Atherosclerosis 346 (2022) 68–74

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Generation of cardio-protective antibodies after pneumococcal polysaccharide vaccine: Early results from a randomised controlled trial

Shu Ren [a], Philip M. Hansbro [b,c,d], Wichat Srikusalanukul [e], Jay C. Horvat [b,c], Tegan Hunter [b,c], Alexandra C. Brown [b,c], Roseanne Peel [a,c,*], Jack Faulkner [c], Tiffany-Jane Evans [c], Shu Chuen Li [b], David Newby [b], Alexis Hure [a,c], Walter P. Abhayaratna [e], Sotirios Tsimikas [f], Ayelet Gonen [f], Joseph L. Witztum [f], John Attia [a,c,g,**], on behalf ofThe AUSPICE investigators

[a] School of Medicine and Public Health, University of Newcastle, Newcastle, NSW, Australia
[b] School of Biomedical Sciences and Pharmacy, University of Newcastle, Newcastle, NSW, Australia
[c] Hunter Medical Research Institute, Newcastle, NSW, Australia
[d] Centenary UTS Centre for Inflammation, Sydney, NSW, Australia
[e] Australian National University Medical School, Canberra Hospital, Canberra, ACT, Australia
[f] Department of Medicine, University of California San Diego, La Jolla, CA, USA
[g] Department of Medicine, John Hunter Hospital, Newcastle, NSW, Australia

ARTICLE INFO

Keywords:
Atherosclerosis
Cardiovascular disease
Anti-oxLDL antibodies
Anti-pneumococcal antibodies
Human clinical trial
Oxidative stress
Pneumococcal vaccine

ABSTRACT

Background and aims: Observational studies have demonstrated that the pneumococcal polysaccharide vaccine (PPV) is associated with reduced risk of cardiovascular events. This may be mediated through IgM antibodies to OxLDL, which have previously been associated with cardioprotective effects. The Australian Study for the Prevention through Immunisation of Cardiovascular Events (AUSPICE) is a double-blind, randomised controlled trial (RCT) of PPV in preventing ischaemic events. Participants received PPV or placebo once at baseline and are being followed-up for incident fatal and non-fatal myocardial infarction or stroke over 6 years.

Methods: A subgroup of participants at one centre (Canberra; n = 1,001) were evaluated at 1 month and 2 years post immunisation for changes in surrogate markers of atherosclerosis, as pre-specified secondary outcomes: high-sensitive C-reactive protein (CRP), pulse wave velocity (PWV), and carotid intima-media thickness (CIMT). In addition, 100 participants were randomly selected in each of the intervention and control groups for measurement of anti-pneumococcal antibodies (IgG, IgG2, IgM) as well as *anti*-OxLDL antibodies (IgG and IgM to CuOxLDL, MDA-LDL, and PC-KLH).

Results: Concentrations of anti-pneumococcal IgG and IgG2 increased and remained high at 2 years in the PPV group compared to the placebo group, while IgM increased and then declined, but remained detectable, at 2 years. There were statistically significant increases in all *anti*-OxLDL IgM antibodies at 1 month, which were no longer detectable at 2 years; there was no increase in *anti*-OxLDL IgG antibodies. There were no significant changes in CRP, PWV or CIMT between the treatment groups at the 2-year follow-up.

Conclusions: PPV engenders a long-lasting increase in anti-pneumococcal IgG, and to a lesser extent, IgM titres, as well as a transient increase in *anti*-OxLDL IgM antibodies. However, there were no detectable changes in surrogate markers of atherosclerosis at the 2-year follow-up. Long-term, prospective follow-up of clinical outcomes is continuing to assess if PPV reduces CVD events.

1. Introduction

Over the last 20 years, evidence has emerged that the pneumococcal polysaccharide vaccine (PPV) may reduce atherosclerosis and related cardiovascular events. Mouse models have demonstrated reductions in atherosclerotic plaque formation after immunisation with heat killed *Streptococcus pneumoniae* [1]. It has been demonstrated that the phosphocholine (PC) moiety present in the capsular polysaccharide cell wall

* Corresponding author. School of Medicine and Public Health, University of Newcastle, Newcastle, NSW, Australia.
** Corresponding author. School of Medicine and Public Health, University of Newcastle, Newcastle, NSW, Australia.
E-mail addresses: roseanne.peel@newcastle.edu.au (R. Peel), john.attia@newcastle.edu.au (J. Attia).

https://doi.org/10.1016/j.atherosclerosis.2022.02.011
Received 19 September 2021; Received in revised form 31 January 2022; Accepted 9 February 2022
Available online 5 March 2022

of *S. pneumoniae* is a molecular mimic of the PC headgroup of oxidised phospholipids found in oxidised low-density lipoprotein (OxLDL) [2]. Indeed, immunisation with PC-KLH (PC coupled to keyhole limpet hemocyanin) or direct infusion of anti-PC IgM (T15 antibody) has been shown to reduce atherosclerosis in mice [2,3]. Epidemiological studies, although not entirely consistent, have shown that IgG autoantibody titres to OxLDL are positively associated but IgM titres are inversely associated with cardiovascular risk [4–14]. Anti-OxLDL antibodies, such as those to PC that would be boosted by immunisation with PPV appear to reduce atherosclerosis via multiple different mechanisms including: blocking the uptake of OxLDL by macrophages thereby reducing the formation of foam cells, reducing cellular toxicity and apoptosis, and reducing both localised and systemic inflammation due to oxidised phospholipids [15–17].

PPV (Pneumovax, Merck Sharpe and Dohme) administration has been associated with the same *anti*-OxLDL antibodies in humans [18], but PCV (protein conjugate vaccine, Prevenar, Pfizer) does not seem to elicit the same degree of response [19]. Some international observational studies have provided evidence for an association between PPV and reduced risk of cardiovascular ischemic events [20,21] while other studies have not [22,23]. A meta-analysis of these studies found a statistically significant reduction of 17% for acute coronary syndrome (ACS) events in those over 65 years of age [24]. However, these observational studies were heterogeneous in terms of sample population, exposures measured, and definition of outcomes [24]. Therefore, a randomised controlled trial is needed to specifically test the effect of the PPV on atherosclerotic cardiovascular risk.

The Australian Study for the Prevention through Immunisation of Cardiovascular Events (AUSPICE) is a multi-centre, randomised, placebo-controlled, double-blind, clinical trial to formally test whether vaccination with PPV protects against fatal and non-fatal acute coronary syndromes and ischemic strokes. Cardiovascular outcomes will be obtained over 6 years of follow-up, through health record linkage with state and national administrative datasets [25].

This paper presents initial, pre-specified, follow-up results from AUSPICE performed in a sub-group of the national study, including anti-pneumococcal and *anti*-OxLDL antibody titre changes over time by vaccination group and changes in several surrogate markers of cardiovascular disease (CVD), including high-sensitivity C-reactive protein (CRP), pulse wave velocity (PWV), and carotid intima-media thickness (CIMT). CRP, an inflammatory biomarker and a strong predictor of future cardiovascular events [26]; PWV, a measure of arterial stiffness [27]; and CIMT, a measure of the extent of carotid artery atherosclerosis [28], are all established surrogate markers of cardiovascular health that correlate with development of CVD and in studies involving HMG-CoA reductase inhibitors have shown significant changes over 2 years [29–32].

2. Patients and methods

The AUSPICE study protocol was published previously [25]. Briefly, female and male participants aged 55–60 years without a history of cardiac or stroke events, or pneumococcal vaccination, but at an increased risk (at least two risk factors: hypertension, hyperlipidaemia, or overweight/obesity) were recruited from 6 study sites around Australia. Participants (n = 4725) were randomly allocated to receive either PPV (Pneumovax, Merck Sharpe and Dohme) or a saline placebo (control) and are being followed-up for atherosclerotic cardiovascular events, i.e. fatal and non-fatal myocardial infarction and stroke, via health record linkage [33]. Participants recruited at the Canberra site formed the subgroup reported in the current paper (n = 1,001). Demographics and characteristics were obtained at baseline; high-sensitivity CRP levels (mg/L; Roche Cobas platform in NATA-accredited ACT Pathology lab) were collected at baseline, 1 month and 2 years of follow-up, and PWV (m/s) and CIMT measurements (μm) were recorded by an independent assessor at baseline and 2 years. Using applanation tonometry (SphygmoCor device, AtCor Medical, Sydney, Australia), PWV was calculated as the ratio of distance between common carotid artery and femoral artery recording sites to the transit time of the waveforms (distance/transit time) [27]. An average CIMT was acquired using ultrasound measurements from anterior, lateral and posterior walls of left and right carotid arteries (Vivid I, GE Medical Systems, USA). CIMT measures were obtained with participants in a seated position and facing forward, and PWV measures when lying flat; all scans were performed by a single, blinded operator, who also read and coded the images. Three anti-pneumococcal capsular polysaccharide immunoglobulin (Ig) antibody titres were measured in 200 randomly selected patients (100 active and 100 control, randomly selected by independent statistician); IgM (U/mL), IgG (mg/L) and IgG2 (mg/L) measures were assessed at baseline, 1 month and 2-year follow-up, using VaccZyme™ Anti-PCP Enzyme Immunoassay kits. Six antibody tests for various *anti*-OxLDL antibodies were performed at baseline, 1 month and 2 years of follow-up, using previously developed in-house assays [1]. These include IgG and IgM (measured in relative light units/100 ms) against:

- phosphocholine complexed to the carrier protein Keyhole Limpet hemocyanin (PC-KLH);
- $CuSO_4$ oxidised LDL (CuOxLDL);
- malondialdehyde-derivatised LDL (MDA-LDL).

For all commercial and in-house assays, the coefficient of variation (CV) was tested for each 96 well plate; any that had a CV>15% was retested.

2.1. Statistical analysis

2.1.1. Antibody titres

Interim analyses were pre-specified in the protocol. For each anti-pneumococcal antibody (IgG, IgG2 and IgM), change from baseline was modelled using repeated measures ANCOVA. Antibody titres at follow-up were log-transformed and modelled against baseline log-transformed titre, vaccination group, time, as well as a two-way interaction term between group and time; an interaction term between group and baseline log-transformed titre was also included to account for non-parallel regression lines. Time was considered categorical to account for unequal spacing of patient assessments. A random intercept was employed to account for correlations of repeated outcome measures within patients and the model was fitted under the assumption of heteroscedasticity using exponential local effects to address increasing variance with increasing value of the transformed antibody measure [34]. Separate models were used for each antibody.

For the various *anti*-OxLDL antibodies, longitudinal mixed model ANCOVA was utilised to compare the repeated values of titres. All time points were measured in triplicate in the same assays. Because there was variability in baseline titres to a given antigen between subjects, and the goal was to see changes in response to the vaccination, we used ratios of titres at one month (time 1) to baseline (time 0) (t1/t0) and ratios of titres at 2 years (time 2) to baseline (time 0) (t2/t0). Both time point ratios were then analysed together (for each antibody) using longitudinal mixed models, with a random effect for participant. Due to the skewed nature of the ratios, the natural log of the ratios was modelled (log-transform). Ratios with a value of zero were therefore set to 0.0001 to allow the natural log to be calculated. The mean ratio (back-transformed to the original scale) was estimated at each time point for each arm (active or control), and the effect of active treatment (compared to control) was calculated as a ratio of ratios.

To check for consistency and any non-linear effects, ratios were also dichotomised as ≤ 1 *vs* > 1 (non-responder *vs* responder respectively). The proportion of responders in each arm was then compared at each time point using chi-squared analyses, and longitudinally using logistic mixed models, with fixed effects for treatment arm, time, and arm*time,

and a random effect for participant. Spearman's correlation coefficients (and *p*-values) between the various *anti*-OxLDL antibody ratios and pneumococcal antibody ratios were also estimated at each time point. Given that groups were randomised, there was no adjustment for potential confounders.

For antibody assays, 100 participants were chosen in each group based on the fact that this would detect a Cohen's d of 0.4 (small to modest effect size) with 80% power at $p = 0.05$.

2.1.2. Surrogate markers of atherosclerosis

To assess change in PWV, CIMT and CRP measures by group over time, natural log transformations of respective values at both time points were generated. Individual analysis of covariance (ANCOVA) linear regression models were utilised to explore change in log-transformed measures from baseline to 2 years. The models included terms for group and the baseline log-transformed value. Robust standard errors were applied due to non-constant variance of residuals.

A random intercept accounted for correlations of repeated outcome measures within patients and robust standard errors were used due to non-constant variance of residuals. Least squares (LS) mean estimates (and confidence intervals) were also produced for each follow-up time point by group. Differences in estimates were calculated to assess both time and group effects.

All statistical analyses were conducted using SAS v9.4 (SAS Institute, Cary, North Carolina, USA); $p < 0.05$ (two-tailed) was used to indicate statistical significance. The multiple testing burden was reduced by using models that incorporated repeated measures, but otherwise no correction was made to *p*-values.

2.2. Ethics approval

Approval for the clinical trial was granted by the Human Research Ethics Committee governing the University of Newcastle (reference H-2014-0064) and the ACT Health Ethics and Governance Committee governing the Canberra Hospital (reference ETH.7.14.177).

3. Results

Of the 1001 participants in the Canberra subgroup of the AUSPICE RCT, 200 were randomly chosen: 100 each from the PPV intervention group and the control group. All 200 randomly chosen participants attended the baseline and 1 month follow-up visits; 19 participants did not attend the 2- year follow-up visit, 9 in the control group and 10 in the active group. Baseline characteristics of the 2 groups were well balanced (Table 1) and were similar to the entire study group [33]; there were no statistically significant differences between the groups at baseline.

3.1. Anti-pneumococcal antibody response

The absolute titres of the anti-pneumococcal antibodies by group and time are shown in Supplementary Table 1. Changes in anti-pneumococcal IgG and IgG2 antibody titres were greater for the active group compared to the control group at both follow-up time points (Fig. 1A and B, respectively). These differences between the active and control groups and the difference over time between the groups were both significant (Table 2).

The titres of anti-pneumococcal IgM antibodies rose from baseline to 1 month and decreased thereafter over 2 years in the intervention group (Fig. 1C). The differences between the intervention and control groups and the difference over time between the groups were both significant (Table 2).

3.2. Anti-OxLDL antibody response

The absolute titres of the *anti*-OxLDL antibodies by group and time are shown in Supplementary Table 2. The titres of the *anti*-OxLDL antibodies were expressed as ratios:

- t1/t0: titre at 1 month/titre at baseline
- t2/t0: titre at 2 years/titre at baseline

and analysed together in a mixed model. All 3 *anti*-OxLDL IgM antibodies (PC-KLH, CuOxLDL and MDA-LDL) were significantly increased at 1 month in the active group compared to the control group but had returned to baseline levels at 2 years (Table 3). In contrast, none of the 3 *anti*-OxLDL IgG antibodies showed any increase above baseline, either at 1 month or at 2 years (Table 3).

The titres were then dichotomised into responders (where $t1/t0>1$) and non-responders (where $t1/t0\leq1$), and the proportion of responders in each group and each time point was analysed. The results confirm the same pattern as previously seen with continuous titres (Table 4). There were more responders to all 3 *anti*-oxLDL IgM antibodies in the intervention group than the control group at 1 month but this response had subsided at 2 years. For example, 22% of the control group were responders at 1 month with respect to anti-PC-KLH IgM antibodies, compared with 50% of the intervention group, an odds ratio of 3.5 (*p*-value = 0.0002) but this was no longer detectable at 2 years with an odds ratio of 1.06 (*p*-value = 0.87). There was also a transient IgG response to PC-KLH at 1 month seen using this analysis (*p*-value = 0.0098) that was not seen when analysing the continuous titre, but this was not sustained at 2 years.

3.3. Correlations between anti-pneumococcal and anti-OxLDL antibodies

To further explore whether those with the greatest antibody response to pneumococcus also had the greatest response to OxLDL, the correlations (at time point 1) between the anti-pneumococcal ratios, and the *anti*-OxLDL ratios, for the control and intervention groups combined, are reported in Table 5.

The rise in anti-pneumococcal IgM antibody ratios between t1 and t0 were moderately correlated to the rise in the *anti*-OxLDL IgM antibody

Table 1
Baseline characteristics for 200 randomly selected participants, 100 in each group.

Characteristics	Class/ Statistics	Treatment Group		
		Control (n = 100)	Active (n = 100)	Total (N = 200)
Gender	Female	42 (42%)	55 (55%)	97 (49%)
	Male	58 (58%)	45 (45%)	103 (52%)
High Blood Pressure	No	22 (22%)	30 (30%)	52 (26%)
	Yes	77 (78%)	69 (70%)	146 (74%)
Overweight	No	18 (20%)	17 (20%)	35 (20%)
	Yes	73 (80%)	66 (80%)	139 (80%)
ATSI status	NO	100 (100%)	100 (100%)	200 (100%)
Weight (kg)	Mean (SD)	93.88 (19.94)	89.34 (14.61)	91.63 (17.61)
	Median (Q1, Q3)	90.00 (81.00, 105.0)	88.00 (78.00, 97.00)	89.00 (79.00, 100.0)
Waist (cm)	Mean (SD)	103.1 (16.28)	101.0 (18.51)	102.1 (17.36)
	Median (Q1, Q3)	101.0 (94.00, 110.0)	98 (92.00, 109.0)	100.0 (93.50, 110.0)
Height (cm)	Mean (SD)	170.9 (9.22)	169.9 (9.31)	170.4 (9.26)
	Median (Q1, Q3)	170.0(165.0, 178.0)	170.0 (164.0, 177.0)	170.0 (164.0, 177.0)
Age (years)	Mean (SD)	58 (2)	58 (2)	58 (2)
	Median (Q1, Q3)	58 (57,60)	58 (56,60)	58 (57,60)

These participants were chosen at random from the 1001 participants at one of the study sites, who all agreed to participate in a substudy collecting blood samples at baseline, 1 month, and 2 years.

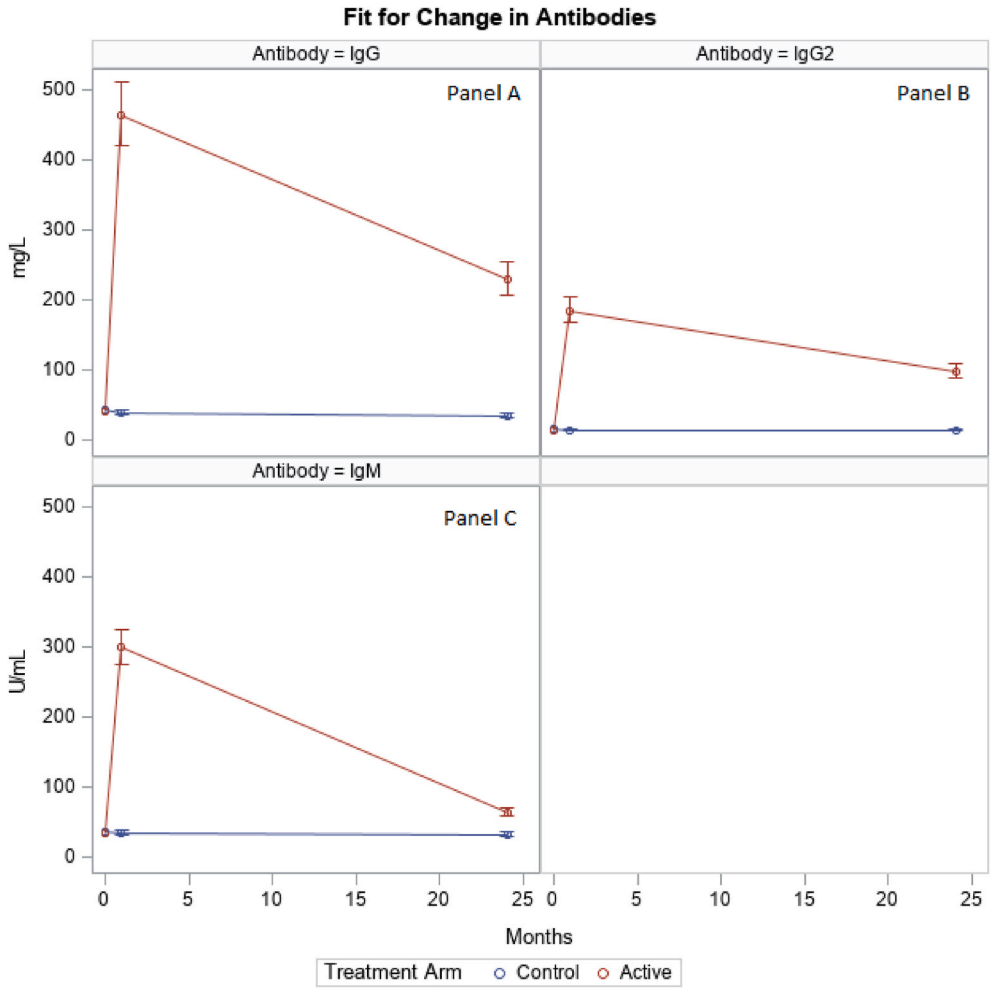

Fig. 1. Plots of antibody titres over time.
Crude means are presented at baseline with corresponding back-transformed LS mean estimates (and 95% confidence intervals) plotted at 1 month and 2 year follow-up time points.

Table 2
Repeated measures ANCOVA model for change in anti-pneumococcal antibody titres (log-transformed values).

Effect	Anti-pneumococcal antibody type					
	IgG (mg/L)		IgG2 (mg/L)		IgM (U/mL)	
	Estimate (95%CI)	*p*-value	Estimate (95%CI)	*p*-value	Estimate (95%CI)	*p*-value
Group (active *vs* control)	3.5 (2.8, 4.2)	<0.0001	3.1 (2.6, 3.6)	<0.0001	2.8 (2.3, 3.4)	<0.0001
Time (2 yrs *vs* 1 month)	−0.13 (−0.20, −0.06)	0.0005	−0.002 (−0.09, 0.08)	0.95	−0.07 (−0.16, 0.01)	0.1
Interaction of group and time	−0.58 (−0.67, −0.47)	<0.0001	−0.64 (−0.76, −0.52)	<0.0001	−1.5 (−1.6, −1.3)	<0.0001

Results of the ANCOVA model for change in anti-pneumococcal antibody titres over time, adjusted for baseline values. The group variable reflects the difference across time between the active *vs* control group, the time variable reflects the difference across groups between the 2 year values and the 1 month value, and interaction term captures whether the difference between time points is different between groups.

ratios; Spearman correlations ranged from 0.16 to 0.39 (*p*-value 0.03 to <0.001). There was little to no correlation with the *anti*-OxLDL IgG ratios. This pattern of correlations was attenuated but still present with the anti-pneumococcal IgG antibodies.

3.4. Surrogate markers of atherosclerosis (PWV, CIMT and CRP)

Fig. 2 presents the surrogate markers of atherosclerosis for the entire Canberra sub-study sample (n = 1001). There were no significant differences between the intervention and control groups for either PWV (ANCOVA *p*-value = 0.84) or CIMT (*p*-value = 0.60) at 2 years (adjusted for baseline values) (panel A). There was also no difference in CRP levels between groups over time (interaction *p*-value = 0.13) (panel B).

4. Discussion

This is the first randomised study addressing the generation of potentially cardioprotective antibodies following PPV with surrogate measures of atherosclerosis over 2 years. In a subset of patients, increases in IgG and IgG2, specific to pneumococcal capsular polysaccharide, persisted at high concentrations at 2 years. IgG2 is representative of a mature type-1 antibody response and shows that the effects of vaccination persist for extended periods. Anti-pneumococcal IgM in the active group declined at 2 years but was still significantly

Table 3
Results for the mixed models of continuous log-transformed antibody ratios, comparing active and control at time 1 and time 2.

Outcome	Time Point	Control Mean (95% CI)	Active Mean (95% CI)	Ratio Active *vs* Control (95% CI)	*p*-value	N
log IgG PC-KLH	T1/T0	0.94 (0.83, 1.07)	1.03 (0.95, 1.12)	1.09 (0.94, 1.27)	0.2511	180
	T2/T0	0.85 (0.72, 1.00)	0.92 (0.81, 1.05)	1.09 (0.88, 1.35)	0.4145	180
log IgG CuOxLDL	T1/T0	0.72 (0.57, 0.90)	0.88 (0.80, 0.96)	1.23 (0.96, 1.56)	0.0996	179
	T2/T0	0.90 (0.79, 1.02)	1.05 (0.93, 1.18)	1.16 (0.98, 1.39)	0.0883	179
log IgG MDA-LDL	T1/T0	0.86 (0.82, 0.90)	0.81 (0.65, 0.99)	0.94 (0.76, 1.16)	0.5390	180
	T2/T0	0.93 (0.86, 1.01)	0.97 (0.89, 1.05)	1.04 (0.92, 1.17)	0.5177	180
log IgM PC-KLH	T1/T0	0.92 (0.89, 0.96)	1.04 (0.99, 1.10)	1.14 (1.07, 1.21)	<.0001	180
	T2/T0	0.88 (0.83, 0.94)	0.90 (0.85, 0.96)	1.02 (0.94, 1.11)	0.6460	180
log IgM CuOxLDL	T1/T0	0.81 (0.75, 0.87)	0.94 (0.86, 1.02)	1.16 (1.03, 1.30)	0.0141	180
	T2/T0	0.85, (0.78, 0.93)	0.84 (0.76, 0.92)	0.98 (0.86, 1.12)	0.7787	180
log IgM MDA-LDL	T1/T0	0.85 (0.80, 0.89)	0.96 (0.92, 1.01)	1.14 (1.06, 1.22)	0.0004	179
	T2/T0	0.85 (0.81, 0.90)	0.86 (0.80, 0.92)	1.01 (0.92, 1.09)	0.9069	179

A ratio of active to control >1 indicates that the response (change in titre) in the intervention (active) group has been greater than the response in the control group. The number of people (N) is less than 200 due to some missing samples at various time points.

Table 4
Results for the logistic mixed model comparing the probability of having an antibody ratio >1 for active *vs* control, at time 1 and time 2.

Outcome	Time Point	Control Proportion (95% CI)	Active Proportion (95% CI)	OR Active *vs* Control (95% CI)	*p*-value	N
IgG PC-KLH	1	0.27 (0.19, 0.38)	0.47 (0.36, 0.57)	2.33 (1.23, 4.41)	0.0098	180
	2	0.30 (0.21, 0.40)	0.30 (0.21, 0.40)	1.00 (0.52, 1.93)	0.9992	180
IgG CuOxLDL	1	0.23 (0.15, 0.33)	0.23 (0.15, 0.33)	1.00 (0.49, 2.07)	0.9946	179
	2	0.45 (0.35, 0.57)	0.47 (0.36, 0.58)	1.08 (0.57, 2.04)	0.8236	179
IgG MDA-LDL	1	0.24 (0.16, 0.34)	0.30 (0.21, 0.40)	1.33 (0.68, 2.62)	0.4022	180
	2	0.49 (0.38, 0.59)	0.39 (0.29, 0.49)	0.66 (0.36, 1.21)	0.1781	180
IgM PC-KLH	1	0.22 (0.15, 0.32)	0.50 (0.40, 0.60)	3.53 (1.83, 6.78)	0.0002	180
	2	0.25 (0.17, 0.36)	0.27 (0.18, 0.37)	1.06 (0.54, 2.07)	0.8714	180
IgM CuOxLDL	1	0.16 (0.10, 0.25)	0.37 (0.27, 0.48)	3.14 (1.52, 6.46)	0.0021	180
	2	0.31 (0.22, 0.41)	0.34 (0.24, 0.45)	1.16 (0.61, 2.22)	0.6536	180
IgM MDA-LDL	1	0.15 (0.09, 0.24)	0.36 (0.26, 0.47)	3.22 (1.54, 6.73)	0.0021	179
	2	0.23 (0.15, 0.33)	0.26 (0.18, 0.36)	1.17 (0.58, 2.36)	0.6591	179

This table presents a dichotomised view of the data in Table 3. The proportion of those who had a ratio >1 over time, indicating an increasing antibody titre over time, are labeled "responders"; the percentage of responders across the intervention and control groups are then compared using an odds ratio, with an OR>1 indicating a greater percentage of responders in the intervention group compared to the control group.

Table 5
Correlations between anti-pneumococcal antibody titre ratios and *anti*-oxLDL antibody titre ratios across both active and control groups, at time 1 (one month).

Anti-pneumococcal ratio	Anti-OxLDL ratio	Spearman correlation	*p*-value
IgG t1/t0	IgG PC-KLH t1/t0	0.20	0.01
	IgG CuOxLDL t1/t0	0.04	0.56
	IgG MDA-LDL t1/t0	0.10	0.17
	IgM PC-KLH t1/t0	0.33	<0.001
	IgM CuOxLDL t1/t0	0.19	0.01
	IgM MDA-LDL t1/t0	0.26	<0.001
IgM t1/t0	IgG PC-KLH t1/t0	0.22	<0.001
	IgG CuOxLDL t1/t0	0.09	0.26
	IgG MDA-LDL t1/t0	0.06	0.45
	IgM PC-KLH t1/t0	0.39	<0.001
	IgM CuOxLDL t1/t0	0.16	0.03
	IgM MDA-LDL t1/t0	0.31	<0.001

The table indicates the correlation coefficients between anti-pneumococcal IgG titres at time 1 (1 month) and various *anti*-oxLDL IgG/IgM titres at time 1, essentially answering the question: are those individuals who respond to the pneumococcal vaccine also the ones who show an increase in *anti*-oxLDL antibodies?

higher than at baseline or compared to the control group. IgM is produced by plasma cells as the first response to vaccination and is expected to decline over time as the antibody response matures. IgG, IgG2 and IgM are important tools for monitoring adaptive immune responses to PPV [35]. The acute increase in IgM that occurs after immunisation is adaptive, and with class switching, these become IgG in a matter of weeks. These results are consistent with existing literature that demonstrates sustained effects of PPV, with IgG titres remaining high up to 5–6 years, but IgM declining during that period [35,36].

It is interesting to note that a sizable proportion of people in both the control and intervention groups showed detectable *anti*-OxLDL antibodies at baseline. This is in line with many epidemiological studies showing similar results [4,9,11,14,37–40].

From this baseline, it is clear that PPV caused increases in all *anti*-OxLDL IgM antibodies at 1 month, i.e. against PC-KLH, CuOxLDL, and MDA-LDL. Previous work has indicated that *anti*-OxLDL IgM antibodies that potentially have cardioprotective effect are likely natural antibodies that are the product of innate B-1 like cells rather than adaptive immunity [15,41]. Natural antibodies are present without needing antigenic stimulation and usually target non-protein epitopes, e.g. carbohydrates and glycolipids. These antibodies may be increased by generalised stimuli to B-1 cells in a non-cognate T-independent manner, hence an antigenic stimulus from PPV may indirectly boost titres of natural *anti*-OxLDL antibodies, as previously demonstrated in mice [42].

Despite this increase in titres at 1 month, there did not appear to be any lasting or detectable change, either in IgG or IgM *anti*-OxLDL antibodies, at 2 years. This is contrary to the prospective study in which *anti*-OxLDL antibody levels remained stable over time [37]. The difference between the studies may be due to continuing pathogen exposure in the latter; our study was randomised and hence the contrast between groups removes the influence of any unknown confounders.

Traditionally, it was thought that bacterial carbohydrate antigens typically induced a T cell-independent antibody response in the form of IgG2 [43]. More recent data provides evidence that there is cell-mediated immunity that occurs alongside humoral responses, in the form of pneumococcus-specific $CD4^+$ Th17 cells [44,45]. By analogy, a similar immune response that occurred with PPV vaccination could

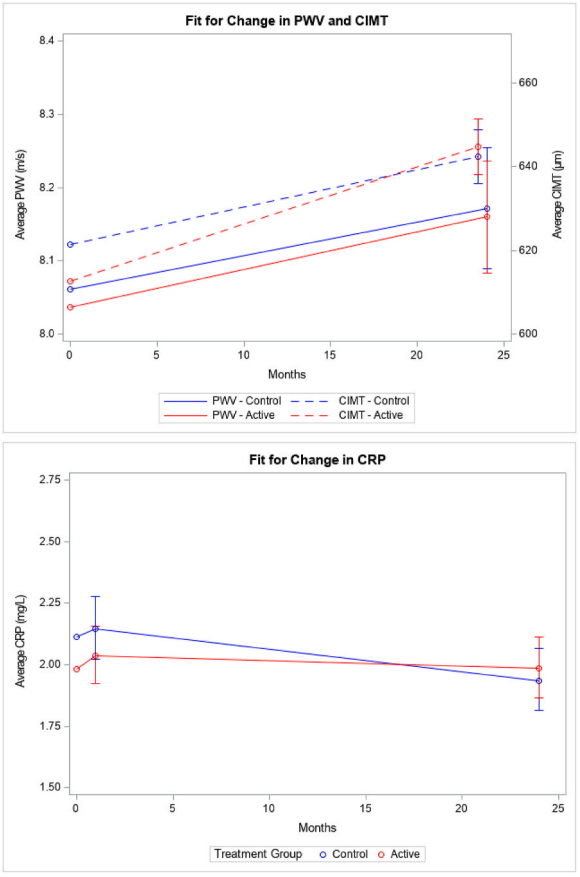

Fig. 2. Surrogate measures of CVD, including all 1001 participants in the Canberra substudy.
(A) Plots of PWV and CIMT measures over time. Crude means were used at baseline and back-transformed LS mean estimates (and 95% confidence intervals) were plotted at 2 years of follow-up. To improve visibility of curves at the 2-year time point, PWV was jittered along the x-axis so estimates/confidence intervals of CIMT could be seen. There were no statistically significant differences between the 2 groups at any time point. (B) Plot includes crude baseline means of CRP with back-transformed LS mean estimates (and 95% confidence intervals) at 1 month and 2 years follow-up. There were no statistically significant differences between the 2 groups at any time point.

mediate cardioprotective effects of PPV long after *anti*-OxLDL antibody titres wane. Indeed, previous studies indicate that the cardioprotective effect seems to persist long-term; high titres of *anti*-OxLDL IgM antibodies in humans were inversely correlated with CIMT and coronary artery stenosis, and low titres were associated with higher risk of stroke and myocardial infraction [46,47]. Compared to control subjects, subjects who received PPV developed higher titres of IgM *anti*-OxLDL antibodies. This was seen in the moderate correlation between the change in anti-pneumococcal IgM titres and *anti*-OxLDL IgM titres.

Despite previous preclinical data showing potential beneficial cardiovascular effects of raising titres to OxLDL, we did not observe any differences in surrogate markers of atherosclerosis, i.e. CRP, PWV, or CIMT, over time, despite PPV clearly engendering an anti-pneumococcal and *anti*-OxLDL response. It is possibly too early to see a change in surrogate markers: however, in the case of HMG-CoA reductase inhibitors, these markers have shown changes in the same time frame as this study, i.e. over 2 years [29–32]. Statins of course have a profound effect in substantially lowering LDL levels, which are the primary driver of atherosclerosis. Raising antibody titres to OxLDL would be expected to have a weaker effect and may take a longer time to manifest. It is important to note that the AUSPICE trial is scheduled to follow-up participants for at least 6 years; clinical endpoints will be obtained via health record linkage at that point and so are not yet available.

Although there are a number of possible explanations for the lack of an effect on sub-clinical atherosclerosis at this interim stage, it should also be noted that the increase in *anti*-OxLDL IgM titres at 1 month, while statistically significant, is rather modest. It is possible that the PPV immunisation is insufficient to generate a robust *anti*-OxLDL response targeting the PC of oxidised phospholipid. A different configuration of the PC antigen that elicits more robust and lasting anti-PC and *anti*-OxLDL titres may be required to test the hypothesis that raising *anti*-OxLDL IgM titres in humans can provide cardioprotective properties. Finally, it is possible that the benefit of PPV occurs more at the thrombosis stage than atherosclerosis/plaque burden, which would not be detected by PWV, CIMT and CRP measurements.

In conclusion, this is the first evidence from a randomised controlled study in humans that PPV elicits an increase, albeit modest, in *anti*-OxLDL IgM antibodies that have previously been shown to have cardioprotective effects in animal models of atherosclerosis. It is possible that PPV boosts the titres of these innate natural antibodies that are present at low levels even without cognate antigenic stimulus. Although the anti-pneumococcal IgG and IgM response is sustained long-term, the *anti*-OxLDL response appears to wane after 1 month. Whether there is a lasting benefit for cardiovascular disease remains to be seen in the long-term follow-up of the AUSPICE study. We are currently collecting 4-year blood samples and atherosclerotic markers to further plot the trajectory of these measures; metabolic markers (fasting glucose and insulin, liver function tests, and quantitative liver ultrasound) will also be assessed at the 4-year follow-up of this sub-group. The full clinical outcomes, i.e. fatal and non-fatal ischemic cardiac and cerebral events, will be obtained via health record linkage at 6 years' follow-up in 2023; this health record linkage will be done electronically by an independent statistician blinded to group allocation.

CRediT authorship contribution statement

Shu Ren: Resources, Writing – original draft, Writing – review & editing. **Philip M. Hansbro:** Writing – review & editing. **Wichat Srikusalanukul:** Investigation, Resources, Writing – review & editing. **Jay C. Horvat:** Investigation, Writing – review & editing. **Tegan Hunter:** Investigation, Writing – review & editing. **Alexandra C. Brown:** Investigation, Writing – review & editing. **Roseanne Peel:** Resources, Writing – review & editing. **Jack Faulkner:** Formal analysis, Writing – review & editing. **Tiffany-Jane Evans:** Formal analysis, Writing – review & editing. **Shu Chuen Li:** Writing – review & editing. **David Newby:** Funding acquisition, Writing – review & editing. **Alexis Hure:** Funding acquisition, Writing – review & editing. **Walter P. Abhayaratna:** Conceptualization, Funding acquisition, Writing – review & editing. **Sotirios Tsimikas:** Funding acquisition, Writing – review & editing. **Ayelet Gonen:** Formal analysis, Investigation, Resources, Supervision, Writing – review & editing. **Joseph L. Witztum:** Funding acquisition, Writing – review & editing. **John Attia:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

AUSPICE was funded by a National Health and Medical Research Council (NHMRC) project grant (APP1062563), and two Ministry of Health, NSW Australia grants (Reference: H19/133660). P.M.H is funded by a fellowship and grants from the National Health and Medical Research Council (NHMRC) of Australia (1079187, 1175134) and by

UTS.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2022.02.011.

References

[1] C.J. Binder, S. Horkko, A. Dewan, et al., Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL, Nat. Med. 9 (2003) 736–743.

[2] J.R. Faria-Neto, K.Y. Chyu, X. Li, et al., Passive immunization with monoclonal IgM antibodies against phosphorylcholine reduces accelerated vein graft atherosclerosis in apolipoprotein E-null mice, Atherosclerosis 189 (2006) 83–90.

[3] G. Caligiuri, J. Khallou-Laschet, M. Vandaele, et al., Phosphorylcholine-targeting immunization reduces atherosclerosis, J. Am. Coll. Cardiol. 50 (2007) 540–546.

[4] B.J. Geller, J.L. Mega, D.A. Morrow, et al., Autoantibodies to phosphorylcholine and cardiovascular outcomes in patients with acute coronary syndromes in the ATLAS ACS-TIMI 46 trial, J. Thromb. Thrombolysis 37 (2014) 310–316.

[5] K. Caidahl, M. Hartford, T. Karlsson, et al., IgM-phosphorylcholine autoantibodies and outcome in acute coronary syndromes, Int. J. Cardiol. 167 (2013) 464–469.

[6] M.L. Bertoia, J.K. Pai, J.-H. Lee, et al., Oxidation-specific biomarkers and risk of peripheral artery disease, J. Am. Coll. Cardiol. 61 (2013) 2169–2179.

[7] G.P. Rossi, M. Cesari, R.D. Toni, et al., Antibodies to oxidized low-density lipoproteins and angiographically assessed coronary artery disease in white patients, Circulation 108 (2003) 2467–2472.

[8] J.T. Salonen, S. Ylä-Herttuala, R. Yamamoto, et al., Autoantibody against oxidised LDL and progression of carotid atherosclerosis, Lancet (Lond. Engl.) 339 (1992) 883–887.

[9] A. Prasad, P. Clopton, C. Ayers, et al., Relationship of autoantibodies to MDA-LDL and ApoB-immune complexes to sex, ethnicity, subclinical atherosclerosis, and cardiovascular events, Arterioscler. Thromb. Vasc. Biol. 37 (2017) 1213–1221.

[10] S. Tsimikas, P. Willeit, J. Willeit, et al., Oxidation-specific biomarkers, prospective 15-year cardiovascular and stroke outcomes, and net reclassification of cardiovascular events, J. Am. Coll. Cardiol. 60 (2012) 2218–2229.

[11] A. Ravandi, S.M. Boekholdt, Z. Mallat, et al., 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. 52 (2011) 1829–1836.

[12] N. Ahmadi, S. Tsimikas, F. Hajsadeghi, et al., Relation of oxidative biomarkers, vascular dysfunction, and progression of coronary artery calcium, Am. J. Cardiol. 105 (2010) 459–466.

[13] S.H. Choi, A. Chae, E. Miller, et al., Relationship between biomarkers of oxidized low-density lipoprotein, statin therapy, quantitative coronary angiography, and atheroma: volume observations from the REVERSAL (Reversal of Atherosclerosis with Aggressive Lipid Lowering) study, J. Am. Coll. Cardiol. 52 (2008) 24–32.

[14] P. Gounopoulos, E. Merki, L.F. Hansen, S.H. Choi, S. Tsimikas, Antibodies to oxidized low density lipoprotein: epidemiological studies and potential clinical applications in cardiovascular disease, Minerva Cardioangiol. 55 (2007) 821–837.

[15] C.J. Binder, N. Papac-Milicevic, J.L. Witztum, Innate sensing of oxidation-specific epitopes in health and disease, Nat. Rev. Immunol. 16 (2016) 485–497.

[16] X. Que, M.-Y. Hung, C. Yeang, et al., Oxidized phospholipids are proinflammatory and proatherogenic in hypercholesterolaemic mice, Nature 558 (2018) 301–306.

[17] X. Sun, J.S. Seidman, P. Zhao, et al., Neutralization of oxidized phospholipids ameliorates non-alcoholic steatohepatitis, Cell Metabol. 31 (2020) 189–206.e8.

[18] B. Suthers, P. Hansbro, S. Thambar, M. McEvoy, R. Peel, J. Attia, Pneumococcal vaccination may induce anti-oxidized low-density lipoprotein antibodies that have potentially protective effects against cardiovascular disease, Vaccine 30 (2012) 3983–3985.

[19] H.W. Grievink, P. Gal, M. Ozsvar Kozma, et al., The effect of a 13-valent conjugate pneumococcal vaccine on circulating antibodies against oxidized LDL and phosphorylcholine in man, A randomized placebo-controlled clinical trial, Biology 9 (2020) 345.

[20] F. Lamontagne, M.P. Garant, J.C. Carvalho, L. Lanthier, M. Smieja, D. Pilon, Pneumococcal vaccination and risk of myocardial infarction, CMAJ (Can. Med. Assoc. J.) 179 (2008) 773–777.

[21] D.T. Eurich, J.J. Johnstone, J.K. Minhas-Sandhu, T.J. Marrie, S.R. Majumdar, Pneumococcal vaccination and risk of acute coronary syndromes in patients with pneumonia: population-based cohort study, Heart 98 (2012) 1072–1077.

[22] H.F. Tseng, J.M. Slezak, V.P. Quinn, L.S. Sy, S.K. Van den Eeden, S.J. Jacobsen, Pneumococcal vaccination and risk of acute myocardial infarction and stroke in men, JAMA : J. Am. Med. Assoc. 303 (2010) 1699–1706.

[23] A.N. Siriwardena, S.M. Gwini, C.A. Coupland, Influenza vaccination, pneumococcal vaccination and risk of acute myocardial infarction: matched case-control study, CMAJ (Can. Med. Assoc. J.) 182 (2010) 1617–1623.

[24] S. Ren, D. Newby, S.C. Li, et al., Effect of the adult pneumococcal polysaccharide vaccine on cardiovascular disease: a systematic review and meta-analysis, Open Heart 2 (2015).

[25] S. Ren, A. Hure, R. Peel, et al., Rationale and design of a randomized controlled trial of pneumococcal polysaccharide vaccine for prevention of cardiovascular events: the Australian Study for the Prevention through Immunization of Cardiovascular Events (AUSPICE), Am. Heart J. 177 (2016) 58–65.

[26] M.K. Chang, C.J. Binder, M. Torzewski, J.L. Witztum, C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: phosphorylcholine of oxidized phospholipids, Proc. Natl. Acad. Sci. Unit. States Am. 99 (2002) 13043.

[27] R.M. Bruno, E. Bianchini, F. Faita, S. Taddei, L. Ghiadoni, Intima media thickness, pulse wave velocity, and flow mediated dilation, Cardiovasc. Ultrasound 12 (2014) 34.

[28] M.W. Lorenz, H.S. Markus, M.L. Bots, M. Rosvall, M. Sitzer, Prediction of clinical cardiovascular events with carotid intima-media thickness, Circulation 115 (2007) 459–467.

[29] L. D'elia, E. La Fata, A. Iannuzzi, P.O. Rubba, Effect of statin therapy on pulse wave velocity: a meta-analysis of randomized controlled trials, Clin. Exp. Hypertens. 40 (2018) 601–608.

[30] U.S. Bedi, M. Singh, P.P. Singh, et al., Effects of statins on progression of carotid atherosclerosis as measured by carotid intimal—medial thickness: a meta-analysis of randomized controlled trials, J. Cardiovasc. Pharmacol. Therapeut. 15 (2010) 268–273.

[31] P. Grigoropoulou, A. Tentolouris, I. Eleftheriadou, et al., Effect of 12-month intervention with low-dose atorvastatin on pulse wave velocity in subjects with type 2 diabetes and dyslipidaemia, Diabetes Vasc. Dis. Res. 16 (2018) 38–46.

[32] P.M. Ridker, E. Danielson, F.A.H. Fonseca, et al., Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein, N. Engl. J. Med. 359 (2008) 2195–2207.

[33] R. Peel, S. Ren, A. Hure, et al., Evaluating recruitment strategies for AUSPICE, a large Australian community-based randomised controlled trial, Med. J. Aust. 210 (2019) 409–415.

[34] J. Nauta, Adjusting for Imbalance in Pre-vaccination State. Statistics in Clinical Vaccine Trials, Springer Berlin Heidelberg, Berlin, Heidelberg, 2011, pp. 57–65.

[35] A.R. Parker, M.A. Park, S. Harding, R.S. Abraham, The total IgM, IgA and IgG antibody responses to pneumococcal polysaccharide vaccination (Pneumovax®23) in a healthy adult population and patients diagnosed with primary immunodeficiencies, Vaccine 37 (2019) 1350–1355.

[36] D.M. Musher, S.B. Manof, C. Liss, et al., Safety and antibody response, including antibody persistence for 5 years, after primary vaccination or revaccination with pneumococcal polysaccharide vaccine in middle-aged and older adults, J. Infect. Dis. 201 (2010) 516–524.

[37] M. Mayr, S. Kiechl, S. Tsimikas, et al., Oxidized low-density lipoprotein autoantibodies, chronic infections, and carotid atherosclerosis in a population-based study, J. Am. Coll. Cardiol. 47 (2006) 2436–2443.

[38] S. Tsimikas, J.L. Witztum, E.R. Miller, et al., High-dose atorvastatin reduces total plasma levels of oxidized phospholipids and immune complexes present on apolipoprotein B-100 in patients with acute coronary syndromes in the MIRACL trial, Circulation 110 (2004) 1406–1412.

[39] S. Tsimikas, H.K. Lau, K.R. Han, et al., Percutaneous coronary intervention results in acute increases in oxidized phospholipids and lipoprotein(a): short-term and long-term immunologic responses to oxidized low-density lipoprotein, Circulation 109 (2004) 3164–3170.

[40] S. Tsimikas, C. Bergmark, R.W. Beyer, et al., Temporal increases in plasma markers of oxidized low-density lipoprotein strongly reflect the presence of acute coronary syndromes, J. Am. Coll. Cardiol. 41 (2003) 360–370.

[41] J. Zou, G. Wang, H. Li, X. Yu, C. Tang, IgM natural antibody T15/E06 in atherosclerosis, Clin. Chim. Acta 504 (2020) 15–22.

[42] C.J. Binder, K. Hartvigsen, M.K. Chang, et al., IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis, J. Clin. Invest. 114 (2004) 427–437.

[43] A. Freijd, L. Hammarström, M.A. Persson, C.I. Smith, Plasma anti-pneumococcal antibody activity of the IgG class and subclasses in otitis prone children, Clin. Exp. Immunol. 56 (1984) 233–238.

[44] E. Ramos-Sevillano, G. Ercoli, J.S. Brown, Mechanisms of naturally acquired immunity to Streptococcus pneumoniae, Front. Immunol. 10 (2019) 358.

[45] R. Malley, Antibody and cell-mediated immunity to Streptococcus pneumoniae: implications for vaccine development, J. Mol. Med. 88 (2010) 135–142.

[46] J. Su, A. Georgiades, R. Wu, T. Thulin, U. de Faire, J. Frostegård, Antibodies of IgM subclass to phosphorylcholine and oxidized LDL are protective factors for atherosclerosis in patients with hypertension, Atherosclerosis 188 (2006) 160–166.

[47] D. Tsiantoulas, C.J. Diehl, J.L. Witztum, C.J. Binder, B cells and humoral immunity in atherosclerosis, Circ. Res. 114 (2014) 1743–1756.