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Transmission electron microscopy for the description of platelet ultrastructure before and after the onset of an antiplatelet therapy ELECTROSTROKE

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ABSTRACT

Introduction Ischemic stroke is the leading cause of adult disability. A strategy based on an efficient antiplatelet therapy has been developed to reduce recurrent stroke and disability. Platelet function assays for the monitoring of antiplatelet therapy are not optimal. The efficiency of antiplatelet agents could be assessed with the estimation of the degree of activation of platelets. The degree of activation of platelets is assessed by the analysis of platelet ultrastructure. The first step in this project consists in describing platelet ultrastructure before and after the onset of an antiplatelet therapy.

Methods and analysis Our pilot descriptive study will include fifty patients hospitalized for an ischemic stroke. Our primary objective is to describe platelet ultrastructure from non-cardioembolic ischemic stroke patients before and after the onset of an antiplatelet agent. The secondary objective is to assess cardiovascular outcomes at 6 months. The primary endpoint of the study is to collect platelet ultrastructural data from the analysis of images of platelets from non-cardioembolic ischemic stroke patients before and after the onset of an antiplatelet therapy. The secondary endpoint is to collect cardiovascular outcomes during the consult scheduled at six months.

Ethics and dissemination This protocol was approved by the French Ethics Committee. The results of our study will be disseminated through scientific publications.

Strengths and limitations of this study

- This is the first study to describe platelet ultrastructure in patients hospitalized for an ischaemic stroke before and after the onset of an antiplatelet therapy (aspirin, aspirine+dypiridamole or Clopidogrel).
- This is a prospective monocentric trial
- This is a descriptive and a pilot study including fifty patients.
- This study combines clinical data with ultrastructure data of platelets
- Electron microscopy is time-consuming but provide rare and specific data

Trial registration number

Keywords: pilot study, platelets, ischemic stroke, transmission electron microscopy

2336 words

INTRODUCTION

Ischemic 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 secondary prevention of stroke is based on the physiopathology of the disease. While anticoagulants are recommended for cardioembolic stroke, antiplatelet therapy is prescribed for the management of acute ischemic stroke.

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

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

The most frequent underlying mechanism for ischemic stroke is linked to platelets activation leading to platelets aggregation and the formation of an arterial occlusive thrombus (1-3).

The physiological role of platelets includes the initial adherence of circulating platelets to the altered vascular wall, the activation of theses platelets, the amplification of the activation, the recruitment of new activated platelets and platelets aggregation (5). This sequence is associated with ultrastructural modifications (22) of platelets: a spherical shape, emission of pseudopodia and lamellipodia, granules centralization, the release of the content of the granules in the open canalicular system (6-7).

Patients who suffer from an ischemic stroke at the acute phase of the disease have activated platelets. An efficient antiplatelet agent inhibits platelet activation and platelet aggregation.

Antiplatelet therapy low biological response (8-10) is monitored with platelet function assays (Light transmission and impedance aggregometry, flow cytometric and VerifyNow assays).

We have assessed biological clopidogrel low responses in ischemic stroke patients (17-18)

Recently the large scales trials ARCTIC and ANTARCTIC have assessed the clinical benefit of laboratory monitoring of antiplatelet therapy (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 validated cut-off value for non-cardioembolic ischemic stroke allowing the discrimination of bad biological responders to antiaggregants.

Current guidelines of European Society of Cardiology for the management of the stable angor do not recommend a daily antiplatelet therapy monitoring before or after an elective angioplasty. This class III-recommendation was supported by a level of evidence A. The daily use of a laboratory monitoring is not recommended anymore (13-15). But the position statement of a panel of European expert (16) was in favor of a laboratory monitoring of antiplatelet therapy for high risk and poor prognosis patients. For this group of patients, optimal assays should be developed in large scale multicentric trials.

When antiplatelet therapy is efficient, platelets activation is inhibited and this inhibition is associated with a specific ultrastructure. So, another option is to use a new morphometric and integrative technique based on the analysis of ultrastructural patterns of platelets membrane and organites. Transmission electron microscopy is a reference technique for the assessment of hereditary platelet ultrastructural defects. Chen and collaborators have standardized and validated ultra-thin section platelet electron transmission microscopy (METPC). This technique was validated (20) by a North-American panel of experts (NASCOLA). Furthermore, platelet ultrastructure has been analyzed by Neumuller and collaborators in order to improve the quality monitoring of platelets concentrates from heathy volunteer's blood stored in blood bank (20). A list of platelet ultrastructural criteria has been established and could be informative about the efficiency of antiplatelet therapy in ischemic stroke. Transmission electron microscopy images of platelets from a Clopidogrel biological bad responder and from a Clopidogrel biological good responder have been displayed in a previous study (19).

Study objectives

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

The secondary objective is to assess stroke outcomes at 6 months.

METHODS AND ANALYSIS

Study design

This is a French descriptive pilot monocentric study. Investigators will provide eligible patients with an informative notice on ELECTROSTROKE study. They will not have access to electron microscopy data. The study will start in 2021. The study outline is displayed in Table 1.

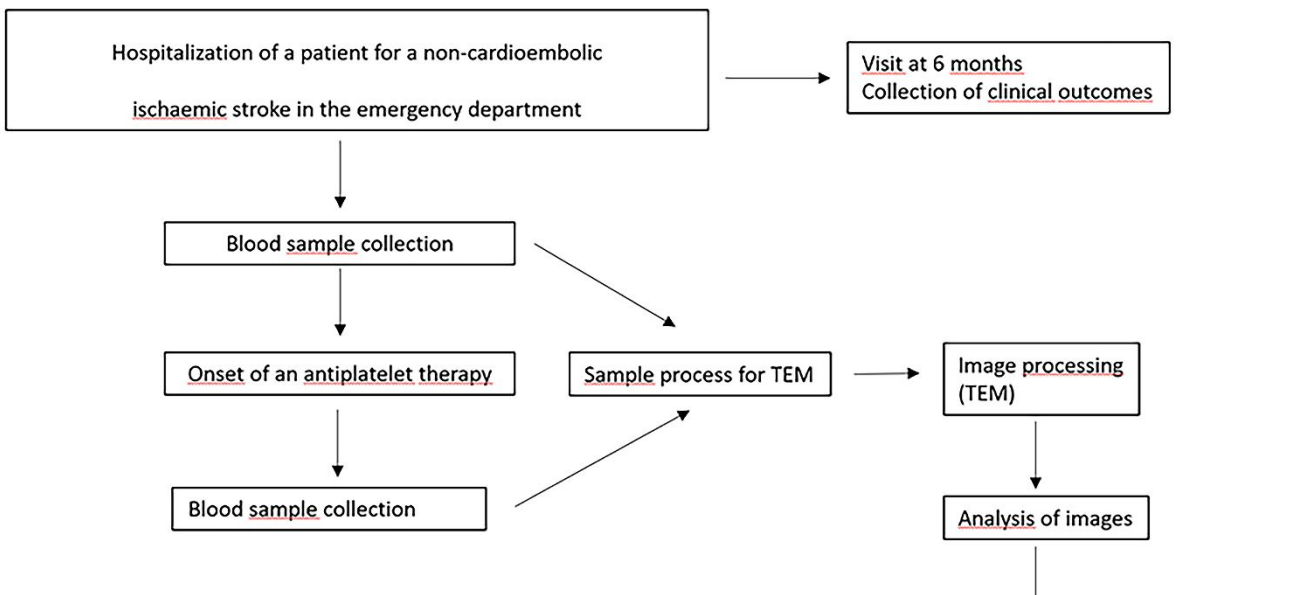

Table 1: Outline of the study.

Study population

Consecutive patients hospitalized in the emergency department of Saint-Etienne University Hospital Centre following a non-cardioembolic ischemic stroke or transient ischemic stroke, will be prospectively recruited. Our study which is descriptive will not interfere with the guidelines for the treatment and management of ischemic stroke. Eligibility criteria are a social security affiliation, a signed-informed consent, patients aged ≥18 years and patients hospitalized for a non-cardioembolic ischemic stroke requiring the onset of an antiplatelet therapy according to the usual guidelines (Table 2). Any contraindication regarding antiplatelet agent(s) and/or at least one excipient according to summary of product characteristics is an exclusion criterion.

Inclusion for Patients
Adult patient> 18 years old
Patients hospitalized in the emergency department
Patient having accepted and signed the consent form
Patients benefitting social security affiliation **Patients hospitalized for a non-cardioembolic ischemic stroke requiring the onset** **of an antiplatelet therapy according to the usual guidelines**
Exclusion
Any contraindication regarding antiplatelet agent(s) and/or at least one excipient according to Summary of Product Characteristics.

Table 2: Inclusion and exclusion criteria for patients hospitalized for a non-cardioembolic ischemic stroke.

Sample collection

Upon patient's written consent, 15ml of blood will be collected in citrated tubes (Sodium Citrate 0.105M / 3.2%, Becton Dickinson, Plymouth, UK) before the onset of an antiplatelet therapy. In vitro activation of platelets can occur during the collection of blood, transport and blood processing. This will be avoided by following usual preanalytical recommendations (23). Blood will be collected before and 5 to 8 days after the onset of an antiplatelet therapy to ensure that the drug had reached a plasmatic steady-state dose

Sample size

Chen and collaborators (20) have included 47 healthy volunteers for the validation of the MET-PC technique. They have described platelet ultrastructure at basal level of platelet activity. In our study, 47 patients are required. In prevision of technical feasibility issues, fifty non-cardioembolic ischemic stroke patients will be included.

Patient involvement

No patient was involved in the protocol design. The ethics committee was consulted on the information notice that will be given to the patients.

Collection of clinical data

Data collected during the study will be recorded on an electronic case report form. During the hospitalization, age, sex, and medical history will be noted. The date of the diagnostic of the disease and biological results will be collected.

Collection of platelet ultrastructural observations

The platelet samples will be processed for electron transmission microscopy. Platelet Rich Plasma (PRP) will be prepared. PRP will be fixed with glutaraldehyde, treated with agar, post-fixed with OsO4, dehydrated and embedded in epoxy resin (20). Serial 70nm ultrathin section will be collected on 200 mesh formvar-coated copper grids. Thin sections will be observed with a transmission electron microscope (Hitachi H-800) at 100kV. The quality and

the resolution of the images will be optimized by assessing the quality of platelet membranes. Ultrastructural observations will be collected according to the list established by Neumuller and collaborators (22). Transmission electron microscopy images from non-cardioembolic ischemic stroke patients before and after the onset of an antiplatelet therapy will be analyzed and ultrastructural characteristics will be collected.

Images analysis

A qualitative analysis will be performed on the basis of ultrastructural criteria:

- round or discoid platelet shape.

- emission of pseudopodia and lamellipodia.

- distribution of alpha and dense granules.

- homogeneous distribution or centralization of mitochondria, glycogen and dense tubular system.

- dilated open canalicular system, and degranulation in the open canalicular system

- peripheral microtubular ring on ultrathin platelet equatorial plane

Images will be analyzed according to a standardized and validated procedure by an independent specialist of analysis of platelet ultrastructure by transmission electron microscopy. A morphological software will be used to standardize the analysis (Amira Software, Thermo Fisher scientific).

(22). These criteria will be described before and after the onset of an antiplatelet treatment. The analysis of platelet ultrastructure before the onset of an antiplatelet therapy is our reference and correspond to the baseline activity.

Collection of clinical outcomes

Cardiovascular outcomes will be collected during the scheduled visit at 6 months.

Statistical analysis

Evaluation criteria are qualitative. Absolute and relative frequencies will be measured with its 95% confidence interval.

Limitations

Transmission electron microscopy

Transmission electron microscopy is time-consuming and cannot be used in routine but it is useful for the identification of specific ultrastructural changes. This pilot and descriptive study could be a first step before the use of less time-consuming techniques.

Imaging analysis

There is a marked heterogeneity of platelets in size, age, maturation and activation (21). This heterogeneity will be precisely described and included in the analysis of the results.

Serial sections

In conclusion,

We propose a reliable ultrathin-section transmission electron microscopy to analyze platelet ultrastructure. This could be helpful for the assessment of the degree of platelet activity and for the monitoring of antiplatelet therapy.

Author contributions

Contributors NM, ME, PG, and PM - conception and design. AG - development of methodology. GL - review of the protocol.

Funding this work was supported by Saint-Etienne hospital grant 20CH026, France.

Competing interest None declared

Patients involvement Patients were not involved in the design or conduct, or reporting, or disseminating of this research.

Patient consent for publication Not required.

conflict of interest disclosure: none of the authors have any conflicts of interest.

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

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Keywords:	Stroke medicine < INTERNAL MEDICINE, Vascular medicine < INTERNAL MEDICINE, Stroke < NEUROLOGY

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

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Abstract

Introduction

Ischæmic 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 fifty consecutive patients hospitalized for an ischæmic 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 trial registration number of our study is NCT05004233. 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 N° 21-031).

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

Strengths and limitations of this study

- This is a pilot and a descriptive study on consecutive patients hospitalized for an non-cardioembolic ischæmic stroke or a transient ischæmic 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

Keywords: pilot study, blood sample, platelets, clopidogrel, ischemic stroke, ultrastructural characterization, transmission electron microscopy, TEM

Background

Ischemic 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 stroke, antiplatelet therapy is prescribed for the management of acute ischemic stroke. Since 2014, guidelines recommend aspirin monotherapy (50-325mg per day) or associated with dipyridamole (2 200mg capsules twice a day) (4).

Clopidogrel (75mg) is also a secondary prevention strategy recommended for ischemic stroke. Regarding ischemic 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 α2β3 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 centralization 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, 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 cutoff 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 standardization.

Recently the large scales trials ARCTIC and 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 ischemic 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 expert (16) was in favor of antiplatelet monitoring assays for high risk and poor prognosis patients.

The main idea of our AAPIX study (clinical trial.gov identifier NCT01955642) was to use Light Transmittance Aggregometry (LTA) and VASP assays in agreement with regulatory recommendations to monitor the antiplatelet therapy for patients hospitalized in the neurovascular unit of Saint-Etienne Hospital for a non-cardioembolic ischemic 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 (PRI=50%), calculated for cardiovascular patients and chosen to discriminate clopidogrel low responders ischemic stroke patients was not optimal. Thus, a reliable and specific cut-off value for ischemic 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 platelets (20) 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 ischemic 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.

Ultrastructural characteristics are assessed by the analysis of transmission electron microscopy images (figure1). **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 ultra-thin sections, the actin-network organization and the organization 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 ischemic stroke and platelets are not uniformly activated during the post-stroke 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 standardized and validated ultra-thin section platelet electron transmission microscopy (METPC). 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 validated (25) by a North-American panel of experts (NASCOLA). **This reliable method will be used in our descriptive pilot project.**

Furthermore, platelet ultrastructure has been analyzed by Neumuller and collaborators in order to improve the quality monitoring of platelets concentrates from heathy 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 ischemic stroke.

Methods

Study population

It is a pilot and descriptive study. Consecutive patients hospitalized in the emergency department of Saint-Etienne University Hospital Centre following a non-cardioembolic ischemic stroke or 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 ischemic stroke requiring the onset of an antiplatelet therapy according to the usual guidelines (Table 1).

Table 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 ischæmic 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 (CEA) and stenting (CAS)

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 (SPC) is an exclusion criterion. Patients requiring carotid artery endarterectomy (CEA) and stenting (CAS) will be excluded.

Study objectives and endpoints

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

The secondary objective is the exploration of the relationship between platelet ultrafine structure 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 ischemic stroke patients before and after the onset of an antiplatelet therapy.

The secondary endpoint is to collect new non-cardioembolic ischemic 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 Figure1.

Sample collection

Upon patient's written consent, 20ml 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 GEHT and of the 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 to 8 days after the onset of an antiaggregant 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. 70nm ultrathin sections will be collected on 200 mesh formvar coated copper grids. 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 Neumuller and collaborators (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 ischemic stroke patients will be included in the study. Chen and collaborators (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% technically 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% confidence interval.

Patient involvement

The trial registration number of our study is NCT05004233. 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 N° 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 identified specific ultrastructural changes. These changes could be analyzed with less time-consuming techniques by electron microscopy transmission negative staining, by immuno-scanning 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 externalization of the P-selectin, the phosphatidylserine, the synthesis or the secretion of agonists leading to the recruitment of multiple signaling 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 α2bβ3 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 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 anti agregant.

Antiagregant, poor biological response to **antiagregant and ultrastructural characteristics**

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

An inefficient antiaggregant 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 reorganization of the OCS and granule centralization. In ELECTROSTROKE, platelets samples will be assessed before and after the onset of an anti agregant treatment. The baseline activity is a key point to draw conclusions about the efficiency of an anti agregant therapy.

Imaging analysis

Images with at least 80 platelets per grids will be 2D analyzed with Image J according to a standardized and validated procedure including particle analysis (surface, Dmin, Dmaj, Dmaj/Dmin ratio) by an independent specialist of analysis of ultrastructural platelets by transmission electron microscopy.

The 3D ultrastructure of platelet organelles will be obtained by 10 serial 70nm cryosections of platelets for at least 3 patients. The images will be processed with Amira/Avizo software (Thermofischer scientific, Waltham, MA, 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 anti agregant therapy. This is a first step to conclude about the efficiency of antiplatelet agents for patients with non-cardioembolic ischemic stroke.

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Contributorship statement. 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.

Competing interests. We declare no competing interest for any author.

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Figure 1: outline of the trial. TEM: transmission electron microscopy

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, 70nm ultrathin sections, contrast with uranyless 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.

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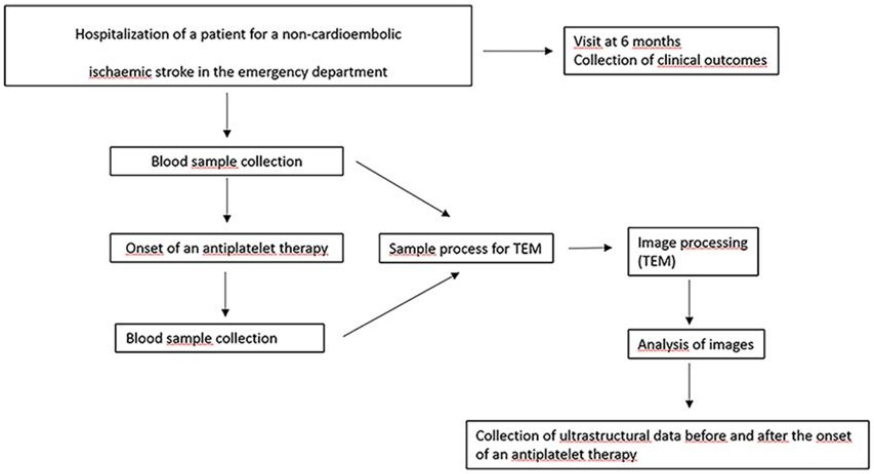

Figure 1: outline of the trial. TEM: transmission electron microscopy

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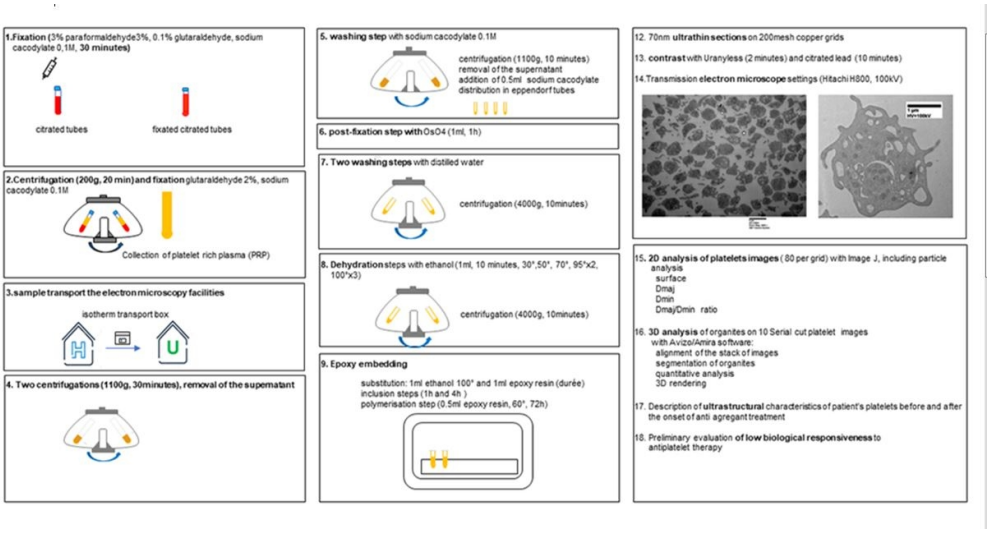

Figure 2: process of platelet samples: collection of citrated blood, fixation of whole blood, preparation of platelet rich plasma (PRP), transport of the samples to the electron microscopy facilities, centrifugation of the PRP, removal of the supernatant, washing step, post fixation step, washing steps,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

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