



Clinical Trial Protocol	
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1. Abstract

Rationale

Stroke is the third leading cause of death and the first cause of physical disability and dementia worldwide. Two main types of stroke exist, ischemic stroke (IS) and intracerebral haemorrhage (ICH), with similar symptoms, but opposite treatment. Among the population of ischemic strokes, occlusion of a large vessel (LVO) gives rise to the most dangerous and deadly stroke subtype. A very effective treatment, called thrombectomy is available for LVO patients, but it has to be administered within 6 hours from stroke onset. Currently, there are no diagnostic methods able to identify LVO patients in the ambulance and direct them to thrombectomy. POCKiT DX are developing a revolutionary innovation that combines blood biomarkers that are highly specific for stroke subtypes with ultra-rapid (<20 minutes) immunoassay detection within a point-of-care device. The panel of blood biomarkers identified by POCKiT DX has already shown 86% sensitivity for LVO identification. The TIME study will aid in the identification and evaluation of the diagnostic performance of the blood biomarker panel designed by POCKiT DX.

Method

The TIME study is a multi-centre, international observational prospective cohort study. Patients referred to the emergency department or stroke unit with a suspected stroke will be enrolled in the study. Up to 2000 patients will be recruited and requested to provide one venous blood sample. Blood samples will be processed on site and frozen. Samples will be analysed retrospectively via standard laboratory assays.

Outcomes

The main outcome of the TIME study will be the evaluation of the clinical diagnostic performance of a panel of blood biomarkers, in conjunction with clinical data, for the identification of large vessel occlusion ischemic stroke subtype. This study will allow the identification and evaluation of a final panel of biomarkers and will prompt the development of a test for LVO stroke diagnosis.

2. Rationale

Stroke affects 16M people in the world every year and 100K in the UK only. More than 30% of these patients die and 90% of survivors develop permanent disabilities as a consequence of stroke. There are two main types of stroke, ischemic and haemorrhagic. Ischemic stroke is caused by the formation of a clot in a blood vessel in the brain and represents ~85% of stroke patients. Haemorrhagic stroke is due to the rupture of a blood vessel in the brain, with consequent bleeding, and it represents ~15% of stroke patients. Both stroke subtypes cause brain damage and their symptoms are very similar. In addition to these two types of stroke, among the population of suspected stroke patients, there are the so-called stroke “mimics”. These are conditions that appear with stroke-like symptoms (e.g. migraine, epilepsy, encephalitis, etc) but that are not stroke.

The deadliest stroke subtype is the one caused by occlusion of large vessels (LVO) in the brain. For these patients, a new treatment is available, called thrombectomy, which is a surgical procedure that mechanically removes the clot via a probe inserted at the level of the groin. Treatment of LVO patients with thrombectomy significantly increases the chances of survival, as well as decreases the extent of disability¹. Thrombectomy is only available in comprehensive stroke centres (CSC), and LVO patients have to be transported specifically to CSC in order to be treated and increase their chances of survival.

Current treatment of stroke patients is dependent on diagnosis via computerised tomography (CT) scan to the head. CT is highly accurate for detection of brain haemorrhages, but is very inaccurate for detection of ischemic stroke or LVOs². In case of a negative result from CT, neurologists can order a further MRI scan to confirm ischemic stroke. If LVO is suspected, patients are transported to the nearest CSC where a further procedure, called CT angiography, is performed. The identification of LVO patients can be a very lengthy process that wastes precious time and makes stroke patients worse.

Fast diagnosis of stroke patients at their first point of admission (i.e. ambulance or emergency department) able to identify LVO patients could quickly direct patients to CSC and significantly improve treatment of the most dangerous stroke subtype. Several studies have investigated the ability of pre-hospital assessment scores based on patient symptoms to be performed in the ambulance³⁻⁶. Despite this, these scoring scales lack the accuracy required for triaging LVO patients with confidence. A more accurate diagnostic test able to complement these assessment scores and direct LVO patients to CSC and CT angiography is much needed⁷.

The aim of the TIME study is to evaluate the clinical diagnostic performance of a panel of blood biomarkers identified by POCkit diagnostics in the identification of LVO stroke patients among the population of suspected stroke. Diagnostic performance of blood biomarkers in conjunction with clinical data and pre-hospital stroke assessment scores (e.g. EMSA) will be evaluated. Results of this study will direct the development of a diagnostic test for directing stroke patients to the right treatment, more rapidly, improving patient outcomes.

3. Aim of the study

The primary aim of this study is to evaluate the clinical diagnostic performance of a panel of blood biomarkers, in combination with clinical data, for the identification of LVO ischemic strokes in the population of suspected stroke patients admitted to ambulance or emergency department. Performance of blood biomarkers together with stroke scaling systems will be evaluated.

The secondary aim of the study is to evaluate the diagnostic performance of blood biomarkers to identify all ischemic strokes in the same patient population.

4. Study endpoints

4.1. Primary outcomes

Primary outcomes of the TIME study will be values of diagnostic accuracy, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of individual/combinations of blood biomarkers and clinical data for identification of LVO ischemic strokes. Together with diagnostic performance, threshold of individual biomarkers, as well as of predictive models of biomarker combinations, will be obtained.

4.2. Secondary outcomes

Secondary outcomes will be values of diagnostic accuracy, sensitivity, specificity, PPV and NPV of individual/combinations of blood biomarkers and clinical data for identification of ischemic strokes vs haemorrhagic strokes and mimics.

5. Study design and methodology

The TIME study is designed as an observational prospective cohort study.

This study is designed to validate candidate biomarkers according to the STARD principles (<http://www.equator-network.org/reporting-guidelines/stard/>).

5.1. Patient recruitment

Patients will be recruited prospectively at their arrival to the emergency department (ED). Patients will be recruited as soon as possible after their arrival to ED and preferably before CT/MRI scans are performed. Patients recruitment will fit into standard clinical procedures for acute stroke management, with minimal disturbance to the patient.

The nurse or physician that is part of the team responsible for the patient's clinical care will identify potential subjects by initiating a "code stroke". Potential subjects will

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subsequently be provided with the patient information sheet. During the visit to the ED the local research assistant and/or physician will answer any remaining questions and inquire if patients are willing to contribute to the research project. If they wish to do so, informed consent will be obtained. All consent will be obtained by a qualified research assistant. Patients will always be given sufficient time to read the documentation and ask questions. Patients will be offered the possibility to take more time to consider participating. A legally authorized representative may also give consent for the patient's participation.

In case the patient is unconscious, or otherwise unable to provide informed consent, this will be considered as a specific circumstance and informed consent procedure may be waived (45 CFR 46.116). Venous blood is routinely withdrawn for standard clinical laboratory tests, and this research study does not involve additional risk for the patient. In addition, this study could not be carried out without the waiver, due to representation of unconscious patients in the target population. The population of LVO strokes is expected to be enriched with patients presenting with more severe symptoms, including unconsciousness. Failure to recruit unconscious LVO patients, due to inability to obtain informed consent, can alter the representation of LVO patients in the final population. This could ultimately result in inappropriate statistical power and inability to observe optimal diagnostic performance. Importantly, identifiable private information of waived consent patients will not be made available to anyone outside the hospital site. If the patient regains consciousness during the stay at hospital site, informed consent will be obtained as per standard consent procedure for conscious patients.

5.2. Sample collection and processing

One 5 mL sample of venous blood will be collected from each consented patient. Blood will be collected using standard procedure. Briefly, blood samples from a single blood draw will be collected into tubes containing sodium EDTA and immediately placed on ice. Blood samples will be processed immediately or within 30 minutes from withdrawal. Blood samples collected into EDTA tubes will be centrifuged at 2000 g for 15 minutes at 4°C. Plasma will be divided into 3 aliquots of ~1 mL each and all plasma aliquots will be frozen at –80°C. Plasma aliquots will be stored at hospital site until transfer to POCkit diagnostics.

5.3. Diagnostic criteria

Patients admitted to the ED with suspected stroke will be recruited in the study. Patient population is expected to be composed of ~50% ischemic strokes (of which ~30-40% could have LVOs), ~40% stroke mimics, and ~10% haemorrhagic strokes.

Final diagnosis of stroke subtype or stroke mimics will be established based on CT-based and MRI-based neurologist report. Diagnosis of ischemic stroke will be based on a positive result from MRI scan. Diagnosis of LVOs will be based on results interpretation from CT angiography. Diagnosis of haemorrhagic stroke (ICH or subarachnoid haemorrhage) will be based on positive results from CT scan and, where possible, MRI scan too. Diagnosis of stroke mimics will be based on negative results from both CT scan and MRI scan. Final diagnosis of stroke mimics (i.e. migraine, encephalitis, epilepsy, etc) will be obtained from

patient data, where possible. All diagnostic results will require interpretation from a qualified stroke neurologist.

5.4. Biomarker measurement

Upon transfer of plasma aliquots from hospital site to POCKiT diagnostics, a panel of plasma proteins and metabolites will be measured by POCKiT diagnostics. Measurement of protein markers will be performed using commercial enzyme-linked immunosorbent assay (ELISA) kits. Plasma metabolites will be measured with commercial enzymatic assay kits. All assays will be performed according to manufacturer's instructions and compliant to ISO13485 and cGMP.

POCKiT diagnostics may sub-contract an external contract research organisation (CRO) to perform biomarker measurement. In this case, blood samples will be transferred from hospital site to the designated CRO.

Interim analyses may be performed upon collection of the first 200, 400, 600, or 1000 samples. In all these cases, transfer of collected blood samples from hospital site to POCKiT diagnostics (or designated CRO) will be required to perform the interim analysis. Results from interim analyses will evaluate the necessity to modify the study protocol or to continue sample recruitment.

6. Study population

A total of up to 2000 patients are expected to be recruited in this study with target enrolment of 1000 patients. In order to be eligible for study participation, subjects must meet the following inclusion and exclusion criteria at the time of consent.

6.1. Inclusion criteria

- 1) Older than 18 years at time of consent;
- 2) Evaluated in the emergency department with suspected and code stroke activated.
- 3) Time from stroke onset < 18 hours

6.2. Exclusion criteria

- 1) Received thrombolytic therapy (e.g. tPA, Alteplase) before collection of blood;
- 2) (Anticipated) inability to provide a blood sample;
- 3) Time from stroke onset > 18 hours.
- 4) At time of consent participating in a Clinical Trial Investigational Medicinal Product (CTIMP)

6.3. Withdrawal criteria

Patients and blood samples will be withdrawn from this study in case:

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- 1) More than 60 minutes have passed from blood withdrawal to beginning of centrifugation;
- 2) Presence of haemolysis in blood plasma;
- 3) Absence of imaging results.

6.4. Sample size estimation

A power analysis was performed aiming at understanding the minimal sample size required to discover given thresholds of sensitivity and specificity for LVO strokes, as well as ischemic strokes.

Two main scenarios have been considered. The test can be used:

- 1) as a rule-in to identify patients with Large Vessel Occlusion (LVO)
- 2) as a rule-in to identify patients who suffered ischemic stroke.

For the first scenario, we assumed a prevalence of 0.28, an expected sensitivity of the test of 99% with a minimum clinically acceptable sensitivity of 95%. Assuming a two-tailed 5% type I error rate (α) and 90% power (β), the required sample size is 646 with a number of LVO cases of 181. Assuming an accepted specificity of 95% with a minimum clinically acceptable specificity of 90%, two-tailed 5% type I error rate (α) and 90% power (β), the required sample size is 366 with a number of LVO cases of 103.

For the second scenario, we assumed a prevalence of 50%, an expected sensitivity of the test of 99% with a minimum clinically acceptable sensitivity of 95%. Assuming a two-tailed 5% type I error rate (α) and 90% power (β), the required sample size is 362 with a number of ischemic strokes detected of 181. Assuming an accepted specificity of 95% with a minimum clinically acceptable specificity of 90%, two-tailed 5% type I error rate (α) and 90% power (β), the required sample size is 526 with a number of ischemic strokes detected of 263.

Based on the power calculation results obtained, we concluded that a sample size of 646 provides adequate statistical power for all considered scenarios. Allowing for 5% of samples being withdrawn from the study because of absence of complete diagnostic results (e.g. imaging) or problems during sample processing, our final sample size estimation is 678 patients with suspected stroke.

Sample size estimation was performed according to Chu and Cole⁹.

Prevalence of LVO/ischemic stroke will be monitored during recruitment to ensure that calculated sample size is appropriate for actual prevalence. A prevalence in the range of 20-40% LVO, and 35-65% ischemic stroke will be accepted. In case observed prevalence will be outside the acceptable range, sample size will be re-estimated and recruitment size adjusted accordingly.

7. Data management

7.1. Data sources and collected data

█ Clinical data

Different types of clinical data will be collected in this study:

- 1) **Final diagnosis:** LVO ischemic stroke, non-LVO ischemic stroke, hemorrhagic stroke, stroke mimic
- 2) **Demographics:** Age, sex, smoking, etc
- 3) **Specific Clinical data:** atrial fibrillation, systolic/diastolic blood pressure, hypertension.
- 4) **Stroke scaling score:** the initial national institute of health stroke scaling (NIHSS) score performed at ED. Scores of individual NIHSS items will be extracted from each NIHSS form and included in the CRF. EMSA score will be calculated from NIHSS form as described by Gropen et al ¹⁰.
- 5) **Stroke onset to blood collection time (OBT).** Time from presumed onset of stroke (as interpreted by local stroke physician) to blood collection will be recorded to evaluate kinetic dynamics of blood biomarkers.
- 6) **Whole blood-to-plasma ratio (WBP).** The volume of whole blood drawn from patient and the volume of plasma resulting after centrifugation will be collected. This information is fundamental because different subjects have different WBPs, and analysis of plasma samples only could create a bias. Recording WBPs from each patient will allow to keep track of the real concentration of biomarkers in whole blood and normalise patients based on their WBPs.

█ Biomarker data

After collection, blood samples will be stored at -80°C until shipment to POCkiT diagnostics. Data on absolute concentrations of blood biomarkers will be obtained using standard procedures (see section 5.4).

Combination of this data with clinical data will allow to build a predictive model for identification of LVOs and/or ischemic stroke (see section 7.4).

7.2. Data management

POCKiT diagnostics is responsible for the data management of this study including quality checking of the data.

All clinical data collected for the purpose of this study are recorded in a Case Report Form (CRF). The CRF will function as the source data if data is not detailed in patient charts or if described in this protocol.

An integrated database for this study will include all clinical data (provided by hospital site within the CRF) and biomarker data (detailed in section 7.1). The clinical database will be locked once all data entry, validation and reconciliation of queries has been completed and a final curation of the collected biomarker data has been completed. The data will subsequently be analysed according to the data analysis plan detailed in section 7.4.

Brain images will only be used to establish final patient diagnosis and will not be part of the CRF. Brain images will not be shared with anyone outside hospital site.

POCKiT diagnostic personnel involved in running subject samples will be blinded to any clinical data pertaining to the nature of these samples.

7.3. Data storage

All electronic data will be stored on a secure server with restricted access to authorised POCKiT diagnostics personnel only. All information will be sent to and -if applicable -from this server through a secure encrypted connection.

Blood samples may be partially or fully utilised as part of the analysis process. Blood samples will be stored at POCKiT diagnostic premises (Cambridge, UK). All samples will be handled in accordance with the Human Tissue Act 2004, and Material Transfer Agreements will be put in place as required by POCKiT diagnostics and hospital site. Upon collection, samples will be stored at hospital site, until shipment to POCKiT diagnostics. Samples will be analysed all at the same time, and only after all samples have been received by POCKiT diagnostics. Samples could be analysed for a period up to 10 years after completion of this study, after which they will be destroyed following standard procedures. During this period data might be used for other studies conducted by POCKiT diagnostics.

Study records and documents at the clinical site, including signed Informed Consent Forms (ICFs), pertaining to the conduct of this study must be retained by the hospital for at least 2 years after study completion unless local regulations or institutional policies require a longer retention period.

No records may be destroyed during the retention period without the written approval of POCKiT diagnostics. No records or data may be transferred to another location or party without written notification to POCKiT diagnostics.

7.4. Data analysis plan

Clinical and biomarker data will be curated after clinical and technical monitoring to assure maximum validity of the data utilised to construct the algorithm. Any subject matching criteria as defined in section "6.3 – Withdrawal criteria" will not be incorporated into the primary analysis. This data is quarantined for a separate sub-group analysis where relevant.

■ Data exploration

Data will be explored by generating box plots of blood biomarker concentrations (plasma or whole blood) and clinical data, according to stroke subtypes. Concentrations of blood biomarkers and clinical data (where applicable) may undergo pre-processing and/or transformations to ensure statistical assumptions are met prior to analysis. Univariate logistic regressions will be used to assess the need of transformations.

■ Whole blood biomarker concentrations

Plasma concentration of individual biomarkers will be adjusted based on whole blood-to-plasma (WBP) ratios, in order to obtain whole blood biomarker concentrations. Values of

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biomarker concentrations (plasma or whole blood) will be reported with corresponding values of standard error of the mean (s.e.m.).

█ *Covariate analysis*

Association between clinical variables and concentration of individual blood biomarkers will be assessed. A linear regression model between biomarker concentration (as the outcome) and key clinical covariates will be generated.

█ *Biomarker kinetics*

To assess the effect of biomarker kinetics on diagnostic performance, patient population will be divided into 3-hour and 6-hour bins based on their stroke onset to blood collection time and multivariate logistic regression will be performed on each population bin separately. Alternatively, the distribution of stroke onset to blood collection times will be assessed and the population will be divided into quartiles or halves, and compared. Results from this analysis will be compared to results obtained on the whole population and will inform if the test will be limited to a specific time window from stroke onset.

█ *Diagnostic performance*

Values of diagnostic accuracy, sensitivity, specificity, positive and negative predictive values for the prevalence reported, positive and negative likelihood ratios will be calculated and reported with confidence intervals. ROC curve will be drawn and the area under the ROC curve will be reported with confidence intervals.

█ *Biomarker diagnostic thresholds*

Thresholds of individual biomarkers already established by POCkIT diagnostics will be validated for their diagnostic performance. If established thresholds are not available, thresholds of individual biomarkers alone, and in conjunction with clinical variables, will be identified by maximising sensitivity while maintaining a minimal specificity of 50% for LVO/ischemic stroke. In addition, another set of thresholds will be identified by maximising specificity, while keeping a minimal sensitivity of 70% for LVO/ischemic stroke.

7.5. Publication policy

The results of this study will be published in peer-reviewed journals and presented at scientific meetings. The study data will, in principle, be published in its entirety unless a sub-analysis is specifically earmarked for separate publication. In addition to these publications the results of study will be published on a free, publicly accessible clinical trial results database.

All publications including manuscripts, abstracts and posters shall be presented to all investigators and to POCkIT diagnostics prior to submission. POCkIT diagnostics shall return a review of the intended publication within 60 days. POCkIT diagnostics can delay publication for up to 6 months from the date of first submission by the author allowing POCkIT diagnostics to protect proprietary information and/or intellectual property rights. Authorship will be determined by mutual agreement and in line with International Committee of Medical Journal Editors authorship requirements.

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The data generated in this study may be disseminated by POCkit diagnostics for the purpose of development and validation of their products. Data may therefore be used in presentations and publications by POCkit diagnostics as well as submission(s) to regulatory authorities.

8. References

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