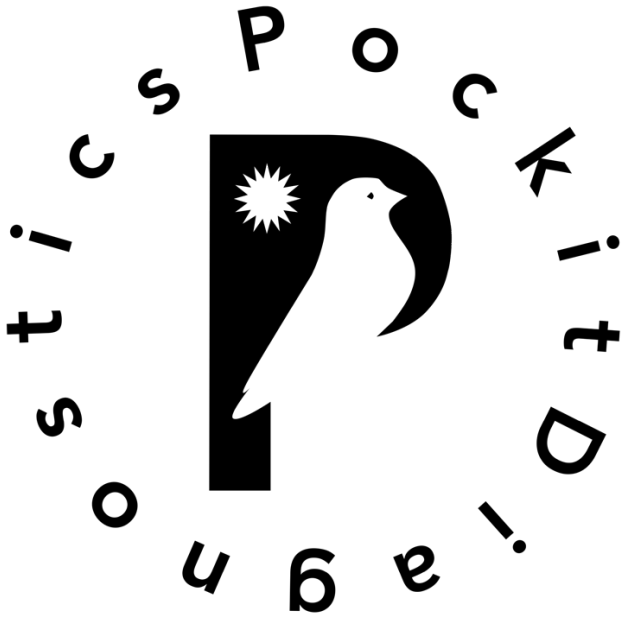

Clinical Trial Protocol	
Protocol Title	Testing of Identification Markers for strokE
Acronym	TIME
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Study Sponsor	Pockit diagnostics Ltd, Future Business Centre, CB4 2HY Cambridge, United Kingdom
Sponsor's Head of Clinical	Edoardo Gaude, PhD, Pockit diagnostics Ltd

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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 different treatment. Among the population of ischemic strokes, large vessel occlusions (LVO) give rise to the most dangerous and deadly stroke subtype. A very effective treatment, called endovascular thrombectomy (EVT) is available for LVO patients, but it is only available at specialised centres around the country. Currently, there are no diagnostic methods able to identify LVO patients in the ambulance and direct them to EVT centres. Pockit diagnostics are developing a revolutionary innovation that combines highly specific blood biomarkers for LVO with stroke severity scales. The diagnostic algorithm identified by Pockit diagnostics has already shown 98% specificity and 95% accuracy for LVO detection. The TIME study will aid in the identification and evaluation of the diagnostic performance of the diagnostic algorithm designed by Pockit diagnostics.

Method

The TIME study is a multi-centre, 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 400 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 validation of the clinical diagnostic performance of Pockit diagnostics' algorithm for LVO detection. This study will validate previously defined diagnostic thresholds for LVO identification.

2. Rationale

Stroke is among the leading causes of death and disability worldwide. Ischaemic stroke caused by large vessel occlusion (LVO) contributes disproportionally to these figures and accounts for 62% of post-stroke disabilities and 96% of post-stroke mortality[1].

Acute ischaemic stroke patients with LVO can be effectively treated with endovascular thrombectomy (EVT)[2], but this treatment is only available in comprehensive stroke centers (CSC) or other EVT-capable centers. Inter-hospital transfer of LVO patients from primary stroke centers to EVT-capable centers significantly delays time to treatment and leads to higher disability rates[3]. Identification of LVO patients in the pre-hospital setting (e.g. within an ambulance) could enable the transfer of LVO patients to EVT-capable centers directly, reducing time to treatment, functional disabilities, and deaths.

Multiple studies have investigated the ability of brief pre-hospital stroke assessment scales to identify LVO strokes in the field. Despite this, these severity scales lack the sensitivity and specificity required for triaging LVO patients with confidence, resulting in false negatives in patients with LVO and milder stroke as well as false positives in patients with stroke mimics or haemorrhagic strokes[4,5]. A more accurate diagnostic test able to complement these assessment scores and direct LVO patients to EVT-capable centers is much needed.

Pockit diagnostics have ventured a novel strategy for the identification of LVO. By combining the measurement of D-dimer and GFAP with pre-hospital stroke severity scales (e.g. FAST-ED[6]), Pockit diagnostics have created a powerful diagnostic algorithm that has demonstrated accuracy of 95% (95%CI 87-99%) and specificity of 98% (95% CI 92-100%) for LVO detection[7].

The aim of the TIME study is to validate the clinical diagnostic performance of previously defined diagnostic thresholds of Pockit diagnostics' algorithm. Results of this study will direct the development of a diagnostic test for identifying LVO patients in the field, directing them to EVT centre, ultimately enabling faster treatment, and improving patient outcomes.

3. Aim of the study

The primary aim of this study is to validate the clinical diagnostic performance of previously defined cut-off points on the plasma concentration of D-dimer and GFAP, combined with stroke severity scales (FAST-ED, FAST, EMSA, RACE, C-STAT).

The secondary aim of the study is to optimise the cut-off points of the diagnostic algorithm for LVO detection, in a larger and inter-racial patient population.

4. Study endpoints

4.1. Primary outcomes

Primary outcomes of the TIME study will be values of diagnostic accuracy, sensitivity, specificity, positive likelihood ratio (LR+) and negative likelihood ratio (LR-) of the model composed of D-dimer, GFAP and stroke severity scales (FAST-ED, FAST, EMSA, RACE, C-STAT) for identification of LVO.

4.2. Secondary outcomes

Secondary outcomes will be optimised values of ut-off points for the different diagnostic algoirthms (one per stroke severity scale evaluated) obtained in a larger interracial population.

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 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 UMMC to the designated CRO.

Interim analyses may be performed upon collection of the first 100, or 200 samples. In all these cases, transfer of collected clinical data from the 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 400 patients are expected to be recruited in this study. 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:

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

The proposed intended use of the test is to rule in patients with LVO to redirect them to EVT-capable centers from a population of suspected stroke, thus we powered the study on specificity. Assuming a prevalence of 20%, a minimum specificity of 90%, a two-tailed 5% type I error rate (**α**) and 90% power (**β**), a sample size of 329 suspected stroke patients with 66 LVO cases would detect a diagnostic specificity of 95% (95%CI: 90%–100%) for each

algorithm. Accounting for 20% of data loss due to missing patient data or sample lysis, a final number of 395 patients will be required.

Sample size estimation was performed according to Chu and Cole[8].

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 10-30% LVO 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:***
    a. NIHSS score and individual NIHSS items used to calculate the score, as collected at the time of patient admission to ED.
    b. FAST-ED score collected by ambulance paramedics;
    c. FAST score collected by ambulance paramedics;
    d. Any other stroke severity scale collected pre-hospitally (e.g. EMSA, RACE).
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).

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. Akaike information criterion (AIC) 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 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 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. Confidence intervals will be estimated via 2000 bootstrapped replicates.

Biomarker diagnostic thresholds

Thresholds of individual biomarkers already established by Pockit diagnostics will be validated for their diagnostic performance. If the accuracy achieved by the model is below acceptable levels (specificity <90% and/or sensitivity<60%) we will aim to refine the model using data from our 395 patient cohort.

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.

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.

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