
replace HbA1c_CEI = 1 if (HbA1c >= 6.3)
replace HbA1c_CEI = . if (HbA1c == .)
```

```
gen VitaminD_CEI = 0
replace VitaminD_CEI = 1 if (VitaminD >= 21.14)
replace VitaminD_CEI = . if (VitaminD == .)
```

```
gen Cortisol_CEI = 0
replace Cortisol_CEI = 1 if (Cortisol >= 444.3)
replace Cortisol_CEI = . if (Cortisol == .)
```

```
gen NTProBNP_CEI = 0
replace NTProBNP_CEI = 1 if (NTProBNP >= 377.2)
replace NTProBNP_CEI = . if (NTProBNP == .)
```

```
gen IL6_CEI = 0
replace IL6_CEI = 1 if (IL6 >= 5.54)
replace IL6_CEI = . if (IL6 == .)
```

```
gen Insulin_CEI = 0
replace Insulin_CEI = 1 if (Insulin >= 50.35)
replace Insulin_CEI = . if (Insulin == .)
```

```
gen NSE_CEI = 0
replace NSE_CEI = 1 if (NSE >= 15.07)
replace NSE_CEI = . if (NSE == .)

gen S100_CEI = 0
replace S100_CEI = 1 if (S100 >= 64)
replace S100_CEI = . if (S100 == .)

gen Urate_CEI = 0
replace Urate_CEI = 1 if (Urate >= 370.8)
replace Urate_CEI = . if (Urate == .)

gen Albumin_CEI = 0
replace Albumin_CEI = 1 if (Albumin >= 40.37)
replace Albumin_CEI = . if (Albumin == .)

gen Ddimer_CEI = 0
replace Ddimer_CEI = 1 if (Ddimer >= 350)
replace Ddimer_CEI = . if (Ddimer == .)

table TOAST_CEI WBC_CEI
table TOAST_CEI Hb_CEI
table TOAST_CEI Hct_CEI
table TOAST_CEI Platelets_CEI
table TOAST_CEI MPV_CEI
table TOAST_CEI NeutroP_CEI
table TOAST_CEI Neutro_CEI
table TOAST_CEI LymphoP_CEI
table TOAST_CEI Lympho_CEI
table TOAST_CEI PT_CEI
table TOAST_CEI INR_CEI
table TOAST_CEI PTT_CEI
table TOAST_CEI TC_CEI
```

table TOAST_CEI TG_CEI
table TOAST_CEI HDL_CEI
table TOAST_CEI LDL_CEI
table TOAST_CEI AST_CEI
table TOAST_CEI ALT_CEI
table TOAST_CEI GLC_CEI
table TOAST_CEI HbA1c_CEI
table TOAST_CEI VitaminD_CEI
table TOAST_CEI Cortisol_CEI
table TOAST_CEI NTProBNP_CEI
table TOAST_CEI IL6_CEI
table TOAST_CEI Insulin_CEI
table TOAST_CEI NSE_CEI
table TOAST_CEI S100_CEI
table TOAST_CEI Urate_CEI
table TOAST_CEI Albumin_CEI
table TOAST_CEI Ddimer_CEI

Appendix J. Biomarkers Univariable Regression STATA Code for Cardioembolic Stroke as an Analysis Example.

```
logistic TOAST_CEI i.WBC_CEI
logistic TOAST_CEI i.Hb_CEI
logistic TOAST_CEI i.Hct_CEI
logistic TOAST_CEI i.Platelets_CEI
logistic TOAST_CEI i.MPV_CEI
logistic TOAST_CEI i.NeutroP_CEI
logistic TOAST_CEI i.Neutro_CEI
logistic TOAST_CEI i.LymphoP_CEI
logistic TOAST_CEI i.Lympho_CEI
logistic TOAST_CEI i.PT_CEI
logistic TOAST_CEI i.INR_CEI
logistic TOAST_CEI i.PTT_CEI
logistic TOAST_CEI i.TC_CEI
logistic TOAST_CEI i.TG_CEI
logistic TOAST_CEI i.HDL_CEI
logistic TOAST_CEI i.LDL_CEI
logistic TOAST_CEI i.AST_CEI
logistic TOAST_CEI i.ALT_CEI
logistic TOAST_CEI i.GLC_CEI
logistic TOAST_CEI i.HbA1c_CEI
logistic TOAST_CEI i.VitaminD_CEI
logistic TOAST_CEI i.Cortisol_CEI
logistic TOAST_CEI i.NTProBNP_CEI
logistic TOAST_CEI i.IL6_CEI
logistic TOAST_CEI i.Insulin_CEI
logistic TOAST_CEI i.NSE_CEI
logistic TOAST_CEI i.S100_CEI
logistic TOAST_CEI i.Urate_CEI
logistic TOAST_CEI i.Albumin_CEI
logistic TOAST_CEI i.Ddimer_CEI
```

Appendix K. Biomarkers Multivariable Regression STATA Code for Cardioembolic Stroke as an Analysis Example.

```
logistic TOAST_CEI i.WBC_CEI i.Hb_CEI i.Hct_CEI i.Platelets_CEI i.NeutroP_CEI
i.LymphoP_CEI i.Lympho_CEI i.PT_CEI i.INR_CEI i.TC_CEI i.TG_CEI i.HDL_CEI
i.LDL_CEI i.AST_CEI i.ALT_CEI i.Cortisol_CEI i.NTProBNP_CEI i.IL6_CEI
i.S100_CEI
```

```
logistic TOAST_CEI i.WBC_CEI i.Hb_CEI i.Hct_CEI i.Platelets_CEI i.NeutroP_CEI
i.LymphoP_CEI i.Lympho_CEI i.PT_CEI i.INR_CEI i.TC_CEI i.TG_CEI i.HDL_CEI
i.AST_CEI i.ALT_CEI i.Cortisol_CEI i.NTProBNP_CEI i.IL6_CEI i.S100_CEI
```

```
logistic TOAST_CEI i.WBC_CEI i.Hb_CEI i.Hct_CEI i.Platelets_CEI i.NeutroP_CEI
i.LymphoP_CEI i.Lympho_CEI i.PT_CEI i.INR_CEI i.TC_CEI i.HDL_CEI i.AST_CEI
i.ALT_CEI i.Cortisol_CEI i.NTProBNP_CEI i.IL6_CEI i.S100_CEI
```

```
logistic TOAST_CEI i.WBC_CEI i.Hb_CEI i.Hct_CEI i.Platelets_CEI i.NeutroP_CEI
i.LymphoP_CEI i.Lympho_CEI i.PT_CEI i.TC_CEI i.HDL_CEI i.AST_CEI i.ALT_CEI
i.Cortisol_CEI i.NTProBNP_CEI i.IL6_CEI i.S100_CEI
```

```
logistic TOAST_CEI i.WBC_CEI i.Hb_CEI i.Hct_CEI i.Platelets_CEI i.NeutroP_CEI
i.LymphoP_CEI i.Lympho_CEI i.PT_CEI i.TC_CEI i.HDL_CEI i.AST_CEI i.ALT_CEI
i.Cortisol_CEI i.NTProBNP_CEI i.S100_CEI
```

```
logistic TOAST_CEI i.WBC_CEI i.Hb_CEI i.Hct_CEI i.NeutroP_CEI i.LymphoP_CEI
i.Lympho_CEI i.PT_CEI i.TC_CEI i.HDL_CEI i.AST_CEI i.ALT_CEI i.Cortisol_CEI
i.NTProBNP_CEI i.S100_CEI
```

```
logistic TOAST_CEI i.WBC_CEI i.Hb_CEI i.Hct_CEI i.NeutroP_CEI i.LymphoP_CEI
i.Lympho_CEI i.PT_CEI i.TC_CEI i.HDL_CEI i.AST_CEI i.ALT_CEI
i.NTProBNP_CEI i.S100_CEI
```

```
logistic TOAST_CEI i.WBC_CEI i.Hct_CEI i.NeutroP_CEI i.LymphoP_CEI
i.Lympho_CEI i.PT_CEI i.TC_CEI i.HDL_CEI i.AST_CEI i.ALT_CEI
```



```
i.NTProBNP_CEI i.S100_CEI
```

```
logistic TOAST_CEI i.WBC_CEI i.NeutroP_CEI i.LymphoP_CEI i.Lympho_CEI  
i.PT_CEI i.TC_CEI i.HDL_CEI i.AST_CEI i.ALT_CEI i.NTProBNP_CEI i.S100_CEI
```

```
logistic TOAST_CEI i.WBC_CEI i.NeutroP_CEI i.LymphoP_CEI i.PT_CEI i.TC_CEI  
i.HDL_CEI i.AST_CEI i.ALT_CEI i.NTProBNP_CEI i.S100_CEI
```

```
logistic TOAST_CEI i.WBC_CEI i.NeutroP_CEI i.LymphoP_CEI i.TC_CEI i.HDL_CEI  
i.AST_CEI i.ALT_CEI i.NTProBNP_CEI i.S100_CEI
```

```
logistic TOAST_CEI i.NeutroP_CEI i.LymphoP_CEI i.TC_CEI i.HDL_CEI i.AST_CEI  
i.ALT_CEI i.NTProBNP_CEI i.S100_CEI
```

```
. estat gof
```

```
. lroc
```

```
. estat classification
```

Appendix L. Stroke Specialists Interviews Summaries.**Representative of Bio-marker X measurement Kit Company**

1. Can you please tell us the details of your company bio-marker X measurement kits (sensitivity, specificity, threshold, robustness, accuracy, reproducibility, price, customers)?
2. What's the hardest part about measuring bio-marker X?
3. Why was that hard?
4. What, if anything, have you done to solve that problem?
5. What don't you love about the solutions you've tried?
6. How often do you experience this problem?
7. How much are you spending to solve this problem now?
8. Where do you find information about this problem?
9. Can you please connect us to a contact you know that is currently working or studying the measurement of bio-marker X?

Representative of Bio-marker X measurement researcher

1. What's the hardest part about measuring bio-marker X?
2. Why was that hard?
3. What, if anything, have you done to solve that problem?
4. What don't you love about the solutions you've tried?
5. How often do you experience this problem?
6. How much are you spending to solve this problem now?
7. Where do you find information about this problem?
8. Can you please connect us to a contact you know that is currently working or studying the measurement of bio-marker X?

Neurologist/ED staff

1. What's the hardest part about Ischemic Stroke Classification?
2. Can you tell me about the last time that happened?
3. Why was that hard?
4. What, if anything, have you done to solve that problem?
5. What don't you love about the solutions you've tried?
6. How often do you experience this problem?
7. How much are you spending to solve this problem now?
8. Where do you find information about Ischemic Stroke Classification?
9. How are the personnel who are dealing with this problem in the hospital?
10. What do you think can be done in order to improve the current Ischemic Stroke classification methods?
11. Can you please connect us to a contact you know that is currently working or studying Ischemic Stroke Classification?

Stroke researcher

1. What's the hardest part about Ischemic Stroke research?
2. Why was that hard?
3. What, if anything, have you done to solve that problem?
4. What don't you love about the solutions you've tried?
5. How often do you experience this problem?
6. How much are you spending to solve this problem now?
7. What do you think can be done in order to improve the current difficulties in Ischemic Stroke research?
8. Can you please connect us to a contact you know that is currently working or studying Ischemic Stroke Classification?

Interview Number 1: Contact details

Date: 24-11-2015

Name: Lau Jin Yi

Designation: Product Specialist

Email: jinyi.lau@transmedicgroup.com

Phone number: +65 9111 0633 / +65 6477 6156

Address: 5 Jalan Kilang Barat 9th Floor Petro Centre Singapore 159349

Interview via: Meeting

Professional experience

Expert in: i-Stat BNP Kit

Experienced with: BNP testing and focus on Point of care system, bed side test.

Professional connection to our project: BNP Test kit, Specialist in Point of Care test

Important insights

1. Transmedic is distributor of Abbott products. They focus on Point of Care testing (bedside test). I-Stat machine is able to test for BNP, results will be ready in 10 minutes. Blood samples, whole blood or plasma samples. There are different types of cartridges (18 selection), purchase suitable cartridge for use. It is a portable machine and thus reduces waiting time.
2. Jin Yi shared the most common test done for patients in ED:
  - Basic Metabolic Status: CHEM8+, results ready in 2 minutes. For evaluation of metabolic status and renal functions of patients.
  - Acute Coronary Syndrome: cTnI, results ready in 10 minutes, For rapid triage of chest pain patients, including those with acute coronary syndrome (ACS).
  - Congestive Heart Failure: BNP, results ready in 10 minutes. For quickly reaching a diagnosis in patients presenting with shortness of breath (acute dyspnea).
  - Lactate: CG4+, results ready in 2 minutes. Useful for the diagnosis and treatment of lactic acidosis and hyperlactataemia
  - Anticoagulation: PT/INR, results ready in less than 5 minutes, For identifying and monitoring of patients on anticoagulant therapy.
3. The cartridges for Cardiac markers are: cTnI, CKMB and BNP.
4. This technology is not commonly used in Singapore. Commonly used in the U.S ED as hospital are far from home. Test is done in the ambulance while sending patient to hospital, takes more than 10minutes for patients to reach hospital.

5. Jin Yi shared a new technology launch 2015, TEG hemostasis analyzer. Used to test for patient bleeding risk, effect on coagulation, patient's platelet function, risk of thrombotic or ischemic events.

Jin Yi mentioned this machine cost 17K. Each cartridge for BNP is \$30. If interested price can be negotiated depending on amount purchase.

Sensitivity and Specificity of BNP

Males (Age Group)						
	All	<45 Years	45-54 Years	55-64 Years	65-74 Years	75+ Years
Sensitivity	71.0% (328/462)	47.1% (8/17)	57.1% (24/42)	57.3% (51/89)	70.6% (115/163)	86.1% (130/151)
95% Confidence Interval	66.6 to 75.1%	23.0 to 72.2%	41.0 to 72.3%	46.4 to 67.7%	62.9 to 77.4%	79.5 to 91.2%
Specificity	94.8% (403/425)	97.2% (104/107)	100.0% (71/71)	97.9% (92/94)	88.7% (102/115)	89.5% (34/38)
95% Confidence Interval	92.3 to 96.7%	92.0 to 99.4%	94.9 to 100.0%	92.5 to 99.7%	81.5 to 93.8%	75.2 to 97.1%

Females (Age Group)						
	All	<45 Years	45-54 Years	55-64 Years	65-74 Years	75+ Years
Sensitivity	80.5% (186/231)	44.4% (4/9)	73.3% (11/15)	50.0% (13/26)	80.6% (58/72)	91.7% (100/109)
95% Confidence Interval	74.8 to 85.4%	13.7 to 78.8%	44.9 to 92.2%	29.9 to 70.1%	69.5 to 88.9%	84.9 to 96.2%
Specificity	88.4% (411/465)	95.9% (94/98)	90.7% (68/75)	89.6% (69.77)	85.7% (114/133)	80.5% (66/82)
95% Confidence Interval	85.1 to 91.2%	89.9 to 98.9%	81.7 to 96.2%	80.6 to 95.4%	78.6 to 91.2%	70.3 to 88.4%

* Representative data, results in individual laboratories may vary from these data.

Image of i-stat blood gas

Figure 12.9. Interview with i-STAT BNP Test Specialist.

Interview Number 2: Contact details

Date: 24-11-2015

Name: Grace Liew

Designation: Account Manager

Email: grace.liew@siemens.com

Phone number: +65 96520212 / +65 64907037 / +65 64907036

Address: The Siemens Centre 60 MacPherson Road Singapore 348615

Interview via: Meeting

Professional experience

Expert in: Biomarkers Machine for testing plasma samples

Experienced with: Biomarkers measurement kit/machine available

Professional connection to our project: Distributor for biomarkers measurement machine and kit

Important insights

Grace shared suitable machine for testing biomarkers.

- Dimension EXL with LM integrated Chemistry System (Reagent Capacity 88 Flex)
- Dimension EXL 200 integrated Chemistry System (Reagent Capacity 44 Flex)
- Stratus CS 200 Acute Care Diagnostic System Specifications (can only test 7 markers, purchase needed cartridge for test)

EXL with LM Vs EXL 200 difference in size of reagent capacity and size of machine, both is the same in functionality. She provided a booklet from Siemens “Biomarkers: Covering the Continuum of Cardiovascular Disease”. It elaborates on common Biomarkers tested for Cardiovascular Disease. Serum/Plasma samples. Within 14 minutes results will be ready for all biomarkers.

1. BNP, CKMB, Troponin I are most common for stroke marker testing. Specialist doctor test D-dimer, hsCRP, Myoglobin, bHCG these 4 are more for cardiac arrest.
2. Dimension EXL (bigger machine) has wider range of markers to test. 72 test in a cartridge to utilize within 30days. EXL is sold in Singapore before for Hospital laboratory.
3. Stratus CS200 (smaller machine), designed for point of care, accepts whole blood no sample preparation is needed. Results will be out in 14 minutes. As mentioned by Grace, this machine is commonly placed at A&E department in U.S. Time for lab test is 2-3 hours in US and Europe as their hospital is bigger due to scattered buildings. Not selling in SG at all. Only marketed to SG recently. Newest technology from Siemens, just launched this year.
4. Accuracy for Dimension EXL is higher than CS200 smaller bench-top machine

Interview Number 3: Contact details

Date: 12-01-2016

Name: Evelyn Wong

Designation: Account Manager

Email: evelyn.wong@thermofisher.com

Phone number: +65 6861 8638 / +65 9115 1938

Address: Thermo Fisher Scientific 33 Marsiling Industrial Estate Road 3 #07-06, Singapore 739256

Interview via: Email

Professional experience

Expert in: D-Dimer Test Kit

Experienced with: Handling NTU for the Invitrogen, Applied Biosystems, Gibco and Molecular Probes products – formerly known as the Life Technology portfolio.

Professional connection to our project: D-Dimer test kit Distributor

Important insights

No Comments given to question asked. Unable to provide much feedback to question asked.

Send me D-Dimer Elisa Kit user guide.

Interview Number 4: Contact details

Date: 12-01-2016

Name: Wen Xian and Patrick Wong

Designation: Product Specialist / Business Manager

Email: wenxian@novadiagscience.com / novadiagscience@gmail.com

Phone number: +65 67472913

Address: Bizlink Centre 59 Ubi Ave 1 #06-11 Singapore 408938

Interview via: Email

Professional experience

Expert in: Medical and dental distributor, distributor of Microalbumin test kit

Experienced with: Medical and Dental products

Professional connection to our project: Microalbumin test kit

Important insights

Wen Xian from NovaDiagnostic shared their Microalbumin kit sensitivity etc. Travis is the company distributing this product, for Singapore NovaDiagnostic is the distributor for them. The Assay insert, calibrators brochure are attached in the email for me.

Their Microalbumin uses immunoturbidimetric method; it is a biochemistry method and therefore not compatible for use with an immunoassay analyzer.

They do not have any customers using this product at the moment. They are unable to answer the interview questions asked about bio-markers etc.

There are no local user using this product, this is a new product they start distributing last year (2015).

SENSITIVITY

The minimum detectable concentration of microalbumin with an acceptable level of precision was determined as 5.11 mg/l.

SPECIFICITY

Urinary analytes other than albumin were dissolved in urine spiked with HSA at two levels.

(mean = 23.4 mg/l and 40.9 mg/l)

The following analytes were tested up to the following levels and found not to interfere:-

ascorbic acid	4 g/l
bilirubin	250 mg/l
creatinine	4 g/l
gentamicin	10 g/l
glucose	40 g/l
paracetamol	5 g/l
potassium chloride	10 g/l
sodium chloride	20 g/l
urea	40 g/l

Note also, that high levels of salicylate (5 g/l) can cause precipitation of urinary protein and give lower than expected values of urinary albumin. (This is, of course, true for all sensitive protein assays).

ASSAY RANGE

The range of this assay is approximately 5.11 - 234 mg/l depending on the lot specific value of calibration in use. In the event of a rerun, the upper limit of the assay range is increased to 440 mg/l.

EXPECTED VALUES^(4,5,6,7,8)

Category	24-h collection (mg/24h)	Timed collection (µg/min)	Spot collection (µg/mg creatinine)
Normal	<30	<20	<30
Microalbuminuria	30-299	20-199	30-299
Clinical albuminuria	300	200	300

It is recommended that each laboratory establish its own reference range to reflect the age, sex, diet and geographical location of the population.

PRECISION**Within run precision**

	Level 1	Level 2	Level 3
Mean (mg/l)	21.2	48.0	116
SD	1.01	1.52	5.12
CV(%)	4.76	3.18	4.41
n	20	20	20

Between run precision

	Level 1	Level 2	Level 3
Mean (mg/l)	28.4	56.8	122
SD	0.616	2.24	10.3
CV(%)	2.17	3.94	8.45
n	20	20	20

CORRELATION

This method (Y) was compared with another commercially available method (X) and the following linear regression equation obtained:

$$Y = 0.94 X - 0.44$$

and a correlation coefficient of $r = 0.99$

40 patient samples were analyzed spanning the range 5.4 to 35.4 mg/l.

Figure 12.10. Interview with Microalbumin Test Specialist.

Interview Number 5: Contact details

Date: 12-01-2016

Name: Adj Assoc Prof Chang Hui Meng

Designation: Senior Consultant

Email: chang.hui.meng@singhealth.com.sg

Phone number: +65 81253629

Address: Singapore General Hospital, Outram Road Singapore 169608, Department of Neurology

Interview via: Call

Professional experience

Expert in: Neurology

Experienced with: Stroke

Professional connection to our project: Neurologist and Sub Specialty in Stroke, done ischemic stroke research

Important insights

Spoke to Prof Chang over the phone.

Shared with Prof Chang the project idea and the subtypes of Ischemic stroke.

She shared that she felt that the whole project idea is too big, is a big idea and it will take a lot of work and time. The need for samples, the time to test the sample and run test on prospective patients, all stroke patients you will have to perform test on them to determine they are ischemic and recruit them to get the samples to test for bio-markers.

She questioned the subtyping- are we going to classify all ischemic stroke and what subtypes, what bio-markers you will be testing, how much literature and research actually proved that these bio-markers are helpful, trust? She shared she read up papers on bio-markers and heard of D-dimer and CRP, she had never heard of BNP or read up about it before (I shared the idea of BNP bio-marker with her).

For Clinical, the idea of subtyping will be good for secondary stroke prevention, good for long term to identify patients with reoccurring stroke.

Her thoughts:

- Using Bio-markers to classify, risk of the bio-markers overlapping.
- Not as straight forward like a pregnancy test kit which tells you yes/no direct answer.

- Many reasons for bio-markers elevation, due to Atrial Fibrillation (AF), rupture, infection etc.
- Timing of test? 4-6hrs, within 24 hrs, there might be changes, patients conditions vary, the bio-markers reading might vary at different time taken.
- Are we going to do something like Oxfordshire Community Stroke Project (OCSP) classification?

She emphasized CT scan and MRI cannot be replaced. It tells clinician the most accurate information.

After sharing with her that we had potential retrospective samples with a Doctor, she advice we do a preliminary test with the samples. Eg, 50 for large vessel, 50 for small vessel, etc. the different types and analysis if the results correlate with the bio-markers and tells you anything. A number too small will not be able to tell you accurate results and not promising also.

From clinical and clinician point of view, they do not use bio-markers clinically at all, bio-markers are not feasible. It is not feasible and not useful.

She suggests to think about making the project more feasible. Whole idea is huge, too big. We should start small, see promising results and move forward to have big ideas, do big. The project is not promising to her, we will have to get preliminary results first before we move forward.

Her thoughts on the approach of interviewing doctors: she is not sure about the idea of us going around calling and looking for doctors to ask them how they feel and to advice on the project, the project is not promising to her, we should think more and make the project more feasible and not sure a big idea before we continue contacting doctors.

Prof Chang shared some doctors names for me to contact if I would like to.

We are welcome to contact and call her to speak to her if we have more questions, she is willing to share and advise us.

Bio-markers representative will be presenting all their kits as best, good, very promising to you as they want you to buy their product.

Interview Number 6: Contact details

Date: 13-01-2016

Name: A/Prof /Dr Christopher Chen Li Hsian

Designation: Associate Professor, Senior Research Fellow

Email: cplhchen@yahoo.com.sg

Phone number: +65 6516 5885

Address: Department of Pharmacology, National University of Singapore

Interview via: Email

Professional experience

Expert in: Neurology, neurochemistry, molecular biology and treatment of stroke and dementia

Experienced with: Neurology

Professional connection to our project: Neurologist background and research in stroke, supervisor for PhD student from NUS. Research title: Retinal and neuroimaging biomarkers of cognitive impairment

Important insights

A/Prof Chen mentioned: It is not the sort of research project I would give my students as I do not believe that it teaches you much.

Questions asked can be found from literature search, should not ask others for answers

He agreed that the classification using bio-markers will help clinicians. But it depends on purpose for: diagnostic, prognostic, mechanistic, surrogate outcomes?

He do not think that blood bio-markers can replace imaging for diagnosis. Prognosis will be a different matter.

Interview Number 7: Contact details

Date: 17-12-2015

Name: Assistant Professor Chester Drum

Designation: Consultant, Department of Cardiology, NUHCS

Assistant Professor, Department of Medicine, Yong Loo Lin School of Medicine, NUS

Email: mdcclld@nus.edu.sg

Phone number: 6601-3001

Address: Cardiovascular Research Institute, MD6, Level 8, 14 Medical Drive, Singapore 117599

Interview via: Meeting

Professional experience

Expert in: Cardiologist MD (USA), PhD (USA)

Experienced with: clinical studies for HF patients management, Had a collaboration with Dr Chris Chen (neurologist) concerning bio-markers measurement for stroke and CVD (there is a direct connection between HF (heart failure), AF (atrial fibrillation) and stroke; this is why they share the same bio-markers).

Professional connection to our project: BNP measurement for HF (Heart failure) patient's management

Important insights**Urine cardiac-Troponin-I test**

1. A new test (new tech) for cTnI have definitely a market, but all the big companies are working on that now
2. The clinical correlation of urine-cTnI test is still undeveloped and invalidated, we should wait for more clinical studies results and knowledge on the use of urine-cTnI test
3. From Dr Chester clinical experience, he had sent outpatients (in his clinic not in the hospital settings) for troponin test only 5 times through his entire medical practice. When he suspects a risk for cardiac event he sends the patients directly to the hospital, which there they do the troponin test very fast and as a part of a full blood test.
4. Dr Chester thinks urine-cTnI would be interesting only after knowing this test's correlation to the patient condition. We need to wait for further developments in the clinical research in this area.

BNP test

1. Dr Chester has a lot of experience with BNP testing. He just finished a 10M grant (him and Dr Chris Chen) for BNP testing for HF.
2. He thinks there is a clinical practice significance to test BNP for HF with our POC platform, but that we should look into the market on what is currently offered for POC BNP testing.
3. He definitely recommends sensing NTproBNP more than BNP.
4. He also has Roche devices, the 2 analyzers like in Dr Raymond Seet lab (cobas c111 and cobas e411).
5. He would like to collaborate for BNP POC sensing for HF low risk patients.
6. Currently he asks his patients to weigh themselves in order to monitor their HF risk (because gaining weight means accumulated fluids and increasing risk for HF).
7. He thinks that an easy to use POC for BNP monitoring at home for low risk patients, would be very significant.
8. He wants to collaborate.

Interview Number 8: Contact details

Date: 17-12-2015

Name: Assistant Professor Wong Ching Chiew Raymond

Designation: Senior Consultant Department of Cardiology, NUHCS

Director Nuclear Cardiology, Department of Cardiology, NUHCS

Director Cardiac Rehabilitation, Department of Cardiology, NUHCS

Director Diagnostic Cardiovascular Laboratory, Department of Cardiology, NUHCS

Programme Director Heart Failure Programme, NUHCS

Assistant Professor Department of Medicine, Yong Loo Lin School of Medicine, NUS

Email: raymond_cc_wong@nuhs.edu.sg

Phone number: N/A

Address: N/A

Interview via: Meeting

Professional experience

Expert in: Senior Consultant in the Department of Cardiology of National University Heart Centre, Singapore

Experienced with: cardiovascular diseases

Professional connection to our project: application for CVD for low risk patients

Important insights

Need for non-invasive cardiac troponin test

1. Preferably cardiac troponin-I
2. Need to reduce the “false-negatives” patients from the Angiography test (type of blood flow test. special dyes are injected into the blood vessels and an X-ray is taken. This test evaluates the size and location of blockages)
3. The patients prefer not to give blood
4. Believes that the patients will give urine sample
5. No time limit, the testing time can be longer than in the ED because those patients don't experience real time life threat

Interview Number 9: Contact details

Date: 17-12-2015

Name: Dr Nathan White MD, MS

Designation: A/P ED MD, university of Washington

Email: whiten4@uw.edu

Phone number: 206.744.8465

Address: box 359702, 325 9th avenue, Seattle, WA 98104

Interview via: Meeting

Professional experience

Expert in: division of emergency department

Experienced with: interdisciplinary collaborations for application for bleeding management in the ED settings

Professional connection to our project: motivated to collaborate for specific needed easy to use sensing

Important insights

1. After he presented an application: a novel polymer that was developed by a PhD student in MIT, for strengthen the cross-link between the clot's fibers of an already formed clots, in order to increase the clot stability.
2. I thought it would be important to sense key proteins of the coagulation system, before injecting the polymer to the circulating system, in order to prevent negative results with patients that suffer from different pathologies (for example, thrombosis), and also to detect the right timing for the injection (for example, in the moment the balance of the coagulation system moves from coagulation to fibrinolysis).
3. He also mentioned that a good target for sensing would be fibrinogen/thrombin/tissue factor, for different application in the ED settings. We should discuss further concerning this direction.

Interview Number 10: Contact details

Date: 17-12-2015

Name: Associate Professor Seet Chee Seong, Raymond

Designation: NUS appointment: Associate Professor, Department of Medicine, Yong Loo Lin School of Medicine

Clinical appointment: Consultant Neurologist, Division of Neurology, National University Health System, Singapore

Research Program Director, Division of Neurology, National University Health System, Singapore

Email: raymond_seet@nuhs.edu.sg

Phone number: (65) 6772 2597

Address: NUHS, MD6, 09-02

Interview via: Meeting

Professional experience

Expert in: Neurologists

Experienced with: clinical and laboratory research in order to improve the care and outcome of acute stroke patients

Professional connection to our project: collaboration

Important insights

1. Potential application is to measure serum bio-markers in order to differentiate between ischemic stroke subtypes
2. Potential bio-markers are troponin-I/T, BNP
3. Access to clinical history and frozen serum samples from around 700 stroke patients from 2009
4. There is a time limit to the test of 24-48h
5. Access to 2 Roche analyzers: cobas c111, cobas e411

Appendix M. NT-proBNP Detection in Paper-based Biosensor Platforms. **Figure 12.11. ELISA NT-proBNP.**

	Sample pad: Unmodified polyester cut into 6mm Ø
	Conjugation pad: Polyester cut into 6mm Ø immobilized with HRP-antibodies diluted in 0.05% PBS-Tween20
	Blocking pad: Polyester cut into 5mm x 70mm strips with chemical activation protocol and covalently bonded with the target protein
	Separation pad: Unmodified polyester cut into 6mm Ø
	PVA film: PVA film in the desired concentration
	Absorption pad: Unmodified absorption pad cut into 5mm x 20mm strips, 3 parts for testing: 1, 2, 3 from the centre outside

Figure 12.12. New Layout of Paper-based Biosensor Platform for NT-proBNP Detection.

Figure 12.13. Effect of Covalent Binding on Protein Detection with HRP-Antibody.

Figure 12.14. Effect of Exposure Time on Antibody Binding to Covalently Bonded NT-proBNP to Polyester.

Overnight Incubation with 100 ng/mL NT-proBNP peptide (blocking layer). Expose to 100 ng/mL HRP-conjugated 15F11 anti NT-proBNP antibody. Wash three times using 0.05% PBST, for 5 minutes each wash. Increased exposure time increases the antibody binding to covalently bonded NT-proBNP on polyester, more filtration of free antibody.

Figure 12.15. Dose Dependency of Covalently Bonded NT-proBNP.

Figure 12.16. Antibody Concentration Effect on Positive/Negative Ratio in New Layout of Paper-based Biosensor Platform: 100 ng/mL NT-proBNP peptide.

Overnight Incubation with 100 ng/mL NT-proBNP peptide (blocking layer). 10, 25, 50, 100 ng/mL HRP-conjugated 15F11 anti NT-proBNP. 6% PVA Films (average weight of 25 mg). 100 μ L Sample, then 100 μ L Buffer (PBS): Negative: only PBS vs. Positive: 100 ng/mL NT-proBNP. After 15 minutes add 100 μ L PBS buffer, wait another 15 minutes for full flow. In the tested conditions no significant difference is seen between positive (100 ng/mL) and negative (0) detection.

Figure 12.17. Effect of Stabilization Step in Polyester Chemical Activation on Positive/Negative Ratio.

Overnight (O/N) Incubation: incubate with 1000 ng/mL protein G for 18 hours in 4 degrees (blocking layer). Additional blocking: Incubate with 1 M glycine for 30 minutes in RT. Additional stabilization: Incubate with 0.3 M sodium cyanoborohydride for 60 minutes at RT. Then expose to HRP-antibody diluted in 0.05% PBS-Tween20. Wash 3 times with 0.05% PBS-Tween20. 100 ng/mL antibody – the ratio between positive to negative signals is the highest after blocking step.

Figure 12.18. Effect of Blocking Reagent in Polyester Chemical Activation on Antibody Binding.

For both 50 and 100 ng/mL antibody concentration, glycine showed higher antibody binding than only Protein-G or BSA. Skim Milk showed less antibody binding than only PBS.

Figure 12.19. Effect of Antibody Concentration on Positive/Negative Ratio in New Layout of Paper-based Biosensor Platform: 5000 ng/mL Protein G.

Overnight Incubation with 5000 ng/mL Protein G (blocking layer). 10, 25, 50, 100 ng/mL HRP-goat anti Human. 6% PVA Films (average weight of 26 mg). 100 μ L Sample, then 100 μ L Buffer (PBS): Negative: only PBS vs. Positive: 5000 ng/mL Protein G. After 15 minutes add 100 μ L PBS buffer, wait another 15 minutes for full flow. A difference between positive and negative signals is visible for 50 and 100 ng/mL antibody concentration.

Figure 12.20. Effect of Protein Concentration in Blocking Layer on Positive/Negative Ratio.

Overnight Incubation with 5,000, 7,500, 10,000 ng/mL Protein G (blocking layer). 100 ng/mL HRP-goat anti Human. 6% PVA Films (average weight of 26 mg). 100 μ L Sample, then 100 μ L Buffer (PBS): Negative: only PBS vs. Positive: 10 μ g/mL Protein G. After 15 minutes add 100 μ L PBS buffer. Wait another 15 minutes for full flow. Control: 10 μ g/mL protein G sample. Highest difference between positive/negative signals is for 7.5K ng/mL protein, however with high SD.

Figure 12.21. Effect of Increased Antibody Concentration on Positive/Negative Ratio: 5000 ng/mL Protein G.

Overnight Incubation with 5000 ng/mL Protein G. 100, 250 and 500 ng/mL HRP-goat anti Human. 6% PVA Films (average weight of 26 mg). 100 μ L Sample, then 100 μ L Buffer (PBS): Negative: only PBS vs. Positive: 5000 ng/ml Protein G. After 15 minutes add 100 μ L PBS buffer. Wait another 15 minutes for full flow. The difference between positive and negative signals is within SD.

Figure 12.22. Effect of Antibody Concentration on Positive/Negative in Ideal Conditions.

Overnight Incubation with 5000 ng/mL Protein G. 100, 250 and 500 ng/mL HRP-goat anti Human. 100 μ L Sample, then 100 μ L Buffer (PBS): Negative: only PBS vs. Positive: 5000 ng/mL Protein G. Looking at the signals from the solution, positive sample (5680 RLU $\pm$ 51) showed a higher signal than in the negative sample (4565 RLU $\pm$ 54), meaning more HRP-antibody is at the positive sample, which can be explained by the immunocomplexes formed. Looking at the signals from the blocking strips, the signal from the negative sample (2965 RLU $\pm$ 210) is slightly higher than in the positive sample (2500 RLU $\pm$ 213), but nearly within standard deviation. The blocking strips were not washed, which can explain this pattern.

Figure 12.23. Effect of Antibody Concentration on Positive/Negative Ratio.

Overnight Incubation with 5000 ng/mL Protein G (blocking layer). 25, 50, 100 and 150 ng/mL HRP-goat anti Human. 100 μ L Sample, then 100 μ L Buffer (PBS): Negative: only PBS vs. Positive: 5000 ng/ml Protein G. After 15 minutes add 100 μ L PBS buffer. Wait another 15 minutes for full flow. First visible pattern is that with increased HRP-antibody concentration immobilized on the conjugation pads, there is a visible increase in the signal detected from all solution, conjugation and blocking samples tested. This fits the theory that since more HRP-antibodies were placed into the sensor, more signal will be visible. However, the signal from the positive vs. negative samples seems to be similar, with differences within standard deviation.

Figure 12.24. Effect of Flow Time on Antibody Release in New Layout of Paper-based Biosensor Platform.

Unmodified blocking strips from polyester. Conjugation pad, polyester pads with 40 μL diluted HRP-goat anti human. 6% PVA Films. 100 μL PBS Sample, then after 15 minutes add 100 μL PBS buffer. Wait another 15 minutes for full flow. Test after the following time points: 0 (no PVA), 5, 10 and 15 minutes.

Figure 12.25. Effect of Flow Direction on Antibodies Release.

Unmodified blocking layer. 25, 50, 100 and 150 ng/mL HRP-goat anti Human. 100 μL PBS sample bottom-up or top-down accordingly. Wait 5 minutes for full flow.

Figure 12.26. Effect of Blocking Pad Filtering in Bottom Up vs. Top Down Flow.

Overnight Incubation with 5000 ng/mL Protein G (blocking layer). 50 and 100 ng/mL HRP-goat anti Human. 100 μ L Sample: Negative: only PBS vs. Positive: 5000 ng/ml Protein G, bottom-up or top-down accordingly. Wait 5 minutes for full flow.

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