


BMJ Open Platelet transmission electron microscopy for the assessment of poor biological response to antiplatelet agent: pilot descriptive and prospective study - ELECTROSTROKE

Nora Mallouk ^{1,2}, Arnaud Garcin,³ Guorong Li,⁴ Magali Epinat,⁵ Claire Szczepaniak,⁶ Olo Franck Hien,¹ Patrick Mismetti,^{2,3,5} Pierre Garnier⁵

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For numbered affiliations see end of article.

Correspondence to

Dr Nora Mallouk;
nora.mallouk@univ-st-etienne.fr

ABSTRACT

Introduction Ischaemic stroke is the leading cause of adult disability. Thus, a strategy based on an efficient antiplatelet therapy has been developed. The monitoring of antiplatelet therapy is now limited to high risk and poor prognosis patients. Indeed, the biological monitoring of the antiplatelet therapy with available platelet function assays do not provide a global integrative approach. Platelet transmission electron microscopy, recently validated for assessing distinct ultrastructural abnormalities is a reliable morphological platelet structural analysis tool which could be used to collect all the ultrastructural platelet characteristics and assess the degree of activation of platelets.

Methods and analysis Our pilot prospective and descriptive study will include 50 consecutive patients hospitalized for an ischaemic stroke. We expect to identify ultrastructural characteristics that will be correlated with the degree of platelet activation to guide clinicians in decision making regarding the antiplatelet therapy strategy.

Ethics and dissemination The French Ethics Committee (comité de protection des personnes d'Ile-de-France VII) approved the information notice that will be given to participants and the protocol of this trial (protocol No 21-031).

The results of the trial will be disseminated through scientific publications.

Trial registration number NCT05004233.

BACKGROUND

Ischaemic stroke is the leading cause of adult disability, the second cause of dementia and the second cause of premature death in France. So therapeutic strategies need to be set up to prevent platelet activation during the acute phase of this disease.^{1–3}

The recommended treatment strategy for stroke secondary prevention is based on the physiopathology of the disease. While anticoagulants are recommended for cardioembolic

Strengths and limitations of this study

- This is a pilot and a descriptive study on consecutive patients hospitalized for a non-cardioembolic ischaemic stroke or a transient ischaemic attack.
- This translational study is based on a patient's platelet ultrastructural analysis, before and after the onset of an antiplatelet agent.
- This pilot study is a first step for the assessment of poor biological response to antiplatelet agent.

stroke, antiplatelet therapy is prescribed for the management of acute ischaemic stroke.

Since 2014, guidelines recommend aspirin monotherapy (50–325 mg per day) or associated with dipyridamole (2 200 mg capsules two times a day).⁴

Clopidogrel (75 mg) is also a secondary prevention strategy recommended for ischaemic stroke.

Regarding ischaemic stroke, the most frequent physio-pathological mechanism is linked to platelets activation leading to platelets aggregation and the formation of an arterial occlusive thrombus.^{1–3}

The physiological mechanisms including the initial adherence of circulating platelets to the altered vascular wall, platelet activation with ultrastructural changes, amplification of the activation, conformational change of $\alpha_2\beta_3$ platelet receptors and linking of fibrinogen and platelets aggregation are well known.⁵

In the acute phase of stroke, platelets activation is associated with specific ultrastructural patterns: the shape of platelets is spherical, the platelets extend different types of cellular protrusions, the granules centralisation occurs and the granules release their content in the open canalicular system.

Finally, platelet aggregation occurs.^{6,7} Antiplatelet agents inhibit platelet activation and platelet aggregation.

The antiplatelet therapy monitoring is based on in vitro assays (Light Transmission Aggregation, flow cytometric assays measuring intraplatelet phosphoVASP or membrane P selectin expression and Verifynow). Platelet activation is reproduced in vitro with agonists (arachidonic acid, ADP).

High levels of clopidogrel biological low responders are reported depending of the assay and of the choice of the cut-off value to discriminate clopidogrel low biological response.⁸ It was demonstrated that low biological response to an antiplatelet agent was correlated to an increase of cardiovascular outcomes during a well-conducted treatment.^{9,10} Most of the studies are based on a small cohort of patients and often lack of methodological, technical and logistic standardisation.

Recently, the large scales trials The Assessment by a Double Randomization of a Conventional Antiplatelet Strategy versus a Monitoring-guided Strategy for Drug-Eluting Stent Implantation and of Treatment Interruption versus Continuation One Year after Stenting (ARCTIC) and Tailored Antiplatelet Therapy Versus Recommended Dose of Prasugrel (ANTARCTIC) have assessed the efficiency and the clinical benefit of an antiplatelet biological monitoring.^{11,12} Unfortunately, this study did not show any benefit for the patients. This lack of benefit was explained by the low predictive value of the assays, the lack of a cut-off value validated for non-cardioembolic ischaemic stroke allowing the discrimination of poor biological response to clopidogrel.

Antiplatelet monitoring assays have been studied in large scale cardiovascular trials. Current guidelines of European Society of Cardiology for the management of stable angor do not recommend daily antiplatelet monitoring assays before or after an elective angioplasty. This class III recommendation was supported by a level of evidence A. The daily use of an antiplatelet therapy monitoring based on biological assays is not

recommended anymore.¹³⁻¹⁵ But the position statement of a panel of European expert¹⁶ was in favour of antiplatelet monitoring assays for high risk and poor prognosis patients.

The main idea of our Evaluation of the Biological Response to Clopidogrel in Patients With Ischemic Stroke (AAPIX) study (clinical trial.gov identifier NCT01955642) was to use Light Transmittance Aggregometry (LTA) and VASP assay (VASP) assays in agreement with regulatory recommendations to monitor the antiplatelet therapy for patients hospitalised in the neurovascular unit of Saint-Etienne Hospital for a non-cardioembolic ischaemic stroke. Seventy-two patients were included in the clinical trial.¹⁷⁻¹⁹ A high level of clopidogrel low responders was reported with both LTA and VASP assays. We have found a lack of correlation between the values of the assays. Furthermore, the low recurrence of new clinical outcomes suggested that the cut-off value used in our study platelet reactivity index (PRI)=50%, calculated for cardiovascular patients and chosen to discriminate clopidogrel low responders ischaemic stroke patients was not optimal. Thus, a reliable and specific cut-off value for ischaemic stroke patients is required. For this, a large scale multicentric trial must be set up.

Another option consists in developing a new morphometric and integrative technique which do not require any agonist induced in vitro activation process as in current antiplatelet monitoring assays.

Electronic microscopy has been extensively used for the study of platelets²⁰ and specifically for the ultrastructural assessment of the degree of platelets activation.^{21,22} Platelet transmission electron microscopy could give an integrative information about the efficiency of antiplatelet therapy in ischaemic stroke patients and could integrate all the antiplatelets prescribed and all the agonists involved.

The first part of this project is a descriptive pilot study based on the collection of platelet ultrastructural characteristics.

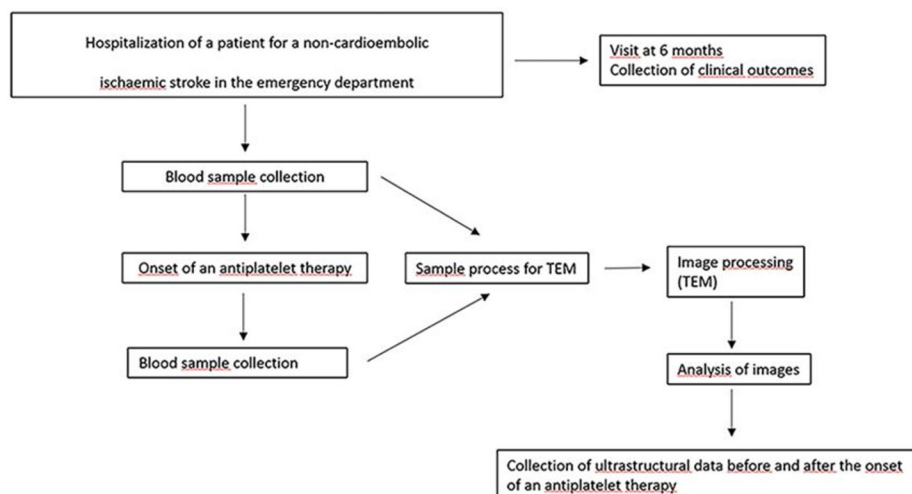


Figure 1 Outline of the trial. TEM, transmission electron microscopy.

Ultrastructural characteristics are assessed by the analysis of transmission electron microscopy images (figure 1). The ultrastructural characteristics of platelets are based on the description of the platelet plasmatic membrane and components of the platelet like the open canalicular system, the dense tubular system, the presence of a peripheral microtubular ring in the equatorial plane of platelet ultrathin sections, the actin-network organisation and the organisation of a bunch of cytoplasmic organelles: mitochondria, lysosomes, alpha and dense granules.^{6 7 23} Specific ultrastructural characteristics could give information about a rest state or a specific and precise degree of platelet activation.

There is a marked heterogeneity of platelets characteristics in patients after ischaemic stroke and platelets are not uniformly activated during the poststroke recovery phase.²⁴

The onset of an efficient antiplatelet therapy will probably induce platelet inhibition. We will collect the ultrastructural characteristics associated with this inhibition. An inefficient antiplatelet therapy will probably not induce platelet inhibition and the ultrastructural pattern of platelet inhibition.

The aim of our pilot study is to describe ultrastructural platelet changes occurring after the onset of an antiplatelet agent from patients hospitalized for a non-cardioembolic stroke.

Transmission electron microscopy is a reference technique for the assessment of hereditary platelet ultrastructural defects. Chen and collaborators have standardised and validated ultrathin section platelet electron transmission microscopy (MET-PC). They have identified the characteristic ultrastructure of platelets at rest with elongated and discoid platelets. Glycogen, alpha granules and the canalicular network are regularly distributed.²⁵ This MET-PC technique was validated²⁵ by a North-American panel of experts. This reliable method will be used in our descriptive pilot project.

Furthermore, platelet ultrastructure has been analyzed by Neumüller *et al.* in order to improve the quality monitoring of platelets concentrates from healthy volunteer's blood stored in blood bank.²⁶ A list of platelet ultrastructural criteria has been established:

- ▶ Round or discoid platelet morphology with protrusions.
- ▶ Alpha or dense granules observation.
- ▶ Homogeneous or irregular distribution of mitochondria, glycogen and dense tubular system.
- ▶ Dilated open canalicular system, degranulation phase.
- ▶ Peripheral or centralized microtubular ring on ultrathin platelet equatorial plane.
- ▶ Platelet aggregated.

We would like to use this ultrastructural characteristic list to assess platelet degree of activation before and after the onset of an antiplatelet treatment for non-cardioembolic stroke patients. This could be useful for the monitoring of an antiplatelet therapy in non-cardioembolic ischaemic stroke.

Box 1 Inclusion and exclusion criteria of the ELECTROSTROKE study

Inclusion criteria

- ▶ Adult patient >18 years old
- ▶ Patients hospitalized in the Emergency department.
- ▶ Patients having accepted and signed the consent form.
- ▶ Patients benefiting social security affiliation.
- ▶ Patients hospitalized for a non-cardioembolic ischaemic stroke requiring the onset of an antiplatelet therapy according to the usual guidelines.

Exclusion criteria

- ▶ Abnormal platelet count.
- ▶ Abnormal platelet function.
- ▶ Any contraindication regarding antiplatelet agent and/or at least one excipient according to Summary of Product Characteristics.
- ▶ Patients requiring carotid artery endarterectomy and stenting.

METHODS

Study population

It is a pilot and descriptive study. Consecutive patients hospitalized in the emergency department of the Centre Hospitalier Universitaire de Saint-Etienne following a non-cardioembolic ischaemic stroke or transient ischaemic attack (TIA), will be prospectively recruited. Inclusions are scheduled between 2022 and 2024.

Eligibility criteria are a social security affiliation, a signed-informed consent, patients aged ≥ 18 years and patients hospitalized for a non-cardioembolic ischaemic stroke requiring the onset of an antiplatelet therapy according to the usual guidelines (box 1).

Abnormal count and abnormal platelet function are exclusion criteria. Any contraindication regarding antiplatelet agent(s) and/or at least one excipient according to Summary of Product Characteristics is an exclusion criterion. Patients requiring carotid artery endarterectomy and stenting will be excluded.

Study objectives and endpoints

The primary objective of this study is to describe platelet ultrastructural criteria from non-cardioembolic ischaemic stroke patients before and after the onset of an antiplatelet agent.

The secondary objective is the exploration of the relationship between platelet ultrastructure changes and stroke outcomes.

The primary endpoint of the study is to collect platelet ultrastructural characteristics from the analysis of transmission electron microscopy images from non-cardioembolic ischaemic stroke patients before and after the onset of an antiplatelet therapy.

The secondary endpoint is to collect new non-cardioembolic ischaemic strokes during a consult at 6 months.

Study design

Investigator will provide eligible patients with an informative notice on ELECTROSTROKE study. The study design is outlined in figure 1.

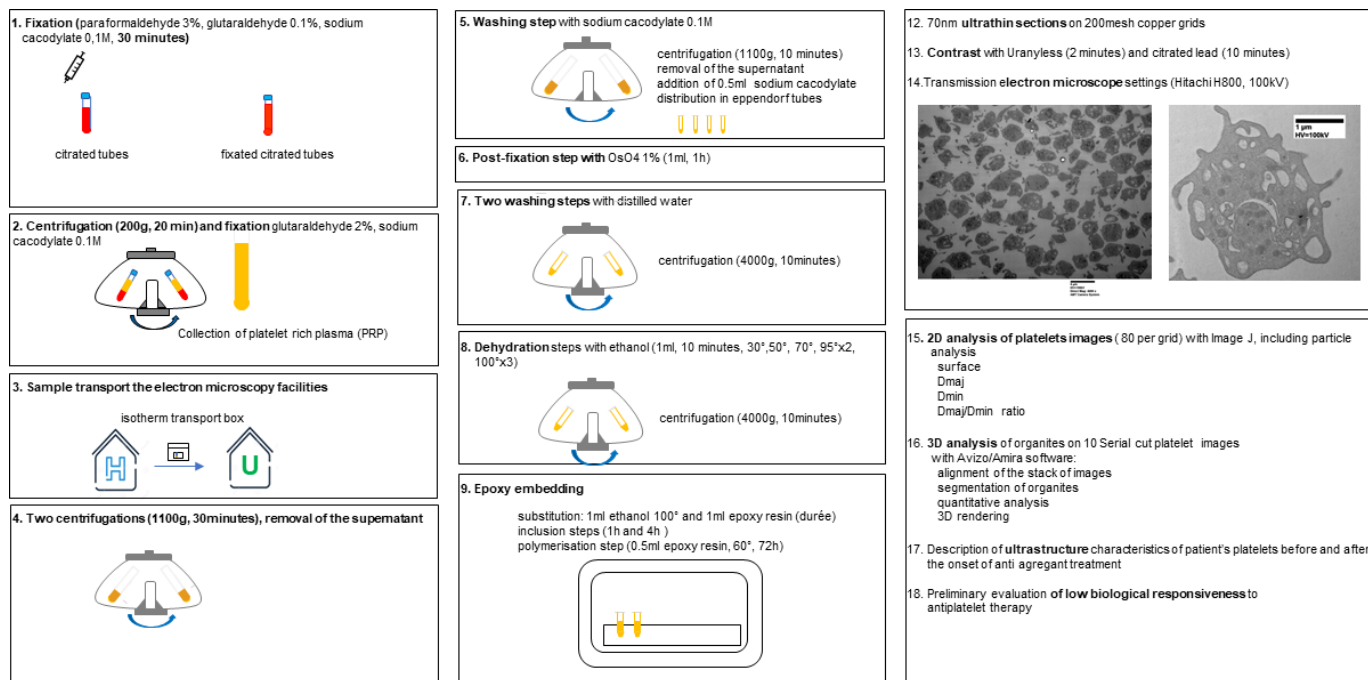


Figure 2 Process of the platelet samples: collection of the citrated blood, fixation of the whole blood, preparation of the platelet rich plasma (PRP), transport of the samples to the electron microscopy facilities, centrifugation of the PRP, removal of the supernatant, washing step, post-fixation step, washing steps, dehydration steps, epoxy embedding, 70 nm ultrathin sections, contrast with Uranylless and lead citrate, transmission electron microscopy observations, 2D and 3D analysis, description of ultrastructural characteristics and preliminary evaluation of low biological responsiveness to an antiplatelet therapy. 2D, two dimensions; 3D, three dimensions.

Sample collection

On patient's written consent, 20 mL of blood, collected in a citrated tube (Sodium Citrate 0.105M/3.2%, Becton Dickinson, Plymouth, UK), will be necessary for each platelet analysis. Artefactual activation processes during the blood collection, the transport and the processing of the blood will be avoided by following the recommendations of GFHT and of the Centre de référence des pathologies plaquettaires (CRPP). Furthermore, blood will be fixed immediately after the collection with 3% paraformaldehyde, 0.1% glutaraldehyde and sodium cacodylate 0.1M immediately after draw.²³

Blood will be collected before and 5–8 days after the onset of an antiagregant therapy in order to ensure that the drug had reached a steady state dose.

Sample preparation

The platelet sample will be processed for electron transmission microscopy (figure 2). Platelet rich plasma (PRP) will be processed. PRP will be fixed with glutaraldehyde 2% and sodium cacodylate 0.1M, post-fixed with OsO₄ 1%, dehydrated and embedded in epoxy resin (Delta Microscopies, France). After their treatment with Uranylless and lead citrate (Delta Microscopies, France) 70 nm ultrathin sections will be collected on 200 mesh formvar coated copper grids (Oxford instrument, France). A grid will be introduced in a transmission electron microscope (Hitachi H-800) and will be observed at 100kV. The quality and the resolution of the images will be optimized by assessing the quality of platelet membranes.

Ultrastructural criteria will be collected according to the list established by Neumüller *et al.*²⁶

Data collection

Data collected during the study will be recorded on an electronic case report form created for patients included in ELECTROSTROKE study. During the hospitalization, age, sex and medical history will be noted. The date of disease diagnosis, pathological results will be collected.

Sample size and statistical analysis

A selected group of non-cardioembolic ischaemic stroke patients will be included in the study. Chen *et al.*²⁵ have included 47 healthy volunteers for the validation of the MET-PC technique and to describe the platelet ultrastructure associated with a basal level of activity. In our study, 47 patients are also required to validate the technique. With a 5% technical feasibility rate, 50 patients will be included.

Evaluation criteria are qualitative. Absolute and relative frequencies of the studied criteria will be measured with its 95% CI.

Patient involvement

The ethics committee (comité de protection des personnes d'Ile-de-France VII) approved the information notice that will be given to participants and the protocol of this trial (protocol No 21–031).

DISCUSSION

Limit of transmission electron microscopy

Of course, thin section transmission electron microscopy is time-consuming and cannot be used in routine but it could help to identify specific ultrastructural changes. These changes could be analyzed with less time-consuming techniques by electron microscopy transmission negative staining, by immunoscanning electron microscopy or by flow cytometry.

Platelet activation steps

The platelet activation is a complex process with multiple events like adhesion of platelets to the subendothelial collagen, platelet shape changes, membrane externalisation of the P-selectin and the phosphatidylserine, the synthesis or the secretion of agonists leading to the recruitment of multiple signalling pathways with the binding of the agonists on specific receptors. The final steps of platelet activation are the diminution of cytosolic cyclic AMP concentration, the conformational change of $\alpha 2b\beta 3$ integrin allowing the binding of fibrinogen inducing the formation of a thrombus by the aggregation of platelets. A fibrin network results from the activation of the coagulation pathways and consolidate the aggregate.

Ultrastructural characteristics according to the degree of activation

The effect of platelet activation is correlated to ultrastructural changes: the discoid shape of platelets, the dilatation of the open canalicular system, the secretion in the open canalicular system (OCS), the centralization of the organelles.²⁶ We would like to use these criteria to establish clearly the degree of activation of platelets. This could give us information about the biological efficiency of the antiplatelet therapy.

Antiplatelet therapy, poor biological response to antiagregant and ultrastructural characteristics

We assume that an efficient antiplatelet agent will inhibit specific ultrastructural changes associated with platelet activation. The antiplatelet agents recommended for the management of ischaemic stroke will interfere with some steps of platelet activation. Aspirin inhibits COX1 and prevents the thromboxane A₂ production from arachidonic acid. P2Y₁₂ receptors are selectively inhibited by clopidogrel. Phosphodiesterase is inhibited by dipyridamole.

An inefficient antiagregant platelets will probably not inhibit platelet activation steps.

We had preliminary results from blood of patients collected 5 to 8 days after the onset of a clopidogrel treatment. The ultrastructural characteristics of platelets from a clopidogrel poor responder and from a clopidogrel good responder have been displayed.¹⁹

The platelets of a good clopidogrel responder were processed and platelets were observed by transmission electron microscopy and displayed ultrastructural characteristics of a basal activity: a discoid shape and the

homogeneous distribution of alpha and delta granules and of the dense tubular system. Transmission electron microscopy images from a poor clopidogrel responder showed moderate activation, with reorganisation of the OCS and granule centralization. In ELECTROSTROKE, platelets samples will be assessed before and after the onset of an antiplatelet therapy. The baseline activity is a key point to draw conclusions about the efficiency of an antiagregant therapy.

Imaging analysis

Images with at least 80 platelets per grid will be two dimensions (2D) analysed with Image J according to a standardized and validated procedure including particle analysis (surface, D_{min}, D_{maj}, D_{maj}/D_{min} ratio) by an independent specialist of analysis of ultrastructural platelets by transmission electron microscopy.

The three-dimension (3D) ultrastructure of platelet organelles will be obtained by 10 serial 70 nm cryosections of platelets for at least three patients. The images will be processed with Amira/Avizo software (ThermoFischer scientific, Waltham, Massachusetts, USA) for the alignment of the stack of images, the segmentation of the organelles, a quantitative analysis and a 3D rendering.

IN CONCLUSION

We propose a reliable ultrathin-section transmission electron microscopy to describe the ultrastructural characteristics of platelets, evaluating the degree of platelet activity before and after the onset of an antiplatelet therapy. This is a first step to conclude about the efficiency of antiplatelet agents for patients with non-cardioembolic ischaemic stroke.

Author affiliations

¹Faculté de Médecine - CMES, Université Jean Monnet-Etienne, Saint-Etienne, France

²Sainbiose INSERM U1059 Université Jean Monnet, Saint-Etienne, France

³Clinical Research Unit Innovation and Pharmacology, Centre Hospitalier Universitaire de Saint-Etienne, Saint-Etienne, France

⁴Department of Urology, Centre Hospitalier Universitaire de Saint-Etienne, Saint-Etienne, France

⁵Neurovascular Unit, Centre Hospitalier Universitaire de Saint-Etienne, Saint-Etienne, France

⁶Centre Imagerie Cellulaire Santé, Université Clermont Auvergne, Clermont-Ferrand, France

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Contributors NM wrote the publication. AG, ME, PG and PM were involved in clinical research. GL improved the English writing. CS and OFH were involved in the setting of the protocol

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Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

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ORCID ID

Nora Mallouk <http://orcid.org/0000-0002-6691-0977>

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