Post-infectious group A streptococcal autoimmune syndromes and the heart

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Abstract

There is a pressing need to reduce the high global disease burden of rheumatic heart disease (RHD) and its harbinger, acute rheumatic fever (ARF). ARF is a classical example of an autoimmune syndrome and is of particular immunological interest because it follows a known antecedent infection with group A streptococcus (GAS). However, the poorly understood immunopathology of these post-infectious diseases means that, compared to much progress in other immune-mediated diseases, we still lack of useful biomarkers, new therapies or an effective vaccine in ARF and RHD. Here, we summarise recent literature on the complex interaction between GAS and the human host that culminates in ARF and the subsequent development of RHD. We contrast ARF with other post-infectious streptococcal immune syndromes - post-streptococcal glomerulonephritis (PSGN) and the still controversial pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS), in order to highlight the potential significance of variations in the host immune response to GAS. We discuss a model for the pathogenesis of ARF and RHD in terms of current immunological concepts and the potential for application of in depth “omics” technologies to these ancient scourges.

Keywords

Group A streptococcus, acute rheumatic fever, rheumatic heart disease, glomerulonephritis, PANDAS, autoimmunity

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1. Introduction

Human infections with *Streptococcus pyogenes* (group A streptococcus, GAS) constitute a major, worldwide health problem, with up to 700 million cases annually [1]. GAS is an anaerobic, gram-positive coccus and its only known reservoir is in humans. The oropharynx and skin are the primary colonization sites for GAS and around 12% of apparently normal individuals harbour GAS as a commensal organism in these locations [2].

GAS has a long history with human disease. Intriguingly, it can cause both infectious and post-infectious, immune-mediated diseases. The former include non-invasive infections, such as pharyngitis and impetigo; invasive infections, such as pneumonia, septic arthritis and necrotising fasciitis; and toxin-mediated syndromes, such as toxic shock syndrome and scarlet fever. Immune syndromes following GAS infection include acute rheumatic fever (ARF), rheumatic heart disease (RHD), post-streptococcal glomerulonephritis (PSGN) and possibly, pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS) (Fig. 1). This review focuses on the clinical and pathological features of these sterile, post-infectious GAS syndromes in order to gain insight into host immunopathogenic mechanisms and hopefully, to suggest new approaches to diagnosis and treatment.

2. Post-streptococcal immune mediated syndromes

2.1 Acute Rheumatic Fever (ARF) and Rheumatic Heart Disease (RHD)

ARF occurs at a median of two weeks after an antecedent GAS infection. The diagnosis is based on the Jones criteria, comprising major and minor manifestations. The five major manifestations in the Jones criteria reflect target tissue involvement: synovium (inflammatory arthritis), heart valves (endocarditis), brain (chorea), skin (erythema marginatum) and subcutaneous tissue (nodules). A diagnosis of ARF is made in the presence of two major, or one major and two minor, manifestations, together with serological evidence of preceding GAS infection. These clinical criteria were initially developed in 1944 and last updated in 1992 [3, 4]. In high disease burden settings, even the updated criteria lack sensitivity and have been adapted by some experts to reduce under-diagnosis [5].

Throat cultures are typically negative for GAS in a patient presenting with ARF. Evidence of preceding streptococcal infection is therefore confirmed by elevated or rising serum antibodies to streptococcal antigens, such as streptolysin O and deoxyribonuclease B. However, these assays lack sensitivity in populations where GAS exposure is endemic and background titres are frequently elevated [6]. Characterisation of the serological response to GAS infection into IgM (recent) versus IgG (longstanding) does not seem to have been adopted clinically, possibly for the same reason. Fever is present in almost all cases of ARF, with the notable exception of isolated chorea. Elevation of the erythrocyte sedimentation rate and the acute phase reactant, C-reactive protein are typical.

Carditis occurs in at least 60% of ARF patients and although any heart valve can be involved, the mitral and aortic valves are most frequently affected [7]. Valve damage is often insidious and factors that determine progression to RHD are poorly understood, although the severity of the initial attack of ARF and the frequency of recurrent episodes are important factors [8]. RHD has a variable clinical course, ranging from asymptomatic valvular dysfunction to cardiac failure. Later complications include infective endocarditis, atrial fibrillation and thromboembolic stroke as a result of atrial enlargement. Progressive valvular stenosis or incompetence frequently requires surgical intervention in early adult life and lifelong medical management.

Sydenham's chorea may appear with the other features of ARF, but often presents later, even up to 6 months after infection. It is characterised by involuntary, rapid and purposeless movements of the face or limbs, often associated with emotional lability. Erythema marginatum and subcutaneous nodules are less common, but more specific features of ARF. However, these dermatological manifestations of ARF are easily missed, especially in pigmented skin. Arthritis occurs in up to 80% of cases of ARF, classically presenting as a migratory polyarthritis of large joints. Inflammatory joint symptoms are typically responsive to anti-inflammatory agents. In the absence of treatment, the duration of arthritis is usually two to four weeks and it does not cause erosive joint damage. In highly endemic areas, including polyarthralgia or monoarthritis as major manifestations increases the sensitivity of

the Jones criteria [5, 6], but can decrease specificity, as there are many potential causes of these presentations, such as viral arthritis.

Current treatment for ARF is supportive and does not prevent ensuing valvular damage [8]. Patients with ARF usually receive penicillin, with the intention of eradicating any residual GAS infection, but symptomatic management of systemic inflammation and any associated heart failure remain the mainstays of treatment. High dose aspirin has been the standard of care for cases with fever and joint symptoms, although better-tolerated non-steroidal anti-inflammatory drugs are increasingly used [9]. In contrast, there is clear evidence that secondary prophylaxis with antibiotics reduces the risk of RHD in patients with a history of ARF [10]. Secondary prophylaxis can also slow the progression and severity of disease in patients with established RHD [11]. The currently recommended strategy for secondary prophylaxis is 3- to 4-weekly intramuscular injections of benzathine penicillin, continuing for at least 10 years from the last ARF episode, or up until the age of 21, whichever is longer [12]. In individuals with documented RHD, penicillin treatment is often extended, in the hope of reducing progression of heart valve damage. However, reliable implementation of long-term secondary prophylaxis, particularly in poorly serviced and socially disadvantaged communities, is often a major logistical challenge.

ARF and RHD have declined in developed countries, although outbreaks still occur.

In contrast, in most developing countries, and in many indigenous populations of

wealthier countries, these diseases remain a significant health problem [1]. For example, it is estimated that RHD affects almost 2% of the Aboriginal population in the Northern Territory of Australia and 3.2% of these people aged 35-44 [13]. Global burden of disease figures estimate that there are over 15 million prevalent cases of RHD in the world, leading to over 345,000 deaths annually. Globally, RHD is thought to be the leading cause of cardiovascular death below the age of 50 [14]. However, even these numbers likely underestimate the true disease burden because of difficulties with case ascertainment [7, 15]. Screening for RHD using echocardiography suggests that many cases of ARF are sub-clinical[16-19].

ARF is a disease arising from poverty, overcrowding and poor living conditions. High-risk populations are exposed to frequent GAS infections that lead to repeated or prolonged ARF episodes, thereby increasing the risk of developing RHD. ARF can occur at any age, but most cases occur in children aged 5-15 years, whereas the prevalence of RHD peaks in early adulthood, with approximately 60% of ARF cases progressing to RHD [1]. Despite high exposure rates, it is rare for children below five years of age to experience a primary episode of ARF, perhaps because repeated exposure to GAS is required to trigger the autoimmune response [20]. Many studies have observed that RHD, in keeping with most autoimmune diseases, is more common in females than males [7].

2.2 Post-Streptococcal Glomerulonephritis (PSGN)

PSGN causes glomerulonephritis, typically manifesting 1 – 3 weeks following pharyngitis and 3 – 6 weeks following impetigo [21]. In temperate, industrialised countries, PSGN usually follows pharyngitis [22]; while in tropical countries, it is more commonly associated with impetigo, often occurring in epidemics [23-26]. PSGN is the most frequent cause of acute nephritis in children globally, typically occurring in children between 3 and 12 years of age, but it can also occur in adults. There are an estimated 470,000 new cases of PSGN annually, the vast majority occurring in developing countries [7]. PSGN most commonly presents with the acute onset of haematuria and oedema [25, 27]. Hypertension occurs in 60–70% of cases and full-blown nephrotic syndrome may occur. Activation of the alternate complement pathway, leading to low serum C3, is an important diagnostic feature in PSGN. Serum C3 levels usually return to normal within 6-8 weeks [28]. Renal failure may occur in PSGN, but is infrequent, and PSGN generally resolves without specific treatment [7, 29]. Although PSGN is considered to have a good prognosis, long-term observational studies in northern Australia raise some concern that childhood PSGN may contribute to chronic renal disease in adulthood [30].

2.3 Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infections (PANDAS)

PANDAS comprises neuropsychiatric manifestations, including the abrupt onset of choreoathetosis (reminiscent of Sydenham's chorea), obsessive compulsive behavior (OCD) or tic disorder, that may follow exposure to GAS [31]. The connection between GAS infection and neurological symptoms in PANDAS remains

contentious [32-36]. In the original description of PANDAS, a temporal relationship between GAS infection, the onset of neuropsychiatric symptoms, and exacerbations was observed [31]. In a subsequent retrospective, case-control study of 144 patients with OCD, tic or Tourette's syndrome [37], patients were twice as likely to have had a GAS infection in the preceding three months and three times more likely to have had multiple GAS infections in the preceding 12 months. A small, uncontrolled prospective study over three years in a primary care setting followed 12 children with putative PANDAS, all of whom had positive throat cultures for GAS at the time of onset of neuropsychiatric symptoms and improved following antibiotic therapy, [38]. Of these, four showed complete resolution within 5-21 days. Recurrent symptoms were observed in the other children, and each new episode was associated with a culture verified GAS infection. This link was supported by a further study of 693 children followed over 8 months, which found that GAS infection was associated with higher rates of choreiform movements and behavioural problems [39]. Choreiform movements and OCD episodes show no association with altered thyroid function [40]. Symptoms were reduced in PANDAS patients who received treatment (including antibiotics, plasmapheresis, or intravenous immunoglobulin) early in disease, supporting potential roles for GAS infection and possibly autoantibodies with effects on the central nervous system (CNS) [38, 41]. More recently, sera from children with tic disorders were used to probe a protein array of over 100 recombinant GAS proteins. This analysis identified 21 proteins that were recognised by sera from tic and pharyngitis patients, but not

in the “no tic” patients, suggesting that a subset of tic disorders could indeed be considered a post-streptococcal disease [42].

However, other prospective observational cohort and case-control studies do not support the temporal relationship between GAS infection and neuropsychiatric symptoms. A cohort of 40 patients who satisfied putative criteria for PANDAS were monitored for clinical exacerbations of neurologic symptoms over a two-year period, with throat swabs taken every four weeks. Of 64 exacerbations, only five were associated with a preceding GAS infection. Although the PANDAS group appeared to be more susceptible to GAS infection and had more exacerbations than the control group, infections and neurological symptoms were not temporally associated [43]. A second longitudinal study of 31 PANDAS patients over 25 months found the PANDAS group had the same rates of exacerbation and infection as the control group. Symptom exacerbations following GAS infection occurred only in the non-PANDAS control group, further challenging the role of GAS infection in causing PANDAS [44]. Other studies have failed to confirm anti-neuronal antibodies in PANDAS patients [45, 46] and showed no benefit with prophylactic penicillin treatment [47]. Clearly, caution and further evidence are required before PANDAS can be considered a clinical syndrome, but skeptics should remain open to examining evidence dispassionately.

3. Genetics of host susceptibility to post-streptococcal immune syndromes

Only a few studies have explored genetic susceptibility to PSGN and PANDAS. Increased frequencies of *HLA-DRW4* [48] and *HLA-DRB1*0311* [49] were reported in Venezuelan and Egyptian cohorts with PSGN compared to healthy controls. In a Turkish study, children with homozygote 'a' alleles, or heterozygote 'a'/'b' alleles, of the endothelial nitric oxide synthase gene intron 4 a/b (eNOS4a/b) variable number of tandem repeats polymorphism, had a greater risk for PSGN than those carrying homozygote 'b' alleles, suggesting a role for eNOSa in PSGN [50]. In a study of PANDAS, 157 first-degree relatives of 54 probands had higher rates of OCD and tic disorder than reported rates in the general population, suggesting a possible genetic influence, although this may be more related to susceptibility to OCD and tic disorders in general [51].

Host susceptibility to ARF and RHD has been more extensively studied. The cumulative, lifetime incidence of ARF is 3–6% in populations exposed to rheumatogenic GAS [8]. During outbreaks in US military camps in the 1950s, 2–3% of patients with GAS pharyngitis developed ARF [52]. In Australian Aboriginals in the Northern Territory, where poor hygiene and overcrowding are near universal and there is endemic exposure to GAS, the cumulative incidence of ARF is thought to be over 5% [8]. Similar rates of ARF occurred in industrialised countries in the first half of the 20th century and still exist in developing countries [53]. These observations suggest that approximately 5% of the population may have an inherited susceptibility to developing ARF and RHD. However, as expected in a complex autoimmune disease, there does not appear to be a simple Mendelian

pattern of inheritance [54]. A systematic review and meta-analysis considered the zygosity status and concordance for RHD across 435 twin pairs [55]. This study estimated the heritability of ARF to be 60%, similar to that of other complex diseases, with a pooled proband-wise concordance risk for ARF of 44% in monozygotic twins, compared to 12% in dizygotic twins.

Many studies have focused on the association of HLA molecules with susceptibility to ARF and/or RHD [56, 57]. HLA class II genes represent the strongest association and more than 30 alleles occur more frequently in RHD; in contrast, a much smaller number of associations have been made with HLA class I genes (Table 1). *HLA-DR7* [64, 77, 79, 81-84] and *HLA-DR4* [58, 61, 62, 69, 72-75] are the most consistently reported HLA class II genes, with association across diverse populations, including American Caucasians, Brazilian-Mullatos, Turkish, Pakistani, Egyptian and others. One study has linked *HLA-DRB1*07* to recurrent streptococcal pharyngitis [77]. In contrast, several HLA class II alleles including *HLA-DR5*, *HLA-DR6*, *HLA-DR51*, *HLA-DR52* and *HLA-DQ* alleles have been linked to protection from RHD (Table 1).

Associations between RHD and other components of the immune response include genetic polymorphisms in tumor necrosis factor, mannose binding lectin and toll-like receptor genes [56, 57, 90-92](Table 2). The most consistent genetic association outside of classical HLA genes has been the *TNF* locus. The first study to implicate this locus used 87 RHD cases and 101 controls of Mexican Mestizo origin to estimate that the G allele at *TNF* position 238 was more frequent in RHD cases (OR = 14.1),

while the A allele at *TNF* position 308 was also more frequent in cases (OR = 10.8)[76]. However, neither allele was associated with mitral valve damage or with multi-valvular lesions. Tumor necrosis factor-induced protein 3 (*TNFAIP3*) more commonly harboured an intronic SNP in a Han Chinese cohort with RHD, providing further support for the involvement of *TNF*-related genes [99].

Unfortunately, genetic associations are not consistent across studies of ARF/RHD. Although this may partly reflect true geographical and ethnic differences, clinical definitions for RHD cases and controls in these studies vary, and most are of limited power. Furthermore, many studies have been restricted to a small number of candidate genes, selected on a traditional view of pathogenesis. Current genotyping and sequencing technologies offer the opportunity to perform unbiased, genome-wide assessments, with the potential to uncover genomic risk scores of greater clinical relevance [55, 112-114].

4. Bacterial factors that contribute to post-streptococcal immune syndromes

At a molecular level, GAS can be typed by sequencing the hypervariable N-terminal regions of the *emm* gene encoding the M protein, the major cell surface glycoprotein of GAS. GAS strains can now be classified into 223 *emm*-types [115]. A number of studies have attempted to link GAS strains or *emm*-types to disease patterns.

Particular *emm*-types are commonly associated with pharyngitis in high-income countries [116] and also appear to be responsible for invasive disease and ARF in the same geographic area. A separate group of *emm*-types is usually associated with

skin infections and glomerulonephritis. Identifying *emm*-types that cause ARF can be difficult due to the delay between infection and the onset of symptoms, with bacterial clearance usually having occurred by the time of diagnosis. Certain GAS *emm* types have been classed as 'rheumatogenic' due to their association with rheumatic fever in developed countries [117-119]. These strains are predominantly found in the oropharynx, rather than the skin. However, recent epidemiological studies in tropical regions challenge the accepted link between 'rheumatogenic GAS', pharyngitis and ARF or RHD [120]. Studies in geographic regions where ARF, RHD and streptococcal infections are endemic, have failed to find dominant, 'rheumatogenic' strains [121-125]. Conventional 'rheumatogenic' GAS strains are in fact rarely found in such areas and the incidence of GAS pharyngitis is low, while the incidence of GAS impetigo and *Streptococcus dysgalactiae* subspecies *equisimilis* (Group G Streptococcus (GGS)) or *Streptococcus equi* subspecies *equi* (Group C Streptococcus (GCS)) pharyngitis is high, making it plausible that atypical streptococcal strains may also be associated with the development of ARF [122, 123, 126].

GAS has evolved multiple virulence mechanisms, some of which are under control of the *covRS* operon [127]. GAS virulence mechanisms target key immune defence systems, such as complement, chemokines and phagocytosis. The cell wall of GAS is composed of complex layers of peptidoglycans, polysaccharides and an outer coating of hyaluronic acid, containing embedded cell surface proteins, including M protein. The M protein (Fig. 2) is a major GAS virulence factor, with great antigenic

diversity. It exists as a dimer, anchored in the bacterial cell wall (Fig. 2)[128, 129]. Most of its protein sequence consists of heptad repeat motifs in which the first and fourth amino acids are typically hydrophobic, and provide core stabilizing residues within a coiled-coil fibrillar structure [130]. The prototypical M5 and M6 proteins contain several internal repeat sequences, known as 'A', 'B', 'C', and 'D' repeats. However, most M proteins, and especially those associated with skin infections in poorer societies, do not possess 'A' or 'B' repeats, instead containing only 'C' and 'D' repeats [115, 128, 131, 132]. Of note, the M protein is not only present on group A streptococcal isolates, but is also found on the closely related *Streptococcus dysgalactiae* subspecies, *equisimilis* (GGS) [133], *Streptococcus equi* subspecies equi (GCS) [134] and *Streptococcus iniae* [135].

M proteins promote bacterial adhesion to epithelial surfaces and keratinocytes and facilitate host invasion [136, 137]. M proteins inhibit complement activation by binding complement regulators, such as C4b binding protein (C4BP), Factor H, and Factor H-like (FHL)-1 [128]. M proteins also mask GAS from immune detection by binding host fibrinogen and albumin, adopting the appearance of "self", while also preventing antibody and complement binding and uptake by phagocytic cells [138, 139]. The binding capacity of 26 representative M proteins has recently been characterised for six host protein ligands (fibrinogen, albumin, C4BP, IgA, IgG and plasminogen). Results from this study grouped numerous *emm*-types into 48 discrete *emm*-clusters containing closely related M proteins that share binding and structural properties. Importantly, the capacity to bind host ligands is related to the

potential virulence of GAS isolates [128, 140]. It is therefore possible that *emm*-cluster typing identifies clinically relevant variations in GAS epidemiology. *Emm*-cluster typing to help characterise post-streptococcal immune syndromes is an avenue for further investigation.

GAS employs multiple strategies to reduce bacterial sequestration and facilitate its dissemination. Streptococcal inhibitor of complement (Sic) binds to and inactivates C5b, prevents the assembly of the membrane attack complex (MAC) and inactivates the antibacterial cathelicidin, LL-37 [141, 142]. Streptococcal C5a peptidase and streptococcal chemokine protease (SpyCEP) degrade the neutrophil chemoattractants C5a and IL-8, respectively [143, 144]. The cationic cysteine protease, streptococcal pyrogenic exotoxin B (SpeB), cleaves fibronectin, degrades vitronectin, activates human metalloproteases, and cleaves anti-GAS antibodies bound to GAS surface proteins [145]. GAS also produces DNase B, which degrades neutrophil extracellular traps (NETS)[146]. Evasion of NETs is thought to promote GAS survival.

GAS, and M1 protein itself, can activate platelets and promote thrombus formation [147-149]. GAS captured within thrombi can be disabled, either by antibacterial peptides produced within the clot, or in conjunction with NETs that form a DNA scaffold for thrombus [150, 151]. Microparticles generated from monocytes stimulated with M protein can engage both the intrinsic and extrinsic coagulation pathways, promoting thrombus formation [152]. GAS promotes activation of

plasminogen to plasmin, and the subsequent stabilization of plasmin, through a number of proteins, including streptokinase [153], Nephritis Associated Plasmin receptor (NAPlr)[154], streptococcal enolase (SEN)[155], plasminogen-binding group A streptococcal M protein (PAM)[156], and PAM-related protein (Prp)[157]. This mechanism most likely evolved to frustrate the sequestration of GAS within the human host. In an ironic biological twist, the thrombolytic actions of streptokinase have been exploited in humans for the treatment of acute vascular thrombosis [158]. Plasmin also activates collagenases, which degrade the extracellular matrix and activate inflammatory mediators [159] - an important pathogenic mechanism in PSGN.

5. Host responses in post-streptococcal immune syndromes

5.1 Complement and immune complex activation

PSGN is considered an immune complex disorder, during which streptococcal proteins and anti-GAS antibodies deposit in renal glomeruli, either as pre-formed immune complexes or forming in situ. Immune complex deposition leads to classical pathway complement activation, triggering inflammatory cell recruitment and tissue damage [160, 161]. Two GAS antigens have been implicated as initiators of PSGN - NAPlr, a streptococcal form of glyceraldehyde 3-phosphate dehydrogenase, and SpeB, a cysteine protease. Both NAPlr and SpeB are frequently detected in renal biopsies of patients with PSGN and antibodies to both have also been detected in the serum [162-165]. NAPlr and SpeB localise on the endothelial side of glomeruli, often co-localizing with glomerular endocapillary neutrophils, and in the mesangium

[166]. NAPlr and SpeB can directly activate the alternative complement pathway [167], bind plasmin [168, 169] and stimulate mesangial cells to produce chemokines, such as CCL-2, and cytokines, such as IL-6. In concert with enhanced expression of ICAM-1, this milieu facilitates renal leucocyte recruitment [170-173]. Binding of NAPlr to plasmin protects it from endogenous inhibitors, such as alpha 2-anti plasmin, promoting extracellular matrix breakdown and cell migration [168, 174].

Increased serum IgM, IgG, and IgA [71, 175], and C1q, C3 and C4 [176, 177] have all been reported in ARF. Circulating immune complexes have been found in both ARF and PSGN, with a predominance of IgM-streptokinase O complexes [178, 179]. In contrast, impetigo seems not to be associated with circulating immune complexes [180]. CRP has also been found in immune complexes in ARF, and may facilitate complement pathway activation [178]. Decreases in complement components, including C3, C4, Factor B and Factor H, and of immunoglobulins have been reported in ARF patient synovial fluid and in the plasma of patients with rheumatic mitral stenosis. These findings suggest that activation of complement can occur in synovial joints and on heart valves in RHD [175, 181].

Polymorphisms in genes of the lectin pathway of complement activation - *MBL2*, *MASP2* and *FCN2* – have recently emerged as risk factors in RHD (Fig. 2)[90, 94, 103-105]. In accord with these findings, deposition of antibody and complement in the heart has been reported in RHD [182]. Interestingly, K/BxN transgenic mice, which are widely used to study pathogenic mechanisms in joint inflammation, may

provide insight into the potential role of complement and Fc receptors in ARF [183]. K/BxN mice harbour transgenic T cells that fortuitously recognise peptides derived from self glucose-6-phosphate isomerase (GPI) presented by the MHC class II molecule, A^g₇. Intriguingly, these mice not only develop arthritis, but also cardiac valvulitis, similar to ARF [184]. Inflammatory arthritis in K/BxN mice is associated with deposition of GPI on the cartilage surface, is dependent on complement C5 deposition on cartilage and on FcR_γ receptors. In contrast, cardiac inflammation required FcR_γ receptors, but was not associated with deposition of GPI or C5 [184]. The binding of circulating autoantibodies to antigens on cardiac valve endothelium via FcR_γ receptors and possibly local complement activation, could therefore parallel PSGN.

5.2 Molecular mimicry in ARF and RHD

Post-streptococcal immune syndromes may be caused by host immune responses directed, at least initially, towards streptococcal virulence mediators. Streptococcal M protein shares an alpha-coiled-coil structure with host proteins found in cardiac tissue, including the cardiac myosin rod region, tropomyosin, keratin, laminin and vimentin [128, 130, 185]. Antibodies recognizing M proteins on GAS cross-react with endogenous alpha-coiled-coil host proteins in RHD [186, 187]. Immunisation with peptides derived from M protein causes cardiac disease in rodent models [188, 189]. GlcNAc, the immunodominant carbohydrate antigen of the GAS cell wall, is also able to induce cross-reactive responses to cardiac proteins, presumably due to structural similarity with host carbohydrate residues [190, 191].

The portion of the M protein that might be primarily responsible for inducing B-cell and T cell autoimmune responses has been identified as the 'B' repeat region in M5 and M6 protein [187, 192-194] although some cross-reactive epitopes were also characterised in the 'A' and 'C' repeats of M5 and M6 (Fig. 2)[185, 187, 193, 195]. M protein epitopes demonstrating cross reactivity with human proteins have been recently reviewed [196, 197]. M5 and M6 strains are *emm*-types associated with oropharyngeal infections in developed countries, but are not commonly found in settings of high ARF disease burden. In the latter case, disease is associated with diverse and poorly characterised 'tropical' M proteins, suggesting that a different set of cross reactive epitopes may be present in these strains [197, 198], particularly given that many of the tropical strains do not contain B repeat regions. Recent data also suggests SpeB and endothelial carbohydrates can be sources of molecular mimicry [199].

GAS-reactive antibodies have been shown to recognise valve endothelium and laminin, perhaps facilitating an initial wave of inflammation in valvular structures [186, 200]. Coxsackie and adenovirus receptor (CAR) and beta 1 adrenergic receptor (B1AR) have also been proposed as surface-exposed, primary targets of cross-reactive anti-GAS antibodies [200]. Antibodies to collagen, elastin, vimentin and several other structural cardiac proteins are also commonly detected in RHD patients [186, 201, 202]. Autoantibodies to intravalvular proteins are most likely generated as a consequence of damage to the endothelium, exposing a range of

intravalvular proteins. Repeated exposure to GAS and the ensuing immune response may also lead to post translational modifications (such as oxidation or nitrosylation) of molecules in the heart, creating neoantigens and causing epitope spreading [203]. Early therapeutic intervention may thus reduce the development of a broad repertoire of pathogenic autoantibodies in RHD. Cardiac myosin has been the primary focus of molecular mimicry studies, although myosin is not found in cardiac valve tissue. It has been proposed that antibody recognition of the S2 subfragment hinge region within human cardiac myosin reflects disease progression in RHD [192]. Antibodies to the S2 sub-fragment emerged as a feature in three divergent populations, regardless of the infecting GAS strain [204]. This finding raises the possibility of a 'universal target epitope' in human cardiac myosin.

Recently, an alternative hypothesis for the pathogenesis of RHD was proposed that does not invoke molecular mimicry. In this scenario, RHD may be the result of an immune response directed at M protein bound to endogenous collagen in the heart [205]. During streptococcal pharyngitis, GAS can gain access to the subendothelial collagen matrix, where M proteins can bind to the CB3 region of type IV collagen, via an octopeptide motif. This interaction may create a neo-epitope that induces an immune response to type IV collagen [206-208]. In murine studies, immunization with M protein containing the octapeptide led to the generation of collagen-reactive antibodies. These antibodies showed negligible recognition of intact M protein, suggesting that the anti-collagen response was not generated through simple molecular mimicry [206-208]. The authors also proposed that while M protein-

collagen interactions may occur at a number of endothelial sites, damaged cardiac valvular endothelium might not heal as quickly, or as completely, as in other tissues, causing prolonged inflammation and angiogenesis, and eventually, fibrosis and calcification of the heart valve. This scenario may therefore be a more severe or persistent variation on the antibody-induced renal inflammation that occurs in PSGN.

Cross-reactive autoantibodies to neuronal cells are hypothesised to be the basis for Sydenham's chorea (and by extension, possibly in PANDAS). IgG antibodies isolated from patients with Sydenham's chorea showed reactivity with both *N*-acetylglucosamine GlcNAc and lysoganglioside on neuronal cells [209]. Moreover, *in vitro*, autoantibodies from patients with Sydenham's chorea (and PANDAS) bind to dopamine D1 and D2 receptors on neuronal cells [210]. Antibody binding induced CaM kinase II signaling in a human neuronal cell line and tyrosine hydrolase activity in rat brain, leading to increased dopamine production [209-211]. Rodents challenged with GAS extract developed PANDAS-like behaviour that was attenuated by antibiotics [212]. These rodents also exhibited an increase in the expression of dopamine receptor D1 and D2 in the striatum and prefrontal cortex, and deposition of IgG in the same neural regions [212]. In other rodent studies, neurological symptoms did not occur when donor sera were depleted of IgG prior to administration, and the severity of neurological symptoms correlated with the level of IgG deposition in the brain [213, 214]. Together, these data argue for the ability of cross-reactive autoantibodies to induce dopamine production and increased

dopaminergic signaling, causing altered neuromuscular function and behavioural effects in ARF. It is therefore tempting to speculate that such functional autoantibodies might contribute to other features of ARF, and possibly RHD.

Cross-reactive antibodies may activate valvular endothelium to induce adhesion molecule expression and allow the subsequent recruitment of inflammatory cells into cardiac tissue during ARF and RHD [215]. Antibodies to GlcNAc, the immunodominant carbohydrate antigen of the GAS cell wall, also exhibit cross reactivity through binding of carbohydrates on valvular endothelium [190, 216]. A study of RHD patients found that 40% had AECA [217], which have been shown to induce the expression of adhesion molecules on endothelial cells [218, 219]. Indeed, the valvular endothelium from RHD patients is activated, with increased expression of the adhesion molecule VCAM-1, which would facilitate inflammatory cellular recruitment [220]. In addition to adhesion molecule expression, AECA can cause activation of IL-1R-associated kinase (IRAK1) and nuclear factor kappa B (NFkB), and stimulate cytokine production from cardiac endothelial cells [219, 221]. In the presence of complement, AECA derived from RHD patients can also induce cytotoxicity in human endothelial cells [186].

ARF and RHD share some interesting similarities with Libman-Sacks endocarditis (LSE), which occurs in the antiphospholipid syndrome (APS), and may also follow infection [222]. Mitral valve inflammation is frequently seen in LSE, with antibody and complement deposition and T cell infiltration of heart valves [223]. Intriguingly,

chorea can also be a manifestation of LSE. The features of LSE are due to the generation of antiphospholipid antibodies, which inhibit antithrombotic phospholipids and β 2-glycoprotein-I (β 2GPI)[224]. There is overlap of antibody reactivities in RHD and APS. One study showed that 24% of RHD patients had anti- β 2GPI antibodies, and conversely, 16.6% of APS patients had antibodies that recognised M protein, with affinity purified anti- β 2GPI cross-reacting with M protein. In addition, anti- β 2GPI from APS patients with chorea recognised GlcNAc [225]. Elevated anti-cardiolipin antibodies are found in the majority of ARF patients, particularly during an acute episode, and in all ARF patients with valvular involvement [226]. AECA may also play a role in LSE [227]. APS and LSE may therefore arise by shared mechanisms, raising (at least in principle) the possibility of anti-coagulation as an approach to preventing progression of valvular disease in ARF and RHD.

5.3 Cellular immunological responses in ARF and RHD

During ARF, there is a transient increase in circulating leucocytes, with elevated CD4+ T cells and B cells representing the most consistently reported observations [71, 228-230]. Both increases and decreases in CD8+ T cells have been reported, as well as increased NK cells [72, 229]. Mild elevation of total leucocyte counts can occur for several weeks after an episode of ARF, suggesting an ongoing inflammatory response [71, 228]. Peripheral blood mononuclear cells (PBMC) from ARF patients have increased responses to GAS antigens *in vitro*, particularly to GAS

cell wall components, which can persist for up to two years after initial diagnosis [231].

The rheumatic heart valve is heavily infiltrated by CD4+ T cells and, to a lesser extent, of CD8+ T cells [232]. Human T cell clones have been generated from cardiac tissue removed at surgery for RHD [187]. Remarkably, proportion of these heart valve-derived T cell clones (approximately 20%) recognised M protein peptides, with strong reactivity for peptides from the B-repeat region of M5 (aa163-177) and M6 (aa151-167, aa176-193). Recognition of N-terminal peptides of M6 by T cell clones has also been reported (aa1-20, aa81-103)(Fig. 2)[187, 195, 233]. A large proportion of T cell clones (63% in one study) show reactivity to a broad range of cardiac myosin peptides, without clear immunodominance [187], perhaps due to the epitope spreading referred to above. Rats immunised with peptides of M5 generated cross reactive CD4+ T cells that caused valvulitis when transferred to naïve recipients, providing proof-of-principle that M peptides can generate heart-valve specific CD4+ T cells [234].

The T cell response to bacterial infections can be shaped by the presence of superantigens; however, both skewed [235, 236] and unskewed T cell receptor repertoires [195, 237] have been reported in the circulation and from excised heart tissue fragments of patients with RHD, as recently reviewed [238]. In the rat autoimmune valvulitis (RAV) model, valvulitis was induced in 45-50% of rats following successive challenges with either formalin-killed GAS, M protein or

peptides of M protein, without the need for viable GAS or superantigens (although adjuvant was required) [188, 193, 239, 240]. Therefore, although skewed V-beta T cell subtypes have been reported in RHD, superantigens do not appear to be critical to immune-mediated valvulitis.

T regulatory cells (T-regs) moderate immune responses and constrain sub-clinical autoimmunity. *In vitro* stimulation of CD4⁺ T cells with GAS induced IL-10 production from T-regs, through the interaction of M protein with CD46 [241]. Inducible T-regs can also be generated following superantigen stimulation *in vitro* [242]. Several studies have reported a reduced number of circulating T-regs in RHD [243, 244] and PANDAS patients [245], with greater reductions correlating with more severe symptoms.

The recruitment of immune cells to foci within heart valves and endocardium is a classic feature of RHD. These foci, called Aschoff nodules, are comprised of lymphocytes and histiocytes surrounding a necrotic, fibrinoid core [246]. Three stages have been described in Aschoff nodules, reflecting sequential increase in cellular complexity and a composition reminiscent of an ectopic lymphoid germinal centre. During the first stage, histiocytes expressing the macrophage marker CD68 are observed, including Anitschkow cells (caterpillar cells), and multinucleated giant cells. These cells produce IL-1 β and TNF [247, 248]. In the second stage, T cells, predominantly CD4⁺, accumulate within the lesion. In the third stage, B-cells and

occasional plasma cells appear within the nodule. At later stages, inflammatory cells diminish in number and are replaced with fibrotic tissue [249].

It is not yet clear which antigen presenting cells (APC) are important for stimulating autoreactive T cells in ARF or RHD, although Anitschkow cells are one obvious possibility. Normal cardiac valve tissue has little expression of MHC II, but aberrant MHC II expression has been reported on valvular fibroblasts and cardiac endothelial cells in rheumatic carditis [250, 251]. MHC II expression on fibroblasts is induced by high levels of IFN γ , which is produced in RHD heart valves [252-254]. A recent study used transgenic mice in which enhanced yellow fluorescent protein was expressed under the CD11c promoter and intriguingly, a network of dendritic cells was observed directly beneath the endothelial layer of the mitral and aortic valves [255]. In addition, monocytes recruited to sites of tissue inflammation can develop into APC [256, 257]. Thus, APC in the rheumatic heart valve may include resident, recruited and non-professional APC, and may change as an acute immune response becomes chronic.

5.4 Cytokines in ARF and RHD

The pathogenesis of post-streptococcal immune syndromes is highly likely to be driven and shaped by cytokines. Increased plasma or serum IL-1 α , IL-2, IL-6, IL-8 and TNF have been found in ARF and RHD patients [258-260]. However, tonsillar cells isolated from RHD patients show reduced levels of IL-1, IL-2, TNF and immunoglobulin production, compared with controls, following T cell activation

[261]. In contrast, circulating PBMC from ARF and RHD patients had increased production of IL-1, IL-2 and TNF to these and other stimuli [261, 262]. Heightened cellular responses correlated with amplified proliferative responses to GAS antigens, persisting for as long as 24 months [231]. These studies suggest immunological responses vary between compartments, but are in keeping with prolonged activation of immune cells in RHD patients.

As outlined above, TNF features in genetic susceptibility studies of RHD. Serum TNF increases during an acute episode of ARF and is elevated in RHD patients with heart failure, suggesting that TNF could play roles at the onset of ARF and during the development of cardiac disease [258, 260, 263]. A role for TNF antagonism in the early phase of ARF has not been explored, but anti-TNF therapy in the setting of chronic heart failure led to adverse outcomes, including increased mortality [264].

TGF- β can inhibit acute inflammatory responses and promote tissue repair [265], including fibrosis [266] and angiogenesis [267]. Heart valvular interstitial cells assume a myofibroblast-like function with activation and produce TGF- β in the inflamed cardiac valve, although recruited inflammatory cells may also contribute [268-270]. Several lines of evidence indicate a role for TGF- β in RHD. Genetic polymorphisms in TGF- β have been reported to increase the risk of developing RHD [271]. TGF- β could also promote neovascularisation in inflamed cardiac valves [272]. Chronic activation of fibroblasts and local production of TGF- β could lead to fibrosis and valvular stenosis in RHD [271]. TGF- β is also a key mediator in the development

of Th17 cells [273], and it could therefore favour Th17 polarisation, as well as contributing to fibrosis and calcification of heart valves.

Several studies indicate that Th1 polarization may be important in ARF and RHD. Elevation of serum IFN γ has been reported in chronic RHD [263] while elevated neopterin, a downstream product of IFN γ , has been observed in the serum and urine of ARF patients [258]. Involvement of IFN γ is also suggested by elevated serum levels of the IFN γ -inducible proteins, CXCL9 and CXCL10. Both CXCL9 and CXCL10 are found in the cerebrospinal fluid of patients with Sydenham's chorea, while CXCL9 mRNA is highly expressed in RHD cardiac valves, suggesting IFN γ can mediate diverse clinical manifestations of ARF [274, 275]. Heart valve-derived T cells that recognise myosin peptides produce IFN γ , in addition to TNF and IL-10 [187]. However, transcriptional profiling of PBMC in RHD patients showed that IFN γ was not as strongly induced by rheumatogenic GAS when compared with non-RHD controls, suggesting blunted Th1 polarisation [276]. Indeed, Th2 polarisation may actually protect the heart from cumulative damage in RHD. Excised valvular heart tissue from RHD patients revealed that the majority of mononuclear cells in non-inflamed myocardium produced IL-4, (78% of fragments had >10% IL-4+ mononuclear cells), while damaged valvular fragments contained mostly IL-4 negative mononuclear cells (18% of fragments had >IL-4+ mononuclear cells)[277].

Th17 polarised cells are implicated in a number of autoimmune diseases, including rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus and

ankylosing spondylitis [278]. Th17 polarised T cells have emerged as possible mediators of ARF and RHD and might be promoted by skin and mucosal infection. Elevated serum IL-17A has been reported in RHD patients, while T cells from RHD patients produce IL-17A when stimulated with mitogens *ex vivo* [243, 279]. When stimulated in culture with GAS, human tonsillar cells produced TGF- β , a growth factor favouring Th17 differentiation [273]. In murine studies of nasal GAS infections, IL-17+ T cells were observed in nasal-associated lymphoid tissue, a tissue analogous to human tonsils, demonstrating that Th17 cells can be generated following GAS infection [280].

5.5 A vaccine against GAS

A vaccine that provides protection from GAS infection and post-infectious GAS syndromes has been a holy grail of research for many years. Much of vaccine research has focused on the M protein, although other targets such as C5a peptidase, fibronectin binding protein and the GAS pilus have also been examined [3]. A multivalent M type-specific vaccine utilising the N terminal portion of the M protein has undergone human clinical trials [5]. This vaccine, initially formulated as a 26-valent vaccine, was found to be safe and immunogenic in humans. It has recently been reformulated into a 30-valent vaccine and further clinical trials are in progress. A vaccine based on the conserved C terminal region of the M protein, the so-called J8 vaccine, is nearing phase I human clinical trials [8].

A major hurdle with the development of GAS vaccines has been immunological safety, specifically the possible induction of autoimmunity and paradoxical recapitulation of ARF and RHD. Vaccine researchers have endeavoured to generate opsonizing antibodies, while minimising T cell activation, by avoiding the N-terminal, purportedly 'rheumatogenic' regions of the M protein. Clearly, better understanding of the influence of host genetics and of the immunopathogenesis of ARF and RHD would greatly assist human vaccine development.

6. Where to next in understanding post-streptococcal immune syndromes?

Surprisingly, there are currently no definitive laboratory-based tests for the various post-streptococcal clinical syndromes and diagnosis remains largely clinical. As outlined above, traditional immunological approaches have yielded limited results. A way forward may lie in the application of the new "omics" technologies - genomics, transcriptomics, proteomics, and metabolomics - to these ancient syndromes. Omics approaches provide an unprecedented breadth of information on disease states due to the concurrent measurement of a multitude of biological parameters. For example, transcriptomics has identified genes sets that distinguish active tuberculosis from other inflammatory diseases [281]; viral from bacterial infections [282, 283]; Kawasaki disease from adenovirus infections [284]; and septicaemic melioidosis from sepsis due to other causes [285]. One study demonstrated that the transcriptional profile of patients infected with GAS was distinct from active tuberculosis, staphylococcus infection, and systemic lupus erythematosus [281]. These data suggest that exploring GAS post-infectious syndromes using 'omics'

approaches will be very worthwhile. Transcriptomics may also identify previously elusive cellular activation pathways that may be amenable to drug treatment, including the repurposing of existing drugs. This approach is now being explored in other diseases [286-288].

Closely monitored human challenge studies are currently being used to examine the immune response to infection with influenza [289], malaria [290], and dengue [291]. A human challenge model of GAS pharyngitis might illuminate the chain of immune events that occur following infection as well as identify the crucial immune responses that protect against GAS-induced, post-infectious sequelae. A human challenge model could greatly expedite the development of potential vaccines for GAS.

7. Immunopathogenesis of post-streptococcal immune syndromes: shared or specific mechanisms?

The clinically distinctive post-streptococcal immune syndromes may result from preferential activation of different components of the immune response, acting within distinct anatomical compartments of hosts with variable genetic and environmentally determined risk. PSGN is caused by glomerular deposition of immune complexes, complement activation and temporary amplification of the actions of plasmin. PSGN appears to be a 'one hit' immune complex disorder that typically resolves without eliciting an ongoing autoimmune response. ARF could likewise result from immune complexes, initially containing GAS-derived antigens,

but depositing (for unknown reasons) in cardiac valve tissue, as well as synovium and skin. Complement may be activated by anti-GAS immune complexes arrayed on the surface of heart valves, similar to PSGN. Functional autoantibodies may be an under-appreciated feature in the post-streptococcal syndromes. AECA activate adhesion molecule expression and may cause cytotoxicity of endothelial cells, as well as local thrombosis, similar to LSE. Functional autoantibodies that gain access to the CNS may induce excessive dopamine production, causing chorea (and possibly PANDAS).

In contrast to PSGN, ARF and RHD seem to persistently engage both humoral and cellular autoimmunity, most likely due to molecular mimicry and epitope spreading [292](Fig. 3). Circulating T cells, activated by GAS exposure, upregulate adhesion molecules, enabling adhesion to valvular endothelium and subsequent trafficking into valve tissue. Endothelial damage may expose intravalvular molecular components and perhaps modify cardiac collagen, myosin, laminin, keratin, tropomyosin and other alpha-coiled coil proteins. These molecules may act as danger signals to local innate immune system cells, [293] and could develop greater immunogenicity if post translationally modified within the local inflammatory milieu. T cells are normally activated through encounter with APCs that have processed antigens for presentation on MHC molecules within secondary lymphoid organs. If this occurs at ectopic sites, such as within inflamed mucosal or epithelial tissues, or in Aschoff nodules, T cell regulation may be perturbed. Dysregulated T cell activation might favour the emergence of anti-self T cell clones and sustained

production of cytokines such as IFN γ and IL-17, recruiting other inflammatory cells and driving RHD. Chronic valvular inflammation would eventually initiate tissue remodelling, including neovascularization of the normally avascular heart valves [277]. Neovascularisation would drive tissue fibrosis and promote easy access for inflammatory cells in future ARF episodes, leading eventually to valve fibrosis and calcification.

Major challenges remain to understand, at both molecular and systems biology levels, how GAS interacts with the susceptible host immune system, resulting in distinctive patterns of target organ inflammation. Doing so is crucial to improving prevention, diagnosis and treatment of the heart disease arising from our ongoing battle with this ancient microbe.

Take home messages

- In susceptible hosts, GAS engages immune pathways that result in post-infectious sequelae, affecting various organs in distinctive ways.
- Genetic risk factors play a role in post-GAS sequelae, but the mechanisms involved remain poorly understood.
- Molecular mimicry between GAS M protein and host cardiac myosin is the most widely accepted cause of ARF and RHD, but additional mechanisms involving a range of alpha coiled-coil endogenous molecules, or other cross reactive targets are likely.
- Immune complexes and complement activation in response to streptococcal antigens are the primary cause of PSGN and may likewise contribute to inflammation of the synovium and heart valves in ARF and RHD.
- Functional antibodies generated by GAS infection can mediate cell signaling in neurons, resulting in chorea and possibly PANDAS; such antibodies may modulate endothelial cell function in cardiac valves and promote thrombosis in ARF, as in Libman Sacks endocarditis.
- Cross-reactive anti-GAS antibodies target various exposed antigens, but some autoreactive antibodies may result from normally sequestered antigens that are revealed, or post-translationally modified, as a result of tissue damage.
- CD4 T cells are important in valvular damage, and may have distinctive activation pathways and cytokine production profiles that influence the outcome of ARF and its progression to RHD.

- The pathogenesis of the different post-infectious GAS syndromes lends itself to the application of intensive, “omics” technologies.

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Figure Titles and Captions

Fig. 1. Target organs of post-streptococcal immune syndromes

Fig. 2. GAS M protein and possible sites for the generation of immunological cross reactivity. Schematic of M5 as a representative M protein containing a non-helical N-terminal region; and A, B, C and D repeat regions anchored to the GAS cell wall. Sequencing of the *emm* gene relating to the N-terminal region of the M protein is used to distinguish M type. Regions of M5 that demonstrate antibody and CD4 T cell cross-reactivity with cardiac proteins are indicated relative to the amino acid position.

Fig. 3. Possible pathogenic mechanisms in post-streptococcal cardiac disease

Cross-section of a heart valve leaflet. Autoreactive antibodies, including AECA and autoreactive T cells are generated by infection with Group A streptococcus in the throat (pharyngitis) or possibly the skin (pyoderma, impetigo) through molecular mimicry and/or anti-collagen responses. AECA have multiple effects, including the activation of endothelial cells leading to adhesion molecule VCAM-1 expression, complement activation leading to cell death, and activation of neuronal cells leading to CaM kinase III signaling. Deposition of complement and immunoglobulin occurs in both RHD and PSGN. Deposition of GAS molecules with functional activity (NAP_Ir and SpeB) occurs in PSGN but has not been identified in ARF or RHD. However, the

presence of M protein in the subendothelial collagen matrix by GAS invasion of endothelial surfaces may lead to the generation of anti-collagen type IV responses. Liberation of structural alpha helical coiled coil peptides including collagen, laminin, keratin and tropomyosin occurs in areas of tissue damage such as valvular lesions. Liberated proteins are presented by antigen presenting cells (APC) either in situ or in the draining lymph node to induce autoreactive CD4+ T cells. These APC are either resident dendritic cells, recruited inflammatory monocytes that have differentiated into APC in the valve interstices or within ectopic Aschoff nodules, or valvular fibroblasts and cardiac endothelial cells that aberrantly express MHC II. The range of reactive T cell and antibody specificities increases over time with epitope spreading. Th1 cytokines, such as IFN γ and chemokines including CXCL9 are generated in ARF and RHD. CXCL9 and CXCL10 are elevated in Sydenham's Chorea and chemokines including CCL6 and IL-6 are increased in PSGN. Prolonged and repeated cycles of inflammation facilitate ongoing tissue damage. In RHD, TGF β from interstitial cells may not only contribute to Th17 generation but to new blood vessel growth, allowing greater access to the valve in successive episodes, as well as stimulating collagen deposition from myofibroblasts, leading to fibrosis.

Table 1. HLA genes associated with RHD

Locus	Effect Size	Country (ethnicity)	Diagnosis	Ref.	Locus	Effect Size	Country (ethnicity)	Diagnosis	Ref.		
CLASS I genes					CLASS II genes (continued)						
A locus	A19	RR 2.39	Kashmir	RHD	58	DR11	OR 3.31	Uganda	RHD	68	
	A33	-	North India	RF/RHD	59	DR6 (DR13 and DR14)	RR 2.6	South Africa (Non-Caucasian)	RF/RHD	66	
	A10	-	Turkey	RF	60		rel odds=0.19	USA (Caucasian)	RHD	72	
B locus	B5	OR 3.46	Egypt	RHD	61		DRB1*06	OR 0.18	Latvia	RHD	79
	B5	RR 0.19	Kashmir	RHD	62	DRB1*13	OR 5.69<	Turkey	RHD	80	
	B14	RR 0.17	Martinique	RF	63	DR7	RR 2.69	Turkey	RHD	64	
	B35	-	Turkey	RF	60		RR 3.8	Brazil (Caucasian/Mulatto)	RF/RHD	81	
	B35	RR 2.33	Martinique	RF	63		RR 2.4	Brazil (Caucasian)	RF/RHD	82	
	B16	RR 3.39	Turkey	RHD	64	DRB1*07	OR 2.78	Turkey	RHD	77	
	B42	RR 0.12	Martinique	RF	63	DRB1*07	OR 2.5	Egypt	RHD	83	
	B51	-	Turkey	RHD	65	DRB1*07	OR 1.76	Pakistan	RHD	84	
	Cw*5	-	Turkey	RHD	65	DRB1*0701	OR 4.18	Latvia	RHD	79	
CLASS II genes					DR10	DR *10-0101	OR 5.75	Egypt	RHD	61	
DR locus					DR16		OR 4.3	USA (Caucasian)	RF	85	
DR1		RR 5.2	South Africa (Non-Caucasian)	RHD	66			OR 3.9	Mexico	RHD	76
		RR 3.12	Martinique	RF	63		DRB1*1602	OR 5.3	Mexico	RHD	76
		-	Brazil (Mulatto)	SC	67	DR51	DRB5	OR 33<	Turkey	RHD	80
	DRB1*01		Turkey	RHD	65	DR52	DRB3	OR 2.66<	Turkey	RHD	80
DR2 (DR15, DR16)		OR 0.42	Uganda	RHD	68	DR53		RR 4.2	Brazil (Caucasian/Mulatto)	RF/RHD	81
		RR 3.86	USA (Non-Caucasian)	RF	69	Allogenotype TaqI DR□		-	Brazil	RF/RHD	86
		-	North India	RF/RHD	59	13.81 kb					
		-	North India	RHD	70						
		RR 0.3	North India	RHD	71						
DR3 (DR17, DR18)		-	North India	RF/RHD	59	DQ locus					
		RR 2.3	North India	RHD	71	DRQA1	DQA1*0101	RR 2.89	South China	RF/RHD	87
		RR 3.14	Turkey	RHD	64		DQA1*0102	RR 0.106	South China	RF/RHD	87
DR4		RR 3.55	USA (Caucasian)	RF	69		DQA1*0103	OR 0.44	Egypt	RHD	83
		rel odds=2.3	USA (Caucasian)	RHD	72		DQA1*0104	RR 2.82	Japan	RHD(MS)	88
		RR 2.74	Kashmir	RHD	58		DQA1*0201	OR 1.93	Egypt	RHD	83
		RR 3.27	Kashmir	RHD	62		DQA1*03	OR 0.462	Turkey	RF	75
		RR 13.6	Saudi Arabia (Arab)	RF/RHD	73		DQA1*0401	OR 3.31	Latvia	RF/RHD	89
		-	Turkey	RF	74		DQA1*0501△	-	Mexico	RHD	76
		-	Egypt	RHD	61	DRQB1	DQB1*0302	OR 3.13	Latvia	RHD	79
		DR*04-02	-	Turkey	RHD	61		DQB1*0401	OR 4.33	Latvia	RHD
	DRB1*04▼	OR 0.42	Turkey	RF	75		DQB1*0501	OR 0.26	Latvia	RHD	79
DR5 (DR11 and DR12)	RR 0.38	Turkey	RHD	64		DQB1*05031	RR 3.23	Japan	RHD(MS)	88	
DR11		-	Turkey	RF/RHD	60		DQB1*0602-8	OR 0.4	Latvia	RHD	79
		OR 0.33	Mexico	RHD	76		DQB1*0603	OR 0.05	Egypt	RHD	83
		OR 0.42	Turkey	RHD	77		DQB1*08	-	Turkey	RHD	65
	DRB1*11	OR 0.42	Turkey	RHD	77	DRQw2		RR 3.76	North India	RHD	70
	DR11-05-DQ7	-	Taiwan	RHD	78						

Table 2. Non-HLA genes associated with RHD

Locus	Description	Effect size	Country (ethnicity)	Diagnosis	Ref.
Inflammatory Mediators and Signaling Molecules					
IL1RN	Interleukin 1 receptor antagonist	2.2	Egypt	RHD	91
		0.11	Brazil	RHD	93
		0.52	Pakistan	RHD	92
IL-6	Interleukin-6	2.6	Pakistan	RHD	92
IL-10	Interleukin-10	3.1, 5.2	Egypt	RHD-MVD	91
MASP2	Mannan Binding Lectin-associated serine	0.25-0.36,	Brazil	RF/RHD	94
PTPN22	Protein tyrosine phosphatase, non-	0.025	India	RHD	95
TNFA	Tumour necrosis factor	9.94	Pakistan	RHD	92
		2.3, 4.7	Egypt	RHD	96
		1.4, 1.9	Brazil	RF/RHD	97
		5.7	Egypt	RHD	91
		3.3/3.4	Turkey	RF/RHD	98
		10.8, 14.1	Mexico (Mestizo)	RHD	76
		1.8	China (Han)	RHD	99
		1.7-6.0	Egypt	RHD	100
TNFAIP3	TNF alpha-induced protein 3	1.49	Taiwan (Chinese Han)	RHD	101
TGFB1	Transforming growth factor-beta1	1.44, 1.78	India	RHD	102
STAT3	Signal transducers and activators of	1.92, 2.31	India	RHD	102
STAT5	Signal transducers and activators of				
Pathogen Recognition					
FCN2	Ficolin 2	1.56	Brazil	RF/RHD	103
MBL2	Mannose binding lectin	3.5	Brazil	RHD	104
		2.26, 2.42	Brazil	RF/RHD	105
		1.98, 1.99	Brazil	RHD	90
TLR2	Toll-like receptor 2	97.1	Turkey	RF	106
TLR5	Toll-like receptor 5	1.7-2.0	China (Han)	RHD	107
FcgammaRIIA	Fc gamma receptor IIA	3.09, 4.98	Turkey	RF	108
Other					
CTLA-4	Cytotoxic T lymphocyte associated	3.11	Turkey	RHD	109
ACE	Angiotensin I-converting enzyme	1.5, 2.12	Taiwan (Chinese Han)	RHD	110
		1.62, 2.08	India	RHD	111

RHD = rheumatic heart disease; MVD =mitral valve disease; RF =rheumatic fever