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# BMJ Open Identifying patients with cerebral infarction within the time window compatible with reperfusion therapy, diagnostic performance of glutathione S-transferase- $\pi$ (GST- $\pi$ ) and peroxiredoxin 1 (PRDX1): exploratory prospective multicentre study FLAG-1 protocol

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## ABSTRACT

**Introduction** Plasma biomarkers may be useful in diagnosing acute cerebral infarction requiring urgent reperfusion, but their performance remains to be confirmed. If confirmed, these molecules could be used to develop rapid and reliable decentralised measurement methods, making it possible to initiate reperfusion therapy before hospital admission. The FLAG-1 large prospective study will constitute a plasma bank to assess the diagnostic performance of two biomarkers: glutathione S-transferase- $\pi$  and peroxiredoxin 1. These molecules are involved in the oxidative stress response and could identify cerebral infarction within a therapeutic window of less than 4.5 hours following the onset of symptoms. Secondary objectives include assessing performance of these biomarkers within 3-hour and 6-hour windows; identifying additional biomarkers diagnosing cerebral infarction and significant criteria guiding therapeutic decisions: ischaemic features of stroke, presence of diffusion/fluid-attenuated inversion recovery mismatch, volume of cerebral infarction and penumbra on cerebral MRI.

**Methods and analysis** The exploratory, prospective, multicentre FLAG-1 Study will include 945 patients with acute stroke symptoms (onset  $\leq 12$  hours, National Institute of Health Stroke Scale score  $\geq 3$ ). Each patient's 25 mL blood sample will be associated with cerebral MRI data. Two patient groups will be defined based on the time of blood collection (before and after 4.5 hours following onset). Receiver operating characteristic analysis will determine the diagnostic performance of each biomarker, alone or in combination, for the identification of cerebral infarction  $< 4.5$  hours.

**Ethics and dissemination** The protocol has been approved by an independent ethics committee. Biological samples are retained in line with best practices and

## Strengths and limitations of this study

- The FLAG-1 Study is an exploratory prospective multicentre interventional study to collect plasma samples from patients with stroke symptoms.
- Time from symptom onset to collection has to be less than 12 hours, and leads to dichotomising included patients (less than and more than 4.5 hours).
- Patients are examined with cerebral MRI with usual sequences to explore cerebrovascular diseases, including perfusion sequence.
- Levels of relevant biomarkers, including enzymes glutathione S-transferase- $\pi$  and peroxiredoxin 1 involved in the oxidative stress response in cerebral infarction, are assessed using ELISA method.
- Receiver operating characteristic analysis will be performed to determine diagnostic performance of biomarkers to identify stroke, onset time, infarction and penumbra volumes.

procedures, in accordance with French legislation. Anonymised data and cerebral imaging records are stored using electronic case report forms and a secure server, respectively, registered with the French Data Protection Authority (Commission Nationale de l'Informatique et des Libertés (CNIL)). Results will be disseminated through scientific meetings and publication in peer-reviewed medical journals.

**Trial registration number** ClinicalTrials.gov Registry (NCT03364296).

## INTRODUCTION

Research into plasma biomarkers of potential interest in the neurovascular field has

generated a huge body of literature. However, most studies only attempt to predict prognosis for patients who had a stroke, and their sample sizes are too small to provide robust results.<sup>1</sup> The potential for use of plasma biomarkers as an aid to stroke diagnosis remains rarely studied. The identification of biomarkers allowing rapid stroke diagnosis would improve patient management in practice, in the same way as troponin levels have improved management of myocardial infarction. A key parameter to determine would be cerebral infarction onset time, which could be estimated from biomarker kinetics. Indeed, stroke onset time is unknown for one-third of patients admitted to hospital.<sup>2 3</sup> This parameter is nevertheless important, as it is associated with the success of reperfusion therapy and risk of haemorrhagic transformation.<sup>4</sup> Previous results from our team revealed the ability of two proteins involved in the oxidative stress response (glutathione S-transferase- $\alpha$  (GST- $\alpha$ ) and peroxiredoxin 1 (PRDX1)) to estimate the time of cerebral infarction onset.<sup>5 6</sup>

Currently, imaging remains the only method by which stroke can be definitively diagnosed in patients presenting acute neurological deficits. In some countries, ambulances are equipped with CT scanner to diagnose stroke before hospital admission, making it possible to initiate intravenous thrombolysis (IVT) at an early stage.<sup>7</sup> However, this practice is quite onerous, and its use remains exceptional. If plasma biomarkers specific for cerebral infarction were identified, rapid and reliable decentralised measurement methods could be developed to facilitate diagnosis before hospital admission. Additional biomarkers estimating significant criteria guiding therapeutic decisions in case of cerebral infarction, such as necrosis volume and penumbra, would also be of considerable interest.

Based on our previous results, we hypothesise that GST- $\alpha$  and PRDX1 plasma levels can identify patients with cerebral infarction within the therapeutic window of less than 4.5 hours since onset. To test this hypothesis, we have developed the exploratory prospective multicentre FLAG-1 Study. During this study, we will constitute a plasma bank with samples from patients with acute neurological deficits for whom cerebral MRI data are available. Moreover, we aim to identify biomarkers for diagnosis of cerebral infarction, and related to criteria such as infarction and penumbra volume, that could guide important treatment decisions.

## METHODS AND ANALYSIS

### Objectives and endpoints

The main objective of the FLAG-1 Study is to determine the diagnostic performance of circulating levels of GST- $\alpha$  and PRDX1, alone or in combination, to identify cerebral infarction within 4.5 hours in a prospective cohort of patients in whom stroke symptoms appeared less than 12 hours before samples were collected. The primary endpoint of the study is the time from symptom onset to

blood sampling, dichotomised as less than and more than 4.5 hours.

Secondary objectives are as follows:

- ▶ Determine the diagnostic performance of GST- $\alpha$  and PRDX1 levels for the identification of cerebral infarction at less than 3 hours and less than 6 hours.
- ▶ Examine how levels of GST- $\alpha$ , PRDX1, S100 calcium-binding protein B (S100b) and glial fibrillar astrocytic protein (GFAP) associate with:
  - Stroke diagnosis.
  - Ischaemic or haemorrhagic features of stroke.
  - The presence of diffusion/fluid-attenuated inversion recovery (FLAIR) mismatch on cerebral MRI.
  - The volume of cerebral infarction and penumbra.

The corresponding secondary endpoints are thus:

- ▶ Time from symptom onset to blood draw dichotomised as less than and more than 3 hours, and less than and more than 6 hours.
- ▶ Stroke diagnosis (vs stroke mimics).
- ▶ Ischaemic features of stroke (vs haemorrhagic features).
- ▶ Presence of diffusion/FLAIR mismatch (vs no diffusion/FLAIR mismatch).
- ▶ Extent of cerebral infarction, and penumbra volume, both quantified by MRI.

### Study setting

FLAG-1 is an exploratory prospective multicentre interventional study, for which 945 patients will be included from six stroke centres in France. The study was awarded funding from the French Health Ministry–Groupement Interrégional de Recherche Clinique et d'Innovation de l'Est (Programme Hospitalier de Recherche Clinique Interrégional—PHRCI-16-050). The first patient was included on 15 October 2018; inclusions are planned until 15 October 2021 (36 months), and the study will end before 15 April 2022. The duration of participation for patients ranges from 1 day to 6 months following diagnosis. Data collection and analysis are planned to be completed by 15 April 2023.

### Population study

Patients will be included if:

- ▶ They have reached the legal age of majority (18 years).
- ▶ They present symptoms consistent with stroke.
- ▶ Their National Institute of Health Stroke Scale (NIHSS) score at inclusion is greater than or equal to 3.
- ▶ Time of symptom onset is known.
- ▶ The symptom-onset-to-inclusion time is less than or equal to 12 hours.

Patients will be not included if:

- ▶ They have a disease interfering with biomarker levels (known oncological disease, cirrhosis, medical history of myocardial infarction or history of stroke in previous 3 months).

- ▶ They present traumatic cerebral lesions (including subdural, epidural or parenchymal haematoma, subarachnoid haemorrhage or contusions).
- ▶ There are contraindications to performing MRI.

As no data are available in the literature that would predict the impact of renal failure on biomarker levels, the study protocol allows inclusion of patients with renal failure. To determine any effect, analysis of circulating biomarker concentrations will be conducted with due consideration of the glomerular filtration rate.

### Intervention

Patients are included after admission to one of the stroke centres with access to MRI, following an assessment by a neurologist, verification of inclusion and non-inclusion criteria, and if the time of stroke onset is precisely specified by the patient, relatives, and/or during the first medical assistance interview. Inclusion will not alter clinical handling of patients with suspected stroke, which will conform to current guidelines.

### Blood collection

To determine biomarker levels, a 25 mL blood sample is collected, ideally at the same time as the standard venous puncture for blood collection during the acute phase of stroke for urgent biological assays.

### Cerebral MRI

Cerebral MRI is performed within 30 min of blood collection with the sequences commonly used to diagnose stroke: diffusion-weighted imaging with apparent diffusion coefficient mapping, FLAIR imaging, T2\*-weighted gradient-recalled echo imaging, time-of-flight MR angiography and dynamic susceptibility contrast-enhanced MR perfusion. In each centre of inclusion, sequences are implemented in line with technical specifications defined in the study protocol to obtain consistent data.

### Follow-up

A final examination is scheduled between 3 and 6 months after inclusion (only for patients for whom cerebral infarction was confirmed by MRI) to complete information relating to investigations to determine stroke aetiology, and to determine the modified Rankin Scale (mRS) score.

### Data collection

Data management is performed by a data manager at Nancy Clinical Investigation Center (CIC-P), France.

### Clinical data

Data are collected relating to:

- ▶ Patient characteristics (sex, age, vascular risk factors including hypertension, diabetes, tobacco use, dyslipidaemia, alcohol consumption, medical history of migraine, atrial fibrillation, coronary heart disease, medical history of stroke, medication).
- ▶ Clinical state at admission (time of symptom onset, NIHSS score).

- ▶ Therapies (reperfusion with IVT and/or mechanical thrombectomy (MT)).
- ▶ Stroke aetiology (ASCOD classification).<sup>8</sup>
- ▶ Degree of dependence (mRS) assessed between 3 and 6 months after inclusion.

### Biological data

The plasma bank is constituted from blood samples. After centrifugation (3000g) of blood samples, EDTA plasma is immediately aliquoted and stored at  $-80^{\circ}\text{C}$  until analysis. Biomarker levels in plasma are determined using the ELISA method. GST- $\alpha$  concentration is determined using the Abbexa colorimetric assay (abx151762) according to the manufacturer's recommendations, on samples diluted 1/5. The PRDX1 level is determined using the Abnova ELISA kit, on samples diluted 1/4, and PRDX1 concentrations are measured by a colorimetric detection system. S100b concentrations are measured by a colorimetric detection system from Millipore (EZHS100B-33K) and GFAP concentration levels are determined using the Meso Scale Discovery GFAP assay (F211M), both in accordance with the recommendations of the kit manufacturers. All measurements are blinded, samples are randomly distributed on 96-well ELISA plates and analysed in duplicate. Assay reproducibility is assessed based on standards, by determining intrarun and inter-run coefficients of variation ( $<10\%$  for results to be valid).

### Imaging data

Imaging parameters include:

- ▶ Diagnosis (stroke or not).
- ▶ Ischaemic or haemorrhagic features of stroke.
- ▶ Infarct volume.
- ▶ Vascular occlusion.
- ▶ Diffusion/FLAIR mismatch.
- ▶ Penumbra volume.

They are determined by a semiautomated method and independent blinded analysis by two neuroradiologists. In case of disagreement on diagnosis, a third assessment is performed in consultation with both radiologists. All imaging analyses are performed in the same centre, University Hospital Nancy (France), following data storage in a secure server (ArchiMed Database).

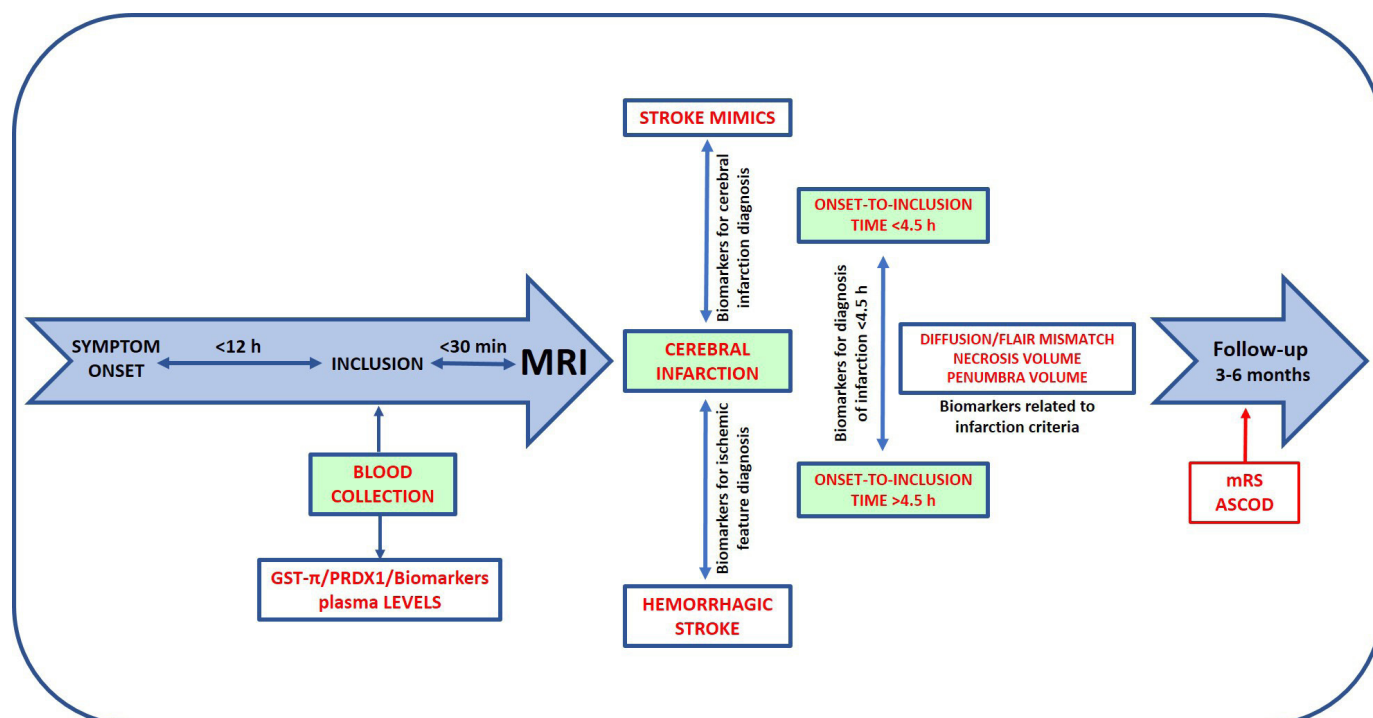
### Statistical analysis

Statistical analysis will be performed at Nancy CIC-P.

### Sample size estimates

A sample size of 756 included patients (378 with cerebral infarction  $<4.5$  hours and 378 with cerebral infarction  $>4.5$  hours) is required to measure a sensitivity of 75% and a specificity of 75% for a two-biomarker strategy (conservative estimation to identify cerebral infarction  $<3$  hours based on Turck *et al*<sup>5</sup> for GST- $\alpha$  alone—68% sensitivity and 82% specificity—and on Richard *et al*<sup>6</sup> for PRDX1 alone—53% sensitivity and 86% specificity), with a precision of 5% (half CI of sensitivity lower than 5%), an alpha risk of 5% (2.5% alpha for sensitivity and 2.5% alpha for specificity), and proportion of





**Figure 1** FLAG-1 Study flow chart. ASCOD: classification of cerebral infarction aetiologies.<sup>8</sup> FLAIR, fluid-attenuated inversion recovery; GST- $\pi$ : glutathione S-transferase- $\pi$ , mRS: modified Rankin Scale, PRDX1: peroxiredoxin 1.

cerebral infarctions <4.5 hours of 50%. Because patients are included before MRI scans are performed, we have to consider that about 20% of initially included patients will present parenchymal haematoma and stroke mimics. Therefore, a total of 945 patients will be included.

#### Analysis to meet the main objective

Patients included in the study are classified into two groups based on the time of blood draw (before and after 4.5 hours following the onset of symptoms). Receiver operating characteristic analysis will be performed, and the area under the curve will be determined for each biomarker with the maximal sum of sensitivity and specificity to determine the diagnostic performance (sensitivity, specificity) of GST- $\pi$  and PRDX1 levels, alone or in combination, to identify cerebral infarction at less than 4.5 hours.

#### Analysis to meet the secondary objectives

To meet secondary objectives, associations will be sought between biomarker levels and dichotomous endpoints using univariate and multivariate logistic regression, and associations between biomarker levels and continuous endpoints will be assessed using linear regression models.

The methods and analysis to be applied in the FLAG-1 Study are summarised in figure 1.

#### Amendments

To facilitate inclusion of patients for whom the onset-to-inclusion period was >4.5 hours, the following amendments have been submitted to the ethics committee for approval.

1. Knowledge of symptom onset time for patients with onset-to-inclusion time >4.5 hours is no longer required. For these patients, to ensure onset-to-inclusion time is between 4.5 and 12 hours at inclusion:
  - Last time patient presented no deficit must be less than 12 hours.
  - Symptoms must have been first recognised more than 4.5 hours before blood draw.
2. Distribution of the sample size according to onset-to-inclusion time has been changed, with 75% and 25% of included patients with onset-to-inclusion time less than and more than 4.5 hours, respectively. Overall sample size is therefore slightly modified, requiring 930 patients to meet the primary objective, including:
  - 558 patients with onset-to-inclusion time of less than 4.5 hours,
  - 186 patients with onset-to-inclusion time of greater than 4.5 hours,
  - 186 patients (20%) presenting haemorrhagic stroke or stroke mimics.

#### Patient and public involvement

None in the FLAG-1 Study.

#### ETHICS AND DISSEMINATION

##### Approval by Research Ethics Committee

The promoter of the FLAG-1 Study is University Hospital Nancy (France). The protocol has been approved by an independent ethics committee (Comité de Protection

des Personnes EST I; approval decision 12 April 2018, under number 2018/27).

### Patient consent

In line with French legislation for interventional studies presenting a moderate risk for included patients, oral consent will be obtained from each patient after presentation of clear written information. For patients who are unable to give consent at the time of inclusion, oral consent will be obtained from their relatives, and if this is not possible, investigators can include patients following the emergency procedure. In these cases, consent is obtained from included patients as soon as possible. Patient information and consent are reported in the medical records, along with a statement that collected data will only be used for ancillary studies in the neurovascular field.

### Data management

All data (including clinical, biological and imaging data) collected for the study are anonymised. Study data will be collected in an electronic Case Report Form (eCRF) created using Ennov Clinical Electronic Data Capture software V.7.5. For each patient included, an eCRF is created with only the patient's initials, month and year of birth, and a number is attributed to ensure confidentiality. Remote access to the eCRFs, via a web browser (https), will be provided to authorised users only, with specific authorisations controlled through a personal login and passwords. Data quality will be ensured by testing for inconsistencies. The database will be locked once discrepancies have been resolved. Data will be provided to the statistical team for analysis in the form of SAS V.9.4 tables (SAS Institute). Analysis will be conducted under the supervision of the trial's methodologist. Data are monitored by the Research and Innovation Unit (Data Management Department) at University Hospital Nancy, independently from investigators. Investigators commit to submitting to audits from the promoter or regulatory authorities.

Throughout the study, plasma samples are sent to and stored at the Biological Resource Center at University Hospital Nancy (France), in line with best practices and procedures set out in the French legislation, until they have been used up, notably in ancillary studies. All cerebral imaging data are anonymised and sent from centres to the 'CIC-Innovations Technologies' at University Hospital Nancy (France), and stored in a secure server (ArchiMed Database) registered at the French CNIL.

Results will be disseminated through communications in scientific meetings and publications in peer-reviewed medical journals.

Due to the minimal risk of adverse events for patients included in this study, no independent data safety monitoring board is required. A steering committee will monitor the study's progress, respond to scientific issues and may suggest amendments.

### DISCUSSION

The FLAG-1 Study aims to set up a major plasma bank consisting of samples from a large cohort of patients who had a stroke associated with comprehensive clinical and imaging data to (1) assess the ability of PRDX1 and GST- $\alpha$ , both involved in oxidative stress, to act as biomarkers for the identification of cerebral infarction within a time frame compatible with reperfusion therapy, (2) identify biomarkers allowing early diagnosis of cerebral infarction, and (3) select biomarkers through which to estimate crucial factors, such as volume of irreversible ischaemia and penumbra, guiding the therapeutic decision to implement cerebral infarction reperfusion.

The timing of the decision to implement reperfusion therapy in patients with cerebral infarction is no longer an impenetrable barrier. Initial studies demonstrating gain of IVT included only patients for whom the timing of symptom onset was precisely known, to determine a therapeutic time window (treatment within 3 hours for NINDS Study, and 4.5 hours for ECASS III).<sup>9 10</sup> Subsequently, a therapeutic time window of 6 hours was used for most MT studies in 2015.<sup>11</sup> Therefore, guidelines stated reperfusion therapy should be used in patients presenting cerebral infarction for whom symptom onset time is known, within the time windows defined by studies.<sup>12</sup> The FLAG-1 Study was developed in this context, with the main objective of assessing the ability of biomarkers to identify patients presenting cerebral infarction within the time window compatible with reperfusion. However, subsequent studies, some including patients for whom the timing of symptom onset was unknown, identified other criteria indicating benefits of implementing reperfusion therapy. These included the diffusion/FLAIR mismatch from the WAKE-UP Study.<sup>13</sup> Results from DAWN<sup>14</sup> and DEFFUSE 3<sup>15</sup> Studies demonstrated dramatic clinical gain for patients treated with MT outside the usual therapeutic time windows, up to 24 hours after the onset of symptoms, leading to immediate modification of guidelines.<sup>16</sup> The issue for the FLAG-1 Study was: is there any remaining need to determine the time of cerebral infarction to decide on the implementation of reperfusion therapy? Analyses of results from patients who had a stroke included in studies assessing outcomes following reperfusion therapy demonstrated that clinical gains and risk of haemorrhagic transformation (ie, efficiency and safety) were closely related to onset-to-treatment time.<sup>4 17</sup> While time must not be considered as an exclusive criterion for excluding cerebral reperfusion therapy, it nevertheless remains one of the most important criteria predicting treatment success.

The secondary objectives of the FLAG-1 Study are as important as the primary objective, as they aim to identify biomarkers in patients who had a stroke related to crucial criteria that could guide physicians in making their decision to implement reperfusion therapy (or not). In practice, evidence of diffusion/FLAIR mismatch provided by MRI is used as a means to consider cerebral infarction within the therapeutic window of 4.5 hours

between onset and examination. However, Thomalla *et al*<sup>18</sup> demonstrated that it was not a reliable criterion to classify patients with certainty within this time window. In contrast, the WAKE-UP Study<sup>13</sup> found it was sufficient to use IVT effectively and safely. The DAWN,<sup>14</sup> DEFFUSE 3<sup>15</sup> and EXTEND-IV<sup>19</sup> Studies all emphasised how the volume of cerebral infarction and penumbra were crucial criteria to guide the decision to implement reperfusion therapy, beyond a simple question of time. These studies included patients with a high potential for long-term functional recovery, with limited irreversible necrosis volumes and presence of penumbra (ie, cerebral tissue with decreased cerebral blood flow allowing neuronal survival but not electrical activity) demonstrated by a radiological/clinical mismatch, or diffusion/perfusion mismatch, and representing reversible ischaemia. The decision to implement reperfusion is thus a compromise between enhancing restoration of cerebral blood flow in penumbra, and the risk of haemorrhagic transformation in necrosis. All patients included in the FLAG-1 Study are assessed by cerebral MRI, and in particular perfusion sequences will be obtained. It will thus be possible to identify biomarkers related to both necrosis and penumbra volume. Along these lines, plasma levels of oxidative stress molecules have been reported to be related to penumbra volume, and we are particularly interested in the ability of PRDX1 and GST- $\alpha$  to estimate this criterion.<sup>20</sup>

The final goal of identifying biomarkers related to cerebral infarction is to allow early diagnosis, before patient admission and imaging. A decentralised immunological method to determine biomarker thresholds could be used to diagnose cerebral infarction, and thus initiate IVT, before hospital admission.<sup>21</sup> To achieve this goal, the FLAG-1 Study aims to identify high-performance biomarkers specifically identifying stroke (as distinct from stroke mimics such as epilepsy, migraine or functional troubles), and ischaemia (as distinct from cerebral haematoma, which is the main contraindication to exclude before initiating IVT). However, any such diagnostic method requires biomarkers identifying cerebral infarction with a high positive predictive value. Previous studies from our team reported increased PRDX1 and GST- $\alpha$  plasma levels within 3 hours of stroke onset.<sup>5 6</sup> These enzymes are produced by astrocytes and endothelial cells, and released by apoptotic cells in response to the oxidative stress inherent to cerebral ischaemia. They promote neutralisation of reactive oxygen species and prevent transmigration of leucocytes across the blood–brain barrier.<sup>6</sup> The FLAG-1 Study will also assess the diagnostic performance of S100b and GFAP plasma levels for the identification of cerebral infarction. Both these molecules are released during cerebral necrosis. Increased plasma levels could be specific evidence of cerebral damage, as their interest to diagnose head injury.<sup>22</sup> We would like to assess the utility of these two biomarkers in the context of cerebrovascular damage. Studies from other groups suggest that S100b can differentiate between patients with cerebral ischaemia and

stroke mimics.<sup>23</sup> This molecule has notably been reported to identify cerebral infarction in patients presenting non-specific clinical signs, such as dizziness.<sup>24</sup> GFAP would be of considerable interest to diagnose haemorrhagic stroke, but it lacks sensitivity for low-volume haematomas.<sup>25–28</sup> Alternative research directions include the identification of biomarkers from other pathways triggered during ischaemia, such as neuronal and glial necrosis, and the inflammatory response. The volume of plasma collected from each patient during the FLAG-1 Study will allow us to assess many other biomarkers through ancillary studies. The literature reports significant results for cerebral infarction diagnosis based on the detection of exosomal miRNAs, glycogen phosphorylase isoenzyme BB and retinol-binding protein 4.<sup>29–31</sup> During ischaemia, cell adhesion molecules are expressed at the endothelial surface to promote migration of leucocytes toward necrotic sites and initiate inflammation. We have already reported increased E-selectin and vascular cell adhesion molecule-1 during the acute phase of stroke.<sup>32</sup> However, all these previous studies share the limitation of a small patient cohort. Larger cohorts, such as that planned for FLAG-1, are needed to confirm the hypotheses.

The FLAG-1 Study is thus the first step to building diagnostic methods. Biomarkers of potential interest for cerebral infarction diagnosis must be validated following clinical studies, first confirming their capacity to allow reliable diagnosis, and thereafter their efficiency and safety with a view to initiating reperfusion therapy.

Through the FLAG-1 Study, we will constitute an extensive bank of plasma samples from patients who had a stroke along with exhaustive clinical and radiological data. This resource will be used to identify biomarkers of interest for early diagnosis of cerebral infarction. The objectives are to identify biomarkers with a good diagnostic performance to identify cerebral ischaemia, and to clearly differentiate between important criteria guiding the therapeutic decision to introduce reperfusion therapy, such as time since stroke onset, volume of necrosis and penumbra. This research could lead to the development of a rapid decentralised diagnostic method in clinical practice, thanks to which it would become possible to initiate reperfusion therapies before hospital admission.

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**Contributors** All authors contributed to the intellectual content of the protocol. AK, SR and SB drafted the manuscript, with J-CS for the processing of biological samples, BG for imaging data and NG for statistical analysis. All authors (AK, NG, J-CS, CS, AW, KL, SM, AA, LH, GM, SB, KD, SR, BG and SR) revised the manuscript critically for important intellectual content and gave their approval for the final submitted form.

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**Competing interests** None declared.

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**Patient consent for publication** Not required.

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