A pilot study to identify serum biomarkers for post-stroke spasticity and related skeletal muscle changes

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This thesis is submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

June 2019

Keele University

ABSTRACT

Spasticity is a common sensory-motor dysfunction observed following a stroke, and it is one of the signs indicating damage to the upper motor neurone system at the spinal or cerebral level. Stroke survivors often experience Some resistance to passive movement of their limbs. Increased resistance to passive movement could be attributed to neural and non-neural mechanisms. Neural resistance to passive movement is often referred to as spasticity. Current methods used clinically to measure spasticity proven to be limited or invalid.

The main objective of the current study was to explore the possible usefulness of GFAP, S100B, NSE, Glutamate, GABA, Purines, CK, LD and Albumin as biomarkers of post-stroke spasticity. By comparing the serum concentrations between spastic and non-spastic groups and identify possible correlations of the biomarkers with the development of spasticity. In addition, it was intended to identify possible correlations of muscle biomarkers with post-stroke muscle weakness.

Although not statistically significant, higher levels of three central nerve system specific biomarkers (GFAP, NSE, S100B) and purines indicate a promising candidate targets for further exploration of associated biomarkers for spasticity following stroke.

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Acknowledgements

I would like to firstly express my sincere gratitude to my lead supervisor Professor Anand Pandyan, his knowledge, patience, encouragement and support is greater appreciated. A special thanks goes to my supervisor, Dr Paul Roach, for his continuous support, particularly during the analysis stages of my PhD. My supervisors' guidance helped and guided me throughout the research and during the writing stages of this thesis, of which I am indebted to.

I would also like to take this opportunity to thank Professor Christine Roffe for her assistance during the clinical part of my study. A special thanks goes to Dr Cameron Lindsay for his training to use the clinical measures. I also appreciate the time given to me by my participants, patients of whom were fundermentally important in my research. A special thanks goes towards the research nurses at the Acute stroke unit, UHNM, who gave me their time and place, and without their assistance, my data collection would not have been possible.

I would like to thank all my friends and colleagues, Ahmad, Amnah, Aphrodite, Fraser, Hamzah, Mohammed and Shallum for their encouragement and support. And finally, I would like to thank my family: my loving and supportive wife, Naiema, son, Mustafa and daughter Joury, who were also supportive of my work and always there for me. Your love and support have been a source of strength, and I could not have accomplished this without you.

Glossary of Abbreviations

ADL - Activities of Daily Living

ATP - Adenosine Triphosphate

BBB - Blood-Brain Barrier

CK - Creatine Kinase

CNS - Central Nervous System

CRF - Case Record Form

CRP - C-Reactive Protein

CSF - Cerebrospinal Fluid

CT - Computerised Tomography

ELISA - Enzyme-Linked Immunosorbent Assay

GABA - Gamma-Aminobutyric Acid

GCP - Good Clinical Practice

GFAP - Glial Fibrillary Acidic Protein

HTA - Human Tissue Authority

ICH - Intracerebral Hemorrhage

LACI - Lacunar Infarct

LDH - Lactate Dehydrogenase

MBP - Myelin Basic Protein

MRI - Magnetic Resonance Imaging

mRS -Modified Rankin Scale

MS - Mass Spectrometry

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NF - Neurofilament

NIHSS - National Institutes of Health Stroke Scale

NSE - Neurone-Specific Enolase

O.D - Optical Density

PACI - Partial Anterior Circulation Infarct

POCS - Posterior Circulation Stroke

PROM - Passive Range of Motion

SAH - Subarachnoid Haemorrhage

sEMG - Surface Electromyography

TACI -Total Anterior Circulation Infarct

TIA -Transient Ischaemic Attack

tPA - Tissue Plasminogen Activator

UMNS - Upper Motor Neuron Syndrome

VLP-1 - Visinin-Like Protein 1

WHO - World Health Organization

CHAPTER 1: INTRODUCTION

1.1 Stroke

Stroke is the second common cause of death, after ischaemic heart disease, and the third leading cause of disability worldwide (Johnson et al., 2016). Over 100 000 people in the United Kingdom suffer from a stroke every year, and this has been a financial strain on the National Health Service costing over £3.6 billion. This figure is likely to increase with an increase in the ageing population of today (Xu et al., 2018). Approximately, a third of stroke survivors remain functionally dependent at one year after a stroke, with the feeling of the disabling impact of a stroke continuing for as long as the stroke survivor lives (Newton et al., 2015).

WHO (1988) defines a stroke as "rapidly developing clinical signs of focal (or global) disturbance of cerebral function lasting more than 24 hours with no apparent cause other than of vascular origin". This is a clinical definition, and it does not depend on brain imaging. The WHO definition includes cerebral infarction, subarachnoid haemorrhage and intracerebral haemorrhage. However, subdural haematoma and other traumatic bleedings are excluded since they are usually caused by trauma.

1.1.1 Classification of stroke

There are two types of strokes, ischemic and haemorrhagic. Ischemic strokes, estimated to be 87% of the entire number of reported strokes, result from a cerebral artery becoming, partially

or entirely blocked thereby decreasing tissue perfusion (Donnan et al., 2008). The extent of the infarction will depend on the size and location of the blockage. Ischemic stroke is created either by a thrombosis, as a consequence of an atherosclerotic plaque separation, or by an embolus originating outside the brain. Atherosclerosis, magnified by arterial hypertension, diabetes mellitus, smoking and raised lipid levels form one of the key processes implicated in the pathogenesis of Ischemic stroke (Wardlaw et al., 2009).

The transient ischemic attack, on the other hand, and according to the new definition is considered to be a brief episode of neurologic dysfunction resulted from the focal brain or retinal ischemia, with clinical manifestations typically lasting less than one hour with no neuroimaging evidence of acute infarction. This new definition is based on evidence from neuroimaging studies showed that even when focal transient neurological symptoms last less than an hour, the risk of permanent tissue injury (infarction) still exists. Even brief ischemia is thought to cause permanent brain injury. (Simmons, Cirignano and Gadegbeku, 2012).

Hemorrhagic stroke accounts for around 13% of all strokes. It is the result of a ruptured cerebral artery with the development of intracranial haemorrhage and sometimes raised intracranial pressure that ultimately leads to the compression of surrounding neuronal tissue, as well as the blocking of the blood flow of surrounding vessels with following ischemia and necrosis (Salman, Labovitz and Stapf, 2009). Hemorrhagic stroke can be categorised as intracerebral haemorrhage if the blood accumulation happens within the cerebral parenchyma or as subarachnoid haemorrhage if the haemorrhage takes place outside the brain (between arachnoid mater and

pia mater) (Laborde et al., 2012). Based on the underlying aetiology of intracerebral haemorrhage, it can be categorised as primary if the rupture of small vessels is spontaneous, or secondary when it is associated with conditions such as coagulopathies, vascular irregularities and tumours (Wilson et al., 2015).

At an individual level, the consequences of a stroke can be destructive. Depending on the affected brain area and the degree of damage, the effects may be extensive (Varona, 2011). The remaining neurological deficits may include loss or weakness of the use of one side of the body (paresis), abnormal muscle activation (spasticity), speech difficulty (aphasia/dysarthria), decrease in mental functioning (cognitive/ perceptual Impairments) and impaired emotional functions (UK Department of Health, 2007). These impairments can affect the ability of the individual to move, (e.g. walking), affect the activities of daily living (ADL), (e.g. feeding, dressing) and reduces the quality of life. It has been found that motor and functional outcomes after stroke correlate with a combination of delimiting sizes and primary locations of lesion more than with lesion sizes only (Alexander et al., 2010).

In addition, a series of secondary medical problems particularly falls, and infections are common post-stroke (Langhorne et al., 2000). The type of the pathology, subtype of clinical stroke, continence and level of consciousness have been documented as prognostic factors for death and impairment (Vohra, Ahmed and Ali, 2000). The exact causes of death in stroke varies. It may be the result of basic pathology, such as an increase in intracranial pressure, which will lead to herniation or disruption of vital cerebral function, or, may be further complications of a stroke,

such as Aspiration Pneumonia. It has been reported that complications of the immobility of ischemic stroke patients, rather than to neurological consequence, accounts for more than a double of the death (Creutzfeldt and Hough, 2015).

1.2 Spasticity

Spasticity is a common sensory-motor dysfunction observed following a stroke, and it is one of the signs indicating damage to the upper motor neurone system at the spinal or cerebral level. The European Working Group, EUSPASM, has defined spasticity as "disordered sensorimotor control, resulting from an upper motor neurone lesion, presenting as intermittent or sustained involuntary activation of muscles" (Pandyan et al., 2005). Based on this EUSPASM definition, the term 'spasticity' can be used to describe most of the 'positive features' associated with the upper motor neuron syndrome. Positive features such as increased reflexes, spasm, clonus, abnormal movement patterns, co-contraction, altered tone and 'abnormal' muscle activity response to an externally imposed passive movement. The response of a relaxed muscle to an externally imposed stretch can present as, velocity-dependent response; position-dependent response; a combination of velocity-dependent and position-dependent response and a clasp-knife-type response. In some patients, increased muscle activity can present as increased resistance to passive movement (Pandyan et al., 2018).

Spasticity is thought to be a result of lost inhibitory control of the spinal reflexes. Spinal reflex activity is tightly regulated, and if inhibitory control is lost, the result is the hyperexcitability of the spinal reflexes. The subsequent adaptations in the spinal networks, because of the primary

lesion, may vary considerably since individual patients have lesions affecting different pathways to a different extent. Changes in spinal cord inhibitory circuits; reciprocal inhibition, plateau potentials and presynaptic inhibition may, in different patients, have different roles (Mukherjee and Chakravarty, 2010).

It is believed that there must be some sort of rearrangement, a kind of neuronal plasticity, that occurs within the spinal cord, as well as at the cerebral level following a stroke. The process of neuroplasticity is believed to happen immediately after a stroke and possibly continue at a heightened level during the first few days or weeks post-stroke. Part of neuroplasticity is sprouting of afferent axons. (Bareyre et al., 2004). Afferent fibres might sprout, attach to previously inhibitory synapses, and convert them to excitatory synapses. The development of denervation hypersensitivity due to upregulation of receptors could be an alternative mechanism (Calabresi et al., 1992).

It is unlikely that spasticity is caused by a single mechanism, but rather by a complicated chain of changes in different interdependent networks. More than one pathophysiologic abnormality contributes to the development of spasticity, and these have been well documented in literature (Sheean, 2002; Nielsen, Crone and Hultborn, 2007).

1.2.1 Complications of spasticity

Patients with spasticity sometimes complain only from stiffness, although other features of spasticity could be present and evident. The elastic and plastic properties of muscle tissues also

change as a result of reduced mobilisation, and this is due to the absence of voluntary movement, making muscle contraction and joint movement more difficult (Ghai et al., 2013). Spasticity may also facilitate fixed positioning and lead to the sensations of heaviness or pain in the affected limb.

Contracture, the permanent loss of range of movement of a joint, is more likely to happen in the presence of spasticity. It is believed that, in patients who have not recovered active movement, any form of position-dependent spasticity, the clasp-knife response and spastic dystonia can all facilitate fixed positioning of the limbs and lead to the development of contracture. Contractures seem to develop more rapidly in patients with spasticity who have no function than patients with spasticity but their function still intact. Patients who recover active movement, do not necessarily have spasticity interfering with active movement and do not develop contractures (Pandyan et al., 2003; Malhotra et al., 2011).

Some disuse atrophy occurs within a few weeks of the injury because of certain changes in the mechanical and physiological properties and the muscular tissue being in a contracted position, and this result in a less efficient muscle function. Spasticity might also lead to a decrease in the functional ability of the affected limb. These limitations directly affect the lives of the stroke sufferers at the primary level, but the effect of spasticity is often noticed as an increased burden on caregivers. It is estimated that in the group of chronic spastic patients, 20–30% will have disabling spasticity and in need of some medical intervention., (Lundstrom, Terent and Borg., 2008).

Skin breakdown can be common in persons with spasticity. Poor positioning may lead to skin breakdown over pressure points. Pressure sore occurs in the hand because of contracture and spasticity. Spasticity in the adductor muscles of the legs makes perineal hygiene difficult and can cause pressure problems, at the location of where the knees rub together (Hughes and Howard, 2013). This would certainly increase the burden on the caregiver, because of the noticeable difficulties in moving, handling, and positioning in routine daily care.

The severity of pain and overall quality of life burden associated with the development of post-stroke spasticity may be reduced or avoided through the initiation of preventive approaches in patients with Upper Motor Neuron Syndrome (UMNS). Early interventions may prevent, slow or limit the progression and the complications of post-stroke spasticity. Studies have suggested that early implementation of rehabilitation program prevents pain development and may also prevent deterioration in contracture which is believed to be linked to spasticity (Malhotra et al., 2013).

1.2.2 Measurement of spasticity

The measures currently available for identifying or classifying spasticity can be categorised into three groups: clinical scales, biomechanical methods, and neurophysiological methods (Johnson, 2005). The biomechanical and neurophysiological measurement methods are mostly used in laboratory settings.

1.2.2.1 Clinical methods

Clinical methods include several tools with different measurement characteristics and assessing different constructs. The Ashworth Scale and its modified version (Ashworth, 1964; Bohannon and Smith, 1987) are still common practice in the clinical setting and are widely used in scientific research. The scales used to measure spasticity by quantification of resistance to passive movement (Dunne, Heye and Dunne, 1995; Sampaio et al., 1997).

The Ashworth scale is as follows:

0- No increase in muscle tone

1- Slight increase in tone giving a catch when the limb is moved

2- More marked increase in tone but limb easily moved

3- Considerable increase in tone - passive movement difficult

4- Limb is rigid in flexion or extension

There is a modified Ashworth scale, that is similar to the Ashworth scale except that it adds a 1+ scoring category to indicate resistance through less than the half of the movement (Appendix I). Due to their inability to distinguish between the neural and non-neural components of the increase in resistance to passive movement, both scales have been proven to be invalid measures of spasticity (Pandyan et al., 2003).

Another scale that claims to quantify muscle spasticity is the Tardieu Scale (Tardieu, Shentoub and Delarue, 1954). This scale evaluates the response of the muscle to stretch applied at certain velocities (AppendixII). Grading in Tardieu method is always performed in a constant position

of the body for a given limb and reaction to stretch is rated at a specified stretch velocity. Because of its ability to assess and compare the muscle response to passive movement at both slow and fast speeds, it has been suggested as a more suitable alternative to the Ashworth Scale for measuring spasticity (Vattanasilp, Ada and Crosbie, 2000).

Here is the quality of muscle reaction in Tardieu Scale:

0 - No resistance throughout passive movement

1- Slight resistance throughout, with no clear catch at a precise angle.

2- Clear catch at a precise angle followed by release

3- Fatigable clonus (<10 secs) occurring at a precise angle

4- Unfatigable clonus (>10 secs) occurring at a precise angle

5- Joint Immobile

The Tardieu Scale is also, confounded by the limitations of its ability to distinguish between the neural (spasticity) and non-neural (mechanical and physiological) components of the increase in resistance to passive movement, making its use as a measure of spasticity per se, questionable at best (Haugh, Pandyan and Johnson, 2006). It would make more sense if the clinical scales were to be used as a measure of the resistance to passive movement, as perceived by the clinician, rather than a measure of spasticity (Fleuren, Bourke and Geurts, 2018).

1.2.2.2 Biomechanical methods

Biomechanical methods to measure spasticity assess muscle activation indirectly, by measuring the resistance to an externally imposed movement. The biomechanical measure can be

performed manually, by instrumented displacement, or by gravity (Vodovnik, Bowman and Bajd, 1984; Biering-Sorensen, Nielsen and Klinge, 2006). However, all biomechanical methods have limited clinical applicability and can only be used to measure resistance to passive movement. Biomechanical methods are unable to distinguish between the neural and non-neural components to the resistance to passive movement (Pandyan et al., 2018).

1.2.2.3 Neurophysiological methods

With neurophysiological measurement methods, electrical activity of involved muscles is measured. The use of surface electromyography (sEMG) for the recording of muscle activity during passive movement can be a valuable addition when applied in a standardised way. Neurophysiological methods include, Hoffmann reflex (H-reflex), H:M ratio and F-wave which measure the efferent response to an electrical stimulus, and tendon tap (T-reflex), manual perturbation, or controlled displacement perturbation which measure the efferent response to a mechanical perturbation.

Most of the neurophysiological methods of measurement are relatively easy to perform. However, due to large inter- and intra-subject variability, parameters related to the magnitude of muscle activity cannot be used reliably. Neurophysiological measurement methods provide more useful information to inform the management of spasticity than any of the clinical scales or the biomechanical measures used in isolation (Pandyan et al., 2018). A combination of biomechanical and neurophysiological measurements is recommended, and it is undoubtedly, more accurate in quantifying spasticity than the clinical scales (Malhotra et al., 2008). However,

biomechanical and neurophysiological measurements usually require specialised equipment and training which might make them impractical and not cost effective. Another limitation is that although they tell us of the presence or absence of spasticity, they do not give sufficient enough information of the causes of spasticity. At a fundamental level spasticity results from changes in signaling within the central nervous system (CNS); changes in signaling may give rise to other markers we can measure using biomarkers analysis.

1.3 Rationale

None of the current clinical measures is sensitive enough to detect early spasticity, and they are unable to identify it before any complications have developed, and by that time patients would have possibly lost their ability to recover. If spasticity has not been detected and treated early, complications will develop which can in turn exacerbate the condition further (e.g., contracture will lead to pressure sores). Finding a way of identifying spasticity earlier is the key to better patient's management, with the prospects of developing future treatments. Considering the insufficient sensitivity of current methods used routinely in the clinical setting and the impracticality of using biomechanical and neurophysiological measures in a clinical setting, it is necessary to establish additional methods that, when coupled with clinical assessment, can improve diagnostic precision. The study of biological markers could represent an additional method for the diagnosis of spasticity. The advantage of using biomarkers include quick testing and, coupled having identified biomarkers for spasticity, possibility of identifying suitable early treatment.

In stroke patients, the time course of development of Spasticity can be within 48 hours or even earlier (Pandyan et al., 2018). It has been reported that more than 90% of patients without arm function will develop spasticity within six weeks post stroke (Malhotra et al., 2011).

If spasticity biomarkers can be identified in the early stages of a stroke, ideally before even muscle overactivity is seen, then we will be able to manage spasticity and stop the limb deformities and pain from emerging. The prevention of secondary complications with early identification and treatment, in addition to helping the patient, may cut down the costs associated with giving such service to the NHS. Biomarkers might provide a more efficient and sensitive method of measurement, helping to elucidate mechanism better, lead to newer treatments and assess the effectiveness of any therapeutic interventions for spasticity.

1.4 Biomarkers

A biomarker has been defined by The Biomarkers Definition Working Group of the National Health Institute as a, "*characteristic that is objectively measured and evaluated as a marker or indicator of normal biological processes, pathogenic processes or pharmacological responses to therapeutic intervention*" (Atkinson et al., 2001).

A variety of methods have been used to develop biomarkers. This has progressed from limited targeted pathological or physiological studies to the identification of total alterations linked to disease processes with the use of methods such as proteomics (Rifai and Gerszten 2006). Biomarkers have been applied in several areas of clinical practice. These include diagnosis, prognosis and monitoring of disease development. Other applications include development and

delivery of personalised treatments and monitoring of clinical responses to treatment (Ilyin, Belkowski and Plata-Salaman. 2004).

A biomarker is considered relevant in clinical practice if it can be measured quickly and precisely at a reasonable cost. Ideally, a biomarker should be of value to patient diagnosis or prognosis and helpful in directing patient management (Lemos, McGuire and Drazner. 2003). C-Reactive Protein (CRP) and Troponins are two of the widely used clinical biomarkers. CRP, which is considered an acute phase protein is non-specific, whereas troponin is a cardiac-specific biomarker although its use is limited in some clinical conditions. It is difficult to identify an ideal biomarker that completely meets the strict parameters needed for the evaluation. It is unlikely for an individual biomarker to work flawlessly well for all phases from disease risk identification before onset to post-disease development. It is important for researchers to develop and place the targeted biomarker to fill a gap or meaningfully complement what is already available clinical information. A biomarker that may be unuseful in assisting with the diagnosis may be quite helpful as a tool to follow the development of a specific pathological process in diseases.

The overall aim of this project was to investigate the potential for serum biomarkers to be able to assess and predict the development of post-stroke spasticity. Therefore, the first objective was to Identify the likely serum biomarkers present in the literature used for the evaluation of neurological dysfunction.

CHAPTER 2: LITERATURE REVIEW

The objective of this literature search is to identify potential biomarkers that can be used for early detection and diagnosis of post-stroke spasticity. In the eventual clinical setting, such biomarkers may also be used to augment the precision of clinical diagnosis, follow disease progression and help with drug development.

2.1 Literature search 1

Electronic databases MEDLINE and CINAHL were searched on 17 June 2018 for all studies detailing the use of biomarkers for post- Stroke spasticity. The search was not restricted by date or language.

2.1.1 Electronic search strategy

The full search strategy has been described in Appendix III.

2.1.2 Results

The literature search retrieved 20 publications none of which related to biomarkers of post-stroke spasticity.

2.2 Literature search 2

The first literature search did not find any publications related to biomarkers of post-stroke spasticity; therefore, a second literature search was performed. The second literature search was looking for biomarkers of stroke. Since spasticity is one of the consequences of stroke, we

believed that such search would provide a collection of stroke biomarkers which could be investigated further and their link to post-stroke spasticity explored.

Electronic databases MEDLINE and CINAHL were searched on 17 June 2018 for all studies detailing the use of biomarkers in Stroke. The search was not restricted by date or language.

2.2.1 Inclusion and exclusion criteria

Studies were eligible for inclusion if they were published in the English language; only assayed blood biomarkers (not Cerebrospinal fluid) and included only stroke (not transient ischemic attack).

2.2.2 Electronic search strategy

The full search strategy has been described in Appendix IV

2.2.3 Results

The search yielded eight thousand, one hundred and ninety-five publications. Studies were included if they were published in the English language; only assayed blood biomarkers (not cerebrospinal fluid, urine or other fluids) and included only stroke (not transient ischemic attack). A number of seven thousand one hundred and thirteen publications did not satisfy the inclusion criteria. Out of the one thousand and eighty-two articles that satisfied the inclusion criteria, only human studies were included, this reduced the number further to seven hundred and sixty-one studies (Appendix V). In this study, not all of the papers we are using to inform this review are

limited to the articles identified in the search. A full review of the reported biomarkers and their possible link to post-stroke spasticity will be discussed next.

2.3 Potential biomarkers

There have not been any studies looking at biomarkers of spasticity, and since UMN lesions are believed to be a consequence of stroke, it might be useful to look at stroke biomarkers and study their link to spasticity. There is the possibility that some of these biomarkers might play a role in the development of spasticity.

An ideal central nervous system (CNS) biochemical marker should have all of the following properties (Ingebrigtsen and Romner, 2002):

1- Central nervous system specificity.

2- Rapid and significant release into blood or CSF after injury.

3- Readily obtainable assay results.

4- Predictability of serious injury from an early sample.

5- Relationship of marker concentration with the degree of injury.

6- Inexpensive.

7- Minimally influenced by confounding factors.

8- Reproducible.

2.3.1 Classification of biomarkers

The literature search yielded 761 articles reporting a range of biomarkers linked to stroke. Stroke biomarkers could be divided roughly into two categories, CNS specific and non-CNS-specific biomarkers. The following are mainly brain-derived proteins or amino acids that had been studied as biomarkers of stroke (Table 1). These are markers of damage to glial and neuronal brain tissue and thus could have the potential to be linked to spasticity. These CNS specific biological markers will be discussed in more detail next.

2.4 Central nervous system (CNS) biochemical markers

Table 1 Central nervous system (CNS) biochemical markers

Biomarker	Role	Origin
Neurone-specific enolase (NSE)	Neuronal damage	Neurone
Protein S100B	Glial damage	Glia
Glial fibrillary acidic protein (GFAP)	Glial protein	Glia
Gamma-Aminobutyric acid (GABA)	Neurotransmitter	Neurones
Glutamate	Neurotransmitter	Neurones
Glycine	Neurotransmitter	Neurones
Spermidine	Modulate NMDA	Neurones
Tau	Neuronal protein	Neurones
Myelin basic protein	Glial damage	Glia
Neurofilaments protein	Structural support	Neurones
Visinin-like protein 1 (VLP-1)	Neuronal damage	Neurones

2.4.1 Neurone-specific enolase (NSE)

Neuron-specific enolase is a cytoplasmic glycolytic enzyme expressed mainly in neurons and neuron-derived cells (Isgro, Bottoni and Scatena, 2015). Four studies have demonstrated significantly lower Neuron-specific enolase concentrations in controls than in patients with acute ischemic stroke (Cunningham et al., 1991; Fassbender et al., 1997; Missler et al., 1997; Oh et al., 2003). However, links between Neuron-specific enolase values and infarction volume, different stroke scale scores and result parameters have been found inconsistent (Cunningham et al., 1991; Butterworth et al., 1996; Fassbender et al., 1997; Missler et al., 1997; Wunderlich et al., 1999).

In a study conducted by Jager (1999), ten thromboembolic stroke patients showed significantly elevated Neuron-specific enolase levels as early as 4 hours after stroke onset. Others could not detect differences at 4 hours but reported a Neuron-specific enolase increase from 8 hours to 72 hours (Fassbender et al., 1997). Three studies showed a significant Neuron-specific enolase increase when blood was drawn within the first 24 hours after ischemic stroke onset (Missler et al., 1997; Hill et al., 2000; Oh et al., 2003). Differences between serum levels in ischemic and hemorrhagic stroke were found in one study at 48 and 72 hours after symptom onset, but not at hospital admission (Cunningham et al., 1996).

One reason might be that measurements of NSE serum concentrations were based on techniques with different detection thresholds, sensitivity and specificity (Table 2). None of the previous investigations had used a fully automated high-end technique. Besides, a variety of

different scales have been applied to the assessment of clinical deficits and functional outcome (Anand and Stead, 2005).

Table 2 Characteristics of NSE studies (Different assays, outcomes and normal ranges reported)

Reference	Assay	Outcomes	Normal NSE range
Cunningham et al. (1991)	Cunningham	CT stroke volume; Glasgow Outcome Scale	5.69-10.99 µg/L
Fassbender et al (1997)	Sangtec	Scandinavian Stroke Scale; CT infarct volume	8.38-9.84 ng/mL
Oh et al (2003)	Roche	MRI infarct volume; NIHSS	4.7- 7.9 ng/dL
Missler et al (1997)	Roche	CT infarct volume; ADLs at dismissal	6.4-15.8 µg/L

2.4.2 Protein S100B

The S-100 is an acidic calcium-binding protein consist of two subunits (α and β). S-100 ββ is present in high concentration in glial and Schwann cells, S-100 αβ in glial cells, and S-100 αα is found in slow-twitch muscle, heart, and kidney (Foster, 2017).

As a result of its broad localisation in various cell types, S100B is believed to be a biomarker of generalised blood-brain barrier dysfunction rather than specific glial damage (Kapural et al., 2002). S100B is released into the cerebrospinal fluid (CSF) on damage to the structure of neuronal cells, but the fundamental mechanism of passage through the blood-brain barrier (BBB) has not been clearly explained. The concentration of S100B is 40 times higher in the cerebrospinal fluid than in serum or plasma. The biomarker is not affected by hemolysis and

has outstanding stability (Kanner et al., 2003). It is frequently reported as a promising biomarker that can be measured in peripheral blood samples (Fassbender et al., 1996; Buttner et al., 1997; Missler et al., 1997; Elting et al., 2000; Herrmann et al., 2000, Wunderlich et al., 2004; Thelin, Nelson and Bellander, 2017).

Studies have demonstrated that following stroke, serum S100B concentrations are increased significantly (Persson et al., 1987; Abraha et al., 1997; Buttner et al., 1997; Elting et al., 2000; Foerch et al., 2003; Wunderlich et al., 2004; Foerch et al., 2005; Jauch et al., 2006; Schulte et al., 2014), with the secretion of S100B increasing up to 48 hours after stroke onset and the highest concentration occurring during the first 24 hours after the onset of cerebral infarction. Elting et al. (2000) reported that patients who had significant neurological deficits and abnormal brain imaging showing large artery cortical infarcts had significantly higher S100B concentrations with maximum variation over time, in comparison with patients who had a transient ischaemic attack (TIA) or normal brain CT at presentation.

Significant correlations between the size of infarction area and S100B concentrations in blood were demonstrated in a variety of clinical or experimental research on focal ischemia. In cases with focal ischemia as a secondary complication, it has been shown that lesion size strongly correlated with S100B concentrations 48 hours after stroke following cardiac surgery (Jonsson et al., 2001). Jauch et al. (2006) have reported a direct correlation of stroke severity to S100B concentrations.

Increased S100B in blood is not specific for cerebral infarction as increases occur in other neurological pathologies including traumatic brain injury and extracranial tumours, thus giving the potential to confound interpretation of results (Raabe et al., 1998; Donato et al., 2001; Saenger and Christenson, 2010). The apparent difficulty for widespread use of S100B in acute conditions includes it's apparent prolonged and delayed release into the blood. It has been shown that S100B increase immediately after stroke onset and peak at 4 days post-stroke (Herrmann et al., 2000). This might be an issue when it comes to the diagnosis of stroke, because of the urgency of tPA treatment, but it is not the case in spasticity diagnosis.

2.4.3 Glial fibrillary acidic protein (GFAP)

Glial fibrillary acidic protein (GFAP) is a monomeric intermediate filament protein thought to be produced almost exclusively in brain astrocytes (Hol and Pekny, 2015). GFAP is found in the white and grey matter of the central nervous system and is considered a highly specific brain damage marker (Missler et al., 1999; Pelinka et al., 2004). Glial fibrillary acidic protein is released rapidly out of the damaged brain and is upregulated through astrocytosis (Herrmann et al., 2000; Yasuda et al., 2004; Yang and Wang, 2015).

Although the exact role of glial fibrillary acidic protein is unknown, it is involved with various neuronal cellular processes and is partially responsible for neurological functions within the blood-brain barrier. Initial clinical studies with glial fibrillary acidic protein reported increased serum concentrations in ischemic stroke patient's vs controls, with peak values around 3 days after symptom onset (Niebro et al., 1994; Herrmann et al., 2000; Foerch et al., 2003; Foerch et

al., 2012). The prolonged release and specificity of glial fibrillary acidic protein led to the hypothesis for its use in stroke differentiation as the onset of intracerebral haemorrhage is typically rapid, and any brain injury should result in leakage of GFAP from astroglial cells.

In a study involved patients admitted to a hospital within 6 hours of onset of stroke symptoms, Blood samples were collected immediately after admission, and patients were diagnosed with hemorrhagic or ischemic stroke based on computerised tomography (CT) scan, or magnetic resonance imaging (MRI) results (Foerch et al., 2006). In this study and using an automated enzyme immunoassay, serum glial fibrillary acidic protein was detectable in 81% of patients with intracerebral haemorrhage, compared with only 5% of patients with ischemic stroke. In addition, serum glial fibrillary acidic protein concentrations were much higher in patients with intracerebral haemorrhage. For the first 24 hours after stroke, GFAP levels in ischemic stroke patients remained within the normal range while it increased between 2-6 hours of stroke onset in hemorrhagic stroke patients. To distinguish between ICH and ischaemic stroke, a time frame was determined to be between 2-6 hours after the onset of stroke (Dvorak et al., 2009).

2.4.4 Gamma aminobutyric acid (GABA)

Gamma-aminobutyric acid (GABA) is the chief inhibitory neurotransmitter in the human central nervous system. Inhibitory neurotransmitters reduce the probability that glutamate stimulation leads to action potential by lowering the resting membrane potential of the neurone (Nuss, 2015). GABA neurotransmission causes hyperpolarisation as a result of increased chloride flux across the postsynaptic membrane (Herbison and Moenter, 2011). These actions are believed to

counterbalance the toxic effects that glutamate cause during cerebral ischemia as it is supported by the neuroprotection of the GABAα receptor agonists in animal stroke models (Lyden, 1997). Using microdialysis technique after permanent middle cerebral artery occlusion in rats and after temporal lobe resection in humans, a sustained increase in Gamma-Aminobutyric acid outflow in the ischemic brain has been detected (Melani et al., 1999). Reduction in Cerebrospinal fluid and plasma levels of Gamma-Aminobutyric acid have been detected in patients with acute ischemic stroke (Blicher et al., 2015).

The dynamics of GABA are unknown but, an increase in neuronal or glial GABA uptake, a decrease in GABA-ergic neuronal activity or an enhanced binding of GABA to its receptors in the ischemic brain have been suggested. The enhanced binding of GABA to its receptors, in particular, may lead to a reversal inflow of GABA from blood across the disturbed blood-brain barrier to the brain tissue and hence the reduction in GABA in the blood (Serena et al., 2001).

2.4.5 Glutamate

Brain tissue releases glutamate in very high concentrations in the core of the cerebral infarction and the penumbral cortex; this leads to a massive influx of calcium that activates many of catabolic processes "which in turn" produce cell death (Lai, Zhang and Wang, 2014). Hypoxia due to the spread of peri-infarct depolarisations, triggered in the core of the ischemic infarct has been suggested as another explanation for glutamate-mediated injury (Busl and Greer, 2010). Glutamate is released in high concentrations in the penumbral cortex a region of reduced perfusion in which neurons are viable for some time after the onset of ischemia (Breton and

Rodriguez, 2012) especially if blood flow is reduced for a long time (Xing et al., 2012). Plasma glutamate level may reflect the extent of glutamate accumulation in an ischemic brain (Davalos et al., 2000). This hypothesis is supported by the finding of a high correlation between plasma, and cerebrospinal fluid concentrations of glutamate in stroke patients (Leibowitz et al., 2012) and the clearly defined increase in plasma glutamate found after 4 hours from the onset of permanent middle cerebral artery occlusion in a rat stroke model (Puig et al., 2000). Early increase of glutamate plasma levels has been reported. Glutamate remains elevated up to 15 days post-stroke and decreases gradually to be within the normal range at 90 days post-stroke (Aliprandi et al., 2005). It has been shown that the amount of glutamate released during experimental ischemia correlates positively with the infarct size. (Carmichael, 2005). Thus, glutamate toxicity may have a role in the progression of penumbra to infarction.

2.4.6 Glycine

Glycine is a well-known inhibitory neurotransmitter in the central nervous system, mainly in the spinal cord, brainstem, and retina. Glycine works by activating the glycine receptors which cause chloride to enter the neuron through the ligand-gated ion channels. This process causes an inhibitory postsynaptic potential (Bowery and Smart, 2006).

In a study performed by Castillo et al., (1997), cerebrospinal fluid and plasma concentrations of glycine were shown to be significantly higher in patients with progressing ischaemic stroke compared to patients with stable ischaemic stroke. Increased levels of glycine in plasma have been detected 4 hours after permanent middle cerebral artery occlusion in a rat stroke model

(Puig et al., 2000). This led to the assumption that glycine levels in the brain are likely to be detected in peripheral blood in large ischemic infarctions. Plasma glycine concentrations in lacunar strokes were found to be within the normal range, and no difference has been detected between patients with progressing and patients with non-progressing lacunar strokes. The difference in glycine level between large infarction and lacunar stroke is thought to be due to a smaller release of glycine in small infarctions (Phillips et al., 2007).

2.4.7 Spermidine

Spermidine is a low molecular weight amine that is found in a very high concentration in the human brain (Park and Igarashi, 2013). Spermidine is mainly found intracellularly with only small quantity detected in the extracellular space or the peripheral circulation. Spermidine is present in nearly all cells of the human brain. (Hougaard, 1992). It has been shown that there is a continuous release of spermidine from cells into the extracellular space (Duan et al., 2011) for the purpose of regulation of cellular concentration (Miller-Fleming et al., 2015). Extracellular spermidine concentration has been shown to be increased after focal cerebral ischemia. However, spermidine showed a significant decrease in concentration after reperfusion of focal cerebral ischemia in rats (Paschen et al., 1991). It has been found that spermidine release plays a significant role in the changes of the blood-brain barrier in focal cerebral ischemia and the development of ischemic oedema (Kindy et al., 1994; Igarashi and Kashiwagi, 2011,). Spermidine is mainly transported in the blood by red blood cells after release from brain tissue (Minois, Carmona-Gutierrez and Madeo, 2011). The transportation mechanism of spermidine across the cell wall in the initial phase of cerebral ischemia is still not fully understood (Yang et al., 2017).

2.4.8 Tau protein

Tau protein is expressed mainly in neurons and axons. It is considered to be a structural microtubule-associated protein. Increased levels of tau protein in cerebrospinal fluid (CSF), have been described in acute ischemic stroke (Hesse et al., 2000), traumatic brain injury (Franz et al., 2003), in cerebral complications after aortic surgery (Shiiya et al., 2004) and neurodegenerative diseases, e.g. Alzheimer's disease (Otto and Wiltfang, 2003).

Bitsch et al. (2002) carried out the first serum analyses of tau protein in acute ischemic stroke. An elevated tau protein levels have been described in 35% of the recruited study patients with peak concentrations within 3–5 days. A correlation of peak tau protein values with infarction volume and functional disability after 3 months was found; nevertheless, Bitsch et al. (2002) failed to show a significant association of peak tau protein values with the severity of clinical deficits.

2.4.9 Myelin basic protein

Myelin basic protein (MBP) is considered to be the second most abundant protein in the human central nervous system (Greene et al., 2012). Its responsible for maintaining the correct structure of the myelin sheath and, it plays a vital role in the process of myelination of nerves in the nervous system (Taveggia, Feltri and Wrabetz, 2010). Myelin sheath protects and improves signal conductance by working as an insulator to increase the velocity of nerve impulses (Husted, 2006).

Serum Myelin basic protein was shown to be elevated up to two weeks in an experimental model of TBI (Rostami et al., 2012). However, its sensitivity as a predictive of injury severity in TBI has been challenged. Berger et al. (2005) reported that myelin basic protein levels are specific for ICH and do not increase immediately after an injury. Recently, spectrometry technology has been used as an alternative method for the characterisation of Myelin basic protein proteolysis following central nervous system injury (Ottens et al., 2008). The level of MBP has been shown to be increased significantly in patients with acute cerebral infarction (Berger et al., 2006).

2.4.10 Neurofilaments protein

Neurofilament (NF) proteins are the dominant structural proteins of axons. NF proteins are composed of three polypeptides, light chain (NFL), medium chain (NFM) and heavy chain (NFH). The release of neurofilament proteins into the extracellular space result from pathological mechanisms that cause axonal damage. Neurofilament proteins then diffused into the cerebrospinal fluid and finally transported into the peripheral blood (Giovannoni, 2010). The levels of neurofilament protein are believed to be a potential and a good surrogate for quantifying central nerve system axonal damage. The utility of neurofilament levels as a biomarker has been reported in several studies looking at neurological diseases characterised by axonal damage such as stroke (Petzold, 2005).

Levels of both, neurofilament light chain and neurofilament heavy chain are acutely increased in relation to acute stroke attacks. Neurofilament proteins reach the highest level in two weeks

after the stroke onset and remain at a high level for at least 15 weeks after stroke onset (Lycke et al., 1998).

2.4.11 Visinin-like protein 1 (VLP-1)

Visinin-like protein 1 (VLP-1) is a calcium sensor protein found in human central nervous system neurons. It has been found to have a widespread distribution in the brain and be abundant in all brain areas except the caudate–putamen (Bernstein and Braunewell., 2009). It has been demonstrated that Visinin-like protein 1 was present in the blood of stroke patients, as well as in the cerebrospinal fluid of an animal model of stroke (Laterza et al., 2006). It is believed that Visinin-like protein 1 takes longer than 24 Hours post-injury to spread from the cerebrospinal fluid into the bloodstream (Hesse et al., 2000).

One study (Laterza et al., 2006) considered Visinin-like protein 1 to be a promising post stroke biomarker even though the samples were obtained retrospectively from routine clinical care and were not systematically collected at a particular time frame after stroke onset.

2.5 Miscellaneous biomarkers reported in stroke studies (non- central nervous system specific)

The following are biological markers reported in stroke studies but are not brain-derived proteins or amino acids. These biomarkers can be detected or their level changes in other pathological processes of the human body and not only limited to central nervous system diseases or injuries. Therefore, it should not be considered ideal (CNS) biomarkers, and hence, their use for studying spasticity cannot be attributed to CNS only (Table 3).

Table 3 Miscellaneous biomarkers reported in stroke studies (non-central nervous system specific)

Biomarker	Study
Inflammatory Biomarkers	
C-reactive protein (CRP)	Rost et al., 2001; Arenillas et al., 2003; Curb et al., 2003; Ballantyne et al., 2005; Di Napoli et al., 2003
Interleukin 6 (IL-6)	Vila et al., 2000; Montaner et al., 2003; Smith et al., 2004; Sotgiu et al., 2006
Tumor necrosis factor alpha (TNF-a)	Castellanos et al., 2002; Sotgiu et al., 2006
Vascular cell adhesion molecule 1 (VCAM-1)	Lynch et al., 2004; Sotgiu et al., 2006
Intercellular Adhesion Molecule 1 (ICAM 1)	Castellanos et al., 2002; Sotgiu et al., 2006
Matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9)	Reynolds et al., 2003; Lynch et al., 2004
Lipoprotein-associated phospholipase A2 (Lp- PLA2)	Ballantyne et al., 2005; Oei et al., 2005; Gorelick, 2008; Wassertheil-Smoller et al., 2008; Elkind et al., 2009; Thompson et al., 2010
Apolipoproteins C-I and Apolipoproteins C-III	Allard et al., 2004
Monocyte chemoattractant protein-1 (MCP-1)	Reynolds et al., 2003
Ferritin	Davalos et al., 2000; Erdemoglu and Ozbakir, 2002
Resistin	Efstathiou et al., 2007
Serum amyloid A	Rallidis et al., 2006
P Selectin	Cha et al., 2002

Haemostasis	
Fibrinogen	Ernst and Resch, 1993; Mauriello et al., 2000; Di Napoli et al., 2001
D-Dimer	Fon et al., 1994; Montaner et al., 2008
Von Willebrand factor (vWF)	Reynolds et al., 2003; Lynch et al., 2004
Cellular fibronectin	Powers et al., 2003
Soluble glycoprotein V	Wolff et al. 2005
Anticardiolipin Antibodies	Tanne et al., 2002
Thrombin-antithrombin complex	Tanne et al., 2006
Fibrinopeptide A	Landi et al., 1987; Feinberg et al., 1996
Factor VIIIC	Landi et al., 1987
Cardiac	
Atrial natriuretic peptide (ANP)	Makikallio et al., 2005
B-type natriuretic peptide (BNP)	Makikallio et al., 2005; Sharma et al., 2006; Montaner et al., 2008
Troponin I	Christensen et al., 2004; Barber et al., 2007
Troponin T	James et al., 2000; Fure et al., 2006; Jensen et al., 2007
Anti-inflammatory	
Adiponectin	Efstathiou et al., 2005
Interleukin-10 (IL-10)	Vila et al., 2003
Cortisol	Davalos et al., 1994
Anticlotting	
Thrombomodulin (TM)	Olivot et al., 2004; Tanne et al., 2006

Dyslipidemia/endothelial damage	
Fatty acid binding protein (FABP)	Pelsers et al., 2004; Wunderlich et al., 2005
Other Biomarkers	
Parkinson disease 7 (PARK7)	Allard et al., 2005
Nucleoside diphosphate kinase A (NDKA)	Allard et al., 2005
B-type neurotrophic growth factor	Reynolds et al.,2003
L-Arginine	Blanco et al., 2006
Beta-thromboglobulin	Feinberg et al., 1996
Insulin-like growth factor (IGF)	Denti et al., 2004
Tissue plasminogen activator (tPA)	Tanne et al., 2006
Homocysteine	Pniewski et al., 2003
Hormetanephrine	Chamorro etal.,2007
Uric Acid	Chamorro et al.,2002; Weir et al.,2003
Plasminogen activator inhibitor	Lip et al., 2002
Purines	Berne et al., 1974; Weigand et al., 1999; Pasini et al., 2000; Suzuki et al., 2000

2.6 Muscle atrophy biomarkers

In stroke patients, lesions that interrupt the descending tracts typically cause weakness of voluntary movements, loss of dexterity and fatigability (negative signs) as acute manifestations. Although the primary lesion leading to spasticity is within the central nervous system, there is no doubt that the peripheral musculature has become abnormal. As a result of the skeletal muscle been affected by spasticity, serum levels of muscle enzymes are sometimes changed. Skeletal muscle sodium-potassium ATPase concentration, for example, is reduced in spastic muscle (Ditor et al., 2004).

Several sources could cause muscle weakness and atrophy, such as denervated conditions, immobilisation and neuromuscular diseases. Muscle atrophy may also take place, secondary to common health problems (Jackman and Kandarian, 2004) or some injuries, such as spinal cord injury (SCI). Ageing (Castro et al.,1999) and various systemic diseases. (Hunter et al., 2004, Sandri et al., 2004) could also cause muscle atrophy.

Furthermore, the condition may be worsened by detraining (Joyner, 2004), starvation (Mitch and Goldberg, 1996), lowered levels of hormones (Franch and Price, 2005), decrease in neuromuscular activity (Fitts et al., 2000), decreases in protein synthesis (Hudson and Franklin, 2002), increases in protein degradation (Kimball et al., 2002), declines in protein content (Jackman and Kandarian, 2004), and several forms of reduced use (Reardon et al., 2001). Muscle atrophy which results from limited movement post stroke could eventually cause muscle

contracture, and as such, the study of muscle atrophy biomarkers could help in understanding the broad aspects of spasticity.

The onset of muscle atrophy is rapid and severe among acute and critically ill patients, starting within 4 hours of hospitalisation. (Kasper et al., 2002). The antigravity or the extensor group muscles are believed to show larger atrophy than non-antigravity or flexor group muscles in the first few weeks during hospitalisation (Fitts et al., 2000). A limb not used for prolonged duration in time leads not only to an impairment of the muscle function (Berg and Tesch, 1996) but also to changes in the muscle morphology During prolonged periods of hospitalisation (Bloomfield, 1997), demonstrated in symptoms such as a decrease of the muscle fibre diameter (Widrick et al., 1997), a reduction in muscle mass (Vandenborne et al., 1998) and a decline in the overall number of muscles fibres (Kasper et al., 2002).

Three catabolic pathways are identified to be associated with the atrophy process during decreased muscular movement /decreased gravitational field:

- ATP-ubiquitin-dependent proteolytic pathways.

Ubiquitination process needs the activation of three ubiquitin-proteasome system enzymes: ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and ubiquitin protein ligase (E3). At first ubiquitin binds to E1 (Adenosine triphosphate (ATP) - dependent process) and then transferred to E2. E3 ligases communicate with E2 after covalently bind protein substrate, which carries activated ubiquitin. Ubiquitin in its turn transferred from E2 to the target protein.

The process replicates till target protein binds a chain of 4-5 ubiquitin particles. Then, the ubiquitinated protein degrades into peptides inside proteasome. The binding can also be reversed by deubiquitinating enzymes (DUBs) (Lecker, Goldberg and Mitch, 2006).

- Lysosomal proteolytic pathways.

Lysosomes are the cell organelles accountable for the elimination of other organelles and aggregated tissues proteins. The large number of autophagosomes in humans with muscle diseases proves that autophagy is an essential feature of muscle cells. Lysosomal enzymes are differently activated during atrophy caused by denervation and unloading. According to biochemical and electron microscope data, lysosomal degradation of proteins is responsible mainly for denervation-induced atrophy (Eskelinen and Saftig, 2009).

- Ca^{2+} dependent proteolytic pathways.

The calcium-dependent proteolytic pathways are comprised by the caspase and calpain families. The calpain family is a large family of non-lysosomal calcium-activated cysteine proteases, and calpastatin is their endogenous inhibitor. Three types of calpains are expressed in human muscle, calpain 1, 2 and 3. It is known, at least partially, that calpain 3 is muscle specific. Deficits in this precise calpain manifest primarily in muscle, showing a muscle distinct function. Calpain 3 is not inhibited by, and thus, it is different from calpains 1 and 2. Calpain 3 is believed to have a regulatory function rather than one of bulk proteolysis of structural proteins. Many members of the caspase family also function as regular proteases degrading structural protein; this has been shown with the role of caspase-3 in breaking of actin during cachexia (Kramerova et al., 2005).

These systems engage differently in the development of muscle atrophy induced by disuse and denervation. It has been noticed that activity of lysosomal proteases does not increase significantly under disuse condition (Kachaeva and Shenkman, 2012).

Human head-down bed-rest lead to a rise in the ubiquitin-binding enzyme, proteasome subunits (Taillandier et al., 1996) and to an accretion of ubiquitinated proteins (Ikemoto et al., 2001), which demonstrates the significant participation of ubiquitin-proteasome system in muscle atrophy under unloading conditions. Ca^{2+} dependent proteases (calpains and caspases) also play a crucial role in skeletal muscle atrophy under disuse (Enns et al., 2007), since Ca^{2+} dependent proteases (calpains and Caspases) do not break down proteins into amino acids or small peptides, they are regarded as being a system of primary protein degradation (Goll et al., 2003). All the three major proteasome catalytic pathways were reported to be involved in the breakdown of muscle tissue after stroke (Springer et al., 2014).

A number of results collected showed that atrophic changes during a space flight or under head-down bed-rest are supplemented by myofibril proteins degradation (Parry and Puthucheary, 2015) and reduction of total muscle protein (Stein, 1999). The loss of muscle structural proteins and reduction of muscle functional characteristics result from the complex effect of activation of different proteolytic systems.

The analysis of plasma levels of muscle enzymes is regarded as an essential part of the evaluation of patients presenting with weakness or atrophy and is essential in controlling the course and response to therapy of specific muscular disorders. Serum enzymes and amino acids that have been used to measure muscle atrophy include:

1- Creatine kinase
2- Aspartate aminotransferase
3- Alanine aminotransferase
4- Lactate dehydrogenase
5- Aldolase
6- Myostatin
7- 3-methylhistidine
8- Albumin

2.6.1 Creatine kinase

Creatine kinase is present in the highest concentrations in serum in response to muscle damage. It is the most broadly used enzyme to diagnose and follow muscle disease. It is the best sensitive indicator and measure of the course of muscle damage (Bohlmeyer et al., 1994). Creatine kinase is found on myofibrils, the muscle cytoplasm and the inner mitochondrial membrane. It is involved in cellular energy transfer and storage (Teixeira and Borges, 2012).

Creatine kinase is a dimer molecule and occurs in three different isoenzyme arrangements (termed MM, MB, and BB). Skeletal muscle has the highest concentration of creatine kinase of any tissue. Normal skeletal muscle creatine kinase is more than 99 percent MM with minute amounts of MB. In comparison, cardiac tissue has the largest concentration of creatine kinase - MB, which estimates around 20 percent of cardiac creatine kinase (Takagi, Yasuhara and Gomi, 2001).

2.6.2 Aspartate aminotransferase, alanine aminotransferase

The aminotransferases catalyse the transformation of the amino acids alanine and aspartate to alpha-ketoglutarate, presenting a source of nitrogen for the urea cycle (Hirotsu et al., 2005). Aspartate aminotransferase, alanine aminotransferase enzymes are generally found in various tissues, and raised serum levels are a nonspecific indicator of disease. Increased serum concentrations values are seen in skeletal muscle, hemolysis and myocardial disease.

2.6.3 Lactate dehydrogenase

Lactate dehydrogenase catalyses the last step of glycolysis, transforming pyruvate to lactate (Phypers and Pierce, 2006). It is located in almost every tissue of the body; as a consequence, raised serum levels are observed in a high variety of disease situations. There are five LD isoenzymes, each consisting of tetrameric orders of M and H chains. The LD1 isoenzyme is more prevalent in cardiac tissue, LD5 predominates in skeletal muscle (Bohlmeyer et al., 1994).

2.6.4 Aldolase

Aldolase is a glycolytic pathway enzyme that is found in all tissues but predominantly in skeletal muscle, brain and liver. Aldolase concentrations are infrequently raised in patients with myositis who have normal creatine kinase levels even though, raised aldolase levels are not as specific or sensitive for muscle disease as creatine kinase levels. (Bohlmeyer et al., 1994).

2.6.5 Myostatin

Myostatin is recognised as a part of the Transforming growth factor alpha (TGF -α) family that may also develop atrophy. Decreased myostatin concentration level lead to skeletal muscle hypertrophy in various species including humans (Lee and McPherron, 2001) while an infusion of cells expressing myostatin into adult mice leads to muscle wasting (Zimmers et al., 2002).

2.6.6 3-Methylhistidine

Serum 3-methylhistidine has also been used as a biological marker of muscle protein breakdown. Actin and myosin are 3-methylated in a human muscle which results in 3-methylhistidine amino acid. The resulting amino acid 3-methylhistidine is not reused for intermediary metabolism or protein synthesis, which makes it a perfect biological marker, as it should signify total protein breakdown based on its biology (Holm and Kjaer, 2010).

2.6.7 Albumin

Albumin is used to assess nutritional status. Reduced albumin is associated with starvation, malnutrition (presumably from lack of essential amino acids), anorexia, malabsorption, prolonged bed rest, intravenous fluids, fast hydration and overhydration.

Albumin is produced by the liver at a rate of 9–12 g/day and catabolized at about the same rate; there is no storage or reserve, and it is not catabolized during starvation (Bharadwaj et al., 2016). It commonly decreases rather quickly in many severe acute diseases or injuries, beginning at about 12-36 hours, with the average maximal albumin decrease being reached in about five days.

The decrease of albumin concentrations is common in ill individuals, and a survey of hospitalised patients shows that a significant proportion of albumin measurements are below healthy reference ranges (Sullivan, 2001). Although some of these reductions are likely dilutional, resulting from the administration of intravenous fluids, others are caused by loss of albumin into urine or by reduced synthesis in the liver caused by hepatic disease (Lee, 2012).

Elevation of the serum albumin level is very unusual other than in dehydration. Most changes involve reduction, although the normal range is somewhat large, and small reductions are thus hidden unless the individual patient's normal levels are known. Serum protein levels are relatively stable except for a gradual decrease after age 70 (Visser et al., 2005). Serum albumin concentration, which reflects the amount of muscle mass (Sanaka et al., 1997), can be employed as a sensitive variable to identify abnormal loss of muscle mass in some Diseases responsible for physical inactivities such as cerebrovascular accidents, renal failure and chronic heart failure.

Use of the albumin test is indicated in patients with moderate to severe cerebrovascular accidents and patients who have severe physical fatigue due to advanced chronic conditions such as malignancy. Those patients usually have disuse and/or neurogenic muscle atrophy

that may hinder them from walking by themselves (Kwon et al., 2007). The lack of using the lower extremities means disuse and/or neurogenic muscle atrophy of the lower limbs which suggests a significant loss of muscle mass.

2.6.8 Purines

Purines are one of two families called nitrogenous bases. The other family of These nitrogen-containing molecules is Pyrimidines. Nitrogenous bases are required to construct the genetic material in every living organism. Current investigation in blood biomarkers has shown promising results for the use of purines as early and sensitive markers of ischemic strokes. Berne et al. (1974) demonstrated the first description of purines release from the *in vivo* ischemic brain. Berne reported that Purines could be released from the ischemic brains into the cerebrospinal fluid. Another study showed that the level of blood purine rises rapidly during the hypoxia state and, when the oxygen supply is restored, returns to the pre-hypoxic level within half an hour (Suzuki et al., 2000). A subsequent study used endarterectomy procedure concluded that, carotid artery clamping induces significant increases in jugular venous purines and that cerebral ischemia can be reflected by changes in purines concentration (Weigand et al. 1999). A study in acute stroke settings showed that stroke is associated with a rapid increase in circulating plasma purines concentration detectable in peripheral veins. The purines increase likely mirrors an increased production from the ischemic brain, and it lasts weeks after the acute event (Pasini et al., 2000). This led to the establishment of purines as biomarkers of ischemic stroke.

2.7 Biomarkers of interest

This review discussed the current state of stroke biomarkers and investigated several potential biomarkers that might be utilised for the diagnosis of post-stroke spasticity and muscle atrophy. The scientific literature reporting studies of biological markers for stroke is immense; however, nothing was looking at spasticity from this perspective. Even though stroke biomarkers investigated are anticipated to be biologically informative about the mechanisms of vascular pathology, their clinical usefulness as sensitive diagnostic and prognostic tests remain unknown. At this point, none of the papers gave us an exact explanation as to why there is disparity regarding biomarkers levels and there are no theoretical bases on which we can explain these differences.

To date, no single biomarker has demonstrated to have sufficient sensitivity and specificity for a clinical diagnostic test. A number of studies have tried a multi-marker panel approach in order to enhance sensitivity and specificity (Laskowitz et al., 2005); however, so far none has been suitably successful in a clinical setting. For a multi-marker panel to be successful, it must present additional information to the clinical diagnosis, yield fast results, and the instrumentation needs to be simple to use and cost-effective.

Even though the reported CNS biomarkers represent a valid candidate to be studied and possibly linked to spasticity, the addition of muscle atrophy biomarkers to the study will undoubtedly improve our understanding of the phenomenon. Spasticity causes atrophy which leads to muscle contracture and as such, it is wise to study the three aspects together to get the full picture. Identification of biomarkers for spasticity will assist our understanding of its

aetiology, present diagnostic and prognostic indicators and play an essential role in developing personalised medicine.

2.7.1 Candidate biomarkers

The following biomarkers have been chosen specifically for the reasons described below:

A. They are sensitive to damage in the neurone (Glutamate, GABA and NSE), glia (S100B and GFAP) or the cell body (CK and LD).

B. Their response has been documented in the literature with respect to stroke.

C. There are viable methods of detecting changes in the serum and there is evidence that this can be done. All assays used were in the linear range of detection i.e., they were sensitive enough to detect at the levels we were looking at.

D. They are upregulated early and have the potential to contribute to early spasticity.

Biomarkers:

1- Glutamate
2- Gamma-aminobutyric acid (GABA)
3- Neurone-specific enolase (NSE)
4- Protein S100B
5- Glial fibrillary acidic protein (GFAP)
6- Creatine kinase
7- Lactate dehydrogenase
8- Albumin
9- Purines

2.7.2 Time course of the selected biomarkers

Most of the candidate biomarkers in the current study are likely to be upregulated in a way consistent with our current understanding of the development of spasticity. Development of spasticity in stroke patients is believed to take place as early as 48 hours post-stroke (Pandyan et al., 2018) and most stroke patients without arm function develop spasticity within six weeks post stroke (Malhotra et al., 2011).

Elevated neuron-specific enolase levels were reported as early as 4 hours after stroke onset, and the increase can still be detected at 72 hours post stroke (Fassbender et al., 1997). NSE is likely to be increased in patients where the damage is extensive. It is believed that, patients who have a more extensive brain damage, are more likely to develop spasticity (Cheung et al., 2016). Serum S100B concentrations were reported to be increased immediately after stroke onset (Persson et al., 1987) and last up to 48 hours post-stroke with the highest concentration occurring during the first 24 hours (Elting et al., 2000).

On the other hand, GABA is the only central nervous system-specific biomarker that has been reported to decrease post-stroke onset immediately. Clinical studies with glial fibrillary acidic protein reported increased serum concentrations as early as 2 hours after stroke onset with peak values around three days after symptom onset (Niebro et al., 1994). Elevated plasma glutamate levels have been found after just 4 hours post-stroke. Glutamate remains elevated up to 15 days post-stroke but after that, decreased to reach a normal level in 3 months period (Aliprandi et al., 2005).

Most of the CNS biomarkers have been studied in the context of acute stroke, and as such, not many were followed for a more extended period. A study in acute stroke settings showed rapid increase in plasma purines concentration and the increase lasted weeks after the acute event (Pasini et al., 2000). Studies looking at CK levels post-stroke have reported mixed results. With one study reporting a gradual increase in the enzyme level within the first three days and a decline afterwards (Ay, Arsava and Saribaş, 2002), and another study reported no significant change in both serum LDH and CK levels (Parakh, Gupta and Jain, 2002). These differences in the reported results of CK, LDH and Albumin, are predicted as these three biomarkers are not central nervous system-specific and as such, the changes reported could be attributed to different causes apart from the stroke. CK, LDH and Albumin serum concentration might provide valuable information concerning muscle weakness, atrophy and the development of contracture.

2.7.3 Biomarkers analysis approaches

Traditionally, antibody-based assays, such as enzyme-linked immunosorbent assay (ELISA) are the primary tool for the targeted quantification of specific protein biomarkers. Mass spectrometry (MS) based assay provides an alternative and complementary method to existing antibody-based assays (Anderson and Hunter, 2006).

2.7.3.1 Immunoassays

The purpose of an immunoassay, such as ELISA, is to identify and quantify specific antigens in a sample, where the presence of this antigen could indicate the existence of the disease. Quantitative data on the concentration can be obtained when compared to a reference standard curve.

2.7.3.1.1 Advantages of immunoassays

1- Equipments required are relatively inexpensive.

2- Training requirements are relatively low.

3- The technique is well understood, trusted and relatively straightforward.

4- Offers good selectivity and sensitivity.

2.7.3.1.2 Disadvantages of immunoassay

1- The availability of a specific antibody is crucial.

2- Limited range of analytes and antigens that can be detected.

3- The daily running costs can be quite high due to the antibody cost and the reagent usage.

4- The immunoassay process is quite long (1-3 hours).

5- Sample volumes, especially in ELISA, can be quite high.

2.7.3.2 Mass spectrometry

Mass spectrometry is an analysis technique used to identify unknown compounds and quantify known materials within a sample. The process involves the conversion of the sample into gaseous ions, which are then categorised by their mass to charge ratios and relative abundances.

2.7.3.2.1 Advantages of mass spectrometry

1- Samples can be analysed in a few minutes.

2- Required sample volumes are very small

3- Low day-to-day reagent costs.

4- The procedure is almost fully automated.

5- Offers the highest precision and sensitivity for the detection and identification.

2.7.3.2.2 Disadvantages of mass spectrometry

1- The technology is perceived as being complex to run with a substantial training requirement.

2- High cost of the preliminary investment in instrumentation.

3- A large number of recruits with large number of samples are needed.

2.7.4 The methods used in the current study

While mass spectrometry is an excellent tool for identifying unknown components in a sample or confirming their presence, ELISA reagents have been deemed to have the upper hand in terms of sensitivity of detection of low abundance proteins (Qian et al., 2008). Specificity on the other hand, is inherent to all immunoassays and is evaluated by determination of the cross-reactivity. Analytes that react with the antibody would decrease in percentage of absorbance; conversely, analytes that do not react with the antibody would produce absorbance near 100% (Jinqing et al., 2011).

Since enzyme-linked immunosorbent assays (ELISA) for the candidate biomarkers already exist, the process of validating biomarker candidates would be a relatively straightforward process. The immunoassays approach was adopted as an analysis method for this study.

CHAPTER 3: METHODOLOGY

3.1 Research question:

Having identified the target biomarkers in order to achieve the aim of this project, i.e. investigate the potential for serum biomarkers to be able to assess and predict the development of post-stroke spasticity, the following objectives were identified.

1) Clinical assessment of chosen biomarkers in patients (spastic and non-spastic)
2) Clinical assessment of spasticity post-stroke in both groups.

3.2 Study type:

Longitudinal Observational Cohort study with repeated measures. Ethical approval was granted from the West Midlands - Coventry & Warwickshire Research Ethics Committee (SMARTCap and SMARTChip studies, REC reference, 14/WM/1034, 16/WM/0164 respectively, Appendix VI, VII).

3.3 Outcome measures

This was a longitudinal observational cohort study with repeated measures. The study used measures with identified time point of measurement. In addition to the serum biomarker's levels and in order to document the associated secondary complications, the following measurements were recorded:

- At the point of admission, the National Institutes of Health Stroke Scale (NIHSS) score and the modified Rankin scale (mRS) score were documented (Appendix VIII, IX).

- In parallel with the time points of drawing blood, non -invasive measurements of spasticity (at the wrist), isometric strength (at the wrist and grip strength) were recorded.

3.4 Study population:

The study aimed to recruit 100 adults aged 18 years and over admitted to the Acute Stroke Unit at University Hospital of North Staffordshire (UHNS) and diagnosed with a stroke. Patients recruitment started as early as 24 hours post-stroke.

3.5 Inclusion criteria:

1- Aged 18 years or over.

2- Patients admitted to hospital with a diagnosis of stroke due to a primary cerebral haemorrhage or infarction.

3- Patient had ongoing symptoms of stroke at the time of enrolment into the study.

4- Capable of providing informed consent directly or, consent obtained from next of kin or legal representative.

3.6 Exclusion criteria:

1- Patients with subarachnoid haemorrhage (SAH).

2- Patient's stroke symptoms that had resolved completely prior to enrolment in the study.

3.7 Study setting:

The recruitment of patients, non-invasive measurement of the secondary sensory motor complications, and collection of samples took place at the acute stroke unit at the University Hospital of North Staffordshire (UHNS). Analysis of the blood samples was either conducted at UHNS labs or Keele University. These centres have Human Tissue Authority (HTA)-approved -80 °C freezers for storing the samples. Venous blood samples collected were stored at the University Hospitals of North Midlands pathology laboratory for a maximum of 4 days for analysis in batches. Thereafter serum samples from the blood were analysed at Guy Hilton Research Centre (Keele University, UK). There was not any quantity of the serum left after the analysis of all sample for all biomarkers was completed. Arrangements for long-term storage for future research and analysis were not needed.

3.8 Identification of potential research participants:

All patients admitted to the UHNS with a diagnosis of stroke were eligible to participate. Potentially eligible patients were identified by a member of their direct healthcare team upon the patient's admission to hospital. After the patients' or consultees' agreement, a stroke research team member visited the patient as soon as possible after admission to assess their eligibility to take part in the study. Patients that satisfied the eligibility criteria and who subsequently consented were enrolled in the study.

3.9 Informed consent:

Informed consent was obtained in compliance with Good Clinical Practice (GCP) and the ethical principles orginated in the Declaration of Helsinki. Wherever possible, valid informed consent was sought directly from the stroke patients after the study (and the risks associated

with the study) were fully explained to the patient and, if appropriate, their next of kin. If the patient was competent but unable to sign because of their impairments, verbal consent, witnessed and signed by an independent observer was documented. Where the patient was competent but only able to make a mark on the paper rather than sign as required, the same procedure was followed.

In patient's lacked capacity to give fully informed consent, an appropriate person was identified to act as a personal consultee. We approached a person with a close personal relationship with the potential subject, for example, their next of kin, spouse or partner (including same-sex partners), adult child or parent, who could give information about the patient's views and wishes. Other relatives or a close friend or past carer were considered (Department of Health Guidance on nominating a consultee for research involving adults who lack the capacity to consent 2008). Confirmation of consent was sought in patients who were recruited with consent from a legal representative but regained competence prior to the end of the trial.

Consent was taken by a member of the research team who knew the protocol and was trained in the procedures of GCP. Due to the nature of this study, patients or their legal representatives had to decide within a few hours of admission to hospital. They were given the opportunity to discuss the study with a relative or friend. Participants or their legal representatives were free to withdraw from the trial at any time without giving reasons and without prejudicing further treatment.

The original signed consent forms were filed in the Case Record Form (CRF). One copy was given to the patient or legal representative, and one was filed in the notes. The participant information sheets and content forms were not available in other languages. If needed, the usual hospital interpreter and translator services were available to assist with a discussion of the trial.

3.10 Subject/patient participation:

Venous blood was taken from all participating patients at 1, 3, 7 and 45 days after admission to hospital. Blood samples were collected by trained clinical staff in accordance with NHS procedures. All participants received routine care throughout the study; treatment was not withheld at any point. Results of the CT head scan done as part of routine clinical practice on the first day and results of further head scans during the study period were recorded.

The following serum biomarkers of acute stroke and muscle atrophy were measured, and their link to spasticity was explored:

1- Glutamate

2- Gamma-aminobutyric acid (GABA)

3- Neurone-specific enolase (NSE)

4- Protein S100B

5- Glial fibrillary acidic protein (GFAP)

6- Creatine kinase

7- Lactate dehydrogenase

8- Albumin

9- Purines

3.11 Spasticity and grip strength measurement protocol:

The study utilised two measurement techniques to quantify both spasticity and muscle weakness and explore their links to the biomarkers panel measured. Spasticity was measured using EMG to quantify muscle hyperactivity while a dynamometer was used to measure grip strength and detect muscle weakness.

Spasticity was measured at the wrist flexors. Participants were seated on a chair or bed with the forearm resting on their side. Participant's forearm was positioned in a parallel direction to the ground and fully supported. The forearm was in mid-pronation-supination, the shoulder slightly abducted and the elbow flexed to approximately 90 degrees. Forearm extensor and flexor muscles location were identified and cleaned with an alcohol wipe. For forearm extensors, the electrode was placed one-third of the distance between the lateral epicondyle of the humerus and the radial styloid with the arm in full pronation. For Forearm flexor muscles, the electrode was attached one-third of the length of the forearm toward the midpoint of a line between the medial epicondyle of the humerus and medial border of the biceps tendon (Hermens et al., 1999).

Surface bipolar electromyography electrodes (SX230, Biometrics Ltd, UK) were placed over the identified flexor and extensor muscles, and the ground reference electrode was placed over the acromion. A flexible electrogoniometer (SG75, Biometrics Ltd, UK) was placed across the medial aspect of the wrist joint for measuring the range of motion. The transducers were all then connected to the DataLOG (MWX8, Biometrics Ltd, UK) for data collection purposes and display of the readings. The patient was instructed to be completely relaxed and recording

of the baseline muscle activity was taken. To measure spasticity, the wrist was first flexed as far as comfortable for the patient. Applying a force transducer, Myometer (M550, Biometrics Ltd, UK) to measure the force applied for passive stretching the forearm manually, on the palmar surface of the hand, the wrist was passively and slowly extended using a slow stretch from full flexion into full extension while manually counting for 3 seconds. The wrist was then held in full extension for 3 seconds before returned again into flexion while manually counting for 3 seconds. After holding the wrist in full flexion for 3 seconds, the movement was then repeated using a fast stretch (duration of stretch being 1 second). The wrist was then held in full extension for 3 seconds before returned in a fast stretch (duration of stretch being 1 second) into flexion. The procedure was repeated once. Muscle activity (measured in millivolts), range of movement (measured in degrees) and Force (measured in Newtons) were taken simultaneously during the externally imposed passive stretch. The data from all transducers were sampled at 1000 Hz and stored in a laptop for analysis. Stiffness and velocity were quantified based on the force, range of movement and duration of displacement measurements. Surface electromyography recordings quantified the quantity of muscle activity. Data were processed and analysed using customised software (Mathcad 15, PTC Inc, USA).

For each patient and to classify muscle action, muscle activity and wrist angles data were graphed as an XY scatter plot. Muscle activity quantified by calculating the area under the muscle activity plot. To determine the resistance to passive stretch (stiffness) of muscle, the angle versus force data was also presented as an XY scatter plot. A grip dynamometer was also used to measure grip strength (measured in Newtons) to quantify any weakness that might develop as a result of a stroke. These measures were taken at 1, 3, 7 and 45 days post-stroke.

This measurement protocol has been used in previous trials (Malhotra et al., 2008). The total measurement time was not greater than 30-minutes. Spasticity measurements mirror time points of blood collection for the biochemistry part of the study. Biomarker's specific protocols will be presented in the next chapters.

3.12 Biomarker-specific protocols

3.12.1 Glutamate assay

3.12.1.1 Introduction

Glutamic acid is one of the twenty proteinogenic amino acids. Salts of glutamic acid and the carboxylate anions are known as glutamates. Glutamate is a vital neurotransmitter which plays a crucial role in long-term potentiation and is essential for memory and learning. Glutamic acid is the precursor of gama aminobutyric acid (GABA) but has the opposite role; it might play a role in the function of the prostate and heart. Glutamic acid is one of the few nutrients that crosses the blood-brain barrier.

3.12.1.2 Intended use

Serum from blood samples was independently analysed in duplicates for glutamate using Glutamate Assay Kit (Fluorometric, ab83389) from Abcam, UK. The glutamate assay kit provides a quick and sensitive method for the measurement of glutamic acid in various biological samples. In the assay, the coupled enzyme system catalyses the reaction between L-Glutamic acid and NADP to produce NADPH, which is specifically recognised by NADPH sensor and recycled back to NADP. During the reaction, a red fluorescence product is produced . A fluorescence microplate reader can read the signals at Ex/Em = 530-570

nm/590-600 nm. With this Glutamate Assay Kit (Fluorometric), as little as 1 µM glutamic acid in a 100 µL reaction volume has been detected.

The kit's package included:

- Component A: Enzyme mix
- Component B: Assay buffer
- Component C: NADP (Nicotinamide adenine dinucleotide phosphate)
- Component D: Glutamic acid
- Component E: Dilution buffer

Additional materials used:

- 96-well microplates: Solid black microplates
- Fluorescence microplate reader

3.12.1.3 Reagent preparation

1. 100 µL of dilution buffer (Component E) was added to the vial of NADP (Component C) to Prepare NADP stock solution (200X).

2. 200 µL of dilution buffer (Component E) was added to the vial of glutamic acid (Component D) to Prepare glutamic acid stock solution (100mM)

3. 10 mL of assay buffer (Component B) was added to the bottle of enzyme mixture (Component A).

4. 50 µL 200X NADP stock solution was added to the enzyme mixture bottle and mixed well.

5. 10 µL of the glutamic acid stock solution was added to 990 µL dilution buffer (Component E) to generate 1 mM glutamic acid standard solution.

6. 200 µL of 1 mM glutamic acid standard solution was taken to perform 1:3 serial dilutions to get 300, 100, 30, 10, 3, 1 and 0 µM serially diluted glutamic acid standards.

7. Serially diluted glutamic acid standards and glutamic acid containing test samples were added into a solid black 96-well microplate.

3.12.1.4 Assay procedure

1. For each well, a quantity of 50 µL of glutamic acid assay mixture was added into glutamic acid standard, test samples and blank control to make the total glutamic acid assay volume of 100 µL/well.

2. Incubation at room temperature for two hours was performed with the plate protected from light.

3. A fluorescence plate reader was used to monitor the fluorescence increase at Ex/Em = 550/590 nm.

3.12.1.5 Calculation of results

The fluorescence in blank wells (which contain the dilution buffer only) was used as a control and was subtracted from the values for those wells with the glutamic acid reaction.

3.12.1.6 Expected values

The expected values for this method were 62.3 – 180.7 µM.

3.12.2 Glial fibrillary acidic protein (GFAP) assay

3.12.2.1 Introduction

Glial fibrillary acidic protein is the main component of astrocyte intermediate filaments in the central nervous system. It has also been found in the glial cells of the enteric nervous system and some Schwann cells in the peripheral nervous systems. GFAP antibodies are the most popular marker for astrocytes in neurological studies, and along with its breakdown products (BDPs), GFAP has been proposed as a useful candidate for biofluid-based markers for numerous neurological conditions especially during traumatic brain/spinal cord injury and stroke.

3.12.2.2 Intended use

Serum samples were independently analysed in duplicates for GFAP using Human GFAP DuoSet ELISA from R&D Systems. For the development of sandwich enzyme-linked immunosorbent assays to measure natural and recombinant human glial fibrillary acidic protein. The reagent diluent recommended may be suitable for most cell culture supernate, serum, and plasma samples. The reagent diluent selected for use can alter the performance of an immunoassay. Reagent diluent optimisation for samples with complex matrices such as serum and plasma may improve their performance in this assay.

The kit's package included:

- Human GFAP capture antibody
- Human GFAP detection antibody
- Human GFAP standard
- Streptavidin-HRP

Additional materials used:

- 96 well microplates
- Plate sealers
- PBS (Phosphate-buffered saline)
- Wash buffer
- Reagent diluent
- Substrate solution
- Stop solution

3.12.2.3 Reagent preparation

Before use, all reagents were brought to room temperature. All components were allowed to sit for a minimum of 15 minutes with gentle agitation after initial reconstitution. Working dilutions were prepared and used immediately.

Streptavidin-HRP: 2.0 mL of streptavidin combined with horseradish peroxidase. Dilution was performed to the working concentration specified on the vial label via reagent diluent.

Mouse anti-human GFAP capture antibody: Reference was made to the lot-specific certificates of analysis (C of A) for amount supplied. Reconstitution was done with 0.5 mL of PBS. Dilution in PBS without carrier protein to the working concentration indicated on the C of A.

Biotinylated sheep anti-human GFAP detection antibody: Reference was made to the lot-specific C of A for amount supplied. Reconstitution was done with 1.0 mL of reagent diluent. Dilution in reagent diluent to the working concentration was done as indicated on the C of A.

Recombinant human GFAP standard: Reference was done to the lot-specific C of A for amount supplied. Reconstitution of each vial was done with 0.5 mL of reagent diluent. A seven-point standard curve using 2-fold serial dilutions in reagent diluent was made. 1000 µL prepared of high standard per plate were assayed at the concentration indicated on the C of A.

3.12.2.4 Assay procedure

1. The capture antibody was diluted to the working concentration in PBS without carrier protein. Immediately a 96-well microplate was coated with 100 µL per well of the diluted capture antibody. The plate was sealed and incubated overnight at room temperature.

2. Aspiration and washing of each well were done with wash buffer; the process was repeated two times for a total of three washes. Using a do squirt bottle, washing was done by filling each well with wash buffer (400 µL). A complete removal of liquid at each step was performed for good performance. Any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels after the last wash.

3. 300 µL of reagent diluent was added to each well to block the plates. Incubation at room temperature was done for a minimum of 1 hour.

4. The aspiration and washing process in step 2 was repeated. The plates were ready for sample addition.

5. A quantity of 100 µL of sample or standards were added in reagent diluent per well. Incubation at room temperature for two hours was done after being covered with an adhesive strip.

6. The aspiration and washing process in step 2 was repeated.

7. A volume of 100 µL of the detection antibody was added to each well after dilution in reagent diluent. Incubation at room temperature for 2 hours was done after being covered with an adhesive strip.

8. The aspiration and washing process in step 2 was repeated.

9. For each well, a quantity of 100 µL of the working dilution of Streptavidin-HRP was added. Incubation for 20 minutes at room temperature was done after being covered with an adhesive strip and avoiding direct light.

10. The aspiration and washing process in step 2 was repeated.

11. For each well, a volume of 100 µL of substrate solution was added. Incubation for 20 minutes at room temperature was done after being covered with an adhesive strip and avoiding direct light.

12. 50 µL of stop solution was added to each well. The plate was tabbed gently to ensure thorough mixing.

13. For each well, the optical density was determined immediately, a microplate reader set to 450 nm was used. Readings were subtracted at 540 nm from the readings at 450 nm. This subtraction was to correct for optical imperfections in the plate.

3.12.2.5 Calculation of results

The duplicate readings were averaged for each sample and standard, and the average zero standard optical density (O.D) was subtracted. By plotting the average absorbance for each standard on the y-axis against the concentration on the x-axis, a standard curve was created and drawing of a best-fit curve through the points on the graph. The data was linearised by plotting the log of the human GFAP concentrations versus the log of the O.D., and the best fit line was determined (Appendix X).

3.12.2.6 Expected values

The expected values for this method were 0 – 0 ng/mL.

3.12.3 Human enolase 2/Neuron-specific enolase assay

3.12.3.1 Introduction

Enolase 2 is also known as neuronal enolase or gamma enolase. Enolase (2-phospho-D-glycerate hydrolase) is a cytoplasmic enzyme which converts 2-phosphoglycerate to phosphoenolpyruvate. It has three members: enolase 1, enolase 2, and enolase 3, which are also termed α, γ, and β enolase, respectively. The αγ and γγ isoenzymes are abundant in neurons and neuroendocrine cells, and therefore, they are also termed neuron-specific enolase. Serum enolase 2 values are low in normal individuals. However, when neuronal damage occurs, it is released from the injured cells into the cerebrospinal fluid and systemic circulation. Elevated serum levels of enolase 2, as study shown, are commonly found between a variety of conditions associated with central nervous system injuries such as stroke, traumatic brain injury, multiple sclerosis, and alzheimer's disease.

The quantikine human enolase 2 immunoassay is a 4.5-hour solid-phase enzyme-linked immunosorbent assay designed to measure human enolase 2 in cell culture supernates, serum, and plasma. It contains E. coli-expressed recombinant human enolase 2 and has been shown to precisely quantitate the recombinant factor. Results obtained using natural human enolase 2 displayed linear curves that were similar to the standard curves obtained using the Quantikine kit standards. These results show that this kit can be used to determine relative mass values for naturally occurring human enolase 2.

3.12.3.2 Principle of the assay

Serum samples were independently analysed in duplicates for NSE using NSE Quantikine ELISA kit from R&D Systems. The assay employs the quantifiable sandwich enzyme-linked immunosorbent assay technique. A microplate has been pre-coated with a monoclonal antibody specific for enolase 2. Standards and samples are pipetted into the wells, and any enolase 2 exist is bound by the immobilised antibody.

An enzyme-linked polyclonal antibody specific for enolase 2 is added to the wells After washing away any unbound substances. A substrate solution is added to the wells following a wash to remove any unbound antibody-enzyme reagent and colour develops in proportion to the amount of enolase 2 bound in the initial step. After the colour development is stopped, The intensity of the colour is measured.

The kit's package included:

- Enolase 2 microplate
- Enolase 2 standard
- Enolase 2 conjugate
- Assay diluent RD1-9
- Calibrator diluent RD5C concentrate
- Wash buffer concentrate
- Color reagent A
- Color reagent B
- Stop solution
- Plate sealers

Additional materials used:

- Microplate reader
- Pipettes and pipette tips
- Distilled water
- Squirt bottle
- 100 mL and 500 mL graduated cylinders
- Horizontal orbital microplate shaker
- Test tubes for dilution of standards

3.12.3.3 Reagent preparation

1- All reagents were brought to room temperature before use.

2- Wash buffer - 20 mL of wash buffer concentrate was diluted into distilled water to prepare 500 mL of wash buffer.

3- Substrate solution - Color reagents A and B were mixed in equal volumes within 15 minutes of use. 200 mL of the resultant mixture was required per well.

4- Calibrator diluent RD5C (1X) - 10 mL of calibrator diluent RD5C concentrate were diluted into 40 mL of distilled water to prepare 50 mL of calibrator diluent RD5C (1X).

5- Enolase 2 standard - The enolase 2 standard was reconstituted with 1.0 mL of distilled water. A stock solution of 200 ng/mL were produced. The standard was mixed to ensure complete reconstitution and allowed to sit for a minimum of 15 minutes.

6- 900 mL of calibrator diluent RD5C (1X) were pipetted into the 20 ng/mL tube. 500 mL of calibrator diluent RD5C (1X) were pipetted into the remaining tubes. The stock solution was used to produce a dilution series. Each tube was mixed thoroughly before

the next transfer. The 20 ng/mL standard served as the high standard. Calibrator diluent RD5C (1X) served as the zero standards (0 ng/mL).

3.12.3.4 Assay procedure

All reagents and samples were brought to room temperature before use. All samples, controls, and standards were assayed in duplicate.

1. Excess microplate strips were removed from the plate frame, returned to the foil pouch containing the desiccant pack, and resealed.

2. 100 mL of assay diluent RD1-9 were added to each well.

3. 50 mL of standard, control, or sample were added per well. Covered with the adhesive strip provided. Incubated at room temperature for two hours on a horizontal orbital microplate shaker set at 500 ± 50 rpm.

4. Each well was aspirated and washed; the process was repeated three times for a total of four washes. Each well was washed by filling with wash buffer (400 mL) using a squirt bottle. It is crucial to ensure the complete removal of any liquid at each step. Any remaining wash buffer was removed by aspiration after the last wash. The plate was inverted and blotted against clean paper towels.

5. 200 mL of enolase 2 conjugate were added to each well and covered with a new adhesive strip. Incubated at room temperature for two hours on the shaker.

6. The aspiration/wash process was repeated as in step 4.

7. 200 mL of substrate solution was added to each well. Protected from light. Incubation was done for 30 minutes at room temperature on the benchtop.

8. 50 mL of stop solution was added to each well. The plate was tapped gently to ensure thorough mixing. The colour in the wells was changed from blue to yellow

9. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm. Readings were subtracted at 550 nm or 570 nm from the readings at 450 nm. This subtraction was to correct for optical imperfections in the plate.

3.12.3.5 Calculation of results

The duplicate readings for each standard, control, and sample were averaged, and the average zero standard optical density (O.D.) was subtracted. A standard curve was constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data was linearised by plotting the log of the Enolase 2 concentrations versus the log of the O.D., and the best fit line was determined by regression analysis.

3.12.3.6 Expected values

The expected values for this method were 1.85 – 4.14 ng/mL.

3.12.4 S100 Calcium-binding protein B (S100B)

3.12.4.1 Introduction

S100B belongs to the S100 subcategory of the EF-hand family of calcium-binding proteins. It is a homodimer that is expressed mainly in the brain by astrocytes, oligodendrocytes and schwann cells. S100B has several intracellular functions, but can also be secreted by cells to exert extracellular functions. Some of the extracellular roles of S100B may be mediated by receptor for advanced glycation end products. Blood levels of S100B can be used to monitor the extent of brain injury

3.12.4.2 Intended use

Serum from blood samples was independently analysed in duplicates for S100B using Human S100B DuoSet ELISA kit from R&D Systems, UK. For the development of sandwich enzyme-linked immunosorbent assays to measure natural and recombinant human S100B calcium binding protein B (S100B). The reagent diluent recommended may be suitable for most cell culture supernate, serum, and plasma samples. The reagent diluent selected for use can alter the performance of an immunoassay. Reagent diluent optimisation for samples with complex matrices such as serum and plasma may improve their performance in this assay.

The kit's package included:

- Human S100B capture antibody
- Human S100B detection antibody
- Human S100B standard
- Streptavidin-HRP

Additional materials used:

- 96 well microplates
- Plate sealers
- PBS (Phosphate-buffered saline)
- Wash buffer
- Block buffer
- Reagent diluent
- Substrate solution
- Stop solution

3.12.4.3 Reagent preparation

1- All reagents were brought to room temperature before use. All components were allowed to sit for a minimum of 15 minutes with gentle agitation after initial reconstitution.

2- Streptavidin-HRP: 2.0 mL of streptavidin were conjugated to horseradish peroxidase. Diluted to the working concentration using reagent diluent.

3- Mouse Anti-Human S100B Capture Antibody: Reconstituted with 0.5 mL of PBS. Diluted in PBS without carrier protein to the working concentration.

4- Biotinylated Mouse Anti-Human S100B Detection Antibody: Reconstituted with 1.0 mL of reagent diluent. Diluted in reagent diluent to the working concentration.

5- Human S100B Standard: Each vial was reconstituted with 0.5 mL of distilled water. A seven-point standard curve using 2-fold serial dilutions in reagent diluent was performed. 1000 µL of high standard was prepared per plate assayed.

3.12.4.4 Assay procedure

1. The capture antibody was diluted to the working concentration in PBS without carrier protein. A 96-well microplate was immediately coated with 100 µL per well of the diluted capture antibody. The plate was sealed and incubated overnight at room temperature.

2. Each well was aspirated and washed with wash buffer; the process was repeated two times for a total of three washes. Each well was washed by filling with wash buffer (400 µL) using a squirt bottle. Any remaining were removed after the last wash by wash buffer by inverting the plate and blotting it against clean paper towels.

3. Plates were blocked by adding of 300 µL of block buffer to each well. Incubation was done at room temperature for a minimum of 1 hour.

4. The aspiration/wash process was repeated.

5. 100 µL of sample or standards were added in reagent diluent, per well. Covered with an adhesive strip and incubated for two hours at room temperature.

6. The aspiration/wash process was repeated.

7. 100 µL of the detection antibody, diluted in reagent diluent, were added to each well. Covered with a new adhesive strip and incubated for two hours at room temperature.

8. The aspiration/wash process was repeated.

9. 100 µL of the working dilution of streptavidin-HRP was added to each well. The plate was then covered and incubated for 20 minutes at room temperature.

10. The aspiration/wash process was repeated.

11. 100 µL of substrate solution were added to each well. Incubated for 20 minutes at room temperature.

12. 50 µL of stop solution were added to each well. The plate was gently tapped to ensure thorough mixing.

13. The optical density of each well was calculated immediately, using a microplate reader set to 450 nm. Readings at 550 nm or 570 nm were subtracted from the readings at 450 nm. This was done to correct for optical imperfections in the plate.

3.12.4.5 Calculation of results

The duplicate readings were averaged for each standard, control, and sample and the average zero standard optical density (O.D.) was subtracted. A standard curve was made by plotting the average absorbance for each standard on the y-axis compared to the concentration on the x-axis and draw a best fit curve over the points on the graph. The data was linearised by

plotting the log of the human S100B concentrations versus the log of the O.D., and the best fit line was determined by regression analysis.

3.12.4.6 Expected values

The expected values for this method were 20 – 150 pg/mL.

3.12.5 Gama aminobutyric acid (GABA) assay

3.12.5.1 Intended use

Serum from blood samples was independently analysed in duplicates for GABA using the GABA ELISA Kit from IBL international, Germany. The kit is for the quantitative detection of GABA in human EDTA plasma, serum and urine. The test is based on the method of competitive enzyme-linked immunoassays. The sample preparation includes the addition of a derivatisation reagent for GABA derivatisation. Afterwards, the treated samples are incubated in wells of a microtiter plate coated with a polyclonal antibody against GABA-derivative, together with assay reagent containing GABA-derivative, In the sample, The target GABA competes with the GABA-derivative during the incubation period for the binding of the polyclonal antibodies on the wall of the microtiter wells. GABA in the sample displaces the tracer out of the binding to the antibodies. Therefore, the concentration of the antibody-bound tracer is inverse proportional to the GABA concentration in the sample. To detect the tracer, a peroxidase conjugate is added to each microtiter well during the second incubation step. The unbound components tetramethylbenzidine (TMB) is added as a peroxidase substrate after washing away.

The enzymatic reaction is ended by an acidic stop solution in the final step. The colour changes to yellow and the absorbance is measured in a photometer at 450 nm. The intensity of the yellow colour is contrary proportional to the GABA concentration in the sample, high GABA concentration in the sample lowers the photometric signal and reduces the concentration of antibody-bound tracer. A dose-response curve of absorbance unit (optical density at 450 nm) vs concentration is generated using the values obtained from the standards. GABA present in the patient samples is determined directly from this curve.

The kit's package included:

- One holder with precoated strips
- Standards diluted in reaction buffer
- Controls diluted in reaction buffer
- Wash buffer concentrate
- Assay reagent
- Peroxidase conjungate
- Conjungate stabilizing buffer
- Reaction buffer
- Derivatisation reagent
- Dimethylsulfoxide
- Dilution buffer
- Tetramethylbenzidine substrate
- Stop solution

Additional materials used:

- Double distilled water
- Precision pipettors and disposable tips
- Foil to cover the microtiter plate
- Horizontal microtiter shaker
- A multi-channel dispenser
- Centrifuge
- Vortex-mixer
- Microtiter plate reader

3.12.5.2 Reagent preparation

The wash buffer concentrate (WASHBUF) was diluted with aqua bidest (100 mL WASHBUF + 900 mL aqua bidest.) and mixed well.

Standards (STD) and controls (CTRL1, CTRL2) were already diluted in reaction buffer (REABUF). The content of a vial of derivatisation reagent (DER) was dissolved in 550 µL DMSO. The vial was then placed on a horizontal shaker for 5 min. The content of a vial of assay reagent (ASYREAG) was melted in 4 mL of diluted wash buffer. The POD conjugate (CONJ) 1:200 was diluted with conjugate stabilising buffer (CONJBUF).

3.12.5.3 Assay procedure

Derivatization of standards, controls and diluted samples were carried out in a single analysis. EDTA serum samples were diluted with reaction buffer by factor 1:4. These vials, containing 400 µL diluted sample, were used for derivatisation.

1. All reagents and all samples were brought to room temperature.

2. 400 µL of ready to use standards, 400 µL of ready to use controls and 400 µL of diluted samples were added to the corresponding vial.

3. 25 µL of freshly prepared derivatisation reagent (DER) was added to each vial mixed well and incubated for 60 minutes on a shaker at room temperature.

4. 500 µL of dilution buffer (CODIL) were added afterwards into each vial, mixed well and incubated for 30 minutes on a shaker at room temperature. 2 x 100 µL of each treated sample (STD, CTRL, SAMPLE) were used in the ELISA as duplicates.

5. Each well was washed five times by dispensing 250 µL of diluted wash buffer into each well. The inverted microtiter plate was tapped on absorbent paper to remove excess solution after the final wash.

6. 2 x 100 µL of standards, controls and samples were taken out of the vial and added into the respective wells of the microtiter plate.

7. 100 µL of dissolved assay reagent (ASYREAG) were added to each well.

8. Incubated overnight at 2-8°C.

9. The contents of each well were aspirated. Each well was washed five times by dispensing 250 µL of diluted wash buffer into each well. After the final washing step, the inverted microtiter plate was tapped on absorbent paper to remove excess solution.

10. 200 µL diluted POD conjugate were added to each well.

11. The plate was covered tightly and incubated for 1 hour at room temperature on a horizontal shaker.

12. Each well contents were aspirated. Each well washed five times by dispensing 250 µL of diluted wash buffer into each well. After the final washing step, the inverted microtiter plate was tapped on absorbent paper to remove excess solution.

13. 200 µL of TMB substrate (SUB) were added to each well.

14. Incubated for 6-12 min at room temperature in the dark.

15. 100 µL of stop solution were added to each well, mixed thoroughly.

16. Absorption was immediately determined with an ELISA reader at 450 nm.

3.12.5.4 Evaluation of results

The test was performed in strict compliance with the instruction's standards, controls, and blood samples were equally diluted in the buffer reagents as per the kit protocol from the supplier.

3.12.5.5 Expected values

The expected values for this method were 0.076 – 0.288 µmol/L.

3.12.6 Purines

3.12.6.1 Intended use

Blood samples were independently analysed in duplicates for Purines using the Sarissa Biomedical SMARTChip biosensor (Sarissa Biomedical Ltd, UK). The sarissa biomedical SMARTChip purine biological sensor is an array of 4 biological sensors that can measure purine levels in the freshly drawn whole blood, enabling the rapid measurement of blood purines at the bedside. It is comprised of electrochemical biosensors fabricated in a planar array that

covers a small circular footprint about 3 mm in diameter. The arrangement allows the biosensor array to be covered by a small droplet of blood 10-15 µL in volume. Two biosensors will be coated with the purine-sensing biological layer.

The other two biosensors in the array will be coated with the enzymes-free biological layer. The latter will establish the zero-level reading in the blood sample and act as a control. Measurements of purines are derived from the mean of the difference in current recorded at the purine biological sensors and the enzyme-free sensors.

3.12.6.2 Measurement of blood purine levels

1- The Sarissa potentiostat was connected to the laptop, and the power turned on; the measurement software loaded and ran correctly. The software guided the researcher through the procedure, and the required data entry.
2- The SMARTChip sensor was removed from its packaging and placed into the docking station. A drop of solution was placed to cover the electrodes on the SMARTChip using the dropper bottle marked “Calibration”. The researcher pressed “OK” once this was done. The calibration step took two minutes, and the machine beeped when this was finished. After calibration was completed, the integral blotter was used to remove the calibration solution and, the electrodes were covered with buffer solution using the dropper marked “Buffer”. The researcher pressed “Continue” - the SMARTChip was stable and ready for use.
3- Blood for purine measurements was sampled via the finger prick method using the lancet provided. This was done to prevent red blood cell lysis that might be caused by blood

samples drawn through needles which in turn might compromise the accuracy of the measurement.

4- Once the finger-tip was pricked, and a droplet of blood was present, it was drawn into the provided capillary tube. The buffer was quickly blotted off the SMARTChip, and the capillary tubes were used to transfer the patient's blood sample onto the SMARTChip and made sure it completely covers the electrodes.
5- Once the blood was placed on the SMARTChip electrode, the research nurse hit the "Go" button. After two minutes (the measurement period) the SMARTChip device was removed from the machine and disposed of via the standard clinical waste procedures.
6- The researcher got an indication of the measurement was complete, but no results were displayed. The data from the measurement were stored in the laptop and analysed later by the technical team.

3.12.6.3 Expected values

The expected values for this method were 2 – 3.2 µM.

3.12.7 Creatine kinase assay

3.12.7.1 Intended use

For in *vitro* diagnostic use in the quantifiable detection of creatine kinase activity in human serum and plasma on the ADVIA Chemistry systems. Such measurements are used mainly in the treatment and diagnosis of myocardial infarction and muscle diseases such as Duchenne progressive muscular dystrophy. Serum from blood samples was independently analysed in duplicates for creatine kinase using Creatine Kinase Assay Kit (CKNAC) from Siemens, USA.

The kit's package included:

- Reagent 1 (Sodium azide)
- Reagent 1 Mix

Additional materials used:

- Sample containers
- System solutions
- Control materials

Serum analysis was run on Siemens ADVIA 2400 analysers.

3.12.7.2 Principles of the procedure

Creatine kinase reacts with creatine phosphate and ADP to form ATP which is coupled to the hexokinase-G6PD (Glucose-6-phosphate dehydrogenase) reaction, generating NADPH (Nicotinamide adenine dinucleotide phosphate). The concentration of NADPH is measured by the increase in absorbance at 340/410 nm.

3.12.7.3 Reagent preparation and use

The reagent was prepared:

1. The contents of the R1 mix vial includes ADP, AMP (adenosine monophosphate), diadenosine pentaphosphate, NADP, HK (Histidine Kinase), G6PD, N-acetyl-L-cysteine and Creatine phosphate) were reconstituted with a portion of R1(Sodium azide).

2. The R1 mix vial was rinsed several times with R1.

3. The entire contents of the R1 mix were transferred to the R1 wedge.

3.12.7.4 Expected values

The following table lists the reference ranges for the method used:

Table 4 CK reference range

Sex	Reference Range (iu/L)
Male	15/185 iu/L
Female	15/165 iu/L

3.12.8 Lactate dehydrogenase assay

3.12.8.1 Intended use

For in *vitro* diagnostic use in the quantifiable detection of lactate dehydrogenase activity in human serum and plasma on ADVIA Chemistry systems. Such measurements are used mainly in the treatment and diagnosis of myocardial and pulmonary infarction. They may also be used to monitor cancer chemotherapy. Serum was independently analysed in duplicates for lactate dehydrogenase using lactate dehydrogenase assay Kit L-P (LDLP) from Siemens, USA.

The kit's package included:

- Reagent 1 (L-lactic acid, Sodium azide)
- Reagent 2 (Nicotinamide adenine dinucleotide)

Additional Materials used:

- Sample containers
- System solutions
- Control materials

Serum analysis was run on Siemens ADVIA 2400 analysers.

3.12.8.2 Principles of the procedure

Lactate dehydrogenase catalyses the conversion of L-lactate to pyruvate in the presence of nicotinamide adenine dinucleotide (NAD). The enzymatic activity of LD is proportional to the rate of production of NADH (hydrogen reduced). The amount of NADH produced is determined by measuring the increase in absorbance at 340/410 nm.

3.12.8.3 Reagent preparation and use

Reagents were ready to use. Before use, the reagent was gently swirled to dislodge bubbles and assure homogeneity.

3.12.8.4 Expected values

The expected values for this method were 115 – 235 iu/L.

3.12.9 Albumin assay

3.12.9.1 Intended use

For in *vitro* diagnostic use in the quantifiable detection of albumin in human serum or plasma on ADVIA® chemistry systems. Serum from blood samples was independently analysed in duplicates for albumin using albumin assay kit (ALBP) from Siemens, USA. Albumin measurements are used in the treatment and diagnosis of numerous diseases primarily involving the liver or kidneys.

The kit's package included:

- Albumin BCP reagent 1 (bromocresol purple, acetate buffer, surfactant and microbial inhibitor)

Additional Materials used:

- ADVIA chemistry albumin BCP calibrator
- Control materials

Serum analysis was run on Siemens ADVIA 2400 analysers.

3.12.9.2 Principles of the procedure

In the ADVIA chemistry ALBP assay, serum or plasma albumin quantitatively binds to BCP (Bromocresol purple) to form an albumin-BCP complex that is measured as an endpoint reaction at 596/694 nm.

3.12.9.3 Preparing reagents

Reagents were ready to use. Before use, the reagent was gently swirled to disrupt bubbles and assure homogeneity. A clean transfer pipette is used to aspirate bubbles or foam if present from the reagent container before use.

3.12.9.4 Calculation of results

The system automatically calculates, and reports results based on the absorbance measurements of the test sample during the test and of the calibrator(s) from calibration.

The instrument calculates the concentration of albumin in g/L.

3.12.9.5 Expected values

The reference range for albumin is 34–50 g/L for adults.

3.13 Follow-up

Follow-up (see above for procedures) was done at 3, 7 and 45 days after admission to hospital. Studies have shown that over 80 per cent of stroke patient would develop spasticity within 6 weeks of stroke based on a neurophysiological measure of muscle activity (Malhotra et al., 2008).

3.14 Outcome measures:

This was an exploratory lab-based study. The principal outcome was serum analysis at 1, 3, 7 and 45 days after stroke onset. In addition, spasticity, strength, stroke severity (NIHSS score) and independence (Modified Rankin Scale) were measured. Measurements mirrored time points of blood collection.

3.15 Size of the study:

As there was no previous data in the published literature, we proposed to recruit a maximum of 100 participants for this observational study. This would have allowed for 10 participants for each independent variable. However, this study only managed to recruit a total of 13 patients.

3.16 Proposed methods of analysis:

Analysis of results was done by simple descriptive statistics (numbers, percentages, means, medians, ranges and standard deviations) and time series graphs. For the time series graphs, we plotted the either the mean absolute deviation (mean absolute deviation is the average distance between each data value and the mean (Kader, 1999)) or the mean of the outcome measure over the time period. In addition, the normalised value of the outcome measure

(calculated using patient value divided by maximum normal value) over the time points of measurement, was also plotted.

The independent sample t-test was used for single comparisons of each biomarker between spastic and non-spastic groups. Statistical analyses were performed using the SPSS for Windows program (version 24). A *p*- value < 0.05 is considered statistically significant and, as the Bonferroni correction was not applied, 95% confidence intervals were also reported. For sample data, the mean and standard error (SE) were used for reporting purposes. For between group differences, the mean difference and the 95% confidence interval (95% CI) of the differences were reported.

3.17 Data analysis location:

The analysis was performed at Keele University and/or UHNS by the applicants and other members of Keele University research staff.

3.18 Data collection tools and source document identification

Recruitment logs of all patients enrolled in the study were held at UHNS. Patients that agree to participate in the study were assigned a unique identifier, which was used to identify all documents associated with that participant for the duration of the study. Participant consent was recorded, in triplicate, on an informed consent form and stored securely at the same location. Access to study data was restricted to members of the study team, and patient identifiable data was limited to those members of staff that require it for the performance of their role.

3.19 Ethical approval

The study took place as a sub-study of two larger studies, SMARTCap and SMARTChip, which were looking at Purines as a biomarker for stroke (REC reference, 14/WM/1034, 16/WM/0164 respectively). Ethical approvals for this study was sought from the West Midlands - Coventry & Warwickshire Research Ethics Committee. No study activities were commenced until favourable ethical opinion had been obtained. Local NHS R&D approvals were obtained before commencement of the study at the UHNS site.

3.20 Data protection and patient confidentiality

The study complies with the Data Protection Act 1998 and Participants were assigned a unique identifier upon enrolment into the study to allow link-anonymisation of patient-identifiable data. Access to patient identifiable data was restricted to members of the study team who required it for the performance of their role. Electronic data was stored on an encrypted and password protected drives, and hard copies of study documents were stored in locked filing cabinets in secure entry-card protected sites.

CHAPTER 4: RESULTS

4.1 Spasticity

Thirteen participants (2 men and 11 women; 9 with right side affected and 4 with left side affected) were recruited for the study. The median age was 77 years (range 45–96). The stroke in 4 patients was classified as haemorrhagic, 3 as PACI, 2 as LACI, one as TACI, one as POCS and 2 as an undetermined type of stroke.

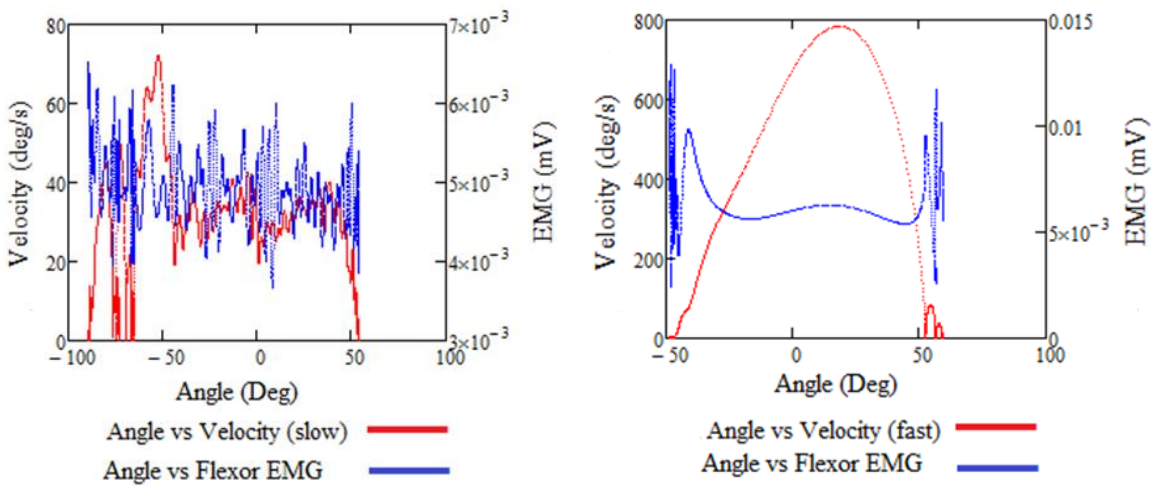
The testing protocol was performed as planned. The velocity during the slow movement was 3 seconds from maximum flexion into maximum extension (manual count) while the velocity during the fast movement was 1 second as per guidance for the modified Ashworth Scale. Seven patients ended up showing no abnormal activity during an externally imposed stretch at 45-days' time point, but six did (Table 5).

Table 5 Time points for spasticity measurements and related patients' characteristics. NO= no spasticity, YES= spasticity, IS= ischaemic, ICH= intracerebral haemorrhage, UNKN= unknown, Y= thrombolysed, N= not thrombolysed.

Patient no	Age/Sex	Day 1 slow	Day 1 fast	Day 3 Slow	Day 3 fast	Day 7 slow	Day 7 fast	Day 45 slow	Day 45 fast	Spasticity	NIHSS Admission	IS/ICH	Thrombolysed
1	70/M	NO	NO	NO	NO	NO	NO	NO	NO	NO	3	IS	Y
2	77/M	NO	YES	NO	NO			NO	NO	NO	6	IS	Y
3	87/F	NO	YES	NO	YES	NO	YES	YES	YES	YES	24	IS	Y
4	81/F	NO	YES	NO	YES	NO	YES			YES	11	ICH	N
5	77/F	NO	YES	NO	YES	YES	YES			YES	2	IS	N
6	80/F	NO	NO							NO	2	IS	N
7	72/F	NO	NO					YES	YES	YES	26	ICH	N
8	86/F					NO	YES	YES	YES	YES	4	IS	Y
9	96/F	NO	NO	NO	YES	NO	NO			NO	15	ICH	N
10	48/F	NO	NO					NO	NO	NO	5	UNKN	N
11	45/F	NO	NO					NO	NO	NO	6	UNKN	N
12	63/F	NO	YES	NO	YES	NO	YES	YES	YES	YES	12	ICH	N
13	59/F	NO	NO							NO	11	IS	Y

Depending on muscle activity detected, pattern responses were classified into four groups:

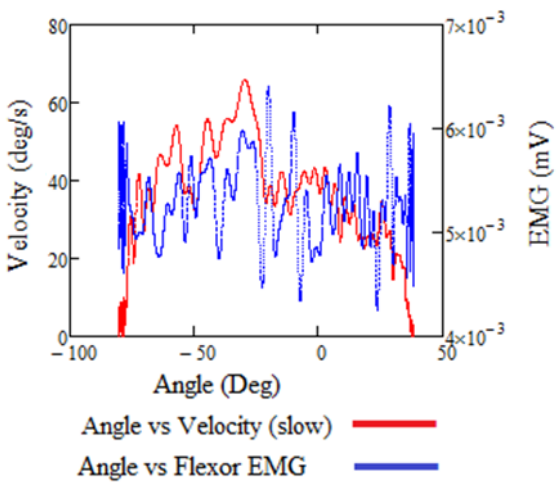
1) No/negligible muscle activity: Negligible muscle activity during both the slow and the fast stretch was seen in 7 out of 13 patients (Figure 1)



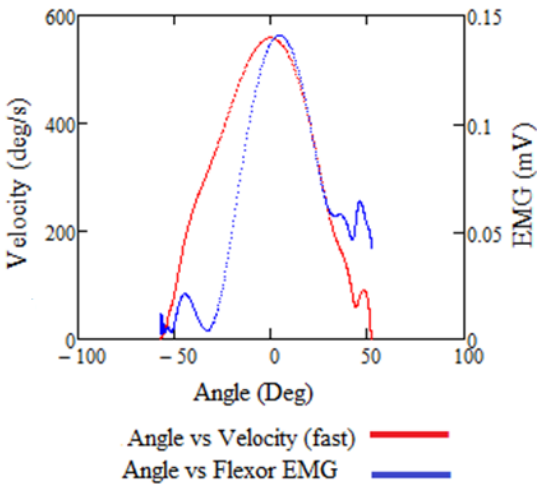
(a) Negligible muscle activity (slow) (b) Negligible muscle activity (slow)

Figure 1 Muscle activity response (annotated as EMG on graphs) to an externally imposed passive extension movement about the wrist joint. The wrist joint was fully flexed and then extended and held. The hold was <5 seconds in duration. Two velocities were used to stretch the joint. The angle is plotted on the x-axis and flexor muscle activity on the y-axis. The muscle patterns demonstrated: (a) negligible activity as the muscle is stretched at a slow speed; (b) negligible activity as the muscle is stretched at a fast speed.

2) Velocity-dependent muscle activity: During the slow stretch, there is negligible muscle activation, but there was a subsequent increase in muscle activity during the fast stretch. This was seen in 1 out of 13 patients (Figure 2).



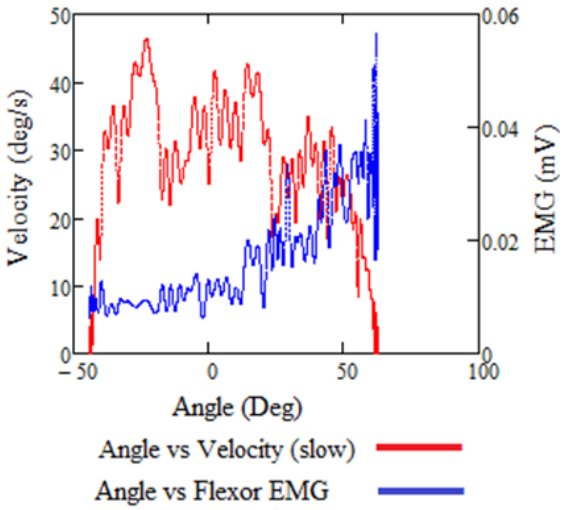
(a) Negligible muscle activity (slow)



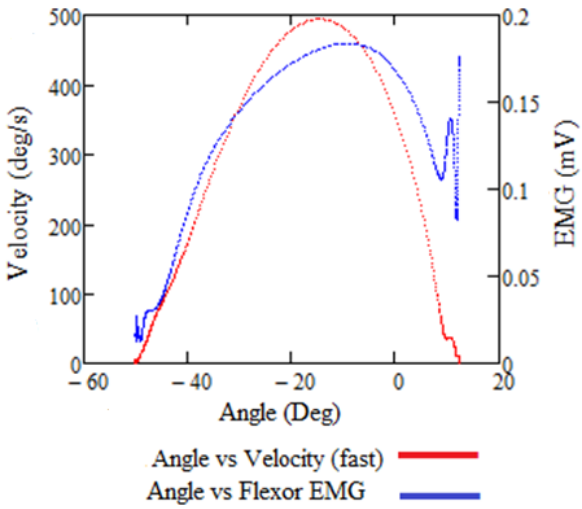
(b) Velocity-dependent muscle activity (fast)

Figure 2 Muscle activity response (annotated as EMG on graphs) to an externally imposed passive extension movement about the wrist joint. The wrist joint was fully flexed and then extended and held. The hold was <5 seconds in duration. Two velocities were used to stretch the joint. The angle is plotted on the x-axis and flexor muscle activity on the y-axis. The muscle patterns demonstrated: (a) negligible activity as the muscle is stretched at a slow speed; (b) increased activity as the muscle is stretched at a fast speed.

3) Position-dependent muscle activity: The muscle activity increased as the muscles are stretched and the activity continued even when the movement was stopped (at end range of a stretch), This was seen in 3 out of 13 patients (Figure 3).



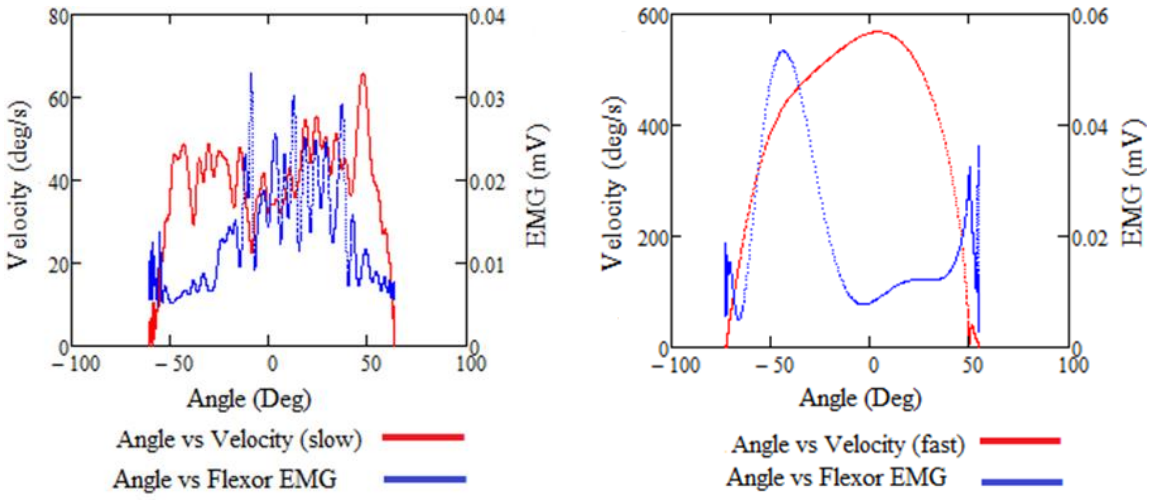
(a) Position-dependent muscle activity (slow)



(b) Position-dependent muscle activity (fast)

Figure 3 Muscle activity response (annotated as EMG on graphs) to an externally imposed passive extension movement about the wrist joint. The wrist joint was fully flexed and then extended and held. The hold was <5 seconds in duration. Two velocities were used to stretch the joint. The angle is plotted on the x-axis and flexor muscle activity on the y-axis. The muscle patterns demonstrated: (a) increased activity as the muscle is stretched at a slow speed and the activity continued even when the movement was stopped (at end range of a stretch); (b) increased activity as the muscle is stretched at a fast speed and the activity continued even when the movement was stopped (at end range of a stretch).

4) Position and velocity dependent muscle activity: Increased abnormal muscle activity during both slow and fast stretch. This increase is independent of velocity. In addition, during the fast stretch, the muscle activity was trigged in the early part of the movement. This was seen in 2 out of 13 patients. Movement-related increase in flexor muscle activity is evident during the range of movement (Figure 4).



(a) Position and velocity dependent muscle activity (slow)

(b) Position and velocity dependent muscle activity (fast)

Figure 4 Muscle activity response (annotated as EMG on graphs) to an externally imposed passive extension movement about the wrist joint. The wrist joint was fully flexed and then extended and held. The hold was <5 seconds in duration. Two velocities were used to stretch the joint. The angle is plotted on the x-axis and flexor muscle activity on the y-axis. The muscle patterns demonstrated: (a) increased activity as the muscle is stretched at a slow speed; (b) increased activity as the muscle is stretched at a fast speed.

4.2 Grip strength

Four patients had zero scores on grip strength measured using a dynamometer while at the same time being spastic (Spastic paralysis). Two out of the four were classified as having sever stroke while the other two were classified as having moderate stroke based on the NIHSS. Two out of the six spastic patients had a minor stroke, and that means they still have some function intact. Improvements in grip strength were evident in four out of seven Non-spastic patients during the follow-up period while spastic group grip strength ended up with a lower score than the baseline measurement or maintained the zero Newton score. The non-spastic group stroke severity ranged from minor to moderate stroke while the spastic group stroke severity ranged from minor to sever stroke based on NIHSS score (Table 6).

Table 6 Grip strength (in Newton) in Spastic (Y) and non-spastic (N) patients

Pt./no	Spasticity	Grip Str 1d	Grip Str 3d	Grip Str 7d	Grip Str 45d	NIHSS Admission	NIHSS Discharge
1	N	58.8	127.5	215.7	225.5	3	2
2	N	58.8	88.2		294.2	6	2
3	Y	0	0	0	0	24	16
4	Y	0	0	0		11	9
5	Y	107.9	39.2	78.5		2	1
6	N	157				2	1
7	Y	0			0	26	18
8	Y			34.3	7.8	4	16
9	N	68.6	47.1	49.0		15	15
10	N	142.2			176.5	5	6
11	N	73.5			245.2	6	6
12	Y	0	0	0	0	12	12
13	N	63.7				11	11

4.3 Passive range of motion.

Patients with spasticity showed a decline in the passive range of motion started at the 3-days' time point. The decrease in PROM continued reaching the lowest at the 45-days' time point. Patients with no spasticity did not show any decline in PROM apart from the limited decrease at the 3-days' time point. The PROM returned to about the baseline value at the 7-days' time point and continued about the same level toward 45-days' time point (Figure 5).

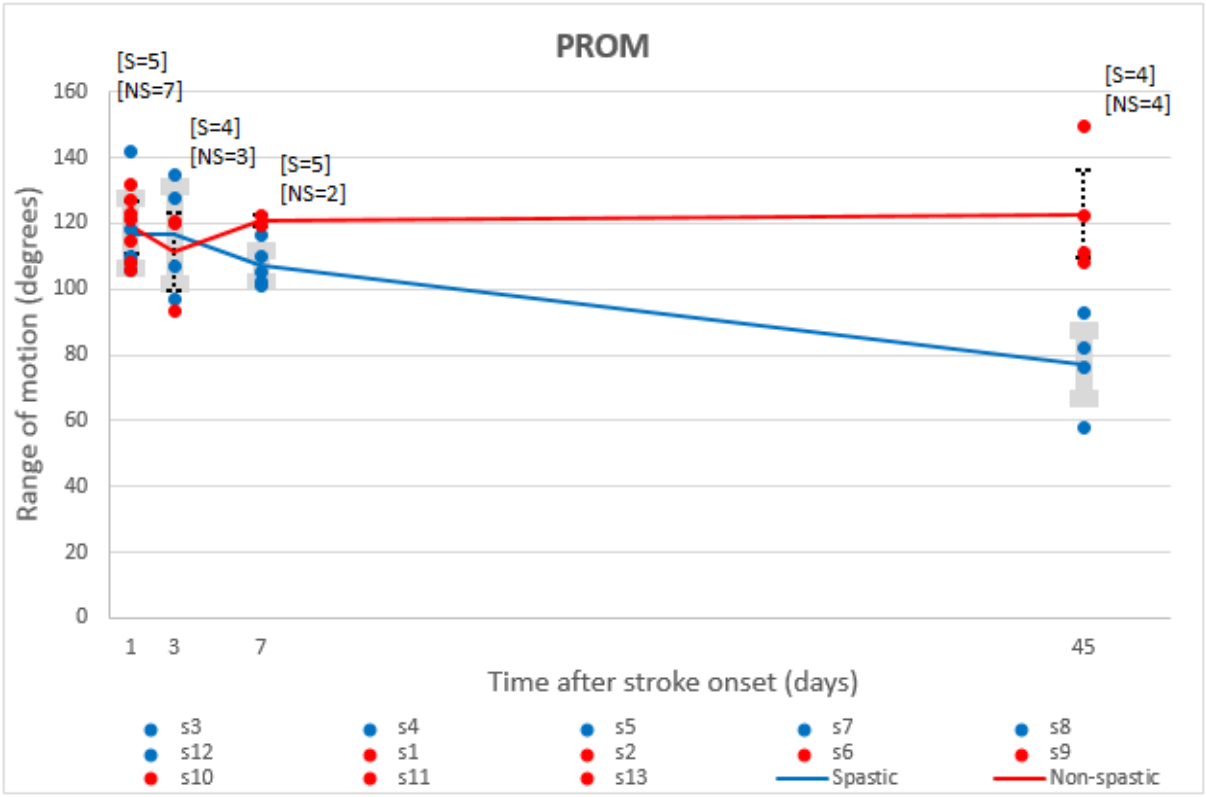


Figure 5 Mean of passive range of motion with mean absolute deviation in both spastic (blue) and non-spastic (red) groups over time (Mean absolute deviation is the average distance between each data value and the mean). The time after stroke onset is plotted on the x-axis and range of motion on the y-axis. Number of patients in each group is also plotted.

4.4 Stiffness and contracture

Patients with spasticity showed an increase in stiffness (calculated using applied force (N) vs passive range of movement (Degree)) at the 7-days' time point. The increase continued to the 45-days' time point. Patients with no spasticity had a peak increase in stiffness at the 3-days' time point and decrease at the subsequent 7-days' time point where it started to increase again toward the 45-days' time point (Figure 6). Both spastic and non-spastic groups increase in stiffness were considered negligible as they were less than the 0.07N/degree cut-off point reported in the literature (Pandyan et al., 2001).

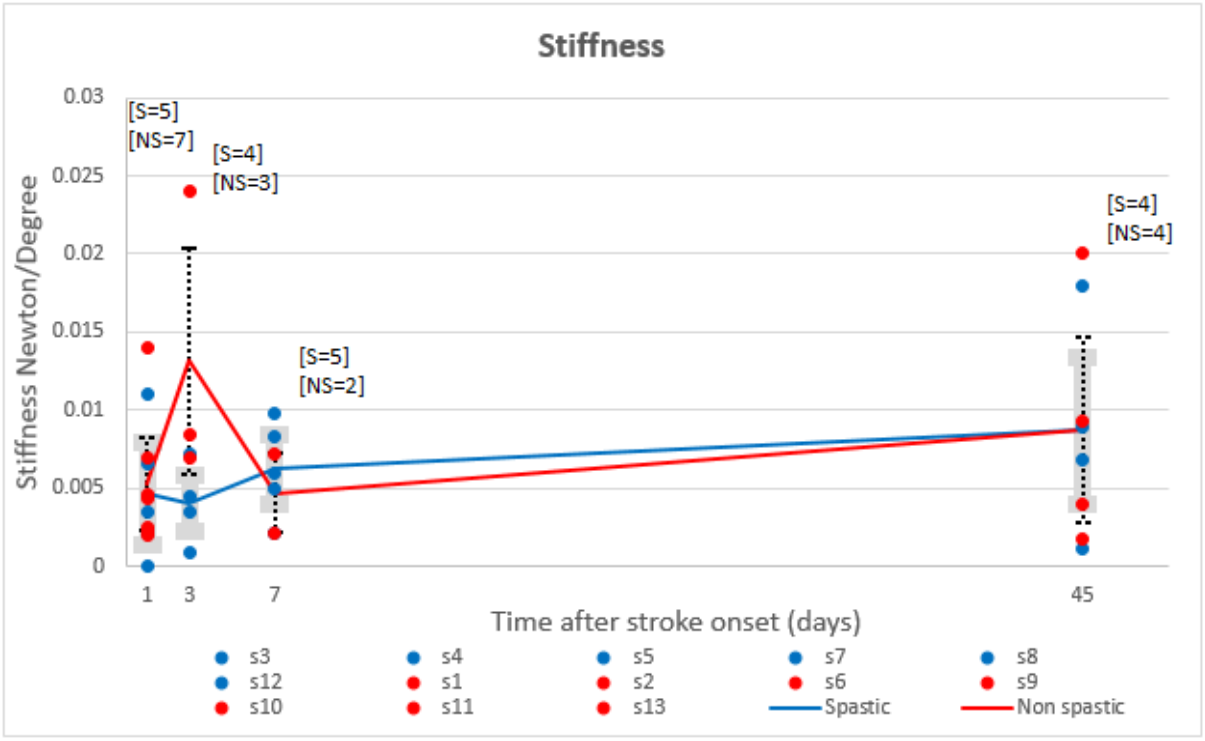


Figure 6 Mean of resistance to passive movement (N/Degree), spastic (blue) and non-spastic (red), with mean absolute deviation over time, (Mean absolute deviation is the average distance between each data value and the mean). The time after stroke onset is plotted on the x-axis and stiffness on the y-axis. Number of patients in each group is also plotted.

There were variable degrees of contracture development within the spastic patients at the 45-days' time point, with subjects no 8 and no 12 losing 44 and 36% of PROM respectively (Figure 7).

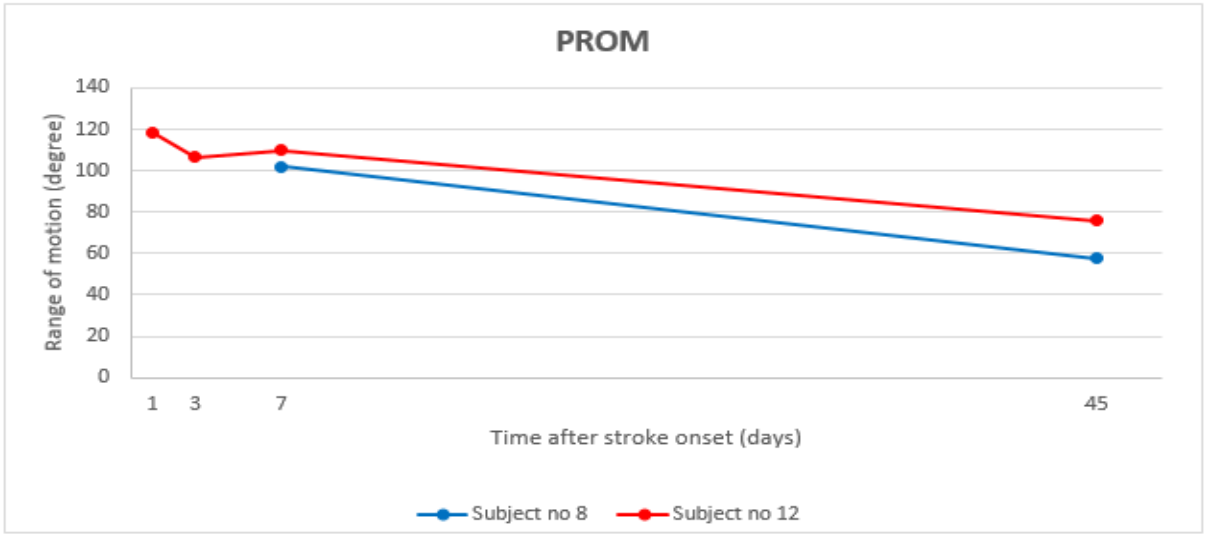


Figure 7 Passive range of motion in two spastic patients, subject no 8 (blue) and subject no 12 (red). The time after stroke onset is plotted on the x-axis and range of motion on the y-axis.

4.5 Spasticity biomarkers

4.5.1 Glutamate assay

Both spastic and non-spastic patients showed within the normal level serum glutamate concentration at all time points. Spastic group glutamate concentration was higher than the non-spastic group at all time points but, within the normal level of serum glutamate. The largest serum glutamate level difference between the two groups was seen at 1-day time point with a mean difference of 24.1 (95% CI: -2.3 to 50.3; *p*= 0.07) again, within the normal level of serum glutamate.

Mean serum glutamate for each time-point for spastic patients was compared with non-spastic patients and normal level glutamate concentrations. Mean absolute deviation was used to show the average distance between each data point and the mean (Figure 8). Both groups showed within the normal level serum glutamate levels.

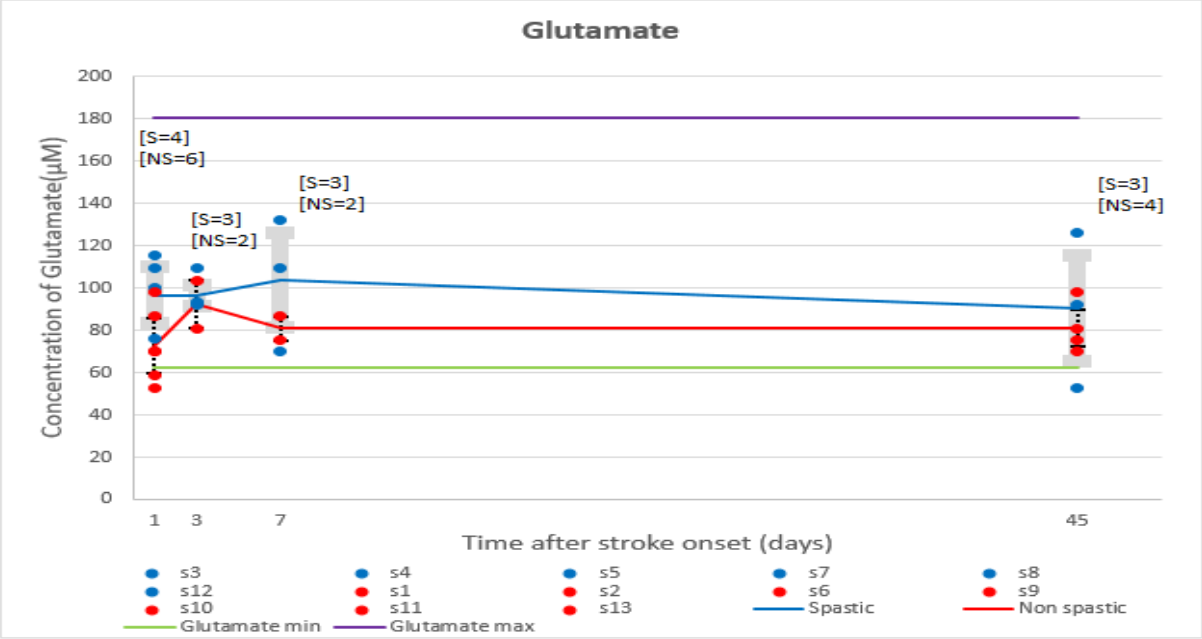


Figure 8 Mean of glutamate with mean absolute deviation in both groups, spastic (blue) and non-spastic (red), at different time points (Mean absolute deviation is the average distance between each data value and the mean). The time after stroke onset is plotted on the x-axis and concentration of glutamate on the y-axis. Number of patients in each group is also plotted.

4.5.1.1 Statistical analysis

4.5.1.1.1 Day 1

Independent t-test

Even though both spastic and non-spastic patients showed within the normal level serum glutamate concentration on day 1, spastic patients had a higher glutamate serum level (mean (M) = 96.7, standard error (SE) = 9.3) than non-spastic patients (M= 72.6, SE= 7). This difference, 24.1, 95% CI (-2.3, 50.3) was not significant t (8) =2.1, p = 0.07. The mean difference between the two groups was highest at this time point.

4.5.1.1.2 Day 3

Independent t-test

Again, both spastic and non-spastic patients showed within the normal level serum glutamate concentration on day 3, spastic patients had a higher glutamate serum level (M= 96.2, SE= 3.8) than non-spastic patients (M= 92.4, SE= 11.3). This difference, 3.8, 95% CI (-95.1, 102.6) was not significant t (3) =0.39, p = 0.8.

4.5.1.1.3 Day 7

Independent t-test

Spastic patients had a higher glutamate serum concentration (M= 103.7, SE= 18.2) than non-spastic patients (M= 81.1, SE= 5.7) Even though both spastic and non-spastic patients showed within the normal level serum glutamate concentration. This difference, 22.7, 95% CI (-53.2, 98.6) was not significant t (3) =0.95, p = 0.41.

4.5.1.1.4 Day 45

Independent t-test

Even though both spastic and non-spastic patients showed within the normal level serum glutamate concentration at this time point, spastic patients had a higher glutamate serum level (M= 90.5, SE= 21.2) than non-spastic patients (M= 81.1, SE= 6.1). This difference, 9.4, 95% CI (-39.9, 58.7) was not significant t (5) =0.49, p = 0.6.

4.5.2 Glial fibrillary acidic protein (GFAP)

Both spastic and non-spastic patients showed above the normal level serum GFAP concentration at all time points. Non-spastic group GFAP concentration was higher than spastic group at 1 and 3-days' time points (Mean difference 0.03; 95% CI: -0.22 to 0.15; p = 0.63 and -0.07; 95% CI: -0.19 to 0.06; p = 0.21 respectively). Spastic group serum concentrations then sharply peaked from this point reaching its highest levels at 7-days' and then subsequently declining towards normal level concentrations at 45-days.

At 7-days, the difference in measured mean serum GFAP in spastic subjects was at its highest compared to non-spastic patients. (mean difference 0.26; 95% CI: -0.66 to 1.20; p = 0.43). Mean GFAP for each time point for spastic patients was compared with non-spastic patients and normal level GFAP concentrations. Mean absolute deviation was used to show the average distance between each data point and the mean (Figure 9).

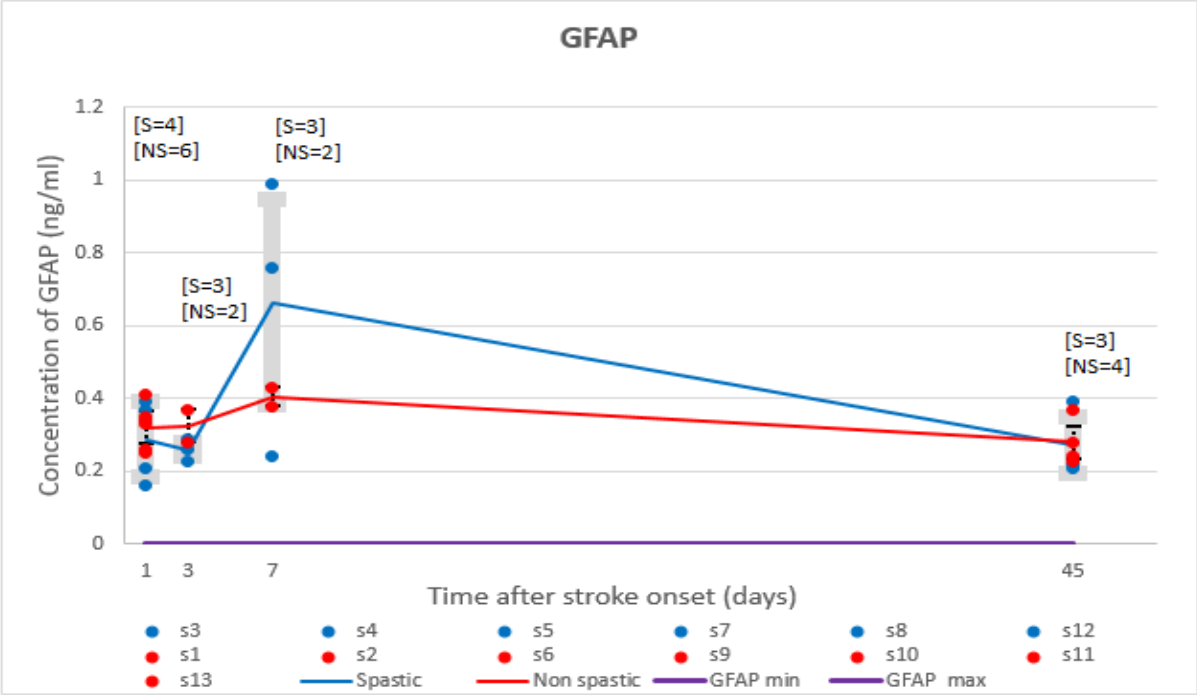


Figure 9 Mean of GFAP with mean absolute deviation in both groups, spastic (blue) and non-spastic (red), at different time points (Mean absolute deviation is the average distance between each data value and the mean). The time after stroke onset is plotted on the x-axis and concentration of GFAP on the y-axis. Number of patients in each group is also plotted.

4.5.2.1 Statistical analysis

4.5.2.1.1 Day 1

Independent t-test

On average, non-spastic patients had a higher GFAP serum level (M= 0.32, SE= 0.02) than spastic patients (M= 0.29, SE= 0.06). This difference, -0.03. 95% CI (-0.22, 0.15) was not significant t (8) = -0.60, *p* = 0.63. Both groups were above the normal serum GFAP concentration.

4.5.2.1.2 Day 3

Independent t-test

Again, on average, non-spastic patients had a higher GFAP serum level (M= 0.33, SE= 0.05) than spastic patients (M= 0.26, SE= 0.02). This difference, -0.07, 95% CI (-0.19, 0.06) was not significant t (3) = -1.6, *p* = 0.21. Both groups were above the normal serum GFAP concentration.

4.5.2.1.3 Day 7

Independent t-test

On average, spastic patients had a higher GFAP serum level (M= 0.66, SE= 0.22) than non-spastic patients (M= 0.41, SE= 0.03). This difference, 0.26, 95% CI (-0.66, 1.17) was not significant t (3) =0.9, *p* = 0.43. Both groups were above the normal serum GFAP concentration. The mean difference between the two groups was highest at this time point.

4.5.2.1.4 Day 45

Independent t-test

On average, non-spastic patients had a bit higher GFAP serum level (M= 0.28, SE= 0.03) than spastic patients (M= 0.27, SE= 0.06). This difference, -0.007, 95% CI (-0.17, 0.15) was not significant t (5) = -0.11, *p* = 0.92. Both groups were above the normal serum GFAP concentration.

4.5.3 Human enolase 2/Neuron-specific enolase immunoassay

The inter- and intra-assay coefficients of variation using the R&D Systems NSE Quantikine® ELISA kit for the measurement of NSE were 4.97 and 4.3 % respectively. All mean NSE

measurements in spastic patients were higher than non-spastic concentrations. Both spastic and non-spastic patients showed above the normal level NSE concentration. Mean NSE concentrations for both groups showed an increasing trend from 24-hours post-stroke peaking at 3-days' and subsequently declining towards normal level concentrations at 45-days' (Figure 10). At 45-days, the difference in measured mean NSE in spastic subjects was at its highest compared to non-spastic patients. (mean difference 4.5; 95% CI: -9.05, 17.95; p = 0.33).

Mean NSE for each time point for spastic patients was compared with non-spastic patients and normal level NSE concentrations. Mean absolute deviation was used to show the average distance between each data point and the mean (Figure 10).

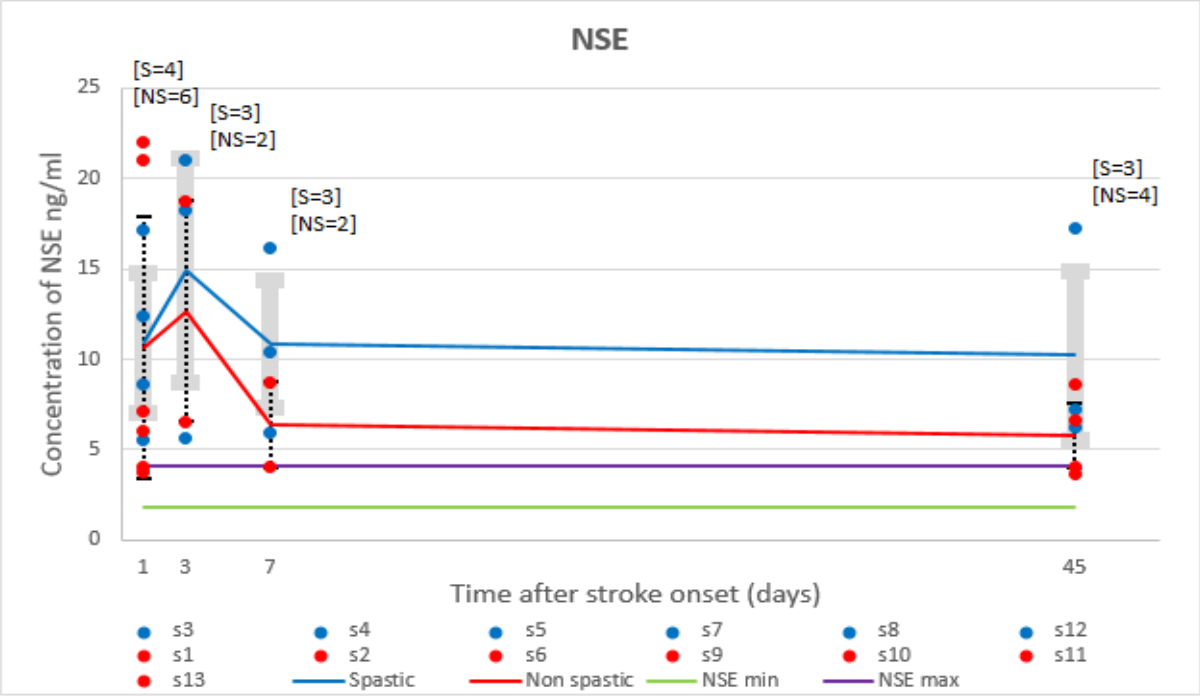


Figure 10 Mean of NSE with mean absolute deviation in both Spastic (blue) and non-spastic (red) patients at different time points (Mean absolute deviation is the average distance between each data value and the mean). The time after stroke onset is plotted on the x-axis and concentration of NSE on the y-axis. Number of patients in each group is also plotted.

4.5.3.1 Statistical analysis

4.5.3.1.1 Day 1

Independent t-test

On average, spastic patients had a bit higher NSE serum level (M= 10.91, SE= 2.51) than non-spastic patients (M= 10.64, SE= 3.47). This difference, 0.28, 95% CI (-10.73, 11.28) was not significant t (8) =0.058, p = 0.96. Both spastic and non-spastic patients showed above the normal level NSE concentration.

4.5.3.1.2 Day 3

Independent t-test

On average, spastic patients had a higher NSE serum level (M= 14.94, SE= 4.73) than non-spastic patients (M= 12.67, SE= 6.10). This difference, 2.27, 95% CI (-21.97, 26.5) was not significant t (3) = 0.30, p = 0.79. Both spastic and non-spastic patients showed above the normal NSE serum levels.

4.5.3.1.3 Day 7

Independent t-test

On average, spastic patients had a higher NSE serum level (M= 10.87, SE= 2.96) than non-spastic patients (M= 6.39, SE= 2.35). This difference, 4.48, 95% CI (-8.88, 17.8) was not significant t (3) = 1.07, p = 0.36. Both spastic and non-spastic patients showed above the normal level NSE concentration.

4.5.3.1.4 Day 45

Independent t-test

On average, spastic patients had a higher NSE serum level (M= 10.23, SE= 3.51) than non-spastic patients (M= 5.8, SE= 1.13). This difference, 4.5, 95% CI (-9.05, 17.95) was not significant t (2) = 1.2, *p* = 0.33. Both spastic and non-spastic patients showed above the normal level NSE concentration. The mean difference between the two groups was highest at this time point.

4.5.4 S100 calcium-binding protein B (S100B)

All mean S100B measurements in spastic patients were higher than non-spastic concentrations apart from the third-day time point measurements when the non-spastic S100B level was higher than the spastic group (mean difference -203.50; 95% CI: -4741 to 4334; *p* = 0.90). Both spastic and non-spastic patients showed above the normal level S100B serum concentration at all time points. Mean S100B concentrations for spastic group showed an increasing trend from the third-day post stroke peaking at 7-days' and subsequently declining towards normal level concentrations at 45-days' (Figure 11). Mean S100B concentrations for non-spastic group showed an increasing trend from the first-day post stroke peaking at 3-days' and subsequently declining towards normal level concentrations at 45-days. At 7-days, the difference in measured mean S100B in spastic subjects was at its highest compared to non-spastic patients. (mean difference 879.8; 95% CI: -10581 to 12341; *p* = 0.6).

Blood samples were collected from acute stroke patients (n = 13) at four-time points (1, 3, 7 and 45-days). Mean S100B for each time-point for spastic patients was compared with non-

spastic patients and normal level S100B concentrations. Mean absolute deviation was used to show the average distance between each data point and the mean.

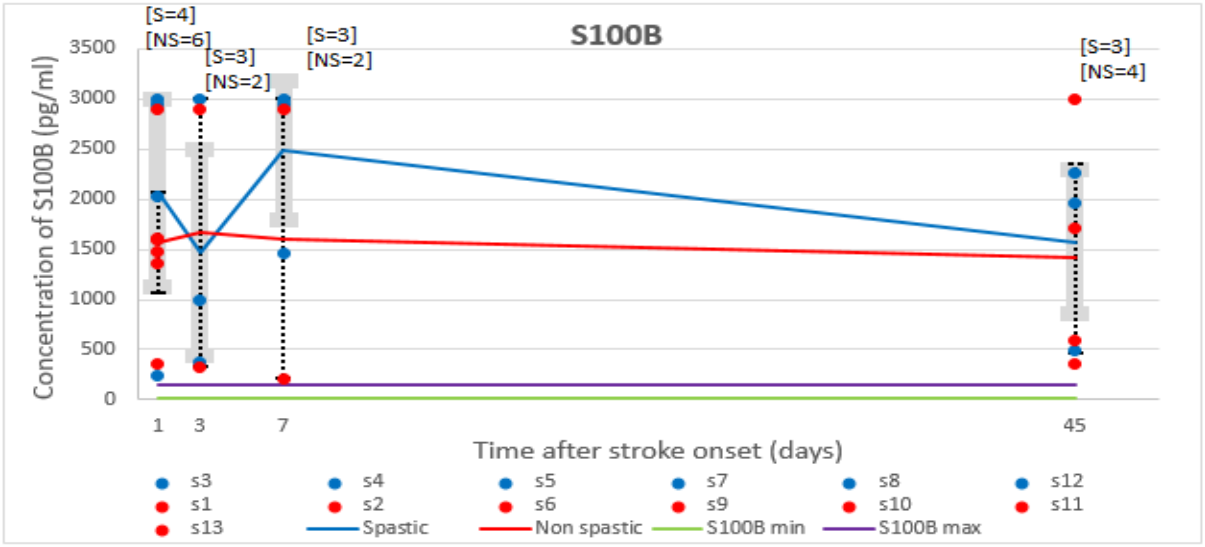


Figure 11 Mean of S100B with mean absolute deviation in both spastic (blue) and non-spastic (red) patients at different time points (Mean absolute deviation is the average distance between each data value and the mean). The time after stroke onset is plotted on the x-axis and concentration of S100B on the y-axis. Number of patients in each group is also plotted.

4.5.4.1 Statistical analysis

4.5.4.1.1 Day 1

Independent t-test

On average, spastic patients had a higher S100B serum level (M= 2064.5, SE= 652.1) than non-spastic patients (M= 1565.3, SE= 345.02). This difference, 499.2, 95% CI (-1050.78, 2049.11) was not significant t (8) =0.743, p = 0.48. Both spastic and non-spastic patients showed above the normal level S100B serum concentration.

4.5.4.1.2 Day 3

Independent t-test

On average, spastic patients had a lower S100B serum level (M= 1460, SE= 790.1) than non-spastic patients (M= 1663.5, SE= 1336.5). This difference, -203.5, 95% CI (-4741, 4334) was not significant t (3) = -0.143, p = 0.90. Both spastic and non-spastic patients showed above the normal serum S100B level.

4.5.4.1.3 Day 7

Independent t-test

On average, spastic patients had a higher S100B serum level (M= 2484, SE= 515.7) than non-spastic patients (M= 1604.5, SE= 1395.5). This difference, 879.8, 95% CI (-10581, 12340.7) was not significant t (1) =0.59, p = 0.64. Both spastic and non-spastic patients showed above the normal level S100B serum concentration. The mean difference between the two groups was highest at this time point.

4.5.4.1.4 Day 45

Independent t-test

On average, spastic patients had a higher S100B serum level (M= 1571, SE= 547.3) than non-spastic patients (M= 1413.8, SE= 605.67). This difference, 157.3, 95% CI (-2028.8, 2343.3) was not significant t (5) =0.185, p = 0.86. Both spastic and non-spastic patients showed above the normal serum S100B level.

4.5.5 Purines

Both spastic and non-spastic patients showed above the normal level blood Purine concentration at baseline and 24-hours' time points. The spastic group showed a higher purine level at both time points than the non-spastic group. The spastic group also showed the highest purine level at 24-hours compared to the baseline value. On the other hand, the non-spastic group showed near normal level Purine concentration at 24-hours. The largest blood purine level difference between the two groups was seen at 24-hours' time point with a mean difference of 5.5 (95% CI: -3.7, 14.7; p = 0.2).

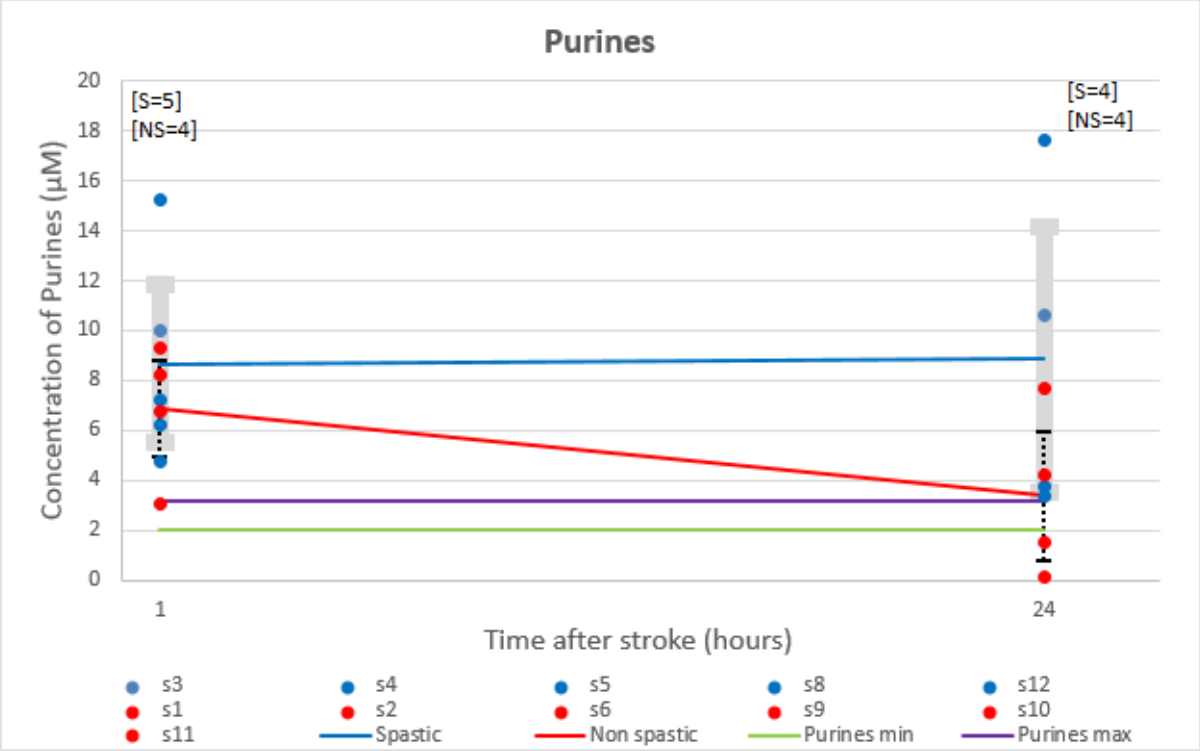


Figure 12 Mean of Purines with mean absolute deviation in both spastic (blue) and non-spastic (red) patients at baseline and 24-hours' time points (Mean absolute deviation is the average distance between each data value and the mean). The time after stroke onset is plotted on the x-axis and concentration of Purines on the y-axis. Number of patients in each group is also plotted.

Mean blood Purines for each time-point for spastic patients was compared with non-spastic patients and normal blood level purine concentration. Mean absolute deviation was used to show the average distance between each data point and the mean (Figure 12). The graph showed that spastic patients have a higher score than the non-spastic on both time points. The spastic group mean absolute deviation showed wide spread and overlapping with the non-spastic group because of one spastic patient with very high reading at each time point.

4.5.5.1 Statistical analysis

4.5.5.1.1 Baseline

Independent t-test

On average, spastic patients had a higher purine blood level (M= 8.7, SE= 1.9) than non-spastic patients (M= 6.9, SE= 1.3). This difference, 1.8, 95% CI (-3.9, 7.5) was not significant t (7) =0.8, p = 0.5. Both spastic and non-spastic patients showed above the normal level blood purines concentration at this time point.

4.5.5.1.2 24 Hours

Independent t-test

On average, spastic patients had a higher purines blood level (M= 8.9, SE= 3.4) than non-spastic patients (M= 3.4, SE= 1.7). This difference, 5.5, 95% CI (-3.7, 14.7) was not significant t (6) =1.5, p = 0.2. Both spastic and non-spastic patients showed above the normal level blood purine concentration at this time point with the non-spastic group being barely above the normal level. The mean difference between the two groups was highest at this time point.

4.5.6 Creatine kinase assay

Both spastic and non-spastic patients showed within the normal level serum creatine kinase concentration at all time points apart from the first-day measurement. On first day measurement, spastic group CK concentration was way over the normal CK level (mean 636.5, 95% CI: -319.6 to 1592.6; $p = 0.94$) normal CK level is between (15-185 iu/L). The largest serum CK level difference between the spastic and non-spastic groups was seen at 1-day time point with a mean difference of 563.64 (95% CI: -392.09 to 1519.37; $p = 0.92$). The rest of the time points measurements were all within the normal serum CK concentration.

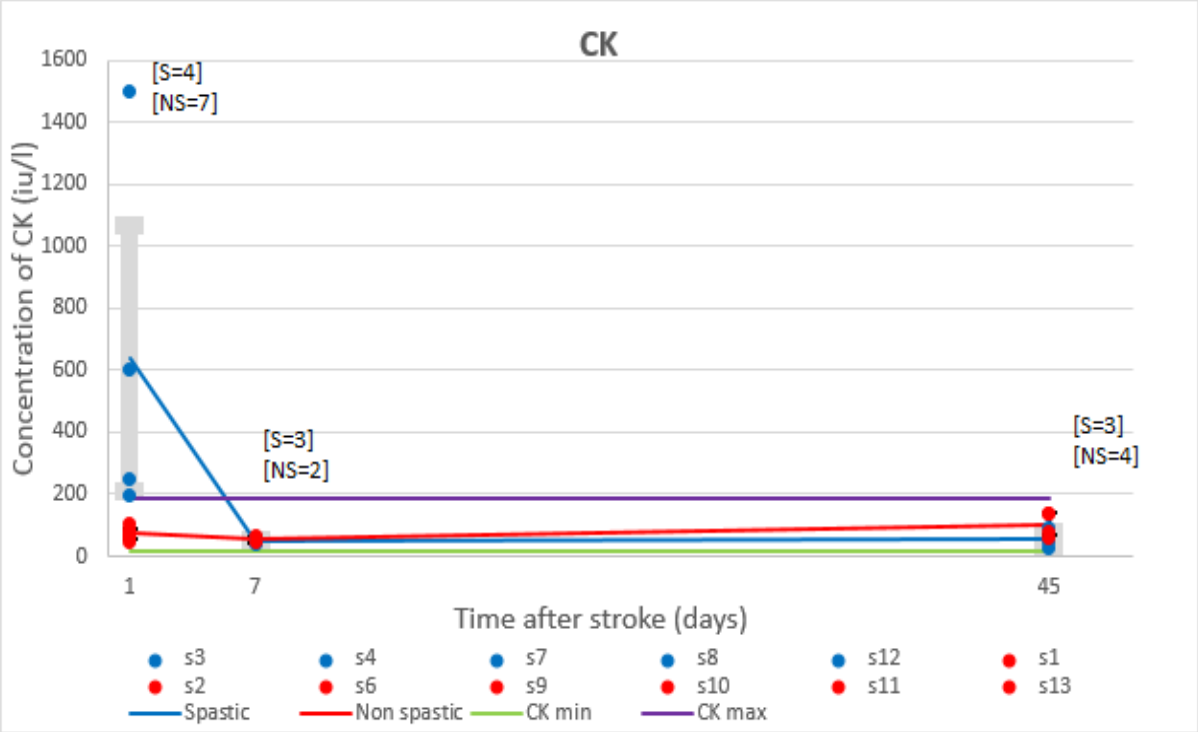


Figure 13 Mean of Creatine Kinase with mean absolute deviation in both Spastic (blue) and non-spastic (red) patients at 1, 7 and 45-days' time points (Mean absolute deviation is the average distance between each data value and the mean). The time after stroke onset is plotted on the x-axis and concentration of CK on the y-axis. Number of patients in each group is also plotted.

Mean serum CK for each time-point for spastic patients was compared with non-spastic patients and normal level CK concentrations. Mean absolute deviation was used to show the

average distance between each data point and the mean (Figure 13). The graph showed that spastic patients have a higher score than the non-spastic on 1-day time points. The spastic group mean absolute deviation showed wide spread on 1-day time point because of one spastic patient with very high reading at that time point. It was not possible to include 3-days' time point values because of a limited number of participants (Only one patient in the non-spastic group).

4.5.6 1 Statistical analysis

4.5.6.1.1 Day 1

Independent t-test

Spastic patients had a higher creatine kinase serum level (M= 636.5, SE= 300.4) than non-spastic patients (M= 72.9, SE= 7.2). Only non-spastic group showed within the normal level serum creatine kinase concentration. This difference, 563.6, 95% CI (-392.1, 1519.4) was not significant t (3) = 1.9, p = 0.2. The mean difference between the two groups was highest at this time point.

4.5.6.1.2 Day 3

It was not possible to run any statistical tests at the 3-days' time point because of the very low sample size (only four patients in total with the non-spastic group limited to only one patient). Both spastic and non-spastic patients showed within the normal level serum creatine kinase concentration. The spastic group had higher creatine kinase serum level though.

4.5.6.1.3 Day 7

Independent t-test

Non-spastic patients had a tad higher creatine kinase serum level (M= 54, SE= 10) than spastic patients (M= 49, SE= 5.3) even though both spastic and non-spastic patients showed within the normal level serum creatine kinase concentration. This difference, -5, 95% CI (-37.2, 27.2) was not significant t (3) =-0.5, p = 0.66.

4.5.6.1.4 Day 45

Independent t-test

Even though both spastic and non-spastic patients showed within the normal level serum creatine kinase concentration at this time point, non-spastic patients had a higher creatine kinase serum level (M= 103, SE= 20.2) than spastic patients (M= 54.2, SE= 20.3). This difference, -48.7, 95% CI (-124.2, 26.9) was not significant t (5) =-1.7, p = 0.16.

4.5.7 Lactate dehydrogenase assay

Both spastic and non-spastic patients showed above the normal level serum lactate dehydrogenase concentration at 3-days' time points. The spastic group lactate dehydrogenase concentration was higher than the non-spastic group at 3-days' time point. Spastic group serum concentrations were also above the normal level at 24-hours post-stroke while the non-spastic group LD level was within the normal level at this time point. Both groups serum LD level was within the normal range at 7 and 45-days' time points. The largest serum lactate dehydrogenase level difference between the two groups was seen at 1-day time point with a mean difference of 76.5 (95% CI: 32.95 to 119.95; p = 0.004) non-spastic LD level was within the normal range though.

Mean serum LD for each time-point for spastic patients was compared with non-spastic patients and normal level LD concentrations. Mean absolute deviation was used to show the average distance between each data point and the mean (Figure 14). It was not possible to include 3 and 7-days' time points values because of a limited number of participants (four patients total with only one non-spastic and three patients total with only one spastic patient respectively).

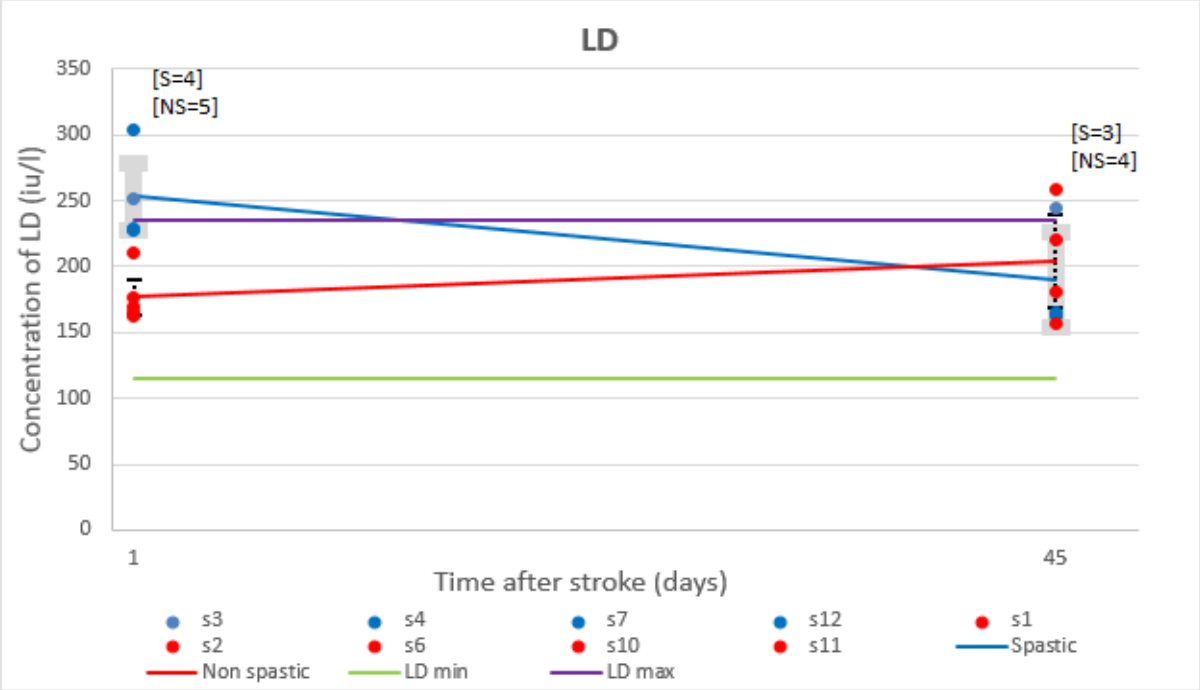


Figure 14 Mean of Lactate dehydrogenase with mean absolute deviation in both Spastic (blue) and non-spastic (red) patients at 1 and 45-days' time points (Mean absolute deviation is the average distance between each data value and the mean). The time after stroke onset is plotted on the x-axis and concentration of LD on the y-axis. Number of patients in each group is also plotted.

4.5.7.1 Statistical analysis

4.5.7.1.1 Day 1

Independent t-test

On average, spastic patients had a higher lactate dehydrogenase serum level (M= 253.3, SE= 17.7) than non-spastic patients (M= 176.8, SE= 8.7). This difference, 76.5, 95% CI (32.95,

119.95) was significant t (7) = 4.2, *p* = 0.004. Spastic group serum LD was above the normal range. Non-spastic group serum LD level was within the normal range though. The mean difference between the two groups was highest at this time point.

4.5.7.1.2 Day 3

It was not possible to run Independent t-test for the 3-days' time point because of a limited number of participants (four patients total with only one non-spastic patient). Both groups serum LD level was over the normal range with the spastic group LD level being higher.

4.5.7.1.3 Day 7

It was not possible to run Independent t-test for the 7-days' time point because of a limited number of participants (three patients total with only one spastic patient). Both groups serum LD level was within the normal range with the non-spastic group being a bit higher than the spastic patient serum LD level.

4.5.7.1.4 Day 45

Independent t-test

On average, non-spastic patients had a higher lactate dehydrogenase serum level (M= 204.2, SE= 22.3) than spastic patients (M= 190.3, SE= 26.8). This difference, -13.9, 95% CI (-102.9, 75.1) was not significant t (5) = -0.4, *p* = 0.7. Both groups serum LD level was within the normal range.

4.5.8 Albumin assay

Both spastic and non-spastic patients showed within the normal level serum albumin concentration at all time points apart from the 3-days' measurement when non-spastic group albumin concentration was below the normal albumin level at 30g/L (normal serum albumin level: 35-50 g/L). The largest serum albumin level mean difference between the spastic and non-spastic groups was seen at 45-days' time point with the non-spastic group showing a higher serum level than the spastic group with a mean difference of -6.25 (95% CI: -14.6 to 2.1; p = 0.1). The spastic group mean serum albumin concentration stayed barely at the same level at all time points of measurements.

Mean serum albumin for each time-point for spastic patients was compared with non-spastic patients and normal level albumin concentrations. Mean absolute deviation was used to show the average distance between each data point and the mean (Figure 15). It was not possible to include 3-days' time point values because of a limited number of participants (Only one patient in the non-spastic group).

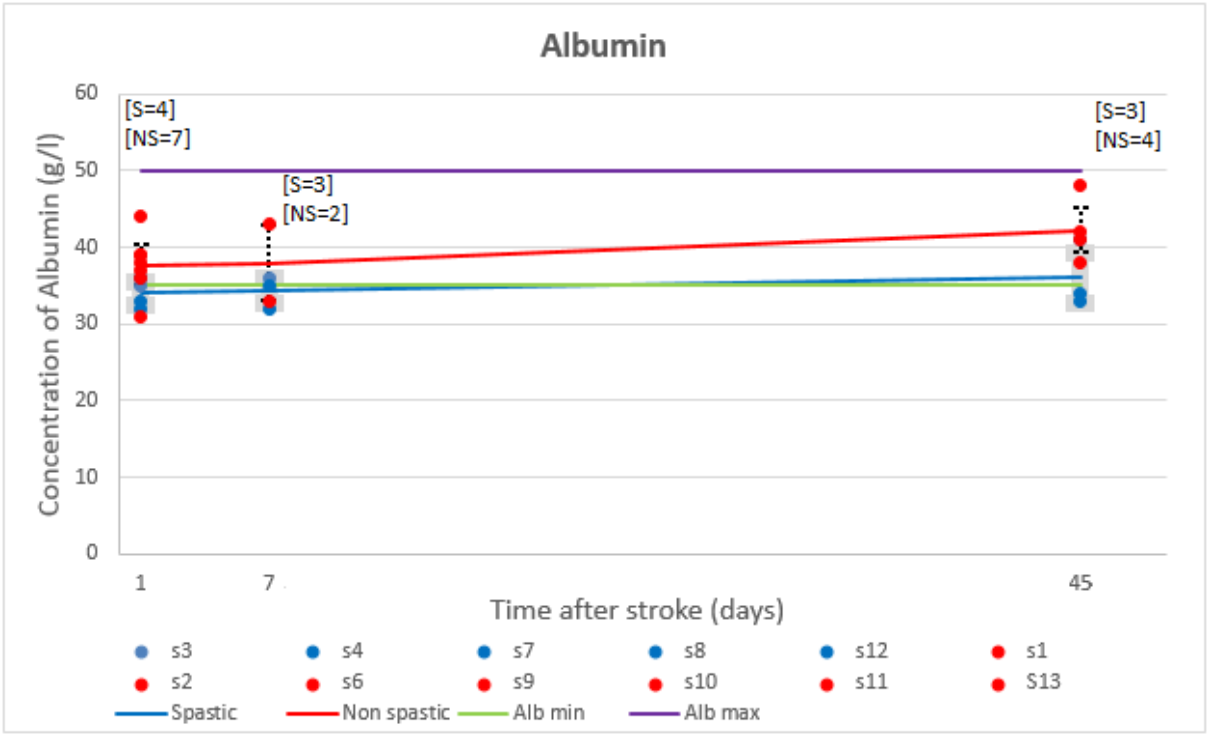


Figure 15 Mean of Albumin with mean absolute deviation in both spastic (blue) and non-spastic (red) patients at 1, 7 and 45-days' time points (Mean absolute deviation is the average distance between each data value and the mean). The time after stroke onset is plotted on the x-axis and concentration of Albumin on the y-axis. Number of patients in each group is also plotted.

4.5.8.1 Statistical analysis

4.5.8.1.1 Day 1

Independent t-test

On average, spastic patients had a lower serum albumin level (M= 34, SE= 0.9) than non-spastic patients (M= 37.7, SE= 1.5). This difference, -3.7, 95% CI (-8.5, 1.05) was not significant t (9) =-1.8, p = 0.1. Only spastic patient's serum albumin level was a bit below the normal range. Non-spastic patient's serum albumin concentration, on the other hand, was within the normal range.

4.5.8.1.2 Day 3

It was not possible to run any statistical tests at the 3-days' time point because of the very low sample size (only four patients in total with the non-spastic group limited to only one patient). The spastic group had within normal mean serum albumin level while the non-spastic patient had below the normal range serum albumin level.

4.5.8.1.3 Day 7

Independent t-test

Even though it was within the normal serum albumin level, non-spastic patients had a higher mean serum albumin level (M= 38, SE= 5) than spastic patients (M= 34, SE= 1.2). This difference, -3.7, 95% CI (-54.9, 47.5) was not significant t (3) =-0.91, p = 0.6. The spastic group, on the other hand, had a bit below the normal range serum albumin concentration.

4.5.8.1.4 Day 45

Independent t-test

Non-spastic patients had a higher mean serum albumin level (M= 42.3, SE= 2.1) than spastic patients (M= 36, SE= 2.5) Even though it was within the normal serum albumin level for both groups. This difference, -6.3, 95% CI (-14.6, 2.1) was not significant t (5) =-1.9, p = 0.1. The mean difference between the two groups was highest at this time point.

4.5.9 Gama aminobutyric acid (GABA)

The experiment was conducted to quantify GABA from the thirteen samples collected and compare the results between the spastic and non-spastic groups. Unfortunately, due to assay complication and limited volumes of samples, no reproducible results were obtained. Based

on previous work done by kanthan et al., (1995), below the normal range serum levels of Gamma-aminobutyric acid was expected in stroke patients. For future studies, more robust methodologies should be investigated as this particular assay, GABA ELISA kit from IBL International, Germany, was not stable.

4.6 Biomarkers kinetics

The characteristics of biomarkers in both the spastic and non-spastic stroke patients varied. In the spastic group, the mean NSE, albumin and lactate dehydrogenase showed an increase with both NSE and LD reaching peak concentration at the 3-days' time point (Figure 16).

The non-spastic group NSE, glutamate, S100B and lactate dehydrogenase serum concentration peaked at the same time point. Both albumin and glutamate were within the normal level. On the 7-days' time point, GFAP, S100B and glutamate reached peak concentration in the spastic group though the glutamate was within the normal serum level. Serum GFAP reached peak concentration in the non- spastic group at the 7-days' time point while both CK and albumin showed an increase but within the normal range (Figure 17).

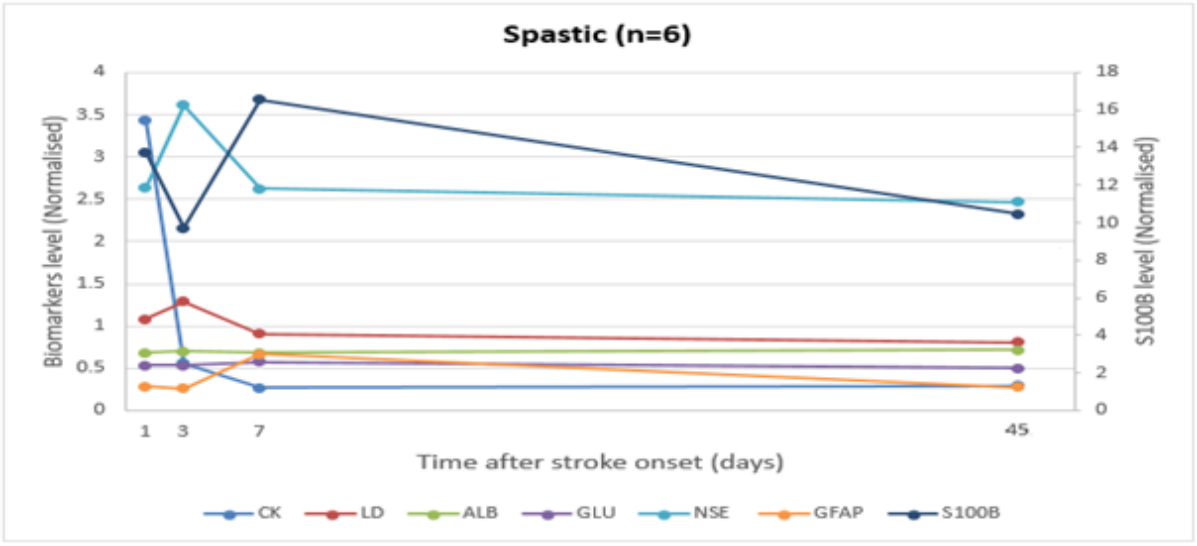


Figure 16 Normalised biomarkers curves in spastic patients calculated using mean patient value divided by maximum normal value. The time after stroke onset is plotted on the x-axis and concentration of biomarkers on the y-axis. S100B is on the second y-axis.

On the 45-days' time point, albumin showed peak concentration in both groups while the CK peak concentration was evident in the non-spastic group. Based on the biomarkers behaviour in the current study, the increase in biomarkers level is more likely to be seen at both, 3 and 7-days' time points than the 1 and 45-days' time point.

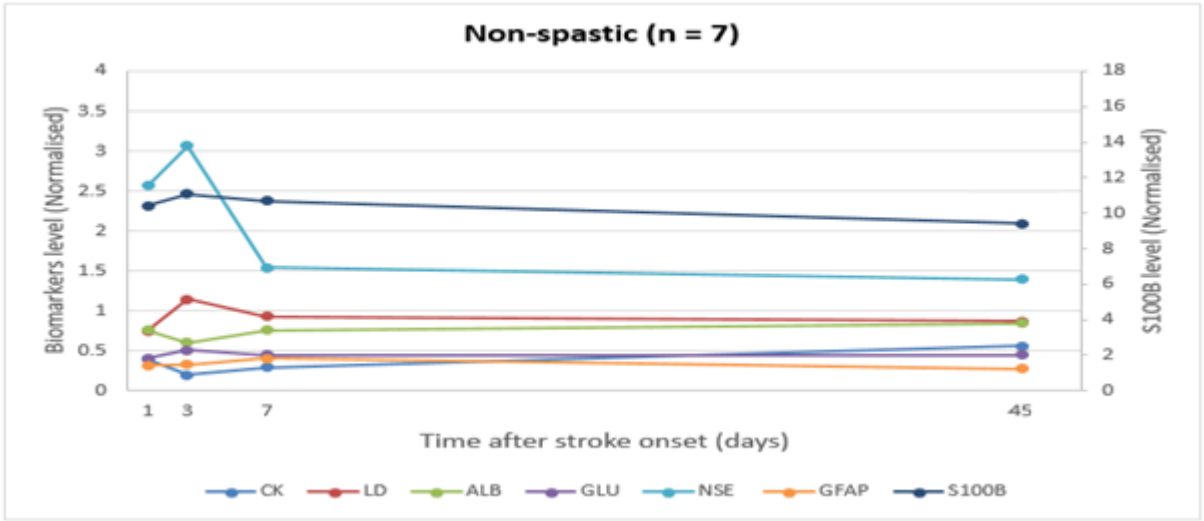


Figure 17 Normalised biomarkers curves in non-spastic patients calculated using mean patient value divided by maximum normal value. The time after stroke onset is plotted on the x-axis and concentration of biomarkers on the y-axis. S100B is on the second y-axis.

4.7 Association between muscle atrophy biomarkers and post-stroke muscle weakness

The evaluation of any muscle weakness that might develop post-stroke was part of the current study. Three muscle weakness related biomarkers were explored; creatine kinase, lactate dehydrogenase and albumin. To quantify and detect any muscle weakness, a grip dynamometer was used to measure grip strength (measured in Newtons). Grip strength measures were taken at 1, 3, 7 and 45-days' post-stroke in parallel with the time points of drawing blood and spasticity measurements.

The characteristics of biomarkers in both the spastic and non-spastic stroke patients varied. In the spastic group, the mean CK was at its highest level at 24-hours post-stroke time point while LD and albumin peaked at 3 and 45-days' time point respectively. Grip strength was at its highest at 24-hours' time point. Grip strength continued to decline with both CK and LD biomarkers while albumin continues to elevate (Figure 18). The increase in serum level of CK or LD and the decrease in serum albumin are all believed to be linked to muscle damage. The decline in grip strength in the spastic group cannot be attributed to muscle damage as the biomarkers behaved completely the opposite. It is important to point out that most of the changes in serum concentration for all the three biomarkers are proportional and take place within the normal ranges. In the non-spastic group, the mean LD was at its highest level at 3-days post-stroke time point while CK and albumin peaked at 45-days' time point. Grip strength was at its highest at 45-days' time point. Grip strength continued to improve in parallel with the increase of both CK and albumin biomarkers and peaked at 45-days' time point (Figure 19).

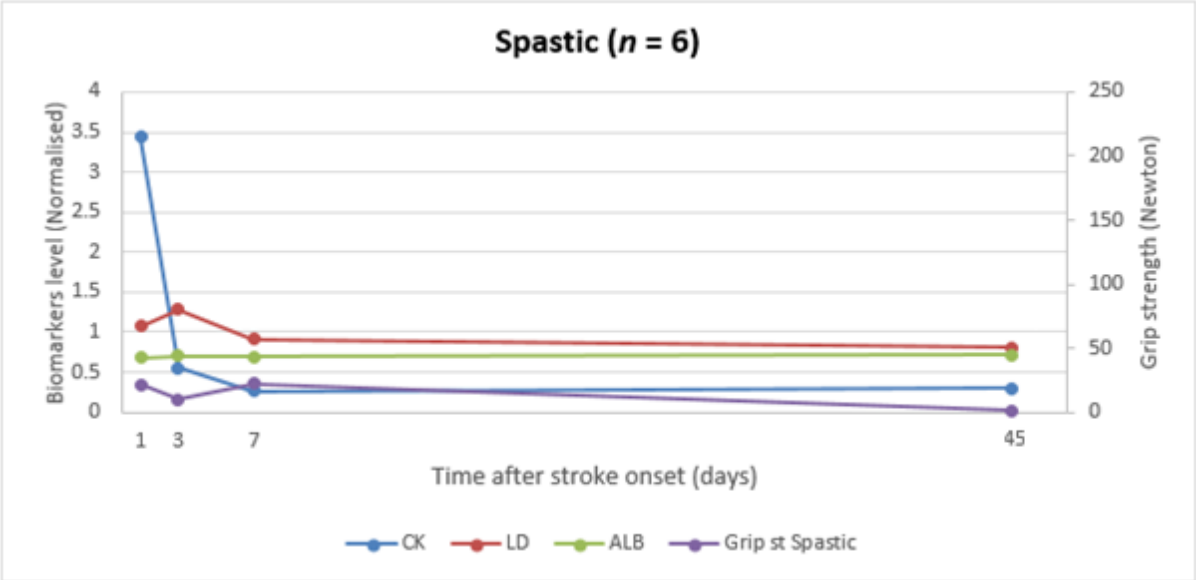


Figure 18 Normalised atrophy biomarkers curves in spastic patients calculated using mean patient value divided by maximum normal value. The time after stroke onset is plotted on the x-axis and concentration of biomarkers on the y-axis. Grip strength is on the second y-axis.

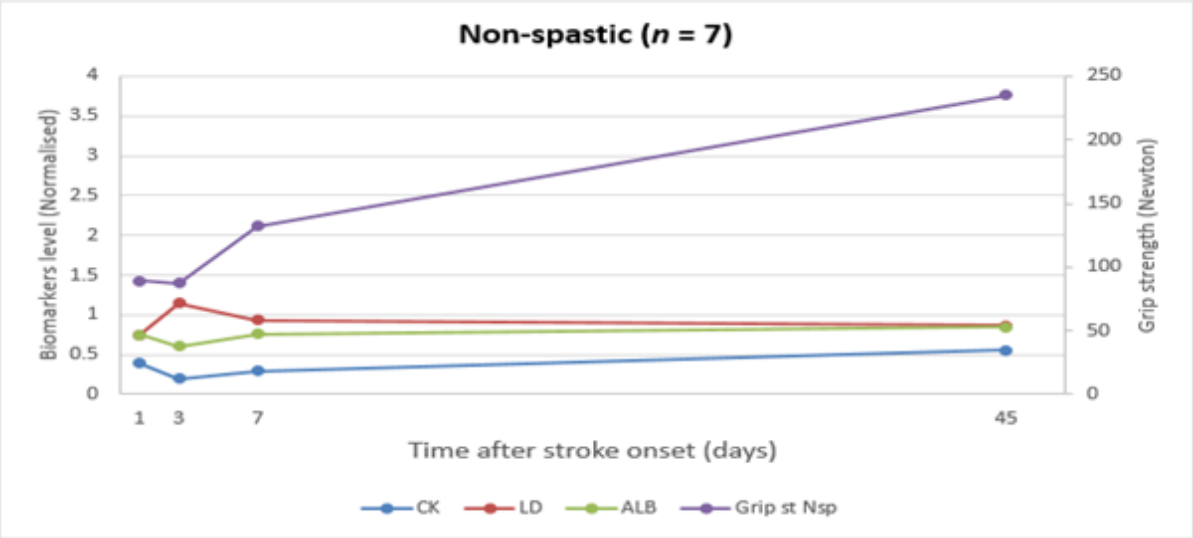


Figure 19 Normalised atrophy biomarkers curves in non-spastic patients calculated using mean patient value divided by maximum normal value. The time after stroke onset is plotted on the x-axis and concentration of biomarkers on the y-axis. Grip strength is on the second y-axis.

4.8 Atrophy biomarkers kinetics based on grip/no grip strength

The characteristics of biomarkers in patients with no grip strength and patients with grip strength varied. In the no grip strength group, the mean CK was at its highest level at 24-hours post-stroke while LD and albumin peaked at 3 and 45-days' time points respectively (Figure

20). It is important to point out that most of the changes in serum concentration for all the three biomarkers took place within the normal ranges.

In patient with grip strength, the mean LD was at its highest level at 3-days post-stroke time point while CK and albumin peaked at 45-days' time point (Figure 21). Both groups had similar biomarker pattern, mostly within normal ranges.

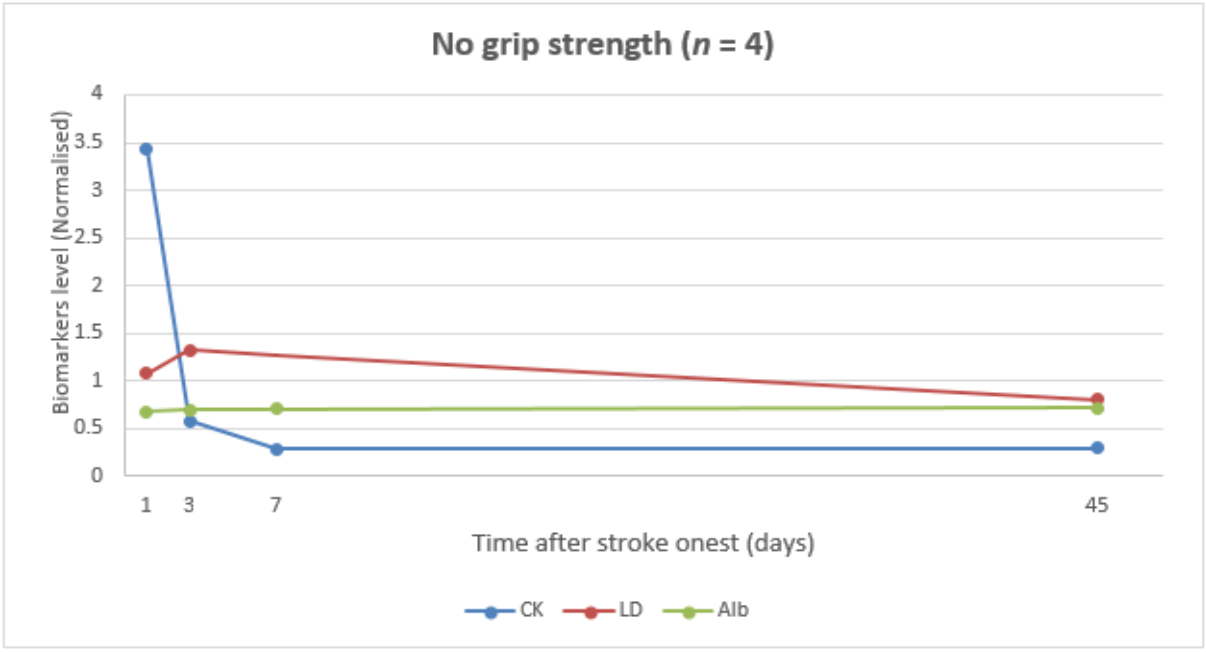


Figure 20 Normalised atrophy biomarkers curves in patients with no grip strength calculated using mean patient value divided by maximum normal value. The time after stroke onset is plotted on the x-axis and concentration of biomarkers on the y-axis.

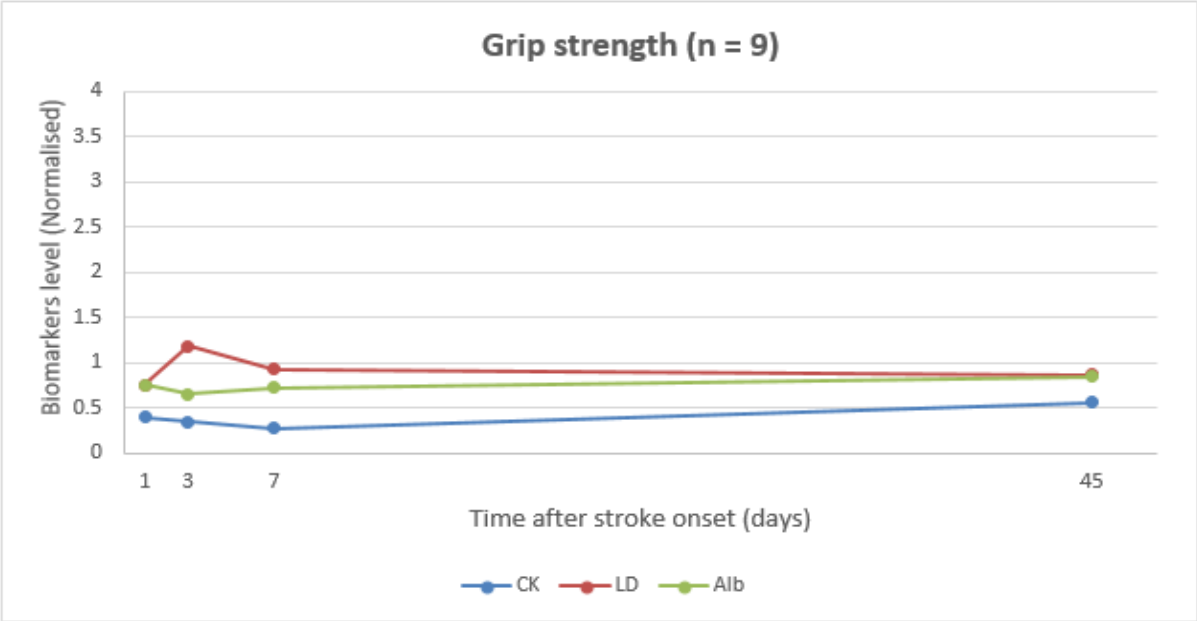


Figure 21 Normalised atrophy biomarkers curves in patients with grip strength calculated using mean patient value divided by maximum normal value. The time after stroke onset is plotted on the x-axis and concentration of biomarkers on the y-axis.

4.9 Association between biomarkers and spasticity or grip strength levels

4.9.1 Spasticity levels

Out of the 6 spastic patients, kinetic of biomarkers with spasticity levels were only available in 4 patients because of the missing data. Patient No 3 showed a decrease in NSE, S100B and CK levels in parallel with decreased spasticity at 3-days' time point. On 7-days' time point and in parallel with increased spasticity, both NSE and S100B were increased. With increased spasticity on 45- days' time point and apart from the increase of NSE, all the remaining biomarkers were decreased (Figure 22).

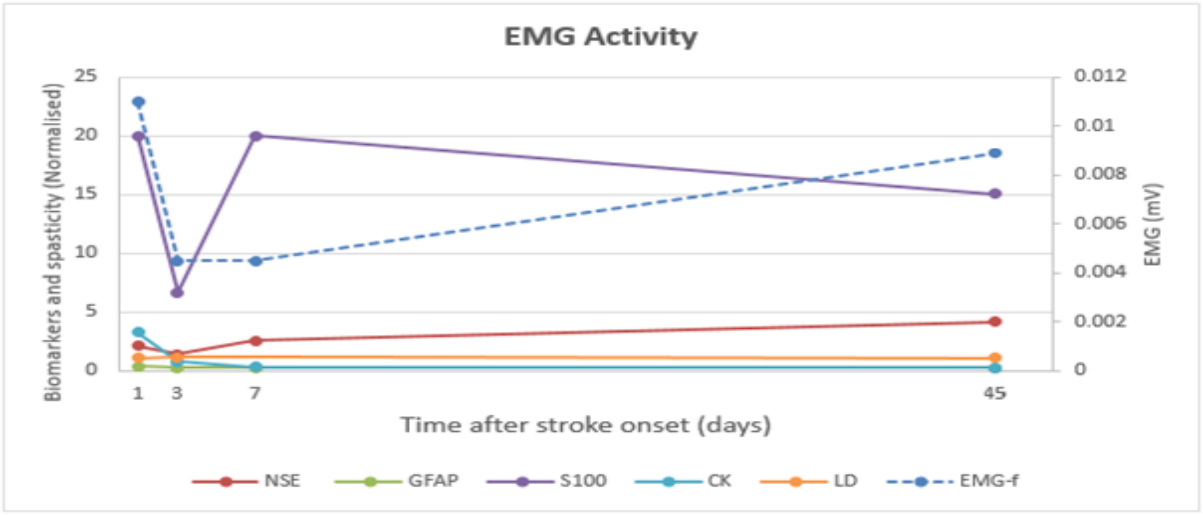


Figure 22 Patient No.3, normalised spasticity and biomarkers levels calculated using patient value divided by maximum normal value. The time after stroke onset is plotted on the x-axis and concentration of biomarkers on the y-axis. EMG activity is on the second y-axis.

Patient No 4 (Spastic) showed a decrease in spasticity on 3-days' time point and at the same time, increased the level of NSE, GFAP, S100B and LD (Figure 23).

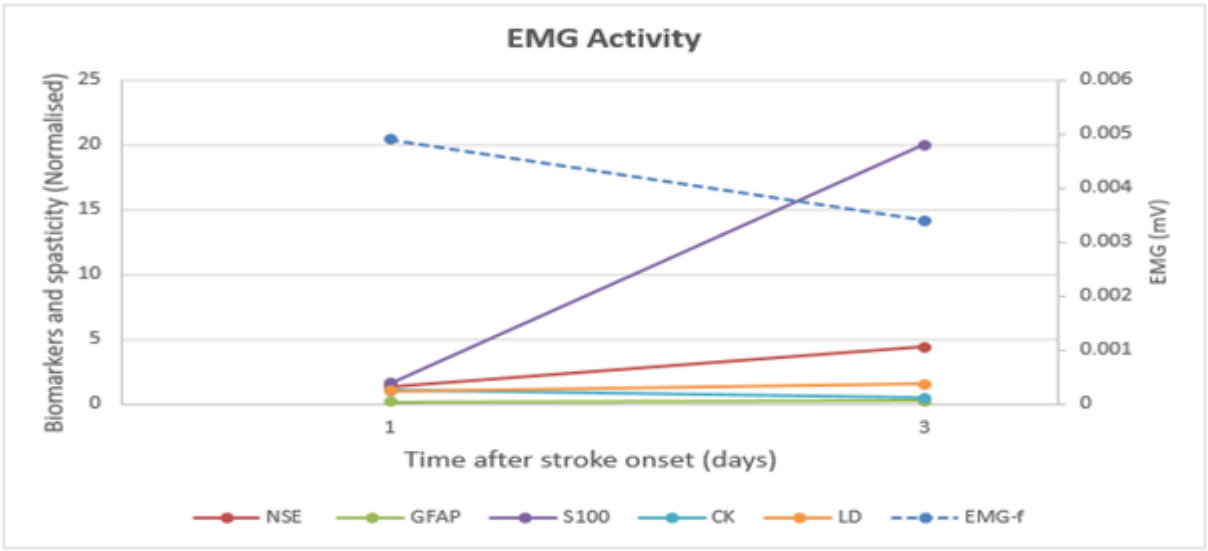


Figure 23 Patient No. 4, normalised spasticity and biomarkers levels calculated using patient value divided by maximum normal value. The time after stroke onset is plotted on the x-axis and concentration of biomarkers on the y-axis. EMG activity is on the second y-axis.

Spastic Patient No 7 and while showing a decrease in spasticity at the 45-days' time point, a parallel decrease in all biomarkers was evident (Figure 24).

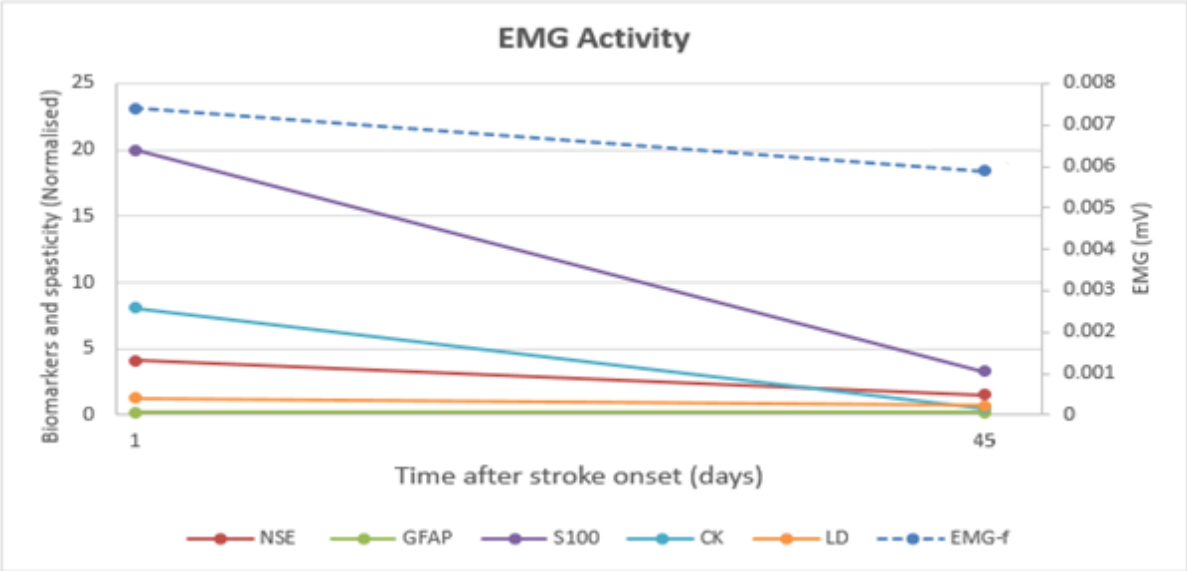


Figure 24 Patient No. 7, normalised spasticity and biomarkers levels calculated using patient value divided by maximum normal value. The time after stroke onset is plotted on the x-axis and concentration of biomarkers on the y-axis. EMG activity is on the second y-axis.

Patient No 12 and in parallel with the increased spasticity at 7-days' time point, an increase of NSE, GFAP and S100B levels were detected. On 45-days' time point, an apparent decrease in all biomarkers was evident even though the spasticity level was increased (Figure 25).

While the increase in some biomarkers was varied, an associated increase in CNS biomarkers with increased spasticity were more likely to be detected at the 7-days' time point.

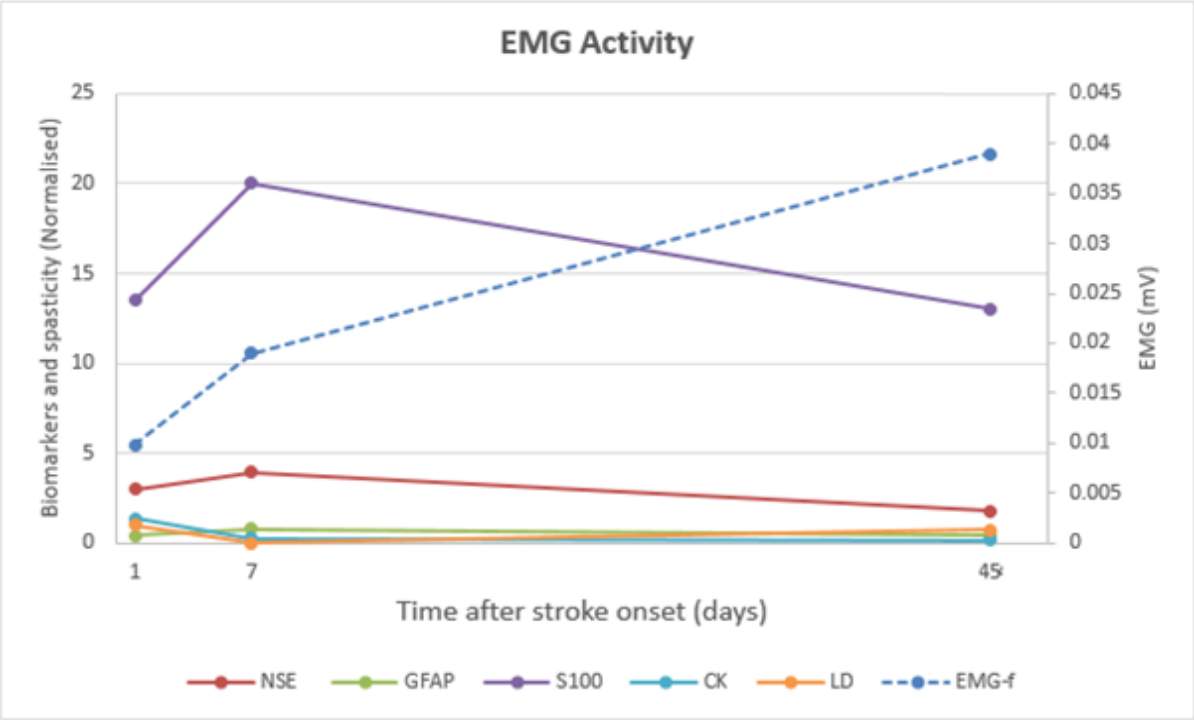


Figure 25 Patient No. 12, normalised spasticity and biomarkers level calculated using patient value divided by maximum normal value. The time after stroke onset is plotted on the x-axis and concentration of biomarkers on the y-axis. EMG activity is on the second y-axis.

4.9.2 Grip strength levels

Only non-spastic patients had changes in grip strength while spastic patients had either zero Newton grip strength or missing data. Out of the 7 non-spastic patients, kinetic of biomarkers with grip strength were only available in 5 patients because of the missing data.

Patient No 1 had an increased NSE, GFAP, S100B and LD levels in parallel with increased grip strength at the 7-days' time point. Only CK and LD were increased at 45-days' time point with increased grip strength (Figure 26). The higher level of S100B in this patient compared with the rest of the group could be attributed to the infarction size as this patient had a left middle cerebral artery occlusion.

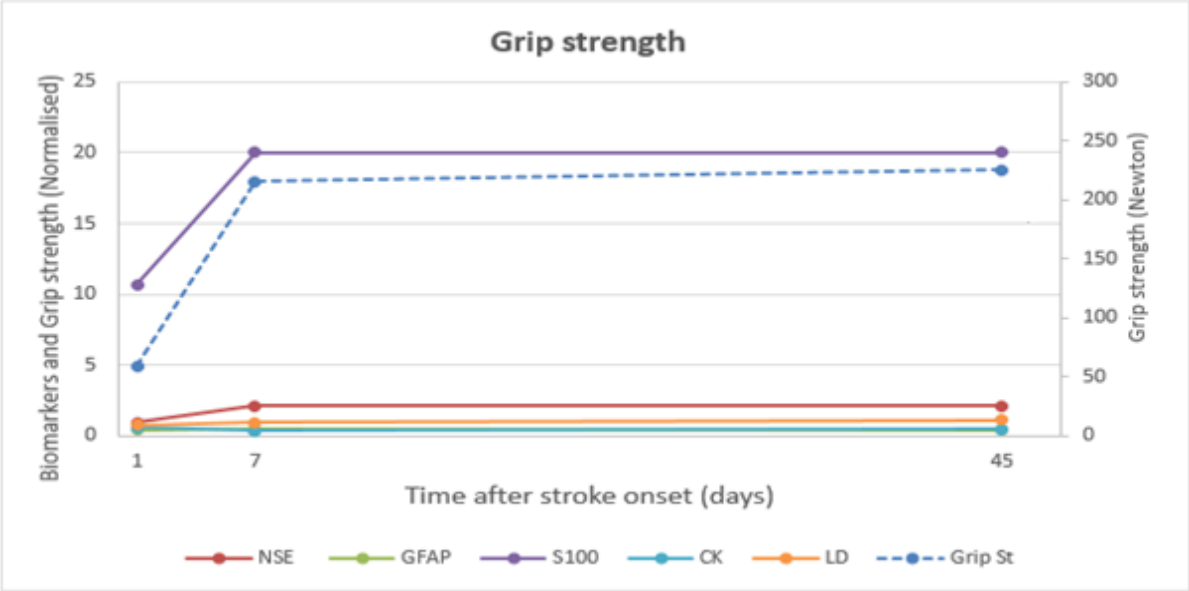


Figure 26 Patient No. 1, normalised grip strength and biomarkers levels calculated using patient value divided by maximum normal value. The time after stroke onset is plotted on the x-axis and concentration of biomarkers on the y-axis. Grip strength is on the second y-axis.

NSE, GFAP and s100B were all decreased in patient No 2 at 45-days' time point while grip strength, CK and LD were increased (Figure 27).

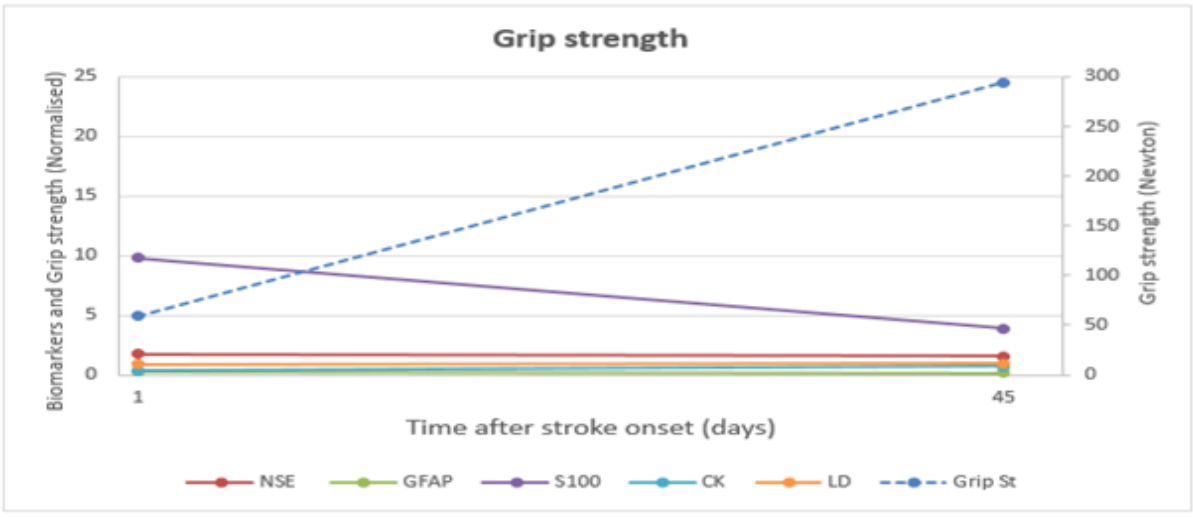


Figure 27 Patient No. 2, normalised grip strength and biomarkers levels calculated using patient value divided by maximum normal value. The time after stroke onset is plotted on the x-axis and concentration of biomarkers on the y-axis. Grip strength is on the second y-axis.

Grip strength was only decreased at 3-days' time point when patient No 9 showed a decrease in NSE, GFAP and CK. While NSE continues to decrease at 7-days' time point, GFAP and CK were both increased in parallel with increased grip strength (Figure 28).

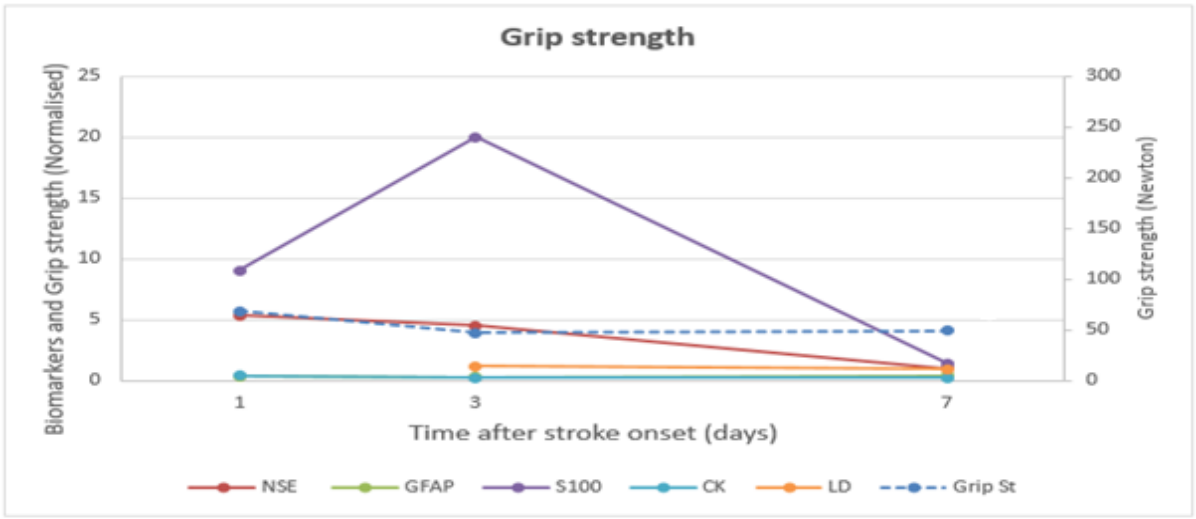


Figure 28 Patient No. 9, normalised grip strength and biomarkers levels calculated using patient value divided by maximum normal value. The time after stroke onset is plotted on the x-axis and concentration of biomarkers on the y-axis. Grip strength is on the second y-axis.

While both GFAP and CK were both increased in patient No 10 at the 45-days' time point, NSE and LD were decreased in contrast with the increased grip strength (Figure 29).

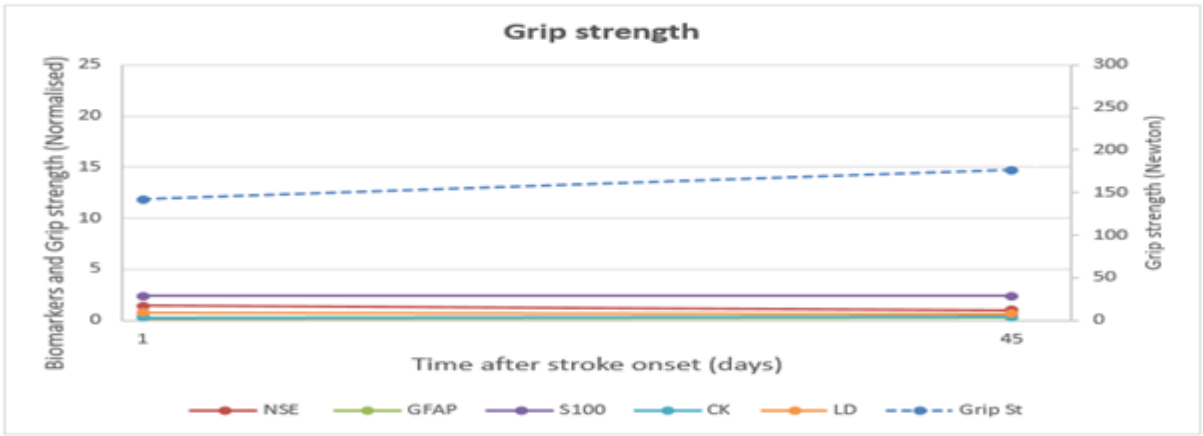


Figure 29 Patient No. 10, normalised grip strength and biomarkers levels calculated using patient value divided by maximum normal value. The time after stroke onset is plotted on the x-axis and concentration of biomarkers on the y-axis. Grip strength is on the second y-axis.

Grip strength increased in patient No 11 at 45-days' time point in parallel with the increase in S100, CK and LD levels. Both NSE and GFAP showed a decrease at the same time point (Figure 30).

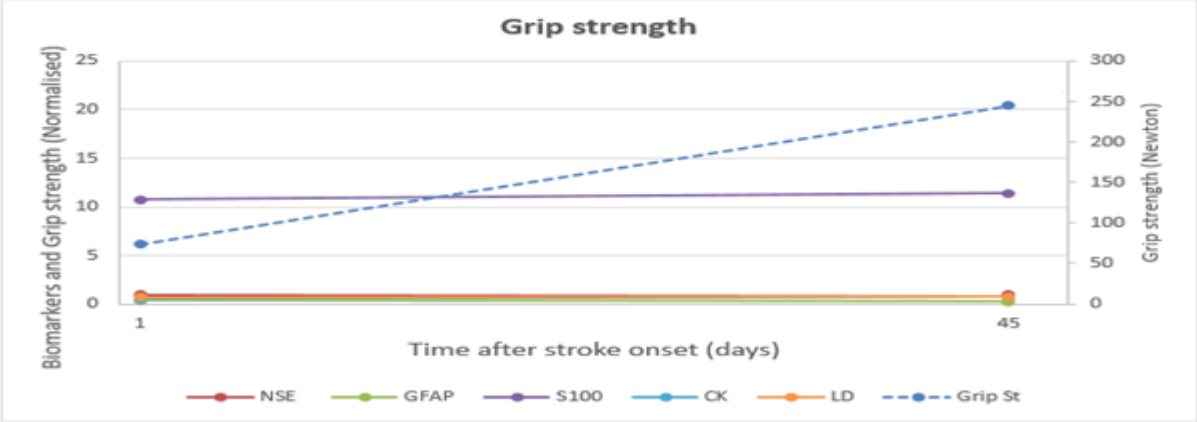


Figure 30 Patient No. 11, normalised grip strength and biomarkers levels calculated using patient value divided by maximum normal value. The time after stroke onset is plotted on the x-axis and concentration of biomarkers on the y-axis. Grip strength is on the second y-axis.

The current data showed that grip strength was improving from 7-days' time point and after. While biomarkers level varied at the 7-days' time point, an evident decrease in most of the CNS biomarkers with contrasting increase of most of the muscle atrophy biomarkers was observed at the 45-days' time point.

CHAPTER 5: DISCUSSION AND CONCLUSIONS

5.1 Discussion

The main objective of the current research is to explore the possible usefulness of GFAP, S100B, NSE, glutamate, GABA, purines, CK, LD and albumin as biomarkers of post-stroke spasticity. Furthermore, it was intended to identify possible correlations of muscle biomarkers with post-stroke muscle weakness and contractures. Within literature and to date, there is no publication that examines the variation of serum biomarkers concentration in post-stroke spasticity, and this is the novelty of the research, contributing to knowledge. A discussion of the results from this study will be outlined here.

With respect to spasticity, some fluctuations in abnormal muscle activity was seen towards the 45-days' time point. Some patients showed abnormal muscle activity as early as 24-hours post-stroke mainly during the fast movement, and this went on to show normal muscle activity at a later stage. This fluctuation in muscle activity could be attributed to the period of shock and recovery that immediately follow lesions of the CNS. During this period, the system will start to present with varying responses and time delays (Pandyan et al., 2018). With respect to grip strength, we saw no pattern between changes in grip strength and biomarkers concentrations. Further studies are needed to explore these relationships as our sample size was small and heterogenous.

This current research showed that, following acute stroke, there was increase in the serum concentration of NSE, GFAP, s100B and purines, and this is consistent with the observation in previous studies (Pasini et al., 2000, Oh et al., 2003, Foerch et al., 2012, Schulte et al., 2014).

NSE, GFAP, purines and S100B protein concentrations demonstrated a differentiation potential between spastic and non-spastic patients. The serum concentration in all four biomarkers was lower in the non-spastic group compared with the spastic group. Serum concentration in all four biomarkers was above the normal range at all time points. These results were statistically not significant due to the limited sample size.

In this study, an elevation of serum NSE concentration was noted as early as 24-hours after stroke onset, again consistent with the earlier reported studies (Missler et al., 1997; Hill et al., 2000; Oh et al., 2003). Peak concentrations occurred in both groups at 3-days' time point, again, similar to the results reported in previous studies (Missler et al. 1997, Cunningham et al. 1996). NSE is suggestive of neuronal damage. Large neuronal damage represents diffused injury which is more likely to lead to spasticity (Ivanhoe and Reistetter, 2004).

The mean peak S100B concentration occurred at 72-hours post-stroke in the non-spastic group which is consistent with reports by Missler et al. (1997) and Wunderlich et al. (1999). On the other hand, mean peak S100B concentration occurred at 7-days' time point in the spastic group and subsequently declined to reach admission levels at 45-days as reported by Missler et al. (1997). The delayed release of the S100B might be due to the penumbra not being salvaged, which in turn affect surrounding cells, leading to disruption of blood-brain barrier and leaks to the peripheral circulation.

In this study, the peak of serum GFAP concentration was noted at 7-days after stroke onset which is different to earlier studies that reported peak serum GFAP concentrations to occur

around 2-days after the onset of stroke (Missler, et al. 1997, Wunderlich, Wallesch and Goertleret, 2006).

The mean peak purine concentration occurred at 24-hours post-stroke in the spastic group, consistent with the report by Suzuki et al. (2000). On the other hand, mean peak purines concentration in the non-spastic group occurred at the baseline time point and subsequently declined to reach a normal level at 24-hours post-stroke. Stroke patients with above the normal level blood purines concentration at 24-hours, after stroke onset, ended up developing spasticity while patients with normal range purines at 24-hours did not. Because of the limited sample size, this result should be taken with caution as it is not statistically significant. It is worth mentioning that purines are not CNS specific biomarker and as such, other factors or comorbidity might affect the results; factors such as time since the last meal and history of gout disease.

The characteristics of the glutamate in both spastic and non-spastic patients varied from those of NSE, GFAP and S100B protein. The mean NSE, GFAP and S100B serum concentrations in both groups were above the normal range at all time points compared to glutamate which stayed within the normal range at all time points in both spastic and non-spastic groups. Our results contrast previous studies reported an increase in serum glutamate concentrations post-stroke (Puig et al., 2000; Aliprandi et al., 2005).

An elevation of serum CK concentration in the spastic group was noted as early as 24-hours after stroke onset and subsequently declined to reach a normal range at 3-days post-stroke. Serum CK level in the non-spastic group stayed within the normal range at all time points. The

results suggest that CK is unlikely to have any links with the development of spasticity as both groups serum concentration remained within the normal range at all time point, apart from the initial increase at a 24-hours' time point in the spastic group. Serum CK concentration also cannot explain the deterioration or improvement in grip strength seen in spastic and non-spastic groups respectively.

Spastic group serum LD concentration elevation was noted as early as 24-hours after stroke onset. The elevation continued at 3-days' time point in both groups and subsequently declined to reach normal range at 7-days post-stroke. Serum LD level in both groups stayed within the normal range at 7 and 45-days' time points. The results suggest that LD is unlikely to have a link with the development of spasticity or muscle weakness since it was within the normal range at most of the time point. Although the non-spastic group showed improvement in grip strength compared to the spastic group, normal serum LD concentration was seen in both groups. Again, due to the limited sample size, this result should be looked at carefully as it is not statistically significant. In addition to that, LD is not a CNS specific biomarker, and as such, other physiological or pathological factors might affect the results.

A decline of serum albumin concentration in the non-spastic group was noted only at 3- days' time point post-stroke and subsequently elevated to reach normal range at 7-days' time point post-stroke. Serum albumin level in both spastic and non-spastic groups stayed within the normal range at all other time points. The absence of meaningful changes in the albumin serum kinetics, making it unlikely to be considered as a potential biomarker in terms of spasticity or muscle weakness development. Normal serum albumin concentration was seen

in both groups even though the non-spastic group showed improvement in grip strength compared to the spastic one.

The current study showed an increase in most CNS tissue injury biomarkers; NSE, GFAP and S100B. It is believed that the loss of cerebral blood flow in the ischemic core during stroke leads to a complete reduction of oxygen and glucose supply to cerebral neurons and other supporting cells. This causes a series of biochemical and metabolic changes that finally lead to massive cell death. The CNS damage will lead to a release of neurons or glia-specific biomarkers such as neuron-specific enolase (NSE), glial fibrillary acidic protein (GFAP), acidic calcium-binding protein (S100B). NSE expression and activity are markedly upregulated in glial and neuronal cells after stroke indicating the enzyme's role in inflammation following these events. GFAP is a structural protein that is released when astrocytes are disintegrated, and the cytoskeletons are degraded (Pelinka et al., 2004). Structural damage to the neuronal cells also leads to the release of S100B into the blood. During a stroke, the blood-brain barrier (BBB) is compromised by endothelial cell death. CNS tissue injury biomarkers have the potential to cross the BBB and thus, their blood level increase.

5.2 Implications for stroke rehabilitation

The findings of this study suggest that there is a link between certain biological markers namely, S100B, GFAP, NSE and purines and the development of post-stroke spasticity. Further research is required before definitive conclusions can be made. However, this research may want to start with a focus on S100B as this particular marker seems the most promising in terms of mechanism of spasticity. Although the EMG showed fluctuations, the biomarker

changes in S100B may have lower levels of variability. It is possible therefore that biomarkers are likely to be more specific.

The biomarker activity in patients with spasticity corresponded to the neurophysiological recordings and occurred before the changes in the passive range of motion and stiffness. Therefore, one could use the biomarker profiles to inform treatment plans for patients who are likely to get musculoskeletal complications after a stroke. There was evidence that the changes in the passive range of movement occurred before stiffness and this is consistent with the literature (Malhotra et al., 2011). The stiffness increases in this study were within normal values (Pandyan et al., 2001) and may reflect the fact that most measurements were taken within 6-weeks of the stroke. Future research with larger samples is needed to confirm these observations.

This study included ischemic and hemorrhagic patients combined, and while studying each type of stroke separately might provide more insight into the related changes and the underlying mechanisms, the small sample size made it impractical. The same implications are for ischemic patients who received thrombolysis treatment. A larger sample study would likely have a more promising chance of answering these questions. The current clinical measures cannot detect early spasticity (e.g., The Modified Ashworth Scale; The Tardieu Scale) used routinely in practice and clinicians face the reality of having to deal with spasticity complications after it already has developed. In situations like this, it is crucial to develop different approaches that could help with the early detection of spasticity and lead to timely interventions and prevention of complications.

The study of biomarkers could provide insight into the physiological and pathological process that accompanies the development of neurological disorder such as spasticity. Not only it is important for identifying the mechanisms behind the development of spasticity at a cellular level and in aiding practitioners to examine its natural progression, it also might be of great importance at identifying and developing targeted drug treatments.

5.3 Limitations of the study

Several potential limitations of the present study must be considered. The results of this study are limited by the small sample size and therefore generalising the results beyond this study population is problematic. One reason for the limited number of recruits is the fact that people are reluctant to consent to invasive studies that involve repeated drawing of blood. This explains why the second sub-study, the Pharmacokinetic sub-study, planned to collect blood samples every hour for six hours ended up recruiting none. This issue can be dealt with by keeping blood collection time points to the minimum and integrating blood collection within the routine blood investigation.

A major problem with the small sample size was that the external validity was limited. There was a significant risk that our findings were affected by readings that were not typical. Further, the small sample size and the presence of 31% of zero values in grip strength has restricted the study of the association between biomarkers and physiological responses of motor recovery. The Bonferroni correction was not applied for the multiple comparisons; however, the 95% Confidence Intervals were reported to reduce the risk of type I error reporting.

The recruitment depended on research nurses who did not have ownership of the study and so poses barriers in recruiting for this project. The ability to use CSF fluid would have significantly improved our ability to identify relevant biomarkers. However, given the challenges we had with recruiting patients for the study of blood this could be more difficult.

This study was limited to a panel of biomarkers studied in the context of stroke and as such, it should not be considered as spasticity specific biomarkers.

5.4 Indications for future research

The main challenge in our study was patient recruitment and in future studies, there may be a need to engage with patient groups to help solve this problem. Further studies are needed to assess and confirm the relationship between certain serum biomarkers and the development of post-stroke spasticity. It is worth mentioning that the biomarkers included in this study, are not specific to spasticity and as such, a different approach might worth adopting. An approach that could widen the scope of the investigation by analysing the blood samples from scratch without preconception of any biochemical markers. Mass spectrometry techniques and its application in the field of clinical proteomics might provide the means to identify a specific biomarker that could be linked to the development of post-stroke spasticity.

5.5 Conclusions

The main objectives of this study were to explore the possible usefulness of a panel of specifically chosen biomarkers as an indicator of post-stroke spasticity and related complications. Serum concentrations in both spastic and non-spastic groups were compared, and the possible correlations of the biomarkers with the development of post-stroke spasticity were identified.

Out of the five-central nerve system specific biomarkers, three showed promising results; GFAP, NSE and S100B. The difference in serum concentration between the spastic and the non-spastic group in all three was at its highest on day seven-time point with the spastic group been higher. Glutamate did not show any promising result with the serum concentration showed within the normal range at all time points. It was not possible to get a meaningful result out of the GABA test as it failed even at calibration.

Purines showed a promising result, and the serum concentration was always higher in the spastic group than the non-spastic group. The largest difference was at 24-hours' time point post-stroke. Creatine kinase was higher in the spastic group at the 1-day time point and was way above the normal range and went to be within the normal range on the subsequent time-points. Non-spastic group CK serum concentration, on the other hand, stayed within the normal range at all time points. Lactate dehydrogenase serum concentration, in the spastic group, was a bit higher than the normal range at one-day time point but went to be within the normal range on the subsequent time points. Non-spastic group LD serum concentration, on the other hand, stayed within normal range at all time points. Albumin serum concentration was higher in the non-spastic group than the spastic at all time points. Both groups were within the normal range though.

In conclusion, although not statistically significant, my research enabled the identification of four promising biomarkers, three central nerve system specific biomarkers (GFAP, NSE, S100B) and purines, that have links to the development of spasticity. Further studies are needed to assess and confirm the relationship between these biomarkers and the development of post-stroke spasticity.

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APPENDICES CONTENT LIST

APPENDIX I - Modified Ashworth Scale

Modified Ashworth Scale Instructions

General Information (derived Bohannon and Smith, 1987):

- Place the patient in a supine position
- If testing a muscle that primarily flexes a joint, place the joint in a maximally flexed position and move to a position of maximal extension over one second (count "one thousand one")
- If testing a muscle that primarily extends a joint, place the joint in a maximally extended position and move to a position of maximal flexion over one second (count "one thousand one")
- Score based on the classification below

Scoring (taken from Bohannon and Smith, 1987):

0	No increase in muscle tone
1	Slight increase in muscle tone, manifested by a catch and release or by minimal resistance at the end of the range of motion when the affected part(s) is moved in flexion or extension
1+	Slight increase in muscle tone, manifested by a catch, followed by minimal resistance throughout the remainder (less than half) of the ROM
2	More marked increase in muscle tone through most of the ROM, but affected part(s) easily moved
3	Considerable increase in muscle tone, passive movement difficult
4	Affected part(s) rigid in flexion or extension

Patient Instructions:

The patient should be instructed to relax.

Modified Ashworth Scale Testing Form

Name:_____ Date:_____

Muscle Tested	Score
_____	____
_____	____
_____	____
_____	____
_____	____
_____	____
_____	____
_____	____
_____	____

Reference for test instructions:

Bohannon, R. and Smith, M. (1987). "Interrater reliability of a modified Ashworth scale of muscle spasticity." Physical Therapy 67(2): 206.

APPENDIX II - Tardieu Scale

TARDIEU SCALE

This scale quantifies muscle spasticity by assessing the response of the muscle to stretch applied at specified velocities.

Grading is always performed at the same time of day, in a constant position of the body for a given limb. For each muscle group, reaction to stretch is rated at a specified stretch velocity with 2 parameters x and y.

Velocity to stretch (V)

V1	As slow as possible
V2	Speed of the limb segment falling
V3	As fast as possible (= natural drop)

V1 is used to measure the passive range of Motion. (PROM). Only V2 and V3 are used to rate spasticity

Quality of muscle reaction (X)

0	No resistance throughout passive movement
1	Slight resistance throughout, with no clear catch at a precise angle
2	Clear catch at a precise angle, followed by release
3	Fatigable clonus (<10secs) occurring at a precise angle
4	Unfatigable clonus (>10secs) occurring at a precise angle
5.	Joint Immobile

Angle of muscle reaction (Y)

Measure relative to the position of minimal stretch of the muscle (corresponding at angle)

Spasticity Angle

R1	Angle of catch seen at Velocity V2 or V3
R2	Full range of motion achieved when muscle is at rest and tested at V1 velocity

Boyd. Graham 1999

- A large difference between R1 & R2 values in the outer to middle range of normal m. length indicates a large dynamic component
- A small difference in the R1 & R2 measurement in the middle to inner range indicates predominantly fixed contracture

Date	Joint/ Muscle	L/R	V	X	R1	R2	Active ROM	Power MRC	Ashworth Rating	°

Ref: Boyd R, Graham K. Objective Measurement of clinical findings in the use of Botox type A for the management of children with Cerebral Palsy. European Journal of Neurology 6(Supp 4): S23-35
Tardieu G, Rondont O, Mensch J, Dallaz J, Montfrais C, Tabary J. Responses electromyographiques a l'etirement musculaire chez l'homme normal. Revue Neurologie – 97(1): 60-61
Gracies J, Marosszeky J, Renton R, Sandaman J, Gandevia S, Burke D. Short term effects of dynamic splints on the upper limb in hemiplegic patients. Archives of Physical Medicine and Rehabilitation. 81: 1547-1555.

Testing Positions

Upper Limb

To be tested in a sitting position, elbow flexed by 90° at the recommended joint positions and velocities.

Shoulder	Horizontal Adductors	V3	
	Vertical Adductors	V3	
	Internal Rotators	V3	
Elbow	Flexors	V2	Shoulder adducted
	Extensors	V3	Shoulder abducted
	Pronators	V3	Shoulder adducted
	Supinators	V3	Shoulder adducted
Wrist	Flexors	V3	
	Extensors	V3	
	Fingers		Angle PII of digit III - MCP
	Palmar Interossei + FDS	V3	Wrist resting position

Lower Limb

To be tested in supine position, at recommended joint positions and velocities

Hip	Extensors	V3	Knee extended
	Adductors	V3	Knee extended
	External Rotators	V3	Knee flexed by 90
	Internal Rotators	V3	Knee flexed by 90
Knee	Extensors	V2	Hip flexed by 30
	Flexors	V3	Hip flexed
Ankle	Plantarflexors	V3	Knee flexed by 30

APPENDIX III - MEDLINE and CINAHL databases search strategy (Search 1)

Spasticity biomarkers Sunday, June 17, 2018 9:18:26 PM

#	Query	Limiters/Expanders	Last Run Via	Results
S17	S7 AND S12 AND S16	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	20
S16	S13 OR S14 OR S15	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	303,727
S15	TI Molecular signature OR AB Molecular signature	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	5,782
S14	TI (biomarker* OR biological marker*) OR AB (biomarker* OR biological marker*)	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	252,105
S13	(MH "Biological Markers+")	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	60,623
S12	S8 OR S9 OR S10	Search modes -	Interface - EBSCOhost Research Databases	86,103

	OR S11	Boolean/Phrase	Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	
S11	TI (muscle* N5 (spasm OR spasms OR rigid* OR tone OR tonus OR hyperton* OR hypermyoton* OR dyston*)) OR AB (muscle* N5 (spasm OR spasms OR rigid* OR tone OR tonus OR hyperton* OR hypermyoton* OR dyston*))	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	17,257
S10	TI (spastic* or high tone) OR AB (spastic* or high tone)	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	40,170
S9	(MH "Spasm+") OR (MH "Dystonia+") OR (MH "Paraparesis+")	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	22,559
S8	(MH "Muscle Spasticity") OR (MH "Muscle Hypertonia+") OR (MH "Muscle Rigidity") OR (MH "Muscle Tonus")	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	21,792
S7	S1 OR S2 OR S3 OR S4 OR S5 OR S6	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and	717,152

			Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	
S6	TI (hemipleg* OR hemipar* OR paresis OR paretic) OR AB (hemipleg* OR hemipar* OR Paresis OR paretic)	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	50,893
S5	(MH "Hemiplegia") OR (MH "Paresis+")	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	23,654
S4	TI ((brain* OR cerebr* OR cerebell* OR intracerebral OR intracranial OR subarachnoid) N5 (haemorrhage* OR hemorrhage* OR haematoma* OR hematoma* OR bleed*)) OR AB ((brain* OR cerebr* OR cerebell* OR intracerebral OR intracranial OR subarachnoid) N5 (haemorrhage* OR hemorrhage* OR haematoma* OR hematoma* OR bleed*))	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	76,306
S3	TI ((brain* OR cerebr* OR cerebell* OR intracran* OR intracerebral) N5	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus	83,586

	(isch?emi* OR infarct* OR thrombo* OR emboli* OR occlus*)) OR AB ((brain* OR cerebr* OR cerebell* OR intracran* OR intracerebral) N5 (isch?emi* OR infarct* OR thrombo* OR emboli* OR occlus*))		with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	
S2	TI(stroke OR poststroke OR post-stroke OR cerebrovasc* OR brain vasc* OR cerebral vasc* OR cva* OR apoplex* OR SAH) OR AB (stroke OR poststroke OR post-stroke OR cerebrovasc* OR brain vasc* OR cerebral vasc* OR cva* OR apoplex* OR SAH)	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	420,994
S1	(MH "Cerebrovascular Disorders+") OR (MH "Basal Ganglia Cerebrovascular Disease+") OR (MH "Brain Ischemia+") OR (MH "Carotid Artery Diseases+") OR (MH "Intracranial Arterial Diseases+") OR (MH "Intracranial Embolism and Thrombosis+") OR	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	418,197

(MH "Intracranial Hemorrhages+") OR (MH "Stroke+") OR (MH "Brain Infarction+") OR (MH "Vertebral Artery Dissection")

MY

EBSCOhost

APPENDIX IV - MEDLINE and CINAHL databases search strategy (Search 2)

Stroke biomarkers

Sunday, June 17, 2018 8:50:32 PM

#	Query	Limiters/Expanders	Last Run Via	Results
S12	S7 AND S11	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	8,195
S11	S8 OR S9 OR S10	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	303,727
S10	TI Molecular signature OR AB Molecular signature	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	5,782
S9	TI (biomarker* OR biological marker*) OR AB (biomarker* OR biological marker*)	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	252,105
S8	(MH "Biological Markers+")	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	60,623
S7	S1 OR S2 OR S3	Search modes -	Interface - EBSCOhost Research Databases	717,152

	OR S4 OR S5 OR S6	Boolean/Phrase	Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	
S6	TI (hemipleg* OR hemipar* OR paresis OR paretic) OR AB (hemipleg* OR hemipar* OR Paresis OR paretic)	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	50,893
S5	(MH "Hemiplegia") OR (MH "Paresis+")	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	23,654
S4	TI ((brain* OR cerebr* OR cerebell* OR intracerebral OR intracranial OR subarachnoid) N5 (haemorrhage* OR hemorrhage* OR haematoma* OR hematoma* OR bleed*)) OR AB ((brain* OR cerebr* OR cerebell* OR intracerebral OR intracranial OR subarachnoid) N5 (haemorrhage* OR hemorrhage* OR haematoma* OR hematoma* OR bleed*))	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	76,306
S3	TI ((brain* OR cerebr* OR cerebell* OR	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and	83,586

	intracran* OR intracerebral) N5 (isch?emi* OR infarct* OR thrombo* OR emboli* OR occlus*)) OR AB ((brain* OR cerebr* OR cerebell* OR intracran* OR intracerebral) N5 (isch?emi* OR infarct* OR thrombo* OR emboli* OR occlus*))		Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	
S2	TI(stroke OR poststroke OR post-stroke OR cerebrovasc* OR brain vasc* OR cerebral vasc* OR cva* OR apoplex* OR SAH) OR AB (stroke OR poststroke OR post-stroke OR cerebrovasc* OR brain vasc* OR cerebral vasc* OR cva* OR apoplex* OR SAH)	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	420,994
S1	(MH "Cerebrovascular Disorders+") OR (MH "Basal Ganglia Cerebrovascular Disease+") OR (MH "Brain Ischemia+") OR (MH "Carotid Artery Diseases+") OR (MH "Intracranial Arterial Diseases+") OR (MH "Intracranial	Search modes - Boolean/Phrase	Interface - EBSCOhost Research Databases Search Screen - Advanced Search Database - AMED - The Allied and Complementary Medicine Database;MEDLINE;PsycINFO;SPORTDiscus with Full Text;AgeLine;CINAHL Plus with Full Text;PsycARTICLES	418,197

Embolism and Thrombosis+") OR (MH "Intracranial Hemorrhages+") OR (MH "Stroke+") OR (MH "Brain Infarction+") OR (MH "Vertebral Artery Dissection")

APPENDIX V- Type of stroke with the number of articles retrieved (Unclassified means it is not clear which type of stroke)

Type of stroke	Number of articles retrieved
Human	
Hemorrhagic	(146)
Ischemic	(482)
Unclassified	(133)
Animal	
Hemorrhagic	(94)
Ischemic	(165)
Unclassified	(62)

APPENDIX VI - Letter of ethical approval National Research Ethics Service (SMARTCap study)

NHS
Health Research Authority

NRES Committee West Midlands - Coventry & Warwickshire
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Tel: 0115 8839521

02 March 2015

Professor Christopher Imray
Consultant Vascular and Endovascular Surgeon
UHCW NHS Trust
UHCW NHS Trust
Clifford Bridge Road
Coventry
CV4 7AL

Dear Professor Imray

Study title:	**SMARTCap: A field-deployable blood test for stroke, capable of detecting brain ischaemia from the earliest stages of pathology**
REC reference:	**14/WM/1067**
Amendment number:	**Amendment 2**
Amendment date:	**20 February 2015**
IRAS project ID:	**156750**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering letter on headed paper		20 February 2015
Notice of Substantial Amendment (non-CTIMP) [Amendment 2]		20 February 2015
Other [Advertisement poster - Control]	1	17 February 2015
Participant consent form [Participant (clean and tracked)]	3	17 February 2015
Participant consent form [Representative (clean and tracked)]	3	17 February 2015
Participant consent form [Control (clean and tracked)]	2	17 February 2015
Participant consent form [Healthy volunteer (clean and tracked)]	3	17 February 2015
Participant consent form [Participant (UHNM only)]	1	17 February 2015
Participant consent form [Representative (UHNM only)]	1	17 February 2015
Participant consent form [Control (UHNM only)]	1	17 February 2015

Participant information sheet (PIS) [Participant or representative (clean and tracked)]	3	17 February 2015
Participant information sheet (PIS) [Control (clean and tracked)]	2	17 February 2015
Participant information sheet (PIS) [Healthy volunteer (clean and tracked)]	3	17 February 2015
Participant information sheet (PIS) [Participant or representative (UHNM only)]	1	17 February 2015
Participant information sheet (PIS) [Control (UHNM only)]	1	17 February 2015
Research protocol or project proposal [clean and tracked]	4	17 February 2015

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

14/WM/1067:	**Please quote this number on all correspondence**

Yours sincerely

pp. V. Sutton

Dr Helen Brittain
Chair

E-mail: NRESCommittee.WestMidlands-CoventryandWarwick@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Ms Isabella Petric*
Cori Jones
Sonia Kandola

NRES Committee West Midlands - Coventry & Warwickshire

Attendance at Sub-Committee of the REC meeting on 20 February 2015

Committee Members:

Name	*Profession*	*Present*	*Notes*
Dr Helen Brittain - Chair	Clinical Psychologist Retired	Yes	
Dr Christopher Jones	Academic FY2 Doctor	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Miss Victoria Strutt	REC Assistant

APPENDIX VII - Letter of ethical approval National Research Ethics Service (SMARTChip study)

NHS
Health Research Authority
West Midlands - Coventry & Warwickshire Research Ethics Committee
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

06 June 2016

Professor Christopher Imray
Consultant Vascular and Endovascular Surgeon
University Hospitals Coventry and Warwickshire NHS Trust
Clifford Bridge Road, Walsgrave
Coventry
CV2 2DX

Dear Professor Imray,

Study title:	**SMARTChip: A field deployable blood test for stroke, capable of detecting brain ischaemia from the earliest stages of pathology**
REC reference:	**16/WM/0164**
IRAS project ID:	**198854**

Thank you for your letter of 2nd June 2016, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager, Ms Rachel Nelson,
NRESCommittee.WestMidlands-CoventryandWarwick@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Mental Capacity Act 2005

I confirm that the committee has approved this research project for the purposes of the Mental Capacity Act 2005. The committee is satisfied that the requirements of section 31 of the Act will be met in relation to research carried out as part of this project on, or in relation to, a person who lacks capacity to consent to taking part in the project.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet completed any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon as an SSA application(s) has been reviewed. In the meantime no study procedures should be initiated at non-NHS sites.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Copies of advertisement materials for research participants		
Covering letter on headed paper [Covering letter]		29 February 2016
Covering letter on headed paper [Covering letter]		01 June 2016
IRAS Application Form [IRAS_Form_17032016]		17 March 2016
IRAS Checklist XML [Checklist_02062016]		02 June 2016
Non-validated questionnaire [Evaluation questionnaire - Patients]	v1.0	29 February 2016
Non-validated questionnaire [Evaluation questionnaire - representatives]	v1.0	29 February 2016
Other [Letter confirming SMARTChip = SMARTCap]		21 January 2016
Other [Sarissa Biomedical IVD000738]		27 June 2014
Other [Response to question A13]	v1.0	04 March 2016
Other [Protocol - clean]	v1.2	01 June 2016
Participant consent form [Assent form - consultees - clean]	v1.3	01 June 2016
Participant consent form [Assent form - consultees UHNM only -	v1.3	01 June 2016

clean]		
Participant consent form [Consent form - controls - clean]	v1.3	02 June 2016
Participant consent form [Consent form - UHNM spasticity sub-study controls - clean]	v1.3	02 June 2016
Participant information sheet (PIS) [Feeding Controls sub study]	1.2	04 April 2016
Participant information sheet (PIS) [PIS - consultees - clean]	1.4	01 June 2016
Participant information sheet (PIS) [PIS - consultees UHNM only - clean]	v1.4	01 June 2016
Participant information sheet (PIS) [PIS - controls - clean]	v1.3	02 June 2016
Summary CV for Chief Investigator (CI) [Chief Investigator's CV]		01 September 2015
Summary CV for student [Student CV - Ali Wasif]		29 February 2016
Summary CV for supervisor (student research) [Academic supervisor's CV - Dr Anand Pandyan]		29 February 2016

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

16/WM/0164	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project.

Yours sincerely,

Dr Helen Brittain
Chair

Email:NRESCommittee.WestMidlands-CoventryandWarwick@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Mrs Katie Bruce*

Ms Isabella Petric

APPENDIX VIII - NIH Stroke Scale

NIH STROKE SCALE

Patient Identification. ___ ___-___ ___ ___-___ ___ ___

Pt. Date of Birth ___ ___/___ ___/___ ___

Hospital _____(___ ___-___ ___)

Date of Exam ___ ___/___ ___/___ ___

Interval: [ ] Baseline [ ] 2 hours post treatment [ ] 24 hours post onset of symptoms ±20 minutes [ ] 7-10 days
[ ] 3 months [ ] Other _____(___ ___)

Time: ___ ___:___ ___ [ ]am [ ]pm

Person Administering Scale _____

Administer stroke scale items in the order listed. Record performance in each category after each subscale exam. Do not go back and change scores. Follow directions provided for each exam technique. Scores should reflect what the patient does, not what the clinician thinks the patient can do. The clinician should record answers while administering the exam and work quickly. Except where indicated, the patient should not be coached (i.e., repeated requests to patient to make a special effort).

Instructions	Scale Definition	Score
1a. Level of Consciousness: The investigator must choose a response if a full evaluation is prevented by such obstacles as an endotracheal tube, language barrier, orotracheal trauma/bandages. A 3 is scored only if the patient makes no movement (other than reflexive posturing) in response to noxious stimulation.	0 = **Alert;** keenly responsive. 1 = **Not alert;** but arousable by minor stimulation to obey, answer, or respond. 2 = **Not alert;** requires repeated stimulation to attend, or is obtunded and requires strong or painful stimulation to make movements (not stereotyped). 3 = Responds only with reflex motor or autonomic effects or totally unresponsive, flaccid, and areflexic.	_____
1b. LOC Questions: The patient is asked the month and his/her age. The answer must be correct - there is no partial credit for being close. Aphasic and stuporous patients who do not comprehend the questions will score 2. Patients unable to speak because of endotracheal intubation, orotracheal trauma, severe dysarthria from any cause, language barrier, or any other problem not secondary to aphasia are given a 1. It is important that only the initial answer be graded and that the examiner not "help" the patient with verbal or non-verbal cues.	0 = **Answers** both questions correctly. 1 = **Answers** one question correctly. 2 = **Answers** neither question correctly.	_____
1c. LOC Commands: The patient is asked to open and close the eyes and then to grip and release the non-paretic hand. Substitute another one step command if the hands cannot be used. Credit is given if an unequivocal attempt is made but not completed due to weakness. If the patient does not respond to command, the task should be demonstrated to him or her (pantomime), and the result scored (i.e., follows none, one or two commands). Patients with trauma, amputation, or other physical impediments should be given suitable one-step commands. Only the first attempt is scored.	0 = **Performs** both tasks correctly. 1 = **Performs** one task correctly. 2 = **Performs** neither task correctly.	_____
2. Best Gaze: Only horizontal eye movements will be tested. Voluntary or reflexive (oculocephalic) eye movements will be scored, but caloric testing is not done. If the patient has a conjugate deviation of the eyes that can be overcome by voluntary or reflexive activity, the score will be 1. If a patient has an isolated peripheral nerve paresis (CN III, IV or VI), score a 1. Gaze is testable in all aphasic patients. Patients with ocular trauma, bandages, pre-existing blindness, or other disorder of visual acuity or fields should be tested with reflexive movements, and a choice made by the investigator. Establishing eye contact and then moving about the patient from side to side will occasionally clarify the presence of a partial gaze palsy.	0 = **Normal.** 1 = **Partial gaze palsy;** gaze is abnormal in one or both eyes, but forced deviation or total gaze paresis is not present. 2 = **Forced deviation,** or total gaze paresis not overcome by the oculocephalic maneuver.	_____

Rev 10/1/2003

NIH STROKE SCALE

Patient Identification. ___ ___-___ ___ ___-___ ___ ___

Pt. Date of Birth ___ ___/___ ___/___ ___

Hospital _____(___ ___-___ ___)

Date of Exam ___ ___/___ ___/___ ___

Interval: [] Baseline [] 2 hours post treatment [] 24 hours post onset of symptoms ±20 minutes [] 7-10 days
[] 3 months [] Other _____(___ ___)

3. Visual: Visual fields (upper and lower quadrants) are tested by confrontation, using finger counting or visual threat, as appropriate. Patients may be encouraged, but if they look at the side of the moving fingers appropriately, this can be scored as normal. If there is unilateral blindness or enucleation, visual fields in the remaining eye are scored. Score 1 only if a clear-cut asymmetry, including quadrantanopia, is found. If patient is blind from any cause, score 3. Double simultaneous stimulation is performed at this point. If there is extinction, patient receives a 1, and the results are used to respond to item 11.	0 = **No visual loss.** 1 = **Partial hemianopia.** 2 = **Complete hemianopia.** 3 = **Bilateral hemianopia** (blind including cortical blindness).	_____
4. Facial Palsy: Ask – or use pantomime to encourage – the patient to show teeth or raise eyebrows and close eyes. Score symmetry of grimace in response to noxious stimuli in the poorly responsive or non-comprehending patient. If facial trauma/bandages, orotracheal tube, tape or other physical barriers obscure the face, these should be removed to the extent possible.	0 = **Normal** symmetrical movements. 1 = **Minor paralysis** (flattened nasolabial fold, asymmetry on smiling). 2 = **Partial paralysis** (total or near-total paralysis of lower face). 3 = **Complete paralysis** of one or both sides (absence of facial movement in the upper and lower face).	_____
5. Motor Arm: The limb is placed in the appropriate position: extend the arms (palms down) 90 degrees (if sitting) or 45 degrees (if supine). Drift is scored if the arm falls before 10 seconds. The aphasic patient is encouraged using urgency in the voice and pantomime, but not noxious stimulation. Each limb is tested in turn, beginning with the non-paretic arm. Only in the case of amputation or joint fusion at the shoulder, the examiner should record the score as untestable (UN), and clearly write the explanation for this choice.	0 = **No drift**; limb holds 90 (or 45) degrees for full 10 seconds. 1 = **Drift**; limb holds 90 (or 45) degrees, but drifts down before full 10 seconds; does not hit bed or other support. 2 = **Some effort against gravity**; limb cannot get to or maintain (if cued) 90 (or 45) degrees, drifts down to bed, but has some effort against gravity. 3 = **No effort against gravity**; limb falls. 4 = **No movement.** UN = **Amputation** or joint fusion, explain: _____ 5a. **Left Arm** 5b. **Right Arm**	_____ _____
6. Motor Leg: The limb is placed in the appropriate position: hold the leg at 30 degrees (always tested supine). Drift is scored if the leg falls before 5 seconds. The aphasic patient is encouraged using urgency in the voice and pantomime, but not noxious stimulation. Each limb is tested in turn, beginning with the non-paretic leg. Only in the case of amputation or joint fusion at the hip, the examiner should record the score as untestable (UN), and clearly write the explanation for this choice.	0 = **No drift**; leg holds 30-degree position for full 5 seconds. 1 = **Drift**; leg falls by the end of the 5-second period but does not hit bed. 2 = **Some effort against gravity**; leg falls to bed by 5 seconds, but has some effort against gravity. 3 = **No effort against gravity**; leg falls to bed immediately. 4 = **No movement.** UN = **Amputation** or joint fusion, explain: _____ 6a. **Left Leg** 6b. **Right Leg**	_____

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NIH STROKE SCALE

Patient Identification. ___ ___-___ ___ ___-___ ___ ___

Pt. Date of Birth ___ ___/___ ___/___ ___

Hospital _____(___ ___-___ ___)

Date of Exam ___ ___/___ ___/___ ___

Interval: [] Baseline [] 2 hours post treatment [] 24 hours post onset of symptoms ±20 minutes [] 7-10 days [] 3 months [] Other _____(___ ___)

7. Limb Ataxia: This item is aimed at finding evidence of a unilateral cerebellar lesion. Test with eyes open. In case of visual defect, ensure testing is done in intact visual field. The finger-nose-finger and heel-shin tests are performed on both sides, and ataxia is scored only if present out of proportion to weakness. Ataxia is absent in the patient who cannot understand or is paralyzed. Only in the case of amputation or joint fusion, the examiner should record the score as untestable (UN), and clearly write the explanation for this choice. In case of blindness, test by having the patient touch nose from extended arm position.	0 = **Absent.** 1 = **Present in one limb.** 2 = **Present in two limbs.** UN = **Amputation** or joint fusion, explain: _____	_____
8. Sensory: Sensation or grimace to pinprick when tested, or withdrawal from noxious stimulus in the obtunded or aphasic patient. Only sensory loss attributed to stroke is scored as abnormal and the examiner should test as many body areas (arms [not hands], legs, trunk, face) as needed to accurately check for hemisensory loss. A score of 2, "severe or total sensory loss," should only be given when a severe or total loss of sensation can be clearly demonstrated. Stuporous and aphasic patients will, therefore, probably score 1 or 0. The patient with brainstem stroke who has bilateral loss of sensation is scored 2. If the patient does not respond and is quadriplegic, score 2. Patients in a coma (item 1a=3) are automatically given a 2 on this item.	0 = **Normal**; no sensory loss. 1 = **Mild-to-moderate sensory loss**; patient feels pinprick is less sharp or is dull on the affected side; or there is a loss of superficial pain with pinprick, but patient is aware of being touched. 2 = **Severe to total sensory loss**; patient is not aware of being touched in the face, arm, and leg.	_____
9. Best Language: A great deal of information about comprehension will be obtained during the preceding sections of the examination. For this scale item, the patient is asked to describe what is happening in the attached picture, to name the items on the attached naming sheet and to read from the attached list of sentences. Comprehension is judged from responses here, as well as to all of the commands in the preceding general neurological exam. If visual loss interferes with the tests, ask the patient to identify objects placed in the hand, repeat, and produce speech. The intubated patient should be asked to write. The patient in a coma (item 1a=3) will automatically score 3 on this item. The examiner must choose a score for the patient with stupor or limited cooperation, but a score of 3 should be used only if the patient is mute and follows no one-step commands.	0 = **No aphasia**; normal. 1 = **Mild-to-moderate aphasia**; some obvious loss of fluency or facility of comprehension, without significant limitation on ideas expressed or form of expression. Reduction of speech and/or comprehension, however, makes conversation about provided materials difficult or impossible. For example, in conversation about provided materials, examiner can identify picture or naming card content from patient's response. 2 = **Severe aphasia**; all communication is through fragmentary expression; great need for inference, questioning, and guessing by the listener. Range of information that can be exchanged is limited; listener carries burden of communication. Examiner cannot identify materials provided from patient response. 3 = **Mute, global aphasia**; no usable speech or auditory comprehension.	_____
10. Dysarthria: If patient is thought to be normal, an adequate sample of speech must be obtained by asking patient to read or repeat words from the attached list. If the patient has severe aphasia, the clarity of articulation of spontaneous speech can be rated. Only if the patient is intubated or has other physical barriers to producing speech, the examiner should record the score as untestable (UN), and clearly write an explanation for this choice. Do not tell the patient why he or she is being tested.	0 = **Normal.** 1 = **Mild-to-moderate dysarthria**; patient slurs at least some words and, at worst, can be understood with some difficulty. 2 = **Severe dysarthria**; patient's speech is so slurred as to be unintelligible in the absence of or out of proportion to any dysphasia, or is mute/anarthric. UN = **Intubated** or other physical barrier, explain:_____	_____

Rev 10/1/2003

NIH STROKE SCALE

Patient Identification. ___ ___-___ ___ ___-___ ___ ___

Pt. Date of Birth ___ ___/___ ___/___ ___

Hospital _____(___ ___-___ ___)

Date of Exam ___ ___/___ ___/___ ___

Interval: [] Baseline [] 2 hours post treatment [] 24 hours post onset of symptoms ±20 minutes [] 7-10 days
[] 3 months [] Other _____(___ ___)

11. **Extinction and Inattention (formerly Neglect):** Sufficient information to identify neglect may be obtained during the prior testing. If the patient has a severe visual loss preventing visual double simultaneous stimulation, and the cutaneous stimuli are normal, the score is normal. If the patient has aphasia but does appear to attend to both sides, the score is normal. The presence of visual spatial neglect or anosagnosia may also be taken as evidence of abnormality. Since the abnormality is scored only if present, the item is never untestable.	0 = **No abnormality.** 1 = **Visual, tactile, auditory, spatial, or personal inattention** or extinction to bilateral simultaneous stimulation in one of the sensory modalities. 2 = **Profound hemi-inattention or extinction to more than one modality;** does not recognize own hand or orients to only one side of space.	_____

_____

_____

Rev 10/1/2003

COOKIE
JAR

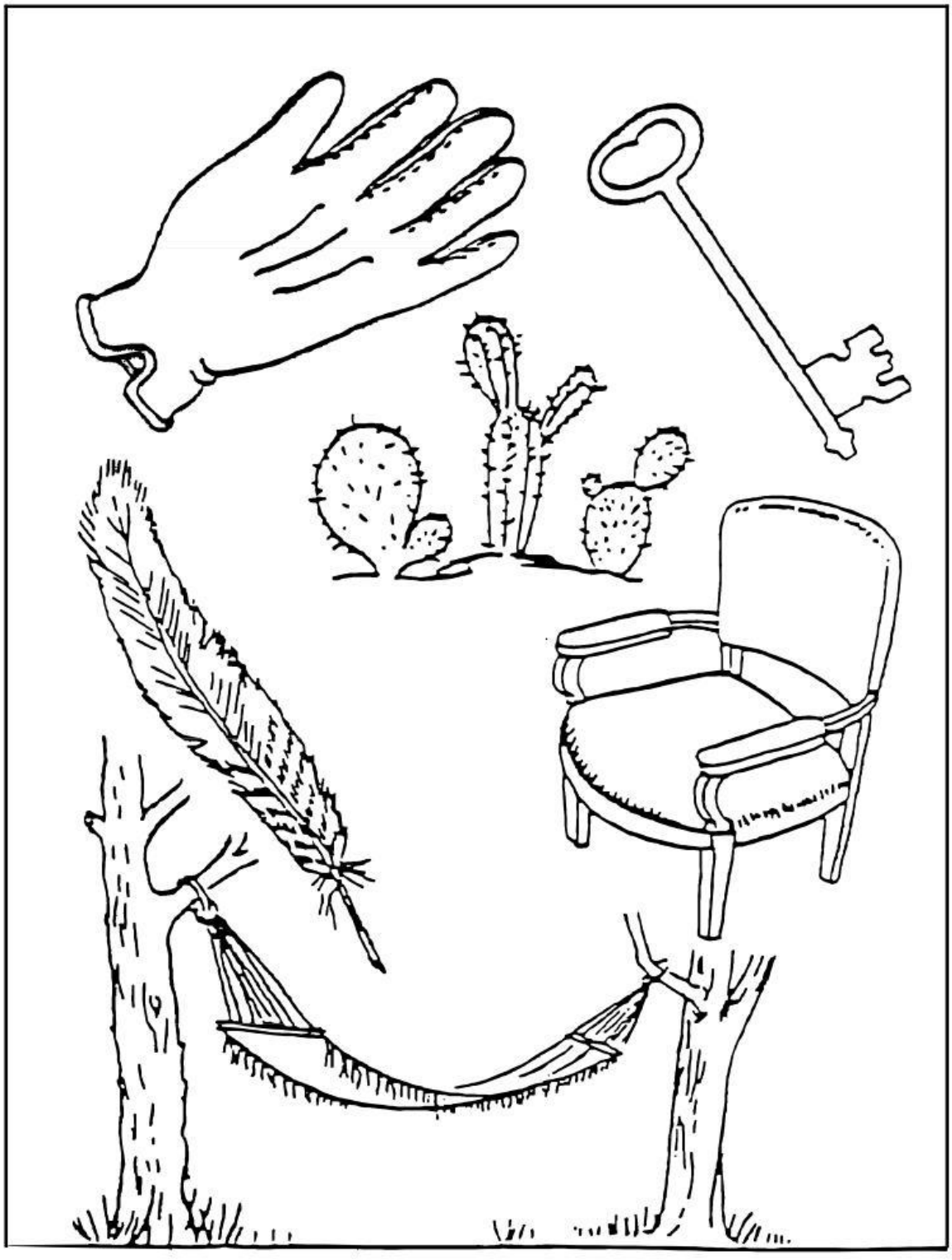
You know how.

Down to earth.

I got home from work.

Near the table in the dining room.

They heard him speak on the radio last night.



MAMA

TIP – TOP

FIFTY – FIFTY

THANKS

HUCKLEBERRY

BASEBALL PLAYER

MODIFIED RANKIN SCALE (MRS)

Patient Name:
Rater Name:
Date:

Score	Description
0	No symptoms at all
1	No significant disability despite symptoms; able to carry out all usual duties and activities
2	Slight disability; unable to carry out all previous activities, but able to look after own affairs without assistance
3	Moderate disability; requiring some help, but able to walk without assistance
4	Moderately severe disability; unable to walk without assistance and unable to attend to own bodily needs without assistance
5	Severe disability; bedridden, incontinent and requiring constant nursing care and attention
6	Dead

TOTAL (0–6): _____

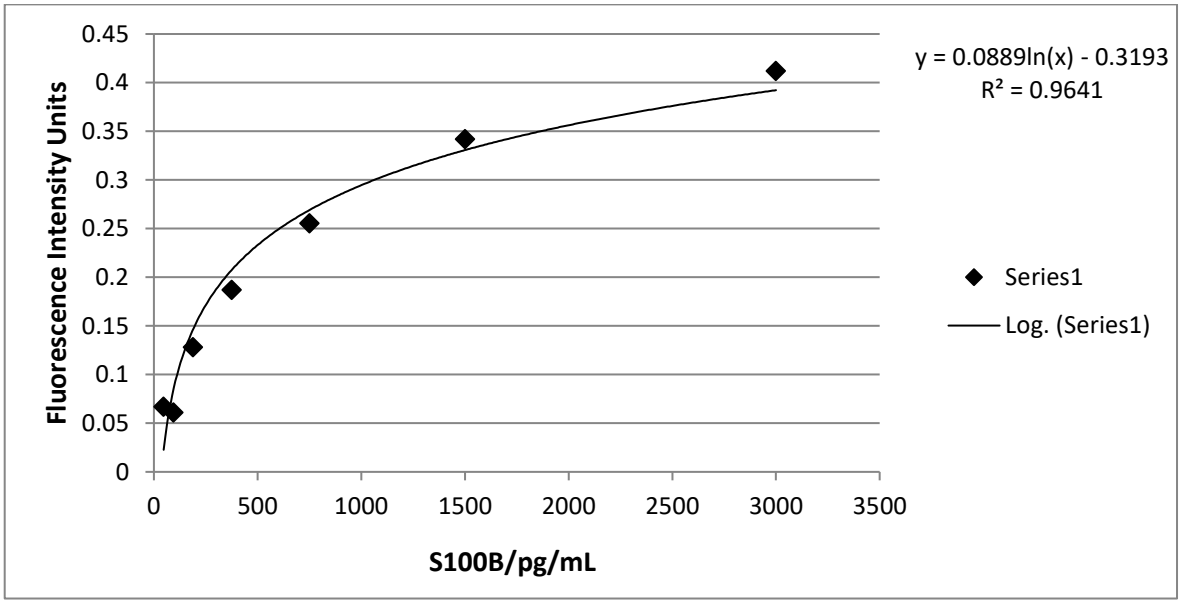
References

Rankin J. "Cerebral vascular accidents in patients over the age of 60."
Scott Med J 1957;2:200-15

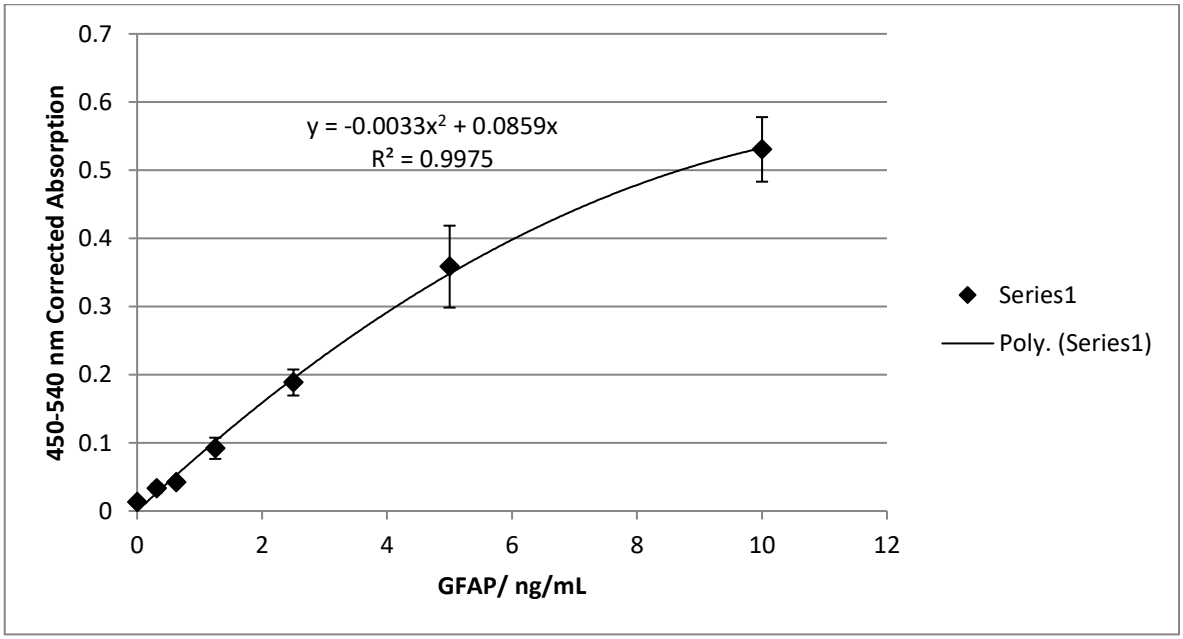
Bonita R, Beaglehole R. "Modification of Rankin Scale: Recovery of motor function after stroke."
***Stroke* 1988 Dec;19(12):1497-1500**

Van Swieten JC, Koudstaal PJ, Visser MC, Schouten HJ, van Gijn J. "Interobserver agreement for the assessment of handicap in stroke patients."
***Stroke* 1988;19(5):604-7**

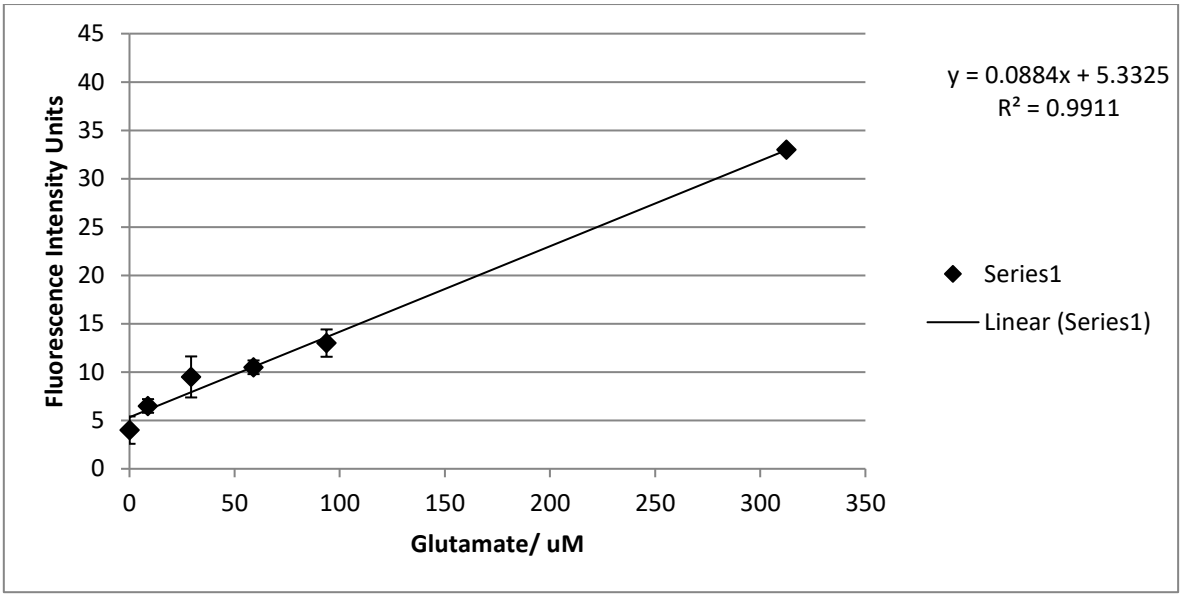
APPENDIX X - Biomarkers standard curves



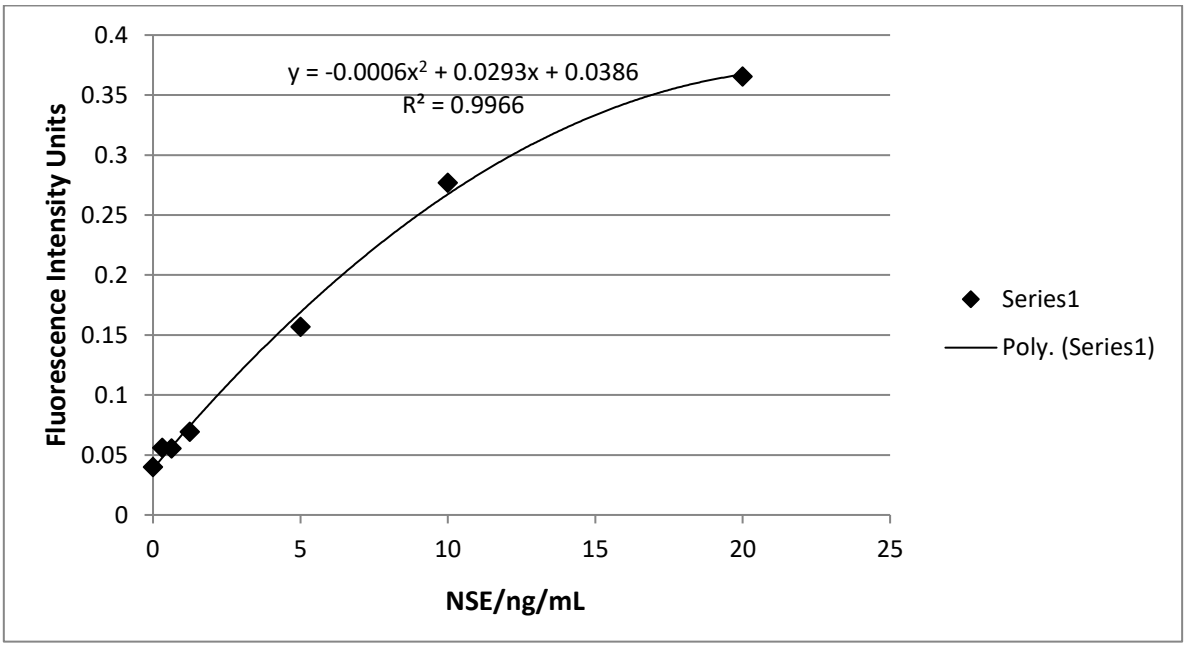
S100B standard curve



GFAP standard curve



Glutamate standard curve



NSE standard curve

abcam®
discover more

ab138883

Glutamate Assay Kit

(Fluorometric)

Instructions for Use

For quantifying Glutamic acid in various biological samples

This product is for research use only and is not intended for diagnostic use.

Table of Contents

1. Introduction

Glutamic acid is one of the 20 proteinogenic amino acids. The carboxylate anions and salts of Glutamic acid are known as glutamates. Glutamate is an important neurotransmitter which plays a key role in long-term potentiation and is important for learning and memory. Glutamic acid is the precursor of GABA but has somewhat the opposite function; it might play a role in the normal function of the heart and the prostate. As one of the few nutrients that crosses the blood-brain barrier, Glutamic acid is used in the treatment of diseases such as depression, ADD and ADHD, fatigue, alcoholism, epilepsy, muscular dystrophy, mental retardation, and schizophrenia.

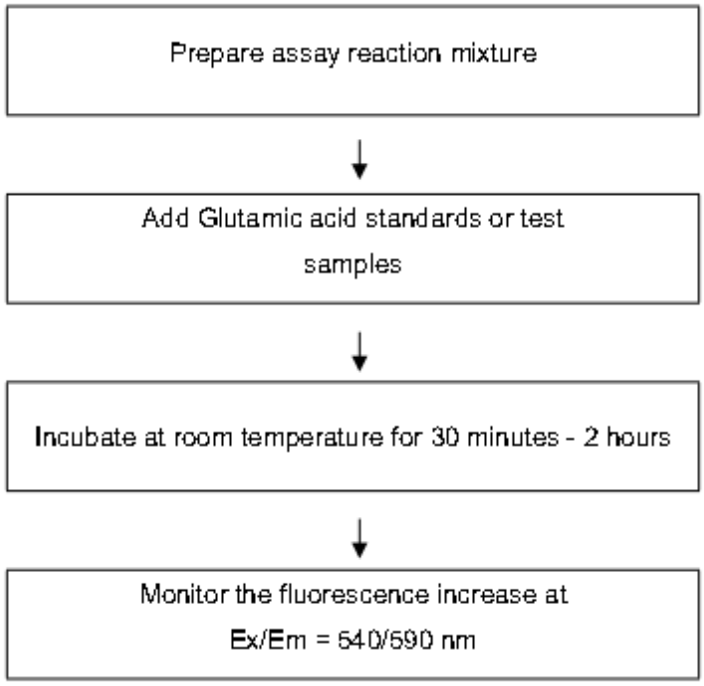
ab138883 provides a quick and sensitive method for the measurement of Glutamic acid in various biological samples. In the assay, the coupled enzyme system catalyzes the reaction between L-Glutamic acid and NADP to produce NADPH, which is specifically recognized by NADPH sensor and recycled back to NADP. A red fluorescence product is produced during the reaction. The signal can be read by either a fluorescence microplate reader at Ex/Em = 530-570 nm/590-600 nm (optimal Ex/Em = 540 nm/590 nm) or an absorbance microplate reader at 576±5 nm. With our Glutamate Assay Kit (Fluorometric), we have detected as little as 1µM Glutamic acid in a 100 µl reaction volume. The assay is robust, and can be readily adapted for a wide variety of applications that require the measurement of Glutamic acid.

Kit Key Features

- **Sensitive:** Detect as low as 1 µM of Glutamic acid in solution.
- **Continuous:** Easily adapted to automation without a separation step.
- **Convenient:** Formulated to have minimal hands-on time. No wash step needed.
- **Non-Radioactive:** No special requirements for waste treatment.

2. Protocol Summary

Summary for One 96-well Plate



Note: Thaw all the kit components to room temperature before starting the experiment.

3. Kit Contents

Components	Amount
Component A: Enzyme Mix	1 bottle (lyophilized powder)
Component B: Assay Buffer	1 x 10 ml
Component C: NADP	1 vial
Component D: Glutamic Acid	1 vial
Component E: Dilution buffer	1 bottle 10 ml

4. Storage and Handling

Keep at -20°C. Avoid exposure to light.

5. Additional Materials Required

- 96 or 384-well microplates: Solid black microplates
- Fluorescence microplate reader

6. Assay Protocol

Note: This protocol is for one 96 - well plate.

A. Preparation of Stock Solutions:

1. Prepare NADP stock solution (200X) by adding 100 µL of Dilution Buffer (Component E) into the vial of NADP (Component C).

   Note: The unused NADP *stock solution should be divided into single use aliquots and stored at -20°C.*

2. Prepare Glutamic acid stock solution (100mM) by adding 200 µL of Dilution Buffer (Component E) into the vial of Glutamic Acid (Component D).

   Note: The unused glutamic acid stock solution should be divided into single use aliquots and stored at -20 °C

B. Preparation of Assay Reaction Mixture:

1. Add 10 mL of Assay Buffer (Component B) into the bottle of Enzyme Mixture (Component A).

2. Add 50 µL 200X NADP stock solution into the Enzyme Mixture bottle and mix them well.

Note: This glutamic acid assay mixture is enough for two 96-well plates. The unused glutamic acid assay mixture should be divided into single use aliquots and stored at -20°C.

C. Prepare serially diluted glutamic acid standards (0 to 1 mM):

1. Add 10 µL of glutamic acid stock solution into 990 µL Dilution Buffer (Component E) to generate 1 mM glutamic acid standard solution.

   Note: Diluted glutamic acid standard solution is unstable. Use within 4 hours.

2. Take 200 µL of 1 mM glutamic acid standard solution to perform 1:3 serial dilutions to get 300, 100, 30, 10, 3, 1 and 0 µM serially diluted glutamic acid standards.

3. Add serially diluted glutamic acid standards and glutamic acid containing test samples into a solid black 96-well microplate as described in Tables 1 and 2.

Table 1. Layout of glutamic acid standards and test samples in a solid black 96-well microplate

BL	BL	TS	TS	….	….						
GLU1	GLU1	….	….	….	….						
GLU2	GLU2										
GLU3	GLU3										
GLU4	GLU4										
GLU5	GLU5										
GLU6	GLU6										
GLU7	GLU7										

Note: GLU= Glutamic Acid Standards, BL=Blank Control, TS=Test Samples.

Table 2. Reagent composition for each well

Glutamic Acid Standards	**Blank Control**	**Test Sample**
Serial Dilutions*: 50 µL	Dilution Buffer : 50 µL	50 µL

**Note: Add the serially diluted glutamic acid standards from 1 µM to 1 mM into wells from GLU1 to GLU7 in duplicate.*

D. Run Glutamic Acid assay:

1. Add 50 µL of glutamic acid assay mixture into each well of glutamic acid standard, blank control, and test samples to make the total glutamic acid assay volume of 100 µL/well.

   Note: For a 384-well plate, add 25 µL of sample and 25 µL of glutamic acid assay mixture into each well.

2. Incubate the reaction at room temperature for 30 minutes to 2 hours, protected from light.

3. Monitor the fluorescence increase by using a fluorescence plate reader at Ex/Em = 530-570/590-600 nm (optimal Ex/Em = 540/590 nm).

   Note: The contents of the plate can also be transferred to a white clear bottom plate and read by absorbance microplate reader at the wavelength of 576 ± 5 nm. The absorption detection has lower sensitivity compared to the fluorescence reading.

7. Data Analysis

The fluorescence in blank wells (with the dilution buffer only) is used as a control, and is subtracted from the values for those wells with the glutamic acid reaction.

Note: The fluorescence background increases with time, thus it is important to subtract the fluorescence intensity value of the blank wells for each data point.

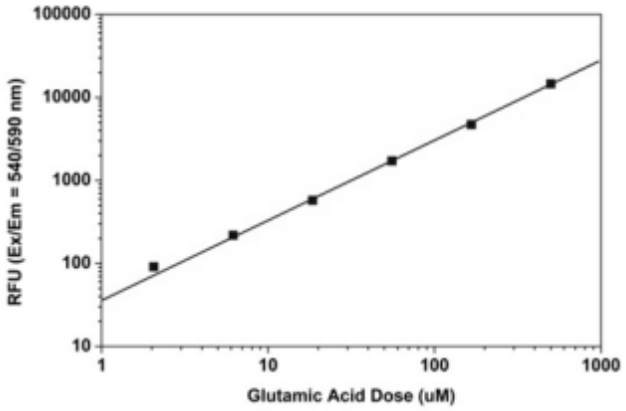


Figure 1. Glutamic acid dose response was measured with ab138883 in a black 96-well plate using a microplate reader. As low as 1 µM glutamic acid was detected with 1 hour incubation.

8. Troubleshooting

Problem	Reason	Solution
Assay not working	Assay buffer at wrong temperature	Assay buffer must not be chilled - needs to be at RT
	Protocol step missed	Re-read and follow the protocol exactly
	Plate read at incorrect wavelength	Ensure you are using appropriate reader and filter settings (refer to datasheet)
	Unsuitable microtiter plate for assay	Fluorescence: Black plates (clear bottoms); Luminescence: White plates; Colorimetry: Clear plates. If critical, datasheet will indicate whether to use flat- or U-shaped wells
Unexpected results	Measured at wrong wavelength	Use appropriate reader and filter settings described in datasheet
	Samples contain impeding substances	Troubleshoot and also consider deproteinizing samples
	Unsuitable sample type	Use recommended samples types as listed on the datasheet
	Sample readings are outside linear range	Concentrate/ dilute samples to be in linear range

Problem	Reason	Solution
Samples with inconsistent readings	Unsuitable sample type	Refer to datasheet for details about incompatible samples
	Samples prepared in the wrong buffer	Use the assay buffer provided (or refer to datasheet for instructions)
	Samples not deproteinized (if indicated on datasheet)	Use the ***10kDa spin column (ab93349)*** or ***Deproteinizing sample preparation kit (ab93299)***
	Cell/ tissue samples not sufficiently homogenized	Increase sonication time/ number of strokes with the Dounce homogenizer
	Too many freeze-thaw cycles	Aliquot samples to reduce the number of freeze-thaw cycles
	Samples contain impeding substances	Troubleshoot and also consider deproteinizing samples
	Samples are too old or incorrectly stored	Use freshly made samples and store at recommended temperature until use
Lower/ Higher readings in samples and standards	Not fully thawed kit components	Wait for components to thaw completely and gently mix prior use
	Out-of-date kit or incorrectly stored reagents	Always check expiry date and store kit components as recommended on the datasheet
	Reagents sitting for extended periods on ice	Try to prepare a fresh reaction mix prior to each use
	Incorrect incubation time/ temperature	Refer to datasheet for recommended incubation time and/ or temperature
	Incorrect amounts used	Check pipette is calibrated correctly (always use smallest volume pipette that can pipette entire volume)

Standard curve is not linear	Not fully thawed kit components	Wait for components to thaw completely and gently mix prior use
	Pipetting errors when setting up the standard curve	Try not to pipette too small volumes
	Incorrect pipetting when preparing the reaction mix	Always prepare a master mix
	Air bubbles in wells	Air bubbles will interfere with readings; try to avoid producing air bubbles and always remove bubbles prior to reading plates
	Concentration of standard stock incorrect	Recheck datasheet for recommended concentrations of standard stocks
	Errors in standard curve calculations	Refer to datasheet and re-check the calculations
	Use of other reagents than those provided with the kit	Use fresh components from the same kit

For further technical questions please do not hesitate to contact us by email (technical@abcam.com) or phone (select "*contact us*" on www.abcam.com for the phone number for your region).

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Tel: +81-(0)3-6231-0940
www.abcam.co.jp

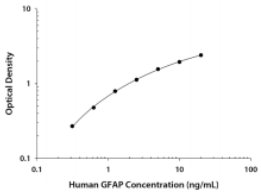
CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y axis against the concentration on the x axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human GFAP concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is only for demonstration purposes. A standard curve should be generated for each set of samples assayed.



SPECIFICITY

The following factors prepared at 200 ng/mL were assayed and exhibited no cross-reactivity or interference.

Recombinant human:
Lamin B1
Menin
Presenilin-1 N-Terminal Fragment
S100A1
S100B

TECHNICAL HINTS & LIMITATIONS

- We recommend the use of R&D Systems' Reagent Diluent Concentrate 2 (Catalog # DY995) to prepare Reagent Diluent for use in this assay.
- The use of high quality Bovine Serum Albumin (BSA) for the Reagent Diluent is crucial for the optimum performance of the DuoSet ELISA Development kit. Impurities such as proteases, binding proteins, soluble receptors or other interfering substances can be found to varying degrees in virtually all BSA preparations and can inhibit or interfere with the detection of certain analytes. If the standard curve appears suppressed, consider evaluating a different preparation of BSA.
- It is suggested to start Reagent Diluent optimization for serum and plasma samples by using PBS supplemented with 1.0-10% animal serum. Do not use buffers with animal serum to reconstitute or dilute the Detection Antibody or Streptavidin-HRP.
- It is important that the Reagent Diluent selected for reconstitution and dilution of the standard reflects the environment of the samples being measured.
- Avoid microbial contamination of reagents and buffers.
- A thorough and consistent wash technique is essential for proper assay performance. Wash Buffer should be dispensed forcefully and removed completely from the wells by aspiration or decanting. Remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- Individual results may vary due to differences in technique, plasticware and water sources.
- It is recommended that all standards and samples be assayed in duplicate.
- The use of PBS from tablets may interfere in this assay.

TROUBLESHOOTING

Note: *For more detailed troubleshooting, please visit:*
www.RnDSystems.com/ELISADevelopment

Poor Standard Curve

- Impure BSA used for Reagent Diluent preparation.
- Improper reconstitution and/or storage of standard.
- Improper dilution of highest standard and standard curve.
- Incomplete washing and/or aspiration of wells.
- Unequal volumes added to wells/pipetting error.
- Incorrect incubation times or temperatures.

Poor Precision

- Unequal volumes added to wells/pipetting error.
- Incomplete washing and/or aspiration of wells.
- Unequal mixing of reagents.

Low or No Color Development

- Inadequate volume of substrate added to wells.
- Incorrect incubation times or temperatures.
- Impure BSA used for Reagent Diluent preparation.

DuoSet® ELISA
DEVELOPMENT SYSTEM

Human GFAP

Catalog Number: DY2594-05 (5 plates)

INTENDED USE

For the development of sandwich ELISAs to measure natural and recombinant human Glial Fibrillary Acidic Protein (GFAP). The Reagent Diluent recommended may be suitable for most cell culture supernate, serum, and plasma samples. The Reagent Diluent selected for use can alter the performance of an immunoassay. Reagent Diluent optimization for samples with complex matrices such as serum and plasma, may improve their performance in this assay.

This kit contains sufficient materials to run ELISAs on at least five 96-well plates, provided the following conditions are met:

- The reagents are prepared as described in this package insert.
- The assay is run as described in the General ELISA Protocol.
- The recommended microplates, buffers, diluents, substrates, and solutions are used.

This package insert must be read in its entirety before using this product.
Refer to the Certificate of Analysis for component concentrations as they may vary.
For research use only. Not for use in diagnostic procedures.

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R&D SYSTEMS
a bio-techne brand

OTHER MATERIALS & SOLUTIONS REQUIRED

DuoSet Ancillary Reagent Kit 2 (5 plates):
(R&D Systems, Catalog # DY008) containing 96 well microplates, plate sealers, substrate solution, stop solution, plate coating buffer (PBS), wash buffer, and Reagent Diluent Concentrate 2.

The components listed above may be purchased separately:

96 well microplates: (R&D Systems, Catalog # DY990).

Plate Sealers: (R&D Systems, Catalog # DY992).

PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2 7.4, 0.2 µm filtered (R&D Systems, Catalog # DY006).

Wash Buffer: 0.05% Tween® 20 in PBS, pH 7.2 7.4 (R&D Systems, Catalog # WA126).

Reagent Diluent: 1% BSA in PBS, pH 7.2 7.4, 0.2 µm filtered (R&D Systems, Catalog # DY995).
Quality of BSA is critical (see Technical Hints).

Substrate Solution: 1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine) (R&D Systems, Catalog # DY999).

Stop Solution: 2 N H_2SO_4 (R&D Systems, Catalog # DY994).

PRECAUTIONS

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

The Stop Solution suggested for use with this kit is an acid solution.

The Color Reagent B suggested for use with this kit may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

CALIBRATION

This DuoSet is calibrated against a highly purified *E. coli* expressed recombinant human GFAP (aa 292 432) produced at R&D Systems.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2 8 °C. Do not use past kit expiration date.

DESCRIPTION	PART #	# VIALS	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human GFAP Capture Antibody	841350	1 vial	Refer to the lot-specific Certificate of Analysis (C of A) for storage conditions.
Human GFAP Detection Antibody	841351	1 vial	
Human GFAP Standard	841352	2 vials	
Streptavidin-HRP	893975	1 vial	

REAGENT PREPARATION

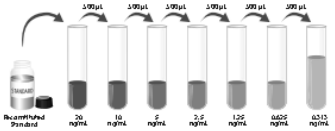
Bring all reagents to room temperature before use. Allow all components to sit for a minimum of 15 minutes with gentle agitation after initial reconstitution. Working dilutions should be prepared and used immediately.

Streptavidin-HRP: 2.0 mL of streptavidin conjugated to horseradish peroxidase. Dilute to the working concentration specified on the vial label using Reagent Diluent.

Mouse Anti-Human GFAP Capture Antibody: Refer to the lot specific C of A for amount supplied. Reconstitute with 0.5 mL of PBS. Dilute in PBS without carrier protein to the working concentration indicated on the C of A.

Biotinylated Sheep Anti-Human GFAP Detection Antibody: Refer to the lot specific C of A for amount supplied. Reconstitute with 1.0 mL of Reagent Diluent. Dilute in Reagent Diluent to the working concentration indicated on the C of A.

Recombinant Human GFAP Standard: Refer to the lot specific C of A for amount supplied. Reconstitute each vial with 0.5 mL of Reagent Diluent. A seven point standard curve using 2 fold serial dilutions in Reagent Diluent is recommended. Prepare 1000 µL of high standard per plate assayed at the concentration indicated on the C of A.



GENERAL ELISA PROTOCOL

Plate Preparation

1. Dilute the Capture Antibody to the working concentration in PBS without carrier protein. Immediately coat a 96 well microplate with 100 µL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.
2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
3. Block plates by adding 300 µL of Reagent Diluent to each well. Incubate at room temperature for a minimum of 1 hour.
4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

Assay Procedure

1. Add 100 µL of sample or standards in Reagent Diluent, or an appropriate diluent, per well. Cover with an adhesive strip and incubate 2 hours at room temperature.
2. Repeat the aspiration/wash as in step 2 of Plate Preparation.
3. Add 100 µL of the Detection Antibody, diluted in Reagent Diluent, to each well. Cover with a new adhesive strip and incubate 2 hours at room temperature.
4. Repeat the aspiration/wash as in step 2 of Plate Preparation.
5. Add 100 µL of the working dilution of Streptavidin HRP to each well. Cover the plate and incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
6. Repeat the aspiration/wash as in step 2.
7. Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
8. Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

Quantikine® ELISA

Human Enolase 2/Neuron-specific Enolase Immunoassay

Catalog Number DENL20

For the quantitative determination of human Enolase 2 concentrations in cell culture supernates, cell lysates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Enolase 2 is also known as gamma enolase or neuronal enolase. Enolase (2-phospho-D-glycerate hydrolase) is a cytoplasmic enzyme that is involved in the glycolytic pathway, in which it converts 2-phosphoglycerate to phosphoenolpyruvate. It has three members: Enolase 1, Enolase 2, and Enolase 3, which are also termed α, γ, and β enolase, respectively. They exist as several dimeric isoenzymes including αα, αβ, ββ, αγ, and γγ. The αγ and γγ isoenzymes are abundant in neurons and neuroendocrine cells, and therefore, they are also termed neuron specific enolase (NSE) (1-2). Human Enolase 2 is 434 amino acids (aa) in length. It shares 83% aa identity with human enolases 1 and 3 and 99% with its mouse orthologue.

Serum Enolase 2 levels are low in normal subjects. However, when neuronal injury occurs, it is released from the injured cells into the cerebrospinal fluid and systemic circulation. Studies have shown that elevated serum levels of Enolase 2 are commonly found among a variety of conditions associated with central nervous system damage such as stroke, traumatic brain injury, multiple sclerosis, and Alzheimer's disease (3-6). In malignant tumors of neuroendocrine origin, Enolase 2 production is increased, which usually also results in elevated serum levels of Enolase 2 (7). Such examples include small cell lung cancer, APUDoma, and neuroblastoma (8-11).

The Quantikine Human Enolase 2 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Enolase 2 in cell culture supernates, cell lysates, serum, and plasma. It contains *E. coli*-expressed recombinant human Enolase 2 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Enolase 2 showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for naturally occurring human Enolase 2.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for Enolase 2 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Enolase 2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for Enolase 2 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Enolase 2 bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Enolase 2 Microplate	893813	96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against Enolase 2.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of zip-seal. May be stored for up to 1 month at 2-8 °C.*
Enolase 2 Standard	893815	200 ng of recombinant human Enolase 2 in a buffer with preservatives; lyophilized.	Aliquot and store for up to 1 month at ≤ -20 °C.*
Enolase 2 Conjugate	893814	21 mL of polyclonal antibody against Enolase 2 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-9	895167	11 mL of a buffered protein solution with preservatives. *Assay Diluent RD1-9 may contain a precipitate. Warm to room temperature, and mix gently to dissolve. If the precipitate does not completely dissolve, mix well during use.*	
Calibrator Diluent RD5C Concentrate	895046	21 mL of a concentrated buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives.	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards.
- Human Enolase 2 Controls (optional; available from R&D Systems).

If using cell lysate samples, the following is also required:

- Cell Lysis Buffer 1 (R&D Systems, Catalog # 890713).

PRECAUTIONS

Calibrator Diluent RD5C contains sodium azide, which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

SAMPLE COLLECTION & STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Cell Lysates - Cells must be lysed prior to assay. See Sample Values section.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma on ice using heparin as an anticoagulant. Centrifuge for 15 minutes at 2-8° C at 1000 x g within 30 minutes of collection. An additional centrifugation step of the plasma at 10,000 x g for 10 minutes at 2-8° C is recommended for complete platelet removal. Assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Enolase 2 is present in platelet granules and is released upon platelet activation. Therefore, to measure circulating levels of Enolase 2, platelet-free plasma should be collected for measurement. It should be noted that many protocols for plasma preparation, including procedures recommended by the National Committee for Clinical Laboratory Standards (NCCLS), result in incomplete removal of platelets from blood.

Note: *Citrate plasma has not been validated for use in this assay. EDTA plasma is not suitable for use in this assay.*

REAGENT PREPARATION

Bring all reagents to room temperature before use.

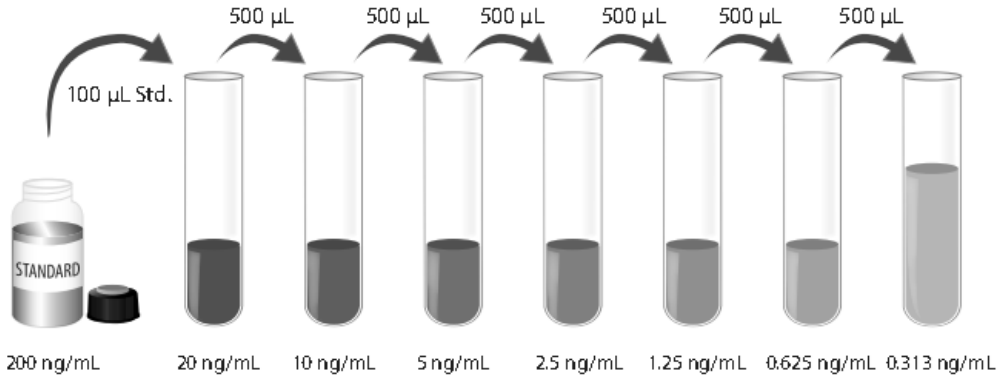
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5C (1X) - Dilute 10 mL of Calibrator Diluent RD5C Concentrate into 40 mL of deionized or distilled water to prepare 50 mL of Calibrator Diluent RD5C (1X).

Enolase 2 Standard - Reconstitute the Enolase 2 Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 200 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5C (1X) into the 20 ng/mL tube. Pipette 500 µL of Calibrator Diluent RD5C (1X) into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 20 ng/mL standard serves as the high standard. Calibrator Diluent RD5C (1X) serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent RD1-9 to each well. *May contain a precipitate. Warm to room temperature, and mix gently to dissolve. If the precipitate does not completely dissolve, mix well during use.*
4. Add 50 µL of Standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Enolase 2 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. **Protect from light.** Incubate for 30 minutes at room temperature **on the benchtop.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

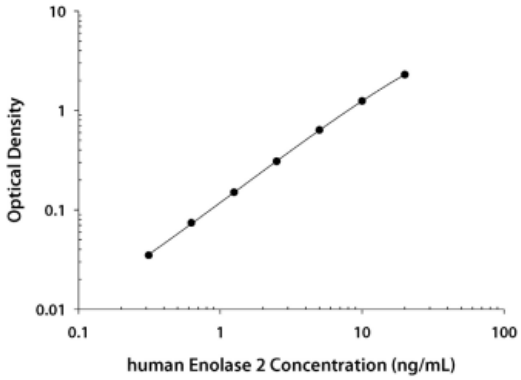
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the Enolase 2 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(ng/mL)	O.D.	Average	Corrected
0	0.014 0.018	0.016	—
0.313	0.051 0.051	0.051	0.035
0.625	0.088 0.092	0.090	0.074
1.25	0.165 0.166	0.166	0.150
2.5	0.322 0.325	0.324	0.308
5	0.650 0.652	0.651	0.635
10	1.259 1.262	1.261	1.245
20	2.280 2.339	2.310	2.294

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	2.15	8.41	13.0	1.96	8.50	12.6
Standard deviation	0.03	0.17	0.36	0.13	0.33	0.55
CV (%)	1.4	2.0	2.8	6.7	3.9	4.3

RECOVERY

The recovery of Enolase 2 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=8)	99	85-114%
Cell lysates (n=8)	105	85-115%
Serum (n=4)	95	85-110%
Platelet-poor heparin plasma (n=4)	95	85-111%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Enolase 2 were serially diluted with Calibrator Diluent RD5C (1X) to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=8)	Cell lysates* (n=4)	Serum (n=4)	Platelet-poor Heparin plasma (n=4)
1:2	Average % of Expected	101	100	100	98
	Range (%)	96-107	98-103	95-102	92-101
1:4	Average % of Expected	106	102	99	101
	Range (%)	103-110	100-103	94-101	96-106
1:8	Average % of Expected	110	103	101	98
	Range (%)	105-115	99-106	96-106	93-107
1:16	Average % of Expected	111	101	101	98
	Range (%)	99-115	98-102	95-107	91-106

*Samples were diluted prior to assay.

SENSITIVITY

Twenty assays were evaluated and the minimum detectable dose (MDD) ranged from 0.013-0.038 ng/mL. The mean MDD was 0.020 ng/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human Enolase 2 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of Enolase 2 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=15)	3.02	1.85-4.14	0.613
Heparin plasma (n=15)	6.74	3.39-13.7	3.37
Platelet-poor heparin plasma (n=20)	2.82	1.97-4.98	0.686

Cell Culture Supernates/Cell Lysates -
U-87 MG human glioblastoma/astrocytoma cells were cultured in MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, 1 mM sodium pyruvate, and 100 µg/mL streptomycin sulfate until confluent.

SK-Mel-28 human malignant melanoma cells were cultured in MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, 1 mM sodium pyruvate, and 100 µg/mL streptomycin sulfate until confluent.

MDA-MD-453 human breast cancer cells were cultured in RPMI supplemented with 10% fetal bovine serum, and 2 mM L-glutamine until confluent.

IMR-32 human neuroblastoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin sulfate until confluent.

After the supernates were harvested, cells were gently washed with cold PBS. Any excess PBS was poured off and discarded. For a T75 flask, 10 mL of Cell Lysis Buffer 1 was added and incubated at room temperature for one hour with gentle agitation. The lysates were then collected and centrifuged at 12,000 x g for 10 minutes to remove insoluble cell debris. The total protein concentration of the lysate supernates was determined by the Bradford method [Bradford, M.M. (1976) Anal. Biochem. **72**:248]. Lysates were aliquoted and stored at -70 °C.

Cell Line	Cell Culture Supernates (ng/mL)	Cell Lysates (ng/mg)
U-87 MG	1.44	221
SK-Mel-28	1.13	245
MDA-MB-453	1.16	121
IMR-32	1.15	44.4

SPECIFICITY

This assay recognizes recombinant and natural human Enolase 2. The factors listed below were prepared at 200 ng/mL in Calibrator Diluent RD5C (1X) and assayed for cross-reactivity. Preparations of the following factors at 200 ng/mL in a mid-range recombinant human Enolase 2 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:
Enolase 1
Enolase 3

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9. Ladenstein, R. *et al.* (1995) Eur. J. Cancer **31a**:637.
10. Burghuber, O.C. *et al.* (1990) Cancer 65:1386.
11. Fizazi, K. *et al.* (1998) Cancer 82:1049.

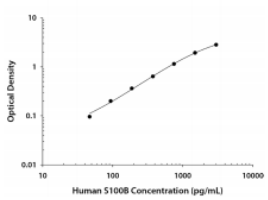
CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y axis against the concentration on the *x* axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human S100B concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is only for demonstration purposes. A standard curve should be generated for each set of samples assayed.



SPECIFICITY

The following factors prepared at 50 ng/mL were assayed and exhibited no cross reactivity or interference.

Recombinant human:
NDR1
p53
RAGE/Fc Chimera
S100A6
S100A4

This Human S100B DuoSet also recognizes bovine S100B, which may be present in Bovine Sera.

TECHNICAL HINTS & LIMITATIONS

- We recommend the use of R&D Systems' Reagent Diluent Concentrate 2 (Catalog # DY995) to prepare the Block Buffer for use in this assay.
- If assaying sample types other than cell culture supernates, each laboratory should develop and validate its own diluent. The diluent must not be used to dilute the Detection Antibody or the Streptavidin-HRP.
- It is important that the Reagent Diluent selected for reconstitution and dilution of the standard reflects the environment of the samples being measured.
- Avoid microbial contamination of reagents and buffers.
- A thorough and consistent wash technique is essential for proper assay performance. Wash Buffer should be dispensed forcefully and removed completely from the wells by aspiration or decanting. Remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- Individual results may vary due to differences in technique, plasticware and water sources.
- It is recommended that all standards and samples be assayed in duplicate.
- The use of PBS from tablets may interfere in this assay.

TROUBLESHOOTING

Note: *For more detailed troubleshooting, please visit:*
www.RnDSystems.com/ELISADevelopment

Poor Standard Curve

- Impure BSA used for Block Buffer preparation.
- Improper reconstitution and/or storage of standard.
- Improper dilution of highest standard and standard curve.
- Incomplete washing and/or aspiration of wells.
- Unequal volumes added to wells/pipetting error.
- Incorrect incubation times or temperatures.

Poor Precision

- Unequal volumes added to wells/pipetting error.
- Incomplete washing and/or aspiration of wells.
- Unequal mixing of reagents.

Low or No color Development

- Inadequate volume of substrate added to wells.
- Incorrect incubation times or temperatures.
- Impure BSA used for Block Buffer preparation.

DuoSet® ELISA DEVELOPMENT SYSTEM

Human S100B

Catalog Number: DY1820-05 (5 plates)

INTENDED USE

For the development of sandwich ELISAs to measure natural and recombinant human S100 Calcium Binding Protein B (S100B). The Reagent Diluent recommended may be suitable for most cell culture supernate, serum, and plasma samples. The Reagent Diluent selected for use can alter the performance of an immunoassay. Reagent Diluent optimization for samples with complex matrices such as serum and plasma, may improve their performance in this assay.

This kit contains sufficient materials to run ELISAs on at least five 96-well plates, provided the following conditions are met:

- The reagents are prepared as described in this package insert.
- The assay is run as described in the General ELISA Protocol.
- The recommended microplates, buffers, diluents, substrates, and solutions are used.

This package insert must be read in its entirety before using this product.
Refer to the Certificate of Analysis for component concentrations as they may vary.
For research use only. Not for use in diagnostic procedures.

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R&D SYSTEMS a biotechne brand

OTHER MATERIALS & SOLUTIONS REQUIRED

96 well microplates: (R&D Systems, Catalog # DY990).

Plate Sealers: (R&D Systems, Catalog # DY992).

PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2–7.4, 0.2 µm filtered (R&D Systems, Catalog # DY006).

Wash Buffer: 0.05% Tween® 20 in PBS, pH 7.2–7.4 (R&D Systems, Catalog # WA126).

Block Buffer: 1% BSA in PBS, pH 7.2–7.4, 0.2 µm filtered (R&D Systems, Catalog # DY995).
Quality of BSA is critical (see Technical Hints).

Reagent Diluent: 50 mM Tris, 10 mM $CaCl_2$, 0.15 M NaCl, 0.05% Brij® 35, pH 7.45–7.55, 0.2 µm filtered.

Substrate Solution: 1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine) (R&D Systems, Catalog # DY999).

Stop Solution: 2 N H_2SO_4 (R&D Systems, Catalog # DY994).

PRECAUTIONS

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

The Stop Solution suggested for use with this kit is an acid solution.

The Color Reagent B suggested for use with this kit may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

CALIBRATION

This DuoSet® is calibrated against S100B purified from bovine brain.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2–8 °C. Do not use past kit expiration date.

DESCRIPTION	PART #	# VIALS	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human S100B Capture Antibody	841420	1 vial	Refer to the lot-specific Certificate of Analysis (C of A) for storage conditions.
Human S100B Detection Antibody	841421	1 vial	
Human S100B Standard	841422	2 vials	
Streptavidin-HRP	893975	1 vial	

REAGENT PREPARATION

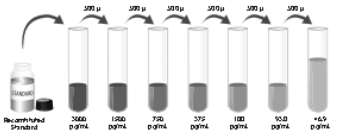
Bring all reagents to room temperature before use. Allow all components to sit for a minimum of 15 minutes with gentle agitation after initial reconstitution. Working dilutions should be prepared and used immediately.

Streptavidin-HRP: 2.0 mL of streptavidin conjugated to horseradish peroxidase. Dilute to the working concentration specified on the vial label using Reagent Diluent.

Mouse Anti-Human S100B Capture Antibody: Refer to the lot specific C of A for amount supplied. Reconstitute with 0.5 mL of PBS. Dilute in PBS without carrier protein to the working concentration indicated on the C of A.

Biotinylated Mouse Anti-Human S100B Detection Antibody: Refer to the lot specific C of A for amount supplied. Reconstitute with 1.0 mL of Reagent Diluent. Dilute in Reagent Diluent to the working concentration indicated on the C of A.

Human S100B Standard: Refer to the lot specific C of A for amount supplied. Reconstitute each vial with 0.5 mL of deionized or distilled water. A seven point standard curve using 2 fold serial dilutions in Reagent Diluent is recommended. Prepare 1000 µL of high standard per plate assayed at the concentration indicated on the C of A.



GENERAL ELISA PROTOCOL

Plate Preparation

1. Dilute the Capture Antibody to the working concentration in PBS without carrier protein. Immediately coat a 96 well microplate with 100 µL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.
2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
3. Block plates by adding 300 µL of Block Buffer to each well. Incubate at room temperature for a minimum of 1 hour.
4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

Assay Procedure

1. Add 100 µL of sample or standards in Reagent Diluent, or an appropriate diluent, per well. Cover with an adhesive strip and incubate 2 hours at room temperature.
2. Repeat the aspiration/wash as in step 2 of Plate Preparation.
3. Add 100 µL of the Detection Antibody, diluted in Reagent Diluent, to each well. Cover with a new adhesive strip and incubate 2 hours at room temperature.
4. Repeat the aspiration/wash as in step 2 of Plate Preparation.
5. Add 100 µL of the working dilution of Streptavidin-HRP to each well. Cover the plate and incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
6. Repeat the aspiration/wash as in step 2.
7. Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
8. Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

Instructions for Use

GABA ELISA

Enzyme immunoassay for the quantitative determination of GABA in human EDTA plasma, serum and urine

REF **ID59301**

Σ **96**

For illustrative purposes only.
To perform the assay the instructions for use provided with the kit have to be used.

Distributed by:

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1. INTENDED USE

The GABA ELISA Kit is intended for the quantitative determination of GABA in human EDTA plasma, serum and urine. It is for research use only.

2. PRINCIPLE OF THE TEST

This assay is based on the method of competitive enzyme linked immunoassays. The sample preparation includes the addition of a derivatization reagent for GABA derivatization. Afterwards, the treated samples are incubated in wells of a microtiter plate coated with a polyclonal antibody against GABA-derivative, together with assayreagent containing GABA-derivative (tracer). During the incubation period the target GABA in the sample competes with the tracer for the binding of the polyclonal antibodies on the wall of the microtiter wells. GABA in the sample displaces the tracer out of the binding to the antibodies. Therefore, the concentration of antibody-bound tracer is inverse proportional to the GABA concentration in the sample.

During the second incubation step, a peroxidase conjugate is added to each microtiter well to detect the tracer. After washing away the unbound components tetramethylbenzidine (TMB) is added as a peroxidase substrate. Finally, the enzymatic reaction is terminated by an acidic stop solution. The color changes from blue to yellow, and the absorbance is measured in a photometer at 450 nm. The intensity of the yellow color is inverse proportional to the GABA concentration in the sample; this means, high GABA concentration in the sample reduces the concentration of antibody-bound tracer and lowers the photometric signal. A dose response curve of absorbance unit (optical density, OD at 450 nm) vs. concentration is generated using the values obtained from the standards. GABA present in the patient samples (serum or EDTA plasma) is determined directly from this curve. For urine samples the ELISA results are normalized to the creatinine concentration in the samples. Therefore, a parallel determination of the creatinine concentration is required.

3. MATERIAL SUPPLIED

Catalog No	Content	Kit Components	Quantity
K7012MTP	PLATE	One holder with precoated strips	12 x 8 wells
K7012ST	STD	Standards diluted in reaction buffer (ready to use)	6 x 1 vial
K7012KO1 K7012KO2	CTRL 1 CTRL 2	Controls diluted in reaction buffer (ready to use)	2 x 1 vial
K7012WP	WASHBUF	Wash buffer concentrate (10-fold)	2 x 100 ml
K7012AR	ASYREAG	Assay reagent (lyophilized)	3 x 1 vial
K7012K	CONJ	POD conjungate (concentrate)	120 µl
K7012KV	CONJBUF	Conjugate stabilizing buffer	24 ml
K7012RP	DERBUF	Reaction buffer	2 x 25 ml
K7012DR	DER	Derivatization reagent	3 x 1 vial
K7012LM	DMSO	Dimethylsulfoxide (DMSO)	2 ml
K7012SL	CODIL	Dilution buffer after derivatization	28 ml
K7012TMB	SUB	TMB substrate	25 ml
K7012AC	STOP	Stop solution	15 ml

4. MATERIAL REQUIRED BUT NOT SUPPLIED

- Double distilled water (aqua bidest.)
- Precision pipettors and disposable tips to deliver 10-1000 µl
- Foil to cover the microtiter plate
- Horizontal microtiter plate shaker
- A multi-channel dispenser or repeating dispenser
- Centrifuge capable of 10000 x g
- Vortex-Mixer
- Standard laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader at 450 nm
  (reference wave length 620 or 690 nm)

5. PREPARATION AND STORAGE OF REAGENTS

- To run assay more than once ensure that reagents are stored at conditions stated on the label. **Prepare only the appropriate amount necessary for each assay**. The kit can be used up to 3 times within the expiry date stated on the label.

- Reagents with a volume less than **100 µl** should be centrifuged before use to avoid loss of volume.

- Dilute the **wash buffer concentrate (WASHBUF)** with aqua bidest. **1:10** before use (**100 ml WASHBUF + 900 ml aqua bidest**.), mix well. Crystals may occur due to high salt concentration in the stock solution. The crystals must be redissolved at room temperature or at 37°C using a water bath before dilution. The WASHBUF is stable at **2-8°C** until the expiry date stated on the label. Diluted buffer solution can be stored in a closed flask at **2-8°C for one month**.

- **Standards (STD) and controls (CTRL1, CTRL2)** are already diluted in reaction buffer (REABUF). Store standards and controls frozen at **-20°C**, thaw before use in the test, and re-freeze immediately after use. Standards and controls can be re-frozen up to 3 times.

- **DMSO** could crystallize at 4°C. Dissolve the crystals at 20-25°C in a water bath.

- Dissolve the content of one vial of **derivatization reagent (DER)** in **550 µl DMSO.** Put the vial on a horizontal shaker for 5 min. Discard any rest of the reagent after use. DER must be **prepared immediately before use**. The ELISA kit can be separated into three performances by providing three DER vials. Please note: DMSO attacks all plastics but not polypropylene products and laboratory glass.

- Dissolve the content of one vial of **assay reagent (ASYREAG)** in **4 ml of diluted wash buffer**. The ELISA kit can be separated into three performances by providing three ASYREAG vials. Dissolved assay reagent can be stored at **-20°C for 4 weeks.**

- Dilute the **POD conjugate (CONJ) 1:200** with conjugate stabilizing buffer (CONJBUF) (**e.g. 110 µl CONJ + 22 ml CONJBUF, prepare only the required amount**). The undiluted POD conjugate is stable at 2-8°C until the expiry date stated on the label. Diluted POD conjugate is not stable over a longer period. It can be stored at **2-8°C for only 5 days.**

- All other test reagents are ready for use. Test reagents are stable until the expiry date (see label of test package) when stored at 2-8°C.

6. PRECAUTIONS

- For research use only.
- Human materials used in kit components were tested and found to be negative for HIV and Hepatitis B. However, for safety reasons all kit components should be treated as if potentially infectious.
- Stop solution is composed of sulfuric acid, which is a strong acid. Even diluted, it still must be handled with care. It can cause acid burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spills should be wiped out immediately with copious quantities of water.
- Reagents should not be used beyond the expiration date shown on kit label.

7. SPECIMEN COLLECTION AND PREPARATION

EDTA plasma, serum and urine

- Venous fasting blood and urine are suited for this test system. Blood samples are stable for one week at 2-8°C. In urine samples GABA is stable for 72 h at room temperature. Therefore urine samples can be sent without cooling. For longer storage, blood and urine samples should be frozen at -20°C. We recommend acidifying the urine samples.
- Lipemic or hemolytic samples may give erroneous results and should not be used for analysis.
- The EDTA plasma, serum and urine samples are diluted for derivatization.

  Samples with visible amounts of **precipitates** should be **centrifuged** at least for 5 min at 10000 x g. The resulting supernatant is used in the assay.
- For sample preparation, a derivatization reagent (DER) for derivatization of GABA is added (details are given in the sample preparation procedure).

8. ASSAY PROCEDURE

Procedural notes

- Quality control guidelines should be observed.

- Incubation time, incubation temperature, and pipetting volumes of the different components are defined by the producer. Any variations of the test procedure that are not coordinated with the producer may influence the test results.

- The assay should always be performed according to the enclosed manual.

Sample preparation procedure

Derivatization of standards (STD), controls (CTRL) and diluted samples (SAMPLE) is carried out in single analysis.

Dilute **EDTA plasma and serum samples** with reaction buffer by **factor 1:4**, i.e. **100 µl** sample + **300 µl** reaction buffer (DERBUF). These vials, containing 400 µl diluted sample, are used for derivatization (see step 2.)

Dilute **urine samples** with reaction buffer by **factor 1:50**, i.e. **20 µl** urine sample + **980 µl** reaction buffer (DERBUF). Take out 400 µl for derivatization (see step 2.)

1.	Bring all reagents and samples to room temperature (18-26°C).
2.	Add **400 µl of ready to use standards (STD), 400 µl of ready to use controls (CTRL)** and **400 µl of diluted samples (SAMPLE)** in the corresponding vial.
3.	Add **25 µl** of freshly prepared **derivatization reagent (DER)** into each vial (standards, controls and samples), mix well and incubate **for 60 min** on a shaker (180-240 rpm) **at room temperature (18-26°C)**.
4.	Afterwards add **500 µl of dilution buffer (CODIL)** into each vial, mix well and incubate for **30 min** on a shaker (180-240 rpm) **at room temperature (18-26°C)**.

2 x 100 µl of each treated sample (STD, CTRL, SAMPLE) are used in the ELISA as duplicates.

Test procedure

5.	Mark the positions of standards (STD)/ controls (CTRL)/ samples (SAMPLE) in duplicate on a protocol sheet.
6.	Take as many microtiter strips (PLATE) as needed from kit. Store unused strips covered at 2-8°C. Strips are stable until the expiry date stated on the label.
7.	Wash each well **5 times** by dispensing **250 µl of diluted wash buffer** into each well. After the final washing step, the inverted microtiter plate (PLATE) should be firmly tapped on absorbent paper to remove excess solution.
8.	For the analysis in duplicate, take **2 x 100 µl of standards (STD) / controls (CTRL) / samples (SAMPLE)** out of the vial and add into the respective wells of the microtiter plate (PLATE).
9.	Add **100 µl** of dissolved **assay reagent (ASYREAG)** into each well. Cover the plate tightly.
10.	Incubate overnight **(15-20 hours) at 2-8°C.**
11.	Aspirate the contents of each well. Wash each well **5 times** by dispensing **250 µl of diluted wash buffer** into each well. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper to remove excess solution.
12.	Add **200 µl** diluted **POD conjugate (CONJ)** into each well.
13.	Cover plate tightly and incubate for **1 hour at room temperature (18-26°C)** on a horizontal shaker (180-240 rpm).
14.	Aspirate the contents of each well. Wash each well **5 times** by dispensing **250 µl of diluted wash buffer** into each well. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper to remove excess solution.
15.	Add **200 µl** of **TMB substrate (SUB)** into each well.

16. Incubate for **6-12 min at room temperature (18-26°C)** in the dark*.
17. Add **100 µl of stop solution (STOP)** into each well, mix thoroughly.
18. Determine **absorption** immediately with an ELISA reader at **450 nm**. If the highest extinction of the standards **(STD)** is above the range of the photometer, absorption must be measured immediately at **405 nm** and the obtained results used for evaluation. If possible, the extinctions from each measurement should be compared with extinctions obtained at a reference wavelength, e. g. 595 nm, 620 nm, 630 nm, 650 nm and 690 nm can be used.

* The intensity of the color change is temperature sensitive. We recommend to observe the color change and to stop the reaction upon good differentiation.

9. EVALUATION OF RESULTS

If the test is performed in strict compliance with the manufacturer's instructions (i.e. with the exact volumes for standards, controls, samples, and with correct sample treatment), standards, controls, and blood samples are equally diluted. Therefore, **no dilution factor is required for the calculation of results from plasma and serum samples.**

For **urine samples** with 1:50 dilution, the values calculated from the calibration curve have to be multiplicated by a factor of 12.5 to obtain the true results. The results must be related to the creatinine content of the urine samples.

$$GABA\left[\frac{\mu g}{g_{creatinine}}\right] = dilution\,factor \times \frac{c_{GABA}\left[\frac{\mu mol}{l}\right]}{c_{creatinine}\left[\frac{mmol}{l}\right]} \times \frac{MW_{GABA}\left[\frac{g}{mol}\right]}{MW_{creatinine}\left[\frac{g}{mol}\right]}$$

Or, simplified: the resulting factor of 11395 is multiplicated with the concentration of GABA [µmol/l] and divided by the concentration of creatinine [mmol/l]:

$$GABA\left[\frac{\mu g}{g_{creatinine}}\right] = 11395 \times \frac{c_{GABA}\left[\frac{\mu mol}{l}\right]}{c_{creatinine}\left[\frac{mmol}{l}\right]}$$

Calculation of results

The following algorithms can be used alternatively to calculate the results. We recommend using the "4-parameter-algorithm".

1. *4-parameter-algorithm*

   It is recommended to use a linear ordinate for optical density and a logarithmic abscissa for concentration. When using a logarithmic abscissa, the zero calibrator must be specified with a value less than 1 (e.g. 0.001).

2. *Point-to-point-calculation*

   We recommend a linear ordinate for optical density and a linear abscissa for concentration.

3. *Spline-algorithm*

   We recommend a linear ordinate for optical density and a logarithmic abscissa for concentration. When using a logarithmic abscissa, the zero calibrator must be specified with a value less than 1 (e.g. 0.001).

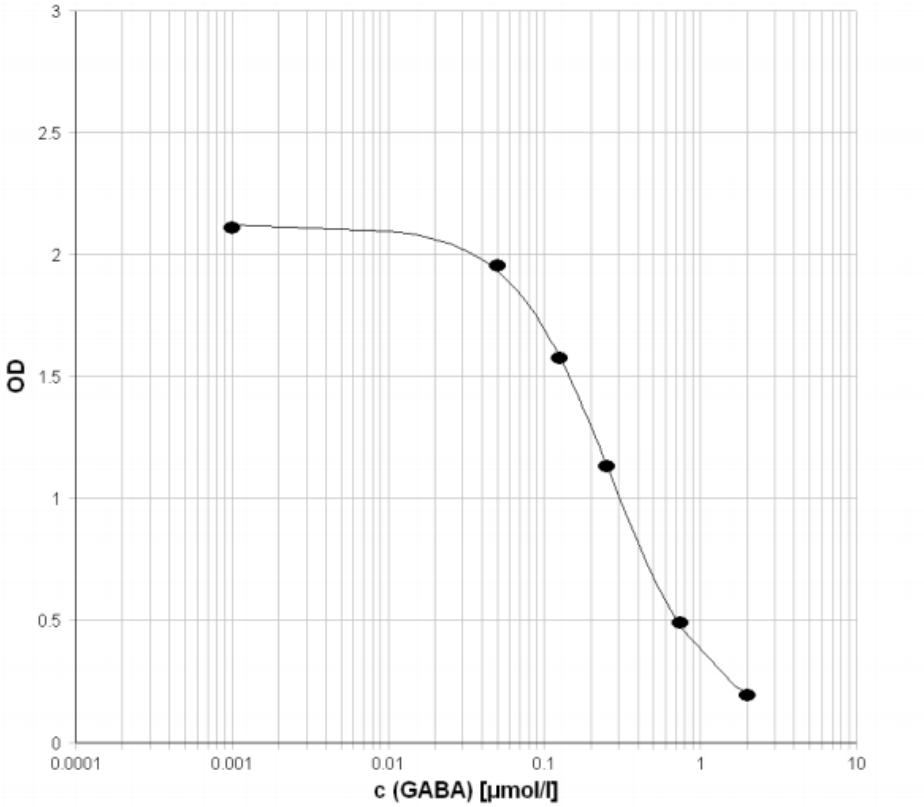
Plausibility of the measured pairs of values should be examined before automatically evaluating the results. If this option is not available within the used program, the pairs of values should be controlled manually.

Controls

Control samples or plasma pools should be analyzed with each run. Results generated from the analysis of control samples should be evaluated for acceptability using appropriate statistical methods. The results for the patient samples may not be valid if within the same assay one or more values of the quality control samples are outside the acceptable limits.

The concentration of controls and patient samples can be determined directly from the calibration curve. In the following, an example of a calibration curve is given, do not use it for the calculation of your results.

Example of calibration curve



Expected results

Based on internal studies with plasma samples of evidently healthy persons (n=20) a mean value of 0.182 µmol/l was calculated. The standard deviation was 0.053 µmol/l.

Plasma mean value ± 2 x standard deviation:	**0.182 ± 0.106 µmol/l**
Normal range:	**0.076 – 0.288 µmol/l**

We recommend each laboratory to develop its own normal range. The values mentioned above are only for orientation and can deviate from other published data.

10. PERFORMANCE CHARACTERISTICS

Cross reactivity

β-alanine	< 0.4 %
α-aminobutyric acid	< 0.01 %

Precision and reproducibility

EDTA plasma:

Intra-assay (n=12)		
sample	GABA [µmol/l]	coefficient of variation (CV) [%]
1	0.122	7.1
2	0.197	9.2

Inter-assay (n=6)		
sample	GABA [µmol/l]	coefficient of variation (CV) [%]
1	0.089	13.5
2	0.420	8.0

Sensitivity

The sensitivity was set as B_0 + 2SD. The zero-standard was measured 48 times.

sample	GABA mean value [OD]	2 x standard deviation (SD)	detection limit [µmol/l]
zero-standard	2.28	0.18	0.024

Recovery

One sample was spiked with different GABA concentrations and measured in this assay. The analytical recovery rate was determined by the expected and measured GABA levels. The expected levels were calculated as the sum of the measured GABA concentration in the original sample and the spiked GABA amount. The mean recovery rate for all concentrations was 99.5 % (n=10).

spike [µmol/l]	GABA measured [µmol/l]	GABA expected [µmol/l]	recovery [%]
0	x = 0.104	x	100.0
0.15	0.252	0.104+0.15 = 0.254	99.2
0.3	0.401	0.104+0.3 = 0.404	99.3

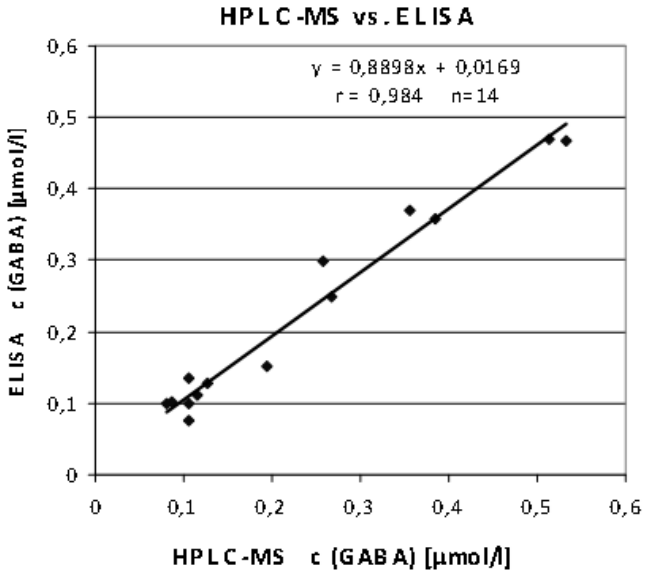
Linearity

The linearity of the ELISA was determined by the dilution of a spiked patient sample. The mean linearity was 107 % (n=10).

dilution	measured [µmol/l]	expected [µmol/l]	recovery [%]
original	0.478	0.478	100.0
1+1	0.252	0.239	105.4
1+3	0.138	0.120	115.5

Correlation with HPLC-MS

14 samples were measured with this ELISA and HPLC-MS. The correlation was r = 0.984.



11. LIMITATIONS

Hemolytic and lipemic samples may give erroneous results. Do not measure hemolytic and lipemic samples.

12. REFERENCES

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- Cai HL, Zhu RH, Li HD, Zhang XH, Hu L, Yang W, Ye HS: Elevated plasma γ-aminobutyrate/glutamate ratio and responses to risperidone antipsychotic treatment in schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry. 2010 Oct 1;34(7):1273-8. Epub 2010 Jul 15.
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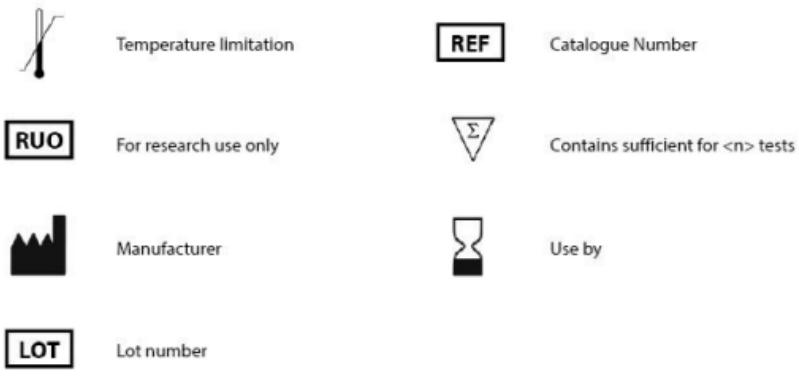
- Vaiva G, Boss V, Ducrocq F, Fontaine M, Devos P, Brunet A, Laffargue P, Goudemand M, Thomas P: Relationship between posttrauma GABA plasma levels and PTSD at 1-year follow-up. Am J Psychiatry. 2006 Aug;163(8):1446-8.

13. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- Test components contain organic solvents. Contact with skin or mucous membranes must be avoided.
- All reagents in the test package are for research use only.
- Reagents should not be used after the date of expiry stated on the label.
- Single components with different lot numbers should not be mixed or exchanged.
- Guidelines for medical laboratories should be observed.
- Incubation time, incubation temperature, and pipetting volumes of the components are defined by the producer. Any variation of the test procedure, which is not coordinated with the producer, may influence the results of the test.

Used symbols:



Symbols / Symbole / Symbôles / Símbolos / Símbolos / Σύμβολα

REF	Cat.-No.: / Kat.-Nr.: / No.- Cat.: / Cat.-No.: / N.º Cat.: / N.–Cat.: / Αριθμός-Κατ.:
LOT	Lot-No.: / Chargen-Bez.: / No. Lot: / Lot-No.: / Lote N.º: / Lotto n.: / Αριθμός -Παραγωγή:
	Use by: / Verwendbar bis: / Utiliser à: / Usado por: / Usar até: / Da utilizzare entro: / Χρησιμοποιείται από:
	No. of Tests: / Kitgröße: / Nb. de Tests: / No. de Determ.: / N.º de Testes: / Quantità dei tests: / Αριθμός εξετάσεων:
CONC	Concentrate / Konzentrat / Concentré / Concentrar / Concentrado / Concentrato / Συμπύκνωμα
LYO	Lyophilized / Lyophilisat / Lyophilisé / Liofilizado / Liofilizado / Liofilizzato / Λυοφιλιασμένο
IVD	In Vitro Diagnostic Medical Device. / In-vitro-Diagnostikum. / Appareil Médical pour Diagnostics In Vitro. / Dispositivo Médico para Diagnóstico In Vitro. / Equipamento Médico de Diagnóstico In Vitro. / Dispositivo Medico Diagnostico In vitro. / Ιατρική συσκευή για In-Vitro Διάγνωση.
	Evaluation kit. / Nur für Leistungsbewertungszwecke. / Kit pour évaluation. / Juego de Reactivos para Evaluació. / Kit de avaliação. / Kit di evaluazione. / Κίτ Αξιολόγησης.
	Read instructions before use. / Arbeitsanleitung lesen. / Lire la fiche technique avant emploi. / Lea las instrucciones antes de usar. / Ler as instruções antes de usar. / Leggere le istruzioni prima dell'uso. / Διαβάστε τις οδηγίες πριν την χρήση.
	Keep away from heat or direct sun light. / Vor Hitze und direkter Sonneneinstrahlung schützen. / Garder à l'abri de la chaleur et de toute exposition lumineuse. / Manténgase alejado del calor o la luz solar directa. / Manter longe do calor ou luz solar directa. / Non esporre ai raggi solari. / Να φυλάσσεται μακριά από θερμότητα και άμεση επαφή με το φως του ηλίου.
	Store at: / Lagern bei: / Stocker à: / Almacene a: / Armazenar a: / Conservare a: / Αποθήκευση στους:
	Manufacturer: / Hersteller: / Fabricant: / Productor: / Fabricante: / Fabbricante: / Παραγωγός:
	Caution! / Vorsicht! / Attention! / ¡Precaución! / Cuidado! / Attenzione! / Προσοχή!
Symbols of the kit components see MATERIALS SUPPLIED. Die Symbole der Komponenten sind im Kapitel KOMPONENTEN DES KITS beschrieben. Voir MATERIEL FOURNI pour les symbôles des composants du kit. Símbolos de los componentes del juego de reactivos, vea MATERIALES SUMINISTRADOS. Para símbolos dos componentes do kit ver MATERIAIS FORNECIDOS. Per i simboli dei componenti del kit si veda COMPONENTI DEL KIT. Για τα σύμβολα των συστατικών του κιτ συμβουλευτείτε το ΠΑΡΕΧΟΜΕΝΑ ΥΛΙΚΑ.	

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Symbols Version 3.5 / 2012-01-20

SIEMENS

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ADVIA® Chemistry
Systems

Siemens Healthcare Diagnostics Inc.

Instructions for Use
2011-02

Creatine Kinase (CKNAC)

System	New Information
ADVIA® 1200	Updates: New document number; Symbols page added
ADVIA 1650/1800	Updates: New document number; Symbols page added
ADVIA 2400	Updates: New document number; Symbols page added

Method Summary

Item	Description
Method Principle	NAC Activated, IFCC
Specimen Type	Human serum and plasma (lithium heparin)
On-board Stability	ADVIA 1200: 10 days ADVIA 1650/1800: 10 days ADVIA 2400: 10 days
Reagent Storage Temperature	2–8°C
Calibration Frequency	N/A
Reagent Blank (RBL) Frequency	Daily
Reaction Type	Rate (RRA)
Measurement Wavelength	340/410 nm
Standardization	IFCC reference method
Analytical Range	Serum/Plasma: 0 – 1300 U/L
Expected Values	Males: 32 – 294 U/L Females: 33 – 211 U/L
Reagent Code	74043
Calibrator	Fixed System Factor Value

Siemens Healthcare Diagnostics Inc., Tarrytown, NY 10591-5097 USA
10493970_EN Rev. D, 2011-02

Intended Use

For *in vitro* diagnostic use in the quantitative determination of creatine kinase activity in human serum and plasma on the ADVIA Chemistry systems. Such measurements are used mainly in the diagnosis and treatment of myocardial infarction and muscle diseases such as Duchenne progressive muscular dystrophy.

Summary and Explanation [1,2]

The Creatine Kinase (CK) method is an adaptation of the IFCC Reference Method. The reaction is based on the modified procedure of Szasz.

Principles of the Procedure

Creatine Kinase reacts with creatine phosphate and ADP to form ATP which is coupled to the hexokinase-G6PD reaction, generating NADPH. The concentration of NADPH is measured by the increase in absorbance at 340/410 nm.

Reaction Equation

$$\textit{Creatine Phosphate} + \textit{ADP} \xrightarrow{\textit{CK, } Mg^{2+}} \textit{Creatine} + \textit{ATP}$$

$$\textit{D-Glucose} + \textit{ATP} \xrightarrow{\textit{HK, } Mg^{2+}} \textit{ADP} + \textit{G6P}$$

$$\textit{G6P} + \textit{NADP}^{+} \xrightarrow{\textit{G6PD}} \textit{6-Phosphoglucono-}\delta\textit{-Lactone} + \textit{NADPH}$$

Reagents

The reagents are packaged as listed below. Components of the package are available as a kit only.

REF (PN) Container Size	Symbol	Contents	Amount	No. of Tests
07498541	CKNAC	Creatine Kinase Reagents		7 x 140
20-mL	CKNAC R1	Reagent 1	7 x 15 mL	
	CKNAC R1 MIX	Reagent 1 Mix	7 x 350 mg	
02096577 (B01-4137-01)	CKNAC	Creatine Kinase Reagents		4 x 270
70-mL	CKNAC R1	Reagent 1	4 x 30 mL	
	CKNAC R1 MIX	Reagent 1 Mix	4 x 700 mg	

Safety data sheets (MSDS/SDS) available on www.siemens.com/diagnostics.

Components and Concentrations

Reagent	Component	Concentration
Reagent 1	Sodium azide	0.02%
Reagent 1 Mix	ADP	2.0 mmol/L
	AMP	5.0 mmol/L
	Diadenosine pentaphosphate	10 µmol/L
	NADP	2.0 mmol/L
	HK	≥ 2.5 U/mL
	G6PD	≥ 1.5 U/mL
	N-Acetyl-L-cysteine	20 mmol/L
	Creatine phosphate	30 mmol/L

NOTE: Sodium azide can react with copper and lead plumbing to form explosive metal azides. If disposal into a drain is in compliance with federal, state, and local requirements, flush reagents with a large amount of water to prevent the buildup of azides.

For *In Vitro* Diagnostic Use.

Reagent Preparation and Use

Prepare the reagent:

1. Reconstitute the contents of the R1 mix vial with a portion of R1.
2. Rinse the R1 mix vial several times with R1.
3. Transfer the entire contents of the R1 mix to the R1 wedge.

On-board Reagent Stability (OBS)

System	Stability
ADVIA 1200	10 days
ADVIA 1650/1800	10 days
ADVIA 2400	10 days

For all systems, unopened reagents are stable until the expiration date printed on the product label when stored at 2° – 8°C. Do not freeze reagents.

For additional details, refer to the *Method's Introduction* section of the system-specific Operator's Guide.

Sample Handling

Siemens Healthcare Diagnostics recommends using serum or plasma (lithium heparin) for this method. The use of hemolyzed samples may cause a significant interference with this method.

For additional details, refer to Sample Collection and Preparation in the *Methods Introduction* section of the system-specific Operator's Guide.

For instructions on how to load reagents and run samples, refer to the *Daily Operations* section of the system-specific Operator's Guide.

Materials Required but not Provided

The following list contains the materials required, but not provided, to perform this method:

- sample containers
- system solutions
- control materials
- reagent container adapters:
  - 20-mL adapter (REF 02404085; PN 094-0159-01) for 40-mL slot (ADVIA 1200/1800)
  - 20-mL adapter (REF 05249323; PN 073-0936-01) for 70-mL slot (ADVIA 1800)
  - 20-mL adapter (REF 00771668; PN 073-0345-02) for 70-mL slot (ADVIA 1650/2400)

For storage and stability information, refer to the package insert.

Calibration

Calibration uses a fixed system Factor Value (FV), which is based on the established molar extinction coefficient of NADPH at 340 nm, adjusted by the patient sample correlation to the IFCC reference method. One unit is defined as that amount of enzyme required to produce 1 µmol of NADPH per minute under the conditions of the assay.

For setup and use instructions, refer to the *Calibration Overview* section of the system-specific Operator's Guide.

Calibration Frequency

No calibration required.

Reagent Blank (RBL) Frequency

Siemens recommends measurement of the RBL daily.

Quality Control

Follow government regulations or accreditation requirements for quality control frequency.

Siemens recommends the use of commercially available quality control materials with at least 2 levels (low and high). A satisfactory level of performance is achieved when the analyte values obtained are within the Acceptable Control Range for the system or within your range, as determined by an appropriate internal laboratory quality control scheme.

The actual frequency of control in a laboratory is based on many factors, such as workflow, system experience, and government regulation. Each laboratory should evaluate the controls based on the frequency established by their laboratory guidelines. When the method is performed, analyze at least 2 levels of controls daily.

Also, assay controls under the following conditions:

- whenever you use a new reagent lot
- following the performance of any system maintenance, cleaning, or troubleshooting procedure
- after performing a new calibration

For more information, refer to the *Quality Control Overview* section of the system-specific Operator's Guide.

Limitations of the Procedure [3]

A number of substances cause physiological changes in serum or plasma analyte concentrations. A comprehensive discussion of possible interfering substances, their serum or plasma concentrations, and their possible physiological involvements is beyond the scope of this document. Consult listed reference for specific details on known potential interfering substances.[3]

As with any chemical reaction, you must be alert to the possible effect on results of unknown interferences from medications or endogenous substances. The laboratory and physician must evaluate all patient results in light of the total clinical status of the patient.

Interferences

Siemens tested the following potential inteferents and found the results shown below:

ADVIA 1200

Interferent	Interferent Level	CKNAC Sample Concentration	Interference*
Bilirubin (conjugated/unconjugated)	25 mg/dL (428 µmol/L)	199 U/L	NSI
Hemolysis (hemoglobin)	125 mg/dL (1.25 g/L)	164 U/L	+9.8%
	250 mg/dL (2.50 g/L)	164 U/L	+21%

ADVIA 1200

Interferent	Interferent Level	CKNAC Sample Concentration	Interference*
Lipemia (from Intralipid)	500 mg/dL (5.7 mmol/L)**	193 U/L	NSI

*NSI = No Significant Interference. A percentage effect ≥10% is considered a significant interference.

**as triolein

ADVIA 1650/1800

Interferent	Interferent Level	CKNAC Sample Concentration	Interference*
Bilirubin (conjugated/unconjugated)	30 mg/dL (513 µmol/L)	107 U/L	NSI
Hemolysis (hemoglobin)	125 mg/dL (1.25 g/L)	167 U/L	+12.6%
	250 mg/dL (2.50 g/L)	167 U/L	+20.9%
Lipemia (from Intralipid)	650 mg/dL (7.4 mmol/L)**	107 U/L	NSI

*NSI = No Significant Interference. A percentage effect ≥10% is considered a significant interference.

**as triolein

ADVIA 2400

Interferent	Interferent Level	CKNAC Sample Concentration	Interference*
Bilirubin (conjugated/unconjugated)	25 mg/dL (428 µmol/L)	209 U/L	NSI
Hemolysis (hemoglobin)	125 mg/dL (1.25 g/L)	166 U/L	+11.8%
	250 mg/dL (2.50 g/L)	166 U/L	+21.7%
Lipemia (from Intralipid)	500 mg/dL (5.7 mmol/L)**	210 U/L	NSI

*NSI = No Significant Interference. A percentage effect ≥10% is considered a significant interference.

**as triolein

Performance Characteristics

Precision [4]

Each sample was assayed 2 times per run, 2 runs per day, for at least 20 days. Precision estimates were computed according to CLSI document EP05-A2, *Evaluation of Precision Performance of Quantitative Measurement Methods*; Approved Guideline.[4]

Data contained in this section represents typical performance for ADVIA Chemistry systems. Your laboratory data may differ from these values.

ADVIA 1200

		Within-Run		Total	
Specimen Type	Level (U/L)	SD	CV (%)	SD	CV (%)
Serum	101	1.8	1.8	4.7	4.6
Serum	166	3.2	1.9	3.8	2.3
Serum	417	4.7	1.1	9.3	2.2

ADVIA 1650/1800

		Within-Run		Total	
Specimen Type	Level (U/L)	SD	CV (%)	SD	CV (%)
Serum	139	1.5	1.1	3.7	2.7
Serum	449	2.7	0.6	13.5	3.0

ADVIA 2400

		Within-Run		Total	
Specimen Type	Level (U/L)	SD	CV (%)	SD	CV (%)
Serum	104	0.8	0.8	1.6	1.6
Serum	441	2.3	0.5	4.1	0.9

Analytical Range

This method is linear from 0 – 1300 U/L for serum and plasma.

Siemens has validated an automatic rerun condition for this method that extends the reportable range up to 3900 U/L on the ADVIA 1200, and up to 7800 U/L on the ADVIA 1650/1800/2400 systems for serum and plasma.

Expected Values [5]

The following table lists the reference ranges for this method:

Sex	Reference Range
Male	32 – 294 U/L
Female	33 – 211 U/L

Siemens provides this information for reference. Each laboratory should establish its own normal range. You can enter normal range values and abnormal range values at the Analytical Parameters (Chemistry) window.

System Correlation

The performance of the applicable method (y) was compared with the performance of the same method on the comparison system (x).

ADVIA 1200

Specimen Type	Comparison System (x)	N	Regression Equation	Sy.x	r	Sample Range
Serum	ADVIA 1650	288	y = 0.981x - 0.1	3.2	1.000	14 – 1193 U/L
Plasma*	ADVIA 1200 (serum)	45	y = 1.000x + 0.0	4.9	0.999	44 – 549 U/L
Serum	Reference method	63	y = 1.000x - 3.3	14.1	0.999	34 – 1058 U/L

*lithium heparin

ADVIA 1650/1800

Specimen Type	Comparison System (x)	N	Regression Equation	Sy.x	r	Sample Range
Serum	Technicon DAX®	151	y = 0.97x + 1.2	3.3	0.999	6 – 700 U/L
Serum	Beckman CX7	145	y = 1.01x + 0.8	6.5	1.000	13 – 1187 U/L
Plasma*	ADVIA 1650 (serum)	56	y = 1.05x - 9.0	7.9	0.997	41 – 507 U/L
Serum	Reference method	47	y = 1.05x + 0.4	9.3	1.000	53 – 1170 U/L

*lithium heparin

ADVIA 2400

Specimen Type	Comparison System (x)	N	Regression Equation	Sy.x	r	Sample Range
Serum	ADVIA 1650	361	y = 1.01x + 1.5	2.2	1.000	6 – 1212 U/L
Serum	Reference method	47	y = 1.05x - 0.6	12.9	0.999	53 – 1170 U/L

Standardization

The ADVIA CKNAC method is traceable to the IFCC reference method, which uses IFCC-455 reference material via patient sample correlation. See the correlation data in System Correlation for the relationship.

Bibliography

1. Recommendations of the German Society for Clinical Chemistry Standard Method for the Determination of Creatine Kinase Activity Revised Draft of 1976. *J Clin Chem Clin Biochem.* 1977;15:255-260.
2. Szasz G, Gruber W, Bernt E. Creatine kinase in serum: Determination of optimum reaction conditions. *Clin Chem.* 1976;22:650-656.
3. Young, D.S. *Effects of Drugs on Clinical Laboratory Tests.* 3rd ed. Washington, AACC Press; 1990.
4. Clinical and Laboratory Standards Institute (formerly NCCLS). *Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline - Second Edition.* Wayne, PA: Clinical and Laboratory Standards Institute; 2004. NCCLS Document EP05-A2.
5. Data on file.

Technical Assistance

For customer support, please contact your local technical support provider or distributor.

www.siemens.com/diagnostics

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Origin: US

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Understanding the Symbols

The following symbols may appear on the product labeling:

Symbol	Definition	Symbol	Definition
	*In vitro* diagnostic medical device		Catalog number
	Manufacturer		Authorized Representative in the European Community
	CE Mark		CE Mark with identification number of notified body
	Consult instructions for use		Caution! Potential Biohazard
	Do not freeze (> 0°C)		Temperature limitation (2–8°C)
	Lower limit of temperature (≥ 2°C)		Upper limit of temperature (≤ -10°C)
	Keep away from sunlight		Use by
	Store upright		Contains sufficient for (n) tests
	Batch code		Printed with soy ink
2010-01	Date format (year-month)		Recycle
	Green dot		

Lactate Dehydrogenase L-P (LDLP)

System	New Information
ADVIA® 1200	Updates: Method Summary; Materials Required but Not Provided; Calibration; Calibration Frequency; Reagent Blank (RBL) Frequency
ADVIA 1650/1800	Updates: Method Summary; Materials Required but Not Provided; Calibration; Calibration Frequency; Reagent Blank (RBL) Frequency
ADVIA 2400	Updates: Method Summary; Materials Required but Not Provided; Calibration; Calibration Frequency; Reagent Blank (RBL) Frequency

Method Summary

Item	Description
Method Principle	Lactate/NAD
Specimen Type	Human serum and plasma (lithium heparin)
On-board Stability	30 days
Reagent Storage Temperature	2–8°C
Calibration Frequency	30 days
Reagent Blank (RBL) Frequency	Daily
Reaction Type	Rate (RRA)
Measurement Wavelength	340/410 nm
Standardization	IFCC reference method
Analytical Range	Serum/Plasma: 20 – 700 U/L
Expected Values	Serum/Plasma: 120 – 246 U/L
Reagent Code	74048
Calibrator	ADVIA Chemistry Enzyme 1 Calibrator (REF 10916057)

Siemens Healthcare Diagnostics Inc., Tarrytown, NY 10591-5097 USA
10494007_EN Rev. E, 2016-05

Intended Use

For *in vitro* diagnostic use in the quantitative determination of lactate dehydrogenase activity in human serum and plasma on ADVIA Chemistry systems. Such measurements are used mainly in the diagnosis and treatment of myocardial and pulmonary infarction. They may also be used to monitor cancer chemotherapy.

Summary and Explanation [1-3]

The procedure for the determination of Lactate Dehydrogenase L–P (LDLP) is based on the method of Amador, Dorfman, and Wacker. The method was automated by Morgenstern, Flor, Kessler, and Klein and modified by Morgenstern, Rush, and Lehman. The LD method is based on the recommendation of Richards, Lubinski, and Vanderlinde to use the TRIS buffer in place of AMP buffer.

Principles of the Procedure

LD catalyzes the conversion of L-lactate to pyruvate in the presence of nicotinamide adenine dinucleotide (NAD). The enzymatic activity of LD is proportional to the rate of production of NADH (reduced NAD). The amount of NADH produced is determined by measuring the increase in absorbance at 340/410 nm.

Reaction Equation

Reagents

The reagents are packaged as listed below. Components of the package are available as a kit only.

REF (PN) Container Size	Symbol	Contents	Amount	No. of Tests
03029628	LDLP	Lactate Dehydrogenase (L-P) Reagents		6 x 670
70-mL	LDLP R1	Reagent 1	6 x 68 mL	
40-mL	LDLP R2	Reagent 2	6 x 33.6 mL	
07502115	LDLP	Lactate Dehydrogenase (L-P) Reagents		7 x 274
40-mL	LDLP R1	Reagent 1	7 x 30 mL	
20-mL	LDLP R2	Reagent 2	7 x 13.6 mL	
07812807 (PN B01-4142-01)	LDLP	Lactate Dehydrogenase (L-P) Reagents		4 x 480
70-mL	LDLP R1	Reagent 1	4 x 49 mL	
70-mL	LDLP R2	Reagent 2	2 x 46 mL	

Safety data sheets (MSDS/SDS) available on siemens.com/healthcare.

Components and Concentrations

Reagent	Component	Concentration
Reagent 1	L-lactic acid	62 mmol/L
	Sodium azide	0.09%
Reagent 2	NAD	27 mmol/L

Note Sodium azide can react with copper and lead plumbing to form explosive metal azides. If disposal into a drain is in compliance with federal, state, and local requirements, flush reagents with a large amount of water to prevent the buildup of azides.

For *in vitro* diagnostic use.

Reagent Preparation and Use

Reagents are ready to use. Before use, gently swirl the reagent to dislodge bubbles and assure homogeneity.

On-board Reagent Stability (OBS)

System	Stability
ADVIA 1200	30 days
ADVIA 1650/1800	30 days
ADVIA 2400	30 days

For all systems, unopened reagents are stable until the expiration date printed on the product label when stored at 2° to 8°C. Do not freeze reagents.

Sample Handling [4,5]

Siemens Healthcare Diagnostics recommends using serum or plasma (lithium heparin) for this method. To avoid significant interferences with this method, do not use hemolyzed samples.

IMPORTANT: Carefully evaluate plasma data because of the possible effects of sample handling on LD levels. Elevations in plasma LD levels can occur as a result of the release of LD from red blood cells or platelets. For this reason, serum is the preferred sample.

To avoid falsely elevated results due to high red blood cell LD levels, separate specimens from the clot as soon as possible. There is considerable disagreement over the optimal storage conditions for the LDH isoenzymes so each laboratory should determine its own suitable storage conditions.

Materials Required but not Provided

The following list contains the materials required, but not provided, to perform this method:

- sample containers
- system solutions
- calibrator (refer to the *Method Summary* section for the REFs)
- control materials
- reagent container adapters:
  - 20-mL adapter (REF 02404085; PN 094-0159-01) for 40-mL slot (ADVIA 1200/1800)
  - 20-mL adapter (REF 05249323; PN 073-0936-01) for 70-mL slot (ADVIA 1800)
  - 20-mL adapter (REF 00771668; PN 073-0345-02) for 70-mL slot (ADVIA 1650/2400)
  - 40-mL adapter (REF 08163594; PN 073-0788-01) for 70-mLslot (ADVIA 1650/2400)

For storage and stability information, refer to the package inserts.

Calibration

To calibrate the ADVIA Chemistry LDLP assay, use the ADVIA Chemistry Enzyme 1 Calibrator (**REF** 10916057).

Enter the lot-specific calibrator values that are provided with each lot of calibrator. Perform the calibration as described in the calibrator instructions for use.

For setup and use instructions, refer to the *Calibration Overview* section of the system-specific Operator's Guide.

Calibration Frequency

Calibrate the assay every 30 days.

Calibrate the assay after the following events:

- When the reagent lot number changes
- When a reagent pack is replaced by a new reagent pack with the same lot number, and the previous reagent pack was recalibrated during use
- When a reagent pack is replaced by a new reagent pack with the same lot number, and an additional reagent blank was run on the previous reagent pack during use
- After replacing critical optical or hydraulic components
- When indicated by quality control procedures

Individual laboratory quality control programs and procedures may require more frequent calibration.

Reagent Blank (RBL) Frequency

Siemens recommends measurement of the RBL daily.

Run an additional RBL when a reagent pack is replaced by a new reagent pack with the same lot number and an additional reagent blank was run during use.

Note Use deionized water as the sample for the RBL in the ADVIA Chemistry LDLP assay.

Quality Control

Follow government regulations or accreditation requirements for quality control frequency.

Siemens recommends the use of commercially available quality control materials with at least 2 levels (low and high). A satisfactory level of performance is achieved when the analyte values obtained are within the Acceptable Control Range for the system or within your range, as determined by an appropriate internal laboratory quality control scheme.

The actual frequency of control in a laboratory is based on many factors, such as workflow, system experience, and government regulation. Each laboratory should evaluate the controls based on the frequency established by their laboratory guidelines. When the method is performed, analyze at least 2 levels of controls daily.

Also, assay controls under the following conditions:

- whenever you use a new reagent lot
- following the performance of any system maintenance, cleaning, or troubleshooting procedure
- after performing a new calibration

For more information, refer to the *Quality Control Overview* section of the system-specific Operator's Guide.

Limitations of the Procedure [6]

A number of substances cause physiological changes in serum or plasma analyte concentrations. A comprehensive discussion of possible interfering substances, their serum or plasma concentrations, and their possible physiological involvements is beyond the scope of this document. Consult listed reference for specific details on known potential interfering substances.[3]

As with any chemical reaction, you must be alert to the possible effect on results of unknown interferences from medications or endogenous substances. The laboratory and physician must evaluate all patient results in light of the total clinical status of the patient.

Interferences

At the Analytical Parameters (Serum) window, you can set up the ADVIA Chemistry system to flag different levels of lipemia (turbidity), hemolysis, and icterus for samples run on the system.

Avoid hemolyzed samples.

Siemens tested the following potential interferents up to the indicated levels and found the results shown below:

ADVIA 1200

Interferent	Interferent Level	LDLP Sample Concentration	Interference*
Bilirubin	25 mg/dL (427 µmol/L)	151 U/L	NSI
Lipemia (from Intralipid)	500 mg/dL (5.7 mmol/L)**	146 U/L	NSI

* NSI = No Significant Interference. A percentage effect ≥10% is considered significant interference.

**as triolein

ADVIA 1650/1800

Interferent	Interferent Level	LDLP Sample Concentration	Interference*
Bilirubin	30 mg/dL (513 µmol/L)	126 U/L	NSI
Lipemia (from Intralipid)	650 mg/dL (7.4 mmol/L)**	126 U/L	NSI

* NSI = No Significant Interference. A percentage effect ≥ 10% is considered significant interference.

**as triolein

ADVIA 2400

Interferent	Interferent Level	LDLP Sample Concentration	Interference*
Bilirubin	30 mg/dL (513 µmol/L)	135 U/L	NSI
Lipemia (from Intralipid)	625 mg/dL (7.1 mmol/L)**	134 U/L	NSI

* NSI = No Significant Interference. A percentage effect ≥ 10% is considered significant interference.

**as triolein

Performance Characteristics

Precision [7]

Each sample was assayed 2 times per run, 1 or 2 runs per day, for at least 20 days. Precision estimates were computed according to CLSI document EP05-A2, *Evaluation of Precision Performance of Quantitative Measurement Methods*; Approved Guideline.[7]

Data contained in this section represents typical performance for ADVIA Chemistry systems. Your laboratory data may differ from these values.

ADVIA 1200

		Within-Run		Total	
Specimen Type	Level (U/L)	SD	CV (%)	SD	CV (%)
Serum	157	1.4	0.9	2.1	1.3
Serum	386	3.5	0.9	5.2	1.3
Serum	488	2.6	0.5	4.4	0.9

ADVIA 1650/1800

		Within-Run		Total	
Specimen Type	Level (U/L)	SD	CV (%)	SD	CV (%)
Serum	145	1.2	0.8	2.4	1.7
Serum	435	2.9	0.7	5.2	1.2

ADVIA 2400

		Within-Run		Total	
Specimen Type	Level (U/L)	SD	CV (%)	SD	CV (%)
Serum	133	1.0	0.7	1.3	1.0
Serum	555	2.7	0.5	3.7	0.7

Analytical Range

This method is linear from 20 – 700 U/L for serum and plasma.

Siemens has validated an automatic rerun condition for this method that extends the reportable range up to 2100 U/L on the ADVIA 1200, and up to 4200 U/L on the ADVIA 1650/1800/2400 systems for serum and plasma.

Expected Values [3]

The expected values for this method are 120 – 246 U/L.

Siemens provides this information for reference. Each laboratory should establish its own normal range. You can enter normal range values and abnormal range values at the Analytical Parameters (Chemistry) window.

System Correlation

The performance of the applicable method (y) was compared with the performance of the same method on the comparison system (x).

ADVIA 1200

Specimen Type	Comparison System (x)	N	Regression Equation	Sy.x	r	Sample Range
Serum	ADVIA 1650	240	y = 1.01x - 4.0	3.3	0.999	93 – 645 U/L
Plasma*	ADVIA 1200 (serum)	30	y = 0.96x + 5.4	6.0	0.964	77 – 199 U/L
Serum	Reference Method	98	y = 1.04x - 0.01	15.2	0.997	53 – 678 U/L

*lithium heparin

ADVIA 1650

Specimen Type	Comparison System (x)	N	Regression Equation	Sy.x	r	Sample Range
Serum	Technicon DAX©	150	y = 1.05x - 6.5	8.8	0.995	75 – 545 U/L
Plasma*	ADVIA 1650 (serum)	49	y = 0.92x + 7.8	8.7	0.917	109 – 214 U/L
Serum	Reference Method	90	y = 0.99x + 10.1	13.2	0.996	76 – 680 U/L

*lithium heparin

ADVIA 2400

Specimen Type	Comparison System (x)	N	Regression Equation	Sy.x	r	Sample Range
Serum	ADVIA 1650	352	y = 1.01x - 0.1	3.2	0.999	82 – 682 U/L
Serum	Reference Method	90	y = 0.97x + 3.5	12.2	0.997	76 – 680 U/L

Standardization

The ADVIA LDLP method is traceable to the IFCC reference method, which uses IFCC-453 reference material via patient sample correlation.

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Technical Assistance

For customer support, please contact your local technical support provider or distributor.

siemens.com/healthcare

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Definition of Symbols

The following symbols may appear on the product labeling:

Symbol	Definition	Symbol	Definition
IVD	In vitro diagnostic medical device	REF REF	Catalog number
	Legal manufacturer	EC REP	Authorized Representative in the European Community
CE	CE Mark	CE 0086	CE Mark with identification number of notified body
	Consult instructions for use		Biological risk
	Keep away from sunlight and heat		Temperature limitation
	Lower limit of temperature		Upper limit of temperature
	Do not freeze (> 0°C)		Up
	Use by	Σ (n)	Contains sufficient for (n) tests
	Recycle	PRINTED WITH SOY INK	Printed with soy ink
Rev.	Revision	YYYY-MM-DD	Date format (year-month-day)
LOT	Batch code		

SIEMENS

CE

ADVIA® Chemistry
Systems

Albumin BCP (ALBP)

Current revision and date*	10699220 Rev. C, 2015-06		
Product Name	ADVIA® Chemistry Albumin BCP Reagent (ALBP)	REF10/11840	REF10/11841
Systems	ADVIA 1200 ADVIA 1650/1800 ADVIA 2400	4 x 800 tests 4 x 800 tests 4 x 700 tests	4 x 1525 tests 4 x 1525 tests 4 x 1325 tests
Materials Required but Not Provided	ADVIA Chemistry Albumin BCP Calibrator Reagent container adapters Commercially available controls	REF10/11842	
Specimen Types	Human serum and plasma (lithium heparin, potassium EDTA)		
Assay Principle	Bromocresol Purple (BCP) dye binding		
Analytical Measuring Range	0.6–8.0 g/dL (6–80 g/L)		
Reagent Storage	2–8°C		
Reagent On-System Stability	60 days		
Reagent Code	/4849		

* In Rev. C or later, a vertical bar in the margin indicates a technical update to the previous version.

Intended Use

For *in vitro* diagnostic use in the quantitative measurement of albumin in human serum or plasma on ADVIA® Chemistry systems.

Albumin measurements are used in the diagnosis and treatment of numerous diseases primarily involving the liver or kidneys.

Summary and Explanation

The ADVIA Chemistry Albumin BCP assay (ALBP) is an adaptation of the bromocresol purple (BCP) dye-binding method reported by Carter[1] and Louderback, *et al.*[2]

Principles of the Procedure

In the ADVIA Chemistry ALBP assay, serum or plasma albumin quantitatively binds to BCP to form an albumin-BCP complex that is measured as an endpoint reaction at 596/694 nm.

$$\text{Albumin} + \text{BCP dye} \xrightarrow{\text{pH 4.9}} \text{Albumin-BCP complex}$$

Reagents

Reagent	Description	Storage	Reagent Stability
REF **10711840**	**ADVIA Chemistry Albumin BCP** 18.0 mL in 20-mL container		
Albumin BCP Reagent 1 ALBP R1	Bromocresol Purple 1.1 mmol/L Acetate Buffer Surfactant Microbial Inhibitor	2–8°C	**Unopened:** Stable until the expiration date on carton. **On-system:** 60 days
REF **10711841**	**ADVIA Chemistry Albumin BCP** 35.0 mL in 40-mL container		
Albumin BCP Reagent 1 ALBP R1	Bromocresol Purple 1.1 mmol/L Acetate Buffer Surfactant Microbial Inhibitor	2–8°C	**Unopened:** Stable until the expiration date on carton. **On-system:** 60 days

Warnings and Precautions

Safety data sheets (MSDS/SDS) are available on www.siemens.com/diagnostics.

H317	**Warning!**
P280, P272, P302+P352, P333+P313, P501	May cause an allergic skin reaction. Wear protective gloves/protective clothing/eye protection/face protection. Contaminated work clothing should not be allowed out of the workplace. IF ON SKIN: Wash with plenty of soap and water. If skin irritation or rash occurs: Get medical advice/attention. Dispose of contents and container in accordance with all local, regional, and national regulations. **Contains:** 2-Chloracetamide; Reagent 1

Dispose of hazardous or biologically contaminated materials according to the practices of your institution. Discard all materials in a safe and acceptable manner and in compliance with prevailing regulatory requirements.

For *in vitro* diagnostic use.

Preparing Reagents

Reagents are ready to use. Before use, gently swirl the reagent to disrupt bubbles and assure homogeneity. If bubbles still exist or foam is present, use a clean transfer pipette to aspirate them from the reagent container prior to use.

Storing and Stability

For all systems, unopened reagents are stable until the expiration date printed on the product label when stored at 2–8°C. Do not freeze reagents.

For additional information, refer to the *Methods Introduction* section of the system operating instructions.

Specimen Collection and Handling

Siemens Healthcare Diagnostics validated serum and plasma (lithium heparin, potassium EDTA) for the ADVIA Chemistry ALBP assay.

Follow these guidelines for specimens used for this assay:

- Specimens should be free of particulate matter.
- Specimens should be as fresh as possible. Specimens may be stored for up to 72 hours at 2–8°C or stored frozen for up to 6 months at -20°C.[4]

The purpose of handling and storage information is to provide guidance to users; however, you may validate your own procedures for handling and storing patient specimens.

Procedure

Materials Provided

Item	Contents	Number of Tests
REF 10/11840	Albumin BCP Reagent (ALBP) Reagent 1:4 x 20-mL container	ADVIA 1200: 4 x 800 tests (3200 tests per kit) ADVIA 1650/1800: 4 x 800 tests (3200 tests per kit) ADVIA 2400: 4 x 700 tests (2800 tests per kit)
REF 10/11841	Albumin BCP Reagent (ALBP) Reagent 1:4 x 40-mL container	ADVIA 1200: 4 x 1525 tests (6100 tests per kit) ADVIA 1650/1800: 4 x 1525 tests (6100 tests per kit) ADVIA 2400: 4 x 1325 tests (5300 tests per kit)

Materials Required but Not Provided

The following materials are required to perform this assay, but are not provided:

Item	Description
REF 10/11842	ADVIA Chemistry Albumin BCP Calibrator
	Control materials (refer to the *Performing Quality Control* section)
REF 02404085 (PN 094-0159-01)	20-mL reagent container adapter for 40-mL slot (ADVIA 1200, ADVIA 1800)
REF 05249323 (PN 073-0936-01)	20-mL reagent container adapter for 70-mL slot (ADVIA 1200)
REF 00/1668 (PN 073-0345-02)	20-mL reagent container adapter for 70-mL slot (ADVIA 1650, ADVIA 2400)
REF 08163594 (PN 073-0788-01)	40-mL reagent container adapter for 70-mL slot (ADVIA 1650, ADVIA 2400)

Assay Procedure

For detailed instructions on performing the procedure, refer to the system operating instructions or to the online help system.

Preparing the System

For detailed instructions on preparing the system, refer to the system operating instructions.

Preparing the Samples

Before placing samples on the system, ensure that samples have the following characteristics:

- Samples are free of fibrin or other particulate matter.
- Samples are free of bubbles.

On-System Stability

The following table lists reagent on-system stability for each applicable ADVIA Chemistry system:

System	Reagent On-System Stability
ADVIA 1200	60 days
ADVIA 1650/1800	60 days
ADVIA 2400	60 days

Performing Calibration

Refer to the package insert supplied with the ADVIA Chemistry Albumin BCP Calibrator (REF 10711842) for handling instructions and values. Enter the lot-specific calibrator values provided with each lot of calibrator on the system calibration set-up screen.

Calibration Frequency

Calibrate the assay every 60 days.

Calibrate the assay after the following events:

- When the reagent lot number changes
- When a reagent pack is replaced by a new reagent pack with the same lot number, and the previous reagent pack was recalibrated during use
- After replacing critical optical or hydraulic components
- When indicated by quality control procedures

Individual laboratory quality control programs and procedures may require more frequent calibration.

Reagent Blank (RBL) Frequency

The ADVIA Chemistry systems measure the RBL during assay calibration. Run an additional RBL on the same reagent pack every 35 days thereafter.

Run an additional RBL when a reagent pack is replaced by a new reagent pack with the same lot number and an additional reagent blank was run during use.

Use deionized water as the sample for the RBL in the ADVIA Chemistry ALBP assay. Place a sample container with deionized water at the cup position assigned to the blank for calibration.

Performing Quality Control

Follow government regulations or accreditation requirements for quality control frequency.

Siemens recommends the use of commercially available quality control material with at least 2 levels (low and high). A satisfactory level of performance is achieved when the analyte values obtained are within the expected control range for the system or within your range, as determined by an appropriate internal laboratory quality control scheme.

The actual frequency of control in a laboratory is based on many factors, such as workflow, system experience, and government regulation. Each laboratory should evaluate the controls based on the frequency established by their laboratory guidelines.

When the assay is performed, analyze at least 2 levels of controls daily.

Also, assay controls under the following conditions:

- Whenever you use a new reagent lot
- Following the performance of any system maintenance, cleaning, or troubleshooting procedure
- After performing a new calibration or an additional reagent blank

For more information, refer to the *Quality Control Overview* section of the system operating instructions.

Taking Corrective Action

If the quality control results do not fall within the expected values or within the laboratory's established values, do not report results. Take the following actions:

1. Determine and correct the cause of the unacceptable control results:
   a. Verify that the materials are not expired.
   b. Verify that required mairtenance was performed.
   c. Verify that the assay was performed according to the instructions for use.
   d. Rerun the assay with fresh quality control samples, and confirm that quality control results are within acceptable limits before running patient samples.
   e. If the quality control results are not within acceptable limits, recalibrate the assay, and repeat the prior step.
   f. If necessary, contact your local technical support provider or distributor for assistance.
2. After corrective action is complete, repeat required testing of patient samples before reporting results.

Perform corrective actions in accordance with your established laboratory protocol.

Results

Calculation of Results

The system automatically calculates and reports results based on the absorbance measurements of the test sample during the test, and of the calibrator(s) from calibration.

The instrument calculates the concentration of albumin in g/dL (common units) and g/L (SI units).

Conversion factor: g/dL x 10 = g/L

Interpretation of Results

Results of this test should always be interpreted in conjunction with the patient's medical history, clinical presentation, and other diagnostic findings.

Limitations

United States federal law restricts this device to sale by or on the order of a physician.

A number of substances cause physiological changes in serum or plasma analyte concentrations. A comprehensive discussion of possible interfering substances, their serum or plasma concentrations, and their possible physiological involvements is beyond the scope of this document. Consult the listed reference for specific details on known potential interfering substances.[4]

As with any chemical reaction, you must be alert to the possible effect on results of unknown interferences from medications or endogenous substances. The laboratory and physician must evaluate all patient results in light of the total clinical status of the patient.

Siemens has determined that there is a possibility for certain ADVIA Chemistry reagents to interact with the ALBP assay when run on the same system. To mitigate these carryover events, the ADVIA Chemistry Systems software provides a Contamination Avoidance process. For further information and instructions to establish this process on your systems, refer to the Customer Bulletin entitled: *Consolidated Directory of Contamination Avoidance Settings for ADVIA Chemistry Systems* (PN 10813375, latest revision).

Expected Values

The reference range[5] for albumin is 3.4–5.0 g/dL (34–50 g/L) for adults.

Siemens provides this information for reference. As with all *in vitro* diagnostic assays, each laboratory should determine its own reference ranges for the diagnostic evaluation of patient results. Consider this range as a guideline only. You can enter normal range values and abnormal range values at the Analytical Parameters (Chemistry) window.

Performance Characteristics

Analytical Measuring Range

The ADVIA Chemistry ALBP assay is linear from 0.6–8.0 g/dL (6–80 g/L).

Results that are below the low end of the assay range are flagged **L**. You should report the test result as < 0.6 g/dL (< 6 g/L).

Results that are above the high end of the assay range are flagged **H**. You should report the test result as > 8.0 g/dL (> 80 g/L).

Sensitivity (LoB, LoD, LoQ)

The ADVIA Chemistry ALBP assay performance at low levels was evaluated according to CLSI guideline EP17-A2.[6] Limit of Blank (LoB) is the highest measurement result that is likely to be observed on a blank sample. The LoB for the ADVIA Chemistry ALBP assay is 0.1 g/dL (1 g/L).

Limit of detection (LoD) is the smallest amount that this assay can reliably detect to determine presence or absence of an analyte. The LoD for the ADVIA Chemistry ALBP assay is 0.6 g/dL (6 g/L).

LoB and LoD values are determined with proportions of false positives (α) less than 5% and false negatives (ß) less than 5%, based on 720 determinations with 240 blank and 480 low-level sample replicates for each ADVIA Chemistry system (ADVIA 1200, 1650, 1800, and 2400 Chemistry systems).

Limit of Quantitation (LoQ) is 0.6 g/dL (6 g/L) based on 480 determinations, with an inter-assay precision of < 10%.

Precision

Each sample was assayed 2 times per run, 2 runs per day, for at least 20 days. Precision estimates were computed according to CLSI document EP5-A2, *Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline.*[7]

Data contained in this section represents typical performance for ADVIA Chemistry systems. Your laboratory data may differ from these values.

Conversion factor: g/dL x 10 = g/L

ADVIA 1200

			Repeatability (Within-Run)		Between-Run		Between-Day		Within-Lab (Total)	
Specimen Type	N	Mean	SD	%CV	SD	%CV	SD	%CV	SD	%CV
Common Units (g/dL)										
Serum Control	80	2.6	0.03	1.1	0.01	0.4	0.04	1.4	0.05	1.8
Serum Control	80	4.0	0.04	0.9	0.01	0.3	0.04	0.9	0.05	1.3
Serum Pool	80	3.5	0.04	1.1	0.00	0.0	0.03	0.9	0.05	1.4
Serum Pool	80	5.2	0.04	0.7	0.02	0.4	0.04	0.7	0.06	1.1
SI Units (g/L)										
Serum Control	80	26	0.3	1.1	0.1	0.4	0.4	1.4	0.5	1.8
Serum Control	80	40	0.4	0.9	0.1	0.3	0.4	0.9	0.5	1.3
Serum Pool	80	35	0.4	1.1	0.0	0.0	0.3	0.9	0.5	1.4
Serum Pool	80	52	0.4	0.7	0.2	0.4	0.4	0.7	0.6	1.1

ADVIA 1650/1800

			Repeatability (Within-Run)		Between-Run		Between-Day		Within-Lab (Total)	
Specimen Type	N	Mean	SD	%CV	SD	%CV	SD	%CV	SD	%CV
Common Units (g/dL)										
Serum Control	80	2.7	0.03	1.1	0.02	0.6	0.00	0.2	0.03	1.3
Serum Control	80	4.0	0.03	0.8	0.00	0.0	0.02	0.6	0.04	1.0
Serum Pool	80	3.5	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0
Serum Pool	80	5.2	0.02	0.5	0.03	0.7	0.05	0.9	0.06	1.2
SI Units (g/L)										
Serum Control	80	27	0.3	1.1	0.2	0.6	0.0	0.2	0.3	1.3
Serum Control	80	40	0.3	0.8	0.0	0.0	0.2	0.6	0.4	1.0
Serum Pool	80	35	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Serum Pool	80	52	0.2	0.5	0.3	0.7	0.5	0.9	0.6	1.2

ADVIA 2400

			Repeatability (Within-Run)		Between-Run		Between-Day		Within-Lab (Total)	
Specimen Type	N	Mean	SD	%CV	SD	%CV	SD	%CV	SD	%CV
Common Units (g/dL)										
Serum Control	80	2.7	0.03	1.2	0.02	0.6	0.06	2.1	0.07	2.5
Serum Control	80	4.0	0.05	1.2	0.00	0.0	0.06	1.5	0.08	1.9
Serum Pool	80	3.5	0.02	0.6	0.00	0.0	0.01	0.4	0.03	0.8
Serum Pool	80	5.2	0.03	0.5	0.02	0.4	0.05	1.0	0.06	1.2
SI Units (g/L)										
Serum Control	80	27	0.3	1.2	0.2	0.6	0.6	2.1	0.7	2.5
Serum Control	80	40	0.5	1.2	0.0	0.0	0.6	1.5	0.8	1.9
Serum Pool	80	35	0.2	0.6	0.0	0.0	0.1	0.4	0.3	0.8
Serum Pool	80	52	0.3	0.5	0.2	0.4	0.5	1.0	0.6	1.2

Assay Comparison

The performance of the ADVIA Chemistry ALBP assay (y) was compared with the performance of the comparison assay on the indicated system (x). Linear regression was used to analyze the method comparison data in the following tables.

ADVIA 1200

Specimen Type	Comparison Assay (x)	N	Regression Equation	Sy.x	r	Sample Range
Serum	Siemens Dimension® Chemistry Analyzer	69	y = 1.01x - 0.02 g/dL y = 1.01x - 0.2 g/L	0.10 g/dL 1.0 g/L	0.999	0.8–7.6 g/dL 8–76 g/L
Plasma* (Lithium Heparin)	ADVIA 1200 (Serum)	47	y = 1.02x + 0.05 g/dL y = 1.02x + 0.5 g/L	0.09 g/dL 0.9 g/L	0.997	0.8–7.8 g/dL 8–78 g/L
Plasma* (EDTA)	ADVIA 1200 (Serum)	47	y = 1.01x - 0.13 g/dL y = 1.01x - 1.3 g/L	0.09 g/dL 0.9 g/L	0.997	0.8–7.7 g/dL 8–77 g/L

* Matrix comparison

ADVIA 1650/1800

Specimen Type	Comparison Assay (x)	N	Regression Equation	Sy.x	r	Sample Range
Serum	Siemens Dimension Chemistry Analyzer	69	y = 0.99x + 0.01 g/dL y = 0.99x + 0.1 g/L	0.07 g/dL 0.7 g/L	0.999	0.9–7.9 g/dL 9–79 g/L
Plasma* (Lithium Heparin)	ADVIA 1650/1800 (Serum)	47	y = 1.01x + 0.04 g/dL y = 1.01x + 0.4 g/L	0.08 g/dL 0.8 g/L	0.998	1.0–7.8 g/dL 10–78 g/L
Plasma* (EDTA)	ADVIA 1650/1800 (Serum)	47	y = 0.99x - 0.06 g/dL y = 0.99x - 0.6 g/L	0.14 g/dL 1.4 g/L	0.993	1.0–7.7 g/dL 10–77 g/L

* Matrix comparison

ADVIA 2400

Specimen Type	Comparison Assay (x)	N	Regression Equation	Sy.x	r	Sample Range
Serum	Siemens Dimension Chemistry Analyzer	68	y = 1.03x - 0.04 g/dL y = 1.03x - 0.4 g/L	0.06 g/dL 0.6 g/L	0.999	0.9–7.9 g/dL 9–79 g/L
Plasma* (Lithium Heparin)	ADVIA 2400 (Serum)	46	y = 1.01x + 0.05 g/dL y = 1.01x + 0.5 g/L	0.07 g/dL 0.7 g/L	0.998	1.0–7.6 g/dL 10–76 g/L
Plasma* (EDTA)	ADVIA 2400 (Serum)	47	y = 1.00x - 0.11 g/dL y = 1.00x - 1.1 g/L	0.10 g/dL 1.0 g/L	0.997	1.0–7.8 g/dL 10–78 g/L

* Matrix comparison

Interferences

At the Analytical Parameters (Serum) window, you can set up the ADVIA Chemistry system to flag different levels of lipemia (turbidity), hemolysis, and icterus for samples run on the system.

Siemens tested the following potential inteferents and found the results shown below:

ADVIA 1200

Interferent	Interferent Level	Albumin Sample Concentration	Interference*
Bilirubin (conjugated)	60 mg/dL (1026 µmol/L)	3.2 g/dL (32 g/L)	NSI
	60 mg/dL (1026 µmol/L)	4.7 g/dL (47 g/L)	NSI
Bilirubin (unconjugated)	60 mg/dL (1026 µmol/L)	3.3 g/dL (33 g/L)	NSI
	60 mg/dL (1026 µmol/L)	4.8 g/dL (48 g/L)	NSI
Hemolysis (hemoglobin)	500 mg/dL (5.0 g/L)	3.1 g/dL (31 g/L)	NSI
	750 mg/dL (7.5 g/L)	3.1 g/dL (31 g/L)	↓12.9%
	1000 mg/dL (10.0 g/L)	4.6 g/dL (46 g/L)	NSI
Lipemia (from Intralipid)	525 mg/dL (5.9 mmol/L)†	3.5 g/dL (35 g/L)	NSI
	638 mg/dL (7.2 mmol/L)†	3.3 g/dL (33 g/L)	↓12.1%
	638 mg/dL (7.2 mmol/L)†	4.8 g/dL (48 g/L)	NSI
	850 mg/dL (9.6 mmol/L)†	4.8 g/dL (48 g/L)	↓10.4%

* NSI = No Significant Interference. A percentage effect of ≥10% is considered a significant interference.
† SI units calculated as triolein.

ADVIA 1650/1800

Interferent	Interferent Level	Albumin Sample Concentration	Interference*
Bilirubin (conjugated)	60 mg/dL (1026 µmol/L)	3.2 g/dL (32 g/L)	NSI
	60 mg/dL (1026 µmol/L)	4.7 g/dL (47 g/L)	NSI
Bilirubin (unconjugated)	60 mg/dL (1026 µmol/L)	3.3 g/dL (33 g/L)	NSI
	60 mg/dL (1026 µmol/L)	4.7 g/dL (47 g/L)	NSI
Hemolysis (hemoglobin)	500 mg/dL (5.0 g/L)	3.1 g/dL (31 g/L)	NSI
	750 mg/dL (7.5 g/L)	3.1 g/dL (31 g/L)	↓12.9%
	1000 mg/dL (10.0 g/L)	4.5 g/dL (45 g/L)	NSI
Lipemia (from Intralipid)	525 mg/dL (5.9 mmol/L)†	3.4 g/dL (34 g/L)	NSI
	638 mg/dL (7.2 mmol/L)†	3.3 g/dL (33 g/L)	↓12.1%
	638 mg/dL (7.2 mmol/L)†	4.7 g/dL (47 g/L)	NSI
	850 mg/dL (9.6 mmol/L)†	4.7 g/dL (47 g/L)	↓10.6%

* NSI = No Significant Interference. A percentage effect of ≥10% is considered a significant interference.
† SI units calculated as triolein.

ADVIA 2400

Interferent	Interferent Level	Albumin Sample Concentration	Interference*
Bilirubin (conjugated)	60 mg/dL (1026 µmol/L)	3.3 g/dL (33 g/L)	NSI
	60 mg/dL (1026 µmol/L)	4.8 g/dL (48 g/L)	NSI
Bilirubin (unconjugated)	60 mg/dL (1026 µmol/L)	3.4 g/dL (34 g/L)	NSI
	60 mg/dL (1026 µmol/L)	4.8 g/dL (48 g/L)	NSI
Hemolysis (hemoglobin)	750 mg/dL (7.5 g/L)	3.2 g/dL (32 g/L)	NSI
	1000 mg/dL (10.0 g/L)	3.2 g/dL (32 g/L)	↓12.5%
	1000 mg/dL (10.0 g/L)	4.6 g/dL (46 g/L)	NSI
Lipemia (from Intralipid)	638 mg/dL (7.2 mmol/L)†	3.4 g/dL (34 g/L)	NSI
	850 mg/dL (9.6 mmol/L)†	3.4 g/dL (34 g/L)	↓14.7%
	638 mg/dL (7.2 mmol/L)†	4.8 g/dL (48 g/L)	NSI
	850 mg/dL (9.6 mmol/L)†	4.8 g/dL (48 g/L)	↓10.4%

* NSI = No Significant Interference. A percentage effect of ≥10% is considered a significant interference.
† SI units calculated as triolein.

CMPF (3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid) present in sera of patients with renal failure has been reported to give falsely low albumin values.[8,9]

Standardization

The ADVIA Chemistry ALBP assay is traceable to ERM-DA470k Reference Material.[10] Assigned values of the ADVIA Chemistry Albumin BCP Calibrator are traceable to this standardization.

Technical Assistance

For customer support, please contact your local technical support provider or distributor.

www.siemens.com/diagnostics

References

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Definition of Symbols

The following symbols may appear on the product labeling:

Symbol	Definition	Symbol	Definition
IVD	In vitro diagnostic medical device	REF REF	Catalog number
	Legal manufacturer	EC REP	Authorized Representative in the European Community
CE	CE Mark	CE 0086	CE Mark with identification number of notified body
	Consult instructions for use		Biological risk
	Keep away from sunlight and heat		Temperature limitation
	Lower limit of temperature		Upper limit of temperature
	Do not freeze (> 0°C)		Up
	Use by	Σ (n)	Contains sufficient for (n) tests
	Recycle	PRINTED WITH SOY INK	Printed with soy ink
Rev.	Revision	YYYY-MM-DD	Date format (year-month-day)
LOT	Batch code		

Trademarks

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10699225 EN Rev. C, 2015-06