Material and methods

AAA patients

Spanish patients

In a first cohort, serum from 62 male patients with an asymptomatic infrarenal AAA was collected during clinical examination (aortic size = 3-5 cm, small AAA). Additionally, serum from 28 male patients with an asymptomatic infrarenal AAA was collected before surgical repair (aortic size > 5 cm, large AAA). Twenty-eight healthy male controls with non-dilated infrarenal aortas (aortic size < 3 cm, confirmed with abdominal ultrasound) and no risk factors were obtained from a screening program undertaken in our area of care. All these samples were obtained from Galdakao Ursansolo Hospital (Bilbao, Spain). In a second cohort, plasma samples were obtained from the biobank of IIS-FJD (Madrid, Spain) including 26 small AAA patients and 39 large AAA patients. Hypertension was defined as systolic blood pressure (sBP) >140 mmHg and/or diastolic pressure (dBP) ≥90 mmHg measured during the examination, after the participant had been sitting for at least 30 minutes, or the participant was already taking hypotensive medication. A patient was considered diabetic if he was under treatment (supervised diet, hypoglycaemic oral medication, insulin) or we found basal glycaemia >120 mg/dL and/or HbA1c >=6.5%. Hypercholesterolemia was defined as total basal cholesterol levels ≥200 mg/dl, LDL levels ≥100 mg/dl or the patients were receiving specific medication or a supervised diet. Cardiac disease included coronary heart disease, valvular disease, cardiomyopathy and arrhythmia. Clinical characteristics are summarized in **Table 1.** The studies were approved by Spanish center's Research and Ethics Committees, and informed consent from the patients and the controls for their inclusion in the study was obtained.

Danish patients

Blood cells were obtained from 186 patients from the randomised population based Viborg Vascular (VIVA) screening trial screening 65-74 year old men for AAA, peripheral arterial disease and unrecognised hipertension (1). Informed consent was obtained from all subjects before participation, and the study was approved by the Local Ethics Committee of the Viborg Hospital, Denmark, and performed in accordance with the Helsinki Declaration. Cases were selected according to initial size and growth rate. Clinical characteristics of the patients are included in **table 1 online.**

AAA tissue and tissue-conditioned media

Sixteen AAA thrombus and wall samples were collected from patients enrolled in the RESAA protocol (2) undergoing surgery (three for MS analysis, ten for ELISA, western-blot and immunohistochemistry and six for homogenization). One part was included in paraffin for immunohistochemistry and the rest was dissected into thrombus and wall (media and adventitia) for incubation in a RPMI protein-free medium. All patients gave their informed written consent and the protocol was approved by a French ethics committee (CPB, Cochin Hospital). Twelve control aortas (six for immunohistochemistry and six for homogenization) were sampled from

dead organ donors with the authorization of the French Biomedicine Agency (PFS 09-007). These control aortic samples were macroscopically normal, devoid of early atheromatous lesions. Different layers of AAA thrombus and wall, as well as healthy walls, were cut into small pieces (5 mm^2) and separately incubated in RPMI 1640 medium containing antibiotics and an antimycotic (Gibco) for 24 hours at 37°C (6 ml/g of wet tissue). The conditioned medium (supernatant containing proteins released by the tissue sample) was obtained after centrifugation as 3,000 g for 10 minutes at 20°C. In some cases, native C3 [purified as previously described in Alcorlo M *et al*, (3)] was incubated for 90 min at 37ºC with 1 µl of thrombus-conditioned media and then subjected to western-blot. In addition, tissues were snap-frozen in N_2 liquid and homogenates (0.2 g) were divided and resuspended for mRNA and protein analysis.

Bidimensional nanoLC-MS/MS analysis

Proteins from AAA–tissue conditioned media obtained from 3 patients were precipitated using 2D clean-up kit (GE Healthcare) and resuspended in Triethylammonium bicarbonate (TEAB) buffer for protein concentration measure by Bradford assay (Biorad). A total of 50 µg of protein was reduced with 5 mM Tris (2-carboxiethyl) phosphine (TCEP) for 1 hour at 60ºC and alkylated using 10 mM s-methylmethenethiosulponate (MMTS) at room temperature during 10 minutes. Later, proteins were digested with trypsin, at 37°C for 5h, at a ratio of 1:50 trypsin to protein. Digested samples were subjected to nano-liquid chromatography coupled to MS for protein identification. Peptides were injected onto a strong cation exchange (SCX) microprecolumn (500 µm I.D. and 15mm BioX-SCX TM, LC Packings, Amsterdam, The Netherlands) with a flow rate of 30µL/min as a first dimension separation. Peptides were eluted from the column as fractions by injecting three salt steps of increasing concentration of ammonium acetate (10, 100 and 2000 mM). Each of the three fractions together with the nonretained fraction was on line injected onto a C-18 reversed phase (RP) nano-column (100 mm I.D. and 12 cm, Mediterranea sea, Teknokroma) and analyzed in a continuous acetonitrile gradient consisting of 0-50% B in 90 min, 50-90% B in 1 min (B=95% acetonitrile, 0.5% acetic acid). A flow rate of 300 nL/min was used to elute peptides from the RP nano-column to an emitter nanospray needle for real time ionization and peptide fragmentation on an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher). An enhanced FT-resolution spectrum (resolution = 30000) followed by the MS/MS spectra from most intense three parent ions (dissociated using CID activation) was analyzed along the chromatographic run (130 min). Dynamic exclusion was set at 1 min.

Database Searching

Tandem mass spectra were extracted by Proteome Discoverer v1.0 software (Thermo Fisher). Charge state deconvolution and deisotoping were not performed. For protein identification, fragmentation spectra were searched against a curated subset of a human database (human_ref.fasta; 2003, April; 39414 entries) using Sequest (Thermo Fisher Scientific version 1.0.43.2) and X-Tandem (The GPM, thegpm.org; version 2007.01.01.1) engines. Sequest and X-Tandem were searched allowing two missed trypsin cleavages, and a tolerance of 15 ppm or 0.8 Da was set for full MS or MS/MS spectra searches, respectively. Methane thiosulfate alkylation of cysteine residues and oxidation of methionine were allowed as variable modifications. Finally, Scaffold

v.3.00.02 software (Proteome Software Inc) was used to validate MS/MS based peptide and protein identifications.

Bioinformatics

Pathway analysis were created using Ingenuity System software (Ingenuity System Software, Inc.). Enriched canonical pathways were calculated as the ratio between the number of genes for one pathway found in the experiment and the total number of genes destinated to that pathway. All the ratios shown are associated to p-values provided by Fisher's exact test. These analyses were derived from all protein identifications obtained from the MS analyses. Predictions of protein secretions were made using a software package, publically available, hosted at the Technical University of Denmark, as described (4).

Quantification of C3 and C3a

Soluble concentrations of C3 in human plasma samples were automatically measured using VITROS chemistry products C3 reagents in the VITROS 5,1 FS and VITROS 5600 Integrated System analyzers, following the manufacturer´s instructions (Ortho-Clinical Diagnostics, Johnson & Johnson). Soluble concentrations of C3 in serum samples from first cohort or in plasma samples from third cohort were assayed automatically by timed nephelometry using a BNII Nephelometer (Siemens9). Both methods were standardized against the international reference preparation CRM 470 (RPPHS). C3 and C3a in conditioned media was measured with commercial kits (EC2101 Assaypro and 550499 BD, respectively) following the manufacturer's instructions.

AP50 assay

To test the hemolitic capacity of the complement system, red blood cells (RBCs) from healthy rabbits were used together with human sera as described (5). Briefly, washed RBCs were resuspended to 1% (v/v) in AP-CFTD buffer (5 mM sodium barbitone pH 7.4, 150 mM NaCl, 7 mM $MgCl_2$, 10 mM EGTA). 40 ul of serum from control or AAA patients, 50 ul of EDTA 0.2 M and 100 ul of VBS (NaCl 0.14 M, Sodium 5,5-diethylbarbiturate 1.45 mM, acid 5,5-diethylbarbituric 2.5 mM) were added to 200 ul of rabbit RBCs 1% (v/v) and incubated at 37°C for 30 minutes. To calculate lysis, 1.8 ml of VBS-EDTA (VBS= and 0.2 M EDTA) were added, cells were pelleted by centrifugation at 2.500 rpm for 10 min and hemoglobin release was measured by absorbance at at 412 nm. Control incubations included 0% lysis (buffer only) and 100% lysis (1.8 ml of H2O instead of VBS= 0.2 M EDTA). Percentage lysis 100*(A412 test sample-A412 0% control)/(A412 100% control-A412 0% control).

DNA isolation and genetic study

Genomic DNA was extracted from peripheral blood using EZ1 DNA Blood 350 µl Kit in an EZ1 Advance Robot (Qiagen) following standard procedures. DNA samples were genotyped for six single nucleotide polymorphisms (SNPs) (CFH Ile62Val, CFH c.1696+2019G>A, CFHR1 Glu175Gln, CFB Leu9His, CFB Arg32Gln/Trp) (6). The genotyping was performed using multiplex PCR and minisequencing methodology (ABI Snapshot; Applied Biosystems). Minisequecing reations were run in an

automated sequencer (model 3730; ABI), and the fragments were analyzed with the appropriate software (GeneMapper Software 4.0; ABI).

Immunohistochemistry

AAA and control aorta samples were fixed in 3.7% paraformaldehyde and embedded in paraffin. Immunohistochemistry was performed using antiC3 (purified as described in 7) and anti-C9 (mAb B7, a generous gift of Prof. Paul Morgan, Cardiff University) as primary antibodies. Negative controls using the corresponding IgG were included for checking non-specific staining. The secondary antibody and ABComplex/HRP were added and sections were stained with 3,30-diaminobenzidine and mounted in DPX. For colocalization of C3 with C9, CD15 (clone Carb-3, DAKO) and CD68 (clone PG-M1, DAKO), immunohistochemistry in serial sections was performed. For colocalization of C3 with vascular smooth muscle cells (alpha-actin, clone 1A4 DAKO), immunohistochemistry followed by immunofluorescence was performed.

Western blot

Equal amounts of proteins from tissue or conditioned medium (30 μg or 5 μL previously normalized to tissue weight: 1 g/6 mL, respectively) were loaded onto 12.5% polyacrylamide gels, electrophoresed and transferred to nitrocellulose membranes. Then they were blocked with 7% milk powder in TBS-T for 1 hour and incubated overnight at 4ºC with antiC3 (7). Then the membranes were washed with TBS-T and incubated with anti-rabbit antibody (1:5000) for 1 hour at RT. After 4 washes, the signal was detected using the ECL chemiluminiscence kit (GE Healthcare).

Real time PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen). One microgram of RNA was used to perform the reverse transcription with the high capacity cDNA archive kit (Applied Biosystems). Real-time PCR reactions were performed on an ABI Prism 7500 sequence detection PCR system (Applied Biosystems) according to the manufacturer's protocol, using the DDCt method. Human mRNA levels for C3 and 18S were done by amplification of cDNA using SYBRw Premix Ex TaqTM (Takara Biotechnology). The primer sequences are: Forward C3 primer: AAGCGCATTCCGATTGAGGA, Reverse C3 primer: AAGACTTCCCCACCAGGTCT. The mRNA levels of C3 were normalized to the 18S mRNA content.

Cell isolation, chemotaxis assay and measurement of NADPH-dependent ROS production

Neutrophils were isolated from venous blood of healthy volunteers (with informed consent), sampled on EDTA. Red blood cells were aggregated by addition of 2% dextran for 20 minutes at 20°C and the upper phase containing leukocytes was centrifuged on Ficoll (20 minutes at 600 g, 20°C) (PAA Laboratories GmbH). The pellet containing neutrophils was submitted to a hypo-osmotic shock to eliminate residual erythrocytes.

Transwell migration assays were performed using 96-well disposable chemotaxis chambers with a 8 µm polycarbonate filter (ChemoTX, Neuroprobe). Briefly, 29 ul of 3 different luminal thrombus conditioned media were added to the lower compartment of each well. Luminal thrombus were preincubated for 30 min at 37ºC in a humidified atmosphere (5% CO_2), in the presence or in the absence of antiC3 or IgG (0.4 ug/ul) in 30 µL of RPMI. Polymorphonuclear cells (200.000) were added to the upper compartment. The chamber was then incubated at 37 °C in a humidified atmosphere (5% CO_2) for 2 h. A standard curve, consisting of a 1:2 dilution cascade of polymorphonuclear cells (top standard, 200.000 cells in 29 µL), was constructed. After incubation, the framed filter was carefully removed and the number of cells that had migrated was determined reading fluorescence at 485ex/530em by comparison with the standard curve. Each experiment was performed in triplicate.

Lucigenin-enhanced chemiluminescence assay was used to determine the NADPH-dependent ROS production in fresh neutrophils as described (8). Briefly, 5 µL of 3 different luminal thrombus conditioned media were incubated for 2 min with 500,000 polymorphonuclear cells. Luminal thrombus were preincubated for 30 min at 37ºC in a humidified atmosphere (5% CO2), in the presence or in the absence of antiC3 or IgG (0,4 ug/ul) in 30 µL of RPMI. The reaction mixture comprised 50 mM phosphate buffer containing 1mM EGTA, pH 7.0, 5 µM lucigenin and 0.1 mM NADPH. The chemiluminescence, which was measured for 5 minutes after the addition of NADPH, was recorded in a luminometer Sirius (Berthold Detection System). No activity could be measured in the absence of NADPH. The ROS production was determined from the ratio of relative light units.

Statistics

Normality of data was checked by probability plots. Normally distributed C3 concentrations and activity are expressed as mean±SEM. $P < 0.05$ was considered to be statistically significant. Difference among the groups in the first cohort (control, small aaa and large aaa) was analyzed by one-way ANOVA test followed by post hoc Tukey Kramer test for multiple comparisons. Differences among the groups of the second cohort and third cohort (small AAA vs large AAA) were analyzed by t-test. Pearson correlation was used to determine correlations between two variables. Logistic or linear regression analysis adjusted by risk factors was conducted with AAA stage (small/large AAA) and aortic size as dependent variables, respectively. The Wilcoxon paired test was used to analyze differences in C3 and C3a levels between thrombus and wall supernatants of the same samples, while non-paired tests were used for pathological wall vs healthy wall supernatants comparisons. All the statistical analyses were performed by using SPSS 11.0 statistical package.

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