Study protocol and statistical analysis plan (SAP)

Atherosclerotic plaque imaging project (APIP)

Vulnerable atherosclerotic plaque: underlying mechanisms and novel tools for its detection

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Table of Contents

Background and objective 3
Study design 3
Study population and inclusion 3
Eligibility criteria 3
Follow-up of study subjects 3
Outcome 4
Cardiovascular events 4
Methods 4
Study enrolment 4
Specimen preparation 4
Plaque analyses 4
Cytokine analysis in plasma and homogenate 5
Histology 5
Ethical considerations 5
Review approval identifiers 5
Statistical analysis protocol (SAP) 6
General 6
Survival analysis 6
RNA-sequencing analysis 6
References 7

Background and objective

The rupture or erosion of an atherosclerotic plaque with thrombosis or embolization often underlie heart attacks and strokes[1]. The early identification of patients with atherosclerotic plaques prone to rupture or erosions, vulnerable plaques (VP), and their treatment before the occurrence of events is, therefore, one of the greatest cardiovascular challenges today. Possible approaches for early detection of VP include imaging techniques allowing visualization of plaque structure, circulating biomarkers and better understanding of the pathophysiologic mechanisms of the disease.

In the atherosclerotic plaque imaging project (APIP) human atherosclerotic plaques (that were removed by endarterectomy) are studied to disclose their underlying structure and mechanisms, finding possible novel therapeutic targets or markers for VP. Plaque structure is also studied with imaging methods and try to develop new ways to detect VP using circulating or imaging markers.

Study design

The study uses an observational, non-randomized design.

Study population and inclusion

The study target population is all patients with carotid stenosis undergoing endarterectomy at the Vascular Dept. of Skåne University Hospital (SUS), Malmö, Sweden. The first patient was included Oct. 26th, 2005 and, at present, 1897 subjects have been enrolled and recruitment will be active until 3500 subjects have been included.

Eligibility criteria

Inclusion criteria: patients older than 18 years old, male or female, that are eligible for carotid endarterectomy due to atherosclerosis and that can provide informed consent.

Exclusion criteria: patients younger than 18 years old that cannot provide informed consent. Patients that are pregnant.

Follow-up of study subjects

The Swedish Cause of Death and National in-patient Health Registers are used to identify postoperative cardiovascular events corresponding to the following codes of the 10th revision of the International Classification of Diseases (ICD-10): G45, G46, I20 to I25, I60 to I69, and I97. These are nationwide validated registers where all causes of death and more than 99% of all somatic (including surgery) and psychiatric hospital discharges are registered[2]. All deaths are verified against the Swedish National Population Register. Follow up of research subjects starts 24h after inclusion, with assessment every 2nd year until date of death or emigration from Sweden, whichever comes first (up to 20 years or according to ethical permits).

Intraoperative events are defined as including all events occurring within the first 24 h after carotid endarterectomy and excluded from the follow-up analysis.

Outcome

Patients suffering more than one episode of the same event (for example, patients with multiple strokes) are classified as suffering multiple events. In these cases, only the first chronological event is taken into account in subsequent survival analysis.

Cardiovascular events

Postoperative myocardial infarction, stroke, transient ischaemic attack, amaurosis fugax, cardiovascular death (according to Swedish National Registers).

Methods

Study enrolment

Patients are preoperatively assessed by an independent accredited neurologist. Indications for surgery are as described by Asciutto et al[3], briefly, plaques associated with ipsilateral symptoms (stroke, transitory ischemic attack, stroke or *amaurosis fugax*) and stenosis, measured by ultrasound based on flow velocities as previously validated[4], >70% or plaques not associated with symptoms and stenosis >80% in the 6 months prior to surgery. The decision on eligibility for carotid endarterectomy is proposed on a multidisciplinary meeting where neurologists, radiologists, vascular surgeons and angiologists participate.

Information about comorbidities and past medical history are obtained through standardized preoperative interviews and review of the medical records. Cardiovascular risk factors are recorded, namely hypertension (systolic blood pressure >140 mm Hg), diabetes and smoking history, as well as use of medications (anti-hypertensive, anti-coagulants, diabetes treatment and lipid-lowering medication).

Blood samples are collected one day before endarterectomy. White blood cell (WBC) counts and plasma levels of high sensitive C-reactive protein (hsCRP), triglycerides, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) and creatinine/GFR are determined by routine laboratory methods. The reminder (EDTA-plasma, serum) is aliquoted and frozen -80°C.

Specimen preparation

Plaques are snap-frozen in liquid nitrogen immediately after surgical removal. One fragment (~2 mm) from the most stenotic region is used for histology. Plaques are homogenized in a buffer consisting of 50 mmol/L Tris-HCl (pH 7.5), 0.25 mol/L sucrose, 2 mmol/L tris(2-carboxyethyl)phosphine HCl, 50 mmol/L NaF, 1 mmol/L Na-orthovanadate, 10 mmol/L Na-glycerophosphate, 5 mmol/L Na-pyrophosphate, protease inhibitor cocktail (Roche Complete, EDTA-free,), 1 mmol/L benzamidine, and 10 mmol/L phenylmethylsulfonyl fluoride according to Gonçalves et al[5].

Plaque analyses

In homogenates, elastin is measured using the Fastin Elastin assay was used (Biocolor, Carrickfergus, Northern Ireland, UK). Collagen is measured using a Sircol soluble Collagen assay (Biocolor, Carrickfergus, Northern Ireland, UK). MMPs (1, 2, 3, 9 and 10) are analysed using the Mesocale human MMP ultra sensitive kit (Mesoscale, Gaithersburg, MD, USA). TIMPs (1 and 2) are analysed using MILLIPLEX MAP Human TIMP Magnetic Bead Panel

(Milliplex, MA, USA). All kits are used according to manufacturer's instructions. Absorbance measurements are performed using a Tecan Elisa plate reader.

RNA is prepared using standard Trizol method and cleared of Ribosomal RNA using Ribo-Zero™ Magnetic Kit (Epicentre). Strand specific paired end RNAseq libraries are prepared with ScriptSeq™ v2 RNA-Seq Library v2 Preparation Kit (Epicentre) and paired end libraries sequenced using high-output kit version 2, HiSeq2000 platform, Illumina, USA.

Further analyses are performed as covered by the ethical permit.

Cytokine analysis in plasma and homogenate

A multiplex assay is used for measuring cytokines according to the manufacturer's instructions (incl. fractalkine, interferon-γ (IFN-γ), interleukin-(IL)-6, monocyte chemoattractant protein-1 (MCP-1), monocyte inflammatory protein-1β, (MIP-1β), Platelet derived growth factor (PDGF)-AB/BB, Regulated on Activation Normal T Cell Expressed and Secreted (RANTES), and tumour necrosis factor-α (TNF-α; the Human Cytokine/chemokine Immunoassay, Millipore Corporation, MA, USA) with analysis by Luminex 100 IS 2.3 (Austin, Texas, USA).

Histology

Plaque fragments are embedded in optimal cutting temperature (OCT) compound and cryosectioned and/or fixed in 4% formaldehyde, dehydrated, embedded in paraffin and sections with a microtome. Stained slides are scanned using ScanScope Console Version 8.2 (LRI imaging AB, Vista CA, USA). Measurements of the area of plaque (% area) for histology stainings were quantified blindly using BiopixiQ 2.1.8 (Gothenburg, Sweden) and QuPath Version: 0.3.0[6].

Ethical considerations

The study was approved by 'the Regional Ethical Review Board in Lund' (Sweden), which has since been re-designated 'the Swedish Ethical Review Authority' (under the Swedish Ministry of Education) and conformed to the principles of the Declaration of Helsinki. Written informed consent is given by all patients.

Individual clinical trial participant-level data (IPD) may not be shared due to General Data Protection Regulation (GDPR; (EU)2016/679) legislation as sensitive personal data is included.

Review approval identifiers

From the Swedish Ethical Review Authority: 472/2005; 472/2005; 2010/480; 2014/904; 2017/89; 2018/63; 27-2020/3.1; 60/2008; 2012/209; 2023-05910-01, 2024-01389-02.

Swedish Data Inspection Board approval: 1572-2005.

Regional Biobank Register [Regionalt Biobanksregister Södra sjukvårdsregionen]: SC392; BD48.

Statistical analysis protocol (SAP)

General

All analyses based on plaque homogenate measurements are normalized against wet weight of the plaque. Presentation of values are as mean and standard deviation (SD) if normally distributed and as median and interquartile range (IQR) if non-normally distributed.

Two-group comparisons are performed with Mann-Whitney test if variables are non-normally distributed, and with students t-test if variables are normally distributed. Pearson's $\chi 2$ test is used for categorical data. Spearman's rho is used for correlation analysis. The Benjamini-Hochberg (BH) procedure is applied to the P values to control for false discovery rate. If not stated otherwise P values less than 0.05 are considered significant.

Survival analysis

Simple and multiple linear regressions are used to explore the relationship between two or more continuous variables, while logistic regression is used in case of dichotomous variables. Freedom from postoperative events is calculated by life-tables according to Kaplan–Meier survival analysis. Correction for potential confounders is done through Cox regression analysis. Values are presented as hazard ratio (HR) with 95% confidence intervals (CI) and statistical significance (p).

RNA-sequencing analysis

Gene expression is quantified using Salmon with gene annotations from Gencode v.27 and imported using tximport. Using count data, differential expression analysis is examined using DESeq2 and counts normalized using DESeq2´s median of ratios and log2 transformed. For orthogonal partial least squares discriminant analysis (OPLS-DA), expression of genes and transcription factors are pulled out from a whole dataset and analysed in SIMCA-P software package (version 14.1, Umetrics, Umeå, Sweden). Prior to analysis, the gene expressions are mean centred and scaled to unit variance. R2Y and Q2Y metrics describe the percentage of variation explained by the model and predictive ability of the model, respectively. Furthermore, overall contribution of each gene to group discrimination is ranked by VIP values (variable influence on projection). Gene with VIP value greater than 1 is considered important in group discrimination. For network analysis, counts are normalized between samples using a trimmed mean of M values (TMM) by the edgeR, resulting in gene expressions as log2-counts per million[7]. Network analysis of human plaque expression data is conducted using the weighted gene co-expression network analysis (WGCNA). Genes with low-expression variance (<1.5) across samples are filtered out[8]. Gene co-expression networks are constructed, and clusters of genes (modules) are identified consequently. Pearson correlation between the first principal component of gene expressions in each module and variable (such as symptomatic) status are calculated. Pathway enrichment in each module is performed using Enrichr on 1721 high-quality pathways from the Elsevier Pathway Collection[9].

More advanced analyses are performed by the team bioinformatician in R.

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