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

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Short title: *F10* disrupting variants protect against MI

Key words: Coagulation factor X, *F10*, Rare mutations, Ischemic heart disease, Myocardial infarction

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Coagulation factor X (FX) is a serine protease playing a pivotal role in the clotting process. It exerts its function by catalyzing thrombin formation, ultimately leading to the generation of fibrin from fibrinogen to produce a stable clot (Figure 1A).[1] This mechanism prevents excessive blood loss after injury; however, it can also cause the generation of pathologic thrombi in blood vessels, blocking blood flow to a tissue and eventually resulting in ischemia and tissue death. An example is the acute coronary syndrome (ACS), the most severe complication of coronary artery disease (CAD). ACS commonly results from atherosclerotic plaque rupture, followed by platelet and coagulation cascade activation, which leads to a thrombus formation in the coronary arteries.[2,3] Since activated FX (FXa) is central in the coagulation cascade, being involved in the initiation, amplification, and propagation phases of clot formation (Figure 1A), its specific inhibition was demonstrated to be effective in the prevention/treatment of life-threatening thrombi formation in arterial atherothrombotic diseases and venous thromboembolism.[3,4] FXa inhibitors (i.e., rivaroxaban, apixaban, edoxaban) were proven to reduce the risk of stroke and systemic embolism in patients with nonvalvular atrial fibrillation, and to treat and/or prevent deep-venous thrombosis and pulmonary embolism. Rivaroxaban was shown to reduce the risk of major cardiovascular events in patients with a recent ACS when co-administered with antiplatelet therapies.[5] Recently, the association between rivaroxaban and aspirin was proposed to improve cardiovascular outcomes even in patients with stable atherosclerotic vascular disease.[6] Therefore, growing evidence emphasizes the role of FXa in the modulation of the residual cardiovascular risk in ischemic heart disease.

Besides coagulation, FX is implicated in inflammation, tissue fibrosis, and vascular remodeling through the interaction with protease-activated receptors (PARs).[7,8] PARs belong to a family of 7-transmembrane, G protein-coupled receptors that are activated by different serine proteases by specific N-terminal cleavage. FXa activates PAR-1 and PAR-2, expressed in endothelial cells (ECs), dendritic cells, leukocytes, fibroblasts, and vascular smooth muscle cells (VSMCs). Recent findings suggested that FXa and its major receptor, PAR-2, play an important role in the pathophysiology of inflammatory diseases, including atherosclerosis (Figure 1B,C).[9-11] In this frame, Hara and colleagues demonstrated that the administration of rivaroxaban reduces atherosclerotic plaque progression in ApoE-deficient mice by decreasing lipid deposition, macrophage accumulation, and MMP-9 expression

within plaques.[10] This indicates that FXa inhibition may attenuate plaque progression/destabilization beyond the influence on coagulation pathway. Importantly, the inflammation response was also affected: after treatment, expression levels of TNF-α, Cox-2, iNOS, MCP-1, and IL-1β were significantly reduced in atherosclerotic plaques and macrophages.[10] Consistently, FXa proteolytic activities were found significantly increased in early atherosclerotic lesions compared to lesions at a later stage, suggesting an important role for this protease also in the initial development of atherosclerosis.[12]

Naturally occurring DNA variants affecting the expression/activity of drug protein targets can give insights in the therapeutic treatment directed against such gene products. Mutations lowering the expression of a drug-target gene are hence particularly interesting, because they may mimic the effect of pharmacological inhibition.[13] According to this notion, variants disrupting the protein function of 2 drug-target genes, *PCSK9* and *NPC1L1*, were demonstrated to be associated with a lower risk of CAD, and clinical trials testing the inhibition of their protein products proved consistent with the genetic findings.[13]

Here, we aimed at evaluating whether rare variants leading to decreased FX levels are associated with a lower risk of ischemic heart disease. We were able to show, for the first time, that rare damaging variants in the *F10* gene are associated with reduced MI risk, thus providing a genetic support to the working hypothesis of clinical trials showing that FX inhibition may be beneficial for the treatment of ischemic heart disease.

The study was conducted on an Italian cohort collected by “The Atherosclerosis, Thrombosis, and Vascular Biology Italian Study Group” (ATVB). The cohort is composed of 2,008 patients with early-onset MI (first event before 45 years) and an equal number of controls, matched for sex, age, and geographical origin. The clinical characteristics of the population are shown in Supplementary Table 1.

Whole-exome sequencing was performed on the ATVB cohort at the Broad Institute (Boston, MA). Exome capture, sequencing, and data processing were previously described.[14] Overall, sequencing of the FX gene (*F10*) was successful for 1,791 cases and 1,750 controls. No null variants (nonsense, frameshift, splicing) were present in the cohort, in line with the constraint score reported in GnomAD repository

(https://gnomad.broadinstitute.org/), i.e. the ratio of the observed/expected (o/e) number of loss-of-function (LoF) variants in the gene (o/e=0.6, 90%CI=0.38-0.97), which indicates a certain degree of LoF mutation intolerance for *F10*. Conversely, 34 different rare missense (listed in Supplementary Table 2) and 20 low-frequency synonymous variants were identified. A total of 86 subjects carried one missense variant in the heterozygous state, including 32 MI cases (1.8%), and 54 controls (3.1%).

The 34 missense variants were analyzed using 5 algorithms, with the aim of predicting their damaging effect (for details, see Supplementary Materials). Only 5 were predicted as damaging by all software (Supplementary Table 2). In parallel, we searched these 34 missense variants in publicly-available databases, finding that that 5 variations (p.E54G, p.G134R, p.E142K, p.G192R, p.G420R) were already described in patients affected by FX deficiency (Supplementary Table 2). Interestingly, only 3 of these (p.E54G, p.G134R, p.G420R) were predicted as damaging by all algorithms, whereas the variants p.E142K and p.G192R were recognized as pathogenic only by 3 and 1 software, respectively.

We therefore performed an initial analysis including all variants identified as disruptive by all the 5 algorithms, plus those previously annotated as pathogenic in FX deficiency. When restricting the analysis to this set, we observed an enrichment in the burden of potentially deleterious variants among controls: in fact, 1.48% of controls carried at least one such rare mutation compared to only 0.78% of cases, (P=0.046, OR=0.51, 95%CI=0.26-0.99) (Figure 2A). This result highlighted a reduced risk of MI in subjects carrying a *F10* deleterious mutation. When considering a broader set of variants (all the identified missense), we still observed a significant enrichment in controls (P=0.013, OR=0.57, 95%CI=0.36-0.89); as expected, when we analyzed all synonymous variants, no significant difference was detected in the distribution of variants between cases and controls (P=0.711, OR=1.08, 95%CI=0.71-1.67).

To corroborate our results, we tried to re-contact all carriers of the novel-identified mutations predicted to be damaging by the 5 algorithms to obtain a fresh blood sample for measuring FX antigen (FX:Ag) level and activity (FX:C). The evaluation was possible for 4 out of 5 variants, due to unavailability of the subject carrying the p.D73E variant. The results confirmed that all mutations diminish FX:C and FX:Ag levels when compared to healthy subjects (Figure 2B), highlighting a mild

disproportion between FX:Ag and FX:C. When compared with the mutation pattern detected in patients with FX deficiency,[15] our findings suggest the hypothesis of an enrichment of "moderate-mild" missense changes with residual procoagulant function. The distribution of the mutated residues on the FXa structure is presented in Figure 2C.

To confirm the enrichment of *F10* variants among controls, we decided to focus on p.E142K, which was the most frequent among the deleterious variants (Supplementary Table 2). Indeed, in the ATVB cohort, we found a total of 35 carriers, corresponding to 0.72% of cases vs 1.26% of controls (P=0.127, OR=0.57, 95%CI=0.26-1.2; Figure 3A). We hence genotyped by high-resolution melting analysis the p.E142K variant (for details, see Supplementary Methods) in an Italian replication cohort. This comprised 1,113 patients with angiographically documented CAD and 457 healthy controls (CAD-free), without any angiographic evidence of atherosclerosis, collected by the Verona Heart Study (VHS). The clinical characteristics of this population are shown in Supplementary Table 3. In the VHS, we identified a significantly higher proportion of heterozygous subjects in the CAD-free group compared to cases (1.54% vs 0.27%, P=0.009, OR=0.18, 95%CI=0.03-0.78) (Figure 3A). A meta-analysis of the 2 cohorts clearly highlighted a protective effect of the p.E142K allele (P=0.010, OR=0.45, 95%CI=0.24-0.88) (Figure 3A,B).

The striking 6-fold increase in the p.E142K frequency observed among angiographically-documented CAD-free subjects suggests the intriguing hypothesis that this variant might impact primarily on FXa functions related to atherogenesis, rather than on those related to thrombosis. Indeed, apart from liver, FX is also produced locally by VSMCs, ECs, and inflammatory cells in atherosclerotic plaques;[11] FXa stimulation is able to initiate senescence in VSMCs/ECs, and to induce the production of inflammatory cytokines, which impairs tissue regeneration by PAR-1 and PAR-2 signaling. By blocking FXa-mediated activation of these receptors, cell senescence and the production of inflammatory mediators are inhibited.[16]

Of course, we have to acknowledge the limits of our work, which is an exploratory study based on a total of "only" 5,100 individuals, and should be hence further replicated in independent cohorts. Unfortunately, we were not able to confirm our results by digging publicly available MI/CAD datasets (i.e., http://www.cardiogramplusc4d.org/data-downloads). Here, data are given on

“aggregate” phenotypes and focused on common rather than rare variants and, as such, signals on *F10* are overall flat. Instead, in the light of our observations (especially in the older VHS cohort), association/burden studies should be performed considering large-effect rare variants on angiographically-documented CAD-free vs CAD individuals.

In conclusion, we showed for the first time that rare variants lowering FX levels are associated with a reduced risk of MI/CAD, thus supporting the role for this coagulation factor in the development of atherosclerosis. The reduced frequency of *F10* mutations in MI/CAD patients also reassures on the potential risk of using FXa inhibition in unknown carriers of mild FX deficiency. Our study further stresses the utility to exploit human genetic variations impacting on drug-target genes as a proxy of the effect of pharmacological inhibition of the gene product in a life-long “experiment of nature”.

SOURCES OF FUNDING

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LEGENDS TO FIGURES

Figure 1. Role of FX/FXa in blood coagulation and atherothrombosis.

A) The panels show a simplified overview of blood coagulation, which has been sub-divided in initiation, propagation, amplification, and clot formation phases. Clotting factors are indicated using Roman numbers, with the corresponding active form specified by "a". Pharmacological inhibitors specifically targeting FXa are also listed. vWF, von Willebrand factor; TF, tissue factor.

B) The figure shows a schematic representation of an artery, highlighting the different stages of the atherothrombotic process (from left to right).

C) The scheme shows in more details the processes characterizing endothelial cell activation up to plaque rupture in atherothrombosis. Thrombin (IIa) and FXa play a fundamental role through the interactions with PARs (protease-activated receptors).

The figure was created using BioRender (https://biorender.com/).

Figure 2. Rare variants lowering the levels of coagulation FX are protective against early-onset MI.

This study was approved by the Institutional Ethical Committees of the participating hospitals. All study participants signed an informed consent and gave information about their clinical history, and cardiovascular risk factors.

A) Association of the burden of rare mutations in the *F10* gene with the risk for early-onset MI. Summary allele counts and carrier frequencies are shown (calculation performed on 1,791 cases and 1,750 controls); only variants with minor allele frequency less than 1% were considered in the burden analysis. The "deleterious" set is defined by missense variations predicted to be possibly damaging by all the 5 algorithms used (LRT score, MutationTaster, PolyPhen-2 HumDiv, PolyPhen-2 HumVar, and SIFT) and those annotated as responsible for FX deficiency in publicly available databases. The "non-synonymous" set comprises all the missense variants; the "synonymous" set comprises the synonymous variants. All the tests were run using EPACTS.

T1: alleles carrying variants with minor allele frequency less than 1%; Freq (%): percentage of cases or controls carrying a T1 allele; OR: odds ratio; CI: confidence interval.

B) FX coagulant activity (FX:C; light grey bars) and antigen levels (FX:Ag; dark grey bars) were measured in plasma of subjects carrying the newly identified missense variants (predicted to be damaging by all prediction programs). All analyzed subjects were not taking any anticoagulant drugs at time of the blood drawn. The normal range for FX:C is between 66% and 126%, and is represented by a light grey box delimited by dashed lines in the graph. The normal range for FX:Ag is between 70% and 150% (represented by solid lines). The protein variations are referred to the transcript NM_000504.3. Details on FX:C and FX:Ag measurements are specified in Supplementary Methods.

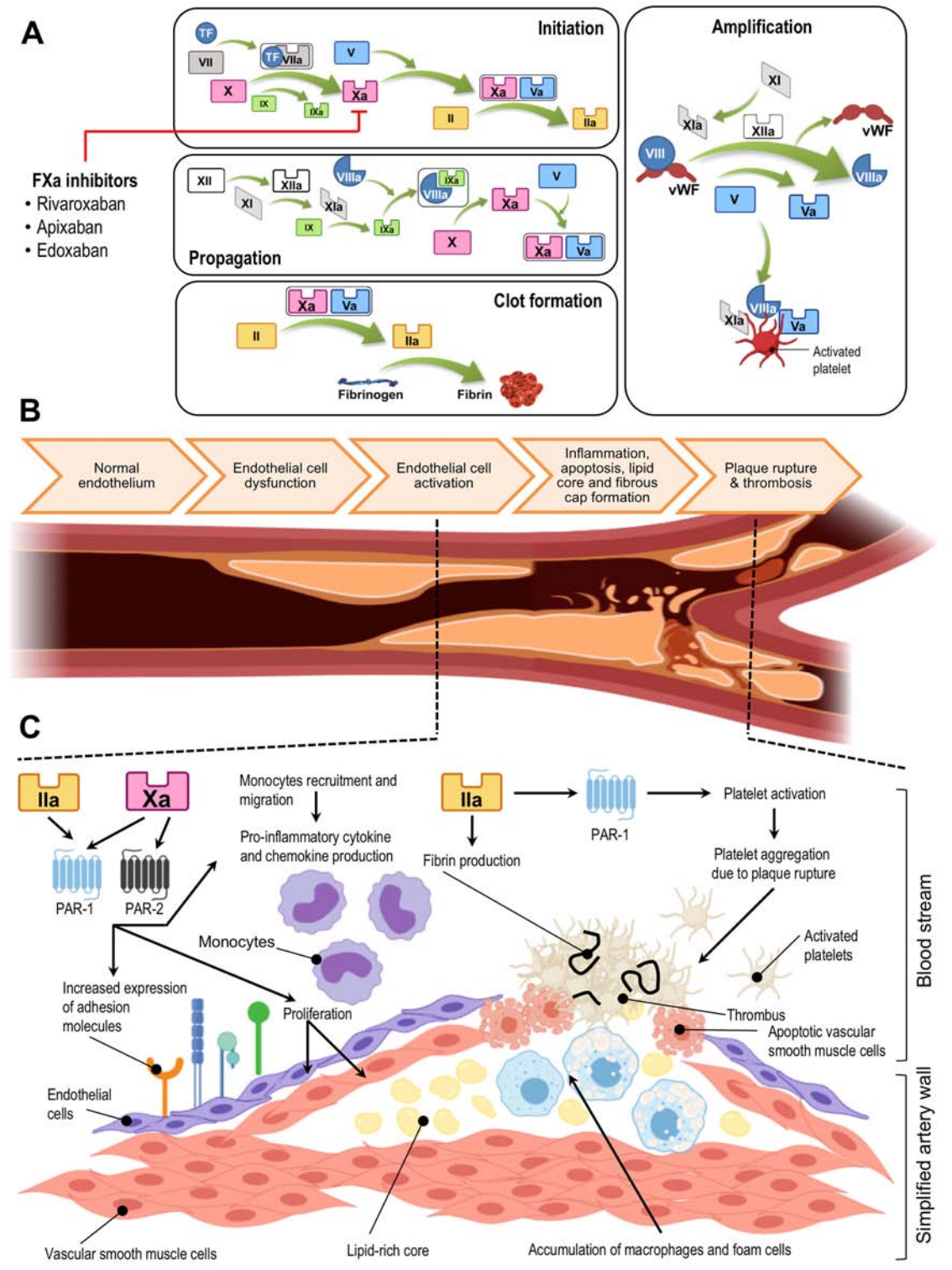
C) Ribbon diagrams of secondary/tertiary structures of the human FXa are shown. The positions of 3 out of 4 newly identified missense mutations are reported (the region harboring the p.E54G variant is not included in the FXa structure). The position of the "frequent" p.E142K variant is also shown. The color code indicates the different FXa chains (shades of blue and green point to the light and heavy chains, respectively). The protein surface is represented to show that 3 out of four mutated residues are exposed to the solvent. Diagrams were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics (http://www.rbvi.ucsf.edu/) software and the Protein Data Bank 1ezq entry. Image credits: Dr. Sonia Caccia (University of Milan; sonia.caccia@unimi.it).

Figure 3. The *F10* p.E142K (rs61753266) variant is protective against MI/CAD.

A) Association analysis. The association between the presence of the p.E142K variant and MI/CAD status (Fisher's exact test) was tested in 2 Italian cohorts (ATVB and VHS). Summary allele counts and carrier frequencies are shown. T1: alleles carrying the p.E142K variant; Freq (%): percentage of cases or controls carrying a T1 allele; OR: odds ratio; CI: confidence interval.

B) Meta-analysis. The meta-analysis was performed using the Mantel-Haenszel fixed-effects model, as already described for rare variants (see Supplementary Materials). The squares indicate the estimated OR for carriers, as compared with non-carriers, in each group. The diamond indicates the combined results.

For all analyses, a $P<0.05$ was considered statistically significant; calculations were performed using the R software (https://www.r-project.org/).


A
Initiation
TF
VII
TF VIIa
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FXa inhibitors
• Rivaroxaban
• Apixaban
• Edoxaban
XII
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VIIIa
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V
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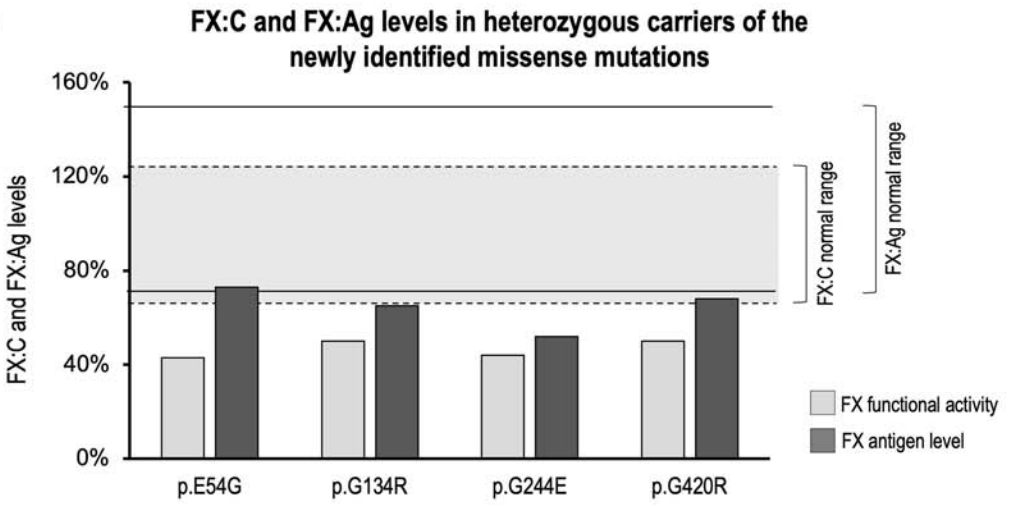
XI
XIa
IX
IXa
X
Xa Va
Propagation
Xa Va
Clot formation
II
IIa
Fibrinogen
Fibrin
Amplification
XI
XIa
XIIa
vWF
VIII
vWF
VIIIa
V
Va
VIIIa
XIa
Va
Activated platelet
B
Normal endothelium
Endothelial cell dysfunction
Endothelial cell activation
Inflammation, apoptosis, lipid core and fibrous cap formation
Plaque rupture & thrombosis
C
IIa
Xa
PAR-1
PAR-2
Monocytes recruitment and migration
Pro-inflammatory cytokine and chemokine production
Monocytes
Increased expression of adhesion molecules
Proliferation
Endothelial cells
Vascular smooth muscle cells
IIa
PAR-1
Platelet activation
Fibrin production
Platelet aggregation due to plaque rupture
Activated platelets
Thrombus
Apoptotic vascular smooth muscle cells
Lipid-rich core
Accumulation of macrophages and foam cells
Blood stream
Simplified artery wall

A

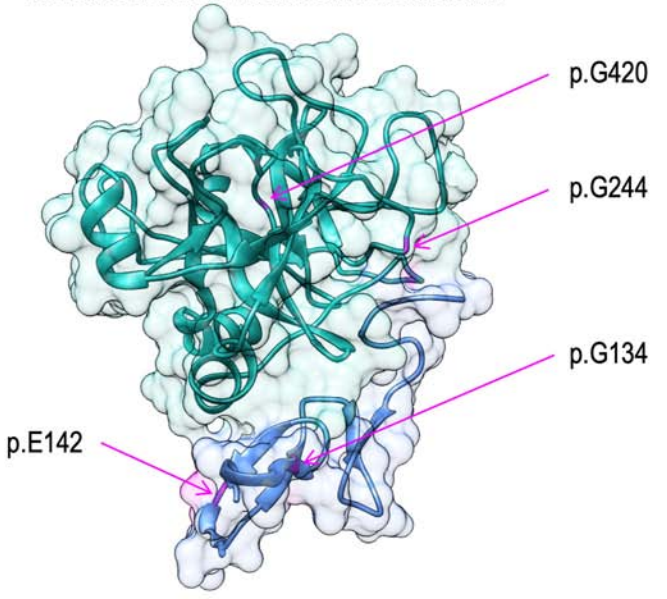
Burden of rare mutations in the *F10* gene in early-onset MI

Mutation set	T1 cases	T1 controls	Freq cases (%)	Freq controls (%)	OR	CI	P
Deleterious	14	26	0.78	1.48	0.51	0.26-0.99	0.046
Non-synonymous	32	54	1.79	3.09	0.57	0.36-0.89	0.013
Synonymous	47	43	2.62	2.46	1.08	0.71-1.67	0.711

B



C



A

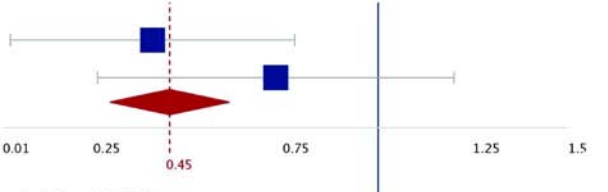
Analysis of the p.E142K variant in the ATVB and VHS populations

Cohort	N cases/ N controls	T1 cases	T1 controls	Freq cases (%)	Freq controls (%)	OR	CI	P
ATVB	1,791/1,750	13	22	0.72	1.26	0.57	0.26-1.20	0.127
VHS	1,105/454	3	7	0.27	1.54	0.18	0.03-0.78	0.009
Overall	2,896/2,204	16	29	0.55	1.32	0.45	0.24-0.83	0.010

B

Meta-analysis of the p.E142K variant in the ATVB and VHS populations

Cohort	Cases		Controls		OR (95% CI)
	Carriers	Total	Carriers	Total	
VHS	3	1105	7	454	0.18 (0.03-0.78)
ATVB	13	1791	22	1750	0.57 (0.26-1.20)
Summary					**0.45 (0.24-0.83)**



2-tailed pooled P = 0.010
P for heterogeneity = 0.12

SUPPLEMENTARY MATERIAL

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

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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[ll]	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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