

SUPPLEMENTARY DATA FILE

Genetic and in vitro inhibition of *PCSK9* and calcific aortic valve stenosis

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Study populations

UK Biobank

UK Biobank is a large prospective cohort of about 500,000 individuals between 40 and 69 years old recruited from 2006 to 2010 in several centers located in the United Kingdom (1). The present analyses were conducted under UK Biobank data application number 25205. We used genotyping data obtained from the second genetic data release, including 488,377 individuals. Samples were genotyped with the Affymetrix UK BiLEVE Axiom array or the Affymetrix UK Biobank Axiom Array. Phasing and imputation were performed centrally using a reference panel combining the Haplotype Reference Consortium (HRC) as a first choice and UK10k and 1000 Genomes Phase 3 samples for SNPs not available in HRC. Samples with call rate <95%, outlier heterozygosity rate, gender mismatch, non-white British ancestry, related samples (second degree or closer), samples with excess third-degree relatives (> 10), or not used for relatedness calculation were excluded. CAVS diagnosis was established from hospital records, using the International Classification of Diseases, 10th revision (ICD10) and Office of Population Censuses and Surveys Classification of Interventions and Procedures (OPCS-4) coding. CAVS was defined as ICD10 code number I35.0 or I35.2. Participants with a history of rheumatic fever or rheumatic heart disease as determined by ICD10 codes I00–I02 and I05–I09 were excluded from the CAVS group. We included all other participants in the control group, except for those with OPCS-4 codes K26 (plastic repair of aortic valve) or K30.2 (revision of plastic repair of aortic valve) or a self-reported diagnosis of CAVS, which were excluded from the analysis. This analysis included 1350 cases and 349,043 controls. We defined CAD as self-reported myocardial infarction, CAD from ICD or OPCS codes (I20 to I25).

EPIC-Norfolk

The EPIC-Norfolk was a prospective population study of 25,639 male and female inhabitants of Norfolk (United Kingdom), aged between 39 and 79 years. The design and methods of EPIC-Norfolk have been described in detail previously (2,3). Data were collected from February 1993 through March 2016. Samples were genotyped with the Affymetrix UK BiLEVE Axiom array or the Affymetrix UK Biobank Axiom Array. The primary outcome was CAVS, participants were identified as having incident AVS if they were hospitalized with AVS (International Classification of Diseases, Tenth Revision [ICD-10], code I35) as an underlying cause or if they died with AVS as an underlying cause. This analysis included 508 cases and 20,421 controls.

Malmö Diet and Cancer Study

The Malmö Diet and Cancer Study (MDCS) is a prospective, population-based cohort study from the city of Malmö in southern Sweden. Data collection, genotyping, sample characteristics, and clinical definitions of prevalent and incident CAVS for MDCS have been described previously (4). This analysis included 682 cases and 5963 controls.

QUEBEC-CAVS

Patients with severe CAVS undergoing aortic valve replacement (AVR) were recruited at the *Institut universitaire de cardiologie et de pneumologie de Québec* (IUCPQ). Only cases with tricuspid nonrheumatic CAVS were included. No severe regurgitation or other severe valvular heart diseases were present. All patients signed an informed consent for the realization of genetic studies. The study was approved by the ethics committee of the IUCPQ. Genome-wide genotyping and quality control in 1009 CAVS cases, demographics, anthropometric measurements, lifestyle factors, previous and current medical history, current medication, and

blood pressure measurements were collected and have previously been published (5).

Participants from the CARTaGENE cohort, which is a population-based cohort from Quebec that includes individuals aged between 40 and 69 years (6), without self-reported CAVS were used as controls. Genotyping was performed using five different microarrays: three different versions of the Global Screening Array, Human Omni 2.5 and Affymetrix Axiom 2.0. Genome-wide genotyping data including rs11591147 was available in for 11,625 participants.

Genetic Epidemiology Research on Aging (GERA)

The GERA cohort is a population-based cohort of more than 100,000 adults who are living in Northern California (7). All participants are members of the Kaiser Permanente Northern California integrated health care delivery system and provided written, informed consent (database of Genotypes and Phenotypes study accession phs000674.v2.p2). The study was approved by the relevant internal review boards at Kaiser Permanente Northern California and the McGill University Health Centre. CAVS cases (n=3469), determined through extracting electronic health records data from January 1996 to December 2015, inclusive, were defined based on the presence of either: an ICD, Ninth Revision (ICD9) code for CAVS (ICD9 424.1), or a procedure code for a prior AVR. Individuals with congenital heart disease (ICD9 746-747) were excluded. Controls (n=41,234) were study participants without an ICD-9 code for CAVS or a procedure code for AVR. This analysis was restricted to participants who self-reported only European descent, as there were insufficient numbers of individuals available of other races/ethnicities.

Estonian Biobank

The Estonian Biobank is a population-based biobank of the Estonian Genome Center at the University of Tartu (EGCUT). All participants are aged 18 and over and closely reflect the age, sex, and geographical distribution of the Estonian population. Detailed overview of the biobank has been described previously (8). CAVS was defined as ICD10 code number I35.0 or I35.2. Controls were age- and smoking-matched to cases. This analysis included 481 cases and 7223 controls.

French Datasets

A large cohort of “isolated” CAVS cases has been constituted by l’institut du thorax in Nantes. Doppler-echocardiography and blood sampling were carried out at the time of enrollment. Patients with severe renal failure, history of rheumatic disease or chest radiation were excluded. A total of 1663 severe CAVS cases were recruited between 2001 to 2017 at Nantes, Rennes and Angers University Hospitals. Most of the patients were referred to surgery after enrolment. The study was approved by the local ethics committee and all patients provided informed consent for the purpose of genetic studies. In parallel, the Cardiovascular department in Bichat university Hospital in Paris has recruited 1500 patients (GENERAC and COFRASA projects). Blood samples and (DNA, blood and tissue bank stored at the level of the Center of Biological Resources). The control populations came from two datasets called D.E.S.I.R. and P.R.E.G.O. (*Population de Référence du Grand Ouest*). D.E.S.I.R. (The Data from the Epidemiological Study on the Insulin Resistance Syndrome) (9) is an epidemiological cohort that is used here as a control general population. The P.R.E.G.O. is a set of 5707 healthy persons selected through the Blood Donor Service, originating from Western France, as a resource dedicated to provide a regional reference population of Western France for national and international research projects

in the field of evolution, population and medical genetics. The patients were genotyped in three waves. In CAVS-France 1, 1329 patients from the *institut du thorax* biobank were genotyped using Axiom Genome-Wide CEU-1 array (Affymetrix, Inc). We used a general population as controls: a subset of 901 individuals from D.E.S.I.R. and 466 from P.R.E.G.O. After quality controls (genotyping rate and heterozygosity) and a selection on individuals (relatedness and demographic stratification), we kept 1261 patients (741 tricuspid, 168 bicuspid, 352 ambiguous), 865 individuals from D.E.S.I.R. and 440 from P.R.E.G.O. In CAVS-France 2, study participants were genotyped using Axiom Genome-Wide PMRA array (Affymetrix, Inc). The dataset is composed of a set of 1478 patients recruited at the Hôpital Bichat and 319 patients from the *institut du thorax* biobank and 2828 controls from P.R.E.G.O. In patients, we observed 946 tricuspid, 317 bicuspid and 534 whose status was ambiguous. We made the same quality controls and selection on individuals as cohort CAVS-France 1 and kept at the end 1181 patients from Hôpital Bichat, 314 patients from the *institut du thorax* biobank (807 tricuspid, 254 bicuspid and 434 ambiguous) and 2707 controls. The French CAVS-France 3 dataset is composed of a set of 379 patients from the *institut du thorax* biobank and 2743 controls from P.R.E.G.O. All patients had tricuspid valve. We made the same quality controls and selection on individuals as cohorts CAVS-France 1 and CAVS-France 2 and we kept at the end 367 patients and 2519 controls.

Supplementary Table 1. Definitions of calcific aortic valve stenosis in the study samples.

Study	CAVS definition
UK Biobank	ICD10 (I35.0 or I35.2) or OPCS-4
EPIC-Norfolk	ICD-10 (I35) or died with CAVS
Malmö Diet and Cancer Study	ICD-8 (424.10, 424.11, 424.19), ICD-9 (424B, 424BA, 424BB), ICD-10 (I35.0 or I35.2), KKÅ (FMA or FMD), Op6 (3074, 3075, 3116, 3117 or 3078)
QUEBEC-CAVS	Aortic valve replacement for CAVS
Genetic Epidemiology Research on Aging	ICD9 (424.1)
Estonian Biobank	ICD10 (I35.0 or I35.2)
French Datasets	Hospital records

Supplementary Table 2. Characteristics of 10 independent single nucleotide polymorphisms at the PCSK9 locus and their effect on low-density lipoprotein cholesterol levels.

SNP	Modelled Allele	Other Allele	MAF	Effect on LDL-C*	Standard error	P-value	Consequence
rs10493176	T	G	0.89	0.0776	0.0102	<0.001	Intronic variant
rs10888896	C	G	0.72	0.0426	0.0049	<0.001	Intronic variant
rs11206510	T	C	0.846	0.0831	0.005	<0.001	Intergenic variant
rs11583974	A	G	0.03	0.0646	0.0117	<0.001	Noncoding transcript/exon variant
rs11591147	G	T	0.98	0.497	0.018	<0.001	Missense variant
rs12067569	A	G	0.03	0.0885	0.01	<0.001	Intronic variant
rs2479394	G	A	0.29	0.0386	0.0041	<0.001	Downstream gene variant
rs2479409	G	A	0.33	0.0642	0.0041	<0.001	Upstream gene variant
rs2483205	C	T	0.53	0.0514	0.0053	<0.001	Intronic variant
rs585131	T	C	0.81	0.0637	0.005	<0.001	Intronic variant
rs7552841	T	C	0.37	0.0368	0.0044	<0.001	Intronic variant

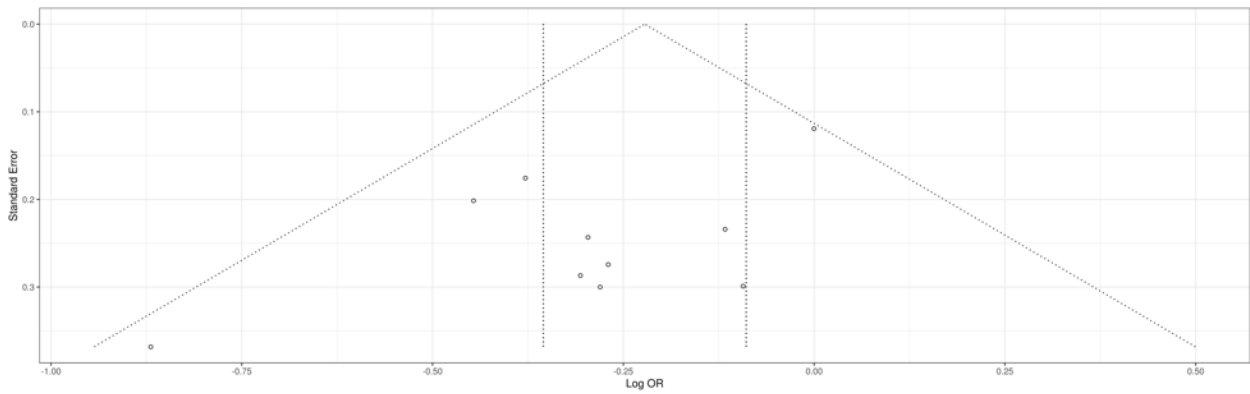
*from the Global Lipids Genetics Consortium and effect size reported per 1-standard deviation increment. MAF indicates modelled allele frequency and LDL-C indicates low-density lipoprotein-cholesterol.

Supplementary Table 3. Demographic and clinical variables of patients used for *ex vivo* and *in vitro* analysis.

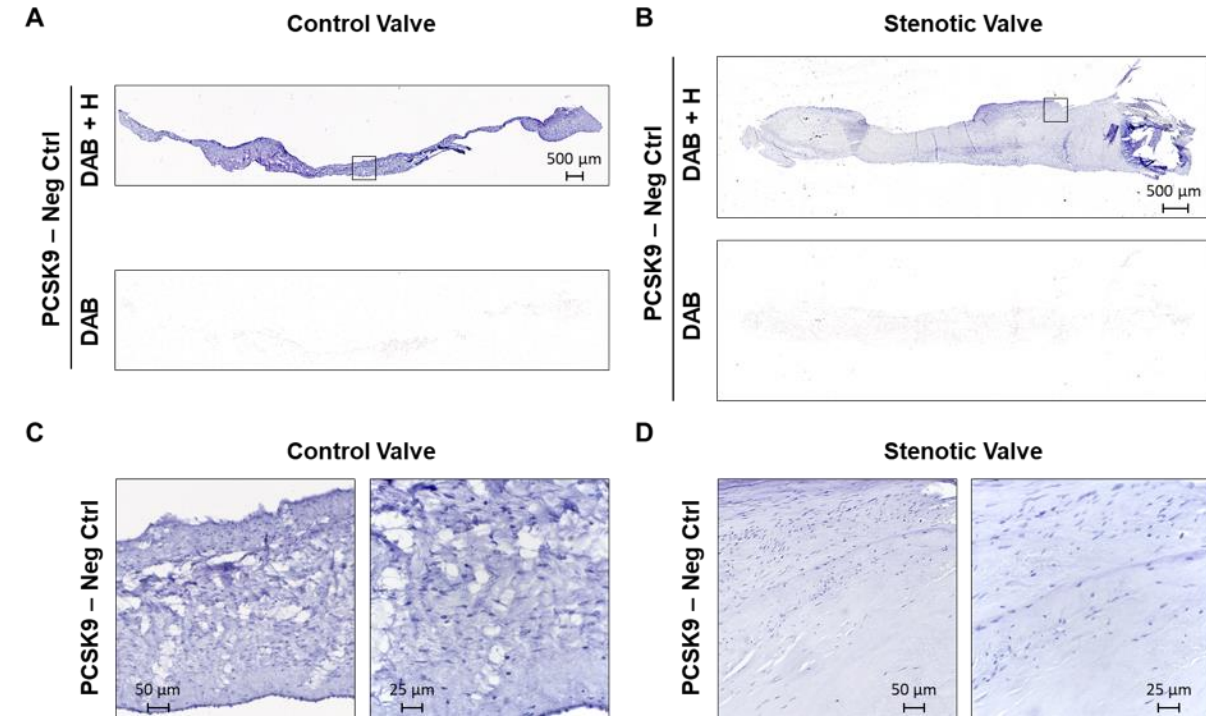
Variables	Controls (n = 10)	AS (n = 22)	p value
Age (years)	36.1 ± 15.3	74.1 ± 5.3	< 0.001
Male sex, n (%)	5 (50)	12 (54.5)	0.811
BMI kg/m ²	24.5 ± 4.3	28.6 ± 4.4	0.037
Diabetes, n (%)	0 (0)	8 (34.6)	0.061
Hypertension, n (%)	3 (42.9)	16 (72.7)	0.148
Dyslipidemia, n (%)	0 (0)	14 (63.6)	0.003
Smokers, n (%)	2 (28.6)	6 (27.3)	0.947
LVEF (%)	-	64.2 ± 5.7	-
AV_Vel max (m/s)	-	4.2 ± 0.5	-
AV_Grad max (mmHg)	-	71.5 ± 16.0	-
AV_Grad med (mmHg)	-	43.3 ± 10.5	-
AV_Area (cm ²)	-	0.79 ± 0.15	-

BMI: body mass index; LVEF: left ventricular ejection fraction; AV_Vel max: maximum aortic valve velocity AV_Grad max: maximum aortic valve gradient; AV_Grad med: medium aortic valve gradient; AV_Area: aortic valve area.

The data are presented as mean \pm SD or number and percentage (%).

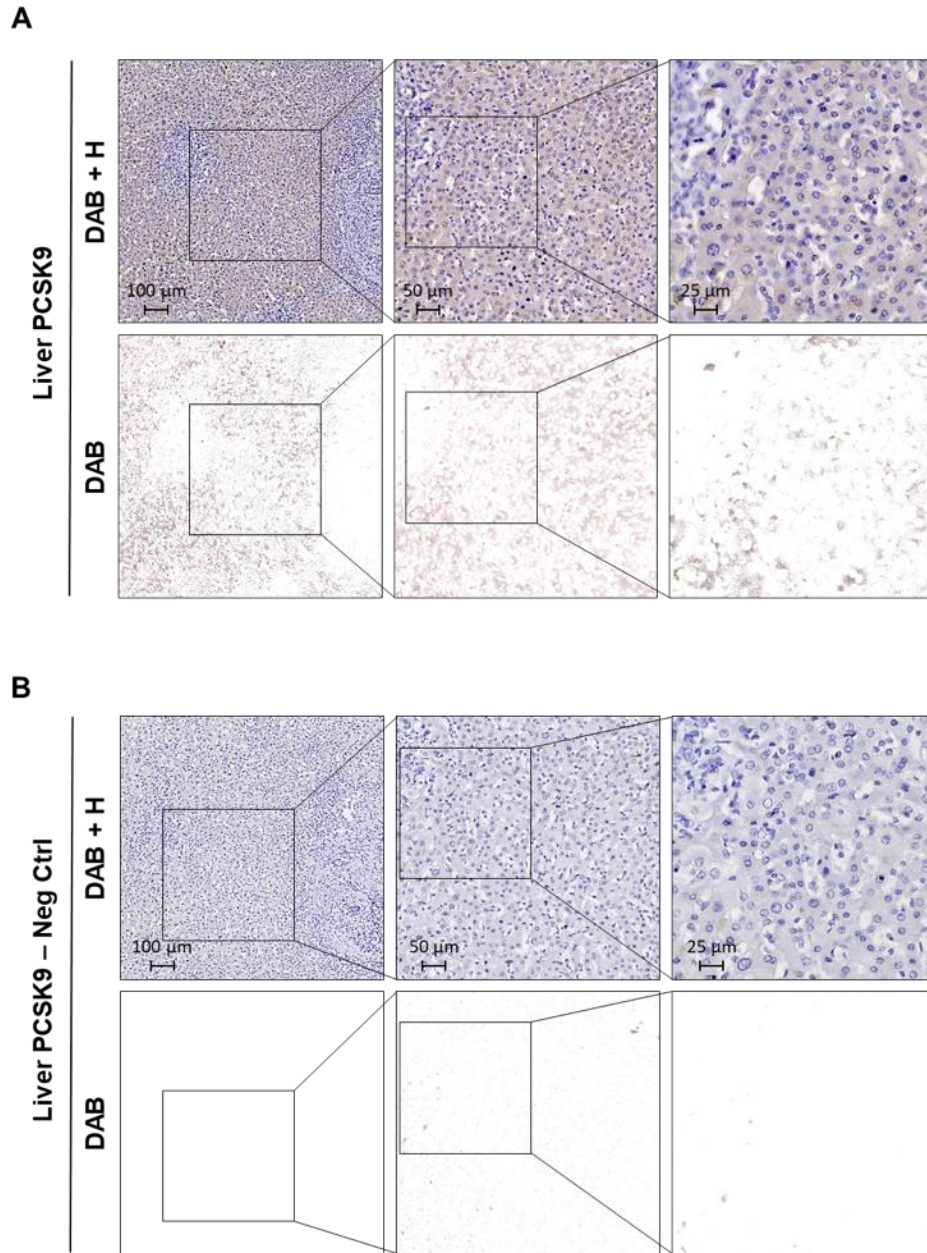


Supplementary Figure 1. Funnel plot of the *PCSK9* R46L meta-analysis of calcific aortic valve stenosis.



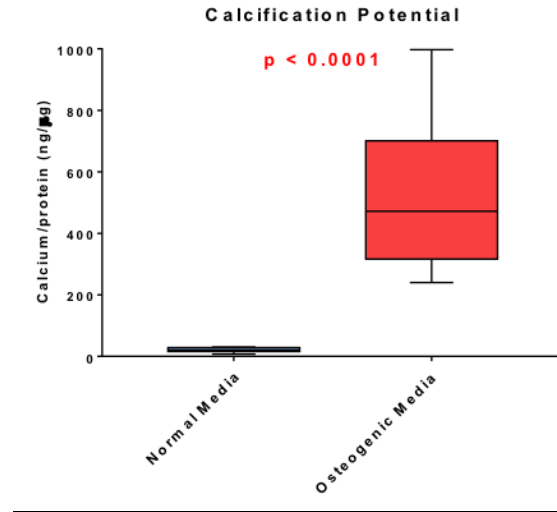
Supplementary Figure 2. PCSK9 immunohistochemistry negative controls. (A)

Representative explanted aortic valve leaflet with normal structure (control valve) and (B) representative calcified aortic valve leaflet (stenotic valve) negative immunohistochemistry controls. The negative controls are presented as 3,3'-Diaminobenzidine (DAB) and hematoxylin (H) counter staining (DAB + H) and as de-convoluted image visualizing only the DAB staining. Panoramic images were taken with a 10x magnification. Black boxes indicate the higher magnification areas. (C and D) High magnification area of aortic valve leaflets (20x left panels and 40x right panels).



Supplementary Figure 3. PCSK9 positive control staining.

(A) Representative liver section is presented in three different magnifications (10x, 20x, and 40x) as 3,3'-Diaminobenzidine (DAB) and hematoxylin (H) counter staining (DAB + H) and as de-convoluted image visualizing only the DAB staining. (B) Negative control is presented in three different magnifications (10x, 20x, and 40x) as DAB + H and as de-convoluted image visualizing only the DAB staining.



Supplementary Figure 4. Valve interstitial cell calcification potential. Box and Whisker plots showing the calcium quantification of valve interstitial cells (VIC) cultured for 7 days in normal or osteogenic media (n = 4). Calcium levels were normalized to total protein content.

Supplementary references

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