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Systems Immunology reveals a linked IgG3-C4 response in patients with Acute Rheumatic Fever

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Abstract

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Acute rheumatic fever (ARF) and chronic rheumatic heart disease (RHD) are autoimmune sequelae of a Group A Streptococcus (GAS) infection with significant global mortality and poorly understood pathogenesis. Immunoglobulin and complement deposition were observed in ARF/RHD valve tissue over 50 years ago, yet contemporary investigations have been lacking. This study applied systems immunology to investigate the relationships between the complement system and immunoglobulin in ARF. Patients were stratified by C-Reactive Protein (CRP) concentration into high ($\geq 10 \mu\text{g mL}^{-1}$) and low ($< 10 \mu\text{g mL}^{-1}$) groups to distinguish those with clinically significant inflammatory processes from those with abating inflammation. The circulating concentration of 17 complement factors and six immunoglobulin isotypes and subclasses were measured in ARF patients and highly matched healthy controls using multiplex bead-based immunoassays. An integrative statistical approach combining feature selection (LASSO) and principal component analysis revealed a linked IgG3-C4 response in ARF patients with high CRP that was absent in controls. Strikingly, both IgG3 and C4 were elevated above clinical reference ranges, suggesting these features are a marker of ARF associated inflammation. Humoral immunity in response to M protein, an antigen implicated in ARF pathogenesis, was completely polarised to IgG3 in the patient group. Furthermore, the anti-M protein IgG3 response was highly correlated with circulating IgG3 concentration, highlighting a potential role for this potent immunoglobulin subclass in disease. In conclusion, a linked IgG3-C4 response appears important in the initial, inflammatory stage of ARF and may have immediate utility as a clinical biomarker given the lack of specific diagnostic tests currently available.

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INTRODUCTION

Acute rheumatic fever (ARF) is a severe post-infectious consequence of a Group A streptococcal (GAS) infection that can result in rheumatic heart disease (RHD). ARF is an acute inflammatory illness in which patients present with fever, migratory polyarthritides, subcutaneous nodules and carditis¹. Recurrences of ARF with carditis can lead to permanent heart valve damage and RHD². Unlike ARF where active inflammation contributes to symptoms, RHD is post-inflammatory and chronic in nature³. Globally there are approximately 33 million people living with RHD and this condition is responsible for over 300,000 deaths per annum⁴. ARF and RHD are generally considered diseases of the developing world, yet they persist in indigenous populations in the developed world. Māori

and Pacific children in New Zealand and Aboriginal children in Australia have among the highest incidences of ARF globally ^{5,6}.

ARF pathogenesis is poorly understood with the current prevailing hypothesis based on the notion that the alpha helical coiled-coil structure of GAS M-protein mimics human proteins with a coiled-coiled structure ¹. This mimicry is thought to induce M-protein specific antibodies and T-cells that cross-react with cardiac myosin, laminin and tropomyosin in the heart and synovium ⁷. However, the role of mimicry is debated, with an alternative hypothesis suggesting that GAS colonisation results in disruption of the extracellular matrix and exposure of cryptic collagen epitopes, which trigger an autoimmune response ⁸⁻¹⁰.

Despite the current uncertainty around triggering antigen(s), the humoral basis for ARF was documented more than five decades ago when Kaplan *et al.* observed immunoglobulin and complement factor C3 deposits on the heart valves of children who had died from ARF with RHD ¹¹. The presence of C3 in these inflamed valves suggests a role for complement in ARF pathogenesis, yet contemporary analyses of the complement system in ARF are lacking. Technological advancements now enable the quantification and/or function of large panels of serum proteins to be determined concurrently in multiplex assays ^{12,13}. When combined with systems level computational approaches that allow for multidimensional analysis, the data generated by these high throughput assays can identify novel combinations of immune features associated with a particular disease state or treatment response ¹⁴⁻¹⁶. The aim of this study was to apply these technologies to enable the first systematic and comprehensive analysis of the relationships between the complement system and immunoglobulin in ARF. The circulating concentration of complement factors that span the initiation (classical, lectin and alternative pathways), amplification and effector phases of the complement cascade, together with six immunoglobulin isotypes and subclasses in a cohort of ARF patients and matched healthy controls is reported. By applying systems serology data modelling techniques ¹⁵, a unique complement-immunoglobulin relationship was revealed that provides new insight into ARF inflammation.

RESULTS

Demographics and stratification by concentration of C-reactive Protein

The 69 participants comprised children diagnosed with ARF according to the New Zealand modification of the Jones criteria ¹⁷ (n=34) and matched healthy controls (n=35). Demographics are shown in Table 1. Single serum samples were obtained from each ARF

participant during their hospital stay and the C-reactive protein (CRP) concentration was used to stratify patients into ‘high’ ($\geq 10 \mu\text{g mL}^{-1}$, clinically significant inflammation) and ‘low’ CRP ($< 10 \mu\text{g mL}^{-1}$, reduced inflammation) groups¹⁸. All 69 participants identified as either Māori or Pacific and the median age across the three groups was 11-11.5 years old, reflecting the peak incidence age for ARF in New Zealand¹⁹.

Circulating complement factors and immunoglobulin

The concentration of the 17 complement factors, 6 different immunoglobulin isotypes and subclasses, and anti-C1q were determined using bead-based immunoassays and ELISA methodologies and compared between the ARF-high and ARF-low CRP groups and healthy controls in a single variable manner. Seven of the 24 analytes were significantly elevated ($P < 0.05$, mean FC > 1.5) in the ARF-high CRP group compared with healthy controls, with levels trending back to control levels in the ARF-low CRP group (Figure 1, Table S1). This included the opsonization pre-cursor C4, complement system regulators factor B and H, and the immunoglobulins IgG1, IgG3, IgG4 and IgA. The clear asymmetry of the Volcano plot for healthy controls compared with the ARF-high CRP group (Figure 1b) illustrates a general trend for elevation of circulating complement factors and immunoglobulin in ARF patients. Indeed, no notable reductions in the circulating concentrations of any analyte were observed in the ARF patient groups compared with the healthy controls, though the concentrations of some (including anti-C1q antibodies, the alternative pathway regulator Factor D, and the effector phase component C9) were essentially unchanged. When clinical reference ranges were applied to the seven significantly elevated analytes, only IgG3 and C4 showed marked elevation in the ARF-high CRP group; 18/20 patients (90%) and 10/20 patients (50%) had IgG3 and C4 concentrations above the upper limit of the reference range, respectively (Figure 1c). These proportions were reduced in the ARF-low CRP group such that for IgG3 11/14 patients (78%) and for C4 4/14 patients (28%) had concentrations above reference ranges. For the remaining five analytes, $\leq 25\%$ of patients in the ARF-high CRP group had serum concentrations above clinically accepted normal ranges.

Identification of Rheumatic Fever Specific Features

To further explore relationships between complement and immunoglobulin in ARF, and to identify key features contributing to differences between groups, “systems immunology” methodology was applied^{15,20}. Multidimensional analysis combining feature selection (the

least absolute shrinkage and selection operator; LASSO) and principal component analysis (PCA) were used to analyze the 27 features comprising the 24 analytes from the univariate analysis together with age, gender and CRP concentration. Four of the 27 features accounted for 82% of the variance between healthy controls and patients in the ARF-high CRP group, when assessing across two PCA dimensions (Figure 2). Near to complete separation of the healthy controls from ARF-high CRP patients is shown in the scores plot, with each point representing individual healthy controls (blue) or ARF (yellow) participants (Figure 2c). The first dimension (PC1) accounted for most of the variance between the groups (64.2%), and the corresponding loadings plot (Figure 2d) shows the contribution of the four features (C4, IgG3, IgA and IgG1) selected by LASSO. The scores plot highlights some heterogeneity amongst ARF patients with the second dimension (PC2) accounting for 17.8% of the variance (Figure 2c). However, the majority of individuals in the ARF group (65%) are skewed towards elevated C4 and IgG3 and scatter with these features in multidimensional space (Figure 2d).

When the three groups were analyzed simultaneously (healthy controls, ARF-high CRP and ARF-low CRP), the same four features separated the participant profiles and accounted for 76.5% of the variance (Supplementary Figure 1). Again, the first dimension (PC1) accounted for most of the variance (59.3%), with the scores and loading plots highlighting a dominant effect of IgG3 and C4 on the rheumatic fever profile.

The proximity of IgG3 and C4 in the loadings plots prompted a correlation analysis of these two features. Scatter plots show that the concentration of serum IgG3 and C4 were significantly correlated in the ARF-high CRP group ($P < 0.05$) but not in healthy controls (Figure 3).

Antigen Specific Humoral Responses in Rheumatic Fever

To determine whether the increase in circulating IgG3 observed in the ARF-high CRP group was due to IgG3 specific for GAS antigens associated with ARF, ELISA detecting the four IgG isotypes were performed. M protein was chosen as an ARF associated antigen since antibodies specific for M protein repeats are proposed to cross-react with cardiac antigens and cause valvulitis^{1,21}. The M6 protein was selected as a representative M protein as *emm6* strains have been associated with ARF in New Zealand²². As a comparator, the conserved GAS antigen Streptolysin-O (SLO) was chosen as it is known to be highly immunogenic but is not implicated in ARF pathogenesis^{23,24}.

A marked difference in the IgG subclass response to the two GAS antigens was observed in the ARF-high CRP participants. For SLO, IgG1 was the most abundant subclass, with IgG2, IgG3 and IgG4 detected at moderate levels. In contrast, the response induced by M6 was dominated by IgG3, followed by IgG1, with IgG2 and IgG4 responses low or undetectable (Figure 4a). When the ratio of IgG3/IgG1 was plotted there was a highly significant difference between SLO and M6 ($P < 0.0001$), such that for all 20 ARF patients (100%) the most abundant anti-M6 subclass was IgG3, while the most abundant anti-SLO subclass was IgG1 (Figure 4b). Six of the 34 healthy control participants had evidence of a prior GAS exposure via clinical streptococcal serology²³. Comparison of the IgG subclass response in these six participants with the ARF-high CRP patients showed reduced M6 IgG3 polarization (Supplementary Figure 2). Indeed, only 3/6 (50%) healthy controls had IgG3 as the most abundant anti-M6 subclass and the ratio of anti-M6 IgG3/IgG1 in the ARF-high participants was significantly elevated compared with controls ($P < 0.01$).

To investigate whether the M6 anti-IgG3 in ARF patients was directed towards the hypervariable (HVR) N-terminus of the M protein, or the more conserved repeat regions implicated in pathogenesis^{7,25}, ELISA were performed with a 50-mer peptide corresponding to the M6 HVR sequence. Four of the 20 participants in the ARF-high CRP group had elevated total IgG to M6 HVR (Supplementary Figure 3), indicative of a previous infection with an M6 GAS strain²⁶. Further ELISA in these four M6-positive ARF patients showed the type-specific, HVR response was dominated by IgG1, in contrast with the IgG3 response for the full-length M6 protein (Supplementary Figure 3). Thus, it can be assumed that most of the IgG3 M protein binding in the ARF-high CRP group is to the more conserved repeat regions of the protein.

Finally, a correlation analysis performed between the circulating levels of IgG3 with antigen specific isotype responses found a significant correlation between anti-M6 IgG3 levels and total IgG3 ($P < 0.05$), but not anti-SLO IgG3 levels and total IgG3 (Figure 4c). This suggests anti-M protein antibodies may contribute to the elevated IgG3 observed in ARF patient sera.

DISCUSSION

This study has examined the circulating levels of complement factors and immunoglobulin in children with ARF utilizing sera obtained during hospital admission. A striking elevation of IgG3 and C4 was observed in ARF patients with clinically significant inflammation ($\text{CRP} \geq 10 \mu\text{g mL}^{-1}$) with 90% and 50%, respectively, having circulating concentrations above reference ranges. Though other immunoglobulins and complement factors were

192 elevated, $\leq 25\%$ of patients had circulating concentrations above reference ranges suggesting
193 these analytes maybe of less clinical importance. The application of a multivariate, systems
194 serology approach to analyse the data set also identified IgG3 and C4 as key features
195 associated with ARF. This, together with a significant correlation between the circulating
196 concentration of IgG3 and C4 suggests an important role for a linked IgG3-C4 response in
197 ARF inflammation.

198 The association of IgG3, the most potent IgG subclass with respect to classical pathway
199 activation ²⁷, and C4, an early classical pathway component, might suggest classical pathway
200 driven complement activation in ARF inflammation. This is supported by studies conducted
201 over 40 years ago that found IgG (total), C3 and C4 were elevated in circulation, yet
202 decreased in the synovial fluid of affected joints in ARF patients ^{28,29}. In the early stages of
203 disease, IgG3-C4 complement driven inflammation may contribute to immune activation at
204 affected tissue sites in ARF. This process appears to reverse as inflammation abates given the
205 concentration of complement and immunoglobulin features in patients with low CRP return
206 to control levels, and decreases in complement and immunoglobulin were observed in a
207 recent proteomic study of post-inflammatory RHD ³⁰.

208 An acute phase response to proinflammatory cytokines, rather than activation of the
209 complement cascade *per se*, may also contribute to C4 elevation. Complement C3 and C4 are
210 well described acute phase reactants – that is plasma proteins which increase in response to
211 inflammatory stimulus and proinflammatory cytokines such as interleukin(IL)-6, IL-1,
212 tumor necrosis factor- α and interferon(IFN)- γ ³¹. Secretion of both IL-6 and IFN- γ is
213 significantly elevated in peripheral blood mononuclear cells from ARF patients stimulated
214 with GAS ³² and circulating concentrations of proinflammatory cytokines are elevated in
215 ARF (reviewed by ³³). Thus, the linked IgG3-C4 response observed here may be a marker for
216 a proinflammatory state in ARF patients.

217 The significant elevation in complement C4 in ARF contrasts with the reduction in C4
218 associated with post-streptococcal glomerulonephritis (PSGN), the other major post-
219 infectious sequelae associated with GAS infection ³³. Indeed, transient depression of C1q,
220 C2, C3 and C4 are hallmarks of PSGN ^{34,35} and are thought to result from immune complex
221 formation with complement consumption, and lead to glomerular immune deposits ³⁴. The
222 opposing observation of increased complement features in ARF suggests very different roles
223 for complement in these two GAS sequelae. Understanding the proposed contributions of (i)
224 IgG3 mediated activation of the classical complement cascade, versus (ii) proinflammatory

cytokines and acute phase responses, to the linked IgG3-C4 response in ARF will require broader interrogation of immune features. Yet, regardless of the contributing mechanisms, IgG3 and C4 may have immediate clinical utility as disease biomarkers. Specific diagnostic tests for ARF are lacking¹⁰, and the incorporation of IgG3 and C4 into the panel of tests currently utilized may aid differential diagnosis, particularly as levels were elevated above reference ranges.

A central question in ARF pathogenesis is the role of M-protein specific antibody cross-reactivity in causing disease⁸. In this study the humoral response to M6 protein was found to be dominated by IgG3, whereas the response to SLO, a conserved antigen not implicated in ARF pathogenesis, was dominated by IgG1. Polarization to an IgG3 humoral response to M protein was previously observed in a study of patients with GAS associated sore throats, cellulitis and glomerulonephritis³⁶. However, the IgG3 predominance was not as marked with 48% of the sera tested having an IgG3:IgG1 ratio >1.0, compared with 100% of ARF participants in this study. A recent investigation of adaptive immunity to two conserved GAS antigens in adults compared with children found polarization to IgG3 increases with age³⁷. It is possible that in ARF, there is dysregulation of the antigen specific IgG3 response at a younger age due to repeated exposure to GAS in at risk populations. This process of “immune priming”³⁸ may contribute to the significant elevation of M protein specific IgG3 observed in this study. Given the diversity of M types epidemiologically associated with ARF²², and that the hypervariable N-terminus of the M6 protein induced an IgG1 dominated response, it is likely that most of the observed IgG3 M protein binding is to the more conserved B- and C- repeat regions of the protein. While these M protein regions have been proposed to contain cross-reactive epitopes to host proteins¹, it is difficult to postulate on any possible link to pathogenesis in the absence of a confirmed and clinically validated target for autoantibodies in ARF³⁹. Nevertheless, the elevation of M protein specific IgG3 in ARF may be a consideration for GAS vaccine development, where the need to generate protective immune responses should be balanced with the potential risks of eliciting a harmful response. In conclusion, this study has utilized multiplex assays combined with multidimensional analysis to identify a linked IgG3-C4 response in patients with ARF. This response may be a marker for patients in a proinflammatory state and, as IgG3 and C4 are elevated above normal reference ranges, may have utility in ARF diagnostics.

METHODS

Study subjects

Human blood samples were obtained from participants recruited as part of the Rheumatic Fever Risk Factors (RF RISK) study conducted in New Zealand between 2014-2017. All participants (or proxies) provided written informed consent and the protocols had appropriate ethical board approval (HDEC 14/NTA/53). This study included sera from participants recruited in the first 18 months (September 2014 – April 2016) of a three-year recruitment period. Patients with first episode ARF were diagnosed according to the New Zealand modification of the Jones criteria¹⁷ and those with a ‘probable/definite’ diagnosis were included, while those categorised as ‘suspect ARF’ were excluded. Control samples were obtained from healthy participants that were matched for age, ethnic identification, area-based measures of sociodemographic deprivation (as assessed by the New Zealand Deprivation Index score⁴⁰) and geographic area (district health board region in which the subject resides). Blood specimens were collected into serum clotting tubes and serum was separated by centrifugation, frozen and stored at -80 °C until analysis.

Measurement of C-reactive protein

The CRP concentration in sera was determined using a custom made singleplex cytometric bead array assay (BioLegend LEGENDplex, San Diego) as per the manufacturer’s instructions. Serum samples were diluted 1:600 and fluorescence was detected using a flow cytometer (BD Accuri C6). Data analysis was performed using Flow Cytometric Analysis Program (FCAP) Array software, version 3 (Becton–Dickinson, New Jersey).

Given the varied length of hospital admission and broad range of disease severity associated with ARF presentation, patients were stratified into those with CRP < 10 µg mL⁻¹ (ARF-low CRP) and CRP ≥ 10 µg mL⁻¹ (ARF-high CRP). As an acute phase protein, CRP is released during systemic inflammation and tissue damage⁴¹ and was used to distinguish participants with clinically significant inflammatory processes from those in which these processes were abating¹⁸. Healthy controls with CRP ≥ 10 µg mL⁻¹ were excluded from further analysis since, in the absence of full clinical information, it was difficult to interpret whether elevated CRP was due to an undiagnosed infection or inflammatory condition or another, unrelated reason. Of the 45 healthy controls recruited in the 18-month timeframe, 35 had CRP < 10 µg mL⁻¹ and were included in all subsequent analysis (Table 1). These 35 healthy controls were screened for prior GAS exposure via streptococcal serology using bead-based immunoassays as previously published²³.

IgG isotype measurements

The concentration of six different immunoglobulin isotypes and subclasses were measured in the sera using magnetic human immunoglobulin isotyping beads (Merck Millipore, Darmstadt). The assays were run in a 5-plex format containing beads for IgG1, IgG2, IgG4, IgM and IgA with a serum dilution of 1:16,000. A singleplex assay was needed for IgG3 that required a serum dilution of 1:50,000 for the resulting Median Fluorescence Intensities (MFI) to fall within the range of the standard curve. Assay plates were read using a Magpix Analyser (Luminex Corporation, Austin) and MFI was converted to concentration using the associated Xponent software (version 4.2, Luminex Corporation, Austin).

Complement System Factors

Two different bead-based platforms were utilized to measure complement system factors. The concentrations for C3a and C4a were determined by Cytometric Bead Array (CBA) assay (Human Anaphylatoxin Kit, Becton–Dickinson, New Jersey) using a serum dilution of 1:6000, while C5a was measured using the same kit and a dilution of 1:300. Fluorescence was detected using a flow cytometer (BD Accuri C6, New Jersey) and conversion to concentration was performed using Flow Cytometric Analysis Program (FCAP) Array software, version 3 (Becton–Dickinson, New Jersey). A further 15 complement factors were measured using human complement magnetic bead panels (Merck Millipore, Darmstadt). One panel included beads for C2, C4b, C5, C5a, C9, Factor D, Mannose-Binding lectin (MBL) and Factor I and required a serum dilution of 1:200, while the second panel included beads for C1q, C3, C3b, C4, Factor B, Factor H and Properdin and required a serum dilution of 1:40,000. Fluorescence was detected using a Magpix Analyser (Luminex Corporation, Austin) and MFI was converted to concentration using the associated Xponent software (version 4.2, Luminex Corporation, Austin). C5a was included in both the CBA and magnetic bead panels, with a strong correlation between the assays (Pearson r 0.86, $P < 0.001$) such that only data from the magnetic bead panel are presented.

The concentration of C1q auto-antibodies in sera were measured using a C1q antibody Enzyme-Linked Immunosorbent Assay (ELISA) kit and a 1:100 dilution as per the manufacturer's instructions (Abnova Corporation, Taipei City).

IgG1 and IgG3 Enzyme-Linked Immunosorbent Assays

The M6 and SLO proteins used in IgG isotype ELISA were prepared using standard recombinant expression techniques in *Escherichia coli*. M6 protein from MGAS10394 was

produced as a mature protein, with the signal sequence and transmembrane removed, via expression in BL21(DE3) pLysS *E. coli* followed by immobilized-metal affinity chromatography (IMAC) as previously described⁴². SLO from MGAS SF370 was produced without the signal sequence, and with P427L and W535F mutations to remove lysin activity, via expression in BL21-AI *E. coli* and IMAC purification as published⁴³. The recombinant His₆-tag was cleaved from M6 and SLO using 3C and rTEV protease, respectively, and both antigens were further purified by gel filtration using a Superdex 200 column (GE Lifesciences, Pittsburgh)⁴². The M6 50-mer peptide corresponding to the N-terminal HVR sequence was obtained commercially (GenScript, Piscataway, New Jersey).

For ELISA using recombinant proteins, Nunc-immunoplates (Sigma-Aldrich, St Louis) were coated with antigen at 5 µg ml⁻¹ and blocked with Phosphate Buffered Saline supplemented with 0.1% Tween-20 and 5% skim milk powder for 1 hour at room temperature (RT). Serum was added at a 1:200 dilution and incubated for 1 hour at RT. IgG binding was detected using isotype specific secondary antibodies labelled with horse-radish peroxidase at 1:500 dilution as recommended by the manufacturer (ThermoFisher Scientific, Waltham). Antigen coating for ELISA using the M6 50-mer peptide was modified according to published protocols²⁶. The resulting absorbance readings were converted to normalized ELISA Units (nEU) in GraphPad Prism (Version 8.0) using a 7-point standard curve constructed with positive control sera. The lowest dilution (1:500) on the standard curve was designated 10 nEU, while the highest dilution (1:1,039,500) was designated 0.01 nEU and the same standard curve was included on each ELISA plate.

Data analysis and statistics

The univariate analysis of immunoglobulin and complement factors utilized a Kruskal-Wallis test for comparison between three groups (healthy control, high CRP cases and low CRP cases), with Dunn's multiple comparisons test. The IgG isotype ELISA were analysed using a Wilcoxon matched pairs signed rank test for comparison of antigen specific Ig in each sera sample. Correlations were calculated using Spearman r. All of these analyses were performed in GraphPad Prism (Version 8.0) and a *P*-value of ≤ 0.05 was considered significant.

Key features that contributed to differences between groups were identified using principal component analysis (PCA)⁴⁴, while least absolute shrinkage and selection operator (LASSO) penalized regression feature selection method⁴⁵, was used to reduce the dimensions of the outcome variables. Cross-validation was performed iteratively to find the optimal value of the

regularized parameters (k-fold cross validation). Prior to analysis, all data were normalized with mean centering and variance scaling.

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REFERENCES

- 1 Carapetis JR, Beaton A, Cunningham MW *et al.* Acute rheumatic fever and rheumatic heart disease. *Nat Rev Dis Primers* 2016; **1**: 1–24.
- 2 Carapetis JR, McDonald M, Wilson NJ. Acute rheumatic fever. *Lancet* 2005; **366**: 155–168.
- 3 Steer AC. Historical aspects of rheumatic fever. *Journal of Paediatrics and Child Health* 2015; **51**: 21–27.
- 4 Watkins DA, Johnson CO, Colquhoun SM *et al.* Global, Regional, and National Burden of Rheumatic Heart Disease, 1990–2015. *N Engl J Med* 2017; **377**: 713–722.
- 5 Milne RJ, Lennon DR, Stewart JM, Vander Hoorn S, Scuffham PA. Incidence of acute rheumatic fever in New Zealand children and youth. *Journal of Paediatrics and Child Health* 2012; **48**: 685–691.
- 6 Parnaby MG, Carapetis JR. Rheumatic fever in indigenous Australian children. *Journal of Paediatrics and Child Health* 2010; **46**: 527–533.
- 7 Cunningham MW. Streptococcus and rheumatic fever. *Current Opinion in Rheumatology* 2012; **24**: 408–416.
- 8 Tandon R, Sharma M, Chandrashekhar Y, Kotb M, Yacoub MH, Narula J. Revisiting the pathogenesis of rheumatic fever and carditis. *Nat Rev Cardiol* 2013; **10**: 171–177.
- 9 Cunningham MW. Rheumatic fever revisited. *Nat Rev Cardiol* 2014; **11**: 123.
- 10 Karthikeyan G, Guilherme L. Acute rheumatic fever. *Lancet* 2018; **392**: 161–174.

- 385 11 Kaplan MH, Bolande R, Rakita L, Blair J. Presence of bound immunoglobulins and
386 complement in the myocardium in acute rheumatic fever. Association with cardiac
387 failure. *N Engl J Med* 1964; **271**: 637–645.
- 388 12 Bongoni AK, Lanz J, Rieben R, Banz Y. Development of a bead-based multiplex assay
389 for the simultaneous detection of porcine inflammation markers using xMAP technology.
390 *Cytometry A* 2013; **83**: 636–647.
- 391 13 Lash GE, Pinto LA. Multiplex cytokine analysis technologies. *Expert Rev Vaccines*
392 2010; **9**: 1231–1237.
- 393 14 Kidd BA, Peters LA, Schadt EE, Dudley JT. Unifying immunology with informatics and
394 multiscale biology. *Nat Immunol* 2014; **15**: 118–127.
- 395 15 Chung AW, Alter G. Systems serology: profiling vaccine induced humoral immunity
396 against HIV. *Retrovirology* 2017; **14**: 57.
- 397 16 Nakaya HI, Pulendran B. Vaccinology in the era of high-throughput biology. *Phil Trans*
398 *R Soc B* 2015. doi:10.1086/650310.
- 399 17 Atatoa-Carr P, Lennon D, Wilson N, New Zealand Rheumatic Fever Guidelines Writing
400 Group. Rheumatic fever diagnosis, management, and secondary prevention: a New
401 Zealand guideline. *N Z Med J* 2008; **121**: 59–69.
- 402 18 Kushner I, Antonelli MJ. What should we regard as an ‘elevated’ C-reactive protein
403 level? *Ann Intern Med* 2015; **163**: 326.
- 404 19 Gurney JK, Stanley J, Baker MG, Wilson NJ, Sarfati D. Estimating the risk of acute
405 rheumatic fever in New Zealand by age, ethnicity and deprivation. *Epidemiol Infect*
406 2016; 1–10.
- 407 20 Chung AW, Kumar MP, Arnold KB *et al.* Dissecting Polyclonal Vaccine-Induced
408 Humoral Immunity against HIV Using Systems Serology. *Cell* 2015; **163**: 988–998.
- 409 21 Fischetti VA. Streptococcal M protein: molecular design and biological behavior. *Clin*
410 *Microbiol Rev* 1989; **2**: 285–314.
- 411 22 Williamson DA, Smeesters PR, Steer AC *et al.* M-Protein Analysis of Streptococcus
412 pyogenes Isolates Associated with Acute Rheumatic Fever in New Zealand. *J Clin*
413 *Microbiol* 2015; **53**: 3618–3620.
- 414 23 Hanson-Manful P, Whitcombe AL, Young PG *et al.* The novel Group A Streptococcus
415 antigen SpnA combined with bead-based immunoassay technology improves
416 streptococcal serology for the diagnosis of acute rheumatic fever. *J Infect* 2018; **76**: 361–
417 368.

- 24 Bensi G, Mora M, Tuscano G *et al.* Multi High-Throughput Approach for Highly Selective Identification of Vaccine Candidates: the Group A Streptococcus Case. *Molecular & Cellular Proteomics* 2012; **11**: M111.015693–M111.015693.
- 25 Smeesters PR, McMillan DJ, Sriprakash KS. The streptococcal M protein: a highly versatile molecule. *Trends in Microbiology* 2010; **18**: 275–282.
- 26 Frost HR, Laho D, Sanderson-Smith ML *et al.* Immune Cross-Opsonization Within emm Clusters Following Group A Streptococcus Skin Infection: Broadening the Scope of Type-Specific Immunity. *Clin Infect Dis* 2017; **65**: 1523–1531.
- 27 Damelang T, Rogerson SJ, Kent SJ, Chung AW. Role of IgG3 in Infectious Diseases. *Trends Immunol* 2019; **40**: 197–211.
- 28 Bhattacharya S, Reddy KS, Sundaram KR *et al.* Differentiation of patients with rheumatic fever from those with inactive rheumatic heart disease using the artificial subcutaneous nodule test, myocardial reactive antibodies, serum immunoglobulin and serum complement levels. *Int J Cardiol* 1987; **14**: 71–78.
- 29 Svartman M, Potter EV, Poon-King T, Earle DP. Immunoglobulins and complement components in synovial fluid of patients with acute rheumatic fever. *J Clin Invest* 1975; **56**: 111–117.
- 30 Mukherjee S, Jagadeeshaprasad MG, Banerjee T *et al.* Proteomic analysis of human plasma in chronic rheumatic mitral stenosis reveals proteins involved in the complement and coagulation cascade. *Clinical Proteomics* 2014; **11**: 35.
- 31 Markanday A. Acute Phase Reactants in Infections: Evidence-Based Review and a Guide for Clinicians. *Open Forum Infect Dis* 2015; **2**: ofv098.
- 32 Kim ML, Martin WJ, Minigo G *et al.* Dysregulated IL-1 β -GM-CSF Axis in Acute Rheumatic Fever That Is Limited by Hydroxychloroquine. *Circulation* 2018; **138**: 2648–2661.
- 33 Martin WJ, Steer AC, Smeesters PR *et al.* Post-infectious group A streptococcal autoimmune syndromes and the heart. *Autoimmunity Reviews* 2015; **14**: 710–725.
- 34 Balasubramanian R, Marks SD. Post-infectious glomerulonephritis. *Paediatr Int Child Health* 2017; **37**: 240–247.
- 35 Eison TM, Ault BH, Jones DP, Chesney RW, Wyatt RJ. Post-streptococcal acute glomerulonephritis in children: clinical features and pathogenesis. *Pediatr Nephrol* 2011; **26**: 165–180.
- 36 Falconer AE, Carson R, Johnstone R, Bird P, Kehoe M, Calvert JE. Distinct IgG1 and IgG3 subclass responses to two streptococcal protein antigens in man: analysis of

- antibodies to streptolysin O and M protein using standardized subclass-specific enzyme-linked immunosorbent assays. *Immunology* 1993; **79**: 89–94.
- 37 Mortensen R, Nissen TN, Blauenfeldt T, Christensen JP, Andersen P, Dietrich J. Adaptive Immunity against *Streptococcus pyogenes* in Adults Involves Increased IFN- γ and IgG3 Responses Compared with Children. *The Journal of Immunology* 2015; **195**: 1657–1664.
- 38 Raynes JM, Frost HRC, Williamson DA *et al.* Serological Evidence of Immune Priming by Group A Streptococci in Patients with Acute Rheumatic Fever. *Front Microbiol* 2016; **7**: 1119.
- 39 Bright PD, Mayosi BM, Martin WJ. An immunological perspective on rheumatic heart disease pathogenesis: more questions than answers. *Heart* 2016; **102**: 1527–1532.
- 40 Salmond C, Crampton P, King P, Waldegrave C. NZiDep: a New Zealand index of socioeconomic deprivation for individuals. *Soc Sci Med* 2006; **62**: 1474–1485.
- 41 Clos Du TW, Mold C. C-reactive protein: an activator of innate immunity and a modulator of adaptive immunity. *Immunol Res* 2004; **30**: 261–277.
- 42 Jones S, Moreland NJ, Zancolli M *et al.* Development of an opsonophagocytic killing assay for group a streptococcus. *Vaccine* 2018; **36**: 3756–3763.
- 43 Chiarot E, Faralla C, Chiappini N *et al.* Targeted amino acid substitutions impair streptolysin O toxicity and group A *Streptococcus* virulence. *MBio* 2013; **4**: e00387–12.
- 44 Jolliffe IT. *Principal Component Analysis*. Springer Series in Statistics. Springer, New York, NY, 1986.
- 45 Tibshirani R. The lasso method for variable selection in the Cox model. *Stat Med* 1997; **16**: 385–395.

Table 1. Demographic characteristics and C-reactive protein concentration of study participants

Characteristic	Healthy control* n=35	ARF High (CRP $\geq$ 10 $\mu\text{g mL}^{-1}$) n=20	ARF Low (CRP < 10 $\mu\text{g mL}^{-1}$) n=14
Age, median (IQR), years	11.0 (8.0-13.0)	11.5 (9.3-13.0)	11.0 (9.0-13.0)
Male sex (%)	23 (66)	13 (65)	7 (50)
Ethnicity			

Māori (%)	13 (37)	7 (35)	3 (21)
Pacific (%)	22 (63)	13 (65)	11 (79)
CRP concentration			
($\mu\text{g mL}^{-1}$)			
Mean [95% CI]	2.17 [1.46-2.88]	49.44 [22.16-76.72]	5.94 [4.43-7.45]
Median [95% CI]	1.38 [0.85-2.23]	23.78 [17.12-53.70]	6.41 [3.18-8.37]

* Participants recruited into the control arm with $\text{CRP} \geq 10 \mu\text{g mL}^{-1}$ were excluded (n=10)

FIGURE LEGENDS

Figure 1. Circulating complement factors and immunoglobulin. (a) Heatmap showing the concentration of analytes in ARF-high (n=20) and ARF-low CRP (n=14) groups compared to healthy controls (n=35). Data represent two technical replicates. Fold change (FC) was determined from the means of each group and *P*-value by Kruskal–Wallis one-way analysis of variance. FC and *P*-value are indicated by coloured boxes and analytes with $\text{FC} > 1.5$ and $P < 0.05$ are marked with a triangle. **(b)** Volcano plot comparing the concentration of analytes in healthy controls with ARF-high CRP patients. FC was determined from the means and *P*-value by Dunn’s multiple comparisons test. Dotted lines indicate FC of 1.5 and *P*-value of 0.05, and analytes with $\text{FC} > 1.5$ and $P < 0.05$ are shown in red. **(c)** Serum concentration of Ig (mg mL^{-1}) and complement factors ($\mu\text{g mL}^{-1}$) in the healthy control (HC), ARF-high CRP (RF-H) and ARF-low CRP (RF-L) groups. Bars represent mean $\pm$ SD and the *P*-values were determined by Dunn’s multiple comparisons test. The upper limit of clinical reference ranges are shown as dashed lines.

Figure 2. PCA and LASSO Identify Rheumatic Fever Specific Features.

(a and b) **(a)** Scores plot for the PCA represents the ARF-high CRP (RF-H; yellow, n=20) and healthy controls (HC; blue, n=35) for each participant tested (dots). **(b)** Features included in the PCA analysis. PC1 and PC2 captured 38.97% of the variance. **(c and d)** **(c)**, PCA and LASSO for the ARF-high CRP (yellow, n=20) and healthy controls (blue, n=35). **(d)** The four features listed on the loadings plot accounted for 82% of the variance between the two groups. See also Supplementary Figure S1.

Figure 3. The linked IgG3-C4 response in Rheumatic fever. Scatter plots comparing the circulating concentration of total IgG3 and C4 in the ARF-high CRP group (RF-H; yellow,

n=20) and healthy controls (HC; blue, n=35). Data represent two technical replicates.
Spearman r and P -values as shown.

Figure 4. IgG responses in Acute Rheumatic Fever Patients. (a) IgG subclasses specific for M6 (grey) and SLO (white) in the ARF-high CRP group (n=20) as measured by ELISA. Data are shown as normalized ELISA units (nEU) and bars represent the median nEU for each IgG isotype. **(b)** Ratio of IgG3:IgG1 in individual sera for the ARF-high CRP group to M6 and SLO proteins. For **(a and b)**, P -values were determined by Wilcoxon matched pairs signed rank test and data are representative of three independent experiments. **(c)** Scatter plots comparing the circulating concentration of total IgG3 with the IgG3 response to M6 and SLO in the ARF-high CRP group. Spearman r and P -values as shown. See also Supplementary Figure S2 and S3.

(a)

(b)

(c)

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