Serum levels of anti-apolipoprotein A-1 auto-antibodies and myeloperoxidase as predictors of major adverse cardiovascular events after carotid endarterectomy

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Introduction

During the last decade, atherosclerosis has undergone a substantial pathophysiological paradigm shift, moving from a disease characterised mostly by a lipid metabolism anomaly, to an inflammatory driven condition, involving both innate and adaptive immunity (1). Furthermore, by fulfilling the "Koch's postulates", recent work suggests that atherosclerotic low-grade inflammation might be even considered as an autoimmune disease (2). As reviewed elsewhere, this hypothesis is supported by the fact that different auto-antibodies have been shown to predict poor cardiovascular (CV) outcome (3), and by the fact that these mediators might directly influence atherosclerotic processes triggering innate immune receptors’ signalling either toward a pro- or an anti-inflammatory response (3). Among the CV relevant autoantibodies, we and others (4-6) have focused on IgG against apolipoprotein A-1 (anti-apoA-1), the major protein fraction of high-density lipoprotein (HDL). High levels of anti-apoA-1 IgG have been initially described in patients with autoimmune diseases associated with an increased CV risk (such as systemic lupus erythematosus (4, 5),

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Summary

We aimed at challenging the prognostic accuracies of myeloperoxidase (MPO) and antibodies anti-apolipoprotein A-1 (anti-apoA-1 IgG), alone or in combination, for major adverse cardiovascular events (MACE) prediction, one year after carotid endarterectomy (CEA). In this prospective single centre study, 178 patients undergoing elective CEA were included. Serum anti-apoA-1 IgG and MPO were assessed by enzyme-linked immunosorbent assay prior to the surgery. Post-hoc determination of the MPO cut-off was performed by receiver operating characteristics (ROC) analyses. MACE was defined by the occurrence of fatal or non-fatal acute coronary syndromes or stroke during one year follow-up. Prognostic accuracy of anti-apoA-1 IgG was assessed by ROC curve analyses, survival analyses and reclassification statistics. During follow-up, 5% (9/178) of patients presented a MACE, and 29% (52/178) were positive for anti-apoA-1 IgG. Patients with MACE had higher median MPO and anti-apoA-1 IgG levels at admission (p=0.01), but no difference for the 10-year global Framingham risk score (FRS) was observed (p=0.22). ROC analyses indicated that both MPO and anti-apoA-1 IgG were significant predictors of subsequent MACE (area under the curve [AUC]: 0.75, 95% confidence interval [95%CI]: 0.61–0.89, p=0.01; and 0.74, 95%CI: 0.59–0.90; p=0.01), but combining anti-apoA-1 IgG positivity and MPO>857 ng/ml displayed the best predictive accuracy (AUC: 0.78, 95%CI: 0.65–0.91; p=0.007). It was associated with a poorer MACE-free survival (98.2% vs. 57.1%; p<0.001, LogRank), with a positive likelihood ratio of 13.67, and provided incremental predictive ability over FRS. In conclusion, combining the assessment of anti-apoA-1 IgG and MPO appears as a promising risk stratification tool in patients with severe carotid stenosis.

Keywords
Autoantibodies, myeloperoxidase, atherosclerosis, major adverse cardiovascular events, carotid stenosis

* These authors equally contributed as first authors to this work.
* These authors equally contributed as last authors to this work.
Material and methods

The Medical Ethics Committee of San Martino Hospital approved the study, and participants provided written informed consent before enrolment. The study was conducted in compliance with the Declaration of Helsinki.

Patient population and study design

The sample size was computed based on an expected prevalence of anti-apoA-1 IgG in patients with severe carotid stenosis of 20% (11), and taking into account a minimum of four-fold increased MACE risk observed in anti-apoA-1 IgG positive patients which was based upon previous published prospective studies on myocardial infarction and RA indicating that the MACE risk increased by four- to five-fold in presence of anti-apoA-1 positivity (8, 9). Accordingly to our power calculation for LogRank test, the minimal sample size requested to detect a four-fold increase in the risk of major event (5 vs. 20%) (8, 9) with a power of 80% and with a two-sided alpha error of 5% was of 164 patients. From April 2010 to April 2011, 96 supplemental patients were included. On those 208 patients available for analyses, hormone, cytokine, or growth factor therapies.

Endpoint definition

The unique and primary endpoint of this study was the occurrence of MACE one year after CEA, which was defined by the occurrence of fatal or non-fatal acute coronary syndromes (ACS) or stroke.

Patient follow-up and study endpoint adjudication

All patients completed the 12-month follow-up. Study endpoint adjudication was independently adjudicated by two of the study coordinators who were blinded to the results of biochemical analyses. Information was obtained during a check-up visit at one year and was further confirmed by checking patients’ medical file, targeting medical history relevant to the study endpoint.

Biochemical analyses

Blood chemistry including plasma glucose, haemoglobin A1c (HbA1c), insulin, triglycerides, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL) cholesterol and high-sensitive C-reactive protein (hs-CRP) were performed by routine autoanalysers.

Anti-apoA-1 IgG serum levels were measured as previously described (7-11). Briefly, Maxi-Sorb plates (Nunc) were coated with purified, human-derived delipidated apoA-1 (20 µg/ml; 50 µl/well) for 1 hour (h) at 37°C. After three washes with phosphate-buffered saline (PBS)/2% bovine serum albumin (BSA; 100 µl/well), all

primary anti-phospholipid syndrome (6), and rheumatoid arthritis (RA) (7, 8), or suffering from advanced atherosclerotic diseases (9-11). For instance, in patients with severe carotid stenosis, the presence of high circulating anti-apoA-1 IgG levels was associated with recognised features of local atherosclerotic plaque vulnerability, such as increased matrix metalloprotease (MMP)-9 expression, increased neutrophils infiltration, and decreased collagen III content (11). Mirroring this observation, passive immunisation of apoE/-/- mice with anti-apoA-1 IgG increased intraplaque inflammatory parameters (11). This study suggested that those autoantibodies might directly increase atherosclerotic plaque vulnerability in vivo (11). Nevertheless, knowing whether anti-apoA-1 IgG could predict poor CV outcome following carotid endarterectomy (CEA) remains elusive.

Therefore, we challenged the potential prognostic value of anti-apoA-1 IgG to predict major adverse cardiovascular events (MACE) one year after CEA in patients with severe carotid stenosis. Also, because the presence of anti-apoA-1 IgG in autoimmune disease have been described to be associated with dysfunctional HDLs (6, 12), known to be generated by myeloperoxidase (MPO)-catalysed apoA-1 oxidation (13, 14), we challenged the MPO prognostic accuracy for MACE prediction, as already demonstrated in myocardial infarction (15-17). Finally, we investigated whether combining anti-apoA-1 IgG with MPO could improve the predictive accuracy of any of those two biomarkers alone for MACE prediction.

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wells were blocked for 1 h with 2% BSA at 37°C. Samples were diluted 1:50 in PBS/2% BSA and incubated for 60 min. Additional patient samples at the same dilution were also added to an uncoated well to assess individual non-specific binding. After six further washes, 50 µl/well of signal antibody (alkaline phosphatase-conjugated anti-human IgG; Sigma-Aldrich, St. Louis, MO, USA) diluted 1:1,000 in PBS/2% BSA solution was incubated for 1 h at 37°C. After six more washes (150 µl/well) with PBS/2% BSA solution, the phosphatase substrate p-nitrophenyl phosphate disodium (50 µl/well; Sigma-Aldrich) dissolved in diethanolamine buffer (pH 9.8) was added. Each sample was tested in duplicate, and absorbance, determined as the optical density (OD) at 405 nm (OD405 nm), was determined after 20 min of incubation at 37°C (VersaMax, Molecular Devices, Sunnyvale, CA, USA). The corresponding non-specific binding value was subtracted from the mean absorbance value for each sample. The positivity cut-off was predefined as previously validated and set at an OD value of 0.6 and 37% of the positive control value, as described earlier (8-12). OD values ranged from 0.1-4.4, and corresponding index values were between 0 and 158.8%. The intra- and inter-assay variation coefficients at the cut-off level were 16% (n=10) and 12% (n=8), respectively.

Serum MPO levels were measured using the colourimetric enzyme-linked immunosorbent assay (ELISA) commercial kit purchased from R&D Systems (Minneapolis, MN, USA). Samples were run in duplicate according to the manufacturer’s instructions. The limit of detection was 1.56 ng/ml. Mean intra- and inter-assay coefficients of variation (CV) were below 8%. For MPO, as the cut-off has been described to vary widely among studies (15-17), and because no data are currently available for MACE prediction following CEA, we defined the cut-off based on post-hoc receiver operating curve (ROC) analysis.

Statistical analyses
Analyses were performed using Statistica™ software (StatSoft, Tulsa, OK, USA) and S-Plus 8.0 for Windows (Insightful Corp., Seattle, WA, USA). For exact logistic regression software StatXact 8.0.0 (Cytel Inc, Cambridge, MA, USA) was used. Fisher exact test and Mann–Whitney U-tests were used for comparisons when appropriate. Ranked Spearman correlations were performed to establish correlations between variables. The prognostic abilities of the Framingham risk score (FRS), anti-apoA-1 IgG, MPO and the sum anti-apoA-1 IgG and MPO over one year were assessed by ROC. The area under the curve (AUC) was given with the 95% confidence interval (CI) obtained using Analyse-It™ software for Excel (Microsoft, Redmond, WA, USA). The AUCs of two curves were compared using the DeLong’s method (20). Sensitivity, specificity, predictive values and likelihood ratios were assessed at a selected cut-off. A cut-off of 20% was used for FRS in order to have equilibrated events per group. No cut-off had been established for MPO, and we selected the cut-off corresponding to the point of the ROC curve the closest to the upper-left corner. The association between the risk of MACE and the indexes (anti-ApoA-1 IgG, FRS and MPO) categorised at these cut-off was assessed using exact logistic regressions and Kaplan-Meier analyses. Univariate odds ratios (OR) and ORs adjusted on the 10-year global FRS (19) (allowing adjusting for traditional CV risk factors within a single continuous variable) are presented. Reclassification statistics using the integrated discrimination index (IDI) compared the predictive performances of FRS, MPO, anti-apoA-1 IgG, and of the anti-apoA-1 IgG and MPO, as recommended by Pencina et al. (21). The predicted risk of MACE according to the categorised variables was calculated from the regression coefficients of logistic models and the integrated discrimination index (IDI) were derived from the predicted risks. The IDI between two models was interpreted as the difference between two models in the mean predicted risk in patients with the event minus the difference in the mean predicted risk in patients without the event. A value of p < 0.05 was considered statistically significant.

Results
Patient demographic characteristics are listed in Table 1. The frequency of high levels of anti-apoA-1 IgG was 29% (52/178). 5% (9/178) of the patients presented a MACE during one-year follow-up: among those, six patients presented an ACS with one fatality, two patients presented an ischaemic stroke, and one patient had a sudden death attributed to the occurrence of malignant arrhythmia.

Traditional risk factors, high-sensitive CRP and their association with subsequent MACE
As shown in Table 1, in patients with MACE during follow-up, the prevalence of known coronary artery disease was higher than in patients with a favourable outcome, the median diastolic pressure was lower as the proportion of patient under aspirin treatment. No difference was observed for the remaining parameters, including traditional CV risk factors. Notably, no significant differences were noted for the median FRS between patients with and without MACE (30 vs. 29.4%, p = 0.22; Table 1), which was confirmed by ROC curve analyses with a non-significant AUC of 0.62 (95%CI: 0.47-0.78, p = 0.19; Table 2). Also, no differences were noted in the median hs-CRP between patients with and without MACE during follow-up (2.23 vs. 2.95 mg/l; p = 0.25; Table 1), and ROC curve analyses confirmed the non-significant nature of this association (AUC: 0.62; 95%CI: 0.39-0.85, p = 0.15; Table 2).

Patients with a symptomatic carotid stenosis prior CEA were not found to be overrepresented in the group who developed a MACE when compared to asymptomatic patients (Table 1), and the degree of carotid stenosis prior surgery was not found to be a significant predictor of MACE upon ROC curve analyses (data not shown). No association was retrieved upon Spearman correlation between FRS and hs-CRP (r = 0.002, p = 0.97).
### Table 1: Patients’ demographic characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Severe carotid stenosis patients (n=178)</th>
<th>Patients with MACE (n=9)</th>
<th>Patients without MACE (n=169)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age in years</strong></td>
<td>72 (67–77; 48–93)</td>
<td>76 (65–78; 54–84)</td>
<td>72 (67–77; 48–93)</td>
<td>0.83</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
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<tr>
<td>Male, % (n)</td>
<td>64 (115)</td>
<td>89 (8)</td>
<td>63 (107)</td>
<td>0.16</td>
</tr>
<tr>
<td>Female, % (n)</td>
<td>46 (63)</td>
<td>11 (1)</td>
<td>37 (62)</td>
<td>-</td>
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<tr>
<td><strong>CV risk factors</strong></td>
<td></td>
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<tr>
<td>Diabetes, % (n)</td>
<td>32 (56)</td>
<td>56 (5)</td>
<td>30 (51)</td>
<td>0.14</td>
</tr>
<tr>
<td>Smoking, % (n)</td>
<td>25 (45)</td>
<td>11 (1)</td>
<td>26 (44)</td>
<td>0.45</td>
</tr>
<tr>
<td>Dyslipidaemia, % (n)</td>
<td>51 (91)</td>
<td>33 (3)</td>
<td>52 (88)</td>
<td>0.32</td>
</tr>
<tr>
<td>Hypertension, % (n)</td>
<td>58 (103)</td>
<td>33 (3)</td>
<td>60 (100)</td>
<td>0.17</td>
</tr>
<tr>
<td>Known CAD, % (n)</td>
<td>44 (34)</td>
<td>56 (5)</td>
<td>17 (29)</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Blood pressure, mmHg</strong></td>
<td></td>
<td></td>
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<tr>
<td>Systolic</td>
<td>135 (130–140;100–190)</td>
<td>130 (130–145;125–170)</td>
<td>135 (130–140;100–190)</td>
<td>0.64</td>
</tr>
<tr>
<td>Diastolic</td>
<td>80 (80–90;59–110)</td>
<td>75 (65–85;60–80)</td>
<td>80 (80–90;59–110)</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>10-year Global FRS at admission, %</strong></td>
<td>29.4 (18.4–30; 4.5–30)</td>
<td>30 (29.4–30;15.6–30)</td>
<td>29.4 (18.4–30;4.5–30)</td>
<td>0.22</td>
</tr>
<tr>
<td><strong>Medical treatment upon admission</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Aspirin, % (n)</td>
<td>49 (87)</td>
<td>11 (1)</td>
<td>51 (86)</td>
<td>0.03</td>
</tr>
<tr>
<td>Clopidogrel, % (n)</td>
<td>22 (39)</td>
<td>11 (1)</td>
<td>23 (38)</td>
<td>0.68</td>
</tr>
<tr>
<td>β-blockers, % (n)</td>
<td>3 (5)</td>
<td>0 (0)</td>
<td>3 (5)</td>
<td>1</td>
</tr>
<tr>
<td>ACE inhibitors, % (n)</td>
<td>40 (70)</td>
<td>22 (2)</td>
<td>40 (68)</td>
<td>0.48</td>
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<tr>
<td>AT-1 blockers, % (n)</td>
<td>23 (41)</td>
<td>0 (0)</td>
<td>24 (41)</td>
<td>0.12</td>
</tr>
<tr>
<td>Insulin, % (n)</td>
<td>2 (4)</td>
<td>0 (0)</td>
<td>2 (4)</td>
<td>1</td>
</tr>
<tr>
<td>Oral anti-diabetic agents, % (n)</td>
<td>8 (15)</td>
<td>11 (1)</td>
<td>8 (14)</td>
<td>0.56</td>
</tr>
<tr>
<td>Diuretics, % (n)</td>
<td>10 (18)</td>
<td>11 (1)</td>
<td>10 (17)</td>
<td>0.63</td>
</tr>
<tr>
<td>Calcium channel blockers, % (n)</td>
<td>28 (50)</td>
<td>0 (0)</td>
<td>30 (50)</td>
<td>0.06</td>
</tr>
<tr>
<td>Statins, % (n)</td>
<td>42 (75)</td>
<td>11 (1)</td>
<td>44 (74)</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>Biological parameters upon admission</strong></td>
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<tr>
<td>Total cholesterol, mg/dl</td>
<td>196 (165–225;100–455)</td>
<td>176 (148–210;100–361)</td>
<td>196.5 (165–226;100–455)</td>
<td>0.19</td>
</tr>
<tr>
<td>HDL, mg/dl</td>
<td>48 (41–62;15–130)</td>
<td>45 (43–60;34–90)</td>
<td>48 (41–62;15–130)</td>
<td>0.83</td>
</tr>
<tr>
<td>LDL, mg/dl</td>
<td>117 (90.8–143;32–349)</td>
<td>98 (78.8–110;35–275.8)</td>
<td>117.5 (90.9–144.2;32–349)</td>
<td>0.12</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>118 (90–63;46–471)</td>
<td>111 (109–194;75–244)</td>
<td>118 (90–162;46–471)</td>
<td>0.42</td>
</tr>
<tr>
<td>MPO, ng/ml</td>
<td>171.4 (62.1–416.8;0.4–6062)</td>
<td>462.7 (236.2–1160;149.8–1245.5)</td>
<td>167.4 (60.5–382.5;0.4–6062)</td>
<td>0.01</td>
</tr>
<tr>
<td>Hs-CRP, mg/l</td>
<td>2.80 (1.18–7.9;0.02–172.4)</td>
<td>2.23 (0.2–6.12;0.02–8.7)</td>
<td>2.95 (1.2–7.92;0.02–172.41)</td>
<td>0.25</td>
</tr>
<tr>
<td>Anti-apoA-1 IgG, index</td>
<td>25 (14.5–42.6, 0–158.8)</td>
<td>53.6 (24.4–57;20.8–59.6)</td>
<td>23.8 (14.4–41.1;0–158.8)</td>
<td>0.01</td>
</tr>
<tr>
<td>Anti-apoA-1 IgG, OD</td>
<td>0.38 (0.23–0.56;0–1.44)</td>
<td>0.48 (0.45–0.70;0.38–1.04)</td>
<td>0.37 (0.23–0.57–0–1.44)</td>
<td>0.02</td>
</tr>
<tr>
<td>Anti-apoA-1 IgG positivity, % (n)</td>
<td>29 (52)</td>
<td>67 (6)</td>
<td>27 (46)</td>
<td>0.02</td>
</tr>
</tbody>
</table>
Table 1: Continued

<table>
<thead>
<tr>
<th>Echographic findings before CAE</th>
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<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carotid stenosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptomatic; % (n)</td>
<td>20 (35)</td>
<td>22 (2)</td>
<td>20 (33)</td>
<td>1.00</td>
</tr>
<tr>
<td>Asymptomatic; % (n)</td>
<td>80 (142)</td>
<td>78 (7)</td>
<td>80 (135)</td>
<td>-</td>
</tr>
<tr>
<td>Stenosis in % of lumen</td>
<td>80 (70–85; 35–100)</td>
<td>80 (67–90; 50–100)</td>
<td>80 (70–85; 35–95)</td>
<td>0.76</td>
</tr>
</tbody>
</table>

All continuous variables are expressed as median (Interquartile Range [IQR]; and range). For continuous variables, U-Mann Whitney test was used for group comparisons between, and bilateral exact Fischer test was used for proportion comparisons between groups. CAD: coronary artery disease. ACE: angiotensin converting enzyme. AT-1: angiotensin-1 receptor.

**Anti-apoA-1 IgG and MPO association with the 10-year global FRS**

Ranked Spearman correlation demonstrated a weak but significant positive association between anti-apoA-1 IgG and FRS (r=0.16, p=0.04), whereas no significant association was found between MPO levels and FRS (r=0.001; p=1). No significant association was retrieved between anti-apoA-1 IgG and MPO levels (r=0.07; p=0.39).

**High serum levels of anti-apoA-1 IgG and MPO are positively associated with MACE 1 year after CEA**

Patients with MACE during follow-up had significantly higher median index of anti-apoA-1 IgG upon inclusion than patients without MACE (53.6 vs. 23.8 %; p=0.01), and the anti-apoA-1 IgG positivity rate was also higher in patients with MACE than those without (67% vs. 27%, p=0.02). Kaplan-Meier analyses confirmed that regardless of MPO values, patients positive for anti-apoA-1 IgG upon inclusion had a significantly worse complication-free survival at one year than those tested negative for those auto-antibodies (88.5% vs. 97.6%, p=0.01; Figure 1A). ROC curve analyses confirmed that anti-apoA-1 IgG was a significant predictor of subsequent MACE with an AUC of 0.74 (95%CI: 0.59-0.90; p=0.01). At the pre-specified cut-off, anti-apoA-1 IgG positivity was found to have a sensitivity (SE) of 73%, a specificity (SP) of 67%, a negative predictive value (NPV) of 98 % and positive predictive value (PPV) of 12% (Table 2). The positive and negative likelihood ratios were 2.46 and 0.46, respectively (Table 2). Finally, translated into exact OR, being positive for anti-apoA-1 IgG increased the risk of subsequent MACE by five-fold (OR: 5.29, 95%CI: 1.08-34.02, p=0.04), which did not remain significant after adjustment for FRS, although close to significance (OR: 4.81, 95%CI: 0.9731.06; p=0.06).

Also, patients with subsequent MACE during follow-up were found to have higher median serum levels of MPO upon admission when compared to patients with a favourable outcome (462.7 vs. 167.4 ng/ml, p=0.01; Table 1). ROC curve analyses confirmed the significant predictive accuracy of MPO for subsequent MACE with an AUC of 0.75 (95%CI: 0.61-0.89, p=0.0002, Table 2). Those analyses indicated that the optimal cut-off for MACE prediction was 857 ng/ml. At this cut-off, MPO was found to have a SE of 44%, a SP of 91%, a NPV of 97%, and a PPV of 21% (Table 2). The positive and negative likelihood ratios were 4.86 and 0.61, respectively (Table 2). Kaplan-Meier analyses demonstrated that irrespective of anti-apoA-1 IgG values, patients with baseline MPO levels above 857 ng/ml had a worse MACE-free survival at one year when compared to patients with baseline MPO levels below or equal to 857 ng/ml (80% vs. 96.7%; p=0.001; Figure 1B). Risk analyses indicated that having baseline values of MPO above 857 ng/ml increased the risk of subsequent MACE by eight-fold (OR: 7.77; 95%CI: 1.39-40.65; p=0.02), and remained of the same order of magnitude after the adjustment for the FRS (OR: 7.00; 95%CI: 1.24-36.63; p=0.03).

AUC differences between MPO and anti-apoA-1 IgG (0.75 vs. 0.74) were not found to be significant (p=0.94) according to the non-parametric method of Delong et al. (20).

**Serum MPO and anti-apoA-1 IgG combination for MACE prediction**

Combining anti-apoA-1 IgG with MPO positivity displayed an AUC for MACE prediction of 0.78 (0.65-0.91, p<0.0001; Table 2), which was not significantly higher than MPO alone, according to the Delong method (p=0.13). Kaplan-Meyer analyses indicated that risk of MACE was 1.8% (2/109) in patients tested negative for both anti-apoA-1 IgG and MPO, 6.7 % (3/45) when patients were tested positive for anti-apoA-1 IgG but negative for MPO (p=0.13, LogRank), 8.3% (1/12) in patients tested positive for MPO but negative for anti-apoA-1 IgG, and 42.1% (18/43) when patients tested positive for both anti-apoA-1 IgG and MPO. The combination of anti-apoA-1 IgG and MPO resulted in a risk of 55.4% (24/43) at the pre-specified cut-off, with a positive likelihood ratio of 13.3 and a negative likelihood ratio of 0.3, respectively (Table 2). Finally, Kaplan-Meier analyses indicated that the optimal cut-off for MACE prediction was 857 ng/ml. At this cut-off, MPO was found to have a SE of 44%, a SP of 91%, a NPV of 97%, and a PPV of 21% (Table 2). The positive and negative likelihood ratios were 4.86 and 0.61, respectively (Table 2). Kaplan-Meier analyses demonstrated that irrespective of anti-apoA-1 IgG values, patients with baseline MPO levels above 857 ng/ml had a worse MACE-free survival at one year when compared to patients with baseline MPO levels below or equal to 857 ng/ml (80% vs. 96.7%; p=0.001; Figure 1B). Risk analyses indicated that having baseline values of MPO above 857 ng/ml increased the risk of subsequent MACE by eight-fold (OR: 7.77; 95%CI: 1.39-40.65; p=0.02), and remained of the same order of magnitude after the adjustment for the FRS (OR: 7.00; 95%CI: 1.24-36.63; p=0.03).

AUC differences between MPO and anti-apoA-1 IgG (0.75 vs. 0.74) were not found to be significant (p=0.94) according to the non-parametric method of Delong et al. (20).

**Serum MPO and anti-apoA-1 IgG combination for MACE prediction**

Combining anti-apoA-1 IgG with MPO positivity displayed an AUC for MACE prediction of 0.78 (0.65-0.91, p<0.0001; Table 2), which was not significantly higher than MPO alone, according to the Delong method (p=0.13). Kaplan-Meyer analyses indicated that risk of MACE was 1.8% (2/109) in patients tested negative for both anti-apoA-1 IgG and MPO, 6.7 % (3/45) when patients were tested positive for anti-apoA-1 IgG but negative for MPO (p=0.13, LogRank), 8.3% (1/12) in patients tested positive for MPO but negative for anti-apoA-1 IgG, and 42.1% (24/43) when patients tested positive for both anti-apoA-1 IgG and MPO. The combination of anti-apoA-1 IgG and MPO resulted in a risk of 55.4% (24/43) at the pre-specified cut-off, with a positive likelihood ratio of 13.3 and a negative likelihood ratio of 0.3, respectively (Table 2). Finally, Kaplan-Meier analyses demonstrated that irrespective of anti-apoA-1 IgG values, patients with baseline MPO levels above 857 ng/ml had a worse MACE-free survival at one year when compared to patients with baseline MPO levels below or equal to 857 ng/ml (80% vs. 96.7%; p=0.001; Figure 1B). Risk analyses indicated that having baseline values of MPO above 857 ng/ml increased the risk of subsequent MACE by eight-fold (OR: 7.77; 95%CI: 1.39-40.65; p=0.02), and remained of the same order of magnitude after the adjustment for the FRS (OR: 7.00; 95%CI: 1.24-36.63; p=0.03).
negative for anti-apoA-1 IgG (p=0.16, LogRank), and 42.9% (3/7) when patients were tested positive for both anti-apoA-1 IgG and MPO (p<0.001, LogRank; Figure 1C).

Being positive for both MPO and anti-apoA-1 IgG had a SE of 33%, a SP of 99%, a PPV of 60%, and a NPV of 97% (Table 2). The positive and negative likelihood ratios were 13.67 and 0.68, respectively (Table 2). The risk in patients positive for both MPO and anti-apoA-1 IgG was strongly increased to 20-fold (OR: 19.57; 95%CI: 1.86-128.25; p=0.01).

Reclassification statistics

Reclassification statistics indicated that the mean predicted risk of MACE in patients presenting a MACE during follow-up was 8.4% for anti-apoA-1 IgG, 11% for MPO, 4.9% for FRS, and 15.9% for the anti-apoA-1 IgG and MPO combination. In patients without MACE during follow-up, the predicted risks of MACE were of 4.8%, 4.8%, 3.1%, and 4.4%, respectively (Table 3).

The IDI values comparing the predictive ability of anti-apoA-1 IgG vs. FRS was 1.8% (p=0.02), and the IDI comparing MPO vs. the FRS was 4.4% (p=0.15).

The anti-apoA-1 IgG/MPO combination when compared to anti-apoA-1 IgG alone and FRS yielded an IDI of 8% (p = 0.008) and 9.7% (p = 0.04), respectively. The combination did not show increased predictive ability when compared to MPO alone, although a trend was observed (IDI: 5.3%, p=0.08; Table 3).

Those results indicate that i) anti-apoA-1 IgG alone and in combination with serum MPO provide incremental predictive ability over FRS, but not MPO, although close to significance, ii) the anti-apoA-1 IgG/MPO combination provides significant incremental predictive ability when compared to either the FRS or anti-apoA-1 IgG alone (Table 3).

Discussion

This preliminary study demonstrates the proof of principle that both serum anti-apoA-1 IgG and MPO, as well as their combination, can provide incremental information over FRS for CV risk prediction in patients undergoing elective surgery for severe carotid stenosis. Those results confirm and extend previous findings demonstrating the CV prognostic value of anti-apoA-1 IgG in other high-risk settings such as RA, myocardial infarction and acute chest pain patients (8-10). Also, by demonstrating the poor CV prognostic associated to high levels of anti-apoA-1 IgG, those results complete our previous observation indicating that high levels of circulating anti-apoA-1 IgG were positively associated with features of atherosclerotic plaque instability in humans and apoE-/-mice (11). If higher levels of MPO were initially shown to be associated with the progression of carotid stenosis in humans (22),

| Table 2: Predictive accuracy of anti-ApoA-1 IgG, FRS and MPO subsequent MACE occurrence according to ROC curve analyses. |
|-----------------------------------------------|----------------|----------------|----------------|
| AUC (95% CI) p-value | Anti-ApoA-1 IgG | FRS | MPO | MPO + anti-apoA-1 IgG |
| 0.74 (0.59–0.90) 0.01 | 0.62 (0.47–0.78) 0.19 | 0.75 (0.61–0.89) 0.01 | 0.78 (0.65–0.91) 0.007 |

* AUC were computed using continuous values of anti-apoA-1 IgG MPO, and of the addition of anti-apoA-1 IgG to MPO. SP: specificity, SE: sensitivity, PPV: positive predictive value, NPV negative predictive value, LR+: positive likelihood ratio, LR-: negative likelihood ratio.
this single-centre prospective study is, to the best of our knowledge, the first to confirm the CV prognostic value of MPO following CEA for severe carotid stenosis.

From a clinical point of view, anti-apoA-1 IgG and MPO alone looked appealing because of their high NPV (96% and 98%, respectively) for subsequent MACE prediction, which is nevertheless mostly explained by the low prevalence (5%) of MACE occurrence in this population, setting the rate of non-event at 95%. On the other hand, their respective SP, PPV and of both biomarkers alone were too low to be clinically useful for rule-in purposes. However, being positive for both MPO and anti-apoA-1 IgG which occurred in 4% (7/178) of patients, markedly increased the risk of subsequent MACE to 42.9%, which was deemed to be significant when compared to patients tested negative for both biomarkers (Log Rank, p<0.001) and translated into a positive LR of 13.69 for MACE prediction, which exceeded the recommended standard value of 10 to be clinically meaningful as a rule-in test (23). This was partly supported by reclassification statistics, which demonstrated a significant predictive ability of the combination over anti-apoA-1 IgG alone (IDI: 8%, p=0.008) and a trend was noted when comparing the combination against MPO alone (IDI: 5.3%, p=0.08). Taken together these results suggest that the combination of low anti-apoA-1 IgG and low MPO levels could be of help to identify patients at very low risk of subsequent CV complications after CEA, whereas in the presence of double positivity, our preliminary results indicate that this biomarker pattern could be of help for identifying a minor subset of patients (4%) at very high risk of subsequent MACE. Nevertheless, due to power limitation, based upon the present study, we cannot conclude to the superiority of any of those two biomarkers over one other. Larger prospective cohort studies are needed to confirm our preliminary results before any definite conclusions or recommendations can be made.

From a pathophysiological point of view, in vitro results support a pro-arrhythmogenic effect of anti-apoA-1 IgG mediated by a protein kinase A-dependent activation of L-type calcium channels (24), as well as a direct pro-inflammatory effect of anti-apoA-1 autoantibodies (8, 11) mediated through TLR2/CD14 complex signalling (25). Another possible pro-inflammatory mechanism inferred to anti-apoA-1 IgG is their ability to specifically promote chemotaxis of human primary neutrophils through unknown
mechanisms, whereas no effect was retrieved for lymphocytes and monocytes (11). Finally, another mutually non-exclusive possibility linking anti-apoA-1 IgG to cardiovascular disease could be related to HDL dysfunction. Indeed, the presence of anti-apoA-1 antibodies were shown to be associated with dysfunctional HDL (5, 6) and related to a decrease in paraoxonase (PON)-1 activity, leading to an increase of pro-inflammatory reactive oxygen species in mice (12). Nevertheless, the possible causal relationship between the presence of anti-apoA-1 IgG and dysfunctional HDL needs to be demonstrated.

In accordance with those observations, passive immunisation of apoE knockout mice with anti-apoA-1 IgG increases atherosclerosis and induces a more vulnerable atherosclerotic plaque phenotype (11). Therefore, it is hypothesised that the superposition of both pro-arythmogenic and pro-inflammatory properties of anti-apoA-1 IgG could partly explain their prognostic value. This hypothesis is currently devoid of published experimental evidence, and is under active investigation in our laboratory. Furthermore, clinical and animal studies indicate that anti-apoA-1 IgG autoantibodies could also impede some HDL-related anti-atherogenic properties, leading to dysfunctional HDL (5, 6, 12, 14). Since MPO-driven apoA-1 modifications, such as tyrosine chlorination, oxidation or nitration have been proposed to be important determinants of dysfunctional HDL generation (14, 15, 26), we hypothesised that the occurrence of anti-apoA-1 IgG could be related to high MPO levels. Nevertheless, the fact that no associations between anti-apoA-1 IgG and MPO were retrieved in the present study does not support the hypothesis that MPO-modified apoA-1 and the presence of anti-apoA-1 IgG are interrelated processes.

This study has several limitations. Firstly, despite being appropriately powered according to our estimations, this single-centre study has a limited sample size and a very limited number of events, giving rise to broad CIs. A second limitation of this work is related to the fact that very stringent exclusion criteria were applied for patient selection, impeding us to apply those preliminary results to patients with atrial fibrillation, autoimmune, liver, renal, or inflammatory bowel disease. The reason for applying such stringent selection criteria was to obtain a cohort suffering strictly from atherosclerosis without any other potential inflammatory or infectious confounding factor, and this feature constitutes strength of this cohort. Another possible limitation may be related to a matrix issue in the sense that serum rather than EDTA plasma was used for the MPO measurements in the present study. Indeed, Shih et al. showed that EDTA plasma was one of the critical pre-analytical requirements for MPO assessment, and should be the preferred matrix for MPO measurements (27). Nevertheless, because the commercial ELISA kit used in the present study was validated for serum MPO assessment, and because abundant data in the literature indicate that serum is an acceptable alternative matrix (15, 28), we believe that our data were not technically biased, especially when all the other pre-analytical MPO-related requirements were met. Thirdly, another limitation is related to the fact that the MPO cut-off used in this study was defined in post-hoc fashion based upon ROC curve analyses. This approach was used because the prognostic value of serum MPO on this kind of patients has never been tested before. Therefore, further studies are required to validate the proposed MPO cut-off. On the other hand, these results confirm that the prospectively chosen anti-apoA-1 IgG cut-off is also clinically relevant in patients following CEA. A fourth limitation is related to the fact that we used the FRS in secondary prevention settings where the relevance of this score has not yet been validated. The reason to use this score was mostly to be able to adjust for most of the traditional CV risk factors within a single integrative continuous value because of the expected

Table 3: Reclassification statistics.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Mean predicted risk</th>
<th>Improvement in predicted risk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MACE</td>
<td>No MACE</td>
</tr>
<tr>
<td>Anti-apoA-1 IgG +</td>
<td>8.4%</td>
<td>4.8%</td>
</tr>
<tr>
<td>MPO &gt; 857 ng/ml (+)</td>
<td>11.0%</td>
<td>4.8%</td>
</tr>
<tr>
<td>FRS &gt; 20%</td>
<td>4.9%</td>
<td>3.1%</td>
</tr>
<tr>
<td>Anti-apoA-1 IgG + and MPO +</td>
<td>15.9%</td>
<td>4.4%</td>
</tr>
</tbody>
</table>

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What is known about this topic?

- Emerging evidence from both basic research and clinical studies suggests that auto-antibodies might play a direct pathogenic role in atherosclerosis.
- High levels of anti-apoA-1 IgG have been shown to be an independent predictor of MACE in acute coronary syndromes and in patients with Rheumatoid Arthritis and to be associated with increased intraplaque features of atherosclerotic plaque vulnerability in humans and mice.

What does this paper add?

- Combination of serum levels of anti-apoA-1 IgG and MPO provide incremental CV predictive ability over the classical FRS in patients with severe carotid stenosis undergoing endarterectomy.
- Although validation from larger clinical study is needed, these serum biomarkers might be useful clinical tools to better assess the CV risk in atherosclerotic patients after elective CEA.

References


Conflicts of interest
None declared.


