Immune-driven inflammation plays an important part in atherogenesis, and is therefore believed to be paramount for cardiovascular (CV) disease (CVD) development, currently considered as the leading cause of death in the Western world. By fulfilling some of the Koch postulates atherogenesis has even been proposed to be considered as an autoimmune disease, raising the hope that CVD could be prevented by immunomodulation. Nevertheless, the role of the immune system and autoimmune reactions in atherosclerosis appears to be a double edge-sword, some of them being pro-atherogenic, while others anti-atherogenic. For this reason, providing that immunomodulation is to become a therapeutic option for atherosclerosis and CVD, it will be crucial to correctly identify patients that will require a boost of the immune response from those requiring a targeted suppression of deleterious autoimmune responses, which could, among others, be achieved by the detection of specific autoantibodies. In this work, I will present the main clinical studies derived from a translational research project dedicated to autoantibodies directed against [...]
"Autoantibodies to apolipoprotein A-1 as a biomarker of cardiovascular autoimmunity"

Thesis submitted to the Medical School of the University of Geneva

for the degree of Privat-Docent

by

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SUMMARY

Immune-driven inflammation plays an important part in atherogenesis, and is therefore believed to be paramount for cardiovascular (CV) disease (CVD) development, currently considered as the leading cause of death in the Western world. By fulfilling some of the Koch postulates atherogenesis has even been proposed to be considered as an autoimmune disease, raising the hope that CVD could be prevented by immunomodulation. Nevertheless, the role of the immune system and autoimmune reactions in atherosclerosis appears to be a double edge-sword, some of them being pro-atherogenic, while others anti-atherogenic. For this reason, providing that immunomodulation is to become a therapeutic option for atherosclerosis and CVD, it will be crucial to correctly identify patients that will require a boost of the immune response from those requiring a targeted suppression of deleterious autoimmune responses, which could, among others, be achieved by the detection of specific autoantibodies. In this work, I will present the main clinical studies derived from a translational research project dedicated to autoantibodies directed against apolipoprotein A-1 (anti-apoA-1 IgG), the major proteic fraction of High Density Lipoprotein (HDL). Those studies demonstrate that both in patients with autoimmune disease and in non autoimmune settings, high levels of anti-apoA-1 IgG are associated with a worse CV prognosis. I will also present current data derived from in vitro and in vivo studies supporting a pro-inflammatory, pro-atherogenic, and thus causal role of those autoantibodies in CVD, suggesting that they could also represent an emerging therapeutic target. In this context, autoantibodies to apoA-1 appears as a promising biomarker of cardiovascular autoimmunity allowing the identification of subset of CVD patients that could benefit from immunomodulation in the future, contributing to the development of personalized medicine in the field of CVD.
ACKNOWLEDGEMENTS

The translational nature of the scientific work presented here is the result of fruitful and long-lasting collaborations between the department of genetics and laboratory medicine and the department of medical specialties, including cardiology, immunology, endocrinology and diabetology divisions of Geneva University Hospitals.

I would like to warmly thank my mentor Professor Denis Hochstrasser for teaching me the key role of laboratory medicine for the proper development of patient-oriented translational medicine, and for providing me with an optimal environment to develop my career, wisely orchestrated between clinical activities in internal and laboratory medicine, and translational research in the field of biomarkers. I’m also grateful to former Professor Francis Waldvogel for his generous and open-minded attitude, allowing me to train and specialize in internal medicine besides research, and routine laboratory medicine training.

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Finally, I would like to dedicate this work to my parents for their generous support during my education and career, and last but not least, to my wife Audrey, and to my children Nathan, Aloys, and Edouard.

“The love of life is necessary to the vigorous prosecution of any undertaking.”

Samuel Johnson
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1. Introduction

1.1. Current epidemiology of cardiovascular diseases and preventive strategies

Despite increasing public awareness and major therapeutic progress, cardiovascular disease (CVD) remains the leading cause of morbidity and mortality worldwide. In the United States, CVD prevalence in the general population is expected to reach 40%, with direct related costs set to reach 800 billion dollars per year in the next two decades [1]. In Europe, CVD causes 47% of all deaths (Figure 1), accounting for 4 million fatalities each year, and costing 196 billions euros a year. Half of them (54 %) being attributed to direct health care costs, whereas the other half (46%) being inferred to indirect losses (Heart Network: www.ehnheart.org).

![Figure 1. Deaths by cause in Europe for the latest available year, and both gender. Adapted from European Heart Network (www.ehnheart.org)](image)

Because the disease progresses asymptptomatically, the first indication that an individual has atherosclerosis is often a severe cardiovascular event. According to statistics obtained in the
USA during the last two decades, the first indicator of atherosclerosis for 30-50% of patients was a sudden, and in many cases fatal, myocardial infarction (MI) [2]. Current guidelines address this problem by identifying high-risk individuals according to the cumulative presence of different Framingham risk factors (smoking, obesity, diabetes, dyslipidemia, and hypertension), with the decision to go forward into preventive treatment made according to the estimated risk. Based upon those clinically-based CV risk stratification tools, individuals identified as at-risk for atherosclerosis and CVD are subjected to treatment that directly addresses the established risk factors, combining lifestyle modification (e.g. smoking, exercise, diet) with anti-platelets therapy (aspirin), medication both to reduce blood pressure (anti-hypertensive agents) and levels of circulating cholesterol (statins).

While this strategy has undoubtedly made some impact, current CV risk stratification tools only have the power to segregate very high-risk individuals from very low-risk individuals, but do lack of sensitivity and specificity in persons deemed at intermediate risk [3]. As a consequence, up to 60% of CV events occur in primary prevention (i.e. in patients with asymptomatic CVD) subjects deemed at low or intermediate risk of CVD (false negative) [4-5], and many others are potentially unnecessarily put on to lifelong prevention medication (false positives) (reviewed in [2, 6]).

For this reason, strong calls have been made to exploit existing knowledge and technology to improve the sensitivity and specificity of risk stratification approaches used to guide preventive therapy [2]. To be effective as public health measures, new approaches would have to be not only sensitive and specific, but also low-cost, non-invasive and adaptable to scale-up and commercialization for widespread use [2, 6]. While solutions involving imaging technologies such as ultrasound, chest computed tomography (CT) and magnetic resonance imaging have been proposed [2], their implementation at population level in primary care is currently difficult to envisage mainly for economical reasons, and also because of health hazards related to radiations exposure.
As a more viable alternative strategy in regards of costs and health issues, much attention has been drawn on different CV biomarkers allowing on a simple blood sample measurement to quantify either the amount of myocardial necrosis, such as cardiac troponins [7], the degree of myocardial stretch, such as natriuretic peptides [8], or the amount of systemic inflammation, such as high sensitive C-reactive protein (hsCRP) [9], to only quote the “usual suspects” in the field. However, the list of CV biomarkers candidates is much longer, as a reflection of the numerous studies published in the field (Figure 2). Nevertheless, only few of those candidates are likely to make their way to clinical practice.

![Figure 2. Annual evolution of publications on cardiac biomarkers since the year 2000. This graphic represents the number of publications per year indexed and retrieved in Pubmed between 2000 and 2012 when the key words “cardiovascular biomarkers” are entered.](image)

To increase the chance of success, it is of paramount importance to demonstrate that the biomarker of interest is causally involved in the disease, because it is hoped that improved knowledge of the pathogenesis of atherosclerosis will lead to the development of new risk stratification approaches based on the detection of biomarkers for atherosclerosis and CVD [6].
1.2. Pathogenesis of atherosclerosis and cardiovascular disease

CVD is causally linked to atherosclerosis, the swelling of artery walls due to the formation of plaque lesions. Plaques are made up of leukocytes, smooth muscle cells and lipid deposits, with the surface of the plaque in contact the arterial lumen covered with a fibrous connective tissue cap. Although atherosclerosis accumulates gradually and asymptptomatically from childhood, it is accelerated by a number of established risk factors, including Framingham risk factors. Atherosclerotic plaques may remain stable as they grow, gradually reducing arterial blood flow as the lumen becomes increasingly obstructed, or may become prone to rupture. When plaque rupture occurs, the highly thrombogenic interior of the plaque is revealed, leading to atherothrombosis. The resulting ischaemia is what causes CVD morbidity and mortality. Depending on the location of the affected artery the outcome can be myocardial infarction (heart), stroke (brain), or claudication (limbs) [4].

1.2.1. Atherosclerosis as an immune-mediated disease

Evidence linking high blood cholesterol to atherosclerosis, together with the presence of lipid deposits within atherosclerotic plaques led to the prevailing view that atherosclerosis was a lipid-related disease. This view was held until the 1990’s, when a series of discoveries led to a paradigm shift in the understanding of atherosclerosis, shifting emphasis from lipid metabolism and transport to inflammation [10-12]. Inflammatory responses are now believed to underlie all of the key steps in atherosclerotic pathogenesis, from the initial modification of healthy arterial endothelium to thrombus formation at the site of plaque rupture, as depicted in Figure 3 below.
According to the current paradigm (reviewed in [2, 10-12]), atherosclerosis is initiated by inflammatory activation of arterial wall endothelial cells, allowing adhesion of circulating leukocytes. Expression of inflammatory chemokines leads to the migration of these leukocytes, predominantly circulating monocytes, across the endothelium and into the tunica intima. At this site the monocytes mature, acquiring a macrophage phenotype and the capacity to ingest native and modified low-density lipoprotein (LDL) particles that exit the blood and permeate the activated arterial endothelium. Following extensive lipid ingestion, these macrophages become ‘foam cells’, which are the main constituents of an early atherosclerotic lesion. Foam cells release further a broad range of cytokines and serving to amplify the inflammatory response, as well as inducing the proliferation of resident smooth muscle cells and promoting local angiogenesis. Chronic inflammation leads to the formation of an advanced atherosclerotic plaque, comprising a mass of foam cells surrounding a ‘necrotic...
core’ of lipids released by dead and dying, capped by a fibrous layer made up of smooth muscle cells and extracellular matrix. Inflammatory responses also play a key role in atherothrombosis, which is recognized to account for up to 80% of acute CV manifestations [13]. Inflammation influences the local extracellular matrix composition through a complex interplay between different matrix-metalloproteinases (MMPs) determining the propensity of the fibrous cap to rupture [14-17]. Furthermore a pro-inflammatory micro-environment also promotes thrombus formation through the activation of coagulation factors promoting thrombus formation leading to acute vessel occlusion [6].

Detailed analysis of the content of atherosclerotic plaques, together with the advent of a wide range of genetically modified mouse strains, has enabled further elucidation of the inflammatory pathogenesis of atherosclerosis [18].

The identification of autoantibodies as well as autoreactive T cells in atherosclerotic plaques [19], and the correlation established in clinical studies between their detection and disease severity provided a clear indication that adaptive immunity plays a role in atherosclerosis (reviewed in [20]). This role was underlined in a number of studies in which ApoE-/- knockout mice, which are predisposed to hypercholesterolemia and atherosclerosis, were crossed with different mouse strains deficient in specific arms of the adaptive immune system. These studies revealed a key pro-atherogenic role for the Th1 subset of CD4 T cells, and an anti-atherogenic role for the regulatory T cell subset (reviewed in [20]), as well as both pro- and anti-atherogenic roles for different B cell subsets [21]. In addition, they highlighted the importance in atherogenesis of signalling through pattern recognition receptors (PRR) of the innate immune system, such as Toll-like receptors (TLR) (reviewed in [18, 20]).

1.2.2. Atherosclerosis as an autoimmune disease?

Grounded on the fact that atherogenesis fulfills several of the “Koch’s postulates (Table 1), atherosclerosis has even been proposed to be of autoimmune etiology [22, 23]. This
hypothesis is based on the following pieces of evidence. Firstly, atherosclerotic plaques are infiltrated by both T cells and antibodies specific for various autoantigens [20], patients suffering from autoimmune disease, such as systemic lupus erythematosus (SLE), antiphospholipid syndrome (APS) and rheumatoid arthritis (RA) display an increased cardiovascular (CV) risk, independently of traditional CV risk factors [24-26]. Secondly, as reviewed elsewhere, patients without autoimmune diseases but established CVD, antibodies directed against various and numerous endogenous epitopes, such as modified LDL, heat-shock proteins (HSP), cardiolipin, have been shown to independently predict CV outcome [27]. Thirdly, in vivo and in vitro evidences demonstrate that some autoantibodies might directly influence atherogenesis and atherosclerotic plaque vulnerability, mostly through innate immune receptors signaling, thereby supporting a causal role of humoral autoimmunity in atherosclerosis [29-31].

<table>
<thead>
<tr>
<th>Basic Koch postulates</th>
<th>Koch postulates transposed to the role of autoimmunity in atherosclerosis</th>
<th>Koch postulates met?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Pathogens must be detected in the diseased host at every stage of the disease</td>
<td>1. Auto-antibodies and auto-reactive T cells can be detected in atherosclerotic plaques and serum of patients in primary or secondary prevention for CVD.</td>
<td>YES</td>
</tr>
<tr>
<td>2. Pathogens must be isolated from the diseased host and grown in culture</td>
<td>2. Autoreactive T cells can be isolated and cultivated from diseased host presenting experimental atherosclerosis.</td>
<td>YES</td>
</tr>
<tr>
<td>3. When inoculated to healthy animals, the pathogens from pure culture must induce the disease</td>
<td>3. Passive or active immunization drastically affect the course of atherogenesis in animal models.</td>
<td>YES</td>
</tr>
<tr>
<td>4. The pathogen must be re-isolated from the diseased animal and correspond to the original pathogen in pure culture</td>
<td>4. Protective autoantibodies of expected specificity can be isolated from animals exposed to active immunization</td>
<td>Partly</td>
</tr>
</tbody>
</table>

Table 1. Koch posultats applied to the role of autoimmunity in atherosclerosis. To establish causality link between a microorganism and an infection, the four Koch postulates must be fulfilled. When applied to the role of autoimmunity in atherosclerosis, the Koch postulates support a causal role between autoimmunity atherosclerosis and CVD. Adapted from [20-28].
Nevertheless, the relationship between autoantibodies and CVD is debated, because some of them have been shown to be anti-atherogenic, whilst other act as pro-atherogenic molecules [27, 28]. The reason for such duality is still elusive and won’t be further discussed in the present work.

Another unresolved question concerns the mechanisms by which tolerance is broken to generate autoimmunity. However, certain lines of evidence point to pathogen molecular mimicry, i.e. cross-reactivity between microbial antigens and components of host structures, including modified LDL and heat shock proteins [32, 33]. In addition, modification of proteins by oxidation can generate new epitopes that are recognized as non-self by the adaptive immune system [32]. However, the presence of a non-self epitope is not normally sufficient to drive an autoimmune response, since in order to effectively prime T cells, antigen presenting cells must concomitantly receive ‘danger signals’ through their PRR. In the case of pathogen molecular mimicry, the PRR ligands are provided by the pathogen in the form of pathogen-associated molecular patterns (PAMPs). In the absence of a pathogen, ‘sterile inflammation’ can be induced when antigen presenting cells are stimulated via their PRR by an analogous set of structures called damage-associated molecular patterns (DAMPs), which are typically released by stressed or necrotic cells (Figure 4).

Hence both pathogen molecular mimicry, as a consequence of infection with e.g. *Chlamydia pneumoniae* or *Helicobacter pylori*, or DAMP-mediated sterile inflammation represent mechanisms by which autoantibodies targeting antigens implicated in atherosclerosis can emerge (reviewed in [12]).
Figure 4. Pathogen associated molecular patterns (PAMPs) derived from altered self as mediators of innate immune signaling. Adapted from Chou MY et al. J Intern Med. 2008;263(5):479-88.

1.4. Autoantibodies as CV risk stratification tools?

Parts of this chapter have been published as:


As mentioned in chapter 1.1, there is a clear need for new biomarkers to improve current CV risk stratification [6, 34]. Driven by the paradigm shift of atherogenesis moving from a lipid-centered to inflammatory-centered etiology, the quest for new potential cardiovascular risk marker to better assess global cardiovascular vulnerability was principally oriented on inflammatory biomarkers, including auto-antibodies [27,28, 29].

Among the advantages identified for some autoantibodies is that they meet the current benchmark specifications requested for novel CV biomarkers [35, 36]. Firstly, their
association to CV outcomes has not only been shown to be independent of traditional CV risk factors (reviewed in [27, 28]), but also to provide incremental predictive information over current CV risk stratification tool as presented later in chapter 3.3. Secondly, the stability provided by their long half-life place them as good candidates for long term prognosis when compared to biomarkers with shorter half-life. Thirdly, their measurement is rather simple, accurate, robust, and can be achieved at moderate costs.

1.5. Autoantibodies as potential therapeutic targets?

Providing that some autoantibodies have been shown to modulate atherogenic processes in antagonistic ways (reviewed in [27, 28]), attempts to induce atheroprotective immunity through active immunization raised the hope that vaccination against different specific antigens (wide variety of modified LDL, HSP, etc…) could be a possible way to provide a lifelong protection against atherosclerosis and CVD. This hypothesis is currently under active investigations in humans [37, 38].

On the other hand, neutralizing the deleterious effects of pro-atherogenic autoantibodies represents another interesting therapeutic modality which could currently be achieved through passive immunization with intra-venous immunoglobulins (IVIG). To this respect, data concerning IVIG administration in humans after MI yield rather contradictory results [38-39], and data restricted to animal model do support an anti-atherogenic role of IVIG in rodents [40-43]. Nevertheless, because of the costs related to IVIG therapy, the widespread administration of IVIG to all MI or CVD patients may well be prohibitive on the long term, even if effective. In the same line of thought, based upon the identification of specific autoantibodies, rather than using IVIG, one could easily foresees the use of specific anti-idiotypic molecules to selectively neutralize the harmful effects of a given pro-atherogenic antibody.
Accordingly, an approach based upon the presence autoantibodies would enable the identification of a subset of CVD patients that could benefit either from immunomodulation (passive or active immunization) or from a specific mimetic peptide-based therapy. Such a strategy could represent an affordable step forward toward personalized medicine in the field of CVD, allowing a more targeted therapeutic intervention.
2. High-density lipoprotein, Apolipoprotein A-1, and its related autoantibodies.

Parts of this chapter have been published as:


Human apolipoprotein A-1 (apoA-1) is a 28-kD protein with 243 amino acid residues encoded by the apolipoprotein multigene superfamily located on chromosome 11q23 [44]. The protein is synthesized as a 24 amino-acid-longer preprosequence of apoA-1, primarily by hepatocytes in the liver and also by enterocytes. Mature apoA-1 constitutes the principal protein fraction of high density lipoprotein (HDL) whose protective role in the cardiovascular (CV) system derives to a great extent from the inverse association of HDL-cholesterol and apoA-1 plasma concentrations with the risk of myocardial infarction. The atheroprotective role of HDL in the cardiovascular system has been attributed to the pleiotropic effects of HDL, including reverse cholesterol transport from resident arterial wall macrophages to the biliary tract for elimination, vasodilatation, anti-thrombotic, anti-coagulant and anti-inflammatory effects [45-46]. Mirroring those versatile properties, mass spectrometry analyses revealed that HDL encompasses very heterogeneous macromolecular complexes of lipids and proteins. Only one third of the up to 80 different proteins identified in HDL is dedicated to lipid transport. The remaining proteins being either acute-phase proteins, proteases, anti-oxidant, anti-thrombotic enzymes or proteins involved in complement regulation [45-46].
On top of being the principal protein fraction of HDL and a limiting factor for HDL formation, apoA-1 per se has many of the HDL-related atheroprotective properties, such as inhibition of immune cells trans-endothelial migration, inhibition of monocyte activation, inhibition of cytokine production induced by T-cell contact, inhibition of lipid peroxidation, and interference with innate immune receptors pro-inflammatory signaling [45]. There is also a growing body of evidence indicating that both acute and chronic inflammatory conditions induce post-translational modifications of apoA-1 transforming HDL and apoA-1 into pro-inflammatory molecules [46]. As described in the next chapter, humoral autoimmunity to apoA-1 and HDL has been reported as possible contributing mechanism underlying the loss of atheroprotective functions of apoA-1 and HDL. For this reason, we and others have focused our work on autoantibodies to apoA-1 (anti-apoA-1 IgG) as an emergent biomarker of CV risk.

3. Experimental Studies

3.1. Synopsis

In this experimental section, this thesis presents six studies published in peer reviewed journals where the applicant was either the first or the last author. In order to respect the temporality of their discovery, this section will starts with chapter 3.2 including a brief introduction dedicated to the initial description of anti-apoA-1 IgG in autoimmune diseases, such as SLE, and APS. This chapter will be then expanded to RA patients, by presenting the first paper.

Chapter 3.3 will be dedicated to the description of anti-apoA-1 IgG in high CV risk populations without autoimmune diseases. This chapter will start with two publications dedicated to acute coronary syndromes patients, followed by one publication on patients with severe carotid stenosis.

The chapter 3.4 will present two mechanistic papers including in vitro and in vivo experiments pointing to those autoantibodies as active mediators of inflammation and atherogenesis.
3.2. Anti-apoA-1 IgG in autoimmune diseases

3.2.1. Anti-apoA-1 IgG in SLE and APS patients

In 1995, using early phage display technology, Merill JT and colleagues reported that sera derived from SLE patients were immunoreactive against a protein displaying 82% DNA sequence homology with human apoA-1, followed by the confirmation that those sera were indeed reactive to human apoA-1 when coated on gamma-irradiated ELISA plates [47]. Further understanding of anti-apoA-1 autoantibody architecture was provided by the same group in 1998 reporting that high levels of anti-apoA-1 IgG were retrieved in a significant subset of SLE (32.5%) and primary APS patients (22.9%) [35]. Those autoantibodies were found to be associated with the presence of anti-beta2glycoprotein I (β2GPI) antibodies, and to display an optimal affinity for mature HDLs [48]. In 2001, Abe and colleagues characterized six different monoclonal anti-apoA-1 antibodies (derived from two SLE patients) displaying a low specificity, as reflected by their broad cross-reactivity to single strand DNA, thrombin, cardiolipin (CL), and to HDL [48-49]. Because of the latter observation, anti-apoA-1 IgG were considered as a possible subgroup of anti-HDL antibodies [49]. The first insight about the potential pathogenicity of this class of autoantibodies in atherogenesis was brought in 2003 by Delgado Alves and colleagues, who demonstrated an inverse correlation between anti-HDL IgG and paraoxonase-1 (PON-1) activity, and with the total antioxidant capacity of the corresponding sera [50]. More specifically, those initial results suggested that that anti-HDL, and later anti-apoA-1 IgG [51], could be related to atherogenesis, through HDL dysfunction [52-53], whose pathophysiological role in atherogenesis started to be recognized [54].
3.2.2. Anti-apoA-1 IgG in Rheumatoid Arthritis

This paper has been published as:

Anti–Apolipoprotein A-1 IgG Predicts Major Cardiovascular Events in Patients With Rheumatoid Arthritis

Nicolas Vuilleumier,1 Sylvette Bas,1 Sabrina Pagano,1 Fabrizio Montecucco,1 Pierre-André Guerne,1 Axel Finckh,1 Christian Lovis,1 François Mach,1 Denis Hochstrasser,1 Pascale Roux-Lombard,1 and Cem Gabay2

Objective. To determine whether anti–apolipoprotein A-1 (anti–Apo A-1) IgG are associated with major cardiovascular events in patients with rheumatoid arthritis (RA).

Methods. We determined anti–Apo A-1 IgG levels and the concentrations of cytokines, oxidized low-density lipoprotein (LDL), and matrix metalloproteinase 1 (MMP-1) MMP-2, MMP-3, and MMP-9 in sera from 133 patients with RA who did not have cardiovascular disease at baseline, all of whom were longitudinally followed up over a median period of 9 years. A major cardiovascular event was defined as a fatal or nonfatal stroke or acute coronary syndrome. The proinflammatory effects of anti–Apo A-1 IgG were assessed on human macrophages in vitro.

Results. During followup, the overall incidence of major cardiovascular events was 15% (20 of 133 patients). At baseline, anti–Apo A-1 IgG positivity was 17% and was associated with a higher incidence of major cardiovascular events (adjusted hazard ratio 4.2, 95% confidence interval 1.5–12.1). Patients who experienced a subsequent major cardiovascular event had higher circulating levels of anti–Apo A-1 IgG at baseline compared with those who did not have a major cardiovascular event. Receiver operating curve analysis showed that anti–Apo A-1 IgG was the strongest of all tested biomarkers for the prediction of a subsequent major cardiovascular event, with an area under the curve value of 0.73 (P < 0.0008). At the predefined and previously validated cutoff levels, the specificity and sensitivity of anti–Apo A-1 IgG to predict major cardiovascular events were 50% and 90%, respectively. Anti–Apo A-1 IgG positivity was associated with higher median circulating levels of interleukin-8 (IL-8), oxidized LDL, and MMP-9 and higher proMMP-9 activity as assessed by zymography. On human macrophages, anti–Apo A-1 IgG induced a significant dose-dependent increase in IL-8 and MMP-9 levels and proMMP-9 activity.

Conclusion. Anti–Apo A-1 IgG is an independent predictor of major cardiovascular events in RA, possibly by affecting vulnerability to atherosclerotic plaque.

In rheumatoid arthritis (RA), the burden of atherosclerosis and subsequent cardiovascular events is increased and represents the primary cause of mortality. RA is currently considered to be an independent risk factor for cardiovascular disease (1–5). A combination of traditional and nontraditional risk factors has been described to account for the accelerated atherosclerosis and the increased cardiovascular risk, but the underlying pathways are not well understood. It is not clear to what extent emergent nontraditional cardiovascular risk factors such as chemokines (6), cytokines (7), autoantibodies (8,9), oxidized low-density lipoproteins (LDLs) (10), and matrix metalloproteinases (MMPs) (11) could contribute to atherosclerosis and atherothrombosis in RA.
High levels of anti-apolipoprotein A-1 (anti-Apo A-1) IgG have been identified in patients with autoimmune diseases associated with high cardiovascular risk, such as systemic lupus erythematosus (SLE) (12), as well as in patients with myocardial infarction (MI) (13,14), in whom anti-Apo A-1 IgG have been shown to be an independent predictor of major cardiovascular events (15). Whether anti–Apo A-1 IgG exist in patients with RA and could predict the occurrence of major cardiovascular events remains unknown.

Therefore, we investigated 1) whether anti–Apo A-1 IgG are present in RA, 2) whether they could predict the occurrence of major cardiovascular events in RA, and 3) whether anti–Apo A-1 IgG are associated with higher levels of circulating mediators of inflammation and plaque instability, such as oxidized LDL, interleukin-6 (IL-6), IL-8, IL-1 receptor antagonist (IL-1Ra), tumor necrosis factor α, monocyte chemotactic protein 1, and MMP-1, MMP-2, MMP-3, and MMP-9, which are the MMPs most consistently associated with increased cardiovascular risk in humans (6,7,11).

**PATIENTS AND METHODS**

The research Ethics Committee of the Geneva University Hospitals approved this protocol, which was performed in compliance with the principles of the Declaration of Helsinki.

**Patient population and study design.** This was a nested cohort study of patients with RA for whom serum samples were available and who were included in the Geneva arthritis biobank patient cohort. The sample size of this study was computed based on preliminary results showing that the prevalence of Apo A-1 IgG in patients with RA was 11% (14), and by extrapolating our previous results from patients with MI (15) to patients with RA. Using a survival model (utilizing the log rank approach), a total sample size of 133 patients was estimated to detect a 3-fold increase in the risk of a major event (12% versus 36%) with a power of 90% and an alpha error of 5%. Inclusion criteria consisted of a diagnosis of RA, regardless of age and sex. Exclusion criteria were the presence of concomitant SLE based on the revised American College of Rheumatology (ACR) criteria (16), antiphospholipid syndrome (APS) according to the Sapporo criteria (17), or a major cardiovascular event (see definition below) before enrollment. Between January 1, 1990 and January 1, 2008, a total of 1,200 patients with a diagnosis of RA according to the revised ACR criteria (18) were followed up at the Geneva University Hospitals, for whom 144 samples were available in the serum biobank. Among those, 133 patients did not have any known cardiovascular event, SLE, or APS at the time of blood collection and were deemed eligible for inclusion in the present study.

The primary outcome was the occurrence of a major cardiovascular event during followup, defined prospectively by the presence of fatal or nonfatal acute coronary syndrome or stroke. The occurrence of major cardiovascular events was adjudicated by a study coordinator who was blinded to the results of biochemical analyses. Information was obtained by checking the medical files of the patients and by contacting the physician in charge of the patient. We also made contact with the patients individually and inquired about the occurrence of cardiovascular events. Only major cardiovascular events confirmed by the medical record and the treating physician were taken into account.

The secondary outcome involved analyzing the relationship between anti–Apo A-1 IgG and several markers of inflammation and plaque stability, such as oxidized LDL, IL-6, IL-8, IL-1Ra, MMP-1, MMP-2, MMP-3, and MMP-9.

**Sample collection.** The median time between RA diagnosis and sample collection was 7 years (range 0–50 years; interquartile range [IQR] 1–14 years). Samples were immediately centrifuged, aliquoted, and frozen at −80°C until analyzed.

**Biochemical analysis.** Determination of human antibodies to Apo A-1 by enzyme-linked immunosorbent assay (ELISA). Anti–Apo A-1 IgG were measured as previously described (14). Briefly, Maxi-Sorb plates (Nunc) were coated with purified, human-derived delipidated Apo A-1 (20 μg/ml; 50 μl/well) for 1 hour at 37°C. After 3 washes with phosphate buffered saline (PBS)/2% bovine serum albumin (BSA; 100 μl/well), all wells were blocked for 1 hour with 2% BSA at 37°C. Samples were diluted 1:50 in PBS/2% BSA and incubated for 60 minutes. Additional patient samples at the same dilution were also added to an uncoated well to assess individual nonspecific binding. After 6 further washes, 50 μl/well of signal antibody (alkaline phosphatase–conjugated anti-human IgG; Sigma-Aldrich) diluted 1:1,000 in PBS/2% BSA solution was incubated for 1 hour at 37°C. After 6 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 at 405 nm (OD405 nm), was determined after 20 minutes of incubation at 37°C (VersaMax, Molecular Devices). The corresponding nonspecific binding value was subtracted from the mean absorbance value for each sample.

The specificity of detection was assessed using previously described conventional saturation tests (13,14) and was further confirmed by Western blot analysis (data not shown). The positivity cutoff was predefined and set at an OD value of 0.6 and 37% of the positive control value, as described earlier (15). OD values ranged from 0 to 1.33.

**Oxidized LDL assessment.** Oxidized LDL levels were determined using a commercially available ELISA kit with 4E6 monoclonal antibodies (Merckodia). Samples were run in duplicate, with results given as the mean.

**Serum C-reactive protein (CRP), creatinine, total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglyceride levels.** CRP, creatinine, total cholesterol, HDL cholesterol, and triglyceride levels were determined using a Dxl autoanalyzer (Beckman Coulter). LDL was conventionally calculated based on the Friedewald equation.

IL-6, IL-8, IL-1Ra, MMP-1, MMP-2, MMP-3, and MMP-9 quantification. The levels of cytokines and MMPs were determined in patient sera and culture supernatants by using a commercially available multiplex beads immunoassay (Fluorokine MAP Multiplex Human Cytokine Panel and Human
MMP Panel; R&D Systems) according to the supplier's instructions, using a Bio-Plex 200 array reader (Bio-Rad) with Luminox MAP Technology.

ProMMP-9 zymographic assay. ProMMP-9 activity was detected by zymography on serum samples and supernatants from in vitro experiments (see below). Gels were copolymerized with gelatin (Sigma). Equal amounts of serum and supernatant (2 μl) and 1 ng of recombinant proMMP-9 standard (Calbiochem) were loaded on 9% sodium dodecyl sulfate–polyacrylamide gels in the absence of reducing agents. After electrophoresis overnight, gels were rinsed twice in 2.5% Triton X-100, incubated for 20 hours in 0.15M NaCl, 10 mM CaCl₂, 0.2% Brij-35, and 50 mM Tris HCl buffer, pH 7.4, at 37°C, stained for 30 minutes in Coomassie blue R250, and destained in acetic acid–ethanol–water (1:3:6). The results of zymography were expressed as proMMP-9 proteolytic activity.

The amount of gelatinase in the serum of patients with RA and donors, as previously described (20). Monocytes were then from the peripheral blood mononuclear cells of healthy blood donors, as previously described (20). In vitro experiments. Human monocytes were isolated from peripheral blood mononuclear cells of healthy blood donors. LPS contamination in anti–Apo A-1 IgG and control IgG was ruled out using the Limulus test (21), which was performed according to the manufacturer’s instructions. The results are expressed as the median value from the 4 different blood donors.

**Statistical analysis.** Analyses were performed using Statistica software (StatSoft). Fisher’s exact test, the chi-square test with Yates’ correction, the Mann-Whitney U test, and the \( r \)-test were used, when appropriate, to compare groups of patients. Spearman’s rank test was used to establish correlations between variables. Receiver operating curve (ROC) analysis was performed using Excel Analyse-it software (Microsoft). Time-to-event analyses were performed by Cox regression analysis, using anti–Apo A-1 IgG as a dichotomous variable. The adjusted Cox regression analysis was corrected for potential confounders, such as age, sex, diabetes mellitus, hypertension, dyslipidemia, smoking, obesity, and RA disease.

### Table 1. Baseline demographic and clinical characteristics of the patients with RA

<table>
<thead>
<tr>
<th></th>
<th>RA patients (n = 133)</th>
<th>Anti–Apo A-1 IgG negative (n = 110)</th>
<th>Anti–Apo A-1 IgG positive (n = 23)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD years</td>
<td>65 ± 16.3</td>
<td>65.2 ± 16.7</td>
<td>65.4 ± 14.8</td>
<td>0.95</td>
</tr>
<tr>
<td>Female sex</td>
<td>95 (71)</td>
<td>83 (75)</td>
<td>12 (52)</td>
<td>0.04</td>
</tr>
<tr>
<td>Cardiovascular risk factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>16 (12)</td>
<td>16 (15)</td>
<td>0 (0)</td>
<td>0.31</td>
</tr>
<tr>
<td>Hypertension</td>
<td>43 (32)</td>
<td>34 (31)</td>
<td>9 (39)</td>
<td>0.46</td>
</tr>
<tr>
<td>Obesity</td>
<td>6 (5)</td>
<td>5 (5)</td>
<td>1 (4)</td>
<td>1</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>10 (8)</td>
<td>7 (6)</td>
<td>3 (13)</td>
<td>0.37</td>
</tr>
<tr>
<td>Positive family history</td>
<td>1 (0.07)</td>
<td>1 (1)</td>
<td>0 (0)</td>
<td>1</td>
</tr>
<tr>
<td>CVD incidence</td>
<td>20 (15)</td>
<td>10 (9)</td>
<td>10 (45)</td>
<td>0.001</td>
</tr>
<tr>
<td>RF positive</td>
<td>96 (72)</td>
<td>83 (75)</td>
<td>13 (57)</td>
<td>0.08</td>
</tr>
<tr>
<td>Disease duration, mean ± SD years</td>
<td>19.7 ± 13.2</td>
<td>19.1 ± 13.4</td>
<td>19.8 ± 12.7</td>
<td>0.80</td>
</tr>
<tr>
<td>Time from sampling to CV event, mean ± SD years</td>
<td>9 ± 5.4</td>
<td>9.4 ± 5.4</td>
<td>9.5 ± 5.8</td>
<td>0.95</td>
</tr>
<tr>
<td>Time from RA diagnosis to sampling, mean ± SD years</td>
<td>9.8 ± 11</td>
<td>10 ± 11.6</td>
<td>8.8 ± 9</td>
<td>0.64</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMARDs</td>
<td>95 (71)</td>
<td>79 (72)</td>
<td>16 (70)</td>
<td>0.8</td>
</tr>
<tr>
<td>Anti-TNFα</td>
<td>27 (20)</td>
<td>25 (23)</td>
<td>2 (9)</td>
<td>0.1</td>
</tr>
<tr>
<td>Anti-CD20</td>
<td>15 (11)</td>
<td>13 (12)</td>
<td>2 (9)</td>
<td>1</td>
</tr>
<tr>
<td>Anti–Apo A-1 IgG positive</td>
<td>22 (17)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Anti–Apo A-1 IgG titer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median OD(_{450 \text{ nm}})</td>
<td>0.23</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IQR (range)</td>
<td>0.06–0.48 (0–1.33)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Except where indicated otherwise, values are the number (%). Normally distributed continuous values are expressed as the mean ± SD, with \( P \) values calculated using the 2-tailed \( t \)-test. Continuous variables with skewed distribution are expressed as the median (interquartile range [IQR]). The 2-sided Fisher’s exact test was used to compare dichotomous variables. RA = rheumatoid arthritis; anti–Apo A-1 = anti–apolipoprotein A-1; CVD = cardiovascular disease; RF = rheumatoid factor; DMARDs = disease-modifying antirheumatic drugs; anti-TNFα = anti–tumor necrosis factor α; OD\(_{450 \text{ nm}}\) = optical density at 450 nm.
duration. Given the relatively limited size of the study sample, Cox regression analyses were also performed between anti-Apo A-1 IgG tertiles to determine whether the major cardiovascular event risk increased proportionally to anti-Apo A-1 IgG titers. Results are presented as crude and adjusted hazard ratios (HRs) with corresponding 95% confidence intervals (95% CIs). Survival analysis was performed by the Kaplan-Meier log rank method. Two-tailed $P$ values less than 0.05 were considered significant; the value for significance was lower when Bonferroni correction was applied to take multiple testing into account.

**RESULTS**

Characteristics of the study subjects. The demographic characteristics of the patients with RA are summarized in Table 1. The incidence of anti-Apo A-1 IgG positivity was 17% in patients with RA versus 1–3% in healthy blood donors, consistent with the results from our previous work (13,14). Male sex was significantly overrepresented in patients positive for anti-Apo A-1 IgG when compared with patients who had negative test results for these autoantibodies (Table 1). Anti-Apo A-1 IgG–positive and anti-Apo A-1 IgG–negative patients with RA did not differ in terms of most of the important potential cardiovascular confounders such as age, traditional cardiovascular risk factors, RA treatment, and disease duration at the time of blood collection (Table 1).

During a median followup of 9 years (IQR 5–15 years), the rate of major cardiovascular events was 15% (20 of 133 patients). During followup, 6 patients died of...
cardiovascular complications (4 fatal MIIs and 2 fatal ischemic strokes), 10 patients experienced a nonfatal acute coronary syndrome (5 patients with ST elevation MI and 5 with non–ST elevation MI), and 4 patients had a nonfatal ischemic stroke.

Baseline characteristics associated with major cardiovascular events during followup. As shown in Table 2, patients who had a major cardiovascular event during followup were older and were more likely to be diabetic, hypertensive, or dyslipidemic, and had higher circulating levels of IL-1Ra and oxidized LDL and lower HDL concentrations. However, a multivariable analysis in which age, sex, diabetes mellitus, hypertension, dyslipidemia, obesity, smoking, disease duration, oxidized LDL, IL-1Ra, HDL, and anti–Apo A-1 IgG were set as confounders, only hypertension ($\chi^2 = 11.3, P = 0.0007$), dyslipidemia ($\chi^2 = 5.1, P = 0.02$), and anti–Apo A-1 IgG ($\chi^2 = 10, P = 0.001$) remained significant predictors of major cardiovascular events. The confounder disease duration was close to significant in terms of predicting major cardiovascular events ($\chi^2 = 3.38, P = 0.06$). For these reasons, only anti–Apo A-1 IgG, traditional cardiovascular disease risk factors (age, sex, diabetes mellitus, hypertension, dyslipidemia, smoking, obesity), and disease duration were considered for further risk analysis.

Association of anti–Apo A-1 IgG positivity with future major cardiovascular events. RA patients positive for anti–Apo A-1 IgG had a significantly higher rate of major cardiovascular events (43% versus 9% of anti–Apo A-1 IgG–negative patients; $P = 0.001$) (Table 1). Translated into a crude HR, the presence of anti–Apo A-1 IgG positivity increased the risk of major cardiovascular events 5-fold (HR 4.7, 95% CI 1.9–11.2). This association remained significant and of the same order of magnitude after adjustment for age, sex, hypertension, dyslipidemia, smoking, diabetes mellitus, obesity, and RA disease duration (HR 4.2, 95% CI 1.5–12.1). The presence of a significant dose response ($P$ for trend = 0.02) suggests that the risk of a major cardiovascular event increases proportionally to increasing anti–Apo A-1 IgG concentrations. Indeed, in the second tertile (for anti–Apo A-1 IgG, OD$_{450\,\text{nm}}$ = 0.12–0.37), the major cardiovascular event risk did not significantly increase (HR 1.3, 95% CI 0.3–6.0, $P = 0.67$), but in the third tertile (for anti–Apo A-1 IgG, OD = 0.38–1.33), the risk of major cardiovascular events increased significantly (2-fold) (HR 2.2, 95% CI 1.2–14.4). Major cardiovascular event–free survival was 86% in RA patients negative for anti–Apo A-1 IgG and 40% in RA patients positive for anti–Apo A-1 IgG ($P = 0.0001$ by log rank test) (Figure 1). Furthermore, patients with RA who had
an incident major cardiovascular event during followup had higher median values for anti–Apo A-1 IgG at baseline when compared with patients without major cardiovascular events (Table 2), and this difference remained significant after Bonferroni correction (data not shown).

**ROC analysis.** ROC analysis showed that the presence of anti–Apo A-1 IgG is a good predictor of major cardiovascular events in patients with RA, with an area under the curve value of 0.73 (95% CI 0.59–0.86, P = 0.0008). At the predefined positivity cutoff, set at an OD value of 0.6, corresponding to the 97.5th percentile of the distribution defined earlier (14) and recently validated prospectively in patients with MI (15), the sensitivity and specificity of anti–Apo A-1 IgG to predict major cardiovascular events were 90% (95% CI 83–94) and 50% (95% CI 28–72), respectively. Negative and positive predictive values were 90% (95% CI 83–94) and 48% (95% CI 26–70), respectively.

**Anti–Apo A-1 IgG and inflammation parameters.** As shown in Table 3, anti–Apo A-1 IgG-positive patients with RA had higher median serum levels of IL-8, MMP-9, oxidized LDL, and proMMP-9 activity, but none of these differences was significant after Bonferroni correction (data not shown).

**Anti–Apo A-1 IgG–stimulated IL-8 and MMP-9 production by cultured human macrophages.** To further explore the possibility of a causal relationship between high levels of circulating anti–Apo A-1 IgG and inflammation mediators such as IL-8 and MMP-9, human monocyte–derived macrophages were cultured with increasing concentrations of commercial anti–Apo A-1 IgG or respective IgG controls for 24 hours and 48 hours, respectively.

As shown in Figure 2, anti–Apo A-1 IgG induced dose-dependent production of IL-8 (P for trend = 0.0005 by Kruskal-Wallis nonparametric test) and MMP-9 (P for trend = 0.03 by Kruskal-Wallis nonparametric test) by human macrophages. The results of trend tests for control IgG were not significant (P = 0.62 and P = 0.75, respectively). For IL-8, the maximal effect was observed at 40 μg/ml, whereas the optimal stimulating concentration for MMP-9 was 10 μg/ml. Control IgG did not significantly stimulate macrophages, and the differences between anti–Apo A-1 IgG and control IgG for IL-8 and MMP-9 production were statistically significant at 20 μg/ml and 10 μg/ml, respectively (P = 0.03 for both) (Figure 2).

Furthermore, the dose-dependent increase in MMP-9 production was accompanied by a concomitant increase in proMMP-9 activity as assessed by zymography (Figure 3A), which followed the same dose-dependent pattern (Figure 3B). There was a significant correlation between MMP-9 levels and proMMP-9 activity in the supernatant of anti–Apo A-1 IgG–stimulated macrophages (r = 0.59, P < 0.01) (data not shown).

**Table 3.** Baseline biologic markers according to anti–Apo A-1 IgG status

<table>
<thead>
<tr>
<th>Biologic biomarkers</th>
<th>Anti–Apo A-1 IgG–negative patients (n = 110)</th>
<th>Anti–Apo A-1 IgG–positive patients (n = 23)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inflammation marker</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP, mg/liter</td>
<td>15.4 (4.6–37.2)</td>
<td>14.1 (6–31)</td>
<td>0.96</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>3.4 (1.2–7.5)</td>
<td>3.5 (1–11)</td>
<td>0.66</td>
</tr>
<tr>
<td>IL-8, pg/ml</td>
<td>7.2 (2.9–13.8)</td>
<td>14 (6.3–29.1)</td>
<td>0.01</td>
</tr>
<tr>
<td>TNFα, pg/ml</td>
<td>1.7 (1.2–2.3)</td>
<td>1.9 (1–2.6)</td>
<td>0.49</td>
</tr>
<tr>
<td>IL-1RA, pg/ml</td>
<td>398 (198–864)</td>
<td>550 (281–1,075)</td>
<td>0.28</td>
</tr>
<tr>
<td>MCP-1, pg/ml</td>
<td>102 (44–201)</td>
<td>89 (46–195)</td>
<td>0.89</td>
</tr>
<tr>
<td>MMP-9, ng/ml</td>
<td>787 (283–1,490)</td>
<td>1,064 (563–2,103)</td>
<td>0.03</td>
</tr>
<tr>
<td>Log proMMP-9 activity, mean ± SD ng/ml</td>
<td>9.88 ± 0.33</td>
<td>10.34 ± 1.46</td>
<td>0.008</td>
</tr>
<tr>
<td>MMP-1, ng/ml</td>
<td>4 (2–8)</td>
<td>4.4 (2–7)</td>
<td>0.95</td>
</tr>
<tr>
<td>MMP-2, ng/ml</td>
<td>141 (127–154)</td>
<td>142 (122–157.6)</td>
<td>0.75</td>
</tr>
<tr>
<td>MMP-3, ng/ml</td>
<td>61.6 (33–80)</td>
<td>63 (33–91)</td>
<td>0.48</td>
</tr>
<tr>
<td>Oxidized LDL, units/ml</td>
<td>46.5 (36–65)</td>
<td>59 (46–72)</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Lipid profile</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mmoles/liter</td>
<td>4.75 (2.1–9.8)</td>
<td>4.55 (3–6.1)</td>
<td>0.4</td>
</tr>
<tr>
<td>HDL, mmoles/liter</td>
<td>1.05 (0.3–2.2)</td>
<td>0.9 (0.5–1.8)</td>
<td>0.07</td>
</tr>
<tr>
<td>LDL, mmoles/liter</td>
<td>2.9 (1.4–4.7)</td>
<td>3 (1.4–4.2)</td>
<td>0.88</td>
</tr>
<tr>
<td>Triglycerides, mmoles/liter</td>
<td>1.03 (0.5–3.3)</td>
<td>1.25 (0.8–1.6)</td>
<td>0.61</td>
</tr>
</tbody>
</table>

*Except where indicated otherwise, values are the median (interquartile range). Anti–Apo A-1 = anti–apolipoprotein A-1; CRP = C-reactive protein; IL-6 = interleukin-6; TNFα = tumor necrosis factor α; IL-1RA = IL-1 receptor antagonist; MCP-1 = monocyte chemotactic protein 1; MMP-9 = matrix metalloproteinase 9; LDL = low-density lipoprotein; HDL = high-density lipoprotein.

† By Mann-Whitney U test.
Figure 2. Anti–apolipoprotein A-1 (anti–Apo A-1) IgG–induced dose-dependent production of interleukin-8 (IL-8) and matrix metalloproteinase 9 (MMP-9) by human macrophages. Bars show the median (range). * and *** = P = 0.03 versus control, by Mann-Whitney U test. LPS = lipopolysaccharide.

Figure 3. Anti–apolipoprotein A-1 (anti–Apo A-1) IgG–induced dose-dependent increase in pro–matrix metalloproteinase 9 (proMMP-9) activity in human macrophage supernatants, as assessed by zymography. A, White band (arrow) on the gel represents the proMMP-9 gelatinolytic activity of human macrophage supernatant in the presence of standard (ST), control medium (NT), and increasing concentrations of control IgG and anti–Apo A-1 IgG (5–40 μg/ml). Dark bands represent the amount of protein stained by Coomassie blue. B, Mean ± SD results of zymography. *** = P = 0.03 versus control, by t-test. NT = not treated.
shown). The absence of LPS contamination of anti–Apo A-1 IgG and the control IgG preparation was confirmed by Limulus assay (0.1 EU/ml for both antibodies) (data not shown).

DISCUSSION

The main findings of this study are that anti–Apo A-1 IgG exist in a significant subset of patients with RA (17%), and that these autoantibodies are predictive of major cardiovascular events in patients with RA, independent of age, sex, diabetes mellitus, smoking, obesity, disease duration, and IL-1Ra and oxidized LDL levels. Furthermore, our results suggest that among all emergent markers of cardiovascular risk tested in the present study, anti–Apo A-1 IgG positivity was the strongest predictor of major cardiovascular events in patients with RA.

Our results show that anti–Apo A-1 IgG positivity tended to be associated with enhanced systemic inflammatory responses, as reflected by higher levels of IL-8, oxidized LDL, MMP-9, and MMP-9 activity (P not significant after Bonferroni adjustment). However, because Bonferroni correction is known to be prone to a lack of power (Type II error) by missing dose-response relationships between variables (22), we tested the ability of anti–Apo A-1 IgG to induce the production of MMP-9 and IL-8 by human monocyte–derived macrophages, which are key players in inflammation and atherosclerosis. Our results show that physiologically relevant anti–Apo A-1 IgG concentrations induced dose-dependent IL-8 and MMP-9 production by human macrophages with the same order of magnitude as LPS (1 µg/ml), whereas control IgG were devoid of any stimulatory effect.

MMP-9 levels have been reported to transiently increase during acute coronary syndrome (23), stroke (24), and heart failure (25), and increased levels are associated with more unstable atherosclerotic plaques (26). Other MMPs such as MMP-1, MMP-2, and MMP-3 have also been associated with increased cardiovascular risk, but to the best of our knowledge, the most consistent data have been observed with MMP-9. These clinical results are corroborated by results of in vitro and in vivo studies, showing that MMP-9 increases atherosclerotic plaque fragility by degrading type IV collagen within the fibrous cap, by increasing transendothelial migration of immune cells within atherosclerotic plaques, and by stimulating the proliferation of vascular smooth muscle cells (11,27). In patients with RA, circulatory MMP-9 levels have been reported to be higher than those in control individuals (28) and are associated with higher disease activity and progression of joint damage (29). The significance of elevated MMP-9 levels in RA with respect to cardiovascular events remains elusive. However, our data do not suggest that MMP-9 is an independent predictor of major cardiovascular events in RA.

In RA joints, IL-8–secreting cells have been detected within rheumatoid synovium at the pannus–cartilage junction (30). IL-8 is a major neutrophil chemotactic, and neutrophil activation is currently considered to play a critical role in the development of joint inflammation (31), suggesting that IL-8 contributes to the mechanisms leading to tissue damage. However, the pathogenic role of IL-8 in RA-related cardiovascular complications is unknown. IL-8 has been shown to be predictive of fatal and nonfatal coronary artery disease in healthy subjects (32), to be a powerful and independent predictor for cardiovascular events in patients with coronary artery disease (33), and to stimulate MMP-9 production (34). These data are part of the compelling body of evidence suggesting an important role of neutrophil activation in atherosclerosis-related cardiovascular complications in humans (35,36). Indeed, neutrophils have been shown to localize within atherosclerotic plaques (37), to enhance local vascular inflammation and myeloperoxidase release (38). Neutrophils increase the production of radical oxygen species, thus promoting oxidized LDL formation (36) and impeding the atheroprotective functions of HDL by mediating specific Apo A-1 chlorination (39).

Oxidized LDL, produced upon lipid peroxidation, has been reported to play a major role in all stages of atherogenesis, including induction of endothelial dysfunction, transendothelial migration of immune cells, and foam cell formation (40). Plasma levels of oxidized LDL have been described as promising diagnostic and prognostic markers of cardiovascular complications (40) as well as markers of the angiographic severity of coronary heart disease in patients with acute coronary syndromes (41,42). In RA, oxidized LDL has been associated with disease that is more active (10), but the association of oxidized LDL with cardiovascular events remains elusive. The results of our study do not suggest that oxidized LDL is an independent predictor of cardiovascular complications in RA. This result contrasts with previous findings in other autoimmune diseases such as SLE and APS, in which high levels of oxidized LDL were associated with arterial disease (43) and...
thrombotic events (44), respectively. In the context of acute coronary syndrome, anti–Apo A-1 IgG positivity was associated with 5-fold higher levels of oxidized LDL compared with anti–Apo A-1 IgG negativity (14). The link between anti–Apo A-1 IgG and oxidized LDL is unclear but could be mediated by IL-8–related lipid peroxidation, possibly through leukocyte myeloperoxidase activation (37,39).

Current thinking supports the notion that RA and atherosclerosis share several common inflammatory proteins intervening with endothelium and hemostatic factors, which in turn leads to plaque formation and rupture (9). Whether some autoantibodies could actively take part in this process remains elusive, and our results showing that anti–Apo A-1 IgG per se are able to induce IL-8 and MMP-9 production by human macrophages add weight to this hypothesis and further underline the role of humoral autoimmunity in RA-related cardiovascular complications.

Our results also raise several questions such as how anti–Apo A-1 antibodies are involved in the increased incidence of cardiovascular events. Anti–Apo A-1 IgG induces production of proinflammation mediators by macrophages, which are involved in atherogenesis and plaque rupture. To the best of our knowledge, Apo A-1 is not expressed by human macrophages. However, anti–Apo A-1 antibodies induce specific concentration-dependent stimulatory responses in cultured macrophages, suggesting that these antibodies may exert their stimulatory effects by interacting with Fcγ receptors or innate immune receptors, as recently described for anticardiolipin antibodies (45). Alternatively, these antibodies may recognize a common conformational epitope expressed by macrophages. Another nonexclusive hypothesis is that anti–Apo A-1 IgG could interfere with autonomic cardiac regulation, which is increasingly recognized as a vector of sudden cardiac death in RA (46). In acute MI, anti–Apo A-1 IgG positivity is associated with a higher basal heart rate, and plasma from patients positive for anti–Apo A-1 antibodies induced a strong positive chronotropic effect in vitro (15). Furthermore, spiking anti–Apo A-1 IgG into control plasma induced a dose-dependent chronotropic response, suggesting that anti–Apo A-1 IgG mediate the chronotropic effects of plasma samples from patients with MI, and may affect autonomic cardiac function in MI (15). However, it remains to be demonstrated whether this applies to cardiovascular events in patients with RA.

Despite this study being appropriately powered, the number of events was still relatively low, which prevented us from drawing definite conclusions. Although risk analyses were adjusted for the traditional cardiovascular risk factors used to compute the Framingham risk score, we could not formally compare the predictive accuracy of the Framingham risk score and that of anti–Apo A-1 IgG, because the exact values for systolic blood pressure were not available for the total study sample. Because the Framingham risk score does not appear to perform particularly well in patients with RA (47), knowing whether anti–Apo A-1 IgG could outperform the Framingham risk score for predicting major cardiovascular events in RA clearly warrants further study. Additionally, the antibodies used for in vitro experiments in this study were of commercial origin and were not extracted from the serum of patients; extraction of serum was not possible given the insufficient amount of material available. However, because we used LPS-free anti-human Apo A-1 IgG, we believe that our results established proof of principle that anti–Apo A-1 IgG per se are sufficient to trigger significant proinflammatory responses. Another limitation of this study is that disease activity measurements were not available. Because disease activity has been shown to predict the cardiovascular outcome in patients with RA (48), analyzing anti–Apo A-1 IgG with respect to the disease activity score is definitely of interest and warrants further study.

Nevertheless, to our knowledge, no simple diagnostic test is currently available to assess cardiovascular risk in patients with RA, and our results suggest that anti–Apo A-1 IgG testing could represent a useful tool for stratifying patients according to the risk of cardiovascular events. Thus, the presence of anti–Apo A-1 IgG may permit the identification of a subset of patients who could benefit from early and specific cardiovascular risk prevention, including the management of traditional risk factors and better control of RA disease activity.

Finally, because the presence of rheumatoid factor (RF) in serum samples is known to induce false-positive results in immunoassays, it can be contended that the high anti–Apo A-1 IgG positivity rate observed in RA could be attributable to this analytical pitfall. The facts that RF-positive patients with RA had significantly lower median values for anti–Apo A-1 IgG (OD = 0.33; P = 0.02 by Mann-Whitney U test), and that no correlation was observed between the presence of RF (in terms of latex titer or IgM RF concentrations) and anti–Apo A-1 IgG titers provide evidence against that hypothesis. Therefore, although it is not formally ex-
cluded, RF interference is very unlikely to have blunted the results and the conclusions of this study.

In conclusion, this study is the first to demonstrate that anti–Apo A-1 IgG predict major cardiovascular events in patients with RA, and that anti–Apo A-1 IgG positivity is associated with higher levels of MMP-9, IL-8, and oxidized LDL, which are 3 markers and mediators of atherosclerotic plaque destabilization. In addition, the results of in vitro experiments showing that anti–Apo A-1 IgG per se function in a manner similar to that of a proinflammatory molecule indicate that anti–Apo A-1 IgG are not only a marker of RA-associated cardiovascular complications but also could contribute to such complications by promoting inflammation and atherosclerotic plaque destabilization.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Vuilleumier had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Vuilleumier, Bas, Lovis, Hochstrasser, Roux-Lombard, Gabay.

Acquisition of data. Vuilleumier, Pagano, Montecucco, Guerne, Finckh, Mach.

Analysis and interpretation of data. Vuilleumier, Pagano, Montecucco, Finckh, Mach, Hochstrasser.

REFERENCES


3.3. Anti-apoA-1 IgG in non-autoimmune conditions

3.3.1. Anti-apoA-1 IgG in acute coronary syndromes

The two related papers have been published as:


Anti-apolipoprotein A-1 IgG as an independent cardiovascular prognostic marker affecting basal heart rate in myocardial infarction

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Aims
To assess the prognostic value of anti-apolipoprotein A-1 (anti-apoA-1) IgG after myocardial infarction (MI) and its association with major cardiovascular events (MACEs) at 12 months and to determine their association with resting heart rate (RHR), a well-established prognostic feature after MI. Anti-apoA-1 IgG have been reported in MI without autoimmune disease, but their clinical significance remains undetermined.

Methods and results
A total of 221 consecutive patients with MI were prospectively included, and all completed a 12-month follow-up. Major cardiovascular events consisted in death, MI, stroke, or hospitalization either for an acute coronary syndrome or heart failure. Resting heart rate was obtained on Holter the day before discharge under the same medical treatment. Neonate rat ventricular cardiomyocytes (NRVC) were used in vitro to assess the direct anti-apoA-1 IgG effect on RHR. During follow-up, 13% of patients presented a MACE. Anti-apoA-1 IgG positivity was 9% and was associated with a higher RHR (P = 0.0005) and higher MACE rate (adjusted OR, 4.3; 95% CI, 1.46–12.6; P = 0.007). Survival models confirmed the significant nature of this association. Patients with MACE had higher median anti-apoA-1 IgG values at admission than patients without (P = 0.007). On NRVC, plasma from MI patients and monoclonal anti-apoA-1 IgG induced an aldosterone and dose-dependent positive chronotropic effect, abrogated by apoA-1 and therapeutic immunoglobulin (IVIG) pre-incubation.

Conclusions
In MI patients, anti-apoA-1 IgG is independently associated with MACE at 1-year, interfering with a currently unknown aldosterone-dependent RHR determinant. Knowing whether anti-apoA-1 IgG assessment could be of interest to identify an MI patient subset susceptible to benefit from apoA-1/IVIG therapy remains to be demonstrated.

Keywords
Anti-apolipoprotein A-1 autoantibody • Myocardial infarction • Prognosis • Resting heart rate • Autoimmunity

Immune-mediated inflammation plays a major role in atherosclerosis and atherothrombosis, two essential features for cardiovascular disease (CVD) development, currently considered as the leading cause of death in the Western world, and predicted to be the first killer by 2020. At a time where the incidence of CVD is increasing, the research of new and modifiable cardiovascular factors is highly warranted. Those efforts are meant to identify a new CVD patient subset potentially susceptible to benefit from innovative therapeutic approaches. To this respect, there is an accumulating evidence showing that humoral autoimmunity might
play an important role in CVD, and that some auto-antibodies could represent emerging cardiovascular risk factors, potentially modifiable by passive immunization.

Lately, we reported that IgG auto-antibodies against apolipoprotein A-1 (apoA-1), the major proteic fraction of high-density lipoprotein (HDL), were present in a significant subset of patients after myocardial infarction (MI) and were significantly associated to higher levels of circulating oxidized low-density lipoproteins (oxLDLs), a major player of atherogenesis. However, the clinical and pathophysiological significance of anti-apoA-1 IgG remains unknown. The present study was performed to assess their prognostic value in MI and to determine their association with resting heart rate (RHR), a well-established cardiovascular prognostic feature after MI. To this respect, some auto-antibodies have been shown to interfere with the heart rate conduction system function in humans, supporting the growing number of observation showing that humoral autoimmunity may play a role in the autonomic nervous system dysfunction impairment commonly observed in autoimmune diseases. Therefore, we investigated whether anti-apoA-1 IgG could directly influence autonomous contractions in vitro using spontaneously beating neonate rat ventricular cardiomyocytes (NRVC) in which mineralocorticoid activation has been shown to induce a positive chronotropic effect through the expression of low threshold T-type calcium channels and of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels.

Methods
The research ethic committee of Geneva University Hospitals approved the protocol, and all patients gave written informed consent before enrolment.

Patient population and study design
The sample size was computed based on an expected prevalence of apoA-1 IgG in acute coronary syndrome (ACS) of 11%. A total sample size of 220 patients was needed to detect a three-fold increase in the risk of a major event (12 vs. 36%) with a power of 80% with an alpha error of 5%.

Between 1 November 2005 and 30 June 2007, 366 consecutive patients were screened at the emergency room (ER) and intensive care unit (ICU) of Geneva University Hospital for an MI, commonly defined by typical clinical presentation associated with or without ST elevation, with subsequently proven angiographic coronary stenosis needing percutaneous coronary intervention, or associated with cardiac necrosis biomarkers elevation. Significant coronary artery disease (CAD) was defined as a culprit lesion obstructing more than 75% of the vessel lumen on coronary angiography. Such lesions were found in 98% of cases. However, when no significant culprit lesion was found upon coronary angiography despite typical clinical presentation associated with a rise of biochemical markers of cardiac necrosis, patients were included in the present study (four patients). Exclusion criteria were Takotsubo disease, the presence of any known auto-immune disease except diabetes mellitus, and inability to give informed consent for any reason, including oro-tracheal intubation.

Among the 366 screened patients, 145 met exclusion criteria, leaving 221 patients available for the analysis. Conventional left ventricular ejection fraction evaluation by echocardiography was performed within 5 days of admission by experienced cardiologists, blinded to the biochemical results. Resting heart rate was assessed as mean heart rate on 24 h recording (Holter) the day before patients’ discharge. At the time of recording, all patients were treated with statins, angiotensin-converting enzyme inhibitors or angiotensin-receptor blocker, beta-blockers, aspirin, and clopidogrel. None was under aldosterone antagonist therapy.

Definition of endpoint
Major cardiovascular events (MACEs) were predetermined and consisted in any death, MI, stroke, or hospitalization for ACS or acute heart failure at 1 year.

Patient follow-up
All patients completed the 12-month follow-up. Outcome was independently adjudicated by two of the study coordinators (E.C., R.N.) who were blinded to the results of biochemical analyses. Information was obtained by contacting patients by telephone and was further confirmed by checking patients’ medical file and contacting the physician in charge of the patient, targeting medical history relevant to the study endpoints.

Sample collection
To avoid interference with the door-to-revascularization policy, samples were taken after percutaneous coronary intervention within the first 24 h of hospitalization. After collection, serum samples were aliquoted and frozen at −80 °C until analyses. Data were collected from patients’ files in the ER and ICU.

Biochemical analysis

Determination of human antibodies to apoA-1 by enzyme-linked immunosorbent assay
Anti-apoA-1 IgG were measured as described previously. The specificity of the detection was assessed using conventional saturation tests described before and further confirmed by western blot (data not shown).

Intra- and inter-assay variations
Repeatability and reproducibility were determined at two levels. At a high level (two-fold the cut-off value), the coefficients of variation were 10% (n = 10) and 17% (n = 10) for intra and inter-assay, respectively. At the cut-off level, the coefficients of variation were 16% (n = 10) and 12% (n = 8) for intra and inter-assay, respectively.

Reference values
As described earlier, upper reference value was set at an absorbance of 0.6 optical densities (OD), corresponding to the 97.5th percentile of a reference population established on 140 healthy blood donors. In order to limit the impact of interassay variation, we developed an index consisting in the ratio between sample net absorbance and the positive control net absorbance × 100. The value corresponding to the 97.5th percentile of the normal distribution was 37 for the index. Accordingly, to be considered as positive, samples had to display an absorbance value above 0.6 OD and an index above 37.

Classical autoantibody measurement
Anti-nuclear antibody (ANA) and rheumatoid factor (RF) measurements were performed in the clinical laboratory for immunology and allergy of the University Hospital of Geneva, using routine indirect immunofluorescence and enzyme-linked immunosorbent assay
Cardiac troponin I, creatin kinase, N-terminal pro brain natriuretic peptide (NT-proBNP), C-reactive protein, creatinin, total cholesterol, triglycerids, and high-density lipoprotein quantification

Cardiac troponin I (cTnI) concentrations were determined on Unicell DXI 800™ (Beckman Coulter, Brea, CA, USA). Creatin kinase (CK), C-reactive protein, creatinin, total cholesterol, triglycerids, and HDL (mmol/L) were determined using a Synchron LX20 pro™ (Beckman Coulter) auto-analyser, and NT-proBNP was determined using Elecsys™ (Roche, Switzerland) automate.

Cell culture and cell contraction frequency

Neonatal cardiac cells were isolated from 1- to 2-day-old Wistar rat ventricles by digestion with low trypsin-EDTA and type 2 collagenase as described previously.22– 24 Briefly, animals were killed (in conformity with the Guide for the Care and use of Laboratory Animals published by the NIH and with the authorization (31.1.1012/19s3/0) of the local County Veterinary Office) and freshly isolated cells were seeded in plastic flasks to allow selective adhesion of cardiac fibroblasts. Thereafter, cardiomyocytes were decanted from the flasks and distributed in laminin-coated 90-mm Petri dishes or in 6-well culture plates. Cells from a same preparation were used for testing the various experimental conditions.

Spontaneously contracting cell monolayers were incubated for the indicated times with a 10% dilution of MI patients plasma or reference plasma spiked with different concentrations (1–20 μg/mL) of mouse monoclonal anti-human apoA-1 IgG (Abcam, Nottingham, UK) and respective IgG controls (polyclonals) of the same genetic background. Thereafter, cardiomyocytes were decanted from the flasks and distributed in laminin-coated 90-mm Petri dishes or in 6-well culture plates. Cells from a same preparation were used for testing the various experimental conditions.

Analyses were performed using Statistica™ software (StatSoft, Tulsa, OK, USA). Fischer exact test, χ² using Yates correction test, and Mann–Whitney U-test were used when appropriate to compare the group of patients. Spearman test was used to assess correlation between variables. Associations between anti-apo-A-1 IgG and composite cardiovascular outcome are presented as odds ratios (OR) with corresponding 95% confidence intervals (95% CI) and using Kaplan–Meier analysis and Cox regression model. Two-sided P-value was considered as significant when below 0.05. For in vitro experiments, Mann–Whitney U-test has been used to determine P-values unless stated otherwise, and results are expressed as median, interquartile range, and range.

Results

Demographic characteristics

Patient demographic characteristics are listed in Table 1. The frequency of positive anti-apoA-1 IgG auto-antibodies was 9% (19 of 221) and 14% (31 of 221) of patients had a MACE during the 1-year follow-up (Table 1).

High levels of anti-apoA-1 IgG are associated with major cardiovascular events at 1 year

One year after the initial event, 31 patients (14%) experienced a MACE. The detailed distribution of MACEs is given in Table 1. Among the three patients died, one patient died from cardiorespiratory arrest, one from abdominal aorta aneurysm rupture, and one from septic shock. On the 19 patients hospitalized for ACS, 10 had a proven MI, and 9 were diagnosed with unstable angina, of whom 5 presented significant angiographic coronary lesion. The remaining nine patients were hospitalized for acute heart failure. No patient had a clinically evident stroke during this 1-year follow-up period. Patients with MACEs had higher median anti-apoA-1 IgG titre upon admission than patients without MACEs (31.4 vs. 20.6 OD; P = 0.004). Complication rate was 37% in patient tested positive for anti-apoA-1 IgG against 12% in patients tested negative for those auto-antibodies, corresponding to a four-fold increase in risk of MACEs (OR, 4.3; 95% CI, 1.54–11.99; P = 0.008), which remained unchanged after adjustment for age, sex, hypertension, diabetes, dyslipidemia, smoking (OR, 4.3; 95% CI, 1.46–12.6; P = 0.007). Kaplan–Meier analysis confirmed that patients positive for anti-apoA-1 IgG upon admission had a significantly worse complication-free survival at 1 year than those tested negative for those auto-antibodies (63.2 vs. 88.5%, P = 0.001; Figure 1). Cox regression analysis showed that each unit increase of anti-apoA-1 IgG relative index increased the risk of complication at 1 year by 3% (P = 0.0003), independently of time (P = 0.39), confirming the proportionality principle of the model.

Anti-apoA-1 IgG positivity is associated with elevated resting heart rate in myocardial infarction patients

As shown in Table 1, MI patients positive for anti-apoA-1 IgG had higher median RHR than those tested negative and Spearman test showed a modest, but significant, correlation between RHR and anti-apoA-1 IgG upon admission (r = 0.18, P < 0.025). No association was retrieved between apoA-1, C-reactive protein levels, and RHR according to Spearman test (data not shown).

Positive chronotropic action of anti-apoA-1 IgG in vitro

We then tested in vitro the chronotropic action of anti-apoA-1 IgG on freshly isolated NRVC that share some properties with cardiac pacemaker cells. Indeed, these cells maintain their ability (acquired during the foetal development) to contract spontaneously in culture and aldosterone increases their beating frequency.22 We
### Table 1 Patients demographic characteristics

<table>
<thead>
<tr>
<th></th>
<th>MI patients (n = 221)</th>
<th>MI patients negative for anti-apoA-1 IgG (n = 202)</th>
<th>MI patients positive for anti-apoA-1 IgG (n = 19)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td>64 (26–86; 55–74)</td>
<td>64 (28–86; 55–74)</td>
<td>62 (26–86; 54–72)</td>
<td>0.65</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male, % (n)</td>
<td>78 (173)</td>
<td>77 (156)</td>
<td>89 (17)</td>
<td>0.38</td>
</tr>
<tr>
<td>Female, % (n)</td>
<td>22 (48)</td>
<td>23 (46)</td>
<td>11 (2)</td>
<td></td>
</tr>
<tr>
<td><strong>BMI, kg/m²</strong></td>
<td>25.8 (18–39.5; 24–29)</td>
<td>26 (17–39; 24–28)</td>
<td>25 (18–39.5; 21–32)</td>
<td>0.63</td>
</tr>
<tr>
<td><strong>Creatinin, µmol/L</strong></td>
<td>84 (47–275; 73–98)</td>
<td>84 (47–275; 73–97)</td>
<td>93 (62–211; 73–106)</td>
<td>0.20</td>
</tr>
<tr>
<td><strong>C-reactive protein, mg/L</strong></td>
<td>6 (1–230; 14–39)</td>
<td>6 (1–230; 3–14.2)</td>
<td>7.2 (1–96; 2.8–13)</td>
<td>0.76</td>
</tr>
<tr>
<td><strong>cTnI, ng/mL</strong></td>
<td>0.5 (0–153; 5–7)</td>
<td>0.4 (0.15–3; 0.1–3)</td>
<td>1.0 (0–80; 0.1–10)</td>
<td>0.29</td>
</tr>
<tr>
<td><strong>CK peak, IU/L</strong></td>
<td>687 (273–751; 1410–1783)</td>
<td>687 (41–7511; 264–1785)</td>
<td>573 (77–3252; 281–1308)</td>
<td>0.79</td>
</tr>
<tr>
<td><strong>NT-proBNP, pg/mL</strong></td>
<td>785 (20–28 285; 283–2057)</td>
<td>778 (36–28 285; 289–2157)</td>
<td>885 (20–14 205; 125–1842)</td>
<td>0.66</td>
</tr>
<tr>
<td><strong>Lipid profile, mmol/L</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>4.8 (1.9–11.8; 4–5.8)</td>
<td>4.9 (1.9–8.8; 4.1–5.8)</td>
<td>4.8 (3.2–11.8; 3.9–6)</td>
<td>0.94</td>
</tr>
<tr>
<td>HDL</td>
<td>1.06 (0.5–2.7; 0.9–1.3)</td>
<td>1.05 (0.5–2.7; 0.9–1.3)</td>
<td>1.1 (0.7–1.7; 0.9–1.4)</td>
<td>0.35</td>
</tr>
<tr>
<td>LDL</td>
<td>3.1 (0–4; 2.4–3.9)</td>
<td>3.2 (0.3–7; 2.4–3.9)</td>
<td>2.9 (1.8–9.5; 2.4–4.3)</td>
<td>0.86</td>
</tr>
<tr>
<td>Triglycerids</td>
<td>1.1 (0.5–7.3; 0.8–1.7)</td>
<td>1.1 (0.1–7.3; 0.8–1.7)</td>
<td>0.9 (0.4–2.3; 0.6–1.5)</td>
<td>0.22</td>
</tr>
<tr>
<td><strong>Comorbidities, % (n)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>53 (117)</td>
<td>53 (108)</td>
<td>47 (9)</td>
<td>0.63</td>
</tr>
<tr>
<td>Diabetes</td>
<td>19 (43)</td>
<td>20 (40)</td>
<td>16 (3)</td>
<td>1</td>
</tr>
<tr>
<td>Dyslipidaemia</td>
<td>46 (102)</td>
<td>47 (94)</td>
<td>42 (8)</td>
<td>0.81</td>
</tr>
<tr>
<td>Smoker</td>
<td>45 (99)</td>
<td>45 (91)</td>
<td>42 (9)</td>
<td>1</td>
</tr>
<tr>
<td>Known CAD</td>
<td>29 (65)</td>
<td>29 (58)</td>
<td>32 (6)</td>
<td>0.79</td>
</tr>
<tr>
<td>Stroke</td>
<td>5 (12)</td>
<td>4 (8)</td>
<td>21 (4)</td>
<td>0.01</td>
</tr>
<tr>
<td>Positive familial history</td>
<td>31 (69)</td>
<td>31 (62)</td>
<td>37 (7)</td>
<td>0.6</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>5 (11)</td>
<td>4 (9)</td>
<td>11 (2)</td>
<td>0.24</td>
</tr>
<tr>
<td>STEMI, % (n)</td>
<td>54 (120)</td>
<td>53 (108)</td>
<td>63 (12)</td>
<td>0.48</td>
</tr>
<tr>
<td>NSTEMI, % (n)</td>
<td>46 (101)</td>
<td>47 (94)</td>
<td>37 (7)</td>
<td></td>
</tr>
<tr>
<td>LVEF*, %</td>
<td>50 (20–60; 10)</td>
<td>50 (25–60; 45–55)</td>
<td>45 (20–60; 40–60)</td>
<td>0.33</td>
</tr>
<tr>
<td>Resting heart rate at discharge, b.p.m.</td>
<td>68 (50–120; 15)</td>
<td>67 (50–97; 60–72)</td>
<td>76 (56–120; 70–90)</td>
<td>0.0005</td>
</tr>
<tr>
<td>MACE rate at 12 months, % (n)</td>
<td>14 (31)</td>
<td>12 (24)</td>
<td>37 (7)</td>
<td>0.008</td>
</tr>
<tr>
<td>MACE details: deaths</td>
<td>1 (3)</td>
<td>1 (3)</td>
<td>0 (0)</td>
<td>1</td>
</tr>
<tr>
<td>ACS</td>
<td>9 (19)</td>
<td>7 (14)</td>
<td>26 (5)</td>
<td>0.01</td>
</tr>
<tr>
<td>Heart failure</td>
<td>4 (9)</td>
<td>3 (7)</td>
<td>11 (2)</td>
<td>0.17</td>
</tr>
<tr>
<td>Stroke</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Anti-phospholipid antibody positivity rate, % (n)</td>
<td>10 (22)</td>
<td>9 (19)</td>
<td>16 (3)</td>
<td>0.41</td>
</tr>
</tbody>
</table>

| **Medication at inclusion, % (n)** |     |     |     |     |
| Aspirin                  | 32 (71) | 32 (65) | 32 (6) | 1 |
| Clopidogrel              | 16 (25) | 11 (23) | 11 (2) | 1 |
| β-Blocker                | 28 (61) | 27 (55) | 32 (6) | 0.78 |
| ACE or AT1 inhibitors    | 39 (86) | 38 (77) | 47 (9) | 0.46 |
| Statin                   | 35 (77) | 35 (71) | 32 (6) | 1 |
| Diuretics (excluding amiloride and MR antagonists) | 19 (43) | 19 (39) | 22 (4) | 0.76 |
| Anti-diabetic agents     | 12 (27) | 12 (25) | 11 (2) | 1 |
| Insulin                  | 7 (16)  | 7 (14)  | 11 (2) | 0.63 |

All continuous variables are reported as median with (range and interquartile range). P-value was calculated according to Mann–Whitney U-test for continuous variable and according to exact bilateral Fischer test for proportions.

ACS, acute coronary syndrome; ACE, angiotensin-converting enzyme; AT1, angiotensin II receptor type 1; LVEF, left ventricular ejection fraction.

*aAvailable in 90% of patients.*
observed that addition of monoclonal mouse anti-human apoA-1 IgG, spiked in reference plasma or culture media, significantly increased the basal contraction rate of cardiomyocytes in a concentration-dependent manner. The maximal response was observed at a concentration of 10 μg/mL (Figure 2A). At this optimal concentration, the effect was statistically significant when compared with reference plasma alone and with reference plasma spiked with control IgG (78 vs. 27.5 b.p.m., P = 0.01; and 78 vs. 38 b.p.m., P = 0.004; respectively; Figure 2B). This chronotropic effect was not seen when anti-apoA-1 IgG was applied to naïve cells, not primed with aldosterone (Figure 2B) or primed with other steroids than aldosterone (data not shown).

The same experiments were repeated using plasma samples obtained from MI patients. Fifteen plasmas from MI patients tested positive for anti-apoA-1 IgG and 15 plasmas from MI patient samples that were negative for those antibodies were randomly taken. Myocardial infarction plasma positive for anti-apoA-1 IgG induced a significantly higher median increase of cardiomyocyte contraction rate than patient plasma negative for anti-apoA-1 IgG (95 vs. 62 b.p.m.; P = 0.04, Figure 2C). When expressed in percentage of baseline to normalize for the baseline variability (30 vs. 36 b.p.m., Figure 2C), Spearman test showed a significant correlation between the titre of anti-apoA-1 IgG and cardiomyocyte contraction rate (r = 0.45, P = 0.02), confirming the dose-dependent relationship between anti-apoA-1 IgG in MI plasma and basal heart rate.

Figure 1 Kaplan–Meier time-to-events plot for cardiovascular outcome at 1 year according to anti-apoA-1 IgG status.

Figure 2 (A) Dose-dependent chronotropic effect of anti-apoA-1 IgG in vitro. Results are expressed as median with interquartile range and range. *P = 0.01 according to Kruskall–Wallis test. **P = 0.7 according to Kruskall–Wallis test. (B) Aldosterone-dependent effect of anti-apoA-1 IgG in vitro. * and **P = 0.004. Results are expressed as median with interquartile range and range. (C) Effect of myocardial infarction plasma on in vitro contraction rate according to anti-apoA-1 IgG status. *P = 0.2 and **P = 0.04. Results are expressed as median with interquartile range and range.
Anti-apoA-1 IgG chronotropic effect on cardiomyocytes is idiotype-dependent and abolished by therapeutic immunoglobulin

The chronotropic response was almost fully abolished when anti-apoA-1 IgG was added together with 1 mg/mL of delipidated apoA-1, demonstrating the idiotype specificity of the anti-apoA-1 IgG effect on cardiomyocyte contraction rate (Figure 3A). This positive chronotropic effect was also strongly abrogated by IVIG 2 mg/mL (Figure 3B). ApoA-1 per se displayed a modest, but significant, positive chronotropic effect on contraction rate when compared with baseline (Figure 3A, P = 0.03), whereas IVIG per se did not (Figure 3B, P = 1).

Discussion

The novel and important finding of the present study is that anti-apoA-1 IgG appears as a new prognostic marker of MACEs 1 year after MI, independently of traditional cardiovascular risk factors, but associated with RHR, another major cardiovascular prognostic feature after MI. Elevated RHR is known to affect the cardiovascular risk by concomitantly increasing myocardial oxygen demand while decreasing its supply and energy stores, accelerating atherosclerosis, and enhancing plaque vulnerability. Although RHR following MI has been considered as a prognostic factor since more than two decades, the recent results of the BEAUTIFUL study allowed determining a reasonable but arbitrary RHR cut-off above which the risk of cardiovascular complications is drastically increased and set at 70 b.p.m. To this respect and even if our patients had a higher median EF than the patients enrolled in the BEAUTIFUL study, it is interesting to note that MI patients who were positive for anti-apoA-1 IgG had RHR above this threshold (median value of 76 b.p.m.), whereas MI patients tested negative for these antibodies were below the threshold (median value of 67 b.p.m., P = 0.0005). Moreover, plasma of MI patients containing high levels of anti-apoA-1 IgG induced in vitro a significant increase of the basal rat cardiomyocytes beating frequency that was more pronounced than the response to plasma without these auto-antibodies (95 vs. 62 b.p.m., P = 0.04). Together with the dose-dependent effect of anti-apoA-1 IgG at physiologically relevant concentrations (1–20 µg/mL), the significant positive correlations between anti-apoA-1 IgG titre with RHR both in vivo and in vitro strongly suggest that the presence of anti-apoA-1 IgG is responsible for the positive chronotropic effect of MI plasma, which was reverted by physiologically relevant concentrations of IVIG and apoA-1. Both IVIG and apoA-1 have been reported as promising CVD therapeutic modalities in mice, but data in humans are scarce. Therefore, knowing whether anti-apoA-1 IgG-positive MI patients could specifically benefit from IVIG or apoA-1 treatment remains highly speculative at the present time, and further work is needed to determine whether anti-apoA-1 IgG is a cardiovascular risk factor or just a marker of cardiovascular risk.

Our results raise several questions. To the best of our knowledge, apoA-1, the antigen against which those auto-antibodies are directed to, is not known to be expressed by rat cardiomyocytes. Accordingly, one can evoke a non-specific effect of those antibodies or even serendipity. However, given the absence of effect of the negative controls, and the concentration-dependency of the response, it is more reasonable to evoke molecular mimicry between a common conformational epitope shared by cardiomyocytes and apoA-1 to account for the aforementioned observations. More importantly, the complete reversion of anti-apoA-1 IgG effect by a saturation test using apoA-1 is the strongest argument, indicating that the chronotropic effect observed was idiotype-dependent, lending further weight to the molecular mimicry hypothesis. To this respect, the fact that the effect was observed only for aldosterone incubation raises several mutually non-exclusive hypotheses about the nature of this epitope. First, since aldosterone is known to increase T-type calcium as well as HCN channels expression and function, which translated into a significant increase in cardiomyocyte contraction rate, it is possible that anti-apoA-1 IgG could interfere with one of those channels function, whose relevance in cardiovascular-related physiopathology is well documented in humans, or with other signalling components induced by the mineralocorticoid receptor. Ongoing in vitro pharmacological and electrophysiological...
studies will resolve those matters. Another non-exclusive hypoth-
thesis could be that those autoantibodies might also interfere with
more classical apoA-1-related properties, such as reverse choles-
terol transport, anti-inflammatory, or anti-oxidant activities,
which in turn might negatively affect atherogenesis and plaque
stability.

Despite being appropriately powered, the number of events in
this study is still relatively low, preventing us to draw definite con-
clusions at the present time and those results need to be repro-
duced before any clinical recommendation can be made. How-
ever, as the rate of cardiovascular complications at 12
months was similar to the one observed in another bigger study
(14 vs. 15%) using similar endpoints, we consider our sample
to be representative of MI pathology. Also, it is noteworthy that
3 of the 19 patients positive for anti-apoA-1 IgG were also positive
for anti-cardiolipin IgG, but not for other anti-β2GP1 autoanti-
bodies, ANA or RF. Even if none of these patients were known
for any other autoimmune disease, we cannot exclude the conco-
mitant presence of anti-cardiolipin in these samples, as cross reac-
tivity between anti-apoA-1 IgG and anti-cardiolipin antibodies has
been described. Because anti-cardiolipin antibodies were not
associated with patients’ outcome in this study (data not shown),
we believe that the reported herein associations are not clouded
by a cross-reactivity confounder. The strength of this study
resides in the fact that the prognostic value of those anti-apoA-1
IgG auto-antibodies has been confirmed in three different statisti-
cal models (multivariate logistic regression, Kaplan–Meier analysis,
and Cox regression) and that we were able to reproduce robustly
the reported association found on this cohort using a relevant in
vitro model, which provides a mechanistic hypothesis to explain
the herein reported prognostic aspect of anti-apoA-1 IgG after MI.

Conclusions
We report anti-apoA-1 IgG as a novel potential prognostic bio-
marker for MACEs in patients in post-MI period, independently
of traditional cardiovascular factors (age, sex, hypertension, dia-
betes, dyslipidaemia, and smoking), but significantly associated
with increased RHR, a major adverse prognostic factor after MI.
The exact chronotropic mechanisms of those auto-antibodies
are not known. Whether anti-apoA-1 IgG could represent a new
and potentially modifiable cardiovascular factor after an MI, or
just a risk marker, remains to be demonstrated in other larger
trials.

Acknowledgements
The authors thank M. Alvarez for her skilful technical help, Dr
M. Frias for his valuable advice concerning rat ventricular cell prep-
eration, and the Geneva University Hospitals Clinical Research
Center (Thomas Perneger) for computation of the required
sample size.

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to M.F.R.) and by Telemaque Foundation (to N.V.).

Conflict of interest: none declared.

Appendix 1
Staining with anti-apoA-1 IgG 1 ug/ml
Secondary ab: FITC labelled anti-Fc
Nucleus stained in red with PI
Magnification 40X

Staining with isotype control IgG 1 ug/ml
Secondary ab: FITC labelled anti-Fc
Nucleus stained in red with PI
Magnification 40X
Staining with anti-apoA-1 IgG 1µg/ml
Secondary ab: FITC labelled anti-Fc
Nucleus stained in red with PI
Magnification 40X

Staining with anti-apoA-1 IgG 1µg/ml
Preincubated with 10µg/ml of apoA-1
for 2h at room temperature
Secondary ab: FITC labelled anti-Fc
Nucleus stained in red with PI
Magnification 40X

References
Non-invasive diagnosis of chylopericardium by cardiac magnetic resonance imaging

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A 24-year-old woman was admitted to the hospital because of massive persistent asymptomatic pericardial effusion. Six months earlier, she had visited her rheumatologist because of polyarthralgias suggesting systemic lupus erythematosus. She had never complained of dyspnoea or cough. On echocardiography, a large, homogeneous pericardial effusion, ‘swinging heart’-type, was found (see Supplementary material, Movie I), with no signs of haemodynamic compromise (Panels A, parasternal long axis, and B, apical four-chamber with severe pericardial effusion [arrow]). A cardiac magnetic resonance imaging (CMR) was performed revealing a massive pericardial effusion (see Supplementary material, Movies II and III). No fibrous tracts were detected. Black blood images without (Panel C) and with fat saturation techniques (Panel D) were acquired and the suppression of the signal intensity of the fluid (asterisks) suggested the presence of fat in it. Pericardiocentesis was planned based on CMR images, obtaining 1 L of dense, intensely chyloous fluid. A chemistry analysis revealed a triglyceride level of 3700 mg/dL. Cytology and cultures yielded negative results. Follow-up MRI and chest computed tomography performed 1 month later showed recurrence of the effusion and findings compatible with lupus pneumonitis, while the patient remained asymptomatic. She was referred for surgery, which included drainage of the chylopericardium, ligation of the thoracic duct, and biopsy of the pericardium, which yielded unspecific results.

In conclusion, CMR is the only non-invasive technique capable of giving the diagnosis of chylopericardium, since it provides a biochemical characterization of pericardial effusion. In clinically stable patients, CMR can be helpful in further diagnosis of pericardial effusion.

Supplementary material is available at European Heart Journal online.

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Autoantibodies against apolipoprotein A-1 and phosphorylcholine for diagnosis of non-ST-segment elevation myocardial infarction

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Objectives. To explore the diagnostic accuracies of anti-apolipoprotein A-1 (anti-ApoA-1) IgG and anti-phosphorylcholine (anti-PC) IgM alone, expressed as a ratio (anti-ApoA-1 IgG/anti-PC IgM), and combined with the Thrombolysis In Myocardial Infarction (TIMI) score for non-ST-segment elevation myocardial infarction (NSTEMI) (NSTEMI-TIMI score) to create a new diagnostic algorithm – the Clinical Autoantibody Ratio (CABR) score – for the diagnosis of NSTEMI and subsequent cardiac troponin I (cTnI) elevation in patients with acute chest pain (ACP).

Methods. In this single-centre prospective study, 138 patients presented at the emergency department with ACP without ST-segment elevation myocardial infarction. Anti-ApoA-1 IgG and anti-PC IgM were assessed by enzyme-linked immunosorbent assay on admission. Post hoc determination of the CABR score cut-off was performed by receiver operating characteristics analyses.

Results. The adjudicated final diagnosis was NSTEMI in 17% (24/138) of patients. Both autoantibodies alone were found to be significant predictors of NSTEMI diagnosis, but the CABR score had the best diagnostic accuracy [area under the curve (AUC): 0.88; 95% confidence interval (CI): 0.82–0.95]. At the optimal cut-off of 3.3, the CABR score negative predictive value (NPV) was 97% (95% CI: 90–99). Logistic regression analysis showed that a CABR score >3.3 increased the risk of subsequent NSTEMI diagnosis 19-fold (odds ratio: 18.7; 95% CI: 5.2–67.3). For subsequent cTnI positivity, only anti-ApoA-1 IgG and CABR score displayed adequate predictive accuracies with AUCs of 0.80 (95% CI: 0.68–0.91) and 0.82 (95% CI: 0.70–0.94), respectively; the NPVs were 95% (95% CI: 90–98) and 99% (95% CI: 94–100), respectively.

Conclusion. The CABR score, derived from adding the anti-ApoA-1 IgG/anti-PC IgM ratio to the NSTEMI-TIMI score, could be a useful measure to rule out NSTEMI in patients presenting with ACP at the emergency department without electrocardiographic changes.

Keywords: acute chest pain, anti-apolipoprotein A-1 autoantibodies, anti-phosphorylcholine antibodies, myocardial infarction, NSTEMI diagnosis.
of myocardial necrosis, it is well known that the sensitivity of cTn within the first 6 h following the onset of symptoms is low [3, 4]. Because of this, prolonged monitoring and repeated blood sampling over a period of 6 h are often required before NSTEMI can be diagnosed. It has been suggested that postponing diagnosis in this way not only increases the risk of complications associated with this condition [2–5], but also contributes to ED overcrowding, the costs of which have been estimated at several billion US dollars each year [6]. Simultaneous assessment of multiple emergent cardiac biomarkers reflecting different underlying pathophysiological processes has produced promising improvements in NSTEMI diagnosis [7–10]. There is also a growing body of evidence to suggest that some autoantibodies could represent possible candidates for cardiovascular (CV) risk stratification, some acting to increase risk and others being protective [11]. Among these candidates, both high levels [above an optical density (OD) of 0.6] of anti-apolipoprotein A-1 (anti-ApoA-1) IgG and low levels [below 27 U mL⁻¹] of anti-phosphorylcholine (anti-PC) IgM have been shown to be independently associated with increased risk of CV disease [12–16]. From a pathophysiological point of view, anti-ApoA-1 IgG antibodies have been shown in vitro to act as positive chronotropic agents in cardiomyocytes [12], to directly promote the release from macrophages of mediators of atherogenesis and plaque vulnerability, such as pro-inflammatory cytokines and matrix metalloproteinase-9 [13, 14], and to negatively affect atherogenesis and atherosclerotic plaque vulnerability in apoE⁻/⁻ mice [14]. By contrast, anti-PC IgM antibodies are considered to be protective, reducing atherogenesis mainly by preventing uptake of oxidized low-density lipoprotein (LDL), which is a key step in the formation of foam cells [15]. Nevertheless, to date, their respective potential contribution to the diagnosis of NSTEMI in the context of acute chest pain has not been evaluated. Therefore, in the present explorative study, we assessed the accuracy of anti-ApoA-1 IgG, anti-PC IgM and the ratio of anti-ApoA-1 IgG to anti-PC IgM for i) NSTEMI diagnostic prediction in patients presenting at the ED with acute chest pain and ii) subsequent cTn positivity prediction following an initial negative cTn sample.

We also studied the relationship between these autoantibodies and the validated prognostic clinical Thrombolysis In Myocardial Infarction (TIMI) score for NSTEMI (NSTEMI-TIMI score) to predict patient outcome at 14 days [17]. Finally, we investigated whether the combination of the autoantibody ratio with the NSTEMI-TIMI score could improve the predictive accuracy of the NSTEMI-TIMI score for NSTEMI prediction.

Material and methods

The research ethics committee of Geneva University Hospitals approved the study protocol, and all patients gave written informed consent before enrolment.

Patient population and study design

As no data were available regarding the prevalence of high titres of anti-ApoA-1 IgG and low titres of anti-PC IgM in patients with acute chest pain, we computed the sample size extrapolating our previously published results [18] on anti-ApoA-1 IgG and myocardial infarction (MI) to this explorative study. Accordingly, assuming that the prevalence of high titres of anti-ApoA-1 IgG should be 10% in the NSTEMI group and 1% in patients with diagnoses other than NSTEMI at discharge [18], a sample of 133 patients was needed to achieve a power of 90% with an alpha error of 5% to detect a difference in the prevalence of anti-ApoA-1 IgG positivity between patients with NSTEMI and those with other diagnoses.

Inclusion criteria consisted of chest pain lasting more than 5 min, regardless of age and gender, without ST-segment elevation on ECG defined by the absence of ST/T abnormalities or dynamic changes, such as nonpersistent ST-segment elevation, ST depression, T-wave abnormalities or no ECG changes. Exclusion criteria consisted of STEMI, chest pain for a duration of less than 5 min, prior hospitalization within 48 h, known autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) or anti-phospholipid syndrome (APS), known HIV or clinically patent signs of heart failure.

Between January and April 2009, 159 patients were screened at the ED of Geneva University Hospital (a primary care hospital) for acute chest pain. Twenty-one patients were excluded: eight patients with STEMI, four with RA, one with APS, five with patent signs of heart failure, one with HIV and two with chest pain for <5 min; thus, 138 patients were eligible for analyses. Medical history was obtained at admission.

Study endpoints

Two predetermined endpoints were considered for this explorative study.
The primary endpoint was a discharge diagnosis of NSTEMI versus chest pain related to unstable angina or to ‘other diagnoses’. Chest pain aetiology was adjudicated by two senior cardiologists blinded to the participants’ biochemical data.

Diagnosis of NSTEMI was established using the universal criteria of type 1 acute myocardial infarction (AMI) based on dynamic changes in cTnI levels in the appropriate clinical context [19], excluding persistent STEMI. Patients were considered to have diagnoses other than NSTEMI when cTnI values were negative, following further investigation by coronary angiography for those with a higher pretest probability of ischaemic origin or noninvasive testing, including treadmill test, cardiac magnetic resonance imaging, stress echocardiography or myocardial perfusion scintigraphy in ambulatory settings, for patients at lowest risk. Patients with possible UA were considered as low-risk NST-ACS and were accordingly labelled 50% as recommended by the recent universal AMI definition proposed by the European Society of Cardiology and the American Heart Association [19]. This cut-off is routinely used at our institution to detect myocardial injury in the setting of ACS.

Anti-Apo-A-1 IgG assessment by enzyme-linked immunosorbent assay

Anti-Apo-A-1 IgG antibodies were measured as previously described [12–14]. Briefly, Maxisorp plates (Nunc™, Roskilde, Denmark) were coated with purified, human-derived delipidated ApoA-1 (20 μg mL\(^{-1}\); 50 μL well\(^{-1}\) for 1 h at 37 °C. After washing, the wells were blocked for 1 h with phosphate-buffered saline (PBS) with 2% bovine serum albumin (BSA) at 37 °C. Then, serum samples diluted 1/50 were incubated for 1 h. Patient serum samples were also added to a noncoated well to assess the individual nonspecific binding. After washing six times, 50 μL well\(^{-1}\) alkaline phosphatase-conjugated antihuman IgG (Sigma-Aldrich, St Louis, MO, USA) diluted 1/1000 in PBS/BSA solution was incubated for 1 h at 37 °C. After washing again six times, the phosphatase substrate p-nitrophanylphosphate disodium (Sigma-Aldrich) dissolved in diethanolamine buffer (pH 9.8) was added. Each sample was tested in duplicate, and absorbance in OD was determined at 405 nm, after incubation for 20 min at 37 °C, using a plate reader (Molecular Devices VersaMax™; Molecular Device, Sunnyvale, CA, USA). The corresponding nonspecific binding was subtracted from the mean absorbance for each sample. The cut-off value for positivity was prospectively defined and set at 0.6 OD and 37% of the positive control value, as previously described [12–14]. At the cut-off level, the intra- and inter-assay coefficients of variation were 16% (n = 10) and 12% (n = 8), respectively.

Anti-PC IgM assessment by enzyme-linked immunosorbent assay

Anti-PC IgM levels were assessed using a commercially available enzyme-linked immunosorbent assay kit (CVDefine™; Athera Biotechnologies, Uppsala, Sweden), with purified PC as antigen, and performed according to the manufacturer’s instructions. Results are expressed in U mL\(^{-1}\), based on a standard curve build on six points. Samples were run in duplicate. Inter-assay coefficients of variation were 2.4% at 12.5 U mL\(^{-1}\) and 2.0% at 25 U mL\(^{-1}\) (n = 4). The intra-assay coefficient of variation at 65 U mL\(^{-1}\) was 5% (n = 8). As the anti-PC IgM cut-off for CV disease risk prediction varies from 17 to 29 U mL\(^{-1}\) according to published reports [15, 16], and because no data...
are currently available for NSTEMI prediction with this autoantibody, we defined the cut-off based on post hoc receiver operating curve (ROC) analysis.

Anti-ApoA-1 IgG/anti-PC IgM ratio determination

The anti-ApoA-1 IgG/anti-PC IgM ratio was determined by dividing the anti-ApoA-1 IgG value (index expressed as percentage of positive control) by anti-PC IgM units (U mL\(^{-1}\)). Results of the ratio are therefore reported as arbitrary units (AU). As for anti-PC IgM, the cut-off was defined post hoc on ROC analysis.

Determination of the clinical autoantibody ratio score

The clinical autoantibody ratio (CABR) score was computed by simple addition of the NSTEMI-TIMI score to the anti-ApoA-1 IgG/anti-PC IgM ratio. The optimal CABR score cut-off for NSTEMI was defined post hoc by ROC analysis.

Statistical analyses

Analyses were performed using statistica™ software (StatSoft, Tulsa, OK, USA). Fisher’s bilateral exact test and Mann–Whitney U-test were used where appropriate. Associations between anti-ApoA-1 IgG, anti-PC IgM, anti-ApoA-1 IgG/anti-PC IgM ratio and study endpoints are presented as the odds ratio (OR) and corresponding 95% confidence interval (95% CI). Multivariable analyses with logistic regression were used to assess associations between variables. In this model, endpoints were set as dependent variables, and NSTEMI-TIMI score [21] (allowing for adjustment for major CV determinants of patient outcome at 14 days within a single continuous variable) was set as the unique confounder because of the limited sample size. The log-normal variation of this model was also used to assess the interdependence of the anti-ApoA-1 IgG/anti-PC IgM ratio and traditional CV risk factors. ROC analyses were performed using analyse-it™ software for Excel (Microsoft, Redmond, WA, USA) to (i) confirm the cut-off values prospectively chosen for anti-ApoA-1 IgG, (ii) determine the best cut-off for anti-PC IgM and for the anti-ApoA-1 IgG/anti-PC IgM ratio and (iii) determine which marker alone, in combination (ratio) or together with the NSTEMI-TIMI score yields the best area under the curve (AUC). AUC comparisons were performed according to the non-parametric approach proposed by DeLong et al. [20]. To further support the ROC curve analyses, reclassification statistics using the integrated discrimination index (IDI) compared the predictive performances of the CABR score with the anti-ApoA-1 IgG/anti-PC IgM ratio and the NSTEMI-TIMI score, as recommended by Pencina et al. [21]. The predicted risk of NSTEMI diagnosis according to the values of variables was assessed by a logistic regression model (the goodness-of-fit was checked by the Hosmer–Lemeshow test), and the IDI was derived from the mean predicted risks. The IDI is interpreted as the difference in the mean risk in patients with the event predicted by two variables minus the difference in the mean risk in patients without the event. It reflects the average gain in sensitivity (SE) minus the average loss in specificity (SP). We report IDI in relative percentage, expressing the CABR-related improvement in discrimination when compared to the NSTEMI-TIMI score and the autoantibody ratio [21].

SE, SP, positive predictive value (PPV), negative predictive value (NPV), positive and negative likelihood ratios (LR+ and LR−, respectively) with the respective 95% CIs are given. Ranked Spearman correlations were performed to establish correlations between variables. A value of \( P < 0.05 \) was considered statistically significant.

Results

Patient demographic characteristics are shown in Table 1. At discharge, 17% (24 of 138) of the patients were diagnosed with NSTEMI. Other diagnoses were considered to account for the symptoms of the remaining patients, as shown in Table 1. One patient with pulmonary embolism classified in the ‘other diagnoses’ group on discharge had elevated cTnI levels on admission.

Traditional risk factors and their association with NSTEMI

Patients with NSTEMI at discharge were older, more likely to be men, and had a higher prevalence of diabetes, hypertension and known coronary heart disease and related treatment when compared to patients with non-NSTEMI-related diagnoses (Table 1).

Autoantibody association with NSTEMI-TIMI score

Ranked Spearman correlation demonstrated a significant association between anti-ApoA-1 IgG/anti-PC IgM ratio and NSTEMI-TIMI score \(( r = 0.29; \ P = 0.005)\). Individually, anti-ApoA-1 IgG and anti-PC IgM were less strongly but still significantly correlated to NSTEMI-TIMI score \(( r = 0.24, \ P = 0.005 \) and \( r = –0.15, \ P = 0.04 \), respectively).
Table 1  *Patient demographic characteristics*

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Acute chest pain (n = 138)</th>
<th>NSTEMI (n = 24)</th>
<th>Other diagnoses (n = 114)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>58 (48–71; 23–93)</td>
<td>69 (62–74; 46–89)</td>
<td>56 (46–69; 23–93)</td>
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</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Male, % (n)</td>
<td>62 (86)</td>
<td>83 (20)</td>
<td>58 (66)</td>
<td>0.02</td>
</tr>
<tr>
<td>Female, % (n)</td>
<td>38 (52)</td>
<td>17 (4)</td>
<td>42 (48)</td>
<td></td>
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<tr>
<td>Cardiovascular risk factors</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Diabetes, % (n)</td>
<td>18 (25)</td>
<td>38 (9)</td>
<td>14 (16)</td>
<td>0.01</td>
</tr>
<tr>
<td>Smoking, % (n)</td>
<td>23 (31)</td>
<td>29 (7)</td>
<td>21 (24)</td>
<td>0.42</td>
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<td>Dyslipidaemia, % (n)</td>
<td>36 (50)</td>
<td>58 (14)</td>
<td>40 (46)</td>
<td>0.11</td>
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<tr>
<td>Obesity, % (n)</td>
<td>14 (19)</td>
<td>13 (3)</td>
<td>14 (16)</td>
<td>1</td>
</tr>
<tr>
<td>Hypertension, % (n)</td>
<td>44 (61)</td>
<td>71 (17)</td>
<td>39 (44)</td>
<td>0.005</td>
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<td>CHD, % (n)</td>
<td>29 (40)</td>
<td>54 (13)</td>
<td>24 (27)</td>
<td>0.005</td>
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<td>Stroke, % (n)</td>
<td>5 (7)</td>
<td>4 (1)</td>
<td>5 (6)</td>
<td>1</td>
</tr>
<tr>
<td>Family history, % (n)</td>
<td>12 (16)</td>
<td>29 (7)</td>
<td>8 (9)</td>
<td>0.008</td>
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<tr>
<td>Blood pressure (mmHg)</td>
<td></td>
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<tr>
<td>Systolic</td>
<td>130 (120–148; 95–200)</td>
<td>130 (117–149; 95–164)</td>
<td>131 (120–148; 97–200)</td>
<td>0.37</td>
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<tr>
<td>Diastolic</td>
<td>75 (90–70; 50–110)</td>
<td>70 (70–80; 60–90)</td>
<td>79 (70–90; 50–110)</td>
<td>0.31</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>75 (66–84; 40–170)</td>
<td>72 (66–80; 47–170)</td>
<td>75 (66–85; 40–130)</td>
<td>0.59</td>
</tr>
<tr>
<td>Body mass index (kg m⁻²)</td>
<td>26.1 (23.9–29.4; 16.4–38.3)</td>
<td>28.1 (25.8–30.1; 20.8–32.9)</td>
<td>25.7 (23.8–29.4; 16.4–38.3)</td>
<td>0.27</td>
</tr>
<tr>
<td>NSTEMI-TIMI score at admission</td>
<td>2 (1–3; 1–6)</td>
<td>4 (3–5; 2–6)</td>
<td>2 (1–3; 1–6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Medical treatment on admission</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin, % (n)</td>
<td>40 (55)</td>
<td>63 (15)</td>
<td>35 (40)</td>
<td>0.02</td>
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<tr>
<td>Clopidogrel, % (n)</td>
<td>8 (11)</td>
<td>8 (2)</td>
<td>8 (9)</td>
<td>1</td>
</tr>
<tr>
<td>β-blockers, % (n)</td>
<td>28 (39)</td>
<td>42 (10)</td>
<td>25 (29)</td>
<td>0.14</td>
</tr>
<tr>
<td>ACE inhibitors, % (n)</td>
<td>23 (32)</td>
<td>42 (10)</td>
<td>19 (22)</td>
<td>0.03</td>
</tr>
<tr>
<td>AT-1 blockers, % (n)</td>
<td>10 (14)</td>
<td>8 (2)</td>
<td>11 (12)</td>
<td>1</td>
</tr>
<tr>
<td>Insulin, % (n)</td>
<td>5 (7)</td>
<td>13 (3)</td>
<td>4 (4)</td>
<td>0.1</td>
</tr>
<tr>
<td>Oral antidiabetic agents, % (n)</td>
<td>20 (27)</td>
<td>33 (8)</td>
<td>17 (19)</td>
<td>0.09</td>
</tr>
<tr>
<td>Diuretics, % (n)</td>
<td>10 (14)</td>
<td>25 (6)</td>
<td>7 (8)</td>
<td>0.01</td>
</tr>
<tr>
<td>Calcium channel blockers, % (n)</td>
<td>10 (14)</td>
<td>17 (4)</td>
<td>9 (10)</td>
<td>0.26</td>
</tr>
<tr>
<td>Statins, % (n)</td>
<td>31 (43)</td>
<td>46 (11)</td>
<td>28 (32)</td>
<td>0.1</td>
</tr>
<tr>
<td>Biological parameters on admission</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol L⁻¹)</td>
<td>4.4 (4.0–5.0; 2.7–6.7)</td>
<td>4.4 (3.9–5.2; 2.8–6.1)</td>
<td>4.4 (4.0–5.0; 2.7–6.7)</td>
<td>0.96</td>
</tr>
<tr>
<td>HDL (mmol L⁻¹)</td>
<td>1.04 (0.79–1.28; 0.65–2.29)</td>
<td>0.98 (0.79–1.28; 0.65–2.29)</td>
<td>1.1 (0.8–1.3; 0.7–1.5)</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>Acute chest pain (n = 138)</td>
<td>NSTEMI (n = 24)</td>
<td>Other diagnoses (n = 114)</td>
<td>P</td>
</tr>
<tr>
<td>----------------------</td>
<td>----------------------------</td>
<td>----------------</td>
<td>--------------------------</td>
<td>----</td>
</tr>
<tr>
<td><strong>LDL (mmol L⁻¹)</strong></td>
<td>2.6 (2.1–3.2; 0.7–4.6)</td>
<td>2.9 (2.1–3.2; 0.7–3.6)</td>
<td>2.5 (2.2–2.9; 1.5–4.6)</td>
<td>0.59</td>
</tr>
<tr>
<td><strong>Triglycerides (mmol L⁻¹)</strong></td>
<td>1.4 (0.9–20.7; −0.3–5.1)</td>
<td>1.5 (0.9–2.1; 0.3–5.0)</td>
<td>1.3 (0.9–2.0; 0.4–5.1)</td>
<td>0.96</td>
</tr>
<tr>
<td><strong>GFR (mL min⁻¹)</strong></td>
<td>66 (60–108; 14–202)</td>
<td>60 (57–88; 29–167)</td>
<td>68 (60–110; 14–202)</td>
<td>0.41</td>
</tr>
<tr>
<td><strong>CRP (mg L⁻¹)</strong></td>
<td>3 (1–10; &lt;1–274)</td>
<td>6.5 (3.5–16.5; 3–237)</td>
<td>2 (1–8; &lt;1–274)</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Initial cTnI value (ng mL⁻¹)</strong></td>
<td>0.02 (0.01–0.04; 0–23)</td>
<td>0.16 (0.07–2.18; 0.01–23)</td>
<td>0.02 (0.01–0.03; 0–0.34)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Initial Elevated cTnI, % (n)</strong></td>
<td>15 (20)</td>
<td>54 (13)</td>
<td>6 (7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Anti-ApoA-1 IgG, index</strong></td>
<td>16 (10–24; 0–92)</td>
<td>23 (18–44; 11–67)</td>
<td>14 (9–23; 0–92)</td>
<td>0.0001</td>
</tr>
<tr>
<td><strong>Anti-ApoA-1 IgG, OD</strong></td>
<td>0.25 (0.14–0.39; 0–1.57)</td>
<td>0.37 (0.26–0.74; 0.15–1.14)</td>
<td>0.22 (0.13–0.33; 0–1.57)</td>
<td>0.0001</td>
</tr>
<tr>
<td><strong>Anti-ApoA-1 IgG positivity, % (n)</strong></td>
<td>12 (16)</td>
<td>38 (9)</td>
<td>6 (7)</td>
<td>0.0002</td>
</tr>
<tr>
<td><strong>Anti-PC IgM, U mL⁻¹</strong></td>
<td>43.1 (26–63.9; 8.5–1714)</td>
<td>32.5 (20.5–50.6; 10–1714)</td>
<td>46.9 (29–69.6; 8.5–216.5)</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Anti-ApoA-1 IgG/anti-PC IgM ratio, Arbitrary units</strong></td>
<td>0.36 (0.16–0.62; 0–6.44)</td>
<td>0.69 (0.48–1.13; 0.02–6.4)</td>
<td>0.28 (0.13–0.52; 0–3.32)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>CABR score</strong></td>
<td>2.4 (1.5–4.1; 1.0–12.4)</td>
<td>4.9 (3.9–5.9; 2.3–12.4)</td>
<td>2.2 (1.3–3.4; 1–6.4)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

**Diagnosis at discharge, % (n)**

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NSTEMI</strong></td>
<td>17 (24)</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Other diagnoses</strong></td>
<td>83 (114)</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Parietal aetiology</strong></td>
<td>7 (9)</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Gastroenterological aetiology</strong></td>
<td>5 (7)</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Pulmonary aetiology</strong></td>
<td>3 (4)</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Supraventricular arrhythmia</strong></td>
<td>4 (5)</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Pulmonary embolism</strong></td>
<td>3 (4)</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Pericarditis</strong></td>
<td>2 (2)</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Psychogenic</strong></td>
<td>3 (4)</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Malignant hypertension</strong></td>
<td>3 (4)</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Undetermined (pulmonary embolism and aortic dissection ruled out)</strong></td>
<td>54 (75)</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

All continuous variables are expressed as median [interquartile range (IQR); and range]. For continuous variables, Mann–Whitney U-test was used for group comparisons and bilateral exact Fisher’s test for proportion comparisons between groups. CHD, coronary heart disease; ACE, angiotensin-converting enzyme; AT-1, angiotensin-1 receptor; cTnI, cardiac troponin I; CRP, C-reactive protein; GFR, glomerular filtration rate; CABR score, Clinical AutoantiBody Ratio score, computed as the anti-ApoA-1 IgG/anti-PC IgM ratio added to the NSTEMI-TIMI score.
Anti-ApoA-1 IgG, anti-PC IgM levels and anti-ApoA-1 IgG/anti-PC IgM ratio and their association with diagnosis of NSTEMI at discharge

The frequency of anti-ApoA-1 IgG positivity was 12% (16/138), and NSTEMI patients had higher median levels of anti-ApoA-1 IgG (index: 23 vs. 14; \( P < 0.0001 \)), lower levels of anti-PC IgM (32.5 vs. 46.9 U mL\(^{-1} \); \( P = 0.02 \)), a higher anti-ApoA-1/anti-PC IgM ratio (0.69 vs. 0.28 AU; \( P < 0.0001 \)) and a higher CABR score (4.9 vs. 2.2; \( P < 0.0001 \)) on admission, compared with patients with other diagnoses at discharge.

ROC curve analyses for prediction of NSTEMI diagnosis based on autoantibodies

ROC curve analyses confirmed that both anti-ApoA-1 IgG and anti-PC IgM levels on admission of patients presenting to the ED with acute chest pain were significant predictors of an NSTEMI diagnosis at discharge, with a better AUC for anti-ApoA-1 IgG when compared to anti-PC IgM (Table 2). These analyses also confirmed that the prospectively defined anti-ApoA-1 IgG cut-off (OD > 0.6 and index > 37%) validated in patients with AMI and RA [13, 14] can also be useful to predict NSTEMI in patients with acute chest pain, with an SP of 93%, an SE of 38%, a PPV of 53% and an NPV of 88%. The LR+ and LR− values were 5.43 and 0.67, respectively (Table 2).

For anti-PC IgM, ROC curve analyses indicated that the best cut-off for NSTEMI prediction was 29 U mL\(^{-1} \), which is the same cut-off previously suggested to be appropriate for MI prediction in a

Table 2  Predictive accuracy of anti-ApoA-1 IgG, anti-PC IgM, anti-ApoA-1 IgG/anti-PC IgM ratio and CABR score for (a) NSTEMI diagnosis and (b) subsequent cTnI positivity in patients with acute chest pain without ST-segment elevation

<table>
<thead>
<tr>
<th>Auto-antibodies and CABR score for NSTEMI prediction</th>
<th>Anti-ApoA-1 IgG</th>
<th>Anti-PC IgM</th>
<th>Anti-ApoA-1 IgG/anti-PC IgM ratio</th>
<th>CABR score</th>
</tr>
</thead>
<tbody>
<tr>
<td>*AUC (95% CI)</td>
<td>0.75 (0.64–0.85)</td>
<td>0.65 (0.53–0.77)</td>
<td>0.79 (0.70–0.89)</td>
<td>0.88 (0.82–0.95)</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.0001</td>
<td>&lt;0.007</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SP, % (95% CI)</td>
<td>93 (87–97)</td>
<td>76 (67–84)</td>
<td>83 (75–90)</td>
<td>74 (66–97)</td>
</tr>
<tr>
<td>SE, % (95% CI)</td>
<td>38 (19–59)</td>
<td>48 (27–69)</td>
<td>57 (35–77)</td>
<td>87 (65–82)</td>
</tr>
<tr>
<td>PPV, % (95% CI)</td>
<td>53 (28–77)</td>
<td>29 (15–46)</td>
<td>41 (24–59)</td>
<td>40 (26–55)</td>
</tr>
<tr>
<td>NPV, % (95% CI)</td>
<td>88 (80–92)</td>
<td>88 (80–94)</td>
<td>91 (83–95)</td>
<td>97 (90–99)</td>
</tr>
<tr>
<td>LR+, % (95% CI)</td>
<td>5.34 (1.98–11.4)</td>
<td>2.02 (1.24–3.59)</td>
<td>3.39 (1.97–5.85)</td>
<td>3.30 (2.34–4.67)</td>
</tr>
<tr>
<td>LR−, % (95% CI)</td>
<td>0.67 (0.54–2.00)</td>
<td>0.68 (0.43–1.01)</td>
<td>0.52 (0.33–0.84)</td>
<td>0.18 (0.06–0.51)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Auto-antibodies and CABR score for subsequent cTnI positivity prediction</th>
<th>Anti-ApoA-1 IgG</th>
<th>Anti-PC IgM</th>
<th>Anti-ApoA-1 IgG/anti-PC IgM ratio</th>
<th>CABR score</th>
</tr>
</thead>
<tbody>
<tr>
<td>*AUC (95% CI)</td>
<td>0.80 (0.68–0.91)</td>
<td>0.52 (0.33–0.70)</td>
<td>0.74 (0.61–0.88)</td>
<td>0.82 (0.70–0.94)</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.0001</td>
<td>0.43</td>
<td>0.0003</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SP, % (95% CI)</td>
<td>90 (83–95)</td>
<td>NA</td>
<td>79 (71–86)</td>
<td>67 (58–75)</td>
</tr>
<tr>
<td>SE, % (95% CI)</td>
<td>40 (12–74)</td>
<td>NA</td>
<td>56 (21–86)</td>
<td>89 (52–100)</td>
</tr>
<tr>
<td>PPV, % (95% CI)</td>
<td>24 (7–50)</td>
<td>NA</td>
<td>16 (5–33)</td>
<td>16 (7–29)</td>
</tr>
<tr>
<td>NPV, % (95% CI)</td>
<td>95 (90–98)</td>
<td>NA</td>
<td>96 (91–99)</td>
<td>99 (94–100)</td>
</tr>
<tr>
<td>LR+, % (95% CI)</td>
<td>3.94 (1.57–9.86)</td>
<td>NA</td>
<td>2.63 (1.34–5.17)</td>
<td>2.71 (1.93–3.80)</td>
</tr>
<tr>
<td>LR−, % (95% CI)</td>
<td>0.67 (0.40–1.10)</td>
<td>NA</td>
<td>0.56 (0.27–1.17)</td>
<td>0.17 (0.03–1.06)</td>
</tr>
</tbody>
</table>

*AUC values were computed using continuous values for anti-ApoA-1 IgG and anti-PC IgM.
SP, specificity; E, sensitivity; PPV, positive predictive value; NPV negative predictive value; LR+, positive likelihood ratio; LR−, negative likelihood ratio; NA, not applicable (anti-PC IgM was not found to be a significant predictor according to AUC).
population-based study [17]. At this cut-off value, SP was 76%, SE was 48%, PPV was 29% and NPV was 88%. The LR+ and LR− values were 2.02 and 0.68, respectively (Table 2).

Finally, ROC curve analyses indicated that the anti-ApoA-1 IgG/anti-PC IgM ratio outperformed anti-PC IgM alone for NSTEMI prediction with an AUC of 0.79. Indeed, AUC comparisons using the nonparametric approach indicated that the anti-ApoA-1 IgG/anti-PC IgM ratio had a higher diagnostic accuracy when compared to anti-PC IgM alone (P = 0.005) but was not superior to anti-ApoA-1 IgG alone (P = 0.46). ROC analyses demonstrated that the best cut-off for the anti-ApoA-1 IgG/anti-PC IgM ratio was 0.64 AU. At this cut-off, SP was 83%, SE was 57%, PPV was 41% and NPV was 91%. The LR+ and LR− values were 3.39 and 0.52, respectively (Table 2).

ROC curve analyses and reclassification statistics for NSTEMI prediction based on NSTEMI-TIMI score, the anti-ApoA-1 IgG/anti-PC IgM ratio and the CABR score

ROC curve analyses indicated that the clinical NSTEMI-TIMI score was a significant predictor of NSTEMI diagnosis at discharge with an AUC of 0.86; that is, patients with a diagnosis of NSTEMI at discharge had a higher NSTEMI-TIMI score on admission (Table 1, Fig. 1). By the addition of anti-ApoA-1 IgG/anti-PC IgM ratio to the NSTEMI-TIMI score, we derived the CABR score, with values ranging from 1.0 to 12.4 (Table 1). Using this CABR score for ROC analysis, we observed an increase in the accuracy of NSTEMI prediction with an AUC of 0.88 (Table 2). According to the nonparametric approach, this increase from an AUC of 0.86 (NSTEMI-TIMI score alone) to 0.88 for the CABR score was significant (P = 0.01), and the CABR score tended to outperform the anti-ApoA-1 IgG/anti-PC IgM ratio (P = 0.05). Further analysis indicated that the best cut-off for the CABR score was 3.3. At this cut-off, SP was 74%, SE was 87%, PPV was 40%, NPV was 97%, LR+ was 3.30 and LR− was 0.18 (Table 2). Fifty (36%) patients had a CABR score above 3.3, and 88 (64%) had a CABR score below or equal to 3.3. These results were further corroborated by reclassification statistics indicating that the mean predicted risk of NSTEMI diagnosis in patients with diagnoses other than NSTEMI on discharge was 11.5% for the CABR score, 14.5% for the autoantibody ratio and 12.1% for the NSTEMI-TIMI score. In patients with NSTEMI diagnosis on discharge, the predicted risks of NSTEMI diagnosis were 42.9% for the CABR score, 28.0% for the autoantibody ratio and 40.1% for the NSTEMI-TIMI score. The IDI values comparing the CABR score to the autoantibody ratio alone and to the NSTEMI-TIMI score alone were 18% (P = 0.002) and 3% (P = 0.008), respectively, indicating that the CABR score provides significant incremental diagnostic ability when compared to either the NSTEMI-TIMI score or the autoantibody ratio.

Anti-ApoA-1 IgG, anti-ApoA-1 IgG/anti-PC IgM ratio and the CABR score as significant predictors of subsequent cTnI elevation

As an important part of the delay in diagnosing NSTEMI can be attributed to the results of cTn measurement in patients who present at the ED within the first 6 h of symptom onset [3, 4], we investigated whether autoantibodies measured at the time the first cTnI value was negative could predict subsequent cTnI positivity. For this purpose, we performed ROC curve analyses in patients with a first negative cTnI result followed by cTnI elevation measured in the second blood sample. Overall, 10 patients had a normal initial cTnI level followed by an elevated level on the second assessment (>0.09 ng mL−1). The adjudicated final diagnosis was NSTEMI in these 10 patients (Table 1).

ROC curve analyses indicated that anti-ApoA-1 IgG and anti-ApoA-1 IgG/anti-PC IgM ratio (but not anti-PC IgM) on admission were significant predictors of subsequent cTnI positivity with respective AUC val-
ues of 0.80 and 0.74 (Table 2). At the cut-off used in the present study both anti-ApoA-1 IgG and the anti-ApoA-1 IgG/anti-PC IgM ratio provided a modest but significant LR+ (3.94; and 2.63, respectively), whereas the LR- values were not significant as the 95% CIs included 1 (Table 2). For subsequent cTnI elevation prediction, ROC curve analyses indicated that the CABR score was also the best discriminator with an AUC of 0.82 (Table 2), which, according to the nonparametric approach, did not significantly outperform the AUCs of anti-ApoA-1 IgG and the ratio of autoantibodies (P = 0.77 and P = 0.06, respectively), but was superior to the NSTEMI-TIMI score alone (P = 0.01). ROC curves for the anti-ApoA-1 IgG/anti-PC IgM ratio, NSTEMI-TIMI score and CABR score are shown in Fig. 2.

At the chosen cut-off value of 3.3, the CABR score had a sensitivity of 89%, an NPV of 99% and an LR- of 0.17 (Table 2). By contrast, SP and PPV were much too low to be clinically meaningful (Table 2).

High anti-ApoA-1 IgG levels, anti-ApoA-1 IgG/anti-PC IgM ratio and CABR score are associated with an increased risk of NSTEMI diagnosis at discharge

Because ranked Spearman correlation indicated that anti-ApoA-1 IgG/anti-PC IgM ratio was only modestly associated with NSTEMI-TIMI score (r = 0.29) and because adding the ratio of autoantibodies significantly improved the NSTEMI-TIMI AUC, we investigated whether the autoantibody ratio could predict the risk of subsequent NSTEMI diagnosis independently of NSTEMI-TIMI score. First, the proportion of patients that tested positive for anti-ApoA-1 IgG was higher in the NSTEMI group compared with patients without ischaemia (38% vs. 6%; P = 0.002; Table 1). In univariate analyses, anti-ApoA-1 IgG positivity and anti-ApoA-1 IgG/anti-PC IgM ratio > 0.64 on admission were respectively associated with a 10-fold (OR: 9.8, 95% CI: 3.2–30.5) and a seven-fold (OR: 6.5; 95% CI: 2.5–17.0) increased risk of NSTEMI diagnosis at discharge, which remained significant after adjustment for the NSTEMI-TIMI score (OR: 6.4, 95% CI: 1.72–24.2 and OR: 5.4, 95% CI: 1.7–17.0, respectively; Table 3). Univariate analysis indicated that anti-PC IgM levels below 29 U mL\(^{-1}\) on admission were associated with a three-fold (OR: 2.9, 95% CI: 1.2–7.5) increased risk of NSTEMI diagnosis at patient discharge; however, this association did not remain significant after adjustment for the NSTEMI-TIMI score (OR: 2.2, 95% CI: 0.70–6.7; Table 3). As shown in Table 3, the CABR score was most strongly associated with the subsequent NSTEMI risk. Indeed, CABR score above 3.3 increased the risk of NSTEMI 19-fold (OR: 18.7; 95% CI: 5.2–67.3), and further confirmed the usefulness of combining the autoantibody ratio with the NSTEMI-TIMI score to markedly increase predictive performance, as suggested by ROC curve analyses.

### Discussion

The novel and important finding of this study is that an autoantibody-based panel relying on the

<table>
<thead>
<tr>
<th>NSTEMIprediction</th>
<th>Univariate odds ratio (95% CI)</th>
<th>Odds ratio adjusted for NSTEMI-TIMI score (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-ApoA-1 IgG positivity</td>
<td>9.8 (3.2–30.5)</td>
<td>6.4 (1.72–24.2)</td>
</tr>
<tr>
<td>Anti-PC IgM &lt;29 U mL(^{-1})</td>
<td>2.9 (1.2–7.5)</td>
<td>2.2 (0.7–6.7)</td>
</tr>
<tr>
<td>Anti-ApoA-1/anti-PC IgM ratio &gt; 0.64</td>
<td>6.5 (2.5–17.0)</td>
<td>5.4 (1.7–17.0)</td>
</tr>
<tr>
<td>CABR score &gt; 3.3</td>
<td>18.7 (5.2–67.3)</td>
<td>–</td>
</tr>
</tbody>
</table>
anti-ApoA-1 IgG/anti-PC IgM ratio could be of clinical value, especially when combined with the NSTEMI-TIMI score, for NSTEMI diagnosis in patients presenting at the ED with acute chest pain but without ST-segment elevation. Indeed, from our ROC curve analyses and reclassification statistics using the IDI, we have demonstrated that the CABR score was not only the strongest predictor of NSTEMI diagnosis (AUC: 0.88), but also provided modest but significantly increased diagnostic ability when compared to the NSTEMI-TIMI score (IDI: 3%, P = 0.008). It is interesting that the CABR score was also found to be a significant predictor of subsequent cTnI positivity when the first cTnI assessment was negative (AUC: 0.82). At the chosen cut-off of 3.3, the CABR score appeared to be a good test to rule out the probability of an NSTEMI diagnosis with an NPV of 97%. Nevertheless, SP, PPV and the LR+ were too low to be clinically useful. Furthermore, at the same cut-off, the CABR score reached the optimal NPV of 99% with a negative LR+ of 0.17, clearly indicating that this test could be useful to rule out myocardial ischaemia in patients with chest pain and a first negative cTnI sample regardless of changes in ECG, such as nonpersistent ST-segment elevation, ST depression or T-wave abnormalities or no ECG changes. This in turn could favourably impact patient flow through the ED, by potentially obviating the need for serial blood sampling in patients with a CABR score ≤ 3.3, or the need to wait for the results of the second cTnI assessment in cases of a normal initial cTnI level. Given the difficulty and costs of rapidly ruling out the diagnosis of NSTEMI, particularly in the absence of ST/T abnormalities or dynamic changes [2–6], these preliminary results need to be confirmed in larger prospective cohorts before any clinical recommendations can be made.

On the other hand, anti-ApoA-1 IgG at the previously validated cut-off [12–14] appeared to be the candidate with the best LR+ of 5.34 and 3.94 for NSTEMI prediction and subsequent cTnI elevation, respectively. Even though these LR+ values did not exceed the recommended standard of 10 to be clinically meaningful as a rule-in test [22], they were above the values for cTnI used in the context of pulmonary embolism risk stratification [23]. Whether measurement of anti-ApoA-1 IgG could serve as a rule-in test should also be further investigated. Moreover, given the well-known difficulty of improving the AUC with single biomarkers of CV risk, despite a strong association with CV disease [23–26], observing such an increase in AUC and risk with the CABR score compared with the autoantibodies or NSTEMI-TIMI score separately is a good indication that these autoantibodies could reflect other pro-atherogenic properties in addition to those of traditional CV risk factors, as previously suggested [12–14, 27]. Indeed, the rationale for examining the anti-ApoA-1 IgG/anti-PC IgM ratio (with or without the NSTEMI-TIMI score) in the context of acute chest pain is that both autoantibodies have been independently associated with increased CV risk [12–16]. High levels of anti-ApoA-1 IgG have been shown to independently predict major adverse CV events in patients with MI or RA and to be associated with more vulnerable atherosclerotic plaque in patients with severe carotid stenosis [12–14]. By contrast, low levels of anti-PC IgM have been consistently associated with a higher risk of CV disease [15, 16]. From a pathophysiologic point of view, anti-ApoA-1 IgG antibodies have been shown in vitro and in vivo to promote inflammation, development of atherosclerosis and plaque vulnerability, whereas anti-PC IgM reduces atherosclerosis [28]. The exact mechanisms of action of these autoantibodies are still under investigation, but appear to involve antagonistic effects on the innate immune receptors of macrophages, with anti-apoA-1 IgG being pro-inflammatory through the engagement of the CD14/Toll-like receptor 2 complex [S. Pagano, N. Satta , D. Werling, V. Offord, P. de Moerloose, E. Charbonney, D. Hochstrasser, F. Mach, P. Roux-Lombard, N.Vuilleumier, unpublished data], whereas anti-PC IgM prevent scavenger receptor-mediated foam cell formation [15]. Whether acute dynamic changes in anti-apoA-1 IgG and anti-PC autoantibody levels precede AMI remains unknown. Nevertheless, based on published data indicating that anti-apoA-1 IgG and anti-PC IgM modulate atherogenesis in the long term but to a moderate degree [12–14, 17, 28], and because autoantibody levels are relatively stable over time, we consider that these autoantibodies are most likely to contribute to an elevated baseline CV disease risk rather than precipitating the acute event itself. However, this hypothesis needs to be proven.

Another interesting finding of this study is that the NSTEMI-TIMI score, in addition to its well-established short-term CV prognostic value [17], could also be important in terms of diagnosis of myocardial necrosis with an AUC to predict myocardial necrosis of 0.86 (95% CI: 0.79–0.93). These results are in line with a recently published meta-analysis including 17 625 patients showing that the TIMI score could be a useful diagnostic option for myocardial ischaemia, with SP and SE values above 95% depending on the cut-off used, in patients presenting at the ED with acute chest pain [29]. However, the results of several
studies have indicated that TIMI score alone is not sufficient for diagnostic purposes as some patients with low TIMI score are still at increased risk of CV disease [30, 31]. In this respect, our results indicate that an autoantibody-based ratio reflecting both pro-/anti-atherogenic processes as well as plaque vulnerability could significantly increase the diagnostic accuracy of clinical parameters for myocardial ischaemia and could therefore represent a different approach for NSTEMI diagnosis in the future.

This study has several limitations. First, despite being appropriately powered, this single-centre study has a limited sample size, as reflected by broad confidence intervals. However, as patient demographics were comparable with those in recent studies including consecutive patients with acute chest pain [29–31], we consider our cohort to be representative of unselected patient samples presenting at the ED with suspicion of NSTEMI. Furthermore, the design of this study does not allow us to quantify the potential benefit of the CABR score in terms of patient flow through the ED and costs associated with a more rapid exclusion of NSTEMI provided by its use. A second limitation of this work relates to analytical issues. Current anti-ApoA-1 IgG and anti-PC IgM assessments take several hours and have a long turn-around time to meet the conventional 60-min requirement for emergency tests; however, this problem could easily be overcome in the near future by the development of fully automated tests [3]. Third, as most of our cut-off values (except for anti-ApoA-1 IgG) have been defined in a post hoc fashion based on ROC curve analyses, these preliminary results and cut-off values must be further confirmed and validated in larger multicentre clinical trials before any clinical recommendations can be made. Nevertheless, the optimal anti-PC IgM cut-off defined in the present study was identical to the value described for MI prediction in a general population-based study [16], suggesting that our post hoc cut-off definition was adequate. Fourth, whether the CABR score can outperform or provide incremental information in addition to other promising biomarkers, such as brain natriuretic peptide, high-sensitivity cTn or copeptin for early rule-out of myocardial ischaemia [8–10] remains to be demonstrated and also warrants further study. Finally, given the exclusion criteria of this study, the potential of the CABR score with regard to NSTEMI diagnosis may not apply to patients with SLE, APS, RA or HIV.

In conclusion, the results of this hypothesis-generating study demonstrate that the CABR score based on an autoantibody ratio coupled to the NSTEMI-TIMI score seems to be a promising complementary partner to cTnI for rapid rule-out of NSTEMI, provided that anti-apoA-1 IgG and anti-PC IgM assays have a turn-around time suitable for emergency tests. This combination of the anti-ApoA-1 IgG/anti-PC IgM ratio and the NSTEMI-TIMI score improved the predictive accuracy for NSTEMI and for subsequent cTnI elevation, when compared to either candidate alone. Because of its good NPV for NSTEMI diagnosis (97%) and subsequent cTnI elevation (99%), a CABR score ≤3.3 could accelerate patient discharge from the ED by removing the need for prolonged monitoring and blood sampling for the majority (60–70%) of patients. These preliminary data should be confirmed in larger multicentre prospective trials, and further cost-effectiveness studies are required to determine the impact of CABR on both patient management in the ED and its related costs. These preliminary results also further support the growing body of evidence indicating that these two autoantibodies could be of clinical value for NSTEMI diagnosis.

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Conflicts of interest

No conflicts of interest to declare.

References

Autoantibodies against apolipoprotein A-1

P-F. Keller et al.


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3.3.2. Anti-apoA-1 IgG in patients with severe carotid stenosis

The related paper has been published as:

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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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

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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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.

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.

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

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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 0-1.44, 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 coefficients at the cut-off level were 16% (n=10) and 12% (n=8), respectively.

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 diagnostic 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>Age in years</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>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<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>
</tr>
<tr>
<td>CV risk factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<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>Blood pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<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>10-year Global FRS at admission, %</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>Medical treatment upon admission</td>
<td></td>
<td></td>
<td></td>
<td></td>
</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>
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<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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<td>AT-1 blockers, % (n)</td>
<td>23 (41)</td>
<td>0 (0)</td>
<td>24 (41)</td>
<td>0.12</td>
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<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>
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<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>
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<tr>
<td>Biological parameters upon admission</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<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>
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; ▶ Table 1). 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 (LRs) were of 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-Meier 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 11.8 % (6/51) in patients tested positive for both anti-apoA-1 IgG and MPO (p=0.28, LogRank).

Table 1: Continued

<table>
<thead>
<tr>
<th>Carotid stenosis</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>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>Echographic findings before CAE</td>
<td></td>
<td></td>
<td></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>
<tr>
<td>Atherosclerotic Plaque size; cm</td>
<td>1.89 (1.50–2.20;0.80–18)</td>
<td>2 (1.5–2;1.5–2)</td>
<td>1.88 (1.50–2.20;0.80–18)</td>
<td>0.89</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.
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: 2.36-148.41; p=0.006), and remained significant after the adjustment for FRS (OR: 15.97; 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 finding 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 prognosis 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),

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Table 2: Predictive accuracy of anti-ApoA-1 IgG, FRS and MPO subsequent MACE occurrence according to ROC curve analyses.

<table>
<thead>
<tr>
<th></th>
<th>Anti-ApoA-1 IgG</th>
<th>FRS</th>
<th>MPO</th>
<th>MPO + anti-apoA-1 IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AUC</strong></td>
<td>0.74</td>
<td>0.62</td>
<td>0.75</td>
<td>0.78</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(0.59–0.90)</td>
<td>(0.47–0.78)</td>
<td>(0.61–0.89)</td>
<td>(0.65–0.91)</td>
</tr>
<tr>
<td><em>p-value</em></td>
<td>0.01</td>
<td></td>
<td>0.01</td>
<td>0.007</td>
</tr>
<tr>
<td><strong>SP, %</strong></td>
<td>73</td>
<td>31</td>
<td>91</td>
<td>65</td>
</tr>
<tr>
<td>(95% CI, n)</td>
<td>(65–79, 123/69)</td>
<td>(24–38, 52/169)</td>
<td>(85–95, 149/164)</td>
<td>(57–73, 107/164)</td>
</tr>
<tr>
<td><strong>SE, %</strong></td>
<td>67</td>
<td>89</td>
<td>44</td>
<td>78</td>
</tr>
<tr>
<td>(95% CI, n)</td>
<td>(30–93, 6/9)</td>
<td>(52–100, 8/9)</td>
<td>(14–79, 4/9)</td>
<td>(40–97, 7/9)</td>
</tr>
<tr>
<td><strong>PPV, %</strong></td>
<td>12</td>
<td>6</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>(95% CI, n)</td>
<td>(5–24, 6/52)</td>
<td>(3–12, 8/125)</td>
<td>(7–46, 4/19)</td>
<td>(5–21, 7/64)</td>
</tr>
<tr>
<td><strong>NPV, %</strong></td>
<td>98</td>
<td>96</td>
<td>98</td>
<td>96</td>
</tr>
<tr>
<td><strong>LR + (95%CI)</strong></td>
<td>2.45</td>
<td>2.72</td>
<td>4.86</td>
<td>2.23</td>
</tr>
<tr>
<td>(1.45–4.13)</td>
<td>(1–1.65)</td>
<td>(2.02–11.66)</td>
<td>(1.49–3.36)</td>
<td>(13.67</td>
</tr>
<tr>
<td><strong>LR - (95%CI)</strong></td>
<td>0.46</td>
<td>0.36</td>
<td>0.61</td>
<td>0.34</td>
</tr>
<tr>
<td>(0.18–1.16)</td>
<td>(0.06–2.32)</td>
<td>(0.34–1.09)</td>
<td>(0.10–1.16)</td>
<td>(0.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.43–1.09)</td>
<td></td>
</tr>
</tbody>
</table>

*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.
Vuilleumier, Montecucco et al. Anti-apoA-1 IgG and myeloperoxidase in carotid atherosclerosis

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 auto-antibodies 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 and only 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 a 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>
<tr>
<td>Anti-apoA-1 IgG + vs. FRS &gt;20%</td>
<td>3.5%</td>
<td>1.7%</td>
</tr>
<tr>
<td>MPO + vs. FRS &gt;20%</td>
<td>6.1%</td>
<td>1.7%</td>
</tr>
<tr>
<td>Anti-apoA-1 IgG + and MPO + vs. FRS &gt;20%</td>
<td>11.0%</td>
<td>1.3%</td>
</tr>
<tr>
<td>Anti-apoA-1 IgG + and MPO + vs. anti-apoA-1 IgG +</td>
<td>7.5%</td>
<td>-0.5%</td>
</tr>
<tr>
<td>Anti-apoA-1 IgG + and MPO + vs. anti-apoA-1 IgG +</td>
<td>4.9%</td>
<td>-0.4%</td>
</tr>
</tbody>
</table>
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.

In conclusion, the results of this hypothesis-generating work set the proof of principle that measuring circulating levels of anti-apoA-1 IgG and MPO provide incremental CV predictive ability over the FRS and could therefore be of relevant for CV risk stratification in patients following CEA. More specifically, the negativity of any of those two biomarkers would allow the identification of the vast majority of patients (>70%) that will have a favourable outcome with a NPV of 98%, whereas being positive for both biomarkers could be used as a rule-in test to identify a small subset (4%) of CEA patients at particular high risk for CV complications. These preliminary data should be confirmed in larger prospective trials, and further cost-effectiveness studies are required to determine the impact of serum anti-apoA-1 IgG and MPO assessment on the management of CEA patients, if any. These preliminary results also support the growing body of evidence pointing to anti-apoA-1 IgG as a valuable humoral autoimmune candidate for CV risk stratification.

Conflicts of interest

None declared.

References


3.4. Anti-apoA-1 IgG as possible mediators of atherogenesis

Those papers have been published as:


Anti-Apolipoprotein A-1 auto-antibodies are active mediators of atherosclerotic plaque vulnerability

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Aims

Anti-Apolipoprotein A-1 auto-antibodies (anti-ApoA-1 IgG) represent an emerging prognostic cardiovascular marker in patients with myocardial infarction or autoimmune diseases associated with high cardiovascular risk. The potential relationship between anti-ApoA-1 IgG and plaque vulnerability remains elusive. Thus, we aimed to investigate the role of anti-ApoA-1 IgG in plaque vulnerability.

Methods and results

Potential relationship between anti-ApoA-1 IgG and features of cardiovascular vulnerability was explored both in vivo and in vitro. In vivo, we investigated anti-ApoA-1 IgG in patients with severe carotid stenosis (n = 102) and in apoE−/− mice infused with polyclonal anti-ApoA-1 IgG. In vitro, anti-ApoA-1 IgG effects were assessed on human primary macrophages, monocytes, and neutrophils. Intraplaque collagen was decreased, while neutrophil and matrix metalloprotease (MMP)-9 content were increased in anti-ApoA-1 IgG-positive patients and anti-ApoA-1 IgG-treated mice when compared with corresponding controls. In mouse aortic roots (but not in abdominal aortas), treatment with anti-ApoA-1 IgG was associated with increased lesion size when compared with controls. In humans, serum anti-ApoA-1 IgG levels positively correlated with intraplaque macrophage, neutrophil, and MMP-9 content, and inversely with collagen. In vitro, anti-ApoA-1 IgG increased macrophage release of CCL2, CXCL8, and MMP-9, as well as neutrophil migration towards TNF-α or CXCL8.

Conclusion

These results suggest that anti-ApoA-1 IgG might be associated with increased atherosclerotic plaque vulnerability in humans and mice.

Keywords

Inflammation  •  Metalloproteases  •  Leucocytes  •  Carotid arteries

Introduction

During the past decades, the concept of global ‘cardiovascular vulnerability’ opened new perspective in cardiovascular prevention.1,2 The possible identification of serum markers reflecting intraplaque vulnerability (easier to be measured than intraplaque parameters) could be of particular importance in both primary and secondary prevention of cardiovascular diseases. Simultaneously, it was
established that atherosclerosis is not only a collection of cholesterol, complicated by smooth muscle cell proliferation, but also a chronic inflammatory disease, involving both innate and adaptive immunity. Cellular immunity is a key player in this process, but humoral immunity and autoantibody production also play an important role. Some autoantibodies seem to be protective, but others are detrimental and associated with accelerated atherosclerosis and cardiovascular diseases.

Among those, we have recently reported the presence of IgG autoantibodies directed against Apolipoprotein A-1 (anti-ApoA-1 IgG), the major fraction of high-density lipoprotein, as an independent predictor of major cardiovascular events, both in rheumatoid arthritis (RA) patients in primary prevention, and in myocardial infarction (MI) patients in secondary prevention. High levels of anti-ApoA-1 IgG were also reported in patients with systemic lupus erythematosus and cardiovascular diseases. In those clinical settings, anti-ApoA-1 IgG positivity was significantly associated with high serum levels of oxidised low-density lipoprotein and matrix metalloprotease (MMP)-9, two other possible systemic markers of atherosclerotic plaque vulnerability.

Therefore, we investigated the potential involvement of anti-ApoA-1 IgG as a potential active factor in plaque vulnerability in humans, mice, and in vitro experiments. Patients without autoimmune or inflammatory diseases and asymptomatic for ischaemic stroke, which underwent carotid endarterectomy (CEA) for severe plaque stenosis, were tested for anti-ApoA-1 IgG. Positive and negative patients were compared for chemokine and MMP serum levels and plaque composition. To strengthen these observations, plaque composition was also assessed in ApoE−/− mice treated with goat polyclonal anti-ApoA-1 IgG. Finally, potential direct pro-atherosclerotic effects of anti-Apo-A-1 IgG were tested in human primary neutrophil, monocyte, and macrophage functions.

Methods

For additional details, please see the online Supplementary data.

Patients and study design

We conducted a cohort study between March 2008 and April 2010 at a single hospital (San Martino Hospital) in Genoa (Italy). Patients (n = 102), which underwent CEA for severe internal carotid stenosis incidentally diagnosed at US Doppler (>70% luminal narrowing) and which did not present personal history of ischaemic cerebral symptoms, were enrolled in the study. Importantly, magnetic resonance imaging with diffusion sequences did not show any signs of cerebral necrosis in all patients enrolled. The degree of luminal narrowing was determined by repeated Doppler echography and angiographic confirmation using the criteria of the North American Symptomatic Carotid Endarterectomy Trial (NASCET). The indication for CEA for asymptomatic patients was based on the recommendations published by the Asymptomatic Carotid Surgery Trial (ACST) and the indication for patients’ symptomatic was according to the recommendations of the European Carotid Surgery Trial (ECST) and the NASCET. The day before endarterectomy, blood samples were obtained by peripheral venipuncture from these patients at fasting state to collect serum and to perform blood parameters.

Medications reported in Table 1 were not modified in the 2 months prior to enrolment.

All patients who developed spontaneous cerebral embolism during 30 min preoperatively and during the dissection phase of the operation (detected by Transcranial Doppler insonation of the middle cerebral artery) were excluded from the study. Other exclusion criteria were malignant hypertension, acute coronary artery disease and unstable angina, any cardiac arrhythmias, congestive heart failure (II, III, and IV NYHA classes), liver or renal disorders or function abnormalities, acute and chronic infectious diseases, autoimmune and rheumatic diseases, rheumatoid factors, antinuclear antibody and anti-extractable nuclear antigen antibody serum positivity, cancer, endocrine diseases, inflammatory bowel diseases and anti-inflammatory (other than aspirin) medications, oral anticoagulant treatments, hormone, cytokine, or growth factor therapies.

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

Animals

Eleven-week-old ApoE−/− C57Bl/6 mice were submitted to passive immunization treatment protocol as described in detail in online Supplementary data.

Determination of human autoantibodies anti-Apo-A-1 by ELISA

Anti-ApoA-1 IgG were measured as previously described. This method was performed as described in the online Supplementary data.

Detection of inflammatory mediators in human serum and cell supernatants

This method was performed as described in the online Supplementary data.

Pro-matrix metalloprotease-9 zymographic assay

This method was performed as described in the online Supplementary data.

Human carotid plaque specimen processing

This method was performed as described in the online Supplementary data.

Immunohistochemistry in human carotid plaques and mouse aortic sinus

This method was performed as described in detail in the online Supplementary data.

Oil Red O staining for lipid content

This method was performed as described in detail in the online Supplementary data.

Sirius Red staining for collagen content

This method was performed as described in detail in the online Supplementary data.

Real-time RT–PCR

This method was performed as described in detail in the online Supplementary data.
Human primary neutrophil isolation and migration assay
This method was performed as described in detail in the online Supplementary data.

Human monocyte isolation and migration assay
This method was performed as described in detail in the online Supplementary data.

Human primary macrophage differentiation and culture
This method was performed as described in detail in the online Supplementary data.

Statistical analysis
Patient characteristics were described 1 day before endarterectomy. Anti-ApoA-1 IgG-positive patients were compared with negative patients using Pearson’s Chi-square test or Fisher’s exact test, when appropriate, for the comparison of qualitative variables and Mann–Whitney non-parametric test (the normality assumption of the variables’ distribution in both groups was violated) for comparisons of continuous variables. The comparisons between upstream and downstream portions of carotid plaques within anti-ApoA-1 IgG-positive and -negative groups were performed using Mann–Whitney U-test. Comparisons between parameters of mouse plaque vulnerability in anti-ApoA-1 IgG-, isotype control IgG-, and vehicle (PBS)-treated mice were performed using Mann–Whitney U-test. For continuous variables, results were expressed as medians [interquartile range (IQR)].

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Anti-ApoA-1 IgG negative (n = 82)</th>
<th>Anti-ApoA-1 IgG positive (n = 20)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, year (IQR)</td>
<td>72.0 (67–76)</td>
<td>71.5 (66.5–81)</td>
<td>0.10</td>
</tr>
<tr>
<td>Males, n (%)</td>
<td>53 (65)</td>
<td>13 (65)</td>
<td>1.00</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg (IQR)</td>
<td>140 (130–150)</td>
<td>145 (130–150)</td>
<td>0.56</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg (IQR)</td>
<td>80 (80–85)</td>
<td>80 (80–81)</td>
<td>0.92</td>
</tr>
<tr>
<td>Waist circumference, cm (IQR)</td>
<td>93.5</td>
<td>86</td>
<td>0.29</td>
</tr>
<tr>
<td>Current smoking, n (%)</td>
<td>21 (26)</td>
<td>10 (50)</td>
<td>0.06</td>
</tr>
<tr>
<td>Type 2 diabetes, n (%)</td>
<td>13 (16)</td>
<td>2 (10)</td>
<td>0.73</td>
</tr>
<tr>
<td>Dyslipidaemia, n (%)</td>
<td>49 (60)</td>
<td>15 (75)</td>
<td>0.30</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>59 (72)</td>
<td>15 (75)</td>
<td>1.00</td>
</tr>
<tr>
<td>Chronic CAD, n (%)</td>
<td>14 (17)</td>
<td>6 (30)</td>
<td>0.21</td>
</tr>
<tr>
<td>Total WBC, n × 10^9/L (IQR)</td>
<td>7.1 (6.2–8.1)</td>
<td>8.0 (6.2–9.7)</td>
<td>0.18</td>
</tr>
<tr>
<td>Neutrophils, n × 10^9/L (IQR)</td>
<td>4.5 (3.5–5.2)</td>
<td>5.3 (4–6.8)</td>
<td>0.08</td>
</tr>
<tr>
<td>Lymphocytes, n × 10^9/L (IQR)</td>
<td>1.77 (1.44–2.14)</td>
<td>1.86 (1.18–2.10)</td>
<td>0.60</td>
</tr>
<tr>
<td>Monocytes, n × 10^9/L (IQR)</td>
<td>0.44 (0.37–0.58)</td>
<td>0.46 (0.39–0.60)</td>
<td>0.42</td>
</tr>
<tr>
<td>Red blood cells, n × 10^12/L (IQR)</td>
<td>4.7 (4.4–5)</td>
<td>4.6 (4.35–4.90)</td>
<td>0.73</td>
</tr>
<tr>
<td>Platelet, n × 10^3/L (IQR)</td>
<td>244 (210–297)</td>
<td>250 (187.5–305)</td>
<td>0.95</td>
</tr>
<tr>
<td>Plasma fibrinogen, g/L (IQR)</td>
<td>3.62 (3.14–4.10)</td>
<td>3.73 (3.04–4.53)</td>
<td>0.38</td>
</tr>
<tr>
<td>Serum ApoA-1, mg/dl (IQR)</td>
<td>167 (122–217)</td>
<td>208.5 (165–222)</td>
<td>0.08</td>
</tr>
<tr>
<td>Serum total-c, mg/dl (IQR)</td>
<td>199 (172–231)</td>
<td>172 (158–198)</td>
<td>0.01</td>
</tr>
<tr>
<td>Serum LDL-c, mg/dl (IQR)</td>
<td>117 (90.8–149)</td>
<td>106.5 (88–126)</td>
<td>0.1</td>
</tr>
<tr>
<td>Serum HDL-c, mg/dl (IQR)</td>
<td>48 (40–60)</td>
<td>47.5 (42.5–55)</td>
<td>0.88</td>
</tr>
<tr>
<td>Serum triglycerides, mg/dl (IQR)</td>
<td>127 (92–175)</td>
<td>102.5 (81.5–138)</td>
<td>0.01</td>
</tr>
<tr>
<td>Serum glycaemia, mg/dl (IQR)</td>
<td>98 (89–112)</td>
<td>101 (92–112)</td>
<td>0.59</td>
</tr>
<tr>
<td>Serum insulin, nU/L (IQR)</td>
<td>9.5 (6.2–11.6)</td>
<td>8.8 (5.5–17.5)</td>
<td>0.88</td>
</tr>
<tr>
<td>Anti-platelets, n (%) aspirin</td>
<td>46 (56)</td>
<td>15 (75)</td>
<td>0.79</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>16 (20)</td>
<td>3 (15)</td>
<td>0.76</td>
</tr>
<tr>
<td>Diuretics, n (%)</td>
<td>4 (5)</td>
<td>3 (15)</td>
<td>0.13</td>
</tr>
<tr>
<td>ACE inhibitors, n (%)</td>
<td>2 (2)</td>
<td>1 (5)</td>
<td>0.48</td>
</tr>
<tr>
<td>ARBs, n (%)</td>
<td>37 (45)</td>
<td>10 (50)</td>
<td>0.89</td>
</tr>
<tr>
<td>Beta-blockers, n (%)</td>
<td>24 (29)</td>
<td>5 (25)</td>
<td>0.79</td>
</tr>
<tr>
<td>Calcium channel blockers, n (%)</td>
<td>23 (28)</td>
<td>8 (40)</td>
<td>0.42</td>
</tr>
<tr>
<td>Statins, n (%)</td>
<td>37 (45)</td>
<td>12 (60)</td>
<td>0.32</td>
</tr>
<tr>
<td>Oral anti-diabetics, n (%)</td>
<td>8 (10)</td>
<td>2 (10)</td>
<td>1.00</td>
</tr>
<tr>
<td>% Carotid lumen stenosis (IQR)</td>
<td>80 (75–90)</td>
<td>75 (70–80)</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Continuous variables are expressed as median [interquartile range (IQR)].

CAD, coronary artery disease; WBC, white blood cells; total-c, total cholesterol; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; ACE, angiotensin converting enzyme; ARBs, angiotensin receptor blockers.
Spearman’s rank correlation coefficients were used to assess correlations between anti-ApoA-1 IgG serum levels and, respectively, intraplaque infiltration of vascular and inflammatory cells, collagen and MMP-9 content, or inflammatory gene mRNA expression (ΔCT) in both upstream and downstream regions of carotid atherosclerotic plaques.

In vitro results were expressed as mean (± SD) (neutrophil and monocyte chemotaxis assays) and as medians (IQR) (macrophage cultures). One-way ANOVA was used for multiple group comparison, while unpaired t-test for two-group comparison. Values of P < 0.05 (two-tailed) were considered significant. All analyses were done with Statistica™ software (StatSoft, Tulsa, OK, USA) and Analyse-it® (Analyse-it Software, Leeds, UK) software.

Results

Patient characteristics

Clinical and demographic characteristics, biological parameters as well as medications in patients with severe internal carotid stenosis and asymptomatic for ischaemic stroke are described in Table 1. Serum anti-ApoA-1 IgG were positive in 20 (19.6%) patients, which are similar to what have been previously observed in patients with acute coronary syndrome.6,8 There was no significant difference between patients negative or positive for anti-ApoA-1 IgG in terms of age, sex, and medications. Although most cardiovascular risk factors were not different between the two groups, total serum cholesterol and triglycerides were increased in anti-ApoA-1 IgG-negative patients (respectively, total cholesterol: 199 vs. 172 mg/dL, P = 0.01 and triglycerides 127 vs. 102.5 mg/dL, P = 0.01).

Systemic levels of inflammatory biomarkers

No significant differences in serum levels for C-reactive protein, TNF-α, CCL2, CCL3, and MMP-8 were observed between anti-ApoA-1 IgG-negative and -positive patients. Although a slight increase in CCL4 and MMP-9 levels was observed in patients positive for serum anti-ApoA-1 IgG, differences were not statistically significant (respectively, CCL4: r = 0.43, P = 0.001; downstream: macrophages: r = 0.40, P = 0.001; downstream: neutrophils: r = 0.50, P = 0.001) (Table 2). Nevertheless, increased gelatinolytic activity for serum pro-MMP-9 was shown in anti-ApoA-1 IgG positive when compared with negative patients (Table 2).

Table 2 Systemic cardiovascular risk markers

<table>
<thead>
<tr>
<th>Cardiovascular markers</th>
<th>Anti-ApoA-1 negative (n = 82)</th>
<th>Anti-ApoA-1 positive (n = 20)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS C-reactive protein, mg/L</td>
<td>1.8 (0.8–5.0)</td>
<td>2.4 (1–3.4)</td>
<td>0.73</td>
</tr>
<tr>
<td>TNF-α, pg/mL</td>
<td>15.6 (15.6–19.8)</td>
<td>15.6 (15.6–23.9)</td>
<td>0.47</td>
</tr>
<tr>
<td>CCL2, pg/mL</td>
<td>15.6 (15.6–65.3)</td>
<td>27.05 (15.6–151.5)</td>
<td>0.30</td>
</tr>
<tr>
<td>CCL3, pg/mL</td>
<td>8.4 (7.7–13.3)</td>
<td>8.7 (7.8–31.9)</td>
<td>0.84</td>
</tr>
<tr>
<td>CCL4, pg/mL</td>
<td>30.0 (17.3–52.6)</td>
<td>37.0 (31.3–67.2)</td>
<td>0.08</td>
</tr>
<tr>
<td>MMP-9, ng/mL</td>
<td>300 (73–615)</td>
<td>549 (218–1027)</td>
<td>0.06</td>
</tr>
<tr>
<td>Pro-MMP-9 activity</td>
<td>20.1 (15.1–28.4)</td>
<td>30.2 (23.8–38.7)</td>
<td>0.009</td>
</tr>
<tr>
<td>MMP-8, ng/mL</td>
<td>7.3 (13–15.5)</td>
<td>10.4 (6–17.5)</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Data are expressed as median [interquartile range (IQR)]. HS C-reactive protein, high-sensitivity C-reactive protein; TNF, tumour necrosis factor; MMP, matrix metalloprotease.

Positive serum levels of anti-ApoA-1 IgG are associated with the increase of atherosclerotic plaque vulnerability in humans

We then looked for a difference in plaque vulnerability between patients positive and negative for serum anti-Apo A-1 IgG. In upstream portions of carotid plaques, collagen III content was decreased in anti-ApoA-1 IgG positive when compared with negative patients (Table 3). Accordingly, intraplaque macrophage and MMP-9 content were significantly increased in anti-ApoA-1 IgG positive when compared with negative patients. A slight increase in MMP-8 mRNA expression was also observed in positive when compared with negative patients, but the difference was not statistically significant (P = 0.08). Similar to upstream regions, in downstream portions of carotid plaques, collagen content was decreased in anti-ApoA-1 IgG positive when compared with negative patients. Accordingly, macrophage and MMP-9 content were increased in anti-ApoA-1 IgG-positive patients. Different from upstream, in downstream portions, neutrophil intraplaque infiltration was markedly increased in anti-ApoA-1 IgG positive when compared with negative patients. No significant differences were observed in other intraplaque parameters, such as lipid, smooth muscle cell and lymphocyte content, or inflammatory gene mRNA expression (Table 3). Circulating anti-ApoA-1 IgG levels inversely correlated with collagen content (upstream collagen III: r = −0.35, P = 0.001; downstream total collagen: r = −0.29, P = 0.008), while positively correlated with macrophage, neutrophil, and MMP-9 content in both upstream and downstream portions of carotid plaques (upstream: macrophages: r = 0.28, P = 0.009; neutrophils: r = 0.31, P = 0.004; MMP-9: r = 0.40, P = 0.001; downstream: macrophages: r = 0.33, P = 0.002; neutrophils: r = 0.43, P = 0.0001; MMP-9: r = 0.42, P = 0.0001) (Table 4). Importantly, no significant correlations were observed between anti-ApoA-1 serum levels and other intraplaque parameters (Table 4). Taken together, these results indicate that atherosclerotic plaque from patients positive for serum anti-Apo A-1 IgG are more vulnerable than those from anti-Apo A-1 IgG-negative patients.

Anti-ApoA-1 IgG treatment increases intraplaque vulnerability in ApoE−/− mice

To confirm these observations, we determined the impact of anti-ApoA-1 IgG levels on mouse plaque vulnerability parameters. We intravenously injected 11-week-old ApoE−/− mice with goat polyclonal anti-human ApoA-1 IgG, respective goat IgG controls, or vehicle (PBS), every 2 weeks over 16 weeks. Mice were fed before and during treatments with standard chow diet to avoid the induction of severe hypercholesterolaemia and render lipid

Table 4 Systemic cardiovascular risk markers

<table>
<thead>
<tr>
<th>Cardiovascular markers</th>
<th>Anti-ApoA-1 negative (n = 82)</th>
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<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS C-reactive protein, mg/L</td>
<td>1.8 (0.8–5.0)</td>
<td>2.4 (1–3.4)</td>
<td>0.73</td>
</tr>
<tr>
<td>TNF-α, pg/mL</td>
<td>15.6 (15.6–19.8)</td>
<td>15.6 (15.6–23.9)</td>
<td>0.47</td>
</tr>
<tr>
<td>CCL2, pg/mL</td>
<td>15.6 (15.6–65.3)</td>
<td>27.05 (15.6–151.5)</td>
<td>0.30</td>
</tr>
<tr>
<td>CCL3, pg/mL</td>
<td>8.4 (7.7–13.3)</td>
<td>8.7 (7.8–31.9)</td>
<td>0.84</td>
</tr>
<tr>
<td>CCL4, pg/mL</td>
<td>30.0 (17.3–52.6)</td>
<td>37.0 (31.3–67.2)</td>
<td>0.08</td>
</tr>
<tr>
<td>MMP-9, ng/mL</td>
<td>300 (73–615)</td>
<td>549 (218–1027)</td>
<td>0.06</td>
</tr>
<tr>
<td>Pro-MMP-9 activity</td>
<td>20.1 (15.1–28.4)</td>
<td>30.2 (23.8–38.7)</td>
<td>0.009</td>
</tr>
<tr>
<td>MMP-8, ng/mL</td>
<td>7.3 (13–15.5)</td>
<td>10.4 (6–17.5)</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Data are expressed as median [interquartile range (IQR)]. HS C-reactive protein, high-sensitivity C-reactive protein; TNF, tumour necrosis factor; MMP, matrix metalloprotease.
levels more similar to that detected in human patients. At sacrifice, the ratio of serum cholesterol sub-fractions and triglycerides was similar in three mouse groups (Supplementary material online, Table S1). Atherosclerotic lesion size in thoracoabdominal aortas was comparable in all groups (lipid deposition on total aorta surface: PBS: 2.5 ± 0.8%; CTL IgG: 3.6 ± 1.3%; anti-ApoA-1: 3.4 ± 0.9%). In aortic roots, atherosclerotic lesion size (as determined by Oil Red O staining) was increased in anti-ApoA-1 IgG-treated mice when compared with PBS or CTL IgG treatments (Table 5, Supplementary material online, Figures S1 and S2). Neutrophil, MMP-8, and MMP-9 contents were also increased in anti-ApoA-1 IgG-treated mice when compared with control groups (Table 5, Supplementary material online, Figures S1 and S2). Accordingly, total collagen content was significantly reduced in anti-ApoA-1 IgG-treated mice when compared with PBS or CTL IgG treatments (Table 5, Supplementary material online, Figure S1). Despite a slight increase in intraplaque macrophage and lymphocyte infiltration in anti-ApoA-1 IgG-treated mice, no significant changes were observed between groups (Table 5, Supplementary material online, Figure S2). The analysis of mRNA expression of mediators of vulnerability [such as macrophage (Cd68), neutrophil (neutrophil elastase, Elane), and Mmp9] and T helper (Th) lymphocyte polarization in mouse abdominal aortas, spleen, and lymphnodes partially confirmed several findings retrieved on histology data (Supplementary material online, Table S4). These results indicate that anti-ApoA-1 IgG treatment was associated with increased plaque vulnerability parameters (i.e. neutrophils and MMPs).

### Table 3 Parameters of intraplaque vulnerability

<table>
<thead>
<tr>
<th>Carotid intraplaque parameters</th>
<th>Anti-ApoA-1 negative (n = 82)</th>
<th>Anti-ApoA-1 positive (n = 20)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque size, cm</td>
<td>1.7 (1.5–2)</td>
<td>1.5 (1.3–1.9)</td>
<td>0.38</td>
</tr>
<tr>
<td>Plaque stenosis, % of lumen</td>
<td>80 (75–90)</td>
<td>75 (70–80)</td>
<td>0.30</td>
</tr>
<tr>
<td>Upstream portion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% lipid</td>
<td>5.98 (2.86–9.37)</td>
<td>7.11 (3.31–10.81)</td>
<td>0.42</td>
</tr>
<tr>
<td>% total collagen</td>
<td>33.58 (26.15–40.06)</td>
<td>33.29 (25.21–39.24)</td>
<td>0.88</td>
</tr>
<tr>
<td>% collagen I</td>
<td>11.54 (9.44–16.9)</td>
<td>13.36 (7.69–15.05)</td>
<td>0.25</td>
</tr>
<tr>
<td>% collagen III</td>
<td>13.17 (10.03–17.27)</td>
<td>9.82 (7.55–12.42)</td>
<td>0.005</td>
</tr>
<tr>
<td>% of smooth muscle cell-rich area</td>
<td>5.39 (3.16–9.37)</td>
<td>5.33 (2.33–5.85)</td>
<td>0.09</td>
</tr>
<tr>
<td>% of macrophage-rich area</td>
<td>4.98 (2.73–9.94)</td>
<td>7.95 (5.59–13.95)</td>
<td>0.02</td>
</tr>
<tr>
<td>Lymphocytes/mm²</td>
<td>2.37 (1.12–5.93)</td>
<td>4.03 (1.62–13.67)</td>
<td>0.14</td>
</tr>
<tr>
<td>Neutrophils/mm²</td>
<td>2.35 (1.12–6.01)</td>
<td>3.27 (2.18–8.44)</td>
<td>0.19</td>
</tr>
<tr>
<td>MMP-8 mRNA, fold increase</td>
<td>1.23 (0.38–3.00)</td>
<td>2.56 (0.66–3.86)</td>
<td>0.08</td>
</tr>
<tr>
<td>% MMP-9</td>
<td>2.99 (1.29–5.87)</td>
<td>6.25 (2.40–12.6)</td>
<td>0.02</td>
</tr>
<tr>
<td>TNF-α mRNA, fold increase</td>
<td>1.20 (0.54–1.95)</td>
<td>1.22 (0.49–1.94)</td>
<td>0.72</td>
</tr>
<tr>
<td>CCL2 mRNA, fold increase</td>
<td>0.93 (0.64–1.39)</td>
<td>1.17 (0.65–1.67)</td>
<td>0.37</td>
</tr>
<tr>
<td>CCL3 mRNA, fold increase</td>
<td>0.91 (0.62–1.72)</td>
<td>1.15 (0.79–1.58)</td>
<td>0.71</td>
</tr>
<tr>
<td>CCL4 mRNA, fold increase</td>
<td>0.88 (0.55–1.53)</td>
<td>1.02 (0.51–1.31)</td>
<td>0.65</td>
</tr>
<tr>
<td>CXCL8 mRNA, fold increase</td>
<td>0.96 (0.52–1.87)</td>
<td>1.32 (0.82–2.00)</td>
<td>0.39</td>
</tr>
<tr>
<td>Downstream portion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% lipid</td>
<td>3.45 (1.64–8.37)</td>
<td>4.78 (1.36–10.05)</td>
<td>0.57</td>
</tr>
<tr>
<td>% total collagen</td>
<td>20.88 (17.20–23.64)</td>
<td>17.90 (12.82–20.49)</td>
<td>0.01</td>
</tr>
<tr>
<td>% collagen I</td>
<td>6.40 (4.27–11.29)</td>
<td>5.45 (2.98–7.04)</td>
<td>0.06</td>
</tr>
<tr>
<td>% collagen III</td>
<td>6.63 (4.11–10.28)</td>
<td>4.58 (3.27–5.84)</td>
<td>0.03</td>
</tr>
<tr>
<td>% of smooth muscle cell-rich area</td>
<td>2.87 (1.62–4.38)</td>
<td>2.69 (1.69–3.88)</td>
<td>0.69</td>
</tr>
<tr>
<td>% of macrophage-rich area</td>
<td>6.88 (2.62–16.12)</td>
<td>11.17 (7.44–18.37)</td>
<td>0.04</td>
</tr>
<tr>
<td>Lymphocytes/mm²</td>
<td>2.09 (0.98–5.37)</td>
<td>2.81 (1.12–11.50)</td>
<td>0.34</td>
</tr>
<tr>
<td>Neutrophils/mm²</td>
<td>2.98 (0.66–8.51)</td>
<td>9.85 (3.88–15.12)</td>
<td>0.002</td>
</tr>
<tr>
<td>MMP-8 mRNA, fold increase</td>
<td>1.13 (0.34–3.25)</td>
<td>1.19 (0.80–2.28)</td>
<td>0.95</td>
</tr>
<tr>
<td>% MMP-9</td>
<td>4.77 (1.94–9.66)</td>
<td>19.37 (6.76–25.69)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TNF-α mRNA, fold increase</td>
<td>1.46 (0.78–2.77)</td>
<td>0.96 (0.62–2.06)</td>
<td>0.08</td>
</tr>
<tr>
<td>CCL2 mRNA, fold increase</td>
<td>1.37 (0.78–2.05)</td>
<td>1.95 (1.15–2.38)</td>
<td>0.07</td>
</tr>
<tr>
<td>CCL3 mRNA, fold increase</td>
<td>1.37 (0.84–2.14)</td>
<td>1.63 (0.58–2.18)</td>
<td>0.87</td>
</tr>
<tr>
<td>CCL4 mRNA, fold increase</td>
<td>1.38 (0.89–2.35)</td>
<td>1.75 (0.64–2.45)</td>
<td>0.91</td>
</tr>
<tr>
<td>CXCL8 mRNA, fold increase</td>
<td>1.16 (0.54–2.28)</td>
<td>0.91 (0.41–1.71)</td>
<td>0.32</td>
</tr>
</tbody>
</table>

Data are expressed as median [interquartile range (IQR)]. MMP, matrix metalloprotease.
not significantly alter primary human monocyte migration during chemotaxis assays did not significantly alter primary human monocyte migration towards classical chemoattractants (CCL2, CCL3, C-reactive protein) (Table 7).

Treatment with anti-ApoA-1 IgG induces the release of CCL2, CXCL8, TNF-α, and matrix metalloprotease-9 in human macrophage supernatants

To determine whether anti-ApoA-1 IgG could modulate the production of leucocyte chemoattractants and plaque vulnerability factors, we tested the effect of goat polyclonal anti-ApoA-1 IgG and respective control on human monocyte-derived macrophages in vitro. Moreover, to verify that human anti-ApoA-1 IgG had the same effects as goat anti-ApoA-1 IgG, we purified IgG from serum of anti-ApoA-1 IgG-positive and -negative patients with severe carotid stenosis and also tested them on monocyte-derived macrophages. Goat anti-ApoA-1 IgG significantly increased CCL2, CXCL8, and TNF-α release when compared with CTL medium or CTL IgG treatments (Table 8). Similarly, IgG from anti-ApoA-1-positive patients induced CCL2, CXCL8, and TNF-α production when compared with treatment with IgG isolated from negative patients (Table 8). Anti-ApoA-1 IgG significantly increased release of MMP-9 and gelatinolytic activity of pro-MMP-9 when compared with treatments with CTL IgG or IgG from negative patients (Table 8). No effect of anti-ApoA-1 IgG was observed on MMP-8 secretion in macrophage supernatants at 48 h of incubation (Table 8). The absence of LPS contamination of human isolated IgG, commercial anti-ApoA-1 IgG, and CTL IgG preparations was confirmed by Limulus assay (0.1 EU/mL for both antibodies, data not shown).

Discussion

In this study, we demonstrated a positive association between serum anti-ApoA-1 IgG levels and features of atherosclerotic intraplaque vulnerability, such as an increased phagocyte (macrophages and neutrophils) and MMP-9 content, and a reduced intraplaque content of collagen in patients with severe but asymptomatic carotid stenosis. Moreover, most of the associations between anti-ApoA-1 IgG positivity and vulnerable atherosclerotic plaque features retrieved in humans (with the exception of intraplaque macrophages content) were reproduced and confirmed in ApoE−/− mice exposed to passive immunization with anti-ApoA-1 IgG. Indeed, even if mice exposed to anti-ApoA-1 IgG did not develop bigger atherosclerotic lesions, those lesions contained higher MMP-9, neutrophil content, and lower total collagen amount, which fulfill some of the possible characteristics of a prone-to-rupture atherosclerotic lesion.17 In order to explore the potential mechanisms underlying these observational data in humans and mice, we investigated in vitro the role of anti-ApoA-1 IgG on both local and systemic processes regulating plaque vulnerability.

Local inflammation was considered by investigating anti-ApoA-1 IgG effects on human monocyte-derived macrophages, which are the most abundant inflammatory cell population in atherosclerotic lesions and have a key role in atherogenesis and plaque vulnerability.18 When exposed to human macrophages, goat polyclonal...
assays, anti-ApoA-1 IgG exposure increased neutrophil migration across atherosclerotic plaques. As shown by chemotaxis migration response to well-known chemoattractants expressed within the cytoplasm of inflammatory cells within atherosclerotic plaques.18,19 On the other hand, MMP-9 selectively degrades gelatin, collagen type IV and V, thereby weakening the fibrous cap making plaques more prone to rupture.20 Therefore, anti-ApoA-1 IgG could be directly involved in plaque vulnerability by promoting the production of pro-atherosclerotic factors.

Concerning the potential vulnerable activity of serum anti-ApoA-1 IgG, auto-antibodies have been shown to promote migration of flowing neutrophils through endothelial cells, presumably by activating β2-integrin-dependant immobilization.12 On the other hand, in the human and mouse models of atherosclerosis, the positive association between anti-ApoA-1 IgG levels and the number of circulating neutrophils has been recently shown to play a crucial role in atherosclerotic plaque vulnerability, this observation supports the role of anti-ApoA-1 IgG in this process.19,21 However, the direct effect of anti-ApoA-1 IgG on neutrophil migration represents a surprising result, for which the mechanism is still unexplained. Other auto-antibodies, namely anti-neutrophil cytoplasmic antibodies, have been shown to promote migration of flowing neutrophils through endothelial cells, presumably by activating β2-integrin-dependant immobilization.12

### Table 5 Parameters of mouse plaque vulnerability

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vehicle-treated mice (n = 10)</th>
<th>CTL IgG-treated mice (n = 14)</th>
<th>Anti-ApoA-1 IgG-treated mice (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil Red O, x 10² µm²</td>
<td>198 (171–216)</td>
<td>216 (185–232)</td>
<td>250 (227–297)</td>
</tr>
<tr>
<td>Total collagen, %</td>
<td>41 (32–43)</td>
<td>40 (35–43)</td>
<td>35 (32–40)</td>
</tr>
<tr>
<td>Macrophage+ area, %</td>
<td>9 (6–11)</td>
<td>10 (6–16)</td>
<td>14 (10–16)</td>
</tr>
<tr>
<td>Lymphocytes/mm²</td>
<td>48.3 (39.5–52.4)</td>
<td>47.1 (29.8–64.3)</td>
<td>49.7 (45.5–59.5)</td>
</tr>
<tr>
<td>Neutrophils/mm²</td>
<td>5 (2–15)</td>
<td>14 (5–19)</td>
<td>20 (14–23)</td>
</tr>
<tr>
<td>MMP-8, %</td>
<td>2 (2–4)</td>
<td>2 (2–4)</td>
<td>8 (4–9)</td>
</tr>
<tr>
<td>MMP-9, %</td>
<td>10 (9–12)</td>
<td>11 (8–16)</td>
<td>21 (16–25)</td>
</tr>
<tr>
<td>MMP-9, %</td>
<td>10 (9–12)</td>
<td>11 (8–16)</td>
<td>21 (16–25)</td>
</tr>
</tbody>
</table>

Data are expressed as median [interquartile range (IQR)].

*P < 0.05 vs. CTL IgG-treated mice.

†P < 0.05 vs. CTL IgG-treated mice.

‡P = NS (not significant).

§P < 0.05 vs. CTL IgG-treated mice.

#P < 0.05 vs. CTL IgG-treated mice.

**P < 0.05 vs. CTL IgG-treated mice.

### Table 6 Control (CTL) isotype IgG- and goat anti-ApoA-1 IgG-treated human primary neutrophil migration in response to classical chemoattractants

#### Migration assay [polycarbonate, chemotaxis index (C.I.)]

<table>
<thead>
<tr>
<th>Human neutrophils</th>
<th>Lower well (chemoattractant)</th>
<th>Upper well (treated cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTL medium</td>
<td>CTL IgG (20 µg/mL)</td>
</tr>
<tr>
<td></td>
<td>CTL medium</td>
<td>Anti-apoA-1 IgG (20 µg/mL)</td>
</tr>
<tr>
<td></td>
<td>CXCL8 (10 nM)</td>
<td>2.49 ± 0.49§</td>
</tr>
<tr>
<td></td>
<td>TNF-α (200 U/mL)</td>
<td>2.42 ± 0.38§</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD (n = 5).

*P < 0.05 vs. CTL medium-treated neutrophil migration towards CTL medium.

†P < 0.01 vs. CTL medium-treated neutrophil migration towards CXCL8.

‡P < 0.05 vs. CTL medium-treated neutrophil migration towards TNF-α.
Anti-ApoA-1 IgG are active mediators of atherosclerotic plaque vulnerability

Table 7 Control (CTL) isotype IgG- and goat anti-ApoA-1 IgG-treated human primary monocyte migration in response to classical chemoattractants

<table>
<thead>
<tr>
<th>Lower well (chemoattractant)</th>
<th>Upper well (treated cells)</th>
<th>CTL IgG (20 μg/mL)</th>
<th>CTL IgG (40 μg/mL)</th>
<th>Anti-ApoA-1 IgG (20 μg/mL)</th>
<th>Anti-ApoA-1 IgG (40 μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL medium</td>
<td>1.0 ± 0.0</td>
<td>0.92 ± 0.06</td>
<td>0.98 ± 0.21</td>
<td>1.01 ± 0.08</td>
<td>0.94 ± 0.22</td>
</tr>
<tr>
<td>CCL2 (10 nM)</td>
<td>2.19 ± 0.38</td>
<td>2.19 ± 0.52</td>
<td>2.26 ± 0.18</td>
<td>2.52 ± 0.45</td>
<td>2.62 ± 0.41</td>
</tr>
<tr>
<td>CCL3 (10 nM)</td>
<td>2.08 ± 0.38</td>
<td>2.19 ± 0.50</td>
<td>2.17 ± 0.39</td>
<td>2.60 ± 0.76</td>
<td>2.62 ± 0.41</td>
</tr>
<tr>
<td>C-reactive protein (40 μg/mL)</td>
<td>2.13 ± 0.56</td>
<td>2.34 ± 0.62</td>
<td>2.11 ± 0.25</td>
<td>2.39 ± 0.42</td>
<td>2.46 ± 0.26</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD (n = 6). No comparisons between groups are statistically significant.

Table 8 Secretion of neutrophil and monocyte chemoattractants, as well as MMP-8 and MMP-9 from human primary macrophages

<table>
<thead>
<tr>
<th>Cell culture assay</th>
<th>Mediator released</th>
<th>Stimulation</th>
<th>CTL medium</th>
<th>LPS (1 ng/mL)</th>
<th>CTL IgG (40 μg/mL)</th>
<th>Anti-ApoA-1 IgG (40 μg/mL)</th>
<th>anti-ApoA-1 IgG-negative patients</th>
<th>anti-ApoA-1 IgG-positive patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CCL2, pg/mL</td>
<td>7 (5–11)</td>
<td>260a (233–2876)</td>
<td>6.9 (4.6–9.5)</td>
<td>291† (225–1047)</td>
<td>10.8 (8–42.9)</td>
<td>173.7‡ (134.7–264.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CXCL8, pg/mL</td>
<td>131 (62–180)</td>
<td>7158† (6591–16840)</td>
<td>51 (73–228)</td>
<td>4189† (4181–11654)</td>
<td>209 (160–313)</td>
<td>1327‡ (788–2274)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TNF-α, pg/mL</td>
<td>BDL</td>
<td>320a (225–456)</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MMP-9, pg/mL</td>
<td>95 (57–168)</td>
<td>326a (54–3635)</td>
<td>1 (1–77)</td>
<td>14.8† (1–173)</td>
<td>71 (34–3192)</td>
<td>139‡ (43–28359)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pro-MMP-9 activity, ng/mL</td>
<td>4.2 (0.4–62)</td>
<td>26a (14–32)</td>
<td>8.7 (4–13)</td>
<td>59† (45–83)</td>
<td>8.4 (3.7–15.3)</td>
<td>40‡ (16.6–59)</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as median and (IQR) (n = 7). BDL, below the detection limit of the assay. For statistical analysis, the lower detection limit value was used.

intraplaque neutrophil infiltration could be also explained by the increased release of neutrophilic chemoattractants (CXCL8 and TNF-α) from infiltrated macrophages (as suggested by our in vitro experiments with human macrophages incubated in an inflammatory microenvironment mimicking atherosclerotic plaque).

To summarize, our human, murine, and in vitro results suggest that anti-ApoA-1 IgG might be associated with increased atherosclerotic plaque vulnerability through two main mechanisms: (i) the increased intraplaque release of chemoattractants and MMP-9 and consequent collagen degradation; (ii) the induction of neutrophil infiltration from the blood stream within atherosclerotic plaques.

Those results may have two important clinical impacts for the management of cardiovascular disease in humans. First, our data suggest that anti-ApoA-1 IgG assessment could potentially represent an emerging surrogate circulating marker of carotid atherosclerotic plaque vulnerability. Given the actual difficulty to assess carotid atherosclerotic plaque vulnerability by currently unstandardized methods and resource demanding imaging modalities, the possibility of developing a cheap, standardized, reproducible, easily available, risk-stratification tool based upon anti-ApoA-1 IgG assessment constitutes a promising approach to better assess carotid atherosclerosis vulnerability. Second, by demonstrating in humans and in mice a direct negative effect of anti-ApoA-1 IgG on atherosclerotic plaque stabilization, it is likely that anti-ApoA-1 IgG could be considered an active factor increasing cardiovascular vulnerability. Given the recent demonstration of passive immunization approach as an emergent
therapeutic modality to treat some atherosclerotic diseases in humans and mice, we can hypothesize that intravenous administration of immunoglobulin (IVIG) or other more specific-targeted antibodies-based therapies could neutralize the deleterious effect of anti-ApoA-1 IgG on atherosclerotic plaque stability.\textsuperscript{24} This potential approach was recently tested \textit{in vitro} on cardiomyocytes, showing that treatment with IVIG abrogated the chronicotrophic effects of anti-ApoA-1 IgG.\textsuperscript{5}

The limitation of the herein study is the relative limited sample size of carotid human specimens (n = 102) due to the application of very stringent exclusion criteria to avoid any potential confounders, related to potential inflammatory and autoimmune co-morbidities, as well as anti-inflammatory medications. Thus, it may be possible that little changes in intraplaque (inflammatory gene and MMP-8 mRNA expression) and systemic (CCL4, MMP-9) parameters failed to reach statistical significance. Nevertheless, this limitation was the trade-off to obtain a pure model of human atherosclerosis in primary prevention, which also constitutes strength of this cohort. Whether our results apply also to unselected patients with carotid atherosclerosis remain to be demonstrated. Also, anti-ApoA-1 IgG positive differed from negative patients only for total serum cholesterol and triglyceride levels (both increased in anti-ApoA-1 IgG-negative patients). Given the role of circulating lipids as a well-known cardiovascular risk factor, this aspect suggests anti-ApoA-1 IgG-negative patients to potentially be at increased cardiovascular risk when compared with positive patients. However, as levels of total cholesterol and triglycerides were not markedly elevated in both groups, we can assume that these alterations in lipid profile did not reasonably induce any remarkable pro-atherosclerotic effects in the study population.\textsuperscript{25} Finally, it must be reminded that there is currently no strict consensus about the exact definition of vulnerable plaque in humans, and about the model system that is best suited to investigate plaque rupture in mice.\textsuperscript{26} The situation is further complicated by the fact that vulnerability criteria used in humans (mostly histological) might not apply to mice.\textsuperscript{23} In this context, observing such a similarity between human and mice results undoubtedly constitutes another strength of the herein study. On the other hand, being focused on assessing the relevance of anti-ApoA-1 IgG in humans \textit{in vivo}, we performed our \textit{in vitro} experiments only on human primary immunocompetent cells, and not mice-derived neutrophils or macrophages. Therefore, we cannot formally extrapolate our \textit{in vitro} results obtained on human cells to mice-derived inflammatory cells. This approach limited the direct relevance of anti-ApoA-1 IgG pro-inflammatory activities to humans instead of the animal model. Another limitation resides in the fact that we did not explore the potential interference of anti-ApoA-1 IgG with more classical ApoA-1-related properties, such as reverse cholesterol transport, anti-inflammatory, or anti-oxidant activities, which in turn might contribute to cardiovascular vulnerability.\textsuperscript{27}

In conclusion, anti-ApoA-1 IgG increased cardiovascular vulnerability in humans and ApoE\textsuperscript{\textminus} mice. Intraplaque markers of vulnerability (such as MMPs, macrophage, and neutrophil content) were increased in anti-ApoA-1 IgG-positive patients and by anti-ApoA-1 IgG treatment in mice. Accordingly, anti-ApoA-1 IgG was inversely correlated with intraplaque collagen content. Furthermore, \textit{in vitro} anti-ApoA-1 IgG increased neutrophil migration in response to classical neutrophilic chemoattractants, expressed in atherosclerotic plaques. In addition, anti-ApoA-1 IgG stimulation was associated with the promotion of the release of monocyte and neutrophil chemoattractants, as well as MMP-9 by human macrophage. These results suggest that anti-ApoA-1 IgG might be associated with atherosclerotic plaque vulnerability. This could explain the poor cardiovascular prognosis observed in anti-ApoA-1-positive patients with MI or RA,\textsuperscript{5,2} but remains to be demonstrated in larger prospective clinical studies.

**Supplementary material**

Supplementary material is available at \textit{European Heart Journal} online.

**Acknowledgements**

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**Conflict of interest:** none declared.

**References**


Anti-ApoA-1 IgG are active mediators of atherosclerotic plaque vulnerability.


Anti-apolipoprotein A-1 IgG in patients with myocardial infarction promotes inflammation through TLR2/CD14 complex

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From the ¹Division of Laboratory Medicine, Department of Genetics and Laboratory Medicine; and ²Division of Angiology and Haemostasis, Department of Internal Medicine, Geneva University Hospital and Faculty of Medicine, Geneva, Switzerland; ³Molecular Immunology Group, Department of Pathology and Infectious Disease, Royal Veterinary College, Hawkshead Lane, Hertfordshire, UK; ⁴Keenan Research Centre, St. Michael’s Hospital, Toronto, Canada, and ⁵Division of Immunology and Allergy, Department of Internal Medicine, Geneva University Hospitals and Faculty of Medicine, Geneva, Switzerland

Abstract. Pagano S, Satta N, Werling D, Offord V, de Moerloose P, Charbonney E, Hochstrasser D, Roux-Lombard P, Vuilleumier N (Geneva University Hospital and Faculty of Medicine, Geneva, Switzerland; Geneva University Hospital and Faculty of Medicine, Geneva, Switzerland; Royal Veterinary College, Hertfordshire, UK; St. Michael’s Hospital, Toronto, Canada; Geneva University Hospital and Faculty of Medicine, Geneva, Switzerland). Anti-apolipoprotein (Apo)A-1 IgG in patients with myocardial infarction promotes inflammation through TLR2/CD14 complex. J Intern Med 2012; 272: 344–357.

Objectives. Toll-like receptor (TLR)-mediated vascular inflammation, inducible by – amongst other factors – auto-antibodies, is increasingly recognized as a potential mediator of cardiovascular disease. We investigated whether anti-apolipoprotein (Apo)A-1 IgG was associated with a pro-inflammatory cytokine profile in myocardial infarction (MI) patients and whether anti-ApoA-1 IgG elicited a pro-inflammatory response by activating TLRs.

Methods. As surrogate markers of atherosclerotic plaque vulnerability, interleukin (IL)-6, tumor necrosis factor (TNF)-α, matrix metalloproteinase (MMP)-9 and MMP-3 levels were assessed in 221 consecutive MI patients. Using human monocyte-derived macrophages (HMDMs) we investigated (i) the anti-ApoA-1 IgG interaction with TLRs using proximity ligation assay and (ii) anti-ApoA-1 IgG-dependent IL-6/TNF-α production. TLR involvement was further confirmed using HEK293-Blue TLR-2/-4 cells and by computational docking simulations.

Results. In MI patients, anti-ApoA-1 IgG positivity was associated with higher levels of IL-6, TNF-α and MMP-9, but lower MMP-3 levels. In in vitro experiments, anti-ApoA-1 antibodies bound to HMDMs in a TLR2-dependent manner, resulting in nuclear translocation of NFκB and a significant increase in TNF-α and IL-6 production. Subsequent functional studies highlighted the importance of CD14 as co-receptor in the anti-ApoA-1 IgG–TLR2-induced cytokine production. Additional bioinformatic studies identified structural homologies between TLR2 and ApoA-1, which may explain the observed cross-reactivity between antibodies against these two molecules.

Conclusions. Anti-ApoA-1 IgG positivity in MI is associated with a high-risk cytokine profile. These auto-antibodies promote inflammation by stimulating the TLR2/CD14 receptor complex, probably because of molecular mimicry, which may contribute to atherosclerosis-related complications in patients.

Keywords: anti-apolipoprotein A-1 auto-antibodies, atherosclerosis, inflammation, molecular mimicry, myocardial infarction, toll-like receptors.

Introduction

Immune-mediated inflammation plays a major role in atherosclerosis and atherothrombosis, two essential pathophysiological events leading to cardiovascular disease (CVD), which is considered to be the leading cause of death in the Western world [1]. In addition to abnormal lipid metabolism, chronic Th-1 immune-mediated inflammation of the arterial wall is actively
involved in atherogenesis driven by, for example, the activation of innate immune receptors, such as Toll-like receptor (TLR)2 and TLR4 [2–4]. Furthermore, accumulating evidence suggests that auto-antibodies can modulate TLR-mediated inflammation and thus may have a role in the pathogenesis of CVD [5–7].

Amongst auto-antibodies of interest, we and others [8] have focused on antibodies against apolipoprotein (ApoA-1), the major protein fraction of HDL. Having demonstrated a higher prevalence of increased auto-antibody titres in patients with acute coronary syndrome (ACS) [9], we also showed an association with higher levels of oxidized LDL (oxLDL), which plays an important part at all stages of atherogenesis [10, 11]. In addition, we reported that a high level of anti-ApoA-1 IgG was an independent predictor of poor cardiovascular outcome after myocardial infarction (MI) and in rheumatoid arthritis (RA) [12, 13] and was associated with more vulnerable atherosclerotic plaques in severe carotid stenosis patients [14]. In addition, high levels of anti-ApoA-1 IgG were associated with high levels of pro-inflammatory molecules in RA [interleukin (IL)-8 and matrix metalloproteinase (MMP)-9] [13] known to be related to increased risk of CVD [15–17]. Finally, we showed that infusion of anti-ApoA-1 IgG per se increased atherosclerosis formation and atherosclerotic plaque vulnerability in ApoE−/− mice [14], further strengthening the evidence in support of a contributory role of anti-ApoA-1 IgG to CVD.

However, the molecular mechanisms underlying a potential direct pro-inflammatory effect of anti-ApoA-1 IgG have not been investigated. Therefore, in this translational study, we examined whether the presence of anti-ApoA-1 IgG was associated with increased concentrations of pro-inflammatory mediators in MI patients and whether a direct interaction of these auto-antibodies with TLRs expressed on macrophages could initiate such a pro-inflammatory response.

Materials and methods

Patient population and study design

The present patient population was derived from a previously published prospective single-centre study of 221 clinically well-characterized MI patients. The present study was approved by the local ethics committee and performed according to the Declaration of Helsinki [12]. All patient samples were collected after percutaneous coronary intervention within the first 24 h after hospitalization. After collection, serum samples were frozen in aliquots at −80 °C until required for analyses.

IgG purification

As a source of human anti-ApoA-1 IgG, IgG fractions were purified from serum samples from MI patients. Owing to the limited amount of available patient material, serum samples from three MI patients who tested positive and three patients who tested negative for anti-ApoA-1 IgG were pooled prior to IgG purification (pool+ and pool−, respectively) and subsequently isolated on a protein G-sepharose column. After elution, IgGs were dried and resuspended in endotoxin-free water. All IgG fractions were below the endotoxin detection limit (<0.25 EU mL−1) using the limulus amebocyte lysate (LAL) Endochrome assay. Further details are available in the Supporting information.

Biochemical analyses

Determination of human antibodies against ApoA-1 by enzyme-linked immunosorbent assay. Levels of anti-ApoA-1 IgG were measured as described previously [12–14]. The cut-off value for positivity was prospectively defined, set at an OD value of 0.6% and 37% of the positive control value [10, 12–14]. The detailed protocol is included in the Supporting information.

Determination of levels of inflammatory factors in human serum. C-reactive protein (CRP) and serum amyloid A (SAA) were determined using auto-analysers (DxI™ and Image™, respectively, both from Beckman Coulter, Brea, CA, USA). Interleukin (IL)-1RA, IL-6, monocyte chemotactic protein-1 (MCP-1), tumour necrosis factor (TNF)-α, MMP-3 and MMP-9 were measured (in pg mL−1) according to the supplier’s instructions, using a Bioplex 200 array reader (Bio-Rad Laboratories, Hercules, CA, USA) with Luminex MAP™ Technology (Luminex Corporation, Austin, TX, USA).

In vitro studies

Stimulation of human monocyte-derived macrophages (HMDMs) by anti-ApoA-1 antibodies. HMDMs were obtained by treating human monocytes with interferon (IFN)-γ for 24 h as previously described [15, 16]. HMDMs were then stimulated in 96-well trays for 24 h with increasing concentrations (5–40 μg mL−1) of polyclonal anti-human ApoA-1/control antibody, with IgG from pool+ or pool− (500 μg mL−1) and with the respective controls. IL-6 and TNF-α levels were...
then measured in cell supernatants with Luminex MAP™ Technology. Experiments were performed with blood from 12 different donors. When indicated, blocking antibodies against TLR2, TLR4, TLR5 and CD14 (10 μg mL⁻¹) were added 30 min before stimulation of HMDMs. Further details can be found in the Supporting information.

**Indirect immunofluorescence measurement of nuclear factor (NF)-κB staining on HMDMs.** To assess the downstream activation of TLRs by different ligands, HMDMs were seeded onto slide chambers and incubated with anti-human ApoA-1 IgG or the respective IgG control (40 μg mL⁻¹), pool+ or pool− IgG (500 μg mL⁻¹), medium alone or lipopolysaccharide (LPS; 100 ng mL⁻¹) as a positive control for 1 h. After fixation, cells were permeabilized, incubated with anti-NF-κB p65 antibody for 1 h and counterstained with DAPI. Images were acquired using an LSM510 confocal microscope. These experiments were repeated using the above-mentioned blocking antibodies 30 min before stimulation. Further details can be found in the Supporting information.

**Assessment of anti-ApoA-1 antibody binding to HMDMs.** To assess the binding of anti-ApoA-1 antibodies to HMDMs, cells were exposed to polyclonal anti-ApoA-1 antibodies or the respective controls. Furthermore, IgG fractions from patients (anti-ApoA-1 IgG positive or negative fraction, i.e. pool+ and pool−, respectively) were assessed for binding. Binding was revealed by indirect immunofluorescence, as described in the Supporting information.

**Quantification of anti-ApoA-1 antibody binding to innate immune receptors expressed by HMDMs or HEK293 cells.** An in situ proximity ligation assay (PLA) with the DuoLink® kit (Olink Bioscience, Uppsala, Sweden) [18, 19] was used to quantify the interaction between anti-ApoA-1 antibodies or control IgG and surface-expressed TLR2, TLR4 and CD14, as previously described [7]; further details are provided in the Supporting information. Human embryonic kidney 293 (HEK293) cells stably expressing human TLR4, MD2 and CD14 (HEK-Blue-4) or human TLR2 and CD14 (HEK-Blue-2; both from InvivoGen, San Diego, CA, USA) were used to verify the specificity of binding [20]. These cells have been shown to be useful for examining auto-antibody-related engagement and activation of TLRs [7]. The detailed protocol is available in the Supporting information.

**Depletion of IgG from polyclonal anti-ApoA-1 antibodies.** To further demonstrate the IgG dependency of the TLR2/CD14 pro-inflammatory signalling reported above, polyclonal anti-ApoA-1 IgG (50 μg mL⁻¹) or control IgG (50 μg mL⁻¹) was mixed with protein G (Roche Diagnostics Rotkreuz Schweiz AG, Rotkreuz, Switzerland) overnight at 4 °C. The following day, the tubes were centrifuged at 12 000 g for 1 min to remove protein G, and the supernatants were tested using HEK-Blue-2 cells as described above.

**Assessment of conformational homology between ApoA-1 and TLR2.** Experimentally derived structures for ApoA-1, TLR2 and three human IgG fragment antigen-binding (Fab) regions were retrieved from the Protein Data Bank with the identifiers 2A01, 2Z7X, 1UJ3, 1CLY and 2VXV, respectively [21]. Chain A from ApoA-1 and TLR2 structures were isolated and used for the subsequent docking simulations. Ligands were removed from antibody structures, and a single representation of the heavy and light chains was isolated for docking.

The anti-TLR2 antibody sequence (Table S2) was transcribed from patent WO 2005/028509 [22]. Antibody homology models were generated by RosettaAntibody [23], Prediction of Immunoglobulin Structure (PIGS) [24] and Web Antibody Modelling (WAM) [25]. Four different PIGS models were generated using the different available methods: model A, ‘same antibody’; model B, ‘best H and L chains’; model C, ‘same canonical structures’; and model D, ‘same antibody and same canonical structures’.

The six complementarity determining regions (CDRs), that is, variable loops of β-strands that bind the antigen, provided by Rosetta Antibody (Table S3) were used to position the antibody on submission to GRAMM-X docking server [26]. The fifteen anti-TLR2 homology models (WAM, RA models 1–10 and PIGS A–D) and three experimentally resolved antibody structures were submitted to GRAMM-X with ligand-binding regions limited to the CDR regions, and the number of ligand residues required set to 20 (optimum value for returning a sufficient number of models; data not shown). WAM, PIGS models A–D and RA model 1 (top ranked) were also submitted to ClusPro 2.0 [27].

Docking results were trimmed to allow an ‘allvsall’ RMSD calculation implemented by ProFit (Dr Andrew CR Martin; http://www.bioinf.org.uk/software/profit/). The resulting distance matrix was used to cluster the 246 models in R (http://www.r-project.org/). The experimentally derived TLR2 was submit-
Mg<sup>2+</sup>, l-glutamine, penicillin and streptomycin were obtained from Meridian Life Science (Saco, ME, USA). Ultrapure ton, TX, USA), and human control IgG was from Academy Bio-Medical Company (Houstoneville, MD, USA). IFN-γ was from Roussel Uclaf (Paris, France).

Goat polyclonal anti-human ApoA-1 IgG was obtained from Academy Bio-Medical Company (Houston, TX, USA), and human control IgG was from Meridian Life Science (Saco, ME, USA). Ultrapure LPS from Escherichia coli was purchased from Alexis Enzo Life Sciences (Lausen, Switzerland), purified lipoteichoic acid (LTA) from Staphylococcus aureus, and synthetic bacterial lipoprotein (Pam3CSK4) and recombinant flagellin from Salmonella typhimurium were from InvivoGen.

Blocking anti-human TLR2 (clone TL2.5) and isotype-matched control antibodies (anti-c-Myc) were purchased from e-Bioscience (San Diego, CA, USA). Anti-human TLR4 clone HTA 125 antibody was from LifeSpan Biosciences (Seattle, WA, USA). Anti-human TLR2 antibody (clone TL2.5) and Low Endotoxin, Azide-Free LEAF-anti-human CD32 antibody were from e-Biosciences, and anti-human TLR5 and blocking anti-human CD14 antibodies were from InvivoGen. Rabbit polyclonal anti-TLR4 (ab47839) and rabbit polyclonal anti-TLR2 (ab47840) antibodies were from Abcam (Cambridge, UK). Rabbit anti-NF-κB (P65) antibody and rabbit control IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cy3-conjugated affinity-pure F(ab)2 donkey anti-human IgG (H+L) and Dylight™ 549-conjugated affinity-pure F(ab)2 donkey anti-goat IgG (H+L) were from Jackson ImmunoResearch Laboratories (West Groves, PA, USA). Human purified ApoA-1 was kindly provided by R. James (Department of Internal Medicine, Clinical Diabetes Unit, Medical Faculty, University of Geneva) and was isolated by ultracentrifugation followed by HDL delipidation as described elsewhere [9, 10].

Reagents. RPMI-1640 medium, foetal calf serum (FCS), phosphate-buffered saline free of Ca<sup>2+</sup> and Mg<sup>2+</sup>, l-glutamine, penicillin and streptomycin were obtained from (Gibco BRL-Life Technologies, Rockville, MD, USA). IFN-γ was from Roussel Uclaf (Paris, France).

Statistical analysis. Analyses were performed using Statistica software (StatSoft, Tulsa, OK, USA). Fisher’s exact test and Mann–Whitney U-test were used when appropriate to compare groups of patients. For in vitro experiments, Kruskal–Wallis test was used to analyse the dose–response relationship and Mann–Whitney U-test was used to determine P values. Results were expressed as median, interquartile range (IQR) and range unless stated otherwise. Two-sided P values <0.05 were considered significant.

Results

The presence of anti-ApoA-1 IgG is associated with an increased systemic inflammatory status in MI patients

Anti-ApoA-1 auto-antibody positivity was determined as previously described [12–14]. The association between anti-ApoA-1 IgG positivity and circulating cytokine/MMP levels is shown in Table 1. Patients who were positive for anti-ApoA-1 auto-antibodies had higher levels of circulating TNF-α, IL-6 and MMP-9 but lower MMP-3 levels, compared to patients who tested negative for these auto-antibodies. No differences were observed for other pro-inflammatory markers (Table 1). The results indicate that the presence of anti-ApoA-1 auto-antibodies in MI was associated with a pro-inflammatory cytokine/MMP profile that is increasingly being recognized as associated with atherosclerotic plaque vulnerability in humans [15, 28–31].

Treatment of HMDMs with anti-ApoA-1 IgG induces production of IL-6 and TNF-α

To further explore a possible causative link between the presence of anti-ApoA-1 antibodies and elevated IL-6 and TNF-α levels in MI patients, we stimulated human macrophages with polyclonal anti-human ApoA-1 IgG and isotype control IgG, as well as IgG fractions purified from pooled serum samples of MI patients who tested either positive (pool+) or negative (pool−) for anti-ApoA-1 IgG. Experiments were performed on four different macrophage preparations for anti-ApoA-1 IgG-controls and on 12 macrophage preparations for IgG fractions prepared from MI patients. To account for the well-known interindividual variation in macrophage reactivity, results are expressed as fold induction compared to baseline. As shown in Fig. 1a,b, exposure of macrophages to anti-ApoA-1 IgG induced a dose-dependent increase in IL-6 and TNF-α, with an eightfold increase in IL-6 and a 15-fold increase in TNF-α production at a concentra-
Table 1  Patient demographic characteristics

<table>
<thead>
<tr>
<th></th>
<th>MI patients (n = 221)</th>
<th>MI patients negative for anti-ApoA–1 IgG (n = 202)</th>
<th>MI patients positive for anti-ApoA–1 IgG (n = 19)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>64 (26–86; 55–74)</td>
<td>64 (28–86; 55–74)</td>
<td>62 (26–86; 54–72)</td>
<td>0.65</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Male, % (n)</td>
<td>78 (173)</td>
<td>77 (156)</td>
<td>90 (17)</td>
<td>0.38</td>
</tr>
<tr>
<td>Female, % (n)</td>
<td>22 (48)</td>
<td>23 (46)</td>
<td>11 (2)</td>
<td></td>
</tr>
<tr>
<td>BMI, kg m⁻²</td>
<td>25.8 (18.0–39.5; 24–29)</td>
<td>26 (17–29; 24–28)</td>
<td>25 (18.0–39.5; 21–32)</td>
<td>0.63</td>
</tr>
<tr>
<td>Creatinine, µmol L⁻¹</td>
<td>84 (47–275; 73–98)</td>
<td>84 (47–275; 73–97)</td>
<td>93 (62–211; 73–106)</td>
<td>0.20</td>
</tr>
<tr>
<td>cTnI, ng mL⁻¹</td>
<td>0.5 (0–153; 5–7)</td>
<td>0.4 (0.15–3; 0.1–3.0)</td>
<td>1.0 (0–80; 0.1–10.0)</td>
<td>0.29</td>
</tr>
<tr>
<td>CKpeak, IU L⁻¹</td>
<td>687 (273–751; 1410–1783)</td>
<td>687 (41–7511; 264–1785)</td>
<td>573 (77–3252; 281–1308)</td>
<td>0.79</td>
</tr>
<tr>
<td>NT–proBNP, pg mL⁻¹</td>
<td>785 (20–28285; 283–2057)</td>
<td>778 (36–28285; 289–2157)</td>
<td>885 (20–14205; 125–1842)</td>
<td>0.66</td>
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<tr>
<td>Lipid profile, mmol L⁻¹</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Total cholesterol</td>
<td>4.8 (1.9–11.8; 4.0–5.8)</td>
<td>4.9 (1.9–8.8; 4.1–5.8)</td>
<td>4.8 (3.2–11.8; 3.9–6.0)</td>
<td>0.94</td>
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<tr>
<td>HDL</td>
<td>1.06 (0.5–2.7; 0.9–1.3)</td>
<td>1.05 (0.5–2.7; 0.9–1.3)</td>
<td>1.1 (0.7–1.7; 0.9–1.4)</td>
<td>0.35</td>
</tr>
<tr>
<td>LDL</td>
<td>3.1 (0.5–4.0; 2.4–3.9)</td>
<td>3.2 (0.5–7.0; 2.4–3.9)</td>
<td>2.9 (1.8–9.5; 2.4–4.3)</td>
<td>0.86</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.1 (0.5–7.3; 0.8–1.7)</td>
<td>1.1 (0.1–7.3; 0.8–1.7)</td>
<td>0.9 (0.4–2.3; 0.6–1.5)</td>
<td>0.22</td>
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<tr>
<td>Comorbidities, % (n)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Hypertension</td>
<td>53 (117)</td>
<td>53 (108)</td>
<td>47 (9)</td>
<td>0.63</td>
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<tr>
<td>Diabetes</td>
<td>20 (43)</td>
<td>20 (40)</td>
<td>16 (3)</td>
<td>1</td>
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<tr>
<td>Dyslipidaemia</td>
<td>46 (102)</td>
<td>47 (94)</td>
<td>42 (8)</td>
<td>0.81</td>
</tr>
<tr>
<td>Smoker</td>
<td>45 (99)</td>
<td>45 (91)</td>
<td>42 (8)</td>
<td>1</td>
</tr>
<tr>
<td>Known CAD</td>
<td>29 (65)</td>
<td>29 (58)</td>
<td>32 (6)</td>
<td>0.79</td>
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<tr>
<td>Stroke</td>
<td>5 (12)</td>
<td>4 (8)</td>
<td>21 (4)</td>
<td>0.01</td>
</tr>
<tr>
<td>Positive familial history</td>
<td>31 (69)</td>
<td>31 (62)</td>
<td>37 (7)</td>
<td>0.60</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>5 (11)</td>
<td>4 (9)</td>
<td>11 (2)</td>
<td>0.24</td>
</tr>
<tr>
<td>STEMI, % (n)</td>
<td>54 (120)</td>
<td>54 (108)</td>
<td>63 (12)</td>
<td>0.48</td>
</tr>
<tr>
<td>NSTEMI, % (n)</td>
<td>46 (101)</td>
<td>47 (94)</td>
<td>37 (7)</td>
<td></td>
</tr>
<tr>
<td>Medication at inclusion, % (n)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Aspirin</td>
<td>32 (71)</td>
<td>32 (65)</td>
<td>32 (6)</td>
<td>1</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>11 (25)</td>
<td>11 (23)</td>
<td>11 (2)</td>
<td>1</td>
</tr>
<tr>
<td>β–blocker</td>
<td>28 (61)</td>
<td>27 (55)</td>
<td>32 (6)</td>
<td>0.78</td>
</tr>
<tr>
<td>ACEor AT1 inhibitors</td>
<td>39 (86)</td>
<td>38 (77)</td>
<td>47 (9)</td>
<td>0.46</td>
</tr>
<tr>
<td>Statin</td>
<td>35 (77)</td>
<td>35 (71)</td>
<td>32 (6)</td>
<td>1</td>
</tr>
<tr>
<td>Diuretics (excluding amiloride and MR antagonists)</td>
<td>20 (43)</td>
<td>19 (39)</td>
<td>21 (4)</td>
<td>0.76</td>
</tr>
<tr>
<td>Anti-diabetic agents</td>
<td>12 (27)</td>
<td>12 (25)</td>
<td>11 (2)</td>
<td>0.63</td>
</tr>
<tr>
<td>Insulin</td>
<td>7 (16)</td>
<td>7 (14)</td>
<td>11 (2)</td>
<td>1</td>
</tr>
<tr>
<td>Inflammatory markers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP, mg L⁻¹</td>
<td>6 (1–230; 14–39)</td>
<td>6 (1–230; 3.0–14.2)</td>
<td>7.2 (1–96; 2.8–13)</td>
<td>0.76</td>
</tr>
<tr>
<td>TNF-α, pg mL⁻¹</td>
<td>0.9 (0–13.2; 0.6–1.3)</td>
<td>0.8 (0–8.4; 0.6–1.2)</td>
<td>1.2 (0.1–13.2; 0.8–2.7)</td>
<td>0.01</td>
</tr>
<tr>
<td>IL-6, pg mL⁻¹</td>
<td>7 (0.2–216; 3–12)</td>
<td>6 (0.2–84; 3–11)</td>
<td>10 (2.3–216.0; 6–18)</td>
<td>0.02</td>
</tr>
</tbody>
</table>
tion of 40 µg mL\(^{-1}\) anti-ApoA-1 IgG. Similarly, pool+ IgG (500 µg mL\(^{-1}\)) induced a significantly stronger IL-6 and TNF-\(\alpha\) production by 4- and 21-folds, respectively, compared to pool\(\n\) IgG (Fig. 1c,d).

Anti-ApoA-1 IgG bind to HMDMs via TLR2

Given the ability of anti-ApoA-1 antibodies to induce sterile inflammation in macrophages, we next used immunofluorescence to evaluate whether anti-ApoA-1 antibodies and patients’ IgG fractions (pool+ and pool\(\n\)) indeed bind to HMDMs (Figs S1 and S2). Binding was quantified by conventional methods of reporting the fluorescent surface area per number of cells in the examined field [7]. To prevent nonspecific binding, Fc\(\n\) receptors II were systematically blocked prior to antibody exposure.

Fig 2 shows that both anti-ApoA-1 IgG and IgG from pool+ bound to a similar extent to the surface of nonpermeabilized HMDMs, but no binding was observed using either control IgG or IgG from pool\(\n\). Taking the initial pro-inflammatory response into account, we next assessed binding specificity by performing competition experiments. TLR2, TLR4 and TLR5 were blocked prior to the addition of anti-ApoA-1 IgG and patient pool+ IgG fraction, and TLR5 served as control for surface-expressed TLRs. As shown in Fig. 2, antibodies to TLR2 and, to a lesser extent, to TLR4 blocked the binding of anti-ApoA-1 and pool+ IgG to HMDMs, whereas no significant inhibition was observed by blocking TLR5. To verify the specificity of blocking, the inhibitory activity of the TLR2, TLR4 and TLR5 antibodies was assessed on HMDMs stimulated with specific TLR2, TLR4 and TLR5 ligands (Table S1). To further confirm that anti-ApoA-1 antibodies bind mainly to TLR2, a PLA was performed, allowing the interaction between the two proteins to be quantified. As shown in Fig. 3, the IgG fraction from pool+ interacted strongly with TLR2 and to a lesser extent with the TLR2 co-receptor CD14. Furthermore, a slight but significant interaction was observed with TLR4, whereas no interaction was seen for either the IgG fraction of pool\(\n\) or TLR5.

Anti-ApoA-1 IgG induces nuclear translocation of NF-\(\kappa\)B in HMDMs

Having established binding of anti-ApoA-1 IgG to TLR2 expressed by HMDMs, we next assessed whether this binding induced subsequent downstream signalling by analysing nuclear translocation of the NF-\(\kappa\)B transcription factor. As shown in Fig. 4, addition of anti-ApoA-1 IgG (40 µg mL\(^{-1}\)), pool+ IgG (500 µg mL\(^{-1}\)) or LPS (100 ng mL\(^{-1}\); positive control) induced strong NF-\(\kappa\)B translocation in HMDMs, whereas no translocation was seen in unstimulated HMDMs or cells treated with control IgG or pool\(\n\) IgG. Furthermore, anti-ApoA-1 IgG-induced NF-\(\kappa\)B translocation was inhibited by anti-TLR2 and anti-CD14 antibodies, but not by anti-TLR5 or anti-TLR4 antibodies (data not shown).

Anti-ApoA-1 IgG-induced IL-6 and TNF-\(\alpha\) production is mediated by the TLR2/CD14 complex

To confirm that anti-ApoA-1 antibodies not only induce NF-\(\kappa\)B translocation, but also lead to enhanced production of pro-inflammatory mediators in a TLR2-dependent fashion, which may explain the elevated levels of cytokines in patients’ serum samples, we
examined whether anti-ApoA-1 IgG-dependent production of IL-6 and tumour necrosis factor (TNF-α) was inhibited by blocking TLR2, TLR4 and TLR5 as well as the cofactor CD14 [32, 33]. Indeed, blocking TLR2 and TLR4 reduced production of both cytokines (although not statistically significantly), whereas blocking CD14 efficiently and significantly abrogated the pro-inflammatory response for both cytokines (Fig. 5); similar results were obtained with pool+ IgG (Fig. 5). By contrast, no effect was seen when blocking TLR5. As the use of HMDMs does not allow for analysis at the specific receptor level and to further demonstrate the need for a functional TLR2/CD14 receptor in the anti-ApoA-1 IgG pro-inflammatory response, HEK293 cells expressing either TLR2/CD14 (HEK-Blue-2 cells) or TLR4/MD2/CD14 (HEK-Blue-4 cells) were used. As with HMDMs, anti-ApoA-1 IgG and the pool+ IgG fraction, but not their respective controls, induced significant and dose-dependent activation of HEK-Blue-2, but not of HEK-Blue-4 cells (Fig. 6). No stimulation was observed with HEK-Blue-4 cells using patients’ IgG (pool+ and pool--; data not shown). Assay
functionality was assessed by stimulating cells with the respective positive controls: LTA for HEK-Blue-2 and LPS for HEK-Blue-4 (Fig. 6). To exclude the presence of LTA contamination in the anti-ApoA-1 IgG, which could account for a positive response, the experiments using HEK-Blue-2 cells were repeated after anti-ApoA-1 IgG depletion. This completely abrogated cell activation \( (P = 0.02) \) (Fig. S2), confirming an IgG-dependent effect. Having confirmed the importance of TLR2 in anti-ApoA-1 IgG-induced stimulation, we next evaluated the importance of CD14 by incubating HEK-Blue-2 cells with 10 \( \mu \)g mL\(^{-1} \) of an anti-CD14 antibody prior to the addition of 100 \( \mu \)g mL\(^{-1} \) pool+ IgG or 1 \( \mu \)g mL\(^{-1} \) LTA. As expected, anti-CD14 antibody completely inhibited the response of HEK-Blue-2 to pool+ IgG and to LTA (Fig. 6c).

These results not only confirm the necessity of a functional TLR2/CD14 complex for an anti-ApoA-1 IgG-induced pro-inflammatory response but also show that the presence of CD14 is required for subsequent TLR2 functionality.

Assessment of conformational homology between ApoA-1 and TLR2

Computational docking simulations were performed to support our hypothesis of a potential interaction between anti-ApoA-1 IgG and TLR2. The potential anti-ApoA-1 IgG-binding interface was resolved for both ApoA-1 and TLR2 to further support the hypothesis of molecular mimicry [34] to account for the cross-reactivity observed between anti-ApoA-1 IgG and TLR2 in vitro (Fig. 7). The docking simulations provided potentially similar binding interfaces for both ApoA-1 and TLR2. Further analysis of the electrostatic surface potential provided concordant re-
results, indicating that the inferred binding sites on the surface of ApoA-1 and TLR2 share similar negatively charged interfaces (Fig. 7a,b). Homology modelling of an anti-TLR2 antibody, using publicly available antibody structure prediction servers, revealed a corresponding complementary positively charged pocket, incorporating the CDRs known to be involved in antibody binding (Fig. 7c,d). The flexible long-looped ApoA-1-binding region is shown to include several amino acids between 82 and 198 and bears similarity to the looped regions displayed on the exterior of the TLR2 solenoid structure. A model of the interaction between ApoA-1 and anti-TLR2/anti-ApoA-1 IgG antibodies is shown in Fig. S3.

Discussion

The major finding of this translational study is that the direct pro-inflammatory effects of anti-ApoA-1 IgG are mediated by binding of the antibody to the TLR2/CD14 complex, resulting in downstream signalling and NF-kB-dependent pro-inflammatory cytokine production. This novel finding is in accordance with previously reported results demonstrating that several auto-antibodies [antiphospholipid (aPL) and anti-heat shock protein (HSP) antibodies] can promote inflammation through engagement of TLR2 and TLR4 [6, 7, 35, 36], as well as with data emphasizing the functional importance of CD14 in aPL antibody signalling [6, 7]. More generally, the importance of CD14 for TLR2 functionality in the present study is consistent with data, indicating that ligand–CD14 complex formation is a prerequisite for binding to and activation of TLR2 and TLR4 [32, 37]. Given the systematic efforts undertaken to rule out contamination in our experimental procedures (negative for LPS in the LAL assay) and because IgG depletion completely abrogated the TLR2-dependent response in HEK-Blue-2 cells, we believe that contamination is very unlikely to have affected the present results. Because of the endogenous nature of anti-ApoA-1 IgG and the ability to interact with innate immune receptors (i.e. key features of damage-associated molecular patterns (DAMPs) [38]), we
hypothesize that such auto-antibodies could represent a new class of DAMPs, thus enhancing atherogenesis.

Furthermore, having previously shown that high levels of anti-ApoA-1 auto-antibodies are an independent predictor of poor cardiovascular outcome in MI and RA patients [12, 13], we demonstrate in the present study that high levels of anti-ApoA-1 antibodies are associated with increased circulating levels of MMP-9, TNF-α and IL-6 and low levels of MMP-3 in MI patients; this cytokine profile is generally associated with an increased risk of CVD. Indeed, high levels of TNF-α and IL-6 have been associated with increased risk of MI according to several prospective epidemiological studies [34, 39], and increased MMP-9 levels combined with low MMP-3 levels have been consistently associated with vulnerable atherosclerotic plaques and increased CVD risk in humans [15, 27, 30].

Our results raise further important questions. Given the well-accepted antibody specificity, the identification of anti-ApoA-1 IgG binding on HMDMs involving TLR2 was somewhat surprising. However, our protein modelling results support the possibility of cross-reactivity of anti-ApoA-1 antibodies with TLR2, owing to the conformational epitope homologies retrieved between looped ApoA-1 sites and the TLR2 exterior solenoid. This is similar to the molecular...
Fig. 6 Implication of TLR2/CD14 complex in the anti-ApoA-1 IgG pro-inflammatory response. HEK-Blue-2 and HEK-Blue-4 cells were exposed for 24 h to either polyclonal anti-ApoA-1/ control IgG or purified IgG fractions derived from pool+ or pool− IgG. (a) HEK-Blue-2 cells exposed to polyclonal anti-ApoA-1 IgG (squares), control IgG (circles) and lipoteichoic acid (LTA) as control (triangles). Anti-ApoA-1 IgG induced a dose-dependent increase in TLR2-dependent reporter gene production (**P = 0.001, Kruskal–Wallis test), in contrast to control IgG (*P = 0.55, Kruskal–Wallis test). Compared to control IgG, 10 µg mL⁻¹ anti-ApoA-1 IgG elicited a significant increase in TLR2-dependent reporter gene production (**P = 0.01; Mann–Whitney U-test). Results are presented as median and range (bars) and represent five independent experiments. (b) HEK-Blue-4 cells exposed to polyclonal anti-ApoA-1 IgG (squares), control IgG (circles) and lipopolysaccharide as control (triangles). In the presence of TLR4 alone, no dose-dependent response of the reporter gene production was achieved upon exposure to anti-ApoA-1 IgG/control IgG (P = 0.96 and P = 1.0, respectively, Kruskal–Wallis test). Results are presented as median and range (bars) and represent five independent experiments. (c) HEK-Blue-2 cells exposed to pool+ IgG (squares), pool− IgG (circles) and LTA as control (triangles). Dose–response experiments were performed using two different donors, whereas pool IgG comparisons at 100 µg mL⁻¹ were made using five different donors. Results are presented as median and range (bars). Pool+ IgG induced a dose-dependent increase in TLR2-dependent reporter gene production (**P = 0.02, Kruskal–Wallis test), in contrast to control IgG (*P = 0.55, Kruskal–Wallis test). Compared to pool− IgG, 100 µg mL⁻¹ pool+ IgG elicited a significant increase in TLR2-dependent reporter gene production (**P = 0.01, Mann–Whitney U-test). Furthermore, when CD14 was blocked 30 min before adding pool+ IgG (100 µg mL⁻¹) and LTA (0.1 µg mL⁻¹), a complete inhibition of reporter gene production was observed (**P = 0.01, Mann–Whitney U-test).
mimicry hypothesis proposed for HSP antibodies in atherosclerosis [40]. Thus, the conformational mimicry hypothesis may explain the observed cross-reactivity between anti-ApoA-1 antibodies and an apparently unrelated protein. As Fcγ receptors were systematically blocked and control IgG did not elicit a response, it is very unlikely that our results are attributable to nonspecific binding. However, we cannot exclude the possibility that TLR2 is stimulated by an immune complex formed by the lipopeptide ApoA-1 and its related antibody, instead of the anti-ApoA-1 IgG alone, as lipopeptides are endogenous agonists of TLR2. This possibility may apply particularly to the IgG fractions from patients, as similar mechanisms were proposed for the β2GPI-dependent activation of TLR by aPL antibodies [41]. However, when western blotting of our pool+ IgG fractions was performed under denaturing conditions, we were not able to retrieve a band of the expected molecular weight for ApoA-1 (data not shown), indicating that our IgG fractions were most probably devoid of ApoA-1. Furthermore, despite the fact that our PLA experiments showed that the strongest interaction of anti-ApoA-1 IgG was with TLR2 and CD14, there was still a significant interaction with TLR4. The reason for this observation could be either the existence of a previously unreported TLR2/TLR4 heterodimer or nonspecific antibody binding generating a signal close to the background value; however, this warrants further investigation. Finally, whether this TLR2/CD14 complex could also be involved in the aldosterone-dependent positive chronotropic action of anti-ApoA-1 IgG reported on ventricular cardiomyocytes in vitro [12] remains an intriguing prospect that is currently under investigation.

We are fully aware of the limitations of this investigation. First, the differences in TNF-α and IL-6 concentrations amongst MI patients who tested positive and those who were negative for anti-ApoA-1 IgG although significant were small, and it could be argued that these differences are not clinically meaningful. However, given the importance of TNF-α and IL-6 with regard to the occurrence of MI [34, 39] and the fact that the presence of anti-ApoA-1 IgG predicts adverse cardiovascular outcome after MI and induces a more vulnerable atherosclerotic plaque phenotype in ApoE−/− mice, these differences are in accordance with published values [34, 39] and further suggest an active role of anti-ApoA-1 IgG in atherogenesis/atherothrombosis in humans [12–14]. Secondly, working with a pool of IgG derived from serum from MI patients and not with anti-ApoA-1 IgG purified from patient serum on an ApoA-1 affinity column represents another limitation of this study and relates to the restricted amount of available patient serum
samples. Nevertheless, because the serum samples from which the pool+ IgG was derived were devoid of any reactivity against nuclear antigens, phospholipids (β2GPI and cardiolipin), phosphorylcholine (the immunodominant epitope of oxLDL) and HSP60 [12] and because the pool+ IgG displayed the same in vitro pro-inflammatory effects as the commercial source of polyclonal anti-human ApoA-1 IgG, we believe that the aforementioned effects of pool+ IgG are attributable to the presence of anti-ApoA-1 IgG in our preparations. The presence of another unrelated and unmeasured auto-antibody in our pool+ IgG that could have confounded our results is unlikely, even if not formally excluded. Thirdly, if our indicated hypothesis of molecular mimicry was supported by our protein modelling approach, our homology modelling was derived from the anti-TLR2 IgG antibody sequence as an anti-ApoA-1 IgG substitute. The rationale of using such a substitute was guided by our in vitro experiments and by the fact that the sequences of our commercial and human anti-ApoA-1 antibodies are currently unavailable. Despite the fact that computational modelling requires ongoing experimental validation, the present homology modelling results were reinforced by the additional use of several experimentally derived IgG Fab structures in subsequent docking simulations and were in complete agreement with our present in vitro data, allowing us to describe an unexpected conformational homology between TLR2 and ApoA-1. Finally, whether such auto-antibodies interfere with HDL-related atheroprotective properties could not be explored in the present study.

In conclusion, the present data clearly show that high levels of anti-ApoA-1 auto-antibodies in MI patients are associated with a pro-inflammatory constellation of cytokines – high circulating levels of TNF-α, IL-6 and MMP-9 and low MMP-3 levels – that has been shown to be related to atherosclerotic plaque vulnerability. Furthermore, our in vitro results demonstrate that anti-ApoA-1 antibodies can directly stimulate the macrophage production of TNF-α and IL-6, through the engagement of TLR2/CD14 complex, possibly 0 as a result of molecular mimicry. Taken together, these results further emphasize the role of humoral autoimmunity as an emergent modulator of atherogenesis through the engagement and subsequent activation of innate immune receptors.

Conflict of interest statement

No conflict of interest was declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Anti-apoA1-TLR2/CD14 in MI-R1

Figure S2. Anti-apoA1 IgG binding to human macrophages.

Figure S3. Anti-apoA1 IgG depletion impact on the reporter gene production in HEKblue-2 cells.

Table S1. Control experiments on human macrophages for TLR2, 4 and 5 pathways.

Table S2. Anti-TLR2 antibody light and heavy chain sequences according to the patent WO 2005/28509.

Table S3. Complementarity determining regions (CDRs) of anti-TLR2 heavy and light chains.

Data S1. Methods.

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4. General discussion

4.1. Summary of the findings

To summarize, the studies presented in this work demonstrate that anti-apoA-1 IgG:

1) are raised in high CV risk populations without autoimmune diseases.
2) are associated with a pro-inflammatory and pro-atherogenic cytokine profile
3) are independent predictors of CV risk
4) are active mediators of atherosclerosis and CVD in vitro and in vivo.

Those four different points will be further developed in the dedicated sections below.

4.1.1. Anti-apoA-1 IgG in other non autoimmune populations

If high levels of anti-apoA-1 IgG were initially described as raised in patients with autoimmune diseases associated with an increased risk of CVD, such as SLE, APS [48-53], and RA [55-57], we reported that anti-apoA-1 IgG could also be detected at high levels in patients without autoimmune disease but with acute clinical manifestations of CVD, such as acute coronary syndrome [58-62], and severe carotid stenosis requiring surgical treatment [63-64].

In addition, we also demonstrated that high levels of anti-apoA-1 IgG were detectable in patients with periodontitis [65] and patients undergoing dialysis [66], two clinical conditions known to be associated with increased CVD risk [67, 68]. Finally, we lately reported the existence of high levels of anti-apoA-1 IgG in obese but otherwise healthy subjects [69].

In those different settings the prevalence of high titre of anti-apoA-1 IgG varies between 10 and 20%, against 0 to 6.5% in healthy blood donors or controls [56, 59, 65]. The clinical relevance of such findings will be presented in the chapter 4.6.
4.1.2. Anti-apoA-1 IgG are associated with a pro-inflammatory and pro-atherogenic cytokine profile

Most studies published so far reported significant associations between high levels of anti-apoA-1 IgG levels and markers of oxidative stress, inflammation and endothelial dysfunction related to atherogenesis and atherosclerotic plaque rupture.

In SLE patients, anti-apoA-1 IgG levels were found to be positively correlated with nitric oxide (r = 0.37, p= 0.007), inversely related to paraoxonase-1 (PON-1) activity (r = -0.31, p = 0.006), and the total anti-oxidant capacity of the sera (r = -0.47, p < 0.0001) suggesting that those autoantibodies could interfere with the anti-oxidant properties of HDL, giving rise to a pro-oxidative micro-environment facilitating atherogenesis [70]. In the same line of thought, RA patients tested positive for those autoantibodies were shown in two different studies to have higher plasma levels of oxLDL levels [55, 56], considered as a major player of all stages of atherogenesis [2,10,11]. Furthermore, RA patients tested positive for anti-apoA-1 antibodies were found to have higher levels of IL-8 and MMP-9 [55], two inflammatory mediators known to be associated with atherogenesis, and atherosclerotic plaque vulnerability in humans [14, 71].

In a retrospective study involving MI patients, we reported a positive association between anti-apoA-1 IgG and serum amyloid A (SAA) protein levels (r:0.76; p=0.006), a multifunctional protein located at the crossroad of inflammation and cholesterol homeostasis [58].

Later on, in a prospective cohort involving 127 MI patients, we retrieved the same relationship between anti-apoA-1 IgG and oxLDL levels than what has been retrieved in RA patients [59]. MI patients considered as positive for anti-apoA-1 IgG had significantly higher median levels of oxLDL when compared to patients tested negative for those autoantibodies (226.5 vs 47.7 U/l; p<0.0001), and a positive correlation between oxLDL and anti-apoA-1
IgG were observed (Spearmann r=0.28, p<0.05) [59]. On the other hand, no association was retrieved with PON-1 activity in this study.

In a prospective study enrolling 221 MI patients, we demonstrated that patients tested positive for anti-apoA-1 antibodies had higher circulating levels of IL-6, TNF-α, and MMP-9, and lower MMP-3 levels [72], a cytokine constellation known to be associated with increased atherosclerotic plaque vulnerability, and worse CV prognosis [73-74]. This increase in MMP-9 levels retrieved in anti-apoA-1 IgG positive patients was associated with an increase in MMP-9 activity [63].

Furthermore, in our periodontitis study, we retrieved a positive correlation between anti-apoA-1 IgG and ADMA levels (Spearmann; r: 0.20, p=0.02) this subgroup of patients, a significant association was retrieved between anti-apoA-1 IgG and ADMA levels [65], a marker of endothelial-dependent dysfunction with strong CV prognostic value [75, 76].

Among other associations retrieved between anti-apoA-1 IgG CV relevant prognostic features is the association with basal heart rate. In one of our prospective MI cohort [60], we demonstrated that when compared to those tested negative for anti-apoA-1 IgG patients tested positive for those antibodies had a higher basal heart rate upon discharge, a well-established CV prognostic feature after MI [77-79].

In conclusion, the most consistent associations retrieved so far between anti-apoA-1 IgG and CV-relevant markers of inflammation concern mostly oxLDLs and MMP-9. Although no causality link can be inferred upon such statistical associations, they were nevertheless instrumental to orient our subsequent in vitro and animal studies as explained in the next paragraph.
4.1.3. Anti-apoA-1 IgG as independent predictors of CV risk

In 2010, we demonstrated that anti-apoA-1 IgG positivity assessed on samples taken within the first 24 hours of patient admission at the hospital for MI was a significant and independent predictor of MACE during 1 year follow-up [60]. The presence of high anti-apoA-1 IgG levels upon admission increased the subsequent risk of MACE by 4-fold, independently of Framingham risk factors (adjusted Odds Ratio (OR): 4.3; 95% CI, 1.46–12.6; p=0.007) [60]. Cox regression analysis demonstrated that for each arbitrary unit increase of anti-apoA-1 IgG, there was a concomitant 3% increase of MACE risk (p=0.0003). All those 221 patients were tested negative for anti-nuclear antibodies and no association with other autoantibodies (rheumatoid factor, anti-β2GPI and anti-cardiolipin antibodies) were retrieved [60].

Those findings were extended in an ancillary study derived from the same cohort of patients aiming at comparing in a “head to head” fashion the prognostic accuracies of other autoantibodies described as potentially relevant for CV event prediction. Among those, we measured antibodies to β2GPI domain I and IV, cardiolipin, heat-shock protein 60 (anti-HSP-60), and phosphorylcholine (anti-PC IgM) [61]. In this study, autoantibodies to apoA-1 were found to be the only autoantibodies to significantly predict subsequent MACE occurrence, although a non significant trend was retrieved for anti-cardiolipin (p=0.05), and anti-HSP60 antibodies p=0.07). In this study the prognostic accuracy as measured by the the area under the curve (AUC) was rather modest (AUC: 0.65, p=0.007) [61], and of the same order of magnitude than the 10-year global Framingham risk score. Risk analyses demonstrated that anti-apoA-1 IgG positivity increased the risk of MACE by 4-fold, independently of the 10-year global Framingham risk score (adjusted hazard ratio: 3.8, p=0.002) [61]. Those preliminary results pointed to anti-apoA-1 IgG as a promising humoral autoimmune candidate for MACE prediction in secondary prevention settings.

Furthermore, in a single centre prospective study involving 138 patients presenting to the emergency room for acute chest pain, we demonstrated that anti-apoA-1 IgG values assessed
on the first sample available had a relatively good diagnostic accuracy for non-ST elevation myocardial infarction (NSTEMI) with an AUC of 0.75 (p<0.0001) that could be increased up to 0.88 when combined with anti-PC IgM and the NSTEMI-TIMI score to generate a clinical antibody ratio (CABR) score [62]. Also, anti-apoA-1 IgG was found to be a good predictor (AUC 0.80, p<0.0001) of subsequent troponin I elevation when the first sample was tested negative, which was the secondary endpoint of this study. Risk analyses indicated that in the presence of high anti-apoA-1 IgG levels the risk of subsequent NSTEMI diagnosis was increased by 6-fold after the adjustment for NSTEMI-TIMI score (OR: 6.4, 95%CI: 1.72-24.2). At the pre-specified cut-off, this test displayed interesting negative predictive value of 88% and 95% for the primary and secondary study endpoints, respectively.

Finally, we demonstrated that anti-apoA-1 IgG were also predictors of MACE at one-year after elective surgery for severe carotid stenosis with an AUC of 0.74 (95%CI:0.59-0.90; p=0.01) [64], and that its combined used with myeloperoxidase could improve the predictive accuracy of the model [64]. In this study, high levels of anti-apoA-1 IgG were associated with a 5-fold increase of MACE during follow-up (exact OR: 5.29; 1.08-34.02; p=0.04), which remained significant after the adjustment for the 10-year FRS according to conventional logistic regression, but not when the exact logistic regression model was applied [64].

Those results will be further developed in the chapter 4.6 dedicated to the clinical relevance.

4.1.4 Anti-apoA-1 IgG as active mediators of atherosclerosis and CVD

Experiments carried out in cellular and animal models indicate that certain autoantibodies contribute directly to the induction of atherogenesis and atherosclerotic plaque vulnerability through their capacity to signal through innate immune receptors, notably Toll-like receptor (TLR) 2 ([29-31]; reviewed in [27]). By analogy, we investigated whether anti-apoA-1 autoantibodies could act through innate immune receptors signaling.
4.1.4a. Anti-apoA-1 IgG elicits sterile inflammation via Toll-like receptor 2/CD14 complex

We recently showed that lipopolysaccharide-free anti-apoA-1 IgG dose-dependently induced the production of a range pro-inflammatory cytokines, such as IL-8, MMP-9, IL-6, TNF-α, and MCP-1 in human monocyte-derived macrophages [55, 63, 72], and that this process was mediated by the TLR2/CD14 complex [72]. In addition, our in silico modeling studies revealed evidence for structural homology between apoA-1 and part of the extracellular domain of TLR2, suggesting a molecular mechanism for this cross-reactivity [72]. Our current understanding on how anti-apoA-1 IgG promotes sterile inflammation through the activation of TLR2/CD14 complex is summarized in Figure 5.

![Diagram showing the interaction between TLR2, CD14, and apoA-1 IgG](image)

**Figure 5.** Anti-apoA-1 IgG elicits a pro-inflammatory response through TLR2/CD14 complex. Anti-apoA-1 IgG specifically bind to TLR2 because of conformational homology between apoA-1 and TLR2. In presence of CD14, the binding of anti-apoA-1 IgG to TLR2 induces a NF-κB-dependent production of pro-inflammatory cytokines.

4.1.4b. Anti-apoA-1 IgG elicits a positive chromotropic effect on cardiomyocytes

Lately, we demonstrated that there was a positive association between levels of anti-apoA-1 IgG and resting heart rate following myocardial infarction, a well-established parameter for CVD prognosis in secondary prevention [60, 79]. In the same study, we showed that in presence of aldosterone, anti-apoA-1 IgG elicits a dose-dependent increase of the spontaneous
contraction rate of neonatal rat ventricular cardiomyocytes (NRVC) [60]. Using patch-clamp electrophysiology combined with a pharmacological approach, we subsequently showed that this positive chronotropic effect was mediated by L-type calcium channel activation, itself induced by the concomitant activation of both the mineralocorticoid receptor-dependent phosphatidyl 3-kinase pathway and the protein kinase A pathway [80]. In support of an activation mechanism involving aldosterone and antibody, we demonstrated that the chronotropic effect can be abrogated by addition of eplerenone, an aldosterone antagonist, and by intravenous immunoglobulins [80, 60]. Hence, there is compelling evidence in support of a role for anti-apoA-1 IgG in the induction of a positive chronotropic effect in cardiomyocytes, but further work will be required to define (i) whether this is a direct or indirect effect, and (ii) if anti-apoA-1 IgG acts directly on cardiomyocytes, which receptor does it engage to activate the protein kinase A pathway (Figure 6).

Figure 6. Current understanding of the mechanism by which anti-apoA-1 IgG elicits chronotropic responses in cardiac myocytes. Stimulation of the mineralocorticoid receptor (MR), either by aldosterone or oxidized glucocorticoids, induces the downstream activation of PI3K, which in turn activates L-type Calcium channels. Anti-apoA-1 IgG has been shown to sensitize L-type calcium channel in protein kinase A (PKA)-dependent manner. The PI3K and PKA activated pathways alone are not sufficient to induce an increase in basal contraction rate, when simultaneously activated, L-type calcium channels are activated, leading to an
increase in the intracellular Ca\(^{++}\). This signal is amplified by the Na\(^{+}\)/Ca\(^{++}\) exchanger, leading to an increase of the prepotential slope of the cells, which ultimately translates into an increased contraction rate.

4.1.4c. Anti-apoA-1 IgG induces atherosclerosis and death in apoE\(-/-\) mice

Animal studies that we have performed provided direct evidence that anti-apoA-1 IgG was sufficient to induce atherosclerosis. Passive immunization of atherosclerosis-prone apoE\(-/-\) mice with anti-apoA-1 IgG increased both atherosclerotic lesion size and histological features of atherosclerotic plaque vulnerability [63]. Furthermore, in recent unpublished work we have shown that infused anti-apoA-1 IgG also increases the mortality rate in apoE\(-/-\) mice (Figure 7, panel A). Consistent with death due to myocardial infarction, mice died suddenly without evidence of prior sickness (e.g. weight loss, changes in activity patterns). Although detailed pathological examination was not performed in this preliminary study, using a subgroup of mice equipped with telemetry device, allowing the continuous electrocardiogram (EKG) recording, enabled us to detect one event in which a typical myocardial infarction EKG pattern occurred before death (Figure 7, Panels B and C).

![Figure 7. Effects of anti-apoA-1 IgG passive immunization in apoE\(-/-\) mice. Panel A: Passive immunization with anti-apoA-1 IgG induces a significant increase in mortality rate (red line) when compared to CTL IgG and vehicle (NaCl). Panels B and C: Baseline electrocardiogram (B) of anti-apoA-1 IgG-recipient mice in which a transmural myocardial infarction was diagnosed based upon typical ST elevation during the study protocol (C, black arrows).](image-url)
Along this line, Srivastava and colleagues demonstrated that using a lupus-prone mice model, the presence of anti-apoA-1 antibodies was associated to a decrease in the anti-oxidant properties of HDL inferred to a decrease in PON-1 activity, leading to an increase of pro-inflammatory reactive oxygen species [81]. Those results support the hypothesis that anti-apoA-1 IgG and HDL dysfunction are two related phenomena. Although the causality link between anti-apoA-1 IgG and HDL dysfunction still remains elusive, those results are aligned with the clinical observations reported earlier in humans [49-52].

**4.2. Clinical relevance:**

As developed in the paragraphs below, the possible clinical relevance of anti-apoA-1 IgG is three-fold as they could be useful as:

1) as an independent prognostic biomarker of CV risk

2) as a biomarker of atherosclerosis and atherosclerotic plaque vulnerability

3) as a new potential therapeutic target

**4.2.1. Anti-ApoA-1 IgG as an independent predictor of CV risk**

Mirroring our *in vitro* and *in vivo* work suggesting that anti-apoA-1 IgG could be active mediators of atherogenesis, our clinical observations indicate that anti-apoA-1 IgG is associated with a poor CV prognosis independently of the presence of traditional CV risk factors. We have observed this feature in patients with acute coronary syndrome (ACS) [60-62], RA [55], and in patients with severe carotid stenosis [64]. In ACS patient the predictive accuracy according to ROC curve revealed areas under the curve (AUC) values ranging between 0.65 and 0.75 [61-62]. If those AUC values are relatively modest and should ideally be above 0.80 [36], they are still of the same order of magnitude than what has been reported for the Framingham risk score which determines patients management [3].
In addition, we have shown that anti-apoA-1 IgG provides incremental prognostic information over traditional cardiovascular risk factors in ACS, in severe carotid stenosis, and in RA patients. When compared to current risk stratification tools (NSTEMI-TIMI score in acute chest pain patients, or the 10-year global Framingham risk score in ACS, RA, or severe carotid stenosis patients), it significantly improved the patient reclassification either in a higher or lower class of CV complication risk [57, 62, 64].

4.2.2. Anti-apoA-1 IgG as a biomarker predictive of atherosclerosis and atherosclerotic plaque vulnerability

Of clinical relevance too, we have also demonstrated that anti-apoA-1 IgG is also detectable in a proportion of healthy people without autoimmune disease and CVD (0-6.5%), albeit at lower levels than seen in patient cohorts [56, 59, 65]. Significantly, in a small case-control study on healthy subjects [69], we demonstrated that anti-apoA-1 IgG levels in the obese subject subgroup were raised to levels previously described in CVD patients, with high levels of anti-apoA-1 IgG being a significant predictor of coronary artery calcifications visualized by chest computed tomography. Because coronary artery calcifications are a major predictor of subsequent cardiovascular events in asymptomatic subjects [82], the results of this preliminary study suggest that anti-apoA-1 IgG may be a valuable biomarker for use in primary prevention to screen for the presence of coronary artery lesions. Indeed in this setting, anti-apoA-1 IgG testing had a negative predictive value of 94% to detect the presence of coronary artery calcification, with an AUC of 0.83 [69].

Along the same line of thought, we demonstrated in periodontitis patients younger than 50 years-old that anti-apoA-1 IgG was the only predictor of a pathological ankle brachial index [65], a measure used to detect peripheral artery disease and known to reflect the global atherosclerosis burden [83, 84].

Extending those results, we also reported that the presence of anti-apoA-1 antibodies in patients with severe carotid stenosis was associated with histological features of
atherosclerotic plaque vulnerability determined on surgical biopsy specimens [63]. Indeed, in this study, we demonstrated that circulating levels of anti-apoA-1 IgG were positively correlated with intraplaque macrophages (r: 0.33, p=0.002), MMP-9 expression (r: 0.43; p=0.0001) and neutrophils (r: 0.42; p=0.0001), and inversely correlated with total collagen content (r:-0.29, p=0.008). Furthermore patients deemed as positive for anti-apoA-1 IgG had within their atherosclerotic lesions significant higher levels of macrophages, MMP-9 expression and neutrophils, and lower levels of total collagen when compared to patients tested negative for those autoantibodies [63]. Interestingly, those finding were mimicked in apoE -/- mice exposed to passive immunisation with anti-apoA-1 IgG when compared to CTL group [63].

Taken together those results indicate that assessing anti-apoA-1 IgG levels could not only be a possible biomarker of atherosclerosis, but could also be used to detect the presence of atherosclerotic plaque vulnerability. Because assessing atherosclerotic plaque vulnerability is currently an unmet clinical need, the possibility of using anti-apoA-1 IgG detection as a simple and affordable surrogate biomarker of atherosclerotic plaque fragility is of patent clinical interest.

4.2.3. *Anti-ApoA-1 IgG as a new potential therapeutic target*

Because current *in vitro* and *in vivo* results indicate that anti-apoA-1 IgG could well be active mediators of atherogenesis, those autoantibodies may represent an emergent therapeutic target. In other words, we speculate that measuring circulating levels of anti-apoA-1 IgG would allow the identification of a patient’s subset that would benefit from a specific therapy aiming at reversing the deleterious effect of those auto-antibodies. To this respect, we have demonstrated that the chronotropic effect of those autoantibodies could be reversed by existing therapeutic compound such as IVIG and eplerenone, a selective MR antagonist.
This implies a thorough understanding of i) the pathophysiological mechanisms involved in the pathogenicity of anti-apoA-1 autoantibodies, and ii) the exact definition of CV-relevant epitope(s) targeted by those autoantibodies. Once determined, those epitopes could be useful both for the detection of anti-apoA-1 IgG by occupying binding sites, and for neutralizing the pathogenic effects of the antibodies (pro-arrhythmogenic and pro-inflammatory effects), which hopefully would translate in reduction of atherogenesis-related complication in humans.

4.3. Limitations

The current limitations concern the three pillars of this translational project, affecting clinical, analytical, and in vitro/in vivo aspects of our studies.

From a clinical point of view, the major limitation reside in the fact that only single center and relatively small cohorts (ranging between 75 and 221 patients) have been used to demonstrate the possible prognostic/diagnostic utility of anti-apoA-1 IgG measure. The present results are therefore preliminary and need to be challenged in larger and multicenter cohorts before any clinical recommendation can be done.

From any analytical point of view, the main limitation is related to the fact that our in house ELISA is based on a direct ELISA format which could be further improved by the development of a sandwich-type ELISA. This in turn implies the exact definition of CV-relevant epitope of apoA-1 to be used in the assay. Such an approach would allow us to circumvent the problem related to the use of whole human purified apoA-1 molecule in our ELISA by using synthetic apoA-1 mimetic peptides and would therefore be more adapted for scale up purposes than using human purified apoA-1. This aspect will be briefly presented in the chapter 4.7.

The limitations concerning our in vitro and animal work are inherent to all experimental models in the sense that the results derived from in vitro and mice models, are not necessarily
predictive of what will really happen in humans. This work does not escape this general rule. Nevertheless, in order to limit the impact of such phenomenon, we chose the so-called reverse translational approach where the experimental studies are driven by clinical results (rather than being driven by basic experimental evidences) to validate the proof of concept, and to provide further mechanistic insights, as illustrated in Figure 8 [85].

**Figure 8. Translational research processes.** In the traditional process of translation, basic research is the first step to identify new mechanisms of disease and potential therapeutic targets that are elaborated in a proof-of-concept, and eventually validated in clinical trials. In reverse translation, the whole process is grounded on a clinical observation that is tested in basic experimental research in order to validate the proof of concept. Adapted from [84].

This reverse approach is currently replacing the traditional translational approach in order to minimize the risk of results over-interpretation leading to project failure, and is currently widely endorsed by the industry for efficiency reasons [85].

### 4.8. Future research directions

The future research directions are directly oriented to circumvent the current limitations of this work.

From a clinical point of view, we are currently exploring the prognostic value of anti-apoA-1 antibodies using the SPUM biomarker cohort which includes 2405 patients hospitalized for MI and followed up for a period of one year. Furthermore, we are currently challenging our
preliminary findings on acute chest pain patients for NSTEMI diagnosis, using the APACE cohort (1250 patients, NCT: 00470587) in collaboration with Professor Christian Mueller from Basel University Hospital [86]. Those two studies will provide benchmark high quality data allowing us to provide answer about the clinical relevance of anti-apoA-1 IgG as cardiovascular risk stratification tool and more importantly, to determine what incremental information to currently existing biomarkers they could provide, if any.

From an analytical perspective, we will aim at defining the apoA-1 epitope(s) against which the CV relevant immune response is directed. Indeed, if the link between CVD and anti-apoA-1 IgG was established using an immunoassay based on purified delipidated apoA-1, the preparation steps involved, which are onerous and time consuming, would present a significant barrier to scale-up and regulatory approval for an anti-apoA-1 IgG detection assay. In collaboration with Professor O. Hartley, we designed shorter peptide fragments derived from apoA-1 that would be suitable for use in the detection of anti-apoA-1 IgG but could be produced in a straightforward, low-cost and scalable manner by chemical synthesis. We have recently identified peptides derived from the C-terminus of apoA-1, named F3L1 (Fig. 9A) which, when stapled to stabilize the alpha-helical conformation of the native protein (Fig. 9B), recapitulated the diagnostic accuracy of immunoassays making use of the intact molecule (Fig. 9C) [87].
**Figure 9. F3L1, a synthetic peptide derived from apoA-1.** Panel A: F3L1 is based on the C-terminal α-helix found in the structure of intact, delipidated apoA-1. Panel B: Taking this region out of the context of the intact protein led to reduced helicity, which could be recovered using a helix-stapling approach. Panel C: The stapled peptide almost fully recapitulates the diagnostic accuracy of intact delipidated ApoA-I for the prediction of NSTEMI (Non ST segment elevation myocardial infarction).

Nevertheless, it must be emphasized that the NSTEMI diagnostic accuracy of immunoreactivity to F3L1 is currently lower than when compared to the whole molecule. A better understanding of the conformation will hopefully allow us to improve the immunoassay and constitutes one of your main priorities.

Finally, from an *in vitro* and *in vivo* experimental perspectives, we will aim at using the best synthethic peptide identified in our immunoassay in order to reverse their deleterious effects. Since the peptides act by occupying the binding site of anti-apoA-1 IgG, we speculated that they might be capable of neutralizing the pathogenic effects of the antibodies. Our preliminary data indicate that this is indeed the case, with the peptide capable of inhibiting the pro-inflammatory and pro-arrythmogenic activities of anti-apoA-1 IgG in vitro (Fig. 10A and 10 B), and those results have been patented [87].
Figure 10. In vitro effects of F3L1 on inflammation and chronotropic response. 


We are currently planning to test them in vivo, aiming at reversing their pro-atherogenic role and to reverse the mortality rate induced by anti-apoA-1 IgG infusion in apoE -/- mice.

On the longterm, it will be of paramount importance to demonstrate that a specific treatment of patients tested postive for anti-apoA-1 IgG will improve patients outcome when compared to the standard of care.
5. Conclusions

To summarize, recent studies demonstrate that IgG autoantibodies against apoA-1 are raised in many diseases associated with a high cardiovascular risk, such as SLE, ACS, RA, severe carotid stenosis, and end-stage renal disease. So far, high levels of anti-apoA-1 IgG were shown to be an independent prognostic marker of poor CV outcome in MI in RA, and in carotid stenosis patients, to display clinically relevant properties for NSTEMI diagnosis in acute chest pain patients, to be associated with atherosclerotic plaque vulnerability in patients with severe carotid stenosis, and to predict coronary artery lesion in obese but otherwise healthy subjects. In most studies reported so far, high levels of anti-apoA-1 IgG are associated with a pro-inflammatory cytokine profile, and in SLE/APS, those autoantibodies have been shown to be associated with the presence of dysfunctional HDLs.

Concomitantly, in vitro data tend to indicate that anti-apoA-1 IgG are active modulators of atherogenesis by i) promoting sterile inflammation through TLR2/CD14 complex, and ii) by eliciting specific neutrophil chemotaxis. Furthermore, intriguing in vitro experiments suggest that those autoantibodies could act as a pro-arythmogenic molecule through an aldosterone-dependent L-Type calcium channels activation that can be reverted by existing therapeutic compounds. In parallel, mice models demonstrate that passive immunization with anti-apoA-1 IgG increases atherogenesis, atherosclerotic plaque vulnerability, death rate, and decrease the anti-oxidant properties of HDL by inhibiting PON-1 activity. Those preliminary results need to be replicated in larger multicentre cohorts and a better understanding of their physiopathological involvement in atherogenesis is required. Nevertheless, the current converging in vitro and animal observations lend weight to the hypothesis that anti-apoA-1 IgG are active mediators of atherogenesis rather than an innocent bystander. If true, those autoantibodies could in the future represent a new possible therapeutic target, whose deleterious effect could be abrogated by synthetic apoA-1 mimetic peptides therapy. In this
context, auto-antibodies to apoA-1 appears as a promising biomarker of cardiovascular autoimmunity allowing the identification of subset of CVD patients that could benefit from specific immunomodulation in the future, substantially contributing to the development of personalized medicine in the field of CVD.
6. References

This chapter includes all references of the main text. References related to experimental studies presented in chapters can be found at the end of each manuscript.


[87] Patent No P1347EP00; Mimetic peptides for prognosis, diagnosis or treatment of a cardiovascular disease.