Relationship of IgG and IgM autoantibodies and immune complexes to oxidized LDL with markers of oxidation and inflammation and cardiovascular events: results from the EPIC-Norfolk Study

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Abstract **Levels of IgG and IgM autoantibodies (AA) to malondialdehyde (MDA)-LDL and apoB-immune complexes (ICs) were measured in 748 cases and 1,723 controls in the EPIC-Norfolk cohort and their association to coronary artery disease (CAD) events determined. We evaluated whether AA and IC modify CAD risk associated with secretory phospholipase A_2 ($sPLA_2$) type IIA mass and activity, lipoprotein-associated PLA_2 activity, lipoprotein (a) [Lp(a)], oxidized phospholipids on apoB-100 (OxPL/apoB), myeloperoxidase, and high sensitivity C-reactive protein. IgG ICs were higher in cases versus controls ($P = 0.02$). Elevated levels of IgM AA and IC were inversely associated with Framingham Risk Score and number of metabolic syndrome criteria (p range 0.02–0.001). In regression analyses adjusted for age, smoking, diabetes, LDL-cholesterol, HDL-cholesterol, and systolic blood pressure, the highest tertiles of IgG and IgM AA and IC were not associated with higher risk of CAD events compared with the lowest tertiles. However, elevated levels of IgM IC reduced the risk of Lp(a) ($P = 0.006$) and elevated IgG MDA-LDL potentiated the risk of $sPLA_2$ mass ($P = 0.018$). This epidemiological cohort of initially healthy subjects shows that IgG and IgM AA and IC are not independent predictors of CAD events but may modify CAD risk associated with elevated levels of oxidative biomarkers.**—Ravandi, A., S. M. Boekholdt, Z. Mallat, P. J. Talmud, J. J. P. Kastelein, N. J. Wareham, E. R. Miller, J. Benessiano, A Tedgui, J. L. Witztum, K-T. Khaw, and S. Tsimikas. **Relationship of IgG and IgM autoantibodies and immune complexes to oxidized LDL with markers of oxidation and inflammation and cardiovascular events: results from the EPIC-Norfolk Study.** ***J. Lipid Res.*** **2011.** 52: **1829–1836.**

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Oxidized low density lipoprotein (OxLDL) plays a major role in initiation and progression of atherosclerosis (1, 2). OxLDL does not represent a single entity because both the protein and the lipid components can be oxidized, which can result in a heterogeneous group of molecules that are not only pro-inflammatory but also immunogenic. OxLDL can initiate cell-mediated and humoral responses resulting in activation of macrophages leading to overexpression of major histocompatibility complex-II molecules, cytokine and chemokine release, and expression of costimulatory molecules (2, 3).

Autoantibodies (AA) against modified LDL have been isolated from both animal and human plasma (4–6). There is evidence from animal studies that increasing levels of AA to modified LDL correlate with atherosclerosis burden (5, 7). For a variety of reasons, the results from human

Abbreviations: AA, autoantibodies; CAD, coronary artery disease; FRS, Framingham Risk Score; HDL-C, high density lipoprotein cholesterol; hsCRP, high sensitivity C-reactive protein; IC, immune complexes; LDL-C, low density lipoprotein cholesterol; Lp(a), lipoprotein (a); Lp-PLA_2, lipoprotein-associated phospholipase A_2; MDA, malondialdehyde; OxLDL, oxidized low density lipoprotein; OxPL, oxidized phospholipid; RLU, relative light units; $sPLA_2$, secretory phospholipase A_2.

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studies have not consistently shown that AA or immune complexes (ICs) are associated with cardiovascular disease or events (8–13). The heterogeneity of findings in human studies is likely due in part to differences in the populations studied, the methodologies measuring AA, and the lack of statistical power in small studies (14). In this study constituting 748 cases and 1,723 controls as part of the EPIC-Norfolk study (15, 16), which is adequately powered to address the hypothesis, we evaluated the association of both IgG and IgM AA and IC with coronary artery disease (CAD) events and assessed their impact on other markers of inflammation in prediction cardiovascular outcomes.

METHODS

Study population

A nested case-control study was designed among participants in the EPIC-Norfolk cohort (15, 16). Briefly, 25,663 healthy men and women aged between 45 and 79 years were recruited from age-sex registers of general practices in Norfolk and enrolled between 1993 and 1997 and followed for an average of 6 years. Participants were identified as having CAD during follow-up if they had a hospital admission and/or died with CAD as the underlying cause. Two controls were matched to each case by sex, age (within 5 years) and time of enrollment (within 3 months). The Norwich Health Authority Ethics Committee approved the study and all participants provided written informed consent.

Measurement of laboratory parameters

Plasma samples were drawn at baseline (1993–1997) in EDTA and were stored at −80°C at the Clinical School, University of Cambridge. We have previously shown that these biomarkers are stable with prolonged freezing or transport to processing sites on ice (17–19).

Plasma levels of total cholesterol, low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), and triglycerides were measured with commercial kits. Chemiluminescence ELISAs were used to measure IgG and IgM autoantibodies to malondialdehyde-modified LDL (MDA-LDL) as initially described (4, 20) and subsequently modified for human applications as described (9, 10, 21, 22). In brief, MDA-LDL (5 μg/ml) was coated on microtiter well plates, plasma (1:200 dilution) added, and IgG or IgG autoantibodies binding to MDA-LDL detected with alkaline phosphatase labeled goat anti human IgG or IgM (Sigma). ApoB-100 ICs were detected by plating murine monoclonal antibody MB47 to bind a saturating amount of human apoB. Plasma (1:50 dilution) was added and IgG or IgG autoantibodies binding to the captured apoB (i.e., apoB-IC) were detected with alkaline phosphatase labeled goat anti human IgG or IgM as above (9, 21). Oxidized phospholipids on apoB-100 (OxPL/apoB) were measured as previously described (23) and subsequently modified (10, 17) using chemiluminescent ELISA. In brief, MB47 was added to microtiter well plates to bind a saturating amount of apoB-100, plasma added (1:50 dilution), and OxPL detected with biotinylated murine monoclonal antibody E06 and read as relative light units (RLU). This measure represents E06 immunoreactivity to OxPL and is not a direct chemical measure of OxPL. Lipoprotein (a) [Lp(a)] (22), myeloperoxidase mass (24), and high sensitivity C-reactive protein (hsCRP) (15) were measured as previously described.

Plasma $sPLA_2$ type IIA mass was performed with an in-house assay as originally described (25) and subsequently reported on in large clinical populations (16, 26, 27). In brief, $sPLA_2$ mass was measured with a sandwich-type ELISA. The measurement has no cross-reactivity with type I, IV, V, or type X $sPLA_2$. The mean intra-assay variation between duplicates was 9.2% and the lower detection limit was 0.4 ng/ml. Plasma $sPLA_2$ activity was measured by a selective fluorometric assay by using fluorescent substrate 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3 phosphomethanol, sodium salt (Interchim, Montluçon, France). One hundred percent hydrolysis of the fluorescent substrate was measured using 0.1 U PLA_2 from bee venom (Sigma Chemical Co). The hydrolysis of substrate in the absence of plasma was used as negative control. All samples were tested in duplicate and plasma activity was expressed as nmol/min per ml. The minimum detectable activity was 0.10 nmol/min per ml. This method is not specific for only $sPLA_2$ type IIA but other calcium-sensitive phospholipases can hydrolyze the substrate used. In contrast to Lp-PLA_2, $sPLA_2$ can also hydrolyze unmodified phospholipids [reviewed in Mallat et al. (28)]. Addition of purified or recombinant Lp-PLA_2 [concentrations up to 26 μg/ml (6 μg in a 230 μL final assay volume), which is two orders of magnitude above the median Lp-PLA_2 physiological range (200 ng/ml)] at the physiological or pathological concentrations in our $sPLA_2$ activity assays resulted in no detectable signal.

Lp-PLA_2 activity was determined with the use of a radiometric assay as reported previously (29).

Statistical analysis

Baseline characteristics were compared between cases and controls taking into account matching between groups. Conditional logistic regression was used for categorical variables and a mixed-effect model was used for continuous variables. Variables with a skewed distribution were log-transformed before being used as continuous variables in analyses but untransformed medians and corresponding interquartile ranges are shown in tables. To determine relationships of IgG and IgM IC and AA with cardiovascular risk factors, we calculated mean risk factor levels per tertile of IC or AA. In addition, odds ratios (OR) and corresponding 95% confidence intervals (CI) as an estimate of the relative risk of incident CAD events were calculated using conditional logistic regression. Analyses took into account the matching for sex, age, and enrollment time and adjusted for diabetes mellitus, smoking (never, previous, current), systolic blood pressure, LDL-C, and HDL-C. Similar analyses were performed that adjusted for the Framingham Risk Score (FRS) instead of the separate risk factors. Analyses were performed for men and women separately and in addition, for sexes pooled. Additional analyses were performed by 3 × 3 analyses according to tertiles of IgG and IgM AA and IC and tertiles of OxPL/apoB, Lp(a), CRP, $sPLA_2$ mass and activity, Lp-PLA_2 activity, and myeloperoxidase mass. Tertiles provided the best balance between discrimination and statistical power. Correlations were computed using Spearman's rank-order method to avoid distributional assumptions and $P < 0.001$ was considered significant to account for multiple comparisons. Statistical analyses were performed using SPSS software (version 12.0.1, Chicago, IL). $P < 0.05$ was considered significant.

RESULTS

Study population

Compared with controls, cases had a higher occurrence of all traditional cardiovascular risk factors, OxPL/apoB, Lp(a), $sPLA_2$ mass and activity, Lp-PLA_2 activity, hsCRP, and myeloperoxidase than controls. IgG IC were higher in cases than controls ($P = 0.02$) but there were no significant

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differences in IgM IC or IgG and IgM AA between cases and controls (**Table 1**).

Relationship between IgG and IgM AA and IC levels and metabolic syndrome criteria and FRS

There was a statistically significant inverse association between the number of individual components of the metabolic syndrome [waist circumference ⩾ 102 cm or 40 inches (male), ⩾ 88 cm or 36 inches(female), TG ⩾ 1.7 mmol/L (150 mg/dl), HDL-C < 40 mg/dl (male), < 50 mg/dl (female), blood pressure ⩾ 130/85 mmHg, fasting plasma glucose ⩾ 6.1 mmol/L or 110 mg/dl)] and tertiles of IgM IC (T1: 2.56 ± 1.02, T2: 2.48 ± 1.01, and T3: 2.30 ± 1.08, $P = 0.004$) and IgM AA (T1: 2.54 ± 1.04, T2: 2.49 ± 1.03, and T3: 2.32 ± 1.06, $P = 0.02$), whereas tertiles of IgG AA and IC were not significantly associated.

Similarly, FRS 10 year estimates were lower with increasing tertiles of IgM IC (T1: 21.4% ± 11.8, T2: 19.5% ± 11.7, and T3: 18.0% ± 10.8, $P = 0.001$) and IgM AA (T1: 21.2% ± 12.2, T2: 19.4% ± 11.0, and T3: 18.3% ± 11.0, $P = 0.005$) were associated with lower calculated. Tertiles of IgG IC and IgG AA were not associated with FRS.

Associations between IgG and IgM IC and AA with demographics, lipoproteins, and biochemical parameters

Covariates were analyzed by tertiles of IgG and IgM IC and AA and only variables with $P < 0.001$ are reported as significant. IgG IC were positively associated with fibrinogen levels and inversely with HDL-C and plasma vitamin C levels. IgM IC was inversely associated with age and triglyceride levels. IgG AA were inversely associated with total cholesterol, LDL-C, VLDL-C, and HDL-C. IgM AA were inversely associated with lipoprotein lipase mass (30).

Relationship between IgG and IgM AA and IC and CAD events

There were no significant associations between tertiles of IgM AA {T1 [OR (95% CI)]: 1.0 (reference), T2: 1.01 (0.81–1.26), T3: 0.91 (0.72–1.15), p-linearity = 0.6}, IgG AA [T1: 1.0, T2: 0.80 (0.64–1.00), T3: 0.94 (0.75–1.18), p-linearity = 0.4)], IgG IC [(T1: 1.0, T2: 0.89 (0.71–1.12), T3: 1.03 (0.82–1.29), p-linearity = 0.7)], and IgM IC [T1: 1.0, T2: 0.99 (0.79–1.24), T3: 0.83 (0.65–1.04), p-linearity = 0.9)] and risk of CAD events.

Relationship between IgG and IgM AA and IC, other oxidative and inflammatory biomarkers, and CAD risk

The relationships between IgG and IgM AA and IC, other biomarkers, and CAD risk were evaluated by three separate statistical analyses in 3 × 3 tables: *1*) linear trends across tertiles; *2*) odds ratios for individual cells compared with the reference cell, which is Tertile 1 for both biomarkers (i.e., top left cell labeled "Reference" which is set by definition to an OR of 1.00), and *3*) interaction tests.

For linear trends across tertiles, there was a linear inverse association between IgM MDA-LDL and risk of CAD events in the highest tertiles of OxPL/apoB (p for linear trend 0.045) and Lp(a) ($P = 0.04$) and between IgM IC levels and CAD risk in the highest tertiles of OxPL/apoB ($P = 0.006$) and Lp(a) ($P = 0.04$) but not in the other tertiles (**Table 2**). A similar inverse association was seen in the subgroup with low CRP levels ($P = 0.02$) but not in any of the other subgroups (Table 2). There were no significant

TABLE 1. Baseline characteristics of the study population

	Controls	Cases	*p*
N	1723	748	
Age, years	65.4 ± 7.8	65.4 ± 7.8	matched
Male % (number)	61.6 (1061)	62.8 (470)	matched
Body mass index, kg/m^2	26.2 ± 3.5	27.3 ± 3.8	<0.001
Waist, cm	91 ± 11	94 ± 12	<0.001
Systolic blood pressure, mmHg	139 ± 18	143 ± 19	<0.001
Diastolic blood pressure, mmHg	83 ± 11	86 ± 12	<0.001
Smoking history			
Current smoker	8.6 (148)	15.5 (116)	
Former smoker	50.8 (876)	51.5 (385)	
Never smoked	40.6 (699)	33.0 (247)	
Total cholesterol, mmol/l	6.3 ± 1.1	6.5 ± 1.2	<0.001
LDL cholesterol, mmol/l	4.1 ± 1.0	4.3 ± 1.1	<0.001
HDL cholesterol, mmol/l	1.4 ± 0.4	1.3 ± 0.4	<0.001
Triglycerides, mmol/l	1.6 (1.2–2.2)	1.9 (1.4–2.6)	<0.001
OxPL/apoB, RLU	1673 (1144–2676)	1902 (1243–3247)	<0.001
Lp(a), mg/dl	8.4 (6.5–13.8)	9.7 (7.0–25.6)	<0.001
$sPLA_2$ mass, ng/ml	10.6 ± 8.4	12.5 ± 10.7	<0.001
$sPLA_2$ activity, nmol/min per ml	4.5 ± 1.2	4.8 ± 1.5	<0.001
Lp-PLA_2 activity, nmol/min/ml	50.6 ± 15.7	53.0 ± 16.5	0.001
hsCRP, mg/dl	1.5 (0.7–3.1)	2.2 (1.0–5.0)	<0.001
Myeloperoxidase, pmol/L	520 (338–837)	553 (353–875)	0.007
IgG immune complexes, RLU	2787 ± 1249	2924 ± 1499	0.02
IgM immune complexes, RLU	1391 ± 1281	1333 ± 1072	0.3
IgG MDA-LDL, RLU	4855 ± 4547	5091 ± 5730	0.3
IgM MDA-LDL, RLU	12888 ± 8950	12845 ± 9310	0.9

Data are shown as n, % (n), mean± SD, or median (95% confidence interval). CI, confidence interval; Lp(a), lipoprotein(a); hsCRP, high sensitivity C-reactive protein; $sPLA_2$, secretory phospholipase A_2; Lp-PLA_2, lipoprotein-associated phospholipase A_2; OxPL/apoB, oxidized phospholipids on apolipoprotein B-100; RLU, relative light unit.

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associations in linear trends between levels of IgG MDA-LDL and risk of CAD (**Table 3**). However, there was a linear association between IgG IC levels and CAD risk in the second tertile of the CRP distribution ($P = 0.014$) and in the second Lp(a) tertile ($P = 0.04$) but not in the highest or lowest CRP or Lp(a) tertiles or in any of the other subgroups (Table 3).

In the 3 × 3 tertile analysis in Tables 2 and 3, the reference cell is compared with the other cells. Significance can be read from the 95%CIs; if the 95%CI does not cross 1, then the OR in that cell is significantly different from the reference cell. Compared with the reference cell, the ORs for CAD were generally significantly higher in the cells with the lowest tertiles of IgM MDA-LDL and the highest tertiles of all the other biomarkers except MPO (Table 2). Similar findings were present for IgM IC. In contrast, compared with the reference cell, the ORs for CAD were generally significantly higher in the

TABLE 2. Odds ratios (95% CI) for CAD events according to tertiles of IgM MDA-LDL and IgM IC and selected biomarkers

		IgM MDA-LDL		
		T1	T2	T3
OxPL/apoB	T1	Reference	1.01 (0.69–1.47)	0.74 (0.48–1.14)
	T2	1.04 (0.70–1.55)	1.16 (0.79–1.70)	1.25 (0.85–1.84)
	T3	1.69 (1.16–2.48)	1.57 (1.06–2.33)	1.28 (0.88–1.85)
Lp(a)	T1	Reference	0.92 (0.63-1.35)	0.78 (0.52–1.19)
	T2	0.85 (0.57–1.26)	1.13 (0.78–1.64)	1.25 (0.84–1.84)
	T3	1.86 (1.28–2.71)	1.71 (1.17–2.51)	1.26 (0.86–1.84)
$sPLA_2$ mass	T1	Reference	1.01 (0.68–1.50)	0.88 (0.58–1.33)
	T2	1.23 (0.83–1.82)	1.45 (0.99–2.12)	1.18 (0.78–1.77)
	T3	2.04 (1.37–3.03)	1.67 (1.12–2.48)	1.65 (1.11–2.45)
$sPLA_2$ activity	T1	Reference	0.94 (0.62–1.44)	0.87 (0.55–1.36)
	T2	1.26 (0.83–1.91)	1.51 (1.00–2.27)	1.15 (0.76–1.74)
	T3	2.13 (1.42–3.19)	2.05 (1.37–3.07)	2.08 (1.37–3.15)
Lp-PLA_2 activity	T1	Reference	0.95 (0.63–1.42)	0.91 (0.61–1.36)
	T2	1.27 (0.86–1.88)	1.47 (1.00–2.15)	1.10 (0.73–1.65)
	T3	1.54 (1.04–2.88)	1.40 (0.95–2.06)	1.45 (0.98–2.15)
hsCRP	T1	Reference	1.47 (1.00–2.16)	1.17 (0.78–1.76)
	T2	1.04 (0.69–1.56)	1.66 (1.13–2.43)	1.96 (1.32–2.89)
	T3	0.90 (0.60–1.35)	0.66 (0.43–1.01)	1.96 (1.33–2.88)
MPO	T1	Reference	1.02 (0.71–1.48)	0.79 (0.52–1.19)
	T2	1.05 (0.72–1.53)	1.30 (0.89–1.88)	0.85 (0.58–1.25)
	T3	1.22 (0.83–1.81)	0.96 (0.65–1.41)	1.29 (0.89–1.85)
		IgM IC		
		T1	T2	T3
OxPL/apoB	T1	Reference	0.78 (0.53–1.16)	0.83 (0.55–1.25)
	T2	1.04 (0.71–1.51)	1.09 (0.75–1.60)	1.16 (0.79–1.71)
	T3	1.72 (1.16–2.54)	1.76 (1.21–2.57)	0.99 (0.69–1.44)
Lp(a)	T1	Reference	0.73 (0.49–1.09)	0.74 (0.50–1.12)
	T2	0.83 (0.56–1.24)	0.96 (0.66–1.40)	1.15 (0.78–1.71)
	T3	1.78 (1.21–2.60)	1.76 (1.22–2.54)	0.97 (0.67–1.42)
$sPLA_2$ mass	T1	Reference	1.23 (0.83–1.84)	0.91 (0.59–1.40)
	T2	1.38 (0.94–2.03)	1.51 (1.02–2.23)	1.32 (0.87–2.00)
	T3	2.63 (1.75–3.96)	1.79 (1.20–2.68)	1.58 (1.06–2.36)
$sPLA_2$ activity	T1	Reference	0.83 (0.53–1.30)	1.09 (0.69–1.71)
	T2	1.53 (1.00–2.35)	1.47 (0.96–2.26)	1.03 (0.66–1.62)
	T3	2.03 (1.34–3.08)	2.84 (1.83–4.38)	1.84 (1.19–2.83)
Lp-PLA_2 activity	T1	Reference	0.73 (0.48–1.11)	0.92 (0.61–1.39)
	T2	1.30 (0.88–1.92)	1.35 (0.91–1.98)	0.94 (0.63–1.41)
	T3	1.33 (0.90–1.96)	1.63 (1.10–2.41)	1.13 (0.76–1.68)
hsCRP	T1	Reference	0.83 (0.55–1.24)	0.67 (0.43–1.04)
	T2	1.24 (0.83–1.85)	1.27 (0.86–1.89)	1.08 (0.71–1.64)
	T3	1.78 (1.20-2.64)	2.08 (1.41–3.08)	1.61 (1.08–2.38)
MPO	T1	Reference	1.05 (0.74–1.51)	0.77 (0.51–1.15)
	T2	1.15 (0.79–1.67)	1.09 (0.77–1.56)	0.96 (0.66–1.40)
	T3	1.35 (0.92–1.99)	1.23 (0.84–1.80)	1.03 (0.72–1.47)

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TABLE 3. Odds ratios (95% CI for CAD events according to tertiles of IgG MDA-LDL and IgG IC and tertiles of selected biomarkers

		IgG MDA-LDL		
		T1	T2	T3
OxPL/apoB	T1	Reference	0.69 (0.46–1.02)	0.83 (0.56–1.22)
	T2	1.16 (0.79–1.69)	0.91 (0.61–1.34)	1.25 (0.74–2.10)
	T3	1.34 (0.91–1.99)	1.24 (0.85–1.83)	1.43 (0.98–2.09)
Lp(a)	T1	Reference	0.75 (0.51–1.12)	1.03 (0.69–1.54)
	T2	1.34 (0.92–1.96)	1.01 (0.68–1.51)	0.90 (0.60–1.32)
	T3	1.57 (1.06–2.33)	1.36 (0.93–2.01)	1.87 (1.27–2.75)
$sPLA_2$ mass	T1	Reference	0.66 (0.44–0.98)	0.87 (0.58–1.31)
	T2	1.27 (0.77–1.65)	1.11 (0.76–1.61)	1.13 (0.77–1.65)
	T3	1.82 (1.23–2.69)	1.32 (0.91–1.94)	1.53 (1.05–2.23)
$sPLA_2$ activity	T1	Reference	1.07 (0.70–1.62)	0.84 (0.54–1.29)
	T2	1.66 (1.10–2.50)	1.13 (0.74–1.73)	1.23 (0.80–1.88)
	T3	2.22 (1.48–3.32)	1.59 (1.05–2.39)	3.06 (2.01–4.66)
Lp-PLA_2 activity	T1	Reference	0.87 (0.58–1.31)	1.26 (0.95–0.64)
	T2	1.93 (0.81–1.76)	1.17 (0.79–1.73)	1.44 (0.98–2.14)
	T3	1.81(1.24–2.66)	1.12 (0.76–1.64)	1.43 (0.96–2.14)
hsCRP	T1	Reference	0.82 (0.55–1.22)	0.70 (0.46–1.07)
	T2	1.29 (0.87–1.90)	0.95 (0.64–1.41)	1.47 (0.99–2.18)
	T3	2.08 (1.43–3.02)	1.62 (1.12–2.34)	1.90 (1.31–2.77)
MPO	T1	Reference	0.84 (0.58–1.21)	0.97 (0.65–1.45)
	T2	1.32 (0.91–1.91)	0.77 (0.53–1.29)	1.07 (0.74–1.53)
	T3	1.12 (0.76–1.64)	1.14 (0.79–1.65)	1.11 (0.78–1.58)
		IgG IC		
		T1	T2	T3
OxPL/apoB	T1	Reference	0.90 (0.60–1.35)	0.81 (0.54–1.21)
	T2	1.10 (0.74–1.62)	1.09 (0.73–1.63)	1.16 (0.77–1.75)
	T3	1.45 (0.97–2.17)	1.16 (0.78–1.72)	1.77 (1.20–2.59)
Lp(a)	T1	Reference	0.81 (0.54–1.2)	0.81 (0.54–1.2)
	T2	0.97 (0.65–1.45)	1.05 (0.80–1.73)	1.19 (0.80–1.77)
	T3	1.54 (1.03–2.31)	1.26 (0.86–1.86)	1.76 (1.20–2.57)
$sPLA_2$ mass	T1	Reference	0.92 (0.62–1.37)	0.99 (0.66–1.51)
	T2	1.25 (0.83-1.90)	1.21 (0.82–1.80)	1.42 (0.96–2.11)
	T3	2.03 (1.35-3.05)	1.48 (1.03–2.32)	1.79 (1.22–2.65)
$sPLA_2$ activity	T1	Reference	0.94 (0.61–1.46)	1.05 (0.67–1.61)
	T2	1.44 (0.95–2.19)	1.41 (0.91–2.13)	1.29 (0.83–1.98)
	T3	2.36 (1.55–3.59)	1.81 (1.19–2.13)	2.61 (1.74–3.93)
Lp-PLA_2 activity	T1	Reference	1.09 (0.72–1.64)	1.26 (0.84–1.89)
	T2	1.64 (1.09–2.46)	1.32 (0.87–2.00)	1.57 (1.05–2.35)
	T3	1.84 (1.24–2.75)	1.54 (1.04–2.31)	1.78 (1.18–2.68)
hsCRP	T1	Reference	0.67 (0.44–1.02)	0.68 (0.45–1.04)
	T2	1.05 (0.70–1.57)	1.17 (0.79–1.73)	1.18 (0.79–1.77)
	T3	1.71 (1.17–2.59)	0.98 (0.66–1.46)	2.09 (1.43–3.05)
MPO	T1	Reference	0.76 (0.51–1.12)	0.68 (0.46–1.01)
	T2	0.91 (0.62–1.33)	0.75 (0.52–1.11)	1.09 (0.74–1.61)
	T3	0.86 (0.58–1.27)	0.98 (0.66–1.46)	1.12 (0.77–1.64)

cells with the highest tertiles of IgM MDA-LDL and the highest tertiles of all the other biomarkers except MPO (Table 3).

For the interaction tests (i.e., does the level of one biomarker affect the association between another biomarker and risk), which are the most statistically stringent analyses and require substantial power, significance was present between IgM IC with Lp(a) ($P = 0.006$) and IgG MDA-LDL with $sPLA_2$ activity ($P = 0.018$) but not for the other sets of biomarkers.

DISCUSSION

This case-control study nested within the prospectively followed EPIC-Norfolk cohort, which represents the highest powered study for CAD events, demonstrated that baseline levels of AA to MDA-LDL and IC are not independently associated with higher risk of fatal and nonfatal CAD. However, they appear to modify the CAD risk of OxPL/apoB, Lp(a), and $sPLA_2$ activity and may be potentially integrated into the risk of other oxidative and inflammatory

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biomarkers. Increased IgM AA and IC levels were also associated with lower number of metabolic syndrome criteria and lower FRS. Overall, these data suggest that IgG and IgM AA and IC may potentially be used in fine tuning risk prediction of other pathophysiologically related biomarkers (15, 16, 24, 26, 31), particularly those that are targets of therapy in clinical trials such as $sPLA_2$ (28).

EPIC-Norfolk represents the most highly powered study in this arena to date in an unselected, initially healthy population and showed no independent association of AA and IC with CAD events. Although many human studies have been published correlating AA and IC to oxidation-specific epitopes to anatomic coronary and carotid and peripheral arterial disease (8, 14), the focus of the current analysis was CAD risk prediction. Relatively few studies have evaluated their prognostic value in predicting CAD events and the results have been inconsistent (6, 10, 12, 13, 32, 33). Initial studies have shown predictive value (10, 12, 13, 33), but more recent studies with appropriate size and multivariable adjustment have shown no independent predictive value (6, 32, 34). Some of the reasons for these disparate findings may be the lack of power, different methodologies for AA and IC measurement, measurement of either IgG of IgM but not both classes, and heterogeneous patient cohorts. The findings from this study are similar to our previous study of 504 patients initially designed to assess the presence of CAD in patients undergoing clinically indicated coronary angiography (6). In a substudy, patients were followed for cardiovascular events (n = 50 events, ~50% were revascularizations) for a median of 4 years. In univariate analysis, IgM OxLDL AA and IgM IC were inversely associated with the presence of angiographically determined CAD, whereas IgG OxLDL AA and IgG IC were positively associated. After statistical adjustment with multivariable analysis for cardiovascular risk factors, neither IgM or IgG AA nor IC was independently associated with angiographically determined CAD or cardiovascular events. In the MIRACL trial, which, unlike EPIC-Norfolk, included patients presenting with acute coronary syndromes, higher baseline levels of IgM IC were an independent predictor of subsequent events at 16 weeks but AA to OxLDL and MDA-LDL were not predictive of future events. Recently, several studies have been published suggesting that low IgM levels to phosphocholine-BSA are associated with cardiovascular events (35). However, further validation of this assay is needed as several studies showed no differences in phosphocholine-BSA titers between cases versus controls, and significant associations were noted only with post hoc analyses.

These studies do not rule out a modulating effect of AA and IC on other risk factors or risk markers integral in atherogenesis. For example, we observed an inverse association between levels of both IgM MDA-LDL and IgM IC and risk of CAD events in the subgroups with high levels of OxPL/apoB and Lp(a). These data suggest that levels of IgM MDA-LDL and IgM IC may modulate the proatherogenic effects associated with markers of oxidation such as OxPL/apoB and Lp(a). Although formal interaction tests were only significant between IgM IC with Lp(a), this stringent statistical test requires substantial statistical power to show differences in interaction terms. Therefore, a potential atherosclerosis-modulating effect of IgM AA and IC should be considered hypothesis generating and is amenable to hypothesis testing in future clinical studies.

Further novel insights derived from this study are the observations that IgM AA and IC are inversely related to the metabolic syndrome and FRS. Although it is not possible to ascribe a causal association in this relationship, one may speculate that patients with high baseline IgM, perhaps mediated through genetic or other factors, may be protected through undefined mechanisms from developing modifiable cardiovascular risk factors. This is consistent with prior data showing that IgM AA are higher in patients considered at lower cardiovascular risk, such as women, nonsmokers, and younger subjects, and are decreased in men, elderly subjects, diabetics, and patients with elevated LDL-C (8, 34, 36, 37). Alternatively, it is also possible that some patients consume such preformed antibodies as atherosclerosis develops, which are ultimately cleared leading to lower plasma levels.

In hypercholesterolemic animal models with homogeneous populations and well-defined atherosclerosis-inducing regimens, AA and IC reflected the extent, as well as the progression and regression, of changes in atherosclerotic lesions induced by dietary and anti-oxidant interventions [reviewed in (8)]. Furthermore, they reflect the response to a variety of immune modulating effects including immunizations with autologous LDL that is oxidatively modified (2), apoB peptides (38), and phosphocholine antigens (PC-KLH) (39). The potential atheroprotective role of human IgM AA and IC is consistent with several complementary animals studies showing the atheroprotective role of natural IgM antibodies (2), such as E06/T15, and the key role of B-1 cell IgM production as manifested by accelerated atherosclerosis in IgM-deficient mice (40). Furthermore, our group has demonstrated that oxidation-specific epitopes constitute a dominant, previously unrecognized target of IgM natural antibodies in both mice and humans and that ~30% of all natural antibodies bind to model oxidation-specific epitopes, atherosclerotic lesions, and apoptotic cells (41). Because oxidative processes are ubiquitous, we hypothesized that these epitopes exert selective pressure to expand natural antibodies, which in turn play an important role in mediating homeostatic functions consequent to inflammation and cell death as demonstrated by their ability to facilitate apoptotic cell clearance.

In conclusion, this epidemiological cohort of initially healthy subjects shows that IgG and IgM AA and IC are not independent predictors of CAD events. However, there was some evidence that higher levels of IgM MDA-LDL and IgM IC may attenuate the proatherogenic effect of oxidative markers such as OxPL/apoB, Lp(a), and $sPLA_2$ activity, suggesting a potential disease-modifying role in atherogenesis.

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