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ORIGINAL ARTICLE

Persistence of Detectable Anti-Pneumococcal Antibodies 4 Years After Pneumococcal Polysaccharide Vaccination in a Randomised Controlled Trial: The Australian Study for the Prevention through Immunisation of Cardiovascular Events (AUSPICE)

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Aim Mouse models have indicated that the pneumococcal polysaccharide vaccine (PPV) can reduce atherosclerosis. This is probably through a process of molecular mimicry, where phosphorylcholine in the capsular polysaccharide of the vaccine elicits antibodies that cross-react with oxidised low-density lipoprotein and reduce plaque. We investigated whether a similar mechanism occurs in humans.

Methods A large national blinded, randomised, placebo-controlled trial of the PPV (Australian Study for the Prevention through Immunisation of Cardiovascular Events [AUSPICE]) is underway with fatal and nonfatal cardiovascular disease (CVD) events as the primary outcome. Participants at one centre agreed to a substudy measuring a number of biomarkers and surrogates of CVD over 4 years, including anti-pneumococcal antibodies (immunoglobulin G and immunoglobulin M), C-reactive protein, carotid intima-media thickness, pulse wave velocity, insulin, fasting blood glucose, glycated haemoglobin, and hepatorenal index.

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Results Antipneumococcal immunoglobulin G and immunoglobulin M were both present and statistically significantly increased in the treated group compared to control at 4 years. However, there were no differences in any of the surrogate measures of CVD or metabolic markers at 4 years.

Conclusions While there were prolonged differences in anti-pneumococcal antibody titres following PPV vaccination, these did not appear to provide any cardioprotective effect, as measured by a range of markers. Final results using the fatal and nonfatal CVD events await the completion of national health record linkage next year.

Trial Registration ACTRN12615000536561.

Keywords Anti-pneumococcal antibodies • Pneumococcal polysaccharide vaccination

Background

For 20 years, evidence has emerged that the pneumococcal polysaccharide vaccine (PPV) may reduce atherosclerosis and related cardiovascular events. Meta-analyses of these studies found a statistically significant reduction in acute coronary syndrome (ACS) events, especially in those aged over 65 years [1,2].

This effect may be potentially mediated via two pathways. A nonspecific effect may occur simply because a pneumococcal infection generates a pro-inflammatory state involving cytokines, metalloproteinases and platelet activation, and vaccination reduces this risk by preventing the infection. Nevertheless, evidence indicates that this cannot be the only mechanism. For example, a study of dialysis patients found that pneumococcal vaccination reduced cardiovascular and all-cause mortality, without reducing hospitalisation due to pneumonia [3]. A specific effect may occur via molecular mimicry [4]. It has been demonstrated that the phosphocholine (PC) moiety present in the capsular polysaccharide cell wall of *Streptococcus pneumoniae* (the pneumococcus) is a molecular mimic of the PC headgroup of oxidised low-density lipoprotein (OxLDL) [5]. Indeed, immunisation with PC (coupled to a carrier) or direct infusion of anti-PC immunoglobulin M (IgM) (T15 antibody) reduced atherosclerosis in mice [5,6]. This may occur via different mechanisms including blocking the uptake of OxLDL by macrophages thereby suppressing the formation of foam cells, reducing cellular toxicity and apoptosis, and reducing both localised and systemic inflammation due to oxidised phospholipids [7–9]. Administration of the pneumococcal vaccine Pneumovax 23 (PPV, Merck Sharpe and Dohme) has been associated with the same anti-OxLDL antibodies in humans [10], but the pneumococcal protein conjugate vaccine (Prevenar, Pfizer) does not seem to elicit the same degree of response [11]. This is probably because the PPV contains PC in the polysaccharide capsules, whereas the pneumococcal protein conjugate vaccine does not. Some international observational studies have provided evidence of an association between PPV and reduced risk of cardiovascular ischaemic events [12,13], while other studies have not [14,15]. However, these observational studies were heterogeneous in terms of the sample population, the exposures measured, and the definition of outcomes [1]. Thus, a randomised controlled trial has been needed to specifically test the effect of the PPV on atherosclerotic cardiovascular risk in humans.

The Australian Study for the Prevention through Immunisation of Cardiovascular Events (AUSPICE) is a multicentre, randomised, placebo-controlled, double-blind, clinical trial formally testing whether vaccination with PPV protects against fatal and non-fatal ACSs and ischaemic strokes. A total of 4,725 participants were recruited at baseline and cardiovascular outcomes will be obtained over ~6 years of follow-up, through health record linkage with state and national administrative datasets [16]. A subgroup of participants at one recruitment site agreed to participate in sub-studies examining the responses to vaccination and surrogate measures of atherosclerosis. The former included anti-pneumococcal immunoglobulin G (IgG), and IgM titres and the latter included high-sensitivity C-reactive protein (CRP, an inflammatory biomarker and a strong predictor of future cardiovascular events [17]), pulse wave velocity (PWV, a measure of arterial stiffness [18]), and carotid intima-media thickness (CIMT, a measure of the extent of carotid artery atherosclerosis [19]). We previously reported the change in these measures between baseline and 2 years [20]. In the intervening time, new research identified a possible effect of anti-OxLDL antibodies on metabolic markers and fatty liver in mouse models [8]. Compared with control mice, transgenic mice secreting the Fc portion of an antibody that binds OxLDL and other phospholipids showed less hepatic inflammation and steatosis, had less fat mass, but had no change in fasting glucose or insulin levels. We therefore added these outcomes to our 4-year time point, measuring fasting glucose, glycated haemoglobin (HbA1c), insulin as well as hepatic steatosis using the hepatorenal index [21]. We report here the updated analyses at 4 years for all the pre-specified markers and the additional metabolic markers.

Methods

The AUSPICE study protocol was published previously [16]. 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 of such events (i.e., having at least two risk factors: hypertension,

hyperlipidaemia, or overweight/obesity) were recruited from six study sites around Australia between February 2017 and November 2018.

Participants (n=4,725) were randomly allocated to receive either PPV 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 [22]. Participants recruited at the Canberra site formed the subgroup reported in the current study (n=1,001) [20].

Measures

Participant characteristics were obtained by questionnaires and physical measurements at baseline [16]. Medications were coded according to the Anatomical Therapeutic Chemical (ATC) codes: C10 for lipid modifying agents, C02 for antihypertensives, and B01 for antithrombotic agents.

C-Reactive Protein

High-sensitivity C-reactive protein (CRP) levels (mg/L; Roche Cobas platform in National Association of Test Authorities [NATA]-accredited Australian Capital Territory [ACT] Pathology laboratory) were collected at baseline, and after 1, 24, and 48 months of follow-up.

Pulse Wave Velocity and Carotid Intima-Media Thickness

Pulse Wave Velocity (PWV) (m/s) and Carotid Intima-Media Thickness (CIMT) measurements (μm) were recorded by an independent assessor at baseline, 2 and 4 years. Using applanation tonometry (SphygmoCor device, AtCor Medical, Sydney, NSW, 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) [18]. 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, Chicago, IL, 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.

Pneumococcal antibody titres

Antipneumococcal capsular polysaccharide Ig antibody titres were measured in 200 randomly selected patients (100 active and 100 control, randomly selected by an independent statistician); IgM (U/mL) and IgG (mg/L) measures were assessed at baseline, 1 month, 2 and 4-year follow-up, using VaccZyme Anti-PCP Enzyme Immunoassay kits.

Metabolic markers

Metabolic markers (fasting glucose, HbA1c and insulin) were assayed by ACT Pathology, a NATA-accredited public pathology provider. Hepatorenal index was measured on a high-resolution ultrasound (GE healthcare, model LOGIQ-E) using the protocol of Marshall et al. [21], and the same operator performed all ultrasounds to minimise interoperator variability.

Statistical Analysis

For the antibody assays, metabolic markers and hepatorenal index measurements, 100 participants were chosen at random in each group (total n=200) 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. For the surrogate outcome measures (CRP, CIMT, PWV) the entire cohort was assessed (total n=1,001).

The analysis of treatment effects at each time point was performed as intention-to-treat using all available cases. Outcome response variables for CRP, CIMT, PWV, and antibody IgG and IgM were fitted as linear mixed models with treatment allocation, time point, and the respective interaction effect as fixed effects. These models included the respective baseline measurement as a fixed effect. Random intercepts at the participant level were included to account for correlated errors introduced by repeated measurements within participants.

The outcome response variables for metabolic markers and hepatorenal index (measured at a single time point) were fitted as simple linear models with treatment allocation as the only fixed effect.

Model diagnostics of the conditional residuals for response variables CRP, CIMT, fasting insulin, and IgG and IgM suggested heteroscedasticity of the conditional residuals, which was remedied by transforming the respective response variables to the scale of natural log. Heteroscedasticity of the conditional residuals was not observed in the remaining outcome response variables and these outcomes were therefore modelled in the original scale. The remaining assumptions for linear models and linear mixed models were examined and deemed appropriate.

The treatment effects for the outcomes modelled in the original scale were presented as the absolute difference of means with 95% confidence intervals (CIs) between the control and treatment arm, at each time point. For the natural log-transformed outcome variables, the treatment effects were presented as the ratio of the exponentiated (back-transformed) natural log-scale means with 95% CI, between the control and treatment arm, at each time point.

In post-hoc analyses, we also looked at correlations between IgG and IgM titres and all the surrogate outcomes, both within treatment groups and pooling across groups.

Statistical analyses were programmed using SAS v9.4 (SAS Institute, Cary, NC, USA). A priori, $p<0.05$ (two-tailed) was used to indicate statistical significance.

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). All participants provided written, informed consent prior to randomisation.

Table 1 **Baseline characteristics of the participants (Canberra centre).**

Characteristic	Response/ statistic	Active vaccine (n=502)	Control (n=499)	Total (N=1001)
Gender	Female	246 (49)	245 (49)	491 (49)
	Male	256 (51)	254 (51)	510 (51)
High blood pressure	Yes	342 (71)	339 (72)	681 (72)
	No	141 (29)	129 (28)	270 (28)
	Missing	19	31	50
Overweight or obese	Yes	491 (98)	473 (95)	964 (96)
	No	11 (2.2)	26 (5.2)	37 (3.7)
Weight (kg)	mean (SD)	93.2 (17.9)	94.2 (18.5)	93.7 (18.2)
	median (Q1–Q3)	91.0 (81.0–103.0)	92.0 (82.0–105.0)	91.0 (81.0–104.0)
Waist (cm)	mean (SD)	105.9 (12.7)	106.8 (13.8)	106.3 (13.3)
	median (Q1–Q3)	105.0 (98.0–113.0)	106.0 (98.0–115.0)	105.0 (98.0–114.0)
Height (cm)	mean (SD)	170.2 (9.9)	170.1 (9.7)	170.2 (9.8)
	median (Q1–Q3)	170.0 (163.0–177.0)	170.0 (163.0–178.0)	170.0 (163.0–177.0)
Age (yrs)	mean (SD)	58 (2)	58 (2)	58 (2)
	median (Q1–Q3)	58 (56–59)	58 (56–59)	58 (56–59)
Lipid modifying drugs	Yes	178 (35)	168 (34)	346 (35)
Antihypertensive agents	Yes	11 (2.2)	14 (2.8)	25 (2.5)
Antithrombotic agents	Yes	7 (1.4)	0	7 (0.7)

All values are n (%) unless otherwise specified.
Abbreviations: SD, standard deviation; Q1, quartile 1; Q3, quartile 3.

Results

The baseline characteristics of the participants in this study are shown in Table 1. The characteristics were evenly balanced between the two groups indicating that randomisation achieved its purpose. The subgroup of 200 participants, selected at random, who were measured for IgG and IgM antibody titres, metabolic markers, and hepatorenal index were very similar in all characteristics to all participants in this study (Supplementary Table 1).

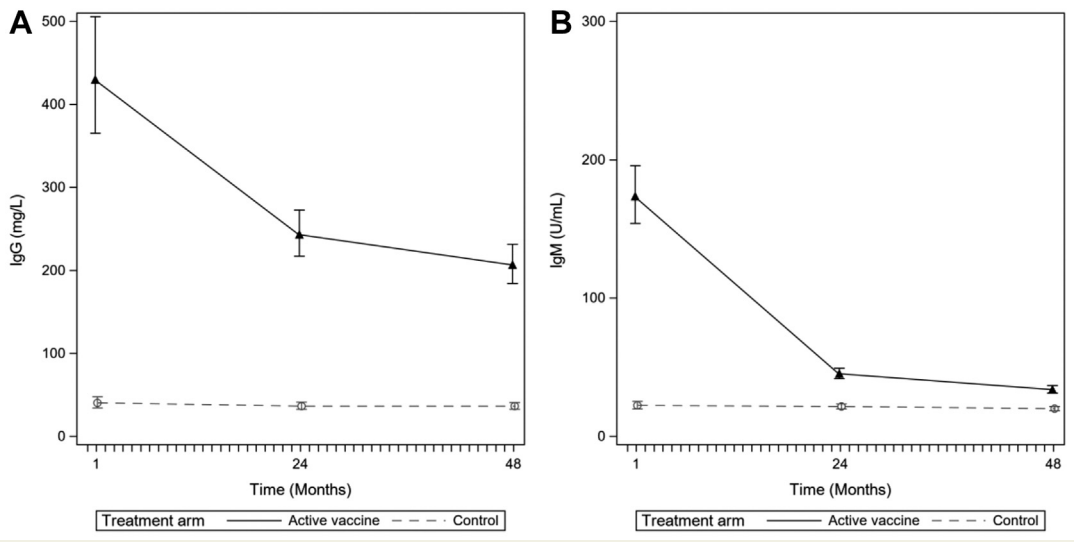

Figure 1 Back-transformed mean estimates of (A) IgG and (B) IgM antibody titres.
Abbreviations: IgG, immunoglobulin G; IgM, immunoglobulin M.

Table 2 **Linear mixed model for differences in IgG and IgM titres over time by treatment arm.**

Outcome		Marginal mean (95% CI)[a]		Treatment effect (95% CI)[a]				
Response	Time point	Active vaccine	Control	Contrast of means	Effect size	Contrast effect p-value	Interaction effect p-value	n
log(IgG)[b]	1-mo follow-up	429.89 (365.54–505.56)	40.64 (34.56–47.80)	Ratio	10.58 (8.41–13.30)	<0.001	<0.001	200
	2-yr follow-up	243.41 (217.12–272.89)	36.76 (32.79–41.21)	Ratio	6.62 (5.63–7.78)	<0.001		
	4-yr follow-up	206.71 (184.43–231.68)	36.59 (32.64–41.02)	Ratio	5.65 (4.81–6.64)	<0.001		
log(IgM)[b]	1-mo follow-up	173.69 (154.02–195.86)	22.53 (19.98–25.41)	Ratio	7.71 (6.50–9.14)	<0.001	<0.001	200
	2-yr follow-up	45.37 (41.83–49.20)	21.60 (19.92–23.43)	Ratio	2.10 (1.87–2.36)	<0.001		
	4-yr follow-up	33.82 (31.19–36.67)	20.09 (18.52–21.79)	Ratio	1.68 (1.50–1.89)	<0.001		

Abbreviations: CI, confidence interval; IgG, immunoglobulin G; IgM, immunoglobulin M.
[a]Adjusted for the respective log-transformed baseline values.
[b]Marginal means are presented in the back-transformed scale.

Antibody Titres

The antibody titres indicated that both anti-pneumococcal IgG and IgM increased within the first month in the vaccination vs the control groups. Although these titres waned over time, they remained statistically significantly higher in the intervention group compared to controls at the 2- and 4-year time points ($p<0.001$; Figure 1). The linear mixed models indicated that the IgG titres in the active group rose to over 10 times that of control at 1 month but were still over 5 times that of control at 4 years ($p<0.001$; Table 2). Likewise, IgM titres rose to over 7 times that of control at 1 month but were still over 1.5 times that of control at 4 years ($p<0.001$; Table 2).

Surrogate Measures of CVD

The changes in CRP, CIMT, and PWV over the 1-month, 2- and 4-year time points are shown in Figure 2. There were no differences in these markers over time between the intervention and control groups, and the linear mixed models essentially showed that the ratio of means between the groups was around unity, i.e., no difference in any of these markers (Supplementary Tables 2–4).

Metabolic Markers

Glucose, HbA1c, and insulin were measured only at the 4-year time point, and these markers showed no difference between the intervention and control groups (Table 3). There was a statistically significant difference in hepatorenal index, with the intervention group having a marginally higher mean index (i.e., higher fat signal, $p=0.032$; Table 4).

Correlation Analyses

IgG and IgM titres were correlated to all the surrogate outcomes, first preserving the treatment groups and then pooling across treatment groups. There were no correlations greater than 0.2 and no significant correlations for any of these post-hoc analyses (see Supplementary Tables 5A and 5B).

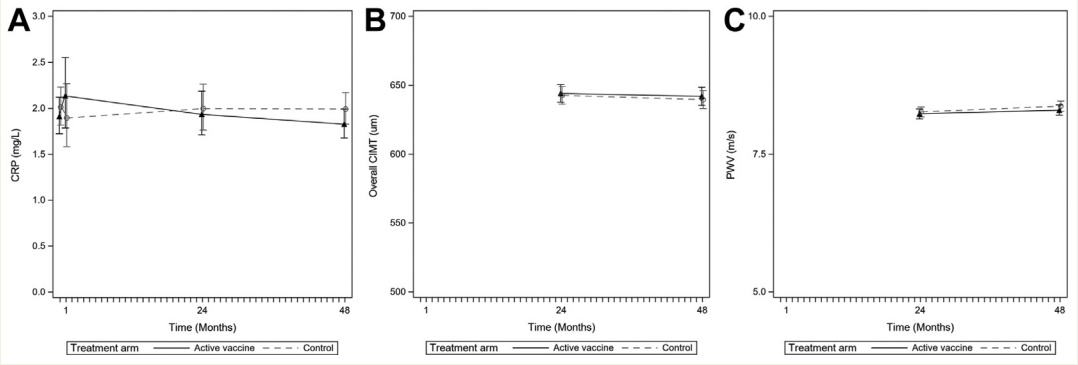

Figure 2 Mean estimates of (A) CRP, (B) CIMT, and (C) PWV in the intervention and control groups at baseline, 1 month, 2 and 4 years.
Abbreviations: CRP, C-reactive protein; CIMT, carotid intima-media thickness; PWV, pulse wave velocity.

Table 3 **Linear model for differences in metabolic marker levels at 4-year follow-up by treatment arm.**

Outcome		Marginal mean (95% CI)		Treatment effect (95% CI)			n
Response	Time point	Active vaccine	Control	Contrast of means	Effect size	Contrast effect p-value	
Glucose (units)	4-yr follow-up	5.47 (5.31–5.63)	5.57 (5.41–5.74)	Difference	–0.10 (–0.33 to 0.13)	0.386	179
HbA1c (units)	4-yr follow-up	5.61 (5.51–5.71)	5.63 (5.53–5.73)	Difference	–0.02 (–0.17 to 0.13)	0.783	178
log(insulin) (units)[a]	4-yr follow-up	9.71 (8.59–10.98)	10.25 (9.06–11.59)	Ratio	0.95 (0.80–1.13)	0.542	171

[a]Marginal means are presented in the back-transformed scale.
Abbreviation: CI, confidence interval; HbA1c, glycated haemoglobin.

Discussion

The idea that anti-OxLDL antibodies, elicited by the pneumococcal PPV vaccine, could cause regression of atherosclerotic plaque, would, if true, be an extremely powerful preventative measure for cardiovascular disease. Although this mechanism has been extensively investigated in mouse models of atherosclerosis [4–6], the data in humans are less robust [23–33]. In this first-ever randomised controlled trial, we show that antipneumococcal antibody titres are still detectable and higher than baseline even 4 years after vaccination; this includes both IgG and IgM. The latter is somewhat surprising given the usual understanding that IgM represent the initial and transient immune response to an antigen. Thus, the detection of IgM antibodies that are still significantly higher than baseline at 4 years in our study heralds the potential for long-lasting effects of the pneumococcal vaccine.

It does not appear however that either the IgG or IgM antibodies detected in these assays retain their anti-OxLDL activity. We previously assayed for this and found that the initial anti-OxLDL activity seen at 1 month was only for IgM (not IgG) and had disappeared at the 2-year mark [20], hence they were not repeated again here.

In accordance with the lack of anti-OxLDL activity, we did not detect any differences in any of the surrogate markers of cardiovascular disease; CRP, as an inflammatory marker; CIMT, as a marker of atherosclerosis; and PWV, as a marker of arterial stiffness, showed no difference between the vaccinated and control groups even 4 years after baseline, despite a large sample size. With 500 participants in each arm, we had 80% power (at p=0.05) to detect a Cohen's d of 0.18, which is a small effect. The relative stability of these markers over time is also congruent with other studies. For example, a study in the United States [34] and one in China [35], in similar aged, nondiseased populations over 4 and 5 years' follow-up, found very similar baseline CIMT (0.62-0.63 mm) and similar changes over time (0.001–0.005 mm/yr) to our study (0.001–0.003 mm/yr).

New work had indicated the possibility that anti-OxLDL antibodies might have metabolic effects: mice generating an Fc fragment with this activity showed no change in fasting glucose or insulin levels but did show significant decreases in hepatic steatosis and fibrosis on histological examination [8]. While this was too invasive to perform on humans, we used ultrasound and calculated the hepatorenal index, which compared density in the liver to density in the ipsilateral renal parenchyma. While there was similarly no effect on fasting glucose, insulin, or HbA1c in humans in our study, there was a borderline significant effect on fatty accumulation in the liver, but this was in the direction of more hepatostatosis in the vaccinated group. However, in the context of many comparisons, this is of questionable significance, especially given that most values were below the threshold of 1.28, which gives 100% sensitivity for detecting fatty liver [21].

The failure to reproduce the results from the mouse models may be due to a number of possibilities: (1) the transient nature of anti-OxLDL activity following pneumococcal vaccination; (2) the late time point for the ultrasound examination, i.e. an effect on fatty accumulation in the liver might have been seen earlier after vaccination; (3) insufficient power to detect a difference, although with 100 in each group, we

Table 4 **Linear model for differences in HRI at 4-year follow-up by treatment arm.**

Outcome		Marginal mean (95% CI)		Treatment effect (95% CI)			n
Response	Time point	Active vaccine	Control	Contrast of means	Effect size	Contrast effect P-Value	
HRI	4-yr follow-up	1.23 (1.17–1.29)	1.14 (1.09–1.20)	Difference	0.09 (0.01–0.17)	0.032	173

Abbreviation: HRI, hepatorenal index; CI, confidence interval.

should have been able to detect a Cohen's d of 0.4, which is a modest effect. With the standard deviation in our measurements, a Cohen's d of 0.4 would equate to a difference of ~0.14 units in the hepatorenal index, which would have been ample to capture a clinically meaningful difference.

Conclusions

The final conclusions on whether the pneumococcal vaccination is cardioprotective, either through an anti-oxLDL pathway, or through an alternative mechanism, will need to await the final clinical outcomes of the trial. The investigators formally closed the study on January 31, 2023, after a mean follow-up of almost 6 years. Cardiovascular outcomes will be assessed through national health record linkage, with final results anticipated in 2024.

Conflicts of Interest

There are no conflicts of interest to disclose.

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Appendices

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.hlc.2023.09.006.

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