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Research paper

ELISA methods comparison for the detection of auto-antibodies against apolipoprotein A1

Miguel A. Frias[a,b,*], Julien Virzi[a,b], Joana Batuca[c], Sabrina Pagano[a,b], Natahlie Satta[a,b], Jose Delgado Alves[c,d], Nicolas Vuilleumier[a,b]

[a] Division of Laboratory Medicine, Diagnostic Department, Geneva University Hospitals, 4 rue Gabrielle-Perret-Gentil, 1205 Geneva, Switzerland
[b] Department of Medical Specialties, Faculty of Medicine, University of Geneva, 1 rue Michel Servet, 1205 Geneva, Switzerland
[c] CEDOC, NOVA Medical School|Faculdade de Ciências Médicas, Universidade NOVA de Lisboa, Lisboa, Portugal
[d] Department of Medicine IV/Immune-mediated Systemic Diseases Unit, Fernando Fonseca Hospital, Amadora, Portugal

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ABSTRACT

Background: Autoantibodies against apolipoprotein A1 (anti-apoA1 IgG) have emerged as an independent biomarker for cardiovascular disease and mortality. Across studies, different ELISA methods have been used to measure the level of circulating anti-apoA1 IgG which could lead to substantial result differences between assays.

Objectives: To make a comparative study of available anti-apoA1 IgG detection methods and to determine whether the choice of matrix sample (serum vs plasma) could influence the results.

Methods: Blood samples were obtained from 160 healthy blood donors and collected on 4 different matrixes (serum, plasma-EDTA, -citrate, -lithium-heparinate). Anti-apoA1 IgG was measured using two homemade (Geneva's and Lisbon's) and one commercial ELISA kits. Passing-Bablok and Bland-Altman were used to compare the results. Anti-apoA1 IgG seropositivity cut-offs were defined according to the user's/manufacturer's criterion.

Results: The current results showed substantial differences between those 3 assays. The dynamic ranges were significantly different, the commercial kit displaying the narrowest one. Passing-Bablok analysis demonstrated important proportional and constant biases between assays. The anti-apoA1 IgG seropositivity rate in Geneva, Lisbon and commercial assays varied between 24.5% and 1.9%. Matrix comparisons demonstrated that the matrix choice (plasma versus serum) influenced anti-apoA1 IgG results as well as the seropositivity rate in an assay-dependent manner. The coating antigen source was identified as important factor underlying results heterogeneity across assays.

Conclusions: These results highlight the impact of the method and the cut-off used on anti-apoA1 IgG results and emphasize the need of standardizing existing assays. Given the important matrix influence, we suggest to use serum as matrix of choice.

1. Introduction

Autoimmune diseases (ADs) represent a major health issue affecting up to 10% of the population (Marson et al., 2015; Ramos et al., 2015; Farh et al., 2015; Cho and Feldman, 2015). Multiple studies have established the relationship between a chronic inflammatory state due to either clinically manifest or quiescent autoimmunity and poor outcomes in various clinical settings (Majka and Chang, 2014; Humphreys et al., 2014). Marking sustained B-cell activation, presence of autoantibodies specifically represents a signature of humoral autoimmunity, which can drive disease pathogenesis even in the absence of overt clinical AD (Solow et al., 2015; Vaarala et al., 1995; Ajeganova et al., 2016). Since the initial description of autoantibodies against apolipoprotein A1 (anti-apoA1 IgG) two decades ago in patients suffering from systemic lupus and anti-phospholipid syndrome (Dinu et al., 1998), anti-apoA1 IgG gained interest as independent biomarkers for incident cardiovascular disease (CVD) complications and/or overall mortality in different populations (Ahmed et al., 2013; Montecucco et al., 2011; Pagano et al., 2016; Vuilleumier et al., 2010a; Chistiakov et al., 2016; Batuca et al., 2017a; Radwan et al., 2014; Antiochos et al., 2017a,b; Batuca et al., 2018; Delgado Alves et al., 2003; Batuca et al., 2007, 2009; Ames et al., 2010; Antiochos et al., 2016). In addition, anti-

* Corresponding author at: Bureau 8C-5-841, Service de médecine de laboratoire, Département Diagnostique, Hôpital Universitaire de Genève, 4, rue Gabrielle-Perret-Gentil, CH-1211 Genève 14, Switzerland.
E-mail address: Miguel.Frias@hcuge.ch (M.A. Frias).

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apoA1 antibodies were identified in patients with systemic lupus erythematosus, anti-phospholipid syndrome where they associated with decreased paraoxonase-1 activity, enhanced endothelial activation, higher disease activity (reviewed in Batuca et al. (2017a)), as well as in the general population where they were found to represent a new independent CV risk factor Antiochos et al., 2016. In parallel, translational studies identified these autoantibodies as mediators of atherogenesis, arrhythmia, myocardial necrosis and death (Montecucco et al., 2011; Pagano et al., 2016; Vuilleumier et al., 2010a; Chistiakov et al., 2016; Antiochos et al., 2017b; Antiochos et al., 2016; Montecucco et al., 2015; Pagano et al., 2012; Vuilleumier et al., 2014), that could be potentially amenable to specific immunomodulation therapeutic modalities (Vuilleumier et al., 2014; Pagano et al., 2015). The clinical relevance of anti-apoA1 IgG has been recently reinforced by data from a phase II, randomized controlled trial, which demonstrated that the Niacin-induced elevation of HDL-C was hampered by a concomitant increase of anti-apoA1 IgG levels associated with a loss of anti-oxidant HDL functions (Batuca et al., 2017a). The reason why Niacin induces the production of anti-apoA1 IgG is still elusive, but this observation could represent a major breakthrough in understanding why therapeutic interventions that increase HDL-C failed to reduce cardiovascular risk so far (Batuca et al., 2017a). Moreover, as elevated anti-apoA1 IgG levels can be found in up to 20% of individuals in the general population (Antiochos et al., 2016), the occurrence of high anti-apoA1 IgG levels might turn into a frequent scenario needing proper interpretation in case these autoantibodies were to be assessed on a more regular basis in coming years.

As current anti-apoA1 IgG assessment methods (Enzyme-linked immunosorbent assay: ELISA) are currently not standardized and display substantial differences in regards of the coating antigen (human purified plasma versus recombinant protein), secondary antibody and overall protocol used (duplicates, systematic sample blank subtraction, etc), significant result differences are expected to occur between these different ELISA methods. Furthermore, to the best of our knowledge, proper matrix difference evaluation for a given ELISA test has never been undertaken.

Therefore, we performed a comparative analysis of three most frequently used ELISA methods for anti-apoA1 IgG detection, and we evaluate the impact of different matrix on the results.

2. Material and methods

For this survey, we chose to compare the most frequent ELISA test used according to the data published in the literature, and we identified two homemade ELISA tests used by two different research groups and one commercial anti-apoA1 IgG ELISA kit. Due to the possible convenience related to the availability of a commercial test, we included it in our survey despite the fact that only one published study used this commercial test so far. The characteristics of these different ELISA tests are summarized in Table 1.

2.1. Study population and samples

Blood samples were obtained from 160 healthy blood donors. In accordance with Geneva cantonal ethical committee and national regulations, written informed consent is obtained from every healthy blood donor prior blood sampling allowing the use of blood or its derivatives for research purpose, including assessing reference intervals for laboratory tests after irreversible anonymisation of data. Blood samples were collected in Becton Dickinson vials in order to obtain serum or lithium-heparinate-, citrate- and EDTA-plasma. After collection, serum was kept at room temperature for 30 min to allow coagulation process. Serum and plasma samples were centrifuged at 2300*g* for 5 min at room temperature, aliquoted and stored at −80 °C until the analyses. Sample aliquots were thawed just prior to assay. For homemade assay, anti-apoA1 IgG measures took place in their respective research groups

Table 1
Characteristics of current ELISA methods included in the survey.

Methods	Research groups	Number of publications	Source of antigen	Coating protocole	Sample handling				Positivity cut-off definition
					Duplicate or uniplicate	Blank substraction	Dilution	Incubation time	
Lisbon Homemade	Delgado et al.	6	Human purified (commercial)	2 h, 37 °C, Polysorp plates in 70% ethanol	Duplicate	Yes	1/200	1 h	> OD to mean + 3SD
Geneva Homemade	Vuilleumier et al.	24	Human purified (homemade)	1 h, 37 °C, maxisorp plates in carbonate buffer pH 9	Duplicate	Yes	1/50	1 h	> 97.5 centile of HBD distribution
Commercial (manufactured by myBiosource, #MBS703406)	El-Lebedy et al.	1	Source not documented	Not documented	Duplicate	Yes	1/101	30 min	OD > 0.1

HBD: healthy blood donor.

Lisbon (Delgado et al.) and Geneva (Vuilleumier et al.) to ensure highly qualified assay realisation. Samples were shipped on dry ice to Lisbon's laboratory. Anti-apoA1 IgG assessment using the commercial assay was performed in Geneva. The study was conducted according to the principles of the Declaration of Helsinki.

2.2. Determination of anti-apoA1 IgG levels

The anti-apoA1 IgG levels were measured with 3 different methods: i) homemade according to Geneva's protocol, ii) homemade, according to Lisbon's protocol and iii) commercial kit (MyBioSource #MBS703406).

The Geneva method: Anti-apoA1 IgG were measured as previously described (Montecucco et al., 2011; Vuilleumier et al., 2010a; Vuilleumier et al., 2010b), using frozen aliquots, stored at −80 °C. Maxisorp plates (Nunc™, Denmark) were coated with purified, human-derived delipidated apoA1 or recombinant human apoA1 (Peprotech EC Ltd., UK) (20 μg/ml; 50 μl/well) dissolved in carbonate buffer (pH 9) for 1 h at 37 °C. After being washed, all wells were blocked for 1 h with 2% bovine serum albumin (BSA) (Merck) in a phosphate buffer solution (PBS) at 37 °C. Participants' samples were also added to a non-coated well to assess individual non-specific binding. After six washing cycles, a 50 μl/well of signal antibody (alkaline phosphatase-conjugated anti-human IgG; Sigma-Aldrich, St. Louis, MO, USA), diluted 1:500 in a PBS/BSA 2% solution, was added and incubated for 1 h at 37 °C. After washing six more times, phosphatase substrate *p*-nitrophenyl phosphate disodium (Sigma-Aldrich) dissolved in a diethanolamine buffer (pH 9.8) was added and incubated for 50 min at 37 °C (Molecular Devices™ Filter Max). Optical density (OD) was determined at 405 nm, and each sample was tested in duplicate. Corresponding non-specific binding was subtracted from mean OD for each sample. The specificity of detection was assessed using conventional saturation tests by Western blot analysis. As previously described (Montecucco et al., 2011; Vuilleumier et al., 2010a,b), elevated levels of anti-apoA1 IgG were set at an OD cut-off of OD > 0.64, corresponding to the 97.5th percentile of a reference population of 140 healthy blood donors. In order to limit the impact of inter-assay variation, we further calculated an index consisting in the ratio between sample net absorbance and the positive control net absorbance × 100. The index value corresponding to the 97.5th percentile of the normal distribution was 37. Accordingly, to be considered as positive (presenting elevated anti-apoA1 IgG levels), samples had to display both an absorbance value > 0.64 OD and an index value ≥37%. The Geneva assay has been validated to detect polyclonal autoantibodies against lipid-free and unmodified apoA1 and reacting with the C-terminal part of the protein (Antiochos et al., 2017b; Pagano et al., 2015; Teixeira et al., 2014).

The Lisbon method: Anti-apoA1 IgG were measured as previously described (Delgado Alves et al., 2003; Batuca et al., 2009; Batuca et al., 2007) using frozen aliquots, stored at −80 °C. Human apoA1 (Sigma-Aldrich, Merck, Portugal) was prepared at 10 μg/ml in 70% ethanol and coated to a half 96-well micro-titer polysorp plates (Nunc, Portugal), for a period of 2 h at 37 °C. Blocking was performed with the addition of 100 μl/well 1% BSA (Sigma-Aldrich, Merck, Portugal) in PBS for 1 h at 37 °C. After washing four times with PBS. Samples, positive, negative controls and standards, were diluted (1:200) in blocking buffer and added in duplicate to each half of the plate for 1 h at 37 °C. The unbound antibodies were removed by repeating the washing step. Alkaline phosphatase conjugated anti-human IgG (1:1000 in the blocking agent) was added for 1 h. Plates were then washed two times with PBS and two times with bicarbonate (BIC) buffer. *P*-nitrophenyl phosphate diluted in BIC buffer pH 9.8 was added and incubated at 37 °C for colour development, and the absorbance read by a Biotrak II plate reader (Amersham Biosciences) at 405 nm after 1 h. All assays were validated by the inclusion of internal quality control samples of known activity and the results were calculated after subtraction of the background in the uncoated half of the plate. The results were expressed as μg/ml determined by the standard curve present in each plate. This curve was prepared with six standards of commercial apoA1 antibody (MIA 1404, Thermo Scientific) in a concentration range of 0.001–0.04 μg/ml. The mean and standard deviation (SD) values of the healthy blood donor were calculated and the samples with OD superior to the mean + 3SD were considered positive. The Lisbon assay also determines anti-apoA1 antibodies when used recombinant apoA1 to coat plates and reacting with the C-terminal part of the protein (Batuca et al., 2017b). Of note, Lisbon's protocol uses a standard curve that allows anti-ApoA1 IgG quantification, nevertheless in this manuscript, we only compared the raw data of OD values, as the two other methods do not express the results in concentration (μg/ml) but in OD.

The commercial assay: Anti-apoA1 IgG was assayed using human anti-apoA1 antibody ELISA kit (myBiosource, Inc., USA, #MBS703406) accordingly to manufacturer's protocol. The intra-assay precision and the inter-assay precision was assessed by manufacturer and the CV% was < 15%. 100 μl of negative or positive control or 100 μl of diluted samples (1:101) were incubated 30 min at 37 °C. After being washed 5 times, 100 μl of HRP-conjugate was added and incubated for 30 min. After a novel washing run, 50 μl of substrate A and 50 μl of substrate B was added to each well and incubated for 10 min at 37 °C. The solution was stopped with 50 μl of stop solution and the optical density (OD) was determined using microplate reader set to 450 nm. Samples were considered positive when sample had a relative absorbance value of 0.1 OD when blank subtraction was performed.

Of note, these methods have been conducted according to the user or manufacturer's instructions and no technical problem was faced.

2.3. Statistics

Passing-Bablok and Bland-Altman tests were used to compare the different methods and were reported with the 95% confidence intervals (95%CI). The correlation between assays and matrixes were also evaluated was by the nonparametric Spearman rank correlation. Exact bilateral Fisher test was used to assess the proportion of anti-apoA1 IgG positivity between methods. Two-sided p value below 0.05 was considered as significant.

3. Results

3.1. Coefficients of variation and distribution of anti-apoA1 IgG values according to the ELISA methods

All assays were validated by the inclusion of internal quality control samples. Inter- and intra-plate coefficients of variation (CV) were evaluated using the three protocols and with the four matrixes. Intra-assay was evaluated in one intermediate range value and inter-assay using two values (low and high) (Table 2). As expected, intra-assay CV was smaller than the inter-assay CV. More specifically, within the Geneva's protocol, intra- and inter- assay values were below 4.3% and 20.8%, respectively. Regarding the matrix of choice, serum had acceptable intra- assay, < 3.7% compared to 3.0%, 4.3% and 3.3% for lithium-heparinate, EDTA- and citrate plasma, respectively. Of note, Lisbon's protocol had 2 inter-assay values for the serum, lithium-heparinate- and EDTA-plasma samples and only one low value for the citrate-plasma. The inter-assay CV varied from 9% to 24.3% in the different matrixes. Regarding the comparison between matrixes, serum had acceptable intra-assay, of 4.8% compared to 3.0%, 2.9% and 2.5% for lithium-heparinate, EDTA- and citrate plasma, respectively. Commercial protocol had broader range inter-assay CV from 5% to 86% in the different matrix. Regarding the comparison between matrixes, serum had an intra- assay, < 9.9% compared to 11.5%, 6.6% and 4.9% for lithium-heparinate, EDTA- and citrate plasma, respectively. Of note, the inter-assay CV in serum of the low and high values in the different assays were as follows: 15.7% and 7.0% (Geneva), 13.9% and 14.3% (Lisbon) and 25.1% and 24.1 (commercial).

Table 2
Coefficient variation of ELISA method calculated during the study.

	Geneva			Lisbon			Commercial		
	inter-assay (%)		intra-assay (%)	inter-assay (%)		intra-assay (%)	inter-assay (%)		intra-assay (%)
Levels	Low	High	intermediate	Low	High	Intermediate	Low	High	Intermediate
Serum	15.67	6.96	3.66	13.90	14.34	4.78	25.06	24.05	9.95
Lithium-heparinate-plasma	20.73	16.09	3.03	21.87	12.50	2.98	25.03	5.95	11.52
EDTA-plasma	10.11	17.80	4.32	24.30	11.40	2.88	13.82	18.83	6.56
Citrate-plasma	17.63	2.71	3.28	8.99	ND	2.46	86.33	45.38	4.94

ND: not determined.

As shown in Fig. 1, the three methods indicated that anti-apoA1 IgG distribution had a non-Gaussian distribution characterized by a right skewed distribution. This figure also illustrated important differences in terms of dynamic range in serum, which was narrow for the commercial assay with values varying between 0 and 0.37 OD. Lisbon protocol displayed a dynamic range varying between 0 and 0.75 OD, and Geneva protocol displayed a dynamic range spanning between 0 and 2.22 OD. Regarding the dynamic range between matrixes, with the Lisbon protocol we observed that the dynamic range of serum samples was narrower than in the other matrixes. The superior limit of serum samples reached 0.75 OD, whereas the superior limits were of 0.82 OD, 1.32 OD and 1.12 OD for EDTA-, lithium-heparinate, and citrate-plasma, respectively. In the other two assays, the dynamic ranges of serum samples were similar to those observed in the other matrixes. The upper limit of serum samples were 2.22 OD and 0.37 OD for the Geneva's and commercial assay, respectively and were 2.01 and 0.481 OD (lithium-heparinate-plasma), 2.02 and 0.344 OD (EDTA-plasma) and 2.22 and 0.235 OD (citrate-plasma) in the other matrixes.

4. Comparative method

Serum and plasma from 160 healthy blood donors were measured using the different tests available. As serum is frequently used for autoantibodies assessment, the OD values obtained in serum samples were compared between the different test using Passing-Bablok (PB) and Bland-Altman (BA) tests. Whether the values of OD were not included in the limit of 95% CI, the two methods were considered significantly different (Fig. 2). When compared to Geneva's tests the PB analyses demonstrated a major proportional bias of 0.35 and 0.03 (should be 1 if values where identical) when compared to Lisbon's and commercial test, respectively. The commercial assay provided a 97% proportional decrease of OD values when compared to the Geneva assay (Fig. 2A), and we noted a 65% proportional decrease of the signal for the Lisbon assay when compared to the Geneva one (Fig. 2B). When compared to the commercial assay, the Lisbon assay showed a major proportional bias increase of 15.5-fold (Fig. 2C). BA confirmed these important inter-assay biases and indicated that they were present on the whole spectrum of anti-apoA-1 IgG concentrations and became significant for OD values above 0.5 when comparing results from Geneva assay with those from Lisbon or the commercial assay (Fig. 2D and E), or when values were above 0.1 OD when comparing the Lisbon assay with the commercial one (Fig. 2F). In addition, we calculated the coefficients of correlation between the different assays that are shown in Table 3. These major inter-assay biases were likely to influence the proportion of patients defined as having high anti-apoA1 IgG levels (anti-apoA1 IgG seropositivity) which was further evaluated.

5. Influence of assays on anti-apoA1 IgG level seropositivity

Given the aforementioned difference observed on PB and BA, we investigated how these differences would influence the prevalence of high anti-apoA1 IgG levels according to method-specific cut-off definitions (see method section). We compared the anti-apoA1 IgG seropositivity rate between assays in serum samples. While Geneva's protocol test showed 39 positive samples (corresponding to 24.5%), the commercial kit identified only 14 positive samples (corresponding to 8.8%) and Lisbon's test only 3 (1.9%) (Table 4). This difference of positive sample ratio was highly significant between Geneva's test and the two others ($p = 0.0003$ and $p < 0.0001$) and significant ($p = 0.0106$) between the commercial and the Lisbon's tests. Among the 3 and 14 positive samples measured by Lisbon's and the commercial test, respectively only 2 and 9 were positive in Geneva's test and none was double positive between Lisbon's and Commercial test. The ratios of positivity concordance (including negative samples) between Geneva's, Lisbon's and commercial tests, respectively corresponded to 76.3% (122/160), 78.1% (125/160) and 89.4% (143/160) (Table 4). In top of methods, specific cut-off variation definition (value > 97.5% of the reference population for the Geneva assay; mean + 3SD for Lisbon assay, OD > 0.1 for the commercial assay), clearly impacts on anti-apoA1 IgG seropositivity rate.

6. Impact of the matrix effect on anti-apoA1 IgG results

The level of anti-apoA1 IgG from the same 160 subjects was evaluated in 4 different matrixes: serum and 3 different kind of plasma (EDTA, lithium-heparinate and citrate). We compared the different plasma preparations to the serum, as the latter is regarded as the matrix of choice for autoantibodies assessment. As shown in Fig. 3, the 3 tests evaluated differed among each other's in term of matrix sensitivity. With the Geneva's protocol (Fig. 3A, B, C), based upon non-significant proportional and constant bias, serum results were considered similar to lithium-heparinate plasma with a constant and proportional bias of 0.02 OD and −2.7%, respectively, but different from EDTA and citrate plasma with proportional bias of +25% and −16%, respectively. With the commercial test (Fig. 3D, E, F), results obtained on serum samples were similar to the ones retrieved on EDTA-plasma with no constant bias and a proportional bias of −2%, but different from those obtained on lithium-heparinate and citrate-plasma with a proportional bias of +20% and −24.3%, respectively. With the Lisbon's protocol (Fig. 3G, H and I), the results obtained in serum were comparable to the ones obtained with lithium-heparinate and EDTA-plasma with constant bias of 0.05 OD and −0.03 OD and proportional bias of +30% and +10.9%, respectively, but significantly different than the ones derived from citrate-plasma which showed a proportional bias of +90%. The coefficients of correlation between the different matrixes were calculated and are shown in Table 5. In Geneva's and Lisbon's tests, results derived from lithium-heparinate plasma provided similar results to serum samples. Furthermore EDTA-plasma provided similar results to serum with Lisbon and the commercial assay, but not with the Geneva assay. Finally, across the three assays, citrate plasma appeared to be the matrix providing the least comparable results to serum.

Taken together these results indicate that matrix choice would also be likely to influence anti-apoA1 IgG seropositivity. In accordance with PB analyses, we found that anti-apoA1 IgG seropositivity rates using Geneva assay were similar on serum and lithium-heparinate-plasma ($n = 39$, 24.5% for both assays), a significant increase in seropositivity

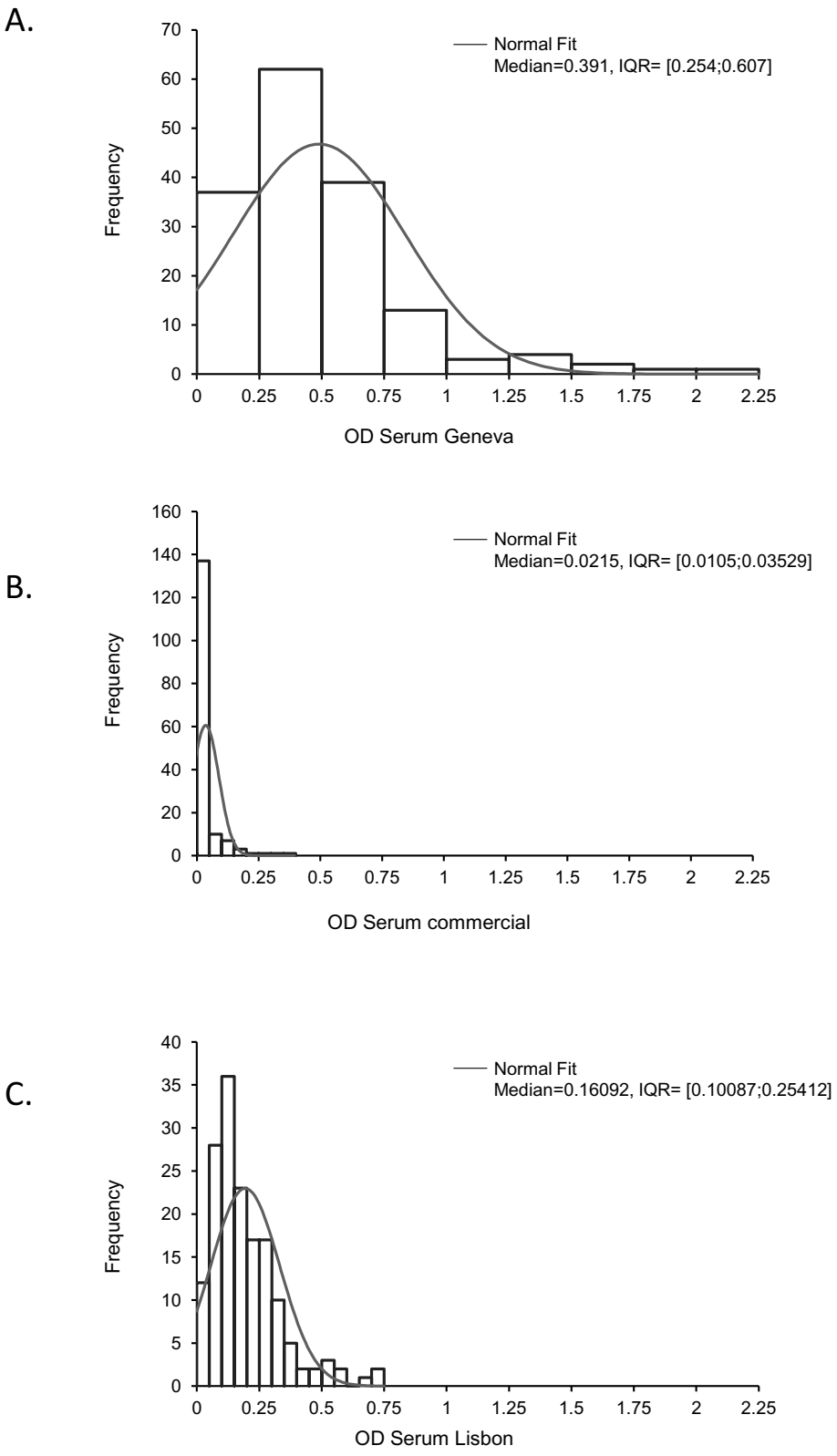

Fig. 1. Distribution values from serum.
The distribution of values expressed in Optical Density (OD) obtained with serum samples are illustrated. A. Data obtained from Geneva's serum protocol. B. Data obtained from commercial's serum protocol. C. Data obtained from Lisbon's serum protocol.

rate was observed using EDTA plasma (n = 75, 46.9%, $p < 0.0001$; Table 4). The seropositivity rate using the Lisbon assay was low (around 2%), and similar across the three different plasma used (Table 4). With the commercial test, no significant differences in seropositivity rates were observed at the exception of citrate plasma displaying a significantly lower positivity rate (Table 4). Finally, no one sample was found to be positive in the 4 matrixes and with the 3 assays, further emphasizing the impact of assay and matrix choice on anti-apoA1 IgG

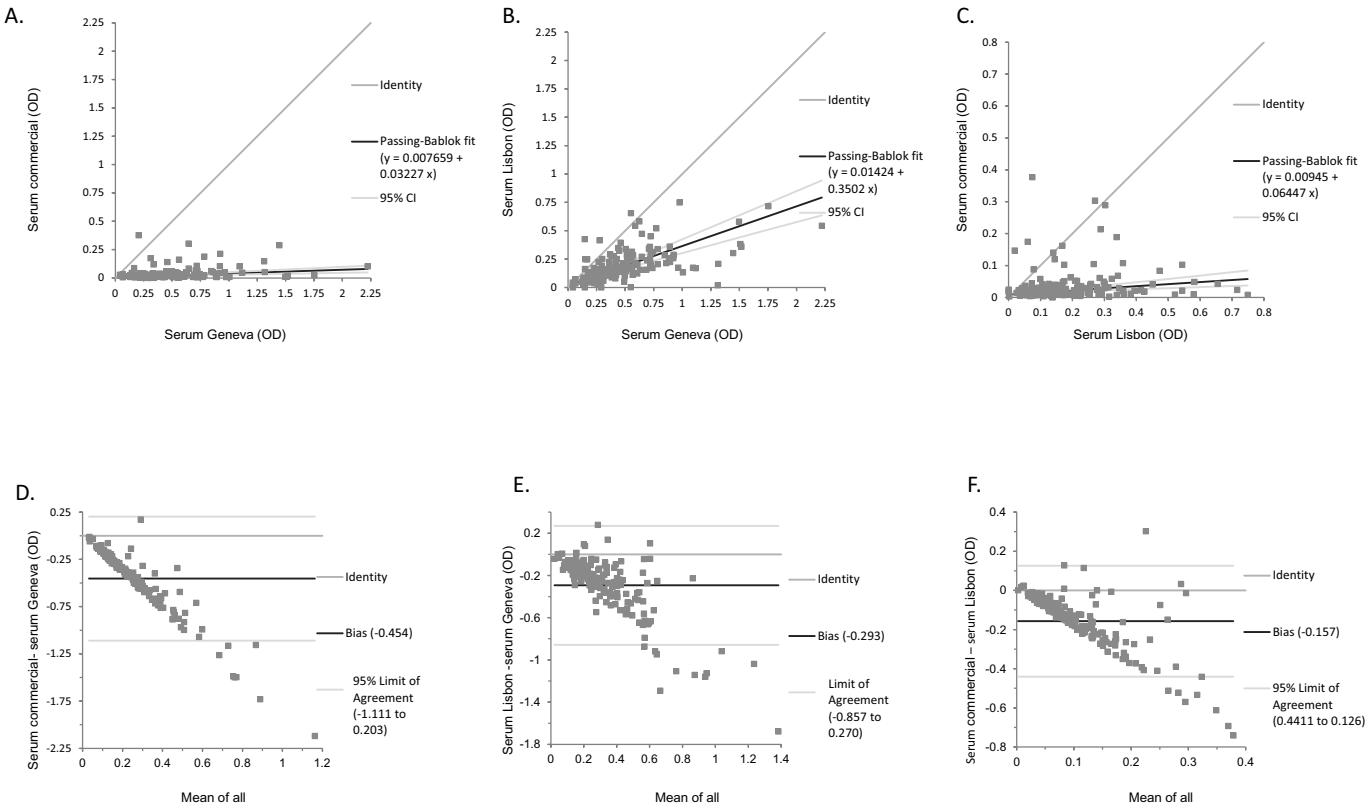

Fig. 2. Method comparison.
The data expressed in Optical Density (OD) obtained with serum samples are compared across the different test. A. PB analysis of Geneva test vs commercial (A), Geneva test vs Lisbon test (B) and Lisbon test vs commercial (C). BA analysis of Geneva test vs commercial (D), Geneva test vs Lisbon test (E) and Lisbon test vs commercial (F).

Table 3
Coefficient of correlation between serum anti-apoA1 values across the different assays.

Assay	Coefficient of correlation (r)	p value
Geneva vs Commercial	0.288	p = 0.0002
Geneva vs Lisbon	0.664	p < 0.0001
Lisbon vs Commercial	0.195	p = 0.0136

Coefficient of correlation and p values have been calculated using nonparametric Spearman's test.

results. This might be explained by the lower percentage of positive subjects.

7. Impact of changing the antigen source in the Geneva assay

As the antigen source is a well-established source of variation between assays, we evaluated the impact of replacing human purified apoA1 by recombinant human apoA1 (Peprotech EC Ltd., UK) in the Geneva assay on 91 samples. As shown in Fig. 4, using recombinant apoA1 instead of human purified apoA1 in the Geneva assay without changing the rest of the standard protocol induced a two-fold proportional bias in presence of the recombinant protein, indicating that the source of the coating antigen is one of the important factors underlying results heterogeneity across assays.

8. Discussion

This is the first study evaluating the different methods for anti-apoA1 IgG measurement. In this comparative study we evaluated the three different methods currently available that have been used so far relating the presence of anti-apoA1 IgG to an increased risk of CVD in different populations (Ahmed et al., 2013; Montecucco et al., 2011; Pagano et al., 2016; Vuilleumier et al., 2010a; Chistiakov et al., 2016; Batuca et al., 2017a; Radwan et al., 2014; Batuca et al., 2009; Antiochos et al., 2016; El-Lebedy et al., 2016). The present study revealed the

Table 4
Anti-apoA1 IgG seropositivity rate according to assays.

	Geneva assay	Lisbon assay	Commercial assay	p value	p value	p value
	Number of positive (%)	Number of positive (%)	Number of positive (%)	L vs G	C vs G	L vs C
Serum	39/160 (24.5%)	3/160 (1.9%)	14/160 (8.8%)	< 0.0001	0.0003	0.0106
Lithium-Heparinate-Plasma	39/160 (24.5%)	4/160 (2.5%)	12/160 (7.5%)	< 0.0001	< 0.0001	0.0698
EDTA-Plasma	75/160 (46.9%) *	3/160 (1.9%)	12/160 (7.5%)	< 0.0001	< 0.0001	0.0312
Citrate-Plasma	13/160 (8.1%) *	5/160 (3.1%)	6/160 (3.8%) *	0.0873	0.1500	0.9900

The number of positive sample was reported to the 160 subjects. *, p < 0.05 vs serum values of the same assay.
L vs G: Lisbon vs Geneva, C vs G: Commercial vs Geneva, L vs C: Lisbon vs Commercial.

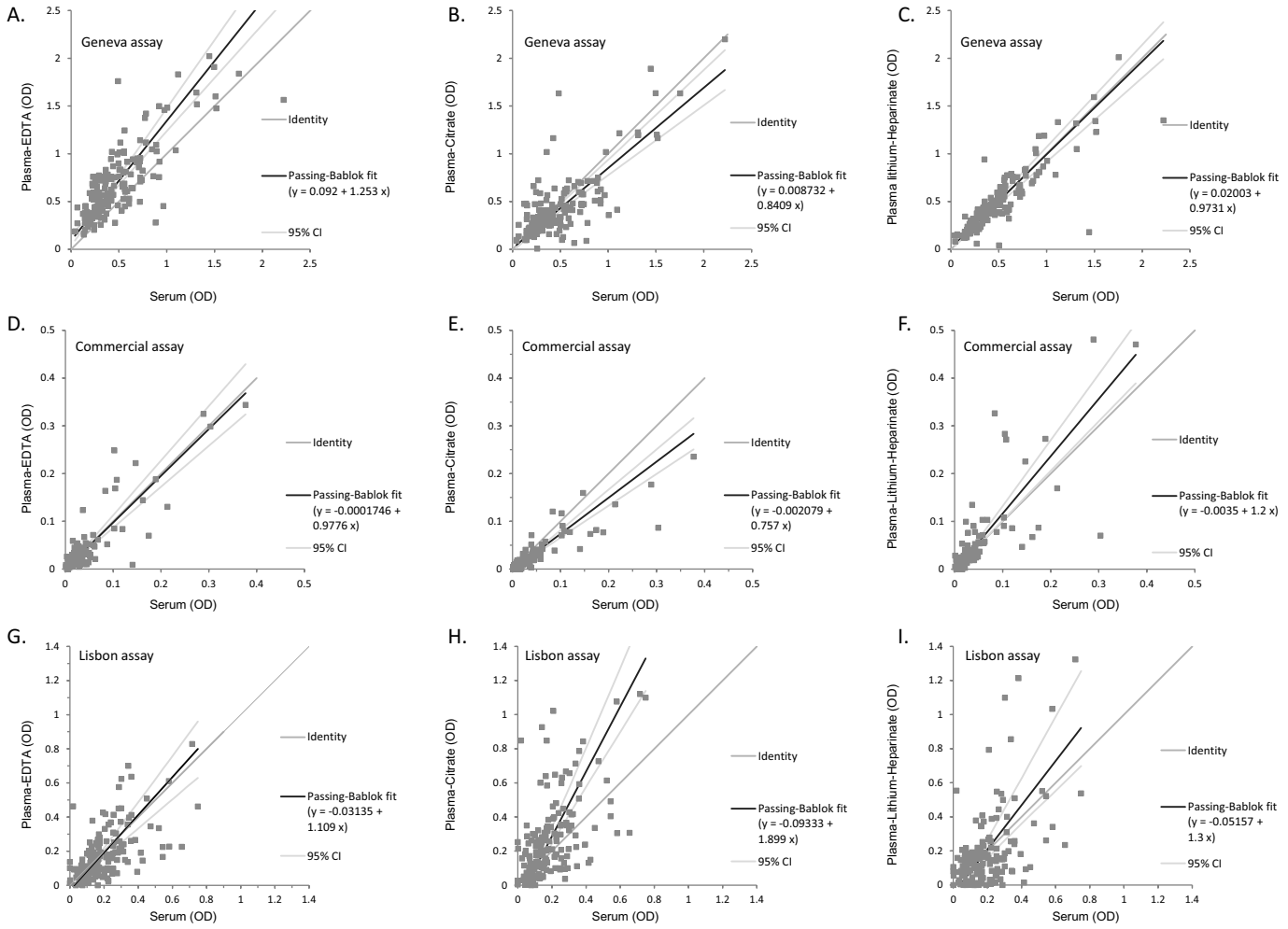

Fig. 3. Matrix comparison.
The data expressed in OD and obtained with serum samples are compared to the different plasma preparation. Geneva's test PB analysis of serum vs EDTA-plasma (A), serum vs citrate-plasma (B) and serum vs lithium-heparinate-plasma (C). Commercial's test Passing Bablok analysis of serum vs EDTA-plasma (D), serum vs citrate-plasma (E) and serum vs lithium-heparinate-plasma (F). Lisbon's test Passing Bablok analysis of serum vs EDTA-plasma (G), serum vs citrate-plasma (H) and serum vs lithium-heparinate-plasma (I).

existence of important differences in the result of anti-apoA1IgG quantification depending on the assay used. Of importance, the results of the assays were also impacted by matrix choice. While the two homemade assays (Geneva and Lisbon) displayed acceptable dynamic range, the commercial test demonstrated a limited dynamic range spanning between 0 and 0.37 OD. The superiority of the two homemade assays is also confirmed by the intra- and inter-assay variation coefficient analysis. While these values are acceptable in these assays, inter- and intra-assay variation coefficient clearly demonstrated an insufficient precision (> 20% for most conditions) for the commercial kit (Table 2). Furthermore, the order of magnitude of the proportional biases calculated using PB analysis encountered was high, varying from −97% up to 15-fold depending on the assay and matrix combination. Of note, the coefficients of correlation between commercial assay and the two other assays were very poor ($r = 0.1947$ vs Lisbon and $r = 0.2879$ vs Geneva), even more considering that the same samples were measured. These major differences can be ascribed to the accumulation of several factors summarized in Table 1 and including the source of apoA1 (commercial source for the Lisbon and the commercial assay, versus purified from human donors' HDL for the Geneva assay), the coating protocol, the sample dilution factor, the nature of the secondary antibody used, and incubation time. Knowing which of these factors have a preponderant effect on the variability observed between the assays is still unclear, but our results clearly indicate that the difference in antigen coating is likely to represent a key factor of variation by modifying the signal by two-fold. As the validated Geneva assay is

Table 5
Coefficient of correlation between serum anti-apoA1 values across the different matrixes of the same assay.

Assay	Coefficient of correlation and p value Serum vs EDTA-plasma	Coefficient of correlation and p value Serum vs Citrate-plasma	Coefficient of correlation and p value Serum vs Lithium-heparinate-plasma
Geneva	0.768 ($p < 0.0001$)	0.639 ($p < 0.0001$)	0.888 ($p < 0.0001$)
Commercial	0.740 ($p < 0.0001$)	0.856 ($p < 0.0001$)	0.823 ($p < 0.0001$)
Lisbon	0.623 ($p < 0.0001$)	0.563 ($p < 0.0001$)	0.415 ($p < 0.0001$)

Coefficient of correlation and p values have been calculated using nonparametric Spearman's test.

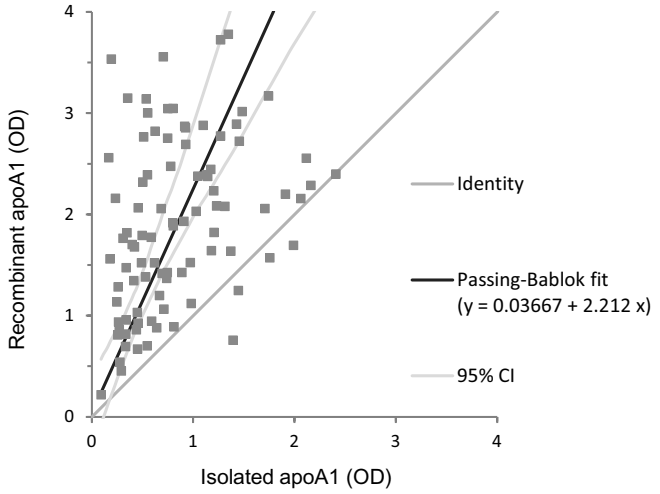

Fig. 4. Impact of changing the antigen source in the Geneva assay.
The data expressed in Optical Density (OD) obtained with serum samples are compared using two different antigen source: human apoA1 recombinant and purified apoA1 from healthy donors. The coefficient of correlation between these values was evaluated by Spearman test and corresponded to $r = 0.4232$ ($p < 0.0001$).

known to detect polyclonal autoantibodies that recognized lipid-free and unmodified apoA1 and targeted more specifically the C-terminal part of the protein (Antiochos et al., 2017b; Pagano et al., 2015; Teixeira et al., 2014), using any other antigen containing a different degree of lipidation is likely to have major impact on the results. Indeed, as lipidation of apoA1 induces conformational changes in the apoA1 molecule susceptible to modify epitope exposure, any lipid-related apoA1 conformational changes is susceptible to modulate the affinity of anti-apoA1 IgG and to induce substantial biases in the results. In addition, the difference of dilution factor between Geneva's and Lisbon's assay may increase the bias between these two tests and explain some of the differences obtain in the dynamic ranges between the two homemade assays.

Consequently, major differences in anti-apoA1 IgG seropositivity rate were found across the assays and varied from 1.9 to 46.9%. It is to point out that the seropositivty rate obtained with the Geneva assay was consistent with what has been retrieved in the general population (19.9%) (Antiochos et al., 2016). In comparison, the results of the Lisbon and commercial assays showed a significant lower seropositivity rate (1.9% and 8.8% vs 24.5%, $p < 0.0001$ and $p = 0.0003$). Lisbon assay previously identified approximately 1–2% of the control population as positive (Batuca et al., 2007), which was also confirmed in this current data set (1.9%). The commercial test was used on type 2 diabetes mellitus (T2DM) patients with CVD and found that 6.1% of healthy subjects, 8.8% of T2DM patients and 35.7% of T2DM with CVD were positive for anti-apoA1 IgG (El-Lebedy et al., 2016). The value in healthy subject is in accordance with the one of this current study (8.8%). Of note, none sample was unanimous and positive for the 3 tests. Among the 14 positive samples measured with the commercial kit and among the 3 positive samples retrieved in the Lisbon test, only 9 and 2 were respectively found to be positive when using the Geneva assay. The concordance of all results (positive and negative) between Geneva, Lisbon and the commercial assay is 76.3% and 78.1%, respectively, and 89.4% (143/160) between the Lisbon and the commercial test. These results indicate that seropositivity rates reported so far with the Geneva assay, varying between 10 and 30% according to the population at risk (Antiochos et al., 2016), could be substantially affected in an assay-dependent manner.

In turn, such differences could potentially blunt the established associations between anti-apoA1 IgG seropositivity and CVD. Nevertheless, regardless of the assay used so far, the association between anti-apoA1 IgG levels and CVD were consistently observed, ruling-out the possibility of analytical artefact underlying such associations (Ahmed et al., 2013; Montecucco et al., 2011; Pagano et al., 2016; Vuilleumier et al., 2010a; Chistiakov et al., 2016; Batuca et al., 2017a; Radwan et al., 2014; Antiochos et al., 2017b; Batuca et al., 2018; Batuca et al., 2009; Antiochos et al., 2016; El-Lebedy et al., 2016). In addition, we observed that the use of a particular matrix (EDTA- and citrate-plasma) may interfere with anti-apoA1 IgG measure. This was particularly the case in Geneva's test where EDTA-plasma and citrate-plasma demonstrated a higher and lower rate of positivity, respectively. On the other hand the use of serum or lithium-heparinate plasma provided similar result to serum in both Geneva and Lisbon assay. Across the three assays, citrate-plasma appeared to be the matrix providing the least comparable results to serum. This study highlights the sensitivity of anti-apoA-1 IgG results to the matrix effect in an assay-dependent manner.

Given the fact that anti-apoA1 IgG assessment has been proposed as a possible biomarker for CV risk stratification (Ahmed et al., 2013; Montecucco et al., 2011; Pagano et al., 2016; Vuilleumier et al., 2010a; Chistiakov et al., 2016; Batuca et al., 2017a; Radwan et al., 2014; Antiochos et al., 2017b; Batuca et al., 2009, 2018; Antiochos et al., 2016; El-Lebedy et al., 2016), the present results highlight the importance of developing standardized anti-apoA1 IgG assays in the future, as results from a given assay cannot be transposed to another one. The initial steps of such standardization process should include i) the use of similar coating antigen, ii) the use of a calibration curve using fully human polyclonal anti-apoA-1 IgG that are currently not commercially available, iii) defining serum as the matrix of choice for anti-apoA1 IgG assessment, iv) the development of an external quality control which is currently inexistent, and v) develop unified criteria to define anti-apoA1 IgG seropositivity.

This study had some limitations. Firstly, samples were measured from a healthy blood donor population from which no clinical information is available. We therefore did not have clinical data to challenge the single and double positive patients. Secondly, the analyses took place in two different places (Geneva and Lisbon) and by three different experimentalists that could have increase the inter-assay difference, but their expertise in the method that could also be considered as quality insurance. Thirdly, given the absence of existing external quality control for anti-apoA-1 IgG, we could not assess the accuracy of these three assays. Finally, we did not assess the impact of storage time and conditions as a potential pre-analytical factor susceptible to affect anti-apoA1 IgG levels.

9. Conclusion

This first anti-apoA1 IgG method evaluation performed on 160 healthy blood donors highlights the impact of existing assays on anti-apoA1 IgG results with major proportional and constant biases between assays. The heterogeneity of the results was influenced by the matrix choice in an assay-dependent manner and the coating antigen source. These observations emphasize the need of standardizing existing anti-apoA1 IgG assays. Meanwhile, we recommend the use of serum as the matrix of choice and to use human purified apoA1 as the optimal source of coating antigen.

Conflict of interest

None.

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Author's contribution

MAF, JDA and NV contributed to the design of the work and wrote the manuscript. MAF, JV, JB performed experiments. MAF, JV, JB, SP, JDA and NV participated in the analyses. All authors reviewed and edited the paper.

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