High Levels of (Un)Switched Memory B Cells Are Associated With Better Outcome in Patients With Advanced Atherosclerotic Disease

John A. L. Meeuwsen, MSc; Amerik van Duijvenvoorde, MSc; Aisha Gohar, MD; Maria O. Kozma, BSc; Sander M. van de Weg, BSc; Crystel M. Gijsberts, MD, PhD; Saskia Haitjema, MD, PhD; Harry Björkbacka, PhD; Gunilla N. Fredrikson, PhD; Gert J. de Borst, MD, PhD; Hester M. den Ruijter, PhD; Gerard Pasterkamp, MD, PhD; Christoph J. Binder, MD, PhD; Imo E. Hoefer, MD, PhD; Saskia C. A. de Jager, PhD

***Background*—**Atherosclerosis is an inflammatory lipid disorder and the main underlying pathology of acute ischemic events. Despite a vast amount of data from murine atherosclerosis models, evidence of B-cell involvement in human atherosclerotic disease is limited. We therefore investigated the association of circulating B-cell subtypes with the occurrence of secondary cardiovascular events in advanced atherosclerotic disease.

***Methods and Results*—**This cohort study consists of 168 patients who were included in the Athero-Express biobank between 2009 and 2011. Before surgery, peripheral blood mononuclear cells were isolated and stored in liquid nitrogen. After gentle thawing of the peripheral blood mononuclear cells, different B-cell subtypes including naïve, (un)switched memory, and $CD27^{+}CD43^{+}$ B1-like B cells, were analyzed by flow cytometry. Univariable and multivariable Cox proportional hazard models were used to analyze associations between B-cell subtypes, circulating antibodies and secondary cardiovascular manifestations during the 3-year follow-up period. Mean age was 70.1±9.6 years, males represented 62.8% of the population, and 54 patients had secondary manifestations during follow-up. High numbers of unswitched memory cells were protective against secondary outcome (hazard ratio, 0.30 [95% CI, 0.13–0.69]; $P<0.01$). Similar results were obtained for the switched memory cells that also showed to be protective against secondary outcome (hazard ratio, 0.33 [95% CI, 0.14–0.77]; $P=0.01$).

***Conclusions*—**A high number of (un)switched memory B cells is associated with better outcome following carotid artery endarterectomy. These findings suggest a potential role for B-cell subsets in prediction and prevention of secondary cardiovascular events in patients with atherosclerosis. **(*J Am Heart Assoc.* 2017;6:e005747. DOI: 10.1161/JAHA.117.005747.)**

Key Words: atherosclerosis • B lymphocytes • carotid endarterectomy • recurrent event

Although the prevention of cardiovascular disease (CVD) has improved in the past decades, it remains one of the major causes of death worldwide.[1] Its main underlying pathology, atherosclerosis, is an inflammatory lipid disorder and the major cause of acute cardiovascular syndromes.[2–4] Many inflammatory cell types, including monocytes, macrophages, mast cells, neutrophils, and T and B cells, have been implicated in the initiation, progression, and destabilization of atherosclerosis.[5] Increased insights into how these inflammatory cells are involved in CVD may lead to the identification of novel biomarkers or therapeutic targets for primary or secondary manifestations of CVD.

The risk of cardiovascular events is particularly high in patients with earlier CVD manifestations.[6,7] For example, the 3-year cumulative incidence of major adverse cardiovascular events in patients undergoing carotid endarterectomy was 13%.[8] Increased inflammation is an important risk factor for recurrent CVD events.[2] For example, different studies showed that high white blood cell counts are associated with the recurrence of CVD events and mortality.[9–13] These studies,

From the Laboratory for Experimental Cardiology (J.A.L.M., A.v.D., A.G., S.M.v.d.W., C.M.G., S.H., H.M.d.R., G.P., I.E.H., S.C.A.d.J.), Department of Vascular Surgery (G.J.d.B.), Laboratory for Clinical Chemistry and Haematology (G.P., I.E.H.), and Laboratory of Translational Immunology (S.C.A.d.J.), University Medical Center Utrecht, Utrecht, the Netherlands; Department of Laboratory Medicine, Medical University of Vienna, Austria (M.O.K., C.J.B.); CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria (M.O.K., C.J.B.); Department of Clinical Sciences, Skåne University Hospital Malmö, Lund University, Malmö, Sweden (H.B., G.N.F.).

Correspondence to: Saskia C. A. de Jager, PhD, Laboratory for Experimental Cardiology, University Medical Center Utrecht, Heidelberglaan 100, 3584CX Utrecht, the Netherlands. P.O. Box 85500, 3508 GA Utrecht, the Netherlands. E-mail: S.C.A.dejager@umcutrecht.nl

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Clinical Perspective

What Is New?

- We show an extensive flow cytometry profiling of B cells in severe cardiovascular patients and associate this profile to risk for future events.
- We show, for the first time, that both unswitched and switched memory B cells associate with better outcome in patients with existing cardiovascular disease.
- Oxidized low-density lipoprotein low-density lipoprotein IgG autoantibody titers strongly correlate to memory B-cell numbers, but oxidized low-density lipoprotein low-density lipoprotein autoantibodies were not predictive for future events.

What Are the Clinical Implications?

- These findings indicate that memory B cells might have value in prediction and prevention of adverse secondary cardiovascular manifestations.

however, mainly consider total white blood cell count or total lymphocyte counts and do not provide additional information regarding cell-specific subtypes.

Besides their well-known role in humoral immunity through antibody production, B cells are important for T-cell activation and cytokine production in maintaining immune homeostasis.[14] The total peripheral B-cell pool in humans mainly consists of naïve B cells, CD43$^+$ B1-like cells,[15–17] unswitched and switched memory cells (mainly expressing immunoglobulin [Ig] M,[18] or IgG and IgA antibodies, respectively[18]), regulatory B cells, and plasma cells. As such, B cells are important in immune homeostasis, but have also been shown to be detrimental in autoimmune diseases like common variable immunodeficiency and systemic lupus erythematosus.[19,20] Interestingly, patients with these B-cell-driven autoimmune diseases are also at high risk for CVD.[19,20]

Evidence for an important role of B lymphocytes in human CVD is limited. In patients with acute myocardial infarction (MI), high levels of the B-cell specific cytokines, chemokine (C-C motif) ligand 7 and B-cell activating factor, predict increased risk of death and recurrent MI.[21] In addition, hypertensive patients with high percentages of CD40$^+$ B cells were at lower risk for stroke,[22] whereas high numbers of CD86$^+$ B cells showed higher risk for stroke.[22] A larger body of evidence is derived from elaborate mouse studies, which identified a subset-specific role for B lymphocytes in atherosclerosis.[23–25] To date, it is generally accepted that (auto)antibody-producing B1 B cells are atheroprotective[26,27] and this protective effect depends on IgM secretion,[28] whereas conventional B2 B cells are generally considered proatherogenic.[29] This proatherogenic phenotype in mice has mainly been attributed to the production of pathogenic IgG antibodies against oxidized low-density lipoprotein (oxLDL) and the immune effector function of these B cells.[27,28,30–33] In human atherosclerosis, however, there is conflicting evidence about the role of autoantibodies directed against oxLDL. On the one hand, oxLDL antibodies have been associated with the presence[34] and progression[35–37] of atherosclerosis and the risk for MI. On the other hand, anti-oxLDL autoantibodies have also been associated with lower oxLDL levels[38] and decreased carotid atherosclerosis as well.[39] In addition, vaccination with a pneumococcal vaccine, mimicking oxLDL epitopes, was shown to attenuate atherosclerotic lesion formation in mice[40] and, according to a recent meta-analysis, also prevents CVD in adults.[41]

Taken together, accumulating evidence points to an important role of B cells in CVD, but human evidence is limited. In order to establish whether a specific B-cell profile is associated with secondary cardiovascular events, we measured circulating B-cell subtypes, including naïve, CD43$^+$ B1-like, unswitched, and switched memory B cells, as well as oxLDL antibodies, in severe atherosclerotic patients derived from the Athero-Express biobank. B-cell subtypes were identified by flow cytometry and associated with the occurrence of secondary cardiovascular manifestations during follow-up after carotid endarterectomy.

Materials and Methods

Study Population

This study includes a subcohort of 168 patients from the Athero-Express biobank, a cohort study of patients undergoing carotid endarterectomy that were included between 2009 and 2011.[42] In addition to the standard procedure, including an extensive patient questionnaire and detailed histological plaque characterization, peripheral blood mononuclear cells (PBMCs) were isolated from blood that was drawn preoperatively. Isolated PBMCs were stored in liquid nitrogen until further analyses were performed. Patient follow-up duration was 3 years or until the occurrence of secondary cardiovascular events (cardiovascular death, stroke, MI, coronary intervention, peripheral intervention [including amputation], or a combination). All events were validated using health records kept by general practitioners. All patients provided written informed consent. The study protocol conforms to the Declaration of Helsinki and has been approved by the Institution's ethics committee on research on humans.

Blood Collection and PBMC Isolation

Twenty milliliters of blood were collected in Li-Heparin blood tubes. A complete blood count profile was determined by a

Table 1. Antibody Characteristics

Marker	Fluorochrome	Clone	µL
IgD	AF488	IA6-2	2.5
CD24	PE	ML-5	2.5
CD19	PE-Cy5	HIB19	2.5
CD38	PE-Cy7	HIT2	2.5
CD43	APC	10G7	2.5
IgM	APC-Cy7	MHM-88	5
CD27	PB	0323	1
CD3	BV510	OKT3	1

Panel of antibodies used. All antibodies are from BioLegend (San Diego, CA). AF indicates Alexa Fluor; APC, allophycocyanin; BV, brilliant violet; Ig, immunoglobulin; PB, pacific blue; PE, (R-)phycoerythrin.

general hematology cell counter (Cell Dyn 1800; Abbott Laboratories, Abbott Park, IL). Directly after collection, the platelet-rich plasma fraction was isolated by centrifugation for 10 minutes at 150*g* at room temperature without brake. The blood volume was restored to its original volume with PBS. Subsequently, blood was gently layered on a Ficoll (17-1440-03; GE Healthcare, Chalfont St. Giles, UK) loaded Leucosep tube (227 290; Greiner bio-one, Alphen aan den Rijn, The Netherlands) and centrifuged at 1000*g* for 15 minutes at room temperature without brake. PBMCs were carefully isolated from the interphase. To remove any residual Ficoll, PBMCs were washed with cold PBS, centrifuged at 330*g* for 10 minutes at 4°C with brake, and resuspended in 1 mL of sterile, serum-free cell freezing medium with DMSO (C6295; Sigma-Aldrich, St. Louis, MO). PBMCs were slowly frozen overnight at −80°C using a Nalgene freezing container and stored in liquid nitrogen until further analyses were performed.

Flow Cytometry

PBMCs were gently thawed and washed with RPMI 1640 ([61870010; Gibco Carlsbad, CA] supplemented with Gluta-Max, 25 nmol/L HEPES, 1% penicillin/streptomycin and 2% FBS [10270-106; Gibco, Carlsbad, CA]). Cells were kept on ice during the whole procedure, unless stated otherwise. To obtain single-cell suspensions, PBMCs were gently filtered over a 40-µm cell strainer (542040; Greiner bio-one), washed

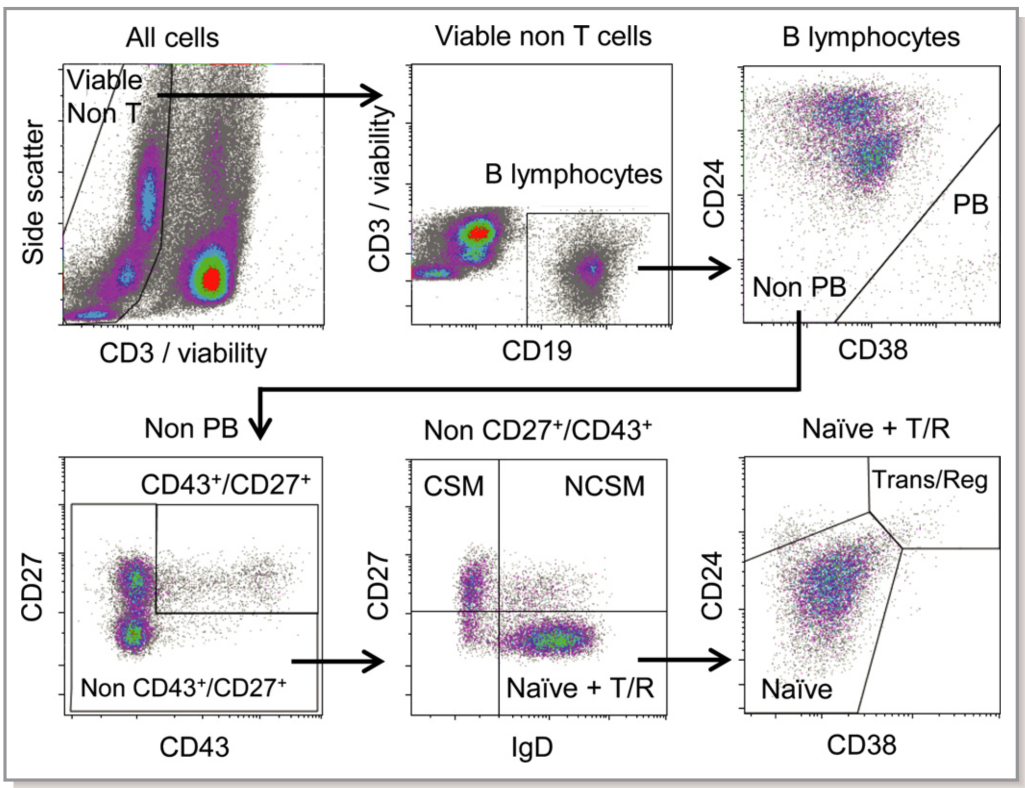

Figure 1. Gating strategy for the selection of different B-cell subtypes from a representative sample. First, dead cells and CD3$^+$ T cells were excluded. Next, from the viable non-T cells, the CD19$^+$ B cells were identified. Then, the CD24^{low}CD38$^+$ plasmablasts (PB) were excluded, and from the non-PB, the CD43$^+$CD27$^+$ cells were selected. Next, based on surface expression of CD27 and IgD, class-switched (CSM) and nonclass switched memory cells (NCSM) were identified. From the IgD$^+$CD27$^-$ cells, the CD38$^+$CD24$^+$ transitional and regulatory (Trans/Reg) could be distinguished from the naïve B cells. An overview of the antibody characteristics is provided in Table 1. Ig indicates immunoglobulin.

Table 2. Baseline Characteristics of the Study Cohort Stratified by Occurrence of Secondary Cardiovascular Events

	Overall (n=168)	No Events (n=114)	Events (n=54)	*P* Value
Age, y	70.1±9.6	69.8±9.3	70.9±10.3	0.50
Sex (% male)	108 (62.8)	72 (61.5)	36 (65.5)	0.74
Current smoker	61 (35.7%)	41 (35.3%)	20 (36.4%)	1
BMI	26.7±4.6	26.7±4.7	26.7±4.4	1
Contralateral stenosis	72 (47.7%)	45 (44.6%)	27 (54.0%)	0.36
Diabetes mellitus	41 (23.8%)	28 (23.9%)	13 (23.6%)	1
Hypercholesterolemia	106 (71.6%)	74 (70.5%)	32 (74.4%)	0.78
Hypertension	149 (86.6%)	100 (85.5%)	49 (89.1%)	0.68
CAD history	62 (36.0%)	41 (35.0%)	21 (38.2%)	0.82
Clinical manifestations				0.26
Asymptomatic	25 (14.5%)	13 (11.1%)	12 (21.8%)	
Ocular	29 (16.9%)	19 (16.2%)	10 (18.2%)	
Stroke	42 (24.4%)	31 (26.5%)	11 (20.0%)	
TIA	76 (44.2%)	54 (46.2%)	22 (40.0%)	
Medication				
Statins	138 (80.2%)	92 (78.6%)	46 (83.6%)	0.57
Beta-blockers	82 (47.7%)	53 (45.3%)	29 (52.7%)	0.46
Anticoagulants	11 (6.4%)	5 (4.3%)	6 (10.9%)	0.11
Laboratory parameters				
Total cholesterol, mmol/L	4.0 [3.3, 4.8]	4.0 [3.4, 4.7]	4.0 [3.3, 4.8]	0.75
Triglycerides, mmol/L	1.5 [1.1, 2.0]	1.4 [1.1, 1.9]	1.5 [1.1, 2.0]	0.54
HDL cholesterol, mmol/L	1.0 [0.9, 1.3]	1.0 [0.9, 1.2]	1.0 [0.9, 1.3]	0.15
LDL cholesterol, mmol/L	2.1 [1.6, 2.8]	2.2 [1.8, 2.9]	2.1 [1.6, 2.8]	0.20
hsCRP plasma, μg/mL	16.0±78.4	17.2±90.7	13.3±37.1	0.78
GFR MDRD, mL/min	69.5±18.3	70.5±17.4	67.3±20.2	0.31
WBC count ($\times 10^6$ cells/mL)	7.2 [5.5, 8.6]	7.0 [5.5, 8.9]	7.4 [5.6, 8.2]	0.99
Lymphocytes ($\times 10^6$ cells/mL)	1.5 [1.2, 2.1]	1.6 [1.2, 2.2]	1.4 [1.1, 1.9]	0.09
Monocytes ($\times 10^6$ cells/mL)	0.8 [0.6, 0.9]	0.8 [0.6, 1.0]	0.8 [0.6, 0.9]	0.59
Granulocytes ($\times 10^6$ cells/mL)	4.6 [3.4, 6.1]	4.4 [3.3, 6.1]	5.1 [3.5, 6.0]	0.34

The demographic characteristics of the study cohort are given for the whole population and also stratified by occurrence of secondary cardiovascular events. Values are presented as mean±SD for normal distributions, number of patients (frequency in percentage) for categorical variables, and median [interquartile range] for non-normal distributions. *P* values are calculated using Student *t* tests, chi-square or Fisher's exact tests, and Kruskal–Wallis tests, respectively. BMI indicates body mass index; CAD history, history of Coronary Artery Disease; Contralateral stenosis, 50% to 100% stenosis of the contralateral carotid artery; GFR MDRD, glomerular filtration rate according to the Modification of Diet in Renal Disease formula; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; TIA, transient ischemic attack; WBC, whole blood cell.

with RPMI again, and centrifuged at 350*g* for 5 minutes at 4°C. Subsequently, cells were resuspended in cold PBS (supplemented with 2% FBS and 20 mmol/L of EDTA), centrifuged at 350*g* for 5 minutes at 4°C, and resuspended in cold PBS with 1% BSA. Subsequently, cells were incubated with antibodies (Table 1) for 30 minutes at room temperature in the dark, washed with PBS (4°C), and centrifuged at 350*g* for 5 minutes at 4°C. Next, cells were incubated for 30 minutes with fixable viability dye eFluor-506 (eBioscience, San Diego, CA), washed, centrifuged, and measured on the flow cytometer (Gallios; Beckman Coulter, Fullerton, CA). Analysis of the flow cytometry data was performed using Kaluza 1.3 software. We selected viable $CD19^+CD3^-$ lymphocytes, excluded plasmablasts ($CD24^-CD38^+$; Figure 1), and gated $CD43^+CD27^+$ cells, which are suggested to resemble B1 B cells.[15] Next, we selected unswitched memory cells ($CD27^+CD43^-IgD^+$) and switched memory cells ($CD27^+CD43^-IgD^-$). From the $CD27^-IgD^+$ B cells, we selected the naïve $CD24^+CD38^+$ B cells (Figure 1). Absolute B-cell numbers were calculated from the ratio measured by

Table 3. Baseline Numbers of Total B Cells and B-Cell Subtypes Stratified By Occurrence of Secondary Cardiovascular Events

B-cell (Sub)Type (cells/μL)	Surface Markers	Overall (n=168)	No Events (n=114)	Events (n=54)	*P* Value
B lymphocytes	$CD19^{+}CD3^{-}$	193 [121, 323]	209 [122, 415]	167 [115, 236]	0.04
B1 like	$CD27^{+}CD43^{+}$	8 [3, 15]	9 [4, 15]	7 [3, 13]	0.39
Unswitched	$CD27^{+}CD43^{-}IgD^{+}$	10 [5, 21]	12 [6, 25]	8 [4, 12]	<0.01
Switched	$CD27^{+}CD43^{-}IgD^{-}$	26 [15, 47]	27 [16, 54]	21 [13, 34]	0.02
Naïve	$CD27^{-}CD43^{-}IgD^{+}$	102 [58, 184]	106 [62, 213]	89 [56, 141]	0.17

The numbers of B cells are presented for the whole cohort and also stratified by occurrence of secondary cardiovascular events during follow-up. Absolute numbers of B cells and B-cell subtypes with their surface markers are indicated as median [interquartile range]. *P* values were calculated using the Kruskal–Wallis test for nonparametric distributions.

flow cytometry multiplied by the absolute number of lymphocytes obtained from the hematology cell counter.

Anti-oxLDL Antibody Measurements

Serum levels of IgM and IgG-α-oxLDL antibodies were measured as described previously.[40,43] In short, serum samples were diluted 500 times for IgM and 2000 times for IgG-α-oxLDL antibody measurements. Antibody titers were determined by chemiluminescent enzyme immunoassays, and values are presented as relative light units per 100 ms.

Statistical Analysis

Normally distributed continuous variables are indicated as means±SDs and compared by Student's *t* tests or a 1-way ANOVA. Non-normal distributed data are presented as medians (interquartile ranges; IQRs) and were compared by Kruskal–Wallis tests. Categorical variables were indicated as percentages and compared by chi-square or Fisher's exact tests where appropriate. As confounders, we selected variables that associate with CVD risk, but also influence B-cell numbers and have been established as confounders in literature, including age, sex, smoking, history of coronary artery disease, and glomerular filtration rate.[22,44–47] We also tested for a sex interaction between the association of B cells and cardiovascular end points.

Univariable and multivariable Cox proportional hazard models were used to study the association of B-cell subtypes and anti-oxLDL antibodies with occurrence of secondary cardiovascular events over time. Next, to visualize this association, subjects were divided into tertiles according to the absolute numbers of B-cell subtypes and plotted against the occurrence of secondary cardiovascular events over time. Data management and statistical analyses were performed with RStudio[48] and the R software package[49] (version 3.2.0.; R Foundation for Statistical Computing, Vienna, Austria). $P<0.05$ was considered as significant.

Results

Clinical Characteristics

Baseline characteristics are shown in Table 2, stratified into patients with and without secondary cardiovascular events. Of the included 168 patients, 54 experienced secondary cardiovascular events during follow-up, which included cardiovascular death (n=11), stroke (n=13), coronary events (n=11), and peripheral intervention (n=35). Risk factors for the presence of CVD, such as age, sex, smoking, and kidney function (glomerular filtration rate), did not differ between patients with or without secondary events (Table 2).

Total B Cells and (Un)Switched Memory B Cells Are Higher in Patients Without Secondary Cardiovascular Events

We measured different B-cell subtypes using flow cytometry. Total B cell numbers (≈200 cells/μL) and B-cell subset numbers corroborate with earlier observations[50] (Table 3). Next, we investigated whether baseline levels of B cells differed between patients who developed secondary cardiovascular events during follow-up after endarterectomy (cases) compared to those without a secondary event (controls; Table 3). Total numbers of $CD19^{+}$ B cells (167 [IQR, 115–236] versus 209 [IQR, 121–415] cells/μL; $P=0.04$), unswitched memory cells (8 [IQR, 4–12] versus 12 [IQR, 6–25] cells/μL; $P<0.01$), and switched memory cells (21 [IQR, 13–34] versus 27 [IQR, 15–54] cells/μL; $P=0.02$) were lower in cases than in controls. Although numbers of naïve B cells or $CD43^{+}CD27^{+}$ B cells also tended to be higher, these differences were not statistically significant between groups. Baseline characteristics of patients stratified by B lymphocyte tertiles are shown in Table 4.

Associations of B-cell subtypes with univariate risk factors

We then investigated whether the numbers of B cells or B-cell subtypes were associated with cardiovascular risk factors. As

Table 4. Baseline Characteristics of the Study Cohort Stratified By Tertiles of Total B Lymphocytes

	Low (n=56)	Intermediate (n=56)	High (n=56)	*P* Value
B Lymphocytes (cells/μL)	96 [67, 121]	193 [168, 214]	430 [326, 506]	
Age, y	72.2±10.5	69.9±9.2	68.0±8.9	0.07
Sex (% male)	36 (64.3)	40 (71.4)	30 (53.6)	0.14
Current smoker	14 (25.5%)	21 (37.5%)	26 (46.4)	0.07
BMI	27.4±4.4	25.4±4.0	27.3±5.2	0.03
Contralateral stenosis	31 (59.6%)	18 (39.1%)	20 (40.8%)	0.07
Hypertension	49 (87.5%)	47 (83.9%)	49 (87.5%)	0.82
Diabetes mellitus	11 (19.6%)	11 (19.6%)	17 (30.4%)	0.30
Hypercholesterolemia	38 (77.6%)	27 (56.2%)	38 (79.2%)	0.02
CAD history	25 (44.6%)	17 (30.4%)	18 (32.1%)	0.23
Clinical manifestations				0.58
Asymptomatic	7 (12.5%)	9 (16.1%)	8 (14.3%)	
Occular	12 (21.4%)	11 (19.6%)	6 (10.7%)	
Stroke	10 (17.9%)	14 (25.0%)	17 (30.4%)	
TIA	27 (48.2%)	22 (39.3%)	25 (44.6%)	
Medication				
Statins	48 (85.7%)	42 (75.0%)	46 (82.1%)	0.34
Beta-blockers	30 (53.6%)	24 (42.9%)	25 (44.6%)	0.48
Anticoagulants	2 (3.6%)	7 (12.5%)	2 (3.6%)	0.11
Laboratory parameters				
Cholesterol, mmol/L	3.8 [3.2, 4.6]	4.1 [3.3, 4.9]	3.9 [3.4, 4.4]	0.71
Triglycerides, mmol/L	1.5 [1.2, 2.1]	1.4 [1.0, 1.9]	1.6 [1.2, 2.0]	0.30
HDL cholesterol, mmol/L	1.0 [0.9, 1.2]	1.0 [0.9, 1.3]	1.0 [0.9, 1.2]	0.72
LDL cholesterol, mmol/L	2.1 [1.6, 2.5]	2.1 [1.6, 3.1]	2.1 [1.6, 2.7]	0.60
hsCRP plasma, μg/mL	5.0 (15.7)	9.7 (29.6)	33.8 (130.8)	0.14
GFR MDRD, mL/min	66.0 (17.7)	74.8 (19.0)	68.8 (17.5)	0.04

The baseline characteristics are depicted for patients with low, intermediate and high numbers of total B lymphocytes. Values are presented as mean±standard deviation for normal distributions, number of patients (frequency in percentage) for categorical variables, and median [inter-quartile range] for non-normal distributions. *P*-values are calculated using a one-way ANOVA, Chi-square or Fisher's exact tests, and Kruskal–Wallis tests, respectively. BMI indicates body mass index; CAD history, history of Coronary Artery Disease; Contralateral stenosis, 50% to 100% stenosis of the contralateral carotid artery; GFR MDRD, Glomerular filtration rate according to Modification of Diet in Renal Disease formula; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; TIA, transient ischemic attack.

expected, total B-cell numbers decreased with age and were higher in women and in current smokers. Specific associations of each B-cell subtype with the cardiovascular risk factors are depicted in Table 5. We selected age, sex, smoking, history of coronary artery disease, and glomerular filtration rate as potential confounders, based on their established prognostic value in CVD.

Cox regression analysis of B cells with secondary cardiovascular events

Because of the known B-cell-related immunological differences between sexes, we tested sex interaction between the association of B cells and cardiovascular end points, but there was no *statistically significant* interaction. Univariable and multivariable Cox proportional hazard regression models were used to calculate the hazard ratios (HRs) for each B-cell subset (Table 6). Patients were then categorized into tertiles according to the absolute number of B cells and B-cell subsets. Interestingly, patients with high numbers of unswitched memory B cells were at lower risk of experiencing a secondary cardiovascular event during follow-up as compared with patients with low numbers (HR, 0.30 [95% confidence interval (CI), 0.13–0.69]; $P<0.01$; Figure 2). Likewise, patients in the highest tertile of switched memory cell numbers were at lower risk than patients in the lowest tertile (HR, 0.33 [95% CI, 0.14–0.77]; $P=0.01$). When we combined

Table 5. Univariable Associations of the Total B Cells and B-Cell Subtypes With Classical Cardiovascular Risk Factors

	B Lymphocytes (Cells/μL)		Naïve (Cells/μL)		Unswitched Memory (Cells/μL)		Switched Memory (Cells/μL)		$CD43^+CD27^+$ (Cells/μL)	
	Beta [95% CI]	*P* Value	Beta [95% CI]	*P* Value	Beta [95% CI]	*P* Value	Beta [95% CI]	*P* Value	Beta [95% CI]	*P* Value
Age, y	−3.14 [−6.35 to 0.07]	0.05	−0.49 [−2.83 to 1.85]	0.68	−0.5 [−0.78 to 0.22]	<0.01	−1.13 [−1.79 to 0.46]	<0.01	−0.4 [−0.61 to 0.19]	<0.01
Sex (male)	−74.19 [−137.92 to 10.47]	0.02	−55.49 [−101.4 to 9.58]	0.02	−1.74 [−7.49 to 4.02]	0.55	−5.35 [−18.98 to 8.28]	0.44	−0.4 [−4.78 to 3.97]	0.86
Current smoker	72.06 [7.83–136.3]	0.03	1.83 [−45.21 to 48.87]	0.94	9.94 [4.34–15.53]	<0.01	39.49 [27.14–51.84]	<0.01	3.39 [−0.98 to 7.77]	0.13
BMI	1.43 [−5.36 to 8.22]	0.68	1.63 [−3.26 to 6.52]	0.51	0.23 [−0.37 to 0.83]	0.45	−0.35 [−1.78 to 1.07]	0.63	0 [−0.45 to 0.46]	0.98
Contralateral stenosis	−24.9 [−85.71 to 35.91]	0.42	−10.17 [−57.28 to 36.93]	0.67	−5.22 [−11.3 to 0.85]	0.09	−3.14 [−12.63 to 6.34]	0.51	−1.35 [−6.02 to 3.32]	0.57
Diabetes mellitus	65.49 [−7.81 to 138.79]	0.08	53.35 [0.61–106.09]	0.05	0.89 [−5.7 to 7.47]	0.79	3 [−12.6 to 18.6]	0.70	1.84 [−3.15 to 6.83]	0.47
Hypercholesterolemia	21.71 [−52.45 to 95.88]	0.56	2.27 [−50.23 to 54.78]	0.93	3.16 [−3.76 to 10.07]	0.37	10.24 [−6.22 to 26.69]	0.22	3.09 [−2.05 to 8.24]	0.24
Hypertension	−42.66 [−133.3 to 47.98]	0.35	−25.09 [−90.52 to 40.35]	0.45	−0.89 [−8.98 to 7.2]	0.83	−13.23 [−32.3 to 5.83]	0.17	2.64 [−3.49 to 8.77]	0.40
CAD history	−53 [−117.68 to 11.69]	0.11	−22.71 [−69.61 to 24.19]	0.34	−5.19 [−10.94 to 0.56]	0.08	−12.09 [−25.72 to 1.53]	0.08	−3.06 [−7.44 to 1.32]	0.17
Total cholesterol, mmol/L	−10.87 [−42.01 to 20.27]	0.49	−14.67 [−37.07 to 7.73]	0.2	1.06 [−1.64 to 3.77]	0.44	1.69 [−4.83 to 8.22]	0.61	1.16 [−0.91 to 3.23]	0.27
Triglycerides, mmol/L	5.68 [−31.34 to 42.7]	0.76	−4.56 [−31.29 to 22.17]	0.74	4.22 [1.07–7.37]	0.01	5.79 [−1.91 to 13.5]	0.14	2.14 [−0.3 to 4.58]	0.09
HDL cholesterol, mmol/L	−3.97 [−122.77 to 114.83]	0.95	−26.37 [−112.05 to 59.31]	0.54	−1.18 [−11.49 to 9.13]	0.82	14.07 [−10.71 to 38.85]	0.26	−2.26 [−10.16 to 5.64]	0.57
LDL cholesterol, mmol/L	−17.96 [−56.83 to 20.91]	0.36	−14.09 [−42.21 to 14.02]	0.32	−1.79 [−4.71 to 1.13]	0.23	−1.97 [−10.12 to 6.19]	0.63	0.2 [−2.2 to 2.6]	0.87
hsCRP plasma, μg/mL	0.04 [−0.39 to 0.47]	0.86	0.03 [−0.28 to 0.34]	0.85	0 [−0.04 to 0.03]	0.91	0.01 [−0.08 to 0.09]	0.87	0 [−0.02 to 0.02]	0.93
GFR MDRD, mL/min	0.59 [−1.2 to 2.38]	0.52	−0.09 [−1.39 to 1.2]	0.89	0.08 [−0.07 to 0.23]	0.31	0.33 [−0.05 to 0.7]	0.09	0.02 [−0.08 to 0.11]	0.73

The associations between risk factors and B cell numbers are presented as beta [95% confidence intervals (CIs)]. For categorical variables, beta is the difference in B-cell numbers (cells/μL) between groups. For continuous variables, beta is the linear regression coefficient relating a 1-unit increase in the variable to the associated change in B-cell numbers. "*P* Value" is the *P* value of the linear or logistic regression model for association. BMI indicates body mass index; CAD history, history of Coronary Artery Disease; Contralateral stenosis, 50% to 100% stenosis of the contralateral carotid artery; GFR MDRD, Glomerular filtration rate according to Modification of Diet in Renal Disease formula; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein.

Table 6. Cox Proportional Hazard Models of Total B Lymphocytes and B-Cell Subtypes

Patients (Events)	Cells/μL		Univariable HR [95% CI]	Univariable *P* Value	Multivariable HR [95% CI]	Multivariable *P* Value
Total B lymphocytes		Continuous	0.98 [0.96–1.00]	0.01	0.98 [0.96–1.00]	0.01
56 (21)	40 to 141	Low	1 (Ref)		1 (Ref)	
56 (21)	142 to 257	Intermediate	0.90 [0.49–1.66]	0.74	0.92 [0.48–1.75]	0.79
56 (12)	258 to 1351	High	0.5 [0.25–1.01]	0.05	0.47 [0.22–1.00]	0.05
Naïve		Continuous	0.98 [0.95–1.00]	0.05	0.98 [0.95–1.00]	0.06
56 (22)	2 to 71	Low	1 (Ref)		1 (Ref)	
56 (19)	72 to 141	Intermediate	0.80 [0.43–1.48]	0.48	0.82 [0.42–1.59]	0.56
56 (13)	141 to 759	High	0.53 [0.27–1.06]	0.07	0.59 [0.29–1.20]	0.15
Unswitched memory		Continuous	0.75 [0.59–0.95]	0.02	0.63 [0.46–0.86]	<0.01
56 (23)	1 to 6	Low	1 (Ref)		1 (Ref)	
56 (21)	7 to 15	Intermediate	0.94 [0.52–1.7]	0.84	0.89 [0.47–1.67]	0.72
56 (10)	16 to 126	High	0.37 [0.18–0.79]	<0.01	0.30 [0.13–0.69]	<0.01
Class switched memory		Continuous	0.86 [0.77–0.97]	0.02	0.80 [0.69–0.93]	<0.01
56 (22)	1 to 17	Low	1 (Ref)		1 (Ref)	
56 (20)	18 to 39	Intermediate	0.87 [0.47–1.59]	0.65	0.74 [0.39–1.42]	0.37
56 (12)	40 to 398	High	0.48 [0.24–0.96]	0.04	0.33 [0.14–0.77]	0.01
All memory		Continuous	0.89 [0.82–0.97]	<0.01	0.84 [0.75–0.93]	<0.01
56 (21)	2 to 26	Low	1 (Ref)		1 (Ref)	
56 (22)	27 to 60	Intermediate	1.05 [0.58–1.92]	0.86	0.88 [0.46–1.69]	0.70
56 (11)	61 to 447	High	0.47 [0.23–0.97]	0.04	0.35 [0.15–0.83]	0.02
$CD43^+/CD27^+$		Continuous	0.96 [0.77–1.18]	0.67	0.84 [0.61–1.16]	0.29
56 (20)	1 to 5	Low	1 (Ref)		1 (Ref)	
56 (19)	6 to 12	Intermediate	0.86 [0.46–1.62]	0.65	0.88 [0.43–1.80]	0.72
56 (15)	13 to 116	High	0.68 [0.35–1.33]	0.26	0.71 [0.33–1.50]	0.36

Cox proportional hazard models are presented for different B-cell (subtype) numbers. In the continuous model, the hazard rate [95% confidence interval (CI)] and *P* values is indicated per increase of 10 cells/μL. In the other models, the intermediate or high tertile is compared to the low tertile in a univariable model or multivariable model adjusted for age, sex, smoking, history of coronary artery disease, and glomerular filtration rate.

both the numbers of switched and unswitched memory cells, patients in the highest tertile of total memory cells were still at lower risk (HR, 0.35 [95% CI, 0.15–0.83]; *P*=0.02) compared with patients in the lowest tertile of total memory cells; however, the combination did not further decrease the risk for recurrent CVD events as the HR was comparable.

Comparable results were obtained when we excluded secondary surgical interventions from the composite end point (25 end points remaining). In multivariable Cox proportional hazard regression models, patients in the highest tertile were at lower risk of events, now only consisting of cardiovascular death, stroke, or MI, as compared with patients in the lowest tertile (HR, 0.35 [95% CI, 0.15–0.82]; *P*=0.01) of unswitched memory cells and (HR, 0.32 [95% CI, 0.13–0.75]; *P*=0.01) of switched memory cells.

Surface IgM Expression Is Higher in Patients With High Numbers of Unswitched Memory Cells

IgM has been shown to be atheroprotective in mouse atherosclerosis models. To assess whether the protective effect of the (un)switched memory B cells is reflected by high expression of IgM, we measured surface expression of IgM. Indeed, in patients with high numbers of unswitched memory cells, surface expression of IgM was also higher as compared with patients with low numbers of unswitched memory B cells (median fluorescence intensity, 62.5 [IQR, 51.2–70.0] versus 53.4 [IQR, 40.4–65.1]; *P*=0.03), suggesting that these patients not only possessed higher numbers of unswitched memory B cells, but that these cells also produce more IgM antibodies.

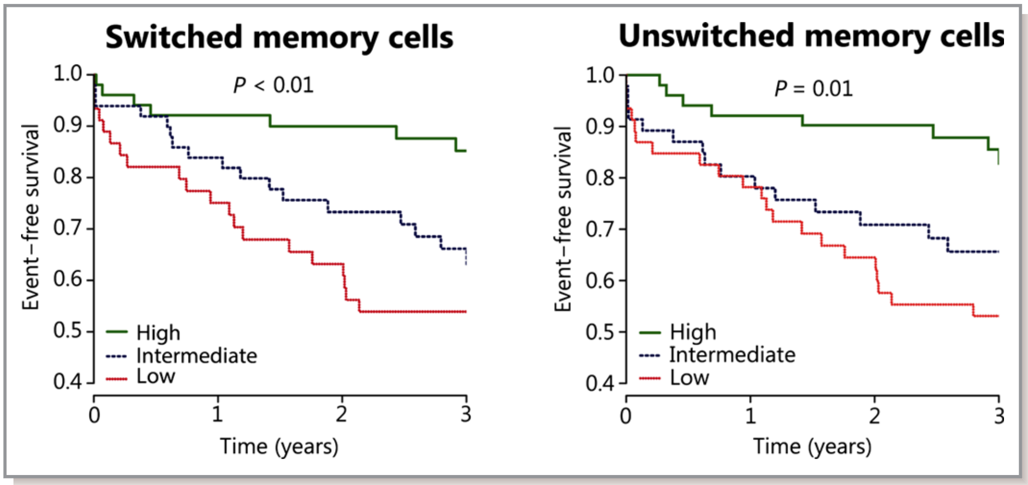

Figure 2. High numbers of (un)switched memory cells associate with decreased risk of secondary adverse cardiovascular events. Cox proportional hazard models are shown by tertiles of (un)switched memory cells. The model is adjusted for age, sex, smoking, history of coronary artery disease, and glomerular filtration rate. The indicated *P* value is derived from comparison of highest to lowest tertile.

Serum Levels of Anti-oxLDL Autoantibodies

To further investigate the antibody production by these (un) switched memory B cells, we determined antibody titers of antibodies directed against oxLDL. Although there was no correlation between IgM-α-oxLDL antibodies and memory B cells, levels of IgG-α-oxLDL antibodies positively correlated with class switched memory cells (spearman's rho=0.28; *P*<0.01; Figure 3). IgM- and IgG-α-oxLDL titers were not significantly increased in patients with a secondary CVD event compared with those without (18 247 [IQR, 8809–35 064] versus 16 982 [IQR, 8251–33 146] relative light units per 100 ms; *P*=0.67 for IgG-α-oxLDL, and 20 328 [IQR 11 706–29 514] versus 22 111 [IQR 13 041–29 698] relative light units per 100 ms; *P*=0.72 for IgM-α-oxLDL). When assessing IgG-α-oxLDL antibodies in a Cox regression model, no association with the risk for secondary CVD events was found (HR, 1.01 [95% CI, 0.98–1.03]; *P*=0.67; data not shown).

Discussion

We investigated the association of circulating B-cell subsets with the occurrence of secondary cardiovascular events in severe carotid atherosclerotic patients undergoing carotid endarterectomy. We found that high levels of (un)switched memory cells were independently associated with the freedom of recurrent cardiovascular events, suggesting that patients with high numbers of (un)switched B cells are protected against secondary cardiovascular manifestations.

Patients with autoimmune diseases, such as systemic lupus erythematosus and common variable immunodeficiency, have an increased risk of developing CVD. Production of (auto) antibodies by B lymphocytes is a hallmark of autoimmune disease and autoantibody formation is also evident in atherosclerotic patients. SLE patients have reduced levels of both switched and unswitched memory cells,[19] and decreased levels of unswitched memory cells are associated with increased levels of SLE autoantibodies.[19] Furthermore, in patients with common variable immunodeficiency, levels of switched memory cells are lower compared with controls.[20] As such low levels of switched and unswitched memory B cells are associated with an unfavorable inflammatory status and, as we

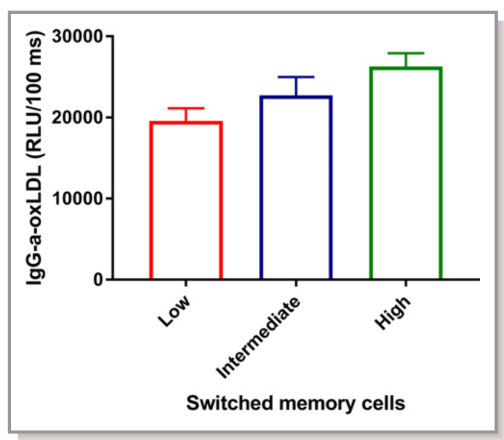

Figure 3. Serum IgG autoantibody titers directed against oxLDL correlate with the numbers of circulating class switched memory cells. Patients are divided in tertiles by numbers of class switched memory cells and corresponding IgG-α-oxLDL titers are shown (*P*<0.01). Data are presented as mean and error bars indicate SEM. Ig indicates immunoglobulin; oxLDL, oxidized low-density lipoprotein.

show here for the first time, with secondary cardiovascular events in patients with severe atherosclerosis.

Similar to our observations regarding the memory B cells, it has previously been shown that patients with high CD40$^+$ B-cell percentages exhibit a lower stroke risk.[22] Accordingly, we also observed a lower rate of cerebrovascular events in patients with high memory cell numbers (0.6%) compared with patients with low numbers (3.6%). However, because of the low prevalence of cerebrovascular events within our population, we were not sufficiently powered to confirm these findings statistically.

The role of IgM antibodies in CVD has gained much attention in the last decades. In mouse models, it was shown that IgM secreted by B1 B cells was responsible for their atheroprotective effect.[28] In contrast to their role in mouse studies,[28,33] CD43$^+$ B cells, suggested to resemble B1 cells in mice,[15] did not associate with protection against secondary events in our study. Contrasting results have been reported for the association of serum IgM levels with primary cardiovascular events.[51–53] In our study, patients with high numbers of unswitched memory cells expressed more IgM on their cell surface, suggesting an increased production and release of IgM antibodies. However, it remains to be elucidated whether the increase of surface IgM expression translates to higher circulating IgM antibody levels and to which antigens this IgM is directed. To investigate whether the protective effect of memory B cells involves autoantibodies directed against oxLDL, we measured oxLDL-specific titers of IgM and IgG. IgM-α-oxLDL antibody titers were not different between patients with and without a follow-up event, nor were they associated with the number of unswitched memory B cells. The number of class switched memory cells did show significant correlations with serum IgG-α-oxLDL antibody titers, indicating that the switched memory cells might indeed be responsible for the production of IgG-α-oxLDL antibodies. However, IgG-α-oxLDL titers were not associated with the occurrence of secondary CVD events, suggesting that oxLDL antibodies are not responsible for the protective effect of memory B cells in our population of severe atherosclerotic patients. These data are in line with other observations that also show no association with secondary CVD events,[54,55] but do not corroborate with other studies showing oxLDL antibodies to be associated with occurrence and severity of CVD.[34–37] Apart from their pivotal role in antibody generation, B cells can also regulate T-cell responses and produce cytokines, suggesting a more-intricate role in cardiovascular disease.[14,56]

In conclusion, we show that high numbers of unswitched and switched memory B cells are associated with lower risk of secondary cardiovascular events in a population with severe CVD. These findings are based on a relatively small patient cohort and should be established in larger patient cohorts. However, our findings do suggest a potential role for B-cell subsets in prediction and prevention of secondary cardiovascular events in patients with atherosclerosis.

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Disclosures

None.

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High Levels of (Un)Switched Memory B Cells Are Associated With Better Outcome in Patients With Advanced Atherosclerotic Disease

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