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ABSTRACT

Objective: We aimed to determine whether autoantibodies against apolipoprotein A-1 (anti-apoA-1 IgG) predict incident coronary artery disease (CAD), defined as adjudicated incident myocardial infarction, angina, percutaneous coronary revascularization or bypass grafting, in the general population. We further investigated whether this association is modulated by a functional CD14 receptor single-nucleotide polymorphism (SNP).

Approach and Results: In a prospectively-studied, population-based cohort of 5220 subjects (mean age 52.6 ± 10.7 years, 47.4% males), followed over a median period of 5.6 years, subjects positive *vs.* negative for anti-apoA-1 IgG, presented a total CAD rate of 3.9% *vs.* 2.8% (p=0.077), and a non-fatal CAD rate of 3.6% vs. 2.3% (p=0.018) respectively. After multivariate adjustment, the hazard ratio (HR) of anti-apoA-1 IgG were: HR=1.36 [(95% Confidence Intervals (CI) 0.94–1.97), p=0.105], and HR=1.53 [(95%CI 1.03–2.26), p=0.034], for total and non-fatal CAD, respectively. In subjects with available genetic data for the C260T *rs2569190* SNP in the CD14 receptor gene (n=4247), we observed a significant interaction between anti-apoA-1 IgG and *rs2569190* allele status with regards to CAD risk, with anti-apoA-1 IgG conferring the highest risk for total and non-fatal CAD in non-TT carriers, whereas being associated with the lowest risk for total and non-fatal CAD in TT homozygotes ("p-for-interaction"=0.011 and "p-for interaction"=0.033 respectively).

Conclusions: Anti-apoA-1 IgG are independent predictors of non-fatal incident CAD in the general population. The strength of this association is dependent on a functional polymorphism of the CD14 receptor gene, a finding suggesting a "gene-autoantibody" interaction for the development of CAD.

ABBREVIATIONS

CAD: coronary artery disease
Anti-apoA-1 IgG: anti-apolipoprotein A-1 autoantibodies
TLR: toll-like receptor
SNP: single nucleotide polymorphism
DAMP: danger-associated molecular pattern
HDL: high-density lipoprotein cholesterol
LDL: low-density lipoprotein cholesterol
SBP: systolic blood pressure
FU: follow-up
eGFR: estimated glomerular filtration rate
CVRF: cardiovascular risk factors
HR: hazard ratio
OD: optical density

INTRODUCTION

Major discoveries in the pathophysiology of atherosclerosis have established the fundamental role of a chronic inflammatory state in the initiation, progression and - finally - rupture of the atherosclerotic plaque. [1] During the last decade, humoral autoimmunity and autoantibodies have been recognized as important modulators of vascular inflammation and atherogenesis. [2] Autoantibodies can be active mediators in the development of coronary artery disease (CAD) [3, 4] and as such, serve as biomarkers for the prediction of incident CAD [5-9] and potentially as biological targets amenable to specific immunomodulatory therapies.

Recently, the atherogenic role of autoantibodies against apolipoprotein A-1 (anti-apoA-1 IgG), the principal protein component of high-density lipoprotein (HDL) has been investigated in clinical studies, showing that anti-apoA-1 IgG are associated with prevalent and incident CAD in subjects with autoimmune diseases [5], subjects at high CV risk [6, 10] or following myocardial infarction [4, 7], independently of established cardiovascular risk factors (CVRFs). Furthermore, we recently showed that anti-apoA-1 IgG are present in up to one-fifth of individuals in the general population and independently associated with prevalent CAD [11] as well as with all-cause mortality [12]. Nevertheless, their predictive value for incident CAD in the general population has not yet been studied.

From a pathophysiological point of view, *in vitro* and *in vivo* studies have demonstrated that anti-apoA-1 IgG *per se* behave as pro-inflammatory, pro-arrhythmogenic and pro-thrombotic molecules, promoting atherogenesis, myocardial necrosis and death in mice. [4, 13] Based upon previous published studies, such events could be related to a chronic low-grade inflammatory state, [3, 14, 15], associations with elevated high-sensitivity C-reactive protein and with increased uric acid levels, [11] impairment of HDL anti-atherogenic properties, [16-18], interference with basal heart rate regulation [7, 11, 15] or breakdown of self-tolerance. [12] However, the main pathophysiological mechanism – reported so far – underpinning the pathogenicity of anti-apoA-1 IgG is their interaction with innate immune system receptors and the activation of the TLR2/TLR4/CD14 complex. [14, 19] In particular, the current paradigm suggests that, due to molecular mimicry of the C-terminal part of ApoA-1 to Toll-like receptor (TLR) TLR2, anti-apoA-1 IgG bind to the TLR2/TLR4 complex, and require a functional CD14 receptor for effective intracellular signaling, NF-κB and MAPK downstream activation, and production of pro-inflammatory cytokines. [13]

These findings point to CD14 receptor, the canonical ligand of lipopolysaccharid (LPS), as a major effector of the anti-apoA-1 IgG deleterious properties. A functional single nucleotide polymorphism (SNP) at position C260T (*rs2569190)* of the CD14 receptor gene has been shown to modulate its transcriptional activity. [20, 21] Among the three groups of CD14 genotypes for *rs2569190* (CC, CT or TT), TT carriers appear to be protected from CD14 ligand-induced inflammation due to a better ability to adequately control the LPS-mediated TLR/CD14–dependent immune response. [22-24] Indeed, previous studies demonstrated that TT carriers were less at risk for Gram-negative bacterial infection and sepsis death, [25, 26] for developing heart failure, [27] as well as atherosclerosis, [28-30] although this latter observation is debated. [31] However, whether TT carriers are also less susceptible to anti-apoA-1 IgG-related atherosclerosis has not been examined.

Thus, our current study had two main aims: firstly, we investigated whether anti-apoA-1 IgG predict incident CAD in the general population. Secondly, because of anti-apoA-1 IgG role as a danger-associated molecular pattern (DAMP), specifically activating CD14-related pathways [4,

[13], we further examined whether the functional C260T *rs2569190* polymorphism in the CD14 receptor gene modulates the anti-apoA-1 IgG-related CAD risk, hypothesizing a protective effect associated with carriage of the T allele.

MATERIALS AND METHODS

Materials and Methods, including characterization analyses related to anti-apoA-1 IgG assay validation are available in the online-only Data Supplement.

RESULTS

Association between anti-apoA-1 IgG and incident CAD

Figure 1 demonstrates the flowchart of the study. Of the initial 6733 participants, 5220 had complete clinical and biological data over a median follow-up (FU) time of 5.6 years and were included in the final sample. Participants that did not participate in FU (21.6%) were more likely to be smokers, hypertensive, overweight with a less favorable lipid profile, compared to those included in the analysis. There were no significant differences in anti-apoA-1 IgG levels or prevalence of anti-apoA-1 IgG positivity between the two groups **(Supplementary Table I)**.

Table 1 provides baseline characteristics of the final sample according to anti-apoA-1 IgG status. Overall, CVRFs were equally distributed between subjects with positive *vs.* negative anti-apoA-1 IgG titers. Among the 157 subjects who developed CAD during FU, 132 had a non-fatal event, and 25 a fatal one. Total Incident CAD rate was 3.9% *vs.* 2.8% (p=0.077), while non-fatal incident CAD rate was 3.6% vs. 2.3% (p=0.018) for subjects with positive *vs.* negative anti-apoA-1 IgG titers. No significant differences were observed with regards to fatal incident CAD.

Table 2 summarizes hazard ratios (HR) for the association of anti-apoA-1 IgG with total, non-fatal and fatal incident CAD. In unadjusted models, we retrieved a trend between anti-apoA-1 IgG positivity and total incident CAD (HR: 1.39, 95% Confidence Intervals (CI): 0.97–1.99, p=0.073), that remained unchanged after adjusting for sex, age, smoking status, diabetes, systolic blood pressure (SBP), LDL and HDL cholesterol, baseline CAD, statin and beta-blocker treatment, and estimated glomerular filtration rate (eGFR) (HR: 1.36, 95%CI: 0.94–1.97, p=0.105). The HRs of one SD increase in log-transformed anti-apoA-1 IgG values for total incident CAD were HR: 1.11, 95%CI: 0.96–1.28 (p=0.159), and HR: 1.09, 95%CI: 0.94–1.27 (p=0.232), in the unadjusted and adjusted analysis respectively. Levels of anti-apoA-1 IgG above OD>0.98 (3rd tertile) were significantly associated with total incident CAD in the unadjusted (HR 1.79, 95%CI 1.09–2.95, p=0.021) and the adjusted analysis (HR: 1.70, 95%CI: 1.03–2.81, p=0.038).

Furthermore, anti-apoA-1 IgG positivity was significantly associated with non-fatal incident CAD both in the unadjusted (HR: 1.58, 95%CI 1.08–2.31, p=0.018) and the adjusted analysis (HR: 1.53, 95%CI 1.03–2.26, p=0.034). Similarly to what was observed for total incident CAD, the HRs of one SD increase in log-transformed anti-apoA-1 IgG values for non-fatal CAD were: HR: 1.15, 95%CI 0.99–1.34, (p=0.072), and HR: 1.14, 95%CI: 0.97–1.33 (p=0.109), in the unadjusted and adjusted analysis respectively. Anti-apoA-1 IgG levels above OD>0.98 (3rd tertile) were strongly associated with non-fatal incident CAD both in the unadjusted (HR: 2.21, 95%CI 1.34–3.67, p=0.002) and the adjusted model (HR: 2.14, 95%CI 1.29–3.56, p=0.003) **(Table 2)**. On the other hand, no associations were observed between anti-apoA-1 IgG positivity or tertiles with fatal incident CAD. Sensitivity analyses after exclusion of subjects with baseline CAD or autoimmune disease yielded similar results for the associations between anti-apoA-1

IgG and total, non-fatal and fatal CAD **(Supplementary Table II)**. Additionally, statistical analyses after excluding adjustment for statin and beta-blocker treatment, or eGFR from the fully adjusted model yielded similar results **(Supplementary Table, III)**.

Interaction between C260T *rs2569190* polymorphism and anti-apoA-1 IgG for incident CAD

Among genotyped subjects (n=4247, **Figure 1**), we further investigated whether the functional C260T *rs2569190* polymorphism in the CD14 receptor gene modulates anti-apoA-1 IgG-related CAD risk. Subjects with missing genetic data tended to have a lower burden of CVRFs and a higher prevalence of anti-apoA-1 IgG positivity **(Supplementary Tables IV & V).**

Characteristics of the genotyped sample, according to the C260T *rs2569190* polymorphism allele status are illustrated in **Supplementary Table VI**. All CVRFs were equally distributed among subgroups, with the exception of an increased prevalence of diabetes and statin treatment in the TT subgroup. Importantly, the C260T *rs2569190* polymorphism *per se* was not associated with total, non-fatal or fatal incident CAD, all-cause mortality or anti-apoA-1 IgG positivity **(Supplementary Table VI)**.

In order to assess differences in anti-apoA-1 IgG-related CAD risk according to the C260T *rs2569190* polymorphism, we created both an additive (CC *vs.* CT *vs.* TT) as well as a recessive (CC/CT *vs.* TT) model and performed a statistical test for the interaction [32] between anti-apoA-1 IgG and carriage of the T allele, for total and non-fatal incident CAD risk. As previously, all analyses were adjusted for sex, age, smoking status, diabetes, SBP, LDL and HDL cholesterol, baseline CAD, statin and beta-blocker treatment and eGFR.

In the case of the additive model (CC *vs.* CT *vs.* TT), we observed a gradient of risk for anti-apoA-1 IgG with regards to CAD across the three predefined C260T *rs2569190* subgroups **(Table 3)**. Specifically, in the subgroup homozygote for the major allele (CC, n=1097), the adjusted anti-apoA-1 IgG HR for total CAD was HR: 2.27; 95%CI: 1.04–4.97, (p=0.039), while it was HR: 1.52; 95%CI: 0.86–2.71, (p=0.152) in the heterozygote subgroup (CT, n=2095), and HR: 0.55; 95%CI: 0.19–1.61, (p=0.275) in the minor allele subgroup (TT, n=1055). Results were similar with regards to the recessive (CC/CT *vs.* TT) model. Notably, in non-TT carriers – representing 75.1% of the cohort, anti-apoA-1 IgG positivity conferred a 1.8-fold risk for total CAD (HR 1.77; 95%CI 1.12–2.80, p=0.014, **Table 3**), while change per 1SD in anti-apoA-1 values yielded a HR of 1.11; 95%CI 0.92–1.34 (p=0.285) for total CAD, in the fully adjusted model. Results were similar with regards to non-fatal incident CAD **(Table 3).**

Testing for the interaction between anti-apoA-1 IgG and C260T *rs2569190* polymorphism with respect to CAD in a fully adjusted analysis, indicated that the observed gradient in anti-apoA-1 IgG-related CAD risk across the different CD14 genotype subgroups in the additive (CC *vs.* CT *vs.* TT) model was statistically significant, for both total and non-fatal CAD risk ("p-for-interaction"=0.011, and "p-for-interaction"=0.033, respectively; **Table 3**), proving substantial heterogeneity in anti-apoA-1 IgG-related CAD risk according to T allele carriage. A forest plot summarizes these findings **(Supplementary Figure 1)**. Furthermore, statistical analyses after excluding adjustment for statin and beta-blocker treatment, or eGFR from the fully adjusted model yielded similar results **(Supplementary Table, VII)**.

Figure 2 describes Kaplan-Meier curves for total and non-fatal CAD according to anti-apoA-1 IgG positivity and C260T *rs2569190* allele status. Participants positive for anti-apoA-1 IgG (panels A and B) presented an increased risk for total and non-fatal CAD compared to those negative for anti-apoA-1 IgG. After splitting the positive anti-apoA-1 IgG group according to homozygous or not carriage of the T allele (CC/CT *vs.* TT), a decrease in the proportion of total

and non-fatal CAD was observed in the anti-apoA-1 IgG positive TT subgroup (panels C and D, green line), falling below the rate of CAD observed in anti-apoA-1 IgG negative subjects (panels C and D, blue line). Conversely, higher proportion of total and non-fatal CAD was observed in anti-apoA-1 IgG positive non-TT carriers (panels C and D, black line), when compared to anti-apoA-1 IgG positive subjects as a whole (panels C and D red line, log-rank: p=0.023, and p=0.017, for total and non-fatal CAD respectively).

DISCUSSION

The main finding of the present study is that anti-apoA-1 IgG are independently associated with non-fatal incident CAD in the general population, with the anti-apoA-1 IgG-related CAD risk being strongly modulated by the C260T *rs2569190* CD14 gene polymorphism. Indeed, after taking CD14 SNPs into account, we observed a significant anti-apoA-1 IgG-related CAD risk gradient, dependent on carriage of the C260T *rs2569190* T allele, with non-TT carriers being at significantly increased risk for both total and non-fatal CAD compared to TT homozygotes. Our results extend current knowledge not only in the field of anti-apoA-1 IgG, but also in the field of personalized CAD prediction in different ways.

Firstly, similarly to what has been shown in high risk populations, [5-7, 33], our current findings argue that anti-apoA-1 IgG positivity is an independent predictor of poor CV outcome in the general population, supporting the notion that preclinical autoimmunity to apolipoprotein A-1 may identify a substantial proportion of individuals at increased risk of CAD. In our study, anti-apoA-1 IgG-related CAD risk was highest in subjects carrying at least one C allele (CC/CT) in the functional C260T *rs2569190* polymorphism, a group that represents roughly three quarters of Caucasian populations. [31] By virtue of being the first study on a "gene-autoantibody" interaction with respect to CAD, our analysis highlights the importance of incorporating genetic data on immune-related polymorphisms when evaluating anti-apoA-1 IgG-related risk and provides insight for future study design on personalized CAD prediction.

Secondly, these results represent a human validation of the key role of CD14 co-receptor in mediating the anti-apoA-1 IgG pro-atherogenic properties as demonstrated so far in animal and *in vitro* models, [4, 13] and reinforce the relevance of these preclinical results to the anti-apoA-1 IgG-associated CAD risk in humans. Conversely, in line with a recent meta-analysis, [31] our findings are equivocal and do not provide definite evidence with regards to the association between TT genotype carriage and CAD risk.

Thirdly, our data highlight the importance of considering the individual genetic information on innate immune receptors for proper assessment of CAD risk associated to biomarkers of humoral autoimmunity. To the best of our knowledge, none of the genetic studies published so far took into account biomarkers (including autoantibodies) for CAD risk prediction, and none of the publications exploring the auto-antibodies-associated CAD risk prediction evaluated the impact of individual genetic background on such risk. By demonstrating a potentially important gene-environment interaction between anti-apoA-1 IgG and the CD14 receptor gene in the pathogenesis of atherosclerosis, our findings may explain the discordant findings regarding both the role of CD14 polymorphisms in CAD risk, [31] and the contrasting results of humoral auto-autoimmunity in CAD risk assessment. [33] Overall, our results provide a "proof-of-concept" that combining genetic data together with serum biomarkers is likely to be required for the implementation of precision medicine in the field of CAD prediction.

In our study, the fact that TT carriers were less at risk to develop anti-apoA-1 IgG-related CAD compared to non-TT carriers, despite TT homozygote status being previously associated with a higher systemic inflammatory profile, [20, 21] merits mention. To this respect, several lines of evidence indicate that TT genotype could confer protection against uncontrolled inflammatory response evoked by long-term DAMP exposure through different and mutually non-exclusive mechanisms. Firstly, previous studies indicate that in the context of chronic low grade CD14/TLR4 stimulation, the higher levels of sCD14 ascribed to TT genotype inhibit systemic LPS-mediated inflammatory responses by down-regulating inflammatory cytokines transcription

[34, 35], and facilitating CD14-related DAMP clearance [24], thus protecting TT carriers against sustained inflammatory responses, through a negative feedback mechanism. Inversely, lower levels of sCD14 observed in CC carriers have been shown to favor vascular wall inflammation and atherogenesis through impaired plasma clearance of endotoxin. [22, 23] C-allele carriers may be less able to prevent anti-apoA-1 IgG-mediated CD14/TLR4 activation, resulting in maintenance of a pro-atherogenic state and a higher risk for developing CAD. [28-30] Lastly, increased expression of CD14 on the vascular endothelium of TT homozygotes [35] could also play a role in atherogenesis, in response to CD14 ligands such as anti apoA-1 IgG. [22, 23, 34, 35]

Several study limitations are noteworthy. Firstly, although great effort was undertaken to maximize the participation rate during follow-up, our results may be subject to attrition bias as drop out rate after mean duration of 5.6 years was about ~20%. Nevertheless, similar losses in follow up are commonly reported in prospective cohorts [36] and are within the conventional participation rate thresholds for cohort studies. [37] Secondly, we didn't directly measure sCD14 in study subjects, in order to confirm the presumed higher sCD14 levels in TT homozygote carriers reported previously. Moreover, as our assay assesses anti-apoA-1 IgG antibodies against native apoA-1, [19, 38] we were not able to measure antibodies against modified forms of apoA-1, such as oxidized apoA-1 (or possibly glycated and carbamylated apoA-1). As these modified forms of apoA-1 were shown to be of relevance for HDL functionality and the pathology of atherosclerosis, [39-41] knowing whether they would elicit a humoral response clinically relevant to human physiopathology is still unclear. Thirdly, due to sample availability, we only measured baseline anti-apoA-1 IgG levels and did not assess the dynamic of anti-apoA-1 IgG levels over time in relation with incident CAD. Moreover, we could not test other clinically relevant antibodies, such as anti-oxidized LDL, anti-phospholipid, anti-nuclear or anti-heat shock protein antibodies, which would have been instrumental to better understanding potential associations with innate immune receptor-related genes of interest. Finally, sample size calculation in our study was performed with regards to the primary outcome of detecting a difference in incident CAD in subjects positive *vs.* negative for anti-apoA-1 IgG. Although the fact that previous evidence suggested an interaction between anti-apoA-1 IgG and the CD14 receptor and that we were able to detect such a – significant – interaction between anti-apoA-1 IgG and the functional C260T *rs2569190* polymorphism in the CD14 receptor gene, it is possible that the current sample size provided less than 80% power for this secondary study outcome. Therefore, this finding of our study requires replication in larger prospective studies.

In conclusion, anti-apoA-1 IgG levels are independent predictors of incident non-fatal CAD in the general population. The strength of this association is significantly modulated by the functional C260T *rs2569190* SNP in the CD14 receptor gene, being the highest in non-TT carriers and the lowest in TT homozygotes. These results imply that preclinical autoimmunity to apolipoprotein A-1 should be evaluated carefully as it may help to improve the identification of individuals at increased risk of CAD in the general population, especially in non-TT carriers representing up to 75% of the population. Our findings indicate that "gene-autoantibodies" interaction studies are likely to be required to better assess the CAD risk related to humoral autoimmunity biomarkers in the general population, a concept that requires further investigation in future studies.

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c) DISCLOSURES

Funding sources played no role in the design and conduct of the study, nor in the collection, analysis and interpretation of the data, nor in the preparation, review, approval of the manuscript or decision to submit for publication. The authors have nothing to disclose.

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Highlights

- **Anti-apoA-1 IgG are independent predictors of non-fatal incident coronary artery disease in the general population.**
- **Anti-apoA-1 IgG could represent a potential novel target for immune-modulating preventive strategies for coronary artery disease.**
- **The strength of the association between anti-apoA-1 IgG and coronary artery disease is dependent on a functional polymorphism of the CD14 receptor gene.**
- **Our findings suggesting a "gene-autoantibody" interaction for the development of CAD, an observation that requires further study.**

FIGURE LEGENDS

Figure 1: Study flowchart
Anti-apoA-1 IgG; autoantibodies against Apolipoprotein A-1, CAD; Coronary Artery Disease

Figure 2: Kaplan-Meier curves for cumulative incident coronary artery disease.
Above, Kaplan-Meier curves for cumulative (A) total and (B) non-fatal incident CAD according to anti-apoA-1 IgG status (red line, positive for anti-apoA-1 IgG; blue line, negative for anti-apoA-1 IgG). Below, Kaplan-Meier curves for cumulative (C) total and (D) non-fatal incident CAD according to anti-apoA-1 IgG and C260T *rs2569190* allele status (black line, positive for anti-apoA-1 IgG and carrying the C allele (CC/CT); green line, positive for anti-apoA-1 IgG and carrying the T allele (TT); blue line, negative for anti-apoA-1 IgG). Data are expressed as the cumulative proportion of the sample presenting with incident CAD (y axis) during study years (x axis). Statistical analysis by Logrank test, for the comparison between anti-apoA-1 IgG negative subjects (blue line) *vs.* anti-apoA-1 IgG positive-TT carriers (green line) *vs.* anti-apoA-1 IgG positive-non-TT carriers (black line).
Anti-apoA-1 IgG; autoantibodies against Apolipoprotein A-1, CAD; coronary artery disease

TABLES

Table 1: Characteristics of the sample, according to anti-apoA-1 IgG status.

Total sample (n=5220)	Anti-apoA-1 IgG		p-value
	Absence (n=4180)	Presence (n=1040)	
Age, years	52.7 ± 10.7	52.2 ± 10.7	0.184
Male sex, n (%)	1985 (47.5)	488 (46.9)	0.744
History of CAD, n (%)	146 (3.5)	43 (4.1)	0.321
Current smoking, n (%)	1086 (26.0)	272 (26.2)	0.909
Diabetes, n (%)	276 (6.6)	58 (5.6)	0.226
Hypertension, n (%)	1389 (33.23)	349 (33.6)	0.841
Autoimmune disease, n (%)	88 (2.1)	32 (3.1)	0.061
Body mass index (kg/m^2)	25.6 ± 4.4	25.7 ± 4.6	0.712
Total cholesterol (mmol/l)	5.58 ± 1.02	5.50 ± 1.03	0.022
HDL cholesterol (mmol/l)	1.64 ± 0.43	1.62 ± 0.46	0.250
LDL cholesterol (mmol/l)	3.33 ± 0.90	3.27 ± 0.92	0.068
Triglycerides (mmol/l)	1.38 ± 1.12	1.36 ± 1.22	0.663*
SCORE CV risk categories, n (%)			
Low risk	2507 (60.1)	643 (62.0)	
Intermediate risk	1160 (27.8)	269 (25.9)	
High risk	311 (7.4)	83 (8.0)	
Very high risk	196 (4.7)	43 (4.1)	0.487
CV drugs, n (%)			
Aspirin	684 (16.4)	160 (15.4)	0.443
Statins	446 (10.7)	98 (9.4)	0.239
Beta blockers	212 (5.1)	70 (6.7)	0.034

Calcium channel blockers	120 (2.9)	33 (3.2)	0.605
ACEi/ARB	511 (12.2)	124 (11.9)	0.354
Diuretics	80 (1.9)	19 (1.8)	0.854
Incident CAD rates, n (%)	117 (2.8)	40 (3.9)	0.077
Non-fatal, n (%)	95 (2.3)	37 (3.6)	0.018
Fatal, n (%)	22 (0.5)	3 (0.3)	0.320

1 Data are expressed as mean ± standard deviation or number of participants and (percentage).

2 Anti-apoA-1 IgG, anti-apolipoprotein A-1 autoantibodies; CAD, coronary artery disease; HDL,
3 high density lipoprotein; LDL, low density lipoprotein; SCORE, Systematic Coronary Risk
4 Evaluation; CV, cardiovascular; ACEi, Angiotensin Converting Enzyme inhibitor; ARB,
5 Angiotensin Receptor Blockers. Statistical analysis for continuous variables by student's t-test or
6 Mann-Whitney test (*) depending on the normality assumption. Statistical analysis for
7 continuous variables by the chi-squared test.

Table 2: Hazard Ratios of anti-apoA-1 IgG for incident total, non-fatal and fatal CAD in the general population

n=5220	Total Incident CAD (n=159)				Non Fatal Incident CAD (n=134)				Fatal Incident CAD (n=25)			
	Unadjusted Model	p-value	Adjusted Model	p-value	Unadjusted Model	p-value	Adjusted Model	p-value	Unadjusted Model	p-value	Adjusted Model	p-value
Positive *vs.* negative	1.39 (0.97–1.99)	0.073	1.36 (0.94–1.97)	0.105	1.58 (1.08–2.31)	0.018	1.53 (1.03–2.26)	0.034	0.54 (0.16–1.80)	0.313	0.56 (0.17–1.91)	0.356
1 SD change in log-transformed anti-ApoA-1 IgG levels	1.11 (0.96–1.28)	0.159	1.09 (0.94–1.27)	0.232	1.15 (0.99–1.34)	0.072	1.14 (0.97–1.33)	0.109	0.88 (0.61–1.29)	0.520	0.87 (0.60–1.27)	0.474
Anti-ApoA-1 IgG levels *												
Negative (OD<0.64)	1 (ref.)		1 (ref.)		1 (ref.)		1 (ref.)		1 (ref.)		1 (ref.)	
1st tertile (0.64<OD≤0.77)	1.18 (0.64–2.19)	0.597	1.39 (0.74–2.59)	0.879	1.32 (0.69–2.53)	0.406	1.50 (0.78–2.89)	0.227	0.60 (0.08–4.43)	0.613	0.75 (0.96–5.92)	0.788
2nd tertile (0.77<OD≤0.98)	1.16 (0.63–2.16)	0.633	0.95 (0.48–1.88)	0.879	1.17 (0.59–2.33)	0.646	0.89 (0.41–1.93)	0.767	1.02 (0.24–4.37)	0.974	1.13 (0.26–4.90)	0.872
3rd tertile (OD>0.98)	1.79 (1.09–2.95)	0.021	1.70 (1.03–2.81)	0.038	2.21 (1.34–3.67)	0.002	2.14 (1.29–3.56)	0.003	no subjects	.	no subjects	.
P-value for linear trend	0.047		0.160		0.012			0.021	.		.	

Results are expressed as adjusted hazard ratios and (95% confidence interval) for subjects positive (OD>0.64) *vs.* negative (OD<0.64) for anti apoA-1 IgG. Statistical analysis by Cox proportional hazards regression adjusted for age,

sex, systolic blood pressure, diabetes, smoking, HDL and LDL cholesterol, baseline CAD, statin, beta-blocker treatment and eGFR. CAD, coronary artery disease; Anti-apoA-1 IgG, anti-apolipoprotein A-1 autoantibodies; SD, standard deviation; OD, optical density; HDL, high density lipoprotein; LDL, low density lipoprotein; eGFR, estimated Glomerular Filtration Rate.

* Subjects with positive Anti-ApoA-1 (n=1040) were divided in tertiles (n=347) of increasing titers: 1st tertile (0.64<OD<0.77), 2nd tertile (0.77<OD<0.98) and 3rd tertile (OD>0.98).

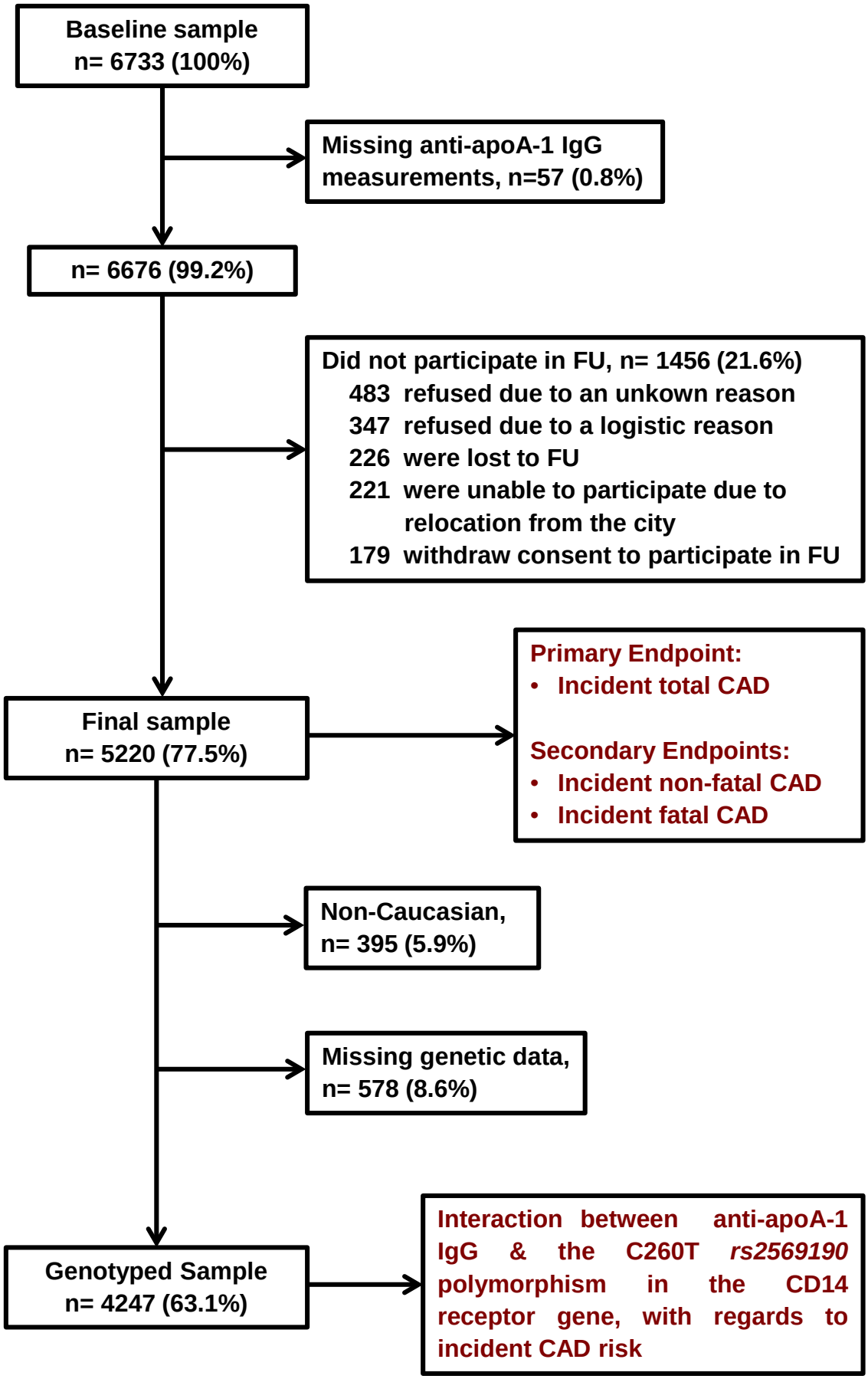
Table 3: Hazard Ratios of anti-apoA-1 IgG for incident total, non-fatal and fatal CAD according to the C260T *rs2569190* polymorphism allele status, in the genotyped population

Anti-apoA-1 IgG HR (95% CI) for CAD	Total Incident CAD (n= 132)				Non Fatal Incident CAD (n= 109)				Fatal Incident CAD (n= 23)			
C260T *rs2569190* allele status	**Unadjusted Model**	**p-value**	**Adjusted Model**	**p-value**	**Unadjusted Model**	**p-value**	**Adjusted Model**	**p-value**	**Unadjusted Model**	**p-value**	**Adjusted Model**	**p-value**
CC (n=1097)	2.08 (0.98; 4.42)	0.056	2.27 (1.04; 4.97)	0.039	2.19 (0.98; 4.87)	0.055	2.38 (1.03; 5.51)	0.042	1.39 (0.14; 13.42)	0.773	1.55 (0.15; 15.81)	0.713
CC/CT (n=3192)	1.67 (1.07; 2.60)	0.023	1.77 (1.12; 2.80)	0.014	1.84 (1.14; 2.95)	0.012	1.95 (1.19; 3.19)	0.008	0.91 (0.26; 3.17)	0.880	0.90 (0.24; 3.32)	0.877
CT (n=2095)	1.55 (0.89; 2.68)	0.120	1.52 (0.86; 2.71)	0.152	1.75 (0.96; 3.16)	0.066	1.73 (0.93; 3.23)	0.084	0.76 (0.17; 3.43)	0.718	0.54 (0.10; 3.00)	0.486
TT (n=1055)	0.58 (0.20; 1.65)	0.306	0.55 (0.19; 1.61)	0.275	0.74 (0.25; 2.14)	0.573	0.74 (0.25; 2.22)	0.592	no subjects		no subjects	
P-value for interaction between anti-apoA-1 IgG & rs2569190 (CC *vs.* CT *vs.* TT)		0.064		0.011		0.135		0.033		n/a		n/a
P-value for interaction between anti-apoA-1 IgG &		0.068		0.020		0.126		0.047		n/a		n/a

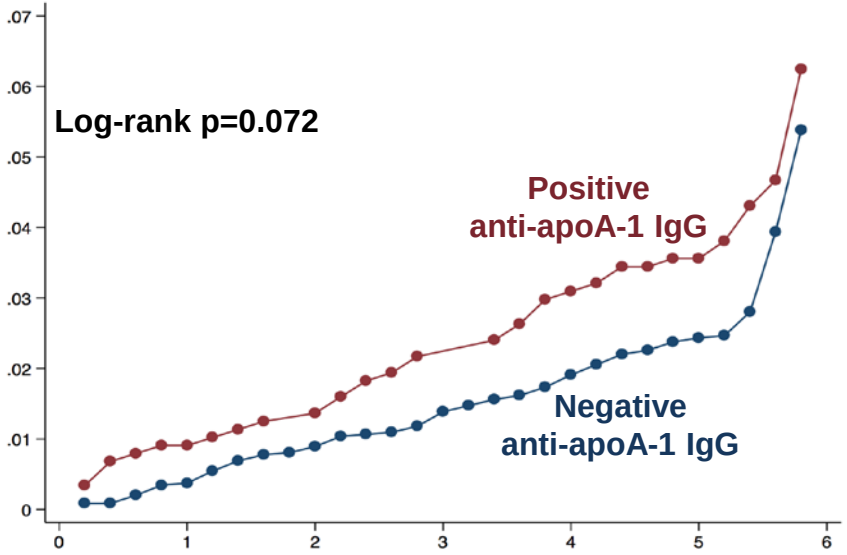
rs2569190 **(CC/CT *vs.* TT)**

Results are expressed as adjusted hazard ratios and (95% confidence interval) for subjects positive (OD>0.64) *vs.* negative (OD<0.64) for anti apoA-1 IgG. Statistical analysis by Cox proportional hazards regression adjusted for age, sex, systolic blood pressure, diabetes, smoking, HDL and LDL cholesterol, baseline CAD, statin, beta-blocker treatment and eGFR. The P value for interaction represents the likelihood of interaction between the C260T *rs2569190* allele status and the relative anti-apoA-1 IgG effect for coronary artery disease.

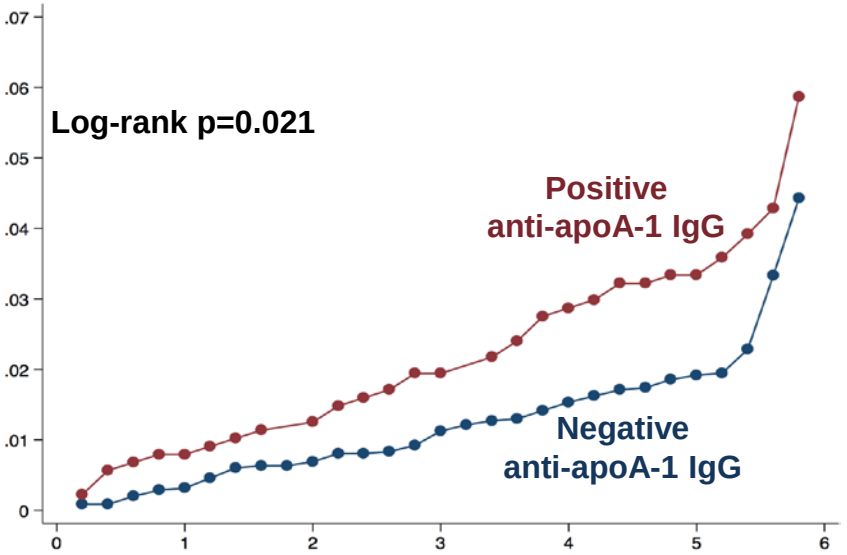
CAD, coronary artery disease; Anti-apoA-1 IgG, anti-apolipoprotein A-1 autoantibodies; HR, hazard ratio; CI, confidence interval; OD, optical density; HDL, high density lipoprotein; LDL, low density lipoprotein; eGFR, estimated Glomerular Filtration Rate.


Baseline sample
n= 6733 (100%)
Missing anti-apoA-1 IgG measurements, n=57 (0.8%)
n= 6676 (99.2%)
Did not participate in FU, n= 1456 (21.6%)
483 refused due to an unkown reason
347 refused due to a logistic reason
226 were lost to FU
221 were unable to participate due to relocation from the city
179 withdraw consent to participate in FU
Final sample
n= 5220 (77.5%)
Primary Endpoint:
• Incident total CAD
Secondary Endpoints:
• Incident non-fatal CAD
• Incident fatal CAD
Non-Caucasian, n= 395 (5.9%)
Missing genetic data, n= 578 (8.6%)
Genotyped Sample
n= 4247 (63.1%)
Interaction between anti-apoA-1 IgG & the C260T rs2569190 polymorphism in the CD14 receptor gene, with regards to incident CAD risk

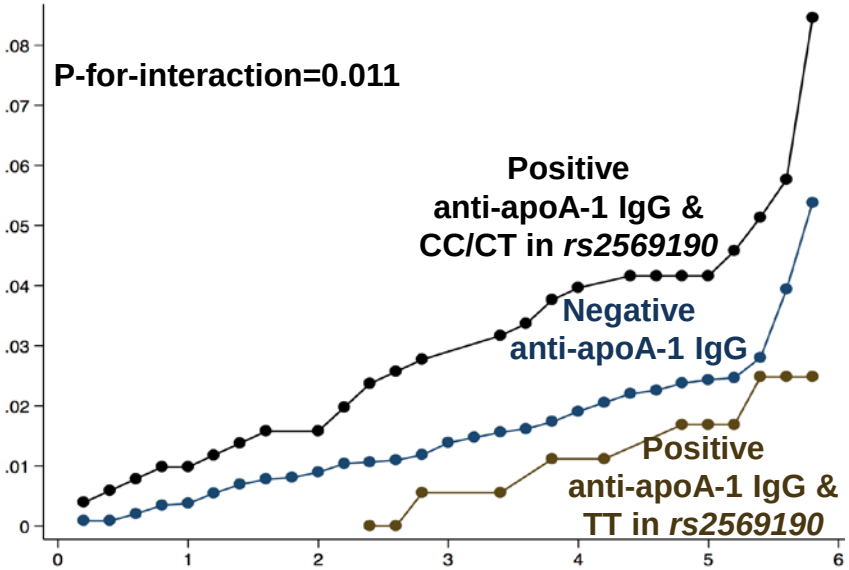
Association between anti-apoA-1 IgG and total incident CAD



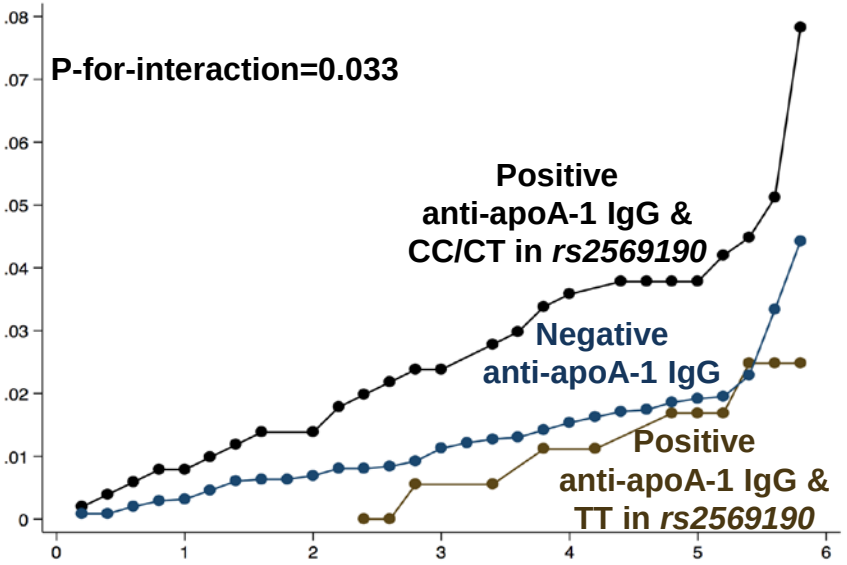
Association between anti-apoA-1 IgG and non-fatal incident CAD



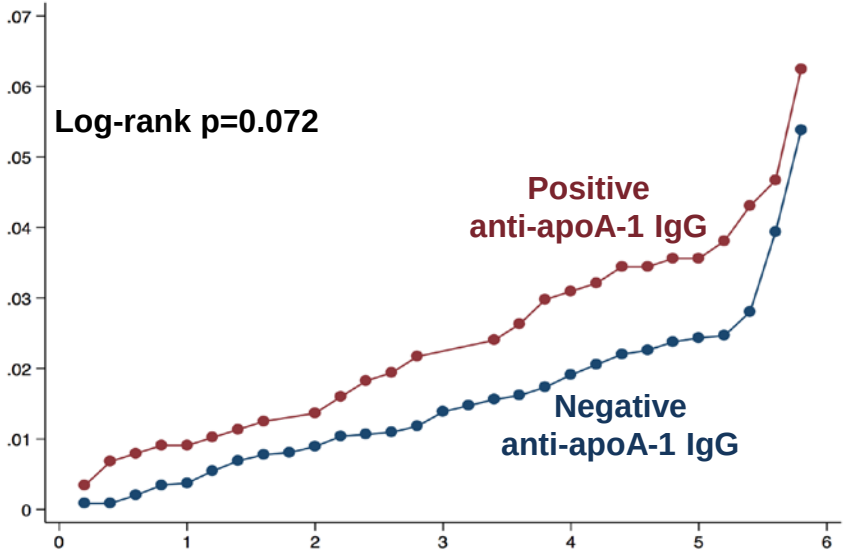
Association between anti-apoA-1 IgG and total incident CAD, according to *rs2569190* allele status



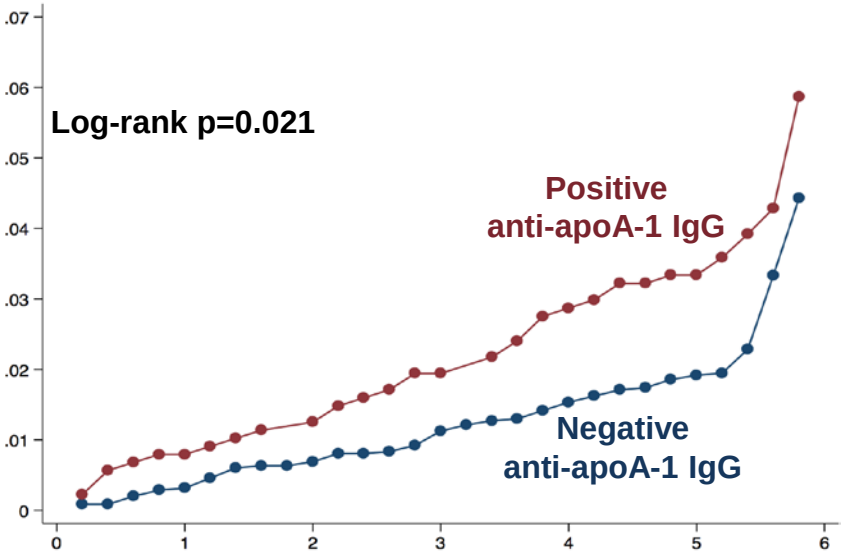
Association between anti-apoA-1 IgG and non-fatal incident CAD, according to *rs2569190* allele status



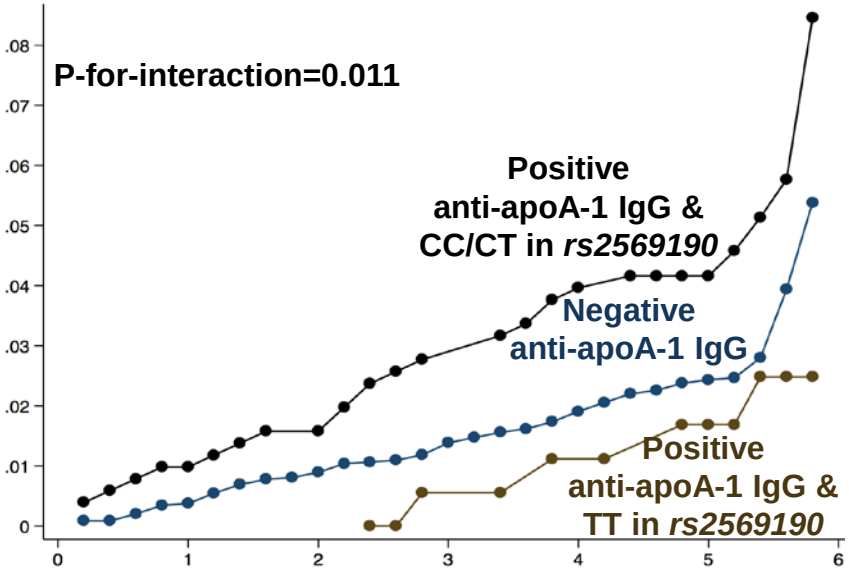
Association between anti-apoA-1 IgG and total incident CAD



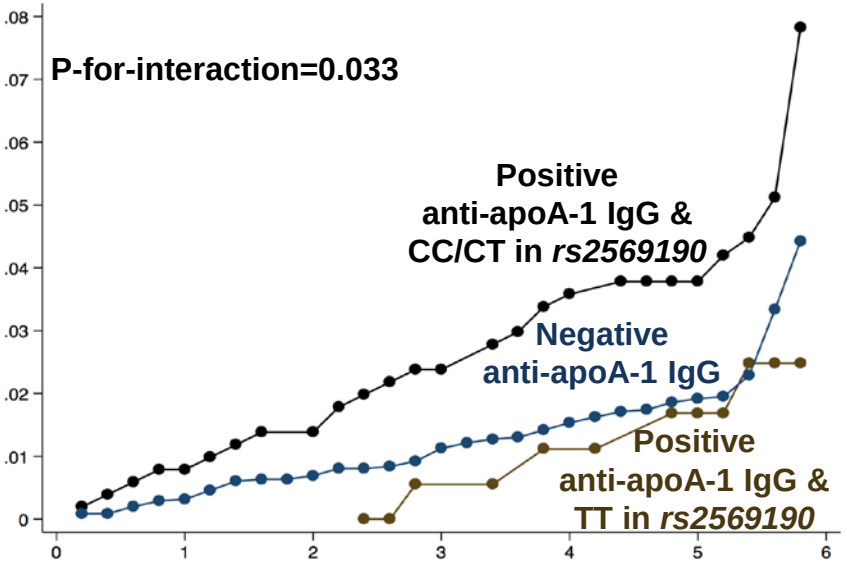
Association between anti-apoA-1 IgG and non-fatal incident CAD



Association between anti-apoA-1 IgG and total incident CAD, according to *rs2569190* allele status



Association between anti-apoA-1 IgG and non-fatal incident CAD, according to *rs2569190* allele status



Impact of CD14 polymorphisms on anti-apolipoprotein A-1 IgG-related coronary artery disease prediction in the general population

Panagiotis Antiochos, Pedro Marques-Vidal, Julien Virzi, Sabrina Pagano, Nathalie Satta, Oliver Hartley, Fabrizio Montecucco, François Mach, Zoltan Kutalik, Gerard Waeber, Peter Vollenweider, Nicolas Vuilleumier

SUPPLEMENT MATERIAL

Supplemental Table I: Baseline characteristics of subjects included in the analysis *vs.* subjects missing follow-up data.

Overall sample (n=6676)	**Subjects included in the analysis (n=5220)**	**Subjects missing follow-up data (n=1456)**	**p-value**
Age, years	52.6 ±10.7	52.6 ± 10.8	0.979
Male sex, n (%)	2496 (47.3)	693 (47.6)	0.841
History of CVD, n (%)	398 (7.5)	90 (6.2)	0.076
Current smoking, n (%)	1382 (26.2)	430 (29.5)	0.011
Diabetes, n (%)	336 (6.4)	100 (6.9)	0.492
Hypertension, n (%)	1756 (33.3)	579 (39.8)	<0.001
Body mass index (kg/m^2)	25.65 ± 4.41	26.34 ± 4.85	<0.001
Total cholesterol (mmol/l)	5.56 ± 1.02	5.62 ± 1.10	0.093
HDL cholesterol (mmol/l)	1.64 ± 0.44	1.60 ± 0.43	0.006
LDL cholesterol (mmol/l)	3.32 ± 0.90	3.37 ± 0.95	0.038
Triglycerides (mmol/l)	1.37 ± 1.14	1.46 ± 1.31	0.010*
SCORE CV risk categories, n (%)	2.07 ± 3.56	2.13 ± 3.40	0.145*
Low risk	3150 (60.4)	817 (57.3)	
Intermediate risk	1429 (27.4)	421 (29.5)	
High risk	394 (7.6)	119 (8.3)	
Very high risk	239 (4.6)	70 (4.9)	0.189
Anti-apoA-1 IgG, n (%)	1040 (19.9)	283 (19.8)	0.920
Anti-apoA-1 OD	0.46 ± 0.33	0.45 ± 0.36	0.377*

Data are expressed as mean ± standard deviation or number of participants and (percentage). CVD, cardiovascular disease; SBP, systolic blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; SCORE, Systematic Coronary Risk Evaluation; Anti-apoA-1 IgG, anti-apolipoprotein A-1 autoantibodies; OD, Optical Density. Statistical analysis for continuous variables by student's t-test or Mann-Whitney test (*) depending on the normality assumption. Statistical analysis for categorical variables by the chi-squared test.

Supplemental Table II: Hazard Ratios of anti-apoA-1 IgG for incident total, non fatal and fatal coronary artery disease in the general population, after excluding subjects with a) baseline coronary artery disease, b) baseline autoimmune disease.

Hazard Ratios (95% CI)	Incident CAD (n=111)				Non Fatal Incident CAD (n=93)				Fatal Incident CAD (n=18)			
a) Excluding subjects with baseline coronary artery disease (n=5031)	**Unadjusted Model**	**p-value**	**Adjusted Model**	**p-value**	**Unadjusted Model**	**p-value**	**Adjusted Model**	**p-value**	**Unadjusted Model**	**p-value**	**Adjusted Model**	**p-value**
Positive *vs.* negative anti-ApoA-1 IgG	1.44 (0.94–2.20)	0.093	1.54 (1.00–2.38)	0.050	1.57 (1.00–2.48)	0.050	1.69 (1.06–2.68)	0.027	0.79 (0.23–2.75)	0.717	0.90 (0.26–3.15)	0.868
Hazard Ratios (95% CI)	**Incident CAD (n=148)**				**Non Fatal Incident CAD (n=124)**				**Fatal Incident CAD (n=24)**			
b) Excluding subjects with baseline autoimmune disease (n=5100)	**Unadjusted Model**	**p-value**	**Adjusted Model**	**p-value**	**Unadjusted Model**	**p-value**	**Adjusted Model**	**p-value**	**Unadjusted Model**	**p-value**	**Adjusted Model**	**p-value**
Positive *vs.* negative anti-ApoA-1 IgG	1.37 (0.95–1.99)	0.095	1.42 (0.98–2.08)	0.077	1.62 (1.10–2.40)	0.015	1.64 (1.11–2.46)	0.017	0.36 (0.09–1.55)	0.172	0.43 (0.12–1.86)	0.256

Results are expressed as adjusted hazard ratios (95% confidence interval). Statistical analysis by Cox proportional hazards regression adjusted for age, sex, systolic blood pressure, diabetes, smoking, HDL cholesterol and LDL cholesterol, statin, beta-blocker treatment and eGFR. CAD, coronary artery disease; OD, optical density; Anti-apoA-1 IgG, anti-apolipoprotein A-1 autoantibodies; eGFR, estimated glomerular filtration rate

Supplementary Table III: Hazard Ratios of anti-apoA-1 IgG for incident total, non-fatal and fatal CAD in the general population.

Final sample (n=5220)	**Total Incident CAD (n=159)**				**Non Fatal Incident CAD (n=134)**				**Fatal Incident CAD (n=25)**			
	Adjusted Model_1	**p-value**	**Adjusted Model_2**	**p-value**	**Adjusted Model_1**	**p-value**	**Adjusted Model_2**	**p-value**	**Adjusted Model_1**	**p-value**	**Adjusted Model_2**	**p-value**
Positive *vs.* negative	1.36 (0.94–1.97)	0.099	1.37 (0.94–1.98)	0.100	1.53 (1.03–2.26)	0.034	1.54 (1.04–2.28)	0.031	0.58 (0.17–1.98)	0.387	0.56 (0.17–1.91)	0.355
1 SD change in log-transformed anti-ApoA-1 IgG levels	1.08 (0.94–1.25)	0.265	1.09 (0.94–1.26)	0.251	1.12 (0.96–1.30)	0.144	1.13 (0.97–1.32)	0.121	0.89 (0.62–1.29)	0.541	0.87 (0.60–1.28)	0.479
Anti-ApoA-1 IgG levels *												
Negative (OD<0.64)	1 (ref.)		1 (ref.)		1 (ref.)		1 (ref.)		1 (ref.)		1 (ref.)	
1st tertile (0.64<OD≤0.77)	1.45 (0.78–2.69)	0.243	1.45 (0.78–2.71)	0.237	1.57 (0.82–3.01)	0.177	1.58 (0.82–3.04)	0.168	0.75 (0.10–5.66)	0.780	0.69 (0.09–5.30)	0.718
2nd tertile (0.77<OD≤0.98)	1.02 (0.52–2.01)	0.958	0.95 (0.48–1.88)	0.882	0.96 (0.44–2.07)	0.910	0.89 (0.41–1.93)	0.766	1.16 (0.27–5.05)	0.839	1.12 (0.26–4.86)	0.880
3rd tertile (OD>0.98)	1.58 (0.96–2.60)	0.074	1.66 (1.01–2.75)	0.047	1.95 (1.17–3.23)	0.010	2.10 (1.26–3.49)	0.004	no subjects	.	no subjects	.
P-value for linear trend		0.254		0.167		0.103		0.022		.		.

Results are expressed as adjusted hazard ratios and (95% confidence interval) for the positive (OD>0.64) vs. negative (OD<0.64) anti apoA-1 antibodies. Statistical analysis by Cox proportional hazards regression adjusted for age, sex, hypertension, diabetes, smoking, HDL cholesterol and LDL cholesterol, baseline CAD, **(Adjusted Model_1)** statin, beta-blocker treatment **(Adjusted Model_2)**.

CAD, coronary artery disease; Anti-apoA-1 IgG, anti-apolipoprotein A-1 autoantibodies; SD, standard deviation; OD, optical density; HDL, high density lipoprotein; LDL, low density lipoprotein; eGFR, estimated Glomerular Filtration Rate.

* Subjects with positive Anti-ApoA-1 (n=1040) were divided in tertiles (n=347) of increasing titers: 1st tertile (0.64<OD<0.77), 2nd tertile (0.77<OD<0.98) and 3rd tertile (OD>0.98).

Supplemental Table IV: Baseline characteristics of subjects with *vs.* without genetic data for the C260T *rs2569190* polymorphism in the CD14 receptor gene.

Final sample (n=5220)	Final genotyped sample (n=4247)	Subjects without genetic data (n=973)	p-value
Age, years	53.4 ± 10.7	48.9 ± 9.9	<0.001
Male sex, n (%)	2014 (47.0)	482 (48.7)	0.315
History of CVD, n (%)	342 (8.0)	56 (5.7)	0.013
Current smoking, n (%)	1106 (25.8)	276 (27.9)	0.173
Diabetes, n (%)	289 (6.7)	47 (4.8)	0.021
Hypertension, n (%)	1480 (34.5)	276 (27.9)	<0.001
Body mass index (kg/m^2)	25.68 ± 4.42	25.51 ± 4.37	0.273
Total cholesterol (mmol/l)	5.59 ± 1.03	5.44 ± 0.99	<0.001
HDL cholesterol (mmol/l)	1.65 ± 0.44	1.60 ± 0.44	0.002
LDL cholesterol (mmol/l)	3.33 ± 0.91	3.25 ± 0.88	0.013
Triglycerides (mmol/l)	1.37 ± 1.12	1.37 ± 1.20	0.162*
SCORE CV risk categories, n (%)			
Low risk	2437 (57.2)	713 (74.7)	
Intermediate risk	1255 (29.5)	174 (18.2)	
High risk	358 (8.4)	36 (3.8)	
Very high risk	208 (4.9)	31 (3.3)	<0.001
Anti-apoA-1 IgG, n (%)	802 (18.81)	238 (24.87)	<0.001
Anti-apoA-1 OD	0.45 ± 0.32	0.51 ± 0.37	<0.001*

Data are expressed as mean ± standard deviation or number of participants and (percentage). CVD, cardiovascular disease; SBP, systolic blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; SCORE, Systematic Coronary Risk Evaluation; Anti-apoA-1 IgG, anti-apolipoprotein A-1

autoantibodies; OD, Optical Density. Statistical analysis for continuous variables by student's t-test or Mann-Whitney test (*) depending on the normality assumption. Statistical analysis for categorical variables by the chi-squared test.

Supplemental Table V: Baseline characteristics of subjects with *vs.* without genetic data for the C260T *rs2569190* polymorphism in the CD14 receptor gene or unable to participate in follow up.

Baseline sample (n=6676)	**Final genotyped sample (n=4247)**	**Subjects without genetic data/unable to participate in follow-up (n=2429)**	**p-value**
Age, years	53.4 ± 10.7	51.1 ± 10.6	<0.001
Male sex, n (%)	2014 (47.0)	1175 (48.1)	0.389
History of CVD, n (%)	314 (7.3)	144 (5.9)	0.025
Current smoking, n (%)	1106 (25.8)	706 (28.9)	0.006
Diabetes, n (%)	289 (6.7)	147 (6.0)	0.243
Hypertension, n (%)	1480 (34.5)	855 (35.0)	0.706
Body mass index (kg/m^2)	25.7 ± 4.4	26 ± 4.7	0.005
Total cholesterol (mmol/l)	5.59 ± 1.03	5.55 ± 1.06	0.084
HDL cholesterol (mmol/l)	1.65 ± 0.44	1.60 ± 0.43	<0.001
LDL cholesterol (mmol/l)	3.33 ± 0.91	3.32 ± 0.93	0.756
Triglycerides (mmol/l)	1.37 ± 1.12	1.42 ± 1.27	0.431*
SCORE CV risk categories, n (%)			
Low risk	2428 (57.2)	1530 (64.3)	
Intermediate risk	1249 (29.4)	595 (25)	
High risk	357 (8.4)	155 (6.5)	
Very high risk	208 (4.9)	101 (4.2)	<0.001
Anti-apoA-1 IgG, n (%)	802 (18.8)	521 (21.8)	0.003
Anti-apoA-1 OD	0.45 ± 0.32	0.48 ± 0.36	<0.001*

Data are expressed as mean ± standard deviation or number of participants and (percentage). CVD,

cardiovascular disease; SBP, systolic blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; SCORE, Systematic Coronary Risk Evaluation; Anti-apoA-1 IgG, anti-apolipoprotein A-1 autoantibodies; OD, Optical Density. Statistical analysis for continuous variables by student's t-test or Mann-Whitney test (*) depending on the normality assumption. Statistical analysis for categorical variables by the chi-squared test.

Supplemental Table VI: Characteristics of the genotyped sample, according to allele status of the C260T *rs2569190* polymorphism in CD14 receptor gene.

C260T *rs2569190* allele status (CC/CT *vs.* TT)	**Overall (n=4247)**		**p-value**	**Anti-apoA-1 IgG positive subjects (n=798)**		**p-value**
	CC/CT (n=3192)	**TT (n=1055)**		**CC/CT (n=585)**	**TT (n=213)**	
Age, years	53.5 ± 10.7	53.5 ± 10.9	0.923	53.2 ± 10.7	53.4 ± 11.3	0.835
Male sex, n (%)	1495 (46.8)	502 (47.6)	0.673	260 (44.4)	108 (50.7)	0.117
History of CAD, n (%)	122 (3.8)	37 (3.5)	0.640	27 (4.6)	8 (3.8)	0.600
Current smoking, n (%)	808 (25.3)	282 (26.7)	0.361	153 (26.2)	51 (23.9)	0.527
Diabetes, n (%)	199 (6.2)	90 (8.5)	0.010	32 (5.5)	15 (7.0)	0.404
Hypertension, n (%)	1099 (34.4)	371 (35.2)	0.663	203 (34.7)	86 (40.4)	0.140
Autoimmune disease, n (%)	80 (2.5)	29 (2.8)	0.666	18 (3.1)	11 (5.2)	0.163
Body mass index (kg/m^2)	25.67 ± 4.46	25.68 ± 4.29	0.973	25.72 ± 4.67	25.90 ± 4.16	0.622
Total cholesterol (mmol/l)	5.59 ± 1.03	5.60 ± 1.02	0.715	5.51 ± 1.00	5.55 ± 1.06	0.654
HDL cholesterol (mmol/l)	1.64 ± 0.44	1.66 ± 0.44	0.501	1.63 ± 0.46	1.65 ± 0.43	0.635
LDL cholesterol (mmol/l)	3.33 ± 0.91	3.34 ± 0.92	0.753	3.27 ± 0.92	3.31 ± 0.88	0.621
Triglycerides (mmol/l)	1.38 ± 1.18	1.36 ± 0.93	0.974*	1.39 ± 1.40	1.27 ± 0.81	0.395*
SCORE CV risk categories, n (%)						
Low risk	1826 (57.3)	602 (57.1)		346 (59.3)	116 (54.5)	
Intermediate risk	952 (29.9)	297 (28.2)		157 (26.9)	65 (30.5)	
High risk	258 (8.1)	99 (9.4)		53 (9.1)	21 (9.9)	

Very high risk	151 (4.7)	57 (5.4)	0.379	28 (4.8)	11 (5.2)	0.682
CV drugs, n (%)						
Aspirin	576 (18.1)	170 (16.1)	0.153	110 (18.8)	29 (13.6)	0.087
Statins	346 (10.8)	143 (13.6)	0.017	60 (10.3)	22 (10.3)	0.976
Beta blockers	185 (5.8)	56 (5.3)	0.553	48 (8.2)	13 (6.1)	0.323
Calcium channel	97 (3.0)	31 (2.9)	0.869	20 (3.4)	5 (2.4)	0.442
blockers						
ACEi/ARB	241 (7.6)	83 (7.9)	0.737	43 (7.4)	14 (6.6)	0.706
Diuretics	63 (2.0)	22 (2.1)	0.822	12 (2.1)	5 (2.4)	0.798
Anti-apoA-1 IgG, n	585 (18.3)	213 (20.2)	0.179	585	213	.
(%)				(100.0)	(100.0)	
Anti-apoA-1 OD	0.45 ± 0.31	0.45 ± 0.33	0.937*	0.94 ± 0.29	0.93 ± 0.28	0.633*
Incident CAD, n (%)	100 (3.1)	32 (3.0)	0.872	27 (4.6)	4 (1.9)	0.077
Non-fatal, n (%)	83 (2.6)	26 (2.5)	0.809	24 (4.1)	4 (1.9)	0.131
Fatal, n (%)	17 (0.5)	6 (0.6)	0.890	3 (0.5)	0 (0.00)	0.295
All-cause mortality, n	125 (3.9)	38 (3.6)	0.653	29 (5.0)	13 (6.1)	0.522
(%)						

Data are expressed as mean ± standard deviation or number of participants and (percentage). Anti-apoA-1 IgG, anti-apolipoprotein A-1 autoantibodies; CAD, coronary artery disease; HDL, high density lipoprotein; LDL, low density lipoprotein; SCORE, Systematic Coronary Risk Evaluation; CV, cardiovascular; ACEi, Angiotensin Converting Enzyme inhibitor; ARB, Angiotensin Receptor Blockers; OD, optical density. Statistical analysis for continuous variables by student's t-test or Mann-Whitney test (*) depending on the normality assumption. Statistical analysis for continuous variables by the chi-squared test.

Supplementary Table VII: Hazard Ratios of anti-apoA-1 IgG for incident total, non-fatal and fatal CAD according to the C260T *rs2569190* polymorphism allele status, in the genotyped population

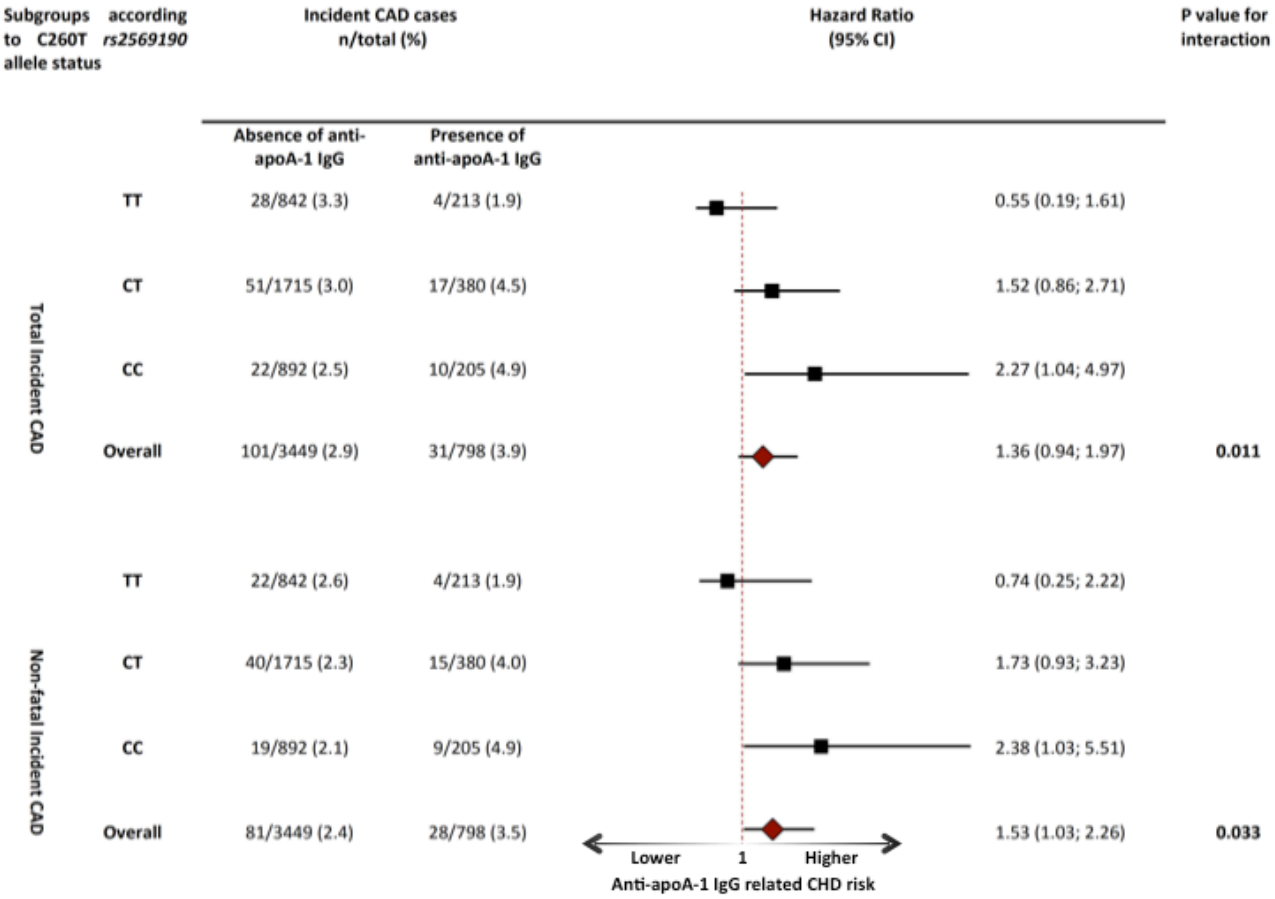
Anti-apoA-1 IgG HR (95% CI) for CAD	**Total Incident CAD (n= 132)**				**Non Fatal Incident CAD (n= 109)**				**Fatal Incident CAD (n= 23)**			
C260T *rs2569190* allele status	**Adjusted Model_1**	**p-value**	**Adjusted Model_2**	**p-value**	**Adjusted Model_1**	**p-value**	**Adjusted Model_2**	**p-value**	**Adjusted Model_1**	**p-value**	**Adjusted Model_2**	**p-value**
CC (n=1097)	2.09 (0.97–4.47)	0.058	2.26 (1.04–4.92)	0.040	2.13 (0.95–4.80)	0.068	2.36 (1.02–5.42)	0.044	1.85 (0.18–18.6)	0.602	1.52 (0.15–15.34)	0.721
CC/CT (n=3192)	1.73 (1.10–2.73)	0.018	1.77 (1.12–2.80)	0.014	1.87 (1.15–3.05)	0.012	1.96 (1.20–3.20)	0.007	0.89 (0.24–3.25)	0.858	0.90 (0.24–3.31)	0.873
CT (n=2095)	1.56 (0.88–2.75)	0.128	1.50 (0.85–2.67)	0.164	1.73 (0.94–3.20)	0.081	1.72 (0.92–3.20)	0.087	0.59 (0.11–3.05)	0.528	0.54 (0.10–2.93)	0.475
TT (n=1055)	0.47 (0.16–1.36)	0.163	0.54 (0.18–1.58)	0.262	0.59 (0.20–1.76)	0.348	0.71 (0.24–2.12)	0.536	no subjects		no subjects	
P-value for interaction between anti-apoA-1 IgG & rs2569190 (CC *vs.* CT *vs.* TT)		0.015		0.010		0.045		0.029		0.183		n/a
P-value for interaction between anti-apoA-1 IgG & rs2569190 (CC/CT *vs.* TT)		0.022		0.025		0.050		0.053		n/a		n/a

Results are expressed as adjusted hazard ratios and (95% confidence interval) for positive

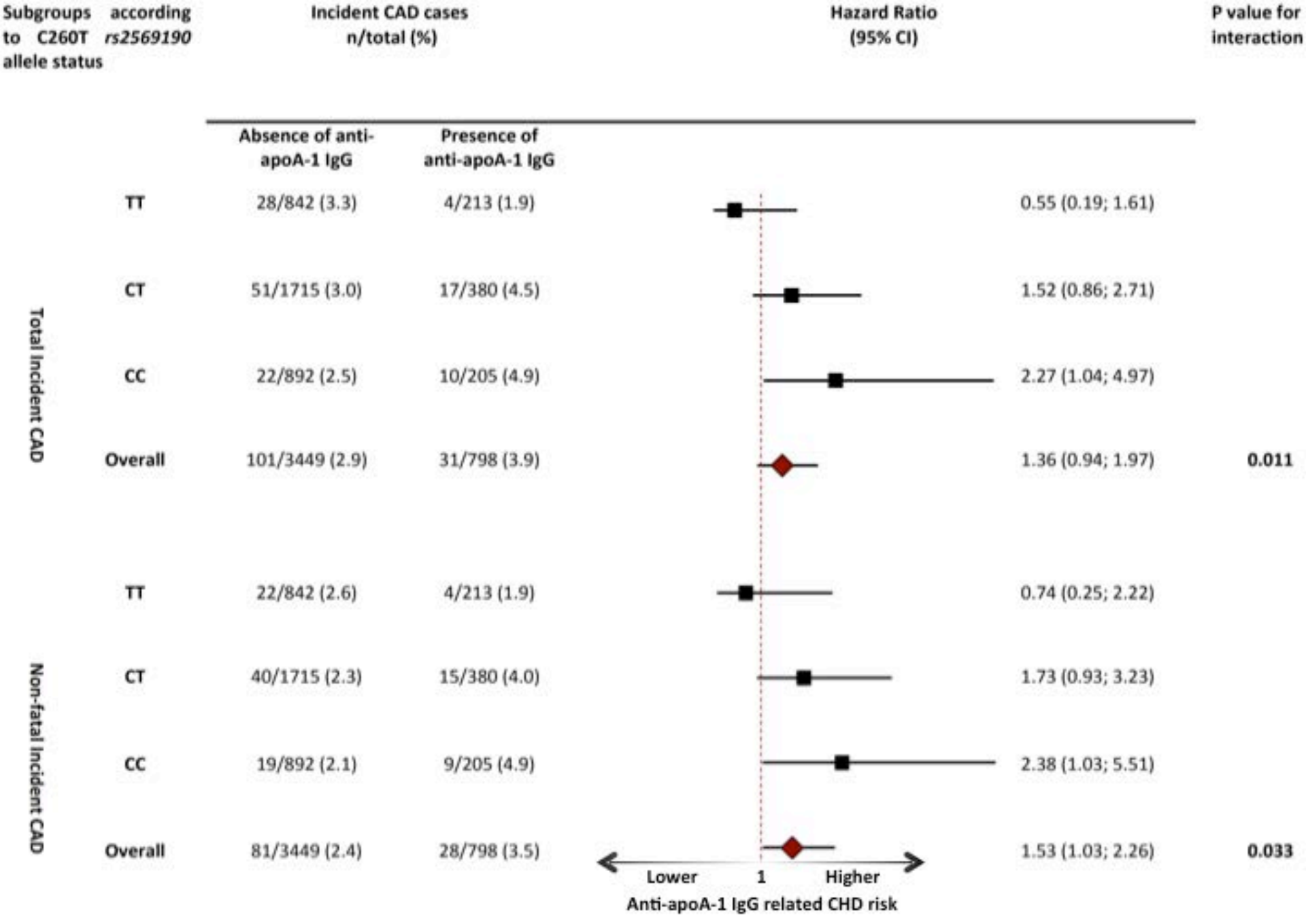
(OD>0.64) *vs.* negative (OD<0.64) anti apoA-1 IgG. Statistical analysis by Cox proportional hazards regression adjusted for age, sex, hypertension, diabetes, smoking, HDL cholesterol and LDL cholesterol, baseline CAD **(Adjusted Model_1)**, statin and beta-blocker treatment **(Adjusted Model_2)**. The P value for interaction represents the likelihood of interaction between the C260T *rs2569190* allele status and the relative anti-apoA-1 IgG effect for coronary artery disease.

CAD, coronary artery disease; Anti-apoA-1 IgG, anti-apolipoprotein A-1 autoantibodies; HR, hazard ratio; CI, confidence interval; OD, optical density; HDL, high density lipoprotein; LDL, low density lipoprotein; eGFR, estimated Glomerular Filtration Rate.

Supplemental Figure I: Forest plot of subgroup analyses according to the C260T *rs2569190* polymorphism allele status, for incident total and non-fatal coronary artery disease.



Hazard ratios of anti-apoA-1 IgG (and 95% confidence intervals) are shown for the endpoint of incident total and non fatal coronary artery disease, according to C260T *rs2569190* polymorphism allele status. Statistical analysis by Cox proportional hazards regression adjusted for age, sex, hypertension, diabetes, smoking, HDL and LDL cholesterol, baseline CAD, statin, beta-blocker treatment and eGFR. The P value for interaction represents the likelihood of interaction between the C260T *rs2569190* polymorphism allele status and the relative anti-apoA-1 IgG effect for coronary artery disease.


Subgroups according to C260T allele status
rs2569190
Incident CAD cases n/total (%)
Hazard Ratio (95% CI)
P value for interaction
Absence of anti-apoA-1 IgG
Presence of anti-apoA-1 IgG
Total Incident CAD
TT
28/842 (3.3)
4/213 (1.9)
0.55 (0.19; 1.61)
CT
51/1715 (3.0)
17/380 (4.5)
1.52 (0.86; 2.71)
CC
22/892 (2.5)
10/205 (4.9)
2.27 (1.04; 4.97)
Overall
101/3449 (2.9)
31/798 (3.9)
1.36 (0.94; 1.97)
0.011
Non-fatal Incident CAD
TT
22/842 (2.6)
4/213 (1.9)
0.74 (0.25; 2.22)
CT
40/1715 (2.3)
15/380 (4.0)
1.73 (0.93; 3.23)
CC
19/892 (2.1)
9/205 (4.9)
2.38 (1.03; 5.51)
Overall
81/3449 (2.4)
28/798 (3.5)
1.53 (1.03; 2.26)
0.033
Lower
1
Higher
Anti-apoA-1 IgG related CHD risk

Impact of CD14 polymorphisms on anti-apolipoprotein A-1 IgG-related coronary artery disease prediction in the general population

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* These authors contributed equally

MATERIALS AND METHODS

Study population and design

We used clinical and biological data from the CoLaus study, a population-based cohort investigating cardiovascular disease risk in 6733 subjects in the general population aged 35 to 75 and living in the city of Lausanne, Switzerland. The recruitment phase of the study took place between 2003 and 2006, where all participants attended the outpatient clinic of the University Hospital of Lausanne. A follow-up visit took place between 2009 and 2012. All study participants at baseline were invited for a follow-up visit at the outpatient clinic of the University Hospital of Lausanne, between 2009 and 2012. Subjects unable to attend had home interviews, were interviewed by phone and/or sent a questionnaire requesting information relevant to study endpoints. Follow-up data collection started after the baseline visit of each participant. The study was approved by the Institutional Ethics Committee of the University of Lausanne and informed consent was obtained from all participants before inclusion in the study, in accordance with the Declaration of Helsinki. A detailed description of the study design, definition of clinical variables and sampling procedures have been described elsewhere.[1]

Briefly, clinical data, and fasting venous blood samples were collected from each participant by trained field interviewers during a single visit lasting about 60 minutes. Blood pressure and heart rate were measured three consecutive times using an automated sphygmomanometer (Omron® HEM-907, Matsusaka, Japan) and the average of the last two measurements was used. Body weight and height were measured with participants standing without shoes in light indoor clothes. Body weight was measured in kilograms to the nearest 100 g using a Seca® scale, which was calibrated regularly. Height was measured to the nearest 5 mm using a Seca® height gauge. Body mass index (BMI) was calculated as weight (kg)/height (m^2). Hypertension was defined as a systolic blood pressure (SBP) ≥140 mm Hg and/or a diastolic blood pressure ≥90 mm Hg and/or the presence of anti-hypertensive treatment. Diabetes mellitus was defined as fasting plasma glucose ≥7.0 mmol/l and/or oral or insulin anti-diabetic treatment. Estimated glomerular filtration rate (eGFR) was calculated by the simplified "Modification of Diet in Renal Disease" prediction equation. [2] Autoimmune disease was defined as history of rheumatoid arthritis or systemic lupus erythematosus, independently of treatment.

Venous blood samples were drawn after an overnight fast, and assays were performed on fresh plasma samples within two hours after blood collection for standard lipid profile, and on unthawed serum aliquots for anti-apoA-1 IgG assessment (see below) that were immediately adequately processed and stored at -80 °C until analysis. [3] Standard lipid profile was performed in the CHUV Clinical Laboratory using a Modular P apparatus (Roche Diagnostics, Switzerland). The following analytical procedures (with maximum inter and intra-batch CVs) were used: total cholesterol by the "CHOD-PAP" method (1.6% – 1.7%); HDL-cholesterol by the "CHOD-PAP/PEG/Cyclodextrin" method (3.6% – 0.9%); triglycerides by the "GPO-PAP" method (2.9% – 1.5%); glucose by glucose dehydrogenase (2.1% – 1.0%); and serum creatinine by the Jaffe kinetic compensated method (2.9% – 0.7%).

Coronary artery disease and death data collection at baseline and during FU

Trained medical doctors actively searched and collected all medical records related to the coronary artery disease (CAD, defined as myocardial infarction, stable or unstable angina, percutaneous coronary revascularization or bypass grafting) in *all* participants who declared, during the baseline and/or follow-up interviews, to have presented any cardiac event or procedure during their lifetime. CAD that occurred during follow-up was classified as incident.
CAD collection in study participants followed a stepwise procedure: 1. The medical record of each participant was checked by hand with the general practitioner and/or the private

cardiologist, by both mail surveys and phone interviews. All CAD-related GPs' reports, reports on outpatient contacts with medical specialists and hospital discharge reports were copied and classified. Collected documents further included related laboratory data, electrocardiograms, cardiac imaging data (echocardiography reports, cardiac radionuclide imaging, magnetic resonance imaging, cardiac CT, stress tests) and coronary angiograms. 2. To further search for presence of CAD that may not have been mentioned by the participant during the follow-up visit, the medical databases of: a. the University Hospital of Lausanne, b. regional hospitals (within a radius of 100 kilometers), and c. the pre-hospital emergency care unit of the City of Lausanne, were checked electronically and then also by hand for CAD-related diagnoses, for *all* study participants.

Data on deceased study participants were likewise collected in a stepwise procedure, by checking electronically and then also by hand the electronic databases of: a. the University Hospital of Lausanne, b. regional hospitals (within a radius of 100 kilometers), c. population registers of the cities where the participants were living in case of returned mail and/or multiple unsuccessful phone contacts, d. the pre-hospital emergency care unit of the City of Lausanne, e. the forensic medicine department of the University Hospital of Lausanne, and f. the "Office Fédérale de la Statistique", the Swiss governmental agency providing death statistics. If a death was confirmed, physicians of the dead participants were asked to send any medical record related to the death. If all previous steps failed to retrieve the cause of death, the physician in charge when the death occurred and/or a family member of the dead participant were contacted to provide information on the cause of death (verbal autopsy).

Study Endpoints

The primary endpoint was total incident CAD as defined by a composite of first-time, fatal or non-fatal myocardial infarction, stable or unstable angina, percutaneous coronary revascularization or bypass grafting for CAD. Separate outcomes of interest included non-fatal as well fatal CAD (definite or probable, in-hospital or out-of-hospital). [4] All CAD events were adjudicated separately by two cardiologists, blinded to all study variables, according to a consensus document edited on behalf of the Joint ESC/ACCF/AHA/WHF Task Force for the "Universal Definition of Myocardial Infarction". [5] All deaths were adjudicated, by 2 internal medicine specialists.

In subjects with available genetic data for the functional C260T *rs2569190* polymorphism in the CD14 receptor gene, the secondary study outcome was to test the interaction between anti-apoA-1 IgG and carriage of the T allele, with regards to incident CAD.

Assessment of anti-apoA-1 IgG levels

Anti-apoA-1 IgG were measured as previously described, [6-8] using the CoLaus study (2003-2006) frozen serum aliquots, stored at -80 °C. Maxisorp plates (Nunc™, Denmark) were coated with purified, human-derived delipidated apolipoprotein A-1 (20 µg/ml; 50 µl/well) for 1h at 37°C. After being washed, all wells were blocked for 1h with 2% bovine serum albumin (BSA) in a phosphate buffer solution (PBS) at 37°C. Participants' samples were also added to a non-coated well to assess individual non-specific binding. After six washing cycles, a 50 µl/well of signal antibody (alkaline phosphatase-conjugated anti-human IgG; Sigma-Aldrich, St Louis, MO), diluted 1:1000 in a PBS/BSA 2% solution, was added and incubated for 1h at 37°C. After washing six more times, phosphatase substrate p-nitrophanylphosphate disodium (Sigma-Aldrich) dissolved in a diethanolamine buffer (pH 9.8) was added and incubated for 20 min at

37°C (Molecular Devices™ Versa Max). As previously published, this assay detects anti-apoA-1 antibodies directed against the native lipid-free apoA-1. [9, 10] Optical density (OD) was determined at 405 nm, and each sample was tested in duplicate. Corresponding non-specific binding was subtracted from mean OD for each sample. The specificity of detection was assessed using conventional saturation tests by Western blot (WB) analysis.
As previously described, elevated levels of anti-apoA-1 IgG were set at an OD cut-off of OD>0.64, corresponding to the 97.5th percentile of a reference population. [6-8] In order to limit the impact of inter-assay variation, we further calculated an index consisting in the ratio between sample net absorbance and the positive control net absorbance × 100. The index value corresponding to the 97.5th percentile of the normal distribution was 37. Accordingly, to be considered as positive (presenting elevated anti-apoA-1 IgG levels), samples had to display both an absorbance value >0.64 OD and an index value ≥37%.

As described before, [9, 10] our ELISA principally detects immunoreactivity (anti-apoA-1 IgG) against unmodified form of apoA-1. In order to further determine whether our assay is specifically dedicated to detect antibodies against native apoA-1 devoid of post-translational modifications (PTM) and does not cross-react with other forms of modified apoA-1, we used our human purified apoA-1 to generate carbamylated apoA-1, glycated apoA-1, and oxidized apoA-1 to be tested as coating antigen in our ELISA, comparing the respective signals produced by Passing-Bablock analyses.

ApoA-1 *carbamylation* was performed according to the protocol by Holzer et al. [11] Briefly, native human purified ApoA-1 was carbamylated with potassium cyanate (50 mmol/L) in phosphate buffered saline (pH 7.4) containing 100 µmol/L diethylenetriaminepentaacetic acid (DTPA) for 4 hours at 37°C. As a control, the same protocol was applied in the absence of potassium cyanate. All preparations were passed through a column (MWCO 10,000 Da) to remove excess reagents and used immediately for experiments. The apoA-1 carbamylation state was then verified using commercial ELISA (OxiSelect Protein Carbamylation Sandwich Elisa kit from Cell Biolabs; ref. STA-877). The quantity of carbamylated apoA-1 generated by this protocol was 33.8 ng/ml against 2.9 ng/ml with the control procedure.

ApoA-1 *glycation* was performed according to the protocol by Nobécourt et al. [12] Briefly, native human purified apoA-1 was exposed to methylglyoxal (MG) (6mmol/l) in phosphate buffered saline containing 100 µmol/L etilendiaminotetracetic acid (EDTA) for 24 hours at 37°C under 5% CO_2. As a control, the same protocol was applied in the absence of MG. All preparations were passed through a column (MWCO 10,000 Da) to remove excess reagents and used immediately for experiments. The apoA-1 glycation state was then verified using commercial ELISA (OxiSelect Methylglyoxal Competitive Elisa kit; Cell Biolabs ref. STA-811). The quantity of MG glycated apoA-1 generated with this protocol was 138.9µg/ml against 0.07 µg/ml with the control procedure.

ApoA-1 *oxidation* was performed according to the protocol by DiDonato et al**. [13]** Briefly, native human purified apoA-1 was oxidized in 60 mmol/l Na[PO_4] buffer (pH 7.4) using $CuSO_4$ (10µmol/l) with 40 µmol/l of H_2O_2 for 24 hours at 37°C. As a control, the same protocol was applied in the absence of $CuSO_4$ and H_2O_2. All preparations were passed through a column (MWCO 10,000 Da) to remove excess reagents and used immediately for experiments. The amount of apoA-1 oxidation state was first verified by the mobility shift on SDS-PAGE gels, and visualized by WB. As shown in **Supplemental Figure II**, the expected higher MW apoA-1

bands induced by oxidation-mediated apoA-1 dimers formation were achieved by combining H_20_2 and $CuS0_4$, allowing to use this procedure to generate oxidized apoA-1 for further experiments.

These results indicate that i) our *in vitro* procedure generated the expected PTM, and that ii) the native apoA-1 used in the present study does not contain substantial amount of carbamylation, glycation or oxidation.

In order to further explore a theoretical potential influence of carbamylation, glycation or oxidation of apoA-1 in our study, we further performed Passing-Bablock analyses using carbamylated, glycated, oxidized apoA-1. Using these modified forms of apoA-1 in our in house ELISA assay, we compared the immunoreactivity signals obtained, with those derived using native apoA-1 on a subset of n=63 randomly selected Colaus subjects and displaying a anti-apoA-1 IgG positivity rate of 17% (11/63), closely corresponding to the anti-apoA-1 IgG positivity rate retrieved on the whole CoLaus cohort (19%).

As shown in **Supplemental Figure III**, using *carbamylated* apoA-1 as coating antigen in our ELISA induced a significant proportional bias of + 22% (slope: 1.22; 95%CI: 1.13-1.33), but no systematic bias (intercept:-0.004; 95%CI:-0.04-0.04). Indeed, taking the same anti-apoA-1 IgG positivity definition, using carbamylated apoA-1 induced a positivity rate of 27% with n=7 discordant cases. Six were false positives ((FP): samples that were negative when using native apoA-1) and one was false negative ((FN): sample that was tested positive using native apoA-1), translating into a significant anti-apoA-1 IgG positivity rate discordance of 64% (0/11 *vs.* 7/11; p=0.003). These results indicate that using carbamylated apoA-1 provides a significant different signal than using native apoA-1 in our ELISA.

As shown in the **Supplemental Figure IV**, using *glycated* apoA-1 as coating antigen in our ELISA, induced a non-significant proportional positive bias of + 8% (slope: 1.08,95%CI:0.97-1.24), and a small statistically significant, but minor bias of 0.07 arbitrary units (Intercept: -0.07; 95%CI:-13 to -0.02). These results indicate that our ELISA is insensitive to apoA-1 MG-induced glycation status.

Lastly, as depicted in **Supplemental Figure V**, using *oxidized* apoA-1 as coating antigen in our ELISA showed that the two methods provide identical results with a no proportional bias (slope: 1.003; 95%CI:0.82-1.34) and no systematic bias (intercept :-0.0006; 95%CI:-0.09-0.09) when compared to using human purified native apoA-1. These results indicate that our ELISA is insensitive to apoA-1 oxidation status.

Lastly, in order to further eliminate the possibility of anti-apoA-1 IgG being directed against glycated, carbamylated, oxidized apoA-1 or PTM-induced cross-linked multimers of apoA-1, we adapted our ELISA protocol, performing additional WB and Liquid Chromatography (LC) – Mass Spectrometry (MS)/MS analyses on pooled serum derived from n=3 study patients tested positive and n=3 tested negative for anti-apoA-1 IgG.

For the *WB analysis*, one microgram of purified delipidated apoA-1 devoid of PTM was resolved by 10% polyacrylamide gel electrophoresis under reducing conditions and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore IPVH 00010), which was then blocked for 1 hour at RT with non-fat dry milk 5% in tris-buffered saline with tween 20 (T-TBS).

Membranes were incubated with pooled sera derived from three anti-apoA-1 IgG positive (OD_{405nm} value: 1.7 AU) patients and three anti-apoA-1 IgG negative patients (OD_{405nm} value: 0.2 AU) diluted (1:50) in non-fat dry milk 5% in T-TBS 2 hours at room temperature. A goat anti-human IgG or rabbit anti-goat horseradish peroxidase conjugated (Dako, Agilent) was used as secondary antibody, diluted 1:7000 in non-fat dry milk 5% in T-TBS for 1 hour at room temperature. The detection was performed using the BM Chemiluminescence Blotting Substrate (POD from Roche). The blot was exposed to horseradish peroxidase-conjugated anti-human Fc IgG to reveal the anti-apoA-1 IgG binding and with horseradish peroxidase-conjugated anti-goat Fc IgG for the commercially available goat anti-human apoA-1 IgG used as positive control.

As shown in **Supplemental Figure VI**, pooled sera from the anti-apoA-1 IgG positive patients displayed a strong signal at 61 kD (panel A), whereas sera derived from anti-apoA-1 IgG negative patients hardly provided a signal on WB (panel B). Polyclonal goat anti-human apoA-1 IgG displayed the expected 29kD MW band (panel C), as well as the one at 61 KD, in accordance to our previous experiments. [9]

We then submitted the identified 61kD MW band to LC-MS/MS analyses. According to our *in-gel digestion* protocol, gel pieces were dehydrated with 100 µl of 50 mM ammonium bicarbonate (AB) in 30% acetonitrile (ACN) for 10 min. This solution was removed and gel pieces were then incubated for 35 minutes at 56°C in 100 µl of 10 mM DTT in 50 mM AB. DTT solution was then replaced by 100 µl of 55 mM iodoacetamide in 50 mM AB and the gel pieces were incubated for 30 min at room temperature in the dark. Gel pieces were then washed for 30 minutes with 100 µl of 50mM AB and for 30 min with 100 µl of 50 mM AB and 30% ACN. Gel pieces were then dried for 45 minutes in a Speed-Vac Concentrator. Dried pieces of gel were rehydrated for 45 minutes at 4°C in 50 µl of a solution of 50 mM AB containing trypsin at 6.25 ng/µl. Extraction of the peptides was performed with 50 µl of 1% trifluoroacetic acid (TFA) for 30 minutes at room temperature with occasional shaking. The TFA solution containing the proteins was transferred to a polypropylene tube. A second extraction of the peptides was performed with 70 µl of 0.1% TFA in 50% ACN for 30 minutes at room temperature with occasional shaking. The second TFA solution was pooled with the first one. The pooled extracts were completely dried by evaporation under speed-vacuum. LC-ESI-MS/MS was performed on a Orbitrap XL Mass Spectrometer (Thermo Fisher Scientific) equipped with a NanoAcquity system from Waters. Peptides were trapped on a home-made 5 µm 200 Å Magic C18 AQ (Michrom) 0.1 × 20 mm pre-column and separated on a home-made 5 µm 100 Å Magic C18 AQ (Michrom) 0.75 × 150 mm column with a gravity-pulled emitter. The analytical separation was run for 40 minutes using a gradient of H2O/FA 99.9%/0.1% (solvent A) and CH3CN/FA 99.9%/0.1% (solvent B) from 5% to 35% A in 20 minutes at a flow rate of 220 nL/min. For MS survey scans, the OT resolution was set to 60000 and the ion population was set to 5×10^5 with an m/z window from 400 to 2000. Four precursor ions were selected for collision-induced dissociation (CID) in the LTQ. For this, the ion population was set to 1×10^4 (isolation width of 2 m/z). The normalized collision energies were set to 35% for CID. Then, for *protein identification*, peak lists (MGF file format) were generated from raw data using the MS Convert conversion tool from ProteoWizard The peaklist files were searched against the *Homo sapiens* database (UniProtKB, release 2017-03, 20184 entries) using Mascot (Matrix Science, London, UK; version 2.5.1). Trypsin was selected as the enzyme, with two potential missed cleavage sites. Precursor ion tolerance was set to 10 ppm and fragment ion tolerance to 0.6 Da. Variable amino acid modifications were oxidized methionine, carbamylated lysine, and glycated lysine, arginine and tryptophan. Fixed amino acid modification was carbamidomethyl cysteine. The Mascot search was validated using Scaffold

4.7.5 (Proteome Software). Protein identifications were accepted if they could be established at greater than 95.0 % probability and contained at least two identified peptides.

Results are shown in **Supplemental Figure VII**. Analysis of this 61 kD band identified apoA-1 with a total of 29 identified peptides spectrum matched (PSM), corresponding to 12 unique peptide sequences, representing a protein sequence coverage of 47% (highlighted in yellow). Among these 29 PSM, 3 PSM with the same sequence ((K)WQEE $\mathbf{M_{ox}}$ ELYR(Q)) were identified with an oxidized methionine (**Supplemental Figure VII**, red frame). The amount of oxidation could not be quantified exactly using this LC-MS/MS system, but as only one oxidized methionin was found, the oxidation status of apoA-1 is likely to be insignificant and most probably generated by the WB and LC-MS/MS process given apoA-1 susceptibility to oxidation. Moreover, among these 29 PSM, no carbamylation or glycation were detected. Of interest, 8 of these PSM corresponded to the C-terminal sequence spanning aa 240 to 265 (data not shown). On this C-terminal sequence no oxidation or other PTM were identified.

These WB & LC-MS/MS findings indicate that the apoA-1 band at 61 KD recognized on WB by pool sera derived from patients tested positive for anti-apoA-1 IgG is very unlikely to result from recognition of glycated, carbamylated, oxidized cross-linked multimers of apoA-1. The most likely explanation for this phenomenon is that anti-apoA-1 IgG preferentially recognize lipid-low apoA-1. However, as the lipid content of our apoA-1 preparation is not assessable by LC-MS/MS, this hypothesis warrants further study.

Taken together, these supplementary characterization analyses point to two main conclusions. The first one is that our native human purified apoA-1 does not contain substantial amount of carbamylation, glycation or oxidation, confirming previous studies that demonstrate that the immune response to apoA-1 measured in our assay is well directed against unmodified apoA-1, devoid of PTM. [9, 10] The second one is that that our assay is not significantly influenced by glycation or oxidation of apoA-1.

Genotyping of the C260T *rs2569190* polymorphism in the CD14 receptor gene.

After exclusion of non-Caucasian participants (n=395) and those with missing genetic data (n=578), the final genotyped sample for the C260T *rs2569190* polymorphism consisted of 4247 individuals **(Figure 1)**. Genotyping was performed using the Affymetrix GeneChip® Human Mapping 500K array set, excluding SNPs with call rate <70% and individuals with call rate <90%. The imputation dataset included 390,631 genotyped SNPs with call rate>0.9, Hardy–Weinberg P-value>10^{-7} and minor-allele frequency (MAF)>1%. Imputation was performed using IMPUTE version 0.2.0 and CEU haplotypes from HapMap release 21. The C260T *rs2569190* polymorphism was imputed with an r2-hat=0.994. The minor allele in the CoLaus study was T (49.3%) and the major allele C (50.7%), which is consistent with previous reports in Caucasian populations. [14]

Statistical analyses

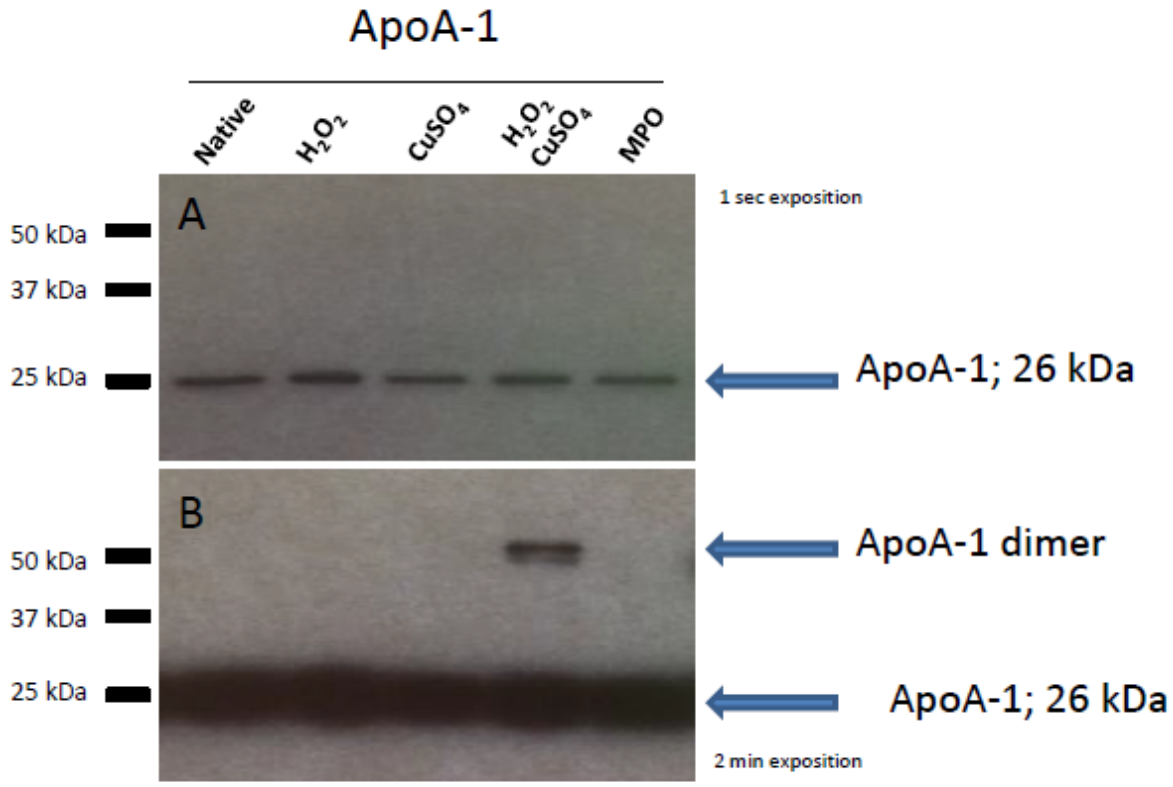
Univariate analysis of continuous variables was performed using the student's t-test or the non-parametric Mann-Whitney test as appropriate, and results were expressed as mean ± standard deviation (SD). Analysis of discrete variables was performed using chi-square test and results were expressed as number of participants and (percentage). Survival curves for incident CAD were produced using the Kaplan-Meier method and compared using the Logrank test. Patients who had no events were censored at the time of death, loss to follow-up or the end of the study

period. Multivariate analysis of the associations between anti-apoA-1 IgG and incident CAD was performed using Cox proportional hazards adjusting for sex, age, smoking status, diabetes, hypertension, low (LDL) and high density lipoprotein cholesterol (HDL), baseline CAD, statin and beta-blocker treatment and eGFR. Results were expressed as hazards ratio (HR) and 95% confidence interval (CI). Adjusted HRs for incident CAD were firstly estimated for anti-apoA-1 IgG positivity as well as across tertiles of increasing anti-apoA-1 IgG values, with anti-apoA-1 IgG negative subjects used as the reference group. As anti-apoA-1 IgG concentration distribution is skewed, values were further natural log transformed and standardized (mean=0 and SD=1) and HR for incident CAD were also assessed per one SD change. The same analyses were repeated in genotyped subjects according to CD14 SNP subgroups and a statistical interaction test was performed to assess differences between these genotype subgroups. [15] Sensitivity analyses were performed after exclusion of subjects with baseline CAD and autoimmune disease.

In genotyped subjects, we first assumed an additive model (CC *vs.* CT *vs.* TT), dividing the sample into three subgroups according to the C260T *rs2569190* allele status. As previously suggested, [14, 16] we then assumed a recessive model (CC/CT *vs.* TT), where C-allele carriers show similar, neutral CD14 gene expression, while homozygotes for the minor allele present increased CD14 gene expression. [17, 18] In both cases, a statistical interaction test was performed to assess the heterogeneity of anti-apoA-1 IgG-related CAD risk, according to carriage of the T allele. [15] Results were expressed as HR (95%CI) within each subgroup and presented as a forest plot. [15] Taking into account the incident CAD rate in CoLaus (157 events or 3%) and a two-sided alpha of 5%, we required 35-59 incident CAD events in subjects positive for anti-apoA-1 IgG to detect an HR of anti-apoA-1 IgG for CAD of 1.5-1.7 with >80% power. Similarly, considering the incident nonfatal CAD rate of 2.5% in our study and two-sided alpha of 5%, we required 32-55 incident non-fatal CAD events in subjects positive for anti-apoA-1 IgG to detect an HR of 1.5-1.7 with >80% power. All analyses were performed using STATA 13.0 (Stata Corp, College Station, Texas, USA). A two-tailed test with $p<0.05$ was considered statistically significant.

316 **FIGURES**

317



318

319 **Figure 1. ApoA-1 oxidation Western Blot**. **Panel A)** one-second exposition, **B)** two-minutes exposition in
320 order to visualize the oxidation-induced apoA-1 dimerization. The expected higher molecular weight
321 apoA-1 bands induced by oxidation-mediated apoA-1 dimers formation were achieved by combining
322 H202 and CuS04.

323

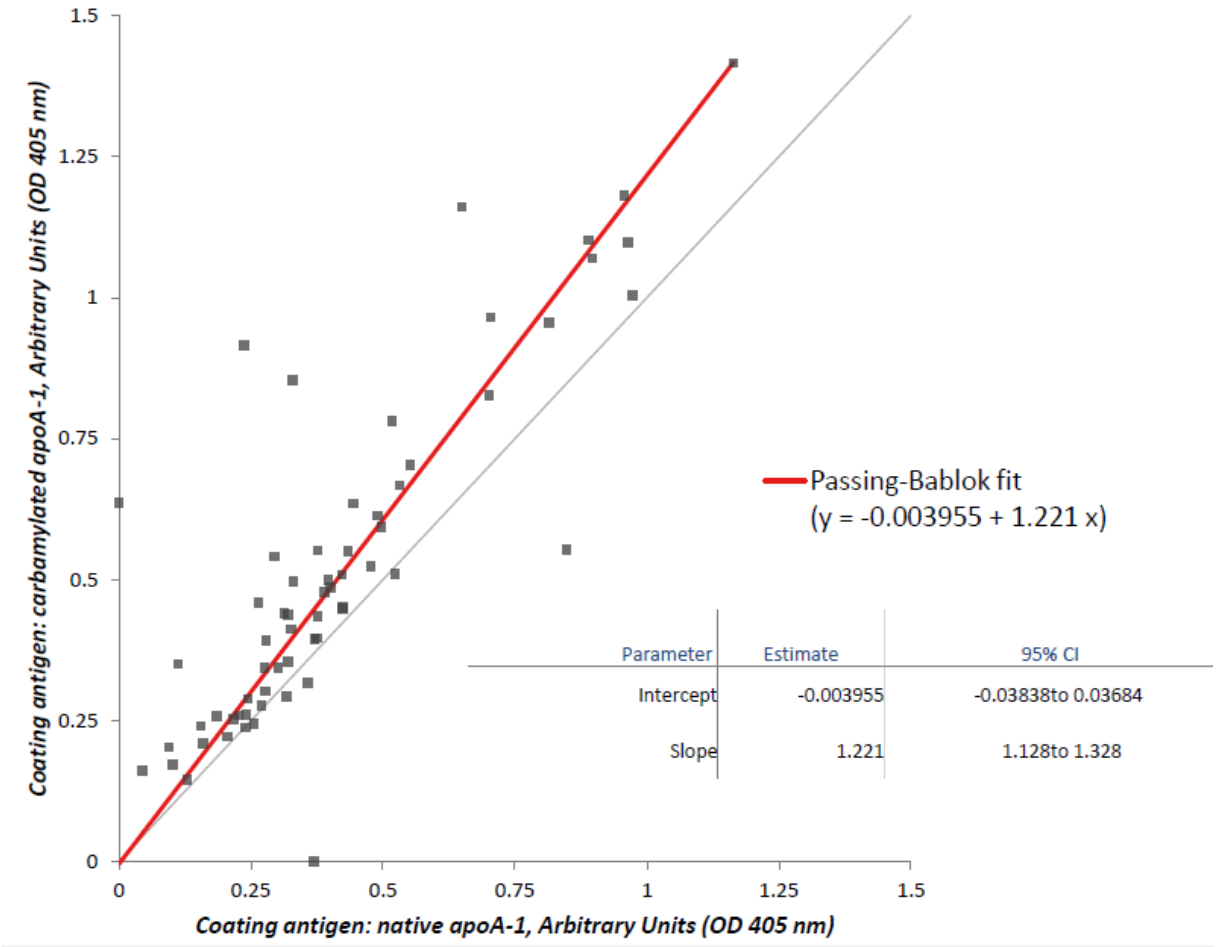


Figure 2. Passing-Bablock analysis comparing the signals obtained using native versus carbamylated apoA-1. The grey line indicates the identity line, the red line indicates the correlation obtained. Using carbamylated apoA-1 as coating antigen in our ELISA, induced a significant proportional bias of + 22% (slope: 1.22; 95%CI: 1.13-1.33), but no systematic bias (intercept: -0.004; 95%CI: -0.04-0.04).

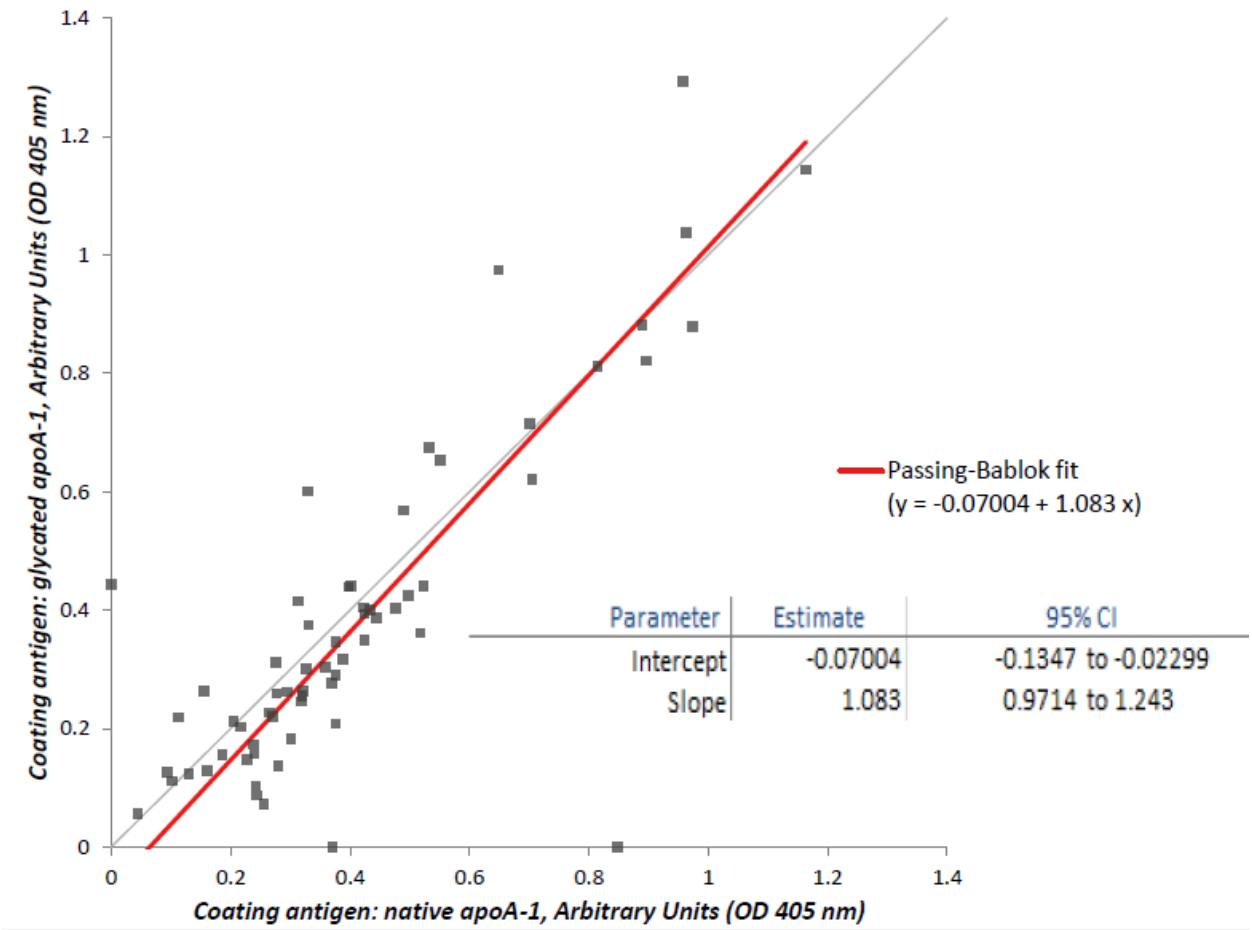


Figure 3. Passing-Bablock analysis comparing the signals obtained using native versus glycated apoA-1. The grey line indicates the identity line, the red line indicates the correlation obtained. Using glycated apoA-1 as coating antigen in our ELISA, induced a non-significant proportional positive bias of + 8% (slope: 1.08; 95%CI: 0.97-1.24), and a small statistically significant, but minor bias of 0.07 arbitrary units (Intercept: -0.07; 95%CI: -13 to -0.02).

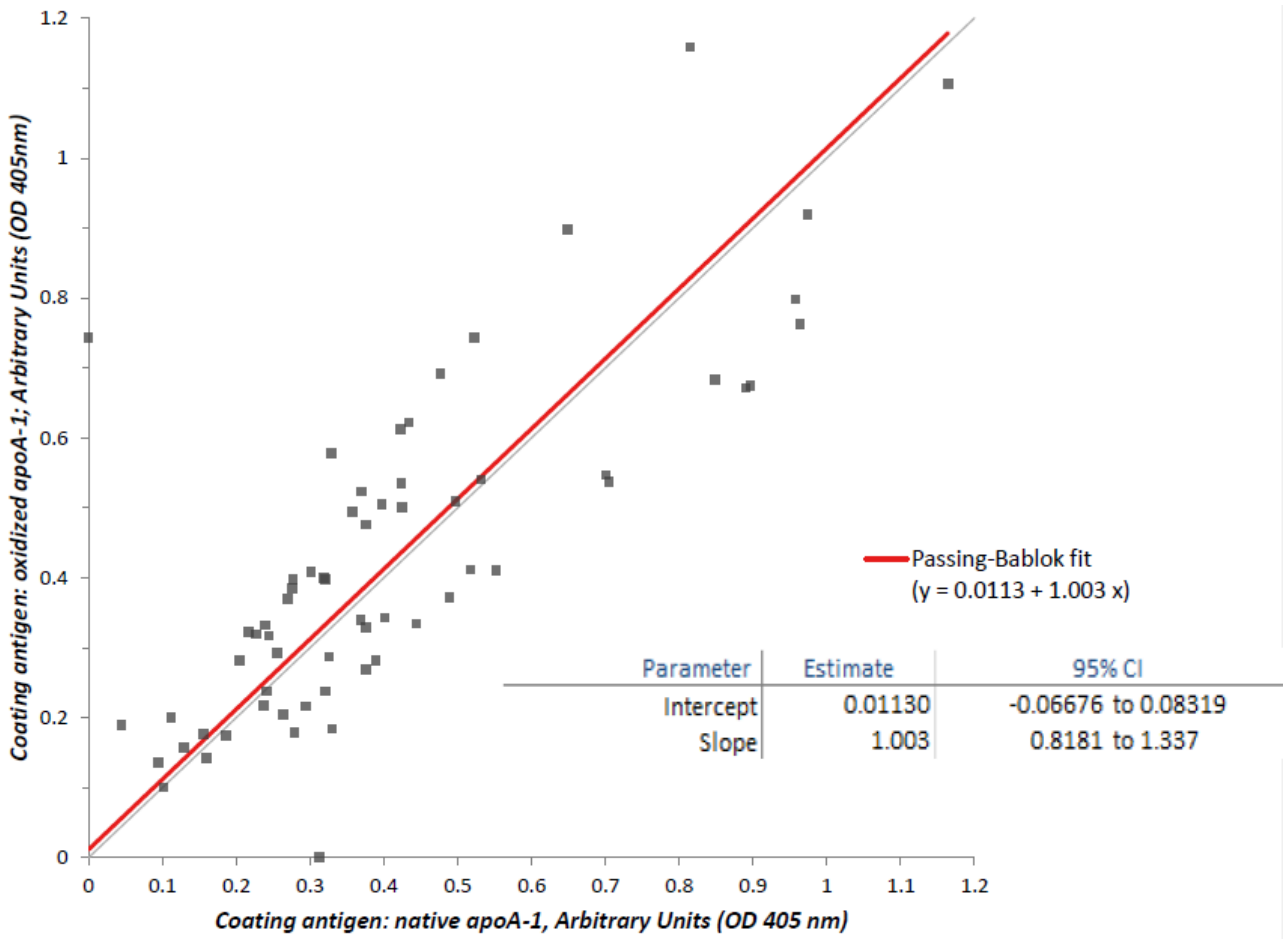


Figure 4. Passing-Bablock analysis comparing the signals obtained using native versus oxidized apoA-1. The grey line indicates the identity line, the red line indicates the correlation obtained. Using oxidized apoA-1 as coating antigen in our ELISA, induced a non significant proportional bias of 0.3% (slope: 1.003; 95%CI: 0.82-1.34) and no systematic bias (intercept: -0.0006; 95%CI:-0.09-0.09).

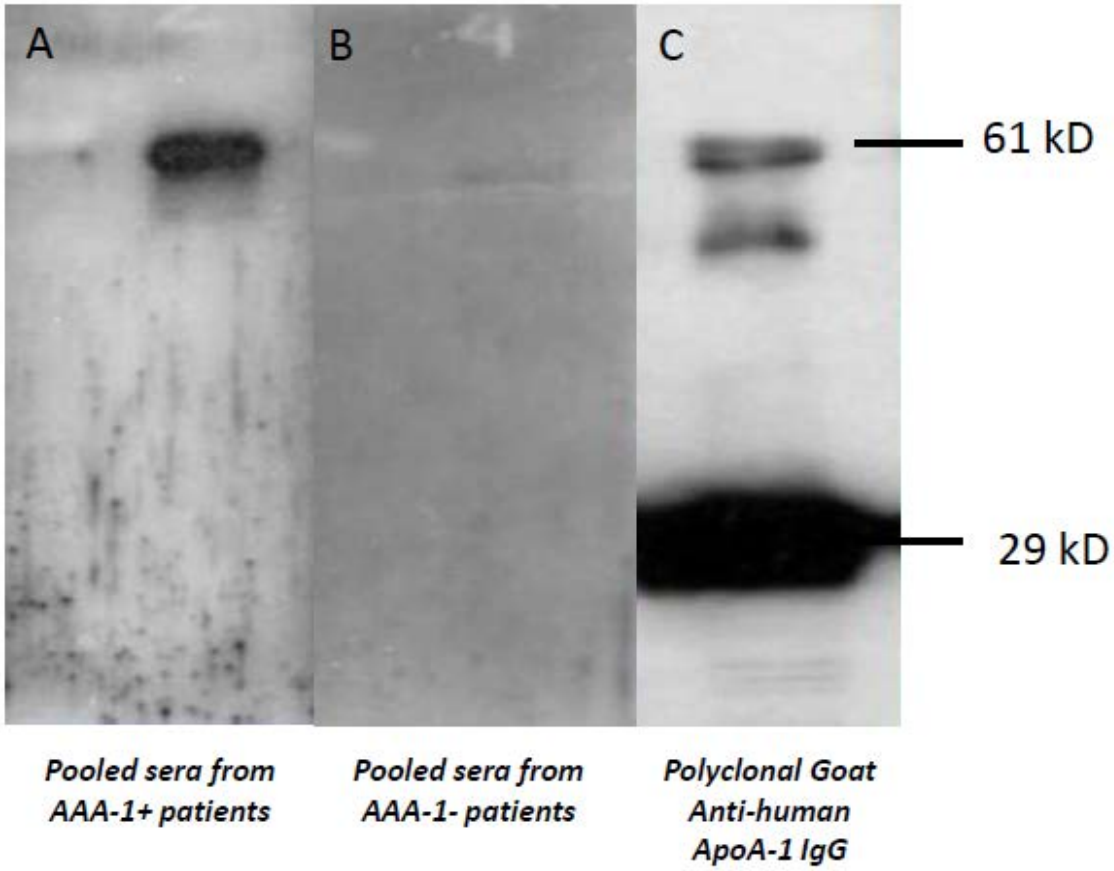


Figure 5. Western blot of anti-apoA-1 IgG. Human apoA-1 was migrated on polyacrylamide gel (10%) and then exposed to pooled sera (dilution 1:50) of patients tested either positive (panel A) or negative for anti-apoA-1 IgG (panel B). The sera from the anti-apoA-1 IgG positive patients displayed a strong signal at 61 kD (panel A), whereas the pooled sera derived from anti-apoA-1 IgG negative patients hardly provided a signal on WB (panel B). Polyclonal goat anti-human apoA-1 IgG displayed the expected 29kD molecular weight band (panel C), as well as a band at 61 KD, further submitted to liquid chromatography – mass spectrometry analyses.

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APOA1_HUMAN (100 %), 30'778.5 Da
Apolipoprotein A-I OS=Homo sapiens GN=APOA1 PE=1 SV=1
12 exclusive unique peptides, 15 exclusive unique spectra, 29 total spectra, 125/267 amino acids (47 % coverage)

M K A A V L T L A V L F L T G S Q A R H F W Q Q D E P P Q S P W D R V K **D L A T**
V Y V D V L K D S G R **D Y V S Q F E G S** **A L G K** Q L N L K **L** **L D N W D S V T S T**
F S K L R E Q L G P V T Q E F W D N L E K E T E G L R **Q E M** **S K D L E E V K** A K
V Q P Y L D D F Q K **K W Q E E M E L Y R** Q K **V E P L R A E L** **Q E G A R** Q K L H E
L Q E K L S P L G E E M R D R A R A H V D A L R **T H L A P Y** **S D E L R** Q R L A A
R L E A L K E N G G A R L A E Y H A K A T E H L S T L S E K **A K P A L E D L R Q**
G L L P V L E S F K **V S F L S A L E E Y** **T K** K L N T Q

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Valid	...	Sequence	Prob	Masc...	Masc...	Masc...	NTT	Modifications	Observed	Actual Mass	Charge	Delta ...	Delta ...	Rete...
☑	✓	(K)DLATVYVDVLK(D)	100 %	40.5	29.2	27.7	2		618.35	1'234.68	2	0.0010	0.84	1760
☑	✓	(K)DLATVYVDVLK(D)	100 %	37.8	29.2	28.0	2		618.35	1'234.68	2	0.0010	0.84	1760
☑	✓	(R)DYVSQFEGSALGK(Q)	100 %	117.0	31.1	102.0	2		700.84	1'399.66	2	0.0016	1.1	1390
☑	✓	(K)LLDNWDSVTSTFSK(L)	100 %	105.1	32.4	99.6	2		806.90	1'611.78	2	0.0027	1.7	1690
☑	✓	(K)LLDNWDSVTSTFSK(L)	100 %	86.7	32.4	86.7	2		806.90	1'611.78	2	0.0040	2.5	1680
☑	✓	(R)QEMSKDLEEVK(A)	100 %	35.8	30.6	33.5	2		668.33	1'334.64	2	0.0014	1.0	1050
☑	✓	(K)WQPYLDDFQK(K)	100 %	46.5	31.2	34.7	2		626.81	1'251.61	2	-0.00039	-0.31	1290
☑	✓	(K)WQPYLDDFQK(K)	100 %	38.3	30.9	34.2	2		626.81	1'251.61	2	0.0012	0.95	1290
☑	✓	(K)WQPYLDDFQKK(W)	100 %	36.8	31.6	22.5	2		690.86	1'379.71	2	-0.00067	-0.48	1200
☑	✓	(K)WQPYLDDFQKK(W)	98 %	31.2	31.6	19.7	2		690.86	1'379.71	2	-0.00067	-0.48	1200
☑	✓	(K)WQPYLDDFQKK(W)	95 %	28.7	31.6	19.9	2		460.91	1'379.71	3	-0.00050	-0.36	1200
☑	✓	(K)WQEEMELYR(Q)	100 %	43.8	25.8	43.8	2	Oxidation (+16)	650.29	1'298.56	2	0.00058	0.45	1200
☑	✓	(K)WQEEMELYR(Q)	100 %	36.0	25.8	36.0	2	Oxidation (+16)	650.29	1'298.56	2	0.00058	0.45	1200
☑	✓	(K)WQEEMELYR(Q)	100 %	33.0	25.8	31.7	2	Oxidation (+16)	650.29	1'298.56	2	0.00058	0.45	1200
☑	✓	(K)WEPLRAELQEGAR(Q)	100 %	35.3	30.8	31.0	2		734.40	1'466.79	2	0.0012	0.84	1150
☑	✓	(K)WEPLRAELQEGAR(Q)	95 %	32.1	30.9	22.3	2		489.94	1'466.78	3	-0.00074	-0.50	1150
☑	✓	(R)THLAPYSDELR(Q)	100 %	38.1	31.3	27.9	2		651.33	1'300.64	2	0.0012	0.89	1120
☑	✓	(R)THLAPYSDELR(Q)	97 %	30.7	31.3	19.7	2		434.56	1'300.64	3	0.0022	1.7	1730
☑	✓	(K)AKPALEDLR(Q)	100 %	43.7	25.0	36.3	2		506.79	1'011.57	2	-0.00063	-0.63	1080
☑	✓	(K)AKPALEDLR(Q)	100 %	32.7	25.0	25.1	2		506.79	1'011.57	2	0.00016	0.16	1080
☑	✓	(K)AKPALEDLR(Q)	98 %	28.0	25.0	20.8	2		506.79	1'011.57	2	-0.00063	-0.63	1080
☑	✓	(R)QGLLPVLESFK(V)	100 %	55.3	27.4	49.0	2		615.86	1'229.70	2	0.00032	0.26	1640
☑	✓	(R)QGLLPVLESFK(V)	100 %	53.2	27.4	41.8	2		615.86	1'229.70	2	0.00032	0.26	1640
☑	✓	(R)QGLLPVLESFK(V)	100 %	51.0	27.3	31.6	2		615.86	1'229.70	2	0.00093	0.76	1620
☑	✓	(R)QGLLPVLESFK(V)	99 %	30.9	26.9	21.3	2		615.86	1'229.71	2	0.0033	2.7	1800
☑	✓	(K)WSFLSALEEYTK(K)	100 %	76.1	30.9	72.3	2		693.86	1'385.71	2	0.0018	1.3	1670
☑	✓	(K)WSFLSALEEYTK(K)	100 %	66.9	30.9	65.3	2		693.86	1'385.71	2	0.0018	1.3	1670
☑	✓	(K)WSFLSALEEYTK(K)	100 %	63.8	30.7	59.9	2		693.86	1'385.71	2	0.00048	0.34	1680
☑	✓	(K)WSFLSALEEYTK(K)	100 %	58.3	30.7	56.7	2		693.86	1'385.71	2	0.00048	0.34	1680

358

359 **Figure 6. Liquid Chromatography – Mass Spectrometry Analyses of the 61kD apoA-1 band. Above :**
360 Highlighted in yellow the 12 exclusive amino acid sequences identified in the 61kD apoA-1 band.
361 Highlighted in green, the only oxidized methionin found. **Below :** Among the 29 peptides spectrum

matched (PSM), 3 PSM with the same sequence ((K)WQEE **M_{ox}** ELYR(Q)) were identified with an oxidized methionine (sequences in the red frame), but no carbamylation or glycation were detected.

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