Soluble Vascular Adhesion Protein-1 Correlates With Cardiovascular Risk Factors and Early Atherosclerotic Manifestations

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Objective—Vascular adhesion protein-1 is an endothelial enzyme that regulates leukocyte traffic and contributes to vascular damage in animal models. The relations of soluble vascular adhesion protein-1 (sVAP-1) with cardiovascular risk factors and markers of subclinical atherosclerosis at a population level have not been studied.

Methods and Results—We developed a new high-throughput method and measured sVAP-1 activities in serum of 2183 persons (The Cardiovascular Risk in Young Finns Study). In women, sVAP-1 activity correlated indirectly with body mass index ($r=-0.15$, $P<0.0001$), triglycerides ($r=-0.13$, $P<0.0001$), C-reactive protein ($r=-0.23$; $P<0.0001$), and brachial artery flow-mediated vasodilatation ($r=-0.076$, $P=0.0089$) and directly with carotid plaques ($r=0.066$, $P=0.023$). None of these correlations was significant in men. In women, all these univariate correlations remained significant after adjustment for body mass index, and direct correlations with LDL-cholesterol ($r=0.094$, $P=0.0014$) and carotid intima-media thickness ($r=0.075$, $P=0.010$) became evident. In men, sVAP-1 activity associated directly with glucose ($r=0.074$, $P=0.020$), intima-media thickness ($r=0.072$, $P=0.025$), metabolic syndrome ($P=0.016$), and type 1 ($P=0.0002$) and type 2 ($P<0.0001$) diabetes. In multivariable analyses, sVAP-1 activity was an independent determinant of carotid intima-media thickness ($P=0.0072$) and plaques [odds ratio 1.71 (95% confidence interval 1.07–2.72, $P=0.025$] in women, but not in men.

Conclusion—sVAP-1 activity correlates directly with intima-media thickness and carotid plaques in general population and may play a role in the pathophysiology of preclinical atherosclerosis. **(*Arterioscler Thromb Vasc Biol*. 2012;32:523-532.)**

Key Words: adhesion molecules ■ atherosclerosis ■ epidemiology ■ risk factors

Migration of leukocytes from the blood into the vascular wall is important for the pathogenesis of atherosclerosis.[1,2] Adhesion molecules on the endothelial surface guide immigration of blood-borne leukocytes to various tissues, including vascular wall. Expression of many of these endothelial adhesion molecules is induced in inflammation. Most endothelial adhesion molecules are also found in plasma as soluble forms.[3] They are normally formed by shedding of the surface-bound molecules or by expression of alternatively spliced variants. Therefore, considerable interest has risen in the possibility of using soluble adhesion molecules as biomarkers for various inflammatory diseases, including atherosclerosis.[4]

Vascular adhesion protein-1 (VAP-1) is an endothelial adhesion molecule involved in leukocyte migration from the blood into sites of inflammation.[5] In contrast to many other adhesion molecules, it is a cell-surface expressed enzyme. It belongs to semicarbazide-sensitive amine oxidases (also known as primary amine oxidases) that harbor oxidase activity in their extracellular domains.[6] VAP-1 oxidatively deaminates primary amines into aldehydes in a reaction that also produces hydrogen peroxide and ammonium. The enzymatic activity of VAP-1 is important for its adhesive function,[7,8] and it also modulates the inflammatory microenvironment through regulation of transcription factors, chemokines, and other adhesion molecules.[9–11] Nevertheless, the biological end-products of VAP-1 catalyzed reaction are potentially cytotoxic at higher concentrations, and they are implied in the development of different vasculopathies. For instance, the involvement of VAP-1 in the production of endogenous aldehydes, reactive oxygen species, and advanced glycation end products and in the regulation of arterial structure have been proposed to lead to vascular damage in cellular and animal models.[12–15]

A soluble form of VAP-1 (sVAP-1) is normally present in plasma.[16] The level of sVAP-1 is increased in certain inflam-

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matory conditions such as diabetes, alcohol-induced hepatitis, and primary sclerosing cholangitis,[17–19] but not in many others.[20] Contradicting reports have been published regarding the correlation of sVAP-1 activity with cardiovascular disorders and risk factors in small clinical patient materials.[21–24]

Analyses of animal models and specific diseases thus suggest that VAP-1 may be involved in cardiovascular damage, but this has never been addressed at a population level. Here we developed a new high-throughput method for determining sVAP-1 activity in serum samples and correlated it to different cardiovascular risk factors and markers of subclinical atherosclerosis in a thoroughly characterized cohort of 2183 young Finns.

Methods

Study Subjects and Serum Samples

The study cohort was from the Cardiovascular Risk in Young Finns Study.[25] It includes 3596 persons recruited in 1980 who have been extensively followed-up at 3- to 6-year intervals. In 2007 serum was collected from 2183 persons (30–45 years old). All sera were drawn after ≥12 hours overnight fast and stored at −70°C. The samples had never been thawed before the sVAP-1 assay. The clinical, laboratory, and ultrasound analyses performed for this cohort are detailed in the Data Supplement, available online at http://atvb.ahajournals.org, and the baseline characteristics are shown in Supplemental Table I. The study protocol was approved by local ethical committees and informed consent was obtained from all subjects.

Measurement of sVAP-1 Activity

The novel sVAP-1 activity assay is based on fluorometric detection of VAP-1 generated hydrogen peroxide (Figure A). The sera and plasma samples used in the assay validation were from 40 healthy volunteers. They were collected in silicon-coated serum, EDTA, heparin, or citrate tubes using a 20-gauge needle, allowed to stand for 30 minutes at room temperature, separated by centrifugation, divided in small aliquots, and stored at −70°C. In certain experiments, the samples were kept at −20 or −70°C, and subjected to freeze-thaw cycles. After each thawing, a small aliquot was separated and stored at the same temperature as the original sample. The sVAP-1 activity was measured from all samples at the same time at the end of the experiment.

The enzyme assays were performed on 96-well (Nunc) and 384-well (Packard BioScience) microtiter plates. The samples were diluted in 0.1 mol/L sodium phosphate buffer, pH 7.4, and incubated for 30 minutes with or without a sVAP-1 inhibitor (hydroxylamine, freshly prepared, final concentration 5 μmol/L). Then the Amplex Red-reagent (Molecular Probes) and horseradish peroxidase (Sigma Aldrich) were added followed by a sVAP-1 substrate (benzylamine, final concentration 0.5 mmol/L). The plates were then immediately measured for 1 hour with 5-minute intervals on a plate spectrofluorometer (Tecan Infinite M200). All incubations were performed at +37°C, and all reagents were preheated to +37°C. Hydrogen peroxide was added to final concentration of 0, 125, 250, 500, 750, and 1000 nmol/L to standard wells. The fluorescence data from the plate reader were transferred to a custom-made Excel-macro to convert it to the sVAP-1 activities of the samples (nmol hydrogen peroxide produced/mL serum/h).

VAP-1–depleted sera with and without spiking with different concentrations of recombinant VAP-1 were used to study the specificity and linearity of the assay. VAP-1 molecules were depleted from the sera using immunoaffinity purifications, as previously described.[16] Recombinant VAP-1 was purified from stable CHO-VAP–1 transfectants using immunoaffinity purification as described.[26]

We found that the optimal final serum dilutions are 1:100 and 1:34 for the 96- and 384-well formats, respectively, and the linear range of the assay is from 2.0 to 86.0 nmol/mL/h. The intraassay and interassay variations were 4% and 10%, respectively, for the 96-well format (tested by measuring triplicates of 9 serum samples in 2 to 4 independent experiments), and 8% and 15%, respectively, for the 384-well format (tested by measuring duplicates of 32 serum samples in 7 independent experiments).

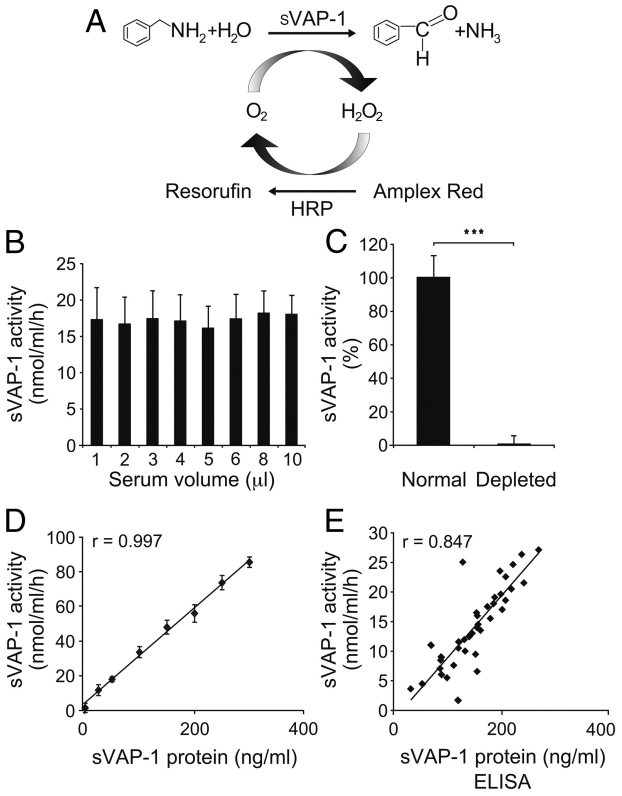

Figure. Soluble vascular adhesion protein-1 (sVAP-1) activity assay for serum. **A**, Principle of the assay. The hydrogen peroxide (H_2O_2) produced during sVAP-1 catalyzed oxidation of benzylamine oxidizes Amplex Red into fluorescent resorufin. **B**, sVAP-1 activity of sera (mean±SD, n=5 individuals) with different volumes of serum added. **C**, sVAP-1 was depleted from serum using immunoaffinity columns and sVAP-1 activity (mean± SD, n=3 individuals) was determined. ***$P<0.0001$. **D**, Vascular adhesion protein-1 (VAP-1) depleted serum was reconstituted with recombinant VAP-1 molecule, and sVAP-1 activities (mean±SD, n=3) were measured. **E**, sVAP-1 protein level in serum was determined by ELISA and correlated to sVAP-1 activities (n=38 individuals).

Statistical Analyses

Values for body mass index (BMI), weight, systolic blood pressure, triglycerides, HDL-cholesterol, C-reactive protein (CRP), intima media thickness (mean and maximal), and risk scores (except Framingham score for disease) were log-transformed to achieve normal distribution (skewness and curtosis both between −1.0 and +1.0). Normal distribution was not achieved with log- or square root transformation for physical activity, alcohol consumption, glucose, maximal bulbus intima-media thickness (IMT), and Framingham score for disease, which were therefore analyzed using nonparametric techniques. sVAP-1 activity and all other continuous parameters analyzed were normally distributed. Sex, smoking, type 1 and 2 diabetes, metabolic syndrome, bulbus plaque, high risk IMT (IMT thickness >90th percentile and/or plaque), recent infection, pregnancy, breast feeding, and the use of oral contraceptives were dichotomized variables, and the phase of menstrual cycle was analyzed in 4 classes.

In univariate analysis, Pearson correlation coefficient *t* test was used for normally distributed parameters, and Spearman correlation

Table 1. Correlations Between sVAP-1 Activity and Background, Clinical and Laboratory Variables

	Women			Men			All		
Variable	n	Correlation Coefficient*	*P*	n	Correlation Coefficient*	*P*	n	Correlation Coefficient*	*P*
Background									
Age (yrs)†	1199	0.025	0.39	983	0.077	0.016	2182	0.047	0.030
Physical activity index (range 5–15 pts)†	1164	0.052	0.076	945	−0.038	0.25	2109	0.010	0.64
Alcohol consumption (drinks per day)†	1176	−0.046	0.12	970	−0.029	0.36	2146	−0.023	0.29
Smoking (no/yes)†	1188	−0.031	0.28	976	0.007	0.83	2164	−0.008	0.70
Recent infection (no/yes)†	1179	−0.076	0.0091	962	−0.108	0.0008	2141	−0.091	<0.0001
Use of oral contraceptives (no/yes)†	1176	−0.14	<0.0001	...	...	...	...	...	...
Breastfeeding (no/yes)†	1182	0.11	0.0002	...	...	...	...	...	...
Clinical									
Log BMI (kg/m^2)	1166	−0.15	<0.0001	974	0.054	0.095	2140	−0.06	0.0053
Log systolic blood pressure (mm Hg)	1191	−0.024	0.41	975	0.048	0.13	2166	0.024	0.26
Diastolic blood pressure (mm Hg)	1191	−0.070	0.016	975	0.020	0.53	2166	−0.023	0.29
Laboratory									
Glucose (mmol/l)†	1199	−0.012	0.69	983	0.074	0.020	2182	0.038	0.075
Log triglycerides (mmol/l)	1199	−0.13	<0.0001	983	0.047	0.14	2182	−0.027	0.21
Total cholesterol (mmol/l)	1199	0.021	0.46	983	0.013	0.68	2182	0.024	0.26
LDL-cholesterol (mmol/l)	1199	0.048	0.095	983	0.011	0.74	2182	0.042	0.049
Log HDL-cholesterol (mmol/l)	1198	0.012	0.67	973	−0.005	0.87	2171	−0.012	0.56
Log CRP (mg/l)	1199	−0.23	<0.0001	983	−0.054	0.090	2182	−0.16	<0.0001

*Pearson correlation for normally distributed parameters and Spearman correlation for the others (marked with†).
sVAP-1 indicates soluble vascular adhesion protein; BMI, body mass index; CRP, C-reactive protein.

coefficient *t* test for nonparametric variables. *t* Test analysis was used for 2 class comparisons and one-way ANOVA for multiple-class comparisons. For multivariable models, linear regression models were used for continuous variables, and logistic regression models for class variables.

Statistical analyses were performed using the SAS software for Windows version 9.2, Microsoft Excel or Origin 8. Probability values <0.05 were considered significant.

Results

Fluorometric Assay to Determine sVAP-1 Activity in Human Blood

We developed a new high-throughput assay for measuring sVAP-1 from human serum samples. sVAP-1 in the serum was used as an enzyme source, and sVAP-1 dependent production of hydrogen peroxide was measured by providing a selective VAP-1 substrate benzylamine without or with a selective VAP-1 inhibitor hydroxylamine to the samples (Figure A). A constant sVAP-1 activity was measurable in serum (Figure B). sVAP-1 activity in sera was practically abolished when VAP-1 was immunodepleted from the sample using monoclonal antibodies against VAP-1 ($P<0.0001$, *t* test; Figure C). Spiking of VAP-1 depleted serum with recombinant VAP-1 protein resulted in a linear ($r=1.00$) increase in sVAP-1 activity (Figure D). A good correlation ($r=0.85$) was found between sVAP-1 enzyme activities and VAP-1 protein concentrations measured by a sandwich ELISA (Figure E).

The assay was then miniaturized into a 384-well format compatible with automatic addition of assay reagents and using only 2.4 μL of serum. The throughput of the assay is 108 samples (plus standards and controls) per a 384 plate, and after adding the serum samples, the assay takes less than 2 hours for completion.

Anticoagulants and Sample Storage Affect sVAP-1 Activity

We determined how the format (serum versus plasma) and the storage of the sample affect the performance of the assay. sVAP-1 activity remained higher in the serum than in plasma samples (Supplemental Figure IA). Heparin-plasma was also compatible with the assay, whereas citrate ($P=0.006$) and EDTA ($P<0.0001$) strongly inhibited the activity. Freeze-thaw cycles and the storage temperature also significantly affected sVAP-1 activity (Supplemental Figure IB–1D).

sVAP-1 Activity in the Normal Population

To determine the sVAP-1 activity in a general population, we used serum samples that had never been thawed and had been stored all the time at −70°C. sVAP-1 activity in the normal population was 13.6±3.3 nmol/mL/h (mean±SD, n=2182, range 7.0–20.2). Men had slightly higher sVAP-1 activities than women (13.8±3.2, n=983 versus 13.5±3.4, n=1199, mean±SD, $P=0.024$). In men, but not in women, sVAP-1 activity increased with age (Table 1). In women, no significant differences in sVAP-1 activity between different phases of the menstrual cycle ($P=0.30$) were found. The correlations of sVAP-1 activity

Table 2. Relations of sVAP-1 Activity With Brachial Artery Flow-Mediated Vasodilation, Carotid Artery IMT and Carotid Artery Plaque

	Women			Men			All		
Variable	n	Correlation Coefficient*	*P*	N	Correlation Coefficient*	*P*	N	Correlation Coefficient*	*P*
Maximal FMD (%)	1181	−0.076	0.0089	972	0.005	0.87	2153	−0.056	0.0092
Maximal bulbus IMT (mm)†	1084	0.062	0.042	889	−0.0002	1.00	1973	0.040	0.073
Bulbus plaque (no/yes)†	1192	0.066	0.023	976	0.044	0.17	2168	0.0099	0.65
Log mean carotid IMT (mm)	1192	0.033	0.25	976	0.066	0.041	2168	0.055	0.010
Log maximal carotid IMT (mm)	1192	0.031	0.28	976	0.072	0.025	2168	0.057	0.0077

*Pearson correlation for normally distributed parameters and Spearman correlation for the others (marked with†).
sVAP-1 indicates soluble vascular adhesion protein; FMD, flow mediated dilatation in brachial artery; IMT, intima media thickness.

to pregnancy ($r=0.007$, $P=0.80$) and to the number of parturitions ($r=-0.019$, $P=0.57$) were not significant either. However, the use of oral contraceptives had an indirect and breast-feeding a direct correlation to sVAP-1 activity (Table 1). The physical activity, alcohol consumption, and smoking had no correlation with sVAP-1 activities in either sex (Table 1). Thus, sVAP-1 activities are slightly higher in men than in women, and high estrogen/progesterone/prolactin levels may be involved in the regulation of sVAP-1 activity. Because of this sex difference, we performed all subsequent analyses separately for women and men.

Correlation of sVAP-1 Activity With BMI and Blood Pressure

We determined the correlation of sVAP-1 activity with multiple other clinical and laboratory risk factors for cardiovascular diseases using univariate analyses. BMI correlated indirectly with sVAP-1 activity in women (Table 1). When compared to sVAP-1 activities of women with normal BMI (BMI 20–25 kg/m^2), lean women (BMI <20 kg/m^2) had higher and severely obese women (BMI >30 kg/m^2) had lower sVAP-1 activities (Supplemental Table II). sVAP-1 activity showed an indirect correlation with the weight ($r=-0.12$, $P<0.0001$, n=1168), but a direct correlation with the height ($r=0.08$, $P=0.011$, n=1169) in women. In men, BMI did not correlate with sVAP-1 activity, but sVAP-1 activity correlated directly with the weight ($r=0.08$, $P=0.015$, n=974) and the height ($r=0.08$, $P=0.017$, n=977). Because BMI is a confounder for multiple cardiovascular risk factors, we included BMI-adjusted data in all subsequent analyses in women.

Systolic blood pressure had no correlation to sVAP-1 activity in either sex, but there was an indirect correlation to the diastolic blood pressure in women (Table 1) that disappeared ($r=-0.023$, $P=0.44$, n=1163) after adjustment with BMI.

Correlation of sVAP-1 Activity and Glucose and Lipids

sVAP-1 activity did not correlate with glucose level in women but did so in men (Table 1). In normoglycemic persons, the correlation was not evident in either sex ($r=-0.030$, $P=0.33$, n=1148 for women, and $r=0.037$, $P=0.27$, n=876 for men), whereas both in hyperglycemic (glucose over 6.0 mmol/L) women ($r=0.29$, $P=0.042$, n=51) and men ($r=0.23$, $P=0.016$, n=107) a direct correlation was seen. sVAP-1 activity was also significantly different between the groups of hyperglycemic and normoglycemic men (Supplemental Table II).

In women, sVAP-1 activity had an indirect correlation with serum total triglycerides, but not with total, LDL, or HDL cholesterol (Table 1). After adjustment for BMI, the indirect correlation with triglycerides was still evident ($r=-0.073$, $P=0.013$, n=1166), and a direct correlation ($r=0.094$, $P=0.0014$, n=1160) with LDL cholesterol became apparent. In men, sVAP-1 activity had no correlation with triglycerides or any of the lipids (Table 1).

Correlation of sVAP-1 Activity and CRP

An indirect linear correlation between high-sensitivity CRP and sVAP-1 activity was found among women (Table 1 and Supplemental Figure IIA–IIC), but not among men. However, in both sexes sVAP-1 activities were found to be significantly lower in subjects with CRP >10 mg/L than in those with CRP <10 mg/L (Supplemental Table II). In women, similar results were seen if CRP value 3 mg/L was used as a cut-off point (Supplemental Table II). Moreover, an indirect correlation between sVAP-1 activity and recent febrile infection was found in both women and men (Table 1 and Supplemental Table II). The indirect correlation between sVAP-1 activity and CRP was evident also in women without (no infection and CRP <10 mg/L; $r=-0.20$, $P<0.0001$, n=1097) or with (reported infection, any CRP value; $r=-0.42$, $P=0.0009$, n=60) history of recent infection.

In multivariable analyses, BMI, waist circumference, triglycerides, HDL cholesterol, age, smoking, physical activity, type 1 and 2 diabetes, recent infection, use of oral contraceptives (in women), and sVAP-1 activity were used in a linear regression model to explain CRP level. In this model, sVAP-1 activity was an independent determinant of the CRP level in both sexes (Supplemental Table III).

sVAP-1 Activity Correlates to Altered Arterial Reactivity and Structure

We then analyzed whether sVAP-1 activity correlates to subclinical atherosclerotic manifestations in a general population. In women, sVAP-1 activity showed an indirect correlation with the brachial artery flow mediated dilatation (maximal change in diameter after a forearm cuff occlusion) measured by ultrasound (Table 2). Moreover, sVAP-1 activity had a direct correlation with the maximal IMT in the

Table 3. Contribution of sVAP-1 Activity to The Multivariable Analysis of IMT

	Women (n=1142)			Men (n=953)			All (n=2107)		
	β	SE	P	β	SE	P	β	SE	P
Log mean IMT									
Log systolic blood pressure	0.16	0.038	<0.0001	0.25	0.054	<0.0001	0.19	0.032	<0.0001
Age	0.0083	0.00076	<0.0001	0.0096	0.00095	<0.0001	0.0090	0.00060	<0.0001
Log HDL cholesterol	−0.075	0.018	<0.0001	−0.0051	0.021	0.81	−0.043	0.013	0.0014
Log BMI	0.090	0.026	0.0005	0.27	0.037	<0.0001	0.15	0.021	<0.0001
sVAP-1 activity	0.0031	0.0011	0.0072	0.0025	0.0015	0.097	0.0031	0.00091	0.0007
LDL cholesterol	0.0095	0.0052	0.072	0.0084	0.0058	0.15	0.0088	0.0039	0.024
Glucose	0.0032	0.0048	0.50	−0.0051	0.0061	0.41	0.00062	0.0038	0.87
Smoking	0.0038	0.010	0.71	0.021	0.011	0.064	0.011	0.0075	0.12
Log CRP	0.00066	0.0038	0.86	−0.0039	0.0052	0.45	−0.00096	0.0030	0.75
Use of contraceptive pills	0.00078	0.011	0.94	...	...	...	...	...	...
Sex	...	...	...	...	...	...	0.0077	0.0070	0.27
Model R^2		0.22			0.24			0.23	
Log maximal IMT									
Log systolic blood pressure	0.16	0.038	<0.0001	0.23	0.055	<0.0001	0.18	0.032	<0.0001
Age	0.0080	0.00076	<0.0001	0.0090	0.00096	<0.0001	0.0085	0.00060	<0.0001
Log HDL cholesterol	−0.068	0.018	0.0001	−0.0028	0.022	0.90	−0.039	0.013	0.0038
Log BMI	0.087	0.026	0.0007	0.25	0.038	<0.0001	0.15	0.021	<0.0001
sVAP-1 activity	0.0028	0.0011	0.013	0.0027	0.0015	0.077	0.0030	0.00091	0.0011
LDL cholesterol	0.010	0.0052	0.052	0.011	0.0059	0.060	0.010	0.0039	0.0070
Glucose	0.0039	0.0047	0.41	−0.0043	0.0062	0.49	0.0013	0.0038	0.73
Smoking	0.0075	0.010	0.46	0.017	0.011	0.13	0.011	0.0075	0.13
Log CRP	−0.00041	0.0037	0.91	−0.0027	0.0052	0.61	−0.0013	0.0030	0.67
Use of contraceptive pills	0.00038	0.011	0.97	...	...	...	...	...	...
Sex	...	...	...	...	...	...	0.010	0.0070	0.14
Model R^2		0.21			0.22			0.22	

Models were additionally adjusted for the study location. sVAP-1 indicates soluble vascular adhesion protein; BMI, body mass index.

bulbus of carotid artery (Table 2). Women with plaque(s) in the carotid bulbus had significantly higher sVAP-1 activities than subjects without plaques (15.5±3.7, n=20 and 13.4±3.4, n=1172, respectively, mean±SD, P=0.0057). Although the mean and maximal IMT (r=0.033, P=0.25, n=1192, and r=0.031, P=0.28, n=1192, respectively) did not correlate with sVAP-1 activity, after adjustment for BMI both correlations became significant (r=0.079, P=0.0073, n=1164, and r=0.075, P=0.010, n=1164, respectively). sVAP-1 activities were higher in women who had high IMT (>90th percentile) and/or bulbus plaque (14.1±3.4 versus 13.4±3.4, n=1069, n=123, P=0.033, mean±SD, t test).

In men, sVAP-1 activity did not correlate with maximal flow mediated dilatation or with bulbus plaques. However, a direct correlation between sVAP-1 activity and the mean and maximal IMT of carotid artery was found (Table 2).

VAP-1 as an Independent Determinant of Carotid IMT and Plaques

Next, the ability of sVAP-1 activity to explain altered arterial function and structure was determined. Age, sex, LDL, and HDL cholesterol, BMI, glucose, oral contraceptives (in women), smoking, systolic blood pressure, and CRP, were chosen as covariants for flow mediated dilatation, IMT, and plaques in linear regression models. The correlation between sVAP-1 activity and flow mediated dilatation in women became diluted (P=0.078) in this model. In contrast, in women, but not in men, sVAP-1 was a determinant explaining both mean and maximal IMT (Table 3).

The same variables were used in logistic regression analyses to study the contribution of sVAP-1 activity to carotid plaques and high risk IMT estimate (IMT over 90th percentile and/or bulbus plaque). sVAP-1 activity was independently associated with both of these early manifestations of subclinical atherosclerosis in women (Table 4).

sVAP-1 Activity and Metabolic Syndrome, and Type 1 and 2 Diabetes

Because sVAP-1 activity directly correlated with early functional and structural alterations in the vasculature, we then determined whether it would associate with established diseases known to involve cardiovascular manifestations and which would already be quite prevalent in our young study population. In women, sVAP-1 activity associated directly

Table 4. Contribution of sVAP-1 Activity to the Multivariable Analyses of Carotid Plaques and High Risk IMT Estimate

	Women (n=1142)		Men (n=953)		All (n=2107)	
	Adjusted Odds Ratio (95 % CI)	*P* Value	Adjusted Odds Ratio (95 % CI)	*P* Value	Adjusted Odds Ratio (95 % CI)	*P* Value
Bulbus plaque*						
Age	2.47 (1.34–4.55)	0.0038	2.10 (1.38–3.20)	0.0005	2.12 (1.50–2.98)	<0.0001
LDL cholesterol	1.85 (1.13–3.02)	0.015	1.62 (1.15–2.28)	0.0053	1.67 (1.28–2.19)	0.0002
sVAP-1 activity	1.71 (1.07–2.72)	0.025	0.84 (0.58–1.21)	0.35	1.08 (0.81–1.43)	0.61
Glucose	1.26 (0.98–1.61)	0.078	0.66 (0.33–1.31)	0.24	1.10 (0.85–1.42)	0.46
Use of contraceptive pills	1.49 (0.95–2.32)	0.080	. . .	. . .	. . .	. . .
Log HDL cholesterol	0.70 (0.39–1.24)	0.22	0.84 (0.56–1.25)	0.40	0.78 (0.57–1.08)	0.14
Log BMI	0.70 (0.39–1.26)	0.23	0.96 (0.56–1.64)	0.88	0.75 (0.51–1.09)	0.13
Smoking	1.26 (0.80–1.97)	0.32	1.11 (0.81–1.53)	0.51	1.13 (0.87–1.46)	0.37
Log systolic blood pressure	1.17 (0.73–1.89)	0.52	1.27 (0.83–1.93)	0.27	1.24 (0.91–1.69)	0.17
Log CRP	1.04 (0.60–1.80)	0.89	0.82 (0.51–1.30)	0.39	0.93 (0.66–1.32)	0.68
Sex	. . .	. . .	. . .	. . .	1.22 (0.63–2.34)	0.56
High risk IMT†						
Age	2.16 (1.69–2.75)	<0.0001	1.81 (1.51–2.16)	<0.0001	1.90 (1.65–2.19)	<0.0001
Log HDL cholesterol	0.69 (0.53–0.88)	0.0031	0.99 (0.82–1.19)	0.91	0.85 (0.74–0.99)	0.03
Log systolic blood pressure	1.29 (1.05–1.60)	0.018	1.43 (1.16–1.76)	0.0007	1.36 (1.17–1.57)	<0.0001
sVAP-1 activity	1.28 (1.04–1.59)	0.020	1.05 (0.88–1.26)	0.56	1.14 (1.00–1.30)	0.051
LDL cholesterol	1.23 (0.99–1.53)	0.065	1.14 (0.96–1.36)	0.12	1.18 (1.04–1.35)	0.012
Glucose	1.14 (0.96–1.34)	0.13	0.90 (0.74–1.10)	0.30	1.02 (0.90–1.17)	0.74
Smoking	1.05 (0.84–1.30)	0.69	1.07 (0.91–1.25)	0.41	1.05 (0.93–1.19)	0.47
Use of contraceptive pills	0.96 (0.75–1.23)	0.73	. . .	. . .	. . .	. . .
Log CRP	1.04 (0.82–1.32)	0.76	0.84 (0.68–1.05)	0.12	0.92 (0.78–1.07)	0.26
Log BMI	0.99 (0.78–1.25)	0.91	1.42 (1.12–1.79)	0.0036	1.17 (0.99–1.37)	0.059
Sex	. . .	. . .	. . .	. . .	1.47 (1.10–1.98)	0.010

Adjusted odds ratios are per 1-SD increase of the variable (except for sex). Models were additionally adjusted for the study locations.
*n of bulbus plaques: women 20, men 34, all 54.
†n of high risk IMT: women 120, men 193, all 313.
sVAP-1 indicates soluble vascular adhesion protein; CI, confidence interval; IMT, intima media thickness.

with type 1 diabetes, but not with metabolic syndrome or type 2 diabetes (Table 5). In men, sVAP-1 activity associated directly with metabolic syndrome. Moreover, sVAP-1 activity associated directly both with type 1 and type 2 diabetes in men (Table 5).

sVAP-1 Activity and Cardiovascular Risk Scores

We finally used cardiovascular risk scores as a surrogate for cardiovascular end points in our study population. In the whole population, sVAP-1 activity correlated directly with Framingham scores for coronary heart disease (Table 6).

Table 5. sVAP Activities in Subjects With Metabolic Syndrome and Diabetes

	Women				Men				All			
	n	Mean	SD	*P**	n	Mean	SD	*P**	n	Mean	SD	*P**
Metabolic syndrome												
No	974	13.5	3.4		744	13.6	3.2		1718	13.5	3.3	
Yes	179	13.3	3.3	0.5	224	14.2	3.4	0.016	403	13.8	3.4	0.15
Type I diabetes												
No	1184	13.4	3.4		962	13.8	3.2		2146	13.6	3.3	
Yes	7	17.5	3.9	0.0017	8	18.0	4.6	0.0002	15	17.7	4.2	<0.0001
Type II diabetes												
No	1183	13.5	3.4		958	13.7	3.2		2141	13.6	3.3	
Yes	7	14.0	4.1	0.64	10	18.0	4.7	<0.0001	17	16.4	4.8	0.028

**t* test for dichotomous comparisons.
sVAP-1 indicates soluble vascular adhesion protein.

Table 6. Correlations of sVAP-1 Activity to Cardiovascular Disease Risk Scores in the Whole Population

Variable	n	Correlation Coefficient*	P
Framingham score (CHD event)	2148	0.052	0.016
Framingham score (CHD)†	2131	0.060	0.0052
SCORE (fatal CHD)	2154	0.047	0.030
SCORE (fatal CVD)	2154	0.045	0.039
FINRISK (CHD event)	2133	0.041	0.061

*Pearson correlation for normally distributed parameters and Spearman correlation for others (marked with†). CHD (coronary heart disease) event includes myocardial infarction and CHD death. CHD includes angina pectoris, coronary insufficiency, and myocardial infarctio. CVD (cardiovascular disease) includes stroke, TIA, congestive heart failure, peripheral vascular disease, coronary insufficiency, angina pectoris, and myocardial infarction.

sVAP-1 indicates soluble vascular adhesion protein; TIA, transient ischemic attack.

sVAP-1 activity also correlated directly with SCORE defining the risk for a coronary or cardiovascular diseases during the next 10 years (Table 6).

Discussion

We report here the development of a new, high-throughput assay for sensitive determination of sVAP-1 activity from serum. We found that at a population level in 30- to 45-year-old Finns sVAP-1 activity correlates with several cardiovascular risk factors and early atherosclerotic manifestations, and often in a sex-dependent manner. In multivariable analyses, sVAP-1 was an independent determinant of carotid plaques and IMT in women.

sVAP-1 is formed by a proteolytic cleavage of the membrane-bound form of VAP-1.[18,27] It catalyzes the oxidation of primary amines in the following reaction: $R\text{-}CH_2{-}NH_2 + H_2O + O_2 -> R\text{-}CHO + H_2O_2 + NH_3$.[28] So far, sVAP-1 activity has been determined with an insensitive photometric method,[29] or with a radioactive method using ^{14}C-labeled amine substrate and detection of the radioactive aldehyde product with liquid scintillation spectrometry after partition with organic solvents.[30] Alternatively, the production of an aldehyde has been followed by high pressure liquid chromatography and fluorometric detection after derivatization.[31] A more robust dye-based method for determination of amine oxidase activity[32] is not sensitive enough for measuring sVAP-1 activity from human serum or plasma. The method developed here relies on the detection of hydrogen peroxide produced in the reaction. Thus, any VAP-1 substrate can be used with this technique. Moreover, our method does not need radiochemicals or complicated analytic instruments, such as high pressure liquid chromatography. Although other enzymes with a similar semicarbazide-sensitive amine oxidases activity (copper-dependent amine oxidases 1 and 2 and lysyl oxidase) could potentially complicate the interpretation of the activity data, our immunodepletion experiments show that >95% of the measured activity actually derives from VAP-1 protein. Thus, this method allows for the first time high-throughput analyses of sVAP-1 activity from human blood.

sVAP-1 activity was slightly higher in men than in women. Physiological changes in sex steroids (menstrual cycle, pregnancy) did not correlate with sVAP-1 activity, but the use of oral contraceptives showed an indirect correlation to sVAP-1 activity. Lactation, on the other hand, had a direct correlation with sVAP-1 activity. This indicates that sex steroids and prolactin, at least at high concentrations, may be involved in the regulation of sVAP-1 activity, most likely in concert with other, unidentified factors. Interestingly, similar effects of sex and contraceptive steroids on serum monoamine oxidase levels, which largely corresponds to sVAP-1 activity, have been noted almost 35 years ago[33] (although both the early enzymological and statistical methodology suffers from certain limitations) but has been ignored ever since. Because the contribution of many cardiovascular risk factors (eg, smoking[34]) differ between men and women, and sex is included as a parameter in all major cardiovascular risk scores, we believe that separate analyses for sVAP-1 in men and women are warranted.

Earlier, sVAP-1 activity has been reported either to show indirect,[17] direct[21,35,36] or no[19,22,23,37] correlation with BMI. Moreover, in some reports no association between triglycerides and sVAP-1 activity has been seen,[35] whereas others have reported either direct[36] or indirect[38] correlations. We found that sVAP-1 activity showed an indirect correlation with BMI and triglycerides, and (after BMI adjustment) a direct correlation with LDL cholesterol in women. In contrast, in men sVAP-1 activity correlated directly with weight, but no correlation with BMI, triglycerides, or lipoproteins was found. The discrepant earlier reports ([17,19,35–38] with 41–49% men, and[21] with 71% men participants) are likely due to the ignorance of the effect of sex to these correlations. Moreover, it is important to take into account the effect of BMI in masking the correlation between sVAP-1 activity and several cardiovascular risk factors, such as LDL cholesterol, and mean and maximal IMT in women.

VAP-1 can contribute to the development of atherosclerosis at several steps,[39] although so far many of these possible mechanisms have been experimentally verified in other models only. VAP-1 in arterial wall can produce hydrogen peroxide and aldehydes,[40] which can be involved in lipid peroxidation and aldehyde modifications of proteins and oligosaccharides. This may lead to endothelial activation and induction of VAP-1.[41] VAP-1 may then be involved in enhanced recruitment of monocytes[42–45] and Th1 CD4 cells[46] into the vessel wall. VAP-1–generated hydrogen peroxide also induces expression of classical endothelial adhesion molecules (P-selectin, MAdCAM-1), chemokines (CXCL8), and inflammatory transcription factors (nuclear factor-κB, p53),[9–11,47] and at high concentrations can be directly cytotoxic. Aldehyde modifications, on the other hand, are involved in production of advanced glycation end-products, for example in glomerulosclerosis,[12–14] and generation of immunogenic epitopes (eg, in LDL), which can further aggravate inflammatory reaction in the vessel wall. Thus, both enzyme-activity dependent and enzyme-activity independent functions of membrane-bound and sVAP-1 have the potential to cause vascular damage and aggravate inflammation.

Consistent with the role of VAP-1 in modulating vascular wall, we found that in women the baseline sVAP-1 activity correlates directly with maximal IMT in the bulbus of carotid artery. After adjustment for BMI, a direct correlation with mean and maximal carotid IMT became evident. sVAP-1 activity also showed a positive association to carotid plaques. Furthermore, we found that sVAP-1 activity is an independent factor determining IMT in women. In this context it is interesting to note that in a transgenic mouse model, overexpression of VAP-1 in smooth muscle cells led to altered morphology of elastin in large arteries, increased pulse pressure, and impaired response to vasodilatory stimuli.[48] Because sVAP-1 activity is a determinant of IMT only in women, we speculate that the deleterious effects of sVAP-1 activity on the arterial wall may be partially dependent on the female sex hormones, which are known to change the vascular endothelium and wall in a sex-specific manner (eg, there is less luminal stenosis and more diffuse, outward enlargening of arteries as a response to athereosclerotic plaques in women).[49]

sVAP-1 had indirect correlations to a few established cardiovascular risk factors. The indirect correlations to BMI and triglyserides may be related to the function of VAP-1 on adipocytes. Membrane-bound VAP-1 is abundantly present on white, but not brown, fat cells, and it regulates fat cell differentiation and glucose uptake.[50,51] Although the exact regulation of the shedding that results in the formation of sVAP-1 from the membrane-bound form is not known, one hypothesis could be that in obese persons more VAP-1 remains on the cell surface to contribute to the fat cell formation and pathological energy metabolism with a concurrent decrease in the soluble form of the molecule.

The indirect correlation between sVAP-1 activity and CRP (both under normal conditions and after a recent infection) was unexpected, because the membrane form of VAP-1 is typically induced on inflammation.[52] Elevated CRP is a traditional cardiovascular risk factor. It should be noted, however, that several recent studies have shown that up to 60% increase in physiological CRP concentration due to genetic polymorphisms does not increase the risk of coronary heart disease.[53,54] This implies that a common factor predisposing to cardiovascular damage may exist, which both induces CRP and inhibits sVAP-1. These results also suggest that increased sVAP-1 activity is not merely a secondary reflection of active inflammation.

The direct correlation of sVAP-1 and IMT, and its indirect correlation with BMI, triglycerides, and CRP, all risk factors of atherosclerosis, is perplexing. However, our multivariable analyses showed that CRP is not an independent risk factor for IMT. When we added triglycerides into the same model, it did not remain significant either (data not shown). These findings are in line with the observations that adjustment for other risk factors weakens or even abolishes the association between triglyceride levels and cardiovascular events[55]; and that fibrates may not be effective in prophylaxis of cardiovascular diseases.[56] In fact, a subfraction of triglycerides within certain lipoproteins may be the most detrimental in respect to atheroma development,[39] and notably sVAP-1 activity did correlate directly with LDL levels. Also many anti-inflammatory therapies, which should decrease CRP, have failed to improve cardiovascular outcomes. Altogether the interconnection between different biochemical risk factors clearly remains incompletely understood at the moment.[39] Nevertheless, baseline or glucose-induced sVAP-1 activity/protein has been shown to have a direct correlation with IMT also in 2 earlier smaller studies with clearly older subjects (mean age 56–57 years; n=25 and 115 subjects) from different ethnic backgrounds.[35,57] Thus, in any case it will be of considerable interest to reanalyze the sVAP-1 correlations with different risk factors and frank cardiovascular morbidity in our follow-up study cohort when new samples become available next year, as well as in other populations.

The strong positive association between sVAP-1 activity and type 1 and type 2 diabetes has been established in many studies.[17,33,58,59] In fact, sVAP-1 activity seems to predict the extent of vascular complications in these diseases,[19,37] and even be an independent risk factor for death in type 2 diabetics.[60] In diabetic patients, sVAP-1 correlates directly with the severity of carotid stenosis and with the carotid plaques.[35] Moreover, the change in sVAP-1 concentration induced by oral glucose tolerance test correlates with carotid IMT in healthy subjects.[57] sVAP-1 activity has also been reported to correlate directly with glucose.[17,22,35,36,59,60] Here we unexpectedly found that there is no correlation between glucose and sVAP-1 activity in normoglycemic persons. This is in line with the report of Li et al, who found no association between fasting plasma glucose and sVAP-1 protein concentrations in patients with glucose values <5.55 mmol/L.[61] The earlier observations[17,22,35,36,47,48] are likely explained by the fact that they have mainly been done in patients with diabetes, because we also noted a clear direct correlation between these 2 parameters in both sexes among hyperglycemic subjects. In any case, it is noteworthy that the association between sVAP-1 activity and type 1 diabetes in both sexes, and type 2 diabetes in men, was also evident in our population-based study.

We are aware that our study suffers from certain inherent limitations. The bivariate analyses should be interpreted with some caution because there are multiple potential confounding factors and multiple correlations were done. Moreover, due to the young age of the persons in our study cohort only very modest cardiovascular disease morbidity or mortality is present (eg, only 1 myocardial infarction, 3 angina pectoris, 5 cerebral blood flow disturbances). Therefore, the patient numbers in disease groups remain small, and many correlations had to be done for risk scores rather than observed cardiovascular end-points of these diseases. We also do not have any other soluble cell adhesion molecule measured from this cohort, and therefore we cannot experimentally compare the performance of sVAP-1 against those. Merely on a theoretical basis, sVAP-1 analyses may have certain benefits: 1) They may be more specific. The other soluble cell adhesion molecules, such as sICAM-1, sVCAM-1, or sE-selectin, are upregulated in almost all types of inflammations and correlate strongly with CRP.[4] sVAP-1 on contrast, is only upregulated in selected inflammatory disorders,[20] and shows indirect correlations with CRP. 2) sVAP-1 has a potential to affect wider repertoire of atherogenic events than the classical

soluble adhesion molecules. In addition to the regulation of leukocyte traffic, which is the only known function for classical adhesion molecules, VAP-1 also contributes to the regulation of local redox-balance and advanced glycation, which are pathogenetically important in arteriosclerosis.[7,9–14] 3) sVAP-1 analyses may confer economical benefits. The consumable costs for sVAP-1 activity assays are much lower than those for ELISAs (eg, 100 USD and 8000 USD for 1000 samples in sVAP-1 assay and in a commercial sICAM-1 ELISA, respectively). In any case, it will be interesting to determine in the future, whether inclusion of sVAP-1 activity into the cardiovascular risk models (with or without other soluble adhesion molecules) would improve their performance.

In conclusion, we have developed here a robust sVAP-1 activity assay that should be useful for several clinical applications. Because sVAP-1 activity is different in men and women, some reevaluation of published sVAP-1 studies is warranted, and the sex difference should be taken into account in future studies. Multivariable analyses showed that sVAP-1 activity is an independent determinant of IMT and carotid plaques in women. The VAP-1 activity may thus be involved in the pathogenesis of early atherosclerotic alterations in the vascular wall.

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Disclosures

None.

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