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Low levels of IgG autoantibodies against the apolipoprotein B antigen p210 increases the risk of cardiovascular death after carotid endarterectomy

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Cover title: Antibodies to oxLDL epitopes and cardiovascular death.

Tables: 3 Figures: 2

Key words: autoantibodies, apolipoprotein B, carotid stenosis, cardiovascular events.

ABSTRACT

Objectives: Autoimmune responses against oxidized-LDL (oxLDL) have been suggested to modulate inflammation in atherosclerosis. Previous studies showed an association between autoantibodies against the apolipoprotein B (apoB) p210 antigen and a lower risk of cardiovascular (CV) events. In the present study we investigated if autoantibodies against p210 at the time of carotid endarterectomy (CEA) predict risk for future CV events.

Methods: Native (nat) and malondialdehyde (MDA)-modified apoB p210 autoantibodies (IgM-p210nat, IgG-p210nat, IgM-p210MDA and IgG-p210MDA) were analyzed by ELISA from plasma samples of 351 patients at the time they underwent CEA. The incidence of postoperative CV events was assessed using national registers.

Results: A total of 52 non-fatal and 15 fatal CV events were registered during the follow-up period (35.1 ± 16.7 months). Patients who suffered from a fatal CV event had significantly lower plasma levels of IgG-p210nat and IgG-p210MDA. Kaplan–Meier curves of event-free survival showed increased CV mortality in patients with levels of IgG-p210nat and IgG-p210MDA below the median (Log Rank 7.813, p .005 and 9.105, p .003 respectively). The association between low levels of p210 IgG and fatal post-operative CV events remained significant when adjusting for age, sex, total cholesterol, HDL cholesterol, smoking habits and hypertension in a Cox Proportional Hazard model (hazard ratios (HR) IgG-p210nat below median: HR 6.7 (95% C.I. 1.5-30.6, p .013) and IgG-p210MDA below median: HR 7.8 (95% C.I. 1.7-35.5, p .008).

Conclusions: The present findings support the notion that autoantibodies against LDL antigens are involved in the atherosclerotic disease process and suggest that CEA patients with low levels of IgG-p210nat and IgG-p210MDA have an increased risk of post-operative CV death.

INTRODUCTION

Carotid endarterectomy (CEA) is effective in preventing neurological events in patients with severe carotid artery stenosis.[1, 2] There is accumulating evidence that the immune system modulates the inflammatory reactions that characterize the development and progression of atherosclerosis.[3] Accumulation of low-density lipoprotein (LDL) occurs in the intima of the arterial wall where it may become oxidized (oxLDL) by multiple mechanisms.[4, 5] OxLDL stimulates monocyte infiltration, as well as smooth muscle cell migration and proliferation in plaques.[6] Moreover, it affects the synthesis and action of several inflammatory circulating mediators such as C-reactive protein (CRP), interleukin (IL)-6, and tumor necrosis factor (TNF)-α.[7, 8]

Epitopes of apolipoprotein B (apoB) 100 generated in association with LDL oxidation have been identified as major targets for immune responses in atherosclerosis.[9-11] Several studies have demonstrated increased titers of antibodies recognizing oxLDL in patients with coronary,[12] cerebral[13] or peripheral artery disease,[14] suggesting that they can serve as markers of the atherosclerotic process.[13, 15-17] However, whether these autoimmune responses have protective or pathogenetic effects remains to be fully elucidated. In the present study we investigated if autoantibodies against p210 at the time of carotid endarterectomy (CEA) predict risk for future cardiovascular (CV) events.

MATERIALS AND METHODS

Patient data

Three hundred-fifty-one patients aged 72.3 ± 8.9 years who underwent CEA between October 2005 and October 2010 at a tertiary university Vascular Department were included in the current analysis. All patients gave their written informed consent and the local ethical committee approved the study. Indications for CEA have been previously described.[18] Patients with ipsilateral carotid artery occlusion, radiation induced primary carotid stenosis or restenosis after previous CEA or endovascular treatment were excluded. Patients were considered to have asymptomatic carotid disease if they had no *amaurosis fugax* (AF), transient ischemic attacks (TIAs) or stroke in the 6 months prior to surgery as assessed clinically by an independent accredited neurologist. The clinical charts of all patients were reviewed in order to gain information about comorbidities and the past medical history and were completed by standardized preoperative interviews. The Swedish Cause of Death and National in-patient Health Registers were used identify postoperative CV events that occurred between January 2005 and December 2010 corresponding to the following codes of the 10th revision of the International Classification of Diseases (ICD-10): G45, G46, I20 to I25. These are nationwide validated registers where all causes of death and more than 99 % of all somatic (including surgery) and psychiatric hospital discharges are registered.[19] In doubtful cases, information was checked through telephone interviews and review of the medical charts. All deaths were verified against the Swedish National Population Register.

Definition of outcomes

Postoperative events were registered and analyzed separately. For the purpose of this study, intraoperative events were defined as including all events occurring within the first 24 hours after CEA. This was done in order to exclude procedure-related events from the follow-up analysis. The primary endpoint of the current study was fatal or non-fatal acute myocardial infarction (AMI) or cerebrovascular event (stroke, transient ischemic attack (TIA) or amaurosis fugax (AF)). In patients suffering of multiple postoperative events of the same type (i.e. multiple AMI, for example), only the first one was taken into account in the survival analysis. A total of 15 fatal (9 AMI and 6 strokes) and 52 non-fatal events (20 AMI, 15 strokes, 11 TIA and 6 AF) were registered during follow-up.

Blood sample analysis

Blood samples were collected on the day before surgery. The following parameters were evaluated: total cholesterol, free triglycerides, serum high-density lipoprotein (HDL)-cholesterol, serum LDL-cholesterol. The plasma levels of high sensitive CRP (hsCRP) were also analyzed in order to exclude the presence of a systemic inflammatory reaction which could have influenced the titers of circulating antibodies against oxLDL epitopes.

Measurement of plasma levels of antibodies recognizing oxLDL epitopes by Enzyme-Linked Immunosorbent Assay (ELISA)

The titres of antibodies against oxLDL epitopes were measured using a capture ELISA as reported elsewhere.[19] Briefly EDTA-plasma was supplemented with the anti-oxidants diethylenetriaminepentaacetic acid (DTPA) and butylated hydroxytoluene (BHT). In the beginning, the 96-holes plates (Nunc MaxiSorp, Nunc, Roskilde, Denmark) were coated at a concentration of 20 mg/ml with the following peptides: native p210 and MDA-modified p210

(TAG Copenhagen A/S, Copenhagen, Denmark), and incubated overnight at 4 ˚C. On the second day, after washing and blocking procedures, 100 µL of plasma diluted 1/100 were added and the plates were incubated for two hours at room temperature and then over night at 4 ˚C. The following day, the plates were washed and the secondary biotinylated rabbit polyclonal antibody to human IgG or goat anti-human IgM (eBioscience Inc., San Diego, CA, USA) were added. After another incubation for 2 h at room temperature the plates were washed and the bound biotinylated antibodies detected by alkaline phosphatase conjugated streptavidin (Sigma), incubated for 2 h at room temperature. The colour reaction was developed by using phosphatase substrate kit (Pierce, Rockford, Illinois) and the absorbance was measured at 405 nm (Tecan Austria GmbH, Grödig, Austria) under lightless conditions during 90 minutes at room temperature for IgM and 120 minutes for IgG, using a reference filter at 570 nm. For practical reasons the anti-oxLDL epitope antibodies were named as follows: IgM against the peptide 210 native (IgM-p210nat); IgM against the MDA-modified peptide 210 (IgM-p210MDA); IgG against the native peptide 210 (IgG-p210nat); IgG against the MDA-modified peptide 210 (IgG-p210MDA). Values of the levels of the different antibodies are given in arbitrary units based on the ratio of the absorbance of the test plasma and pooled reference plasma obtained from healthy controls. The intra- and inter-assay coefficient of variation for the p210 autoantibody ELISAs are 5-10% and 10-15% respectively.[20]

Statistical analysis

Continuous variables are presented as mean ± standard deviation (SD) when normally distributed and median followed by interquartile range (IQR) in parenthesis if non normally distributed. Categorical variables are presented as percentages. Pearson´s Chi-square test was used for categorical variables. All the anti oxLDL antibodies except the subtype IgM-

p210MDA were non-normally distributed. Student's *t*-test and Pearson´s correlation were used for continuous variables whenever normally distributed, while Mann-Whitney U test and Spearman´s rank correlation were used for non-normally distributed variables. Linear and logistic regression analysis (with age, sex, total cholesterol, HDL cholesterol, smoking habits, the presence of hypertension included as correction variables) were used to explore the relationship between two or more variables. Freedom from postoperative events was calculated by life-tables according to Kaplan-Meier survival analysis and is presented as survival estimate ± standard error. Correction for the above mentioned variables was done through Cox regression analysis. In the subgroup of symptomatic patients, the time between the occurrence of preoperative symptoms and CEA was included in the regression model. A P-value of $< .05$ was considered statistically significant. Statistical analysis was performed using SPSS 22.0 (IBM Corp., Amonk, NY, USA).

RESULTS

Two thirds of the patients had experienced neurological symptoms preoperatively (table 1), with a median time between the occurrence of symptoms and CEA of 16 (IQR 8-27) days. The overall mean stenosis degree was 84.1 ± 13.1 %. Patients with symptomatic carotid stenosis had lower levels of IgG-p210MDA (table 2). Of the comorbity factors analyzed, the presence of a diabetes was associated with lower levels of IgG-p210nat (0.54 IQR 0.38-0.73 versus 0.61 IQR 0.48-0.79, p <.05). There were no significant correlations between apoB p210 antibodies and plasma lipoproteins or hsCRP.

Apo B p210 autoantibodies and incidence of post-operative CV events

A total of 67 CV events were registered during the post-operative follow-up period (35.1 ± 16.7 months). Fifteen of these events were fatal. Patients who suffered from a fatal CV event had significantly lower plasma levels of IgG-p210nat and IgG-p210MDA, whereas no difference was observed for patients with non-fatal CV events (table 3). We next categorized patients based on the median values of IgG-p210nat and IgG-p210MDA autoantibodies. Kaplan–Meier curves of event-free survival showed increased CV mortality in patients with below median levels of IgG-p210nat and IgG-p210MDA (Log Rank 7.813, p .005 and 9.105, p .003 respectively; figure 1 and 2). Remarkably, out of the 15 subjects that suffered a fatal CV event only two had either IgG-p210nat or IgG-p210MDA while 1 patient had both antibodies levels above the median. The association between low levels of IgGs-p210 and CV mortality remained significant when controlling for age, sex, total cholesterol, HDL-cholesterol, smoking habits and the presence of hypertension (tables 4 and 5) in a Cox Proportional Hazard model (hazard ratios IgG-p210nat below median: HR 6.7 (95% C.I. 1.5-30.6, p .013) and IgG-p210MDA below median: HR 7.8 (95% C.I. 1.7-35.5, p .008).

DISCUSSION

The present study demonstrates that CEA patients with low levels of autoantibodies against the LDL antigen p210 at the time of surgery have increased post-operative risk of CV death. This association was found to be independent of both the presence of pre-operative plaque-related CV events as well as other CV risk factors. Patients with IgG-p210nat and IgG-p210MDA below the median level had a 6.9 and 8.2-fold increased risk for post-operative CV death, respectively. These observations suggest that CEA patients with low level of IgGs-p210 represent a group with high post-operative risk that may require more intense preventive treatment. However, this possibility needs to be confirmed in larger studies. The mean levels for IgG-p210nat and IgG-p210MDA among those with a fatal CV event was 0.47 and 0.66, respectively. As the values are expressed as a ratio between test plasma and pooled healthy control plasma this means that IgG-p210nat levels were reduced by 53% as compared to the pooled healthy control plasma and that IgG-p210MDA levels were reduced by 34%. For those without a fatal CV event the corresponding reductions were 38% and 12%, respectively. This can be compared with observations from a recent study on high risk individuals without clinically manifest cardiovascular disease demonstrating about a 10% reduction in p210 IgG and IgM levels as compared to healthy control plasma.[20] Interestingly, we did not observe any association between p210 autoantibodies and the incidence of non-fatal CV events during follow-up. This could mean that a possible protective effect of these antibodies is on the outcome of CV events rather than on their occurrence. However, this possibility needs to be addressed in future separate studies.

It is important to keep in mind that observational studies do not provide information about causality. However, the present finding of an association between autoantibodies against apo B peptides and CV death are well in line with accumulating evidence suggesting that immune responses against plaque antigens play an important role in atherosclerosis and that LDL is a

major target for these immune responses.[16, 17, 21, 22] Although initial studies argued for a pro-atherogenic effect of adaptive immunity in atherosclerosis[23] it has now become evident that the role of the immune system in the disease process is much more complex and that both pathogenic and protective responses are involved. Suppressive regulatory T cells protect against atherosclerosis through release of anti-inflammatory cytokines and suppression of autoreactive T cells in the artery wall.[24] There is also evidence that some antibodies recognizing structures in oxidized LDL protect against atherosclerosis. One such antigen is phosphatidylcholine (PC) which is present on the surface of oxidized LDL.[25] Moreover, immunization of hypercholesterolemic mice with PC-containing *Streptococcus pneumoniae* vaccine were associated with an increase in PC-specific antibodies and reduced atherosclerosis.[26] A human recombinant IgG specific for the MDA-modified apo B peptide p45 inhibits the development of atherosclerosis in hypercholesterolemic mice and induces regression of lesions when combined with cholesterol lowering.[27, 28] Studies performed on cultured cells have shown that immune complexes of this antibody and oxidized LDL inhibit cytokine release from monocytes through interaction with the inhibitory FcγIIb receptor.[29] Treatment of obese, hypercholesterolemic non-human primates with this antibody resulted in lower plasma levels of pro-inflammatory cytokines.[29]

The association between antibodies against oxLDL and CV disease has been extensively studied. Results from studies analyzing antibodies against the intact oxLDL particle have provided inconclusive and partly contradictive results possible due to difficulties in obtained standardized antigens in the detection assays used.[30] A more coherent picture has emerged from studies using single LDL-derived antigens such as PC- and apoB-derived peptides. We have previously shown that high levels of IgG against native as well as aldehyde modified p45 and p210 are associated with less severe coronary and carotid disease as well as with a lower risk for CV events.[31-33] In a recent large observational cohort study of high-risk

individuals in five European countries McLeod and coworkers[20] demonstrated increased carotid intima-media thickness and a more rapid progression of carotid disease in those with low levels of autoantibodies against native and MDA-modified p210. There is also evidence for an association between low levels of anti-PC IgM and CV events.[34] Taken together these experimental and clinical studies provide support for a protective role of antibodies recognizing antigens in oxLDL. The present findings are well in line with this notion and provide further evidence for an important role of these antibodies also in subjects with advanced disease such as CEA patients.

There are some limitations of the present study that should be considered. Taking into account the observational character of the study we can only speculate on the possible functional role of antibodies against p210. The number of patients included was limited and the findings need to be confirmed in larger cohorts with longer observational intervals. Furthermore, studies were not carried out to provide further information on the possible presence and extension of atherosclerotic plaques in other territories. An additional limitation of the current study is that we lack data regarding changes in antibody levels during follow-up. However, we have previously reported that p210 antibody levels remain relatively stable over a 12- month period,[35] but it is currently unknown if the CEA can influence the antibodies levels. Finally, there are no published reference values for p210 antibody levels but in a recent European multicenter study involving over 3000 subjects without clinically manifest cardiovascular disease and an average age of 65 years the mean value for IgG-p210nat was 0.88 and for IgM-$p210_{MDA}$ it was 0.92.[20] Accordingly, the levels of both antibodies were markedly lower in the present cohort with more severe CV disease.

SUMMARY

In conclusion our findings show that CEA patients with low levels of IgG against the native or MDA-modified apoB peptide p210 have an increased risk of post-operative CV death. Our observations add further support for the notion of a protective role of humoral immune responses against peptide antigens in oxLDL Additionally our data suggests that determining IgG-p210nat and IgG-p210MDA could be of interest to identify CEA patients in need of more intensive post-operative preventive treatment.

SOURCES OF FUNDINGS

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DISCLOSURES

None.

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LEGENDS

Figure 1:

Kaplan-Meier survival analysis with median IgG p210nat plasma levels as cut-off. Postoperative time to CV death expressed in months. Numbers (N) at risk in the two groups at different time points are indicated below the figure.

Figure 2:

Kaplan-Meier survival analysis with median IgG p210MDA plasma levels as cut-off. Postoperative time to CV death expressed in months. Numbers (N) at risk in the two groups at different time points are indicated below the figure.

Table 1:

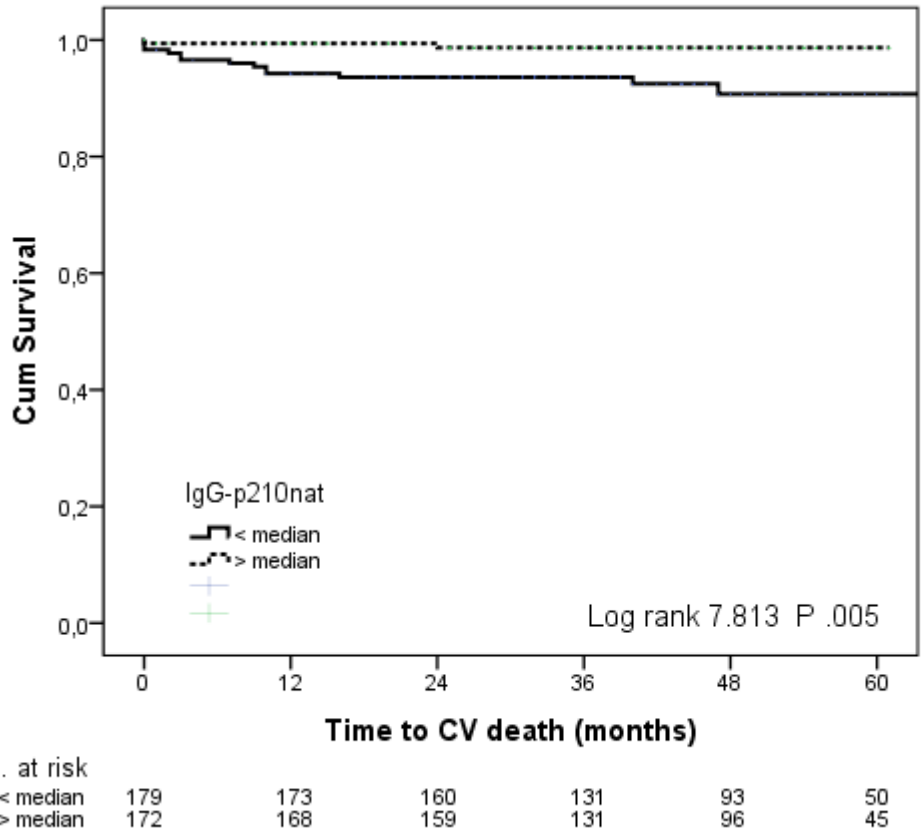
Clinical characteristics of the study cohort.

Table 2.

Autoantibody levels in asymptomatic and symptomatic patients.

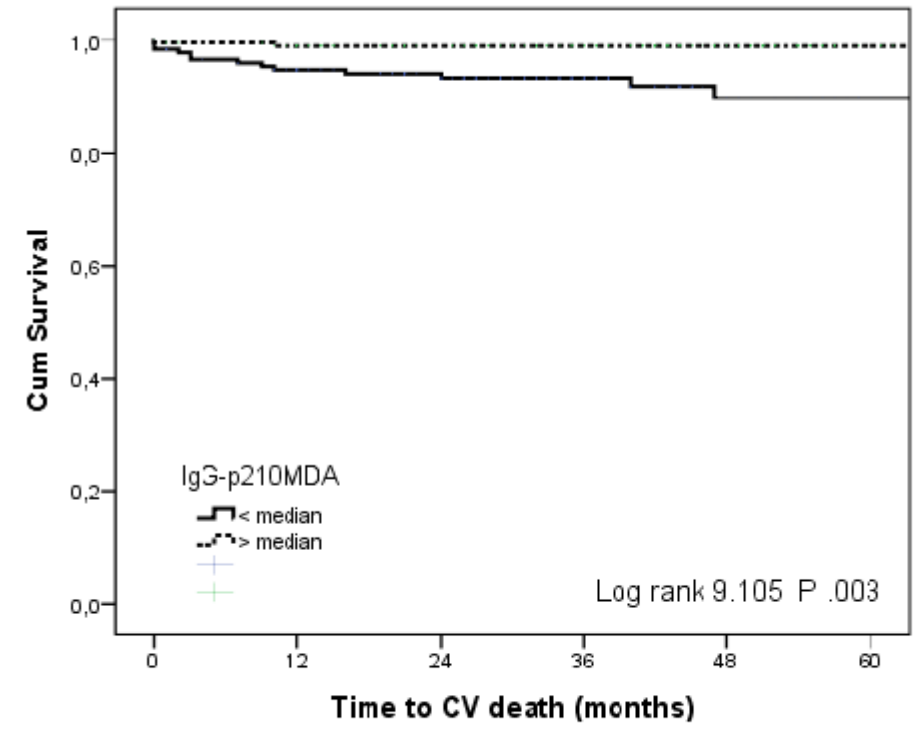
Table 3.

Autoantibody levels related to the occurrence of postoperative events.



N. at risk

< median	179	173	160	131	93	50
> median	172	168	159	131	96	45



N. at risk

< median	174	169	153	125	86	39
> median	177	172	166	137	103	56

Table 1. Clinical characteristics of the study cohort

	All (351)	CV death (15)	no CV death (336)	P
Age (years)	70.52 ± 8.57	74.4 ± 6.95	70.35 ± 8.6	.065
Male	65.8 (231)	73.3 (11)	65.5 (220)	.782
Preoperative symptoms	67.2 (236)	73.3 (11)	67 (225)	.781
Coronary artery disease *	38.2 (134)	40 (6)	38.1 (128)	.968
Peripheral artery disease †	12 (42)	13.3 (2)	11.9 (40)	.697
Hypertension ‡	71.2 (250)	80 (12)	71.1 (239)	.737
Diabetes	23.9 (84)	40 (6)	23.2 (78)	.211
Inflammatory disease	3.5 (6)	0.7 (1)	0.1 (5)	.348
Smoking				
no	20.5 (72)	13.3 (2)	20.8 (70)	
current	30.8 (108)	6.7 (1)	31.8 (107)	.039
past §	48.7 (171)	80 (12)	47.3 (159)	
Overweight (BMI >25)	60.7 (213)	66.7 (7)	61.3 (206)	.287
Statins use	86.9 (305)	73.3 (11)	87.5 (294)	.119
Cholesterol (mmol/L)				
Total	4.43 ± 1.12	4.47 ± 1.29	4.40 ± 1.15	.981
HDL	1.18 ± 0.42	1.08 ± 3.72	1.19 ± 0.42	.303
LDL	2.66 ± 1.06	2.91 ± 1.12	2.64 ± 1.03	378
Triglycerides (mmol/l)	1.42 ± 0.69	1.17 ± 0.58	1.41 ± .071	.140
hsCRP (mg/L)	6.42 ± 9.84	4.40 ± 2.86	6.40 ± 9.47	.906
Creatinine (mmol/L)	91.49 ± 28	130.40 ± 62.21	89.74 ± 24.21	.001

Categorical variables are expressed in percentages, continuous variables as median ± standard deviation. (n) number of patients;. * stable and/or unstable angina, history of myocardial infarction and/or coronary artery surgery/angioplasty; † previous arterial surgical/endovascular intervention at the lower extremities, including intervention at iliac arteries; ‡ systolic pressure > 140 mmHg; § not active smoking for at least 6 months before surgery.

Table 2. Autoantibody levels in asymptomatic and symptomatic patients.

	Asymptomatic	Symptomatic	P
IgM-p210 nat	0.67 ± 0.26	0.65 ± 0.26	.489
IgG-P210 nat	0.65 ± 0.33	0.59 ± 0.24	.709
IgM-p210 MDA	0.76 ± 0.25	0.76 ± 0.27	.823
IgG-p210 MDA	0.93 ± 0.32	0.85 ± 0.32	.016

Data expressed as mean ± standard deviation.

Table 3. Autoantibody levels related to the occurrence of postoperative events.

Event (n. of patients)	IgM-p210 nat	P	IgG-p210 nat	P	IgM-p210 MDA	P	IgG-p210 MDA	P
CV events (total)								
Yes (67)	0.65 ± 0.29	.904	0.55 ± 0.27	.066	0.77 ± 0.30	.681	0.85 ± 0.35	.314
No (284)	0.66 ± 0.25		0.63 ± 0.28		0.76 ± 0.25		0.88 ± 0.32	
CV fatal								
Yes (15)	0.61 ± 0.29	.468	0.47 ± 0.22	.016	0.69 ± 0.27	.273	0.66 ± 0.24	.003
No (336)	0.66 ± 0.26		0.62 ± 0.28		0.76 ± 0.26		0.88 ± 0.32	
CV non-fatal								
Yes (52)	0.67 ± 0.29	.779	0.58 ± 0.28	.373	0.80 ± 0.31	.323	0.90 ± 0.36	.765
No (284)	0.66 ± 0.25		0.63 ± 0.28		0.76 ± 0.25		0.88 ± 0.32	
Cerebrovascular events								
Yes (36)	0.69 ± 0.29	.437	0.60 ± 0.25	.657	0.79 ± 0.30	.465	0.83 ± 0.32	.309
No (315)	0.66 ± 0.25		0.62 ± 0.28		0.76 ± 0.26		0.88 ± 0.32	
AMI								
Yes (29)	0.63 ± 0.30	.766	0.54 ± 0.30	.157	0.74 ± 0.32	.674	0.91 ± 0.38	.870
No (322)	0.66 ± 0.25		0.62 ± 0.27		0.76 ± 0.26		0.87 ± 0.32	

Values are mean ± standard deviation. **CV events included AMI (n=29), stroke (n=21) TIA (n=11) and AF (n=6). CV death included 9 AMI and 6 strokes.**

Table 4
IgG-p210 nat. Multiple regression analysis. CV death as dependent categorical variable.

						CI for Exp(B)	
	B	**St. error**	**Wald**	**P-value**	**Exp(B)**	Lower bound	Upper bound
Constant							
IgG-p210 nat (<median)	1.910	.771	6.138	.013	6.752	1.490	30.592
Age	.051	.036	2.001	.157	1.052	.981	1.129
Gender	-.388	-650	.357	.550	.678	.190	2.424
Cholesterol (mmol/L)	.239	.241	.980	.322	1.270	.791	2.038
HDL-cholesterol (mmol/L)	-1.128	.979	1.328	.249	.324	.048	2.204
Smoking							
No+Yes 0 / Ex 1	.705	.805	.766	.381	2.024	.418	9.809
No+Ex 0 / Yes 1	-1.204	1.271	.897	.343	.300	.025	3.621
Hypertension	.229	.677	.114	.735	1.257	.333	4.741

Table 5
IgG-p210 MDA. Multiple regression analysis. CV death as dependent categorical variable.

						CI for Exp(B)	
	B	**St. error**	**Wald**	**P-value**	**Exp(B)**	Lower bound	Upper bound
Constant							
IgG-p210 MDA (<median)	2.054	.774	7.046	.008	7.797	1.711	35.517
Age	.058	.037	2.474	.116	1.060	.986	1.140
Gender	.010	.668	.0002	.989	1.010	.273	3.737
Cholesterol (mmol/L)	.273	.249	1.210	.271	1.314	.808	2.139
HDL-cholesterol (mmol/L)	-1.574	1.062	2.195	.138	.207	.026	1.662
Smoking							
No+Yes 0 / Ex 1	1.008	.808	1.557	.212	2.740	.563	13.346
No+Ex 0 / Yes 1	-.921	1.262	.533	.466	.398	.034	4.722
Hypertension	.062	.686	.008	.928	1.064	.277	4.079