Universitat de Girona

Detection of patients with carotid stenosis at high risk of developing vascular complications by the identification of novel biomarkers

FINAL DEGREE PROJECT

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2. ABBREVIATIONS

CEA	Carotid endarterectomy
CS	Carotid stenosis
CRF	Case Report Form
ELISA	Enzyme-linked immunosorbent assay
HDJT	Hospital Doctor Josep Trueta
Hs-PCR	High-sensitivity C-reactive protein
ICA	Internal carotid artery
Lp-PLA$_2$	Lipoprotein-associated phospholipase A_2
MRI	Magnetic resonance imaging
PAI-1	Plasminogen activator inhibitor-1
PTX3	Pentraxin 3
TIA	Transient ischemic attack

3. ABSTRACT

Background: Currently, stroke is one of the leading causes of death worldwide and the first cause of long-term morbidity in our country. The main risk factor to develop an ischemic stroke is the presence of an atherosclerotic plaque in the wall of the carotid artery. Nowadays the factor that makes subjects with carotid stenosis cross the line from clinical silent to cardiovascular disease is unknown. The available data suggest that there is an interaction between local and systemic factors. So far, several studies have been carried out trying to find a novel biomarker that could detect those subjects that are at high risk of developing a vascular event into the brain to achieve an objective measure for their clinical management. However, no biomarker has been qualified for regular clinical use in carotid artery disease.

Objective: The aim of the study is to determine the relationship between the expression of PAI-1, PTX3 and Lp-PLA_2 in the carotid plaques of patients with carotid stenosis and the presence or absence of neurological symptoms.

Methods: This protocol is a cross-sectional and before and after 1-year follow-up study design in adult subjects with significant carotid stenosis (> 50%) who undergo carotid endarterectomy. The study will include 204 participants, 136 who have developed neurological symptoms and 68 that remain asymptomatic. Recruitment of participants will last 3 years. Participants will be selected as they are admitted in the emergency department or in the outpatients' department of neurology in the Hospital Doctor Josep Trueta (HDJT) in Girona. Preoperative and postoperative studies and appointments are established in order to do a follow-up of patients and collect all the data.

Keywords: management of carotid stenosis; ischemic stroke prevention; biomarkers; hs-CRP; PAI-1; PTX3; Lp-PLA_2

4. INTRODUCTION

4.1 BACKGROUND

4.1.1 Definitions of ischemic stroke and transient ischemic attack

Stroke is a clinical syndrome produced by an alteration of the cerebral artery circulation in population with a previous silent cardiovascular disease. It is characterized by a sudden development of focal or global neurological signs and symptoms lasting more than 1 hour or leading to death. Ischemic stroke is defined as an acute episode of neurological dysfunction caused by cerebral or retinal vascular injury as a result of infarction of the central nervous system tissue. Thus, it is necessary to prove the presence of tissue damage by means of a neurological scan (CT or MR). However, Transient ischemic attack (TIA) is defined as a brief episode of neurological dysfunction caused by a focal disturbance of cerebral or retinal ischemia lasting less than 1 hour and without evidence of acute cerebral infarction in the neurological scan(1,2).

4.1.2 Epidemiology

Currently, a stroke is the second leading cause of death worldwide and the first cause of long-term morbidity (as measured in disability-adjusted life-years). Moreover, it seems to be the health trends until 2030 while life expectancy will increase(3).

EPICES study showed that 87% incidence of all strokes in Spanish population are ischemic and within them, 28% are caused by large-artery atherosclerosis of internal carotid artery (ICA)(4). Therefore, carotid atherosclerosis is one of the main risk factors for ischemic stroke and TIA.

TIA is associated with an early risk of established stroke with a probability from 15% to 20% at 90 days(5) and large-artery atherosclerosis has specially elevated the risk of early recurrence for ischemic stroke within the first 30 days(6).

4.1.3 Atherosclerosis process

Atherosclerosis is considered a systemic chronic disease. Lesions begin with the deposition of lipids in the endothelium of medium and large arteries and its consequent oxidative modifications. Recruitment and differentiation of monocyte-derived macrophages leads to foam cell formation (macrophages with massive amounts of cholesterol esters). Its interaction with T cells (expressing both Th1 and Th2 cytokines) establishes a chronic inflammatory process. Smooth muscle cells migrate from the medial to the intimal lamina of the artery wall and synthesize extracellular matrix proteins that form a fibrous plaque. Necrosis of macrophages and smooth muscle cells lead to the formation of a necrotic core that increases the potential for thrombosis. Macrophage secretion of matrix metalloproteinases acts degrading extracellular matrix proteins. Together with neovascularization they contribute to a weakening of the fibrous plaque. Plaque rupture exposes plaque lipids and tissue factor to blood components, initiating coagulation, platelet adherence and the formation of thrombus. Evolution of advanced plaques involves repetitive cycles of microhaemorrhage and thrombosis that can cause occlusive arterial disease(7).

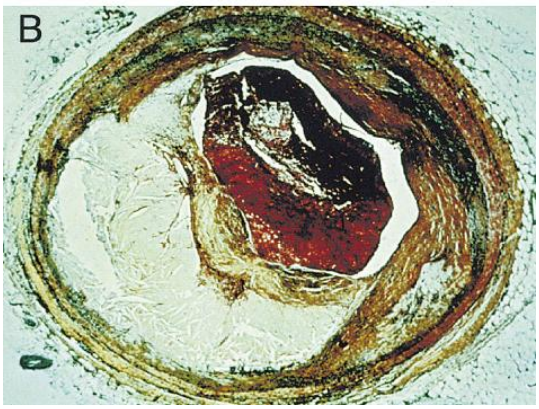

Figure 1: Late atherosclerotic lesion(7).

4.1.4 Management of carotid artery stenosis

It is well-known that carotid stenosis is a modifiable risk factor for ischemic stroke. However, population screening is not recommended because it is difficult to identify those asymptomatic patients who would benefit from carotid plaque removal to reduce the risk of stroke(8). Recently, in November 2015, a systematic review of all international guidelines of carotid stenosis management has published their results, showing a high variability between guidelines that leads to unclear recommendations and identify important biases in quality and relevant evidence(9). In accordance to this review, the target population of guideline procedural endorsements, as they clearly achieved a statistically significant carotid endarterectomy (CEA) benefit, should be patients with life expectancy of more than 3 to 5 years and one of the following characteristics:

- Asymptomatic men < 75 to 79 years with > 60% stenosis.
- Symptomatic women with 70 to 99% stenosis randomized within 2 weeks of their last ischemic event.
- Symptomatic men with 50 to 69% randomized within 2 weeks of their last ischemic event.
- Symptomatic men with 70 to 99% stenosis and without near-occlusion randomized within 12 weeks or longer from their last ischemic event.

Stenosis quantification for these criteria follows The North American Symptomatic Carotid Endarterectomy Trial (NASCET) method in which moderate stenosis is defined as < 70% of the luminal diameter and sever stenosis is defined as 70-99% of the luminal diameter by catheter angiography(10). These patients should also satisfy the average-CEA-risk criteria which is 30-day peri-CEA rate of stroke or death of < 3% for asymptomatic carotid stenosis (CS) and 30-day peri-CEA rate of stroke or death of < 6% for symptomatic CS.(9)

Although there is not a consensus about the recommendation for medical treatment as first choice, some guidelines propose that it should be beneficial (level I class c evidence) in patients with asymptomatic CS < 60% by angiography or < 70% by validated duplex ultrasound and in patients with symptomatic CS and a high-CEA-risk because of multiple comorbidities(8,9).

4.2 JUSTIFICATION

Stroke has a huge worldwide socioeconomic impact because it's high prevalence, hospitalization rate and long-term sequelae in survivors. The direct lifetime costs per ischemic stroke have been estimated at 43,129€. Rehabilitation and chronic nursing care account for a largest part of these costs. The number of stroke patients and the healthcare costs will rise continuously as a higher proportion of patients will survive strokes in western countries(11).

ICA stenosis is an important public health issue as approximately 15% of men and women over the age of 80 have a significant grade of ICA stenosis (≥ 50% of diameter reduction). Prevalence of severe asymptomatic carotid stenosis in the general population ranges from 0% to 3.1%(12). Routine diagnostic methods (such as duplex ultrasound or magnetic resonance angiography) for stratifying asymptomatic subjects according to stroke risk remain ineffective(8).

Although two randomised trials, the Asymptomatic Carotid Atherosclerosis Study (ACAS)(13) and the Asymptomatic Carotid Surgery Trial (ACST)(14), concluded that CEA conferred a 50% relative risk reduction in a 5-year risk of stroke compared to medical treatment in patients with asymptomatic CS of > 70%, CEA remains statistical risky with a number-needed-to-treat (NNT) to prevent one stroke in 1 year being as high as 100(15).

Acute cardiovascular events generally result from plaque rupture and thrombosis, but atherosclerosis disease does not follow a one-way progression. Fibrotic plaques can become vulnerable and those with intra-plaque haemorrhage can remain silent. Therefore, both symptomatic and asymptomatic patients can present similar histological features in their carotid plaques. No strong and reliable correlation has been established between plaque type and symptoms(16–18). So, what makes plaques cross the line from clinical silent to cardiovascular disease? Available data suggest that atherosclerosis plaque destabilization and rupture involves an interaction between local (vulnerable plaque) and systemic factors (vulnerable blood)(19). Furthermore, hemodynamic condition (disturbed flow) also plays a relevant role in plaque formation. Because of its particular topography, internal carotid artery is the main source of cerebral embolism in atherothrombotic stroke. Persistence of low wall shear stress (frictional force applied to the vessel wall by the movement of blood) in advanced atherosclerotic lesions has been correlated with increased plaque vulnerability(20).

Taking into account all these findings, the proposal of this study is to identify subjects (vulnerable patients) with a high likelihood of developing atherosclerosis complications. More precisely, the identification of suitable biomarkers that could provide a useful criteria to ensure best risk stratification and management of either asymptomatic subjects with CS who would benefit from surgical treatment as primary prevention or symptomatic patient with CS and a high risk of stroke recurrence.

According to literature review, the following inflammatory biomarkers have been selected to be analyzed in this study as they have shown to be actively involved in the atherosclerotic process. Nowadays, none of them has qualified for regular clinical use in carotid artery disease.

High-sensitivity C-reactive protein (hs-PCR) is a nonspecific biomarker of inflammation that affects endothelial cells from the vessels wall and vascular smooth muscle cells. It has emerged as a powerful independent predictor of future cardiovascular disease(21). Previous studies have suggested that elevated levels of serum hs-CRP in patients with high-grade carotid stenosis may be higher in symptomatic patients and in unstable plaques as they were correlated with a higher number of inflammatory cells (macrophages and T lymphocytes) and may potentially identify patients at risk for developing a neurological deficit (22–24). However, some other studies showed elevated levels of serum hs-CRP in patients with carotid stenosis but differences between symptomatic and asymptomatic patients did not reach statistical significance(25,26). One trial showed that hs-CRP was sensitive to identify patients with vascular risk, but didn't yield a significant difference between subclinical and advanced stages of atherosclerosis. Their conclusion was that hs-CRP was not a good option to monitor disease progression(27). Different results were found in another study which suggested a positive correlation between high baseline levels of hs-CRP and the morphological and clinical progression of atherosclerosis(28). There is disagreement between studies. More research is needed to clarify hs-CRP's role in the atherosclerotic disease and risk of stroke.

Plasminogen activator inhibitor-1 (PAI-1) is a serine protease secreted and expressed by endothelial cells. It has an important role as an inhibitor of fibrinolytic activity and as recently suggested, for the maintenance of endothelial integrity(29). One study showed a disparity of circulating biomarkers depending on the location of atherosclerosis (intracranial or extracranial arteries). PAI-1 was correlated with moderate to severe intracranial atherosclerotic disease in asymptomatic patients(30). It's involvement in the onset of neurological symptoms remains unclear.

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is considered an independent biomarker for cardiovascular disease. It is a serine lipase that specifically hydrolyzes

oxidized phospholipids such as low density lipoprotein (LDL) and results from the generation of lysophosphatidylcholine (lysoPC)(31). Detection of patients at high risk for a stroke is difficult. In 2008, an international recommendation panel incorporated Lp-PLA_2 as a new parameter into the stratification of cardiovascular disease-risk assessment guidelines to identify patients at moderate to high risk who will benefit from intensification of lifestyle and lipid-modifying therapies(32). Mannheim et al. showed that local expression of Lp-PLA_2 and its product lysoPC is increased in the carotid plaques of symptomatic patients(33). However, another study showed significantly higher plasma levels of Lp-PLA_2 in patients with unstable plaques but no differences between symptomatic and asymptomatic patients were found(24). Recently, it has been suggested that Lp-PLA_2 could be associated with recent development of symptoms in subjects with carotid stenosis(26).

Pentraxin 3 (PTX3) is a member of the pentraxin superfamily. Endothelial cells, smooth muscle cells, monocytes and macrophages are able to produce PTX3 in response to an inflammatory stimulation(34). These findings suggest its local effect in the atherosclerotic disease. In 2011 one study showed no relationship between PTX3 and carotid artery symptomatology(35). However, recent studies have suggested higher PTX3 levels in patients with vulnerable carotid plaque(17,36).

5. HYPOTHESIS

Levels of PAI-1, PTX3 and Lp-PLA_2 will be higher in symptomatic than in asymptomatic carotid plaques of patients undergoing CEA. Serum levels of hs-CRP, PAI-1, PTX3 and Lp-PLA_2 will be more elevated in patients with symptomatic carotid stenosis and they will show a positive correlation with their expression in the carotid specimens. The selected biomarkers will show a positive correlation with histological and neuroimaging features of plaque vulnerability.

6. OBJECTIVES

6.1 MEAN OBJECTIVE

The aim of the study is to determine the relationship between the expression of PAI-1, Lp-PLA_2 and PTX3 in the carotid plaques of patients with carotid stenosis who undergo carotid endarterectomy and the presence or absence of neurological symptoms. In addition, a proteomic analysis of the carotid specimens will be performed in order to find new potential inflammatory biomarkers.

6.2 SECONDARY OBJECTIVES

- Serum levels of hs-CRP, PAI-1, Lp-PLA_2 and PTX3 will be tested in order to analyse its correlation with their expression in the carotid specimens and with the presence or absence of neurological symptoms.
- Determine the relationship between the expression of the selected biomarkers and the histological findings in the carotid specimens.
- Correlate the morphological findings of vulnerable plaque with both histological and neuroimaging study.
- Follow-up of all patients at 3 and 12 months after surgery to evidence changes in the expression of serum biomarkers and monitor carotid artery by Doppler ultrasound study.

7. MATERIAL AND METHODS

7.1 STUDY DESIGN

This study will be designed as a cross-sectional study. However, for the last secondary objective which concerns the patients follow-up, this study will be designed as a before and after 1-year follow-up study. The total length of the study period will be four years and it will be performed in the Hospital Doctor Josep Trueta in Girona.

7.2 STUDY POPULATION

The target population of the study will be adult subjects diagnosed with significant carotid artery stenosis (>50%) who undergo carotid revascularization procedure (CEA). Participants will be divided in two groups depending on the presence or absence of neurological symptoms:

- Symptomatic patients will be defined as those who have experienced one of the following:
    - Ischemic stroke: neurological event lasting more than 1 hour
    - TIA: reversible neurological event lasting less than 1 hour.
- Asymptomatic patients will be defined as those who have carotid artery alteration in a Doppler ultrasound and magnetic resonance imaging (MRI) but have never experienced a vascular event into the brain.

7.2.1 **Inclusion criteria:**

- Patients with unilateral or bilateral carotid artery stenosis.
- Symptomatic patients with more than 50% and less than 99% of ICA stenosis.
- Asymptomatic patients with more than 70% and less than 99% of ICA stenosis.

7.2.2 **Exclusion criteria:**

- When the cause of carotid stenosis is not related to the presence of atherosclerosis.
- Previous ipsilateral CEA which means a restenosis of carotid artery.
- Complete occlusion of the carotid artery.
- CEA surgery delayed more than 30 days from the onset of symptoms.
- History of cardiovascular disease. Some examples are acute coronary syndrome (< 4 weeks) or atrial fibrillation. To avoid the possible confusion of cardiac origin instead of carotid atherosclerosis.
- Clinical evidence of infection defined as axillary body temperature > 37ºC.
- Any undercurrent disease that could cause an elevation of serum biomarkers as a response of its basal physiopathology. Examples of such are chronic inflammatory diseases or trauma.
- History of malignancy or patients who had received cancer treatment in the last 6 months.
- Invasive procedures during the last 3 months (e.g. surgery or catheterization).
- Expectation of poor surgical risk or any major life-threatening condition.

7.3 SAMPLING

7.3.1 **Sample selection**

A non-probabilistic consecutive sampling method will be used. This sampling consists of selecting patients diagnosed with ICA stenosis, who meet the inclusion and exclusion criteria, as they are admitted in the HDJT. Symptomatic patients will be collected from the Emergency Department when they consult for an acute ischemic stroke or TIA. Selection of asymptomatic patients will be done in the outpatients' department of neurology when the ICA stenosis will have been found by accident by a

physician from a different department or when a patient with a lot of cardiovascular risk factors consult for a Doppler study. Candidates will be informed about the study and invited to participate voluntarily by signing the informed consent (Annex 1). In the case of symptomatic patients, it will be done once they are controlled and out of risk in the specialized Stroke Unit of the Hospital.

7.3.2 **Sample size**

A sample size for each biomarker has been calculated according to the data found in the literature review. Hs-CRP biomarker has shown to need the biggest sample size. Thus, as its sample covers all the other biomarkers requirements, it will be used as the sample size of this study. Accepting an alpha risk of 0.05 and a beta risk of 0.2 in a two-sided test, 68 subjects are necessary in the group of asymptomatic CS and 136 subjects in the symptomatic CS group to recognize a statistical significance. A drop-out rate of 5% has been anticipated. Sample size has been calculated using GRANMO application.

As this study also has a before and after design, repeated measurements have been calculated in order to ensure that the selected sample size covers both designs. The number of subjects that are necessary in each group has shown to be equal or lower. Therefore, sample size has not been modified.

7.4 VARIABLES AND MEASUREMENTS

Several objectives have been taken into account for this study. Some variables are repeated in different objectives but not as the same type of variable; sometimes as the independent variable and others as the dependent one. For that reason and in order to facilitate its explanation and interpretation, variables have been classified as principal variables of the study and co variables.

7.4.1 **Principal variables:**

- Analysis of PAI-1, Lp-PLA_2 and PTX3 in the carotid specimens. Carotid plaque specimens will be collected in the operating room, trying carefully to remove it in a single piece. Plaques will be divided into two pieces. The first piece will be used for the study of PAI-1, Lp-PLA_2 and PTX3 and for the proteomic analysis. The second piece will be used for the histopathologic and immunohistochemical analysis. Both pieces will be immediately frozen and stored at -80ºC until their study. Quantification of PTX3, Lp-PLA_2 and PAI-1 positive cells will be established from immunohistochemical analysis by using monoclonal anti-PTX3 and anti-Lp-PLA_2 antibodies, and polyclonal anti- PAI-1 antibody(29,33,36). Results will be expressed as the number of cells per 10 fields.

- Proteomic analysis of carotid specimens. A proteomic analysis of the carotid specimens will be performed in order to find new potential inflammatory biomarkers. Proteomic analysis will be performed by using 2D-PAGE and MALDI-TOF or HPLC and tandem MS/MS. More detailed information about proteomic analysis is attached in Annex 2.

- Histopathologic analysis of carotid specimens. Plaque specimens will be fixed in 10% formalin, sectioned in 3.0 mm transverse slices, decalcified with 2.5% nitric acid to prevent artifacts, and embedded in paraffin. The paraffin-embedded specimens will be sectioned at 5-µm thickness and stained with haematoxylin and eosin (HE). Morphological features will be examined with an optic microscope. Criteria for plaque definition are based on the histological classification of atherosclerosis made by the American Heart Association(37) and complemented with a systematic review from 2010(38).

- Stable lesions: small lipid core, large fibrous cap of conjunctive tissue, extracellular lipids and laminated acellular collagen, without any endothelial disruption.
- Vulnerable lesions: large and altered lipid core (>40% of total volume), thin fibrous cap (<100μ), ulceration of the endothelial surface, recent intra-plaque haemorrhage or an intra-plaque thrombosis.

- Immunohistochemical analysis of macrophages and lymphocytes in the carotid specimens. For immunohistochemical study of tissue, the thin paraffin-embedded sections will be deparaffinised in xylene, rehydrated through graded alcohols, and preincubated in 10% hydrogen peroxide for 10 minutes. Samples will be then incubated with specific antibodies for 60 minutes at room temperature. Antibodies will be monoclonal for macrophages (CD68) and activated T lymphocytes (HLA-DR), and polyclonal for T lymphocytes (CD3). Subsequently, samples will be incubated with biotin-labeled secondary antibodies. Finally, blocks will be stained with streptoavidin and examined with an optic microscope. Differential cell count will be performed with a standard morphometry method. Results will be expressed as the number of cells per 10 fields(23).

- Serum analysis of hs-CRP, PAI-1, Lp-PLA_2 and PTX3. Blood samples will be taken the same day of surgery. Venous blood collection will be centrifuged at 3000rpm for 15 min and stored at -80 ºC until their analysis. Levels of hs-CRP, PAI-1, Lp-PLA_2 and PTX3 will be measured by using a specific commercial high-sensitivity enzyme-linked immunosorbent assay (ELISA) kits(26,30,36).

- Presence or absence of neurological symptoms. Symptomatic versus asymptomatic carotid stenosis as previously defined.

- Evaluation of morphological plaque features by MRI. MRI study is routinely performed in the medical care assistance as part of the vascular study before CEA surgery. MRI study is formed by a specific MRI protocol for plaque analysis, standard multimodal brain study of parenchyma and MR angiography. Carotid plaques will be identified as stable or vulnerable according to the same features defined in the histopathological analysis. More detailed information about MRI protocol is attached in Annex 3.

- Evolution of carotid arteries by serum analysis of biomarkers. Carotid arteries will be evaluated at 3 and 12 months after CEA surgery by measuring blood sample levels of hs-CRP, PAI-1, Lp-PLA_2 and PTX3. As mentioned before, it will be used a specific commercial high-sensitivity ELISA kits. Serum levels of biomarkers before and after plaque removal will be compared.

- Evolution of carotid arteries by Doppler ultrasound study. Carotid arteries will be evaluated at 3 and 12 months after CEA surgery by Doppler ultrasound study in order to monitor carotid progression. Two different options of progression are contemplated. On one hand, carotid artery can remain clean and without stenosis. On the other hand, carotid artery can suffer a restenosis. Stenosis quantification will be performed according to the recommendations made by the Spanish Society of Neurosonology (Annex 4)(15). Doppler examination will include common carotid artery, internal carotid artery and intracranial arteries on the axial and longitudinal planes by B mode and colour Doppler methods, starting at the origin and including the bifurcation point. Grades of restenosis will be defined as < 50%, from 50% to 69% and from 70% to 99%.

7.4.2 **Co variables:**

Sociodemographic variables:

- Date of birth (years)
- Sex (Male/Female)

Clinical variables:

- Body Mass Index (BMI): normal weight (18,5–24,9Kg/m^2), overweight (25-29,9Kg/m^2) or obesity (≥30Kg/m^2).
- Smoking habit: smoker, non-smoker (those who have never smoked) or ex-smoker (those who have been 5 years without smoking).
- Hypertension (yes/no): systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg or history of medical treatment of hypertension.
- Diabetes mellitus (yes/no): use of hypoglycemic agent, fasting serum glucose >126 mg/dl or HbA1c > 6,5 %.
- Dyslipidemia (yes/no): total cholesterol ≥200 mg/dL, triglyceride level ≥ 150mg/dL or low-density lipoprotein ≥130 mg/dL.
- Coronary artery disease (yes/no): documented in the clinical history of participants.
- Peripheral artery disease (yes/no): documented in the clinical history of participants.
- Drug history: dose and duration of antiplatelet drugs, medication for hypertension, diabetes and lipid-lowering agents.

7.5 DATA COLLECTION

Data obtained from participants at baseline and during the following visits will be registered in the Case Report Form (CRF) (Annex 5), and according to this form, data will be reported to the study database. All variables are included.

Data will be collected by the research team. It will be formed by three neurologists, three radiologists and two laboratory researchers. All of them are currently working in the HDJT. Each member will be previously taught and trained to be familiar with the methodology of collecting data.

During the first evaluation of participants, sociodemographic and clinical features will be obtained. A fast blood screening of serum glucose, glycated haemoglobin (HbA1c), total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL) and triglycerides will be assayed by routine laboratory techniques in all patients without known history of diabetes or hypercholesterolemia.

Doppler ultrasound and MRI are routinely done before surgery as the vascular study for grading ICA stenosis. Doppler criteria (Annex 4) that will be used in this study are the same ones that the neurologists' team currently use for their daily clinical practice. Thus, the neurologists' team is very well-trained and with large experience in this type of examination. Data will be stored and digitized. MRI data will be obtained in two separate times. First MRI appointment is necessary to apply a specific protocol for plaque analysis while the second appointment is necessary to perform the standard multimodal study of brain parenchyma and MR angiography. Radiologists will be blinded to the histological findings and clinical information of participants.

CEA surgery will be performed by the vascular surgeons' team of the HDJT. There is an established protocol (Annex 6) for this procedure that both Vascular and Neurology Departments agreed for their daily clinical practice. Thus, vascular surgeons are very

experienced and trained to follow the same surgery methodology to avoid inter-professional differences.

Blood samples analysis and histopathologic and immunohistochemical analysis of carotid specimens will be performed in the laboratory of the HDJT and data collection will be acquired by the two laboratory researchers. Proteomic study of carotid specimens will be performed in the laboratory of Hospital Clínico Universitario de Santiago de Compostela. To maintain sample quality, carotid specimens will be sent to the Hospital Clínico Universitario de Santiago de Compostela ensuring adequate transport conditions by keeping frozen samples at -80ºC. Results will be sent back by e-mail and the two laboratory researchers will be responsible for its data collection. Laboratory researchers will be blinded to the clinical and neuroimaging details of participants.

Two follow-up visits at 3 and 12 months after CEA surgery will be scheduled for each participant in order to evaluate carotid artery progression. Serum analysis of biomarkers and Doppler ultrasound study will be performed in both appointments. Data will be obtained by following the same methodology mentioned before.

A statistical specialist will be responsible for evaluating data quality and consistence, and to create the database.

The following table summarizes when and which data will be collected and who will collect it.

Month	Appointment	Collected data	Study member
-1	Study inclusion First evaluation Vascular study	Reading the information sheet Signature of the informed consent Baseline data Doppler ultrasound data	Neurologist
	Vascular study	Specific MRI protocol for plaque analysis	Radiologist
		Cranial MR data MR angiography data	
0	CEA Surgery	Before surgery: blood collection After surgery: carotid plaque collection	Nursery Vascular surgeon
1	Laboratory analyses	Serum biomarkers data Carotid plaque data: - Proteomic study - Histopathologic study - Immunohistochemical study	Laboratory staff
3	Follow-up	Serum biomarkers data Doppler ultrasound data	Laboratory staff Neurologist
12	Follow-up	Serum biomarkers data Doppler ultrasound data	Laboratory staff Neurologist

8. STATISTICAL ANALYSIS

Statistical analysis will be performed with the Statistical Package for Social Sciences (SPSS) for Windows program.

Univariate analyses:

Results will be expressed as frequencies (n) or percentages (%) for categorical variables and as mean ± standard deviation (SD) or median and interquartile range (IQR) for continuous variables depending on whether or not they are normally distributed. Non-parametric variables will be mathematically transformed to improve symmetry.

Bivariate analyses:

Proportions will be compared with the x^2 test. The *t* test or Mann-Whitney U test will be used to compare the significance of observed differences between two groups. Non-parametric Spearman's correlation coefficient will be calculated to investigate the correlation between the expression of biomarkers in serum samples and in the carotid plaques specimens.

Multivariate analyses:

Finally, logistic regression model will be applied to evaluate the independent relation between the expression of biomarkers and the presence of neurological symptoms, while also contributing on the adjustment of occurrence of patient confusion-causing variables.

For all analyses, a *p* values of <0.05 will be defined as statistically significant and highly significant for values <0.001. Confidence intervals will be expressed as 95%.

9. ETHICAL CONSIDERATIONS

This project will be evaluated and approved by the Clinical Research Ethics Committee (CEIC) of the “Hospital Doctor Josep Trueta” and by the Autonomous Community Authorities.

Ethical Principles for Medical Research Involving Human Subjects defined in the World Medical Association Declaration of Helsinki will be accurately considered in this study to ensure the human rights and ethical tenets.

All participants will be appropriately informed and will be given an information sheet (Annex 1) about the study before being included. Subjects will have to voluntarily sign the informed consent (Annex 1).

To guarantee and protect confidentiality of all participants, information collection during the course of this study will be performed in accordance to “Ley Orgánica 15/1999, de 13 de diciembre, de Protección de Datos de Carácter Personal” and “Real decreto 1720/2007, de 21 de diciembre por el que se aprueba el Reglamento de desarrollo de la Ley Orgánica 15/1999”.

This study will respect biomedical regulation according to “Ley 14/2007, de 3 de julio, de Investigación Biomédica” for invasive procedures.

Participants have the right to access, modify, oppose or remove their personal data contained in the file as well as to leave the study at any time.

10. STUDY LIMITATIONS

Several limitations should be acknowledged:

- As this study has a cross-sectional design it will not allow us to establish causal inferences. Therefore, it will establish frequencies.
- Since it will be used a non-probabilistic consecutive sampling method a selection bias can be produced.
- Results obtained in the acute phase of disease should be considered critically, since the acute process within the central nervous system may directly influence the concentration of circulating biomarkers. For that reason, this study includes a before and after design with a follow-up of serum biomarkers expression in order to evaluate their course after plaque removal.
- Data collection may be another limitation as members from different departments are involved in it. To avoid mistakes and missing data, all members will be previously trained to use the CRF correctly. Data collection of Doppler study could be considered another limitation. However, neurologist researchers are very well-trained and with large experience in this type of examination. The methodology applied for this study is the same one that they are currently using in their daily clinical practice. Therefore, it will greatly reduce inter-professional variability.
- Possible loss of participants during the follow-up may be a limitation in this study, but as stroke is a disabling disease and both appointments are part of the medical care we expected it will be insignificant. Patients' death is the main reason of participants' loss that this study predicts. Ambulance transport will be provided to patients with limited mobility. If patients do not attend the appointment, the hospital will try to contact with them via phone call during the following days to arrange a new appointment.

11. WORK PLAN AND CRONOGRAM

PERSONNEL OF THE RESEARCH TEAM

The research team will be composed by three neurologists (NRL1, NRL2 and NRL3), three radiologists (R1, R2 and R3), two laboratory researchers (L1 and L2) and a statistical specialist (SS). The principal researcher will be NRL1.

STUDY STAGES

The study has been designed in four stages that are described below:

- **Stage 1. Coordination (6 months):**
    - Activity 1: *protocol elaboration*. This phase has been accomplished in a period of 3 months.
    - Activity 2: *obtaining the ethical approval from the Clinical Research Ethics Committee.* The general coordinator of the study (NRL1) will be the responsible of this activity.
    - Activity 3: *organizational meeting.* The NRL1 will explain all the objectives and will distribute tasks to the rest of the research team. NRL 1 will provide them the appropriate information to ensure that the study will be adequately conducted.
    - Activity 4: *training for using the CRF.* The NRL1 will teach the other members how to collect data in the CRF.
    - Activity 5: *database elaboration*. The SS will be in charge to create the database for this study.

- **Stage 2. Study conduct (48 months):**
    - Activity 6: *research team meetings.* There will be three meetings during the time the study takes place, in order to solve problems and ensure the quality of recruiting and data collection. Moreover, interpretation of preliminary results and quantification of the number of participants will be discussed so as to determine if the estimated sample size will be achieved.
    - Activity 7: *pilot experiment of the CRF during the first months of data collection.* Problems or mistakes will be identified and a definitive CRF will be created.
    - Activity 8: *participants' recruitment.* NRL1, NRL2 and NRL3 will recruit participants during 3 years. Sample selection will be made applying inclusion and exclusion criteria.
    - Activity 9: *Participants' evaluation and data collection.* This period will start simultaneously with participant's recruitment but will end 1 year after the last participant is included in the study. Thus, the global period of this stage lasts 4 years. Data will be introduced in the database after each appointment.
    - Activity 10: *data quality assurance and control.* The SS will be responsible for maintaining a good quality of data and make sure all the information of each participant is correctly introduced in the database.

- **Stage 3. Data analysis and interpretation (3 months):**
    - Activity 11: *statistical analysis.* Data will be analyzed by the SS. Several analyses will be performed throughout the study in order to control its progress. Final analysis will be performed when all data have been collected.

- Activity 12: *interpretation and discussion of the results.* The results will be interpreted and discussed by the NRL1, R1, L1 and SS.

- **Stage 4. Publication and dissemination of the results (3 months):**
  - Activity 13: *publication of the results.* Final research findings and conclusions will be written and published in journal articles.
  - Activity 14: *dissemination of the results.* Attendance to conferences to present the results of the study.

			1st year		2nd year		3rd year		4th year		
ACTIVITIES	**PERSONNEL**	Jul-Dec	Jan-Jun	Jul-Dec	Jan-Jun	Jul-Dec	Jan-Jun	Jul-Dec	Jan-Jun	Jul-Dec	Jan-Jun
Protocol elaboration	NRL1										
Obtaining ethical approval	NRL1										
Organizational meeting	ALL										
CRF training	ALL										
Database elaboration	SS										
Research team meetings	ALL										
CRF pilot experiment	ALL										
Participants' recruitment	NRL1, NRL2 and NRL3										
Participants evaluation and data collection	ALL										
Data quality assurance and control	SS										
Statistical analysis	SS										
Interpretation and discussion of the results	NRL1, R1, L1 and SS										
Publication of the results	ALL										
Dissemination of the results	NRL1										

Stage 1: Coordination (6 months)

Stage 2: Study Conduct (48 months)

Stage 3: Data analysis and interpretation (3 months)

Stage 4: Publication and dissemination of the results (3 months)

12. FEASIBILITY

The research study will be carried out at HDJT in Girona in which all the study members are currently working. The neurologists' team have fifteen years of experience in the research of cerebrovascular diseases. Proteomic analysis of carotid specimens will be carried out in the Hospital Clínico Universitario de Santiago de Compostela with which there is a committed relationship among researchers of both centres since they have worked together several times. Transport costs of specimens will be included in the budget of proteomic analysis. The hospital will provide all the necessary means that are part of the medical healthcare such as personnel salaries, vascular studies or CEA surgeries, and also computer devices and programs to elaborate the database and to carry out the statistical analysis.

It is estimated that in the HDJT approximately 65 patients per year undergo CEA surgery. The calculated sample size to accomplish the aim of the study should be 204 participants, so we expected than in 3 years of data collection we will meet our goal.

13. BUDGET

All staff members that will visit and attend participants during the study, the follow-up appointments, fast blood screenings, vascular studies (including Doppler ultrasound and MRI) and CEA surgeries are not considered in this estimated budget because they are part of the National Health System.

ITEM	COST PER UNIT	NUMBER OF UNITS	TOTAL COSTS
MATERIALS AND SERVICES			
Information sheet and informed consent printing	0.30 €/unit	1unit/participant	0.30€
			x 204 participants
			61.2 €
Data management and statistical analysis	35 €/hour	50 hours	1,750 €
			x 1 person
			1,750 €
Serum hs-PCR (ELISA)	2.62 €/unit	3units/participant	7.86 €
Serum PAI-1 (ELISA)	18 €/unit	3units/participant	54 €
Serum Lp-PLA_2 (ELISA)	16.72 €/unit	3units/participant	50.17 €
Serum PTX3 (ELISA)	16.16 €/unit	3units/participant	48.48 €
			x 204 participants
			32,744.04 €
Histopathologic and immunohistochemical analysis	x 204 carotid specimens		25,160 €
Proteomic analysis	x 204 carotid specimens		56,355 €
			81,515 €
Article publication charges	1,000 €/unit	1 publication	1,000 €
Article translation	1,000 €/unit	1 translation	1,000 €
			2,000 €
TRAVEL EXPENSES AND ALLOWANCES			
Conferences	1,000 €/unit	2 conference	2,000 €
			2,000 €
		TOTAL COSTS:	**120,070.24 €**

14. IMPACT ON THE NATIONAL HEALTH SYSTEM

As previously discussed, carotid artery stenosis is considered to be the main risk factor to develop ischemic stroke. Ischemic stroke has a huge socioeconomic impact in the national healthcare as it is the first cause of long term morbidities in our country.

If the results obtained in this study are relevant and our hypotheses are validated, we will have made an important step forward in the identification of novel biomarkers that would be able to detect vulnerable carotid plaques, and consequently, patients at high risk of developing atherosclerotic complications. Therefore, new strategies to ensure best risk stratification and management of both asymptomatic and symptomatic patients with carotid stenosis could be provided. Thus, long term morbidities would be reduced and the patients' and their families' quality of life would improve.

Moreover, results could be applied in the research of atherothrombotic disease that affects different locations such as ischemic heart disease or peripheral artery disease.

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16. ANNEXES

ANNEX 1: INFORMATION SHEET AND INFORMED CONSENT

FULL D'INFORMACIÓ AL PACIENT

Títol de l'estudi:

Detecció de pacients amb estenosis carotídia que presenten un alt risc de desenvolupar complicacions vasculars a través de la identificació de biomarcadors

Benvolgut/da,

L' ictus és una de les causes més importants de mort i discapacitat en el nostre medi. L'estenosi de l'artèria caròtida per una placa d'ateroma és una de les principals causes d'ictus isquèmic. L'estenosi acostuma a ser asimptomàtica en la població general ja que es tracta d'un procés crònic i silent. A dia d'avui es desconeix el factor que desencadena la ruptura de la placa d'ateroma i provoca l'oclusió per un trombus d'alguna de les arteries que irriguen el cervell, i per tant, l'aparició d' un conjunt de signes i símptomes neurològics.

El/la convidem a participar en un estudi d'investigació sobre l'anàlisi de possibles marcadors biològics de risc de desenvolupar símptomes neurològics en pacients amb estenosis de l'artèria caròtida per una placa d'ateroma. Tindrà una durada d'un any i es realitzarà a l'Hospital Doctor Josep Trueta de Girona.

A continuació li presentem un formulari en el qual s'inclou un resum amb la informació sobre l'estudi per tal que pugui decidir si està interessat/da o no a col·laborar-hi. Llegeixi detingudament i prengui's el temps que cregui convenient. Li recordem que la seva participació és totalment voluntària, i que si decideix no participar, això no afectarà de cap manera el tracte dels professionals sanitaris cap a vostè.

1. Quina és la finalitat de l'estudi?

   El propòsit d'aquest estudi és analitzar uns marcadors biològics determinats en les seves mostres de sang i de placa ateromatosa extreta de l'artèria caròtida interna durant el procés quirúrgic anomenat endarterectomia per tal de

determinar si aquests marcadors estan relacionats amb el desenvolupament de clínica neurològica o no. Uns marcadors així, permetrien detectar persones asimptomàtiques amb estenosis carotídia d'alt risc i per tant susceptibles de patir un ictus i, en aquells/es pacients que ja l'haguessin sofert, saber si presenten un alt risc de recaiguda. En ambdós casos permetria plantejar un tractament més avinent i agressiu per evitar les possibles complicacions de la ruptura de la placa d'ateroma.

2. <u>En què consistirà la meva participació?</u>

Vostè ha estat diagnosticat d'estenosi carotídia i el seu neuròleg li ha indicat una intervenció quirúrgica per tal de treure la placa d'ateroma que ocupa la llum de la seva artèria.

La seva participació en aquest estudi constarà de diverses parts:

- Primer de tot, un dels neuròlegs de l'equip d'investigació li realitzarà una entrevista en la qual recollirà dades de caràcter personal i clínic. En aquesta mateixa visita se li realitzarà un estudi vascular de les artèries del coll i del cap amb una prova anomenada Doppler. El Doppler és una tècnica totalment innòcua i sense riscs.
- En el curs del seu estudi pre-quirúrgic, li realitzaran una Ressonància magnètica. Firmant el consentiment informat, accedeix a deixar-nos utilitzar les seves dades obtingudes amb aquesta prova.
- El mateix dia de la intervenció quirúrgica programada pel seu neuròleg, se li extraurà una mostra de sang que serà congelada fins al seu posterior estudi.
- Un cop finalitzada la cirurgia, es congelarà la placa d'ateroma extreta per al seu posterior estudi.
- Per últim, se li farà un seguiment als 3 i als 12 mesos de la intervenció per tal de repetir l'estudi vascular amb Doppler i l' analítica de sang (a cada una de les visites).

Totes les mostres biològiques recollides durant l'estudi s'emmagatzemaran a l'Hospital Dr Josep Trueta. Les mostres s'utilitzaran per a la finalitat de l'estudi i s'introduiran en el biobanc de l'hospital (en cas que així ho consenti).

3. La meva participació serà confidencial?

   La informació recollida en aquest estudi serà introduïda en una base de dades computeritzada per a la seva posterior anàlisis. Les dades de caràcter personal i informació recollida en l'estudi són totalment confidencials i queden protegides d'acord amb la legislació vigent sobre la protecció de dades de caràcter personal (Llei Orgànica 15/1999 del 13 de desembre). Els resultats d'aquest estudi s'utilitzaran per a la seva presentació en congressos mèdics o la publicació en revistes científiques.

4. Quins són els possibles riscs o inconvenients de participar en aquest estudi?

   No es preveuen riscs ni inconvenients per participar en aquest estudi.

5. Puc retirar-me o canviar d'opinió una vegada iniciat l'estudi?

   Si, la seva participació en aquest estudi és voluntària, pel que pot demanar l'eliminació de totes les mostres recollides que es trobin emmagatzemades i de la informació relacionada amb aquestes en qualsevol moment de l'estudi i sense necessitat d'especificar el motiu. Si així ho decidís, això no repercutiria en les seves cures mèdiques.

6. A qui em puc dirigir per demanar més informació?

   En cas que tingui qualsevol dubte o vulgui més informació, no dubti en contactar amb el seu metge investigador de referència o trucar al següent número de telèfon: **972-257638 (de 8:00 a 17:00h)**.

FULL DE CONSENTIMENT INFORMAT

Títol de l'estudi:

Detecció de pacients amb estenosis carotídia que presenten un alt risc de desenvolupar complicacions vasculars a través de la identificació de biomarcadors

Jo (Nom i cognoms):

_____

- He llegit detingudament i he entès el full d'informació que se m'ha entregat.
- He rebut suficient informació sobre l'estudi.
- L'investigador m'ha explicat de manera entenedora tot el procediment.
- He pogut fer preguntes sobre l'estudi i tots els meus dubtes han estat resolts de manera satisfactòria.
- Entenc que totes les meves dades seran tractades de manera estrictament confidencial.
- Entenc quin serà el meu paper com a participant de l'estudi.
- Entenc que la meva participació és voluntària, i que en qualsevol moment de l'estudi puc canviar d'opinió sense haver de donar cap explicació i que, independentment de la meva decisió, la meva atenció mèdica i els meus drets legals no es veuran afectats.

Per tant, accepto voluntàriament participar en aquest estudi d'investigació i permeto que les meves mostres biològiques siguin introduïdes en el biobanc de l'hospital.

Signatura del participant Signatura de l'investigador

_____ _____

Girona, _____ de _____ de 20____

HOJA DE INFORMACIÓN AL PACIENTE

Título del estudio:

Detección de pacientes con estenosis carotidea que presentan un alto riesgo de padecer complicaciones vasculares mediante la identificación de biomarcadores

Estimado/a,

El ictus es una de las causas más importantes de muerte i discapacidad en nuestro medio. La estenosis de la arteria carótida por una placa de ateroma es una de las principales causas de ictus isquémico. La estenosis acostumbra a ser asintomática en la población general ya que se trata de un proceso crónico y silente. Hoy en día se desconoce el factor que desencadena la ruptura de la placa de ateroma y provoca la oclusión por un trombo de alguna de las arterias que irrigan el cerebro, y en consecuencia, la aparición de un conjunto de signos y síntomas neurológicos.

Le/la invitamos a participar en un estudio de investigación sobre el análisis de posibles marcadores biológicos de riesgo de desarrollar síntomas neurológicos en pacientes con una estenosis en la arteria carótida interna por una placa de ateroma. Su duración será de un año y se realizará en el Hospital Dr Josep Trueta de Girona.

A continuación le presentamos un formulario en el que se incluye un resumen con la información sobre el estudio para que pueda decidir si está interesado/a o no en colaborar. Lea detenidamente y tómese el tiempo que crea necesario. Le recordamos que su participación es totalmente voluntaria, y que si decide no participar, esto no afectará al trato de los profesionales sanitarios hacia su persona.

1. ¿Cuál es la finalidad del estudio?

   El propósito de este estudio es analizar unos marcadores biológicos determinados en sus muestras de sangre i de placa de ateroma extraída de la arteria carótida durante el proceso quirúrgico llamado endarterectomía para determinar si estos marcadores están relacionados con el desarrollo de clínica neurológica o no. Unos marcadores así, permitirían detectar personas asintomáticas con estenosis carotidea de alto riesgo y por lo tanto susceptibles

de padecer un ictus y, en aquellos/as pacientes que ya lo hubiesen sufrido, permitiría saber si presentan un alto riesgo de recaída. En ambos casos permitirían plantear un tratamiento más concreto y agresivo para evitar las posibles complicaciones de la ruptura de la placa de ateroma.

2. ¿En qué consistirá mi participación?

Usted ha sido diagnosticado de estenosis carotidea y su neurólogo le ha indicado una intervención quirúrgica para quitar la placa de ateroma que ocupa la luz de su arteria.

Su participación en este estudio constará de varias partes:

- Para empezar, uno de los neurólogos del equipo de investigación le realizará una entrevista en la que recogerá datos de carácter personal y clínico. En esta misma visita se le realizará un estudio vascular de las arterias del cuello y de la cabeza con una prueba llamada Doppler. El Doppler es una técnica totalmente inocua y sin riesgos.
- En el curso de su estudio pre-quirúrgico, le realizaran una Resonancia magnética. Firmando el consentimiento informado, accede a dejar-nos utilizar sus datos obtenidos con esta prueba.
- El mismo día de la intervención quirúrgica programada por su neurólogo, se le realizará una extracción de sangre que será congelada hasta su posterior estudio.
- Una vez finalizada la cirugía, se congelará la placa de ateroma extraída para su posterior estudio.
- Para terminar, se le hará un seguimiento a los 3 y 12 meses de la intervención para repetir el estudio vascular con Doppler y la analítica de sangre (en cada una de las visitas).

Todas las muestras biológicas recogidas durante el estudio se almacenarán en el Hospital Dr Josep Trueta. Las muestras se utilizaran para la finalidad del estudio i se introducirán en el biobanco del hospital (en caso que así lo consienta).

3. ¿Mi participación será confidencial?

La información recogida en este estudio será introducida en una base de datos computerizada para su posterior análisis. Los datos de carácter personal e

información recogida en el estudio son totalmente confidenciales y quedas protegidos de acuerdo con la legislación vigente sobre la protección de datos de carácter personal (Ley Orgánico 15/1999 del 13 de diciembre). Los resultados de este estudio se utilizaran para su presentación en congresos médicos o la publicación en revistas científicas.

4. ¿Cuáles son los posibles riesgos o inconvenientes de participar en este estudio?

   No se prevén riesgos ni inconvenientes para participar en este estudio.

5. ¿Puedo retirar-me o cambiar de opinión una vez empezado el estudio?

   Sí, su participación en este estudio es voluntaria, por lo que puede pedir la eliminación de todas las muestras recogidas que estén almacenadas y de la información relacionada con estas en cualquier momento del estudio y sin necesidad de especificar el motivo. Si así lo decidiese, esto no repercutiría en sus curas médicas.

6. ¿A quién puedo pedir más información?

   En caso de duda o que quiera más información, no dude en contactar con su médico investigador de referencia o llame al siguiente número de teléfono: **972-257638 (de 8:00 a 17:00h)**.

HOJA DE CONSENTIMIENTO INFORMADO

Título del estudio:

Detección de pacientes con estenosis carotidea que presentan un alto riesgo de padecer complicaciones vasculares mediante la identificación de biomarcadores

Yo (Nombre y apellidos):

_____

- He leído detenidamente y he entendido toda la hoja de información que se me han entregado.
- He recibido suficiente información sobre el estudio.
- El investigador me ha explicado de manera clara todo el procedimiento.
- He podido realizar preguntas sobre el estudio y todas mis dudas han sido resueltas de manera satisfactoria.
- Entiendo que todos mis datos serán tratados de forma estrictamente confidencial.
- Entiendo cuál será mi papel como participante del estudio.
- Entiendo que mi participación es voluntaria, y que en cualquier momento del estudio puedo cambiar de opinión sin tener que dar ninguna explicación y que, independientemente de mí decisión, mi atención médica y mis derechos legales no se verán afectados.

Por lo tanto, acepto voluntariamente participar en este estudio de investigación i permito que mis muestras biológicas sean introducidas en el banco del hospital.

Firma del participante Firma del investigador

_____ _____

Girona, _____ de _____ de 20____

ANNEX 2: WHAT IS PROTEOMICS?

Proteomics is the study and characterization of the entire set of proteins expressed by a genome (proteome). Proteomics is a scientific approach used to detect all protein species expressed within body fluids as well as cells and tissues. Proteomic technologies are suited for the discovery of protein biomarkers which are indicators of normal biological processes, pathogenic processes, or pharmacologic response to a therapeutic intervention(39,40).

The process of biomarker discovery:

To minimise subject-to-subject variability and ensure sample quality, sample collection and storage conditions must be standardised(39). In this study, all carotid specimens will be immediately frozen and stored at – 80ºC until analysis.

Sample preparation involves the process of protein concentration in order to reduce protein complexity and ensure the visualization of lower abundant proteins. Resins and cibacron blue are used to remove immunoglobulins and albumin concentration respectively (they represent 60% of the total serum proteins). Final step in sample processing involves digestion of proteins into peptides using proteases or chemical agents(39,40).

The next step is the separation of proteins. The most common method is two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2D-PAGE) which separates plaque proteins depending on the isolectric point and molecular weight. Protein spots can then be visualized using staining methods which allow the identification and quantification of protein spots differentially expressed between the two groups of study. Although this technique has a low cost, it is sometimes insufficient to visualize all protein species (e.g., those in low abundance). Other methods for

protein separation are high performance liquid chromatography (HPLC) or two-dimensional liquid chromatography (2D-LC)(40).

The final step is the identification of the proteins of interest in our samples. Currently, mass spectrometry (MS) is the chosen method for this purpose. This method offers a high analytical sensitivity and the capacity for high-throughput protein identification. Different techniques have been developed:

- Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS generates a peptide mass fingerprint (PMF) from the sample of interest.
- Ion trap mass spectrometer subject peptides to fragmentation in the tandem mass spectrometric (MS/MS) mode which is the connection of two MS in series. First MS isolate the peptide fragment of interest and the second MS produce its fragmentation by the internal energy or induced by collisions with a gas. This method has a higher sensitivity and is capable to identify low abundant proteins(39,40).

Therefore, 2D-PAGE and MALDI-TOF will be used as first option for protein identification and analysis in the carotid specimens. When these techniques are insufficient, HPLC and tandem MS/MS will be used.

ANNEX 3: MRI PROTOCOL

The MRI protocol applied in the HDJT for patients with carotid stenosis that undergo CEA surgery is explained below. It has been used an article from the journal of Radiology(41) to contrast the methodology.

Carotid artery will be imaged with a 1.5-T scanner.

1) **Specific MRI protocol** to analyse plaque morphology and composition which includes:
    - Three-dimensional T1-weighted turbo field-echo sequence (3D T1-TFE).
    - Three-dimensional time-of-flight sequence (3D TOF).
    - Multisection T2-weighted turbo spin-echo sequence (T2-TSE).
    - Contrast material-enhanced two-dimensional T1-weighted turbo spin-echo sequences (T1-TSE). The contrast-enhanced T1-weighted TSE sequence will be performed approximately 7 minutes after the injection of 0,1mmol per kilogram body weight gadopentetate dimeglumine. Dynamic contrast-enhanced MR imaging will be performed between acquisition of pre- and post-contrast T1-TSE images.
    - Three-dimensional dynamic contrast-enhanced T1-weighted fast field-echo (T1-FFE) images will be acquired by obtaining 10 transverse over-contiguous sections for 16 time frames with a typical separation of 25 seconds between frames.
    - Fat suppression will be used to reduce signal from subcutaneous fatty tissues.

2) **Standard multimodal brain study of parenchyma** to ensure there is no brain damage in the case of asymptomatic CS participants.
    - T1-weighted

- T2-weighted
- FLAIR
- Diffusion (DWI)
- Perfusion (PWI)

3) **MR angiography** to confirm stenosis gradation made by Doppler ultrasound and to evaluate brain circulation for CEA procedure.

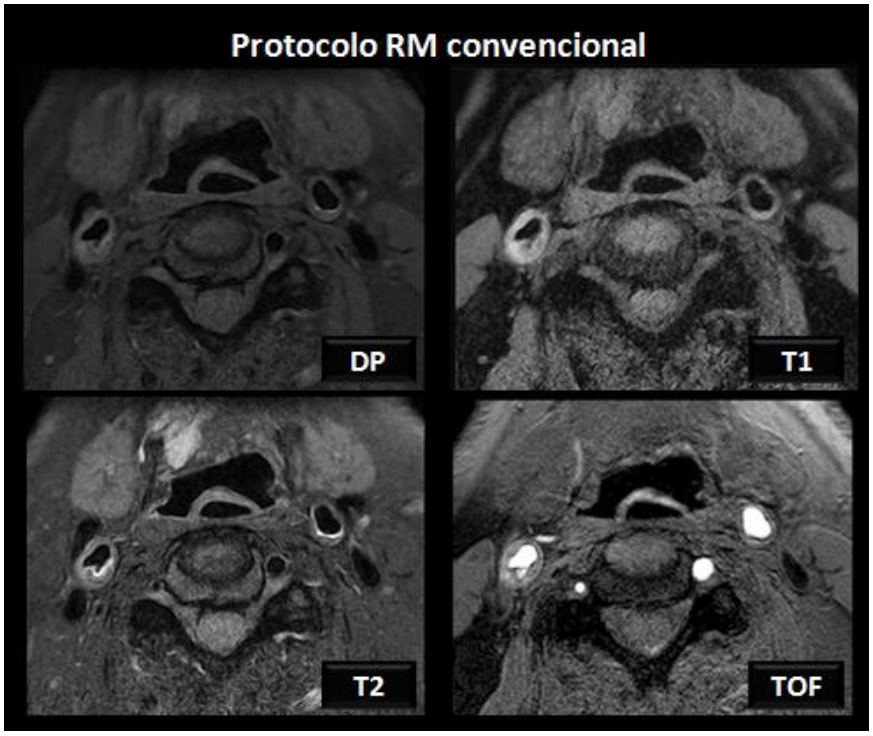

Figure 2: MR image sequences of carotid artery study. Image obtained from the Department of Radiology-IDI of the HDJT.

ANNEX 4: STENOSIS QUANTIFICATION BY DOPPLER ULTRASOUND

Doppler ultrasound study will be performed according to the recommendations made by the Spanish Society of Neurosonology (15). Doppler examination will included common carotid artery, internal carotid artery and intracranial arteries on the axial and longitudinal planes by B mode and colour Doppler methods, starting at the origin and including the bifurcation point.

Hemodynamic parameters will be used to quantify the amount of stenosis:

- Direct signs: peak systolic velocity and end diastolic velocity. ICA is considered normal when peak systolic velocity is < 125 cm/s and end diastolic velocity is < 40 cm/s.
- Indirect signs: peak systolic velocity in the post-stenotic region of extracranial ICA and ophthalmic artery, and collateral circulation of intracranial arteries.

Tabla 1 Criterios hemodinámicos para establecer el grado de estenosis carotídea

Criterios	Grado de estenosis arterial					
	< 50%	50-69%	70-79%	80-89%	≥ 90%	Oclusión
Signos directos						
VSM	< 125	125-230	> 230	> 300	Variable	NA
VDF	< 40	40-100	> 100	Variable	Variable	NA
Signos indirectos						
VSM postestenosis en ACI	Normal	Normal	≥ 50	< 50	< 30	NA
Flujo colateral en AO	No	No	No/↓/invertido	↓/invertido	↓/invertido	↓/invertido
Flujo colateral en PW	No	No	No/presente	Presente	Presente	Presente
Índices						
Relación entre VSM_{ACI}/VSM_{ACC}	< 2	≥ 2	> 4	> 4	Variable	NA

ACC: arteria carótida común; ACI: arteria carótida interna; AO: arteria oftálmica; NA: no aplicable; PW: polígono de Willis; VDF: velocidad diastólica final; VSM: velocidad sistólica máxima.

Figure 3: Direct and indirect signs for the quantification of carotid stenosis(15).

Therefore, significant stenosis will be defined as:

- More than 50% but less than 70% if peak systolic velocity is between 125 and 230 cm/s and end diastolic velocity is between 40 and 100 cm/s.
- More than 70% but less than 99% if peak systolic velocity is >230 cm/s and end diastolic velocity is >100 cm/s.

ANNEX 5: CASE REPORT FORM

CASE REPORT FORM

Participant number: _____

Name (initials): _____ **Surname (initials):** _____

Provenience: ☐ Emergency Department

☐ Primary care physician

☐ Different Departments from the Hospital

Presence of neurological symptoms: yes ☐ no ☐

If yes, symptomatic ICA stenosis: Right ☐ Left ☐

BASELINE DATA

SOCIODEMOGRAPHIC FEATURES

- **Birth date:** ____/____/_____
- **Gender:** Male ☐ Female ☐

CLINICAL FEATURES

- **Smoking habit:** smoker ☐ non-smoker ☐ ex-smoker ☐
- **Body Mass Index (BMI):** normal weight ☐ overweigh ☐ obesity ☐
- **Hypertension:** yes ☐ no ☐
- **Diabetes mellitus:** yes ☐ no ☐
- **Dyslipidemia:** yes ☐ no ☐
- **Coronary artery disease:** yes ☐ no ☐
- **Peripheral artery disease:** yes ☐ no ☐

- **Drug history (dose and duration):**

  ☐Antiplatelet: _____

  ☐Antihypertensive: _____

  ☐Statins: _____

  ☐Lipid-lowering agents: _____

  ☐Antidiabetic: _____

  ☐Others: _____

  ☐None

  ☐Unknown

VASCULAR STUDY

DOPPLER ULTRASONOGRAPHY

- **Carotid vascular study:**
  - **Left carotid artery:** ☐Normal ☐< 50% stenosis ☐50-69% stenosis ☐70-99% stenosis ☐Occlusion
  - **Right carotid artery:** ☐Normal ☐< 50% stenosis ☐50-69% stenosis ☐70-99% stenosis ☐Occlusion
  - **Lesions in other supra-aortic arteries**: ☐yes ☐ no

- **Transcranial vascular study:** ☐Normal
  ☐Intracranial asymptomatic stenosis
  ☐Intracranial symptomatic stenosis
  ☐Occlusion of intracranial artery
  ☐Microangiopathy

MAGNETIC RESONANCE IMAGING

<table>
<tr><td rowspan="2">Specific MRI protocol to analyse plaque morphology</td><td>Lipid core: ☐small ☐large (>40%)
Fibrous cap: ☐large ☐thin (< 100 μ)
Ulceration: ☐yes ☐ no
Intra-plaque haemorrhage: ☐yes ☐ no
Intra-plaque thrombosis: ☐yes ☐ no</td></tr>
<tr><td>☐Stable plaque ☐Vulnerable plaque</td></tr>
<tr><td>Standard multimodal brain study of parenchyma</td><td>☐ Presence of brain damage
☐ Absence of brain damage</td></tr>
<tr><td>MR angiography</td><td>ICA stenosis: ☐Right ☐Left

Grade of stenosis: ☐ Normal
☐ < 50% stenosis
☐ 50-69% stenosis
☐ 70-99% stenosis
☐ Occlusion</td></tr>
</table>

LABORATORY ANALYSIS

BLOOD SAMPLE STUDY

Biomarkers analyses	
Hs-CRP	mg/L
PAI-1	ng/mL
Lp-PLA_2	ng/mL
PTX3	ug/L

CAROTID PLAQUE STUDY

Immunohistochemical analysis of biomarkers (number of cells per 10 fields)	PAI-1	
	Lp-PLA_2	
	PTX3	
Proteomic analysis		
Histopathologic analysis	**Lipid core:** ☐small ☐large (>40%) **Fibrous cap**: ☐large ☐thin (< 100 μ) **Ulceration:** ☐yes ☐ no **Intra-plaque haemorrhage:** ☐yes ☐ no **Intra-plaque thrombosis:** ☐yes ☐ no	
	☐Stable plaque ☐Vulnerable plaque	
Immunohistochemical analysis (number of cells per 10 fields)	Macrophages	
	T Lymphocytes	

FOLLOW-UP DATA

3 months	**Blood sample study**	Hs-CRP	mg/L
		PAI-1	ng/mL
		Lp-PLA$_2$	ng/mL
		PTX3	ug/L
	Doppler ultrasound	☐normal ☐restenosis: ☐ < 50% ☐ 50% – 69% ☐ 70% - 99%	

12 months	**Blood sample study**	Hs-CRP	mg/L
		PAI-1	ng/mL
		Lp-PLA$_2$	ng/mL
		PTX3	ug/L
	Doppler ultrasound	☐normal ☐restenosis: ☐ < 50% ☐ 50% – 69% ☐ 70% - 99%	

ANNEX 6: CEA PROTOCOL

Hospital Universitari de Girona Doctor Josep Trueta

PROTOCOL D'INGRÉS PER A LA REALITZACIÓ D'ENDARTERECTOMIA

Objecte: Establir de forma protocolaritzada els tractaments i procediments a realitzar durant l' ingrés hospitalari de pacients ingressats per a endarterectomia carotídia.

Àmbit d'aplicació: Secció de Neurologia.

Personal que ho ha de fer:

- Neuròlegs de la secció de neurologia
- Infermeria de la secció de neurologia
- Cirurgià Vascular
- Anestesista
- Auxiliars de clínica de la secció de neurologia

ENDARTERECTOMIA CAROTÍDIA

Procediment Preoperatori:

- El pacient ingressarà 48 hores prèviament a la intervenció quirúrgica.
- Es revisarà si està realitzat el preoperatori sol·licitat pel servei d'anestèsia:
  - Rx tòrax
  - Analítica: hemograma, coagulació i bioquímica bàsica
  - ECG
  - Proves creuades i 2 concentrats d'hematies en reserva operatòria

  Si no està realitzat, ho sol·licitarà de forma urgent el neuròleg de guàrdia.

- Tractament:

  a. Es mantindrà el tractament amb AAS 300 mg/dia.
  b. El tractament amb clopidogrel s'haurà suspès 15 dies abans de la IQ, i s'haurà substituït per AAS 300 mg/dia.
  c. En els casos que el pacient estigui prèviament anticoagulat, s'haurà substituït el tractament amb acenocumarol per HBPM a dosis

anticoagulants (servei d'anestèsia) al domicili, administrant-se la darrera dosi a les 20 hores del dia anterior a la IQ. Se sol·licitarà analítica amb proves de coagulació el dia anterior a la IQ.

d. El tractament amb heparines de baix pes molecular s'administrarà a dosis profilàctiques el dia anterior a la IQ abans de les 20 hores.

- Es revisarà si s'ha realitzat RM crani amb seqüències de difusió, si no les té, se sol·licitarà RM crani urgent prèvia a la intervenció quirúrgica, per tal de descartar l'aparició de noves lesions isquèmiques.
- Es revisarà si s'ha realitzat Eco-Doppler de troncs supraaòrtics i Dúplex transcranial amb estudi de reserva hemodinàmica i detecció de microembòlies.
- Es reforçarà la informació sobre la intervenció quirúrgica al pacient i a la família.
- Es realitzarà estudi neuropsicològic previ a la intervenció quirúrgica (es pot realitzar previ a l' ingrés).
- Es col·locarà una via llarga perifèrica i es realitzarà la RX tòrax posteriorment.
- Es realitzarà rasurat ampli de l'àrea quirúrgica el dia anterior de la IQ.

Procediment dia intervenció:

- Es deixarà al pacient amb dieta absoluta (6 hores abans de la IQ). En pacients no diabètics s'administrarà SF 500 ml/12 hores per mantenir la via llarga. En pacients diabètics s'administrarà SG 10% 500ml/12 hores en dosiflow i pauta d'insulina ràpida cada 6 hores segons protocol d'Unitat d'Ictus en pacient diabètic.
- Dutxa preoperatòria a les 7 hores del matí.
- A les 8 hores del matí (1 hora abans de la intervenció quirúrgica) s'administrarà cefazolina (Kefol®) 1 gram i.v. en dosi única.

Procediment postoperatori:

- El pacient arribarà procedent de la sala de reanimació 24 hores després de la intervenció quirúrgica i serà ingressat, preferentment a la Unitat d'Ictus.
- Si el pacient ingressa a la UI, la monitorització neurològica i hemodinàmica es mantindrà en controls habituals.
- Si el pacient ingressa a la Unitat Convencional, es mantindrà el control de les constants vitals i de l' apòsit cada 8 hores.
- Es retirarà el catèter perifèric central.

- Es retirarà la sonda vesical.
- Es practicarà la revisió de la ferida quirúrgica i el canvi de l'apòsit de forma diària. L'objectiu es detectar sagnat, hematoma de la paret o bé infecció de la ferida quirúrgica.
- S'iniciarà la ingesta de líquids i dieta oral.
- El pacient es podrà seure a l'arribada a la planta de neurologia i aixecar-se per anar al lavabo a les 48 hores de la intervenció quirúrgica.
- Es realitzarà la identificació i quantificació del volum drenat en el redon.
- Entre les 24-48 hores es retirarà el redon (quan no dreni la ferida).
- *Tractament:* S'iniciarà el tractament amb AAS 300 mg/dia i heparines de baix pes molecular (fragmin®) 5000 UI/dia sc el mateix dia de la intervenció quirúrgica. Si el pacient requereix clopidogrel o anticoagulació, es mantindrà el tractament amb heparines de baix pes molecular i AAS durant 4 dies i si no hi ha cap contraindicació o complicació quirúrgica posteriorment s'iniciarà el tractament necessari.
- El cirurgià vascular revisarà diàriament la ferida quirúrgica.
- Es realitzarà RM crani posterior a l'endarterectomia, mentre el pacient estigui ingressat, per tal de descartar l'aparició de lesions isquèmiques agudes.

Procediment a l'alta:

EDUCACIÓ SANITÀRIA DEL PACIENT I DE LA FAMÍLIA:

- Informació referent a la cura tòpica diària de la ferida i de l'apòsit.
- Observació de la ferida (tumefacció, envermelliment, increment de la temperatura).
- Hàbits higiènics (dutxa diària) i de control dels seus factors de risc.
- Control de la tensió arterial durant les primeres setmanes.

ADMINISTRATIVAMENT:

- Se citarà per a estudi neuropsicològic i per a estudi ultrasonogràfic de control (codi DOPD) als 3 mesos (el mateix dia).

MANEIG DE LES COMPLICACIONS

Hipotensió:

L'aparició d' hipotensió (TA<110/50mmHg) durant i posteriorment a la intervenció quirúrgica no és infreqüent, degut a la manipulació del si carotidi. El tractament dependrà de si el pacient es troba simptomàtic o asimptomàtic.

- Si el pacient està asimptomàtic s'administrarà serumteràpia (excepte si el pacient presenta insuficiència cardíaca).
- Si la hipotensió és simptomàtica és preferible administrar expansors plasmàtics (1 sèrum de 500 mL de voluven®) de forma ràpida. Si el pacient no s'estabilitza s' utilitzarà efedrina (1 ampolla de 50 mg diluïda en 9 cc de SF, i s'administrarà d'1 a 2 cc i.v. progressivament, fins a milloria de la pressió arterial).

Hipertensió:

Es tractarà, de forma intensiva, les xifres de pressió arterial >185/105, per tal d'evitar l'aparició d'una síndrome d'hiperperfusió o sagnat per la ferida quirúrgica, seguint els tractaments habituals per la hipertensió arterial segons el protocol de la Unitat d'Ictus.

Bradicàrdia:

La bradicàrdia (FC< 50 bpm) simptomàtica es tractarà amb atropina 0.5-1 mg (1 ampolla de 1 mL) i.v. en bolus lent. Si no es controla avisar a cardiologia.

Síndrome d' hiperperfusió:

Es produeix per paràlisi de l' autorregulació en relació amb la isquèmia crònica. És més freqüent i, per tant, han de vigilar-se de forma més estreta els pacients amb un infart cerebral recent, estenosi crítica, flux col·lateral insuficient, oclusió carotídia contralateral, hipoperfusió ipsilateral crònica amb vasoreactivitat exhausta i que presentin xifres elevades de pressió arterial abans i després de la intervenció quirúrgica.

S'haurà de sospitar en pacients amb cefalea ipsilateral associada a crisis focals motores. Davant la sospita clínica es realitzarà:

- Control estricte de la pressió arterial (< 185/105), segons protocol de la Unitat d'Ictus.
- Les crisis epilèptiques es tractaran amb fenitoïna i.v., segons protocol de la Unitat d'Ictus.
- Se suspendrà l' anticoagulació i antiagregació, fins aconseguir un control adequat de la pressió arterial.
- Es pot valorar l'administració de corticoides intravenosos (Dexametasona 4 mg (1 mL/ampolla) i.v/8 hores).
- En el cas de presència de crisis focals, focalitat neurològica o cefalea intensa es practicarà TC cranial urgent i es valorarà la realització de Dúplex transcranial amb estudi de reserva hemodinàmica.

Hematoma intracranial:

L' hemorràgia cerebral és la manifestació més greu de la síndrome d' hiperperfusió. Davant la sospita es realitzarà TC cranial urgent. El tractament és el de l'hematoma intracranial d'altre origen. S'haurà de suspendre el tractament anticoagulant o antiagregant i mantenir la pressió arterial < 185/105.

AIT/Ictus isquèmic:

En la majoria dels casos el dèficit neurològic es detectarà quan el pacient sigui despertat de l'anestèsia. Davant la sospita es realitzarà TC cranial urgent. Es realitzaran les mesures habituals d'un infart cerebral agut.