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Study Protocol for: Imaging della Placca Carotidea (IMPLAC) study

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The aim of the present study will be to evaluate the relation between carotid artery plaque characteristics, cardiovascular risk factors and brain atrophy/WMH burden analyzed, quantitatively as number and volume of lesions and as brain volumes, and progression over 18 months of follow up in subjects asymptomatic for cerebrovascular disease with a carotid artery stenosis <70%. We will characterize the carotid artery plaque using ultrasound, contrast enhanced ultrasound and computed tomography angiography. Cerebral volumes and WMH were evaluated quantitatively using brain magnetic resonance imaging. All baseline imaging and clinical evaluation will be performed within one month from enrollment.

Patient enrollment

Asymptomatic subjects with carotid stenosis <70% according to Doppler velocity measurement presenting to the Neurophysiology and the Vascular Surgery outpatient clinics of Ospedale San Raffaele (Milan, Italy) will be evaluated for enrollment. Exclusion are: age <18 or > 85 years, pregnancy, history of allergic diathesis, previous stroke or transitory ischemic attack, previous carotid endarterectomy (CEA) or stenting, vasculitis, history of alcohol or drug abuse, life expectancy of <18 months, presence of cognitive impairment preventing the patient from providing informed consent, history or current AF or previous cardiac surgery as potential confounding causes of cerebral ischemic damage. These criteria were selected to limit the inclusion of potential confounding causes of cerebral damage. Table 1 provides a schematic for the exclusion criteria. All subject will have to sign a written informed consent to take part in the study.

Exclusion criteria for the IMPLAC study
Age <18 or >85 years
Contraindications to CTA (eGFR<60 mL/min; history of allergic reaction to iodinated contrast media)
Pregnancy or child-bearing potential
Specific contraindication to MRI: • Claustrophobia • Sickle cell anemia • Systemic mastocytosis • Implanted cardiac devices (PM, ICD) • Vascular clips • Vertebral distractors • Infusion pumps • Neurostimulators • Liquor derivations • Any device which could be dispositioned in the presence of a strong magnetic field
Dementia
Life expectancy less than study follow up (18 months)
History of drug abuse, alcohol abuse or any psychiatric or social condition which may contraindicate the participation to a clinical study

Vertebral artery occlusion
Previous revascularization of the carotid artery
Current anti-coagulation
Atrial fibrillation not necessitating anticoagulation
Known PFO necessitating anti-platelet treatment
Previous cerebrovascular accidents
Previous infections to the CNS
Previous surgery to the CNS
History of anoxic damage to the CNS
Previous cardiac surgery or positioning of intracardiac devices (excluded coronary stents)
History of autoimmune vasculitis

Table 1. Exclusion criteria for the IMPLAC study. CTA: computed tomography angiography. CNS: central nervous system. PFO: patent foramen ovale.

Clinical evaluation and biochemical measurements

At enrolment, all subjects will undergo clinical evaluation including careful medical history, and physical examination, comprising cardiac ausculatation for the detection of irregular heartbeat and two blood pressure measurements. The history wil be focused on the identification of cardiovascular risk factors (CVRFs) and of current treatment. Whole blood will collected on the same day using two 5mL EDTA-anticoagulated vacutainer tubes. For each patient, glycaemia, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and triglycerides will be measured using a colorimetric method using the Cobas Mira Plus analyzer (Horiba, ABX, France).

A patient will be defined as suffering from hypertension in compliance with the definition of the European Society of Cardiology (ESC)/European Society of Hypertension, i.e. systolic blood pressure (BP) ≥140mmHg or diastolic BP ≥90mmHg. A clinical history of hypertension with current BP medication will also be considered diagnostic. A subject will be considered as suffering from resistant hypertension in the presence of uncontrolled hypertension or a history of poorly controlled BP and a current BP treatment with at least three drugs including a diuretic. A fasting blood glucose >126 mg/dL, a glycaemia >200 mg/dL two hours after an oral glucose load of 75g, HbA1C levels over 6.5% or current treatment with oral anti-diabetic agents or insulin will be taken into account for the diagnosis of diabetes. Hypercholesterolemia is defined according to ESC guidelines: total cholesterol ≥200 mg/dL and /or LDL-C ≥120 mg/dL. Current lipid lowering therapy will also be taken into consideration for the definition of hypercholesterolemia. To evaluate the cardiovascular risk profile of our patients, we employed the Framingham Risk Score (FRS) to estimate the probability of a first acute myocardial infarction at 10 years. The variables taken into consideration are age, systolic BP, current smoke, BP medication, total cholesterol and HDL-C. A patient with a FRS>20% is considered at high risk. The presence of diabetes mellitus or manifest cardiovascular disease confer high risk independently from calculated FRS.

Carotid artery ultrasound

All patients will undergo bilateral Duplex ultrasound evaluation of the carotid arteries. Examinations will be performed using a dedicated ultrasonography equipment (Logiq S8, GE Healthcare, UK) with a 7-MHz linear probe (7L, GE). The patient is positioned supine, with the head tilted 45° toward the side opposite to the one being imaged. A transverse sweep is recorded from the lower neck to the common carotid artery (CCA) bifurcation into the external carotid artery (ECA) and internal carotid artery (ICA) in order to identify atherosclerotic lesions, defined as focal lesions exceeding 2 mm in thickness. Each of the segments is identified according to the Rotterdam Study criteria: the 15 mm caudal to the bifurcation was defined as carotid bulb, while cranially we identified ICA and ECA. Longitudinal images are then recorded using anterior, posterior and lateral (45°) views.

Common carotid intima-media thickness (CC-IMT) measurement is performed bilaterally in lateral projection images of the CCA in a semi-automated manner. Briefly, the measures performed on 350 consecutive points taken from approximately 10 mm proximal from the bulb are averaged. We carefully avoided inclusion of CCA atherosclerotic plaques in the measurement of CC-IMT. We will record the highest mean CC-IMT for each patient.

Doppler velocity measurements are made on longitudinal views at the site of any identifiable lesions within CCA, carotid bulb or ICA. The degree of stenosis is evaluated by velocimetric criteria according the Society of Radiologists in Ultrasound Consensus Conference. The plaque determining the highest stenosis is considered the main lesion. For the main lesion, we calculated the degree of stenosis according to the European Carotid Surgery Trial (ECST) criteria, as previously described. Briefly, the narrowest diameter and the estimated normal diameter of the artery at the site of the main plaque were measured, and the degree of stenosis was calculated as [1-(narrowest diameter/estimated normal diameter)]%.

For each patient, Total Plaque Area (TPA) is measured off-line. Briefly, two independent operators (M.M. and F.M) measured the two dimensional area of each identifiable lesion by tracking around the lesion perimeter. The sum of all lesions areas is taken as TPA. The number of segments, CCA, bulb, ICA and ECA bilaterally, involved by atherosclerosis was also registered as an indicator of the extension of the disease.

Finally, each plaque is classified according to its echogenicity, as previously described by Purcelot et al in 1999. Briefly, plaques are considered class I if they were uniformly hypoechoic, class II if they were heterogeneous, mainly hypo-echoic, class III if they were heterogeneous, mainly hyperechoic, class IV if they were uniformly iso-hyper-echoic or class V if they were hyperechoic and displayed posterior acoustic shadow. For subsequent analysis, class I and II were considered a single category, namely lipid-rich plaque, while classes III to V were considered together as fibrocalcific plaques.

Contrast enhanced ultrasound (CEUS) examination

CEUS will be performed on the same day of standard ultrasound evaluation of the carotid arteries using the same equipment. Preset constrast-specific modality (pulse inversion) is employed, adjusting image settings in order to maximize contrast signal visualization and using low mechanical index (0.08-0.12). Five ml of sodium hexafluoride (SonoVue, Bracco Imaging, Italy) were diluted 1:3 in saline. A 3 ml bolus is injected through a peripheral vein and then flushed with 5 ml saline. Plaque neovascularization will assessed offline by 3 independent operators as previously described by Coli et al in 2008. Each lesion is graded as $CEUS^-$ or $CEUS^+$ depending on absence or presence of neovascularization signal, respectively. $CEUS^+$ plaques were defined as plaques in which contrast bubbles, identified as moving bright spots, reached the plaque core or in which contrast signal was seen throughout the entire lesion. For each patient, the total number of neovascularized plaques will be recorded as a semi-quantitative index of neoangiogenetic activity.

Computed Tomography Angiography (CTA)

All patients will be examined with a 64-slice CT scanner (VCT Lightspeed, GE Healthcare, USA). Fifty ml of non-ionic, iso-osmolar contrast material (Iodixanol, 320 mg of iodine per millilitre, Visipaque 320; GE Healthcare, USA) prepared at 37°C will be injected into an antecubital vein through a 20 or 18-gauge catheter at a rate of 5 ml/s, followed by the injection 50 ml of saline. Contrast administration is performed using a dual-shot injector (Nemoto Kyorindo, Japan). The degree of stenosis in CCA, carotid bulb and ICA will be analyzed according to North American Symptomatic Carotid Endarterectomy Trial (NASCET) and ECST criteria. Briefly, for what concerns the NASCET method, the narrowest luminal area at the site of the stenosis and the area of normal artery distal to the plaque are measured, and the degree of stenosis was calculated as [1-(narrowest area/normal area)]. For the ECST, the method is analogous as for what is described above for duplex imaging, but areas are used instead of diameters. The axial data and multiplanar reconstruction (MPR) were used to determine the grade of stenosis, to calculate the plaque volume and the composition of the plaque, defined on the basis of plaque density in Hounsfield units (HU).

Brain magnetic resonance imaging

In all subjects, the following sequences of the brain will be collected during a single session using a 1.5 Tesla scanner (ACHIEVA Philips Medical Systems):

- Two-dimensional (2D) FLAIR (repetition time [TR]/echo time [TE]=11000/140 ms, inversion time [TI]=2800 ms, echo train length [ETL]=47, flip angle [FA]=90º, matrix size=384x253, field of view [FOV]=230x230 mm^2, 44 axial 3 mm-thick slices, number of excitations [NEX]=3, SENSE=1.5, acquisition time [TA]=7'20").
- 2D T2-weighted turbo spin-echo (TSE) (TR/TE=8897/100 ms, ETL=15; flip angle [FA]=90º, matrix size=384x242, FOV=230x230 mm^2, 44 axial 3mm-thick slices, NEX=3, SENSE=1.5, TA=4:9).
- 3D T1-weighted transient field echo (TFE) (TR/TE=7/3.2 ms, TI=900 ms, FA=8°, matrix size=256x256, FOV=256x256 $mm^{2,}$, 192 sagittal 1.2 mm-thick slices, NEX=1, SENSE=2, TA=6'47").

FLAIR-hyperintense lesions, i.e. WMH, will be identified by consensus by two experienced observers blinded to carotid arteries CT and ultrasound evaluation. FLAIR lesion volume (LV) is quantified using a local thresholding segmentation technique (Jim 6, Xinapse Systems, West Bergholt, UK; http://www.xinapse.com/). FLAIR lesion volume and number is reported for the whole brain and for each hemisphere separately. Using 3D T1-weighted images, normalized total brain volumes (NBV), WM volumes (WMV) and GM volumes (GMV) will be measured using the SIENAx software. To avoid a possible bias in image segmentation due to the presence of T1-hypointense lesions, a binarized lesion mask from FLAIR lesions is created and transformed to the original space of the 3D T1-weighted image. After visual inspection and an eventual manual editing, WM lesions are refilled using the tool in FSL 5.0.5 software.

Follow up evaluation

Patients will be called for follow up evaluation after 18 months from enrolment. Follow up evaluation will comprise a clinical examination and a clinical history aimed at identifying potential relevant cardiovascular events, i.e. cerebrovascular accidents. All patients will undergo a ultrasound evaluation, CEUS and cerebral imaging using the above specified protocols.