Rare variants lowering the levels of coagulation factor X are protective against ischemic heart disease

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SUPPLEMENTARY MATERIAL

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SUPPLEMENTARY METHODS

Definition of disrupting variants and statistical analysis

We searched the FX gene (*F10*) for deleterious variants (nonsense, frameshift, splicing, or disrupting missense mutations). To define as deleterious all those missense variants that could potentially impair FX protein function, we used both a bioinformatics and a data-mining approach. Rare missense variants were considered damaging if: a) they were predicted to be deleterious or possibly deleterious by all the 5 prediction algorithms used: LRT (likelihood ratio test),[1] MutationTaster,[2] PolyPhen-2 HumDiv, PolyPhen-2 HumVar,[3] and SIFT;[4] and/or b) were annotated as responsible for FX deficiency (OMIM #227600) in publicly available databases (ClinVar,[5] FX deficiency database [https://www.isth.org/?MutationsRareBleedin]).

We performed the analyses on different sets of variants: 1) deleterious variants, defined by a combination of *in-silico* and data-mining analysis, as just described; 2) all non-synonymous variants; 3) all synonymous variants, as a negative control.

The positions of variants were based on the cDNA reference sequence for *F10* (NM_000504.3) with the ATG initiation codon numbered as residue 1 (p.Met1).

All the analyses were performed considering only those variants having a minor allele frequency (MAF) <1%, and were carried out using the EPACTS software (http://genome.sph.umich.edu/wiki/EPACTS), the “b.collapse” option (logistic Wald Test between binary phenotypes and collapsed variables), and correcting for the first 5 principal components of ancestry.

The meta-analysis was performed using the Mantel-Haenszel fixed-effects model, as already described for rare variants, as detailed in.[6]

A $P<0.05$ was considered to indicate statistical significance.

Evaluation of FX antigen level and activity on plasma of selected subjects

The FX coagulant activity (FX:C) was measured with a one-stage prothrombin time (PT) assay performed on an ACL3000 automated analyzer (Instrumentation Laboratory, Milan, Italy). The FX:C values were calculated using, as a reference, plasma pooled from 40 healthy subjects (20 men, and 20 women who were not pregnant and were not taking oral contraceptives). The reference plasma was assigned an arbitrary FX:C value of 100%.

FX antigen levels (FX:Ag) were measured using an in-house enzyme immunoassay. Microtitre plates were coated overnight at room temperature with rabbit anti-human FX polyclonal antibody (Dako, Ely, UK) diluted 1:800. A standard curve was produced using

serial dilutions of pooled normal plasma. Samples were incubated for 2h at room temperature. Plates were washed and incubated again with a 1:650 dilution of horseradish peroxidase conjugated rabbit anti-human FX polyclonal antibody used in the coating step for 2h at room temperature. The enzymatic activity was detected by ortho-phenylene-diamine; the reaction was stopped with a solution of H_2SO_4 and optical density was determined at 492nm. FX:Ag levels were calculated using as a reference the same pooled plasma used for the FX:C assay.

Genotyping of the rs61753266 (p.E142K) variant

The rs61753266 (p.E142K) variant was genotyped in the Verona Hearth Study (VHS) cohort by high-resolution melting (HRM) analysis, using the LightCycler 480 (Roche, Indianapolis, USA) and the Precision Melt Supermix (Biorad, Hercules, United States), following the manufacturer's instructions. Amplicons were analyzed with the Gene Scanning Software (Roche). All samples identified as heterozygous by HRM analysis were further confirmed by direct sequencing using the BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Thermo Fisher Scientific, Waltham, USA), and the ABI-3500 Genetic Analyzer (Thermo Fisher Scientific).

Supplementary Table 1. Clinical characteristics of the ATVB population.

Characteristics	Cases (n=1,791)	Controls (n=1,750)	P
Age (years)*	39.5 ± 4.9	39.6 ± 4.9	
Male sex (%)	89.4	86.8	
Diabetes (%)	5.5	0.5	<0.001†
Hypercholesterolemia (%)	60.5	48.7	<0.001‡
Hypertension (%)	27.1	9.1	<0.001‡
BMI >25 (%)	62.8	42	<0.001‡
Current smokers (%)	45.1	30.2	<0.001‡

* Data are shown as mean ± standard deviation. Age at onset for cases.
† Continuous data were tested using 2-tailed Student *t* test.
‡ Categorical data were tested using a χ^2 test.
Subjects were considered to have diabetes if they were reported to have type I or II diabetes; hypertension was defined as diastolic blood pressure ≥90mmHg, or systolic blood pressure ≥140mmHg or current use of antihypertensive medication; hypercholesterolemia was defined by total cholesterol concentration ≥200 mg/dL or ongoing statin therapy.
BMI: body mass index.
Details about enrollment criteria were described elsewhere.[7,8]
Statistical analyses were performed using the using the R software (https://www.r-project.org/).

Supplementary Table 2. List of all rare missense variants identified in the *F10* gene in the ATVB cohort.

Position (hg19)*	dbSNP identifier	Protein variation†	N cases/ N controls	Literature‡	Bioinformatics prediction§
13:113777177_G/A	n.a.	p.R3H	0/1		0/5
13:113777234_A/G	rs778995263	p.E22G	1/0		0/5
13:113783785_G/C	rs5961	p.Q30H	1/2		0/5
13:113783838_T/C	rs750510185	p.M48T	1/0		2/5
13:113783856_A/G	**rs121964944**	**p.E54G**	**0/1**	**9,10**	**5/5**
13:113783914_C/G	**rs766511333**	**p.D73E**	**0/1**		**5/5**
13:113792784_A/G	rs764589800	p.N82S	1/0		1/5
13:113793676_G/A	rs767111216	p.D88N	1/0		3/5
13:113793724_G/A	rs778616029	p.G104S	1/1		4/5
13:113795244_C/T	rs763662689	p.L128F	1/0		0/5
13:113795262_G/A	**rs368225671**	**p.G134R**	**0/1**	**10,11**	**5/5**
13:113795286_G/A	rs61753266	p.E142K	13/22	10,12	3/5
13:113795316_G/A	rs3211772	p.A152T	0/1		4/5
13:113795320_G/A	rs370999670	p.R153H	1/3		0/5
13:113795338_A/G	rs776162435	p.D159G	0/1		3/5
13:113795343_G/A	rs375847622	p.G161S	0/1		2/5
13:113798212_G/C	rs148472205	p.V184L	0/2		0/5
13:113798236_G/A	rs3211783	p.G192R	0/2	10‖	1/5
13:113798266_C/T	rs772057533	p.P202S	1/0		1/5
13:113798272_G/A	rs775409712	p.D204N	1/0		0/5
13:113798281_G/A	rs753682438	p.D207N	1/0		0/5
13:113798308_G/A	rs144711550	p.D216N	2/3		0/5
13:113798330_C/T	rs566300775	p.T223M	0/1		3/5
13:113798393_G/A	**rs761589067**	**p.G244E**	**0/1**		**5/5**
13:113803236_G/A	rs149212700	p.R291Q	3/3		3/5
13:113803266_C/T	rs145282353	p.A301V	0/1		1/5
13:113803311_A/G	rs144679674	p.K316R	1/0		1/5
13:113803336_C/T	rs747057515	p.P321L#	0/1		2/5
13:113803337_G/A	rs373791924	p.V325M	1/0		3/5
13:113803380_C/T	rs201675411	p.A339V	0/1		1/5
13:113803461_G/A	rs143715673	p.R366H	0/1		3/5
13:113803622_G/A	**rs750759634**	**p.G420R**	**1/0**	**13,14**	**5/5**
13:113803673_G/A	rs776671034	p.V437I	0/1		2/5
13:113803772_G/A	rs200826349	p.G470S	0/2		1/5

Variants predicted to be deleterious by 5 out of 5 algorithms are bolded.
* Position is according to the human genome release GRCh37/hg19, February 2009;
† Protein variation is referred to the transcript NM_000504.3;
‡ Retrieved from: FX deficiency database [https://www.isth.org/?MutationsRareBleedin]), ClinVar;

[§] Prediction performed with: LRT, MutationTaster, PolyPhen-2 HumDiv, PolyPhen-2 HumVar, and SIFT;
[‖] Found in the double homozygous state with Ala274Ser;
[#] Protein variation is referred to the transcript NM_001312675.1;
n.a. not annotated in dbSNP146.

Supplementary Table 3. Clinical characteristics of the VHS population.

Characteristics	CAD-Free (n=454)	CAD (n=1105)	*P*
Age (years)*	59.5±12.5	61.3±10.0	0.006†
Male sex (%)	63.7	79.8	<0.001‡
BMI (kg/m^2)	25.2±4.5	26.4±4.7	<0.001†
Hypertension (%)	44.0	65.9	<0.001‡
Smoking (%)§	42.9	68.6	<0.001‡
Diabetes (%)	9.2	19.5	<0.001‡
Hypercholesterolemia (%)‖	58.9	70.7	<0.001‡

Data are presented as mean ± standard deviation or %. Only data concerning angiographically examined individuals are shown.
* Age at onset for cases and age at examination for controls.
† Continuous data were tested using 2-tailed Student t test.
‡ Categorical data were tested using a χ^2 test.
§ Current and former smokers were aggregated in the single category of smokers.
‖ Hypercholesterolemia was defined by total cholesterol concentration >5.2 mmol/L or presence of lipid-lowering therapy.
Details about enrollment criteria were described elsewhere.[15,16]
Statistical analyses were performed using the using the R.

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