SUPPLEMENTARY APPENDIX

MATERIALS AND METHODS

Cloning of a phage display library of cDNA's preferentially expressed in ruptured human atherosclerotic plaques

For construction of the phage display library we amplified the inserts of a suppression subtractive hybridization (SSH) library containing >3000 ruptured plaque enriched cDNA clones previously generated in our laboratory (15) using primers containing EcoRI recognition sites (s: 5'-TCGAGCGGAATTCCGGGCAGGT as: 5' AGCGTGGTCGAATTCGAGGTAC). After digestion PCR-products were gel-purified and ligated into the vectors pSP6A, pSP6B and pSP6C (16) , also digested with EcoRI. Ligation mixtures were used to transform Escherichia coli TG1 cells by electroporation to obtain the phage display cDNA libraries pSP6A-rup, pSP6B-rup and pSP6C-rup.

Serological antigen selection of phage VI-displayed cDNA libraries

The procedure was performed essentially as described by Somers et al (21). In brief, sera used for the selection procedure were adsorbed by repeated passage through a column of Sepharose 6 MB (Pharmacia) coupled to E. coli TG1 and bacteriophage infected E. coli TG1 lysates to remove antibodies reactive to antigens related to the phage-host infection. Following adsorption, pooled serum was stored at –20 °C. Subsequently, pooled (n=10) pre-absorbed serum was used for affinity selection. An immunotube (Nunc) was coated with rabbit anti-human IgG (Dako) at a concentration of 10 μg/ ml in coating buffer (0.1M sodium hydrogen carbonate pH 9.6) overnight (o/n) at 4° C. The tubes were washed and blocked with 2% Marvel (skimmed milk)/PBS (MPBS). Phages from the pSP6A-rup, pSP6B-rup and pSP6C-rup libraries were prepared as described previously (29). Approximately 7 x 10^{12} phages of each library were blocked in MPBS, incubated with the pre-adsorbed serum and added to the immunotube. After

2.5 hrs incubation at RT, the immunotube was washed 20 times with PBST (0.1 % (v/v) Tween 20 in PBS) and 20 times with PBS. Bound phages were eluted with 1 ml 100 mM triethylamine (TEA), neutralized with 0.5 ml 1.0 M Tris-HCl, pH 7.4 and then used to infect exponentially growing *E. coli* TG1 cells. In total four rounds of selection were performed. After each round input and output phages were titrated to monitor enrichment of specific clones. After round three and four, individual clones were subjected to phage ELISA (enzyme-linked immunosorbent assay) and the diversity and insert size of individual clones was determined by PCR using primers 343: 5' CGCCAGGGTTTTCCCAGTCACGAC and 346: 5' CTCTCTGTAAAGGCTGC.

ELISA of cDNA displaying phage

A 96-well flat-bottom micro-titer plate (Falcon) was coated o/n at 4° C with 200 µl rabbit anti-human IgG (10 µg/ ml in coating buffer) / well. Subsequently, the plate was washed twice with PBST and twice with PBS, blocked with 2% MPBS for 2 hrs at RT, and washed again 3 times with PBST and 3 times with PBS. In a 96 wells round bottom plate (Costar), 50 µl pre-adsorbed pooled serum (diluted 1:100 in 2% MPBS) was added to 100 µl crude phage supernatant diluted 1:1 in 4%MPBS. The mixture was incubated at 37° C for 1 hr followed by 30 min incubation at RT. Next, the pre-incubated serum-phage mixture was transferred to the blocked and washed rabbit anti human IgG coated micro-titer plate and incubated at 37° C for 1 hr followed by 30 min incubation at RT. After washing, 150 µl (1:5000 in 2% MPBS) horseradish peroxidase (HRP) conjugated anti-phage (anti-M13) monoclonal antibody (Amersham) was added and incubated 1 hr at RT. After washing, 100 µl 3,3',5,5'-tetramethyl-benzidine dihydrochloride chromogen (TMB) solution (10 mg/ml) was added to each well. The reaction was stopped by the addition of 50 µl 2 N H_2SO_4/well. Plates were read at 450nm in a Novapath micro-titer plate reader (Biorad). ELISA using individual patient sera was essential as described above. However, adsorption of sera against bacterial and phage-related proteins was not performed, as no

difference in background signals between adsorbed and non-adsorbed sera was observed. Each determination was done in four fold. Both the inter- and intra assay variability were less than 5%.

ELISA to determine IgG levels

A 96-well flat-bottom micro-titer plate (Falcon) was coated o/n at 4° C with 100 µl rabbit anti-human IgG -$F(Ab)_2$ (DAKO, 1:1000 in coating buffer) / well. Subsequently, the plate was washed 3 times with PBST and PBS, blocked with 2% MPBS for 30 min at RT, and washed again 3 times with PBST and PBS. Wells were then incubated for 1 hr at with 100 µl serum diluted 1:300 in 2% MPBS at 37° C for 1 hr followed by a 30 min incubation at RT. After 3 washings with PBST and PBS, 150 µl (1:8000 in PBS) HRP conjugated anti-human IgG monoclonal antibody (Sigma) was added per well and incubated for 45 min at 37 ˚C. After three washings with PBS, specific binding was visualized and read using TMB staining as described above. Each determination was done in triplicate. The coefficient of variation between triplicate tests was < 5%. Purified human IgG (Sigma Aldrich) was used as a standard.

SUPPLEMENTARY REFERENCES

29. Marks, J.D., Hoogenboom, H.R., Bonnert, T.P., McCafferty, J., Griffiths, A.D., and Winter, G. 1991. By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J Mol Biol* **222**:581-597.

Supplemental Table IA: Clinical information of patients with proven ruptured (R) and proven stable (S) peripheral atherosclerotic lesions.

Patient ID	Age	Gender	Plaque origin	Plaque stage
R1	56	m	Femoral artery	Ruptured
R2	68	f	Carotid artery	Ruptured
R3	57	m	Carotid artery	Ruptured
R4	67	m	Carotid artery	Ruptured
R5	70	f	Carotid artery	Ruptured
R6	55	m	Carotid artery	Ruptured
R7	72	m	Femoral artery	Ruptured
R8	56	m	Femoral artery	Ruptured
R9	71	m	Femoral artery	Ruptured
R10	49	m	Carotid artery	Ruptured
R11	64	m	Iliac artery	Ruptured
R12	71	f	Carotid artery	Ruptured
R13	72	m	Femoral artery	Ruptured
R14	80	m	Abdominal aorta	Ruptured
R15	65	m	Abdominal aorta	Ruptured
R16	39	f	Femoral artery	Ruptured
R17	79	f	Carotid artery	Ruptured
R18	49	m	Abdominal aorta	Ruptured
R19	79	m	Carotid artery	Ruptured
R20	68	m	Carotid artery	Ruptured
R21	65	m	Femoral artery	Ruptured
R22	73	m	Carotid artery	Ruptured
R23	66	m	Carotid artery	Ruptured
R24	58	m	Femoral artery	Ruptured
R25	68	m	Femoral artery	Ruptured
R26	64	m	Carotid artery	Ruptured
R27	65	f	Carotid artery	Ruptured
R28	72	m	Femoral artery	Ruptured
R29	68	f	Carotid artery	Ruptured
R30	50	f	Abdominal aorta	Ruptured
R31	64	m	Femoral artery	Ruptured
R32	46	m	Carotid artery	Ruptured
R33	79	m	Carotid artery	Ruptured
R34	61	m	Femoral artery	Ruptured
R35	54	m	Abdominal aorta	Ruptured
R36	62	m	Femoral artery	Ruptured
R37	85	m	Carotid artery	Ruptured
R38	62	f	Carotid artery	Ruptured
R39	64	m	Carotid artery	Ruptured
R40	53	f	Carotid artery	Ruptured
R41	59	m	Carotid artery	Ruptured
R42	68	m	Carotid artery	Ruptured
R43	65	m	Abdominal aorta	Ruptured
R44	51	m	Femoral artery	Ruptured
R45	65	m	Femoral artery	Ruptured

Patient ID	Age	Gender	Plaque origin	Plaque stage
S1	62	m	Femoral artery	Stable
S2	72	f	Carotid artery	Stable
S3	58	m	Carotid artery	Stable
S4	77	m	Carotid artery	Stable
S5	70	m	Femoral artery	Stable
S6	55	f	Carotid artery	Stable
S7	67	f	Carotid artery	Stable
S8	57	f	Carotid artery	Stable
S9	68	f	Carotid artery	Stable
S10	38	f	Carotid artery	Stable
S11	57	m	Femoral artery	Stable
S12	72	m	Abdominal aorta	Stable
S13	55	m	Popliteal artery	Stable
S14	68	m	Carotid artery	Stable
S15	72	m	Abdominal aorta	Stable
S16	67	f	Carotid artery	Stable
S17	72	m	Femoral artery	Stable
S18	71	m	Carotid artery	Stable
S19	58	m	Carotid artery	Stable
S20	76	m	Abdominal aorta	Stable
S21	70	m	Carotid artery	Stable
S22	72	f	Carotid artery	Stable
S23	51	f	Iliac artery	Stable
S24	72	m	Femoral artery	Stable
S25	79	f	Carotid artery	Stable
S26	64	m	Femoral artery	Stable
S27	67	m	Femoral artery	Stable
S28	74	m	Carotid artery	Stable
S29	75	m	Carotid artery	Stable
S30	62	f	Carotid artery	Stable

Summary clinical condition patients with femoral, iliac and popliteal artery disease

Plaque stage	Ankle-Brachial pressure index (ABPI)	Complete occlusion diseased artery	Pressure in digit I (mm Hg)	E1 and/or E12 positive
Ruptured (n=16)	0.41 +/- 0.15	86%	13.4 +/- 18.7	69%
Stable (n=9)	0.62 +/- 0.16	11%	62.0 +/- 23.3	0%
	p = 0.2238	p = 0.0032	p = 0.0007	p = 0.0032

Summary clinical condition patients with carotid artery disease

Plaque stage	Stenosis (%)	Stroke	TIA n > 1	TIA n=1	E1 and/or E12 positive
Ruptured (n=23)	83.5 +/-13.2	61%	43%	0%	70%
Stable (n=18)	81.1 +/- 11.3	0%	0%	75%	0%
	p = 0.537	p = 0.0001	p = 0.0175	p < 0.0001	p < 0.0001

Summary clinical condition patients with abdominal aortic disease (AAA)

Plaque stage	Diameter (mm)	Symptomatic	E1 and/or E12 positive
Ruptured (n=6)	64.8 +/-6.1	100%	83%
Stable (n=3)	59.3 +/- 3.5	33%	0%
	p = 0.2619	p = 0.0952	p = 0.0476

Supplemental Table IB: Detailed characteristics of coronary artery disease patients.

AMI: acute myocardial infarction, UAP: unstable angina pectoris and SAP: stable angina pectoris.

[*] *Canadian Cardiovascular Society (CCS) classification of angina pectoris (27).*

AMI Patients	Age	Gender	Kilip Classification	TnT at admission	Peak TnT
AMI 1	81	m	I	0.19	4.31
AMI 2	76	f	I	<0.01	18.53
AMI 3	70	m	I	0.22	83.88
AMI 4	59	m	I	0.07	2.04
AMI 5	71	m	I	0.07	2.83
AMI 6	57	m	I	0.08	0.08
AMI 7	56	m	I	0.01	0.86
AMI 8	71	m	I	0.13	4.41
AMI 9	78	f	I	<0.01	0.63
AMI 10	67	m	I	0.10	1.04
AMI 11	64	m	I	0.06	7.94
AMI 12	72	f	I	1.41	19.80
AMI 13	65	f	II	0.11	3.78
AMI 14	37	m	I	<0.01	1.73
AMI 15	60	m	I	0.03	5.55
AMI 16	71	f	I	0.03	0.65
AMI 17	79	m	I	0.04	2.50
AMI 18	73	m	I	<0.01	11.57
AMI 19	54	m	I	<0.01	16.33
AMI 20	46	m	I	<0.01	7.22
AMI 21	92	f	I	<0.01	7.60
AMI 22	66	m	I	<0.01	0.88
AMI 23	60	m	I	<0.01	2.53
AMI 24	62	f	I	0.02	10.39
AMI 25	60	m	I	0.13	6.97
AMI 26	58	m	I	<0.01	1.03
AMI 27	54	m	I	0.12	16.03
AMI 28	78	f	I	0.06	2.27
AMI 29	75	m	I	0.09	1.40
AMI 30	64	m	I	0.09	0.56
AMI 31	69	m	I	<0.01	10.00
AMI 32	49	m	I	<0.01	0.48
AMI 33	43	f	I	<0.01	4.44
AMI 34	81	m	I	0.30	0.30
AMI 35	53	m	I	<0.01	18.00
AMI 36	79	f	I	1.31	1.31
AMI 37	55	m	II	0.46	5.44
AMI 38	64	m	I	<0.01	0.73
AMI 39	39	m	I	<0.01	1.12
AMI 40	78	m	I	1.31	8.78

UAP Patients	Age	Gender	"Braunwald" classification (17)
UAP 1	72	f	IIIB
UAP 2	66	f	IA
UAP 3	48	f	IIA
UAP 4	74	m	IIA
UAP 5	53	f	IIIB
UAP 6	71	m	IA
UAP 7	57	m	IB
UAP 8	57	m	I
UAP 9	53	f	I
UAP 10	48	m	I
UAP 11	47	f	I
UAP 12	78	f	I
UAP 13	76	m	I
UAP 14	46	f	I
UAP 15	82	m	IIIB
UAP 16	58	m	I
UAP 17	65	m	IIB
UAP 18	74	m	IIA
UAP 19	54	f	I
UAP 20	84	m	IIIB
UAP 21	86	m	IIIB
UAP 22	49	f	IIIB
UAP 23	64	m	IIIB
UAP 24	83	f	IIB
UAP 25	80	m	I
UAP 26	76	f	I
UAP 27	72	f	IIB
UAP 28	54	m	I
UAP 29	75	f	IA
UAP 30	52	f	I

SAP Patients	Age	Gender	CCS* classification
SAP 1	59	m	III
SAP 2	76	m	III
SAP 3	64	m	II
SAP 4	56	m	II
SAP 5	59	m	IV
SAP 6	64	m	III
SAP 7	69	m	IV
SAP 8	67	m	II
SAP 9	75	m	I
SAP 10	44	m	II
SAP 11	66	m	IV
SAP 12	46	m	III
SAP 13	67	m	I
SAP 14	86	m	III
SAP 15	53	m	II
SAP 16	58	m	III
SAP 17	61	m	II
SAP 18	57	m	III
SAP 19	76	m	II
SAP 20	54	f	III
SAP 21	59	m	II
SAP 22	67	m	II
SAP 23	78	m	II
SAP 24	49	m	III
SAP 25	76	f	II
SAP 26	55	m	III
SAP 27	66	m	II
SAP 28	49	m	III
SAP 29	67	m	I

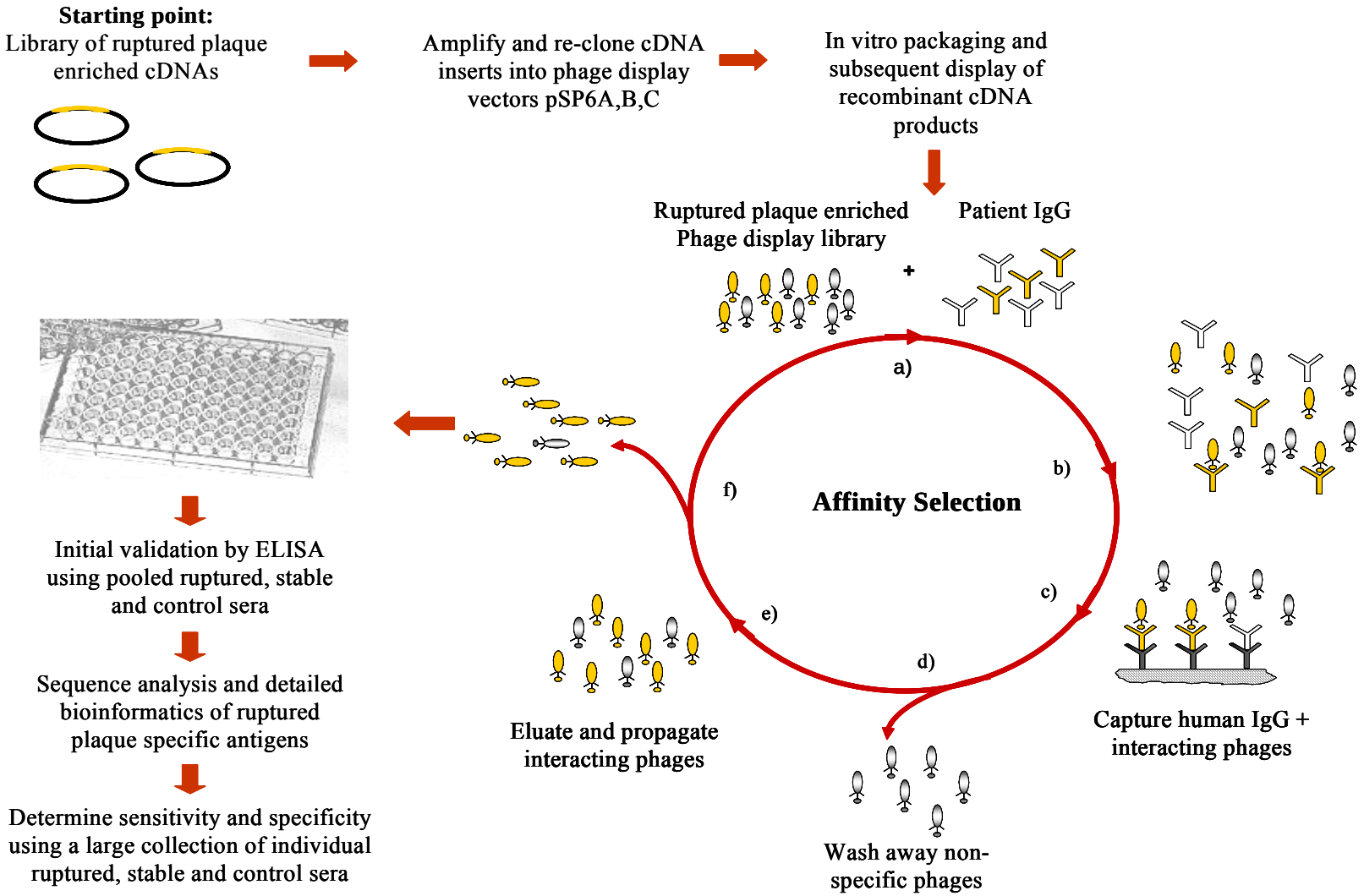

Figure 1. Schematical representation of the identification and initial validation of ruptured plaque specific antigens. *To create an phage display expression library of cDNAs predominantly expressed in ruptured atherosclerotic lesions, a SSH library of over 3000 ruptured plaque enriched cDNAs previously generated in our laboratory (17) , was re-cloned into phage display vectors pSP6A, B and C. Using these vectors, the SSH cDNA fragments are expressed as a fusion protein with the minor coat protein pVI of filamentous phage M13. To enrich this library with clones expressing cDNA products reacting with serum from patients with proven ruptured atherosclerotic lesions four cycles of affinity selection (a-f) were performed.* [a] *A phage-displayed peripheral ruptured plaque enriched cDNA repertoire is incubated with pooled serum of patients with proven peripheral ruptured plaques.* [b] *Ruptured plaque derived antigens displayed on phage (represented in yellow) bind to ruptured serum derived patient IgG (in yellow).* [c] *Phage antigen- IgG complexes (yellow) are captured on a surface coated with polyclonal anti-human IgG (black).* [d] *Non-relevant phages are washed away,* [e] *while phages specifically interacting with serum IgG are eluted, propagated and used for further selection rounds.* [f] *After four rounds of selection, single clones were randomly picked and propagated into 96-wells plates. Next, their reactivity to pooled ruptured (n=10), stable (n=10) and age and sex matched control (n=10) sera, was determined by ELISA. Subsequently, the sensitivity and specificity of two putative ruptured plaque specific antigens was validated on a large collection of individual peripheral ruptured (n= 38), stable (n=23) and normal (n=10) sera.*