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Rheumatic fever: 21st century clinical and experimental insights

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Published in
Frontiers in Cardiovascular Medicine
Frontiers in Medicine
Frontiers in Immunology

ISSN 1664-8714
ISBN 978-2-8325-2366-7
DOI 10.3389/978-2-8325-2366-7

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Rheumatic fever: 21st century clinical and experimental insights

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Citation

Sampaio, R. O., Guilherme, L., Nascimento, B. R., Beaton, A., eds. (2023). *Rheumatic fever: 21st century clinical and experimental insights*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-2366-7

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frontiers | Frontiers in Cardiovascular Medicine

TYPE Editorial
PUBLISHED 24 April 2023
DOI 10.3389/fcvm.2023.1190372

OPEN ACCESS

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RECEIVED 20 March 2023
ACCEPTED 04 April 2023
PUBLISHED 24 April 2023

CITATION
Nascimento BR, Beaton AZ, Guilherme L and Sampaio RO (2023) Editorial: Rheumatic fever: 21st century clinical and experimental insights. Front. Cardiovasc. Med. 10:1190372. doi: 10.3389/fcvm.2023.1190372

Editorial: Rheumatic fever: 21st century clinical and experimental insights

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KEYWORDS

rheumatic heart disease, epidemiology, pathophisiology, treatment, acute rheumatic fever, immunology and inflammation

Editorial on the Research Topic
Rheumatic fever: 21st century clinical and experimental insights

Editorial

The global burden of Rheumatic Heart Disease (RHD) is still high worldwide, and is becoming progressively more concentrated in under-resourced regions (1). As the disease is strictly linked to the socioeconomic background, actions and conditions that ultimately converge to mitigate RHD burden (1) have been more effective in higher-income regions, contributing to its uneven global distribution. Among cardiovascular diseases, RHD accounts for 1.6% of all deaths, resulting in 306,000 fatalities yearly (1), according to updated estimates. Noticeably in the past 2 decades, several research initiatives have been promoted for a deeper understanding not only about RHD, but also about the exaggerated immune event in response to a streptococcal event that precedes RHD, so called acute rheumatic fever (ARF). Thanks to advocacy led by several medical societies and international organizations, there has been growing interest—and consequently funding—for research projects and healthcare programs focused in different fundamental topics, such as: pathophysiology, immune and inflammatory mechanisms, biomarkers, novel diagnostic approaches as echocardiographic (echo) screening and targeted case finding, educational and health promotion interventions, management of early and late phases of disease, novel devices and medications, vaccines and many others.

Beyond allowing for a better understanding of the pathological pathways and processes of ARF and RHD (2), the ongoing programs are globally spreading the importance of RHD and the possibility of its elimination in a lifetime through relatively feasible and simple approaches, bringing together different sectors. Nonetheless, there still remain fundamental gaps in our understanding of ARF and RHD, and some of them were

addressed in the special issue of Frontiers in Cardiovascular Medicine: "*Rheumatic Fever: 21st Century clinical and experimental insights*".

Important data has been presented related to basic science and pathophysiological pathways linked to ARF and RHD. A deep discussion about RHD pathology, immune mechanisms, inflammatory processes at the cell and tissue level, and other underlying mechanisms was provided by Passos et al. in a comprehensive review Passos et al. Evaluating patients pre and post mitral valve commissurotomy due to advanced mitral stenosis (MS), Silva et al. demonstrated that an association between the decrease of specific cytokines and changes in T cell activation with hemodynamic improvement post intervention and long-term outcomes, raising new possibilities for soluble biomarkers of better recovery Silva et al. In this sense, ongoing studies, supported by different funding sources, hold promise in the search for an easily titerable and reproducible biomarker for ARF/RHD, to overcome the need for the combination of clinical observations technological advanced diagnostic tools and non-specific lab tests. As shown by Salie et al. in a meta-analysis of 24 studies, currently available antigens in response to streptococcal infection are not consistently associated with an ARF diagnosis, and further research with more strep proteins is needed Salie et al. Similar observations were reproduced by McGregor et al.: a multi-platform approach to profile circulating autoantibodies denoted marked heterogeneity in autoantibody profiles among ARF patients, although novel candidates were pointed out, in addition to those previously implicated, as myosin and collagens McGregor et al. A translational pilot study by Kirvan et al. involving 23 children (10 with RHD, 6 with Sydenham chorea and 7 with uncomplicated pharyngitis) suggested that group A carbohydrate, N-acetyl-β-$_{D}$-glucosamine-specific IgG2 may be an important autoantibody in initial stages of the pathogenesis of streptococcal sequelae, emerging as a future candidate for a biomarker in early disease stages Kirvan et al. Again here, data is still preliminary and derived from limited patient samples. Looking for strategies to overcome these uncertainties, in the perspective article by McMillan et al. the authors propose that with the sharing of multi-region serial blood samples, antibody array technology and T-cell tetramers could lead to the identification of highly specific peptides McMillan et al. However, given the complexity and heterogeneity of tissue involvement by ARF/RHD—a condition restricted to humans—this requires optimal animal models. Rafeek et al. widely discuss the requirements of an ideal animal model—noticeably small rodents—which may potentially mimic the diagnostic criteria features of ARF/RHD, allowing for research on immune responses, biomarker assessment, treatment evaluation and ultimately vaccine development Refeek et al.

On the epidemiological side of research, following the publication of several echocardiographic screening studies from almost all endemic regions of the world in the past 2 decades, doubts still remain about the clinical management of individuals found to have latent RHD in screening. Although the benefits of secondary prophylaxis have been demonstrated by the GOAL trial (3), implementation of such a strategy require further investigations about how to stratify the risk for progression. Adding to the body of evidence about secondary prophylaxis, Torres et al. present a well-characterized cohort of 593 Brazilian children with past ARF, in which 59% evolved with RHD. Regression of mitral and aortic lesions was strongly associated with prophylaxis, and no patients receiving penicillin had progression of valve involvement Torres et al. Also in this issue, Zimmerman et al. present a novel assessment of the risk of latent RHD among schoolchildren with a previous negative screening. Screen-negative individuals (3–5 years prior) had a statistically similar risk of having RHD in a serial echocardiographic screening, although there was a non-significant trend towards a 40% lower risk in this group compared to children with previous positive screening Zimmerman et al. However, the decision whether serial screening should be recommended should consider these data, but also warrants further investigation.

Adding to the knowledge about the incidence of ARF in non-endemic countries, the study by Marino et al. in Monza, Italy, depict a 10-year incidence of ARF in a retrospective analysis of 70 reported cases. The mean rate in schoolchildren between 5 and 14 years was 5.7/100,000, considerably above the threshold proposed by the World Heart Federation for low-risk areas Marino et al., and reinforcing the need for constant monitoring of disease burden even in places where it near eradicated. On the other hand, the data by Opara et al. depict the economic burden posed by RHD in under sourced areas as Uganda, with overall direct and indirect costs of around USD 78 per patient/year, markedly affecting the poorer areas, where the utilization of financial coping mechanisms is frequent Opara et al.

In terms of imaging, much has been learned from studies that have been applying multi-modality methods and technology-based add-ons and tools, previously validated in other structural heart diseases, for patients with RHD. Beyond diagnostic refinement and planning of interventional procedures, advanced imaging has been contributing for the understanding of the underlying pathological mechanisms of the disease. As an example of this matter, Rosa et al. show preliminary data from with 25 patients suggesting that myocarditis due to ARF reactivation may be a cause of echo-detected left ventricular dysfunction regardless of the degree of valvular involvement Rosa et al. Given the possible reversibility with corticosteroid treatment, the acknowledgement of this relationship is crucial, and serial examinations should be considered in this patient subset. Expanding the possibilities of cardiac imaging, tridimensional (3D) echocardiography is also being progressively more applied for RHD management. As pointed out in a comprehensive review paper by Vieira et al., up-to-date 3D echocardiography is capable to provide additional anatomical and morphofunctional information about patterns of rheumatic valvular involvement, being relevant not only for diagnostic purposes but also for prognostication and guidance of invasive procedures—as mitral commissurotomy—and correction of peri-procedural complications Vieira et al. The authors postulate that, with adequate equipment and training, 3D echo is a ready-to-use technique for rheumatic patients with valvular abnormalities. Indeed, 3D imaging has been enabling for significant advances as a guidance for interventional cardiology

TABLE 1 Manuscripts published in the special issue: rheumatic fever: 21st century clinical and experimental insights.

Pathophysiology/Immunology:	Reference:
Review on pathophysiology and immunology	Passos et al.
Cytokines and changes in T cell activation after intervention	Silva et al.
Antigens in response to Streptococcal infection (metanalysis)	Salie et al.
Heterogeneity in autoantibody profile in ARF	McGregor et al.
N-acetyl-β-$_D$-glucosamine-specific IgG2 autoantibody in early stages of ARF	Kirvan et al.
Identification of specific peptides using multiregion blood samples	McMillan et al.
Animal model for ARF	Refeek et al.
Epidemiology	
Regression of valve disease after Penicillin prophylaxis	Torres et al.
Risk of latent RHD in schoolchildren with previously negative screening	Zimmerman et al.
Incidence of ARF in Northern Italy	Marino et al.
Costs of RHD in Uganda	Opara et al.
The "Cairo Accord" and other global health initiatives for reducing RHD burden	Kotit et al.
Echocardiography/Treatment	
Myocarditis during ARF reactivation	Rosa et al.
3D echocardiography for RHD	Vieira et al.
Valve-in-valve interventions in patients with RHD	Lopes et al.

ARF, acute rheumatic fevere; RHD, rheumatic heart disease.

procedures. Although the pathological process of RHD differs widely from that of non-rheumatic degenerative valve disease, especially in terms of calcification, involvement of the sub valvular apparatus and adjacent structures, and especially the younger age of patients, percutaneous valve replacement has been more commonly indicated in selected cases. Besides case reports and series showing good results of transcatheter aortic valve replacement (TAVR) in rheumatic patients (4), valve-in-valve procedures are being tested in bioprosthetic valve dysfunction. In the case series presented by Lopes et al., including interventions in mitral, aortic and tricuspid positions, RHD patients had similar procedural success compared to non-rheumatic individuals, but 30-day mortality rates were higher. At 20 months, however, cumulative mortality rates were superimposable Lopes et al., suggesting that this may be an option to reduce the morbidity of redo procedures in young patients living with RHD, but randomized data are required.

Finally, the broad and growing scope of research initiatives related to RHD worldwide, notably boosted by implementation science and international collaboration have resulted in outstanding magnification of accords, statements and resolutions that contribute to pave the road to eradicate the disease in a lifetime. As an example, the "2017 Cairo Accord" exemplary defined policy priorities for fighting ARF/RHD and built on a recent series of broad initiatives and calls to action, as detailed in the review paper by Kotit et al. Along with a series of other fundamental statements, the accord culminated in the recognition of ARF/RHD as global health priorities in the global stage following the 2018 World Heart Assembly. Including a broad span of ongoing research on this topic, from basic science to clinical and population studies, this special issue of Frontiers in Cardiovascular Medicine (a summary of the published articles is presented in **Table 1**) is aimed at contributing to the superb scientific moment faced by ARF/RHD, also as a call-to-action for continuing collaborative efforts needed for the mitigation of this complex disease of the poor.

Author contributions

Conception and design of the research: AZB, BRN, LG, ROS. Analysis and interpretation of data: BRN, LG, ROS. Writing of the manuscript: BRN. Critical revision of the manuscript for intellectual content: All authors. Authors responsible for the overall content as guarantors: BRN, AZD, ROS. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Cardiovascular Medicine

REVIEW
published: 18 January 2021
doi: 10.3389/fcvm.2020.612716

Rheumatic Heart Valve Disease Pathophysiology and Underlying Mechanisms

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Rheumatic heart valve disease (RHVD) is a post-infectious sequel of acute rheumatic fever resulting from an abnormal immune response to a streptococcal pharyngitis that triggers valvular damage. RHVD is the leading cause of cardiovascular death in children and young adults, mainly in women from low and middle-income countries. It is known that long-term inflammation and high degree of fibrosis leads to valve dysfunction due to anatomic disruption of the valve apparatus. However, since public and private investments in RHVD studies are practically inexistent the number of publications is scarce. This disease shows different natural history and clinical presentations as compared to other degenerative heart valve diseases. Although more than five decades passed after the pioneering studies on the pathogenesis of RHVD, it is still unclear how self-tolerance mechanisms fail in this disease, and how humoral and cellular inflammatory responses are interconnected. Despite that pathological mechanisms have been already proposed for RHVD, none of them are able to explain the preferential involvement of the mitral valve. This review focuses on pathophysiology and underlying mechanisms of RHVD.

Keywords: rheumatic heart disease, mitral valve, inflammation, autoimmunity, pathogenesis

OPEN ACCESS

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Specialty section:
This article was submitted to Heart Valve Disease, a section of the journal Frontiers in Cardiovascular Medicine

Received: *30 September 2020*
Accepted: *21 December 2020*
Published: *18 January 2021*

Citation:
Passos LSA, Nunes MCP and Aikawa E (2021) Rheumatic Heart Valve Disease Pathophysiology and Underlying Mechanisms. Front. Cardiovasc. Med. 7:612716. doi: 10.3389/fcvm.2020.612716

INTRODUCTION

Rheumatic Heart Valve Disease

Heart valve disease (HVD) is generic term that includes several etiologic entities with different pathophysiologic mechanisms that lead to anatomic disruption of the valve apparatus (1). Functional abnormalities due to alteration in matrix architecture and cellular components impair the proper directionality of blood flow through the heart chambers resulting in heart failure (2).

Overall, HVD are slowly progressive disorders that affect mainly the aging population (>65 years), reaching epidemic proportions worldwide (3). Despite increased life expectancy over the last several decades, calcific aortic valve disease (CAVD), and degenerative mitral valve disease are the two most common types of non-rheumatic valve disease (4). Due to vast health impact in developed world, CAVD has sustained significant research interest and a greater number of studies as compared to the other valvular disorders.

On the other hand, in low and middle-income countries, rheumatic heart valve disease (RHVD) is the leading cause of cardiovascular death in children and young adults (5, 6). Even though the observed progress in research on RHVD pathogenesis with findings that have challenged a variety of historical paradigms, a number of key scientific questions remain. Public and private investments

in RHVD studies are low and therefore the number of publications is limited. RHVD could be viewed as a marker of inequality and social injustice for countless populations living in poverty. Over the last years, defense groups are making efforts for identification and removal of barriers to the translation of existing knowledge into policy, programs, and practice to provide high-quality care for patients with RHVD (7).

HVD require a substantial allocation of health resources, and there is no effective drug therapy to prevent or treat these pathologies. In addition, these valvular diseases show different natural histories and clinical presentations. This review focuses on pathophysiology and underlying mechanisms of RHVD.

PATHOLOGY OF RHVD

Gross Pathomorphological Findings

RHVD is a harmful post-infectious sequel of acute rheumatic fever (ARF) resulting from an abnormal immune response to a streptococcal pharyngitis that triggers valvular damage (8). The first episode of ARF is often associated with only mild manifestations and can occur at any age in genetically predisposed individuals (9). Recurrent *Streptococcus pyogenes* infection, which boosts immune response leads to RHVD. Thus, although RHVD first occurs in childhood, its incidence peaks in adulthood, usually between the ages of 25–45 years (10). In developing countries, social determinants of the disease such as inadequate housing, lack of access to primary health care, education, and availability of cardiologic diagnostic tools hamper the diagnosis. Most of children are undiagnosed and therefore do not receive antibiotic as secondary prophylaxis to prevent new *S. pyogenes* increasing their chances to develop RHVD. Women and girls may experience less access to primary and secondary prophylaxis as compared with men and boys in low-income countries, and this could also contribute to differences in RHVD rates between females and males (9). In addition, women have a closer involvement in childcare and therefore higher *S. pyogenes* exposure.

The mitral valve is affected in almost all RHVD cases, with regurgitation in the early stages, and stenosis in later stages (11). RHVD can also affect aortic valves, however, calcific degeneration is an outcome usually associated with aortic valve. During initial phase of rheumatic disease, echocardiographic exams can detect small verrucous nodules caused by the presence of thrombi along the lines of heart valve closure. These lesions are not able to produce leaflet destruction and therefore valve function is relatively normal. On the other hand, development of long-term inflammation after single or multiple episodes of rheumatic fever can lead to valve dysfunction in untreated genetically predisposed patients. As general pathomorphological findings, mitral valve specimens from patients at end state disease are thick and stiff due to a high degree of fibrosis (**Figures 1A,B**). As this process stretches over decades, different morphological changes dominate the various phases. While leaflets are usually minimally fibrose and pliable in three quarters of patients younger than 30 years of age, they are scared and ridged in two thirds of patients older than 40 years. Different morphological manifestations also lead to different clinical symptoms. While chordal shortening

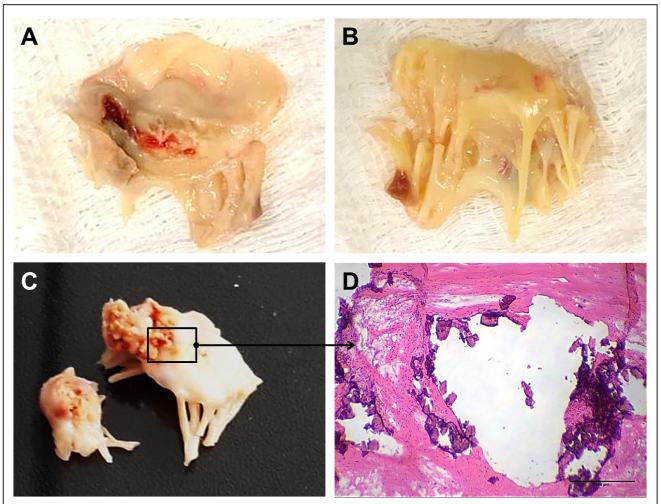

FIGURE 1 | Gross pathology and histological aspects of rheumatic mitral valve at the end stage of RHVD. **(A,B)** Atrial and ventricular sides of mitral valves excised from female, 49 year-old patient, showing thick leaflets with retraction. **(C)** Mitral valve excised from male, 61 year-old, showing calcification. **(D)** Representative Hematoxylin and Eosin staining of anterior mitral valve leaflet showing presence of nodular calcification. Scale bar = 500 μm.

is dominant in 90% of patients with mitral stenosis, it only occurs in 3% of patients with pure mitral regurgitation (MR). Annular dilatation is found in 90% of patients with pure MR but only in 30% of cases with pure stenosis. In most late cases, the valve commissures are fused and often endothelium surface erosion is observed. Chordae tendineae show fusion and shortening (**Figure 1B**), which may reduce subvalvular chordal space (12, 13). Calcification occurs in some cases of RHVD (**Figures 1C,D**), however fibrosis and inflammation are major findings (**Figures 2A–C**) (14, 15). Accumulating studies had shown that lipids may trigger vascular calcification associated with atherosclerosis. Therefore, it is possible that RHVD patients who develop valve calcification have altered cholesterol profile. It still remains a gap of knowledge in RHVD research.

MECHANISMS OF MITRAL VALVE DISEASE

Immune Response in RHVD

Mitral valves are populated by two major types of cells: valvular endothelial cells (VEC), covering the leaflets, on both atrial and ventricular sites; and valvular interstitial cells (VIC), quiescent fibroblast-like cells, which are important in the homeostatic remodeling of matrix constituents (16–19). In disease state, cell composition shifts toward contractile and collagen-producing myofibroblast-like cells leading to fibrotic changes and stiffness of leaflets (**Figure 2A**) (19). Chronic inflammatory processes are dominant in RHVD resulting in accelerated loss of valve function (**Figure 2B**). Mitral valve is composed of three layers of specialized connective tissue between the two endothelial layers and its architecture substantially changes during RHVD progression. Anatomical features of mitral valve can be associated with its preferential involvement

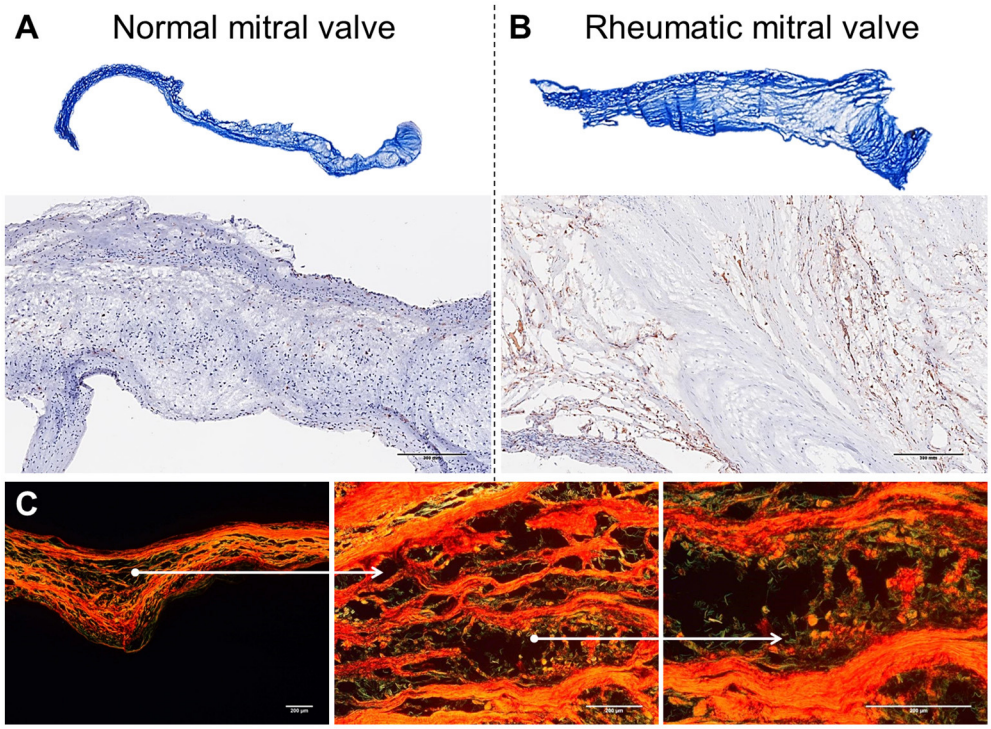

FIGURE 2 | Histological comparison between normal mitral valves and rheumatic mitral valves. **(A)** Masson trichrome staining showing focal fibrosis and thickening of rheumatic mitral valves. Representative images are shown. **(B)** Representative immunohistochemistry staining for $CD45^{+}$ cells evidencing a high frequency of leukocytes. Scale bars = 300 mm. **(C)** Picrosirius red staining showing uneven collagen deposition. Representative images are shown. Scale bars = 200 μm.

in this disorder. However, the underlying mechanisms of mitral valve predominant involvement in RHVD are unknown.

Studies related to immunopathogenesis of rheumatic fever as well as of the RHVD have been conducted since 1960 when the presence of autoantibodies in the serum of patients with throat infection by group A β-hemolytic Streptococci has been demonstrated (20). Although more than five decades passed after the pioneering studies on the pathogenesis of RHVD, little progress has been made in respect of the cellular and molecular aspects, which lead to the destruction of the valve tissue. In RHVD patients, the generation of an antibacterial immune response starts in the pharyngeal epithelium by innate immune components such as neutrophils, macrophages, and dendritic cells. These cells recognize and process bacterial antigens and present them to B lymphocytes, culminating in the production of immunoglobulins that are able to recognize epitopes in several host sites and activate T cells.

Still a mystery the mechanisms involved in the loss of self-tolerance in RHVD and how immune system target heart valvular tissue, especially mitral valve. Autoimmune valvular carditis are mainly described in literature as associated with human rheumatic conditions, however, it is possible that autoreactive antibodies can be associated with pathology of other HVD, including CAVD. Ectopic calcification and autoimmunity has been also explored in atherosclerosis (21, 22).

Multiple bacterial antigens are involved in RHVD damage. M, T and R proteins and N-acetylglucosamine (GlcNac), a group A β-hemolytic Streptococci carbohydrate (GAS), are the main epitopes described to be associated with molecular mimicry. These molecules share structural similarity with host cardiac myosin, laminin, vimentin, and tropomyosin. Many studies point to M protein as the most virulent protein (23, 24). Although myosin is present in the myocardium but not in the valvular tissue, anti-myosin antibodies respond against GlcNac epitope due to similar structures of common alpha-helical sequences and glycosylated proteins (25). It is assumed that myosin is an intracellular protein and therefore immunologically inaccessible, and thus does not participate as the initial target of cross-reactive antibodies. However, after initial mild endothelial breakdown, intracellular epitopes may contribute to robust amplification of the immune response due to increased availability of binding sites for anti-myosin antibodies.

Investigations have highlighted an important participation of GlcNac antigens in RHVD pathogenesis, since persistent levels of anti-GAS antibodies were correlated with valvulitis. It is also observed that after valve replacement the serum levels of anti-GAS were reduced (23, 26). In addition, unlike to some of the cross-reactive intracellular proteins, GlcNac are cell-surface antigens that are more exposed/accessible to antibody recognition (27). In non-rheumatic valve disease, the triggering

factors leading to cardiovascular calcification, as in CAVD are still under investigation (22).

Although pan-carditis occurs in the early disease stages, it is reversible, and only the valvular damage is permanent, especially in mitral valve. Mitral valve damage is initiated by circulating autoantibodies that bind to the endothelial surface of the valves, leading to increased expression of vascular cell adhesion protein 1 (VCAM-1). The activated endothelium facilitates the infiltration of T lymphocytes into the valvular subendothelium at the endocardium site, leading to edema and elongation of the chordae tendinae (28, 29). Due to tissue injury, components of ECM are exposed and anti-collagen antibodies are produced. These antibodies can deposit in the valve contributing to pro-inflammatory environment. All these changes cause the heart valves to be a vulnerable immune-privileged site for injury. The role of anti-collagen antibodies will be addressed in more details below.

It is important to comment that typically the first streptococcus throat infection does not trigger an episode of rheumatic fever. Recent studies have shown that continuous infections maintain the germinal center reaction and affinity for antibody maturation (30, 31). As such, preexisting immune complexes would capture more immunoglobulin leading to amplification of the immune response, which further favors the recognition of several self-antigens and propagate tissue damage. Thus, repeated infections feed the disease onset (32). To date, no evidence exists that the isolated presence of valve-reactive antibodies in the serum of RHVD patients is sufficient to produce the valve lesion, suggesting the importance of cellular response besides humoral components. In addition, autoantibodies are often found in patients after uncomplicated streptococcal pharyngitis and in healthy individuals.

Humoral and cellular response acts together in autoimmune diseases. It is known that women produce more immunoglobulin than men, and the X chromosome contains over 1,000 genes, while the Y chromosome only has about a 100 (33). Many of the X-linked genes are relate to the immune system, such as CD40L, CXCR, OGT, FOXP3, TLR7, TLR8, IL2RG, BTK, and IL9R. Also, sex hormones can directly or indirectly affect the immune response by modulating gene expression through ERα stimulation (34). Immune cells express estrogen and androgen receptors, and engagement of these receptors affects lymphocyte responses (35). ARF usually occurs during childhood equally in males and females, however, RHVD has higher prevalence in adult women. Thus, is likely that endogenous hormones are key mediators of disease progression. Overexpressed X-linked immune genes and estradiol probably act in a synergistic manner, leading to a greater female-biased predisposition in RHVD.

Emerging evidence suggests that the transfer of T-cell lines from M-protein vaccinated Lewis rats to naïve animals can induce valvulitis in recipient animals. These data support that T cells are sufficient to induce inflammation, not requiring the presence of cross-reactive antibodies to trigger valvulitis (36). Although some studies showed that the presence of antibodies is not crucial in triggering RHVD pathogenesis, it is important to emphasize that the ability of antibodies to become self-reactive will depend on the combination of factors such as genetic background, recurrence of infections, and strain virulence. These variables make it even more challenging to fully understand the mechanisms associated with the development of valve lesions.

The inflammatory infiltrate described in the rheumatic mitral valve of patients in the end state of disease are predominantly composed of mononuclear cells (**Figure 3C**), mainly helper (Th) - CD4, and cytotoxic—T CD8 lymphocytes, macrophages and B cells (37, 38). The effector function and therefore the contribution of these cells in the pathogenesis of RHVD is largely associated with the profile of cytokines and other soluble mediators produced by them that lead to differentiation of VICs to activated collagen-producing myofibroblast (39).

So far, T $CD4^+$ lymphocytes have been the most investigated cell population in RHVD since they are present at high frequency in the inflammatory infiltrates and have high cross reactivity against cardiac myosin epitopes. In addition, these cells are able to differentiate into various repertoires of subpopulations producing diverse cytokines leading to the development of different degrees of cross-reactivity (40–43).

Th1 cytokines are pro-inflammatory soluble mediators involved in host defense and also crucial to autoimmunity. In the RHVD context, some studies have evaluated the expression of different inflammatory cytokines in the valvular tissue, however cell sources of these cytokines and contributions of each cellular subpopulation to the cytokine production remain unknown.

Among the Th1 cytokines, TNF-α, IFN-γ, IL-1, IL-2, and IL-6 have already been shown to be associated with disease progression. *In vitro* studies have demonstrated that TNF-α exhibits a high chemotactic potential promoting cell attraction to the site of inflammation, whereas IFN-γ can induce processing and presentation of autoantigens (38, 44, 45). IL-1 is a key cytokine in autoimmune disease and has also been shown to be associated with inflammatory damage, especially in the acute phase of rheumatic disease (46, 47). In a Brazilian population study, polymorphism in IL-Ra and IL-6 genes was associated with susceptibility to RHVD (48, 49). IL-6 was linked to B cell antibody production and suggested to be involved in RHVD pathogenesis (50). Systemic cytokine levels were associated with severity of RHVD and co-regulated expression of IL-6 and TNF-α was associated with severe valve dysfunction (51).

IL-2 is an essential cytokine initiating generation of regulatory T cells (Tregs), which play a vital role in the maintenance of immune tolerance. Low levels of IL-2 and deficiency of circulating Tregs were associated with rheumatic mitral valve disease (44, 52, 53). Moreover, patients who presented multiple valve impairment showed a greater deficiency in the number of Tregs (54).

Th17 cells are T $CD4^+$ cells producing high amounts of IL-17. This cell subset plays opposite roles as compared to Tregs in autoimmune diseases. In the context of RHVD, it was shown to be associated with progression of the disease toward chronic state in an experimental model (55). Peripheral blood cells from patients with rheumatic mitral valve disease showed an increased number of Th17 cells and high serum levels of IL-17 as compared to healthy individuals (52).

Some cytokines with a typical Th2 response mediating activation and regulation responses against allergen toxins,

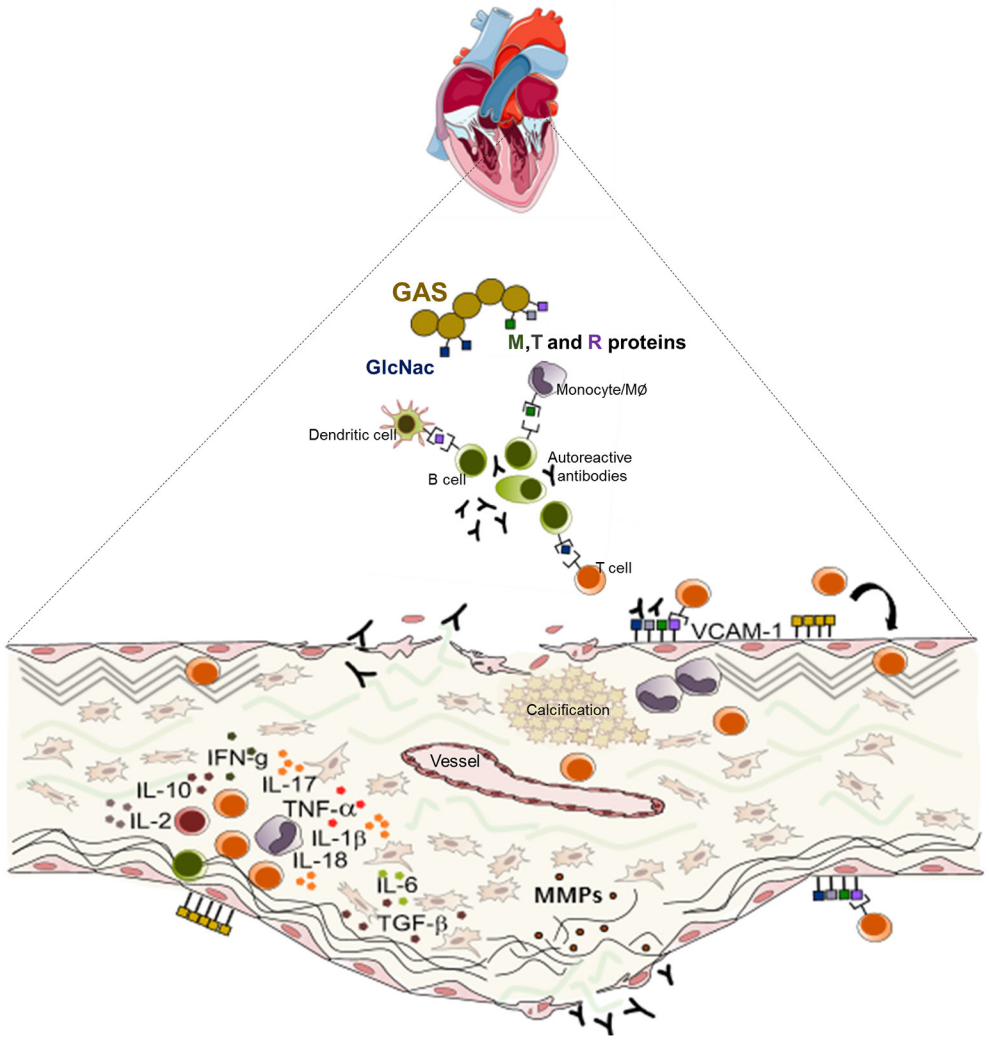

FIGURE 3 | Schematic representation of the mechanisms of the pathogenesis of rheumatic heart valve disease. Following group A Streptococcus (GAS) invasion of the pharyngeal epithelium, GAS recognize and process bacterial antigens and present them to B lymphocytes. Activated B cells produce antibodies that are able to recognize epitopes in several sites in the host and also activate T lymphocytes. In the heart, cross reactive T cell clones and antibodies act against heart valve constituents leading to an intense inflammatory process culminating in valve dysfunction.

extracellular parasites and bacteria, have also been studied in RHVD. In these patients, IL-10 is present at high levels and has a direct correlation with T CD8$^+$ lymphocyte response. IL-10 acts as a chemoattractant for these cells and creates a favorable milieu for the growth of its precursors. On the other hand, Th2 cytokines such as IL-4 and IL-5 are present in very low concentrations or not detectable in RHVD (56–58).

The role of cytokines in the site of inflammation is still underexplored since the vast majority of studies were done using peripheral blood, and the expression of cytokines at the lesion site could be underestimated. Analysis of valve tissue is essential for the understanding of inflammatory mechanisms *in situ*. Studies performed in peripheral blood and valvular tissues from RHVD patients, point to a predominance of T CD4$^+$ cells as compared to the number of T CD8$^+$ cells. However, the proportion of circulating T CD4$^+$ and T CD8$^+$ cells appears to vary between stages of the disease, as evidenced by increased frequency of T CD8$^+$ cells in patients with chronic stage disease (37, 38, 44). The role of T CD8$^+$ cells in the pathogenesis of RHVD, however, remains scant.

B lymphocytes are present in the inflammatory infiltrates of rheumatic mitral valves. While their contribution to the pathogenesis of the disease is often associated with the production of antibodies in the early disease stages, it is likely that these cells also play a role of effector cells participating in chronic lesion development (38, 59).

Macrophages are known to play a high pathogenic role in cardiovascular diseases. A recent study showed that pro-inflammatory macrophages (M1) exhibit effector potential through activation of NLRP3 inflammasome (60) leading to

the production of IL-1β and IL-18, an important pathway in the pathogenesis of rheumatic diseases (61, 62). IL-1β in turn, induces release of matrix metalloproteinases (MMPs), recruitment and proliferation of resident fibroblasts, and secretion of TGF-β and IL-6 resulting in the development of fibrosis (63).

Collagen Remodeling and Calcification Process in RHVD

More recent studies have proposed that the immune response in RHVD may not be merely related to molecular mimicry or failure of the immune system, but rather associated with collagen autoimmunity as proposed for Goodpasture and Alport syndromes (64). In these diseases, the production of autoantibodies against basement-membrane collagen (type IV) on host endothelium is the triggering step of pathological processes. In Streptococci infection, M protein binds to CB3 region of collagen IV leading to the formation of a complex that promotes conformational changes in collagen structure initiating an anti-collagen response (65–67). Thus, a ubiquitous protein can become a self-antigen that contributes to imbalance between collagen deposition and collagen degradation, culminating in subsequent fibrosis of the valve apparatus in the RHVD.

Mitral valves of rheumatic patients have a higher deposition of collagens Type I and Type III, evidence of fibrosis, when compared to non-rheumatic mitral valve controls (**Figures 2A–C**) (68). Among numerous cytokines involved in the inflammatory process in RHVD, the high expression of TGF-β has been shown to be positively associated with valvular fibrosis (69) by contributing to myofibroblast activation and collagen production (39).

MMPs are a major group of proteases that regulate matrix remodeling during fibrogenic process accompanying chronic inflammation. MMP-1 has a high affinity to fibrillar collagens and is able to initiate collagenolysis. High concentrations of MMP-1 in patients' plasma, and gene polymorphism are contributing risk factors for RHVD (68, 70). The majorities of studies addressing the role of MMPs were performed in human plasma and myocardium tissues during acute episodes of rheumatic fever and are scarce in the chronic disease phase, particularly in valves (71–73).

Proinflammatory MMPs also play a role in the modulation of calcification by elastin degradation. Exposure of elastin and matrix-bound cytokine generation after tissue injury create a milieu, which promotes the smooth muscle cell changes into osteoblastic phenotype (74, 75). Calcification is a very common finding in rheumatic mitral valves, however, the cellular mechanisms responsible for the calcification in RHVD are not well-understood (**Figure 3B**). Previous study reported that mineralization occurs in areas of inflammation and neoangiogenesis, which express vascular endothelial growth factor (VEGF) (15). This molecule is able to regulate bone remodeling by attracting endothelial cells and by stimulating osteoblast differentiation (76). Another potential mechanism involved in mitral valve mineralization in RHVD is through calcification-competent extracellular vesicles derived from smooth muscle cells, VICs or macrophages (77–79). Calcification in RHVD seems to be triggered by inflammatory process as observed in CAVD (2, 22).

Together, the immune response triggered by pharyngeal GAS infection results in a cascade of cellular and humoral events that culminate in the production of antibodies and generate self-reactive clones of lymphocytes capable of interacting with valve components leading to leaflet tissue degeneration. Schematic presentation of the mechanisms of RHVD pathogenesis is shown in **Figure 3**.

Neoangiogenesis in RHVD

Neoangiogenesis is a common feature of HVD acting as a facilitator of inflammation by allowing the entry of immune cells and soluble inflammatory factors into the valvular tissue. Besides, vascular networks promote the weakening of valve tissue by changing the normal architecture (80, 81). A variety of growth factors can regulate angiogenesis, including vascular endothelial growth factor (VEGF)-A and MMPs that degrade fibrillar collagen or proteoglycan proteins allowing endothelial cells sprouting vessels by migration (82). Thus, angiogenesis besides contributing to tissue remodeling also can compromise mechanical stability of extracellular matrix. For the first time, we shown that mitral valves from patients with RHVD show an abundant neovascular network characterized by accumulation of large immature vessels, which is lacking in CAVD tissue (**Figures 4A,B**).

Besides blood vessels, lymphatic vasculature network was also observed in heart valves from patients with end stage RHVD as detected by colocalization of lymphatic endothelial cell receptor (LYVE-1) and podoplanin well-known markers of lymphatic endothelial cells (83) (**Figures 4C,D**). These vessels play role in lymph transport, tissue interstitial fluid absorption and serve as an entrance point for immune cells, favorable for optimal tissue function. and homeostasis (84). Autopsy results of adult heart valves show that lymphatic vessels in pathologic conditions such as RHVD present in a high (83). It is likely that neolymphogenesis is beneficial in the early phases of disease to maintain tissue homeostasis, but if become uncontrolled during the disease progression, may lead to pathological maladaptation. Persistent local inflammation impairs lymphatic contraction causing altered fluid transport (85). In addition, accumulation of collagen and fibrogenic molecules produced by smooth muscle cells and fibroblasts into the perilymphatic space causes capillary fibrosis and impairs vascular function (86). Thus, although lymphatic vessels are accumulating in lesions in large numbers, they are not able to exert their function properly.

In addition to growth factors and cytokines, hormones also participate in regulating angiogenesis. Estradiol influences hyaluronic acid synthesis, a major ECM ligand for the LYVE-1, suggesting an indirect effect on lymphatic homeostasis (87, 88). Also, while estrogen is described to reduce cardiovascular risk in women, it has been reported as a risk factor for disorders of lymphatic vascular system. Recently it has been shown that estrogen receptor alpha (ERα) directly regulates lymphoangiogenic genes promoting lymphatic endothelial cell migration and sprouting (89).

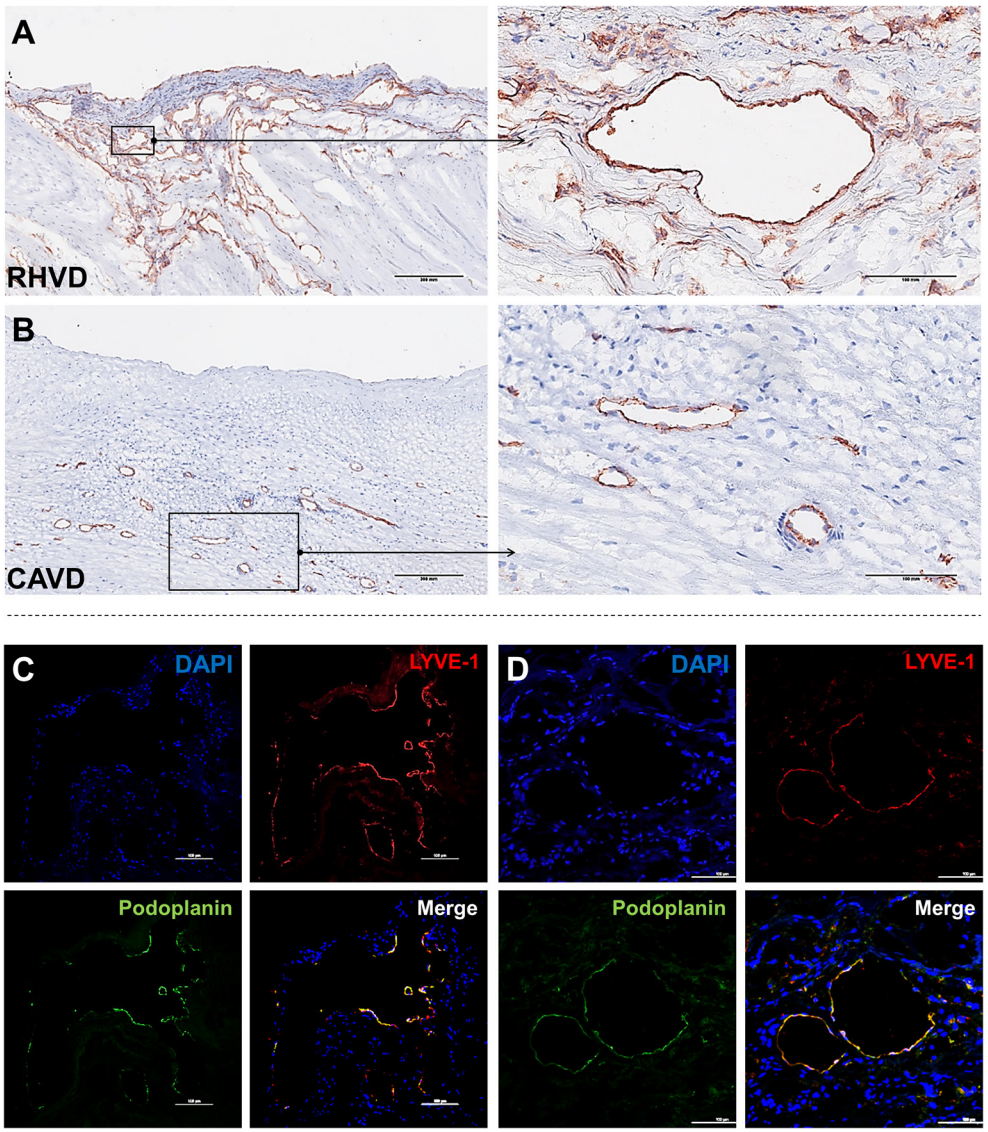

FIGURE 4 | Histological comparison between neoangiogenesis in rheumatic mitral valve and calcific aortic valve. **(A,B)** Representative immunohistochemistry image for CD31$^+$ staining evidencing presence of immature vessels in RHVD mitral valve and calcific aortic valve interstitium. Scale bars = 300 mm. **(C)** Representative immunofluorescence image showing cell co-expressing LYVE-1 and podoplanin demonstrating presence of lymphatic vessels in rheumatic mitral valves. Scale bars = 100 μm.

Thus, excessive neolymphoangiogenesis observed in rheumatic mitral valves obtained predominantly from female patients, could be induced by estrogen which in turn can aggravate vessel sprouting in association with proinflammatory milieu.

FINAL CONSIDERATIONS

No therapies are available to prevent or treat RHVD. Antibiotic prophylaxis is given to prevent repetitive episodes of ARF, and potentially limit the disease progression to severe valve dysfunction. However, there is no robust evidence of efficacy of secondary antibiotic prophylaxis in preventing recurrences of ARF (9). Additionally, there is no treatment to alter the likelihood or the severity of RHVD after an episode of ARF (8). Corticosteroids or intravenous immunoglobulins were tested in clinical trials to reduce the risk of heart valve lesions in patients with ARF, however, little evidence of benefit was found (90). Since RHVD results from an abnormal immune response, therapies targeting immune system could be more effective to avert valvular damage. Therefore, more research is needed to find specific immune components associated with the RHVD pathogenesis that will provide a more precise and effective therapeutical interventions to treat this devastating condition.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

EA was supported by National Institutes of Health (NIH) grants R01HL 136431, R01HL 141917, and R01HL 147095.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cardiovascular Medicine

BRIEF RESEARCH REPORT
published: 03 February 2021
doi: 10.3389/fcvm.2020.604826

Decreased Cytokine Plasma Levels and Changes in T-Cell Activation Are Associated With Hemodynamic Improvement and Clinical Outcomes After Percutaneous Mitral Commissurotomy in Patients With Rheumatic Mitral Stenosis

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Specialty section:
This article was submitted to Heart Valve Disease, a section of the journal Frontiers in Cardiovascular Medicine

Received: 10 September 2020
Accepted: 18 November 2020
Published: 03 February 2021

Citation:
Silva VR, Neves EGA, Passos LSA, Cristina de Melo F, Teixeira-Carvalho A, Nassif MCL, Junqueira LL, Aikawa E, Dutra WO and Nunes MCP (2021) Decreased Cytokine Plasma Levels and Changes in T-Cell Activation Are Associated With Hemodynamic Improvement and Clinical Outcomes After Percutaneous Mitral Commissurotomy in Patients With Rheumatic Mitral Stenosis. Front. Cardiovasc. Med. 7:604826. doi: 10.3389/fcvm.2020.604826

Mitral stenosis (MS) is a consequence of rheumatic heart disease that leads to heart failure requiring mechanical intervention. Percutaneous mitral commissurotomy (PMC) is the treatment of choice for the intervention, and currently there are no soluble markers associated with hemodynamic improvement after PMC. This study aims to determine the changes in cytokine/chemokine plasma levels, as well as T cell activation after PMC, and to investigate their association with immediate hemodynamic improvement and clinical outcomes. Plasma samples from eighteen patients with well-defined MS who underwent PMC and 12 healthy controls were analyzed using BioPlex immunoassay. We observed that 16 out of the 27 (60%) molecules assessed were altered in patients' plasma pre-PMC as compared to control group. Of those, IL-1β, IL-12, IL-6, IL-4, PDGF, and CCL11 showed significant decrease after PMC. Stratifying the patients according to adverse outcome after a 28-month median follow up, we detected a significant reduction of IL-1β, IL-12, IL-6, IL-4, IFN-γ, CXCL-10, VEGF, FGF and PDGF post-PMC in patients without events, but not in those who presented adverse events during the follow-up. Patients with adverse outcomes had lower IL-10 pre-PMC, as compared to the ones without adverse events. In addition, the frequency of CD8+ activated memory cells was increased after PMC, while the frequency of CD4+ activated memory cells did not

change. Our results show an association between the decrease of specific cytokines and changes in T cell activation with hemodynamic improvement post-PMC, as well as with long-term outcomes, suggesting their possible use as soluble markers for hemodynamic recovery after MS intervention.

Keywords: rheumatic heart disease, mitral stenosis, percutaneous mitral commissurotomy, cytokines, T cells

INTRODUCTION

Rheumatic heart disease (RHD) is the major sequel of acute rheumatic fever (ARF) and is an important cause of cardiovascular mortality in children and young adults in low- and middle-income countries. There are an estimated 33 million individuals currently living with RHD, accounting for over a million premature deaths annually (1). We have demonstrated that patients with RHD have higher serum levels of inflammatory mediators than healthy controls, which is a clear evidence of ongoing inflammation (2–4). It is also known that expression of inflammatory cytokines increases in patients with significant pressure or volume overload due to valvular heart disease or chronic heart failure (5–7). Several studies have demonstrated that T-cells play an important role in the pathogenesis of RHD. It has been shown that CD4+ T cells are the main cell type found in the inflammatory infiltrates associated with damaged valves (5–7), suggesting that they play an important role in disease progression. On the other hand, a defective response of CD8+ T cells in chronic RHD patients suggests that these cells display an important immunoregulatory role in protection against RHD (8).

RHD is the major cause of mitral stenosis (MS) which is a progressive and fatal disease, if untreated. Percutaneous mitral commissurotomy (PMC) intervention is the main treatment in patients with significant MS (8–10). While it has been demonstrated that PMC decreases platelet activation and endothelial dysfunction, the alterations in plasma levels of inflammatory markers have been controversial (11–13). Currently there are no systemic markers that allow monitoring hemodynamic improvement, nor predict outcomes after PMC. We aimed to evaluate the plasma levels of cytokines pre- and post-PMC using a multiplex assay, as well as the frequency of T-cell activation using flow cytometry, in a well-defined group of severe MS patients to determine the impact of these changes on immediate results after PMC and long-term outcomes. Our results show a clear association between hemodynamic improvement and favorable outcome with a decrease in the circulating levels of IL-1β, IL-6, IL-12, IL-4 and PDGF post-PMC, as well as with changes in T-cell activation, suggesting that these measures might be useful to monitor PMC-induced hemodynamic improvement and clinical outcomes.

PATIENTS, MATERIALS AND METHODS

Patients

We included 18 consecutive symptomatic patients with severe rheumatic MS (mitral valve area $\leq$1.5 cm^2) and favorable valve morphology who underwent PMC between October 17th−27th 2016 at the Federal University of Minas Gerais (UFMG) Hospital. Exclusion criteria were presence of left atrial thrombus, moderate-to-severe mitral regurgitation, concomitant severe aortic valve disease or any other contraindication to PMC. The clinical characteristics of the study population are summarized in **Appendix 1**. At the time of enrollment, patients had an average age of 49 $\pm$ 2 years, and 89% were females.

Echocardiographic examination was performed in all patients before and 24 h after the procedure. PMC was performed according to the antegrade, trans-septal technique with Inoue balloon catheter (Toray Medical Corporation; Tokyo, Japan).

The control group was composed of 12 healthy volunteers. Peripheral blood samples were collected immediately before PMC, and 4–11 days after the procedure. The date of enrollment in the study was defined as the date on which PMC was performed. Adverse outcome was defined as the need for mitral valve intervention, either percutaneous or surgical, death related to MS, or new onset of atrial fibrillation. Patients with event-free during the follow-up were considered with favorable outcome. Informed consent was obtained from all participants and the UFMG ethical review board approved the protocol. The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

Measurement of Plasma Cytokines, Chemokines, and Growth Factors

Plasma concentrations of cytokines/chemokines/growth factors were measured using Luminex immunoassay kit (Bio-Plex Pro™ Human Cytokine 27-plex Assay, Bio-Rad, Hercules—CA, USA). Results were presented as mean fluorescence intensity (MFI).

Analysis of Frequency of T-cell Activation by Flow Cytometry

Purification of peripheral blood mononuclear cells (PBMC) and immunofluorescence staining was performed as previously described (14). Briefly, heparinized blood was applied over a Ficoll-Hypaque (GE Healthcare Life Sciences) gradient, centrifuged at 600 g for 40 min, at room temperature, and PBMC were collected at the interface between the plasma and Ficoll. Cells were washed 3 times by centrifugation with PBS and resuspended in PBS at a concentration of 10^7 cells/ml. Cells were plated at 2 $\times$ 10^5 cells/well in a 96 U-bottom plate, incubated with a 40 μL mix of monoclonal antibodies CD4-Percp-Cy5.5 (clone OKT4, 1:20 dilution), anti-CD8-APCCy7 (clone SK1, 1:20 dilution), anti-CD45RO-APC (clone UCHL1, 1:40 dilution) and anti-CD69-PECy7 (clone FN50, 1:20 dilution) for 15 min at 4°C, washed with PBS, and fixed for 20 min with 2% formaldehyde.

Samples were acquired on the FACS CANTO II and analyzed using FlowJo software (Tree Star, Ashland, OR, USA). Gating strategy was performed selecting the lymphocyte population in a forward vs. side scater graph (**Figure 3A**). Further gating on CD4+ (**Figure 3B**) or CD8+ cells (**Figure 3C**) were performed, and the expression of CD69 and CD45RO were concomitantly evaluated in the gated CD4+ and CD8+ cells, to determine the frequency of activated cells (**Figures 3B,C** for CD4 and CD8, respectively). All antibodies were obtained from BioLegend (San Diego, CA, USA).

Gene Co-expression Network for Human Immune System Cell Types

Co-expression network was constructed through direct and indirect relationships of CD8+ T cells and plasma soluble factors measured by Luminex immunoassay. Pairwise correlation prediction was determined based on Immuno-Navigator (sysimm.ifrec.osaka-u.ac.jp/immuno-navigator/) and network constructed using NetworkAnalyst.ca. Resulting high-scoring genes that display high correlation of expression in T CD8-derived genes were used to identify hub genes and functional enrichment pathways. Regulatory interactions emerging as a result of interconnections in the network were generated using Kyoto Encyclopedia of Genes and Genomes (KEGG).

Statistical Analysis

The sample size was performed to achieve 80% power for detecting a reduction of up to 30% in IL-6 concentration after PMC with a two-sided type-I error rate of 5% (2). Statistical analyses were performed using SPSS™ software version 25 for Windows (SPSS Inc., Chicago—IL, USA) and GraphPad Prism™ version 7.00 for Windows (GraphPad Software, La Jolla—CA, USA). Continuous variables were expressed as mean ± standard deviation (SD) or as median and interquartile range when appropriate. Categorical variables were expressed as frequency and percentage. We used the Mann-Whitney test to access differences between study group and control group. Wilcoxon signed-rank test was used to evaluate differences within the study group pre and post-PMC. A two-tailed *P*-value of <0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

To assess the hemodynamic improvement after PMC, valve area, transmitral gradient, left atrial pressure and pulmonary pressure were measured before and immediately after the procedure. After PMC, mitral valve are increased from 1.06 ± 0.2 to 1.90 ± 0.3 cm² ($p = 0.001$) (**Figure 1**), and transmitral mean gradient decrease from 9.7 ± 4.3 to 4.6 ± 1.2 mmHg ($p = 0.009$). Similarly, left atrial pressure invasively measured decrease from 19.6 ± 4.4 to 17.1 ± 4.3 mmHg ($p = 0.007$), and mean pulmonary pressure decrease from 23.2 ± 3.9 to 21.2 ± 5.2 mmHg ($p = 0.007$).

Plasma levels of soluble molecules in control group and in MS group pre- and post-PMC are presented in **Appendix 2–4**.

First we compared the levels of growth factors, chemokines and cytokines between control group and MS patients pre-PMC (**Appendix 2**). We observed that there were no significant differences in levels of growth factors (GM-CSF, G-CSF, VEGF, FGF) and proliferative cytokines (IL-2, IL-7, IL-15) among MS group and control group, except for PDGF, that was significantly higher in patients pre-PMC (**Appendix 2**). We attribute de absence of difference in levels of growth factors and proliferative factors among patients and controls to the late chronic stage of the disease. Levels of all chemokines measured were higher in MS patients pre-PMC than control group, possibly related to ongoing inflammation (**Appendix 2**). Levels of TNF-α, IL-10, IL-5, and IL-9 did not change comparing both groups (**Appendix 2**). However, levels of IL-1β, IL-1RA, IL-4, IFN-γ, and IL-17 were higher in MS pre-PMC compared to control group (**Appendix 2**). The higher levels of inflammatory cytokines IL-1β, IL-17, and IFN-γ in MS compared to control group are suggestive of the ongoing inflammation. In particular, IL-17 is a potent cytokine related to autoimmune diseases, and previously associated with severity of RHD (12). The high levels of down-regulatory cytokines IL-1RA and IL-4 suggest that these cytokines may attempt to counterbalance inflammation in this

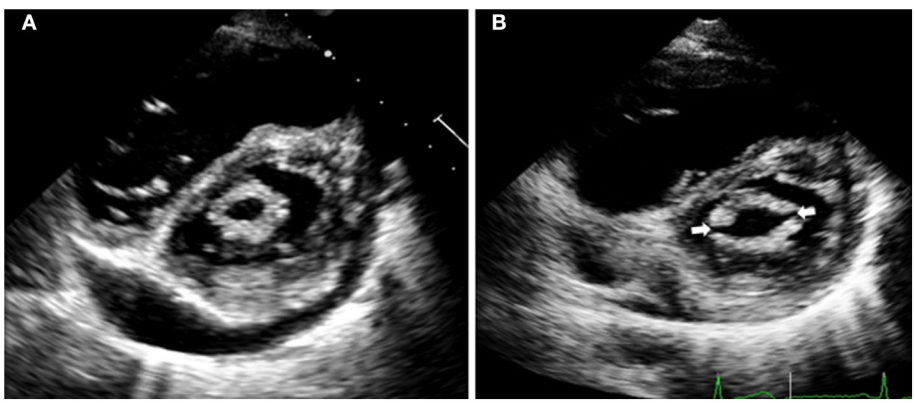


FIGURE 1 | Echocardiographic images of a patient with severe mitral stenosis before percutaneous mitral commissurotomy **(A)**. After the procedure **(B)**, both commissures are open (arrows), which increases the mitral valve area resulting in immediate hemodynamic improvement.

phase of disease. Interestingly, levels of IL-12p70, IL-13 and IL-6 were lower in MS group as compared to controls (**Appendix 2**).

We then compared the levels of circulating growth factors, chemokines and cytokines between MS patients pre-PMC and post-PMC (**Appendix 3**). Comparing levels of growth factors and proliferative cytokines in patients pre- and post-PMC, we demonstrated that while the levels of GM-CSF and G-CSF did not change (**Appendix 3**), a decrease in plasma levels of PDGF, VEGF, FGF basic, IL-2, IL-7 and IL-15 was observed post-PMC (**Appendix 3**), probably due to hemodynamic improvement. Of note, levels of VEGF were negatively correlated with mitral valve area ($p = 0.052$, $r^2 = 0.03$). Rajamannan et al. (11) hypothesized that the mechanism of valvular calcification included neoangiogenesis in valve tissue and demonstrated the presence of VEGF in rheumatic valves. Evaluating the levels of chemokines, only CCL11 decrease in patients post-PMC as compared to pre-PMC (**Appendix 3**). Given CCL11 is involved in recruitment of polymorphonuclear cells, especially neutrophils, which are the first cells to respond in inflammatory settings, it is reasonable to think that the hemodynamic improvement due to adequate relief of valve obstruction may have a prompt neutrophil response. In addition, given the observed decrease in CCL11, IL-4 and IL-5 post-PMC, it is possible to hypothesize that PMC decreases activation of polymorphonuclear cells, high producers of these cytokines. Indeed, there were strong correlations between levels of CCL11 and both IL-4 ($r = 0.87$) and IL-5 ($r = 0.88$). Evaluating the levels of cytokines, we observed that the levels of mostly T cell-derived inflammatory cytokines (IL-17, IFN-γ) did not change comparing post- and pre-PMC (**Appendix 3**). Post-PMC, levels of IL-1β, IL-12p70, IL-6, IL-4, and IL-5 had a significant decrease, as compared to pre-PMC (**Appendix 3**). Consistent with our findings, previous studies have demonstrated that levels of IL-6 and TNF-α decrease in this phase of the disease (13, 14). Of note, the levels of IL-12, IL-1β, and IL-6, inflammatory cytokines produced primarily by monocytes/macrophages and related to innate response, were decreased. Improvement of hemodynamic parameters after PMC may be responsible for this decrease, and the improvement might interfere first with innate immune response and later in adaptive response. We expected a decrease in levels of TNF-α and IFN-γ, as demonstrated by Cagli et al., who showed that TNF-α progressively decreased at the 24th h until the 4th week after PMC (15). However, Cagli *et al* evaluated only patients in sinus rhythm and with no other comorbidity; therefore, it is possible that, in our group, the levels of TNF-α follow a different kinetics than the one observed by those authors. The fact that levels of down-regulatory cytokines IL-10 and IL-1RA did not change after PMC (**Appendix 3**) is probably associated with the reduction in inflammation observed post-PMC. The lower levels of IL-10 pre-PMC were associated with adverse outcomes (No adverse outcome IL-10 pre-PMC = 16, and adverse outcome IL-10 pre-PMC = 14; $p = 0.04$), emphasizing the role of IL-10 in controlling inflammation and possibly preventing restenosis over time.

Finally, we then compared the levels of growth factors, chemokines and cytokines between control group and MS patients post-PMC (**Appendix 4**). PDGF was the only molecule among growth factors and proliferative cytokines that was higher in patients pre-PMC compared to controls. After PMC, although its levels have fallen, they were still higher compared to controls (**Appendix 4**). On the other hand, while all the chemokines presented higher plasma levels in patients pre-PMC compared to controls and only CCL11 had a significant decrease in plasma

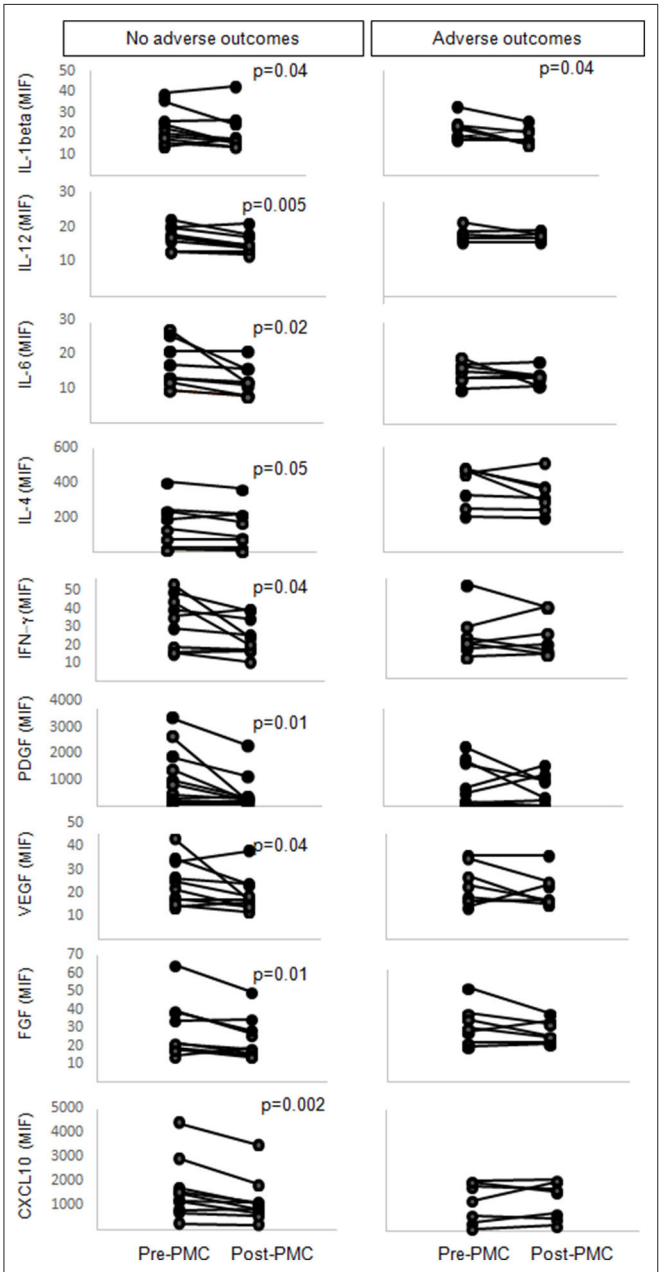
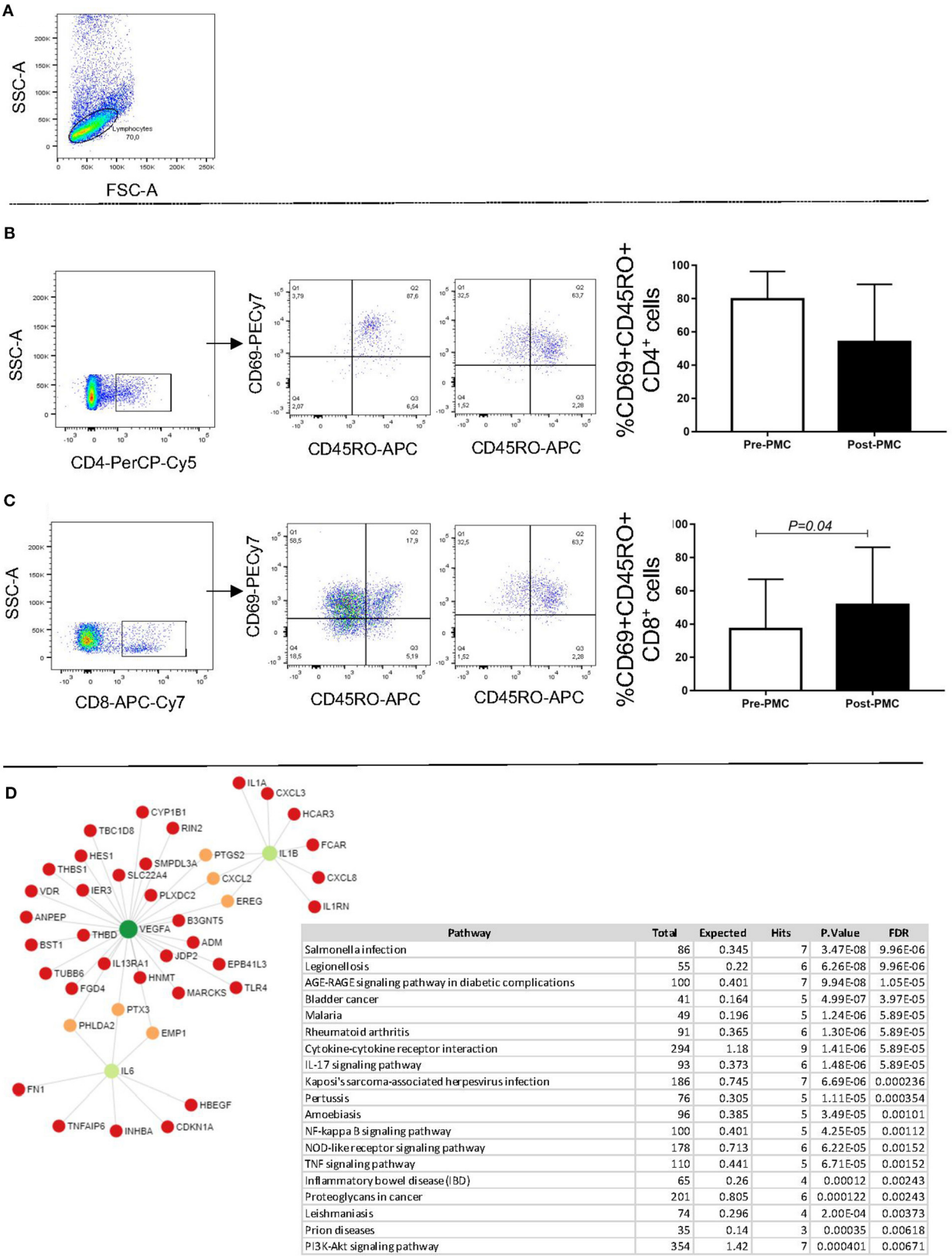


FIGURE 2 | Levels of IL-1β, IL-12, IL-6, IL-4, IFN-γ, PDGF, VEGF, FGF-basic and CXCL10 in plasma from mitral stenosis patients pre- and post-percutaneous mitral commissurotomy (PMC). Patients were followed-up for a median of 28 months and categorized in groups without or with adverse outcomes. Statistically significant differences between levels of soluble factors pre- and post-PMC are indicated.



Pathway	Total	Expected	Hits	P.Value	FDR
Salmonella infection	86	0.345	7	3.47E-08	9.96E-06
Legionellosis	55	0.22	6	6.26E-08	9.96E-06
AGE-RAGE signaling pathway in diabetic complications	100	0.401	7	9.94E-08	1.05E-05
Bladder cancer	41	0.164	5	4.99E-07	3.97E-05
Malaria	49	0.196	5	1.24E-06	5.89E-05
Rheumatoid arthritis	91	0.365	6	1.30E-06	5.89E-05
Cytokine-cytokine receptor interaction	294	1.18	9	1.41E-06	5.89E-05
IL-17 signaling pathway	93	0.373	6	1.48E-06	5.89E-05
Kaposi's sarcoma-associated herpesvirus infection	186	0.745	7	6.69E-06	0.000236
Pertussis	76	0.305	5	1.11E-05	0.000354
Amoebiasis	96	0.385	5	3.49E-05	0.00101
NF-kappa B signaling pathway	100	0.401	5	4.25E-05	0.00112
NOD-like receptor signaling pathway	178	0.713	6	6.22E-05	0.00152
TNF signaling pathway	110	0.441	5	6.71E-05	0.00152
Inflammatory bowel disease (IBD)	65	0.26	4	0.00012	0.00243
Proteoglycans in cancer	201	0.805	6	0.000122	0.00243
Leishmaniasis	74	0.296	4	2.00E-04	0.00373
Prion diseases	35	0.14	3	0.00035	0.00618
PI3K-Akt signaling pathway	354	1.42	7	0.000401	0.00671

FIGURE 3 | Analysis of the percentage of circulating CD4+CD45RO+CD69+ and CD8+ CD45RO+CD69+ T cells pre and post PMC, and network interactions. PBMC were obtained from the patients before and after PMC, and processed for flow cytometry analysis, as described in Material and Methods. **(A)** Shows a

(Continued)

FIGURE 3 | representative dot plot with the selection of the lymphocyte gate. **(B)** Shows the selection of CD4+ T cells, representative dot lots showing the frequency of CD69+CD45RO+ cells, and bar graphs for the analysis before (white bars) and after (black bars) PMC. **(C)** Shows the selection of CD8+ T cells, representative dot lots showing the frequency of CD69+CD45RO+ cells, and bar graphs for the analysis before (white bars) and after (black bars) PMC. Data are expressed as average and standard deviation, and statistical significance is indicated by the *p*-value. **(D)** Co-expression network of soluble factors overrepresented in plasma from patients without adverse outcomes in a background of T CD8-derived genes. KEEG enriched pathways analysis obtained from gene co-expression network.

levels post-PMC, the levels of CXCL10, CCL11, and IL-8 were not statistically different from controls (**Appendix 4**). IL-5 and IL-1β, that presented a significant decrease after PMC, demonstrated levels post-PMC statistically not different from controls, but IL-4, that also demonstrated significant decrease after PMC, still presented higher levels than controls (**Appendix 4**). Levels of IL-17, IFN-γ, and IL-1RA continued statistically higher post-PMC compared to controls (**Appendix 4**). IL-10 levels were not different from controls neither pre nor post-PMC (**Appendix 4**). Interesting, levels of TNF-α, IL-9, IL-6, IL-12p70, and IL-13 were lower in patients post-PMC compared to controls (**Appendix 4**).

We then stratified the patients according to clinical outcomes at long-term follow-up and compared the levels of soluble factors pre- and post-PMC in those groups. During a median follow-up of 28 months (range, 4–40), 10 patients (55.5%) were clinically stable, asymptomatic, whereas 8 (44.4%) patients presented adverse outcomes, including five mitral valve replacements, one repeat PMC, and two onset of atrial fibrillation. The analysis showed that levels of IL-1β decreased in patients with and without adverse outcomes (**Figure 2**), while levels of IL-12, IL-6, IL-4, IFN-γ, PDGF, VEGF, FGF, CXCL10 were decreased only in patients who did not display adverse events, post-PMC as compared to pre-PMC (**Figure 2**). This decrease was not significant in patients with adverse events (**Figure 2**). All these molecules, except for CXCL-10, presented decreased levels post-PMC, even before considering PMC outcome after follow-up, reinforcing their possible usefulness as markers of PMC-induced hemodynamic improvement and favorable outcome.

In order to determine the influence of PMC on T cell activation, we evaluated the expression of CD69 by CD4+CD45RO+ and CD8+CD45RO+ T cells. The expression of CD45RO is associated with pre-activated/memory cells (16). Our data showed that, while not statistically significant, there was a slight reduction in the frequency of CD4+CD45RO+CD69+ cells post-PMC (**Figure 3**). On the other hand, the percentage of CD8+CD45RO+CD69+ cells significantly increased after PMC (**Figure 3**). The observed increase in a pre-activated/memory CD8+ T cell population, previously associated with immunoregulatory functions in RHD (8), together with a tendency to a reduction in the frequency of memory/activated CD4+ cells associated with pathology in RHD (5–7), might reflect the clinical amelioration after PMC. Further studies to determine the functional characteristics of these cells before and after PMC will provide important additional information on their role in MS.

To extend the findings, we then turned to the Immuno-Navigator database to verify co-expression between T CD8-derived genes and the nine altered soluble factors in patients without adverse outcomes. VEGF, IL-1beta and IL-6 were the highly connected genes in a T CD8 co-expression network and therefore might be more relevant to the functionality than other nodes in the network. However, the network analysis shows that VEGF, IL-1β and IL-6 display direct influence in several other genes. Thus, the observed decrease in the levels of these factors after successful PMC will also interfere with the overall inflammatory response. The top 20 KEGG enrichment analyses identified common pathways of infectious diseases, as well as an autoimmune disease, rheumatoid arthritis. Among all enriched inflammatory pathways, we found AGE-RAGE, IL-17, NF-kappa B, NOD-like receptor and TNF signaling pathways, which are under the influence of the three identified hub genes. This entices future studies to further investigate the role of these node molecules as biomarkers and/or potential intervention candidates.

PMC is a safe and effective procedure for treating patients with MS, which results in the alleviation of valve obstruction with hemodynamic and symptoms improvement. Despite its wide use, there are currently no soluble markers to evaluate PMC-induced hemodynamic improvement, nor clinical outcomes. Using cytokine levels to assess outcome of percutaneous coronary intervention has shown promising results (17). Our study is the first to evaluate the impact of PMC on plasma levels of a broader range of cytokines, chemokines and growth factors, together with the frequency of activated circulating T cell subpopulations. Importantly, we showed a clear association between the levels of particular circulating molecules with hemodynamic improvement immediately after PMC, as well as with clinical outcomes. These data suggest useful immunologic prognostic markers of amelioration after PMC and at long term follow up.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Federal University of Minas Gerais UFMG - Ethical Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

VS: patient care, procedure, and follow up and data analysis and writing. EN: experimentation and data preparation. LP: data analysis and methodology. FC: material collection and experimentation. AT-C: supervision of experimentation. MN:

patient care and follow up. LJ: patient care and procedure. EA: bioinformatic analysis and critical revision of the manuscript. WD: conceptualization, data analysis and interpretation, and writing. MN: conceptualization, data analysis and interpretation, patient care, and writing. All authors contributed to the article and approved the submitted version.

FUNDING

This work was partly funded by grants from CNPq (National Council for Scientific and Technological Development). LP was a CNPq fellow at the time of the work and EN, AT-C, WD, and MN are CNPq fellows. EA lab was funded by the National Institutes of Health grants (R01HL136431, R01HL147095, R01HL141917). WD lab is funded by NIH (RO1 AI138230).

ACKNOWLEDGMENTS

The authors would like to thank the medical students of the Federal University of Minas Gerais for their assistance in patient selection.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

APPENDIX

Appendix 1 | Baseline characteristics of the study population.

Characteristics	Mitral stenosis group ($n = 18$)	Control group ($n = 12$)
Age (years)	49.28 ± 2.45	17.25 ± 0.13
Female sex, n (%)	16 (88.9%)	4 (33.3%)
Age at diagnostic (years)	35.6 ± 3.28	–
Functional class NYHA:		
I and II, *n* (%)	12 (66.7%)	–
III and IV, *n* (%)	6 (33.3%)	–
Wilkins score	7 (7–8)	–
Atrial fibrillation, *n* (%)	8 (44.4%)	–
Prior embolic events, *n* (%)	2 (11.1%)	–
Heart failure at admission, *n* (%)	9 (50.0%)	–
Diabetes mellitus, *n* (%)	4 (22.2%)	–
Hypertension, *n* (%)	8 (44.4%)	–
Prior valve intervention, *n* (%)	5 (27.8%)	–
Diuretics, *n* (%)	14 (77.8%)	–
Beta-blockers, *n* (%)	14 (77.8%)	–
Digital, *n* (%)	2 (11.1%)	–
Warfarin, *n* (%)	6 (33.3%)	–
Aspirin, *n* (%)	2 (11.1%)	–
Antibiotic prophylaxis		
Current, *n* (%)	4 (22.2%)	–
Previous, *n* (%)	10 (55.6%)	–

Data are presented as mean ± SD or absolute numbers (percentage).
MS, mitral stenosis; NYHA, New York Heart Association.

Appendix 2 | Levels of soluble factors in control group and study group pre-PMC.

Variables	Control group	Pre-PMC group	*P*-value
Growth factors			
GM-CSF	18 (15–19)	17 (13–21)	0.55
G-CSF	33 (30–42)	51 (33–77)	0.10
VEGF	22 (21–27)	23 (17–34)	0.84
FGF basic	20 (18–21)	24 (19–37)	0.10
PDGF	**124 (50–193)**	**820 (199–1,810)**	**0.001**
Proliferative factors			
IL-2	16 (15–17)	17 (13-23)	0.53
IL-7	23 (22–24)	22 (20–23)	0.57
IL-15	24 (23–26)	20 (19–27)	0.11
Chemokines			
CXCL10	**685 (365–1,104)**	**1,280 (690–1,971)**	**0.03**
CCL2	**45 (24–54)**	**171 (69–357)**	**<0.001**
CCL3	**33 (25–46)**	**375 (98–6,394)**	**<0.001**
CCL4	**329 (318–441)**	**1,265 (736–2,304)**	**<0.001**
CCL11	**520 (454–925)**	**2,533 (448–3,614)**	**0.03**
CCL5	**1,662 (1,427–1,824)**	**2,574 (1,738–2,995)**	**0.02**
IL-8	**28 (26–36)**	**65 (33–467)**	**0.01**
Cytokines			
TNF-α	28 (24–37)	22 (16–31)	0.13
IL-10	18 (16–22)	16 (13–22)	0.09
IL-5	16 (14–20)	18 (14–24)	0.37
IL-9	47 (43–49)	42 (34–49)	0.18
IL-1 β	**17 (16–18)**	**23 (18–26)**	**0.01**
IL-6	**19 (18–20)**	**13 (13–18)**	**0.02**
IL-12p70	**18 (17–25)**	**16 (14–18)**	**0.01**
IL-17	**26 (24–29)**	**62 (29–93)**	**<0.001**
IFN-γ	**16 (13–16)**	**23 (17–42)**	**<0.001**
IL-1ra	**21 (21–25)**	**270 (155–1,057)**	**<0.001**
IL-4	**29 (23–34)**	**136 (49–178)**	**0.003**
IL-13	**19 (17–19)**	**15 (13–18)**	**0.01**

Data are presented as median and interquartile range (Q1–Q3). PMC, percutaneous mitral commissurotomy. Bold values represent statistically significant differences.

Appendix 3 | Levels of soluble factors in study group pre and post-PMC.

Variables	Pre-PMC group	Post-PMC group	*P*-value
Growth factors			
GM-CSF	17 (13–21)	14 (12–18)	0.06
G-CSF	51 (33–77)	39 (31–56)	0.14
VEGF	**23 (17–34)**	**17 (16–24)**	**0.03**
FGF basic	**24 (19–37)**	**22 (17–29)**	**0.01**
PDGF	**820 (199–1,810)**	**314 (210–1,033)**	**0.02**
Proliferative factors			
IL-2	**17 (13–23)**	**14 (13–19)**	**0.01**
IL-7	**22 (20–23)**	**19 (18–23)**	**0.03**
IL-15	**20 (19–27)**	**20 (18–27)**	**0.03**
Chemokines			
CXCL10	1,280 (690–1,971)	1,088 (625–1,815)	0.05
CCL2	171 (69–357)	127 (48–324)	0.20
CCL3	375 (98–6,394)	212 (83–1,513)	0.06
CCL4	1,265 (736–2,304)	1,018 (576–1,586)	0.16
CCL11	**2,533 (448–3,614)**	**1,966 (301–2,971)**	**0.01**
CCL5	2,574 (1,738–2,995)	2,377 (1,732–3,004)	0.40
IL-8	65 (33–467)	29 (25–58)	0.14
Cytokines			
TNF-α	22 (16–31)	19 (14–27)	0.10
IL-10	16 (13–22)	14 (12–24)	0.88
IL-5	**18 (14–24)**	**15 (13–20)**	**0.01**
IL-9	42 (34–49)	35 (30–40)	0.10
IL-1 β	**23 (18–26)**	**17 (16–24)**	**0.02**
IL-6	**13 (13–18)**	**12 (11–15)**	**0.004**
IL-12p70	**16 (14–18)**	**14 (14–16)**	**0.01**
IL-17	62 (29–93)	40 (31–73)	0.06
IFN-γ	23 (17–42)	21 (17–37)	0.13
IL-1ra	270 (155–1,057)	282 (143–654)	0.31
IL-4	**136 (49–178)**	**99 (49–176)**	**0.01**
IL-13	15 (13–18)	14 (13–16)	0.09

Data are presented as median and interquartile range (Q1–Q3). PMC, percutaneous mitral commissurotomy. Bold values represent statistically significant differences.

Appendix 4 | Levels of soluble factors in control group and study group post-PMC.

Variables	Control group	Post-PMC group	*P*-value
Growth factors			
GM-CSF	**18 (15–19)**	**14 (12–18)**	**0.02**
G-CSF	33 (30–42)	39 (31–56)	0.40
VEGF	**22 (21–27)**	**17 (16–24)**	**0.03**
FGF basic	20 (18–21)	22 (17–29)	0.56
PDGF	**124 (50–193)**	**314 (210–1,033)**	**0.002**
Proliferative factors			
IL-2	16 (15–17)	14 (13–19)	0.09
IL-7	**23 (22–24)**	**19 (18–23)**	**0.03**
IL-15	**24 (23–26)**	**20 (18–27)**	**<0.001**
Chemokines			
CXCL10	685 (365–1,104)	1,088 (625–1,815)	0.10
CCL2	**45 (24–54)**	**127 (48–324)**	**0.002**
CCL3	**33 (25–46)**	**212 (83–1,513)**	**<0.001**
CCL4	**329 (318–441)**	**1,018 (576–1,586)**	**<0.001**
CCL11	520 (454–925)	1,966 (301–2,971)	0.06
CCL5	**1,662 (1,427–1,824)**	**2,377 (1,732–3,004)**	**0.04**
IL-8	28 (26–36)	29 (25–58)	0.50
Cytokines			
TNF-α	**28 (24–37)**	**19 (14–27)**	**0.01**
IL-10	18 (16–22)	14 (12–24)	0.08
IL-5	16 (14–20)	15 (13–20)	0.61
IL-9	**47 (43–49)**	**35 (30–40)**	**<0.001**
IL-1 β	17 (16–18)	17 (16–24)	0.72
IL-6	**19 (18–20)**	**12 (11–15)**	**<0.001**
IL-12p70	**18 (17–25)**	**14 (14–16)**	**0.01**
IL-17	**26 (24–29)**	**40 (31–73)**	**0.01**
IFN-γ	**16 (13–16)**	**21 (17–37)**	**0.001**
IL-1ra	**21 (21–25)**	**282 (143–654)**	**<0.001**
IL-4	**29 (23–34)**	**99 (49–176)**	**0.01**
IL-13	**19 (17–19)**	**14 (13–16)**	**<0.001**

Data are presented as median and interquartile range (Q1–Q3). PMC, percutaneous mitral commissurotomy. Bold values represent statistically significant differences.

frontiers in Cardiovascular Medicine

ORIGINAL RESEARCH
published: 12 February 2021
doi: 10.3389/fcvm.2021.632621

Determining the Risk of Developing Rheumatic Heart Disease Following a Negative Screening Echocardiogram

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Heart Valve Disease, a section of the journal Frontiers in Cardiovascular Medicine

Received: 23 November 2020
Accepted: 18 January 2021
Published: 12 February 2021

Citation:
Zimmerman M, Scheel A, DeWyer A, Nambogo J-L, Otim IO, Tompsett A, Rwebembera J, Okello E, Sable C and Beaton A (2021) Determining the Risk of Developing Rheumatic Heart Disease Following a Negative Screening Echocardiogram. Front. Cardiovasc. Med. 8:632621. doi: 10.3389/fcvm.2021.632621

Background: Screening echocardiograms can detect early-stage rheumatic heart disease (RHD), offering a chance to limit progression. Implementation of screening programs is challenging and requires further research. This is the first large-scale study assessing the risk of RHD among previous screen-negative children.

Methods: This retrospective cohort study, conducted in Gulu, Uganda, performed school-based echo screening on children ages 5–18 years. Surveys were used to determine which children underwent initial screening 3–5 years prior. Age, gender, and disease severity were compared between cohorts. Relative risk (RR) of RHD was calculated for those with a prior screen-negative echo (exposed cohort) compared to those undergoing first screening (unexposed cohort).

Results: Echo screening was completed in 75,708 children; 226 were excluded, leaving 1,582 in the exposed cohort and 73,900 in the unexposed cohort. Prevalence of new RHD was 0.6% (10/1,582) and 1% (737/73,900), in the exposed and unexposed cohorts, respectively. The RR of RHD was 0.64 (95% CI 0.3–1.2, $p = 0.15$), a nearly 40% reduced risk of RHD in those with a prior negative echo. There was no difference in age or gender between RHD cohorts. All cases in the exposed cohort were borderline/mild; 2.6% of cases in the unexposed cohort had moderate/severe disease.

Conclusion: There was no statistical difference in RHD prevalence between previous screen-negative children and children with no prior echocardiogram, however, there was a trend toward decreased risk and severity. This information has important implications for the design of screening programs and the use of screening echocardiograms in endemic RHD regions.

Keywords: rheumatic heart disease, pediatrics, echocardiography, screening, global health

INTRODUCTION

Rheumatic Heart Disease (RHD) is a common cause of cardiovascular morbidity and mortality in children and young adults globally. In 2017, the worldwide prevalence of RHD was over 39 million, with 249,000 RHD-related deaths (1, 2). In low-resource settings, clinical RHD is typically diagnosed at late stages of disease, most commonly with heart failure, resulting in high rates of morbidity and mortality (3). Screening echocardiograms (echos) have the ability to detect RHD at earlier stages and thereby create an opportunity to prevent further advancement of disease (4–6). In addition to the many logistic and financial barriers that exist for implementing echo screening programs in endemic regions, several knowledge gaps remain that require attention before echo screening can be recommended as public policy.

One key question is whether or not echo screening and early detection of disease will improve outcomes. An ongoing randomized control trial is designed to answer that question by evaluating the impact of Penicillin prophylaxis on RHD progression over time (GOAL Trial, Clinicaltrials.gov NCT03346525). In addition to studying the utility of screening, the optimal frequency and timing of screening has not been clearly delineated. Knowing the optimal frequency of screening would simultaneously cut unnecessary cost and use of resources, while minimizing the risk of disease progression between screening echos. Prior studies have evaluated small cohorts of children with mild or no RHD to evaluate progression over time, and have shown variable results (7–9). Our study is the first to follow a large cohort of children in an RHD endemic region to determine the risk of progression from normal to abnormal, based on screening echos, over time. This study was designed to determine the risk of RHD following one prior negative screening echo, with the hypothesis being that children with a prior negative screening echo will have lower rates of RHD detection than those never screened before. RHD is caused by a combination of environmental, genetic, and host factors (10). While any child can, in theory, develop RHD at any time, we know that some children are at higher risk. Therefore, a population with a single normal echocardiogram likely has a lower risk than the general population of later developing RHD.

METHODS

Study Design

This retrospective cohort study was conducted in Gulu District, Uganda, located in Northern Uganda, with known endemic rates of RHD. The study enrolled children, ages 5 to 18 years, who attended primary and secondary schools in Gulu District. Initial screening echos were performed between 2013 and 2015 on 8,009 children, as part of three separate studies looking at the utility of hand-held echo to detect RHD (11–13). A second independent echo screening period was performed in primary and secondary schools between June and Sept of 2018. This extensive school-based screening was performed in 75,708 children in 165 schools in both rural and urban areas where prior screening had occurred. The second screening was embedded in a larger study to identify children with RHD in the community.

During the second screening, a short 4-question interview was conducted by members of the research team with each child to determine prior participation in echo screening. When children self-reported a prior echo screening, they were asked for their name, age, and prior school and class. This data was used to ensure adequacy of recall. All children with positive screening echos in the schools subsequently underwent a complete confirmatory echocardiogram, analogous to the process in the initial screening studies. World Heart Federation (WHF) criteria were used to confirm the diagnosis of RHD, evaluate valve morphology, and determine the severity of disease (14). Children were excluded from the study if: (1) they had a known diagnosis of RHD, diagnosed prior to the first screening period (based on the National Ugandan RHD registry), (2) had a diagnosis of other heart disease, (3) were outside the target age range (5–18 years), or (4) failed to return for the confirmatory echocardiogram.

Sample Size

Sample size was determined using standard estimations for cross-sectional and cohort studies (15). "Exposed" was defined as having a prior negative screening echo during the initial screening period, while "unexposed" was defined as having no prior screening echo. Using previously published data, we assumed a conservative baseline population prevalence of 2.0%. Using independent *t*-tests, a sample size of 2,316 would be required in each group to detect a difference of 50% (1% in the exposed population), with an alpha of 0.05 and power (1-B) of 0.8 (16). This goal sample size seemed feasible, as 8,009 children were initially screened in the 2013–2015 timeframe, and the planned volume of the second screening period was >70,000 in Gulu District.

Statistical Analysis

Statistical analysis was performed using SPSS statistical analysis software. Age, gender, and details of the initial screening echo (school name and class) were collected by in-person interviews for those in the exposed cohort, and for all participants with a positive screening echo. Median and interquartile range (IQR) were calculated for age in the exposed cohort and in those with RHD in both the exposed and unexposed cohorts. Within the exposure group, RHD and non-RHD groups were compared. Age was compared by independent *t*-tests and gender was compared using chi-square analysis. RHD prevalence was calculated for both exposed and unexposed cohorts. The absolute risk difference and relative risk (RR) were determined with 95% confidence intervals (CI). Statistical significance was deduced when the 95% CI of a RR did not include one and $p < 0.05$.

Ethics

This study was covered under an existing Institutional Review Board (IRB) through Mulago Hospital at Makerere University, as part of the National RHD Outreach Program (REC REF 2013-072).

RESULTS

A total of 75,708 children underwent echo screening during the second echo screening period. Sixty-five children were excluded from the study: 36 were diagnosed with another form of heart disease (32 with congenital heart disease; 4 with cardiomyopathy), 5 were outside the study age range (<5 years or >18 years of age), and 24 had been previously diagnosed with RHD before the time of the initial screening echo (**Figure 1**). A total of 75,643 subjects were initially included, 1,587 (2.1%) of whom had a previous negative screening echo, making up the exposed cohort, and 74,056 (97.9%) with no prior screening echo, making up the unexposed cohort. Two subjects were reclassified from exposed to the unexposed cohort, as they had been clinically diagnosed with RHD at the Gulu Regional Referral Hospital (GRRH) in between the initial and second screening periods and had not in fact undergone an initial screening echo as part of initial screening studies. An additional 3 (0.1%) subjects were excluded from the exposed cohort, and 158 (0.2%) were excluded from the unexposed cohort for not returning for confirmatory echo (**Figure 1**). This left 1,582 subjects in the exposed cohort and 73,900 in the unexposed group. In the exposed cohort, the median age was 13 years (IQR 10–16) and 693 (43%) were male. Time from the initial echo to the second screening echo ranged from 44 to 60 months (3.6–5 years). To confirm adequacy of recall, a subset (33%) of the exposed cohort data was crosschecked with prior records and found to have accurate recall.

In the exposed cohort, there were 10 new cases of RHD; a prevalence of 0.6% (10/1,582). There were 737 new cases of RHD in the unexposed cohort; a prevalence of 1% (737/73,900). The relative risk of developing RHD if a child had a prior negative screening echo compared to those with no prior screening echo was determined to be 0.64 (95% CI 0.3 to 1.2, $p = 0.15$). The relative risk reduction was 0.4, or a 40% reduced risk of newly diagnosed RHD in those who had a previously negative screening echo compared to those who had never been screened before. There was no difference in age or gender between those who developed RHD and those without RHD in the exposed cohort (**Table 1**) or when comparing the RHD positive cases in the exposed and unexposed cohorts (**Table 2**). Of the 10 new cases of RHD in the exposed cohort, 9 (90%) were borderline, 1 (10%) was mild definite RHD, and none were moderate or severe. Of the 737 new cases of RHD in the unexposed cohort, 566 (76.8%) were borderline, 152 (20%) were mild definite, and 19 (2.6%) were moderate or severe RHD (**Table 2**).

DISCUSSION

This is the first population-based study to examine the risk of developing RHD over time in those with a previously normal screening echo. Our data demonstrate no statically significant difference in rates of RHD development between those with a previously negative screening echo and those never screened before. However, there was a trend demonstrating lower rates of RHD and less severe RHD in those with a prior negative screening echo, and a 40% risk reduction of detecting RHD on a follow up screening echo, when a child had a negative screening echo in the past 3–5 years. While not powered to study severity of disease, this study found that children with a previously negative screening echo did not develop advanced RHD.

Three prior studies also report on the development of RHD in children with a previously normal echocardiogram. An Australian study evaluated the progression of minor echocardiographic changes over time. The study included a comparison group of 325 children with initially normal echos and found that 19 (5.9%) had evidence of RHD at an average follow up of 3.5 years (9). A second study out of South-Pacific New Caledonia followed a cohort 114 children with RHD, diagnosed by echo screening and 227 healthy controls (8). After a median follow-up period of 2.58 years, 31 (13%) of the healthy controls had evidence of RHD on echo screening, where only 2 were definite RHD and 29 were borderline RHD (8). Both studies demonstrate very high rates of RHD development in children who once had a normal echocardiogram, even higher than baseline population prevalence. It would require further study to fully understand such high rates of conversion from normal to abnormal echo findings in these populations.

In a third study out of Fiji, Engelman et al. reported clinical outcomes for 70 screen-negative cases with a median of 7.4 years and found no deaths or RHD-related admissions (7). Albeit in a small cohort size, this demonstrates that over a longer period of time, complications related to RHD may be rare in those with a previously negative screening echo. Similarly, our study found no advanced disease among those who had a previously negative screening echo. While our study was not designed to show differences in disease severity, the results, along with Engelman's study, do suggest that children with prior negative screen are unlikely to develop severe disease over a 3–7 year timeframe. This can help inform future research efforts and planning for currently existing screening programs.

The overall prevalence of RHD in children screened in this study was only 1%, which was lower than anticipated. Previous screening studies in this region have shown a prevalence of 2.5–3% (13, 17, 18). While this study was not designed to understand this difference, it is possible that multiple prior echo screening studies, which included RHD education in schools, health centers, and in the community, may have increased awareness and subsequently reduced community rates of new RHD through improved primary and secondary prevention. The lower overall prevalence in both cohorts in this study may have affected the ability to detect a significant difference between the cohorts. This study was designed with a conservative baseline RHD population estimate of 2%, and hypothesized a 50% risk reduction between the cohorts. If the baseline prevalence in our study had been 2%, the findings would have been statistically significant, even if the prevalence of the exposed cohort increased up to 1%. For the cohort who underwent a prior screening echo, there were no ongoing health campaigns or programs specifically targeting this population that would have led to a significant change in the risk of developing RHD overtime. In addition, multiple studies in this region have continued to show poor health-seeking behavior in children and adults with symptoms of acute rheumatic fever (19, 20). Therefore, it is unlikely that

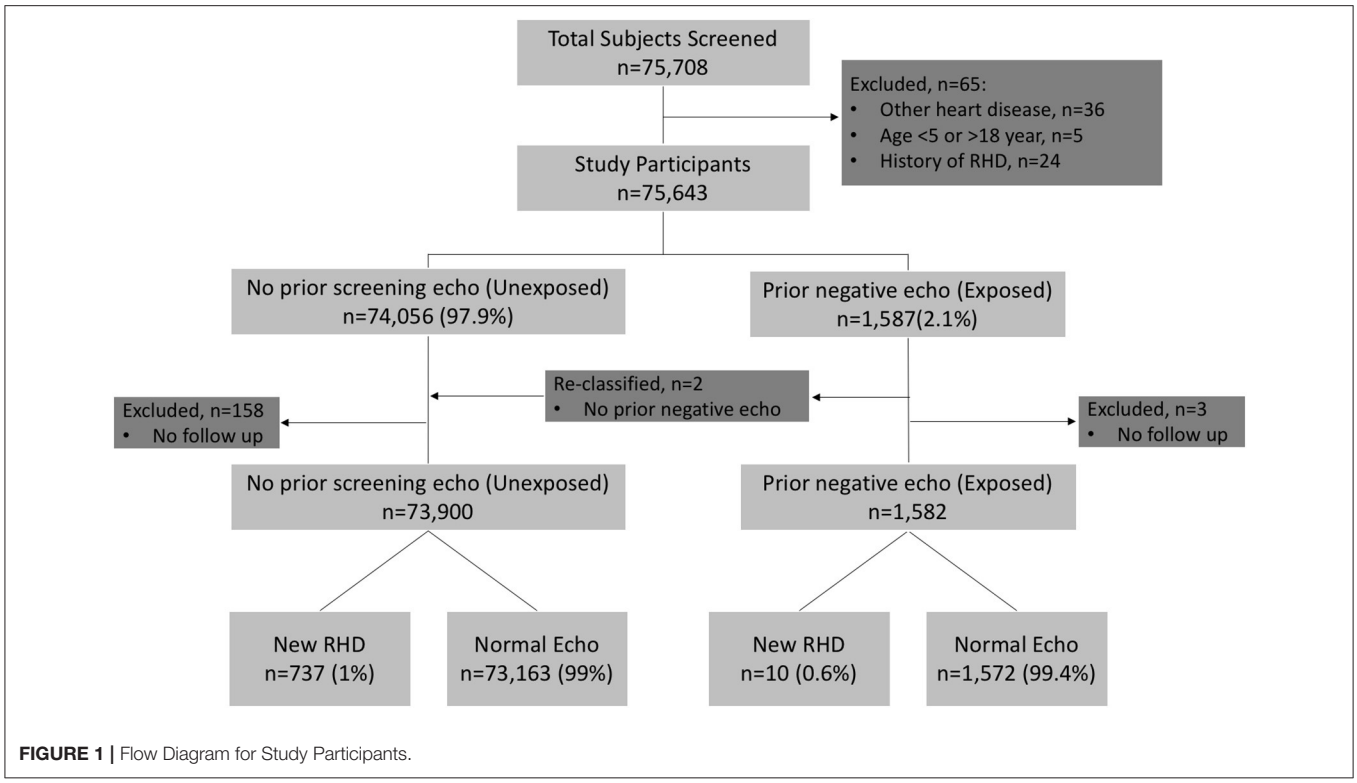

FIGURE 1 | Flow Diagram for Study Participants.

TABLE 1 | Demographic data of children with a prior negative screening echo (exposed cohort) with and without RHD.

	Exposed with RHD (n = 10)	Exposed without RHD (n = 1,572)	*p-value
Age (years), mean (std dev)	14.3 (1.2)	13.5 (1.8)	0.199
Gender			
Male (%)	4 (40%)	678 (43%)	0.841
Female (%)	6 (60%)	893 (67%)	

**To assess the differences between participants in the exposed cohort with RHD vs. without RHD, p-values were calculated using chi-square tests for categorical variables and independent t-tests for continuous variables.*
RHD, Rheumatic heart disease; Std dev, Standard deviation.

community intervention or education altered the rates of RHD in those with a previously negative screening echo any more that in those without a prior screening echo.

The pragmatic design of this study led to multiple limitations. Most importantly, our primary endpoint was underpowered as a result of finding fewer than anticipated children who had prior echo screening, and due to a lower baseline RHD prevalence in newly screened children. Second, this study relied on recall from children to determine if they had a previous screening echo 3–5 years prior. As is true in most developing regions of the world, children in Northern Uganda have limited, or no, health records, medical record numbers, or social security numbers. In addition, their birth dates and name spelling are frequently inconsistent and unreliable. As a result, recall was used in lieu of more reliable data. Cross-checking one-third of the exposed sample found recall to be accurate, but we were unable to cross-check for false-negative reporting. Finally, the echo screening was performed by healthcare professionals with varying levels of ultrasound experience, including sonographers, cardiologists, and cardiology fellows from both the U.S. and Uganda. However, the same staff was used throughout screening, so missed cases of RHD by inexperienced staff would have led to non-differential misclassification. In effect, this may have skewed the results toward the null hypothesis: no difference in risk between the cohorts.

Despite these limitations, this study adds important and novel information to the field of echocardiographic screening for the diagnosis of RHD, at a time when the use of echo screening is being debated as a universal measure in endemic settings. The RHD community lists secondary prevention as one of the four crucial pillars to decrease the burden of RHD, as described by the late Mayosi (21), but this cannot be accomplished without identification of disease. Echocardiographic screening is known to detect subclinical disease, but further research is needed to support determine the proper use and implementation in endemic regions. Studies such as this one are critically examining

TABLE 2 | Demographic data of children with new RHD, exposed and unexposed.

	RHD in exposed ($n = 10$)	RHD in unexposed ($n = 737$)	*p-value
Age (years), mean (std dev)	14.3 (1.2)	12.6 (2.9)	0.069
Gender			
Male (%)	4 (40%)	318 (43%)	0.883
Female (%)	6 (60%)	419 (57%)	
Severity of RHD			
Borderline/mild (%)	10 (100%)	718 (97.4%)	0.606
Moderate/severe (%)	0 (0%)	19 (2.6%)	

**To assess the differences between participants in the exposed cohort with RHD vs. without RHD, p-values were calculated using chi-square tests for categorical variables and independent t-tests for continuous variables.*
RHD, Rheumatic heart disease; Std dev, Standard deviation.

the use of echo screening, so that it can be properly utilized as a vital tool in the domain of secondary prevention. This data provides valuable information about intervals of echo screening over time, as well as the lack of disease progression, and lack of disease severity, in those with previously negative screening echos. RHD largely remains a disease of the poor and disadvantaged, and therefore, resource allocation is vital in these communities. Further research in this realm will help distinguish proper screening intervals, appropriate age of screening, and how best to screen large populations to efficiently and economically incorporate screening into RHD endemic regions of the world to effectively reduce the burden of disease.

CONCLUSION

Our study found no statistical difference in RHD prevalence between children with previously normal echocardiograms and children who had never been screened, however, there was a trend toward decreased risk. These data call for a large-scale controlled cohort study to evaluate the risk of developing RHD after a negative screen, powered to look at differences in age at first negative screen, the interval between screenings, and the severity of disease. This information has important implications for the design of population screening programs and the use of echocardiographic screening in endemic RHD regions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board (IRB), Mulago Hospital at Makerere University, as part of the National RHD Outreach Program (REC REF 2013-072). Written informed consent for participation was not provided by the participants' legal guardians/next of kin because: Echocardiographic screening was performed under the national RHD Outreach Program.

AUTHOR CONTRIBUTIONS

MZ, CS, and AB prepared the written manuscript. MZ, AS, AD, J-LN, IO, AT, JR, EO, CS, and AB reviewed and edited the manuscript. AS, AD, J-LN, IO, JR, and EO contributed to data gathering. AS, AD, and AT contributed to data organization. MZ, CS, and AB contributed to data analysis. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

BRIEF RESEARCH REPORT
published: 24 February 2021
doi: 10.3389/fmed.2021.621668

Acute Rheumatic Fever: Where Do We Stand? An Epidemiological Study in Northern Italy

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Acute rheumatic fever (ARF) is a non-septic complication of group A β-hemolytic streptococcal (GAS) throat infection. Since 1944, ARF diagnosis relies on the Jones criteria, which were periodically revised. The 2015 revision of Jones criteria underlines the importance of knowing the epidemiological status of its own region with updated data. This study aims to describe ARF features in a retrospective cohort retrieved over a 10-year timespan (2009–2018) and to report the annual incidence of ARF among children in the Province of Monza-Brianza, Lombardy, Italy during the same period. This is a multicentric cross-sectional/retrospective study; 70 patients (39 boys) were diagnosed with ARF. The median age at diagnosis was 8.5 years (range, 4–14.2 years). Overall, carditis represented the most reported major Jones criteria followed by arthritis and chorea (40, 27, and 20 cases, respectively). In order to calculate the annual incidence of ARF, only children resident in the Province of Monza-Brianza were included in this part of the analysis. Therefore, 47 patients aged between 5 and 14 years were identified. The median incidence during the study time was 5.7/100,000 (range, 2.8–8.3/100,000). In the Province of Monza-Brianza, we found an incidence rate of ARF among children aged 5–14 years constantly above the threshold of low-risk area as defined in the 2015 revision of Jones criteria. Therefore, the diagnosis of ARF should be based on the moderate–high-risk set of Jones criteria. However, given the burden of secondary prophylaxis, expert opinion is advisable when the diagnosis of ARF is uncertain.

Keywords: acute rheumatic fever, group A β-hemolytic streptococcus, carditis, Jones criteria, penicillin

OPEN ACCESS

Edited by:
Bruno Ramos Nascimento, Universidade Federal de Minas Gerais, Brazil

Reviewed by:
Yosef Uziel, Meir Medical Center, Israel
Emmy Okello, Uganda Heart Institute, Uganda

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Specialty section:
This article was submitted to Rheumatology, a section of the journal Frontiers in Medicine

Received: *26 October 2020*
Accepted: *14 January 2021*
Published: *24 February 2021*

Citation:
Marino A, Cimaz R, Pelagatti MA, Tattesi G, Biondi A, Menni L, Sala M, Calzi P, Morandi F, Cortinovis F, Cogliardi A, Addis C, Bellù R, Andreotti M and Varisco T (2021) Acute Rheumatic Fever: Where Do We Stand? An Epidemiological Study in Northern Italy. Front. Med. 8:621668. doi: 10.3389/fmed.2021.621668

INTRODUCTION

Acute rheumatic fever (ARF) is a non-septic complication of group A β-hemolytic streptococcal (GAS) throat infection. ARF is a multisystem autoimmune disease causing inflammation in different sites (joint, heart, basal ganglia, skin); it affects typically school-aged children (particularly between 5 and 14 years) without sex differences (1).

Several factors influence the likelihood of ARF developing such as genetic predisposition and the virulence of the infecting GAS strain. It has been estimated that <3% of patients who have a GAS tonsillopharyngitis develops ARF (2). The prompt identification and treatment of GAS pharyngitis lowers the risk of this condition. ARF pathogenesis is not completely clear, and there is no sound experimental model. Nevertheless, the GAS type-specific antigen, the M protein, has been found to eventually cause, in a predisposed host, tissue inflammation through the formation of autoantibodies and the activation of cell-mediated immunity by acting as a superantigen. Furthermore, the immunological pathogenesis is supported by the typical latency of about 3 weeks between GAS pharyngitis and ARF onset (3, 4). The genetic predisposition for ARF might be acquired (44% of concordance among monozygotic twins), although this inheritance is polygenic with variable penetrance (5, 6). Joint involvement represents the most frequent clinical manifestation along with heart valvulitis, while chorea is less common (7). Heart involvement may result in permanent damage of valves resulting in rheumatic heart disease (RHD) and represents the major cause of acquired heart disease in the developing world (8, 9). The other ARF manifestations resolve without permanent consequences.

The incidence of ARF is highly variable depending on the geographic areas (10). Middle East, Asia, Eastern Europe, and Australia show a higher incidence (7.5–194 cases/100,000/year) compared to US (0.61 cases/100,000/year), even if an overall downward trend has been observed (11, 12). Since 1944, ARF diagnosis relies on the Jones criteria, which were periodically revised (13–15). The last revision of 2015 was principally driven by the different disease burden around the world and the increasing role of echocardiography. Furthermore, the growing use of over-the-counter drugs has been listed as one of the potential factor impacting clinical presentation. In the 2015 revision, patients were stratified as having low or moderate–high risk for ARF according to the local disease incidence, with cutoff for low incidence being <2 per 100,000 school-aged children (usually 5–14 years old) per year or an all-age prevalence of RHD of ≤1 per 1,000 per year. Therefore, two sets of criteria have been provided according to the risk of ARF (15). Furthermore, the presence of subclinical carditis was considered as a major manifestation. The 2015 revision of Jones criteria underlines the importance of knowing the epidemiological status of its own region with updated data. ARF resurgence in Italy has been suggested by several studies (1, 16, 17).

The province of Monza-Brianza is one of the 12 provinces of Lombardy, a region of northwest of Italy. Despite being the smallest province of Lombardy in terms of size (405.49 km^2), the Province of Monza-Brianza has one of the highest population densities of the region (residents/km^2, 2.18; population of 878,267 to 1 January 2020 from ISTAT data). This study aims to describe ARF features in a retrospective cohort retrieved over a 10-year timespan (2009–2018) and to report the annual incidence of ARF among children in the Province of Monza-Brianza, Lombardy, Italy during the same period.

MATERIALS AND METHODS

This is a multicentric cross-sectional/retrospective study involving Hospitals of the Province of Monza-Brianza (Desio, San Gerardo of Monza, Vimercate, and Carate Hospital) in addition to two nearby collaborating Hospitals (Merate and Lecco Hospital). The inclusion criteria were the diagnosis of ARF between January 1, 2009 and December 31, 2018 and a disease onset before the age of 18 years. Only hospitalized patients were included in the present study. ARF diagnosis was established according to the criteria then in place. ARF cases were first identified by analyzing hospital discharge records (HDRs) according to the International Classification of Disease, Ninth Revision, Clinical Modification (ICD-9 CM). The following ICD-9 CM codes were used: 390, 391.0, 391.1, 391.2, 391.8, 391.9, 392.0, and 392.9. A chart review of each retrieved case was then performed in order to acquire demographical and clinical data and to avoid overlaps and duplicates. All patients underwent echocardiography during the acute phase of the disease. Subclinical carditis was defined as positive echocardiography in the absence of clinical features. Details on the retrieved cohort (demographic, clinical, laboratory, and echocardiographic data) were collected in a customized database. To calculate the annual incidence of ARF in the Province of Monza-Brianza, patients not resident in this province were excluded from the analysis as well as those younger than 5 years and older than 14 years. This subgroup of patients was compared with the age-matched population of the same area of the same year. School-aged children (5–14 years) population for each year was abstracted from the National Institute of Statistics (ISTAT) (http://demo.istat.it/). A descriptive analysis was then undertaken (Excel 2011). Since anonymized data were used, according to local regulations, no ethics committee approval was deemed necessary.

RESULTS

Over the 10-year timespan, 70 patients (39 boys) were diagnosed with ARF. The majority were Caucasians ($n = 60$). The median age at diagnosis was 8.5 years (range, 4–14.2 years). The major features of the cohort are shown in **Table 1**.

Overall, carditis represented the most reported major Jones criteria followed by arthritis and chorea (40, 27, and 20 cases, respectively). Erythema marginatum was quite rare, and in three out of four cases, it was present together with carditis. No subcutaneous nodule was recorded. Among associations of major criteria, arthritis and carditis was the most frequent ($n = 14$), followed by carditis and chorea ($n = 10$). Carditis without other major criteria was recorded 13 times, while isolated arthritis was recorded 12 times and chorea 9 times. The mitral valve was the most commonly affected heart valve, either as an isolate finding ($n = 19$) or along with aortic ($n = 12$) or tricuspid valve involvement ($n = 2$); conversely, the aortic valve was affected more rarely ($n = 6$). Subclinical carditis was identified in 16 patients.

Increased inflammatory markers were the most frequently recorded minor criterion, followed by arthralgia and fever (39, 35,

TABLE 1 | Acute rheumatic fever (ARF) features.

ARF features	No. of patients (%)
Major criteria	
Arthritis	27 (39%)
Carditis	40 (57%)
Subclinical carditis	16 (40%*)
Mitral valve	19 (47.5%)
Aortic valve	6 (15%)
Tricuspid valve	1 (2.5%)
Bivalvular	14 (35%)
Chorea	20 (29%)
Erythema marginatum	4 (6%)
Subcutaneous nodules	0
Minor criteria	
Fever	32 (46%)
Arthralgia	35 (50%)
Increased inflammatory markers	39 (56%)
Prolonged PR interval	3 (4%)

**It refers to patients with carditis only.*

and 32 cases, respectively). Prolonged PR interval was observed in three cases, two of them with associated valvulitis. Information on acute treatment was available for 48 subjects. Ibuprofen was the most used non-steroidal anti-inflammatory drug (NSAID): 17 patients received ibuprofen compared with 7 patients treated with acetylsalicylic acid. Corticosteroids were used in 28 cases. The use of corticosteroids was mainly driven by the presence of carditis (20 cases, 8 with bivalvular involvement and 8 patients with concomitant chorea) and, less frequently, to treat chorea alone (6 cases) or arthritis and erythema marginatum (one case each). Secondary prophylaxis for GAS infection with intramuscular injection of penicillin was administered in all the patients according to their weight (600,000 U for patients <27 kg, 1,200,000 U for patients >27 kg). Secondary prophylaxis was administered every 3 weeks in 36 patients and every 4 weeks in 29 patients, respectively (no information available for 5 patients).

Data about the suggested duration of secondary prophylaxis were available for 59 patients. The majority ($n = 40$) were advised to continue GAS prophylaxis until 21 years of age. In this group, 22 patients had carditis. Two patients with severe bivalvular carditis were recommended to continue secondary prophylaxis until 40 years of age and lifelong, respectively. Discontinuation of GAS prophylaxis before 21 years of age was suggested to 17 patients (10 with carditis). Indeed, four patients (all with carditis) were recommended to continue secondary prophylaxis until 18 years of age, 3 patients (all with carditis) for 10 years, and 10 patients (3 with carditis) for 5 years.

In order to calculate the annual incidence of ARF, only children resident in the Province of Monza-Brianza were included in this part of the analysis. Of the remaining 49 children, 2 were excluded because of their age (both 4 years old). Therefore, over a 10-year time period, 47 patients aged between 5 and 14 years were identified. The geographical distribution of ARF cases sorted by patient's residence is shown in **Figure 1**. The median incidence during the study time was 5.7/100,000 (range, 2.8–8.3/100,000) (**Table 2**). The number of cases for each year did not show an upward trend over 10 years, as well as the annual incidence per 100,000 school-age children (**Figure 2**).

DISCUSSION

The improvements in living conditions and hygiene along with the increased accessibility to the healthcare system were the main reasons for the decline of ARF incidence worldwide during the nineteenth century, together with the increased use of antibiotics, especially in industrialized countries.

Nowadays, globalization might have altered this trend. Indeed, migratory flows have contributed to the resurgence of several diseases such as those related to infectious agents.

To date, updated epidemiological studies on ARF incidence in developed countries are scanty. Furthermore, the 2015 revision of Jones criteria makes the need for up-to-date information on ARF incidence more compelling.

Herein, we described ARF features over a 10-year-period in an area with a high population density such as the Province of Monza-Brianza, Lombardy, Italy. Heart involvement and arthritis were the most common manifestations, either isolated or in association; however, chorea was not rare. The median incidence of ARF among school-aged children residents in this province of northern Italy between 2009 and 2018 was 5.7/100,000 (range, 2.8–8.3/100,000), configuring children living in this area as a moderate–high-risk population for ARF. However, it has to be noted that the annual incidence of ARF depends not only on the number of ARF cases per year but also on the number of school-aged children (5–14 years) of the same year in the area. In this regard, we observed an upward trend of the school-aged population in the Province of Monza-Brianza (by 13,000 over 10 years) (**Table 2**). Overall, the annual incidence of ARF was constantly above the cutoff point of low-risk population (2/100,000); nevertheless, no upward trend was documented.

The age at onset and the slightly male prevalence observed in our study are comparable to previous similar studies (1, 7, 17, 18). Carditis and arthritis were the predominant features of ARF in our cohort, as frequently observed. The rate of carditis in our cohort was similar to what was reported by Breda et al. (1) and Fabi et al. (18) (49 and 57%, respectively), whereas Grassi et al. (7) documented a higher incidence of valvulitis (76%). However, we reported a lower rate of arthritis compared to other studies (39 vs. 53–66% of other studies) (1, 7, 18). This might be partially explained by the high rate of chorea observed in our population (29 vs. 5.7–21% of other studies), giving the subacute nature of this manifestation in ARF (1, 7, 18). The same assumption might be postulated for the lower rate of increased inflammatory markers compared to other studied (1, 7, 18).

With regard to ARF treatment, the majority received ibuprofen, confirming the clinicians' habit to avoid aspirin in children, given its safety profile. Corticosteroids were prescribed more frequently to patients with carditis, as expected. Furthermore, in agreement with recent findings (19), the majority of patients with chorea received corticosteroids.

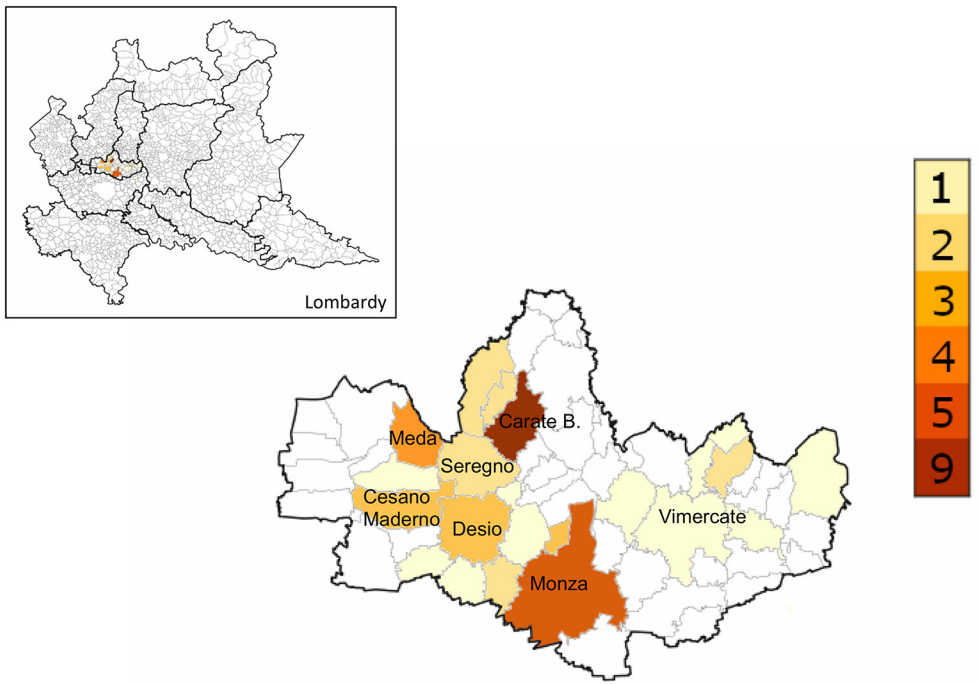

FIGURE 1 | Geographic distribution of acute rheumatic fever cases in the Province of Monza-Brianza between 2009 and 2018.

TABLE 2 | Cases of acute rheumatic fever (ARF) among children (5–14 of age) of the Province of Monza-Brianza.

Year	No. of cases	Age-matched population	No. of cases per 100,000
2009	2	72,104	2.8
2010	6	72,679	8.3
2011	5	80,507	6.2
2012	6	82,063	7.3
2013	5	83,770	6.0
2014	5	84,758	5.9
2015	3	85,259	3.5
2016	5	85,576	5.8
2017	6	85,826	7.0
2018	5	85,430	5.9

The burden of the recommended GAS secondary prophylaxis in children is still concerning. After several years of continuous prophylaxis, family and children often prefer to discontinue these injections despite being suggested otherwise.

In this multicentric cohort, the recommended duration of GAS secondary prophylaxis was predominantly until 21 years of age, although some patients, even with carditis, were recommended to discontinue the prophylaxis before. Conversely, the frequency of administrations varied between 3 and 4 weeks according to each center. This highlights the lack of uniformity among participating centers that might be secondary to scarce knowledge of up-to-date information related to secondary prophylaxis and to the epidemiological status of our region.

A recent multicentric retrospective study showed that a significant number of children with ARF had a low adherence to secondary prophylaxis (20, 21). Nevertheless, the prophylaxis adherence was not associated with ARF recurrences or RDH (20, 21). In this regard, updated trials on GAS secondary prophylaxis in ARF are advisable since the evidence of current recommendations dates back 50 years (22–24).

So far, available updated data on ARF rates in Italy are scarce and difficult to compare given the different methodologies used. There is no national registry for ARF. Up-to-date information about ARF incidence is abstracted by monocentric experience or regional studies.

Breda et al. (1) conducted an epidemiological study between 2000 and 2009 in a region of Central Italy (Abruzzo). The overall incidence rate of ARF was 4.1/100,000 (range, 2.26–5.58/100,000) among children aged 0–18 years with a significant increase in the incidence of about 10% during the observation time (1).

Licciardi et al. (17) reported the incidence of ARF among children (age range, 5–14 years) between 2007 and 2016 in the Northwest of Italy (Turin). They documented a rate of ARF steadily above the threshold of low-risk population ranging between 3.2 and 9.6 per 100,000. Similar to our findings, they did not identify an increasing trend of ARF cases over the study time (17).

Recently, Munteanu et al. reported the rate of hospitalization of ARF in Lombardy by analyzing hospital discharge records between 2014 and 2016 (24). They found an overall rate of hospitalization of 4.24 cases per 100,000 children and adolescents (<18 years). In contrast with our findings, they reported an upward trend in ARF cases. However, the same authors stated that the short duration of the study observation in their cohort

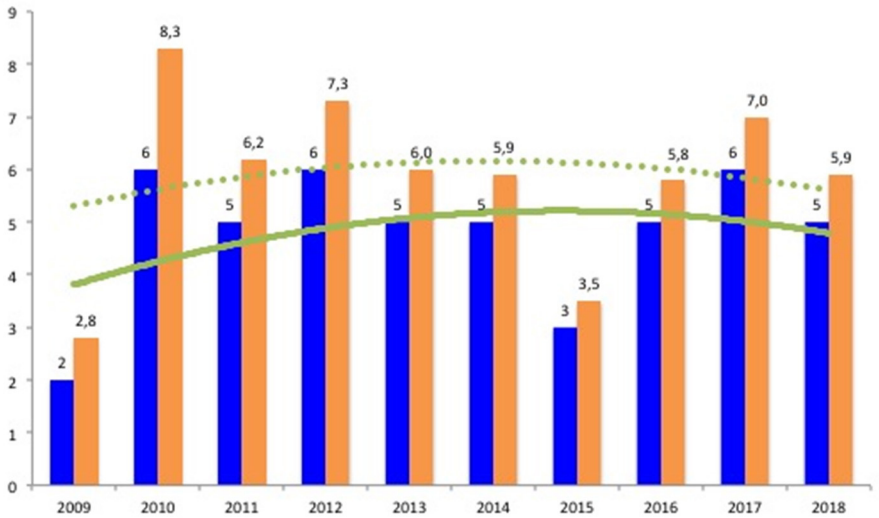

FIGURE 2 | Acute rheumatic fever cases sorted by year with relative incidence (number of cases/100,000/year) among children resident in the province of Monza-Brianza. Blue bars: number of acute rheumatic fever (ARF) cases for each year. Orange bars: annual incidence of ARF/100,000. Continuous and dashed lines reflect trends for annual cases and incidence, respectively.

might have influenced the results as well as the possibility that some ARF cases reported in this study are not incident cases but prevalent ones (25). Our study presents several limitations that are in part intrinsic in its retrospective design. The lack of information about the follow-up of these patients is one limitation. Furthermore, multicentric studies, despite benefitting of larger numbers, are more prone to biases due to the lack of uniformity in clinical approach and data reporting.

In conclusion, we reported principal features of ARF patients between 2009 and 2018 in the Province of Monza-Brianza, a highly populated area of Northern Italy. We found an incidence rate of ARF among children aged 5–14 years constantly above the threshold of low-risk area as defined in the 2015 revision of Jones criteria. Therefore, the diagnosis of ARF should be based on the moderate–high-risk set of Jones criteria. However, given the burden of secondary prophylaxis, expert opinion is advisable when the diagnosis of ARF is uncertain.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AM conceptualized and designed the study, drafted the initial manuscript, and reviewed and revised the manuscript. AB, GT, LM, MS, PC, FM, FC, RB, MA, AC, and CA designed the data collection instruments and collected data. RC, TV, and MP conceptualized and designed the study, coordinated and supervised data collection, and critically reviewed the manuscript for important intellectual content. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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Conflict of Interest: The open access publication fee was covered by PEDIATRICA SPECIALIST srl.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cardiovascular Medicine

MINI REVIEW
published: 05 May 2021
doi: 10.3389/fcvm.2021.675339

Requirements for a Robust Animal Model to Investigate the Disease Mechanism of Autoimmune Complications Associated With ARF/RHD

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The pathogenesis of Acute Rheumatic Fever/Rheumatic Heart Disease (ARF/RHD) and associated neurobehavioral complications including Sydenham's chorea (SC) is complex. Disease complications triggered by Group A streptococcal (GAS) infection are confined to human and determining the early events leading to pathology requires a robust animal model that reflects the hallmark features of the disease. However, modeling these conditions in a laboratory animal, of a uniquely human disease is challenging. Animal models including cattle, sheep, pig, dog, cat, guinea pigs rats and mice have been used extensively to dissect molecular mechanisms of the autoimmune inflammatory responses in ARF/RHD. Despite the characteristic limitations of some animal models, several rodent models have significantly contributed to better understanding of the fundamental mechanisms underpinning features of ARF/RHD. In the Lewis rat autoimmune valvulitis model the development of myocarditis and valvulitis with the infiltration of mononuclear cells along with generation of antibodies that cross-react with cardiac tissue proteins following exposure to GAS antigens were found to be similar to ARF/RHD. We have recently shown that Lewis rats injected with recombinant GAS antigens simultaneously developed cardiac and neurobehavioral changes. Since ARF/RHD is multifactorial in origin, an animal model which exhibit the characteristics of several of the cardinal diagnostic criteria observed in ARF/RHD, would be advantageous to determine the early immune responses to facilitate biomarker discovery as well as provide a suitable model to evaluate treatment options, safety and efficacy of vaccine candidates. This review focuses on some of the common small animals and their advantages and limitations.

Keywords: animal model, acute rheumatic fever, rheumatic heart disease, sydenham chorea, lewis rats, autoimmunity, Group A streptococcus

OPEN ACCESS

Edited by:
Luiza Guilherme, University of São Paulo, Brazil

Reviewed by:
Francesca Bartoli-Leonard, Brigham and Women's Hospital and Harvard Medical School, United States
Adrien Lupieri, Brigham and Women's Hospital and Harvard Medical School, United States

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Specialty section:
This article was submitted to Heart Valve Disease, a section of the journal Frontiers in Cardiovascular Medicine

Received: *02 March 2021*
Accepted: *09 April 2021*
Published: *05 May 2021*

Citation:
Rafeek RAM, Sikder S, Hamlin AS, Andronicos NM, McMillan DJ, Sriprakash KS and Ketheesan N (2021) Requirements for a Robust Animal Model to Investigate the Disease Mechanism of Autoimmune Complications Associated With ARF/RHD. Front. Cardiovasc. Med. 8:675339. doi: 10.3389/fcvm.2021.675339

INTRODUCTION

The concept of comparative medicine developed based on the theory that animal species share physiological, anatomical and behavioral characteristics similar to human (1). This concept led to the use of different model organisms in all fields of biomedical research (2) and they continue to play a vital role in translational research for the advancement of human and animal health. The use of animal models to investigate human disease has its origins over 2,400 years ago. By the beginning of the twentieth century the use of animal models became more experimental rather than observational (1). Animals have contributed immensely in elucidating the disease mechanisms and the development of therapeutics including vaccines. An animal model, in which the immunopathological mechanisms or outcome of disease resembles those that occur in humans, is a logical adjunct to investigate human diseases. Thus, in this review we summarize the current animal models available to investigate the pathogenesis of Acute Rheumatic Fever (ARF), Rheumatic Heart Disease (RHD) and associated post-streptococcal autoimmune complications.

Post-streptococcal autoimmune disorders are complex immune mediated disease mostly affecting children and young adults following exposure to Group A streptococcal (GAS) infection. These includes ARF, RHD, Sydenham Chorea (SC) and possibly, pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS) (3–5). After 1–3 weeks of an untreated GAS infection, ∼1–3% of individuals develop non-suppurative post streptococcal complications including ARF, which may lead to RHD and cardiac failure (6). ARF affects multiple organs and primarily involve the joints, skin, brain and the heart. Except for cardiac damage most other manifestations are transient. After the initial or repeated episodes of ARF, about 30–45% of patients develop RHD (5) which poses an important public health problems in low to middle-income countries, and First Nation Peoples of high-income countries. Indigenous Australians (Aboriginal and Torres Strait Islander people) and New Zealanders (Māori and Pacific Islander populations) have among the highest rates of ARF in the developed countries (5, 7). RHD is the most common acquired cause of cardiac damage (8) affecting children between the ages of 5 and 15 years old (9). A gender propensity for ARF has not been widely observed although some studies have found RHD to be prevalent among females (10). The epidemiology of ARF/RHD is highly diverse and is relatively rare where access to modern medical care is readily available. However, it has not been completely eradicated with annual incidence of ARF varying from <0.5/100,000 in developed countries to >100/100,000 in developing countries (11). It is estimated that annually, approximately half a million new ARF cases are diagnosed globally (11). On the other hand, the overall prevalence of RHD is highest in sub-Saharan Africa, South Asia and Oceania. In 2015, 33.4 million people were reported to be have RHD with ∼297,300–337,300 deaths in RHD endemic regions. In non-endemic regions it was 221,600 cases (12).

Variety of host, bacterial, socioeconomic and environmental factors contribute to the prevalence and incidence of ARF/RHD (5). Environmental factors includes climatic factors, sanitation, poor hygiene, overcrowding and house hold conditions (5). In addition better living conditions led to decrease in the incidence of ARF/RHD (13, 14). Malnutrition and poverty are two other important factor among children contributing to repeated exposure to streptococcal and the spread of infection (13, 15). Poor healthcare system due to low socioeconomic status and inadequate awareness of the disease in the community leads to misdiagnosis or late diagnosis and treatment of GAS infection and ARF/RHD (5, 14, 16). In addition, a strong predisposition of genetic factors including genetic polymorphisms in many human leukocyte antigen (HLA) class II alleles in the development of ARF/RHD have also been described (17).

PATHOGENESIS OF ARF/RHD

The pathophysiology of post-streptococcal complication is not fully understood, however antigenic mimicry between GAS antigens and host proteins is partly considered as factor that triggers autoimmunity. It may also be affected by several environmental, genetic and socioeconomic factors. Although an autoimmune process has long been considered to be responsible for the initiation of ARF/RHD, it is only in the last few decades that the mechanisms involved in the pathogenesis of this post streptococcal conditions have been unraveled partly due to experimentation on animal models. Studies have shown that molecular mimicry of streptococcal antigens enable the generation of antibodies that bind to both GAS antigens and cross-react with host tissue proteins including cardiac myosin, collagen I and IV, tropomycin, laminin, vimentin, and keratin (18).

Further studies have demonstrated that human collagen IV, one of the major components of the basal membrane, a layer of extracellular matrix secreted by epithelial cells, can also be involved in the pathogenesis of ARF/RHD by acting as an autoantigen after forming a complex with GAS antigens (19). Several studies have demonstrated that GAS strains are capable of binding and aggregating to human collagen (6, 20–22). Collagen IV binds to cells and other molecules via an N-terminal Cyanogen Bromide fragment 3 (CB3) (19). GAS binds to CB3 of collagen via the octapeptide (AXYLZZLN) epitope of M protein and aggregate to form an antigenic complex with human collagen IV (19). The octapeptide region of M protein, which interact with collagen, is designated as PARF (peptide associated with rheumatic fever). The autoantigenicity of the M protein-collagen complex induces ARF/RHD. Higher levels of anti-collagen antibodies were found in the sera of ARF patients than healthy controls (22). In addition studies showed that injection of mice with GAS proteins also induce a collagen autoantibody response. However, these antibodies did not cross-react with the respective M protein. This observation leads to the understanding that the collagen autoimmunity caused by PARF motif of M protein does not depend on molecular mimicry (22).

ANIMAL MODELS OF ARF/RHD

Post streptococcal autoimmune complications including ARF/RHD is uniquely a human condition and humans are the only host and reservoir for GAS. Thus, modeling post-streptococcal autoimmune complications in animal is challenging. However, animals are the only experimental models used to investigate the characteristic signs, pathogenesis and pathophysiology specific to ARF/RHD. Animals including guinea pigs, rabbits, pigs, sheep, goats, cattle, cats, dogs, and non-human primates have been used as experimental model to understand the disease mechanism of ARF/RHD and to investigate the rheumatogenic potential of GAS M proteins (23–36). In the last two decades these animals were replaced by mice and rats due to lower costs, ease of handling and observation of pathological, immunological and functional changes comparable to ARF/RHD patients (**Table 1**).

The early experiments on rheumatic myocarditis were carried out in rabbits based on the hypothesis that ARF/RHD was caused either by direct streptococcal infection or by direct damage to heart tissues by streptococcal toxins. However, none of the rabbits showed similar pathology to rheumatic myocarditis in these studies (23). A study by Gross et al. as early as in 1929 examined the development of rheumatic myocarditis induced by live and killed streptococci isolated from patients with ARF/RHD in seven different animals including rabbits, guinea pigs, dogs, cats, swine, sheep, and calves. These studies failed to induce myocarditis in any of these animals (23). However, some rabbits showed accumulation of lymphocytes and mononuclear cells in their myocardium, low-grade pericarditis with mononuclear cells, acute focal interstitial myocarditis and large, irregular, thrombotic mass on the posterior cusp of the mitral valve. Similarly, guinea pigs showed focal interstitial accumulations of lymphocytes and large mononuclear cells in the myocardium, whereas dogs and cats had no gross or microscopic pathological cardiac lesions. Only one of the pigs in the study developed transient arthritis which disappeared after only a few days. The only positive pathological finding in sheep was a few interstitial foci of lymphocytes and mononuclear cells in the myocardium of the left ventricle (23).

To investigate the role of cellular immune response in RHD, Yang et al. (25) injected Guinea pigs with heat killed GAS and/or GAS M protein. Animals developed valvulitis and myocarditis with infiltration of T and B cells, macrophages and fibroblast into the myocardium and mitral valve (25). Myocardial and endothelial damage due to infiltration of granulocytes, macrophage and lymphocytes were observed in New Zealand White Rabbits injected with GAS M proteins (28, 29, 37) (**Table 1**). In addition, GAS pharyngeal spray on non-human primate (rhesus monkey, *Macaca mulatta*) induced typical RHD lesions as well as evidence of myocarditis and valvulitis along with infiltration of lymphocytes, histiocytes, Anitshkow cells, and plasma cells (38). Later, subcutaneous injection of GAS membrane antigens to rhesus monkeys' showed similar histological changes with endocardial and sub endocardial infiltration of mononuclear cells (39). Despite numerous attempts, relevant animal model for ARF/RHD still remains elusive (**Table 1**).

RODENT MODELS OF ARF/RHD

Rodent models due to ease of handling, small body size, large litter sigs, short life span and cost are considered ideal for biomedical research (1). The Swiss-Webster mice were the first rodent model of ARF/RHD (32). These mice developed cardiac lesions similar to ARF when infected with GAS cell wall fragments. MRL+/+ mice injected with *N*-terminal peptides of GAS M5 protein developed myocarditis (40). Moreover, myocarditis and CD4+ lymphocyte infiltration was detected in BALB/c (41, 42), Swiss mice (43), A/J mouse (44) and DBA/2 (45) mouse strains following the injection of GAS antigens and/or cardiac myosin. A more robust animal model for ARF/RHD was developed by immunizing Lewis rats with GAS M protein (26, 46). Upon injection of GAS antigens, or cardiac myosin, animals developed myocarditis and/or valvulitis similar to patients with ARF/RHD with antibody and T-cell responses that cross-reacted with host cardiac proteins.

RAT AUTOIMMUNE VALVULITIS MODEL OF ARF/RHD

Lewis rats were used to scrutinize myocarditis by injection of cardiac myosin. Marked cellular infiltration consisting of mononuclear cells, neutrophils, fibroblasts, and multinucleated giant cells were observed in the experimental allergic myocarditis (EAM) (47). Quinn et al. in 2001 developed the Lewis rat autoimmune valvulitis (RAV) model following exposure to streptococcal antigens to investigate the pathogenesis of ARF/RHD (26). This Lewis rat model has become the dominant animal model used to investigate the pathogenesis of ARF/RHD and to determine the safety of experimental GAS vaccine candidates (27, 30, 33–36).

Lewis rats immunized with recombinant M6 (rM6) protein demonstrated valvulitis and focal myocarditis, which were histologically similar to pathological lesions observed in patients with RHD (26). Later studies by Gorton et al. (30) reported valvulitis and myocarditis with infiltration of CD4+ cells, CD68+ macrophages and Anitschkow cells in the myocardium and mitral, aortic and tricuspid valves of Lewis rats following injection with recombinant M5 proteins (**Table 1**). Antibody and T cell responses to recombinant GAS M protein and the subsequent interactions with cardiac tissue have been predominantly investigated using a RAV model (**Figure 1B**) (30–34, 48). Furthermore, studies on Lewis rats indicated the role of infiltrating CD4+ cells and macrophages in the disease process. In addition to these histological changes, Lewis rats also demonstrated electrocardiographic and echocardiographic changes following exposure to killed GAS and recombinant GAS M proteins induce cardiac functional abnormalities comparable to patients with ARF (**Figure 1D**) (35, 36).

The hallmark features of ARF/RHD includes lesions in myocardium and valves (**Figure 1E**). In Lewis rats repeat

TABLE 1 | Immunopathological changes in small animals and rodents investigated as experimental model for post streptococcal complications.

	Antigens (route of injection)	Histological changes	Antibody response	T-cell and cytokine response	Tissue cross reactivity	References
Cardiac pathology						
Mice	Cell wall fragments of GAS (*i.p.*)	**Myocarditis, valvulitis** MNCs, anitschkow cell, PMNCs	Anti-GAS IgG collagen IV reactive IgG	N/A	Basement membrane collagen	(20, 43)
	Recombinant proteins/peptides of GAS (*f.p., s.c.*)	**Myocarditis, valvulitis**	Anti-GAS IgG	CD4+ (M)	Myosin	(77)
Lewis rats	Whole GAS and/or SDSE (*f.p., s.c.*)	**Myocarditis, valvulitis** monocyte, fibroblast, aschoff like cell lymphocyte, macrophages	Anti-streptococcal IgG Anti-myocardial IgG, antistreptolysin O	CD3+ (M), CD68+ (M), IFN-γ ↑ (B), IL-17A ↑ (B), IL-4 ↑ (B)	Myocardial protein, valvular protein, cardiac myosin, collagen	(31, 32, 35, 36)
	Recombinant proteins/peptides of GAS and/or SDSE *(f.p., s.c.)*	**Myocarditis, valvulitis** T-cell, MNCs, PMNCs, anitschkow cell	Anti-myosin IgG Anti- collagen IgG	CD3+ (M), CD4+ (M), CD8+ (M), CD68+ (M), IFN-γ ↑, IL-17A ↑, IL-4 ↑	Myosin, valvular protein, collagen	(26, 27, 30, 34–36)
	Serum from GAS exposed rats (*s.c.*)	**Myocarditis, valvulitis** T-cell, MNCs, PMNCs, anitschkow cell	Anti-myosin IgG	CD4+ (M)	Myosin, valvular protein, collagen	(36)
Guinea pig	Whole GAS (*s.c., f.p., i.m., i.v.*)	**Myocarditis, valvulitis** B-cell, macrophages, MNCs, fibroblast, cytotoxic lymphocytes	Anti-streptococcal IgG	N/A	Cardiac myofibre, sarcolemma	(23, 25)
	Cell wall fragments of GAS (*s.c., f.p., i.m.*)	**Myocarditis, valvulitis** B-cell, macrophages, fibroblast, cytotoxic lymphocytes	Anti-streptococcal IgG	N/A	Cardiac myofibre, sarcolemma	(25)
Rabbit	Whole GAS (*s.c., f.p., i.m., i.v., i.d., i.p.*)	**Myocarditis, valvulitis** lymphocyte, MNCs, leukocyte, aschoff bodies, fibroblast, fibrin, collagen	N/A	N/A	Skeletal muscle	(23–25)
	Cell wall fragments of GAS (*s.c., f.p., i.m., i.v., i.d.*)	Myofibrosis with degeneration of sarcoplasm, lymphocyte, macrophages, granulocyte	Anti-myosin IgG, Anti-sarcolemmal Ig	T-cell, IL-6 ↑, C3 (CV)	Sarcolemmal membrane protein, myosin	(25, 28, 29, 78)
Neurobehavioral changes						
Mice	Whole GAS (*s.c., i.n*)	**Antibody deposition** deep cerebellar nuclei globus pallidum, thalamus, periventricular areas	Anti-GAS IgG	CD4+, CD68+Iba1+ Th1 ↑, Th17 ↑, IL-17A ↑, IFN-γ ↑(B)	N/A	(57, 61, 62, 67)

(Continued)

TABLE 1 | Continued

	Antigens (route of injection)	Histological changes	Antibody response	T-cell and cytokine response	Tissue cross reactivity	References
	Serum from GAS exposed mice (*s.c.*)	**Antibody deposition** hippocampus periventricular area	Anti-GAS IgG1	IL-4 ↑ (B)	N/A	(58)
Lewis rats	Whole GAS (*s.c.*)	**Antibody deposition** striatum, thalamus, and frontal cortex	Anti-GAS IgG Anti-dopamine IgG	N/A	Dopamine D1R and D2L receptors	(59)
	Serum from GAS exposed rats (*s.c.*)	**Antibody deposition** striatum	Anti-GAS IgG	N/A	Dopamine D1R and D2L, serotonin transporter	(60)

f.p., foot pad; i.v., intra venous; i.m., intra muscular; i.d., intra dermal; s.c., sub cutaneous; i.p., intra peritoneal; CV, Cardiac Valve; C, Complement; B, Blood; GAS, Group A streptococci; IFN, Interferon; IgG, Immunoglobulin G; IL, Interleukin; MNC, Mononuclear Cell; M, Myocardium; SDSE, Streptococcus dysgalactiae subspecies equisimilis; PMNC, Polymorphonuclear cells; Th, helper T-cell; TNF, Tumor necrosis factor; ↑, Elevated; N/A, Not Assessed.

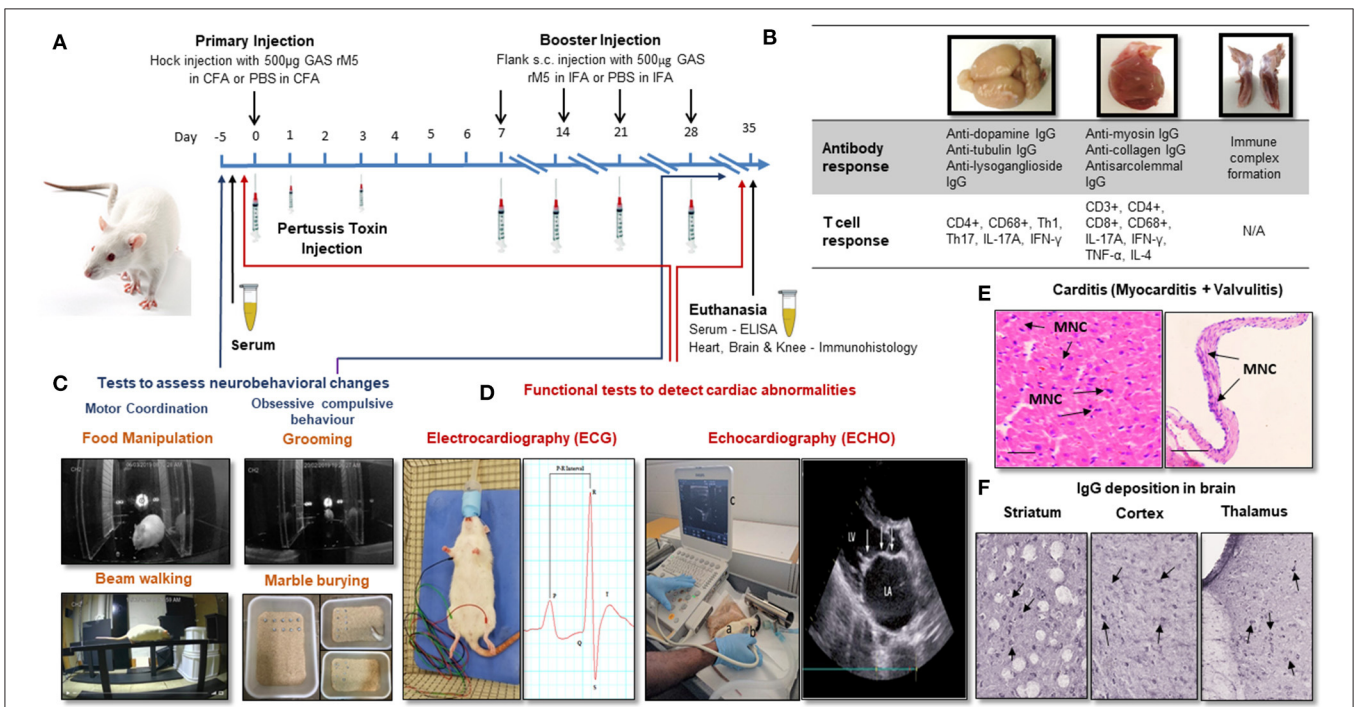

FIGURE 1 | Procedures for the induction of carditis and neurobehavioral changes in Lewis rats. **(A)** Carditis, valvulitis and neurobehavioral changes can be induced by injection of Lewis rats with GAS antigens followed by *Bordetella pertussis* toxin injection and booster injection of GAS antigens. **(B)** Antibody and T cell response can be assessed in brain, heart and knee joints following injection of GAS antigens. **(C)** Standard behavioral tests to assess neurobehavioral changes following exposure to GAS antigens. **(D)** ECG and ECHO changes will demonstrate impairment cardiac function. **(E)** Characteristic mononuclear cell infiltration in the myocardium and valvular tissue (arrows) can be demonstrated in the histological sections of cardiac tissue from rats injected with GAS antigens. **(F)** IgG deposition can be demonstrated in sections of brain following incubation with sera from rats exposed to GAS antigens. CFA, Complete Freund's Adjuvant; IFA, Complete Freund's Adjuvant; PBS, Phosphate Buffered Saline; GAS rM5, Recombinant M5 protein of Group A streptococcus; ELISA, Enzyme Linked Immunosorbent Assay; Th, Helper T-cell; TNF, Tumor Necrosis Factor; IFN, Interferon; IgG, immunoglobulin G; IL, interleukin; MNC, Mononuclear Cell.

injection with whole-killed GAS or recombinant GAS M proteins induced tissue cross-reactive antibodies and T cells (26, 27, 30, 31, 33, 34, 46, 48). Moreover, the involvement of Th-17 cells and associated regulators observed in the pathological process may potentially be considered as biomarkers for RHD (**Figure 1B**) (49, 50). In a separate experiment, in response to different

streptococcal antigens, including both GAS and *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE/GGS), Lewis rats developed typical histological lesions with infiltration of inflammatory cells into cardiac tissue providing experimental evidence that streptococci other than GAS could trigger and/or exacerbate post-streptococcal carditis (**Table 1**). Lewis rats were also used to assess the preclinical immunogenicity and safety of a GAS M protein-based vaccine candidate (51, 52). Therefore, the Lewis rat model is not only useful in elucidating the pathophysiological mechanisms in ARF/RHD, but also provides an opportunity to identify, validate streptococcal epitopes that are truly pathogenic to ARF/RHD. It also enables the assessment of safety and efficacy of GAS antigen based prototype vaccine candidates (51, 52).

ANIMAL MODELS OF NEUROBEHAVIORAL COMPLICATIONS ASSOCIATED WITH STREPTOCOCCAL INFECTION

The two major neurobehavioral complications associated with post GAS infections are Sydenham chorea (SC) and pediatric autoimmune neuropsychiatric disorders associated with streptococcus (PANDAS) (53). SC is a neurological movement disorder described in ARF and is one of the major criterions for the diagnosis ARF (18). PANDAS is a sudden onset of obsessive-compulsive disorder (OCD) associated with GAS infection and not known to be clinically associated with ARF (54). The complex immunopathological mechanisms that mediated immune damage following GAS infections that leads to SC and PANDAS remain unclear (55). However, it has been shown that antibodies against GAS cross-react with neurotransmitter receptors (D1 and D2 dopamine receptors), signaling kinases and ion channels, located primarily in the basal ganglia of the brain in susceptible hosts due to molecular mimicry (56).

In the past many studies have been carried out to develop an animal model to investigate the post streptococcal neurobehavioral disorders (**Table 1**) (57–62). Initial experiments were carried out by infusion of serum from patients with suspected streptococcal related neuropsychiatric disorders directly in to the striatum of rats. However, not all such studies succeeded in modeling these stereotypic behaviors in mice and rats (63–66). In 2004, Hoffman et al. (57) injected female SJL/J mice with purified GAS M6 protein along with Freund's adjuvant and observed that a group of mice developed motor and behavioral problem. These investigators conducted another study by the passive transfer of sera from mice injected with GAS to naïve mice, which also developed in similar neurological and behavioral changes (58). In both these studies immunological analysis of the brain tissue showed anti streptococcal antibody deposition in deep cerebella nuclei and hippocampus.

A recent study by Brimberg et al. (59) observed neurobehavioral and immunological changes akin to SC and PANDAS in male Lewis rats following exposure to GAS antigens. Behavioral changes included impairment in handling food, traversing the narrow beam and obsessive-compulsive behavior (**Figure 1C**) (59). Lewis rats developed behavioral and neurological conditions similar to SC and PANDAS after passive transfer of serum from rats exposed to GAS infection (60). These studies showed elevated levels of antibodies against GAS M protein and cross-reactive antibodies against brain in the peripheral blood and brain, similar to antibodies present in SC and PANDAS patients (59, 60). Antibodies derived from GAS exposed animals have shown strong reactivity with D1 and D2 dopamine receptors and activated calcium/calmodulin-dependent protein kinase II signaling in brain tissue (59, 60). Similarly, *in vitro* studies demonstrated that monoclonal antibodies against N-acetyl-β-D-glucosamine and lysoganglioside GM1 induced the activity of calcium/calmodulin-dependent protein kinase II, which is potentially implicated as an important mediator of learning and behavior (56). Recent studies in C57BL/6, C57BL/6J, or SJL/J female mice following intranasal GAS challenge have demonstrated a breakdown in the Blood Brain Barrier (BBB) enabling the migration of GAS specific Th17 cells from nasal-associated lymphoid tissue to the brain, with the microglial activation and IgG deposition in the striatum (62, 67). Elevated levels of pro inflammatory cytokines including IL17A+ IFN-γ+ due to GAS autoimmunity disrupts the BBB to allow circulating autoantibodies and Th17 and Th1 cells to enter the brain, which targets neurons and trigger neurobehavioral changes (**Table 1**) (67, 68). In addition genetically modified mice lacking Th17 lymphocytes (SJL/J, ROR$\gamma t^{+/GFP}$ and ROR$\gamma t^{GFP/GFP}$ mice) have shown reduced BBB leakage, microglial activation, and antibody infiltration into the brain following intranasal challenge with GAS (**Figure 1B**). This demonstrates the importance of Th17 lymphocytes in BBB leakage and infiltration of autoantibodies into the brain tissue (67). Thus, rodent models are very useful for assessing the disease mechanisms associated with central nervous system to precisely determine sequential events following infection with GAS.

NEED FOR AN ANIMAL MODEL TO INVESTIGATE MULTIPLE COMPLICATIONS ASSOCIATED WITH ARF/RHD

Post streptococcal autoimmune sequelae is a multisystem disorder affecting multiple organs including heart, brain, joints, connective tissues and skin (5). The immunopathology due to autoimmune response defers between organs. In the heart it is due to the pathological process initiated by the cross-reactive anti-GAS antibodies and T cells against host proteins (69). In the brain the disease is associated with IgG deposition (**Figure 1F**) (70). Whereas, in joints the pathogenesis is due to the immune complexes that bind to the synovial membrane and/or collagen in joints (5), and erythema marginatum might be due to cross-reactivity of anti-GAS antibody with keratin (71) and subcutaneous nodules might be due to a delayed hypersensitivity against GAS antigens (5). ARF patients can develop a combination of clinical symptoms that can lead to serious consequences. Approximately 30% of the patients with ARF can suffer from both cardiac and neurobehavioral

complications (3). Moreover, due to the heterogeneity of ARF/RHD, an animal model might reflect a specific phenotype of the diverse complications from those observed in human disease. Therefore, an animal model which can reflect both cardiac and neurobehavioral conditions would be a remarkable advancement in ARF/RHD research, not only to investigate the pathophysiology but also to assess the safety and efficacy of vaccine candidates and treatment modalities. Furthermore, in compliance with more stringent animal welfare considerations (e.g., 3Rs rules', for **r**eplacement, **r**eduction and **r**efinement) determining different aspects of a disease in a single animal will minimize the number of animals needed for research (**Figure 1**). Recently we have achieved this goal by modeling both cardiac and neurobehavioral changes in Lewis rats and rats injected with GAS shown impairments in fine motor control, gait and balance and obsessive-compulsive behavior similar to SC and PANDAS together with functional and immunological changes previously observed in the RAV model (72). Moreover, post-streptococcal complications including RHD and neurobehavioral changes such as SC are prominent in females (10, 73–75), thus most of the studies on RHD have been conducted in female mice or rats. However, neurobehavioral studies described in the literature have either been conducted on male or female mice but solely on male rats. Our recent observations demonstrated that there were no significant difference in using both genders of Lewis rats to simultaneously model carditis and neurobehavioral changes (72). To further validate multiple complications associated with ARF/RHD, more studies are warranted on the Lewis rat model.

LIMITATIONS OF ARF/RHD ANIMAL MODELS

While significant advances in animal models of ARF/RHD have been made in the last decade, there is still a paucity in pre-clinical studies on other complications associated with ARF/RHD including neurobehavioral changes, arthritis and skin manifestations. Arthritis is observed in ∼50–70% of patients with ARF and is a major Jones Criterion for the diagnosis of ARF. However, none of the animal studies have investigated GAS induced autoimmune process in subcutaneous tissue and joint tissue in any of these models.

CONCLUSION

Laboratory models are important to determine the early events leading to chronic disease. In particular when clinical studies are not possible during the early stages. In a credible animal model symptoms of physiological, anatomical and behavioral conditions must be comparable to those observed in human disease. In addition, an animal model should be reliable and the changes observed must be reproducible across laboratories. An animal model of ARF/RHD and associated neurobehavioral complications should possess functional and pathological changes encompassing motor deficits as well as compulsive and stereotyped behaviors similar to SC. Since genotypes, sex and age difference affects the development of autoimmune complication; selection of an appropriate animal model is important to investigate the pathogenesis of ARF/RHD and associated complications. Several animal models have been tested to investigate the onset, and progression of ARF/RHD. The Lewis rat model characterized by us and others, is a reliable model to investigate early events that lead to cardiac valvular pathology. Together with advances in novel imaging technologies and integrated computational approaches our model will provide the means to address these challenges (76). Importantly, comparison of experimental results with clinical observations to extrapolate the sequential event that follow infection with GAS leading to autoimmune complications requires prudence and caution.

AUTHOR CONTRIBUTIONS

RAMR, SS, ASH, NMA, KSS, DJM, and NK wrote the main manuscript. All authors have read and approved the manuscript.

ACKNOWLEDGMENTS

RAMR is recipient of International Postgraduate Research Award (IPRA) from University of New England.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cardiovascular Medicine

ORIGINAL RESEARCH
published: 11 May 2021
doi: 10.3389/fcvm.2021.643317

StreptInCor, a Group A Streptococcal Adsorbed Vaccine: Evaluation of Repeated Intramuscular Dose Toxicity Testing in Rats

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Streptococcus pyogenes infections continue to be a worldwide public health problem, causing various diseases in humans, with rheumatic fever and rheumatic heart disease being the most harmful manifestations. Impetigo and post-streptococcal glomerulonephritis are also important sequelae of skin infections. We have developed a candidate vaccine epitope (StreptInCor) that presents promising results in diverse animal models. To assess whether the StreptInCor alum-adsorbed vaccine could induce undesirable effects, a certified independent company conducted a repeated intramuscular dose toxicity evaluation in Wistar rats, a choice model for toxicity studies. We did not observe significant alterations in clinical, hematological, biochemical, anatomical, or histopathological parameters due to vaccine administration, even when the animals received the highest dose. In conclusion, repeated intramuscular doses did not show signs of macroscopic or other significant changes in the clinical or histopathological parameters, indicating that StreptInCor can be considered a safe candidate vaccine.

Keywords: ***S. pyogenes*, vaccine, safety, histopathology, toxicity**

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Edited by:
Kadaba Sriprakash, QIMR Berghofer Medical Research Institute, Australia

Reviewed by:
Manisha Pandey, Griffith University, Australia
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Specialty section:
This article was submitted to Heart Valve Disease, a section of the journal Frontiers in Cardiovascular Medicine

Received: *17 December 2020*
Accepted: *22 March 2021*
Published: *11 May 2021*

Citation:
de Sá-Rocha LC, Demarchi LMMF, Postol E, Sampaio RO, de Alencar RE, Kalil J and Guilherme L (2021) StreptInCor, a Group A Streptococcal Adsorbed Vaccine: Evaluation of Repeated Intramuscular Dose Toxicity Testing in Rats. Front. Cardiovasc. Med. 8:643317. doi: 10.3389/fcvm.2021.643317

INTRODUCTION

Streptococcus pyogenes, also known as group A streptococcus (GAS), is a Gram-positive bacterium responsible for some human diseases, such as pharyngitis, impetigo, and post-streptococcal complications, including glomerulonephritis and rheumatic fever (RF) and severe sequelae of rheumatic heart disease (RHD). The highest incidence of infections by GAS occurs in children between 5 and 15 years of age, especially in children living in poor sanitation conditions (1). Bowen et al. performed a systematic review and estimated that more than 162 million children younger than 15 years had impetigo and the broader condition pyoderma in low-income and low-middle-income countries (2). A meta-analysis study conducted by Shaikh et al. estimated that the prevalence of GAS infection among children of all ages who present with sore throat was 37%, and in those younger than 5 years, it was 24%. The prevalence of GAS carriage among well children with no signs or symptoms of pharyngitis was 12% (3). A recent study reported the occurrence of ∼319,000 deaths from RHD-related complications. Although the health-related burden of RHD has declined worldwide, high disease rates persist in the world's poorest regions (1).

RF generally develops after the occurrence of untreated streptococcal pharyngitis and affects ∼0.3–3% of susceptible individuals. Among the manifestations of RF, RHD is the most important sequelae and affects 45% of RF patients, and it is capable of causing serious damage to the aortic and mitral valves, leading to cardiac dysfunction (4). In Brazil, RHD accounts for 90% of cardiac surgeries in children (5).

Although not fully elucidated, RF/RHD develops from the involvement of autoimmune mechanisms mediated by molecular mimicry in genetically predisposed individuals. Genetic susceptibility is related to several gene polymorphisms of placebo innate and adaptive immune responses (6–8).

In the case of RHD, the activation of T and B lymphocytes may lead to the production of antibodies and T lymphocytes reactive against some epitopes of the N-terminal region of the M protein, which may react against myocardial and valvar tissue proteins due to their similarity to pathogen epitopes (9–13).

GAS strains are currently identified by sequencing the hypervariable N-terminal region of the *emm* gene encoding the M protein, and they have already been classified into more than 220 *emm* types, with some identified as rheumatogenic strains (14–16). These findings suggest that not all strains contain the epitopes capable of triggering RF/RHD but are probably involved with other manifestations, such as impetigo or glomerulonephritis.

Since the pioneering studies conducted by Rebecca Lancefield in 1962, the M protein has been the target of choice for a streptococcal vaccine (17). The M protein is formed by two coil-coiled chains with a flexible structure located on the bacterial wall and represents an important virulence factor that is highly immunogenic. Unlike the polymorphic N-terminal region of the M protein, the C-terminal region is highly conserved among the different serotypes (15, 18).

Vaccines based on both the N- and C-terminal regions of the M protein have been proposed in the last 14 years using new technologies, such as recombinant proteins or synthetic peptides (19–22).

The present work focused on StreptInCor, a candidate vaccine that contains 55 synthetic amino acid residues of the C-terminal region of the M protein. The formulation of StreptInCor with aluminum hydroxide (alum) was tested in inbred, outbred, and transgenic mice harboring human HLA class II alleles. High titers of specific antibodies and no cross-reactions with cardiac proteins or deleterious effects in vaccinated animals were observed (23–25).

The aim of the present toxicology work was to verify whether the candidate vaccine triggers toxic effects and identify target organs and deleterious effects on animal physiology and hematological, biochemical, anatomical, and histopathological parameters.

MATERIALS AND METHODS

Repeated intramuscular (IM) dose toxicity tests under conditions of good laboratory practice (GLP) and following national (26) and international standards were conducted by TECAM - Tecnologia Ambiental (São Roque, SP, Brazil), and they were identified as 5517/2015IM, protocol 27 (27, 28). All procedures were in accordance with national guidelines of the National Animal Experiment Placebo Council (CONCEA) and Committee for Animal Care and Use (COBEA) and international requirements based on the "Guide for the Care and Use of Laboratory Animals" (29).

The Wistar rats were kept in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and Credenciamento Institucional para Atividades com Animais em Ensino ou Pesquisa (CIAEP)/CONCEA under number 01.0242.214.

The experimental design of the rat IM toxicity tests is shown in **Figures 1**, **2**.

StreptInCor Candidate Vaccine

PolyPeptide Laboratories Inc. (Torrance, CA, USA) manufactured the synthetic peptide StreptInCor based on good manufacturing practice (GMP) standards. The vaccine formulation was prepared at Butantan Institute (São Paulo, Brazil). StreptInCor in different doses are emulsified in aluminum hydroxide as adjuvant, as follows: lot 15055 (50 μg/ml, low dose); lot 15056 (100 μg/ml, medium dose); lot 15057 (200 μg/ml, high dose); and lot 15054 (placebo formulation).

Animals

Outbred male and female Wistar rats (*Rattus norvegicus*) aged 7–9 weeks were bred at TECAM - Tecnologia Ambiental.

Both acute IM toxicity tests and repeated IM dose toxicity tests were evaluated. Only rats deemed healthy after evaluation by a veterinarian and without any clinical manifestation were selected for the study. The animals were acclimated 5 days before the studies and kept in monitored rooms at average temperature and humidity conditions of 20.8°C and 71.2%, respectively. The rats received species-specific rations (Nuvilab CR-1, Quimtia S.A., Colombo, PR, Brazil) and filtered water *ad libitum*. They were maintained in a 12-h light/dark cycle with ∼10–12 air changes per hour.

Administration of the StreptInCor Candidate Vaccine

The candidate vaccine was formulated at the concentrations of 50, 100, and 200 μg/ml of StreptInCor, plus alum (0.5 mg/ml of aluminum hydroxide), thimerosal (0.004 mg/ml), and phosphate buffered saline solution.

Previously identified animals received one IM injection of 250 μl of StreptInCor or placebo solution on the thigh muscles of their hind limbs, which had been trichotomized.

The toxicity due to acute immune response was evaluated in Experimental Group I, which included 50 Wistar rats (25 males and 25 females). On day 0, the rats received only one IM injection of 250 μl of StreptInCor with different concentrations, as follows: 50 μg/ml (low dose) (five males and five females); 100 μg/ml (medium dose) (five males and five females); and 200 μg/ml (high dose) (10 males and 10 females). Animals of placebo subgroup (five males and five females) received only one IM injection of 250 μl of a saline plus aluminum hydroxide solution, on day 0.

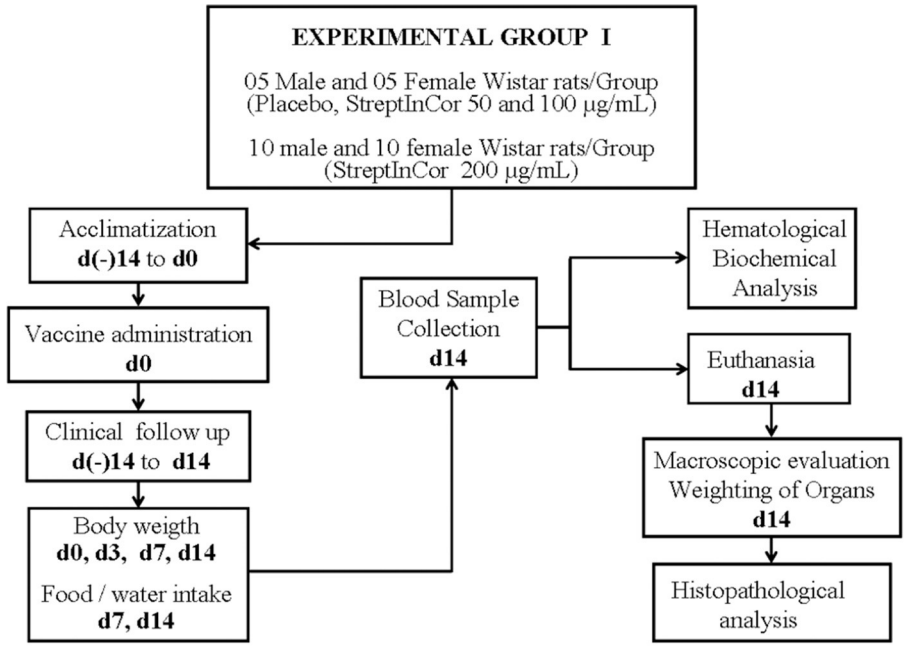

FIGURE 1 | Experimental design.

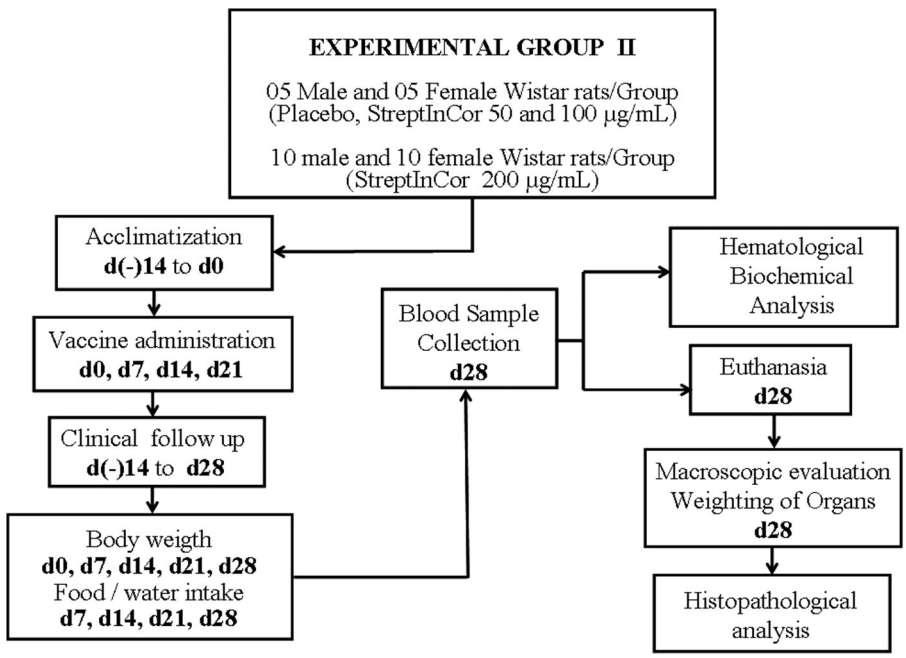

FIGURE 2 | Experimental design.

The toxicity effect of StreptInCor repeated doses was evaluated in Experimental Group II, which included 50 Wistar rats (25 males and 25 females). On days 0, 7, 14, and 21, the animals received one IM injection of 250 µl of StreptInCor with different concentrations, as follows: 50 µg/ml (low dose) (five males and five females); 100 µg/ml (medium dose) (five males and five females); and 200 µg/ml (high dose) (10 males and 10 females). Rats of placebo subgroup (five males and five females) received one IM injection of 250 µl of a saline plus aluminum hydroxide solution, on days 0, 7, 14, and 21.

A recovery group of rats underwent the same protocol of the Experimental Group II protocol and recovered for 2 weeks before euthanasia on day 42. They underwent two additional measurements for body weight and food consumption, on days 35 and 42.

Clinical Follow-Up

The animals were individually monitored throughout the acclimation as well as during the experimental period. Experienced veterinarians classified the severity of clinical signs as mild, moderate, or severe based on physical examination. Clinical observations included changes in the fur, skin, eyes, and mucous membranes; occurrence of secretions and excretions; and autonomic activity (lacrimation, piloerection, changes in the pupils, and respiratory pattern). The veterinarians were also vigilant for possible changes in gait, posture, and reaction to manipulation as well as the presence of tonic or clonic movements and stereotypies, such as excessive grooming, repetitive circulatory movements, self-mutilation, and walking backwards. Finally, local tolerance was assessed by the presence of edema, erythema, desquamation, wounding, alopecia, and any other signs of local irritation and/or inflammation.

The body weights of the animals were measured on days 0, 3, 7, and 14 for Group I and on days 0, 7, 14, 21, and 28 for Group II. Food consumption was measured on days 7 and 14 for Group I and on days 7, 14, 21, and 28 for Group II. The recovery group had two additional measurements for body weights and food consumption (days 35 and 42).

Euthanasia

All animals were sedated by an IM injection of a combination of xylazine (8 mg/kg) and ketamine (65 mg/kg). Blood samples were collected for hematological and biochemical analyses through cardiac puncture after deep anesthesia and prior to euthanasia.

TABLE 1 | Hematology data from Wistar rats vaccinated with StreptInCor at different doses (50, 100 or 200 μg/mL) compared with placebo.

Males	**Placebo**	**50 μg/mL**	**100 μg/mL**	**200 μg/mL**
RBC (10^{12}/L)	9.36 ± 0.14	7.78 ± 0.13*	8.34 ± 0.12	8.26 ± 0.20
Hemoglobin (g/L)	172.60 ± 6.33	145.80 ± 2.52	150.60 ± 2.77	154.50 ± 4.33
Hematocrit (fraction)	0.52 ± 0.02	0.44 ± 0.01	0.46 ± 0.01	0.45 ± 0.01
MCV (fL)	55.29 ± 1.51	56.40 ± 1.02	54.92 ± 1.06	54.89 ± 0.73
MCH (pg)	18.43 ± 0.50	18.76 ± 0.28	18.07 ± 0.45	18.70 ± 0.18
MCHC (g/L)	333.30 ± 0.21	332.70 ± 2.04	329.00 ± 2.23	340.80 ± 2.33
Platelets (10^9/L)	910.80 ± 116.40	913.50 ± 51.40	856.20 ± 85.00	773.90 ± 123.00
Leukocytes (10^9/L)	6.03 ± 0.75	5.50 ± 0.35	7.46 ± 0.76	5.36 ± 0.42
Lymphocytes(10^9/L)	4.97 ± 0.63	4.52 ± 0.32	6.14 ± 0.67	4.26 ± 0.33
Monocytes (10^9/L)	0.13 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.10 ± 0.01
Granulocytes (10^9/L)	0.94 ± 0.16	0.86 ± 0.05	1.19 ± 0.15	1.01 ± 0.11
Lymphocytes (fraction)	0.82 ± 0.02	0.82 ± 0.01	0.82 ± 0.02	0.80 ± 0.02
Monocytes (fraction)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Granulocytes (fraction)	0.15 ± 0.02	0.16 ± 0.01	0.16 ± 0.02	0.19 ± 0.02
Females	**Placebo**	**50 μg/mL**	**100 μg/mL**	**200 μg/mL**
RBC (10^{12}/L)	7.65 ± 0.01	7.77 ± 0.26	7.53 ± 0.19	7.78 ± 0.19
Hemoglobin (g/L)	141.20 ± 1.24	142.00 ± 3.05	136.60 ± 2.98	144.00 ± 2.70
Hematocrit (fraction)	0.42 ± 0.01	0.43 ± 0.01	0.41 ± 0.01	0.43 ± 0.01
MCV (fL)	54.54 ± 0.18	55.24 ± 0.72	54.99 ± 0.38	55.10 ± 0.58
MCH (pg)	18.46 ± 0.13	18.29 ± 0.27	18.15 ± 0.11	18.54 ± 0, 21
MCHC (g/L)	338.50 ± 1.88	331.20 ± 1.27	330.10 ± 1.65	336.60 ± 1.78
Platelets (10^9/L)	802.00 ± 48.14	947.40 ± 93.40	769.60 ± 52.30	800.80 ± 112.20
Leukocytes (10^9/L)	4.08 ± 0.37	7.40 ± 0.47*	2.44 ± 0.38	4.15 ± 0.30
Lymphocytes (10^9/L)	3.16 ± 0.30	6.00 ± 0.40*	1.78 ± 0.34	3.41 ± 0.29
Monocytes (10^9/L)	0.09 ± 0.01	0.13 ± 0.01	0.06 ± 0.00	0.06 ± 0.00
Granulocytes (10^9/L)	0.84 ± 0.17	1.26 ± 0.07	0.60 ± 0.06	0.68 ± 0.03
Lymphocytes (fraction)	0.77 ± 0.03	0.81 ± 0.05	0.71 ± 0.05	0.81 ± 0.01
Monocytes (fraction)	0.02 ± 0.00	0.02 ± 0.00	0.024 ± 0.00	0.02 ± 0.00
Granulocytes (fraction)	0.21 ± 0.03	0.17 ± 0.00	0.27 ± 0.05	0.17 ± 0.01

*Each value represents the mean ± SEM of placebo, StreptInCor at 50, 100 and 200 μg/mL. RBC, red blood cells; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration. Dunn's Multiple Comparison test: *significant differences: males- StreptInCor (50 μg/mL) and placebo ($p < 0.05$) and leukocytes and lymphocytes from females treated with StreptInCor (50 μg/mL) and placebo ($p < 0.05$).*

The veterinarians euthanized the rats by exsanguination after confirmation of deep anesthesia. After 2 weeks (day 14) and 4 weeks (day 28), rats from Groups I and II were euthanized, respectively. Rats from recovery group were euthanized after 6 weeks, on day 42.

Hematological and Biochemical Analyses

Hematological tests, including the red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), white blood cell count, and platelet count were evaluated using HEMATOClin 2.8 Vet (QUIBASA Bioclin, Brazil).

Commercial kits for biochemical parameters were used to determine the serum levels of glucose, total cholesterol, urea, creatinine, alanine aminotransferase, aspartate aminotransferase, albumin, globulin, total protein, sodium, chloride, and potassium. The results were obtained by spectrometry in liquid medium by using the semiautomatic TP Analyzer (Thermo-Plate, Brazil).

Necropsy, Wet Organ Weight, and Histopathological Analysis

Necropsies of all the animals were performed by veterinarians who collected tissue samples for histological analysis. Organs such as the brain, liver, kidneys, adrenal glands, heart, gonads (testis and epididymis or ovary), thymus, and spleen were collected; and their relative organ weight (weight of organ as a percentage of the total body weight of each rat) was calculated and compared with the relative organ weight of the placebo group rats. After macroscopic evaluation, tissue samples of the brain, lung, stomach, esophagus, small intestine, large intestine, liver, pancreas, kidney, adrenal glands, heart, gonad (testis or ovary), urinary bladder, femur, thymus, spleen, and lymph nodes were fixed in 10% buffered formalin. Fixed samples were dehydrated in alcohol baths in a concentration gradient, cleared in xylene, and embedded in paraffin blocks. Five micro sections were cut and mounted on slides and stained by the hematoxylin and eosin method.

Statistical Analysis

For each parameter analyzed, the data are expressed as the mean ± the standard error of the mean (SEM) between the different animals of each group. All data were examined by the D'Agostino and Pearson omnibus normality tests to verify whether the data could be analyzed by parametric tests. When the data passed the normality test, we employed a one-way analysis of variance and Tukey's post-test to compare all pairs of columns. If the data did not pass the normality test, we used the Kruskal–Wallis test and Dunn's post-test to compare all pairs of columns. We used GraphPad Prism software version 5.01 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) to analyze the data, and p-values < 0.05 were considered significant.

RESULTS

Clinical signs of toxicity or intolerance at the site of administration and mortality were not observed during the

TABLE 2 | Biochemistry data from Wistar rats vaccinated with repeated injections of StreptInCor at 50, 100 or 200 μg/mL doses compared with placebo.

Males	**Placebo**	**50 μg/mL**	**100 μg/mL**	**200 μg/mL**
Urea (mmol/L)	16.94 ± 1.91	15.60 ± 0.46	21.49 ± 1.15	16.26 ± 0.86
Creatinine (μmol/L)	42.43 ± 3.92	29.88 ± 8.64	57.11 ± 16.32	37.66 ± 3.18
AST (IU/L)	125.20 ± 15.18	78.40 ± 18.79	115.90 ± 12.64	115.40 ± 14.50
ALT (IU/L)	30.46 ± 8.72	27.94 ± 5.48	34.78 ± 1.72	32.39 ± 2.67
Glucose (mmol/L)	9.94 ± 1.63	12.57 ± 0.77	12.39 ± 1.34	12.43 ± 0.57
Cholesterol (mmol/L)	2.24 ± 0.42	1.85 ± 0.11	1.78 ± 0.05	1.75 ± 0.10
Albumin (g/L)	31.30 ± 0.86	34.46 ± 1.59	29.76 ± 1.11	29.09 ± 0.57
Globulin (g/L)	38.06 ± 3.81	41.08 ± 4.18	43.22 ± 5.39	35.66 ± 1.46
Total Protein (g/L)	69.36 ± 4.67	75.54 ± 5.58	72.98 ± 6.48	64.75 ± 1.97
Females	**Placebo**	**50 μg/mL**	**100 μg/mL**	**200 μg/mL**
Urea (mmol/L)	21.32 ± 1.40	16.72 ± 1.15	13.94 ± 1.51	15.79 ± 1.08
Creatinine (μmol/L)	47.38 ± 8.03	56.22 ± 2.45	56.40 ± 5.67	55.13 ± 3, 0
AST (IU/L)	125.60 ± 16.28	125 ± 60, 25.53	109.20 ± 6.15	81.28 ± 5.04
ALT (IU/L)	26.00 ± 4.91	31.52 ± 3.32	23.76 ± 4.96	30.71 ± 1.45
Glucose (mmol/L)	9.41 ± 0.55	10.73 ± 1.09	10.07 ± 0.67	11.19 ± 0.58
Cholesterol (mmol/L)	1.68 ± 0.09	1.74 ± 0.13	1.38 ± 0.15	1.83 ± 0.10
Albumin (g/L)	33.20 ± 1.32	40.44 ± 8.62	31.30 ± 0.91	33.27 ± 0.82
Globulin (g/L)	33.92 ± 2.62	30.46 ± 1.86	30.94 ± 1.37	32.27 ± 0.82
Total Protein (g/L)	67.12 ± 3.88	70.90 ± 8.68	62.24 ± 2.07	65.54 ± 1.47

Each value represents the mean ± SEM of placebo, StreptInCor at 50, 100, and 200 μg/mL. AST, aspartate aminotransferase; ALT, alanine aminotransferase. Dunn's Multiple Comparison test, no statistically significant differences were observed.

TABLE 3 | Relative organ weights from the male and female Wistar rats after administration of StreptInCor at 50, 100, 200 μg/mL doses or placebo.

Males	**Placebo**	**50 μg/mL**	**100 μg/mL**	**200 μg/mL**
Heart	0.33 ± 0.02	0.30 ± 0.01	0.30 ± 0.01	0.31 ± 0.02
Liver	3.36 ± 0.40	3.70 ± 0.14	3.85 ± 0.15	3.75 ± 0.93
Kidney	0.71 ± 0.05	0.67 ± 0.03	0.69 ± 0.03	0.71 ± 0.02
Adrenal	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
CNS	0.64 ± 0.07	0.57 ± 0.02	0.58 ± 0.01	0.58 ± 0.03
Spleen	0.20 ± 0.02	0.24 ± 0.01	0.20 ± 0.01	0.21 ± 0.01
Thymus	0.16 ± 0.01	0.20 ± 0.02	0.18 ± 0.01	0.16 ± 0.01
Epydidimis+Testicle	1.24 ± 0.08	1.57 ± 0.22	1.60 ± 0.15	1.58 ± 0.09
Females	**Placebo**	**50 μg/mL**	**100 μg/mL**	**200 μg/mL**
Heart	0.32 ± 0.01	0.35 ± 0.03	0.33 ± 0.01	0.32 ± 0.01
Liver	3.64 ± 0.16	3.82 ± 0.13	3.48 ± 0.13	3.47 ± 0.13
Kidney	0.76 ± 0.01	0.75 ± 0.02	0.76 ± 0.04	0.78 ± 0.02
Adrenal	0.03 ± 0.01	0.03 ± 0.01	0.048 ± 0.01	0.36 ± 0.00
CNS	0.85 ± 0.02	0.79 ± 0.03	0.86 ± 0.13	0.84 ± 0.02
Spleen	0.23 ± 0.01	0.23 ± 0.01	0.23 ± 0.01	0.25 ± 0.01
Thymus	0.19 ± 0.03	0.22 ± 0.02	0.24 ± 0.00	0.21 ± 0.01
Ovary	0.08 ± 0.01	0.07 ± 0.01	0.10 ± 0.03	0.07 ± 0.01

Each value represents the mean ± SEM between rats immunized with StreptInCor 50, 100, and 200 μg/mL and placebo. No statistically significant differences were observed by Dunn's Multiple Comparison test.

acute toxicity study. In addition, the animals used in this experiment showed body weight gains from 220 to 300 g during acute phase (Group I) and 180 to 250 g during chronic phase (Group II) consistent with the observed food intake.

Animals from all groups displayed chronic granulomatous inflammation in skeletal muscle at the site of injection: 66.7% of the animals in the placebo (two males and two females); and 66.7% (two males and two females), 16.7% (one female), and 50.0% (two males and one female) of the animals treated with 50, 100, and 200 μg of StreptInCor, respectively. Considering that animals from all groups studied in the acute toxicity test showed good tolerance to StreptInCor, we maintained the same concentrations to evaluate the repeated dose toxicity.

Clinical Follow-Up

No mortality or clinical signs related to the injection of StreptInCor at 50, 100, or 200 μg/ml were recorded. Food intake and water intake were similar between the placebo and experimental groups. In addition, the weight gains were similar among animals of all groups, being around 220 g in placebo and 150 g in immunized animals.

Hematological and Biochemical Analyses

The repeated injections at different doses of the candidate vaccine did not interfere markedly with the hematology data; however, we observed that erythrocytes from the male rats that received 50 μg/ml differed from those obtained in the placebo group ($p < 0.05$). On the other hand, females vaccinated with 50 μg/ml presented an increased leukocyte count ($p < 0.05$) than the placebo group due to an increased number of lymphocytes ($p < 0.05$) (**Table 1**).

Metabolic alterations due to the injection of placebo or the different doses of StreptInCor candidate vaccine were not observed (**Table 2**).

Necropsy, Wet Organ Weight, and Histopathological Analysis

No macroscopic alterations were observed. The organ wet weights as well as the relative weights were similar among the experimental groups and placebo animals (**Table 3**).

The main histological finding observed in all experimental groups was chronic granulomatous inflammation in the skeletal muscle at the site of injection in both the placebo and immunized animals, as follows: 70% of rats in the placebo group (four males and three females); and 70% (four males and three females), 40% (one male and three females), and 65% (six males and seven females) of animals that received 50, 100, and 200 μg of StreptInCor, respectively.

Histological analysis of the other tissues did not show alterations compared with that of placebo animals, and this response was dependent of the StreptInCor dose (**Figures 3**, **4**).

DISCUSSION

The present work is part of a set of experiments performed to develop an effective and safe GAS vaccine capable of inducing protection against oropharyngeal infections, RF/RHD, necrotizing fasciitis, and toxic shock syndrome. *Streptococcus pyogenes* is a strictly human pathogen and normally does not induce disease in animals. This fact causes some difficulties to

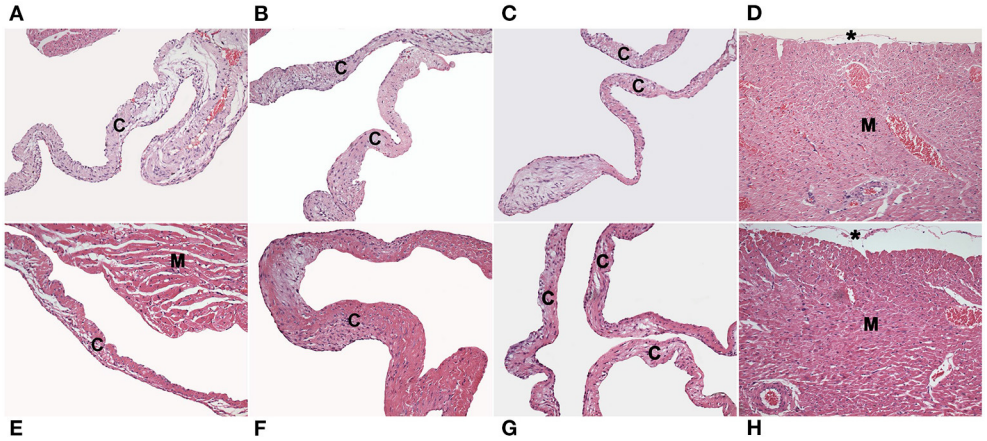

FIGURE 3 | Photomicrographs of histological sections of the tricuspid **(A)**, mitral **(B)** and aortic **(C)** valves, and myocardium **(D)** from Wistar rats injected with placebo. The tricuspid **(E)**, mitral **(F)** and aortic **(G)** valves, and myocardium **(H)** from rats injected with a high dose of StreptInCor (200 μg/ml). There is no necrosis, inflammatory infiltrate, neovascularization, or fibrosis in the valve cusps (c), myocardium (M), or epicardium (*). Hematoxylin and eosin staining, ×200.

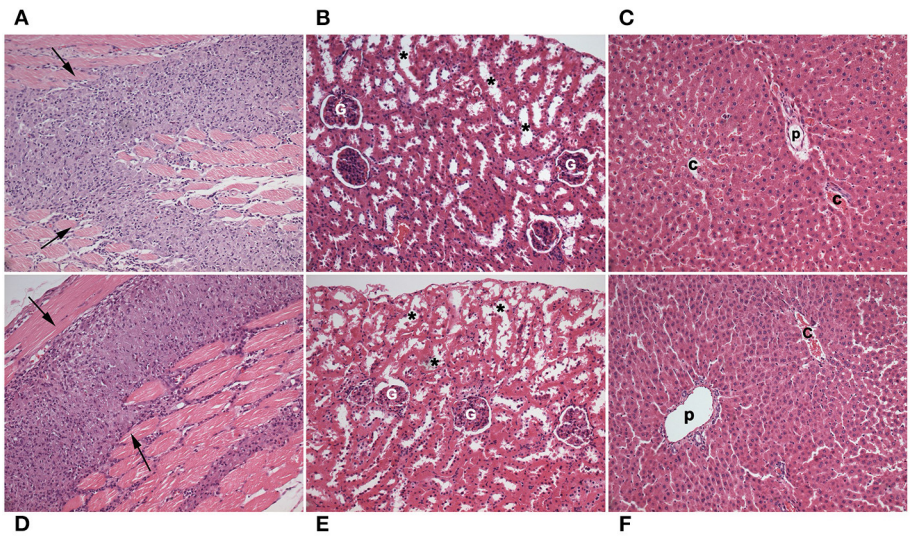

FIGURE 4 | Photomicrographs of histological sections of skeletal muscle **(A)**, kidney **(B)**, and liver **(C)** from Wistar rats injected with placebo, and of skeletal muscle **(D)**, kidney **(E)**, and liver **(F)** from rats injected with high dose of StreptInCor (200 μg/mL). Chronic granulomatous inflammation (arrows) in skeletal muscle at the site of placebo **(A)** and StrepInCor **(D)** injections. There are not necrosis, inflammatory infiltrate, neovascularization, or fibrosis in renal or hepatic tissues. Hematoxylin and eosin staining, ×200. **(G)** Glomerulus; (*) proximal tubules; (p) portal tract; (c) centrilobular hepatic vein.

develop an ideal animal model. Despite of it, encouraging results from studies with mice (Swiss, Balb-c, and HLA class II Tg mice) (23–25) and minipigs (30) showed that StreptInCor candidate vaccine is safe.

In this study, we conducted non-clinical safety tests to determine whether StreptInCor candidate vaccine could induce adverse reactions in other animal species.

Because no adverse effects or mortality were observed at any of the concentrations of StreptInCor (50, 100, and 200 μg/ml) in the acute toxicity study (single dose, 14 days), the same formulations were tested in the repeated dose toxicity studies in two species, non-rodents (30) and rodents (Wistar rats), as described here.

No mortality or major changes related to feeding, hydration, or weight gain were observed among the animals. The most important difference in relation to the placebo group was the increased number of leukocytes (lymphocytes, monocytes, and granulocytes) in the females that received 50 μg of StreptInCor.

Repeated immunizations at intervals of 7 days are indicated for evaluation of toxicity effects. Of note, this protocol is not adequate for the evaluation of humoral response (31, 32).

Macroscopic analysis of tissues and organs showed no toxicological effects of the administration of StreptInCor in Wistar rats. The microscopic examination showed the presence of chronic granulomatous inflammation at the site of the StreptInCor and placebo injections (skeletal muscle) due to the presence of the adjuvant (aluminum hydroxide) in the formulations (33).

Adjuvants are important for enhancing the specific immune response against antigens in vaccines. Aluminum hydroxide induces the migration of neutrophils and eosinophils as well as dendritic cells and monocytes/macrophages to the site of inoculation (34, 35). Eosinophils are able to secrete preformed IL-4 and may play an immunomodulatory role in the activation of B cells elicited by aluminum hydroxide (36). Dendritic cells and macrophages function as inducers of adaptive immunity by presenting antigens to T cells (33, 37).

A major concern with the development of an M protein-based GAS vaccine is the possibility of inducing an autoimmune response, such as RF/RHD. Antibodies raised against the N-terminal region have been linked to RF/RHD pathogenesis (4, 18, 38). The C-terminal region that is conserved did not induce cross-reactive antibodies. In this context, in previous studies on isogenic, outbred, and HLA class II-transgenic mice immunized with StreptInCor (24, 25), no heart-tissue cross-reactive antibodies or deleterious reactions in the heart (myocardium and valves) or other tissues were observed. The repeated dose toxicity test performed on Wistar rats presented here showed no signs of toxic reaction in the heart or any of the organs evaluated after the administration of the StreptInCor candidate vaccine.

CONCLUSIONS

The results of repeated StreptInCor candidate injection in doses ranging from 50 to 200 μg/ml on Wistar rats presented here indicate that the StreptInCor candidate vaccine is non-toxic and well-tolerated in rodent animal models.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by National Animal Experiment Control Council (CONCEA) and Committee for Animal Care and Use (COBEA) and international requirements based on the Guide for the Care and Use of Laboratory Animals.

AUTHOR CONTRIBUTIONS

LS-R, EP, and RA: performed animal experimentation and analysis of data. RS: contributed with manuscript preparation and analysis of results. LD: histopathology analysis and manuscript preparation. JK: director of Immunology lab. LG: analysis of the data and manuscript preparation. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from Banco Nacional de Desenvolvimento (BNDES), No. 14.2.1159.1, 2015–2020, Brazil.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cardiovascular Medicine

PERSPECTIVE
published: 14 May 2021
doi: 10.3389/fcvm.2021.674805

In Search of the Holy Grail: A Specific Diagnostic Test for Rheumatic Fever

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Specialty section:
This article was submitted to General Cardiovascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: 02 March 2021
Accepted: 22 April 2021
Published: 14 May 2021

Citation:
McMillan DJ, Rafeek RAM, Norton RE, Good MF, Sriprakash KS and Ketheesan N (2021) In Search of the Holy Grail: A Specific Diagnostic Test for Rheumatic Fever. Front. Cardiovasc. Med. 8:674805. doi: 10.3389/fcvm.2021.674805

Current diagnosis of Acute Rheumatic Fever and Rheumatic Heart Disease (ARF/RHD) relies on a battery of clinical observations aided by technologically advanced diagnostic tools and non-specific laboratory tests. The laboratory-based assays fall into two categories: those that (1) detect "evidence of preceding streptococcal infections" (ASOT, anti-DNAse B, isolation of the Group A *Streptococcus* from a throat swab) and (2) those that detect an ongoing inflammatory process (ESR and CRP). These laboratory tests are positive during any streptococcal infection and are non-specific for the diagnosis of ARF/RHD. Over the last few decades, we have accumulated considerable knowledge about streptococcal biology and the immunopathological mechanisms that contribute to the development, progression and exacerbation of ARF/RHD. Although our knowledge is incomplete and many more years will be devoted to understanding the exact molecular and cellular mechanisms involved in the spectrum of clinical manifestations of ARF/RHD, in this commentary we contend that there is sufficient understanding of the disease process that using currently available technologies it is possible to identify pathogen associated peptides and develop a specific test for ARF/RHD. It is our view that with collaboration and sharing of well-characterised serial blood samples from patients with ARF/RHD from different regions, antibody array technology and/or T-cell tetramers could be used to identify streptococcal peptides specific to ARF/RHD. The availability of an appropriate animal model for this uniquely human disease can further facilitate the determination as to whether these peptides are pathognomonic. Identification of such peptides will also facilitate testing of potential anti-streptococcal vaccines for safety and avoid potential candidates that may pre-dispose potential vaccine recipients to adverse outcomes. Such peptides can also be readily incorporated into a universally affordable point of care device for both primary and tertiary care.

Keywords: rheumatic fever, rheumatic heart disease, diagnostic test (MeSH), group A streptococcus, M protein

"*Despite the increase in knowledge of rheumatic fever, no specific diagnostic test has been forthcoming. This is a distinct deterrent to the advancement.…" T. Duckett Jones (1944)* (1)

INTRODUCTION

In 2005, the WHO estimated that annually over 700 million people worldwide suffered from two of the commonest forms of Group A Streptococcal (GAS) infection—GAS pharyngitis and GAS pyoderma. Of the over 500,000 annual deaths due to complications of GAS infections, well over 65% were attributed to rheumatic heart disease (RHD) (2, 3). Acute rheumatic fever (ARF) and RHD, which are autoimmune sequelae of GAS pharyngitis and/or pyoderma, have ceased to be major public health problems in high-income countries. However, in some of these countries it is still highly prevalent among Indigenous populations and occasional outbreaks of ARF/RHD occur among the wider population. In middle and low-income countries, which account for more than 80% of the world's population, poverty, household overcrowding, and poor access to timely medical care continue to be associated with high incidence/prevalence of this disease. Control efforts in these countries continue to be fraught with several confounding factors, namely paucity of accurate data, unavailability of preventive measures such as safe and effective vaccines, non-specific diagnostic tools, and lack of treatment options. The diagnosis of ARF is mostly based on a clinical algorithm initially described in 1944 (1) with later modifications. The current Modified Jones Criteria for diagnosis of ARF take into account as to whether the patient resides in a low, moderate or high -risk population (4) (**Figure 1**). Although, a specific unequivocal laboratory diagnostic test has been long envisioned and possibly within our reach, it is yet to be realised.

THE UTILITY OF THE JONES CRITERIA FOR DIAGNOSIS OF RHEUMATIC FEVER

Over seven decades have passed since Duckett Jones set forth a well-defined group of "major and minor criteria" for "the diagnosis of rheumatic fever" in his seminal publication (1). This was during the pre-antibiotic era when salicylates were the therapeutic agent of choice for treating ARF. These criteria were intended to be useful "until the aetiology of rheumatic fever is known or there is a specific diagnostic test." They were developed to avoid confusion and misdiagnosis of acute ARF/RHD and provide a rational basis to develop programs for prevention and patient care. Since then, the additions and modifications made to the original criteria, which now form the "Revised Jones Criteria" (4, 5) still do not prevent misdiagnosis (6–9).

In response to the falling incidence of ARF in the USA, changes were made to improve the specificity of the criteria at the expense of sensitivity. This resulted at times in an underdiagnosis of the disease in high-incidence populations. The consequences of under-diagnosis in these populations, in generally low resource environments, could be considerable and possibly greater than those of over-diagnosis. In 2006, the first version of the Australian Rheumatic Fever Guidelines incorporated additional criteria, and of subclinical carditis, aseptic monoarthritis and polyarthralgia as major manifestations in high-risk groups. Subsequently in 2012, monoarthralgia was included as a minor manifestation. In 2015, the American Heart Association (AHA) further revised the Jones criteria to separate moderate-high and low-risk populations, and to include echocardiography as a tool to diagnose cardiac involvement (4). They noted that the new guidelines aligned more closely with the Australian guidelines and these 2015 re-revised Jones criteria were endorsed by the World Heart Federation. Of the laboratory tests, in addition to a positive throat culture and elevated or rising titre of anti-streptolysin O (ASOT) which were described by Jones we have now added anti-DNase titre. However, these are non-specific laboratory tests that are used to determine an exposure to streptococcal infection and are of little use in the definitive diagnosis of ARF/RHD, particularly in regions where streptococcal infection is endemic (6–9). Therefore, a robust specific diagnostic test that can be used in the laboratory setting is required to overcome misdiagnosis of ARF/RHD. We need to acknowledge that the scientific information that has been accumulating over the last 75 years on the molecular and cellular mechanisms involved in the pathogenesis of the disease process have not translated to the development of a specific low-cost diagnostic test for ARF/RHD. Such a test could significantly contribute to the accuracy of ARF/RHD diagnosis, in particular in regions where ARF/RHD is still rampant. Indeed, the availability of such a specific diagnostic test could make the Jones Criteria redundant in the diagnosis of ARF/RHD as Duckett Jones himself may have intended.

PROBLEMS WITH THE CURRENT DIAGNOSIS OF ARF

In several countries, with increased awareness of hygiene and relatively low-density living conditions, the incidence of ARF has declined. However, for the majority of world populations living in socio-economically deprived conditions, the incidence of ARF is still high (4). Although in some countries ARF is a reportable disease, the data may greatly underestimate the incidence because of uncertainty in diagnosis or misdiagnosis.

Firstly, the Jones criteria, rely on a set of criteria that together are used of clinical diagnostic purposes. Several of these criteria are associated with other conditions and can lead to misdiagnosis. Another problem lies in the accepted association of GAS infection, solely in the setting of pharyngitis with ARF. Thirdly a lack of a specific diagnostic laboratory marker for ARF further hinders early confirmation of the diagnosis. Several epidemiological observations suggest that in some populations, skin infections may also pre-dispose to ARF (10). Furthermore, *Streptococcus dysgalactiae* subspecies *equisimilis* (SDSE) may also be an aetiological agent for ARF (11). Indeed, in many humid regions skin infection, and SDSE throat isolation rates are common. In such regions, the GAS isolation rates from throat swabs do not correlate with the reported high incidences of ARF

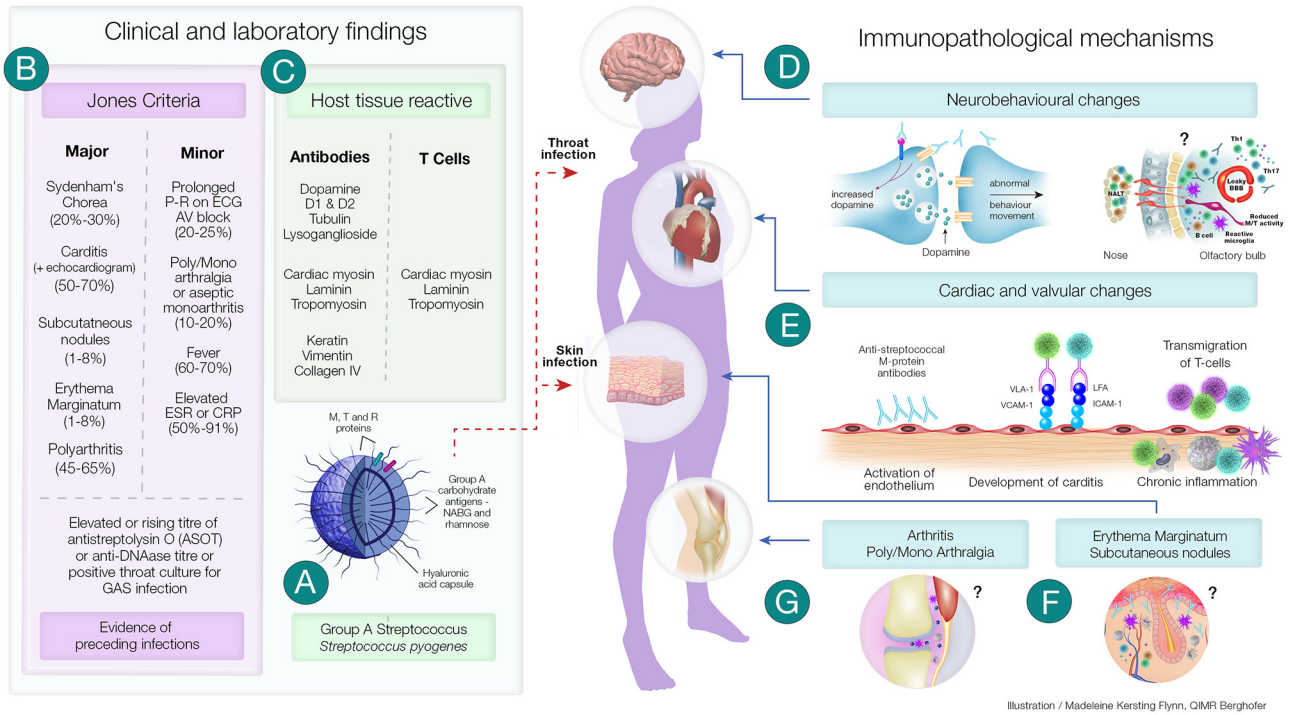

FIGURE 1 | Clinical and Immunopathological Features of Acute Rheumatic Fever and its Complications. **(A)** Group A streptococci (GAS) have several virulence factors that enables it cause a variety of superficial and deep-tissue infections. In a proportion of individuals following pharyngitis or pyoderma, one of the post-streptococcal autoimmune sequalae that can develop is acute rheumatic fever (ARF). **(B)** A group of universally accepted clinical manifestations and non-specific laboratory investigations form the Revised Jones Criteria. Laboratory investigations include evidence of recent streptococcal infection (as assessed by rising titre of anti Streptolysin O (ASOT) or anti-DNase titre or positive throat culture for streptococcal infection). Evidence of preceding streptococcal infection in addition to either two major or one major and two minor criteria confirms the diagnosis of ARF. **(C)** Both clinical and experimental studies have shown the presence of antibodies and $CD4^+$ T cells generated in ARF have the ability to cross-react with both streptococcal proteins and host tissue proteins. The autoimmune inflammatory process initiated by these antibodies and T cells lead to several of the immunopathological changes observed in ARF. **(D)** Sydenham's Chorea, a group of neurobehavioral abnormalities observed in ARF has been found to involve anti-dopamine, anti-β-tubulin and anti-lysoganglioside antibodies reacting with D1 and D2 dopamine receptors, signalling kinases and ion channels and causing these abnormalities. Recent studies have also shown that the $CD4^+$T cells may be involved in breaching the blood brain barrier and facilitating the entry of antibodies and inflammatory cells. **(E)** *Streptococcus* specific antibodies can upregulate VCAM-1 and ICAM-1 on vascular and valvular endothelial cell. Activation of these cells lead to transmigration of activated streptococci specific T-cells into heart tissue leading to cross-reactive responses with tissue proteins perpetuating inflammatory responses including neovascularisation and the appearance of granulomatous lesions in cardiac tissue. **(F)** Although direct experimental evidence is space, anti-streptococcal antibodies that cross-react with laminin, tropomyosin, vimentin and keratin in the skin may cause the characteristic rash—erythema marginatum observed in ARF. Furthermore, the formation of subcutaneous nodules may be due to delayed hypersensitivity type responses against streptococcal antigens. **(G)** Anti-streptococcal antibodies could also form immune complexes which bind to the synovial membrane and/or collagen in joints leading to inflammation of the synovial tissue causing arthralgia and arthritis. Repetitive streptococcal infections drive the autoimmune process leading to chronic inflammation and carditis, culminating in rheumatic heart disease and if untreated it is followed by congestive cardiac failure and death. ?Mechanisms not well-characterised; ICAM-1, Intercellular adhesion molecule-1; LFA, Leukocyte associated function antigen; Jones Criteria, (% of patients presenting with the specific feature); Th1 and Th17 $CD4^+$, T cell subsets; VLA-1, Very late antigen-1; VCAM-1, Vascular cell adhesion molecule-1.

(12, 13). Moreover, many subjects carry GAS and SDSE in their throat, which often could erroneously contribute to high throat isolation rates of these bacteria and may not have contributed to the pathogenesis of ARF. Notwithstanding the above difficulties, most clinicians adhere to the traditional revised Jones criteria to diagnose ARF.

Understanding ARF pathogenic mechanisms may help in identifying a suitable diagnostic marker for ARF. Unfortunately, GAS is a human-specific pathogen and ARF manifests only in humans. Fortunately, however, recent work using the Lewis Rat model for RHD is changing this scenario. Histopathological presentation of heart tissues in the rats injected with GAS M5 strain is akin to that observed in RHD patients (14–22). Since ARF precedes RHD, it is reasonable to assume that the RHD-like histological changes in Lewis Rats exposed to GAS may have progressed through stages that exhibit partly analogous manifestations to ARF in humans. One such major manifestation relates to neurobehavioral changes as in Sydenham's Chorea (SC). Indeed, in our recent study (23) we have also demonstrated neurobehavioral changes in rats injected with whole killed GAS. To develop a standalone specific laboratory diagnostic test for ARF/RHD, it is imperative that the early mechanisms leading to the development of the disease process is well-characterised.

THE AETIOPATHOGENESIS OF RHEUMATIC FEVER AND ITS COMPLICATIONS

The post-streptococcal autoimmune process that leads to ARF/RHD is multifactorial. Genetic pre-disposition, the type or strain of streptococcal pathogen, frequency of infection and the site of infection contribute to the disease process. However, the relative importance of each of these factors remains unknown. Although several studies in different regions showed that ARF/RHD may be linked to specific MHC antigens, both Genome Wide Association studies (24) and transcriptome based studies (25) are still in their infancy in ARF/RHD research and yet to provide a clear mechanistic pathway in disease pathogenesis (26).

Streptococcal pharyngitis and skin infections activate humoral and cell-mediated immune responses against streptococcal virulence factors. Some of the antibodies and T cells generated during the infection process cross-react with host tissue proteins, a hallmark of ARF/RHD immunopathogenesis. To develop a specific and low-cost diagnostic that can be used in ARF/RHD endemic countries, it is essential to have a good understanding of the molecular and cellular aetiopathogenesis of rheumatic fever and its complications. While epidemiological and clinical studies on patients with ARF/RHD have contributed to our understanding of the burden of this human-specific disease, decades of microbiological, immunological and animal studies have complemented the clinical finding by enabling hypothesis driven research to verify and understand the mechanisms involved in the multisystem clinical manifestations.

Antibodies generated against GAS M protein and *N*-acetyl-beta-D-glucosamine cross-react with cardiac tissue proteins. It has been demonstrated that monoclonal antibodies against these antigens, derived from patients with ARF (27), cross-react *in vitro* with human myosin and valvular endothelium. Furthermore, injection of recombinant streptococcal M protein induces autoantibody and autoreactive T cell that leads to carditis and valvulitis in the Lewis autoimmune valvulitis model (18). Antibodies and T cells derived from these rats also activate aortic endothelial cells in culture (21) facilitating transmigration of activated T cells across the endothelial barrier. These, observations have added further evidence that cross-reactive antibodies generated against streptococcal proteins that bind to tissue proteins is a major mechanism involved in the immunoinflammatory process observed in ARF/RHD leading to carditis (**Figure 1**). There is also evidence that structural similarities between tissue proteins, such as laminin and vimentin, could be the basis of antibody-mediated damage to valve structures. T-cell clones derived from valvular lesions from patients react with myosin and valve-derived proteins (28) and T cells from rats injected with GAS M protein release inflammatory cytokines upon exposure to these and streptococcal antigens *in vitro*. The Th1/Th17 inflammatory response may also facilitate epitope spreading within the valvular tissue and further expose other tissue antigens such as vimentin and collagen perpetuating and amplifying the inflammatory process (29). Chronic inflammation leads to characteristic changes observed in cardiac tissue in ARF/RHD including neovascularisation, giant cell formation and fibrosis.

There is considerable evidence that some streptococcal M protein N-terminus domains bind the CB3 region of Collagen type IV and initiate an autoantibody response to collagen establishing an inflammatory process leading to the spectrum of disease presentation observed in ARF/RHD (30). These autoantibodies do not cross-react with streptococcal M proteins (31, 32). The proponents of this concept view that the pathology in ARF/RHD points to the sub-endothelial collagen matrix being the primary site of the inflammatory process due to the systematic targeting of collagen matrix by these antibodies. Due to the distinctive structure of the heart valves and the endothelium, the inflammatory process progresses to valvular scaring. This in turn leads to the haemodynamic changes that progress to RHD. Of the several autoantibodies observed in patients with ARF/RHD (33, 34) some cross-react with streptococcal proteins ("molecular mimicry") and the others do not ("collagen neoantigen"). Although, the proponents of the "molecular-mimicry" and the "collagen neoantigen" may consider the mechanisms that initiate the disease process to be distinct, it is conceivable that both these mechanisms contribute to the disease process that culminates in host tissue damage.

Antibodies against GAS *N*-acetyl-beta-D-glucosamine cross-react with neuronal cells in the basal ganglia, leading to deposition of immune complexes causing excessive release of dopamine that form the basis of the symptomatology observed in SC (35, 36). Recent studies in mouse models have also shown GAS infection of the olfactory epithelium can cause breaches in the blood brain barrier and facilitate T cell infiltration into the brain (37, 38). However, further work on the development and clinical progression remains to be done. The migratory and transient manifestations observed in joints and the characteristic rash and subcutaneous nodules have been partly attributed to the accumulation of immune complexes that cause these clinical manifestations and are part of the "major" Jones Criteria used in the diagnosis of ARF. However, compared to the pathogenesis of carditis, there is a paucity of experimental data on the pathogenesis of SC, arthritis, erythema marginatum and the development of subcutaneous nodules in ARF.

ENABLING THE DESIGN AND DEVELOP A NOVEL DISEASE SPECIFIC DIAGNOSTIC TOOL

Point-of-care rapid antigen tests are currently available to diagnose GAS infection. While these tests are diagnostic for a current GAS infection, they are not diagnostic for ARF/RHD. It has also been shown that a rise in anti-Streptolysin O titre (ASOT) is less prominent in recurrent ARF than during the first episode (6). Moreover, lab-based detection of serum ASOT

or Anti-DNase B (ADB) antibodies used to diagnose a recent streptococcal infection are non-specific and of little value in regions of high streptococcal endemicity. A recent attempt using a Triplex assay combining the ASOT and ADB with anti SpnA antibodies noted that while promising, this only confirms recent GAS infection and not ARF/RHD (39). This highlights the importance of identifying robust markers of ARF/RHD that can be incorporated into point-of-care tests to be used in low-income countries. Such point-of-care tests simplified in a lateral flow assay format can also overcome the limited laboratory facilities available in low-income countries.

The autoimmune nature of ARF indicates that specific peptides present in GAS antigens trigger the host responses that ultimately results in ARF/RHD. Despite decades of research, the identity of the specific epitopes which contribute to ARF/RHD, and which could form the basis of a diagnostic, are yet to be identified. The challenges in identifying these peptides are immense. Above, we outlined how the lack of an animal model until recently has precluded lab-based immunological studies or ARF/RHD. From a host perspective, we do not know if the same epitopes are responsible for ARF/RHD in different patients. Several studies also suggest host susceptibility to ARF/RHD is associated with HLA type (40). As ARF/RHD occurs after a GAS infection, it is also not possible to recover the specific GAS isolates responsible for case of the autoimmune process that triggers ARF.

Our group is addressing this deficit through combining our RAV model with advances in peptide array technology. Peptide arrays are a recent advance that enable the systematic screening of thousands of peptides for reactivity with specific sera samples. Using this technology, we have screened pooled sera from patients with ARF and controls for reactivity with 186 20 mer peptides derived from three M proteins. Sixteen peptides, all of which contained all or part of the same conserved sequence motif reacted with the sera (**Figure 2**). This peptide motif, (ASRQGLRRDLDASREAKKQV; P20) is found in the conserved C-terminal region of all three proteins represented on the array. Control sera were not reactive against these peptides. To establish whether the observed peptide reactivity in human was due to the M protein, we also probed the array with antisera raised against M5 protein generated in our RAV model. We again saw reactivity with the same set of peptides (**Figure 2**). Subsequent bioinformatic analysis has revealed P20 to be 100% conserved in 72 of 175 different M-types. Moreover, the same conserved motif is present in an M-protein from a SDSE isolate that was isolated from an individual presenting with ARF (11). When these GAS and SDSE M-proteins were injected subcutaneously into separate groups of Lewis rats, antibodies to the same motif were found to be predominant.

The epitope we identified is congruent with the amino acid region used to define Class I and Class II proteins (41), and within various C-repeat regions. Our peptide results are not proof that anti-P20 antibodies contribute to ARF/RHD but suggest that the higher titres may be used as a serological marker of ARF/RHD in some patient groups. Expanding our research to include individual sera samples from well-characterised patient cohorts (including patients with recent streptococcal infection), and expansion of our peptide arrays to represent all the peptides present in the various M-protein types, and indeed all peptides encoded in GAS genome, represents a generational change in the way that antibodies from ARF/RHD patients reactive with GAS epitopes can be identified and used for diagnostic purposes. Peptide arrays also have one other advantage over previous approaches. The screening of one array requires very small amounts of sera (<50 μl). Current ARF/RHD sera collections can therefore be leveraged against these arrays, leaving valuable human sera remains available for other research studies. Well-catalogued serial patient samples from several regions are required to identify the specific peptide/s that would serve as targets for both specific diagnostic development and mechanistic studies. Moreover, if future studies also included collections of DNA samples, variable autoimmune immune responses following GAS infection can be linked to human genetic variation. Given the complexity of ARF/RHD such an exhaustive and comprehensive approach may be the only path to a true specific diagnostic and greater understanding of these diseases.

HOW DOES DETECTING ANTIBODIES TO DISEASE SPECIFIC STREPTOCOCCAL EPITOPES BE OF VALUE IN DEVELOPING VACCINES FOR STREPTOCOCCAL INFECTIONS?

A rat model of myocarditis and valvulitis was developed (14) and it was subsequently demonstrated that immunisation with a pool of 15 peptides from the C-repeat region of the M-protein induced mononuclear cell infiltration into the hearts of Lewis rats (16). A further study, however, found that while peptides from the A-repeat region of the M-protein induced significant myocarditis, peptides from the B-repeat region induced mild carditis and that peptides from the C-repeat region did not induce any carditis (42). Studies in 2016 (43) and 2020 (44) then showed that immunisation of rats with the leading peptide vaccine candidate from the C-repeat region of the M-protein, "J8," "J14," and "p*17" did not induce cardiomyopathy.

$CD4^+$ T-cells are the most common T-cell subset identified in diseased valves (45). The only human T-cell epitopes that have been identified as a possible cause of valvulitis are from the A-repeat region. One hundred and sixty three human T-cell clones were generated from diseased valves and tested against M-protein peptides from the A-repeat region and against heart proteins (46). Twenty percent of the clones recognised M-protein peptides and these only reacted with three A-repeat region peptides. Some also reacted with heart proteins. Since T-cell epitopes from the A-repeat region of the M-protein have been implicated in pathogenesis, the frequency and phenotype of T-cells specific for these epitopes could be assessed using combinatorial tetramer staining. This technology has been used to detect and characterise $CD4^+$ T-cell influenza-specific epitopes

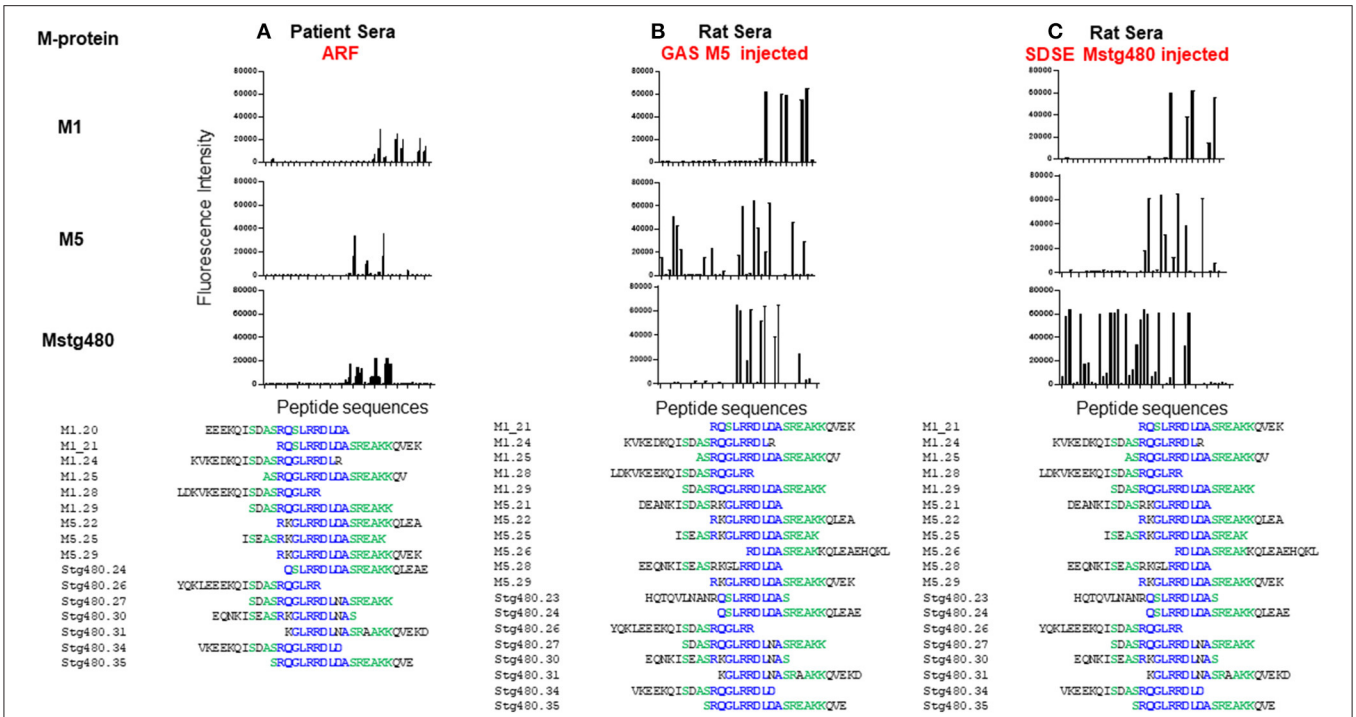

FIGURE 2 | Antibody reactivity with peptides from streptococcal M protein. Fluorescence intensity (RFI) of peptides screened with; **(A)** pooled human sera from patients with ARF ($n = 7$), **(B)** pooled sera from rats following five injections of GAS M5 ($n = 3$), and **(C)** pooled sera from rats following five injections of SDSE Mstg480 ($n = 3$). Rat serum samples were collected 224 days following initial injections. Peptide microarrays consisting of 186 overlapping 20 mer peptides derived from M1, M4, M5, and Stg480 M-proteins were constructed by JPT technologies. Sera samples were diluted in blocking buffer (1:200) and incubated with the peptide array for 1 h at 30°C. The primary sera were removed, arrays washed and incubated with fluorescently labelled anti-human-IgG or anti-rat-IgG antibody at 0.1 μg/ml for 1 h. After washing and drying the microarrays were scanned using a high resolution fluorescence scanner (635 nm) to obtain fluorescence intensity profiles. The y-axis represents the fluorescence intensity (FI) of experimental sample with FI from negative control sera subtracted. The x-axis represents peptides from M-proteins shown on the left Peptides with a FI >5,000 were aligned using Clustal Omega. Amino acids present in >75% of aligned sequences are coloured blue. Peptides present in >50% of aligned sequences are coloured green. (Deidentified pooled human serum samples were collected under ethics approval #HREC/15/QTHS/134 and rat serum under #JCUA2083).

at frequencies as low as 1 per million in peripheral blood (47). The same technology could identify A-repeat region-specific $CD4^+$ T-cells and may provide laboratory diagnosis of some patients with active rheumatic valvulitis.

As stated, with the enormous burden of GAS infection, a streptococcal vaccine is urgently needed. How else can we harness the recent discovery of the ARF/RHD rat model for safe vaccine design? In the sections above we describe identification in the GAS M protein derived peptides that reacted with sera derived from rats injected with recombinant M5 (**Figure 2**). These peptides are largely similar to or overlap with the peptides that are reactive to ARF human sera (**Figure 2**). This important observation, namely similarity of reactions between the rat model and patients has implications to further assess safety of M protein-based vaccine candidates. So far, the M-protein based peptide vaccine candidates have been painstakingly designed with the aim of deletion of deleterious T cell epitopes to make the vaccine candidates safe. While the efficacy of such candidates has been amply tested, there has been no direct assessment of these candidates for propensity to cause or exacerbate reactions that could lead to ARF. Additionally, we also need to demonstrate that the human cardiac tissue cross-reactive antibodies in ARF and in the GAS-injected rats react to similar or overlapping peptides of these tissue proteins.

CONCLUSION

Since the clinical criteria for diagnosis was first described (1), we have gained significant understanding of the pathogenesis of rheumatic fever and its complications that provide an adequate foundation to develop prototype antibody-based specific diagnostics. It is our view that by exploiting available technologies and having access to serial serum samples of patients with rheumatic fever from different regions coupled with studies on the rat autoimmune valvulitis model, it is possible to test and identify a specific antibody-based assay to simplify the diagnosis of rheumatic fever. The COVID-19 pandemic has demonstrated that rapid detection tools, basic infection control measures, international information and resource sharing can provide a platform to adequately mitigate an infection regardless of the economic status of individual nations. While it remains

to be seen whether developing a specific and low-cost diagnostic test is achievable by the centenary of the first publication of the diagnostic criteria for rheumatic fever, the pursuit of the holy grail may rely on the proposition that "simplicity is the ultimate sophistication."[1]

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by #HREC/15/QTHS/134. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. The animal study was reviewed and approved by #JCUA2083.

[1]Aphorism attributed to the renaissance polymath Leonardo da Vinci (1452–1519).

AUTHOR CONTRIBUTIONS

All authors were involved in the conceptualisation of the content of the manuscript and contributed equally in preparing and editing the manuscript.

ACKNOWLEDGMENTS

The authors would like to thank Madeleine Kersting Flynn, a Biomedical Illustrator and Dr. Katja Fischer of QIMR Berghofer Medical Research Institute for facilitating the artwork (**Figure 1**). The authors acknowledge Drs. Suchandan Sikder, Catherine Rush, Brenda Govan (James Cook University, Australia) and Clinical Nurse Consultant Louise Axford-Haines (Queensland RHD Surveillance and Communicable Disease Control, Australia) and Dr. Andrew White (Paediatrician at the Townsville University Hospital) for their support enabling the determination of antibody reactivity using peptide arrays (**Figure 2**). MG is a recipient of an Australian National Health and Medical Research Council Investigator Fellowship and RAMR an Australian International Post-graduate Research Award.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cardiovascular Medicine

ORIGINAL RESEARCH
published: 04 June 2021
doi: 10.3389/fcvm.2021.674435

Left Ventricular Remodeling Following Balloon Mitral Valvuloplasty in Rheumatic Mitral Stenosis: Magnetic Resonance Imaging Study

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OPEN ACCESS

Edited by:
Roney Orismar Sampaio,
University of São Paulo, Brazil

Reviewed by:
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Sapienza University of Rome, Italy
Matthew Bersi,
Washington University in St. Louis, United States

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Specialty section:
This article was submitted to Heart Valve Disease, a section of the journal Frontiers in Cardiovascular Medicine

Received: 02 March 2021
Accepted: 27 April 2021
Published: 04 June 2021

Citation:
Samaan AA, Said K, Aroussy WE, Hassan M, Romeih S, El Sawy A, Fawzy ME and Yacoub M (2021) Left Ventricular Remodeling Following Balloon Mitral Valvuloplasty in Rheumatic Mitral Stenosis: Magnetic Resonance Imaging Study. Front. Cardiovasc. Med. 8:674435. doi: 10.3389/fcvm.2021.674435

Background: Rheumatic heart disease affects primarily cardiac valves, it could involve the myocardium either primarily or secondary to heart valve affection. The influence of balloon mitral valvuloplasty (BMV) on left ventricular function has not been sufficiently studied.

Aim: To determine the influence of balloon mitral valvuloplasty (BMV) on both global and regional left ventricular (LV) function.

Methods: Thirty patients with isolated rheumatic mitral stenosis (MS) were studied. All patients had cardiac magnetic resonance imaging (CMR) before, 6 months and 1 year after successful BMV. LV volumes, ejection fraction (EF), regional and global LV deformation, and LV late gadolinium enhancement were evaluated.

Results: At baseline, patients had median EF of 57 (range: 45–69) %, LVEDVI of 74 (44–111) ml/m^2 and LVESVI of 31 (14–57) ml/m^2 with absence of late gadolinium enhancement in all myocardial segments. Six months following BMV, there was a significant increase in LV peak systolic global longitudinal strain (GLS) (−16.4 vs. −13.8, $p < 0.001$) and global circumferential strain (GCS) (−17.8 vs. −15.6, $p = 0.002$). At 1 year, there was a trend towards decrease in LVESVI (29 ml/m^2, $p = 0.079$) with a significant increase in LV EF (62%, $p < 0.001$). A further significant increase, compared to 6 months follow up studies, was noticed in GLS (−17.9 vs. −16.4, $p = 0.008$) and GCS (−19.4 vs. −17.8 $p = 0.03$).

Conclusions: Successful BMV is associated with improvement in global and regional LV systolic strain which continues for up to 1 year after the procedure.

Keywords: mitral stenosis, balloon mitral valvuloplasty, myocardial tagging, left ventricular function, left ventricular remodeling, left ventricular deformation, cardiac magnetic resonance imaging

INTRODUCTION

Although, the incidence of rheumatic fever and its complications has declined in developed countries, the disease is still a major health problem in many developing countries (1). It is estimated that up to 30 million schoolchildren and young adults have chronic rheumatic heart disease worldwide, and nearly a third of these have mitral stenosis (MS) (2).

The procedure of balloon mitral valvuloplasty (BMV) has been first described in 1984 by a Japanese cardiac surgeon called Kanji Inoue (3). The main mechanism of successful BMV relies on splitting of the fused commissures and in comparison to surgical commissurotomy it has comparable success rates with better long-term outcomes (4, 5).

Impaired left ventricular (LV) systolic function has been reported in around 30% of patients with MS (6), while in some recent studies, underlying abnormal LV contractility has been described using tissue Doppler Imaging (TDI) and Speckle Tracking Echocardiography (STE) in MS patients with apparently normal LV systolic function (7, 8).

Changes taking place in LV following BMV have been a subject of investigation and debate over the past years. Different diagnostic tools including cardiac catheterization and angiocardiography (9), echocardiography (10), TDI (11), and STE (12) were used earlier for that purpose. Whereas, some of these studies showed improved LV function after BMV (13, 14) others failed to show any change following the procedure (15, 16).

PURPOSE OF THE STUDY

The present study aimed to determine the impact of BMV on LV volumes, global, and regional function using CMR, 6 months and 1 year following BMV in patients with isolated rheumatic MS.

METHODS

The Study Population

The study was an observational prospective cohort study that took place at a tertiary referral hospital. The study included 30 consecutive patients with isolated rheumatic MS who underwent successful BMV, and a control group of 12 healthy volunteers without known prior cardiovascular disease.

BMV was considered indicated for symptomatic patients with severe MS (mitral valve area (MVA) < 1.5 cm^2) and favourable valve morphology (Wilkin's score of 11 or less and the absence of commissural calcifications) in the absence of left atrial thrombus or moderate-to-severe mitral regurgitation (17). A successful BMV was defined as an immediate final MVA of more than 1.5 cm^2 or a 40% increase in MVA with no mitral incompetence beyond mild severity and with no procedure-related complications (18, 19). Exclusion criteria for this study included conditions that may alter LV function e.g., coronary artery disease, uncontrolled systemic hypertension, more than mild aortic valve disease, or any advanced systemic disease as well as patients with contraindications to CMR e.g., claustrophobia or metallic implants. The study was conducted after the approval of institutional Research Ethics Committee of Aswan Heart Centre, Aswan, Egypt, with complete adherence to all required institutional safety measure. The study protocol was illustrated to all the patients and a written informed consent was obtained from all the participants before being enrolled in the study.

Patients referred to the hospital were first screened for eligibility for this study. Eligible patients had baseline workup that included clinical evaluation, electrocardiogram (ECG), echocardiography study [transthoracic and trans-oesophageal (TEE)], CMR imaging and invasive haemodynamic study before BMV. Only patients who had a successful procedure were recruited and then had two follow up studies at 6 months and 1 year after the procedure. Medical treatment, in the form of beta-blockers and a small dose of diuretics, was initiated at least 6 months pre-procedural and was kept unchanged throughout the follow-up period. On the other side, individuals in the control group were evaluated clinically and had ECG and trans-thoracic echocardiography to exclude any cardiovascular disease and then had a complete CMR imaging evaluation.

Echocardiography Study

Transthoracic echocardiography images were obtained *via* Philips iE33 with 2.5 MHz sector transducer. All studies were done according to the criteria provided by the American Society of Echocardiography (ASE) (20). LV end-systolic and end-diastolic volumes, EF, left atrial volume, MVA (planimetry), mean pressure gradients, Wilkin's score and degree of mitral regurgitation were estimated. TEE was performed for all the patients to exclude left atrial appendage thrombus before valvuloplasty.

Invasive Haemodynamic Measurements

Haemodynamic assessment was performed *via* right and left heart catheterization immediately before BMV. Tracings were obtained using AXON Sensis XP (Siemens Healthcare Systems) using a standardized protocol under stable conditions. Left atrial pressure (LAP), mean pressure gradient, systemic vascular resistance (SVR), pulmonary artery pressure (PAP), and cardiac index were measured.

Balloon Mitral Valvuloplasty

All patients had percutaneous BMV by antegrade trans-septal approach with the use of the Inoue balloon catheter (Toray Medical Co., Tokyo, Japan) using a standardized technique (3). Immediate post-procedural haemodynamic assessment of trans-mitral pressure gradients and left atrial pressure was performed.

Cardiac Magnetic Resonance Imaging

CMR examination was performed using 1.5 Tesla Siemens Aera (Siemens Medical System, Erlangen, Germany), with 25-m T/M maximum gradient strength and a phased array cardiac coil of 48 channels.

Image Acquisition

Systolic and diastolic volumes were assessed using a retrospective ECG-gated steady-state free precession (SSFP) sequence during breath-holding. Vertical long-axis 2- and 4- chamber views and short-axis views consisting of 12–14 contiguous slices were

acquired, covering both ventricles from the base of the heart to the apex.

For the tissue characterization (viability) studies, a bolus of 0.2 mmol/kg of Gadolinium was infused at an injection rate of 4 ml/s followed by a bolus of 20 ml of normal saline, infused at the same rate. Delayed enhancement 3D acquisitions were acquired in diastole 10 min after Gadolinium injection with a time of inversion (TI) of 175–300 ms. Tissue characterization studies were performed only once for MS patients at baseline.

Myocardial Tagging

Tagging MR images were performed in the short heart axis orientation and then in apical four-chamber, apical two-chamber and apical long-axis planes. Three short-axis planes were obtained (basal, mid and apical). A segmented two dimensional electrocardiographically triggered fast low angle shot pulse sequence (field of view 240 × 320 mm^2, matrix 216 × 256, TR/TE 9.0/4.0, flip angle 15°) was used in the cine mode. A rectangular grid with a spacing of 8 mm was applied. The acquisition window for one cardiac phase was 70–90 ms. resulting in a temporal resolution of 35–45 ms.

Image Analysis

Ventricular volumes and function were analyzed using the SYNGO software (Siemens health system). After the determination of the end-diastolic and the end-systolic frame on the first basal slice to show circumferential myocardium at both diastole and systole, the endocardial contour was traced manually. All volumes were calculated automatically by summing the areas in the entire series of short-axis cine images (**Figure 1**). Volumes were then indexed to body surface area (BSA) and LV ejection fraction (EF) was calculated (21).

Strain Measurement

Tagged MRI images were analyzed quantitatively using the software HARP (Harmonic Phase Imaging, version 5, Diagnosoft). After adjusting the Region of Interest (ROI) and manual defining of the endocardial and epicardial borders, myocardial strain curves throughout the cardiac cycle were automatically generated for the left ventricular 17 segments. Peak systolic longitudinal strain was measured for different myocardial segments from the tagged apical 2, 3, and 4 chambers views while peak systolic circumferential strain was estimated from tagged short axis views. Strain was measured as the change in myocardial segment length relative to its end-diastolic length. Global strain values were calculated as the mean value of strain measurements of the LV segments. Negative strain values denoted myocardial shortening (22, 23) (**Figures 2**, **3**).

STATISTICAL ANALYSIS

Statistical analysis was performed using Statistical Package for Social Sciences, version 16 (SPSS 16). Firstly, all variables were tested for normality using Kolmogrov-Smirnov test; If the test was significant, non-normality was accepted otherwise double-check using graphs, skewness and kurtosis were required to confirm normality. All the quantitative variables in this research were not normally distributed and accordingly are presented as median (range). Qualitative data are presented as number (percentage).

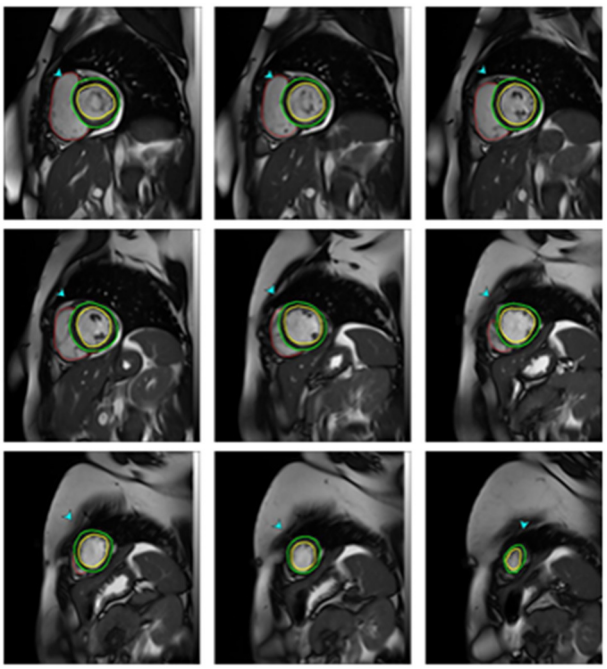

FIGURE 1 | CMR Measurements of ventricular volumes in one of mitral stenosis patients. End diastolic short-axis images from the apex to the base with epicardial (green lines) and endocardial (red lines) contours drawn for the left ventricle and endocardial contours for the right ventricle.

Variables were compared between two related samples using Wilcoxon test. Categorical variables were compared using Chi-square analysis. Bivariate correlations were performed using Spearman correlation coefficient. Probability value of <0.05 was considered statistically significant.

Delta (Δ) for a specific parameter was calculated by subtracting the value of this parameter at follow up from its corresponding value at baseline study.

RESULTS

Among 78 patients screened for eligibility of BMV, 51 patients had BMV over a 1 year period. Three patients had unsuccessful procedure where two patients had post-procedural severe mitral regurgitation and were referred to surgery, and the procedure failed to attain a satisfactory valve area in the third one. Eighteen patients were excluded due to various reasons including pregnancy, claustrophobia, and refusal to participate in the study. The remaining 30 patients, who had a successful procedure, were included in the study and had two follow up visits at 6 and 12 months after BMV.

The demographics of the study population, clinical characteristics, baseline echocardiographic and heamodynamics data of the patients are summarized in **Tables 1**, **2**.

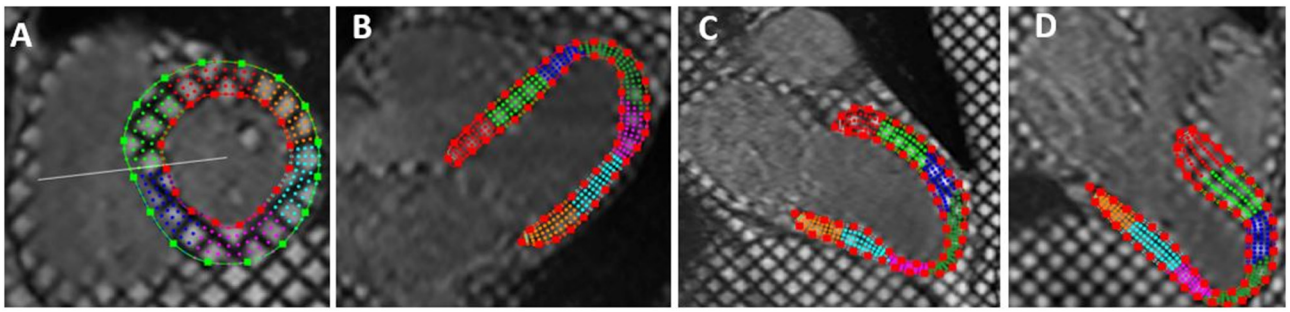

FIGURE 2 | Tagged cardiac magnetic resonance imaging with epicardial and endocardial border tracing in: short axis **(A)**, horizontal long axis (apical four chambers) **(B)**, vertical long axis (apical two chambers) **(C)**, and left ventricular outflow view (three chambers views) **(D)**. The short axis images were used for estimation of circumferential strain while long axis views were used for estimation of longitudinal strain.

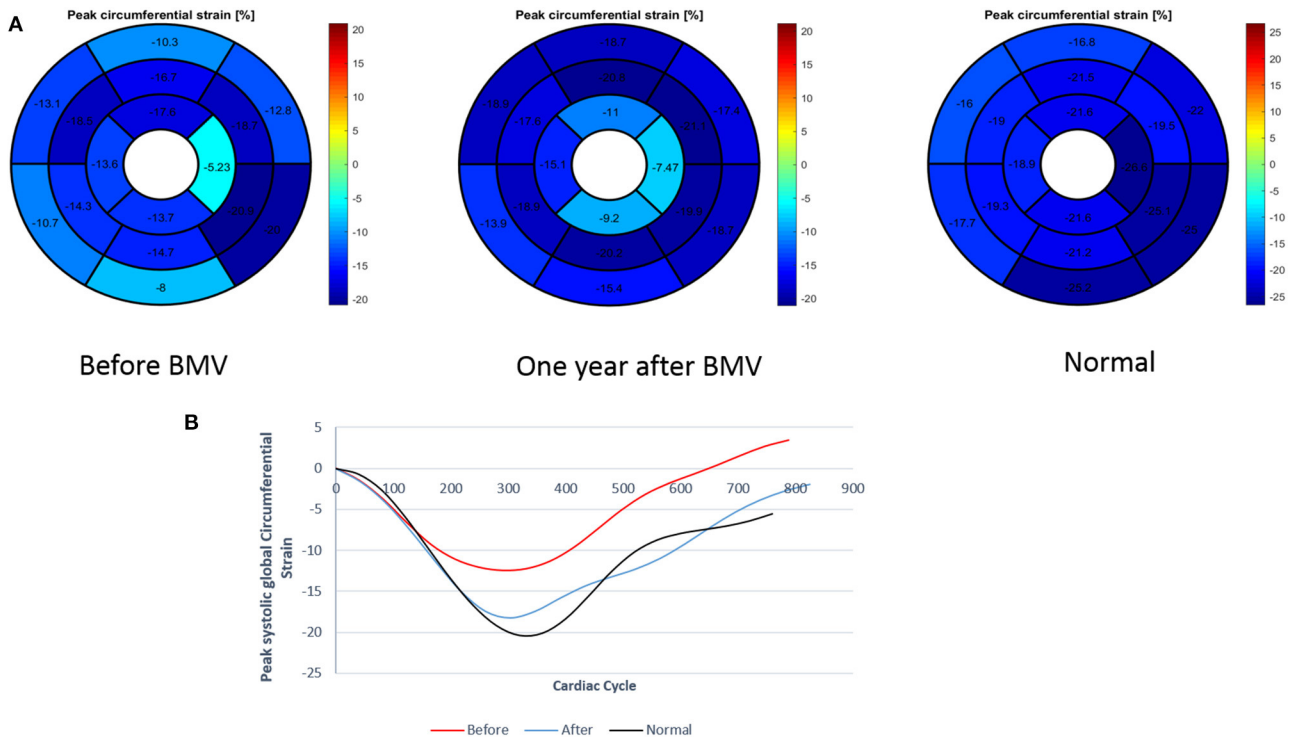

FIGURE 3 | (A) Measurement of regional and global circumferential strain (Bull's eye plot) in a patient with mitral stenosis before and 1 year after BMV with comparison to a control individual **(B)** Graph representation of GCS in a patient with mitral stenosis before and after BMV in comparison to a control individual.

LV Volumes and Function in MS Patients vs. Control Group

Compared to the control group, patients with MS had similar LVEDVI (74 vs. 71 ml/m^2, $p = 0.32$), significantly larger LVESVI (31 vs. 22 ml/m^2, $p = 0.007$) and significantly lower LV ejection fraction (57 vs. 64%, $p = 0.004$). MS patients had lower regional peak systolic longitudinal and circumferential strain in all LV myocardial segments comapred to control group. Peak systolic global circumferential strain (GCS) and global longitudinal strain (GLS) were significantly lower in MS patients (−23.2 vs. −15.6%, $p = <0.001$ and −22.7 vs. −13.8%, $p = <0.001$, respectively).

Tissue Characterization

Late gadolinium enhancement studies showed no evidence of myocardial fibrosis in all MS patients.

Changes Following Balloon Mitral Valvuloplasty

Following BMV, as evaluated by trans-thoracic echocardiography, there was a significant increase in MVA [1.9 (1.7–2.3) vs. 0.9 (0.6–1.3) cm^2, $p < 0.001$], a significant drop in mean pressure gradients [5 (2–8) vs. 12.5 (8–24) mmHg, p

TABLE 1 | Baseline clinical, echocardiographic, and hemodynamic data of the patients.

Variable	Value
Age (years)	33 (23–41)
Female gender [*n* (%)]	22 (73.3%)
NYHA	
II [*n* (%)]	17 (56.6%)
III [*n* (%)]	13 (43.3%)
Atrial fibrillation ryhthm [*n* (%)]	3 (10%)
Echocardiographic data	
MVA (cm^2)	0.9 (0.6–1.3)
Mean pressure gradient (mmHg)	12.5 (8–24)
Wilkin's score	7 (6–10)
PASP (mmHg)	47.5 (25–120)
Heamodynamics data	
HR (bpm)	72 (64–88)
LAP (mmHg)	30 (14–35)
LVEDP (mmHg)	12 (8–19)
Mean trans-mitral pressure gradient (mmHg)	14 (9–28)
Mean PAP, (mmHg)	40 (19–72)
CO (L/min)	3.6 (2.5–5.7)
Cardiac index (L/min/m^2)	2 (1.5–3.1)
SVR (Woods units)	20.4 (14.3–38.2)

MVA, Mitral valve area; NYHA, New York Heart Association; PASP, pulmonary artery systolic pressure; HR, heart rate; LAP, left atrial pressure; LVEDP, left ventricular end diastolic pressure; mean PAP, mean pulmonary artery pressure; CO, cardiac output; SVR, systemic vascular resistance. Values are expressed as median and range. Data are presented as median (range) or number (%).

TABLE 2 | Age and gender in both control group and MS patients.

Parameter	Control group ($n = 12$)	Baseline MS patients ($n = 30$)	*P*-value
Age (years)	31 (21–39)	33 (23–41)	0.81
Female gender, [*n* (%)]	9 (75%)	22 (73.3%)	0.89

< 0.001] and a significant drop in PASP [35 (20–50) vs. 47.5 (25–120) mmHg, $p = 0.002$].

LV Volumes and Ejection Fraction

No significant change was seen in LVEDVI at 6 months and 1 year following BMV. At 1 year, a trend toward a significant decrease in LVESVI was seen (29 vs. 31 ml/m^2, $p = 0.079$) associated with a significant improvement in LVEF (62 vs. 57%, $p = 0.002$) (**Table 3**).

Deformation Analysis

- **Regional strain:** At 1 year follow up, an improvement in peak circumferential systolic strain values was noted in all LV myocardial segments; this improvement showed statistical significance in 15 segments and trend toward significance in the remaining two segments. Similarly, all LV myocardial segments showed significant improvement in peak longitudinal systolic strain values at 1 year (**Supplementary Tables 1**, **2**).
- **Global strain:** A significant improvement was shown in LV GLS at 6 months (−16.4 vs. −13.8%, $p = 0.001$) with a further improvement at 1 year (−17.8 vs. −16.4%, $p = 0.008$). Similar changes were observed in GCS with a significant improvement at 6 months (−17.8 vs. −15.6%, $p = 0.002$) and a further improvement at 1 year (−19.4 vs. −17.8%, $p = 0.03$). However, at 1 year following BMV, both GLS and GCS values remained significantly lower than those of the control group (−17.8 vs. −22.7%, $p < 0.001$ and −19.4 vs. −23.2%, $p < 0.001$, respectively) (**Figures 4**, **5**).

Patients with lower pre-procedural left ventricular GLS and GCS experienced significantly higher improvement in 1 year post-procedural strain values ($r = -0.7$, $p < 0.001$ and $r = -0.4$, $p = 0.013$ respectively) (**Figure 6**). No significant correlation was found between changes in LV deformation parameters and changes in MVA, mean pressure gradient across the mitral valve or LV volumes.

DISCUSSION

Rheumatic heart disease represents a significant medical challenge in the developing countries. BMV remains the procedure of choice for patients with rheumatic mitral stenosis who have a favorable valve morphology. This type of intervention is still frequently asked for in the developing countries though it is uncommonly needed in the developed countries owing to the remarkably declined incidence and prevalence of RHD in these regions. Surgical intervention, mostly *via* valve replacement, is reserved for patients who have markedly thickened or calcified valves in whom BMV wouldn't be feasible or in the presence of associated significant mitral incompetence.

This study describes the extent, timing and pattern of left ventricular remodeling following BMV. Although, rheumatic heart disease affects primarily heart valves, there is a continuing debate whether the myocardium is affected primarily by the rheumatic process due to molecular mimicry between myosin heavy chain and bacterial proteins or secondary to chronic changes in cardiac output due to valvular disease (24). Furthermore, the influence of relief of the hemodynamic burden of MS on LV function has not been systematically studied. MRI imaging provides an extremely powerful tool to study left ventricular function non-invasively. In addition, including a control group helped define the normal values of myocardial deformation which are known to vary among different racial groups.

Changes in LV Volumes After BMV

Several previous studies described the change in ventricular volumes following BMV. Mohan et al., using angiocardiography in one study (25) and echocardiography in another one (10), showed no significant change in both LVEDV and ESV following BMV. Both Sengupta et al. (16) and Pamir et al. (15) also showed no significant short term changes in ventricular volumes following BMV. On the contrary, using ventriculography,

TABLE 3 | LV volumes, EF, strain and torsion values in control group and in MS patients at baseline, 6 months and 1 year after BMV.

Parameter	Control group	Patients at baseline	6 months follow-up	1 year follow-up	$P^{(1)}$	$P^{(2)}$	$P^{(3)}$	$P^{(4)}$
LVEDVI (ml/m^2)	71 (64–85)	74 (44–99)	73 (48–117)	73(48–119)	0.32	0.45	0.75	0.65
LVESVI (ml/m^2)	22 (15–35)	31 (14–57)	30 (20–57)	29 (17–54)	**0.007**	0.14	**0.079**	0.12
LVEF (%)	64 (58–67)	57 (45–69)	58 (50–68)	62 (53–72)	**0.004**	0.244	**<0.001**	**0.002**
LV GLS (%)	−22.7 (−21.1– −24.8)	−13.8 (−10.2– −18.5)	−16.4 (−13.7– −23.1)	−17.8 (−13– −23.9)	**<0.001**	**0.001**	**<0.001**	**0.008**
LV GCS (%)	−23.2 (−22.2– −24.9)	−15.6 (−10.5– −19)	−17.8 (−13.8– −21.9)	−19.4 (−13.6– 23.9)	**<0.001**	**0.002**	**<0.001**	**0.03**

$P^{(1)}$: Baseline vs. control group, $P^{(2)}$: Baseline vs. 6 months follow up, $P^{(3)}$: baseline vs. 1 year follow up, $P^{(4)}$: 6 months vs. 1 year follow up. LVEDVI, left ventricular end-diastolic volume index; LVESVI, left ventricular end-systolic volume index; EF, ejection fraction; GCS, global circumferential strain; GLS, global longitudinal strain. Bold values refer to statistical significance.

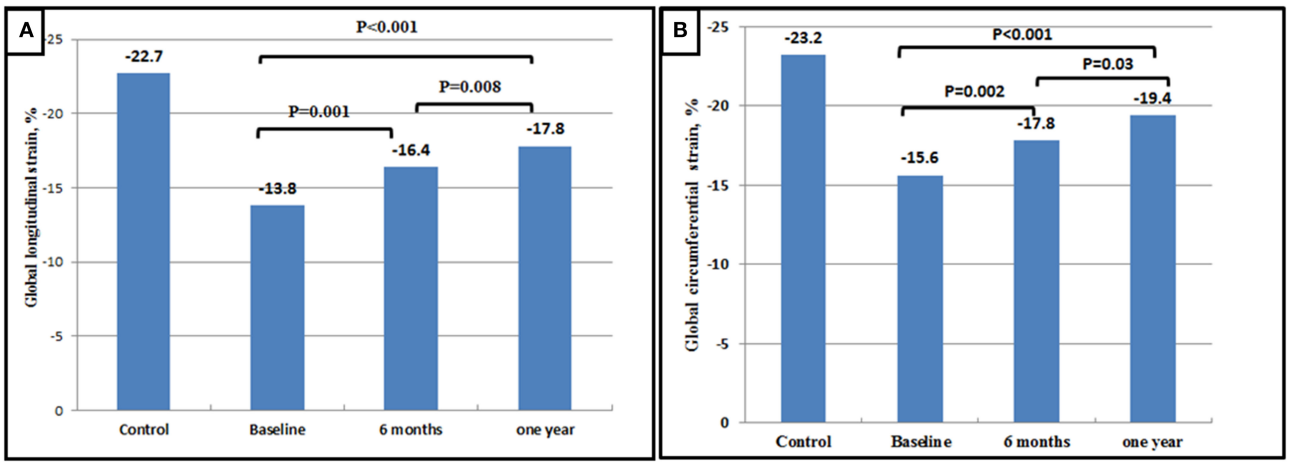

FIGURE 4 | Bar graphs showing Changes in LV GLS after BMV **(A)** and Changes in LV GCS after BMV **(B)**.

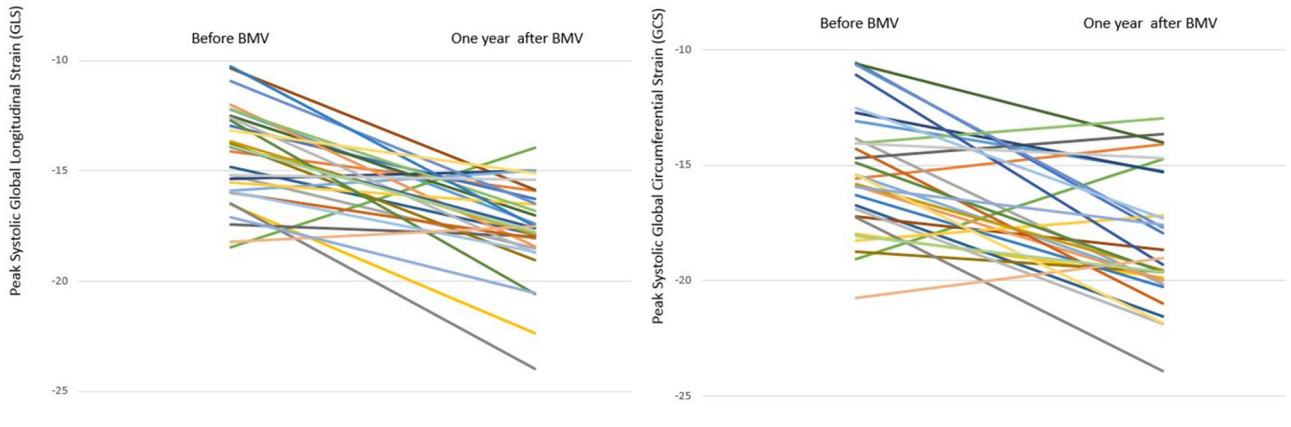

FIGURE 5 | Line chart representing changes in GLS and GCS in individual mitral stenosis patients 1 year after BMV.

Goto et al. (13) demonstrated an immediate increase in LVEDVI after successful BMV and recently, Sengupta et al. (26) showed a significant increase in LVEDVI 72 h after BMV in an echocardiography study. Only a few studies evaluated the long term effects of BMV on ventricular volumes. Fawzy et al. (9), using angiography, followed 17 MS patients after BMV and showed a significant increase in LVEDVI immediately after BMV with a further increase at a mean of 12 months follow up. In the present study, using CMR, we demonstrated a decreased LVESVI with no significant change in LVEDVI following BMV.

Changes in LVEF After BMV

In the present study, as estimated by CMR, a late significant improvement in LVEF was observed at 1 year following BMV.

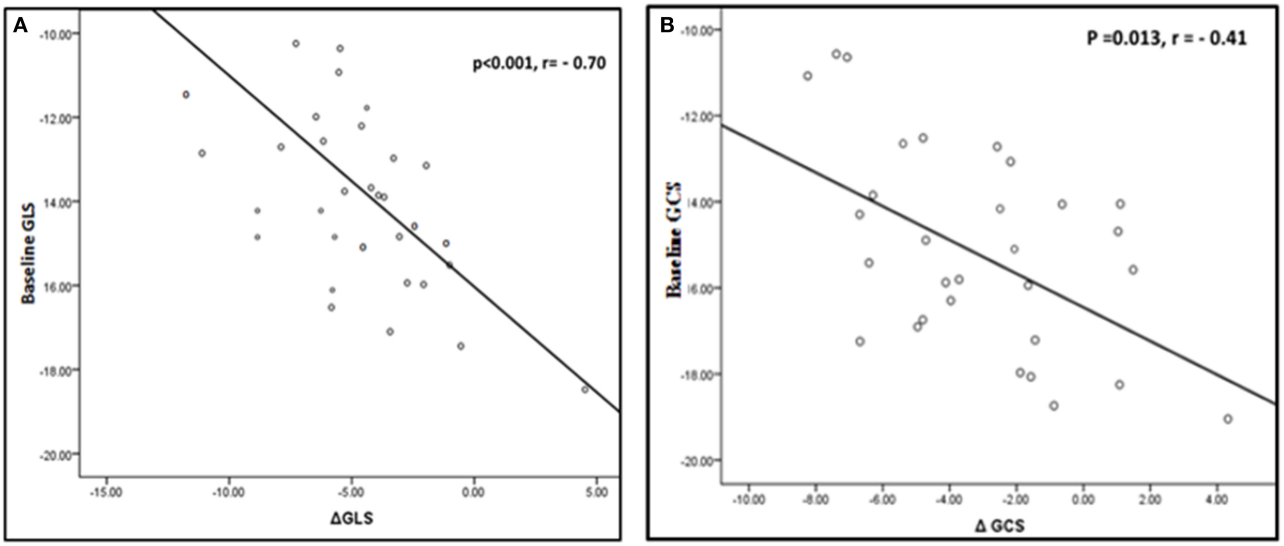

FIGURE 6 | Correlation between change in LV deformation parameters and baseline values. **(A)** Correlation between change in GLS and baseline GLS. **(B)** Correlation between change in GCS and baseline GCS.

Using ventriculography, both Mohan et al. (25) and Pamir et al. (15) could not find any significant change in LVEF acutely after BMV. Using echocardiography, Sengupta et al. (16) and Akcakoyun et al. (11) showed no significant acute change in LVEF following BMV. Improved LVEF immediately after BMV was reported by Goto et al. (13), Fawzy et al. (9), and Razzolini et al. (14) using cardiac catheterization and angiography, and by Tischler et al. (27) using echocardiography, and was attributed to improved LV loading conditions. A few studies investigated the long term effect of BMV on LVEF. Following the immediate increase in LVEF after BMV, both Fawzy et al. (9) and Tischler et al. (27) showed a further increase at a mean of 12 and 11 months follow up, respectively.

Changes in Left Ventricular Deformation After BMV

We demonstrated a significant improvement in both GLS and GCS at 6 months with a further improvement in both parameters at 1 year.

A few previous studies investigated the change in LV deformation following BMV. Sengupta et al. (16), using TDI, showed a significant improvement in mitral annular peak systolic and peak early diastolic excursion 72 h following BMV. Bektaş et al. (28) showed a significant improvement in basal lateral, septal, anterior, and inferior systolic strain 7 days after BMV. Recently, STE was used in two studies for evaluation of changes in LV deformation following BMV. Roushdy et al. showed significant improvement in LV GLS immediately after BMV, a change that was maintained at 3 months follow up (12). Sengupta et al. (26) reported significant improvement in both GLS and GCS 72 h after BMV.

In our study, the improvement in LV deformation after BMV followed by the late improvement in global ejection fraction suggests that myocardial dysfunction in mitral stenosis is reversible and is probably due to long-standing heamodynamic alteration rather than rheumatic inflammatory process. However, at 1 year both GLS and GCS values remained significantly lower than those of the control group. Though, that might be explained by the presence of a residual mild mitral stenosis, it may also suggest an underlying myocardial factor to be contributing to LV abnormalities. Whether the deformation parameters will continue to increase to reach the levels of the control group at a later stage or will remain as impaired as they are, is a question that needs further investigation.

Suggested Mechanisms of LV Abnormalities and Their Improvement After BMV

Loading Conditions

- **After-load:** Increased after-load in MS patients has been described in many previous studies (6, 9, 29). The reduced stroke volume in these patients could result in a compensatory peripheral systemic vasoconstriction leading to increased SVR. To the best of our knowledge, the long term effect of BMV on SVR was investigated in only one previous study where Fawzy et al. (9) showed a significant drop of SVR in MS patients 1 year after BMV.
- **Pre-load & pattern of LV filling:** In previous studies, LVEDV, used as a surrogate for LV pre-load, has been described to be either reduced, normal (29, 30) or even increased (31, 32). Abnormal pre-load might be associated with LV dysfunction in these patients. Recently, with the advent of techniques used for intra-cardiac flow visualization, it was shown that LV systolic function depends not only on the amount of diastolic filling but also on the pattern of filling (33, 34). The flow within the cardiac chambers has been shown to

follow a complex sequence characterized by the formation of spiral rings and that pattern is essential to maintain a normal systolic and diastolic performance (34). Sengupta et al. (26, 34) have recently suggested that mitral stenosis might disturb that normal pattern of filling which in turn might contribute to the systolic dysfunction seen in MS patients. Improved pattern of ventricular filling may be one of the factors associated with improved LV function following BMV. This theory, however, requires further investigation.

Myocardial Factor

Besides the disturbed loading conditions, it has been debated whether the intrinsic myocardial contractility is normal or impaired in MS patients (6, 29, 35). The myocardial factor as a cause of LV dysfunction in MS patients was best supported in a previous pathological study where ultra-structural pathological changes were observed in myocardial biopsies obtained from MS patients (36). While these pathological alterations were linked by some to a previous rheumatic myocardial process, others related these changes to the chronic abnormalities in ventricular filling (37, 38).

In the present study, the absence of late gadolinium enhancement in all MS patients excludes myocardial fibrosis as a contributing factor of LV dysfunction. On the other hand, it took up to 1 year to see a significant change in LVEF after relieving mitral obstruction and this lag may be the time needed for the aforementioned ultra-structural pathological alterations to reverse after correcting the long-standing abnormal loading conditions.

STUDY LIMITATIONS

The small number of patients included in the study is one of the main limitations. Another limitation is that the recruited patients represented just a subgroup of MS patients where only patients with favourable valve morphology were included. Patients with heavily affected valves were excluded as well as elderly patients and those with any other valvular affection or comorbidities. In clinical practice, MS patients occasionally present with other conditions and associations that might be interfering with the ventricular recovery observed following BMV in this study.

CLINICAL IMPLICATIONS

This study elucidates LV abnormalities associated with rheumatic MS, proves that the associated LV dysfunction is reversible following BMV and clarifies the time frame of LV recovery. In MS patients who present with significantly impaired LV, physicians may hesitate to dilate the valve lest increasing blood flow may have a detrimental effect on LV. However, this study is reassuring that LV undergoes gradual favourable remodeling following BMV and, in addition, patients with lower baseline myocardial strain values appeared to achieve a higher significant improvement at 1 year.

CONCLUSIONS

BMV results in continued slow favourable LV remodeling. This strengthens the recent strategy to make this form of treatment available for the very large number of patients who need it worldwide.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Magdi Yacoub Heart Foundation Research Ethical Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS: collecting data and writing the manuscript. KS and SR: conception of the idea, supervising the methodology, and revising the manuscript. WA, MF, and MY: critically revising the manuscript. MH: analysis of the data and statistics. AE: collecting the data. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.674435/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cardiovascular Medicine

ORIGINAL RESEARCH
published: 10 June 2021
doi: 10.3389/fcvm.2021.676694

Rheumatic Myocarditis: A Poorly Recognized Etiology of Left Ventricular Dysfunction in Valvular Heart Disease Patients

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Heart Valve Disease, a section of the journal Frontiers in Cardiovascular Medicine

Received: *05 March 2021*
Accepted: *19 May 2021*
Published: *10 June 2021*

Citation:
Rosa VEE, Lopes MP, Spina GS, Soares Junior J, Salazar D, Romero CE, Lottenberg MP, de Santis A, Pires LJNT, Gonçalves LFT, Fernandes JRC, Sampaio RO and Tarasoutchi F (2021) Rheumatic Myocarditis: A Poorly Recognized Etiology of Left Ventricular Dysfunction in Valvular Heart Disease Patients. Front. Cardiovasc. Med. 8:676694. doi: 10.3389/fcvm.2021.676694

Background: Heart failure occurs in ∼10% of patients with acute rheumatic fever (RF), and several studies have shown that cardiac decompensation in RF results primarily from valvular disease and is not due to primary myocarditis. However, the literature on this topic is scarce, and a recent case series has shown that recurrent RF can cause ventricular dysfunction even in the absence of valvular heart disease.

Methods: The present study evaluated the clinical, laboratory and imaging characteristics of 25 consecutive patients with a clinical diagnosis of myocarditis confirmed by 18F-FDG PET/CT or gallium-67 cardiac scintigraphy and RF reactivation according to the revised Jones Criteria. Patients underwent three sequential echocardiograms at (1) baseline, (2) during myocarditis and (3) post corticosteroid treatment. Patients were divided according to the presence (Group 1) or absence (Group 2) of reduced left ventricular ejection fraction (LVEF) during myocarditis episodes.

Results: The median age was 42 (17–51) years, 64% of patients were older than 40 years, and 64% were women. Between Group 1 ($n = 16$) and in Group 2 ($n = 9$), there were no demographic, echocardiographic or laboratory differences except for NYHA III/IV heart failure (Group 1: 100.0% vs. Group 2: 50.0%; $p = 0.012$) and LVEF (30 [25–37] vs. 56 [49–62]%, respectively; $p < 0.001$), as expected. Group 1 patients showed a significant reduction in LVEF during carditis with further improvement after treatment. There was no correlation between LVEF and valvular dysfunction during myocarditis. Among all patients, 19 (76%) underwent 18F-FDG PET/CT, with a positive scan in 68.4%, and 21 (84%) underwent gallium-67 cardiac scintigraphy, with positive uptake in 95.2%, there was no difference between these groups.

Conclusion: Myocarditis due to rheumatic fever reactivation can cause left ventricular dysfunction despite valvular disease, and it is reversible after corticosteroid treatment.

Keywords: myocarditis, rheumatic fever, rheumatic heart disease, valvular heart disease, heart failure

INTRODUCTION

Rheumatic fever is a prevalent disease, mainly in low- and middle-income countries but also in specific populations in developed countries. Data on the prevalence of rheumatic fever are probably underreported due to (a) the cost of screening, (b) difficulties in acute rheumatic disease diagnosis and (c) data on surgery or mortality representing rheumatic fever incidence from 2 decades ago. Chronic valvular heart disease is the most feared consequence of rheumatic fever, leading to a decreased quality of life, hospitalizations, and surgical procedures, primarily in young adults (1–5).

Heart failure occurs in ∼10% of patients with acute rheumatic fever with carditis and is described mainly during rheumatic fever reactivation (6–9). Valvulitis due to the involvement of the endocardium is the predominant manifestation of rheumatic fever carditis despite myocarditis and pericarditis occurrence. Furthermore, several studies have shown that heart failure in rheumatic fever patients results solely from valvular disease and is not due to primary myocarditis (10–14).

A recent case series of patients with predominantly rheumatic valvular disease undergoing heart transplantation showed that 27.7% had non-diagnosed myocarditis leading to refractory heart failure and ultimately heart transplantation. These patients had normofunctional valve prostheses and left ventricular dysfunction, suggesting that recurrent rheumatic fever may cause subacute myocarditis, a condition difficult to diagnose (15).

The aim of the present study was to evaluate the clinical, laboratory, and echocardiographic profiles of patients with rheumatic fever reactivation and clinical diagnosis of myocarditis, confirmed using fluorine-18-fluorodeoxyglucose positron emission tomography (18F-FDG PET/CT) or gallium-67 cardiac scintigraphy.

MATERIALS AND METHODS

Study Protocol and Population

This was a single-center, retrospective study assessing 25 consecutive patients between 2005 and 2020 with a diagnosis of rheumatic fever reactivation according to the revised Jones Criteria (16). Because the inhabitants of Brazil are considered a high-risk population (4), we selected the following major manifestations in this study: (i) carditis; (ii) monoarthritis, polyarthritis, or polyarthralgia; (iii) chorea; (iv) erythema marginatum; and (v) subcutaneous nodules. The minor manifestations were as follows: (i) monoarthralgia; (ii) fever; (iii) erythrocyte sedimentation rate ≥30 mm/h and/or C-reactive protein (CRP) ≥3.0 mg/dL; and (iv) prolonged PR interval. The presence of Aschoff bodies in the histological examination was considered a definitive criterion for rheumatic fever reactivation.

Rheumatic carditis diagnosis was confirmed in patients with at least one of the four clinical findings: (i) significant murmur ($n = 4$), (ii) cardiac enlargement ($n = 16$), (iii) cardiac decompensation ($n = 4$), or (iv) pericardial friction rub or effusion ($n = 1$) (5). To corroborate myocardial involvement, patients underwent 18F-FDG PET/CT or gallium-67 cardiac scintigraphy. Patients without myocardium involvement in both imaging tests were excluded from the present analysis. Differential diagnoses of myocardial involvement were assessed and excluded. Clinical data included age, sex, symptoms, medications, documented diagnosis of traditional cardiovascular risk factors, and comorbidities, such as hypertension, diabetes mellitus, and coronary artery disease. Patients also underwent laboratory tests and at least three sequential echocardiograms at (1) baseline, (2) during myocarditis, and (3) post corticosteroid treatment. To understand the impact of carditis on left ventricular function, patients were divided into two groups according to the left ventricular ejection fraction course during myocarditis episodes as follows:

- Group 1: reduction of left ventricular ejection fraction during the myocarditis episode;
- Group 2: no reduction in left ventricular ejection fraction during the myocarditis episode.

The study protocol was reviewed and approved by the local institutional ethics committee.

Echocardiography

All transthoracic Doppler-echocardiographic exams were analyzed in a central echocardiography laboratory at our institution. All exams were performed using a commercially available ultrasound system (Vivid 9, GE Healthcare, Milwaukee, WI, USA or EPIQ 7, Koninklijke Philips N.V., Amsterdam, Noord-Holland, Netherlands). Cardiac chambers were measured using the American Society of Echocardiography standards (17).

Fluorine-18-fluorodeoxyglucose positron emission tomography (18F-FDG PET/CT) imaging: Images were acquired on a positron emission tomography scanner coupled to computed tomography [Gemini TF 64 TOF (Philips Healthcare)] 1 h after intravenous administration of 18F-FDG (370 MBq). To suppress normal myocardial glucose utilization, patient preparation consisted of a high-fat and low- or no-carbohydrate diet 24 h prior to the exam, followed by 8–12 h of fasting. Tomographic reconstruction was performed in both modalities in the axial, sagittal, and coronal planes. FDG uptake in the myocardium was considered positive for inflammation. Visual and quantitative evaluation (standard uptake value, SUV) was performed.

Gallium-67 Cardiac Scintigraphy

Planar images of the thorax (anterior, lateral, and posterior views) were acquired 72 h after the intravenous injection of 150–185 MBq of gallium-67 citrate using an Infinia gamma camera (GE Healthcare). The intensity of gallium-67 uptake in the heart was compared with that in the ribs and sternum, and any evidence of gallium-67 cardiac uptake (qualitative evaluation) was considered positive for active inflammation.

Outcomes

The endpoints analyzed were 30-day mortality, vasoactive drug use, surgery during myocarditis and late cardiac death.

TABLE 1 | Baseline clinical and laboratory data of the study population.

Variable	Overall ($n = 25$)	Group 1 (LVEF reduction during carditis) ($n = 16$)	Group 2 (No LVEF reduction during carditis) ($n = 9$)	*p*-value
Clinical data				
Age, years	42 (17–51)	42 (37–57)	41 (22–51)	0.452
Age ≥40 years	16 (64.0)	11 (68.8)	5 (55.6)	0.821
Female sex	16 (64.0)	11 (68.8)	5 (55.6)	0.821
Hypertension	7 (28.0)	4 (25.0)	3 (33.3)	1.000
Diabetes mellitus	4 (16.0)	3 (18.8)	1 (11.1)	1.000
Dyslipidemia	4 (16.0)	2 (12.5)	2 (22.2)	0.946
Coronary artery disease	–	–	–	–
Previous stroke or TIA	3 (12.0)	2 (12.5)	1 (11.1)	1.000
Atrial fibrillation	17 (68.0)	10 (62.5)	7 (77.8)	0.734
Symptoms				
Heart failure NYHA I/II	3 (12.0)	–	3 (37.5)	0.050
Heart failure NYHA III/IV	20 (80.0)	16 (100.0)	4 (50.0)	**0.012**
Arthralgia	3 (12.0)	1 (6.3)	2 (25.0)	0.513
Fever	3 (12.0)	–	3 (33.3)	0.069
Medications				
ACE inhibitors or ARB	11 (44.0)	6 (37.5)	5 (55.6)	0.650
β-Blockers	17 (68.0)	12 (75.0)	5 (55.6)	0.580
Antiplatelet agents	5 (20.0)	2 (13.3)	3 (33.3)	0.516
Furosemide	17 (68.0)	12 (75.0)	5 (55.6)	0.580
Spironolactone	10 (40.0)	6 (37.5)	4 (44.4)	1.000
Statins	3 (12.0)	2 (12.5)	1 (11.1)	1.000
Digoxin	9 (36.0)	6 (37.5)	3 (33.3)	1.000
Oral anticoagulation	17 (68.0)	12 (75.0)	5 (55.6)	0.580
Penicillin	9 (36.0)	5 (31.3)	4 (44.4)	0.821
Laboratory data				
Hemoglobin, g/dl	12.5 (11.7–13.9)	12.0 (11.5–13.3)	13.5 (12.6–14.0)	0.121
Hematocrit, %	38 (37–41)	37 (34–38)	40 (39–42)	0.083
Leukocytes, /mm^3	6,020 (4,110–8,305)	5,690 (388–9,035)	7,540 (4,760–8,372)	0.301
C-reactive protein, mg/dl	6.29 (2.26–23.12)	11.11 (4.24–46.00)	4.36 (1.57–11.20)	0.370
eGFR, ml/min/1.73 m2	77.6 (55.3–84.0)	77.6 (51.4–84.0)	76.4 (59.7–82.6)	0.860

Values are median (interquartile range), or n (%). ACE indicates angiotensin-converting enzyme; ARB, angiotensin receptor blocker, eGFR, estimated glomerular filtration rate; LVEF, left ventricular ejection fraction, NYHA, New York Heart Association; and TIA, transient ischemic attack. Bold values denote statistical significance.

Statistical Analysis

Continuous variables are presented as medians (interquartile ranges), and categorical variables are presented as percentages. The Shapiro-Wilk test was used to test the normality of variables. The Mann-Whitney test was applied for continuous variables, and Fisher's exact test or the Chi-squared test was applied for categorical variables. Generalized estimating equations were used to analyze repeated echocardiographic measures (gamma or binary logistic model as appropriate). The *post-hoc* analysis was performed with a Bonferroni test. Kaplan-Meier curves and log-rank test of the time-to-event data were used to evaluate late cardiac mortality. All tests were two-tailed, and $p < 0.05$ was used to indicate statistical significance. All analyses were conducted using the SPSS statistical package, version 20 (IBM, Armonk, NY).

RESULTS

Patient Characteristics

The main baseline clinical and laboratory data are shown in **Table 1**. Among the 25 patients with myocarditis included in the study, the median age was 42 (17–51) years, 64% of the patients were older than 40 years, and 64% were women. We found a low incidence of comorbidities with the exception of atrial fibrillation (found in 68%), and no patient had coronary artery disease. There were no demographic or laboratory differences between Group 1 ($n = 16$) and Group 2 ($n = 9$). However, Group 1 patients had

TABLE 2 | Baseline echocardiographic, 18F-FDG PET/CT and Gallium-67 cardiac scintigraphy data.

Variable	Overall ($n = 25$)	Group 1 (LVEF reduction during carditis) ($n = 16$)	Group 2 (No LVEF reduction during carditis) ($n = 9$)	p-value
Echocardiography during myocarditis				
LVEF, %	36 (27–53)	30 (25–37)	56 (49–62)	**<0.001**
LVEDD, mm	62 (48–70)	58 (48–68)	65 (47–73)	0.601
LVESD, mm	46 (38–58)	49 (40–58)	45 (32–56)	0.512
LVEDV, mL	145 (93–229)	118 (93–210)	198 (97–268)	0.382
LVESV, mL	76 (55–116)	72 (52–181)	83 (56–115)	1.000
LV mass, g/m^2	132 (95–165)	123 (96–162)	141 (95–207)	0.773
Pulmonary artery systolic pressure, mmHg	56 (51–64)	57 (52–64)	53 (38–64)	0.301
Moderate/severe rheumatic aortic regurgitation	9 (36.0)	3 (18.8)	6 (66.7)	0.050
Moderate/severe rheumatic mitral regurgitation	14 (58.3)	7 (46.7)	7 (77.8)	0.285
Moderate/severe functional tricuspid regurgitation	17 (73.9)	11 (73.3)	6 (75.0)	1.000
Moderate/severe right ventricular dysfunction	11 (47.8)	9 (60.0)	2 (25.0)	0.245
Normofunctional valve prosthesis, mitral bioprosthesis stenosis or native mitral stenosis	16 (64.0)	11 (68.8)	5 (55.6)	0.821
Pericardial effusion				0.267
Discrete	5 (20.0)	4 (26.7)	1 (12.5)	
Moderate	1 (4.0)	–	1 (12.5)	
18F-FDG PET/CT	$N = 19$	$N = 13$	$N = 6$	
Positive scan	13 (68.4)	10 (76.9)	3 (50.0)	0.520
SUV max	4.5 (3.35–8.37)	4.15 (3.12–7.22)	7.64 (3.50–7.64)	0.371
Gallium-67 cardiac scintigraphy	$N = 21$	$N = 13$	$N = 8$	
Positive Gallium-67 imaging	20 (95.2)	12 (92.3)	8 (100.0)	1.000
Gallium uptake				0.070
Discrete	12 (60.0)	5 (41.7)	7 (87.5)	
Discrete/moderate	8 (40.0)	7 (58.3)	1 (12.5)	

Values are median (interquartile range), or n (%).18F-FDG PET/CT indicates fluorine-18-fluorodeoxyglucose positron emission tomography; LV, left ventricular; LVEDD, left ventricular end-diastolic diameter; LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; LVESD, left ventricular end-systolic diameter; LVESV, left ventricular end-systolic volume; and SUV, standardized uptake value. Bold values denote statistical significance.

more NYHA III/IV heart failure than Group 2 patients (100.0 vs. 50.0%, respectively; $p = 0.012$).

Rheumatic Fever Reactivation Diagnosis

Carditis was considered a major manifestation for all patients. In addition to rheumatic carditis, six patients had two minor manifestations (CRP ≥3.0 mg/dL + fever in three patients; and CRP ≥3.0 mg/dL + monoarthralgia in three patients). Two patients had rheumatic carditis confirmed by myocardial biopsy. Rheumatic carditis was highly suspected in the remaining patients.

Complementary Tests

The main baseline data from echocardiography, 18F-FDG PET/CT and gallium-67 cardiac scintigraphy are shown in **Table 2**. The criteria for rheumatic carditis were the presence of cardiac enlargement in the 16 patients in Group 1, significant murmur in four patients in Group 2, pericardial effusion in one patient in Group 2 and cardiac decompensation in four patients in Group 2. During myocarditis, Group 1 patients had a lower left ventricular ejection fraction than Group 2 patients (30 [25–37] vs. 56 [49–62]%, respectively; $p < 0.001$), as expected. There was no other significant echocardiographic difference between the groups. Normofunctional valve prostheses, mitral bioprostheses with stenosis or native mitral stenosis were found in 64.0% of patients. In 19 patients, 18F-FDG PET/CT was performed, with positive scans in 68.4% and a median SUV ratio of 4.5 (3.35–8.37), and there was no difference between groups regarding the SUV ratio (**Figure 1**). Gallium-67 cardiac scintigraphy was performed in 21 patients, with positive uptake in 95.2%, and there

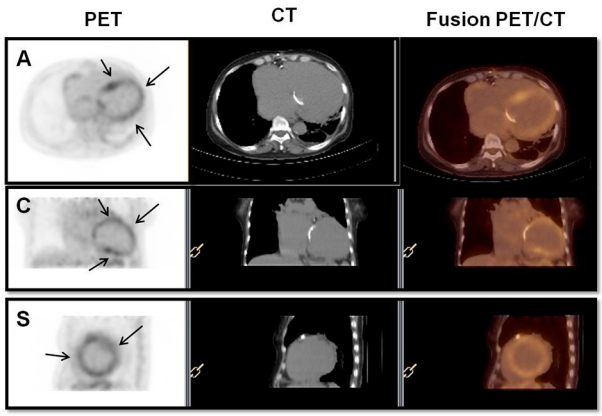

FIGURE 1 | Positive ^{18}F-FDG PET/CT scan. Fluorine-18-fluorodeoxyglucose positron emission tomography (18F-FDG PET/CT) (PET, CT, and fusion PET/CT) showing diffuse FDG uptake in the LV myocardium (arrows), indicating active inflammation. The intensity of uptake was discrete, and SUVmax was 3.3. A, axial; C, coronal; S, sagittal; and SUV, standardized uptake value.

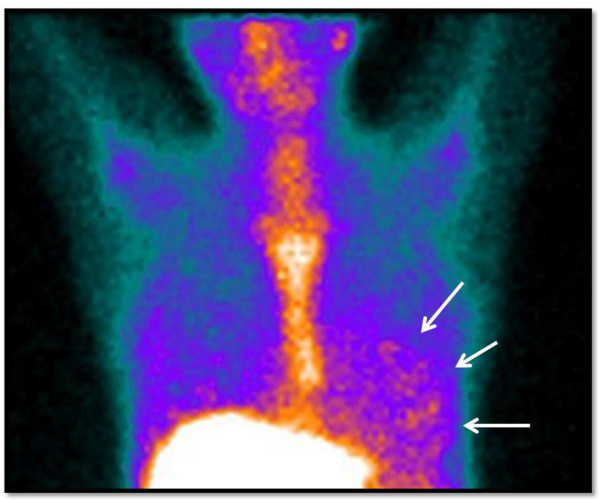

FIGURE 2 | Positive gallium-67 cardiac scintigraphy (anterior view of the thorax). Image obtained 72 h after radiopharmaceutical administration, showing diffuse and mild gallium-67 uptake in cardiac projection (arrows), indicating an inflammatory myocardial process. Physiological gallium-67 uptake was observed in the liver and bones (sternum and ribs).

was no difference between groups regarding gallium-67 uptake (**Figure 2**).

Sequential Echocardiographic Findings

A comparison of Group 1 and Group 2 baseline echocardiography and echocardiography during myocarditis and post-treatment echocardiography is shown in **Table 3**, **Figures 3**, **4**. There was a difference within subjects in relation to the three echocardiograms and between groups regarding the left ventricular ejection fraction ($p < 0.001$ and $p = 0.011$, respectively). The *post-hoc* analysis showed that differences existed between baseline echocardiography and echocardiography during myocarditis ($p < 0.001$) as well as between echocardiography during myocarditis and post-treatment echocardiography ($p = 0.020$) within Group 1 subjects. When comparing Group 1 to Group 2, differences were related to echocardiography during myocarditis ($p < 0.001$) as previously described. Thus, Group 1 patients showed a significant reduction in left ventricular function during carditis with further improvement after treatment.

Correlation Between Echocardiographic Data During Myocarditis

There was no correlation between left ventricular ejection fraction and moderate/severe rheumatic mitral regurgitation ($p = 0.375$), moderate/severe rheumatic aortic regurgitation ($p = 0.437$), moderate/severe functional tricuspid regurgitation ($p = 0.320$), and moderate/severe right ventricular dysfunction ($p = 0.053$).

Clinical Outcomes

The clinical outcomes are shown in **Table 4**. All patients were treated with corticosteroids, and 76.0% were treated during hospitalization. Valvular heart surgery during myocarditis was performed in 12% of patients, and there was no 30-day mortality. The median follow-up was 10.8 (2.6–30.8) months, and late cardiovascular deaths occurred in 20% of patients, with no difference between the groups (log-rank $p = 0.829$). Causes of late death were septic shock ($n = 2$), endocarditis ($n = 1$), cardiogenic shock ($n = 1$), and complications of myocardium biopsy ($n = 1$).

DISCUSSION

The main findings of the present study were as follows: (1) rheumatic fever reactivation can cause myocarditis and left ventricular dysfunction in the absence of severe valvular heart disease; and (2) in these cases, the left ventricular ejection fraction improves after corticosteroid treatment.

Rheumatic heart disease is the most common consequence of a prevalent and difficult-to-diagnose disease. Approximately 20% of rheumatic fever patients may present reactivation episodes in 10 years, causing death, hospitalization and worsening of valvular heart disease severity (18). In addition, the diagnosis of acute rheumatic fever is complex. There is no definitive diagnostic test, and clinical criteria show high sensitivity and low specificity (16). These factors, together with the difficulty of accessing health services in low-income countries, explain the relatively low acute-phase diagnostic rate and low number of patients included in studies (1–5).

Regarding rheumatic carditis, diagnostic criteria are vague and depend only on the presence of valvulitis (16). In addition, several studies and guidelines claim that valvular disease is the cause of cardiac decompensation in acute rheumatic fever and not myocardial dysfunction itself (5, 10–14), which raises several questions. It is unknown if patients with valve prostheses have

TABLE 3 | Comparison of baseline echocardiography, echocardiography during myocarditis and post-treatment echocardiography of patients with and without reduction of left ventricular ejection fraction during carditis episode.

Variable	Group 1 (LVEF reduction during carditis) ($n = 16$)			Group 2 (No LVEF reduction during carditis) ($n = 9$)			P (WS)	P (BG)
	Baseline echocardiography	Echocardiography during myocarditis	Post-treatment echocardiography	Baseline echocardiography	Echocardiography during myocarditis	Post-treatment echocardiography		
LVEF, %	57 (49–64)	30 (25–37)	45 (30–54)	54 (39–61)	56 (49–62)	56 (43–62)	**<0.001**	**0.011**
LVEDD, mm	56 (48–64)	58 (48–68)	50 (48–65)	60 (52–62)	65 (47–73)	56 (44–65)	**0.039**	0.754
LVESD, mm	39 (31–48)	49 (40–58)	36 (32–56)	41 (28–50)	45 (32–56)	40 (29–54)	0.053	0.563
LVEDV, mL	154 (97–194)	118 (93–210)	118 (87–224)	187 (16–222)	198 (97–268)	107 (76–191)	0.268	0.822
LVESV, mL	66 (39–90)	72 (52–181)	45 (41–173)	78 (45–127)	83 (56–115)	42 (26–88)	0.240	0.369
LV mass, g/m^2	102 (93–134)	123 (96–162)	98 (88–135)	132 (86–172)	141 (95–207)	109 (75–169)	0.149	0.632
Pulmonary artery systolic pressure, mmHg	46 (33–58)	57 (52– 64)	55 (42–59)	50 (39–58)	53 (38–64)	49 (42–49)	**0.019**	0.885
Moderate/severe rheumatic aortic regurgitation	1 (6.7)	3 (18.8)	1 (7.1)	4 (50.0)	6 (66.7)	4 (57.1)	0.835	0.053
Moderate/severe rheumatic mitral regurgitation	1 (6.7)	7 (46.7)	–	3 (42.9)	7 (77.8)	3 (50.0)	**<0.001**	**<0.001**
Moderate/severe functional tricuspid regurgitation	6 (42.9)	11 (73.3)	5 (38.5)	2 (28.6)	6 (75.0)	2 (33.3)	**0.005**	0.718
Moderate/severe right ventricular dysfunction	1 (6.7)	9 (60.0)	3 (25.0)	1 (12.5)	2 (25.0)	1 (16.7)	**0.013**	0.663

Values are median (interquartile range), or n (%). BG indicates between groups; LV, left ventricular; LVEDD, left ventricular end-diastolic diameter; LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; LVESD, left ventricular end-systolic diameter; LVESV, left ventricular end-systolic volume; and WS, within subjects. Bold values denote statistical significance.

acute rheumatic myocarditis, and if they do, it is unknown if these patients are protected from cardiac decompensation.

An unexpected finding from a previous study refutes this hypothesis. In this previous case series of patients with valvular heart disease undergoing heart transplantation, Aschoff bodies were found in the histological examination of 27.7% of the recipients' hearts. Those patients did not have a prior diagnosis of reactivated rheumatic myocarditis at the time of transplantation, and they had normofunctional valve prostheses and left ventricular dysfunction, suggesting a subacute myocarditis diagnosis (15). Unlike the cited study, all patients in the present study were treated with corticosteroids, including patients with a reduction in the left ventricular ejection fraction. Despite the lack of pathological confirmation of rheumatic fever reactivation, rheumatic myocarditis was an exclusion diagnosis, and the improvement of left ventricular ejection fraction after treatment was highly suggestive of rheumatic fever. Notably, no patient had a history of coronary artery disease or other cardiomyopathy.

All patients underwent confirmation of myocarditis using 18F-FDG PET/CT or gallium-67 cardiac scintigraphy. 18F-FDG PET/CT is a new tool for the diagnosis of inflammation. F-18 2-fluoro2-deoxy-D glucose (18F-FDG) is an analog of glucose, and like glucose, it is taken up by activated inflammatory cells that accumulate at the sites of infection or inflammation. Thus, diffuse myocardial uptake is highly suggestive of myocarditis. Because the heart uses a mixture of free fatty acids and glucose for energy production under normal resting conditions and to obtain information regarding the inflammatory process in the myocardium, it is necessary to inhibit physiological myocardial glucose uptake. For this purpose, we used a preparation that consisted of a high-fat and low- or no-carbohydrate diet 24 h before 18F-FDG administration. Data on the role of 18F-FDG PET/CT in the context of acute rheumatic fever are scarce. However, previous studies have reported low sensitivity but high positive predictive value of 18F-FDG PET/CT in the context of acute rheumatic fever (19, 20). Gallium-67 cardiac scintigraphy is also a marker of cardiac inflammation, and the accuracy of this technique varies according to the etiology of myocarditis (21). Data on the role of gallium-67 cardiac scintigraphy in acute rheumatic fever patients have shown good sensitivity and positive predictive value for the diagnosis and evaluation of the therapeutic results (22). Most of the 20 patients undergoing gallium-67 cardiac scintigraphy showed discrete uptake, which confirmed previous studies demonstrating that rheumatic fever is characterized predominantly by interstitial inflammatory changes with minimal damage to myocardial cells and, thus, with low levels of troponin T or I (12, 23, 24). It is important to note that 18F-FDG PET/CT or gallium-67 cardiac scintigraphy was not mandatory for rheumatic carditis diagnosis. These procedures were used only to demonstrate that active inflammation occurs not only in the endocardium but also in the myocardium. Rheumatic carditis is an exclusion criterion, and the improvement of symptoms and reverse

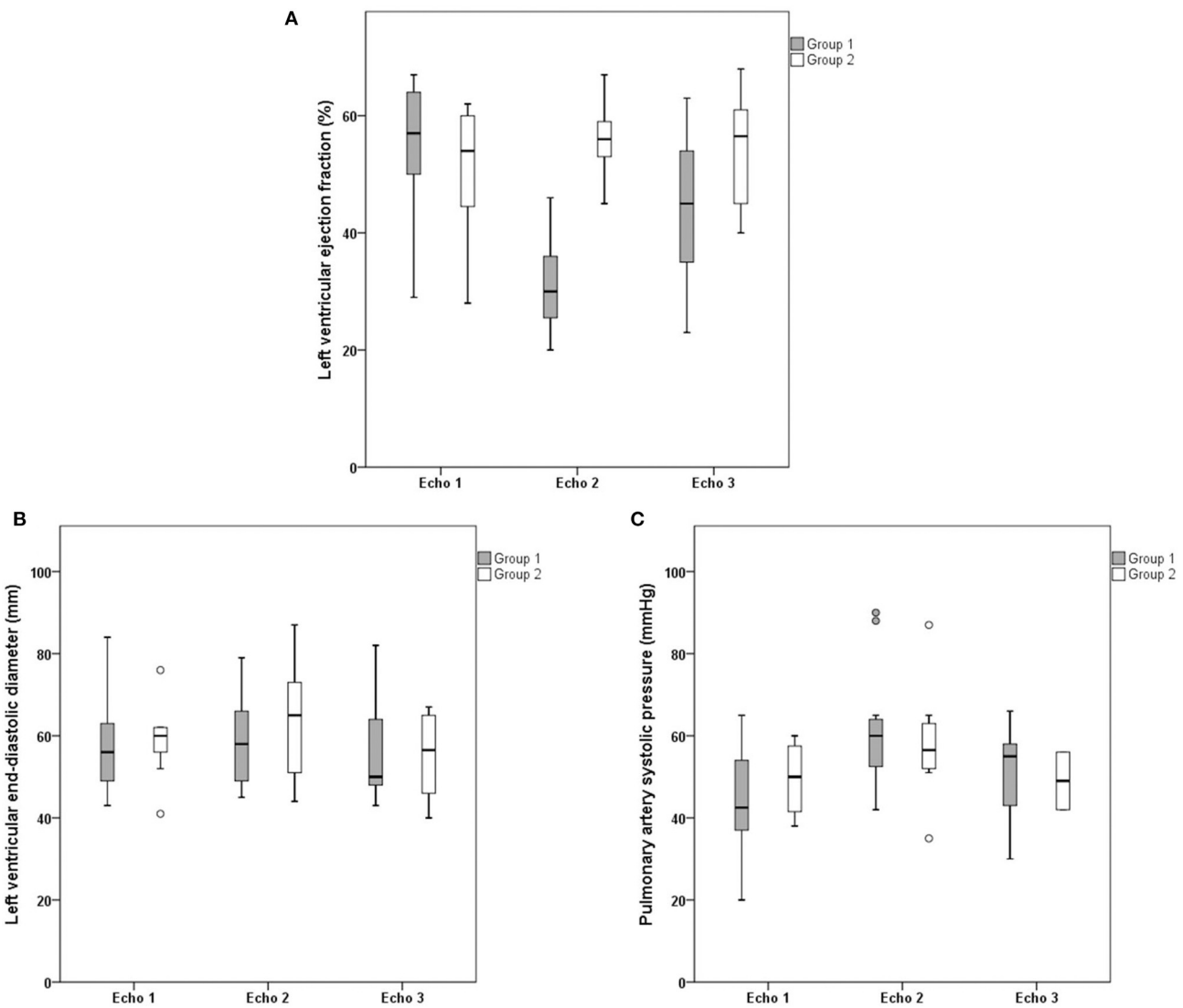

FIGURE 3 | Sequential echocardiographic findings: left ventricular ejection fraction, left ventricular end-diastolic diameter and pulmonary artery systolic pressure. Comparison of baseline echocardiogram (Echo 1), echocardiogram during myocarditis (Echo 2) and postcorticosteroid treatment echocardiogram (Echo 3) between Group 1 (left ventricular ejection fraction reduction during carditis) and Group 2 (no left ventricular ejection fraction reduction during carditis). Comparison of **(A)** left ventricular ejection fraction, **(B)** left ventricular end-diastolic diameter, and **(C)** pulmonary artery systolic pressure. Solid horizontal line indicates mean value. Gray and white boxes indicate 1 SD, and vertical lines indicate highest and lowest mean values.

cardiac remodeling after corticosteroid treatment retrospectively reinforced the diagnosis.

Our patients differed from those with acute rheumatic myocarditis reported in the literature. Most previous studies have included first episodes in children, while the patients in our study had reactivation and were mostly (64%) older than 40 years (10–14). In addition, moderate/severe mitral regurgitation showed an increased incidence during myocarditis episodes. However, unlike the literature, there was no correlation between mitral regurgitation and left ventricular ejection fraction in the present study, and only three patients required surgical treatment during carditis. Another important aspect was the high prevalence of atrial fibrillation (68%). Despite the low median age of the studied population (42 [17–51] years), chronic rheumatic heart disease is associated with an increased prevalence of atrial fibrillation, ranging from 4.3 to 79.9% (25).

The present study had several limitations. First, this was a single-center study with a relatively small number of patients (albeit large for this clinical entity). Second, this was a retrospective study with all inherent bias due to its nature. Although left ventricular dysfunction was not related to valvular disease itself in most of patients, it is difficult to rule-out valvular disease as a cause of heart failure in the these patients due to the high prevalence of atrial fibrillation and due to the study design.

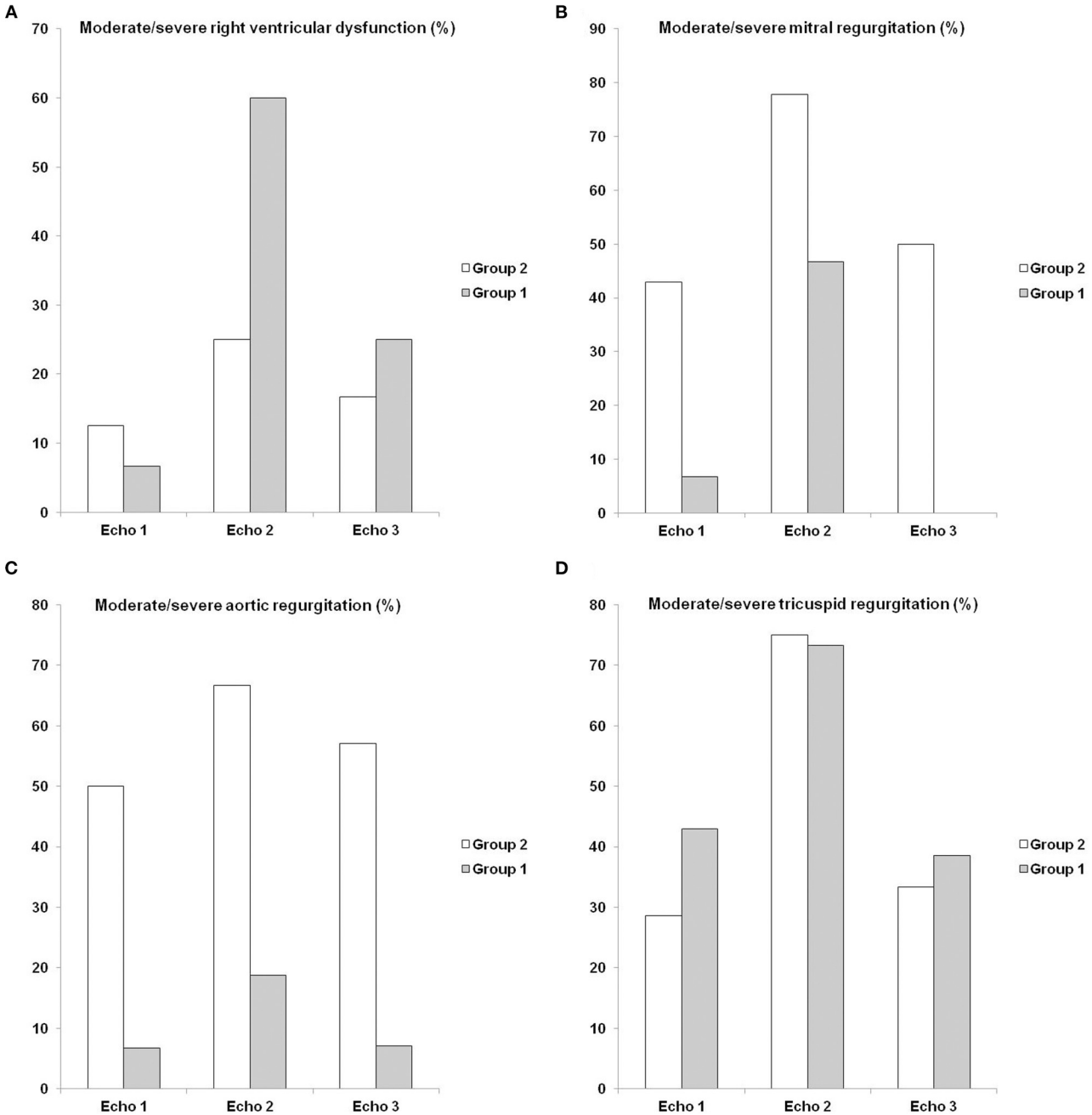

FIGURE 4 | Sequential echocardiographic findings: moderate/severe right ventricular dysfunction, moderate/severe mitral regurgitation, moderate/severe aortic regurgitation and moderate/severe tricuspid regurgitation. Comparison of baseline echocardiogram (Echo 1), echocardiogram during myocarditis (Echo 2) and postcorticosteroid treatment echocardiogram (Echo 3) between Group 1 (left ventricular ejection fraction reduction during carditis) and Group 2 (no left ventricular ejection fraction reduction during carditis). Comparison of **(A)** moderate/severe right ventricular dysfunction, **(B)** moderate/severe mitral regurgitation, **(C)** moderate/severe aortic regurgitation, and **(D)** moderate/severe tricuspid regurgitation. Gray and white bars indicate percentage (%) of each group.

Unfortunately, we were also unable to evaluate some important data, e.g., electrocardiogram analysis. Third, patients who did not undergo 18F-FDG PET/CT or gallium-67 cardiac scintigraphy and those whose tests were negative were excluded from the analysis. This bias may have contributed to underestimating 30-day mortality.

In conclusion, this study showed that myocarditis due to rheumatic fever reactivation may cause left ventricular

TABLE 4 | Clinical outcomes.

Variable	Overall ($n = 25$)	Group 1 (LVEF reduction during carditis) ($n = 16$)	Group 2 (No LVEF reduction during carditis) ($n = 9$)	*p*-value
In-hospital care	19 (76.0)	12 (75.0)	7 (77.8)	1.000
Vasoactive drugs	12 (48.0)	8 (50.0)	4 (44.4)	1.000
Surgery during carditis	3 (12.0)	3 (18.8)	–	0.457
30-day mortality	–	–	–	–
Late cardiovascular death	5 (20.0)	2 (12.5)	3 (33.3)	0.466

Values are n (%). LVEF indicates left ventricular ejection fraction.

dysfunction, which is reversible after corticosteroid treatment. In addition, heart failure was not related to valvular disease itself in 64% of patients. These findings contradict the statement that heart failure in the acute-phase of carditis only occurs in patients with severe valvular lesions. However, due to the inherent limitations of the present study, further prospective research is needed to confirm these findings.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the data, analytic methods, and study materials will not be made available to other researchers for purposes of reproducing the results or replicating the procedure. Requests to access the datasets should be directed to Vitor Rosa, vitoremer@yahoo.com.br.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by CAPPesq - Comissão de Ética para Análise de Projetos de Pesquisa do HCFMUSP. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

VR, GS, JS, LG, AdS, JF, and RS: design of the study. MLop, DS, CR, and MLot: data collection. VR and RS: data analysis. VR: drafting. VR, MLop, GS, JS, AdS, LP, JF, RS, and FT: approval of the final version. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cardiovascular Medicine

ORIGINAL RESEARCH
published: 23 June 2021
doi: 10.3389/fcvm.2021.676098

Improvement of Rheumatic Valvular Heart Disease in Patients Undergoing Prolonged Antibiotic Prophylaxis

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OPEN ACCESS

Edited by:
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Reviewed by:
Matthew Bersi, Washington University in St. Louis, United States
Francesca Bartoli-Leonard, Brigham and Women's Hospital and Harvard Medical School, United States

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Specialty section:
This article was submitted to Heart Valve Disease, a section of the journal Frontiers in Cardiovascular Medicine

Received: *04 March 2021*
Accepted: *05 May 2021*
Published: *23 June 2021*

Citation:
Torres RPdA, Torres RFdA, Crombrugghe Gd, Moraes da Silva SP, Cordeiro SLV, Bosi KA, Smeesters PR and Torres RSLdA (2021) Improvement of Rheumatic Valvular Heart Disease in Patients Undergoing Prolonged Antibiotic Prophylaxis. Front. Cardiovasc. Med. 8:676098. doi: 10.3389/fcvm.2021.676098

Secondary prophylaxis of rheumatic heart diseases is efficient in reducing disease recurrence, heart damage, and cardiac impairment. We aimed to monitor the clinical evolution of a large Brazilian cohort of rheumatic patients under prolonged secondary prophylaxis. From 1986 to 2018, a cohort of 593 patients with rheumatic fever was followed every 6 months by the Reference Center for the Control and Prevention of Rheumatic Fever and Rheumatic Cardiopathy (CPCFR), Paraná, Brazil. In this cohort, 243 (41%) patients did not present cardiac damage (group I), while 350 (59%) were diagnosed with rheumatic heart disease (RHD) (group II) using the latest case definition. Among group II, 233 and 15 patients had impairment of the mitral and aortic valves, respectively, while 102 patients had impairment of both valves. Lesions on the mitral and aortic valves presented a regression in 69.9 and 48.7% of the patients, respectively. Active patient recruitment in the reference center and early detection of oropharyngeal GAS were important factors for optimal adherence to the prophylactic treatment. Patients with disease progression were associated with noncompliance to secondary prophylaxis. No patients undergoing regular prophylaxis presented progression of the rheumatic cardiac disease. Eighteen valvular surgeries were performed, and four (0.7%) patients died. This study confirmed that tailored and active efforts invested in rheumatic heart disease secondary prevention allowed for significant clinical improvement.

Keywords: secondary prophylaxis, rheumatic heart disease, benzathine penicillin G, Group A β-hemolytic *Streptococcus*, carditis, recurrence

INTRODUCTION

Acute rheumatic fever (ARF) is an inflammatory, autoimmune disease induced by a throat infection caused by *Streptococcus pyogenes* (Group A β-hemolytic *Streptococcus*—GAS) in genetically predisposed individuals. Global prevalence of rheumatic heart disease (RHD) is estimated to be 33.4 million and is responsible for about 319,400 deaths per year (1). Most cases of ARF occur in

low- and middle-income countries where limited resources are often available for optimal health programs. In Brazil, a significant amount of financial resources from the Brazilian Unified Health System (SUS) is intended to assist and treat ARF and RHD patients (2, 3).

Promising progresses have been recently made toward a safe and protective vaccine against streptococcal infections (4, 5). However, prophylaxis remains, so far, the most effective treatment option to prevent RHD recurrences. Benzathine penicillin-based prophylaxis, every 3–4 weeks, remains the treatment of choice since GAS continues to be fully susceptible to penicillin (6, 7).

Secondary prophylaxis of RHD is known for modifying the natural history of the disease, allowing for the prevention of disease recurrence and consequently the prevention of further development of heart damage and/or cardiac impairment (8–10). Nevertheless, less is known about the impact of secondary prophylaxis on the recovering of cardiac damage in patients who regularly undergo a prolonged prophylactic treatment. The aim of this study is to describe the RHD secondary prevention program in the Brazilian state of Paraná and monitor the cardiac evolution of rheumatic patients undergoing prolonged and regular secondary prophylaxis.

MATERIALS AND METHODS

This prospective cohort study monitors the clinical evolution of patients with confirmed ARF diagnosed on the basis of the 2015 revised Jones criteria (11). The patients were diagnosed, registered, and followed up at the Center of Reference for the Control and Prevention of Rheumatic Fever and Rheumatic Cardiopathy (CPCFR) of the Health Secretariat of the State of Paraná/Brazil from July 1986 through June 2018.

All included patients had a confirmed history of ARF or presented with one or more morphological features of RHD according to the World Heart Federation (WHF) criteria (12). Doppler and morphological echocardiogram findings are detailed in **Supplementary Table 2**.

RHD patients were classified according to the severity of their valvulopathy into mild, moderate, or severe according to the most recent definition (13, 14). Patients with borderline RHD according WHF guidelines (12), those who remained under prophylaxis treatment for <2 years, and those receiving oral penicillin treatment were excluded from the study.

Patients were divided into two groups. Group I included patients without cardiac damage, and group II included patients who presented cardiac damage consistent with RHD, either as an isolated manifestation or in association with arthritis, chorea, erythema marginatum, and subcutaneous nodules (**Supplementary Table 1**).

All patients were invited to follow a secondary antibiotic prophylaxis using intramuscular (IM) benzathine penicillin G every 21 or 28 days as recommended by the American Heart Association (AHA) (6) and to undergo a follow-up clinical and echocardiographic evaluation every 6 months. To improve compliance, reminder messages were sent to those who missed their semester consultation. Throat swab was performed at each consultation searching for the presence of GAS. In case of positive culture, discussion was undertaken with the patients to recall the importance of treatment compliance.

Adherence to the prophylactic treatment was checked at each consultation during follow-up. The patients receiving penicillin injections every 21 days were considered as compliant and included in the "regular prophylactic treatment group" when receiving at least 13 doses/year (more than 76% of the doses), with a maximum delay of 7 days, i.e., 28 days between the doses. Patients who received <13 doses/years and/or had spaced their doses (more than 28 days between doses) were included in the "irregular prophylactic treatment group."

The patients receiving penicillin injections every 28 days were included in the "regular prophylactic treatment group" when receiving at least 10 doses/years (more than 76% of the doses), with a maximum delay of 7 days, i.e., 35 days between the doses. Patients who received <10 doses/years and/or had spaced their doses (more than 35 days between doses) were included in the "irregular prophylactic treatment group."

The progression of the cardiac impairment was assessed by comparing the type and severity of valvular heart damage when the patient first registered at the CPCFR with the type and severity of the valvular heart damage at discharge. Progression was defined as a change in diagnostic (RHD or normal) or a modification in the severity of RHD cases (mild, moderate, or severe cases).

To determine possible associations between the categorical variables, chi-square (χ^2) and t tests were used to compare means; correction was performed by Fisher's exact test. The significance level of $p < 0.05$ was adopted.

TABLE 1 | Rheumatic fever patients' characteristics (group I and II).

Characteristic *N* (%)	
Gender	
Female	309 (52.1)
Male	284 (47.9)
Age at screening, years	2–21 years (median of 9 years)
Duration of follow-up, years	2–26 years (median of 10 years)
Duration of follow-up detailed:	
2 to <3 years: 11 (1.9%)	
3 to <5 years: 70 (11.8%)	
5 to <6 years: 32 (5.4%)	
6 to <10 years: 165 (27.8%)	
10 to <11 years: 31 (5.2%)	
≥11 years: 284 (47.9%)	
Time of follow-up necessary to achieve total recovery of valve lesions:	
Recovery of mitral valve (*N* = 104):	Recovery of aortic valve (*N* = 10):
After 2 years = 40 (38.5)	After 2 years = 5 (50.0)
After 5 years = 53 (50.9)	After 5 years = 5 (50.0)
After >5 years = 11 (10.6)	After >5 years = 0 (0.0)

This study was approved by the Research Ethics Committee at the Pequeno Príncipe Hospital under CAAE-02153912.2.0000.0097.

RESULTS

This study has monitored 593 ARF patients from the 709 registered at the CPCFR during the study period. One hundred and sixteen patients (16.4%) were excluded for not meeting the inclusion criteria: 70 (9.9%) for not complying with at least 2 years of follow-up, 26 (3.7%) for presenting borderline RHD, and 20 (2.8%) for oral penicillin V prophylaxis. Three hundred and nine patients (52.1%) were female and 284 (47.9%) were male. Patients included in the study were between 2 and 21 years old at the beginning of follow-up. The follow-up time ranged from 2 to 26 years with almost half of the patients (47.6%) followed for more than 11 years (**Table 1**).

All patients started their prophylactic treatment with IM penicillin every 3 weeks, as recommended by the AHA for populations with particularly high incidence of rheumatic fever (6). A minority of the patients, 62 (10.4%), spaced their prophylactic treatment to every 4 weeks when they completed 21 years of age. GAS isolation in the culture of the patients' oropharynx helped identify non-compliance to the secondary prophylaxis in 92 (15.5%) patients. These patients received more attention and guidance from the CPCFR team to raise their awareness about the risks of recurrence and progression of the disease.

Group I consisted of 243 patients (41.0%) without cardiac lesions. At the beginning of the follow-up, 207 of these patients (85.2%) had presented a single episode of ARF, and 36 (14.8%)

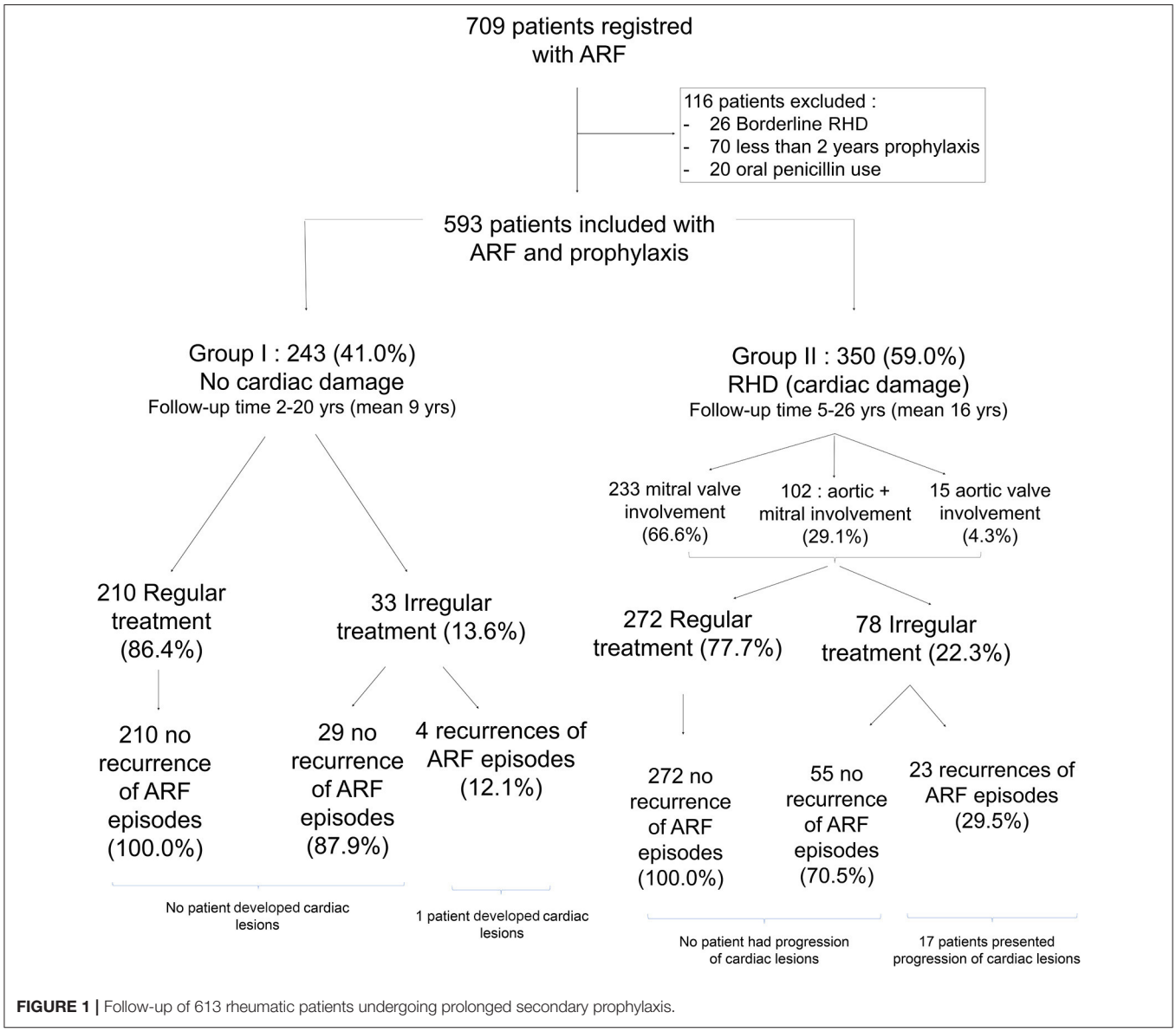

FIGURE 1 | Follow-up of 613 rheumatic patients undergoing prolonged secondary prophylaxis.

had presented more than one episode. The follow-up time of this group ranged from 2 to 20 years (median of 9 years). Most patients (210; 86.4%) were compliant to prophylaxis and included in the "regular prophylactic treatment group," while 33 (13.6%) were not always compliant and therefore included in the "irregular prophylactic treatment group." Four patients (12.1%) from group I had a recurrence of ARF: two with arthritis, one presented two recurrences of chorea, and one developed carditis and mild mitral regurgitation (MR). All these episodes of recurrences occurred in the "irregular prophylactic treatment group" (**Figure 1**).

Group II consisted of 350 patients (59.0%) with cardiac lesions consistent with RHD (12). At the start of the follow-up, 206 patients (58.9%) had presented a single episode of ARF, while 144 (41.1%) had presented more than one episode. Most patients in group II presented mitral valve involvement (233; 66.6%), 15 patients (4.3%) presented aortic valve involvement, and 102 patients (29.1%) presented coexistence of mitral and aortic involvement (**Table 2**). The follow-up time of this group ranged from 5 to 26 years of age (median of 16 years). Two hundred and seventy-two patients (77.7%) were included in the "regular prophylactic treatment group" while 78 (22.3%) underwent irregular prophylactic treatment. In the irregular treatment group, we observed 23 (29.5%) recurrences of ARF episodes, leading to a worsening of the cardiac lesions in 17 patients (**Figure 1**). Gender analyses of mitral and aortic lesion evolution did not show significant differences (**Table 3**). Although recurrences were observed in all age groups, the highest frequency, even if age difference is not statistically significant, was overall observed among adolescents (14 to 16 years old) (**Table 3**).

Many patients from group II benefitted from the secondary prophylaxis. The patients presenting aortic regurgitation and/or mitral regurgitation were those who benefitted most from regular prophylaxis, presenting the highest proportion of recovery of their valve lesions. Two hundred thirty-four patients (69.9%) presented regression of the mitral valve lesions including 104 (31.0%) with a total regression. Fifty-seven patients (48.7%) presented regression of the aortic valve lesions including 10 (8.5%) with a total regression and 47 (40.2%) with a partial regression. The regeneration of the mitral valve was significantly higher than the aortic valve regeneration ($p < 0.001$) (**Tables 1**, **2**). Forty (38.5%) patients of the 104 with mitral valve recovery were observed after 5 years of follow-up, whereas half of the 10 with aortic valve recovery were observed after only 2 years of follow-up (**Table 1**). Although some patients presenting mitral stenosis (MS), double mitral lesion (DML), mitral stenosis combined with aortic regurgitation, as well as double aortic lesion (DAL) combined with mitral regurgitation have benefitted from the secondary prophylaxis, none of these patients achieved total regression of the preexisting lesions. Nine (50%) of the 18 surgeries performed were done on these patients. The 17 patients presenting recurrence of ARF episodes resulting in progression of their valve damage were part of the irregular prophylactic treatment group. No patients undergoing the regular prophylactic treatment presented recurrence of ARF, and none of them presented progression of the valve damage. Four patients (1.1%) with irregular prophylaxis died (**Table 2**).

DISCUSSION

To our knowledge, this study describes the largest cohort of rheumatic patients undergoing secondary prophylaxis and the longest follow-up time. The 593 patients were followed up for up to 26 years, with almost half of the patients being followed for more than 11 years, with clinical, microbiological, and echocardiographic monitoring.

Although ARF and RHD represent a significant matter of concern for low- and middle-income countries, few countries have efficient prevention program in place. The REMEDY study (Global Rheumatic Heart Disease Registry) reports this reality. The lack of efficient prevention program leads to patients being unfortunately diagnosed at an advanced stage of cardiac valve disease too often presenting pulmonary hypertension and/or other complications (15). A similar situation has been described in Fiji, where only 6.3% of the patients with RHD received ≥80% of the prescribed injections and only 2% of the patients received regular antibiotic prophylaxis (16). Without prophylaxis, the disease evolves rapidly leading to hospitalization and surgical intervention.

When a prevention program is in place, one of the key goals is to obtain, and maintain, the patient's adherence to secondary prophylaxis (1, 17, 18). Usual obstacles to strong secondary prophylaxis program include difficulties related with the patient's registration, recording of injection dates, injection-associated pain, lack of IM penicillin in remote health centers, and limited training of the health care workers (19–21). According to Dassel et al., some protection is already provided to patients who receive 40% of the prescribed doses for secondary prophylaxis; however, a lower percentage (<20%) of doses is associated with a fourfold increase in the odds of having a recurrence (22). The proportion of regular treatment adherence was relatively high in our study (81.3%). Program prioritization and significant human resource dedicated to such program is likely to play an instrumental role for this overall good adherence. Although we could not find an epidemiological report confirming our assertion, our personal experience suggests that the interval's extension between doses (4 weeks rather than 3 weeks) may have convinced some patients not to abandon their prophylaxis treatment. Additionally, detection of oropharyngeal GAS was an important tool for maintaining optimal adherence on the long term.

None of the patients undergoing regular prophylactic treatment presented recurrence of ARF episodes or progression of the heart disease. On the contrary, significant rates of ARF recurrence were observed among patients who received irregular prophylactic treatment (12.1 and 29.5% in groups I and II, respectively). In comparison, the cumulative incidence of recurrences identified in Australian indigenous communities with a low rate of adherence to secondary prophylaxis was 3.8% in the 1st year, 14.9% in the 5th year, and 20.1% in the 10th year of

TABLE 2 | Group II: evolution of the cardiac impairment in 350 patients with rheumatic heart disease undergoing prophylactic treatment.

Diagnosis at baseline N (%)		Diagnosis at follow-up N (%)										
		Total improvement		Partial improvement		Unchanged		Progression		Surgeries		Death
		Mitral	Aortic	Mitral	Aortic	Mitral	Aortic	Mitral	Aortic	Mitral	Aortic	
Mitral valve involvement												
MR[a]	218 (62.3)	93 (42.7)		59 (27.1)		56 (25.7)		8 (3.7)		2 (0.9)		3 (1.4)
MS[b]	3 (0.9)					1 (33.3)				2 (66.7)		
DM[c]	12 (3.4)			4 (33.3)		1 (8.3)		1 (8.3)		6 (50.0)		
Sub-Total	233 (66.6)											
Aortic valve involvement												
AR[d]	15 (4.3)		2 (13.3)		1 (6.7)		9 (60.0)		1 (6.7)		2 (13.3)	
Sub-Total	15 (4.3)											
Mitral valve involvement												
MR and AR	98 (28.0)	11 (11.2)	8 (8.2)	66 (67.3)	43 (43.8)	13 (13.3)	45 (45.9)	4 (4.1)	2 (2.0)	4 (4.1)		1 (1.0)
MS and AR	2 (0.6)				1 (50.0)	1 (50.0)				1 (50.0)	1 (50.0)	
MR and DAL[e]	2 (0.6)			1 (50.0)	2 (100.0)			1 (50.0)				
Sub-Total	102 (29.1)											
Total Mitral valve involvement 335 (95,7)		104 (31.0)		130 (38.8)		72 (21.5)		14 (4.2)		15 (45)		3 (1.4)
Total Aortic valve involvement 117 (33,4)			10 (8.5)		47 (40.2)		54 (46.2)		3 (2.6)		3 (2.6)	1 (1.0)

[a]MR, Mitral Regurgitation; [b]MS, Mitral Stenosis; [c]DML, Double Mitral Lesion (MS + MR); [d]AR, Aortic Regurgitation; [e]DAL, Double Aortic Lesion (AS + AR).

TABLE 3 | Evolution of mitral and aortic lesions in group 2 ($N = 350$).

		Mitral lesions ($N = 335$)				Aortic lesions ($N = 117$)		
	Number of patients	Number (%) of cardiac lesion regression (partial or complete)	Number (%) of cardiac lesion unchanged or worsened	*p*-value	Number of patients	Number (%) of cardiac lesion regression (partial or complete)	Number (%) of cardiac lesion unchanged or worsened	*p*-value
Female	174	121 (69.5)	53 (30.5)	0.905	60	28 (46.7)	32 (53.3)	0.712
Male	161	113 (70.2)	48 (29.8)		57	29 (50.9)	28 (49.1)	
Age at disease onset				0.161				0.579
0–4 years old	29	24 (82.8)	5 (17.2)		0	0	0	
5–9 years old	189	132 (69.8)	57 (30.2)		62	32 (51.6)	30 (48.4)	
10–14 years old	116	78 (67.2)	38 (32.8)		54	25 (46.2)	29 (53.7)	
≥15 years old	1	0	1 (100.0)		1	0	1 (100)	

Of note, the same patient may present with different valves lesions. Fisher's exact test was used to analyse differences between sex and age at diagnostic.

the study (23). Approximately 50% of the Australian indigenous RHD population required surgery within 2 years and 10% died after 6 years of their initial diagnosis (18).

Adherence to secondary prophylaxis also resulted in cardiac recovery in a significant number of patients, including some discharged with no echocardiographic abnormalities. Cases with follow-up interval longer than 5 years were more likely to improve. In our study, the patients who most benefitted from the secondary prophylaxis were those who did not present carditis at the start of the treatment (Group I). A study by Haran et al. reports that four of the six patients with no initial valvular involvement developed valvular alterations during the 27-months follow-up study; half of them were compliant to secondary prophylaxis (24). Even if resistance to penicillin is not a concern for GAS so far, the potential long-term effect of prolonged antibiotic prophylaxis should be monitored.

In our study, patients who presented rheumatic cardiac lesions (Group II) also benefitted from secondary prophylaxis. Total or partial regression of the cardiac lesions was observed in 69.8 and in 48.7% of the patients with mitral and aortic damage, respectively. Regeneration of the mitral valve (the most frequent lesion in our patients) was significantly higher than the aortic valve. Previous studies also shown that cardiac impairment improved in 43.5–51% of the patients undergoing prophylactic treatment, with the highest improvement for mitral regurgitation (>70% improvement) (8–10). In Northern Australia, 17 RHD patients were monitored, with a 70% rate of adherence to secondary prophylaxis; five patients presented total regression, two partial regression, seven kept their former lesions, and three were reported with progression of their valvular disease (24). In Pakistan, 21 RHD patients were monitored for 10 years; among them, only six adhered to secondary prophylaxis, presenting regression of their preexisting valvular lesions. The other patients were reported with progression in the severity of their first lesions or with development of new valvular lesions with a high death rate (23%) (25). In Brazil, 462 rheumatic patients were followed up for 13.6 years. More than one third of them presented recurrences by non-adherence to secondary prophylaxis (26).

Our results from Brazil shows that dedicated efforts for secondary prevention of ARF and RHD allow for significant clinical improvement. Close follow-up including clinical, microbiological, and echocardiographic monitoring is needed for a prolonged period of time to reach that goal.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Pequeno Príncipe Hospital, under CAAE-02153912.2.0000.0097. Written informed consent to participate

in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

RPAT and RSLAT conceived the project. RPAT, RSLAT, and RFAT conducted the study according to the protocol. RPAT, RSLAT, RFAT, PRS, and GC analyzed the results. RPAT, RSLAT, RFAT, PRS, GC, SPMS, SLVC, and KAB wrote and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.676098/full#supplementary-material

Supplementary Table 1 | Most prevalent manifestations presented by 593 patients at the start of the follow-up. *ARF, Acute rheumatic fever; **EM, Erythema Marginatum; ***SN, Subcutaneous Nodules.

Supplementary Table 2 | Criteria for the diagnosis of RHD patients*. *Gewitz et al. (11).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cardiovascular Medicine

MINI REVIEW
published: 24 June 2021
doi: 10.3389/fcvm.2021.694393

What Lies Ahead for Young Hearts in the 21st Century – Is It Double Trouble of Acute Rheumatic Fever and Kawasaki Disease in Developing Countries?

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Rheumatic heart disease (RHD), the principal long-term sequel of acute rheumatic fever (ARF), has been a major contributor to cardiac-related mortality in general population, especially in developing countries. With improvement in health and sanitation facilities across the globe, there has been almost a 50% reduction in mortality rate due to RHD over the last 25 years. However, recent estimates suggest that RHD still results in more than 300,000 deaths annually. In India alone, more than 100,000 deaths occur due to RHD every year (Watkins DA et al., N Engl J Med, 2017). Children and adolescents (aged below 15 years) constitute at least one-fourth of the total population in India. Besides, ARF is, for the most part, a pediatric disorder. The pediatric population, therefore, requires special consideration in developing countries to reduce the burden of RHD. In the developed world, Kawasaki disease (KD) has emerged as the most important cause of acquired heart disease in children. Mirroring global trends over the past two decades, India also has witnessed a surge in the number of cases of KD. Similarly, many regions across the globe classified as "high-risk" for ARF have witnessed an increasing trend in the incidence of KD. This translates to a double challenge faced by pediatric health care providers in improving cardiac outcomes of children affected with ARF or KD. We highlight this predicament by reviewing the incidence trends of ARF and KD over the last 50 years in ARF "high-risk" regions.

Keywords: epidemiology, fever, heart, incidence, Kawasaki disease, rheumatic, trend

OPEN ACCESS

Edited by:
Roney Orismar Sampaio, University of São Paulo, Brazil

Reviewed by:
Vitor Emer Egypto Rosa, Universidade de São Paulo, Brazil
John Bernard Lawrenson, Stellenbosch University, South Africa

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Specialty section:
This article was submitted to General Cardiovascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: *13 April 2021*
Accepted: *01 June 2021*
Published: *24 June 2021*

Citation:
Banday AZ, Mondal S, Barman P, Sil A, Kumrah R, Vignesh P and Singh S (2021) What Lies Ahead for Young Hearts in the 21st Century – Is It Double Trouble of Acute Rheumatic Fever and Kawasaki Disease in Developing Countries? Front. Cardiovasc. Med. 8:694393. doi: 10.3389/fcvm.2021.694393

INTRODUCTION

Globally, acute rheumatic fever (ARF) remained the most important cause of acquired heart disease in children until the later part of the 20th century. Improvements in general standard of living, hygiene, sanitation, health care facilities, better understanding of the disease, appropriate use of antimicrobials, and directed public health policies resulted in a significant decrease in incidence of ARF and prevalence of rheumatic heart disease (RHD) in developed countries (1, 2). On the other hand, Kawasaki disease (KD) is increasingly being recognized in many developed and developing countries. KD is now the commonest

cause of acquired heart disease in children in developed countries and the incidence of KD in developing countries also seems to be rising (3).

However, in many under-developed and developing regions, a significant burden of ARF/RHD still exists due to poor standard of living, suboptimal hygiene and sanitation facilities (4). Also, KD has clinical features overlapping with many infectious diseases and no specific diagnostic tests for KD are available. It is possible that children with KD in developing countries may end up being empirically treated with antimicrobials given the tremendous burden of infectious diseases in these countries (5). Additionally, timely intravenous immunoglobulin therapy remains a challenge due to concerns of limited availability and high costs involved in its procurement. Consequently, a higher proportion of children with KD in developing countries may suffer cardiac complications (as compared to the developed world) (6–10).

In this review, we note the trends in the incidence of ARF and KD [before the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic] in regions that have been traditionally described to have "high-risk"/"high-burden" for ARF/RHD. We highlight that the incidence of KD is increasing in these areas while a significant burden of RHD remains. So there is a dual challenge (of KD and ARF) in a majority of regions in the developing world that needs to be tackled to reduce the burden of cardiovascular morbidity in children.

HISTORY (INCLUDING DIAGNOSTIC CRITERIA)

ARF

The first descriptions indicative of acute rheumatic fever (ARF) and its cardiac sequel date back to the first century A.D (11, 12). Sydenham in late 1600s provided a detailed description of ARF and, for the first time in history, he differentiated this illness from gout (13). The first probable description of ARF in modern English language was published around 1700 (14). Around the turn of the 20th century, the complete spectrum of ARF in the pediatric population was described (15, 16). The year 1944 was a landmark in history of rheumatism when Dr. T. Duckett Jones, for the first time, proposed diagnostic criteria for ARF (17, 18). The Jones criteria have been revised on numerous instances with the latest version having been published in 2015 (19, 20). The latest version incorporates monoarthralgia as a minor criterion, and monoarthritis and polyarthralgia as major manifestations in high-risk settings. Additionally, lower cutoffs for fever and erythrocyte sedimentation rate have been promulgated for such populations (**Supplementary Table 1**). These substantial revisions will enhance the recognition of ARF in high-risk populations across the globe (20).

KD

The first descriptions of Kawasaki disease (KD) were published by Dr. Tomisaku Kawasaki in 1967 and 1974 in Japanese and English language, respectively (21–23). In 1975, Kato et al. published the first English language description of coronary artery aneurysms in KD detected by coronary angiography (24, 25). Two sets of diagnostic criteria are currently employed for KD – the American Heart Association (AHA) and the Japanese criteria. These diagnostic criteria are essentially based on the seminal observations made by Dr. Kawasaki in the 1960s. The latest version of the AHA diagnostic criteria has been published in 2017 (3) and the latest version of Japanese criteria has been published in the English Language in 2020 (26). Essentially, both these sets of criteria incorporate fever, rash, bilateral non-exudative conjunctival injection, oral mucosal changes, cervical lymphadenopathy, and edema or periungual skin peeling of hands or feet as major manifestations of the disease (**Supplementary Tables 2**, **3**). However, clinical judgment is imperative as KD may present only with fever and coronary artery abnormalities, especially in young infants (3). The recent Japanese criteria have been updated to augment the diagnosis of KD – for example, reactivation of Bacillus Calmette–Guérin vaccination site, most commonly seen infants and young children, has been added as a major manifestation (26). Similar modifications to the AHA criteria may increase the sensitivity of KD diagnosis. Analogously, revisions have already been made by the AHA to the modified Jones criteria (in 2015).

TREND OF INCIDENCE

India

ARF

In a school-based study from rural South India in 1970s, the average incidence of rheumatic fever and newly diagnosed rheumatic heart disease was estimated to be 110/100,000 schoolchildren per year (27). In a population-based study from rural North India in late 1980s, the average incidence of first episode of ARF in children 5–15 years of age was estimated to be 54/100,000 per year (overall ∼79/100,000) (28). Surveys conducted in 1980s and early 1990s estimated the annual incidence of ARF in pediatric age group to be 38–70/100,000 (29). In early 1990s, a study from North India estimated the annual incidence of first episode of ARF to be 19/100,000 school children (overall 32/100,000) (30). In a North Indian survey of ∼16,000 children between 5–15 years of age, no cases of ARF were reported during the observation period of 2007 and 2008 (29). Multicentric studies have noted a declining trend in the prevalence and burden of RHD in India; however, studies on the incidence of ARF are sparse (**Figure 1**) (31).

KD

Prior to 1990, there were only three reports of KD from India. Subsequently, hospital-based incidence studies were undertaken predominantly from Chandigarh, North India (32). In children below 15 years, the incidence of KD was estimated to be 0.51/100,000 in 1994. The incidence gradually increased in the subsequent years and was estimated to be 4.54/100,000 in 2007 (33). In children <5, the average annual incidence of KD during 2009–14 was 5.35/100,000 (34). The incidence of KD in children <5 years at Chandigarh has been estimated

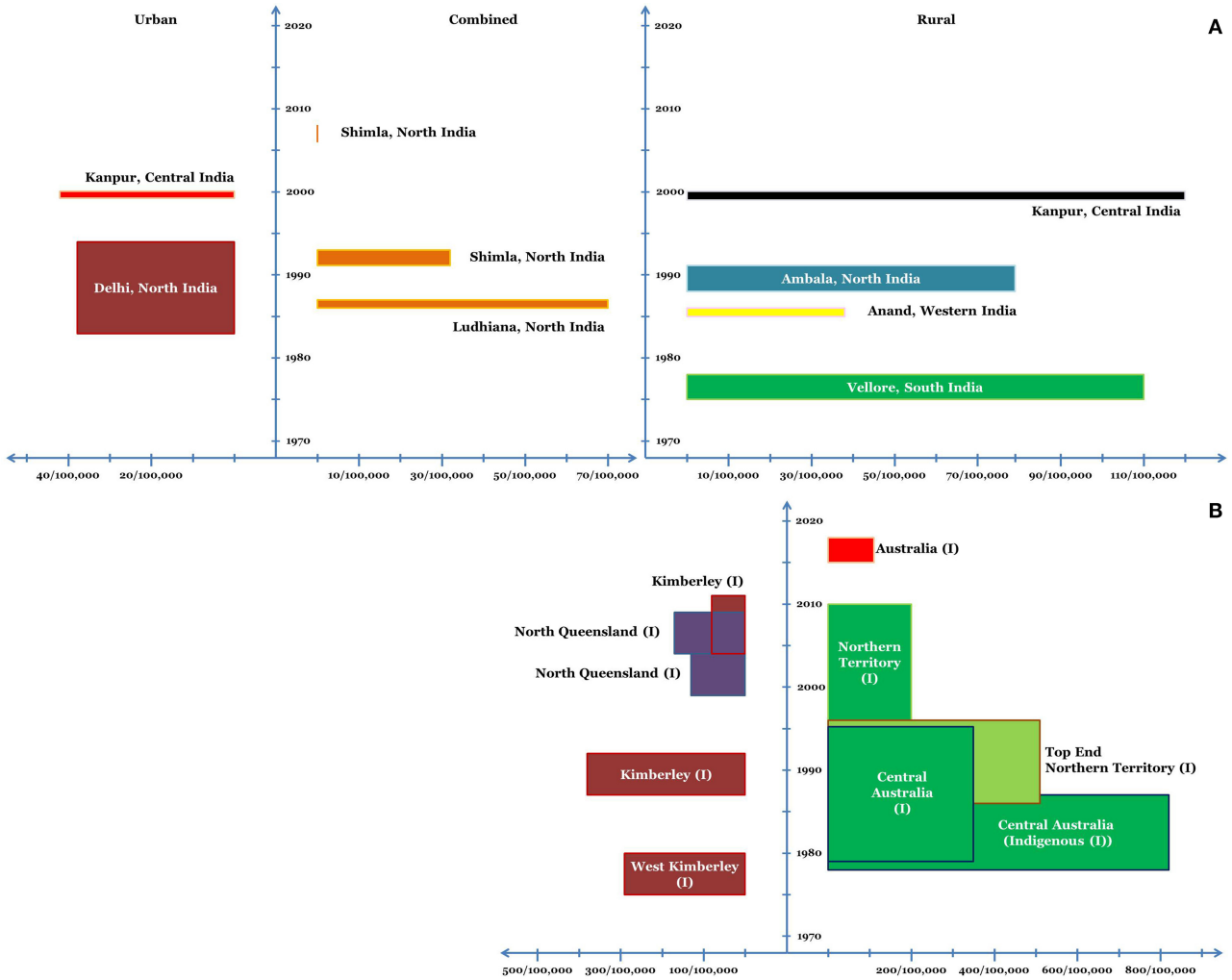

FIGURE 1 | Incidence of ARF in various regions of India **(A)** and Australia **(B)** in school-age children. (I) refers to the indigenous population. The x-axis depicts the incidence and the y-axis depicts the timeline. The dimension of the bars along the x-axis (length of the bars) depicts the average annual incidence of ARF. The dimension of the bars along the y-axis (width of the bars) corresponds to the duration for which the said average annual incidence was noted.

to be 5.64/100,000 and 10.6/100,000 during 2015 and 2019, respectively (unpublished observations) (**Figure 2**). Other centers in India have also witnessed a similar increase in the number of cases with KD in the last decade, although nationwide estimates for incidence are lacking (35–37).

China

ARF

English language reports on incidence of ARF in China are scarce. In early 1990s, the annual incidence of ARF in the pediatric population in South Western China was estimated to be 12.87/100,000 (38). In a large survey across five provinces in mainland China conducted between 1992–1995, the average annual incidence of ARF in children 5–18 years of age was reported to be 20.05/100,000 (39, 40). Although population-based data reflecting the trend of ARF incidence in China are sparse, hospital-based data have noted a decreasing trend similar to other regions of the world (39, 41).

KD

Shanghai and Beijing

A number of studies have been conducted in China to assess the epidemiology of KD. By the turn of the 21st century, KD had already overtaken ARF in the annual incidence, at least in Shanghai and Beijing. In Shanghai, the average annual incidence of KD in children below five has gradually increased over the last two decades which was 27.32/100,000 during 1998–2002, 46.32/100,000 during 2003–07, 50.5/100,000 during 2008–12, and 94.7/100,000 during 2013–17 (42–44). In Beijing, the average annual incidence of KD has been similar to Shanghai; for example, during 1995–99 it was 22.9/100,000 (45), while during 2000–04 it was 49.4/100,000 (46). However, reports suggest

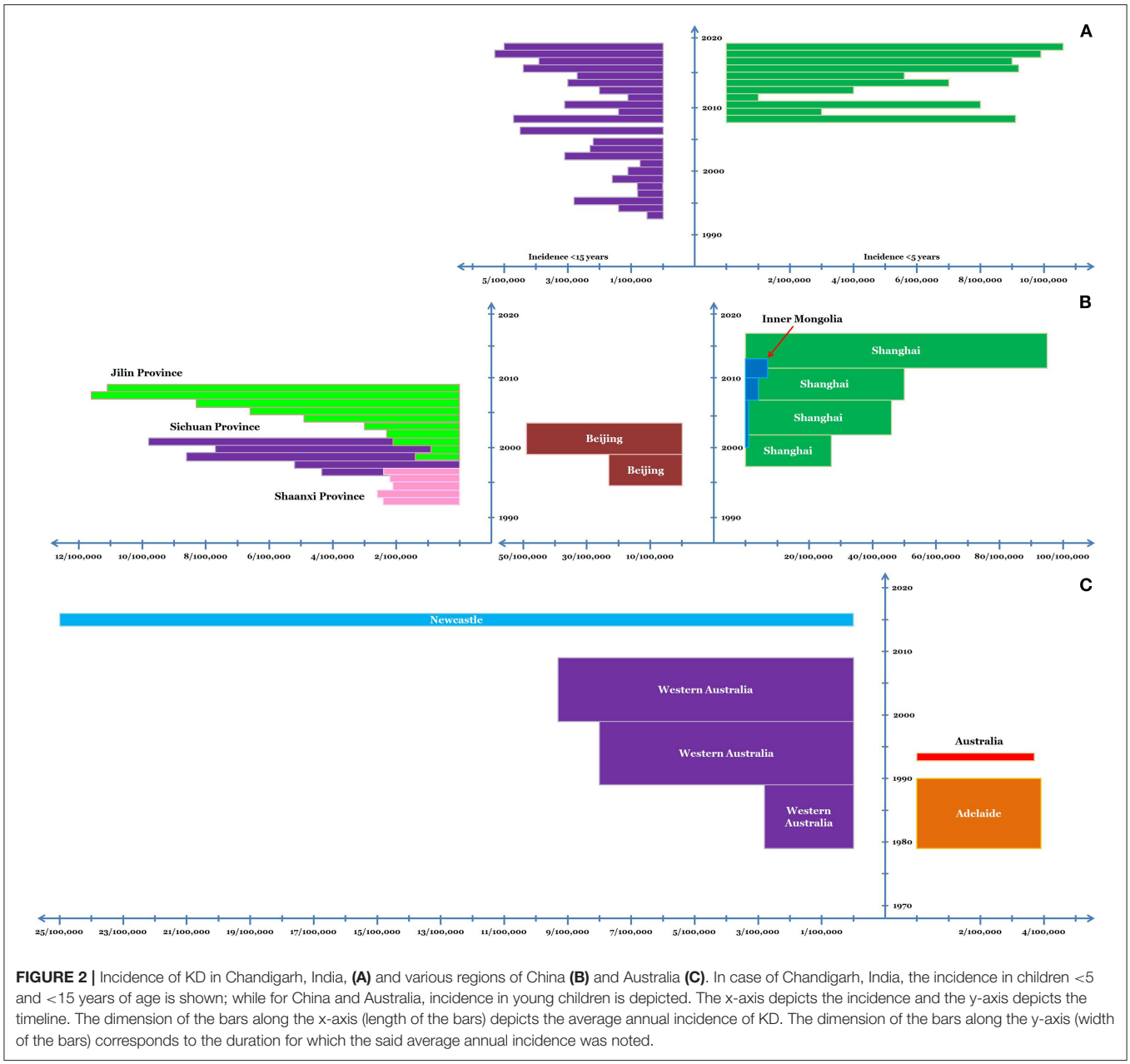

FIGURE 2 | Incidence of KD in Chandigarh, India, **(A)** and various regions of China **(B)** and Australia **(C)**. In case of Chandigarh, India, the incidence in children <5 and <15 years of age is shown; while for China and Australia, incidence in young children is depicted. The x-axis depicts the incidence and the y-axis depicts the timeline. The dimension of the bars along the x-axis (length of the bars) depicts the average annual incidence of KD. The dimension of the bars along the y-axis (width of the bars) corresponds to the duration for which the said average annual incidence was noted.

significant variability in the incidence of KD among different regions in China.

Other Regions

The average annual incidence of KD in Jilin Province during 1999–2008 was 5.04/100,000 children under five (47). Similarly, in Inner Mongolia, the mean annual incidence of KD during 2001–13 was 3.55/100,000 children under 5 (48). Nonetheless, these surveys did note an increasing trend in the disease incidence during these periods. In Shaanxi province, the annual incidence of KD remained fairly constant at ∼2.34/100,000 children <5 years of age during mid-1990s (49). However, an increasing trend in the incidence of KD was noted in Sichuan Province where the incidence of KD in children <5 years was 4.26/100,000 and 9.81/100,000 in 1997 and 2001 respectively (50) (**Figure 2**).

Australia

ARF

Western Australia

In the late 1970s, hospital-based studies from West Kimberley (Western Australia) estimated the annual incidence of ARF to be 230–350/100,000 in Indigenous school children (51). During 1988–92, the annual incidence of ARF in Indigenous school-children (5–14 years) in the Kimberley region was 375/100,000 (52). The annual incidence in Indigenous population in the age

group of 15–29 years was also high at 258/100,000 (52). Hospital-based incidence data, however, suggested a decrease in incidence of ARF in the region. During 1988–92, the annual incidence as per 100,000 hospitalized school-children was 278 (52). The annual age-standardized hospitalization rates for ARF during 2005–11 ranged between 50–100/100,000 Indigenous population in Kimberley (except for 2008, when it was <50/100,000) (53). In the same study, a significant yearly decrease of ∼9% in the age-standardized hospitalizations of ARF/RHD was noted during 2003–11 (continued decrease noted for both ARF and RHD) (53, 54).

Queensland

The annual incidence of ARF in Indigenous children (5–14 years) in north Queensland during 1999–2004 was 133/100,000. The highest incidence was noted in Northern Peninsula Area and Torres Strait Health Service District (349/100,000 children), whereas, no ARF case was detected in Innisfail District (55). A significant increase in the incidence of ARF was noted over the next 5 years (2004–09) with the annual incidence in children 5–14 years of age being 155/100,000. This finding also reflected that the transition from intensified to usual surveillance in the year 2004 had no adverse bearing on the notification of ARF (56).

Northern Territory

During 1978–87, the incidence of ARF in Central Australian Indigenous children (5–14 years) was estimated to be 815/100,000 (57). From 1987–96, the incidence in Indigenous children in Top End Northern Territory was estimated to be 224/100,000. However, the incidence in Indigenous communities where complete data was available was higher at 508/100,000 and in Non-Indigenous children (5–14 years) the incidence was 1.3/100,000 (58). In a combined study assessing the epidemiology of ARF in Northern Territory during 1979–96, the incidence of ARF in 5–14 year old indigenous children in Top End and Central Australia was estimated to be 245/100,000 and 351/100,000 respectively (59). During 1997–2010, the annual incidence of the first episode of ARF in the Indigenous children (5–14 years) of the Northern Territory was 194/100,000 (60). Notably, in this large and well-designed study, a decreasing trend in the annual incidence of ARF was not noted on multivariate analysis (60).

The annual incidence of the first episode of ARF across 5 Australian Jurisdictions during 2015–2017 was 107.6/100,000 for Indigenous children (5–14 years) and 1/100,000 for non-Indigenous children (61). More than 80% of the ARF episodes were reported from North Australia (61) (**Figure 1**).

KD

During 1979–90, the average annual incidence of KD in Adelaide was 3.9/100,000 children aged 0–5 years, with highest incidence being noted in the year 1986 (7.7/100,000) (62). In 1994, the incidence of KD in Australian children <5 years was noted to be 3.7/100,000 (63). A large study analyzing the 30-year epidemiology of KD in Western Australia from 1979–2009 noted a gradual increase in the incidence of KD in children <5 years of age. The average annual incidence rate for the said population during the consecutive decades of the study was 2.82, 7.96, and 9.34 per 100,000 respectively (64). A hospital-based study from Newcastle estimated the average annual incidence of KD (in children <5 years) to be ∼25/100,000 during 2015–16 (65) (**Figure 2**). In a nationwide study, an increase in the hospitalizations due to KD (0–19 years) was noted during the 25-year period from 5.2/100,000 in early 1990s to 12.4/100,000 in 2017–18 (66). Notably, very few Indigenous children have been diagnosed to have KD in these large studies (64, 66).

Africa

ARF

Most of the studies have focused on the prevalence of RHD from Africa; however, epidemiological studies assessing the incidence of ARF are quite scarce. It has been estimated that half of the global RHD population under the age of 15 years lives in Africa (67). In the year 1990, the annual incidence of ARF in school-going children was estimated to be 30/100,000 (68). In Algerian children and adolescents (aged 4–19 years), the annual incidence of ARF was estimated to be 11.1/100,000 in 1997 and 6.2/100,000 in 2000 (69). A systematic review on the global burden of group A streptococcal disease by Carapetis et al. has noted a lesser incidence of ARF in sub-Saharan and North Africa in comparison to other "high-risk" regions (70). Poor case ascertainment and documentation seem to be the likely reasons for the lesser incidence of ARF in Africa (70).

KD

Similar to ARF, the actual incidence of KD in many African countries is not known. Variable incidence rates amongst children below 5 years of age have been documented from different African countries, for example, 3.15/100,000 from Algeria, 0.95/100,000 from Tunisia, and 4.52/100,000 from Morocco (71). Although incidence studies of KD are largely lacking, the increasing number of publications from Africa are encouraging and portend enhanced detection of KD in near future (7, 8).

Latin America

ARF

The highest population-based annual incidence of ARF (∼360/100,000 children aged 10–20 years) in Latin America has been documented in Belo Horizonte, Brazil during the year 1992 (69, 72). However, the study was carried out in a limited population only and the duration of the study was from March to December 1992 (72). Other studies from the region have noted at least a 5-fold lower incidence of ARF. In Cuban province of Pinar del Rio, a marked reduction in incidence of ARF (in the age group 5-25 years) was noted during 1986 to 1996 wherein the incidence rate decreased from 18.6/100,000 to 2.5/100,000, respectively (73). In the French Caribbean islands of Martinique and Guadeloupe, the incidence of ARF (in the age group of <20 years) in

1982–83 was 19.6/100,000 and 17.4/100,000, respectively. By 1992, the incidence was reduced by about 4- to 5-fold in both the regions as a result of a decade-long educational program (74). A study from Chile has noted a gradual decline in the incidence of ARF from 3/100,000 inhabitants in 1979 to 0 in 1998 (75). During 1994–99, a hospital-based study from Mexico noted incidence of first episode of ARF to be 660/100,000 cases admitted to the hospital (700/100,000 children and adolescents aged 5–20 years) (76). The incidence of ARF during this period was noted to be lower than the early 1970s when it was 1060/100,000 subjects (76, 77).

KD

The exact incidence of KD in many Latin American countries remains unknown. For precise estimation of disease burden, a research network was officially formed in 2013 comprising of 20 countries (78). Using the nationwide hospital-based data, the incidence of KD in Chilean children <5 years of age was estimated to be 5.7/100,000 in 2001–2004, 8.4/100,000 in 2005–2007, and 10.4/100,000 in 2009–2011 (79, 80). The highest incidence of KD (19.8/100,000) was noted in Eastern Metropolitan Health District region that, notably, had the highest socioeconomic status in the country (80).

The trends in the incidence of KD and ARF in regions classified as "high-risk" for ARF are summarized in **Supplementary Tables 4**, **5**.

DISCUSSION

KD and ARF are the two most common causes of acquired heart disease in the pediatric population (1–3). ARF is an important complication of β-hemolytic group-A streptococcal infection that may lead to life-long cardiac morbidity primarily due to sequelae of valvular involvement (2). In contrast, KD is a medium vessel vasculitis of unknown etiology that has the predilection to involve coronary arteries and myocardium (3, 81). As we have reviewed, the incidence of ARF is on the decline in majority of the "high-risk" regions. This could be due to general improvement in health and sanitation facilities and effective public health programs. However, robust population-based data on the incidence of ARF are lacking from many high-risk regions with the most notable exception of Northern Australia – the region with the highest incidence of ARF amongst the Indigenous population. Even within Northern Australia, the incidence of ARF is seemingly high in regions where there is stringent data collection (58). Based on this corollary, KD may be under-recognized in the Indigenous population (64, 66). The robust ARF surveillance network may be leveraged to know the incidence of KD specifically amongst the Indigenous population. It would be intriguing to know the epidemiology of KD in populations with the highest incidence of ARF.

The most notable cardiac sequel of KD is the formation of coronary artery aneurysms. These aneurysms may get thrombosed in the acute stage and even lead to myocardial infarction (3). Myocardial involvement (myocarditis), increasingly thought to be universal in KD, may be severe enough to lead to severe cardiogenic shock (81). Long-term cardiac complications of KD include the risk the premature coronary artery disease, early myocardial infarction, and possibly cardiomyopathy (3, 81). It is imperative to mention that the diagnosis of KD may easily be missed especially in developing countries. Lack of awareness, poor healthcare infrastructure, and increased burden of infectious diseases (many of tropical infections may mimic KD) are some of the major contributing factors. The available diagnostic criteria for KD are based on constellation of clinical features and there is lack of a specific diagnostic test. Besides, epidemiological data collection in many developing countries remains suboptimal. These issues can further compound the problem of underdiagnosis of KD in developing countries. Nevertheless, increase in the incidence of KD has still been noted in the developing countries which has been attributed to industrialization and urbanization, besides the increase in the general awareness regarding the disease (82, 83). In the developed world, has already overtaken ARF as the leading cause of acquired heart disease in children. The developing countries also seem to be on a similar trend.

Recognition of KD and ARF in the pediatric population would help to reduce the burden of acquired heart disease in a significant proportion of the economically productive age group. This would have immense implications for the developing countries many of which face significant economic constraints (82). Surveillance networks simultaneously assessing the incidence of KD and ARF would be an effective option to tackle the major chunk of acquired heart disease in children in these regions.

Finally, multisystem inflammatory syndrome in children (MIS-C) post-SARS-CoV-2 usually presents with KD-like features. Myocardial dysfunction is seen in approximately half, whereas, coronary artery involvement is seen in about one-tenth (84). The SARS-CoV-2 pandemic has resulted in a spike in the incidence of KD in many countries around the globe with may further add to the burden of acquired heart disease in children in the near future. On a global stage, especially in the developing countries, the young hearts faced a dual challenge of ARF and KD even before the SARS-CoV-2 pandemic. MIS-C post-SARS-CoV-2 has compounded the challenge of mitigating the problem of acquired heart disease in children. A dedicated collaborative effort is required on a global level to manage the predicament of acquired heart disease in children.

AUTHOR CONTRIBUTIONS

AB: writing of initial draft of manuscript, editing and revision of manuscript at all stages of its production, review of literature, drawing of figures, and final approval. SM: writing of initial draft of the manuscript, contributed to editing of manuscript, review of literature, and final approval. PB, AS, and RK: contributed to editing of manuscript, data collection, and final approval. PV: contributed to editing of manuscript,

critical revision of the manuscript at all stages of production, review of literature, and final approval. SS: contributed to editing of manuscript, revision of the manuscript, and its final approval. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.694393/full#supplementary-material

Supplementary Table 1 | American Heart Association (2015) Revised Jones criteria for the diagnosis of acute rheumatic fever (ARF).

Supplementary Table 2 | American Heart Association (2017) criteria for diagnosis of Kawasaki disease (KD).

Supplementary Table 3 | Japanese criteria (2020) for diagnosis of Kawasaki disease (KD).

Supplementary Table 4 | Summary of trends in incidence of acute rheumatic fever in "high-risk" regions.

Supplementary Table 5 | Summary of trends in incidence of Kawasaki disease in children below 5 years of age (unless specified otherwise) in regions classified as "high-risk" for acute rheumatic fever.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cardiovascular Medicine

REVIEW
published: 02 July 2021
doi: 10.3389/fcvm.2021.690227

The "Cairo Accord"- Towards the Eradication of RHD: An Update

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Rheumatic heart disease (RHD) is the most common cause of acquired heart disease in children and young adults. It continues to be prevalent in many low- and middle-income countries where it causes significant morbidity and mortality. Following the 2017 Cairo conference "Rheumatic Heart Disease: from Molecules to the Global Community," experts from 21 countries formulated an approach for addressing the problem of RHD: "The Cairo Accord on Rheumatic Heart Disease." The Accord attempts to set policy priorities for the eradication of acute rheumatic fever (ARF) and RHD and builds on a recent series of policy initiatives and calls to action. We present an update on the recommendations of the Cairo Accord and discuss recent progress toward the eradication of RHD, including contributions from our own Aswan Rheumatic Heart Disease Registry (ARGI).

Keywords: Cairo accord, neglected disease, eradication, vaccine, echocardiography, screening, rheumatic heart disease, RHD

OPEN ACCESS

Edited by:
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Reviewed by:
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Antonino S. Rubino, University of Campania Luigi Vanvitelli, Italy

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Specialty section:
This article was submitted to Heart Valve Disease, a section of the journal Frontiers in Cardiovascular Medicine

Received: *02 April 2021*
Accepted: *07 June 2021*
Published: *02 July 2021*

Citation:
Kotit S, Phillips DIW, Afifi A and Yacoub M (2021) The "Cairo Accord"- Towards the Eradication of RHD: An Update. Front. Cardiovasc. Med. 8:690227. doi: 10.3389/fcvm.2021.690227

INTRODUCTION

Rheumatic heart disease (RHD) is a late consequence of acute rheumatic fever (ARF) following group A streptococcal (GAS) infection (1). It is the most common cause of progressive acquired heart disease in children and young adults (2, 3). Although once common throughout the world, the disease burden is now almost entirely limited to low and middle income countries and the poor indigenous populations of some wealthy countries (3–5). RHD continues to be an important cause of mortality and disability and yet remains a neglected disease (6–8). While there is strong evidence linking RHD to poor socio-economic (9, 10) and environmental conditions (11, 12), the underlying pathogenesis, particularly the reasons for host susceptibility (13–18) and bacterial rheumatogenicity (19, 20), remain poorly understood.

Developing appropriate strategies to deal with this problem remains a top priority in public health. In response, the Cairo Accord was formulated in 2017 following a meeting entitled "Rheumatic Heart Disease - From Molecules to The Community" (21, 22) and attempts to set criteria and priorities for addressing the problems related to RHD and achieving the eradication of RHD through different approaches. The Accord, in turn, builds and expands on a continuing series of initiatives around the world (**Figure 1**) (21, 24–41).

It stresses the need for a comprehensive systematic approach to the problem incorporating basic science, epidemiological, and clinical components. The recommendations include establishing standardized data collection procedures and databases for epidemiological studies; establishing clear echocardiographic procedures for diagnosis through research-driven studies; improving research into the interaction between and dependence on host genetics and pathogen source on the

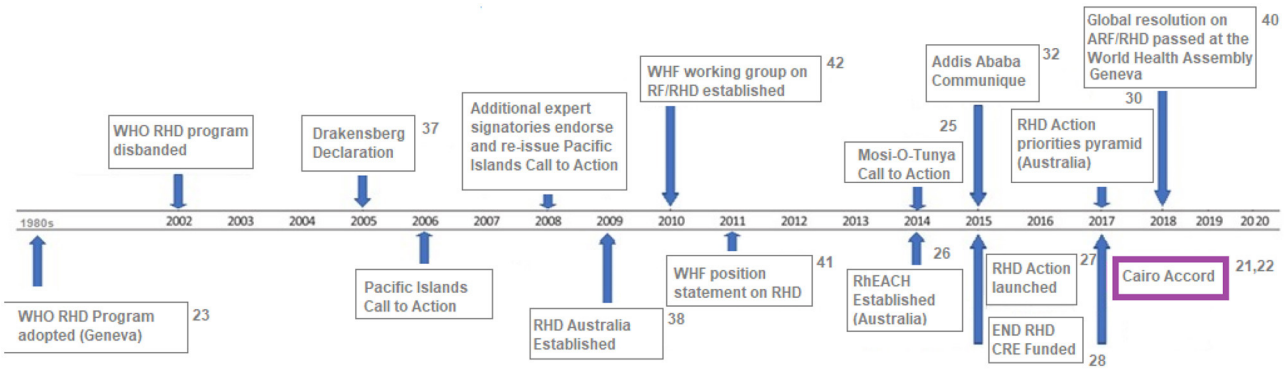

FIGURE 1 | Global and regional RHD-specific policy activity and calls to action, 1980 – 2020 [adapted from (23)].

prevalence of RHD; the development of a vaccine; and improved engagement from international experts in valve surgery.

We here present an update of the areas enumerated by the Cairo Accord and discuss progress toward the eradication of RHD, including our own Aswan Rheumatic Heart Disease Registry (ARGI).

THE "CAIRO ACCORD": UPDATE ON THE RECOMMENDATIONS

1. **"Call for obtaining more accurate data on the epidemiology and natural history of the disease by strengthening the existing databases, and by ensuring that different databases are capable of cross-communication and data exchange."**

Generating accurate data on the epidemiology and natural history of RHD is a vital tool for both the prevention and control of the disease as well as for programme planning and advocacy activities. Compared with many other diseases there has been a lack of routine data on disease occurrence and hitherto much has depended on national mortality recording systems or hospital-based records. The past few years have, however, seen considerable development and strengthening of registries at global, national and hospital-based level.

Global Databases

An encouraging number of studies now report estimates of the global prevalence of RHD. For example, Watkins et al. (42) combined multiple data sources with modeling techniques to evaluate global prevalence and mortality of RHD over a 25-year period. Although RHD has declined in most countries, it remains a major problem in sub-Saharan Africa, South Asia, and Oceania (**Figure 2**). During 2015, the authors estimate that globally there were 319,400 deaths and 33.4 million cases of RHD as well as the loss of 10.5 million disability-adjusted life-years (DALYs). However, the authors stress the continuing poor quality of data and misclassification of causes of death, which is a particular problem in low and middle-income countries (LMICs) where weak health systems are associated with a high prevalence of RHD.

Another welcome addition from the Institute for Health Metrics and Evaluation (IHME) is a review of "Rheumatic heart disease burden, trends, and inequalities in the Americas." In this report they estimated age-adjusted incidence, mortality and DALYS in 37 counties throughout South America. In spite of the considerable drop in both incidence and mortality during the period of the study (1990-2017), there were marked regional inequalities related mainly to socioeconomic factors and facilities for RHD prevention and control (4). A particular problem in the estimation of the true burden of the disease is the use of surveys of schoolchildren in many population studies. Because RHD is linked with poverty, surveys carried out in schoolchildren will necessarily under-represent disease prevalence as poor children with RHD are less likely to attend school (5). Furthermore, because the peak incidence of RHD occurs after school age, school-based studies will necessarily underestimate the true burden of the disease.

National Databases

Although progress in establishing national databases has been disappointingly slow, recent encouraging achievements have been the addition of RHD to the list of notifiable diseases in Queensland, Australia starting from September 2018 (43), and following a similar initiative in Western Australia in 2015 (44). Furthermore, a RHD action plan (2018-2021) has been developed to ensure that treatment and management is carried out in the best way possible, to aid preventive measures, and to assist the co-ordination of care (45). A multijurisdictional Australian study of acute rheumatic fever (ARF) and RHD using multiple person-linked longitudinal administrative data sources is a good example of what can be achieved. ARF and RHD cases were retrieved from linked ARF/RHD registers, inpatient hospitalizations and RHD-coded death registry data

Abbreviations: ARF, Acute Rheumatic Fever; RHD, Rheumatic Heart Disease; ARGI, Aswan Rheumatic Heart Disease Registry; DALYs, disability-adjusted life-years; LMICs, middle-income countries; GAS, group A streptococcus; GWAS, genome-wide association studies; IM, intramuscular; BPG, benzathine benzylpenicillin G.

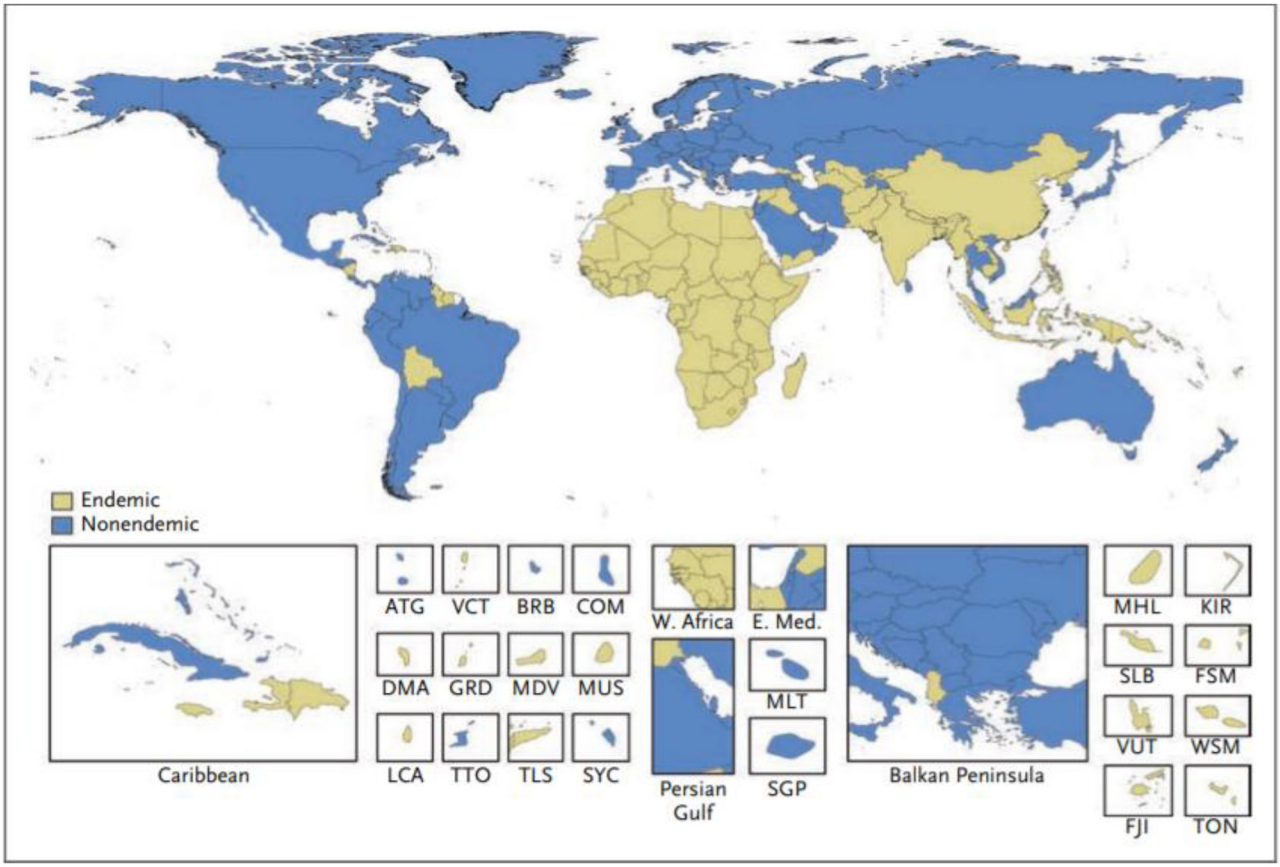

FIGURE 2 | Classification of countries as having an endemic or non-endemic pattern of rheumatic heart disease. A country was classified as having an endemic pattern of disease if its estimated childhood mortality due to rheumatic heart disease was greater than 0.15 deaths per 100,000 population among children 5 to 9 years of age. ATG, Antigua and Barbuda; BRB, Barbados; COM, Comoros; DMA, Dominica; E. Med., Eastern Mediterranean region; FJI, Fiji; FSM, Federated States of Micronesia; GRD, Grenada; KIR, Kiribati; LCA, Saint Lucia; MDV, Maldives; MHL, Marshall Islands; MLT, Malta; MUS, Mauritius; SGP, Singapore; SLB, Solomon Islands; SYC, Seychelles; TLS, Timor-Leste; TON, Tonga; TTO, Trinidad and Tobago; VCT, Saint Vincent and the Grenadines; VUT, Vanuatu; W. Africa, West Africa; WSM, Samoa (42).

in 5 Australian jurisdictions (mid-2001–2018) to create a comprehensive database for characterizing the ARF/RHD patient population and estimating the burden of ARF and RHD. Using linked data provides more reliable estimates of disease burden as the linked dataset allows for a person's records to be followed across different data collections, compensating for the incompleteness of data from a single source. The longitudinal nature of the data allows an accurate estimation of disease onset and progression. Importantly this study suggested that previous data on disease burden had underestimated prevalence, while demonstrating substantial ethnic and subnational disparities in occurrence of the disease (see **Figures 3**, **4**). This method has potential applicability elsewhere (46, 47) as a means of generating reliable data on the burden of disease.

Hospital Based Databases

The increasing availability of both surgical and non-surgical treatment for RHD has underscored the need for the development of databases for the long-term follow-up of patients. Hospital-based registries have an important role in improving disease management, quality of care and clinical outcomes, monitoring anticoagulation, reducing loss to follow-up and the management of women with RHD during pregnancy in joint cardio-obstetric clinics. They can also be linked with national RHD registration systems. However, where they have been set up they do demonstrate the poor outcomes of current management. The Remedy study, a 2-year follow-up of a multicenter study of individuals with RHD from 14 LMICs in Africa and Asia, highlights the problems of treating the disease in resource-poor settings. Despite the young median age of patients (28 yr), the authors reported a 2-year case fatality rate of 16.9%, a mortality rate of 116.3/1000 patient-years in the first and a rate of 65.4/1000 patient-years in the second year (48). Other studies in sub-Saharan Africa report similar high mortality, for example the Mulago National Referral hospital in Uganda reported a 1-year mortality rate of 17.8% (49). In Aswan, a comprehensive database (ARGI) has been established since 2011 comprising over 1750 patients with RHD treated by medical,

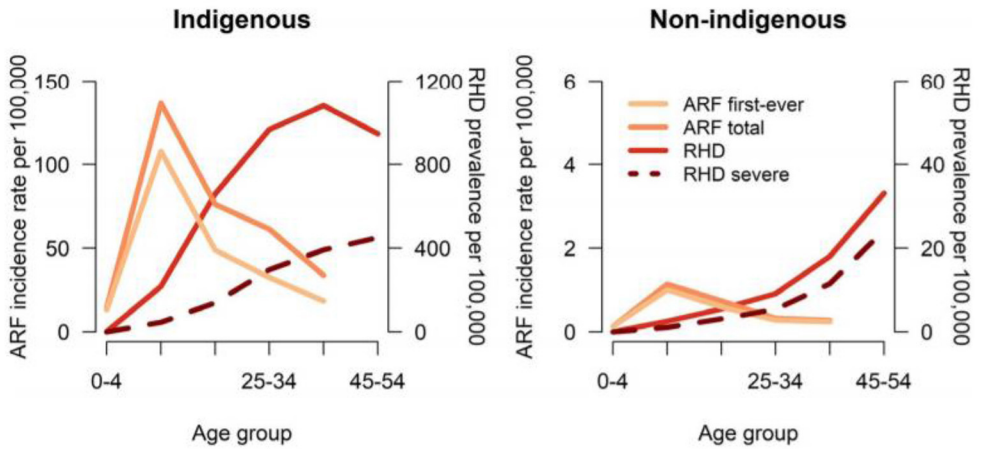

FIGURE 3 | Age-specific incidence of ARF and prevalence of RHD in 5 Australian jurisdictions, by Indigenous status, 2015 to 2017. ARF total includes first-ever episodes of ARF plus ARF recurrences. Severe RHD includes RHD cases who were recorded as having been in heart failure, received at least 1 cardiac valvular intervention or were recorded on RHD register as being severe. ARF or RHD includes any live person with a history of either ARF or RHD. ARF, acute rheumatic fever; RHD, rheumatic heart disease (46).

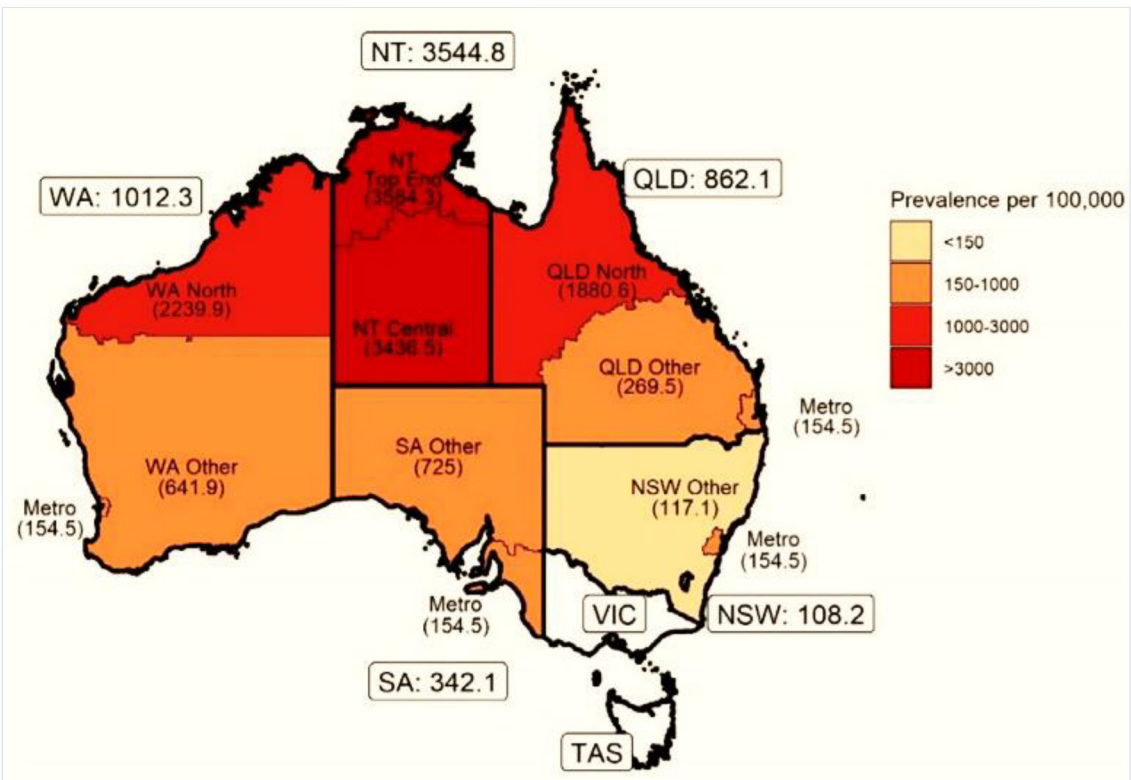

FIGURE 4 | Age-standardized prevalence (per 100 000) of ARF or RHD in indigenous populations by state and residence. NSW, New South Wales; NT, Northern Territory; QLD, Queensland; SA, South Australia; TAS, Tasmania; VIC, Victoria; WA, Western Australia [modified from (46)].

interventional or surgical procedures with plans to follow them up to improve outcomes.

Setting up RHD registries in resource poor countries, however, is a formidable challenge and progress is likely to be slow in the context of poorly-developed health systems already struggling to cope with both the traditional burden of communicable disease and the rising burden of non-communicable disease. One way forward may be the use of mobile and cloud technologies, which

when combined with low-cost mobile phones and computer tablets and accessible data storage could be used to create electronic patient registers for RHD control programmes (50).

Socioeconomic and Environmental Factors

The link between poverty and RHD is well-established (9, 51), and have led to various initiatives to address the disease (52–54). These include the provision of improved housing to reduce overcrowding and education. However, there is a clear need for a deeper investigation of the role of socioeconomic or environmental factors as they may identify specific poverty-linked determinants of RHD which may be amenable to public health intervention. Although ambitious, some possible approaches are emerging. Because ARF is a disease of childhood with a maximal incidence between 5 and 15 years of age, it is likely that any causative environmental factors operate during early childhood, a period of life which has tended to be neglected by most studies of RHD. Historical mortality studies carried out in the UK point to the operation of adverse environmental factors in early life. These studies demonstrated strong geographical associations between high rates of chest infection during infancy and RHD in later adult life. They were not explained by known risk factors such as domestic overcrowding (10). As air pollution is known to be strongly linked with chest infection during infancy, these associations raised the possibility that air pollution could be a factor in RHD susceptibility. The hypothesis was supported by the finding of strong geographical correlations between estimates of exposure to domestic air pollution in early childhood, based on coal consumption which was the major source of air pollution at that time, and adult mortality from chronic RHD (11, 12). Again these associations were not explained by overcrowding or related socio-economic factors. A role for air pollution is also mechanistically plausible as it affects epithelial integrity and has potent immunomodulatory effects, being implicated in the pathogenesis of other autoimmune diseases. If this association were valid, reducing domestic air pollution, which is widespread in many of the countries with high RHD prevalence and a major WHO public health priority, could be an important contributor to primary prevention of the disease.

2. **"Confine the use of echocardiographic screening programmes to research until further evidence regarding its impact on prognosis and cost-effectiveness is made available."**

The past decade has seen considerable development of echocardiographic screening as a tool to identify subclinical or latent cases of RHD in the community. This has required agreement on standardized diagnostic criteria which have been developed by The World Heart Federation (WHF, 2012) (55). Several studies now show that echocardiographic screening is much more sensitive than auscultation, providing a detection rate up to 10 times greater than clinical examination alone. It is an important tool to estimate the burden of disease in populations and its use in community-based studies has provided more accurate data on disease prevalence, and important insights in uncovering the full spectrum of RHD (56, 57).

Controversial, however, is the use of echocardiography as a screening tool to identify cases of mild RHD that might be treated early to prevent clinical complications. A recent meta-analysis combined data from 12 studies following up patients with latent RHD. The authors' estimated prevalence progression for latent RHD (defined variously as worsening of the WHF grade or clinical severity of disease) was 5%/year, while in the fewer studies reporting on the progression of borderline RHD, progression was as low as 2%/year (58). Although these low rates do call into question the use of echocardiography for screening, there is clearly a need for longer term studies of the natural history of latent RHD and for identifying clinical predictors of disease progression.

The major disadvantage of echocardiography is the need for expensive equipment and a high degree of training, which is unlikely to be accessible outside specialist referral hospitals unless simpler screening techniques can be developed. Recently developed possibilities include the single parasternal-long-axis-view-sweep of the heart (SPLASH), which has been shown to be highly sensitive and specific with the potential to improve significantly the efficiency of RHD screening (59), and the use of cheaper, hand-held echo devices as screening tools. Nevertheless, the relatively high cost and complexity of screening have to be weighed against mitigating the risk of complications or mortality from RHD in the context of other demands on health services.

3. **"Enhance and coordinate research efforts on the genetics of rheumatogenic streptococcal strains and affected patients. The influence of ethnicity and epigenetics should be included in future studies."**

Rheumatogenic Streptococcal Strains

Development of a streptococcal vaccine for the prevention of RHD has been hampered by the extensive global genomic heterogeneity of group A streptococcal (GAS) bacterial populations. This is driven by "homologous recombination and overlaid with high levels of accessory gene plasticity" (**Figures 5**, **6**) (19). It is now clear that the concept of certain strains having enhanced rheumatogenic potential should be extended to include RHD caused by strains apart from those classically described. Recent studies show that there are still significant gaps in our understanding of the pathogenesis of rheumatic fever pathogenesis which will need to be addressed in order to develop a viable GAS vaccine (20). perhaps using reverse vaccinology as outlined later in this review (60).

Host Genetic Susceptibility

Although, twin and family studies have long suggested that RHD might have a heritable component, until recently the evidence for this was limited. The search for susceptibility genes has been greatly enhanced by the advent of genome-wide association studies (GWAS) through which a very large number of variants can be tested for associations with RHD. A GWAS carried out on aboriginal Australians has provided evidence that variation at the HLA class 2 (DQA1-DQB1) locus is a major risk factor for RHD. The authors showed that human myosin cross-reactive epitopes of rheumatogenic streptococci were able to bind with

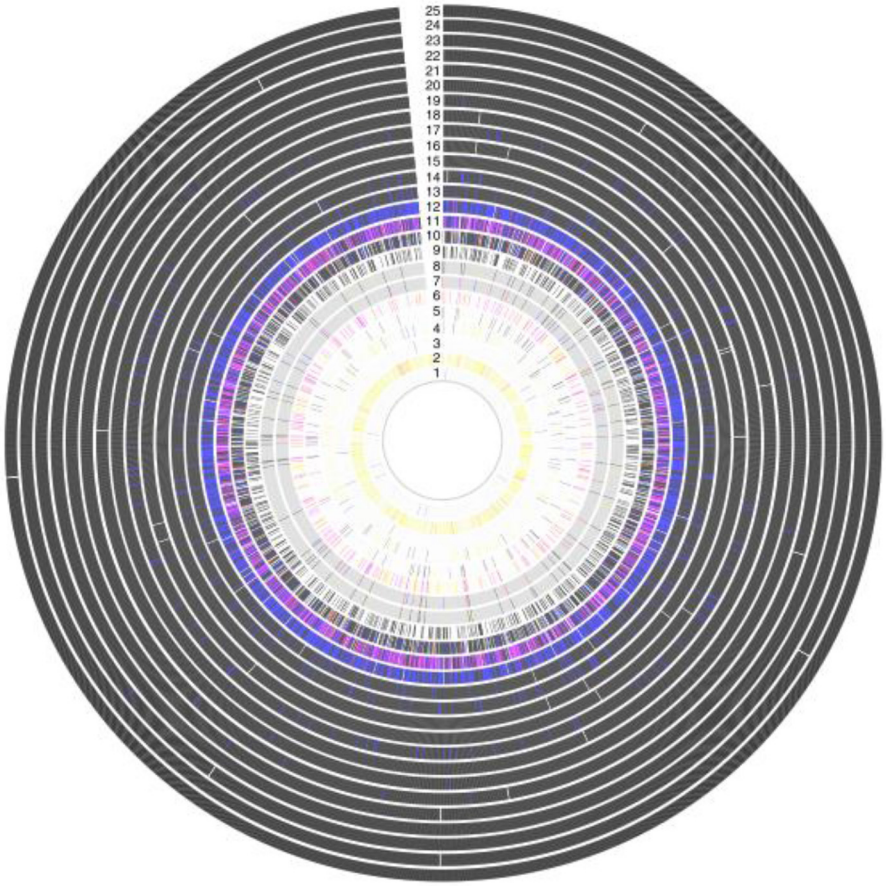

FIGURE 5 | Antigenic variation within vaccine targets, showing a high grade of variation in Amino acid sequence within 25 protein antigens. Each ring represents a single antigen with protein similarity color coded according to pairwise BLASTp similarity: black (>98%); blue (95–98%); red (90–95%); pink (80–90%); yellow (70–80%); gray (<70%); and white (protein absence). Rings correspond to: (1) R28; (2) Sfb1; (3) Spa; (4) SfbII;(5) FbaA; (6) SpeA; (7) M1 (whole protein); (8) M1 (180-bp N terminal); (9) SpeC; (10) Sse; (11) Sib35; (12) ScpA; (13) SpyCEP; (14) PulA; (15) SLO; (16) Shr; (17) OppA; (18) SpeB; (19) Fbp54; (20) SpyAD; (21) Spy0651; (22) Spy0762;(23) Spy0942; (24) ADI; and (25) TF (19).

higher affinity to the DQA1/DQB1 alpha/beta dimers of the risk haplotypes than in protective haplotypes (13). In contrast, a GWAS of New Caledonian and Fijian populations showed that the immunoglobulin heavy chain locus was significantly associated with RHD; (14, 15) the reasons for the differences are unclear.

Circulating exosomes and their cargoes, including mRNA and long non-coding RNA (lncRNA), are now thought to play an essential role in many cardiovascular diseases. Although not yet implicated in the pathogenesis of RHD, the first transcriptome analysis has recently identified differentially expressed lncRNAs and mRNAs in circulating exosomes from RHD patients which may be a route for identifying novel potential biomarkers and therapeutic targets (16).

MicroRNAs (miRNA) are fundamental for normal development, differentiation and growth control and are now implicated in many diseases. They are believed to be involved in valvular heart disease (VHD) related pathways including cell cycle control, inflammation and fibrosis, and are possible diagnostic and prognostic biomarkers for patients as well as therapeutic targets (16). Next generation sequencing of miRNAs has shown that the interleukin 1β and interleukin 1 receptor 1 might be involved in RHD. Two miRNAs, hsa-miR-205-3p and hsa-miR-3909 and their target genes IL-1β and IL1R1, appear to be specifically involved in disease progression, suggesting a potential augmentation of the IL1 pathway. However, more studies are needed on miRNA sequencing and quantitative reverse transcription–PCR analysis of miRNA expression levels (17). In another study of RHD, miR-1183 was demonstrated to be differentially expressed showing that significantly higher expression levels of miR-1183 through regulation of the anti-apoptotic protein, BCL-2, might affect myocardial apoptosis and remodeling (18).

Animal Models

Animal models of autoimmune cardiac valve inflammation and fibrosis associated with arthritis have shown that T-cell receptor transgenic mice spontaneously develop systemic

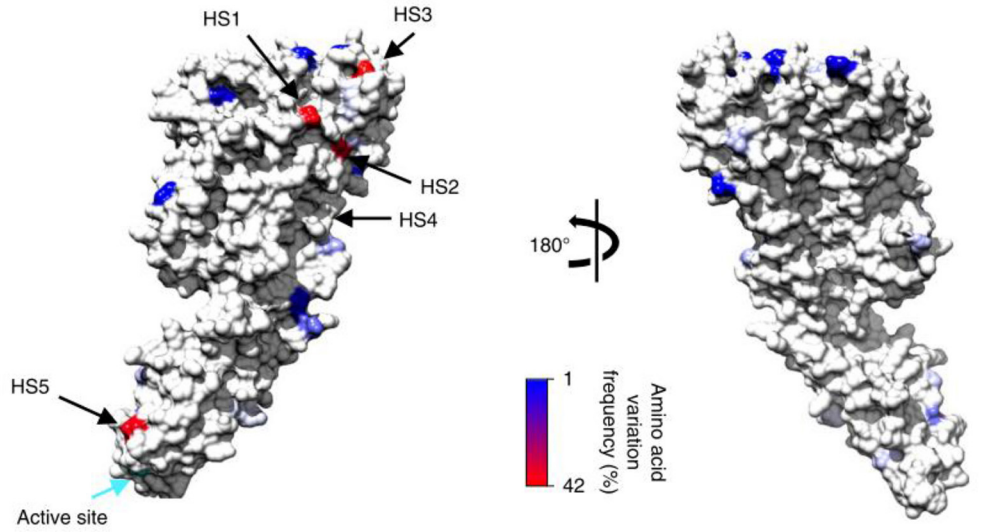

FIGURE 6 | Modeled consensus amino acid sequence and population-derived polymorphisms of the mature streptolysin O protein onto the corresponding crystal structures based on identified polymorphism locations and polymorphism frequencies within GAS genomes for the streptolysin O. Plotted against each structure is the amino acid variation frequency within GAS genomes, as represented in the color gradient from 1% (blue) to 42% variable (red); invariant sites are colored light gray. The positions of the top five most variable surface hot spots (HS) are annotated. Active sites for each enzyme are also indicated (cyan arrow) (19).

autoantibody-associated autoimmunity, leading to fully penetrant fibroinflammatory mitral valve disease (MVD) and arthritis. The key drivers of autoimmune MVD in these K/B.g7 mice are also present in humans. Key inflammatory molecules that drive MVD in this model were Syk, TNF, interleukin-6, very late antigen−4, and vascular cell adhesion molecule– are shown in **Figure 7** (61, 62).

4. **"Enhance and coordinate global efforts to produce a vaccine. Strategies to accelerate the production of an effective vaccine (e.g., reverse vaccinology) should be explored and utilized."**

Gandhi and colleagues reviewed the potential vaccine targets in S. pyogenes and possible *in silico* approaches in developing a RHD vaccine (**Figure 8**) (60). This included reverse, subtractive and pan-genome vaccinology.

Work carried out in the Australian Northern Territories, has led to the development of a possible S. pyogenes "30mer" vaccine which is composed of 30 pharyngitis-associated type-specific antigens from the S. pyogenes M protein. The authors note that the effectiveness of the 30mer vaccine is "dependent on emm type cross protection in humans, which cannot currently be estimated with any certainty. If there is cross protection in accordance with cross opsonization data, then the 30mer vaccine could be reasonably predicted to provide good protection against all emm clusters except D1-5, and possibly variants, which together comprise 53.5% of the known samples. Therefore, a potential strategy would be to combine the 30mer vaccine with a vaccine(s) specifically targeting emm cluster D1-5, and possibly emm55. Accordingly, determination of the correspondence between cross opsonization data and clinical efficacy is critical for defining future directions prior to the consideration of testing" (63).

A randomized clinical trial in Queensland, using a novel acetylated peptide-protein conjugate vaccine candidate MJ8VAX (J8-DT) has been tested in humans and shown safety and immunogenicity with an increase in vaccine-specific antibodies which decreased over time. The vaccine was delivered intramuscularly to healthy adults. No serious adverse events were reported over 12 months but further investigations and changes in the formulation of the vaccine candidate will be required to evaluate the possibility for enhancing immunogenicity and assessing the number of doses needed for protection against GAS infection (64).

5. **"Develop biomarkers for early diagnosis and follow up of disease progression."**

Levels of streptococcal antibody (antistreptolysin O, ASO), and/or antideoxyribonuclease B, ADB, titers), required as evidence of a preceding group A streptococcal infection appear to vary by population (65). However, neither ASO nor ADB antibody responses are sensitive nor specific enough to arbitrate whether or not there has been recent GAS infection. Recently, a novel antigen (SpnA) showed improved ability to detect a previous GAS exposure in ARF. Its sensitivity was 88% compared with 75% for streptolysin-O and 56% for DNaseB. Furthermore, SpnA can be combined with anti-streptolysin-O and anti-DNaseB in a multiplex single cytometric immunoassay to enhance efficiency and accuracy of ARF diagnosis (66).

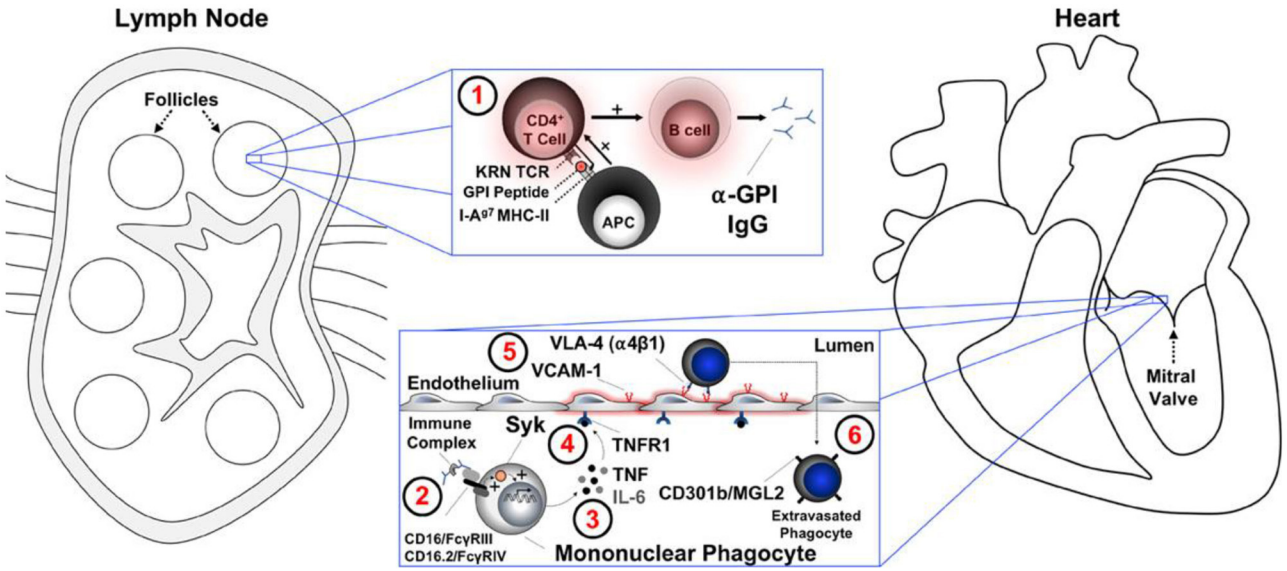

FIGURE 7 | A summary working model for the initiation of cardiac valve inflammation and fibrosis in K/B.g7 mice. Anti–glucose-6-phosphate isomerase (α-GPI) IgG autoantibodies produced systemically in the secondary lymphoid tissue (spleen and lymph nodes) (1) activate valve-infiltrating mononuclear phagocytes (MNPs) in a spleen tyrosine kinase (Syk)–dependent process (2) resulting in local production of TNF and IL-6 from CX3CR1+CD301b/MGL2+ MNPs. Tumor necrosis factor (TNF) receptor−1 (TNFR1)–mediated activation of the valve stroma (3) promotes vascular cell adhesion molecule−1 (VCAM-1) upregulation (4) and subsequent recruitment of additional circulating CX3CR1+ MNPs via very late antigen−4 (VLA-4, α4β1 integrin) in a feed-forward process (5). Interstitial MNPs assume a tissue-reparative phenotype characterized by expression of CD301b/MGL2, resistin-like molecule-α, and arginase-1 (6). APC indicates antigen-presenting cell (e.g., dendritic cell); FcγR, Fc receptor; I-Ag7 MHC-II, major histocompatibility complex–II from the non-obese diabetic mouse strain; IgG, immunoglobulin G; IL-6, interleukin-6; KRN TCR, transgenic T-cell receptor; and MGL2, macrophage galactose N-acetylgalactosamine-specific lectin 2 (49).

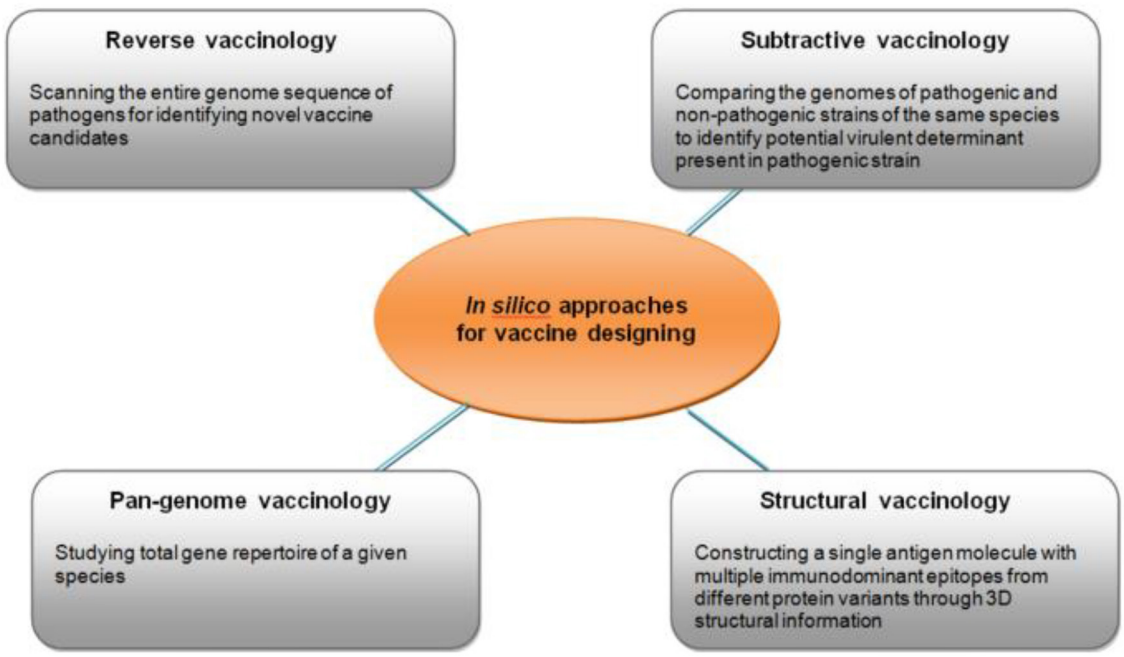

FIGURE 8 | Different types of *in silico* approaches for identifying potential targets for vaccine development (60).

A recent study in an RHD endemic area suggests that rapid nucleic acid molecular testing could detect GAS in the pharynx weeks after an infection. This could allow a higher detection rate of GAS than currently available throat swab cultures and improve management and antibiotic use in high-prevalence communities. However, further work will be required to explore apparent

cross-reactivity with non-group A streptococcal strains, and the ability to detect nonviable GAS persisting after acute infection in patients with poststreptococcal syndromes (67).

Because levels of circulating inflammatory cytokines are associated the severity of RHD, measurements of cytokines in RHD patients may have potential as prognostic markers and provide a means for risk stratification. Recent evidence suggests that the co-regulated expression of IL-6 and TNF-α is associated with a worse clinical presentation and severe valve dysfunction, whereas IL-4 and IL-10 predict subsequent adverse outcomes (68).

6. **"Provision of high-quality penicillin to affected areas – for both primary and secondary prevention – continues to be an important priority. In parallel, longitudinal studies that provide robust evidence for the benefit of secondary prophylaxis on disease progression should be performed."**

Penicillin has been the mainstay for the prevention of recurrences of rheumatic fever (secondary prophylaxis) as well as for treatment of symptomatic GAS infections (primary prevention). However, it must be recognized that disappearance of rheumatic heart disease from the developed world has led to neglect of the supply, manufacture, and accessibility of penicillin together with a lack of research into its effective dose and regimen and the cost benefits of different types of prophylaxis. Accordingly, clinical trials are sparse and current practice is based on historical trials carried out in a manner which today would not be considered acceptable.

Although there is good evidence that secondary prophylaxis with intramuscular (IM) penicillin is both effective and cost-effective at the community level, pharmacokinetic studies of benzathine benzylpenicillin G (BPG) administration in children or adolescents with RHD has shown that concentrations of BPG between injections are largely insufficient (69). Furthermore, while recurrent ARF most commonly occurs in the context of missed or late penicillin doses, a small number of individuals experience ARF recurrence despite acceptable compliance. Risk factors might include environmental factors, host immune factors, penicillin dosing, and pharmacokinetic considerations (70), but currently available data have not identified ways of predicting which individuals are most at risk. Poor compliance is a continuing problem, as demonstrated by a recent study funded by the Egyptian Ministry of health, WHO, WHF, and the African Union of 17050 individuals, reporting that 61.7% were non-compliant to the bi-weekly regimen of long-acting penicillin prophylaxis, thereby compromising efforts for the prevention of disease complications (71).

Another problem with penicillin injections have been reports of fatalities. These do not appear to be related to anaphylaxis but might be due to production problems, substances used in manufacture, or the administration technique of BPG, although most are probably related to the underlying cardiac disease which makes individuals sensitive to reflex vasovagal syncope (**Figure 9**) (72).

We clearly need to know more about the pharmacokinetic/pharmacodynamic relationships between BPG administration and clinical outcomes. Specifically, there is a need for insights into the pharmacokinetic and pharmacodynamic characteristics of BPG and the formulation of improved secondary prophylaxis strategies and regimens (69, 70, 73). Currently, there is an ongoing randomized controlled trial to determine the impact of BPG prophylaxis in children with latent rheumatic heart disease (GOAL trial) (2018-2021). The purpose is to see whether secondary prophylaxis with 4-weekly injectable BPG improves outcomes for children aged 5 to 17 years in Northern Uganda diagnosed with latent RHD (74).

Biodegradable polymer matrices have been investigated as a way of reducing administration frequency and injection pain while improving adherence. Two different approaches have been evaluated, namely a parenteral injectable depot and monolithic surgical implant. The use of highly degradable poly(lactic acid) (PLA) and poly(lactic-co-glycolic) (PLGA)-based polymers were successful as measured by degradation rate and the release of contents. However, the acidic by-products of the degradation of PLA-based polymers also resulted in substantial penicillin degradation. Polycaprolactone (PCL) was studied as an alternative release matrix and although having favorable release behavior, it required an unacceptably large implant for the delivery of the required dose of drug. As yet, biodegradable PLGA-type systems do not appear to be suitable for the development of sustained release BPG treatments, and alternative approaches to degradable polymer systems are required (75).

Finally, although primary prevention has to be an important goal, there is still a lack of evidence that systematic screening and treatment of sore throats is cost effective (76). The widespread use of penicillin is difficult for resource-poor countries and the current political pressure to limit the use of antibiotics and prevent the emergence of resistance reinforces the need to identify alternative strategies.

7. **"Conduct studies that determine the potential value of anti-inflammatory/immunosuppressive therapy after acute rheumatic fever. Other important areas where evidence is needed include optimal stroke prevention strategies in patients with atrial fibrillation and/or mitral stenosis, and pharmacological management of those with heart failure."**

Progress in this area has been modest. However, recent work has shown that the immune response to GAS in peripheral blood mononuclear cells is characterized by a dysregulated interleukin-1β-granulocyte-macrophage colony-stimulating factor (GM-CSF) cytokine axis, whereby persistent interleukin-1β production is coupled to overproduction of GM-CSF and selective expansion of CXCR3+CCR4–CCR6– CD4 T cells (66). Because hydroxychloroquine is a well-established and safe treatment for autoimmune diseases such as rheumatoid arthritis where GM-CSF plays a pivotal role, it is possible that this drug could be repurposed to reduce the risk of RHD after ARF (77).

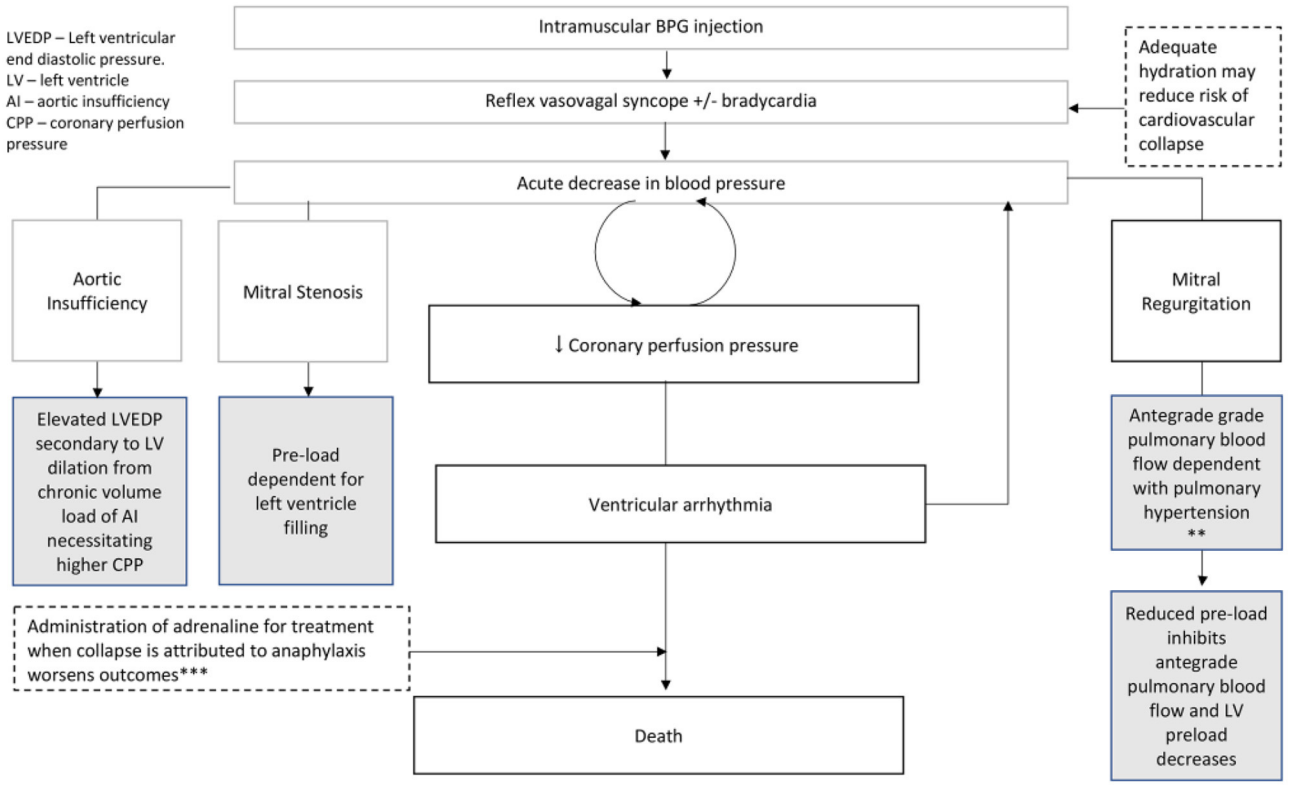

FIGURE 9 | Potential mechanism of sudden death following intramuscular benzathine penicillin injection in common valve lesions of RHD. *Gray boxes reflect contributory or predisposing characteristics of valve lesions. Dashed boxes indicate other potential contributing/mitigating factors. *Plausible mechanism in this case series based on: very rapid onset, no respiratory or skin symptoms and no positive response to epinephrine. BPG, benzathine penicillin G (72).

8. **"Accelerate the development of regional Centers of Excellence equipped with both physical and human resources to deal with prevention and treatment of the disease. Linking these centers into various regional and global research networks will significantly enhance their performance and impact."**

Sadly, the urgent need for development of regional Centers of Excellence equipped with both physical and human resources to deal with prevention and treatment of the disease remains unmet. Moreover, RHD research receives very little global funding despite its substantial disease burden. A study based on the Global Burden of Disease Study (78) with funding from the G-FINDER database (79), shows that across the range of evaluated diseases, RHD receives the least funding relative to its importance (80). Collaboration and the formation of multidisciplinary partnerships are essential in the fight against RHD and will need to involve both northern and southern partners.

A study performed by the Egyptian Ministry of health, also emphasizes the common occurrence of misdiagnosis and inappropriate primary prevention (71). Even the current International Classification of Disease (ICD-10) has limitations in its use for registering RHD (81). Survey work in recent years has suggested that RHD is widespread throughout both rural and urban areas of sub-Saharan Africa and other resource-poor countries. Here decentralization of integrated care is key and it seems feasible and practicable decentralize follow-up of patients with RHD to non-communicable disease clinics (82). Initiatives such as the PEN-plus partnership aim at establishing training sites and national operational plans to address the problems of managing diseases such as RHD in resource-limited situations (83). Finally, the COVID-19 pandemic presents both direct and indirect increased risks for people living with RHD. The COVID-19 pandemic is a significant challenge causing interruptions in secondary prophylaxis and reducing access to care, with potentially severe consequences. The pandemic is also likely to cause delay in surgical and catheter-based intervention as well as progress in RHD research, advocacy and country programmes (84).

9. **"Maximize the use of valve repair through educational programmes and exchange of expertise."**

Rheumatic mitral valves are eminently repairable, especially in children (**Figure 10**) (85), with postoperative results sometimes outperforming balloon mitral valvuloplasty (86) and with

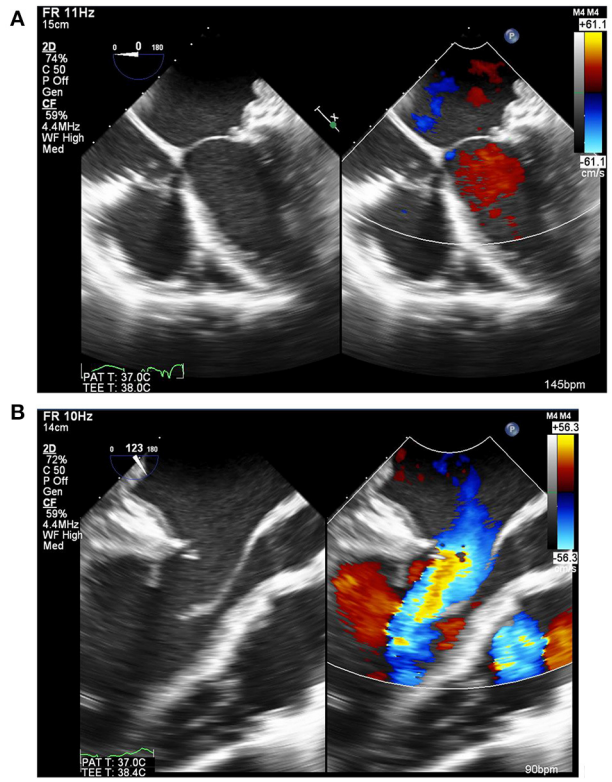

FIGURE 10 | Echocardiogram of the mitral valve after repair by peeling of the anterior leaflet coupled with a pericardial posterior annuloplasty. This showed good coaptation **(A)**, laminar flow through the valve and excellent mobility of the leaflet **(B)** [Courtesy of Aswan Heart Center (AHC) database].

re-intervention rates similar to those of MV replacement (87–89). Rheumatic mitral valve repair has the benefit of lower early and long-term mortality together with freedom from valve related complications. These benefits are not attributable to avoidance of anticoagulation alone, as the use of bioprosthetic valves in rheumatic valve replacement was associated with more valve related adverse events than valve repair or mechanical valve replacement in a recent report from Ethiopia (90). Valve repair should therefore be the primary goal in rheumatic heart disease, when feasible (88). However, patients should be carefully selected for mitral valve repair, particularly those with previous percutaneous transvenous mitral commissurotomy (91).

Yet despite recent evidence, the majority of patients with rheumatic mitral valve disease are still treated with valve replacement. This is due to the peculiar anatomical/pathological features of rheumatic valves (like fibrosis, retraction, calcific degeneration, and subvalvular apparatus involvement) unpredictable disease progression over time and historically poor outcomes of repair. Additionally, due to its rarity in high income countries, the majority of surgical expertise in valve repair has focused on degenerative and connective tissue disorders while rheumatic disease has not received similar attention.

To improve the adoption of mitral repair for rheumatic valve disease, techniques need to be made more reproducible and results followed up. This can be achieved by identifying selection criteria for repair candidates (91–93), standardizing a methodical approach to rheumatic mitral valve repair which can be taught and audited (88, 94, 95) and, importantly, strengthening local surgical programs in endemic areas, with expert valve repair surgeons providing technical guidance as well as mentorship on systematic follow-up and audit (90, 96). Collaboration of local surgical programs, through mutual site visits, workshops, or regional meetings, can have a significant effect on the wide adoption of rheumatic valve repair and the improvement of its techniques.

Surgical techniques for rheumatic aortic valve disease are still evolving. There is a need to evaluate current practice by following up large numbers of patients, focusing specifically on ventricular function and quality of life, which is often significantly impaired. The Ross procedure, where the diseased aortic valve is replaced with the patient's own pulmonary valve while a pulmonary allograft is used to replace the patient's own pulmonary valve, seems to be the best option for young patients as it is associated with reduced mortality, a positive impact on left ventricular function, better exercise tolerance and quality of life as well as having the benefit of avoiding anticoagulation. However, the number of patients which can be treated is limited by homograft availability (97).

A number of reports regarding surgery for rheumatic valve disease have been published recently, mostly from Asia. Fu and colleagues from Beijing, China have published their own experience (93), as well as a metanalysis (88), comparing outcomes between repair and replacement. They concluded that the repair group had better early mortality and less valve related events with similar reoperation rates. Kim et al. from Seol, Korea who reported their outcomes of 1171 patients undergoing surgery for rheumatic mitral valve disease found no difference in mortality between repair and replacement with significantly lower valve related complications in the repair group, again, without difference in the rate of reoperation. Waikittipong et al. published data on long-term outcomes after rheumatic mitral repair, reporting freedom from reoperation of 96, 90, and 82%, at 5, 10, and 15 years respectively (98). Krishnamurty et al. reported a 94% survival rate, 20 years after mitral valve repair in children with freedom from reoperation not statistically different from replacement but a rate of major adverse cardiac and cerebrovascular events (MACCE) that is significantly lower (87).

Taking a different point of view, Chen et al. (Taiwan) have performed propensity matching for 5086 adult patients who underwent surgery for rheumatic mitral valve disease with a mean follow up of 6 years could not find a superiority for mitral valve repair over replacement in terms of long-term outcomes. However, this paper compared 489 repairs and 4597 replacements. The same sentiment was shared with Russel et al. from Australia who found no difference between repair and replacement for rheumatic valves and no difference between surgery for rheumatic and non-rheumatic valves (99). Pillai et al. also looked at outcomes for concomitant aortic

and mitral valve replacement and reported a survival rate of 95, 93, and 93% at of 1, 5, and 10 years, respectively with freedom from MACCE of 94, 89, and 85% (100). A collaborative study of a Canadian team visiting Ethiopia has looked at outcomes of surgery for rheumatic valve disease and found acceptable rates of major valve-related adverse events albeit significantly higher in bioprosthetic valves (90).

Lu and colleagues have published interesting articles on standardizing repair techniques for rheumatic mitral repair as well as their results in commissurotomy, as the main technique, for treating mitral stenosis. They have also proposed a grading system to identify features that make rheumatic mitral valves suitable for repair. They compared their mid-term results of 921 patients receiving repair (221) vs. replacement (700) and found increased incidence of heart failure in the replacement group as well as increased valve related complications and worse quality of life.

It remains evident that outcome of rheumatic mitral valve (MV) repair is excellent in terms of early and long-term survival as well as freedom from valve-related complications, commonly seen in rheumatic MV replacement (88). Until rheumatic valve disease is medically preventable the cardiac surgical community is responsible for improving the surgical treatment offered to these patients. This can be achieved by case selection and improving the adoption of valve repair as well as standardizing techniques, auditing outcomes and following results. This is exemplified by the regular workshops carried out by the Aswan Heart Centre which attracts cardiac surgeons from all over the region to discuss and exchange experience in rheumatic mitral valve repair as well as surgery for atrial fibrillation. The workshop discusses the current literature, the anatomical and physiological concepts of mitral valve pathology in health and disease and the various surgical techniques to repair these pathologies. It is augmented by presentations of recorded videos and live cases as well as wet lab sessions for surgeons in training (101).

10. **"Develop tissue-engineered valve substitutes, including percutaneous valves that are both affordable and simple to implant."**

The technology and concepts involved in tissue engineering of heart valves continue to evolve, with more concentration on *in situ* tissue engineering (**Figure 11**) (102–105).

These techniques rely on understanding and exploiting the basic mechanisms involved in valve morphogenesis (**Figure 12**) (106) and normal valves (**Figure 13**) (107) with the use scaffolds capable of recruiting, housing, and instructing appropriate host cells and ECM (**Figure 14**) (108).

The absence of the desired control over the organization of regenerated tissue, as governed by the *in vivo* remodeling processes highlights the need for a more in-depth understanding of the long-term *in situ* remodeling processes in large animal models to improve predictability of outcome and outcome toward rational scaffold design and robust and safe clinical application (102).

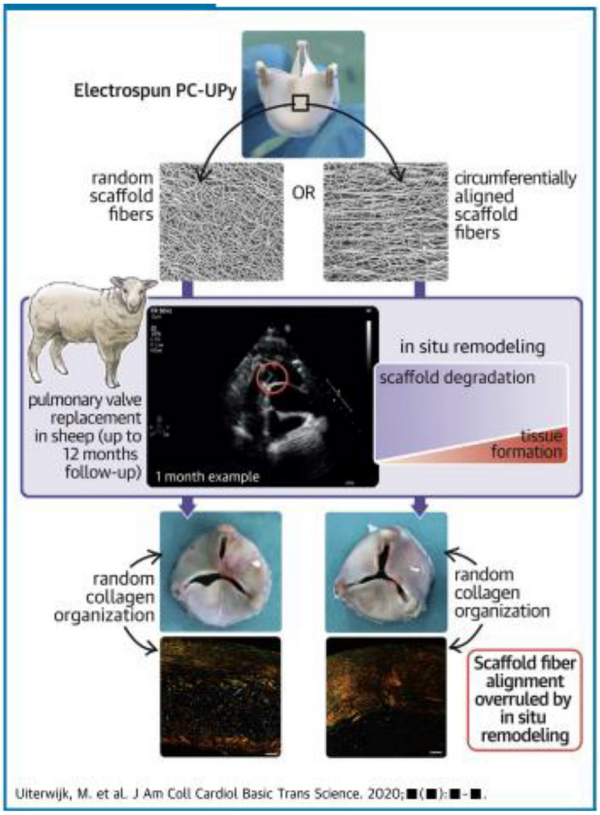

FIGURE 11 | Concept of *in situ* tissue engineering heart valves: electrospun tri-leaflet valve scaffolds of ureidopyrimidinone-polycarbonate with either a random fiber alignment (rTEHV) or with fibers with a predominant alignment in the circumferential direction of the valve leaflet (aTEHV). Electrospun tubular conduits are sutured onto a crown-shaped polyether ether ketone supporting ring (outer diameter 20 mm, inner diameter 18 mm) to create a tri-leaflet valvular shape. The scaffold microstructure is analyzed via scanning electron microscopy (SEM), and fiber alignment is quantified. *In vitro* valve functionality is evaluated in a hydrodynamic pulsatile duplicator system under physiological pulmonary conditions and sterilized by gamma irradiation. Subsequently, surgical orthotopic pulmonary valve replacement is performed in female Swifter sheep (102).

Parallel progress in the design of affordable percutaneous tools for insertion of these valves is being made (109–111).

DISCUSSION

Although there has been considerable progress and much has been learned since the Cairo Accords were formulated, much remains to be done to enhance the global priority of ARF and RHD. There have been encouraging developments in the setting up and strengthening of registries and it is hoped that these will help fill major gaps in our understanding of the natural history of rheumatic heart disease and may help identify why RHD is so closely linked with socioeconomic development. Longitudinal studies are still needed to evaluate the clinical significance of asymptomatic RHD and to assess whether

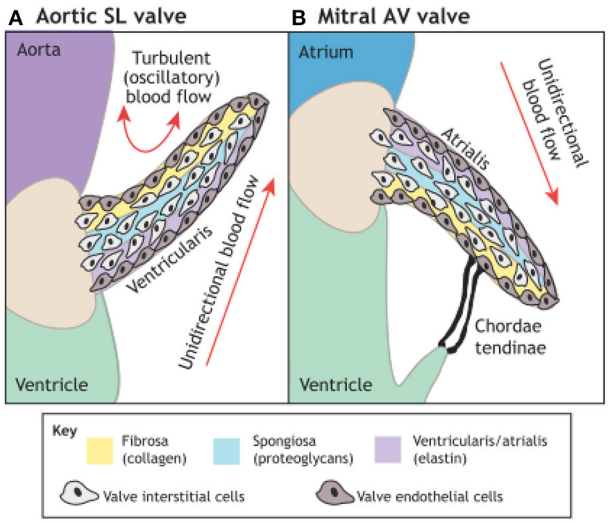

FIGURE 12 | The microarchitecture of adult cardiac valves **(A,B)**. The stratified structure of aortic **(A)** and mitral **(B)** cardiac valves ensures unidirectional blood flow. Valve leaflets are composed of three layers of stratified extracellular matrix, including an elastin-rich ventricularis layer in the SL valves or the atrialis layer in the AV valves (purple), a proteoglycan-rich spongiosa layer (light blue) and a collagen-rich fibrosa layer (yellow), interspersed with valve interstitial cells (VICs) and sheathed in a monolayer of valve endothelial cells (VECs). The direction of pulsatile blood flow in relation to valve leaflets is indicated (red arrows). The microarchitecture of an aortic valve leaflet ensures blood moves from the ventricle to the aorta. The microarchitecture of a mitral valve leaflet is supported by the chordae tendineae, which ensure blood flow from the atrium to the ventricle. AV, atrioventricular; SL, semilunar (106).

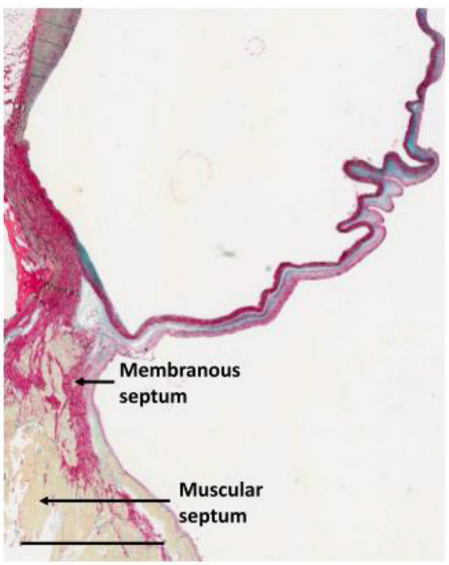

FIGURE 13 | Section through the anterior half of the non-coronary sinus approaching the interventricular septum illustrating the concept of the trilammellar sliding hypothesis. Scale bar is 3 mm (107).

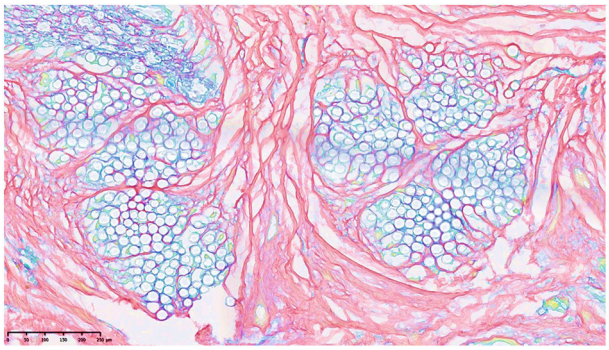

FIGURE 14 | Forming of collagen between 2 membranes in response to the integrated jacket of PCL (gray circles) showing the possibility of manipulation of collagen in tissue engineering. The stain is picrosirius (108).

echocardiographic screening of populations for early disease could play a role in the control of RHD although the available data suggests that echocardiographic screening may find its place as a research tool. Although progress has been made on the genetics of rheumatogenic streptococcal strains as well as genetic determinants of susceptibility in patients, the work carried out underlines the complexity of the processes involved and the difficulties of translation into clinical practice. Encouraging progress, however, has been made in the development of polyvalent streptococcal vaccines but these will need large–scale funding for appropriate clinical trials.

While it is clear that secondary prophylaxis with penicillin continues to play a key role in disease control, current practice is still dependent on clinical protocols developed nearly 70 years ago. Better understanding of the pharmacokinetic and pharmacodynamic characteristics of penicillin and its possible alternatives are essential for improved strategies and regimens, especially in the context of pressures to reduce the use of antibiotics. Early diagnosis and improved diagnostic biomarkers are expected to impact on early detection and prognosis, while the potential value of anti-inflammatory and immunosuppressive therapy should be extensively studied for its effect on the treatment and prognosis of ARF. Because of the complexity of the disease and the involvement of several clinical and academic disciplines, collaboration between the different RHD initiatives is essential, with cross-communication between researchers and mechanisms for data exchange. The establishment of regional and global research networks should be promoted, to enhance the impact of the strategies. Finally, while surgery has always played a central role in RHD treatment, there is much potential for the development of new technology and surgical exchange programmes should be encouraged. One important area is the need to evaluate rheumatic valve repair and the development of improved tissue-engineered valve substitutes as the most effective treatment option for valve disease, especially in the young.

CONCLUSION

This review emphasizes the need for continuing to pursue the target of eradicating RHD with vigor, and illustrates the value of

applying a comprehensive systematic approach as articulated in the Cairo Accord.

AUTHOR CONTRIBUTIONS

SK and MY contributed to the conception and design of the study. SK wrote the first draft of the manuscript. SK, DP, and MY wrote the main manuscript. AA wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

ACKNOWLEDGMENTS

We would like to extend our special appreciation to Prof Bongani Mayosi for his initiative and contribution to the Rheumatic Heart Disease: from Molecules to the Global Community conference and The Cairo Accord on Rheumatic Heart Disease.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 15 July 2021
doi: 10.3389/fimmu.2021.702877

Mapping Autoantibodies in Children With Acute Rheumatic Fever

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OPEN ACCESS

Edited by:
Bruno Ramos Nascimento, Universidade Federal de Minas Gerais, Brazil

Reviewed by:
Joselyn Rwebembera, Uganda Heart Institute, Uganda
Bryce Binstadt, University of Minnesota, United States

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Specialty section:
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

Received: 30 April 2021
Accepted: 02 July 2021
Published: 15 July 2021

Citation:
McGregor R, Tay ML, Carlton LH, Hanson-Manful P, Raynes JM, Forsyth WO, Brewster DT, Middleditch MJ, Bennett J, Martin WJ, Wilson N, Atatoa Carr P, Baker MG and Moreland NJ (2021) Mapping Autoantibodies in Children With Acute Rheumatic Fever. Front. Immunol. 12:702877. doi: 10.3389/fimmu.2021.702877

Background: Acute rheumatic fever (ARF) is a serious sequela of Group A *Streptococcus* (GAS) infection associated with significant global mortality. Pathogenesis remains poorly understood, with the current prevailing hypothesis based on molecular mimicry and the notion that antibodies generated in response to GAS infection cross-react with cardiac proteins such as myosin. Contemporary investigations of the broader autoantibody response in ARF are needed to both inform pathogenesis models and identify new biomarkers for the disease.

Methods: This study has utilised a multi-platform approach to profile circulating autoantibodies in ARF. Sera from patients with ARF, matched healthy controls and patients with uncomplicated GAS pharyngitis were initially analysed for autoreactivity using high content protein arrays (Protoarray, 9000 autoantigens), and further explored using a second protein array platform (HuProt Array, 16,000 autoantigens) and 2-D gel electrophoresis of heart tissue combined with mass spectrometry. Selected autoantigens were orthogonally validated using conventional immunoassays with sera from an ARF case-control study (n=79 cases and n=89 matched healthy controls) and a related study of GAS pharyngitis (n=39) conducted in New Zealand.

Results: Global analysis of the protein array data showed an increase in total autoantigen reactivity in ARF patients compared with controls, as well as marked heterogeneity in the autoantibody profiles between ARF patients. Autoantigens previously implicated in ARF pathogenesis, such as myosin and collagens were detected, as were novel candidates. Disease pathway analysis revealed several autoantigens within pathways linked to arthritic and myocardial disease. Orthogonal validation of three novel autoantigens (PTPN2, DMD and ANXA6) showed significant elevation of serum antibodies in ARF ($p < 0.05$), and further highlighted heterogeneity with patients reactive to different combinations of the three antigens.

Conclusions: The broad yet heterogenous elevation of autoantibodies observed suggests epitope spreading, and an expansion of the autoantibody repertoire, likely plays a key role in ARF pathogenesis and disease progression. Multiple autoantigens may be needed as diagnostic biomarkers to capture this heterogeneity.

Keywords: autoantibody, rheumatic fever, protein array, immunoassay, autoantigen, streptococcus A, group A Streptococcus

INTRODUCTION

Acute rheumatic fever (ARF) is a serious multi-focal autoimmune sequela of Group A *Streptococcal* (GAS) infection, presenting with a combination of signs and symptoms including one or more of the major manifestations used for diagnosis as part of the Jones criteria (1, 2); arthritis, carditis, Sydenham's chorea, erythema marginatum and subcutaneous nodules. Approximately 60% of ARF cases progress to chronic rheumatic heart disease (RHD), which can cause permanent heart valve damage (3), with an estimated 33 million people living with RHD globally (4). Although ARF rates declined over the twentieth century, the disease persists in low-income countries and amongst disadvantaged communities in some high-income countries, with Indigenous Māori and Pacific children in New Zealand and Aboriginal children in Australia having some of the highest incidences in the world (5, 6).

The clinical manifestations of ARF usually develop 2-4 weeks after a GAS pharyngitis infection, with growing evidence also implicating GAS skin infections in disease (7). The pathogenesis pathway for ARF remains poorly understood. The current hypothesis involves "molecular mimicry", wherein antibodies initially targeting specific GAS components are proposed to cross-react with human tissue (8, 9). This is largely based on M-protein specific antibodies and T-cells which cross-react with cardiac myosin, laminin and tropomyosin antigens found in the heart and synovium (9). The role of molecular mimicry remains the subject of debate, with an alternative hypothesis suggesting that infection with GAS causes disruption of the extracellular matrix, which exposes cryptic collagen epitopes, and triggers an autoimmune response (10, 11). Additional autoantibodies could then be generated due to increased inflammation, subsequent tissue damage and epitope spreading (12).

There are few contemporary studies investigating the broader autoantibody response in ARF and a lack of application of unbiased approaches to study the ARF autoantibody repertoire. Protein–microarray technologies enable quantification of autoantibody responses to large proportions of the human proteome (13). These technologies have been used to identify novel autoantibodies and associated disease pathways in a broad range of immune-mediated diseases, including lupus, some cancers and the recently described Multisystem Inflammatory Syndrome in Children (MIS-C) that can develop following SARS-CoV-2 infection (14–16). This study aimed to apply high-content protein-microarray technology to ARF to enable a comprehensive analysis of the disease's autoantibody landscape. This unbiased array-based approach was taken to inform antibody-driven pathogenesis and identify possible novel disease biomarkers.

MATERIALS AND METHODS

Protein Microarrays

Human Protorrays (Protein microarray platform v5.0) were performed following the manufacturer's instructions to detect serum autoantibodies (ThermoFisher, Massachusetts, USA). Samples were diluted 1:500 and antibody binding detected with an Alexa Fluor 647 labelled goat anti-human IgG antibody. Arrays were scanned using a GenePix4000B microarray scanner and array grids aligned using the GenePix Pro 5.0 software (Molecular Devices). Raw data were background corrected using the "saddle" correction (17) from the Bioconductor limma package (18), and data were quantile normalized followed by differential expression statistical analysis using linear models and empirical Bayes statistics with the limma package. Proteins antigens with $p < 0.05$ and a fold-change of > 2.0 were considered significant. For proteins with duplicated identifiers, (proteins with more than one variant on the arrays) variants with the highest absolute fold-change were kept for further analysis. Autoantigens were cross-validated using HuProt v3.0 arrays conducted by CDI Laboratories (Baltimore, USA). Serum antibodies were detected using an Alexa Fluor 532-labelled anti-human IgG secondary and data were quantile normalized as previously described for HuProt arrays (19). Proteins with $p < 0.1$ and fold–change >1.5 were considered significant.

Array Analysis and Visualizations

Analysis and visualizations were carried out in R (version 4.0.2) within R studio[19] (version 1.2.5042) using the tidyverse suite of packages (20). Upset plots were produced using ComplexHeatmap package (21). Venn diagrams were produced using jvenn (22). Heatmaps and hierarchical clustering (using the average Euclidean distance method) were carried out using Morpheus (https://software.broadinstitute.org/morpheus). Disease pathway analysis of differentially bound proteins was carried out using Metascape using custom analysis for enrichment in DisGeNet disease pathways (23, 24). Tissue specificity of proteins was elucidated using "Normal tissue data" downloaded from the human tissue atlas (HPA) from the URL (https://www.proteinatlas.org/about/download) (25). Data were filtered from the HPA using both the "reliability score" and "level". The Compartments database was also used for filtering proteins *via* the "confidence score" (26). Filtering parameters applied were; an enhanced or supported

"reliability score" and high or medium expression "level" in heart muscle, as well as a "confidence score" of >4 for plasma membrane expression or extracellular space.

Enzyme-Linked Immunosorbent Assays and Interpretation

Selected antigens identified were orthogonally validated in a larger cohort of serum samples using ELISA (**Supplementary Table 1**). Antigens were obtained commercially: NM_080423 (protein tyrosine phosphatase non-receptor type 2, PTPN2) (Origene, Maryland, USA), NM_004021 (dystrophin - dp140 variant, DMD) (Origene, Maryland, USA), NM_001155.5 (Annexin VI, ANXA6), (R&D Systems, Minneapolis, USA). For ELISA, Nunc-immunoplates (Sigma-Aldrich, Missouri, USA) were coated with antigen at 2.5 µg/ml (ANXA6) or 2 µg/ml (PTPN2 and DMD) at 4°C overnight and blocked with Phosphate Buffered Saline (PBS) supplemented with 0.5% human serum albumin (Albinorm, Octapharma, Stockholm, Sweden) for 1 hour at 37°C. Following three washes with PBS/0.1% Tween-20, serum was added at a 1:200 dilution in PBS/0.1% human serum albumin for 1 hour at 37°C. IgG binding was detected using goat anti-human IgG labelled with horse-radish peroxidase (Abcam, Cambridge, UK) diluted 1:10,000 in PBS/0.1% human serum albumin, developed with 3,3′,5,5′-Tetramethylbenzidine (TMB) and stopped with 1M HCl. The optical density (OD) at 450 nm was measured using an EnSight absorbance reader. Two serum samples with high and low OD readings for each antigen were included on every ELISA plate as internal positive and negative controls. A CV of <15% between assay runs was set as acceptance criteria.

To compare the individual antigens, and combination of antigens, for performance in discriminating ARF from control groups, the receiver operating characteristic curve (ROC) and area under the curve (AUC) values (from a logistic regression model) for each antigen or combination of all three antigens were calculated by comparing ARF and control samples using the pROC package (27). Confidence intervals for AUC and differences in AUC were obtained using bootstrapping (n=2000) implemented in the pROC package.

Study Participants

Human sera were obtained from several studies conducted in New Zealand. Each had appropriate ethical approval, and all participants (or their proxies) provided written informed consent. All ARF cases were diagnosed according to the New Zealand modification of the Jones criteria (1, 28). The sera for ProtoArrays were from a study conducted in the Waikato District Health Board region (2012 to 2015; ethics CEN/12/06/017) including ARF (n=3), GAS pharyngitis (n=3), as well as ethnically matched healthy controls (n=3) from the Auckland arm of the children of SCOPE study (29). The sera for the HuProt arrays (ARF with carditis (n=7), ARF without carditis (n=5) and matched healthy controls (n=6)) and ELISA orthogonal validation (ARF n=79 and controls n=85) were from participants recruited as part of the Rheumatic Fever Risk Factors (RF RISK) study (30). This nationwide study conducted between 2014 and 2017 (ethics 14/NTA/53) included first-episode ARF patients and closely matched healthy controls (30). Controls were matched by age, ethnic identification, socioeconomic deprivation (using the New Zealand Deprivation Index score (31)) and geographic area. Sera from children with GAS positive pharyngitis (n=39) used for orthogonal validation ELISAs were recruited as part of a paediatric study investigating GAS skin and throat infections conducted in the Auckland region (2018-2019; ethics 17/NTA/262) (32).

RESULTS

Global Analysis of Autoantibody Reactivity in Acute Rheumatic Fever

The ProtoArrays initially utilized to profile the autoantibody response in ARF contain over 9000 human proteins expressed in insect cells. As autoantibodies are present in all individuals (33–35), serum binding from ARF patients (n=3) was compared to that of healthy children (n=3) and children with GAS positive pharyngitis (n=3) as controls. Following array QC and normalization, the total antibody reactivity or fluorescence intensity, was determined for each array. ARF arrays showed an increased number of total reactivities compared to controls ($p < 0.0001$) (**Figure 1A**), suggesting an overall increase in autoantibodies in ARF patient sera. The total reactivity observed on the ARF arrays was markedly increased compared to both the GAS positive pharyngitis and healthy controls (**Supplementary Figure 1**), and as the overarching goal was to identify ARF specific autoantibodies rather than those associated with GAS pharyngitis, the control groups were combined for the subsequent data analysis. The antibody reactivity signals for ARF patients were filtered to include only proteins with a > 2.0 fold-increase in fluorescence intensity compared to the mean of the combined controls (healthy and GAS pharyngitis). This selected for autoantibodies with stronger reactivity in ARF and enabled individual ARF patient's autoantibody profile to be compared. A total of 1013 autoantibodies showed > 2.0 fold-increased reactivity in ARF compared to the combined controls, with each of the ARF patients having similar numbers of proteins with an increased signal (687 in patient A1, 556 in patient A2 and 541 in patient A3) (**Figure 1B**). Nearly half (47%, 480/1013) of the autoantibodies were unique to an individual patient, with only 23% (238/1013) shared between all three ARF patients and the remaining 29% (295/1013) present in two ARF patients but not the other. Taken together, these results show a global increase in autoantibodies in ARF sera with marked heterogeneity in the autoantibody profiles for each of the ARF patients assessed.

Autoantibodies Target Proteins in Relevant Disease Pathways

To identify differentially bound proteins in sera from ARF patients and perform pathway analysis, proteins with significantly elevated fluorescence intensity in the ARF group compared with the combined control group were selected

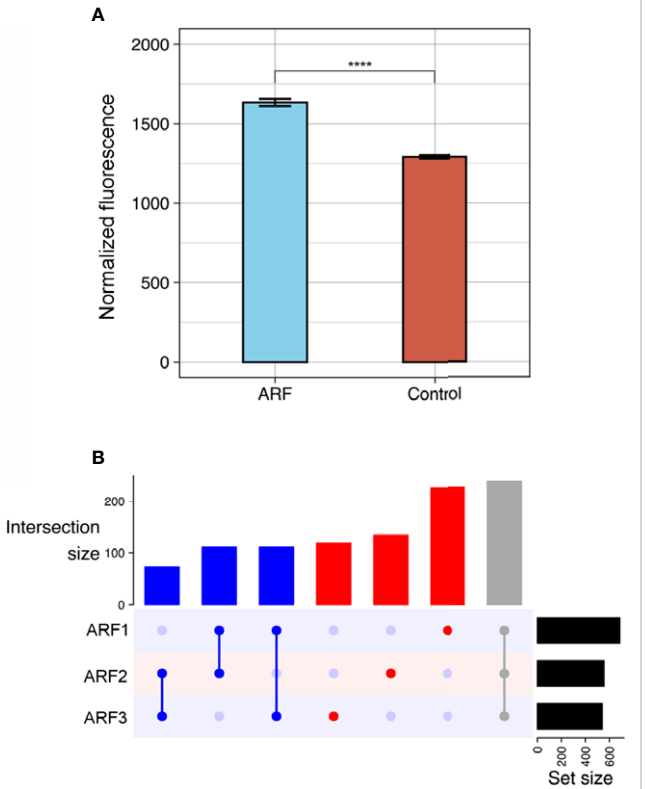

FIGURE 1 | Global autoantibody analysis in ARF using ProtoArrays. **(A)** Overall antibody binding intensities against human proteins in ARF patients (blue, n=3) and grouped controls (red, n=6). Controls comprise both healthy children (n=3) and children with GAS positive pharyngitis (n=3). Bars indicate mean and standard error. ****p < 0.0001 using two sample Wilcoxon test. **(B)** Upset plot showing number of shared autoantibodies between ARF patients (ARF1, 2 and 3). Autoantibodies shared between all three ARF patients are indicated in grey, those shared between at least two patients in blue and those unique to an individual patient in red. The number of autoantibodies in each category is indicated on vertical bar charts with respective colours. Upset plots include only antibodies that showed at least a two-fold enrichment when compared with the mean of grouped controls. Total number of auto-antibodies identified using this threshold per patient is indicated on horizontal bar charts (Set size).

($p < 0.05$ and fold-change > 2.0). In total, 841 proteins were bound significantly more by ARF serum IgG compared to 693 proteins in controls (**Figure 2A** and **Supplementary Data**). This is in line with the prior global analysis suggesting higher overall autoantibodies in the ARF group. Encouragingly, some proteins that have previously been implicated in the pathogenesis of ARF and RHD were identified (10). These included extracellular matrix proteins; fibronectin (36) and collagens (10, 37, 38) (FSD1L, COL-/2/9/14-A1) as well as intracellular proteins involved in muscle contraction; tropomyosins and myosin (39, 40) (TPM2/3 and MYL6) (**Figure 2A**). As the tropomyosin that previously linked to ARF is cardiac tropomyosin (TPM1), a sequence alignment was performed with the TMP2 and TMP3 isoforms identified in this study. This showed high sequence identity between TPM1 and TPM2 (85.563%) and TPM3 (91.197%) (**Supplementary Figure 2**).

To explore disease connections, the 841 proteins bound by ARF IgG were subjected to disease pathway analysis. This identified three noteworthy pathways, which were both significantly enriched ($p < 0.01$, fold-enrichment > 1.5) (**Figure 2B** and **Supplementary Data**), and related to two of the major criteria used to diagnose ARF (carditis and arthritis). The "Myocardial Ischemia", "Autoimmune arthritis" and "Juvenile Rheumatoid Arthritis" disease pathways contain 33, 11 and 13 proteins targeted by autoantibodies in ARF sera, respectively. Unsupervised hierarchical clustering on these pathway proteins shows serum from ARF patients clustering separately from the control groups with respect to fluorescence intensity (**Figure 2C**). This indicates that ARF autoantibodies target a diverse range of proteins that are enriched for in relevant disease pathways.

Multi-Platform Validation of Array Hits

To further explore and validate the ARF autoantibody repertoire, including antigens identified *via* the ProtoArray analysis, additional high-content arrays (HuProt arrays, > 16,000 human proteins, expressed in yeast cells) were conducted using a distinct cohort of patients; ARF patients with carditis (n=7), ARF patients without carditis (n=5) and healthy controls (n=6). A focussed analysis of the HuProt array data identified 158 human proteins bound significantly more by serum IgG in carditis patients compared to healthy controls ($p < 0.1$ and fold change >1.5) (**Figure 3A**). Interestingly, one of these proteins, ANXA6, was previously identified in a pilot mass spectrometry analysis of 2–Dimensional Electrophoresis (2-DE) separated human heart lysate and ARF sera conducted in our laboratory (**Supplementary Methods**). Nine of the 158 proteins identified *via* the HuProt analysis overlapped with those identified by the ProtoArrays (**Figure 3B**). When these nine proteins were filtered for expression in heart muscle [using human protein atlas data (25)] as well as localization in or near the plasma membrane [using the compartments database (26)] just two proteins remained; PTPN2 and DMD (**Figure 3B** and **Supplementary Data**). When the same analysis and filtering was applied to the control groups, five overlapping proteins were identified, but none of these passed the filters for expression location. Plotting normalized fluorescence values from the HuProt arrays for PTPN2 and DMD plus ANXA6 illustrates the increased autoantibodies in ARF compared to healthy controls (**Figure 3C**).

Orthogonal Validation Using ELISA

To orthogonally validate hits from the arrays, ELISAs were performed with DMD, PTPN2 and ANXA6 as antigens. These antigens represent different aspects of ARF disease mechanisms including an immune cell signalling protein [PTPN2 (41)], a central component of the extracellular matrix in muscle fibre [DMD (42)] and a protein abundantly expressed in cardiomyocytes and chondrocytes during osteoarthritis [ANXA6 (43, 44)]. A large cohort was used for validation comprising sera from children with first-episode ARF (n=79), closely matched healthy controls (n=85), as well as children with GAS pharyngitis (GAS positive throat swab and elevated streptococcal serology, n=39)

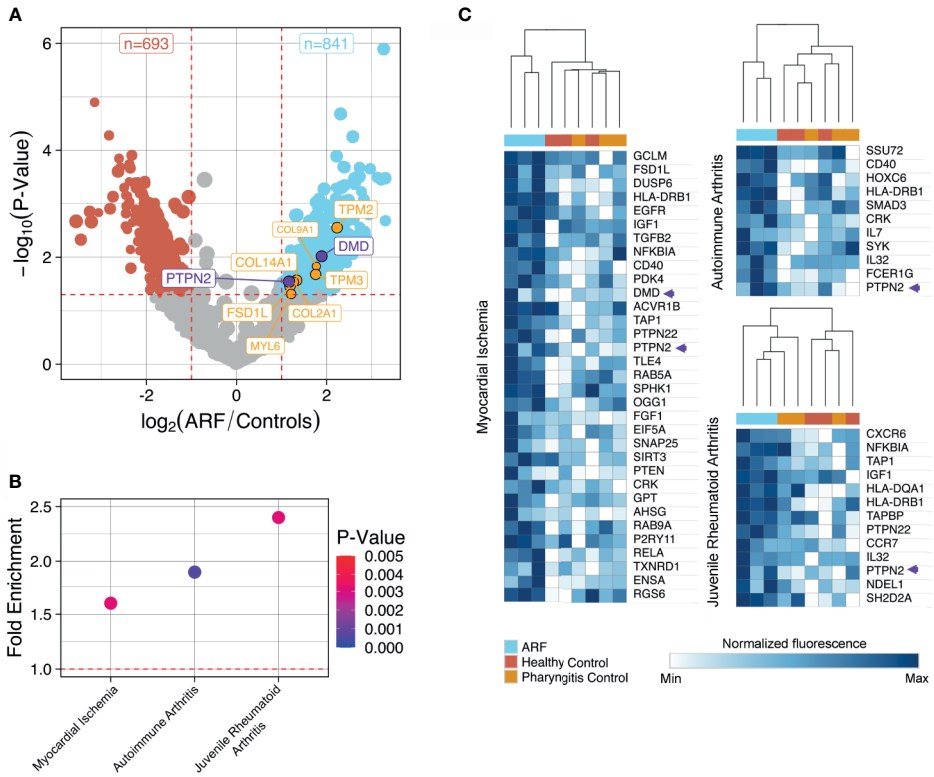

FIGURE 2 | Autoantibody disease pathway analysis using ProtoArray data. **(A)** Volcano plot showing fold-change differences in autoantibody signals between ARF patients (n=3) and controls (n=6). Controls comprise both healthy children (n=3) and children with GAS positive pharyngitis (n=3). The size of the dots and annotations relates to the fluorescence intensity of individual autoantibodies. Red dashed lines indicate cut-offs for significant differences ($p < 0.05$, fold-change >2). Blue dots represent autoantibodies showing significantly increased binding in ARF patients compared to controls whilst red dots represent autoantibodies showing significantly increased binding in controls compared to ARF patients. Orange annotated autoantibodies have historically been implicated in the pathogenesis of ARF and/or RHD. Purple annotated autoantibodies are novel and of interest for downstream analysis (see **Figure 3**). **(B)** Disease pathway analysis conducted on 841 autoantigens with significantly increased binding in ARF patient sera in part (A). Three significant ($p < 0.005$) disease pathways are shown in relation to fold enrichment of proteins in pathways (compared to what would be expected by chance). Dot color intensity corresponds to p-value and dashed red line indicates a fold-enrichment of 1, which would represent no enrichment. **(C)** Heat-maps showing individual autoantibody reactivities to proteins belonging to disease pathways identified in part (B). Color intensity corresponds to $\log_2$ normalized fluorescence values from ProtoArrays of each individual ARF patient (blue columns), healthy controls (red columns) and children with GAS positive pharyngitis (orange columns). A relative color scheme was applied using the min and max values in each row to plot relative colors. The dendrogram represents results of hierarchical clustering on columns. Autoantibody reactivities indicated by purple arrows relate to novel autoantibodies of interest for downstream analysis annotated.

(**Supplementary Table 1**). Significantly elevated autoantibodies were observed in the ARF patient group compared to both healthy controls and the GAS pharyngitis group for all three antigens (**Figure 4A**). The lack of reactivity to these antigens in the GAS pharyngitis group confirms that these autoantibodies are associated with disease, and not with the prior GAS infection. Receiver Operator Curves (ROC) were generated to assess the predictive performance of each of the three antigens alone as well as in combination to distinguish ARF sera from the combined controls (healthy and GAS pharyngitis) (**Figure 4B**). The area under the curve (AUC) metric showed DMD had the best predictive performance (AUC = 0.857, CI:0.805-0.904 followed by PTPN2 (AUC = 0.787, CI:0.718-0.847) and ANXA6 (AUC = 0.642, CI:0.565-0.716). DMD performed significantly better than PTPN2 ($p < 0.05$) which, in turn, performed significantly better than ANXA6 ($p < 0.001$). There was no significant gain in performance, above DMD alone, when combining all three assays (AUC = 0.861, CI:0.809-0.908, p = 0.583).

To further explore the biomarker potential of these three antigens, cut-offs for positivity were determined using the Youden index (45). This method identifies an optimal cut-off from the ROC curve that maximizes sensitivity and specificity and resulting values were DMD = 0.190, PTPN2 = 0.274 and ANXA6 = 0.275 (**Figure 4A**). These cut-offs were applied and the ARF patients were categorized as having positive or negative reactivity to each antigen in the form of an autoantibody barcode (**Figure 4C**). In keeping with the superior predictive performance of DMD in the ROC analysis, this antigen yielded the highest number of positive ARF cases (62/79, 78%). However, the barcode also illustrates that patients with first episode ARF have every combination of biomarkers tested ranging from positive for one, two or three antigens through to negative for

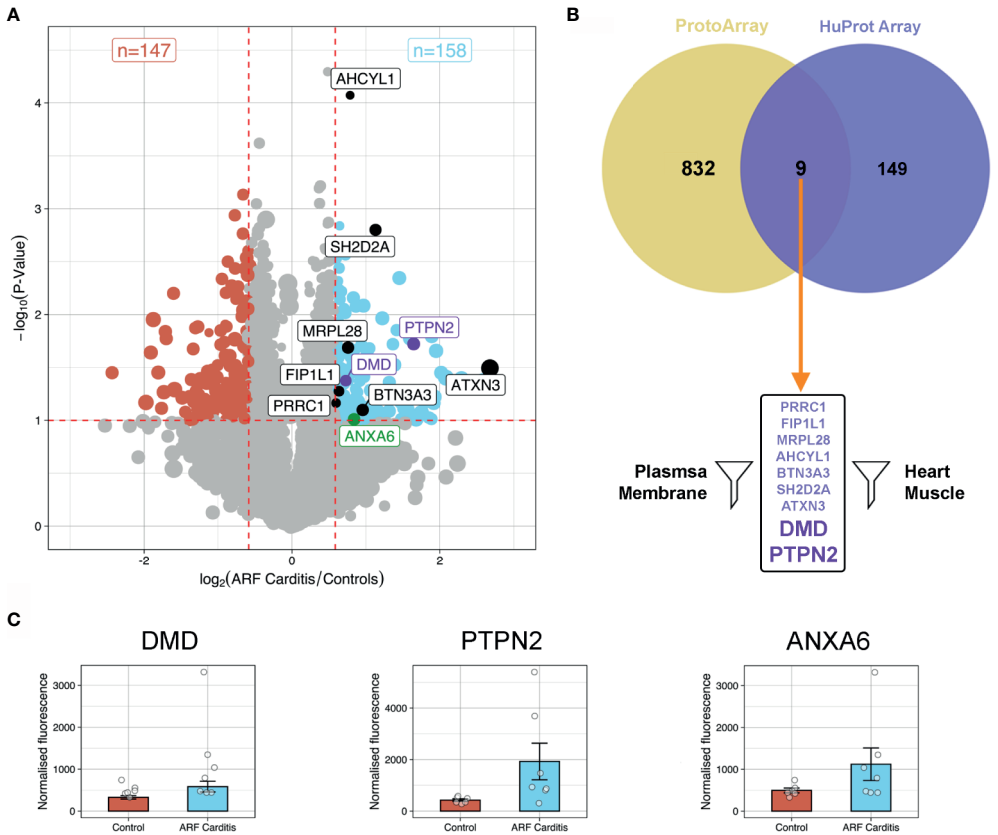

FIGURE 3 | Autoantibody cross-validation using HuProt array **(A)** Volcano plot showing fold-change differences in autoantibody signals between ARF patients with carditis (n=7) and healthy controls (n=6). The size of the dots relates to the fluorescence intensity of individual autoantibodies. Red dashed lines indicate cut-offs for significant differences ($p < 0.1$, fold-change >1.5). Blue dots represent autoantibodies showing significantly increased binding in ARF patients compared to controls whilst red dots represent autoantibodies showing significantly increased binding in controls compared to ARF patients. Black and purple annotated autoantibodies are those also identified in ProtoArray analysis (see **Figure 2**), with purple dots relating to novel autoantibodies of interest for downstream analysis. Green annotated dot was also identified by 2-DE gel. **(B)** Venn diagram showing the nine overlapping autoantibodies between; 158 proteins identified in HuProt analysis from part (A) in violet; and 841 autoantibodies identified in ProtoArray analysis from **Figure 2A** in yellow. Following filtering for proteins localized to plasma membrane and present in heart muscle, autoantibodies targeting two proteins were identified indicated by large purple text. **(C)** Bar graphs showing mean and standard error of normalized fluorescence values representing autoantibody reactivities to DMD (left), PTPN2 (middle) and ANXA6 (right), from healthy controls (blue, n=6) and ARF patients with carditis (red, n=7).

all three antigens. This further highlights the heterogeneity of autoantibody responses in ARF.

DISCUSSION

This study has comprehensively investigated the serum autoantibody repertoire in ARF patients using multiple approaches. High content arrays revealed an overall increase in autoantibodies in ARF, with a large proportion of the antibodies unique to individual patients. The current pathogenesis models for ARF following GAS infection are centred on molecular mimicry and the development of autoantibodies to host coiled-coil proteins and extracellular matrix disruption and exposure of cryptic epitopes (10, 11). While the array analysis in this study did identify autoantibodies to myosin, tropomyosin and collagens that might support mimicry and extracellular matrix disruption, the breadth of the autoantibody reactivity observed also points to epitope spreading playing a role in pathogenesis (12).

Epitope spreading, that is the involvement of antigens beyond those that initially trigger the autoimmune response, is thought to be central to the pathogenesis of other systemic autoimmune diseases such as rheumatoid arthritis (46). It has also been suggested to have a role in ARF pathogenesis (12), and is in keeping with the systemic and heterogeneous nature of symptoms associated with the disease including poly-migratory arthritis, carditis, subcutaneous nodules and in some, neurological symptoms or chorea (1, 8). The pathway analysis applied to the array data in this study enabled filtering of the large number of autoantibodies identified and revealed antigens associated with disease pathways relevant to ARF symptoms; "Myocardial Ischemia", "Autoimmune Arthritis" and "Juvenile Rheumatoid Arthritis". Yet even within these pathways a diverse

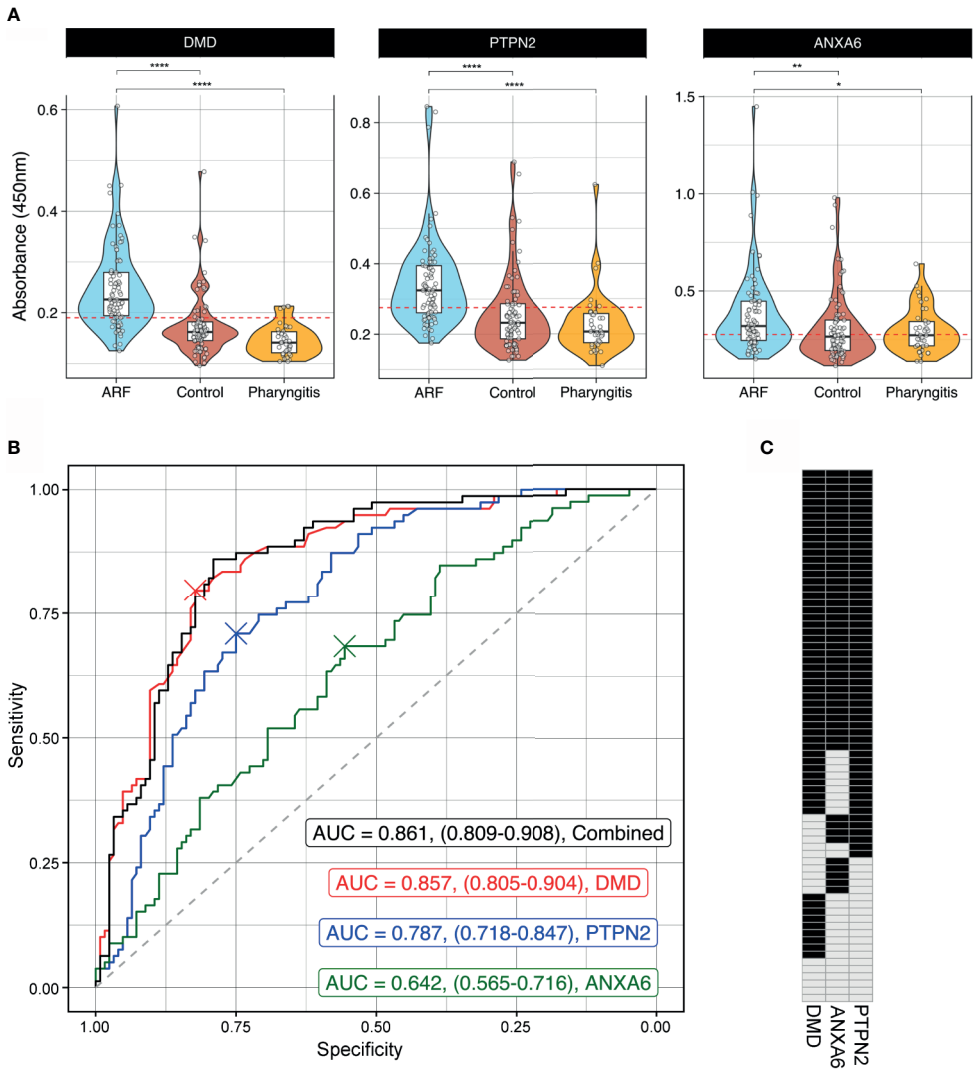

FIGURE 4 | Orthogonal validation of autoantigens by ELISA **(A)** Combined violin and box and whisker plots showing ELISAs targeting DMD (left), PTPN2 (middle) and ANXA6 (right) using sera from ARF patients (blue, n=79), matched healthy controls (red, n=85) and children with GAS positive pharyngitis (orange, n=39). For box and whisker plots the lower and upper hinges correspond to the first and third quartiles (the 25th and 75th percentiles). The whiskers extend from the hinge to the largest and smallest value no further than 1.5 x inter-quartile range from the respective hinge. The violin plot extends from the highest to the lowest value showing density of data. Red dashed line represents the cut-off for positivity positive based analysis in part (B). *p < 0.05, **p < 0.01, ****< 0.0001 using two sample Wilcoxon test. **(B)** Receiver Operator Curves (ROC) of ELISA results from DMD (red), PTPN2 (blue) and ANXA6 (green) as well as all three antigens combined (black). Grey dashed line represents the line of no discrimination, which would indicate a test with no predictive power. The AUC for each analysis is indicated with confidence intervals obtained using bootstrapping in brackets. The crosses represent the optimal cut-off for each autoantigen ELISA. **(C)** Barcode of all 79 ARF patients indicating positive (black) or negative (grey) reactivity to all three autoantigens. Cut-off for positivity was determined from the ROC analysis in part (B) and is represented visually as a red dashed line in part (A).

range of antigens were targeted by patient serum antibodies, suggesting that a loss of tolerance and epitope spreading may occur in relevant tissues. This is consistent with an ARF pathogenesis model in which a loss of tolerance, driven by inflammation, enhances a dysregulated immune response. There is increasing evidence to suggest that repeated GAS infections prime the immune response for a loss of tolerance in ARF (47–49), and it follows that the presence of inflammation observed in ARF patients (12, 50) could enhance the dysregulated autoimmune response, counteracting tolerance mechanisms, and contribute to epitope spreading and further damage in specific tissues.

In order to validate the analysis of the high content protein arrays, the presence of autoantibodies to three of the antigens identified, DMD, PTPN2 and ANXA6, was assessed in a large ARF cohort. While there was a significant increase in autoantibodies to each of these three antigens in ARF, there was also variability at an individual patient level such that a continuum of reactivity was observed, ranging from autoantibodies to all three

antigens in some patients to an absence of autoantibodies to the three antigens in others. The three proteins validated in this study were selected based on their detection across multiple analyses and represent different aspects of potential pathways and tissues involved in ARF. In particular immune signalling [PTPN2 (41)], cardiac tissue [ANXA6 (43), DMD (42)] and joint tissue [ANXA6 (44)]. However, all appear to be associated with the plasma membrane rather than being fully extracellular antigens. It is therefore possible that these antigens are not involved in initiating disease, but rather are exposed as a result of inflammation driven tissue damage and epitope spreading. It is important to note that in autoimmune disease in general not all autoantibodies are pathogenic, and only those targeting cell surface proteins are generally thought to cause clinical manifestations (51). In a similar vein, the anti-myosin autoantibodies observed in ARF (8, 12, 36, 40) could well be the result of tissue damage and cardiomyocyte burst given the intracellular location of myosin within the myocardium (52).

This study has several limitations. The ProtoArrays antigens are expressed in insect cells such that the glycosylation patterns on the extracellular antigens will differ from those that maybe present in human tissue. Similarly, membrane associated antigens may be mis-folded or under-represented on both of the array platforms utilised given the uniform approach required to express and purify such large numbers of antigens in parallel. To overcome this limitation, and expand the antigen space examined in the context of ARF, alternative approaches such as Phage Immunoprecipitation Sequencing [PhiP −Seq (53)] and Rapid Extracellular Antigen Profiling [REAP (54)] of the human proteome could be considered in future studies. Finally, the initial array analysis was based on small patient numbers and it is possible that additional ARF autoantibodies would be detected with larger cohorts. Despite this, the analysis and filtering approach applied to the array data successfully identified three novel ARF autoantigens, each validated in a large patient cohort, supporting the use of high content arrays as a discovery tool.

In conclusion, this study has utilized high content protein array platforms to assess autoantibodies present in ARF in an unbiased fashion. The broad yet heterogenous elevation of autoantibodies in ARF patients support a pathogenesis model in which tissue damage and inflammation leads to a loss of tolerance to endogenous proteins and subsequent epitope spreading. Whilst autoantibodies have diagnostic potential, a panel comprising multiple antigens will likely be needed to capture individual heterogeneity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Health and Disability ethics committee with the following ethics approval numbers (CEN/12/06/017, 14/NTA/53, 17/NTA/262). Written informed consent to participate in this study was provided by the participants' or their legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

NM, RM, NW, WM, and MB conceived the study. NM, RM, MT, LC, PH-M, JR, WF, DB, MM, PA, and JB performed the experiments and/or acquired the data. RM, NM, MT, and LC analyzed the data. RM and NM wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by funding from the Maurice Wilkins Centre, the Heart Foundation of New Zealand (small project grant #1576) and Cure Kids (project grants #3545 and #3585). NM was funded by a Heart Foundation Senior Research Fellowship for part of the study period (#1650). The RF RISK study, from which samples were obtained, was funded by the Heath Research Council of New Zealand (HRC) Rheumatic Fever Research Partnership (Ministry of Health, Te Puni Kōkiri, Cure Kids, Heart Foundation, and HRC).

ACKNOWLEDGMENTS

We thank Connie Talbot at the John Hopkins School of Medicine (USA) and Vicky Fan at NZGL (New Zealand) for bioinformatics assistance. We also thank Anita Bell previously of the Waikato District Health Board Public Health Unit and staff in paediatric departments across the North Island of New Zealand for kind support and assistance with patient recruitment. All members of the RF RISK Study (in addition to the authors on this manuscript) are gratefully acknowledged.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.702877/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cardiovascular Medicine

SYSTEMATIC REVIEW
published: 20 July 2021
doi: 10.3389/fcvm.2021.691646

Utility of Human Immune Responses to GAS Antigens as a Diagnostic Indicator for ARF: A Systematic Review

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Background: Previous studies have established that streptococcal antibody titer is correlated with a diagnosis of acute rheumatic fever (ARF). However, results vary in the usefulness of GAS antibodies, particularly anti-streptolysin-O (ASO) and anti-DNase B, in confirming a recent GAS infection. Therefore, we sought to provide, from published studies, an evidence-based synthesis of the correlation of streptococcal serology to establish the usefulness of immunological data in aiding the diagnosis of ARF. These findings are anticipated to have implications where echocardiography is not freely available, especially where ARF is rampant.

Methods: We conducted a comprehensive search across a number of databases. Applying a priori criteria, we selected articles reporting on studies, regardless of study design, that evaluate the levels of antibodies against GAS-specific antigens in ARF subjects against control values or a published standard. Data were extracted onto data extraction forms, captured electronically, and analyzed using Stata software. Risk of bias was assessed in included studies using the Newcastle-Ottawa Scale (NOS).

Results and Conclusion: The search strategy yielded 534 studies, from which 24 met the inclusion criteria, reporting on evaluation of titers for SLO ($n = 10$), DNase B ($n = 9$), anti-streptokinase (ASK) ($n = 3$) amongst others. Elevation in titers was determined by comparison with controls and upper limit of normal (ULN) antibody values as determined in healthy individuals. Meta-analysis of case-controlled studies revealed moderate odds ratio (OR) correlations between ARF diagnosis and elevated titers for SLO (OR = 10.57; 95% CI, 3.36–33.29; 10 studies) and DNAse B (OR = 6.97; 95% CI, 2.99–16.27; 7 studies). While providing support for incorporating SLO and DNase B in the diagnosis of ARF, we present the following reflections: an elevation in SLO and DNase B levels are not consistently associated with an ARF diagnosis; increasing the number of GAS proteins in the test is warranted to improve sensitivity; paired (acute and convalescent) samples could provide a more accurate indication of a rising titer. Use of community-based controls as a standard is not a reliable marker by which to gauge recent GAS infection.

Keywords: GAS antigens, anti-streptolysin-O, anti-DNase B, ARF diagnosis, systematic review

OPEN ACCESS

Edited by:
Bruno Ramos Nascimento, Universidade Federal de Minas Gerais, Brazil

Reviewed by:
Sasha A. Singh, Brigham and Women's Hospital and Harvard Medical School, United States
Sarvesh Chelvanambi, Brigham and Women's Hospital and Harvard Medical School, United States

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Specialty section:
This article was submitted to Heart Valve Disease, a section of the journal Frontiers in Cardiovascular Medicine

Received: *06 April 2021*
Accepted: *23 June 2021*
Published: *20 July 2021*

Citation:
Salie MT, Rampersadh K, Muhamed B, Engel KC, Zühlke LJ, Dale JB and Engel ME (2021) Utility of Human Immune Responses to GAS Antigens as a Diagnostic Indicator for ARF: A Systematic Review. Front. Cardiovasc. Med. 8:691646. doi: 10.3389/fcvm.2021.691646

INTRODUCTION

Acute rheumatic fever (ARF), which develops within 2–6 weeks after a preceding non-invasive group A streptococcal (GAS) infection such as streptococcal pharyngitis or scarlet fever, affects ∼300,000–500,000 people across the globe each year, the majority of whom live in developing countries. ARF symptoms include fever, arthritis, carditis, rash (erythema marginatum), subcutaneous nodules, and/or Sydenham's chorea (1–3). Since these symptoms are related to other diseases, the Jones criteria has been used since 1944 as a clinical standard in the diagnosis of ARF and rheumatic heart disease (RHD); amongst its criteria, is laboratory evidence of recent streptococcal infection, either through culture or an elevation in serum streptococcal antibodies (3). An accurate diagnosis of ARF ensures proper treatment and reduces the risk of recurrent disease and the development of rheumatic heart disease (4). ARF is often underdiagnosed, by as much as 50%, as reported in a Fijian hospital-based study which compared clinical data against primary care records from health care clinics (5). Okello similarly reports a likelihood of under-representation of the actual number of cases presenting to primary care in Uganda (6), thus highlighting the need to develop simple and practical approaches to diagnosing ARF in primary care in low-resource settings.

The Jones criteria (7) has recently been updated by the American Heart Association (AHA) to suit all types of populations with appropriate recommendations (3, 8). With recent advancements in medical technologies such as echocardiography and Doppler flow assessments, analyzing images of the heart valves has been made easier, with clinicians following the guidelines as described in the Jones criteria (3). However, these tools may not be readily available in areas where ARF is rampant. The Jones criteria has guidelines as to assess the remaining major clinical manifestations which, however, may be difficult to evaluate as these symptoms could be mistaken for or attributed to other illnesses. Symptoms of arthritis may be found in septic arthritis, juvenile idiopathic arthritis, lyme disease, or sickle cell anemia (9). Carditis may be found in mitral valve prolapse, fibroelastoma, cardiomyopathy, or Kawasaki disease. Symptoms associated with chorea may be found in Wilson disease, tic disorder, encephalitis, or other autoimmune diseases such as systemic lupus erythematosus and systemic vasculitis. Therefore, included in the Jones criteria are other mechanisms, such as evidence of a preceding GAS infection to eliminate any doubt in the diagnosis of ARF (9).

The Jones criteria describe evidence of a preceding GAS infection with any one of three scenarios; a positive throat culture for GAS, a positive GAS carbohydrate (GAC) antigen test, or a rise in GAS antibody titers [anti-streptolysin O (ASO) or anti-DNase B] that requires paired samples (at diagnosis and 3 weeks post-suspected diagnosis) (9). These GAS-specific antigens form part of the arsenal of GAS for survival and infiltration of the bacteria into human tissues; thus, these antibodies are present in the sera of GAS-infected individuals (10–12). Streptolysin O (SLO) is a cytolytic toxin released by GAS for cell lysis (13, 14). DNase B is an extracellular virulent protein with DNA-degrading activity (15, 16). These two virulent factors, along with GAC, are mentioned within the Jones criteria; however, there are many other GAS-specific antigens in recent times that have shown potential as putative biomarkers relating to the presence of GAS (17–24).

Few countries have specific guidelines in terms of an ARF diagnosis, specifically including a preceding GAS infection (**Supplementary Table 1**). We sought, through a comprehensive systematic review of published studies, to conduct an evidence-based synthesis of the utility of streptococcal serology in aiding the diagnosis of ARF. Primarily, our review aimed to assess available published literature regarding the association of antibodies against GAS antigens with ARF. We anticipate that our findings could have implications for the design of future diagnostic tests to confirm recent GAS infection in suspected ARF patients.

METHODS

Search Strategy and Selection Criteria

According to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (25), we performed a systematic literature review from two peer-reviewed databases (PubMed and Scopus) with predefined search terms (**Supplementary Table 2**). This review asks the following question: What is the utility of GAS serology, specifically SLO and/or DNase B, in providing evidence of a recent GAS infection in diagnosing ARF? In addition, we further sought to explore the potential of other GAS-specific antigens which may provide additional support of a recent GAS infection. The search strategy incorporated both free term text and Medical Subject Headings (MeSH) adapted to suit the particular database. Keywords incorporated a combination of terms relating to group A streptococcus, GAS antigens, SLO, DNase B, serology, immune response, and acute rheumatic fever. Results were complemented by hand searching and citation searches in Google Scholar. The search was not restricted to publication dates or language. Additionally, gray literature including theses and conference proceedings, were also considered for inclusion.

Studies were included if immunological assays were used to evaluate the expression of antibodies evoked by GAS-specific antigens in ARF cases and controls within the same population or the use of a control standard based on the titers of healthy individuals (upper limit of normal, ULN) from the same region. Cases needed to be clinically diagnosed as ARF (peer-reviewed guidelines were not a prerequisite), while controls were documented as those having no history of ARF or RHD. In addition, longitudinal studies evaluating immune responses at more than one time point following new GAS acquisitions were also included. Case reports, narrative reviews, opinion pieces and publications lacking expression data, or referenced methodology and/or accepted guidelines, were excluded from the review. Duplicated studies of datasets and participants were removed, with the final, most recent, publication of the data assessed for inclusion.

Data Extraction and Article Management

Two reviewers (TS, KR) independently applied the search strategy to the relevant databases. Articles were managed using the Rayyan QCRI web/mobile application (26). Titles and abstracts were evaluated to exclude studies that did not describe the expression of GAS-specific antibodies. Thereafter, full texts of the included titles and abstracts were retrieved and further evaluated against the inclusion criterion (**Supplementary Table 3**). Rayyan QCRI has a built-in "blind" filter function which prevented the reviewers from observing the other's judgements. Discrepancies were resolved through discussion, involving an arbitrator (third reviewer, ME/BM) where necessary.

Two reviewers (TS, BM) extracted data using a standardized data extraction form and any contradictions were solved through discussion with another of the reviewers (ME). Search results from the databases listed above, published and unpublished studies were managed with Endnote X9 referencing software. Briefly, data extraction consisted of recording the study demographics (number of study participants, geographical region), diagnostic measures, GAS-specific antigens and relevant antibody titer measurements describing elevation. The risk of bias assessment tool (**Supplementary Table 4**) established by Wells et al. (27) was adapted in questions specific for use in this review, for assessing bias amongst included articles. Using the Newcastle-Ottawa Quality Assessment Scale (NOS) for case-control studies, which were characterized as being of a low or high risk of bias. A study with a low risk of bias is considered to be of high-quality and a low-quality study, with a higher risk of bias. Risk of bias was incorporated into the evaluation of heterogeneity in the pooled analyses.

Data Analysis

Odds ratio estimates together with their 95% confidence intervals (CIs) were calculated to represent the association between GAS-specific antigens and ARF. The Mantel-Haenszel method was used to pool together odds data from individual studies (28). Variability between studies was evaluated both by assessing forest plots visually, and formally by the heterogeneity tests using χ^2-based Q and I^2 statistic (29). As expected, the studies varied in the constitution of participants and in the types of assays conducted; thus, a random-effects model was used for analysis (30).

We conducted statistical data analyses using Stata version 14.1 (StataCorp, College Station, TX, USA) to estimate the combined effect size (odds ratio and 95% CIs) between GAS antigens and ARF and to generate comparative effect forest plots. Studies were analyzed in subgroups based on the inclusion of an accepted guideline at the time of diagnosis of ARF cases. Where a meta-analysis was not feasible, either because data were too heterogeneous or insufficient to allow for meaningful pooling, we compiled a narrative report of the results. Antigens, for which only a single study was available, thus precluding conducting a meta-analysis, were presented as an odds ratio with its 95% confidence interval (CI). We utilized the respective authors' definition of ULN in defining a rising titer.

RESULTS

Overview of Search Strategy and Included Articles

Our search strategy yielded 534 articles which reduced to 479 after excluding duplicates (**Figure 1**). An additional five studies were included through citation searching, thus leaving 484 for consideration for this review. Following screening of titles and abstracts, 53 articles were deemed potentially relevant and available for full-text evaluation. Twenty-four studies met the inclusion criteria, of which 14 were amenable to meta-analysis. **Table 1** shows the characteristics of the included studies. The included articles were published between 1955 and 2020 with sample sizes (cases and controls) ranging from 43 to 2,118 enrolled participants. Studies were conducted in local and university hospitals, clinics, outpatient departments, and schools situated in the study areas. Studies were conducted in the USA ($n = 7$), Japan ($n = 5$), India ($n = 4$), Egypt ($n = 3$), with one article from each of Pakistan, Trinidad, Madagascar, Ethiopia, UK, Australia, and New Zealand. Participants ranged in age from 1 to 89 years. All the articles narrowed their target population to a specific age group, mainly that of children. Only one article (41) made an effort to obtain participants from any age group so as to reflect the national population. A list of the excluded studies with accompanying reasons are detailed in **Supplementary Table 5**.

The overall quality of studies was moderate, with 12 studies deemed as having a low risk of bias (i.e., a high NOS score; **Supplementary Table 4**). The included studies clearly described the phenotypes of patients, providing an acceptable case definition and guideline or diagnostic algorithm. Studies diagnosed ARF according to the Jones criteria, with controls examined as having no prior history of ARF or RHD from the same population and age-matched to the cases. Seven of the studies classed as of high risk of bias (NOS <5) were completed before 1990 with authors failing to clearly define the controls or cases with appropriate diagnostic guidelines (33–35, 37, 39, 43, 52). One study Zainab et al. (53) recruited no controls but instead used an ULN cut-off published previously from within the same region.

Association of SLO Antibody With ARF

Ten studies (11 populations; controls, $n = 1{,}972$; cases, $n = 1{,}947$) providing data on Anti-SLO titers were amenable to meta-analysis (**Figure 2**).

Overall, ARF cases showed a greater association of an SLO antibody rise in comparison to controls [odds ratio (OR), 10.57 (95% CI [3.36, 33.29]; (I^2, 77.7%))]. In a subgroup analysis according to whether a guideline for the diagnosis of ARF was used or not, the association was not found to be statistically significant among studies not utilizing a guideline. Amongst studies not included in the meta-analysis due to the absence of a control group, each report a rise in anti-SLO titer: Zainab (53) in 24 of 50 cases (48%) of ARF diagnosed according to the Jones criteria (published standard = 200 IU/ml), Saini (48) in 15 of 26 (58%) cases of ARF as per the Jones criteria guideline (published

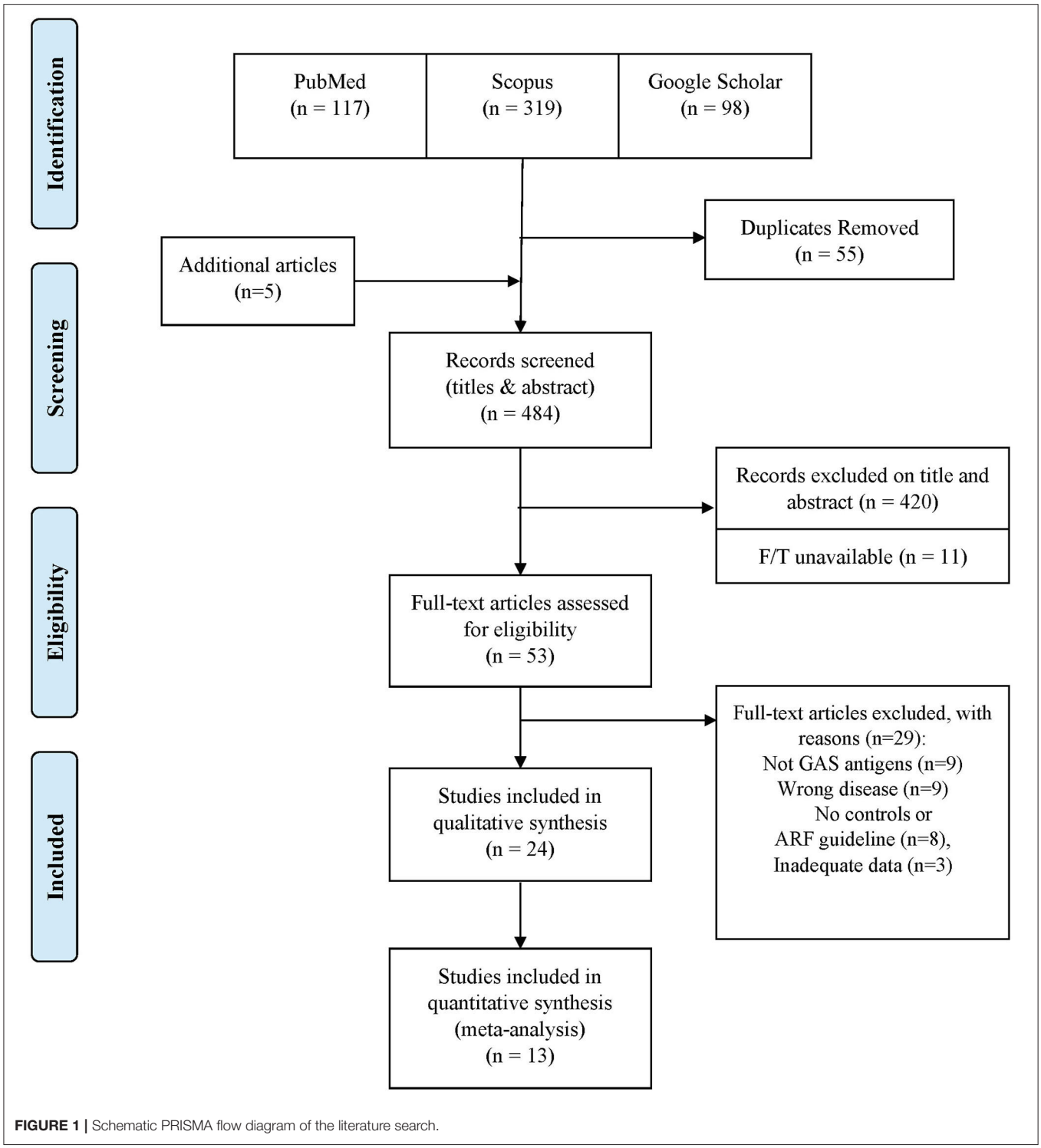

FIGURE 1 | Schematic PRISMA flow diagram of the literature search.

standard = 262 IU/ml), Hokonohara (39) in 24 of 67 cases of ARF (36%) against published standard = 240 IU/ml.

Association of DNase B Antibody With ARF

Nine studies provided data on Anti-DNase B titers, of which seven (cases, $n = 164$, controls, $n = 1{,}031$) were amenable to meta-analysis (**Figure 3**). DNase B antibody levels were significantly increased in ARF cases [OR, 6.97 (95% CI [2.99, 16.27]; (I^2, 67.4%))] in comparison to controls. This result was consistent across all studies, irrespective of the use of a guideline in diagnosing ARF cases. The two studies not included in the meta-analysis did not have control groups as a comparator. Saini

TABLE 1 | Characteristics of included studies.

Study ID	Country	Setting	Diagnostic guideline	Antigen/Detection method	Participants	Age
[a]Ayoub et al. (31)	Grenada/USA	ND	Jones criteria	SLO, DNase B, and GAC—assays described in previous publication (not available)	Grenada: RF ($n = 32$), controls ($n = 30$), Florida: RF ($n = 32$), controls ($n = 32$)	5–32 years
[b]Das et al. (32)	USA/India	ND	NCS	DNase B—ELISA vs. DNA methyl green micromethod	ARF (20), controls ($n = 20$)	ND
[a]Fujikawa and Ohkuni (33)	Japan	ND	RF and RHD Guideline of Japanese Circulation Society	SLO—streptozyme test, DNase B—hemoprobe B test, GAC—ASP kit, SE—enzyme antibody-antigen reaction	RF ($n = 8$), controls ($n = 354$)	6–15 years
[a]Fujikawa and Okuni (34)	Japan	ND	RF and RHD Guideline of Japanese Circulation Society	SLO, DNase B, and SK—multiple enzyme test (streptozyme test)	RF ($n = 21$), controls ($n = 178$)	6–15 years
[a]Fujikawa et al. (35)	Japan	ND	RF and RHD Guideline of Japanese Circulation Society	SLO—described previously, DNase B—hemoprobe B test	RF ($n = 46$), controls ($n = 278$)	3 age groups: <6, 6–16, and >16 years
[b]Gomaa et al. (36)	Egypt	Outpatient RF Clinic	Jones criteria	SLO—turbidimetric immunoassay and ELISA	ARF ($n = 80$), controls ($n = 80$)	ARF-−14.5 years (mean), control-−15.2yrs (mean)
[a]Halbert et al. (37)	USA	ND	NCS	SLO—agar precipitin technique	RF ($n = 33$), non-RF ($n = 35$)	ND
[a]Hanson-Manful et al. (38)	New Zealand	Hospitals	Jones criteria	SLO—turbidimetric technique using SLO kit, DNase B—enzyme inhibition assay, SpnA—bead-based immunoassay	ARF ($n = 16$), controls ($n = 36$)	ARF-−10.6 years (mean), Controls-−6yrs (mean)
[a]Hokonohara et al. (39)	Japan	ND	NCS	SLO—described by other author DNase B—hemoprobe B test, GAC— hemagglutination method	RF ($n = 28$), controls ($n =$ NCS)	5–16 years
[d]Hysmith et al. (40)	USA	University associated clinics	–	SLO, DNase B, SCPA, Mrp, J14, SpyCEP, SSE, SOF, SpyAD, and FBP54—ELISA	PIDs ($n = 41$)	6–15 years
[d]Johnson et al. (12)	USA	University associated clinics	–	SLO and DNase B—ELISA	PIDs ($n = 160$)	6–15 years
[a]Julie et al. (41)	Madagascar	Hospital	NCS	SLO—latex agglutination technique	ARF ($n = 1,690$), control ($n = 428$)	1–89 years
[d]Kaplan et al. (42)	USA	NCS	–	SLO, DNase B, and NADase—assays described in previous publication (not available)	PIDs ($n = 49$)	3–6 years
[a]Kawakita et al. (43)	Japan	Elementary school	NCS	SLO—spectrophotometric method, DNase B—micro method, NADase—reduction by alcohol dehydrogenase	ARF ($n = 3$), controls ($n = 361$)	6–11 years
[a]Kotby et al. (44)	Egypt	Hospital	Jones criteria	SLO—rapid latex agglutination	ARF ($n = 60$), controls ($n = 200$)	3 age groups: <6, 6–10, and >10 years
[b]Read et al. (45)	Trinidad	Hospital	Jones criteria	SLO—antibody titre kit	RF ($n = 44$), controls ($n = 34$)	ND
[b]Read et al. (46)	USA	Hospital	Rheumatic Fever Service of The Rockefeller University Hospital	SLO—*in vitro* cellular migration of white blood cells	RF ($n =$ NCS), controls ($n =$ NCS)	ND

(Continued)

TABLE 1 | Continued

Study ID	Country	Setting	Diagnostic guideline	Antigen/Detection method	Participants	Age
[c]Sagar et al. (47)	India	ND	Jones criteria	SCI, SCPA, and PSA—ELISA	RF ($n = 24$), controls ($n = 25$)	ND
[a]Saini et al. (48)	India	Hospital	Jones criteria	SLO—NCS	ARF ($n = 26$), controls ($n = 84$)	5–15 years
[d]Shet et al. (49)	USA	NCS	–	SLO, DNase B, and SCPA—ELISA	PIDs ($n = 202$)	2–12 years
[b]Tewodros et al. (50)	Ethiopia	ND	NCS	SK—ELISA	ARF ($n = 11$), controls ($n = 10$)	3–12 years
[b]Thakur and Prakash (51)	India	ND	NCS	GAC—ELISA	ARF ($n = 50$), controls ($n = 50$)	ND
[a]Widdowson et al. (52)	UK	Outpatient clinic	NCS	SLO—spectrophotometric method, DNase B—micro method	RF group ($n = 6$), controls ($n = 44$)	16–18 years
[a]Zainab et al. (53)	Pakistan	Hospital	Jones criteria	SLO—kit human tex ASOT	ARF ($n = 50$) (Historic control values)	5–15 years

ND, no data; NCS, not clearly stated; RF, rheumatic fever; PIDs, participants.
SLO, streptolysin O; GAC, group A carbohydrate; SE, streptococcal esterase; SK, streptokinase; SpnA, GAS nuclease A; SCPA, C5a peptidase; Mrp, M-related peptides; J14, C-repeat M peptide; SpyCEP, serine protease; SSE, serine esterase; SOF, serum opacity factor; SpyAD, GAS adhesion and division protein; FBP54, fibronectin-binding protein; SCI, collagen-like surface protein; PSA, putative surface antigen.
[a]*Meta-analysis.*
[b]*Mean titer data.*
[c]*Single article-antigen.*
[d]*Longitudinal data.*

(48), used a published standard of 134 IU/ml and reported an elevation of anti-DNase B titers in 85% (22 of 26) of cases while Hokonohara (39), had 41 cases of ARF and showed a 60% ($n = 25$) titer elevation.

A sensitivity analysis revealed a reduction in the association of anti-SLO levels with ARF in studies with low-risk scores for bias [OR, 4.87 (95% CI, 1.07–22.17); I^2, 93.1%]. Anti-DNase B meta-analysis comprising studies of a low risk of bias, revealed a non-statistically significant association between antibody titers and ARF.

Association of Other GAS-Specific Antigens With ARF: Streptokinase and GAS Carbohydrate

Three studies provided data on anti-streptokinase (ASK) titers, of which only two (controls, $n = 532$; cases, $n = 29$) were amenable to meta-analysis (**Figure 4**) while two studies (controls, $n = 416$; cases, $n = 72$) provided data on Anti-GAC (AGAC) titers (**Figure 4**).

ASK antibody levels were significantly increased in ARF patients [OR, 5.09 (95% CI [2.07, 12.50], (I^2, 13%))] while GAC responses showed no significance in elevation between cases and controls [OR, 2.79 (95% CI [0.87, 8.99]; (I^2, 67.2%))]. Hokonohara (39), not included in the meta-analysis, showed an elevation of 54% (36 of 67) for ASK and 45% (19 of 41) for AGAC titers in cases of ARF.

Narrative Review of Studies Not Included in the Meta-Analysis

Four studies (12, 40, 42, 49) reported on the longitudinal assessment of human immune responses to GAS-specific antigens following a new GAS acquisition. Kaplan (42), in evaluating the immune response of 49 participants (aged 3–6 years) against SLO, DNase B, and NADase antigens, showed that GAS pharyngeal-infected participants had an elevated response to GAS antigens compared with participants without infection: SLO, 57% increase vs. 22%, DNase B, 50 vs. 11% and NADase, 43 vs. 22%. Johnson (12) reported on the immune response of 160 participants (aged 6–15 years), from whom 3,491 cultures and 1,679 serum samples were obtained. Over the study period they identified 58 new GAS acquisitions in 45 participants, of which 34.5% ($n = 20$) of the participants showed a significant increase in SLO and DNase B titers. Thirty-six (62.1%) GAS acquisitions were associated with an increase in antibody titer to SLO or DNase B, while 28 showed an increase to SLO and 28 for DNase B. Thus, had only SLO or only DNase B antibody titers been analyzed, eight GAS acquisitions would have been missed. Johnson (12), provided evidence that of 54 serum samples for ASO and 51 samples for anti-DNase B showing an increase in titer following a new GAS acquisition, ∼60% were below the ULN described, resulting in the mischaracterization of preceding GAS infections. Furthermore, in amongst GAS carriers, 239 samples had an increased ASO titer above the ULN and 307 samples for anti-DNase B, while only 9.6% (ASO) and 6.5% (anti-DNase B) of these were associated with a true titer increase following a GAS acquisition. It was also shown that in the absence of a culture-positive for GAS, ASO, and anti-DNase B titers were still higher than the ULN for extended periods of time. The study by Hysmith (40) presents the most recent and comprehensive investigation of antibody responses following GAS acquisition. From 41 participants (aged 6–15 years) over a period of 2 years, 51 new GAS acquisitions were documented, with 34 showing an increase in antibody titers against SLO and/or DNase B, illustrating an overall sensitivity of 67% in predicting a new

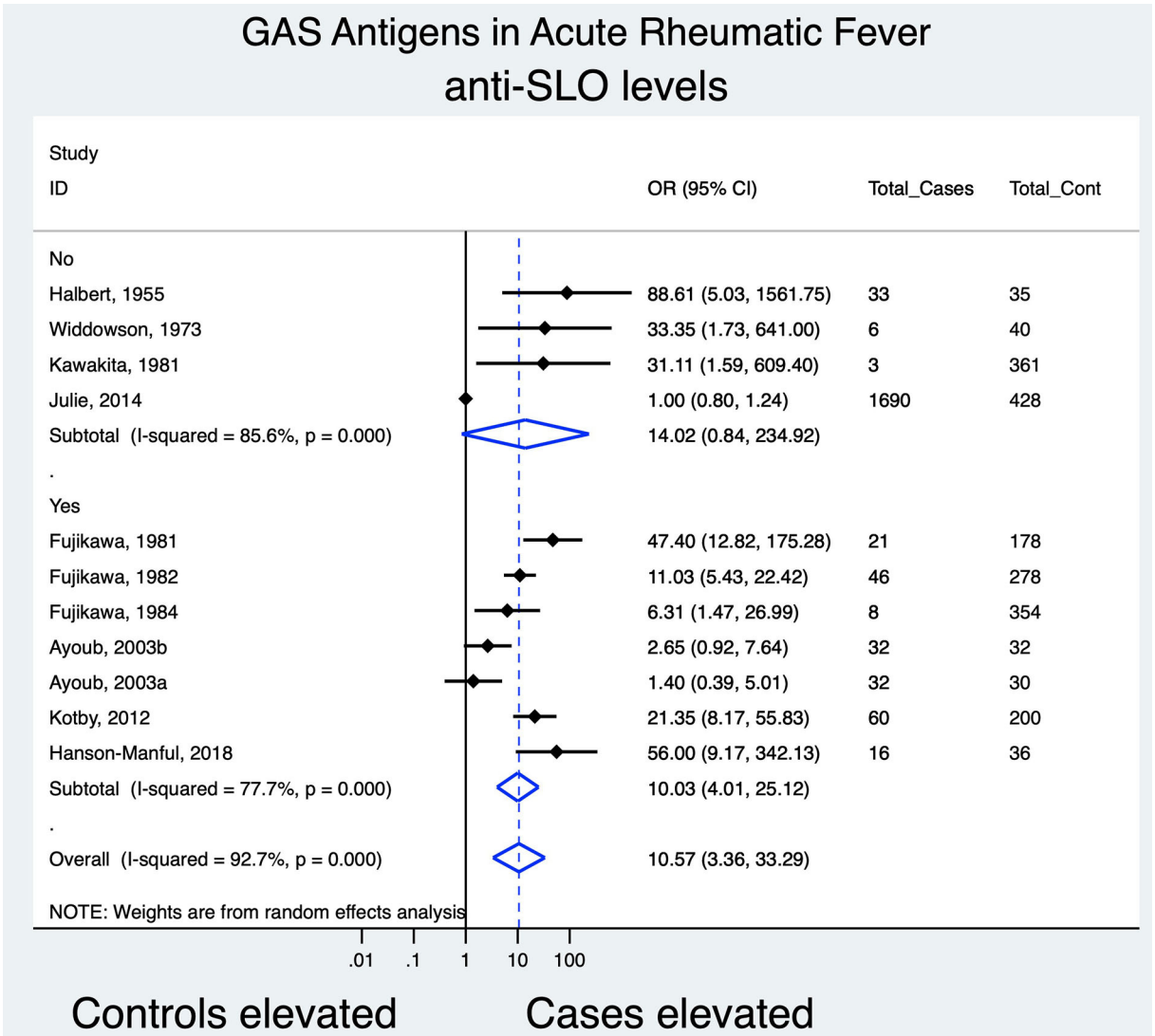

FIGURE 2 | Forest plot evaluating the odds of association between anti-SLO titers and ARF; subgrouping based on whether a guideline was used to diagnose ARF cases.

GAS acquisition. Adding increases in antibody levels to GAS SCPA (C5a peptidase) and one additional GAS-shared antigen to SLO and DNase B antibody level increases, improved the overall sensitivity to 76 and 98%, respectively.

Studies that only reported average mean titers were summarized in **Supplementary Table 6**, provided data were available for SK (50), SLO (36, 45, 46), GAC (51), and DNase B (32). In all the studies, the average mean titers from cases of ARF were considerably higher in comparison to that of the controls.

Studies reporting on less common GAS antigens are summarized in **Supplementary Table 7**, with the use of GAS-specific antigens: GAS nuclease A (Spn A) (38), collagen-like surface protein (SCI), putative surface antigen (PSA), SCPA (54), streptococcal esterase (SE) (33), Nicotinamide adenine dinucleotidase (NADase) (43), and superoxide dismutase (SOD) (55), in which only Spn A showed a significant OR (95% CI, 56.00 [9.17; 342.13]).

DISCUSSION

We have presented a comprehensive review of the literature on group A streptococcal antibody responses and their utility in making the clinical diagnosis of ARF. Our meta-analysis provides evidence for a significant association between ARF and anti-SLO, anti-DNase B, and ASK, thus indicating the usefulness of these immunological markers in supporting an ARF diagnosis. This finding is supported by individual studies showing a consistently higher average mean titer in ARF cases over controls. However, there is currently no evidence of an association between GAC antibodies and ARF.

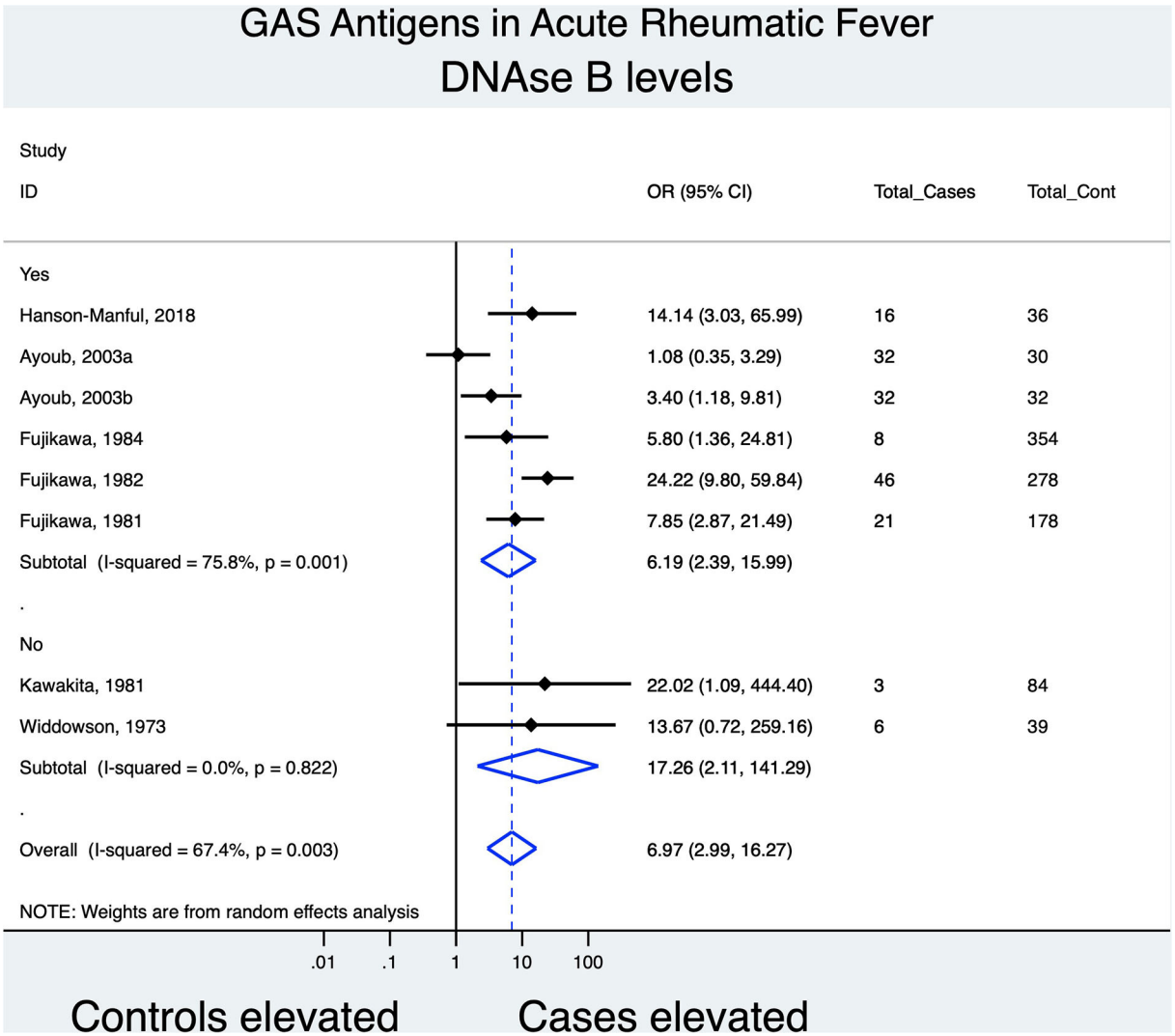

FIGURE 3 | Forest plot evaluating the odds of association between anti-DNase B titers and ARF; subgrouping based on whether a guideline was used to diagnose ARF cases.

We grouped our findings according to whether peer-reviewed guidelines were used in the clinical diagnosis of ARF. Excluding studies which did not employ a guideline, i.e., those classified as having a higher risk of bias in terms of case definition, reduced the combined estimate of association for anti-SLO and anti-DNase B with ARF from OR 10.57 (95% CI [3.36, 33.29]) to OR 10.03 (95% CI [4.01, 25.12]) and OR 6.97 (95% CI [2.99, 16.27]) to OR 6.19 (95% CI [2.39, 15.99]), respectively. This may indicate caution in terms of investigating only a single antigen in establishing an ARF diagnosis.

Given differences in techniques used to measure antibody titers across the studies, sample size variation, regional differences resulting in the variation of ULN titer levels and published standards used (**Table 1**), a high degree of heterogeneity was to be expected; hence we employed the random-effect model for the meta-analysis. Sensitivity analyses of studies with a low risk of bias score revealed a reduction in, although still significant, the association of anti-SLO levels with ARF while anti-DNase B analyses showed no statistically significant association between antibody titers and ARF. Unfortunately, the dearth of studies precluded conducting further meaningful subgroup analyses.

We provide a summary of literature meeting our inclusion criteria, but not amenable to meta-analysis through a narrative review. Additional single antigen studies provide further support for the significant association of SK, SLO, GAC, DNase B, Spn A, and SE with ARF but not for SCI, PSA, SCPA, and SOD. For completeness, though not encompassing ARF cases, we included four studies reporting on the longitudinal assessment of human immune response to GAS-specific antigens following a new GAS acquisition in acute and convalescent samples. The studies provided meaningful data in terms of the effectiveness of SLO,

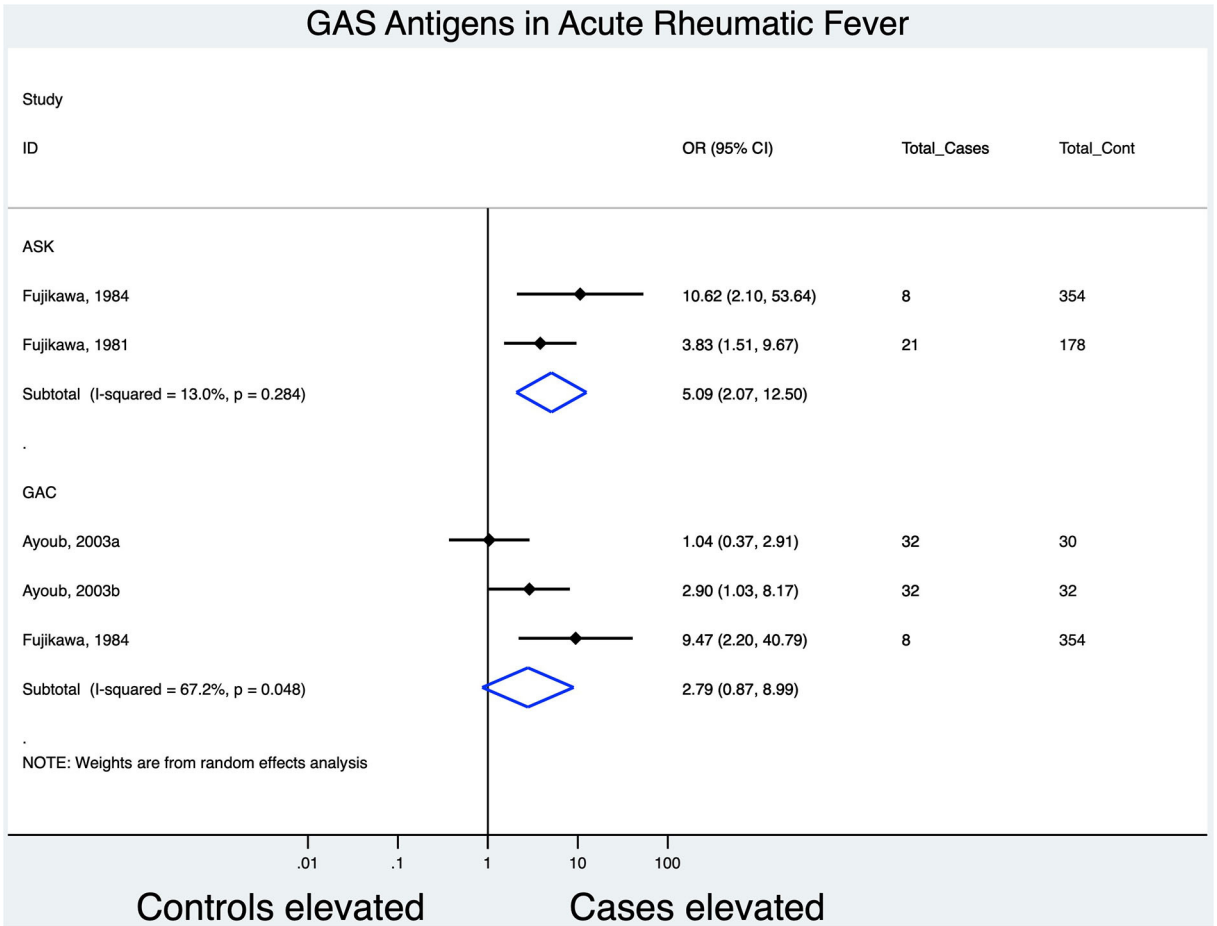

FIGURE 4 | Forest plot evaluating the odds of association between anti-ASK and anti-GAC titers and ARF; subgrouping based on antigen.

DNase B, NADase, and other antigens in detecting a preceding GAS infection. These findings suggest the need to employ an array of antigens to increase the sensitivity of assays confirming a preceding GAS infection, that could be amended within a ARF diagnostic guideline.

Within the studies reporting study limitations, the use of the ULN to describe elevation in titers as evidence of a recent infection risk confounding given its dependence on the controls used within the study. Numerous reports suggest that the ULN of antibody titers against GAS antigens varies with age and geographical location. It has also been reported that titers are lower in adults in comparison with children (56, 57). Johnson (12) and Hysmith (40) identified a number of cases where the participant showed an increase in titer following a new GAS acquisition where the peak titers did not exceed the ULN described for the specific population. Given that cases of GAS carriers demonstrated prolonged elevated responses that exceeded the ULN, caution is warranted since using ULN solely to describe elevation may result in false-negative or false-positive GAS-associations. Thus, these studies strongly suggest that evaluating the rise in titer in paired sequential samples as the most effective way in describing a preceding GAS infection.

This systematic review employed rigorous methods as proposed by the Cochrane Collaboration (58) in synthesizing published resources on the utility of GAS serology in confirming a preceding GAS infection. However, the availability of individual patient data would have further enhanced our findings. As is often the case, data were not reported so as to allow inclusion of some studies into the meta-analysis. Also, there remains a lack of studies in this area.

CONCLUSION AND FUTURE RESEARCH

Providing evidence for a preceding GAS infection remains a challenge. Future studies to evaluate serological tests for evidence of a preceding GAS infection should be designed to overcome the major limitations of the existing evidence base. This can be readily accomplished by ensuring a well-defined case definition as in clear symptoms of ARF with paired sequential sampling of the target population. Furthermore, utilizing an array of GAS

antigens is more likely to provide greater sensitivity in providing evidence of a recent infection.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MS, BM, and ME were jointly responsible for the conceptualization of the study. MS, KR, BM, and KE contributed to the search strategy and performed data extraction. MS and ME designed and executed the analyses, wrote the first draft, and revised drafts of the manuscript. JD and LZ contributed to the interpretation of the findings. All authors have read and approved the final manuscript.

FUNDING

This work was based on the research supported in part by the National Research Foundation of South Africa (grant number 116287). Any opinion, finding, conclusion, or recommendation expressed in this material was that of the authors, and the NRF does not accept any liability in this regard. MS, KR, BM, KE, JD, and ME were supported by grant (NW17SFRN33630027) from the American Heart Association. JD received funds from the U.S. Public Health Service (NIH grants R01 AI-132117 and R01 AI-010085).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.691646/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cardiovascular Medicine

REVIEW
published: 20 July 2021
doi: 10.3389/fcvm.2021.676938

3D Echocardiography for Rheumatic Heart Disease Analysis: Ready for Prime Time

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Rheumatic heart disease (RHD) remains to be a very important health issue worldwide, mainly in underdeveloped countries. It continues to be a leading cause of morbidity and mortality throughout developing countries. RHD is a delayed non-suppurative immunologically mediated inflammatory response to the throat infection caused by a hemolytic streptococcus from the A group (*Streptococcus pyogenes*). RHD keeps position 1 as the most common cardiovascular disease in young people aged < 25 years considering all the continents. The disease can lead to valvular cardiac lesions as well as to carditis. Rheumatic fever valvular injuries lead most commonly to the fusion and thickening of the edges of the cusps and to the fusion, thickening, and shortening of the chordae and ultimately to calcification of the valves. Valvular commissures can also be deeply compromised, leading to severe stenosis. Atrial and ventricular remodeling is also common following rheumatic infection. Mixed valvular lesions are more common than isolated valvular disorders. Echocardiography is the most relevant imaging technique not only to provide diagnostic information but also to enable prognostic data. Further, it presents a very important role for the correction of complications after surgical repair of rheumatic heart valvulopathies. Three-dimensional (3D) echocardiography provides additional anatomical and morphofunctional information of utmost importance for patients presenting rheumatic valvopathies. Accordingly, three-dimensional echocardiography is ready for routine use in patients with RHD presenting with valvular abnormalities.

Keywords: rheumatic disease, echocardiography, valvulopathy, three dimensional echocardiography, diagnosis

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Heart Valve Disease, a section of the journal Frontiers in Cardiovascular Medicine

Received: *11 March 2021*
Accepted: *15 June 2021*
Published: *20 July 2021*

Citation:
Vieira MLC, Branco CEdB, Gazola ASL, Vieira PPAC, Benvenuti LA, Demarchi LMMF, Gutierrez PS, Aiello VD, Tarasoutchi F and Sampaio RO (2021) 3D Echocardiography for Rheumatic Heart Disease Analysis: Ready for Prime Time. Front. Cardiovasc. Med. 8:676938. doi: 10.3389/fcvm.2021.676938

INTRODUCTION

Epidemiology of Rheumatic Heart Disease: Distribution in the US and Worldwide

Brief Clinical Features and Pathogenesis

Rheumatic fever (RF) and rheumatic heart disease (RHD) continue to be very important health issues across different continents, affecting mainly developing or average income countries, as determined by the World Heart Federation position statement and by the Health Statistics Census from those nations (1, 2). Globally, RHD is the most common cardiovascular disease in young

people aged < 25 years (1). Therefore, RHD continues to be a very important cause of morbidity and mortality in developing countries. This scenario is very different and diverse in high-income countries. In such countries, RHD has mostly been eradicated, but a new burden of disease is possible due to migration flow (1). Another problem is that RHD may be under-observed and underdiagnosed in developing countries due to the scarcity of related studies, very small private and public investments, and the need for comprehensive register-based control programs (1–4). RF caused by Group A streptococcus/hemolytic streptococcus (GAS) (*Streptococcus pyogenes*) can be observed in cases of throat infection after tonsillitis in high-income countries. However, such infections in developing or average-income countries can lead to carditis and permanent valve damage as a consequence of repeated attacks of RF (1, 3). Worldwide, RF or RHD may be the cause of death in 233,000–500,000 patients per year (2). In 2015, an estimated 319,400 deaths occurred due to RHD from 33.4 million cases across different continents, and 10.5 million disability-adjusted life-years were lost due to RHD (5).

Clinical manifestations of acute RF can be pleomorphic, including fever, arthritis, carditis, chorea, subcutaneous nodules, and erythema marginatum. The prevalence rates of RHD in school-age children in developing countries vary from one to five cases per 1,000, mainly in sub-Saharan Africa (6). In some African and Asian countries, the rate of subclinical carditis due to RF has been better understood with the use of portable echocardiography for disease screening in schoolchildren, which provides additional diagnostic information for clinical evaluation (6, 7). Mitral valve involvement was observed in most cases (6). After the use of echocardiographic screening protocols, the prevalence of RHD was considerably higher (10 times) (8). Another important issue is that RHD prevalence may be higher in children over 15 years of age, as suggested by data from Australian aborigines (7, 8). Evidence of subclinical RHD is crucial since patients can develop chronic valvular disease. The World Heart Federation standardized echocardiographic criteria for rheumatic carditis in 2012, using three different categories (definite RHD, borderline RHD, and normal), based on 2D, continuous-wave, and color-Doppler echocardiography (9, 10).

It is known that ∼60% of RHD patients who present with heart failure during their first acute RF attack demonstrate valvular disease 10 years later (11, 12). Prognostic factors are highly dependent on the occurrence of carditis and the recurrent attack rate. It is well-understood that the burden of RHD has decreased globally, despite the significant rates throughout the poorer regions of the Earth.

The pathogenesis of acute rheumatic carditis seems to be related to the immune-mediated response following GAS infection, the production of antibodies against GAS pharyngitis, and GAS production of aggressive elements such as streptococcal M protein, which can trigger an autoimmune response.

The activation and infiltration of T cells into the valves produce a disarray of the normal valvar cell arrangement and changes in valvar collagen. There is also activation of inflammatory cytokines, granulomatous inflammation, and CD4+ T cell-mediated delayed hypersensitivity against GAS. The pathognomonic lesions of the rheumatic process in the endocardium of the heart valve and in the myocardium are called Aschoff bodies and are observed as granulomatous inflammation. Valve lesions may be progressive, leading to chronic sequelae such as valvular stenosis and insufficiency, as well as to carditis, heart failure, and pulmonary hypertension. In addition, these aggressive effects depend on evidence of a susceptible host, as well as the level of host autoimmune response.

TRIDIMENSIONAL ECHOCARDIOGRAPHY

Echocardiography, in its various modalities, is a diagnostic imaging investigation technique that has clinical applications in countless scenarios for various heart diseases (13–48).

From the first observations in M-mode, echocardiography has had several advances in technique with the advent of two-dimensional (2D) transthoracic echocardiography with the Doppler technique (and its various presentations), transesophageal echocardiography, strain from speckle-tracking technique and echocardiography, and 3D transthoracic and transesophageal echocardiography with pocket transducers and cell phone acquisition using nanotechnology and miniaturization (**Figures 1–15**).

3D echocardiography results in multi-structural cardiac observation from multiple planes and the rotation of not only the observation planes but also the cardiac structure in relation to a specific investigation plane (13–48). 3D echocardiography is in fact echocardiography in five dimensions, taking into account the three orthogonal planes of structural observation (posteroanterior, lateral medial, and upper inferior), the temporal plane, and the dimension of cardiac flows. It represents an important advance in the anatomical observation of cardiopathies and helps in understanding their pathophysiology and in determining prognostic implications in different clinical situations.

3D echocardiography provides very important clinical applications for the understanding of heart valve diseases, cardiomyopathies, and congenital heart diseases; it is essential for operative planning in cardiac surgeries, for percutaneous transcatheter procedures in the operating room/hybrid room for the treatment of valvular heart disease or congenital heart disease, and for the correction of complications related to surgical treatment of valve diseases (e.g., stenosis of biological prostheses and periprosthetic leaks) (13–48).

3D echocardiography was developed to overcome the limitations of conventional 2D echocardiographic analysis. 3D analysis allows the observation of cardiac structures without the use of mathematical formulae and geometric inferences for cardiac chamber measurement (which is employed during 2D evaluation). 3D echocardiography makes volumetric quantification of the cardiac chambers more realistic and closer to actual anatomical observation. It provides a greater proximity to measurements made by nuclear magnetic resonance (26, 28, 30, 32–34), both from the point of view of cavitary volumes and performance (biventricular ejection fraction and atrial emptying

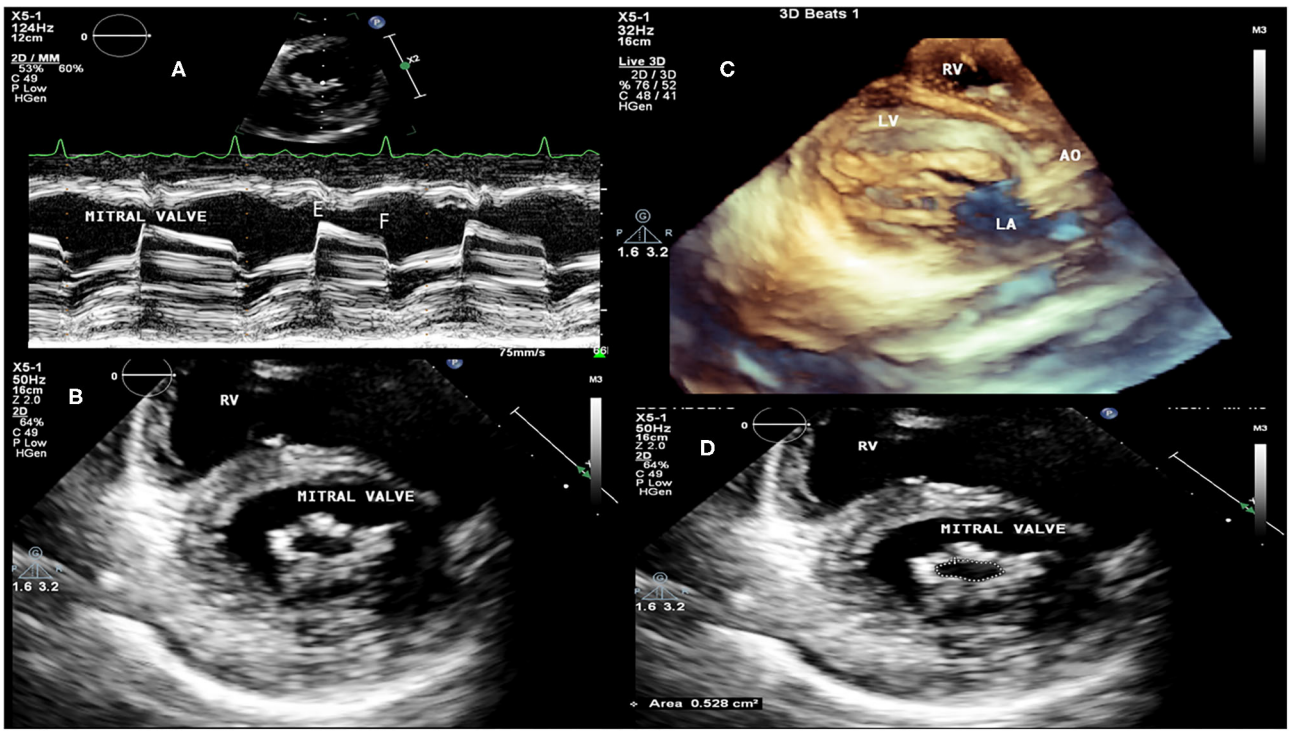

FIGURE 1 | RHD (mitral valve stenosis). **(A)** M mode transthoracic echocardiography. Demonstration of patient with severe mitral valve stenosis (rectification of E to F slope), presenting atrial fibrillation. **(B)** 2D transthoracic echocardiography, transversal view, thickening of mitral valvular leaflets, "buttonhole" shape of the mitral valve. **(C)** 3D transthoracic echocardiography, mitral valve chordae fusion. **(D)** 2D transthoracic echocardiography, transversal view, measurement of the mitral valve area by planimetry: 0.528 cm^2. RV, right ventricle; LV, left ventricle; AO, aorta; LA, left atrium.

total, passive and active) (31–41), and the measurement of left ventricular mass (30, 38). The use of 3D echocardiography reduces errors in cardiac volumetric measurements, minimizing the foreshortening of the cardiac chambers (which can be a very important limitation concerning 2D echocardiography). The incorporation of three-dimensional echocardiography in cardiac structural analysis also allows cardiac observation from new physiological analysis indices (such as the sphericity and conic index of the left ventricle for prediction of left-ventricle remodeling) and for studying the annular planarity index of the mitral valve ring, as well as the measurement of atrioventricular coupling, concerning dyssynchrony during cardiac resynchronization therapy for heart failure (20, 23, 24).

The idea of 3D echocardiography arises from the reports of Baum and Greenwood during their observations of the human orbit in 1954 (49). In 1974, Dekker et al. demonstrated the possibility of 3D observation of cardiac structures (50). Since then, many researchers, such as Raab and Pearlman, have been involved in the development of 3D echocardiography, even in association with the color Doppler technique by Raqueno and Schott for 3D cardiac structural reconstruction (51, 52). 3D transesophageal echocardiography has also advanced from different investigations in the 80s and 90s, with different groups coordinated by Wollschlager, Pandian, Li, Nandian, Levine, Roelandt, and Picard taking account of different clinical situations (53–57). Currently, we have observed great contributions in real-time 3D echocardiography by investigators such as Lang, Mor-Avi, Badano, Muraru, Kisslo, García Fernández, Peres de isla, and Zamorano (13–16, 21, 22, 33, 42). In this sense, 3D echocardiography seems to be absolutely ready for prime use in RHD and multiple valvulopathy aggression, for a better understanding of cardiac function (biventricular ejection fraction), and to present better anatomical correlation for biventricular remodeling and biatrial enlargement.

CARDIAC VALVES

Rheumatic Lesions and the 3D Echocardiography Approach

Mitral Valve Anatomy

The mitral valve apparatus comprises a 3D complex of different structures that enables emptying of the left atrium and filling of the left ventricle during the diastolic phase of the cardiac cycle (58–67). It involves elements such as the mitral valve annulus, leaflets, commissures, chordae tendineae, papillary muscles, and the LV wall with its attached papillary muscle. Any changes in these structures can cause remodeling of the left atrium (mitral stenosis) or the left ventricle (mitral regurgitation), leading to mechanical changes in the left chambers following the onset of RF, and causing modifications over time.

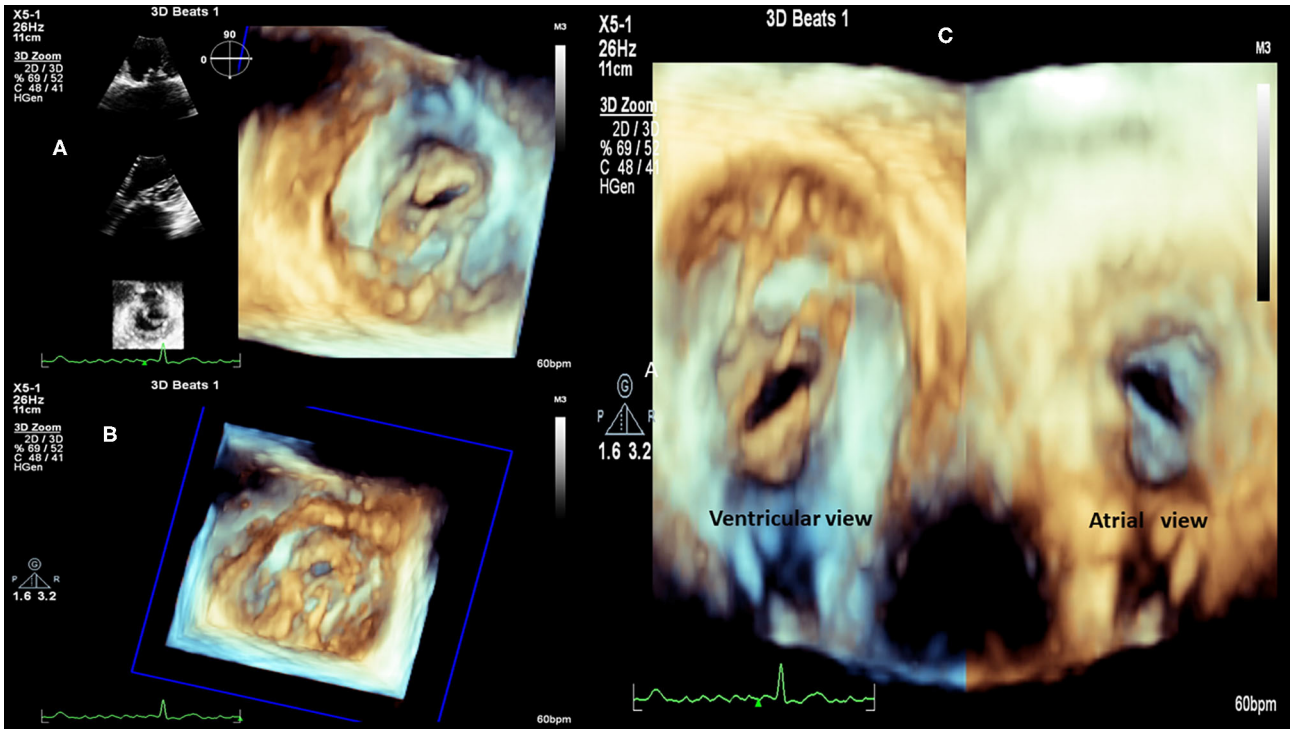

FIGURE 2 | RHD (Mitral valve stenosis). **(A)** 3D transthoracic echocardiography, left atrium view, 2D reference structures, patient with severe valve mitral stenosis. **(B)** 3D transthoracic echocardiography, patient with severe valve mitral stenosis. **(C)** 3D transthoracic echocardiography, view from left atrium (right) and from the left ventricle (left), patient with severe valve mitral stenosis.

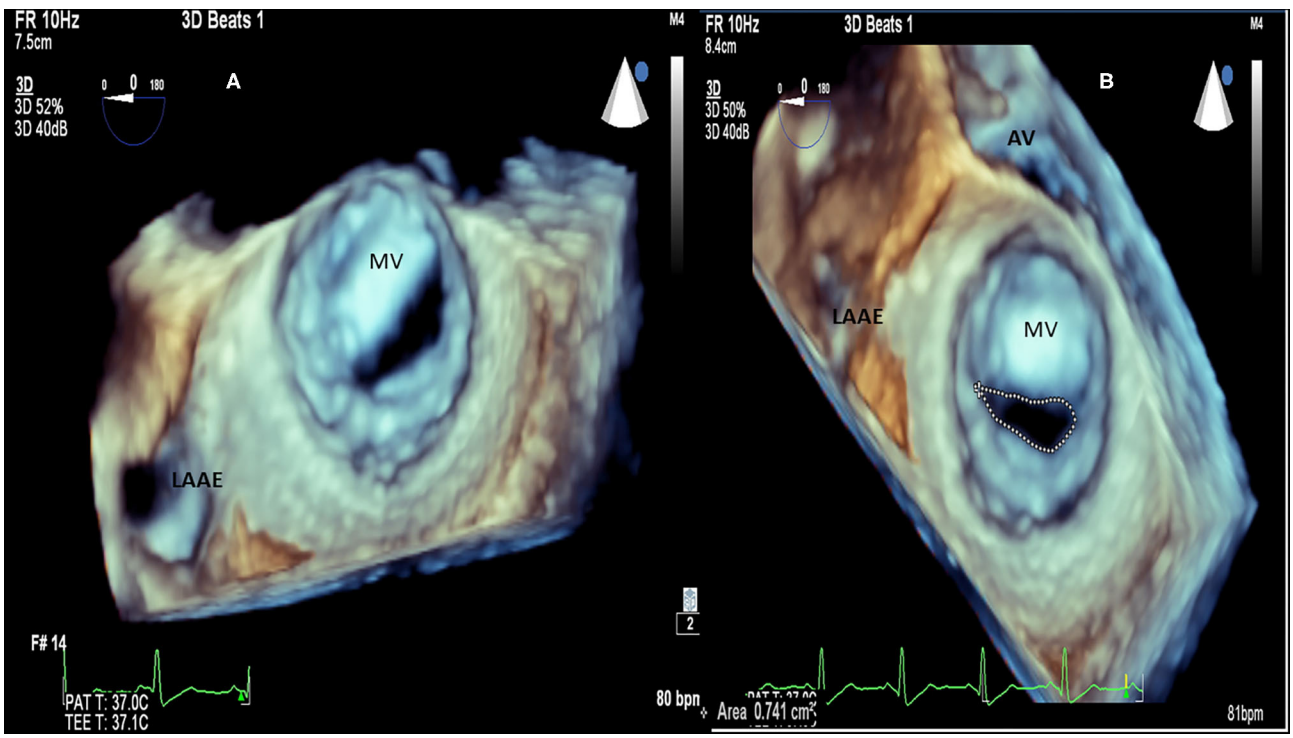

FIGURE 3 | RHD (Mitral valve stenosis). **(A)** 3D transesophageal echocardiography. Demonstration of patient with severe mitral valve stenosis, "fishmouth" shape of the mitral valve. **(B)** 3D transesophageal echocardiography, same patient, measurement of the mitral valve area by planimetry: 0.741 cm^2. LAAE, left atrium appendage; AV, aortic valve; MV, mitral valve.

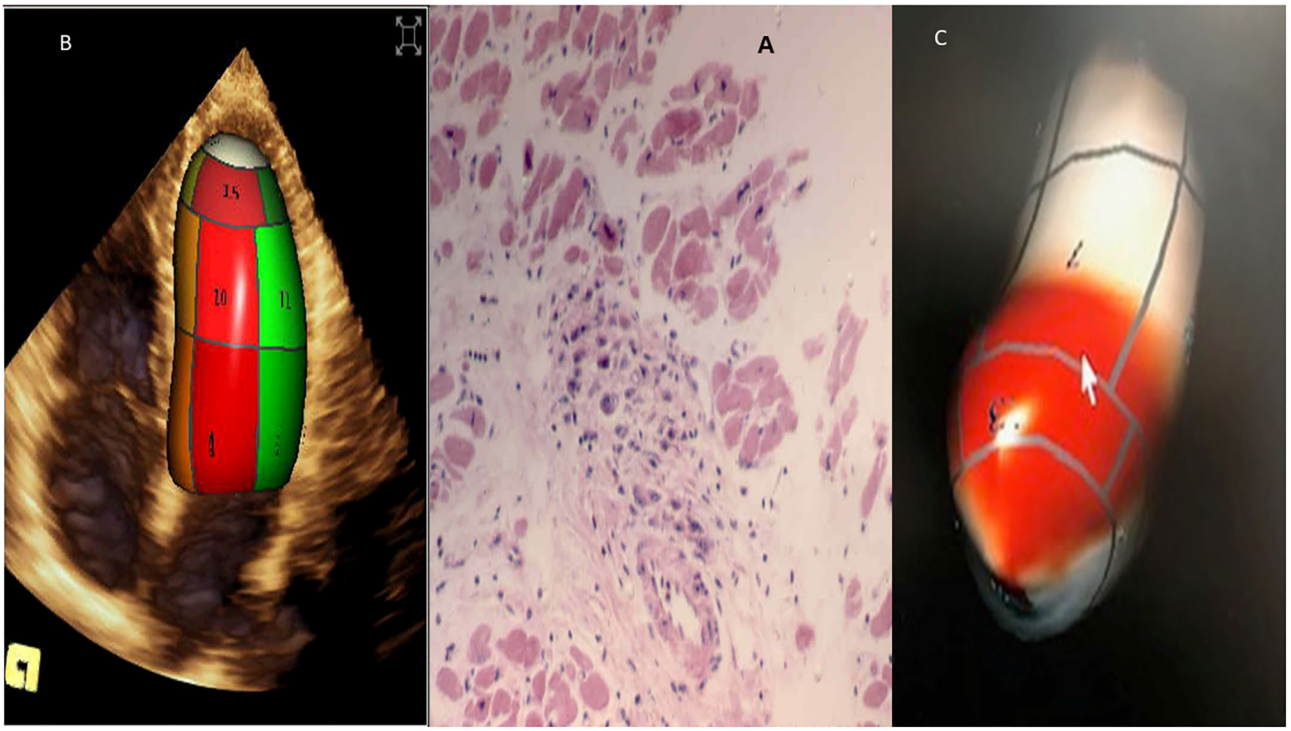

FIGURE 4 | Acute rheumatic fever (carditis). **(A)** Myocardial Aschoff bodies of rheumatic fever (Hematoxylin and eosin stain). **(B)** 3D transthoracic echocardiography depicting normal left ventricle. **(C)** 3D left ventricle presenting myocardial dysfunction, in red abnormal left ventricular synchronicity. 2D Global longitudinal: −12%.

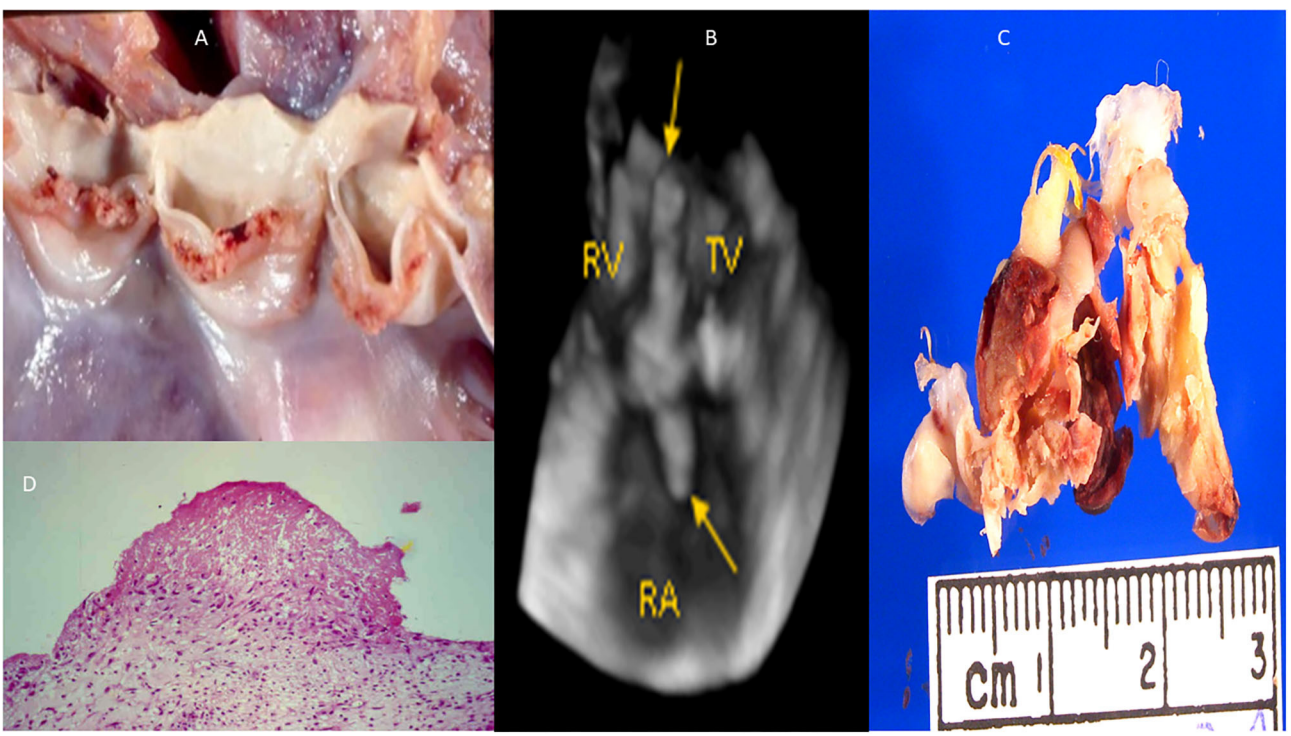

FIGURE 5 | Infective endocarditis (aortic valve and tricuspid valve). **(A)** Macroscopy: small vegetations all over the aortic valve. **(B)** 3D transthoracic echocardiography depicting vegetations over the tricuspid valve (arrows). **(C)** Macroscopy: vegetations all over the tricuspid valve. **(D)** Microcospy: small fibrine thrombi in organization. RV, right ventricle; RA, right atrium; TV, tricuspid valve.

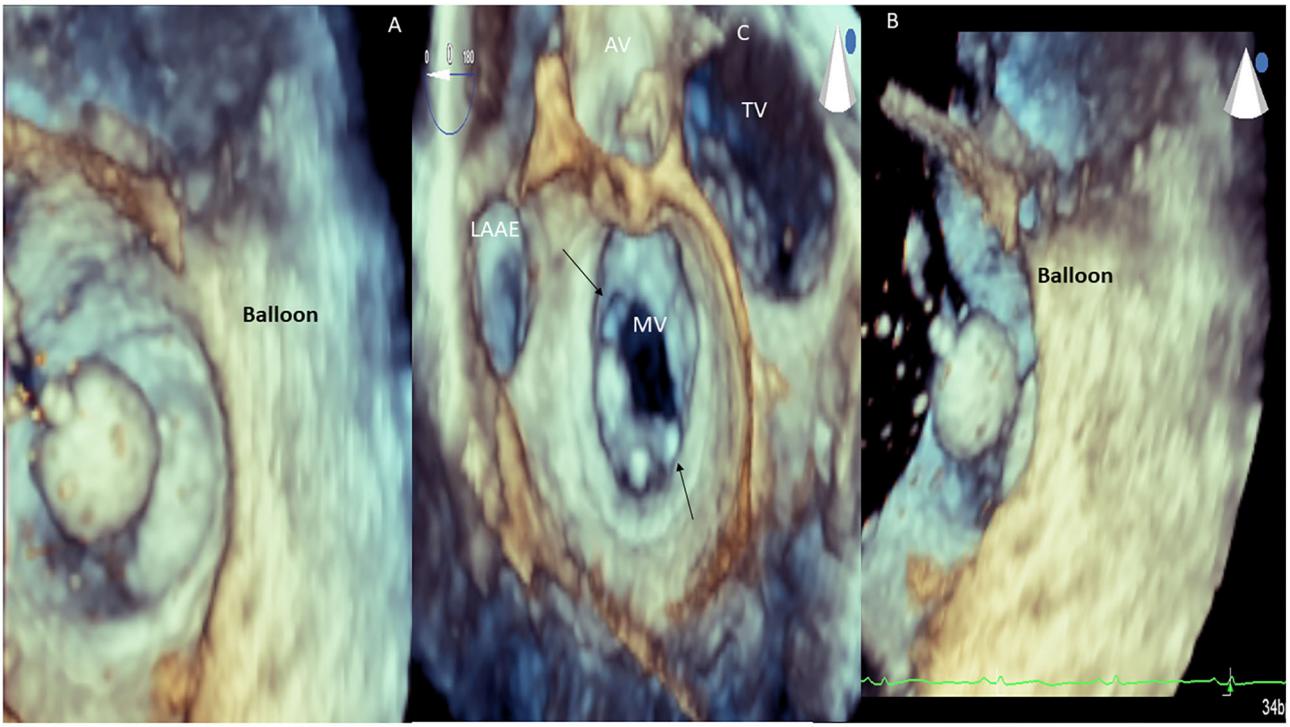

FIGURE 6 | RHD (mitral valve stenosis). 3D transesophageal echocardiography. Demonstration of balloon valvuloplasty in patient with severe mitral valve stenosis **(A,B)**. 3D transesophageal echocardiography. Demonstration of mitral valve commissural opening (arrows, **C**) after balloon valvuloplasty. LAAE, left atrium appendage; AV, aortic valve; MV, mitral valve; TR, tricuspid valve.

Fully understanding and detailed investigation of the normal mitral valve apparatus is key to better determining the level of RF aggression, imbalance, and distortion.

RHD can affect the mitral valve, causing pure mitral stenosis (25% of all patients) or combined mitral stenosis and regurgitation (40%) (**Figure 1**). RHD most commonly affects not only the mitral valve but also the aortic or tricuspid valve (multi-valvular aggression) (**Figures 9**, **14**). The disease causes different forms of stenosis of the mitral valve apparatus (commissural, cuspal, chordal, or combined). Thickening of different components of the mitral valve apparatus can occur alone as commissural (30%), cuspal (15%), or chordal (10%), or in combination, and is associated with calcification of the elements. RF aggression most commonly leads to the fusion and thickening of the edges of the cusps and of the chordae. Valvular commissures can be deeply compromised, leading to severe stenosis. The mitral valve ring can also become more rigid due to extension of aggression and from calcium deposits. Therefore, the stenotic mitral valve will have a funnel shape and its orifice can be observed as being a "fish mouth" or "buttonhole." The degree of calcification is highly related to the increase in transvalvular gradients. When there is less fusion of the valvular commissures and predominant chordae injury, mitral regurgitation is more common. The progression of rheumatic aggression can lead to extensive fibrosis, thickening, and calcification of the entire mitral valve apparatus (**Figures 3**–**5**), with even calcification of the wall of the enlarged left atrium.

Two decisive situations that can definitely change not only the mitral valve apparatus anatomy but also the function, and most importantly, impact prognosis, are RF attacks (carditis) (**Figure 4**) and infective endocarditis. Infective endocarditis can be restricted to the components of the mitral valve apparatus, or not uncommonly, spread to other cardiac valves (**Figures 5**, **12**). Echocardiography is fundamental for the diagnosis of thrombi that is not uncommon in rheumatic patients presenting mitral valve stenosis mainly when atrial fibrillation is observed. However, discrepancy between echocardiographic and histological findings is not unusual, mainly in situations where the differential diagnosis with infective endocarditis is considered.

Mitral Valve Stenosis

3D Echocardiography

Echocardiography was incorporated into the revised Jones criteria for the identification of rheumatic aggression in 2015 (68), as carditis in the scenario of subclinical carditis or valvulitis (mainly observed as mitral valve insufficiency or aortic valve insufficiency). Mitral valve stenosis occurs when the transmitral mean gradient is higher than 4 mmHg, and there are features related to rheumatic disease aggression. The most characteristic valvular features related to rheumatic aggression are thickening and calcification of the mitral valve apparatus and commissural fusion leading to the

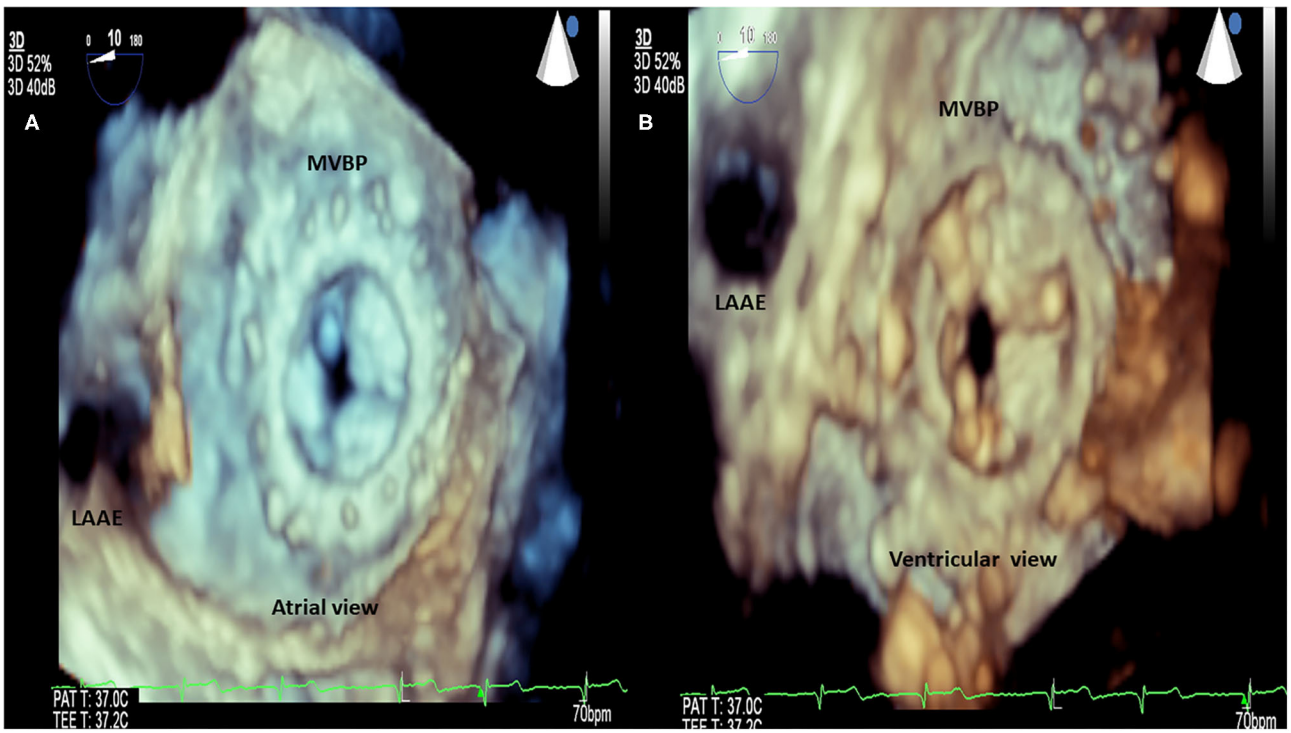

FIGURE 7 | RHD (mitral valve stenosis, stenotic bioprosthesis). 3D transesophageal echocardiography. Demonstration of mitral valve bioprosthesis stenosis. **(A)** Atrial view. **(B)** Ventricular view. LAAE, left atrium appendage; MVBP, mitral valve bioprosthesis.

restriction of the valvular opening (aspect of "hockey-stick" diastolic opening doming of the valvular cusps). International guidelines recommend that evaluation and analysis of the mitral valve as well as cardiac prosthesis after surgical correction of rheumatic lesions should be undertaken using 3D echocardiography (69, 70). Therefore, the use of 3D echocardiography can provide better spatial analysis for more comprehensive diagnosis and treatment (e.g., for valvuloplasty, native valve replacement, damaged prosthetic valve change, or valve-in-valve procedures) (69, 70) (**Figures 8–11**).

A score to predict the feasibility and success of mitral valve percutaneous valvuloplasty was originally described by Wilkins et al. in Boston, USA, in 1988, in 22 patients with mitral valve stenosis, with cross-sectional and 2D echocardiography (71). In the 21st century, 3D echocardiography has made it possible to analyze a combination of mitral valve anatomical variables to build a new echocardiographic score for mitral stenosis assessment (72). In this 3D score, with a maximum of 31 points (6 for thickness, 6 for mobility, 10 for calcification, 9 for subvalvular apparatus involvement), mitral valve stenosis can be graded as mild (<8), moderate (8–13), or severe (≥14). It seems that 3D echocardiography can better observe subvalvular involvement (72) and provide commissural observation after valvuloplasty (73–75).

Considering percutaneous balloon mitral valvuloplasty, 3D echocardiography-based scores provided additive information that could predict post-procedural outcome and suboptimal results. 3D echocardiographic analysis enabled a more comprehensive observation of post-procedural posterior-commissural splitting when compared to 2D observation (74).

Although non-invasive multimodality diagnostic investigation has gained tremendous new advances, invasive hemodynamic measurements still present a diagnostic hallmark concerning RHD. The comparison between non-invasive and hemodynamic evaluation is of great concern and interest to cardiac surgeons and clinical cardiologists. For patients presenting mitral valve stenosis, a combination of 3D echocardiography and invasive information (mitral valve navigation system) proved to provide a better correlation to invasive measurement of the mitral valve area measured by the Gorlin equation, when compared to the three-dimensional planimetry method (75).

Another important issue that 3D echocardiography could provide additional information on is related to the analysis of the left atrium (volumes and function) (19, 76, 77). For instance, after balloon valvuloplasty, improvements in left atrial reverse remodeling (decreased volumes) and left atrial emptying fraction (increased) were observed 72 h and 12 months after the procedure (76). Patients presenting with large left atria can have very low emptying fractions leading to irregular rhythms, similar to atrial fibrillation. In addition, 3D echocardiography could provide important prognostic information concerning the shape of the left atrium and the likelihood of embolic cerebrovascular events in mitral

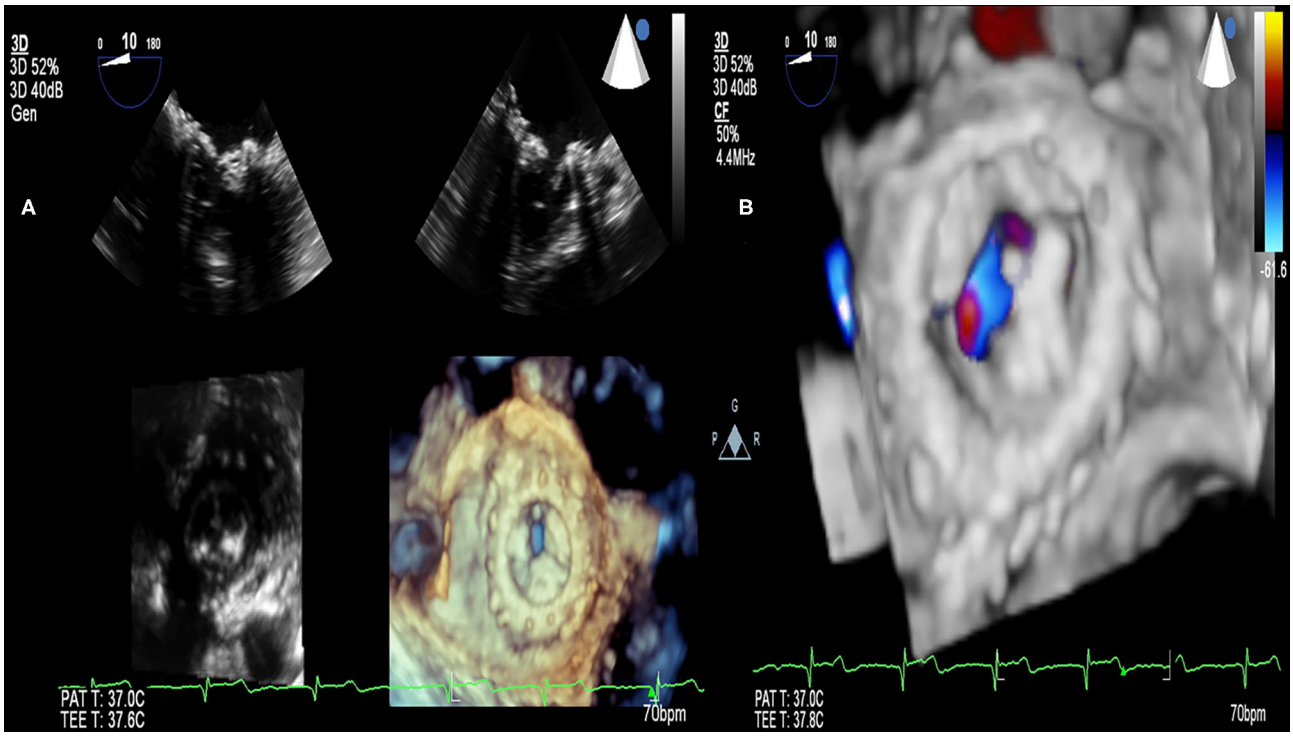

FIGURE 8 | RHD (mitral valve stenosis, stenotic bioprosthesis). **(A)** 3D transesophageal echocardiography. Demonstration of mitral valve bioprosthesis stenosis (comparison with 2D transesophageal echocardiography). **(B)** 3D transesophageal echocardiography. Demonstration of mitral valve bioprosthesis stenosis (color Doppler).

stenosis (77). It was observed that a more spherical LA shape, as determined by the use of 3D echocardiography, was associated with an increased risk of embolic cerebrovascular events (77). The mitral valve area can be analyzed by echocardiography using different analyses: two-dimensional planimetry, continuity equation, pressure half time (PHT) technique, proximal isovelocity approach (PISA), and three-dimensional evaluation. 3D echocardiographic analysis enables multiplane guidance for mitral valve evaluation, providing an accurate measurement of the mitral valve area, and overcoming the sources of errors of other echocardiographic techniques without employing mathematical equations or calculus.

3D echocardiography can also provide automatic information concerning the left atrium appendage, when considered to be percutaneously closed.

Mitral Valve Insufficiency

3D Echocardiography

Mitral valve rheumatic disease regurgitation caused by rheumatic valvular lesions is observed as thickening, calcification, scarring of the cusps, chordal shortening leading to valvular lack of coaptation, and restriction of valvular movement. Complete mitral valve rheumatic disease regurgitation analysis should employ different echocardiographic techniques for complete anatomical and morphological investigation (to differentiate organic from functional mitral valve insufficiency) and to grade the insufficiency. Thus, we should try to obtain parameters such as vena contracta, vena contracta area, regurgitant volume, regurgitant fraction, and regurgitant orifice.

3D echocardiography enables multi-angular spatial observations, mainly in the very particular "en face" view of the mitral valve. It also allows not only the ventricular view but also the ventricular approach, as well as simultaneous observations from the left atrial and ventricular views. 3D transesophageal observation enables a perfect analysis, as in the surgical view from the operating room. In addition, 3D color flow echocardiography enables accurate identification of the origin of the regurgitant jet and the quantification of the number of jets. The quantification of 3D mitral regurgitation vena contracta is employed as a semiquantitative method to grade insufficiency and can be used to overcome two-dimensional PISA limitations (64, 78, 79). 3D echocardiographic analysis of mitral insufficiency is closely correlated to magnetic resonance evaluation (80) and could be employed to predict mitral valve surgical repair results (81). The measurement of the effective regurgitant orifice area (ERO) with the 2D PISA technique underestimates the values of the vena contracta area obtained by the 3D PISA technique, the more elliptic or more asymmetric it is (78, 82).

3D echocardiographic analysis of the mitral valve is very important and valued for transcatheter treatment of mitral disease while managing periprosthetic leaks and valve-in-valve procedures.

In acute attacks of RF, the presence of small vegetations on the line of closure of the insufficient mitral valve may

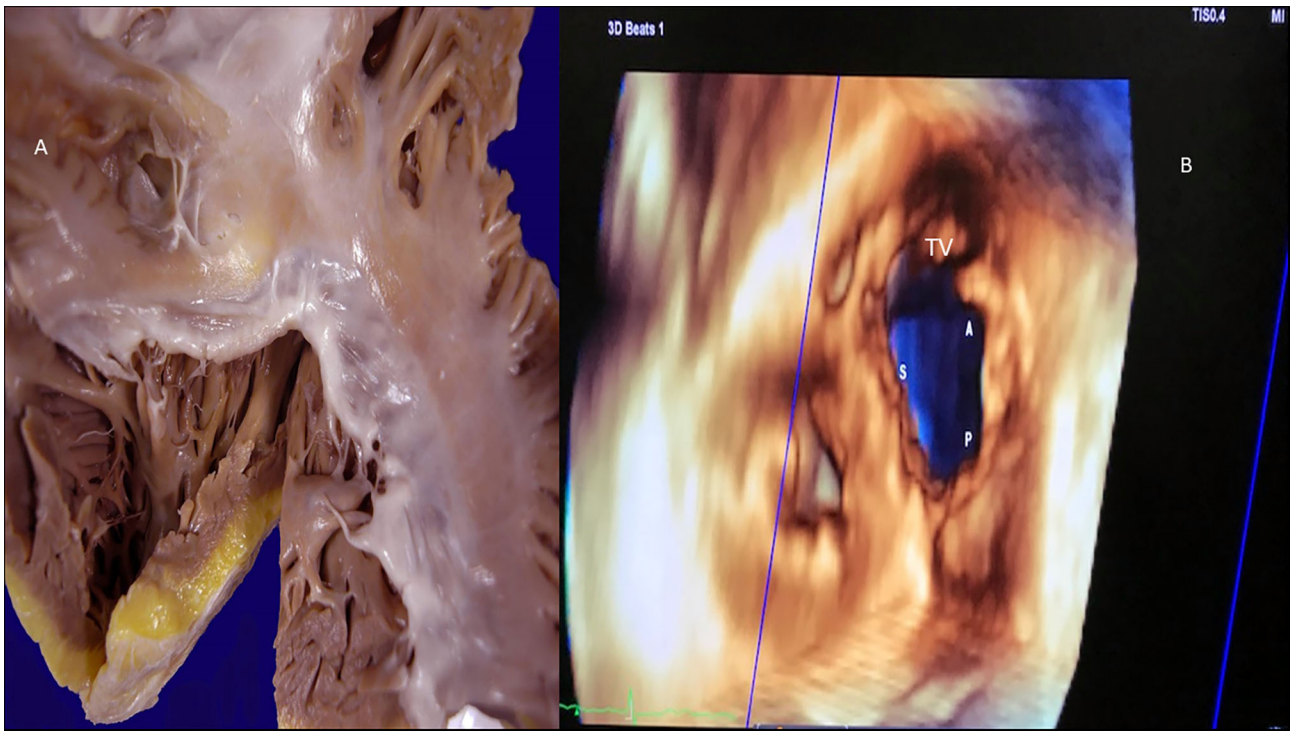

FIGURE 9 | Tricuspid valve rheumatic analysis. **(A)** Macroscopy: the tendinous cords are thickened, fused, and retracted (lack of intercordal spaces), and the anteroseptal commissure is fused. The leaflets show thickening of the free edges. **(B)** 3D echocardiography (right atrial view). Demonstration of lack of coaptation leading to an important regurgitation. S, septal cusp; A, anterior cusp; P, posterior cusp.

lead to a misleading gross and echocardiographic diagnosis of infective endocarditis, even more because the acute RF attack is a febrile disease.

Also, 3D echocardiography can be currently employed to provide automatic information from the mitral valve apparatus, concerning the annulus dimensions, annulus height, tenting area, planar and non-planar angles, shape, sphericity index, intertrigonal and intercommissural distances, and information from the leaflet coaptation (area, width).

Aortic Valve Anatomy

Similar to the mitral valve, echocardiographic analysis of the aortic valve requires detailed knowledge of the aortic valve and aortic root anatomy, including the anatomical complex of the aortic root. It comprises the sinuses of Valsalva, the valve leaflets, and the fibrous triangular inter-leaflets. 3D echocardiography allows direct and real-time visualization of all components of the aortic valvar complex, ensuring excellent anatomical proximity (83, 84). The use of 3D echocardiography allows for accurate measurements of the height of the coronary ostia and the diameters of the aortic root (aortic annulus, sinuses of Valsalva, and sinotubular junction), which are important parameters for percutaneous procedures such as percutaneous implantation of aortic prosthesis (TAVR) (84). An excellent correlation was observed between the diameters obtained by 3D echocardiography and those derived from aortic angiotomographic analysis. Aortic valve insufficiency (47%) is more common than aortic stenosis (14%) in patients with rheumatic heart disease (85). The aortic valve area, assessed by echocardiography, is 4.0 ± 0.8 cm^2. On analysis, measurement with 3D echocardiography provides greater accuracy, showing better reproducibility. 3D echocardiographic analysis also allows better observation of the etiology of aortic valve disease, allowing for a better structural observation of anatomical changes such as rheumatic disease lesions, biscuspid valves, quadricuspid valves, degenerative changes such as Lambl's excretions, and tumors such as papillary fibroelastomas (86). Multiplanar 3D transesophageal analysis of the rheumatic aortic valve can undoubtedly add anatomical information for imaging diagnosis.

Aortic Valve Stenosis

3D Echocardiography

Rheumatic aortic valve disease can lead to commissural fusion, fibrosis, thickening and calcification of the leaflets, retraction of the leaflet edges, and turning of the systolic aortic orifice into a more rounded or triangular shape. The assessment of the severity and relevance of aortic stenosis must take into account anatomical and functional aspects. Thus, it is important to analyze the aortic valve morphology, the presence and distribution of calcium along the valve commissures and leaflets, and the central or peripheral distribution of calcium. We must also obtain the valve area as well as the maximum and medium transvalvular gradients, the maximum speed of the

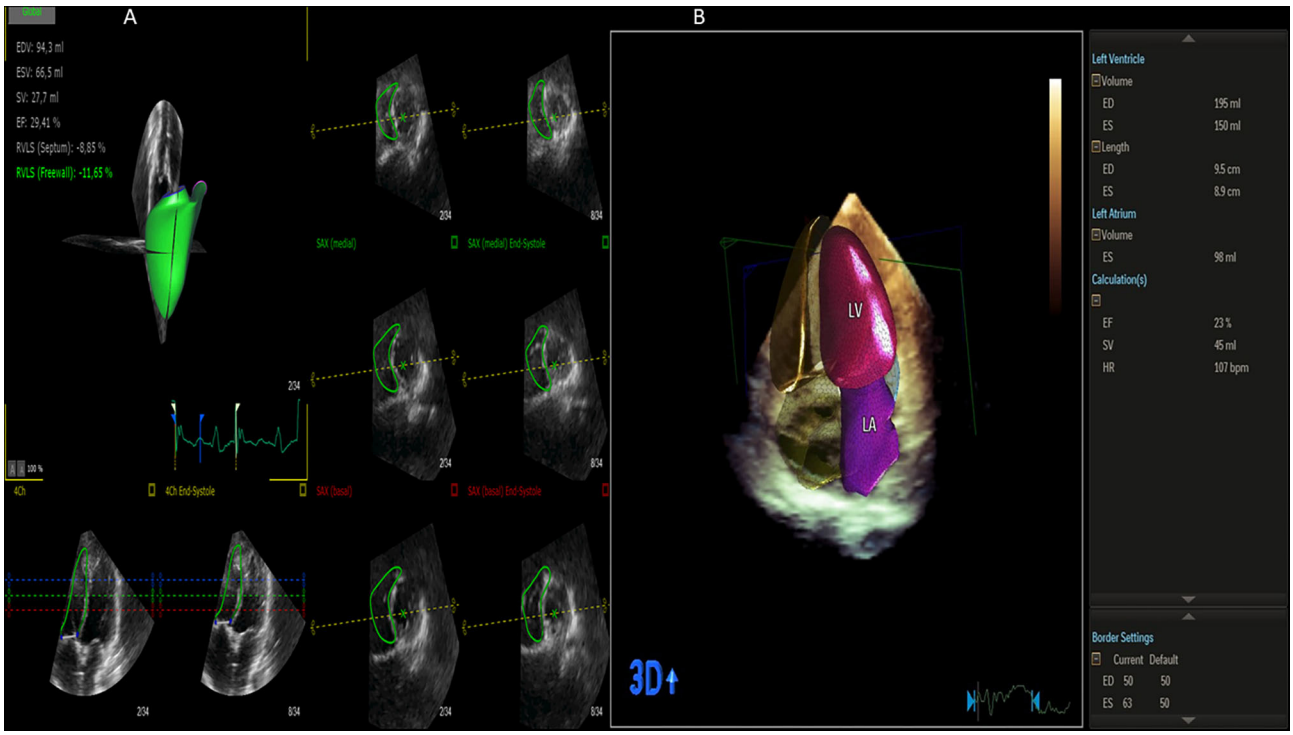

FIGURE 10 | Left and right ventricle analysis by 3D echocardiography (artificial intelligence). **(A)** Right ventricle analysis. **(B)** Left ventricle and left atrium analysis.

left ventricular outflow tract, the contractile performance of the left ventricle (ejection fraction and myocardial deformation), the degree of left ventricular hypertrophy, analysis of left ventricular diastolic function, the analysis of arterial valve impedance (especially in hypertensive patients), the analysis of associated heart valve diseases (mitral and tricuspid regurgitation), the degree of hypertension, and the analysis of the performance of the right ventricle.

Analysis of the aortic valve area is of great importance in analyzing the severity of aortic stenosis. A widely used method for measuring the aortic valve area is the continuity equation using 2D echocardiography. In this method, a circular shape is assumed for the ventricular outflow tract, which, in a large number of cases, is different from the actual elliptical anatomical shape (87). This inference can cause an underestimation of the aortic valve area. Planimetry of the left ventricular outflow tract using 3D echocardiography allows for greater accuracy in the assessment of the aortic valve area from an en-face perspective (42). 3D planimetry of the aortic valve area provides accurate results, even when compared to invasive measurements of the aortic valve area, with several studies documenting superiority over the 2D method (42, 88).

Another method for assessing the aortic valve area consists of using the left ventricular stroke volume measured by 3D analysis (42). To measure the aortic valve area, stroke volume is divided by the aortic valve time–velocity integral (42). The accuracy of this method has proven to be adequate and even superior to measurements performed using the continuity equation and other 2D volumetric methods.

Measurement of the aortic area using the 3D LV stroke volume method is as follows:

1. Left-ventricle stroke volume, measured by 3D echocardiography
2. Aortic valve velocity time integral (TVI)

Aortic Valve Area

Left-Ventricle Stroke Volume (cm^3)

TVI (Aortic Valve) (cm)

This method, using left-ventricle stroke volume for the evaluation of the aortic area, has better correlation with invasive methods (Gorlin equation) than bidimensional methods.

The analysis of the severity of aortic stenosis using 3D echocardiography has better correlation with measurements using other imaging methods (computed tomography and magnetic resonance imaging), when compared with 2D echocardiographic analysis (89). In addition, left ventricular mass analyzed by 3D echocardiography is better correlated with magnetic resonance evaluation than 2D echocardiographic evaluation. The use of 3D echocardiography can lead to reclassification of aortic valve stenosis severity in 10–25% of cases (90).

Aortic Valve Insufficiency

3D Echocardiography

The use of echocardiography for quantification and evaluation of the hemodynamic and anatomical impact of aortic valve insufficiency allows accurate observation and determination of

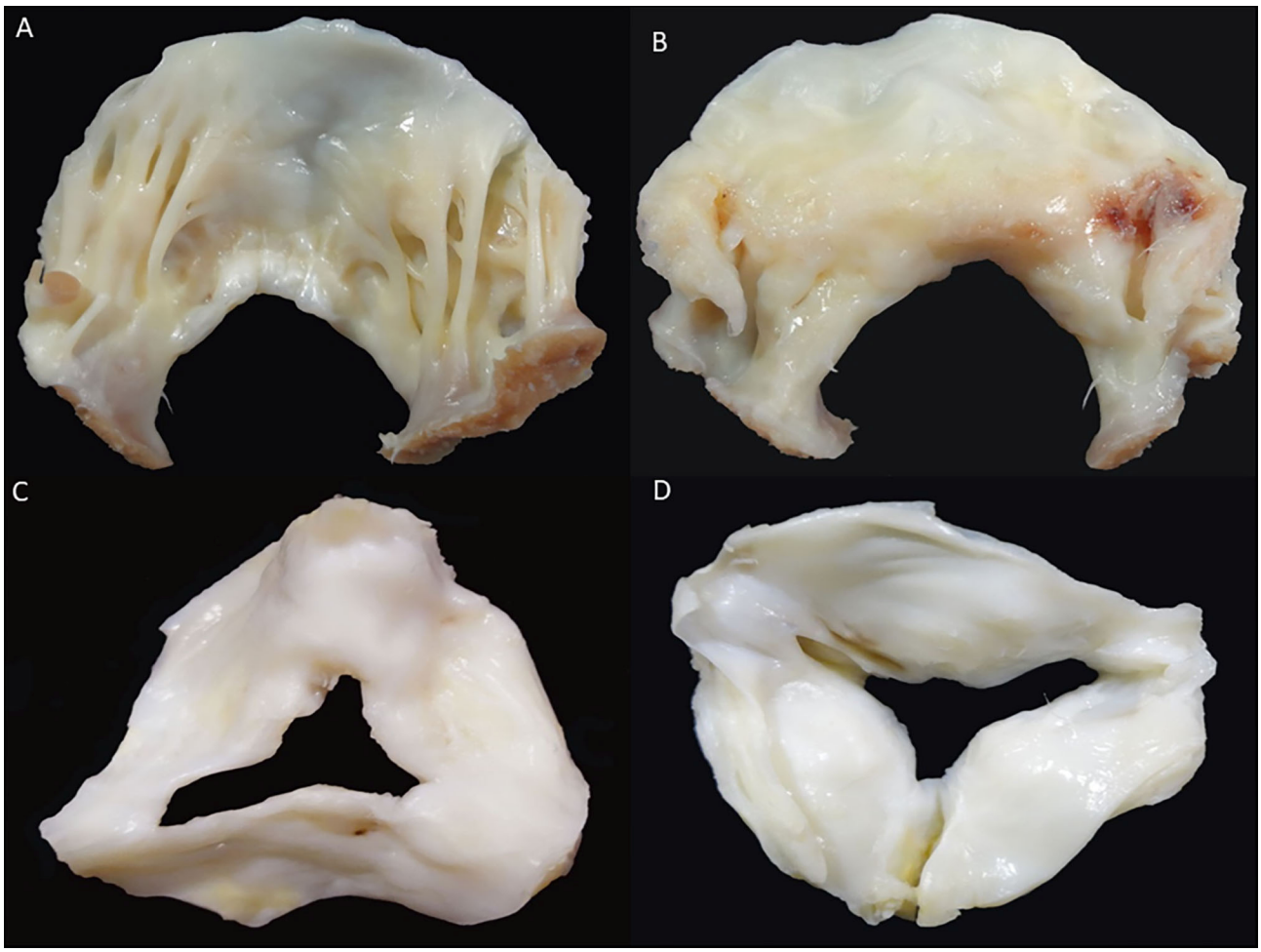

FIGURE 11 | Rheumatic heart disease (Mitral and aortic valves). **(A)** Mitral valve: ventricular surface of the anterior leaflet showing diffuse fibrous thickening. The tendinous cords are thickened, fused, and retracted (lack of intercordal spaces). **(B)** Mitral valve: atrial aspect of the same valvar leaflet showing diffuse thickening and a scar of previous valvular commisurotomy (arrow). **(C)** Aortic valve: surgical specimen showing leaflets thickening and comissural fusion, arterial view. **(D)** Aortic valve: leaflets thickening, commissural fusion and calcification, ventricular view.

the temporal moment of the natural history of patients with aortic insufficiency. The morphofunctional analysis of the patient with aortic insufficiency should take into account the etiological evaluation of the valve disease, the diameters of the ascending aorta, the diameters of the left ventricle, myocardial performance analysis (left ventricular ejection fraction), the quantification of severity of insufficiency with measurement of the contracted vena, the observation of the width of the jet in the left ventricular outflow tract, the measurement of the regurgitant fraction, the regurgitant volume, and the ERO.

The use of 3D echocardiography provided new insights into the analysis of the severity of aortic valve insufficiency (70, 89, 91). Apart from the anatomical approach, 3D echocardiography has also enabled better understanding of the bidimensional vena contracta measurement (2D color echocardiography could lead to inaccurate evaluation and incorrect geometric inferences for the analysis of the regurgitant orifice, which, while initially thought to be plane and circular, has in many cases proved to be elliptical) (91). Thus, the use of a 3D approach increases the accuracy of analysis of the aortic valve regurgitant lesion, enabling the direct measurement of the vena contracta area of the regurgitant jet. Furthermore, for transcatheter aortic valve approaches in rheumatic patients who have undergone previous surgical correction and who present with complications such as periprosthetic leak, the use of 3D echocardiography is of utmost importance to guide transcatheter procedures (plug implantation).

Tricuspid Valve Stenosis and Regurgitation

3D Echocardiography

Isolated tricuspid aggression in RHD is rare and is most commonly associated with mitral and aortic disease. The normal tricuspid area varies from 4 to 6 cm^2. Tricuspid rheumatic valve disease demonstrates similar findings to those observed in mitral valve aggression. Thus, fusion and thickening of the cusps, diastolic cuspal doming, thickening, shortening, fusion,

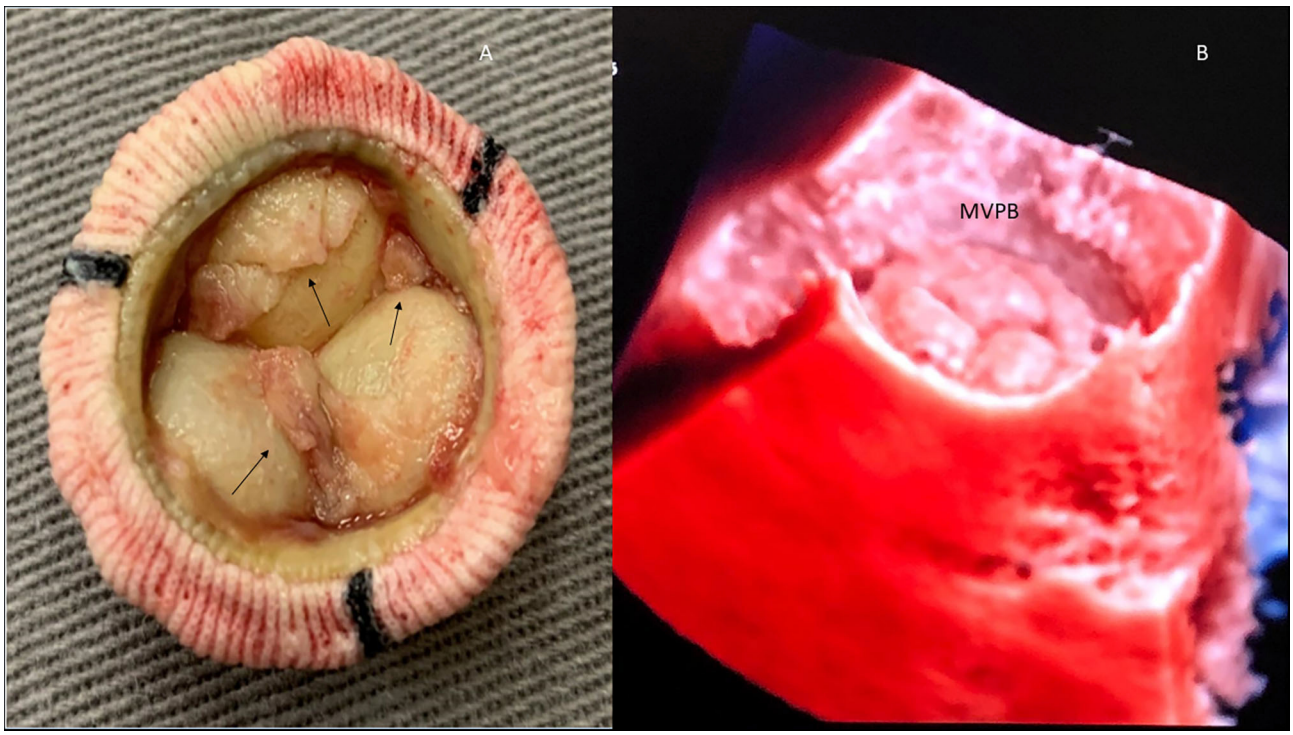

FIGURE 12 | Mitral valve bioprosthesis (MVBP). **(A)** Macroscopy: MVBP in a patient with infective endocarditis. Vegetations (arrows). **(B)** Normal MVBP demonstrated by 3D echocardiography (transluminescence technique).

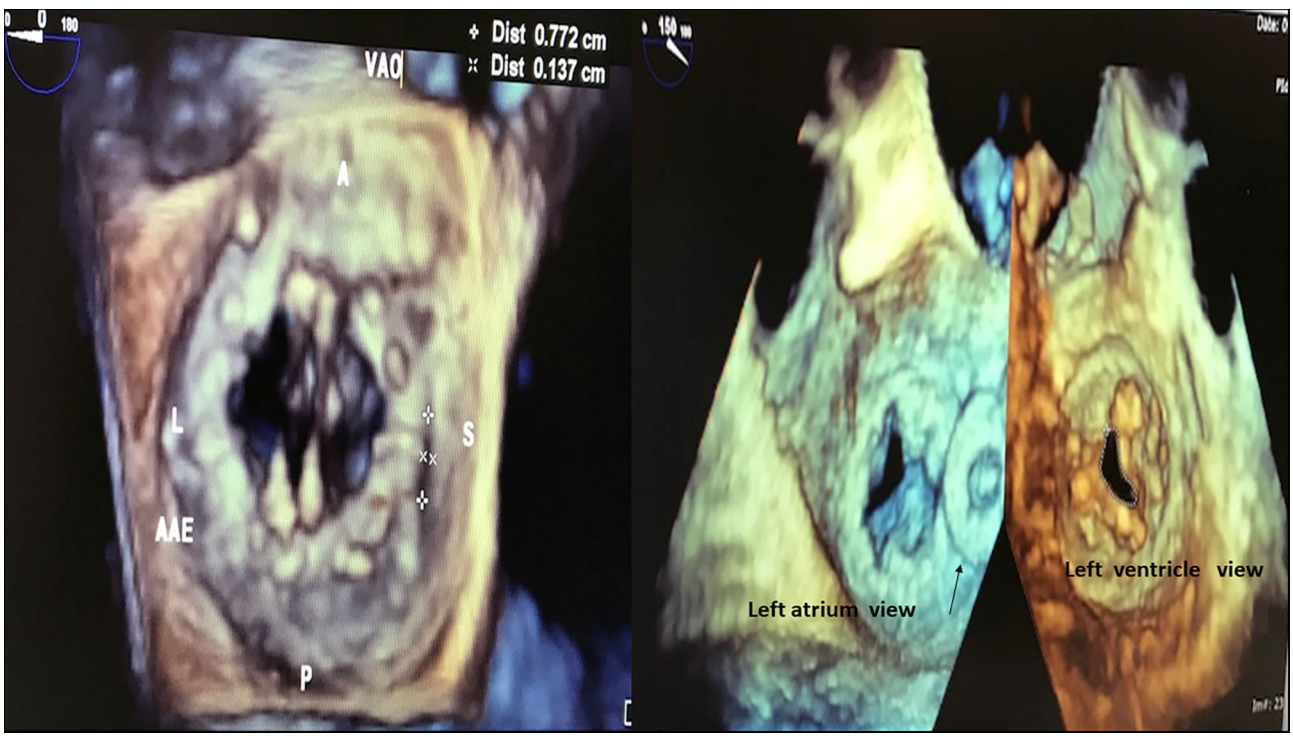

FIGURE 13 | Mitral valve mechanical prosthesis (left) and bioprosthesis (right). Left: Mitral valve mechanical prosthesis presenting periprosthetic leak (7.7 × 1.3 mm) located close to the septal ring. Regions of the prosthetic ring: A, anterior; P, posterior; L, lateral; S, septal; AAE, left atrial appendage; VAO, aortic valve. Right: Mitral valve stenotic bioprosthesis (area: 0.85 cm^2). Arrow: Amplatzer plug used to treat previous periprosthetic leak.

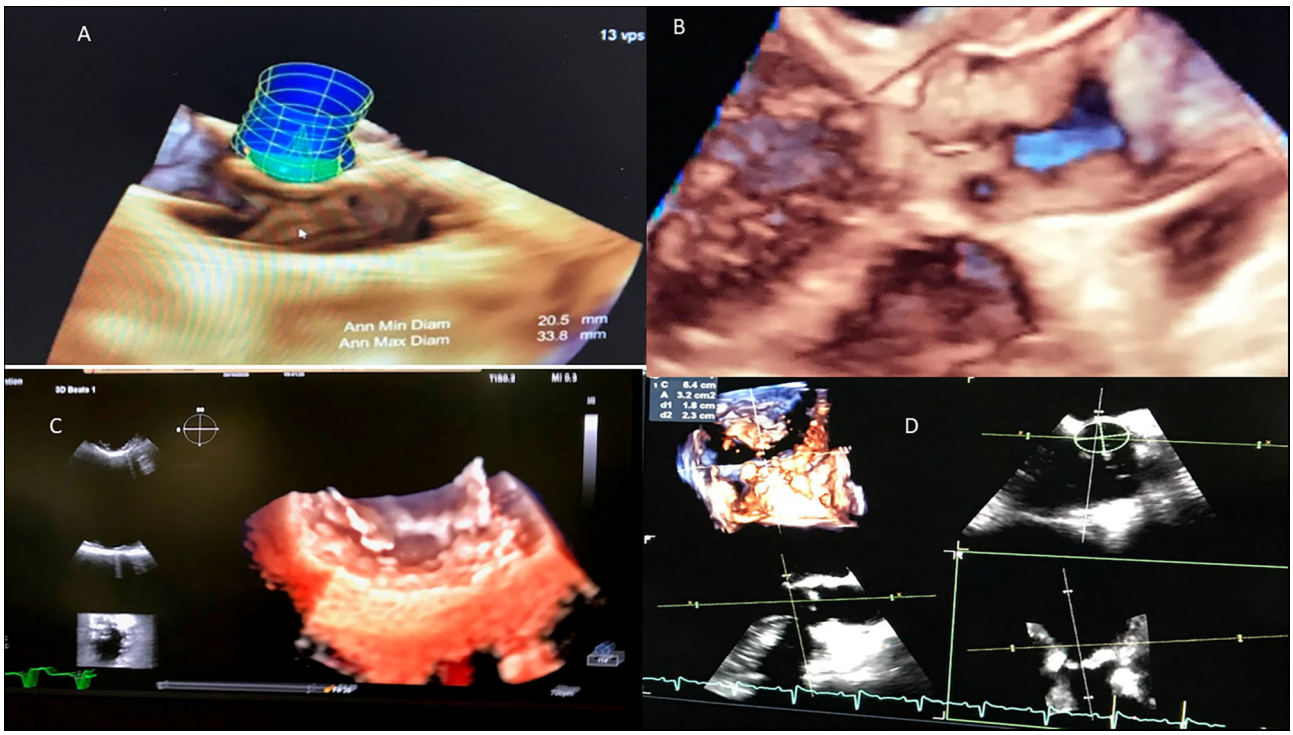

FIGURE 14 | RHD (mitral valve and aortic valve). **(A)** Ventricular surface of the anterior leaflet showing diffuse fibrous thickening. The tendinous cords are thickened, fused, and retracted (lack of intercordal spaces). **(B)** Atrial aspect of the same valvar leaflet showing diffuse thickening and a scar of previous valvular commissurotomy (arrow). **(C)** Surgical specimen showing leaflets thickening and commissural fusion, arterial view. **(D)** Leaflets thickening, commissural fusion and calcification, ventricular view.

and retraction of the chordae, commissural thickening and fibrosis, and calcification/scarring of the different elements of the valvular apparatus can be found over time after rheumatic onset (**Figure 12**). These valvular alterations can lead to stenosis, insufficiency, or combined tricuspid lesions (more common). As in other rheumatic valvular diseases, tricuspid disorders should be analyzed considering anatomical and morphofunctional features. Tricuspid valve stenosis is considered to be severe when the tricuspid area is <1.0 cm^2; the diastolic mean transvalvular gradient is ≥ 5 mmHg when the tricuspid PHT is ≥ 190 ms; and inflow velocity integral is ≥ 60 cm, with consequent right atrial enlargement. The use of 3D echocardiography can lead to a better understanding of tricuspid rheumatic anatomy and derived atrium changes, right ventricular functional and geometric modifications, and evidence of thrombi in the right atrium (92–97). As for left atrium analysis and remodeling comprehension in different clinical scenarios, a global analysis of the right chambers and tricuspid valve elements is better when done using 3D echocardiography (92–97). The tricuspid area can be analyzed from an atrial or ventricular perspective, enabling an en-face wider view of the valve.

This en-face view provides a simultaneous view of the three leaflets, leading to the measurement of the vena contracta area of the tricuspid regurgitant jet.

A 3D vena contracta area cutoff value of 0.61 cm^2 can determine severe tricuspid regurgitation, sensitivity of 78%, and specificity of 97% (97). The measurement of ERO with the 2D PISA technique underestimates the values of the vena contracta area obtained by the 3D PISA technique, in particular for eccentric jets (97).

Another important piece of information derived from 3D echocardiography is the evaluation of right-ventricle performance (3D right ventricle volumes and ejection fraction).

Another important condition that can be very well-visualized with the addition of 3D echocardiography is tricuspid lesions due to infective endocarditis (**Figure 13**).

Pulmonic Valve Stenosis and Regurgitation

3D Echocardiography

Rarely is isolated pulmonic valve aggression observed after RHD establishment. Whenever present, it is more commonly associated with mitral valve stenosis (98). The pulmonic valve is considered to have severe stenosis when the pulmonic valvular area is <1.0 cm^2 in adults, with a peak transpulmonic flow velocity of >4 m/s, along with the demonstration of restriction of leaflet mobility with a doming appearance on systole, calcification, and thickening of the leaflets. The use of 3D transesophageal echocardiography may add diagnostic information to anatomical investigation of the pulmonic valve, when compared to the 2D echocardiographic approach (99).

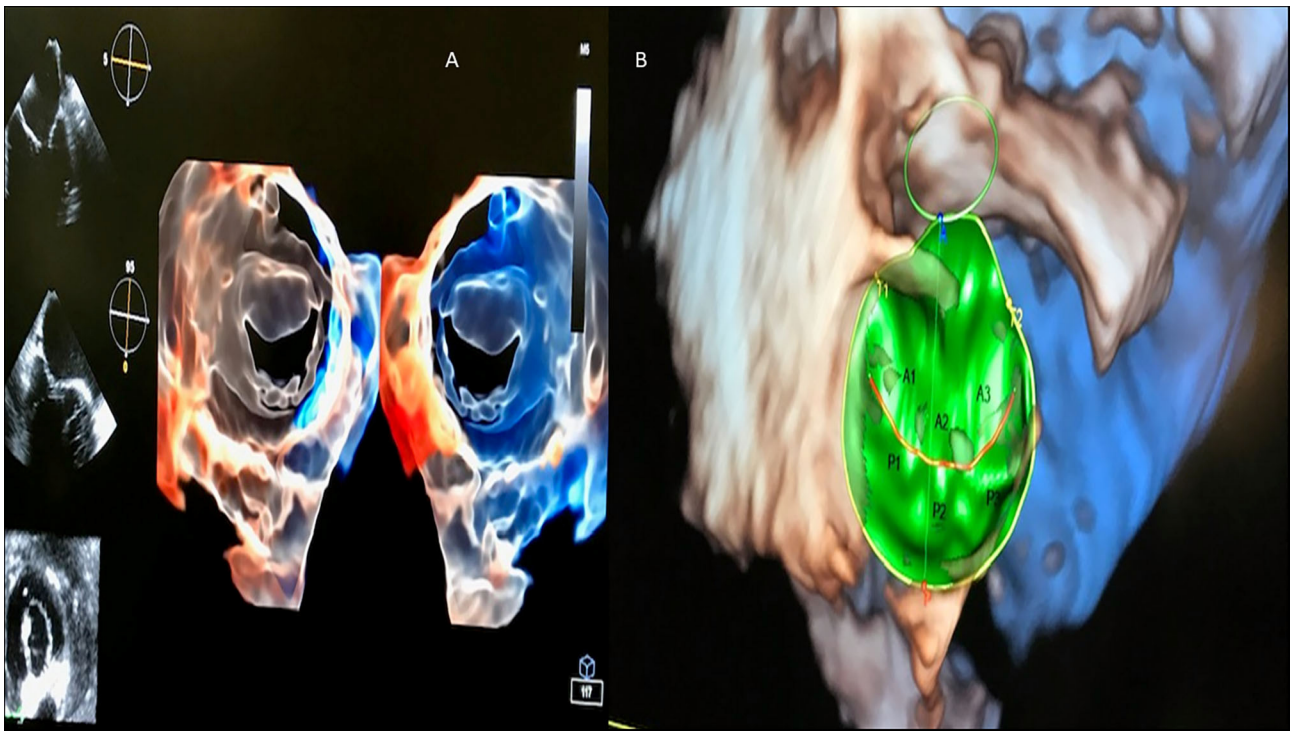

FIGURE 15 | 3D transesophageal echocardiography automatic acquisition (in green). **(A)** Mitral valve (transluminescence technique from atrial and ventricular views); **(B)** Mitral valve (surgeon view), depicting valvular segments (A1, A2, A3, P1, P2, P3) from anterior **(A)** and posterior cusps **(B)**.

Mixed Rheumatic Valve Disease

3D Echocardiography

Mixed rheumatic valvular disease is common during the follow-up of patients with rheumatic valvular disease (100). Multiple valvular disease is considered whenever two or more valvular disorders are present and the functional valve disease is secondary to primary organic valvular involvement. In this sense, it is important for both anatomical evaluation and morphofunctional multivalvular occurrence, considering valvular lesions as well as chamber remodeling (atria and ventricle).

In this sense, the use of 3D echocardiography in valvular heart disease will provide valvular anatomical information as well as biventricular functional analysis (ejection fraction), atria volumes, and emptying fractions.

LIMITATIONS

3D echocardiography techniques are subject to the physical limitations of ultrasound, such as inadequate image quality, planar variation within the acquired image, the occurrence of image artifacts, gain adjustments (such as considering the dependence of valvular flow orifice sizes), the occurrence of cardiac arrhythmias, and breathing variations during image acquisition. Also, the possible occurrence of artificial thickening of the cardiac structures displayed in volume rendering, the unreliability for tissue characterization, and the interobserver bias (which is considered to be decreased with the use of automatic quantification and artificial intelligence) are of great importance, in addition to the need for specific training to obtain and analyze the images. In developing countries, 3D echocardiography equipment is still limited by cost. In the future, a better analysis of RHD patients should be considered, taking into consideration a multimodality approach (considering 3D echocardiography, computed tomography, and cardiac magnetic resonance).

CONCLUSION

RHD is a very important health issue worldwide, mainly in underdeveloped countries. Echocardiography is the most relevant imaging technique providing diagnostic information, enabling prognostic data, and presenting a very important role in the correction of complications after surgical repair of rheumatic heart valvulopathies. Accordingly, 3D echocardiography is ready for routine use in patients with RHD presenting with valvular abnormalities.

AUTHOR CONTRIBUTIONS

MV, CB, AG, PV, LB, LD, PG, VA, FT, and RS: planning, conduct, and reporting. MV: guarantor. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cardiovascular Medicine

ORIGINAL RESEARCH
published: 30 July 2021
doi: 10.3389/fcvm.2021.636280

Household Economic Consequences of Rheumatic Heart Disease in Uganda

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Background: Rheumatic heart disease (RHD) has declined dramatically in wealthier countries in the past three decades, but it remains endemic in many lower-resourced regions and can have significant costs to households. The objective of this study was to quantify the economic burden of RHD among Ugandans affected by RHD.

Methods: This was a cross-sectional cost-of-illness study that randomly sampled 87 participants and their households from the Uganda National RHD registry between December 2018 and February 2020. Using a standardized survey instrument, we asked participants and household members about outpatient and inpatient RHD costs and financial coping mechanisms incurred over the past 12 months. We used descriptive statistics to analyze levels and distributions of costs and the frequency of coping strategies. Multivariate Poisson regression models were used to assess relationships between socioeconomic characteristics and utilization of financial coping mechanisms.

Results: Most participants were young or women, demonstrating a wide variation in socioeconomic status. Outpatient and inpatient costs were primarily driven by transportation, medications, and laboratory tests, with overall RHD direct and indirect costs of $78 per person-year. Between 20 and 35 percent of households experienced catastrophic healthcare expenditure, with participants in the Northern and Western Regions 5–10 times more likely to experience such hardship and utilize financial coping mechanisms than counterparts in the Central Region, a wealthier area. Increases in total RHD costs were positively correlated with increasing use of coping behaviors.

Conclusion: Ugandan households affected by RHD, particularly in lower-income areas, incur out-of-pocket costs that are very high relative to income, exacerbating the poverty trap. Universal health coverage policy reforms in Uganda should include mechanisms to reduce or eliminate out-of-pocket expenditures for RHD and other chronic diseases.

Keywords: rheumatic heart disease, catastrophic health expenditure, universal health coverage, cost of illness, household survey

OPEN ACCESS

Edited by:
Leonardo Roever, Federal University of Uberlandia, Brazil

Reviewed by:
Paulo M. Dourado, University of São Paulo, Brazil
Andre Rodrigues Duraes, Federal University of Bahia, Brazil

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Specialty section:
This article was submitted to General Cardiovascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: *01 December 2020*
Accepted: *08 June 2021*
Published: *30 July 2021*

Citation:
Opara CC, Du Y, Kawakatsu Y, Atala J, Beaton AZ, Kansiime R, Nakitto M, Ndagire E, Nalubwama H, Okello E, Watkins DA and Su Y (2021) Household Economic Consequences of Rheumatic Heart Disease in Uganda. Front. Cardiovasc. Med. 8:636280. doi: 10.3389/fcvm.2021.636280

INTRODUCTION

Rheumatic heart disease (RHD) has declined dramatically in wealthy countries over the past three decades, in part due to penicillin-based preventive measures and improvements in social determinants of infectious diseases (1). However, RHD continues to be endemic in lower-resourced regions such as Southeast Asia and Sub-Saharan Africa (2). Rheumatic heart disease is thought to pose a severe threat to the economic well-being of affected households because of its longitudinal nature and associated recurrent out-of-pocket (OOP) costs, including OOP costs of advanced medical and surgical care. The magnitude of these costs is particularly high in low-income countries like Uganda, where health services that are not financed by development assistance partnerships are often paid for OOP (3).

High OOP costs and their consequences, including catastrophic health expenditure, borrowing, and selling assets to pay for healthcare, and intra-household labor substitution, threaten to keep households in the so-called "poverty trap" (4). More broadly speaking, high rates of medical impoverishment threaten the macroeconomic growth agenda in low-income countries. Indeed, reducing OOP payments has become a key feature of universal health coverage (UHC) reforms. Unfortunately, the scarcity of public resources constrains the ability of many governments to provide adequate financial protection for all but the highest-priority health conditions and services—and to date, RHD has typically been neglected in national priorities (5).

Even though there are good theoretical reasons as to why RHD could be associated with excess household economic burden, there are only a handful of primary costing studies to date that have attempted to quantify the economic burden of RHD from the societal, health sector, and household perspectives (6–12). Only one study (non-peer reviewed) has assessed the household economic impact of RHD in a country where the condition remains endemic (South Africa) (13), and the health system and financing capabilities of South Africa are relatively advanced as compared to most other African nations, limiting the applicability of this study's findings to different settings.

In 2018, the 71st World Health Assembly adopted a global resolution on RHD (14), making this condition a high priority for national UHC systems for the first time. In response to this resolution, the Uganda Ministry of Health has begun to work with the Uganda Heart Institute to develop a national RHD strategy and policies. Around the same time, the American Heart Association made a 4-year investment in RHD research in Uganda, focusing on filling gaps in epidemiological and health services research. The present study, conducted under the auspices of the above collaborations, sought to quantify the economic burden of RHD in Uganda from the household perspective. On a local level, this study aimed to guide the allocation of resources in Uganda to address RHD, particularly by understanding the potential scope for public finance of RHD-related healthcare.

METHODS

Overview

This study used the cost-of-illness method (15). We used a prevalence-based approach to cost estimation and thus designed the study as a cross-sectional survey. In brief, we collected data on direct and indirect RHD-related costs among 87 Ugandan households affected by RHD, looking at all costs incurred over the previous 12 months. We also estimated the prevalence of catastrophic health expenditure among these households and inquired about the use of specific coping mechanisms (e.g., selling assets) to smooth consumption over time.

Recruitment of Subjects

Participants in this study were recruited from the Uganda National RHD Registry. This registry was established in 2010 as a central database for all patients diagnosed with RHD clinically and by echocardiogram in Uganda (16). As of this writing, the registry has enrolled 2,727 participants. For our study, we sampled from among registry enrollees who were receiving RHD care at one of three referral hospitals that served the district in which they lived. The hospitals chosen were Lira Regional Referral Hospital (Lira district, Northern Region), Mbarara Regional Referral Hospital (Mbarara district, Western Region), and Mulago National Referral Hospital (Wakiso district, Central Region).

We pre-screened the Uganda National RHD Registry database for enrollees who were receiving care at the three hospitals mentioned previously and did a stratified random sample of subjects for inclusion in this study. Edited to reflect that we have a few subjects under age 10. Minors were required to provide assent and informed consent from their guardians. There were no clinical exclusion criteria.

Our original target sample size was 100 subjects, distributed approximately equally across the three sites (sampling strata). This target had been chosen to obtain a precision of ±5% on critical descriptive statistics. Eighty-seven subjects had been recruited as of March 2020, at which time the ethical review committees of the responsible institutions suspended nearly all research activities due to the SARS-CoV-2 outbreaks in the United States and Uganda. Because of the uncertain timeframe for resumption of study activities, we decided to close out the study and analyze data for the 87 subjects who were already enrolled.

Data Collection Procedures

Eligible subjects were contacted by a research nurse who explained the reason they had been identified as a potential participant and described the study objective and procedures. Subjects agreeing to participate were scheduled to undergo an in-person survey that was usually conducted at the subject's place of residence. Some subjects (e.g., minors) did not have sufficient information on household finances; in these cases, a household representative with knowledge of finances was also asked to participate in the survey. Written informed consent (and assent, when relevant) was obtained from all individuals who provided responses to the surveys.

After documentation of consent, a research nurse administered a standardized survey instrument that contained two modules, an "individual" module focused on the costs incurred by the patient receiving care for RHD and a "household" module focused on household demographics, income, expenditures, and assets. The survey instrument was adapted from previous studies and was piloted on several subjects prior to finalization. **Supplementary Material** contains the entire survey instrument.

Surveys were conducted from December 2018 through February 2020 and in the participants' preferred language (Luo, Runyankore, Luganda, or English). Following survey completion, participating households were reimbursed 20,000 Ugandan Shillings (about 5.5 United States dollars) for their time.

Data Analysis

Cost estimates obtained in the surveys were initially recorded in current Ugandan Shillings. The research team then standardized these costs to 2019 mid-year United States dollars (US$) using exchange rates and consumer price indices from the most recent World Development Indicators dataset (2020 update) (17).

We first analyzed the survey data using descriptive statistics, linking every inpatient or outpatient episode for RHD that was recalled over the previous 12 months back to a unique patient/household identifier. Costs of healthcare episodes were divided into direct medical costs, direct non-medical costs, and indirect costs (4). We further disaggregated direct medical costs into those due to laboratory tests, consultations, medicines, and in the case of inpatient care, bed tariffs. We disaggregated direct non-medical costs into those due to transportation, accommodation, and food expenses. Indirect (i.e., opportunity) costs were calculated using reported time spent receiving or providing RHD-related care and were disaggregated into costs of ill participants and their caretakers, respectively. The human capital approach was used to estimate indirect costs: reported hours of work missed were multiplied by the Uganda national minimum wage (converted into an hourly rate) (18, 19).

We also estimated the prevalence of catastrophic health expenditure (CHE) among study subjects. We computed total annual direct expenditure on RHD-related care for each subject and compared this expenditure to total annual household expenditure. Two thresholds for CHE were used: RHD expenditure greater than or equal to 10% or 25% of household expenditure (20).

Next, we estimated the prevalence of common coping mechanisms that occurred following utilization of RHD-related care. We asked participants about the use of three mechanisms: (i) taking out one or more loans, (ii) receiving financial assistance from family or friends, and (iii) selling assets. Again, the recall window for these events was the previous 12 months.

Finally, we assessed the relationship between households' use of coping mechanisms and the demographic and socioeconomic characteristics of household members affected by RHD. We used multivariate Poisson regression models to test a pre-specified set of covariates (see **Table 4** for list). The final adjusted model included all covariates.

Other Information

Survey data were managed by a REDCap account hosted by Children's National Medical Center and were exported to Microsoft Excel (v2104) and R (v3.6.3) for data cleaning and analysis (21).

The sponsor of this research was not involved in the design, review, collection of data, analysis, and interpretation of data, or drafting of this manuscript.

RESULTS

Characteristics of Study Subjects and Households

We conducted surveys of 87 individuals with RHD and their households. Of these, 33 were residing in the Northern Region, 20 in the Western Region, and 34 in the Central Region. **Table 1** summarizes the demographic characteristics of the patients and households. The "typical" participant was a young adult woman who achieved at least primary or secondary level education but who was currently unemployed and did not have private health insurance. Notably, socioeconomic characteristics varied widely across participants from the three regions. Participants living in the Northern Region generally demonstrated the lowest income and educational attainment, and participants living in the Central Region the highest.

Among households included in this study, the average monthly income per person was US$ 130, though it varied from US$ 28 in the Northern Region to US$ 280 in the Central Region. The average monthly household expenditure was US$ 123 in total, of which 52% were non-food expenditures. Less than half of households had electricity, 37% owned a vehicle (car, motorcycle), and 93% had at least one mobile phone.

Direct and Indirect Costs Incurred

Direct and indirect costs were incurred across 27 inpatient visits from 21 participants and 408 outpatient visits among all the 87 participants. Overall annual costs were estimated at US$ 78 per person per year, inclusive of both direct and indirect costs.

As highlighted in **Table 2** and **Figure 1**, overall direct costs of outpatient care were comprised predominately of transportation and medications expenses and, to a lesser extent, food and laboratory tests expenses. Notably, OOP medication costs were lower in the Central Region, which probably relates to the greater availability of free medications at public facilities in the Region.

Direct costs of inpatient care, highlighted in **Figure 2**, were substantially higher than outpatient costs but were also comprised predominately of medications, laboratory tests and transportation expenses. Indirect costs were a more substantial contributor to inpatient total costs than to outpatient costs. In the setting of limited inpatient cost data, the composition of OOP costs for inpatient care differed across the three regions, with medications, transportation, and laboratory tests costs having outsized importance in the Northern, Western and Central regions, respectively.

TABLE 1 | Baseline characteristics of sampled participants.

	Northern region ($n = 33$)	Western region ($n = 20$)	Central region ($n = 34$)	All regions ($n = 87$)
AGE				
Mean (*S.D.*)[a]	22 (17)	28 (16)	34 (13)	28 (16)
Female, %	64	60	79	69
EDUCATION, *n* (%)				
None	5 (15)	1 (5)	3 (9)	9 (10)
Primary only	23 (70)	8 (40)	7 (21)	38 (44)
Secondary or more	5 (15)	11 (55)	24 (71)	40 (46)
EMPLOYMENT, *n* (%)				
Unemployed	28 (85)	13 (65)	24 (71)	65 (75)
Self-employed	5 (15)	4 (20)	4 (12)	13 (15)
Formally employed (full-time/part-time)	0 (0)	3(15)	6 (18)	9 (10)
HOUSEHOLD ASSETS, *n* (%)				
Electricity	5 (15)	6 (30)	26 (77)	37 (43)
Mobile phone	30 (91)	19 (95)	32 (94)	81 (93)
Any vehicle	8 (24)	10 (50)	14 (41)	32 (37)
HOUSEHOLD SIZE				
Mean (*S.D.*)	7.0 (4.0)	5.2 (1.7)	1.2 (1.2)	4.3 (3.7)
AVERAGE 30-DAY HOUSEHOLD EXPENDITURES (USD)[b], MEAN (*S.D.*)				
Non-food expenditure	44.80 (74.49)	45.39 (59.09)	94.18 (106.43)	64.68 (88.64)
Food	37.53 (58.65)	56.77 (46.06)	81.22 (56.75)	58.77 (57.91)
MONTHLY HOUSEHOLD INCOME PER PERSON (USD)[b]				
Mean (*S.D.*)	27.81 (57.64)	40.39 (49.18)	276.81 (506.04)	126.28 (335.40)
PRIVATE HEALTH INSURANCE				
n (%)	0 (0)	0 (0)	4 (12)	4 (5)

[a] *Standard deviation,* [b] *currency reported in 2019 USD.*

Catastrophic Health Expenditure

Table 3 presents our estimates of the prevalence of catastrophic health expenditure (CHE) in this sample. At the more conservative 25% threshold, 20% of households experienced CHE, ranging from 3% in the Central Region to 32% in the Western Region. At the more liberal 10% threshold, 35% of households experienced CHE, ranging from 9% in the Central Region to 53% in the Western Region. Put another way, households in the Northern and Western regions were five to ten times more likely to experience CHE as compared to households in the Central Region.

Coping Strategies

Households in the Northern and Western regions demonstrated greater reliance on asset sales and loans than the Central Region, reflecting heavy financial burden due to RHD OOP costs and CHE (**Figure 3A**). By contrast, participants in the Central Region demonstrated greater reliance on financial assistance from extended family or friends, the latter of which probably reflects greater access to financial resources in the community in this relatively wealthier region.

Across all regions, nearly four out of five households used one or more coping strategies in the past year (**Figure 3B**). The use of multiple coping strategies was also particularly notable: nearly half of households used at least two coping strategies, and one in five used all three. Again, households in the Northern and Western regions demonstrated a greater reliance on coping strategies, including the use of multiple strategies, compared to the Central Region.

In the regression analysis, we found that the only significant association with coping strategies was the magnitude of the direct OOP costs that patients incurred (**Table 4**). However, regional differences in utilizing coping strategies that observed in **Figure 3B** were not statistically significant in regression.

DISCUSSION

This study sought to quantify the economic cost of RHD-related healthcare among 87 patients and their households from three diverse districts in Uganda. We found that the total annual cost of receiving care for RHD was US$ 78 per patient, inclusive of both direct and indirect costs. Medications and transportation costs appeared to be the major determinants of high OOP costs. About one-third (35%) of households affected by RHD experienced CHE during the past year, and nearly four in five households coped with these costs using some combination of formal and informal borrowing and asset sales. Unsurprisingly, we found a strong association between the magnitude of OOP costs and the probability of using one or more coping strategies. Our research

TABLE 2 | Costs[a] accrued from seeking RHD care.

	Outpatient		Inpatient		Combined	
	Mean	**Percentage of subtotal (%)**	**Mean**	**Percentage of subtotal (%)**	**Mean**	**Percentage of subtotal (%)**
ALL REGIONS (*N* = 87)						
Direct Medical Costs						
Laboratory tests	4.20	19	3.29	32	7.49	23
Consultations	0.62	3	1.15	11	1.77	5
Medicines	17.34	78	4.37	42	21.71	67
Bed tariffs	N/A	N/A	1.48	14	1.48	5
Subtotal	*22.16*	*39*	*10.28*	*50*	*32.45*	*42*
Direct Non-Medical Costs						
Transportation	22.49	81	3.17	47	25.66	74
Accommodation	–	0	1.41	21	1.41	4
Food and other expenses	5.24	19	2.15	32	7.39	21
Subtotal	*27.73*	*48*	*6.74*	*33*	*34.46*	*44*
Indirect Costs						
Time costs of person ill	3.05	42	2.84	82	5.89	55
Time cost of caretakers	4.26	58	0.64	18	4.90	45
Subtotal	*7.31*	*13*	*3.48*	*17*	*10.79*	*14*
TOTAL	57.20	74	20.50	26	77.70	100
NORTHERN REGION (N = 33)						
Direct Medical Costs						
Laboratory tests	0.49	1	4.53	30	5.01	10
Consultations	–	0	0.12	1	0.12	0
Medicines	33.31	99	9.98	67	43.28	89
Bed tariffs	N/A	N/A	0.36	2	0.36	1
Subtotal	*33.79*	*42*	*14.99*	*50*	*48.78*	*44*
Direct Non-Medical costs						
Transportation	28.61	84	4.27	52	33	78
Accommodation	–	0	–	0	–	0
Food and other expenses	5.49	16	3.97	48	9	22
Subtotal	*34.10*	*43*	*8.24*	*28*	*42.34*	*39*
Indirect Costs						
Time costs of person ill	4.86	40	5.32	81	10.18	55
Time cost of caretakers	7.27	60	1.21	19	8.49	45
Subtotal	*12.13*	*15*	*6.54*	*22*	*18.67*	*17*
TOTAL	80.03	73	29.76	27	109.79	100
WESTERN REGION (*n* = 20)						
Direct Medical Costs						
Laboratory tests	14.26	38	–	0	14.26	37
Consultations	2.68	7	0.27	18	2.94	8
Medicines	20.44	55	1.20	82	21.65	56
Bed tariffs	N/A	N/A	–	0	–	0
Subtotal	*37.38*	*36*	*1.47*	*22*	*38.85*	*35*
Direct Non-Medical costs						
Transportation	43.30	77	5.08	100	48.39	79
Accommodation	–	0	–	0	–	0
Food and other expenses	13.13	23	–	0	13.13	21
Subtotal	*56.43*	*54*	*5.08*	*76*	*61.52*	*56*
Indirect Costs						
Time costs of person ill	4.57	46	0.17	100	4.75	47
Time cost of caretakers	5.31	54	–	0	5.31	53

(Continued)

TABLE 2 | Continued

	Outpatient		Inpatient		Combined	
	Mean	**Percentage of subtotal (%)**	**Mean**	**Percentage of subtotal (%)**	**Mean**	**Percentage of subtotal (%)**
Subtotal	*9.88*	*10*	*0.17*	*3*	*10.05*	*9*
TOTAL	103.69	94	6.73	6	110.42	100
CENTRAL REGION ($n = 34$)						
Direct Medical Costs						
Laboratory tests	1.89	98	4.01	37	5.90	46
Consultations	–	0	2.68	25	2.68	21
Medicines	0.03	2	0.79	7	0.82	6
Bed tariffs	N/A	N/A	3.42	31	3.42	27
Subtotal	*1.92*	*25*	*10.90*	*56*	*12.82*	*47*
Direct Non-Medical Costs						
Transportation	4.31	93	0.98	16	5.29	48
Accommodation	–	0	3.62	58	3.62	33
Food and other expenses	0.35	7	1.65	26	2.00	18
Subtotal	*4.65*	*60*	*6.26*	*32*	*10.91*	*40*
Indirect Costs						
Time costs of person ill	0.41	36	2.00	81	2.40	67
Time cost of caretakers	0.72	64	0.46	19	1.18	33
Subtotal	*1.13*	*15*	*2.46*	*13*	*3.58*	*13*
TOTAL	7.70	28	19.61	72	27.31	100

[a]*Costs reported in 2019 USD as per person per year. Itemized costs add up to 100% of their respective sub-divisions. For example, laboratory tests, consultations, medicines and bed tariffs represent 100% of direct medical costs. Combining subtotals of direct medical, direct non-medical and indirect costs adds up to 100% of total costs. Likewise, combining outpatient and inpatient costs adds up to 100% of total costs.*

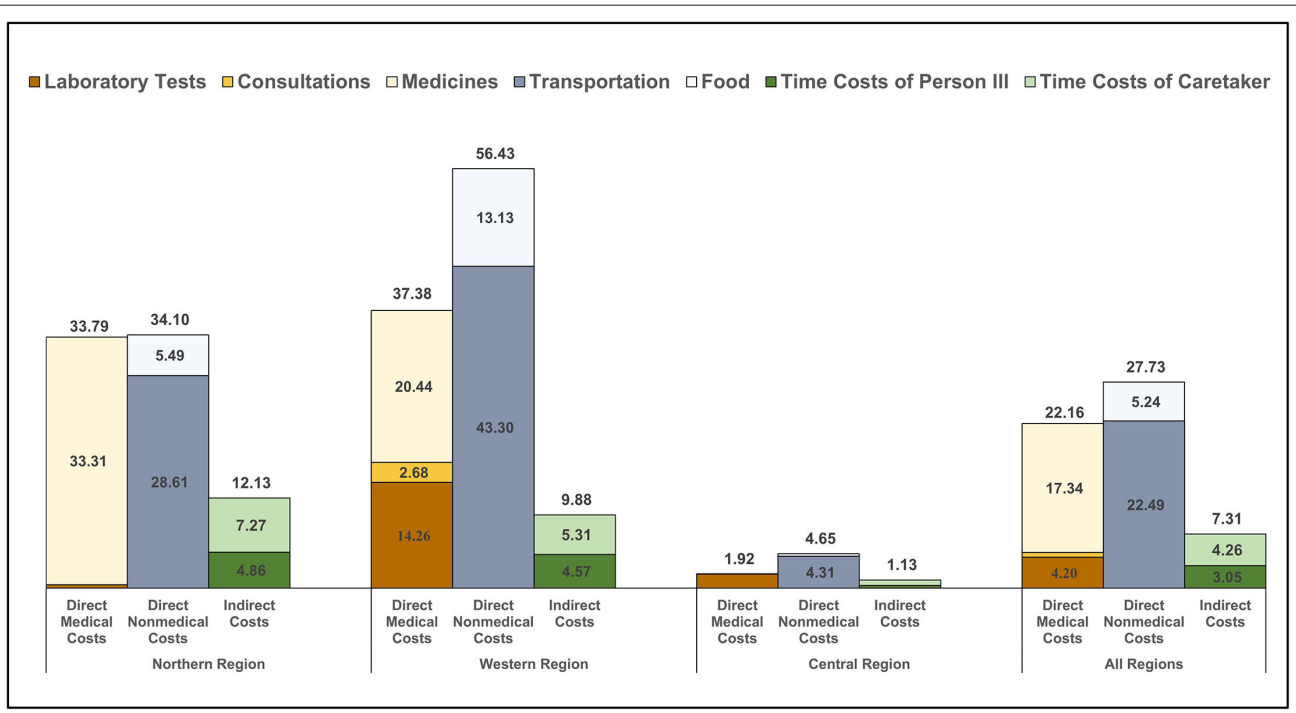

FIGURE 1 | Outpatient costs incurred from receiving care for RHD. The bar graphs show the level and distribution of average costs per person per year for outpatient encounters ($n = 408$). Thirty-three participants from the Northern Region represented 214 visits; 20 participants from the Western Region represented 147 visits; and 34 participants from the Central Region represented 47 visits. Costs are disaggregated by region and by component and include direct and indirect costs, though the latter were low for outpatient care. Costs are presented in 2019 United States dollars.

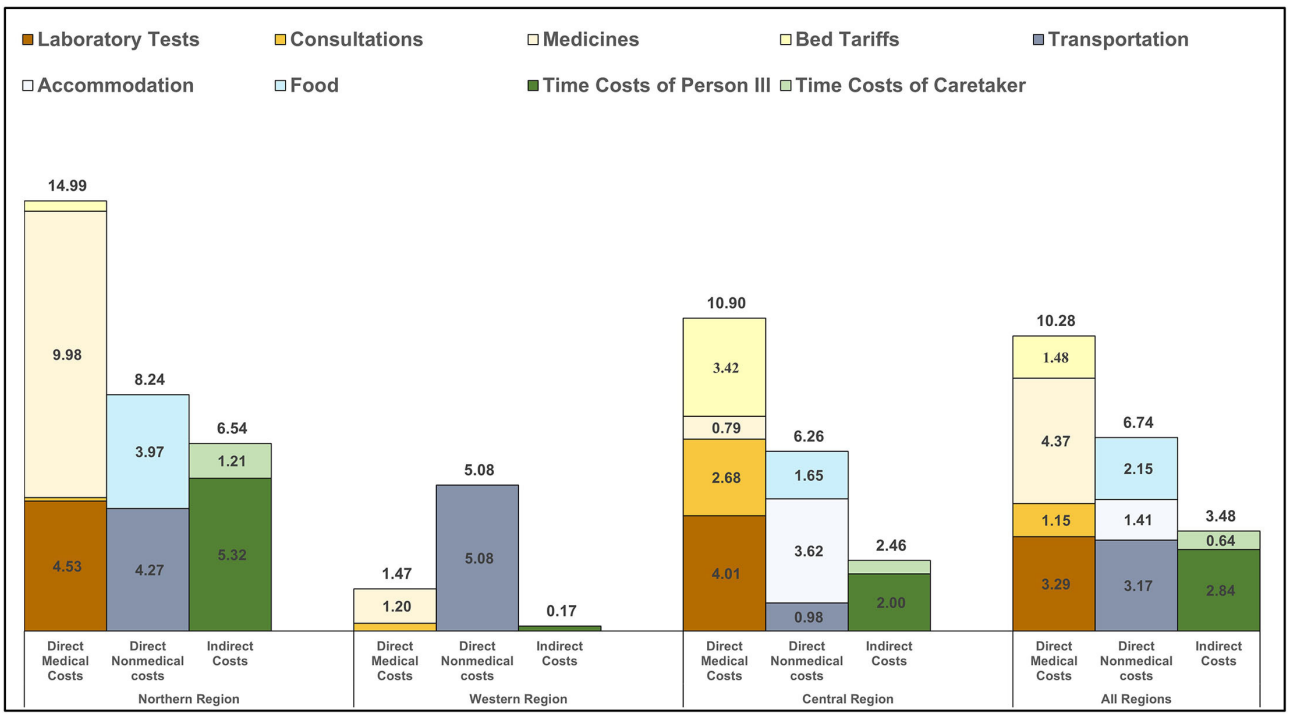

FIGURE 2 | Inpatient costs incurred from receiving care for RHD. The bar graphs show the level and distribution of average costs per person per year for inpatient encounters ($n = 27$). Eleven participants from the Northern Region represented seventeen visits; two participants from the Western Region represented two visits; and eight participants from the Central Region represented eight visits. Costs are disaggregated by region and by component and include direct and indirect costs. Note: costs are presented in 2019 United States dollars.

TABLE 3 | Percentage of households experiencing catastrophic health expenditure by region.

	Northern region ($n = 33$)	Western region ($n = 19$)	Central region ($n = 34$)	Total ($N = 86$)
10% Threshold (%)	52	53	9	35
25% Threshold (%)	30	32	3	20

confirms that RHD is a neglected disease of poverty that results in high healthcare costs, distortions in household economic decision-making, and exacerbation of medical poverty traps.

This study is the second manuscript and the first peer-reviewed study to look at the household economic impact of RHD in the African region. Further, this study adds to the global literature documenting the high costs of chronic diseases to households in low- and middle-income countries—costs which are driven by the long-term nature of healthcare and the high costs of medicines, many of which are not publicly financed (22). Previous research by Oyebamiji in South Africa painted a somewhat more optimistic picture that reflects important differences in health system arrangements between South Africa, an upper-middle-income country, and Uganda, a low-income country. For example, none of the subjects in the South African study incurred direct medical costs (due to a robust free healthcare policy), and the prevalence of coping strategies was much lower than in our study (9). These differences show the positive impact that progressive financing policies can have on households affected by RHD and other chronic health conditions.

Consistent with studies of other health conditions in a range of African countries, transportation costs were a major determinant of OOP costs in our study (23, 24). Because RHD prevention and treatment services are only currently offered at regional referral hospitals in Uganda, and often only in the context of research studies, our study subjects often had to travel significant distances and thus incur considerable costs to receive routine services like monthly antibiotic injections. In addition, retention and medication adherence among individuals enrolled in the Uganda National RHD Registry has been reported to be as low as 41%, which means that it is quite possible that distance to health facilities is a major deterrent to high-quality RHD care (25).

Persons affected by RHD are generally young (average age of 28 years in our study) and can experience substantially better lifetime productivity and quality of life when they have access to regular healthcare, including interventional procedures or heart valve surgeries when their condition deteriorates. Our study demonstrates the strong, mutually reinforcing linkage between chronic disease and poverty. For example, three-quarters of the participants in our study were unemployed and usually had to sacrifice many hours per month traveling to and from

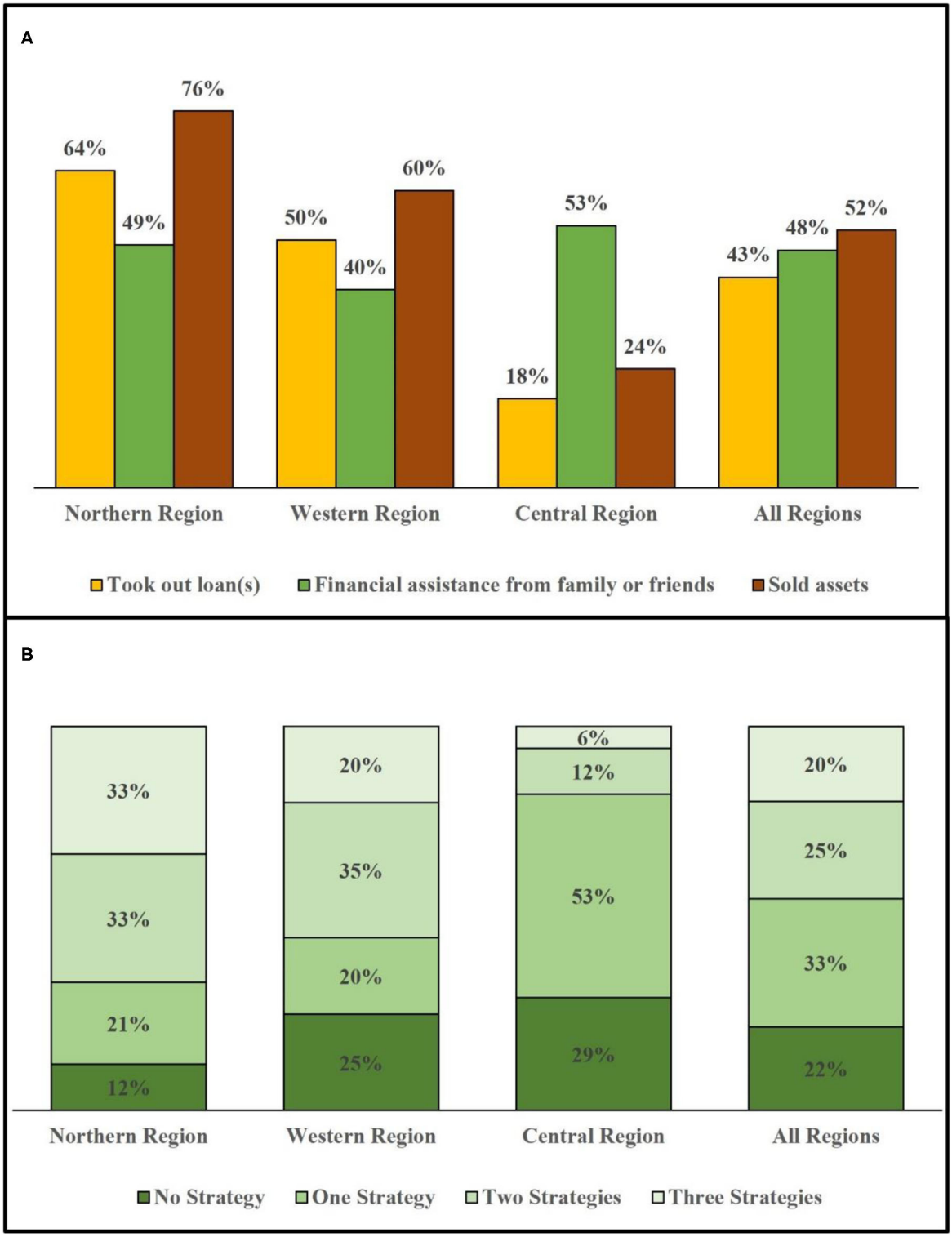

FIGURE 3 | Utilization of financial coping strategies by region. The bar graphs show distribution of financial coping strategies used by households across regions ($n = 87$). This represents the 33 households in the Northern Region, 20 households in the Western Region, and 34 households in the Central Region that were sampled in this study. **(A)** Frequency of each of three coping strategies used. **(B)** Reliance on zero, one, two, or all three coping strategies. Note: Due to rounding, the sum for the Northern region adds to 99%, and the actual number with more decimal points adds to 100%.

TABLE 4 | Effect of demographic and socioeconomic factors on utilization of coping strategies.

	IRR[a]	Robust S.E.[b]	95% CI	*p*-value
DEMOGRAPHIC				
Age	0.9978	0.0044	(0.9892, 1.0064)	0.6105
Gender (reference group: male)	0.8503	0.1063	(0.6655, 1.0863)	0.1945
Secondary education: yes vs. no	0.8053	0.1384	(0.5749, 1.1279)	0.2077
Region (ref: Northern)				
Western	0.8466	0.1452	(0.6049, 1.1848)	0.3315
Central	0.7476	0.1531	(0.5004, 1.1168)	0.1554
SOCIOECONOMIC				
Household income per capita	0.9997	0.0004	(0.999, 1.0004)	0.4083
Employment (reference group: unemployed)	0.8796	0.1881	(0.5785, 1.3375)	0.5486
Private insurance: yes vs. no	1.1793	0.3577	(0.6508, 2.1369)	0.5867
Total direct RHD costs	1.0025	0.0005	(1.0014, 1.0035)	0.0000
INTERCEPT	1.8023	0.2267	(1.4085, 2.3062)	0.0000

[a]*Incident rate ratio,* [b]*standard error.*

health facilities. Since RHD is a disease of poverty, cross-cutting efforts to improve healthcare access and affordability among vulnerable populations in Uganda would prove beneficial for persons with RHD.

While this study does not directly impact clinical decision-making surrounding RHD, it has implications for the organization of cardiovascular health services in Uganda and other countries with similar health system arrangements. Specifically, the current model of highly centralized cardiovascular services shifts many of the costs of RHD from the system onto households and therefore requires immediate reform. We conclude that the Uganda Ministry of Health should experiment with decentralized models of care, at least for services that do not require an in-person, specialized workforce, such as the capture of echocardiography images and administration of prophylactic antibiotics (26). This may have implications for clinical training and expected competencies of non-specialist medical providers. For instance, nurses at local health centers could perform routine focused RHD-screening echocardiograms for remote interpretation. This could assist in the triage of secondary prophylaxis and referral to a cardiologist or surgeon at a regional referral health center. Such referrals may at times be accommodated by telehealth to minimize the barrier of geographical distance.

Additionally, the Government of Uganda does not generally charge user fees for services received at public healthcare facilities. But regional hospitals are authorized to run private patient services and therefore are allowed to charge for certain services within the public hospital. Likewise, medications dispensed from these facilities are typically free of charge—in principle. In practice, though, supply chains for medications are weak, and stock-outs are frequent, especially for medications for cardiovascular diseases (27). The absence of essential medications at public facilities pushes patients to seek care at private pharmacies where these medications are more widely available but are offered on a cost-recovery basis. We strongly suspect that this phenomenon explains our findings regarding the contribution of medication costs to the total OOP costs of RHD care. An important first step in ensuring UHC for RHD will be to invest in supply chains for medications related to RHD, many of which are used for other cardiovascular diseases (e.g., beta-blockers and angiotensin-converting enzyme inhibitors) or for infectious diseases (e.g., benzathine penicillin and azithromycin). This investment would represent a modest subset of the total, long-term cost of a comprehensive RHD program in Uganda. It would, however, provide immediate financial protection to individuals already known to have RHD and would pre-emptively address a key bottleneck to program scale-up.

LIMITATIONS

Our study has a number of important limitations. Our sample size was relatively small and was truncated prematurely due to the ongoing global SARS-CoV-2 pandemic. We sampled participants who were receiving care at three regional referral hospitals and who therefore had the ability to travel potentially far distances. While we attempted to capture the range of socioeconomic and health system variations in the country, our estimates are not statistically generalizable to national averages and may not capture those who are unable to travel to receive RHD care. The accuracy of household survey data is known to decline with long recall periods, so we expect some measurement error to exist for events that were reported to have occurred greater than a few months ago (we chose a 12-month recall period to ensure we captured costly but infrequent events such as hospitalizations). Finally, the prevalence-based approach to conducting a cost-of-illness study may miss important dimensions of costs as compared to an incidence-based approach, such as the evolution of costs with disease progression or inter-temporal consumption smoothing. Despite these important limitations, our study provides crucial emerging insights into the economic consequences of RHD in countries where the disease remains endemic. Our approach and data collection tools (**Supplementary Material**) could prove useful to other researchers, advocates, and health ministries that are seeking to implement the 2018 global resolution on RHD or to build the economic case for greater public investment in RHD control programs.

FUTURE DIRECTIONS

Future research on the household economics of RHD (and other chronic diseases) would benefit from the longitudinal approach to further capture how RHD costs and its socioeconomic consequences evolve over time. These studies should further consider the use of cohorts sampled from the general population, rather than disease registries that are biased toward patients

with more severe disease and an ability or means to travel long distances.

CONCLUSIONS

We demonstrate that Ugandan households seeking care for RHD incur OOP that are very high relative to income. These costs are enhanced in lower-income regions and exacerbate the poverty trap. To achieve UHC in Uganda, the government will need to enact a series of policy reforms that address the major sources of financial hardship faced by individuals affected by RHD and other chronic diseases.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article is available upon request to the authors, without undue reservation.

ETHICS STATEMENT

This study was approved by the Makerere University School of Medicine Research and Ethics Committee (REC RF 2018-082) and by the Uganda National Council for Science and Technology (SS 5081). Written informed consent to participate in this study was provided by the participants or, in the case of minors, their legal guardian or next of kin. In addition, the University of Washington Human Subjects Division approved an earlier version of this study (STUDY00002855) that did not include subjects under the age of ten.

AUTHOR CONTRIBUTIONS

DW conceived the study and acquired funding for the research. YS designed the study with input from DW. JA, RK, and HN collected the data. MN and EN organized the database and supervised the data collection. YD and YK conducted the statistical analysis. AB, EO, DW, and YS reviewed and interpreted the results. CO drafted the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

FUNDING

This study was supported by the American Heart Association (17SFRN33670611, 17SFRN33630027).

ACKNOWLEDGMENTS

We thank Xinpeng Xu for replication of our results that was originally analyzed in R (v3.6.3), using Stata (v15.1). We thank Xinpeng Xu and Emily Chu for valuable comments on the manuscript. We thank Ryan Hoskins for insightful discussions. We also thank Rachel Sarnacki, Peter Lwabi, Craig Sable, Linda Mary Oyella, and Pulle Jafes for helpful team support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.636280/full#supplementary-material

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frontiers in Cardiovascular Medicine

ORIGINAL RESEARCH
published: 04 August 2021
doi: 10.3389/fcvm.2021.694339

Transcatheter Valve-in-Valve Procedures for Bioprosthetic Valve Dysfunction in Patients With Rheumatic vs. Non-Rheumatic Valvular Heart Disease

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OPEN ACCESS

Edited by:
Kai Hang Yiu, The University of Hong Kong, SAR China

Reviewed by:
Neil Ruparelia, Hammersmith Hospital, United Kingdom
Tanja Katharina Rudolph, Heart and Diabetes Center North Rhine-Westphalia, Germany
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Specialty section:
This article was submitted to Heart Valve Disease, a section of the journal Frontiers in Cardiovascular Medicine

Received: *13 April 2021*
Accepted: *29 June 2021*
Published: *04 August 2021*

Citation:
Lopes MP, Rosa VEE, Palma JH, Vieira MLC, Fernandes JRC, de Santis A, Spina GS, Fonseca RdJ, de Sá Marchi MF, Abizaid A, de Brito FS Jr, Tarasoutchi F, Sampaio RO and Ribeiro HB (2021) Transcatheter Valve-in-Valve Procedures for Bioprosthetic Valve Dysfunction in Patients With Rheumatic vs. Non-Rheumatic Valvular Heart Disease. Front. Cardiovasc. Med. 8:694339. doi: 10.3389/fcvm.2021.694339

Background: Bioprosthetic heart valve has limited durability and lower long-term performance especially in rheumatic heart disease (RHD) patients that are often subject to multiple redo operations. Minimally invasive procedures, such as transcatheter valve-in-valve (ViV) implantation, may offer an attractive alternative, although data is lacking. The aim of this study was to evaluate the baseline characteristics and clinical outcomes in rheumatic vs. non-rheumatic patients undergoing ViV procedures for severe bioprosthetic valve dysfunction.

Methods: Single center, prospective study, including consecutive patients undergoing transcatheter ViV implantation in aortic, mitral and tricuspid position, from May 2015 to September 2020. RHD was defined according to clinical history, previous echocardiographic and surgical findings.

Results: Among 106 patients included, 69 had rheumatic etiology and 37 were non-rheumatic. Rheumatic patients had higher incidence of female sex (73.9 vs. 43.2%, respectively; $p = 0.004$), atrial fibrillation (82.6 vs. 45.9%, respectively; $p < 0.001$), and 2 or more prior surgeries (68.1 vs. 32.4%, respectively; $p = 0.001$). Although, device success was similar between groups (75.4 vs. 89.2% in rheumatic vs. non-rheumatic, respectively; $p = 0.148$), there was a trend toward higher 30-day mortality rates in the rheumatic patients (21.7 vs. 5.4%, respectively; $p = 0.057$). Still, at median follow-up of 20.7 [5.1–30.4] months, cumulative mortality was similar between both groups ($p = 0.779$).

Conclusion: Transcatheter ViV implantation is an acceptable alternative to redo operations in the treatment of patients with RHD and severe bioprosthetic valve

dysfunction. Despite similar device success rates, rheumatic patients present higher 30-day mortality rates with good mid-term clinical outcomes. Future studies with a larger number of patients and follow-up are still warranted, to firmly conclude on the role transcatheter ViV procedures in the RHD population.

Keywords: heart valve prosthesis, rheumatic heart disease, bioprosthesis, mitral valve, aortic valve, transcatheter valve-in-valve, transapical access, transeptal access

INTRODUCTION

Rheumatic heart disease (RHD) is a prevalent condition, especially in low- and middle-income countries. The Global Burden of Disease study estimated 10.5 million disability-adjusted life years and 319,499 deaths in 2015 due to RHD (1). In Brazil, the estimated annual incidence reaches 30,000 new cases per year, leading to a high cardiac mortality of −8% (2–4). Of note, RHD population has singular characteristics comparing to other etiologies of valvular heart disease. In general, RHD patients are operated at a younger age and undergo several open-heart surgeries during their lifetime, due to structural valve degeneration (SVD) which occurs earlier in these patients who are first-time operated at a very young age. The current standard treatment for degenerated bioprosthesis involves redo open-heart surgery. However, for many RHD patients, with multiple co-morbidities, such as left ventricular dysfunction, pulmonary artery hypertension and prior surgical procedures, a conventional reoperation poses additional risks.

Transcatheter valve interventions have been established as an alternative to conventional surgical interventions in recent years, initially for patients with severe aortic stenosis of various surgical risk profiles. More recently, this procedure has also been evaluated in patients with bioprosthetic valve failure [valve-in-valve (VIV)] in aortic, mitral and tricuspid positions (4–6), with acceptable clinical outcomes in the short- and long-term follow-up (1, 7–13). The aim of this study was therefore to evaluate the clinical characteristics and outcomes in rheumatic vs. non-rheumatic patients undergoing ViV procedures for severe bioprosthetic valve dysfunction.

MATERIALS AND METHODS

Study Population

Single center prospective study including consecutive patients undergoing transcatheter ViV implantation, from May 2015 to September 2020. All cases were thoroughly discussed by the institutional Heart Valve Team, and patients were elected for transcatheter approach based on (i) preoperative risk assessment (STS $\geq$ 8.0% or EuroSCORE II $\geq$ 6.0%), (ii) presence of comorbidities, (iii) number of previous surgical interventions, (iiii) frailty and other clinical conditions.

Rheumatic etiology of the native valve disease was considered according to the referred clinical history, previous echocardiographic and surgical findings. Exclusion criteria were: (i) active endocarditis, (ii) presence of prosthetic valve thrombosis or thrombus in the left ventricle, and (iii) paravalvular regurgitation. The occurrence of thrombus in the left atrial appendage was considered a relative contraindication and evaluated individually. The study protocol was reviewed and approved by the local institutional ethics committee. All patients provided written informed consent for the procedures.

Preoperative Planning

All patients underwent transthoracic echocardiographic analysis and, whenever necessary, a tridimensional transesophageal was performed. A cardiac gated thoracic computed tomography was also performed in order to obtain adequate measurement of the degenerated bioprosthetic valve's internal diameter (i.e. the True ID), in addition to other important measurements as previously described (14–16). Measurements were performed under multi-planar reconstruction, using OsiriX® Platform, and 3D reconstruction was performed to calculate the ideal fluoroscopic angulation for valve deployment. Coronary angiography was performed routinely, and precluded, at the Heart Team's discretion, if renal function was critical.

Valve-in-Valve Procedure

Transcatheter procedures were performed routinely in hybrid operating room, according to standard techniques. Procedures were guided by transesophageal echocardiography and fluoroscopy using prosthesis metallic rings to position the transcatheter valve.

The self-expandable CoreValve and Evolut R (Medtronic, Minneapolis, MN), the balloon-expandable Sapien XT and Sapien 3 (Edwards Lifesciences, Irvine, CA) and the balloon-expandable Inovare (Braile Biomedica, Sao Jose do Rio Preto, SP) prostheses were used, at the discretion of the operator.

Data Collection and Analysis

Pre and postoperative data were prospectively collected and entered into our institutional database. Data regarding 30-days outcomes and follow-up were retrospectively analyzed according to the Mitral Valve Academic Research Consortium (MVARC-2) and Aortic Valve Academic Research Consortium-2 (VARC-2) (17, 18). Continuous variables were presented as mean $\pm$ SD or median (interquartile range). Categorical variables were presented as percentages. Kolmogorov-Smirnov test was used to test normality of the variable. *T* test or Mann-Whitney test was applied for continuous variables, and Fisher exact test or χ^2 test was applied for categorical variables, as appropriate. Log transformation was applied to normalize the distribution of STS score, creatinine, left ventricular end-diastolic diameter and left ventricular end-diastolic volume. Age, left ventricular end-systolic diameter, left ventricular end-systolic volume and

left ventricular ejection fraction were analyzed using Mann-Whitney test. A logistic regression analysis was used to evaluate the predictors of device success. For mitral procedures, a MVARC modified criteria of device success was used as follow: absence of (i) procedural death, (ii) malposition/embolization/migration, (iii) second transcatheter heart valve, (iv) left ventricular outflow tract obstruction and (v) stroke. (19). For the aortic procedures, the VARC-2 criteria was used: (i) absence of procedural mortality, (ii) correct positioning of a single prosthetic heart valve into the proper anatomical location, (iii) no prosthesis–patient mismatch, (iv) mean aortic valve gradient <20 mmHg and (v) no moderate or severe prosthetic valve regurgitation (18). For the tricuspid ViV procedures, the following criteria was used: (i) absence of reintervention, endocarditis or valve thrombus, (ii) absence of moderate or severe regurgitation and (iii) absence of mean gradient ≥10 mmHg (4). Time-to-event analyses were performed using Kaplan-Meier estimates and groups were compared using log-rank test. All analyses were conducted using the statistical package SPSS, version 20 (IBM, Armonk, NY).

RESULTS

Patient Characteristics

The main baseline clinical, laboratory and echocardiographic data are shown in **Table 1**. Among 106 patients included, 65.1% ($n = 69$) had rheumatic etiology and 34.9% ($n = 37$) had non-rheumatic etiology. The main non-rheumatic etiologies were mitral valve prolapse (32.4%), degenerative aortic stenosis (21.6%), congenital valve disease (16.2%) and post-endocarditis (13.5%). There were no demographic differences between rheumatic and non-rheumatic patients, except for more female sex (73.9 vs. 43.2%, respectively; $p = 0.004$) and atrial fibrillation (82.6 vs. 45.9%, respectively; $p < 0.001$) in rheumatic patients. There was also no difference regarding the median number of previous surgeries between rheumatic and non-rheumatic patients, nonetheless when stratified by the number of procedures, rheumatic patients had a greater number of ≥2 previous surgeries than non-rheumatic (68.1 vs. 32.4%, respectively; $p = 0.001$). In addition, rheumatic patients had smaller left ventricular end-diastolic diameter than non-rheumatic patients (50.0 ± 6.6 vs. 54.6 ±10.7 mm, respectively; $p = 0.020$), and higher pulmonary artery systolic pressure (62.3 ± 19.7 vs. 52.7 ± 17.1 mmHg, respectively; $p = 0.029$). No other echocardiographic differences were seen between groups. The mechanisms for surgical biosprosthetic failure were similar between the groups, as detailed in **Table 1**.

Procedural Data

The main baseline procedural data are shown in **Table 2**. We found no difference between rheumatic vs. non-rheumatic patients regarding time between bioprosthesis implantation and ViV procedure (12 [9–16] vs. 12 [7–15] years, respectively; $p = 0.871$). Rheumatic patients underwent more mitral valve-in-valve procedure than non-rheumatic (81.2 vs. 45.9%, respectively; $p = 0.025$), while non-rheumatic patients underwent more aortic and tricuspid ViV procedures, both in 27.0% of patients, respectively. Examples of ViV procedures with the different

TABLE 1 | Baseline clinical, laboratory and echocardiographic data of the study population.

	Rheumatic (*N* = 69)	Non-rheumatic (*N* = 37)	*P* value
Clinical data			
Age, years	63 ± 10	59 ±22	0.684
Body surface area, m²	1.61 ± 0.14	1.66 ± 0.19	0.170
Female sex	51 (73.9)	16 (43.2)	0.004
Diabetes	10 (14.5)	7 (18.9)	0.753
Hypertension	36 (52.2)	22 (59.5)	0.608
Poor mobility	16 (23.2)	6 (16.2)	0.554
Atrial fibrillation	57 (82.6)	17 (45.9)	<0.001
Coronary artery disease	14 (20.3)	8 (21.6)	1.000
Previous heart failure hospitalization	13 (18.8)	8 (21.6)	0.931
Number of previous surgeries	2 (3–1)	1 (2–1)	0.103
Total of previous surgeries			0.061
1	20 (29.0)	22 (59.5)	
2	22 (31.9)	4 (10.8)	
3	20 (29.0)	6 (16.2)	
4	3 (4.3)	2 (5.4)	
5	2 (2.9)	2 (5.4)	
6	2 (2.9)	1 (2.7)	
≥2 previous surgeries	47 (68.1)	12 (32.4)	**0.001**
STS-PROM score, %	7.93 ±5.47	6.61 ±5.07	0.174
Symptoms			
NYHA class			0.547
I	4 (5.8)	6 (16.2)	
II	18 (26.1)	7 (18.9)	
III	30 (43.5)	14 (37.8)	
IV	17 (24.6)	10 (27.0)	
Angina	9 (13.0)	3 (8.1)	0.535
Laboratory			
Hemoglobin, mg/dl	11.98 ± 1.94	12.41± 2.14	0.315
Creatinine, mg/dl	1.24± 0.55	1.24 ± 0.47	0.935
Baseline echocardiography			
LVED diameter, mm	50.03 ± 6.60	54.68 ±10.72	**0.020**
LVES diameter, mm	33.82 ± 6.18	38.32 ± 11.52	0.147
LVED volume, ml	117.47 ± 41.55	137.80 ± 71.32	0.254
LVES volume, ml	51.02 ± 36.50	63.53 ± 55.15	0.226
LVEF, %	59.8 ±9.6	55.0 ± 11.3	0.265
PSAP, mmHg	62.3 ± 19.7	52.7 ± 17.1	**0.029**
Valve Failing Mechanism			
Aortic	**(*N* = 6)**	**(*N* = 10)**	0.271
Stenosis	2 (33.3)	6 (60)	
Regurgitation	3 (50)	4 (40)	
Mixed	1 (16.7)	-	
Mitral	**(*N* = 59)**	**(*N* = 17)**	0.170
Stenosis	27 (45.8)	7 (41.2)	
Regurgitation	24 (40.7)	10 (58.8)	
Mixed	4 (6.8)	-	

Values are n (%), mean (±SD) or median [IQR]. NYHA, New York Heart Association; eGFR, estimated glomerular filtration; STS-PROM, Society of Thoracic Surgeons predicted risk of mortality; LVED, Left ventricular end-diastolic; LVES, Left ventricular end-systolic; LVEF, left ventricular ejection fraction; PSAP, pulmonary systolic arterial pressure. The bold values refer p-values <0.05 (statistically significant).

TABLE 2 | Procedural data of the study population.

Procedure data	Rheumatic (*N* = 69)	Non-rheumatic (*N* = 37)	*P* value
Device success*	52 (75.4)	33 (89.2)	0.148
Pre-dilatation	1 (1.4)	6 (16.2)	**0.007**
Post-dilatation	23 (33.3)	8 (22.2)	0.337
Access			**0.008**
Transapical	63 (92.6)	26 (70.3)	
Jugular	1 (1.4)	5 (13.5)	
Transfemoral	2 (2.9)	3 (8.1)	
Transeptal	2 (2.9)	3 (8.1)	
Position			**0.025**
Mitral	56 (81.2)	17 (45.9)	
Aortic	6 (8.7)	10 (27.0)	
Tricuspid	0	10 (27.0)	
Multiple	7 (10.1)	0	
Length of in-hospital stay, days	18 (30–10.5)	16 (24.5–8)	0.425

*Values are mean ± SD, median (interquartile range), or n (%). *According to the modified Mitral Valve Academic Research Consortium (MVARC-2) without hemodynamic criteria (SIMONATO) and the Aortic Valve Academic Research Consortium-2 (VARC-2) (9, 10, 19). The bold values refer p-values <0.05 (statistically significant).*

transcatheter valves in the various positions are shown in **Figure 1**. Most of RHD patients underwent transapical access (92.6%) while non-rheumatic patients had transapical access in 70.3%, jugular access in 13.5%, and both transfemoral and transeptal in 8.1% of patients, respectively ($p = 0.008$). Despite the differences in procedural characteristics, device success rate was similar between the groups (75.4 vs. 89.2% in rheumatic vs. non-rheumatic, respectively; $p = 0.148$). There was no significant predictor of device success in the univariable analysis (**Supplementary Table 1**).

Clinical Outcomes and Follow-Up

The main procedural outcomes are shown in **Table 3**. There were no significant differences between groups. The most frequent post-procedural complications were major bleeding and the need for packed red blood cells transfusion in 10.4 and 14.2% of patients, respectively. Also, up to 15.1% of the patients presented acute kidney injury, needing dialysis in 2.8% of them, and valve-related dysfunction requiring valve surgery occurred in 5 patients, being 4 (5.8%) and 1 (2.7%) in rheumatic vs. non-rheumatic patients respectively ($p = 0.656$). At 30-days, there was a trend toward higher mortality rates in rheumatic vs. non-rheumatic patients (21.7 vs. 5.4%, respectively; $p = 0.057$). In the univariable analysis (**Supplementary Table 2**) rheumatic etiology was significantly associated with 30-day mortality (OR 4.861, 95% CI 1.047–22.573, $p = 0.044$). At a median follow-up of 20.7 [5.1–30.4] months, rheumatic etiology was not associated with mid-term mortality (HR 0.873, 95% CI 0.337–2.259, $p = 0.779$; **Figure 2**). Post-procedure echocardiographic data, as prosthesis mismatch and prosthetic paravalvular leak (PVL), are demonstrated in **Supplementary Table 3**.

In our sample, five cases have required further surgery: (1) exploratory thoracotomy due to hemothorax because of a laceration of an intercostal vein; (2 and 3) left ventricle laceration; (4) hemostasis revision; (5) hypertensive pneumothorax during central venous puncture. One patient required a second valve implantation due to migration of the prosthesis into the left atrium (without embolization). There seems to be no relationship between complications and valve disease etiology, however the number of patients who required new interventions is quite small and does not allow us from drawing firm conclusions in that regard. Clinical, laboratory, echocardiographic data and 30-day outcomes of patients that underwent mitral ViV procedure are shown in the **Supplementary Table 4**. In these patients, there was a higher incidence of baseline atrial fibrillation (88.9 vs. 58.8%; $p = 0.008$), and a higher number of previous surgeries in the rheumatic group ($p < 0.001$). In the analysis excluding tricuspid cases (**Table 4**), we found several differences between the two groups (rheumatic vs. non-rheumatic) related to age, sex, atrial fibrillation, creatinine, LVED diameter, LVEF, mechanical assisting device and number of previous surgeries (all with $p < 0.05$).

DISCUSSION

The main findings of this initial series, comparing transcatheter ViV procedures in patients with rheumatic vs. non-rheumatic severe bioprosthetic valve disfunction, were that although RHD patients presented a higher risk profile that included higher rates of female sex, atrial fibrillation and pulmonary hypertension, procedural success rates were similar between rheumatic vs. non-rheumatic patients. Also, despite a trend toward higher rates of mortality in the short-term, over a median follow-up of 20 months rheumatic etiology was not associated with increased mortality.

RHD is the main cause of acquired heart disease and cardiovascular mortality in young people worldwide. It is a condition of global importance as it is estimated ∼36 million affected patients, ensuing in ∼250,000 deaths per year, most often in underserved populations (20–22). These patients undergo cardiac surgery at younger age and are more frequently women with a higher burden of comorbidities (1). This is also the case of our study, so that RHD patients were ∼2-fold more frequently women with atrial fibrillation, alongside a 10 mmHg higher mean PSAP. Furthermore, such rheumatic patients are frequently selected to a biological rather than mechanical valve, due to sociocultural context, comprising difficulties in keeping adequate warfarin control and risks of complications associated with the mechanical prosthesis (1, 7, 22). In developing countries, warfarin anticoagulation presents many logistic difficulties, including lack of facilities in close proximity to monitor the international normalized ratio (INR), employment activities with a greater risk of trauma and the large number of females in child-bearing age who become pregnant (23). These difficulties with anticoagulation, together with the more widespread availability of transcatheter valves, have encouraged in the last years the use of bioprosthetic valves by >70%, including new surgical valves

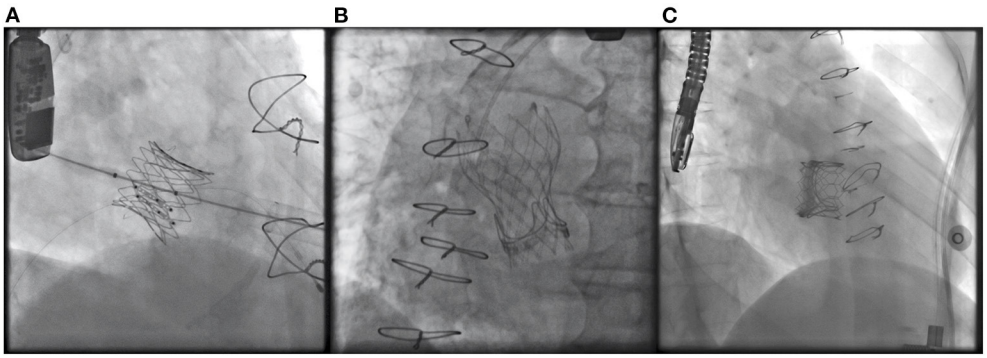

FIGURE 1 | Case examples of a **(A)** balloon expandable Inovare bioprosthesis implanted in the tricuspid position. **(B)** Self-expandable Evolut R bioprosthesis implanted in the aortic position. **(C)** Balloon expandable Sapien 3 valve implanted in the mitral position.

TABLE 3 | 30-day clinical outcomes.

Outcomes	Rheumatic (*N* = 69)	Non-rheumatic (*N* = 37)	*P* value
Procedure mortality	1 (1.4)	1 (2.7)	1.000
In-hospital myocardial infarction	4 (5.8)	1 (2.7)	0.656
Major vascular complication	3 (4.3)	1 (2.7)	1.000
Major bleeding	7 (10.1)	4 (10.8)	1.000
Red blood cells transfusion	11 (15.9)	4 (10.8)	0.667
Acute kidney injury	10 (14.5)	6 (16.2)	1.000
Sepsis	19 (27.5)	6 (16.2)	0.285
Valve-related dysfunction requiring second valve implantation	0	1 (2.7)	0.349
Valve-related dysfunction requiring valve surgery	4 (5.8)	1 (2.7)	0.656
New pacemaker implantation	1 (1.4)	1 (2.7)	1.000
New-onset atrial fibrillation	6 (8.7)	6 (16.2)	0.332
Left bundle branch block	1 (1.4)	0	1.000
Mechanical assisting device			**0.087**
IABP	5(7.2)	3 (8.1)	
ECMO	0	2 (5.4)	
IABP + ECMO	0	1 (2.7)	
30-day re-hospitalization	0	1 (2.7)	0.206
30-day mortality	15 (21.7)	2 (5.4)	0.057

Values are mean ± SD, median (interquartile range), or n (%). IABP, intra-aortic balloon pump; ECMO, extracorporeal membrane oxygenation. The bold values refer p-values <0.05 (statistically significant).

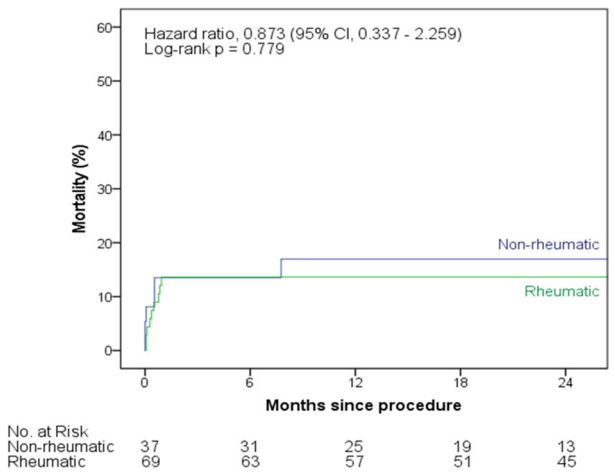

FIGURE 2 | Kaplan-Meier survival curves at 2-year follow-up, according to rheumatic vs. non-rheumatic valve disease.

with improved anticalcification treatment (24). Nonetheless, the use of bioprosthetic valves in rheumatic patients have a lower long-term performance and durability compared to other valve disease etiologies (25, 26). Thus, rheumatic patients often present with multiple previous open-heart surgeries, due to the natural biological valve prosthesis degeneration, also with higher rates (∼20%) of simultaneous severe multivalvular disease requiring intervention (7, 27, 28).

In our study, the median number of prior surgical procedures was 2-fold higher in the RHD patients, so that 68.1% of them had ≥2 prior surgeries vs. 32.4% of non-rheumatic. Collectively such factors, including higher risk profile, together with multiple prior surgical procedures, can magnify the risks of morbidity and mortality up to ∼2–3-fold (26–29). Not surprinsingly, despite similar rates of device success between rheumatic vs. non-rheumatic patients, mortality at the short-term was somewhat higher in RHD patients (21.7 vs. 5.4% in rheumatic vs. non-rheumatic patients, respectively). These relatively high mortality rates are similar to prior literature, where 30-day and 1 year mortality rates in mitral and aortic ViV procedures were ∼8 and ∼20%, respectively, although especific data in RHD patients undergoin transcatheter procedures are lacking (6, 19, 29, 30).

Furthermore, mortality risk for reoperation in patients with degenerated bioprosthesis ranges from 1.5 up to 23% (31). Valve surgery mortality increases proportionally to the number of previous operations, reaching the prohibitive value of 40% in the fourth mitral valve replacement (7, 32). Recent

TABLE 4 | Baseline clinical, laboratory, echocardiographic data and 30-day outcomes of patients undergoing mitral or aortic valve-in-valve procedure (excluding tricuspid valve-in-valve procedure).

	Rheumatic (*N* = 69)	Non-rheumatic (*N* = 27)	*P* value
Clinical data			
Age, years	65 (57–70)	72 (65–81)	**0.023**
Body surface area, m²	1.6 (1.5–1.7)	1.7 (1.6–1.8)	0.069
Female sex	51 (73.9)	12 (44.4)	**0.013**
Diabetes	10 (14.5)	7 (25.9)	0.236
Hypertension	36 (52.2)	20 (74.1)	0.084
Poor mobility	16 (23.2)	5 (18.5)	0.823
Atrial fibrillation	57 (82.6)	13 (48.1)	**0.002**
Coronary artery disease	14 (20.3)	8 (29.6)	0.478
Previous heart failure hospitalization	14 (20.3)	5 (18.5)	1.000
Total of previous surgeries			**<0.001**
1	22 (31.9)	23 (85.2)	
2	22 (31.9)	3(11.1)	
3	19 (27.5)	1 (3.7)	
4	4 (5.8)	-	
5	1 (1.4)	-	
6	1 (1.4)	-	
STS-PROM score, %	6.61 (3.6–11.0)	5.64 (3.9-10.5)	1.000
Symptoms			
NYHA class			0.676
I	4 (5.8)	2 (7.4)	
II	18 (26.1)	4 (14.8)	
III	30 (43.5)	13 (48.1)	
IV	17 (24.6)	8 (29.6)	
Angina	9 (13)	3 (11.1)	1.000
Laboratory			
Hemoglobin, mg/dl	12.3 (10.4–13.5)	11.8 (11.0–13.4)	0.744
Creatinine, mg/dl	1.11 (0.9–1.3)	1.32 (1.0–1.5)	**0.023**
Baseline echocardiography			
LVED diameter, mm	50 (45.0–54.2)	57 (48–63)	**0.033**
LVES diameter, mm	33 (29.5–36.0)	39 (29.5–44.5)	0.06
LVED volume, ml	113 (88.0–132.2)	132.5 (94.5-167.0)	0.797
LVES volume, ml	42 (31–54)	60 (32.7–70.0)	0.139
LVEF, %	61 (56.2–66.0)	55 (45–63)	**0.037**
PSAP, mmHg	59.5 (45.5–73.5)	55 (45–63)	0.364
Procedure data			
Device success*	52 (75.4)	23 (85.2)	0.440
Pre-dilatation	1 (1.4)	3 (11.1)	0.066
Post-dilatation	23 (33.3)	6 (22.2)	0.413
Access			0.558
Transapical	63 (92.6)	26 (96.3)	
Jugular	1 (1.5)	-	
Transfemoral	2 (2.9)	1 (3.7)	
Transeptal	2 (2.9)	-	
Length of in-hospital stay, days	18 (10–30)	16 (8–25)	0.514
Outcomes			
Procedure mortality	1 (1.4)	1 (3.7)	0.486
In-hospital myocardial infarction	4 (5.8)	1 (3.7)	1.000
Major vascular complication	3 (4.3)	-	0.557
Major bleeding	7 (10.1)	4 (14.8)	0.497
Red blood cells transfusion	11 (15.9)	4 (14.8)	1.000
Acute kidney injury	10 (14.5)	6 (22.2)	0.373
Sepsis	19 (27.5)	6 (22.2)	0.783
Valve-related dysfunction requiring second valve implantation	-	1(3.7)	0.281
Valve-related dysfunction requiring valve surgery	4 (5.8)	1 (3.7)	1.000
New pacemaker implantation	1 (1.4)	1 (3.7)	0.486
New-onset atrial fibrillation	6 (8.7)	6 (23.1)	0.083
Left bundle branch block	1 (1.4)	-	1.000
Mechanical assisting device			**0.038**
IABP	5 (7.2)	3 (11.1)	
ECMO	-	2 (7.4)	
30-day re-hospitalization	3 (5.6)	4 (22.2)	0.061
30-day mortality	15 (21.5)	2 (7.4)	0.139

*Values are n (%) or median [IQR]. *According to the modified Mitral Valve Academic Research Consortium (MVARC-2) without hemodynamic criteria and the Aortic Valve Academic Research Consortium-2 (VARC-2) (9, 10, 18, 19). ECMO, extracorporeal membrane oxygenation; eGFR, estimated glomerular filtration; IABP, intra-aortic balloon pump; LVED, Left ventricular end-diastolic; LVEF, left ventricular ejection fraction; LVES, Left ventricular end-systolic; NYHA, New York Heart Association; PSAP, pulmonary systolic arterial pressure; STS-PROM, Society of Thoracic Surgeons predicted risk of mortality. Bold values denote statistical significance.*

meta-analysis showed that ViV is associated with lower rate of MACE, bleeding and short hospitalization complications when compared to re-do surgery, being a reasonable treatment option (33). Besides, ViV procedure does not contraindicate further surgical or transcatheter procedures in the future, which is one of the advantages of transcatheter procedure. Nonetheless, it is important to consider several challenges when a surgical procedure is foreseen after a transcatheter procedure. For instance, in the context of aortic position several challenges may be encountered regarding the smaller annular size, possibly associating with worse hemodynamics in the future in case of a TAVI-in-TAVI, in addition to coronary access and eventually coronary obstruction. These issues have been evaluated recently in some registries worldwide (13), but data is lacking on ViV in patients with RHD vs. those with non-rheumatic etiology.

Of note, despite the higher short-term mortality seen in our study, at a median follow-up of 20 months rheumatic etiology

was not significantly associated with longer term mortality. Most of our patients were treated using the transapical approach, that is known to jeopardize transcatheter outcomes, as compared to transfemoral and transeptal approaches (34). Therefore, the relatively low number of events at 30-days and in the follow-up precluded us from drawing firm conclusions on whether the rheumatic factor itself or the higher burden of comorbidities are resposible for such relatively higher mortality rates in the rheumatic patients, and this will have to be the scope of future larger studies. Likewise, future studies evaluating the different approaches and potential for transfemoral and transeptal approaches in improving clinical outcomes should also be evaluated in the near future.

LIMITATIONS

This is a single-center, non-randomized data-base analyses, that despite the prospective data collection, has the limitations associated with the study design. For instance, there was no data available regarding ViV procedure duration in each group and post-procedure hemodynamic data (such as prosthesis-patient mismatch, gradients, and PVL) were evaluated only by echocardiography and not invasively. The number of patients was relatively small, albeit large for this clinical entity and procedure, with limited number of events that precluded the performance of a multivariable analysis. Therefore, the differences in baseline characteristics, such as the higher prevalence of pulmonary hypertension and atrial fibrillation in rheumatic group, may have influenced the distinct 30-day mortality rates, besides the lack of association in the long-term mortality in the univariable analysis. However, it is important to emphasize that our study is a real-world registry that represent the current practices. Learning curve may have also played a role in the different outcomes, as shown in our series of the first 50 cases undergoing mitral ViV procedures (7).

Another important point is the lack of data on transcatheter valve durability in rheumatic patients undergoing ViV procedure and given our median follow-up of 20 months merit additional evaluation in future larger studies with longer-term follow-up. Of note, recent studies in the field have shown good valve durability up to 8-years in aortic ViV procedures (35) and up to 4-years in mitral procedures (19).

CONCLUSION

In conclusion, transcatheter ViV implantation is an acceptable alternative to redo operations in the treatment of patients with RHD and severe bioprosthetic valve dysfunction. Despite similar device success rates, rheumatic patients present higher 30-day mortality rates with good mid-term clinical outcomes. Future studies with a larger number of patients and follow-up will have to conclude on the role of transcatheter ViV procedures in RHD population.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comissão de Ética para Análise de Projetos de Pesquisa—CAPPesq. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ML, VR, and HR were the main responsible for the analysis of the data and paper writing. JP, AA, HR, MV, and FB performed the valve-in-valve procedures. JF, AS, RF, MS, and GS were responsible for data collection in medical records. FT, RS, and HR were responsible for the idealization of the study. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.694339/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Cardiovascular Medicine

TYPE Original Research
PUBLISHED 06 February 2023
DOI 10.3389/fcvm.2022.919700

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Heart Valve Disease, a section of the journal Frontiers in Cardiovascular Medicine

RECEIVED 13 April 2022
ACCEPTED 29 December 2022
PUBLISHED 06 February 2023

CITATION
Kirvan CA, Canini H, Swedo SE, Hill H, Veasy G, Jankelow D, Kosanke S, Ward K, Zhao YD, Alvarez K, Hedrick A and Cunningham MW (2023) IgG2 rules: N-acetyl-β-D-glucosamine-specific IgG2 and Th17/Th1 cooperation may promote the pathogenesis of acute rheumatic heart disease and be a biomarker of the autoimmune sequelae of *Streptococcus pyogenes*. *Front. Cardiovasc. Med.* 9:919700. doi: 10.3389/fcvm.2022.919700

IgG2 rules: N-acetyl-β-D-glucosamine-specific IgG2 and Th17/Th1 cooperation may promote the pathogenesis of acute rheumatic heart disease and be a biomarker of the autoimmune sequelae of *Streptococcus pyogenes*

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Antecedent group A streptococcal pharyngitis is a well-established cause of acute rheumatic fever (ARF) where rheumatic valvular heart disease (RHD) and Sydenham chorea (SC) are major manifestations. In ARF, crossreactive antibodies and T cells respond to streptococcal antigens, group A carbohydrate, N-acetyl-β-$_D$-glucosamine (GlcNAc), and M protein, respectively, and through molecular mimicry target heart and brain tissues. In this translational human study, we further address our hypothesis regarding specific pathogenic humoral and cellular immune mechanisms leading to streptococcal sequelae in a small pilot study. The aims of the study were to (1) better understand specific mechanisms of pathogenesis in ARF, (2) identify a potential early biomarker of ARF, (3) determine immunoglobulin G (IgG) subclasses directed against GlcNAc, the immunodominant epitope of the group A carbohydrate, by reaction of ARF serum IgG with GlcNAc, M protein, and human neuronal cells (SK-N-SH), and (4) determine IgG subclasses deposited on heart tissues from RHD. In 10 pediatric patients with RHD and 6 pediatric patients with SC, the serum IgG2 subclass reacted significantly with GlcNAc, and distinguished ARF from 7 pediatric patients with uncomplicated pharyngitis. Three pediatric patients who demonstrated only polymigrating arthritis, a major manifestation of ARF and part of the Jones criteria for diagnosis, lacked the elevated IgG2 subclass GlcNAc-specific reactivity. In SC, the GlcNAc-specific IgG2 subclass in cerebrospinal fluid (CSF) selectively targeted human neuronal cells as well as GlcNAc in the ELISA.

In rheumatic carditis, the IgG2 subclass preferentially and strongly deposited in valve tissues (n = 4) despite elevated concentrations of IgG1 and IgG3 in RHD sera as detected by ELISA to group A streptococcal M protein. Although our human study of ARF includes a very small limited sample set, our novel research findings suggest a strong IgG2 autoantibody response against GlcNAc in RHD and SC, which targeted heart valves and neuronal cells. Cardiac IgG2 deposition was identified with an associated IL-17A/IFN-γ cooperative signature in RHD tissue which displayed both IgG2 deposition and cellular infiltrates demonstrating these cytokines simultaneously. GlcNAc-specific IgG2 may be an important autoantibody in initial stages of the pathogenesis of group A streptococcal sequelae, and future studies will determine if it can serve as a biomarker for risk of RHD and SC or early diagnosis of ARF.

KEYWORDS

acute rheumatic fever, Th17 cells, IgG subclass, autoimmunity, group A streptococci

Introduction

Acute rheumatic fever (ARF) is a multisystem inflammatory sequela of group A streptococcal (GAS) pharyngitis where streptococcal antigens provoke autoimmune responses in susceptible individuals and target the heart, brain, and joints (1–3) causing rheumatic heart disease (RHD), Sydenham chorea (SC), or polymigrating arthritis. A resurgence of ARF was observed in the mid-1980s in the United States and currently continues unabated in developing countries (4–11). The pathogenesis of ARF is not completely understood but is believed to be mediated by autoimmune and inflammatory mechanisms initiated by streptococcal infection where molecular mimicry generating crossreactive autoantibodies and T cell responses play a role in the clinical manifestations in a susceptible host (12–16). Diagnosis of rheumatic fever is based on revised Jones criteria that include clinical observations as well as evidence of recent streptococcal infection, identification of antistreptococcal antibodies in blood, or positive throat culture for group A streptococci (1).

Rheumatic heart disease is the most serious manifestation of ARF and a leading cause of acquired pediatric heart disease worldwide where patients develop valvulitis or myocarditis (3, 17–20). In RHD, development of valvular lesions appear to result from antibody deposition with upregulation of vascular cell adhesion molecule 1 (VCAM-1) at the surface of the valve followed by infiltration of primarily CD4+ and some CD8+ T lymphocytes that promote inflammation, fibrosis, and scarring of the valves disrupting cardiac function (21–28). T cells from RHD have been found to secrete proinflammatory cytokines, including IFN-γ and TNFα (16, 29). RHD patients develop valvulitis and heart murmur characterized by mitral and aortic regurgitation (20, 30–34). Severity of heart damage is related to the extent of valvular involvement, and injury to the heart valves may require their replacement (3).

Sydenham chorea (SC) is the principal neurologic manifestation of ARF characterized by involuntary movements and neuropsychiatric disturbances, which may develop in 10–30% of ARF cases (35–38). SC is a basal ganglia disorder which is characterized by autoantibodies that target dopaminergic neurons in the basal ganglia and activate calcium/calmodulin-dependent protein (CaM) kinase II activity as well as increased tyrosine hydroxylase production and dopamine synthesis and release in human neuronal cells (14, 39, 40). Patients with SC produce autoantibodies that recognize and signal the dopamine 2 long receptor (D2LR), which serves as a biomarker for the disorder (41–44) and accompanying rare autoimmune psychoses (45). Expression of SC derived human monoclonal antibody (mAb) V genes in transgenic mice demonstrated that the SC autoantibody targeted dopaminergic neurons in the ventral tegumental area or the substantia nigra of the basal ganglia (40).

There are many gaps in our knowledge of the stages and causes of RHD and SC. Although autoimmunity and molecular mimicry have been a hallmark of group A streptococcal sequelae for decades, there are many other important pathogenic mechanisms that may contribute to the autoimmune state either simultaneously or in stages to produce valvular heart disease severity and outcomes. These mechanisms and how they work together and produce disease are yet to be completely understood and new biomarkers identified. In addition to the mimicry between streptococci and heart, RHD may be caused or exacerbated by release of collagen from damaged tissues (3, 46), development of anti-myosin and anti-collagen antibodies (47) and collagen reactive T cells (48), as well as a fibrotic response to elevated transforming growth factor beta-1 (TGF-β1) in RHD tissues (49). To explain the left sided mitral valve association with heart valve injury, an alternate hypothesis has been proposed suggesting that initial group A streptococcal infection provokes inflammatory signaling (TNFα and IL-6), inducing epigenetic changes that prime gene expression in the endothelial and interstitial cells of cardiac valves (49). Epigenetic priming of valve tissue and exposure to hemodynamic stress attributable to transvalvular pressure gradients (TVPGs) are necessary prerequisites for the initiation and progression of valve disease. Acute valvulitis and progression to RHD preferentially occur in the valves exposed to high TVPGs (49). The valves that are normally not exposed to high

Abbreviations: GAS, group A streptococcus; ARF, acute rheumatic fever; RHD, rheumatic heart disease; SC, Sydenham chorea; IFN-γ, interferon gamma; TNFα, tumor necrosis factor alpha; mAb, monoclonal antibody; CSF, cerebrospinal fluid.

hemodynamic stress only develop lesions characteristic of RHD when exposed to elevated TVPGs. TGF-β1 signaling plays a key role in initiating and sustaining the fibrosis responsible for chronic RHD. This mechanism provides an explanation for both the preferential valve involvement seen in ARF and RHD and the reactivation and progression of valve disease (49). Class II human leukocyte antigen (HLA) molecules predisposition has been proposed to affect RHD, and different HLA alleles have been reported to be associated with RHD susceptibility in different ethnic populations (50–55) while alterations at the C4 complement factor (56), TGF-β1 (49), mannose binding protein, and FcR loci may be pathogenic (13, 57). It is not clear if all of these factors work together simultaneously or if certain ones follow others. Certainly, we do know that autoimmunity and inflammation in the beginning of disease would lead to fibrosis which is an important effect of Th17 cells.

Previous studies have also found humoral mechanisms which may play a role in the pathogenesis of ARF and RHD. The Moreland laboratory has correlated an elevated IgG3-complement C4 protein inflammatory response that is distinctly elevated in ARF from a New Zealand Maori cohort compared to normal control subjects. The IgG3 response, which directs robust complement-mediated cell lysis, was elevated against the serotype specific M protein. The IgG3 response could be directed by IL-21 from an increased T_{FH} cell subset which may lead to brain and heart crossreactive autoimmune responses against the streptococcal M protein in ARF. Of interest, elevated concentrations of IL-21 were found in the mitral valves of RHD patients, indicating a potentially important role for this cytokine in group A streptococcal sequelae (58).

Our current study provides a more in depth understanding of the immunopathogenesis of ARF where GlcNAc-specific IgG2 was the dominant subclass of GAS directed pathogenic responses in RHD and SC, separating ARF from uncomplicated pharyngitis where antibodies do not cause disease. Furthermore, our study links Th17 cells/IL-17A with rheumatic carditis and demonstrates the coincidence of IL-17A and IFN-γ with IgG2 deposition, the most prominent IgG subclass on RHD derived valve tissues. Similarly, IgG2 present in CSF from rheumatic chorea recognized human neuronal cells to identify pathogenic responses in SC. In both RHD and SC, a significant and preferential sera IgG2 response recognized GlcNAc, the dominant group A carbohydrate epitope. Anti-GlcNAc IgG2, in comparison with the other subclasses, was highly elevated and distinct, and clearly separated ARF from pharyngitis. Taken together, our novel human findings suggest that IgG2 may be a pathogenic subclass in RHD and SC and that Th17/Th1 cells cooperate to play an important role in development of IgG2 in ARF. Our novel human study supports past studies of elevated immune responses against the group A carbohydrate which has been associated with a poor valvular prognosis and replacement outcomes (59).

Further, there are no disease related biomarkers for RHD. Understanding and defining new biomarkers that might be used in underdeveloped resource settings has been a worldwide goal. Understanding and defining new biomarkers that might be used in underdeveloped resource settings has been a worldwide goal. We herein define a potential serum biomarker which may identify children at risk of rheumatic fever and rheumatic heart disease and potentially explain how the response to the group A streptococcus could lead to RHD. Our hypothesis, reiterated, is that the development of an autoimmune state in RHD and SC may in part result from secretion of specific IgG subclasses directed at antigens related to carbohydrate moieties either by mimicry or direct design and such antibodies directed to tissues by specific autoreactive T helper cell subsets. The Th1 cell subset and its IFN-γ production promotes IgG2 (60–62), the IgG subclass directed against carbohydrate antigens in humans. Determination of the IgG2 subclass deposited in diseased tissues can provide insights into mechanisms of disease as well as the nature of streptococcal and host antigens that promote RHD and SC, and by their distinction and separation from uncomplicated streptococcal pharyngitis become a new potential biomarker of ARF and RHD in humans.

Materials and methods

Subjects, sera, CSF, RHD valve tissues and study design

Samples were obtained from patients and healthy volunteer subjects enrolled in research protocols at the University of Oklahoma Health Sciences Center, University of Utah School of Medicine, University of Witwatersrand, and the National Institutes of Health. The protocols were reviewed by institutional review boards (IRBs) at the respective institutions, and appropriate assent/consent obtained prior to collection of clinical data and samples. Specifically, the research study protocols were approved for the study of human subjects by the institutional review boards at the National Institutes of Health Combined Neuroscience Institutional Review Board, Bethesda, MD, USA; and at the University of Oklahoma Health Sciences Center Institutional Review Board for Protection of Human Subjects, Oklahoma City, OK, USA. In all studies, each parent and child gave written consent or assent, respectively, for the investigation. Parents gave written consent for their children to participate (witnessed by a member of the NIMH human subjects protection team). All children 7 years and older gave written assent to participate and those 6 and under gave verbal assent. Samples were de-identified and coded to obscure identity and diagnosis prior to laboratory storage or shipment. All serum and tissue samples from outside Oklahoma were deidentified prior to receipt by Dr. Cunningham's laboratory and patients were not in any case identifiable by her laboratory.

Blood was collected from patients at the time of diagnosis of ARF/RHD/SC based on the revised Jones criteria for each individual patient studied. SC patients were collected and diagnosed at the NIMH and in Oklahoma University Hospital and the RHD patients were collected during the Utah ARF outbreak and diagnosed at the University of Utah College of Medicine and Hospitals and patients with ARF and RHD were diagnosed at the Oklahoma University Hospital. **Table 1** describes the number of patients who are included in the study including the numbers of each type of patient, source, ages and diagnosis. Jones criteria are mentioned in **Table 1**. Figures also list the numbers of patients included but the scatter plots show the direct result of each patient which can be followed on the scatter plot graphs shown in the results.

RHD, polymigrating arthritis, and SC patients were assessed by the revised Jones criteria and were GAS culture positive or anti-streptolysin O (ASO) titer positive. RHD and polymigrating arthritis sera were collected in the Departments of Pediatrics, Infectious Diseases, Cardiology, and Pathology at the University of Utah College of Medicine, Salt Lake City, UT, USA. SC sera and CSF samples were

TABLE 1 Acute rheumatic fever: Pediatric patient cohort[bc].

ARF manifestation[abc]	Number of sera	Source of sera
Carditis/RHD	10	7 sera from OUHSC clinics/hospital
		3 sera from Utah ARF outbreak
Chorea	6	1 sera from OUHSC clinics/hospital
		5 sera from the NIMH repository
Arthritis	3	1 sera from OUHSC clinics/hospital
		2 sera from Utah ARF outbreak
Controls		
Uncomplicated Pharyngitis[bd]	7	7 sera from the OUHSC clinics
Healthy Children (ASO < 150)	8	8 sera from the OUHSC clinics

[a]Diagnosis by revised jones criteria.
[b]Positive ASO titer >200 Todd Units in pharyngitis and/or ARF pediatric groups (OUHSC serology laboratory).
[c]Age range 8–17 for pediatric ARF and pediatric control groups.
[d]Diagnosis of pharyngitis by positive throat culture and/or positive ASO titer (OUHSC serology laboratory).

collected at the National Institutes for Mental Health. Additional RHD, SC, uncomplicated pharyngitis, and healthy control sera were collected from the Department of Pediatrics, Divisions of Cardiology and Neurology at the University of Oklahoma Health Sciences Center. Sera were all pediatric cases, and ARF sera ranged from 8 to 17 years old. All serum or CSF samples were stored after collection at −135°C until testing in our assays.

Valve specimens were obtained during surgery or autopsy from 4 patients with rheumatic heart disease at the University of Witwatersrand, Johannesburg, South Africa. The patient ages ranged from 8 to 15 years old. Rheumatic valves were compared with anatomically and functionally normal mitral valves from autopsy of middle-aged individuals after myocardial infarction.

Histology

Examination of hematoxylin-eosin (H&E) stained sections were characterized according to the cellular infiltration, fibrosis (scarring), neovascularization, and mineralization in the valves. Selected heart tissues had evidence of chronic valvulitis, defined by the presence of inflammatory cellular infiltrates of mononuclear cells and neutrophils with scarring, neovascularization, and absence of significant mineralization.

Cell lines

The human SK-N-SH (ATCC HTB-11) neuroblastoma line was obtained from the American Type Culture Collection. Cells were routinely cultured with F12-DMEM (Gibco-BRL) media containing 10% fetal calf serum (FCS), 1% penicillin and streptomycin, and 0.1% gentamicin at 37°C, 5% CO_2.

Immunohistochemistry

Heart tissues were embedded with paraffin to produce slides containing 5-μm tissue sections. Tissue sections were deparaffinized in a 3:1 mixture of Hemo-D:xylene and rehydrated in graded ethanol and washed in PBS for 5 min. For studies of the cytokines in tissues, deparaffinized sections were subjected to antigen retrieval using citrate buffer (10 mM Citrate Buffer, pH 6.0 for 20 min in hot steam) (63). Sections were washed in PBS for three minutes and blocked with Protein Blocker (BioGenex) diluted 1:10 in distilled water with 5% bovine serum albumin then probed with goat anti-human IL-17A, anti-IFN-γ, or isotype control at 15 μg/ml (R&D Systems, Minneapolis, MN, USA) overnight at 4°C. Following overnight incubation, slides were washed three times for 3 min in PBS and then incubated with biotin-conjugated rabbit anti-goat IgG (1:1,000; Abcam) overnight at 4°C. After washing slides in PBS, alkaline phosphatase-conjugated streptavidin (1:1,000 in PBS; Jackson ImmunoResearch) was incubated on tissues for 30 min at room temperature. Slides were washed again in PBS three times for three minutes and antibody binding was detected with Fast Red substrate (BioGenex) (64) followed by counterstaining with Mayer's hematoxylin (Biogenex). Non-RHD heart tissue served as a negative control. Stained tissues were read by at least 3 individuals and graded 0±, 1+, 2+, 3+, and 4+ with the range from zero or no staining or = /− or very weak staining, 1+ clear visible but light staining; 2+ or moderate staining, 3+ moderate to heavy staining, and 4 + the strongest staining visible and all read in a Olympus dual light and fluorescence microscope with computer photography work station and camera attached to the microscope.

Heart tissues from RHD were tested for heart tissue-bound antibody which was detected using biotin-conjugated rabbit anti-human IgG1, IgG2, IgG3, and IgG4 monoclonal antibodies (Sigma Chemical Co.) incubated at 4°C overnight. Tissues were washed in PBS three times for 3 min and incubated with alkaline phosphatase-conjugated streptavidin (1:1,000 in PBS; Jackson ImmunoResearch). Antibody binding was detected using Fast Red Substrate (Biogenex, San Ramon, CA, USA). Non-RHD heart tissue served as a negative control. Stained cells were read by at least three individuals and graded 0, ±, 1+, 2+, 3+, and 4+ as shown in the **Figures 5–7** with the darkest staining registered as a 4+ and the weakest as ± or 1+ accordingly.

To determine if the CSF bound to human neuronal cells, SK-N-SH cells, a human neuronal cell line, was plated in four-chamber tissue culture slides at 1×10^4 cells/chamber overnight in DMEM-F12 medium under standard cell culture conditions. The four-chamber slides were washed with ice cold PBS and blocked with 5% BSA in PBS then incubated with a 1:100 dilution of CSF in DMEM-F12 medium overnight at 4°C. The slides were then washed in PBS and cell-bound antibody was detected using biotin-conjugated rabbit anti-human IgG1, IgG2, IgG3, and IgG4 monoclonal antibodies (Sigma Chemical Co.) for 30 min at 37°C followed by alkaline phosphatase-conjugated strepavidin (1:1,000; Jackson ImmunoResearch). Antibody binding was detected using Fast Red Substrate (Biogenex, San Ramon, CA, USA) and cells counterstained with Mayer's hematoxylin (Biogenex). Non-SC disease CSF served as a negative control. Stained tissues were read by at least three individuals and graded 0, ±, 1+, 2+, 3+, and 4+ as shown in **Figure 4** with the darkest staining registered as a 4+ and the

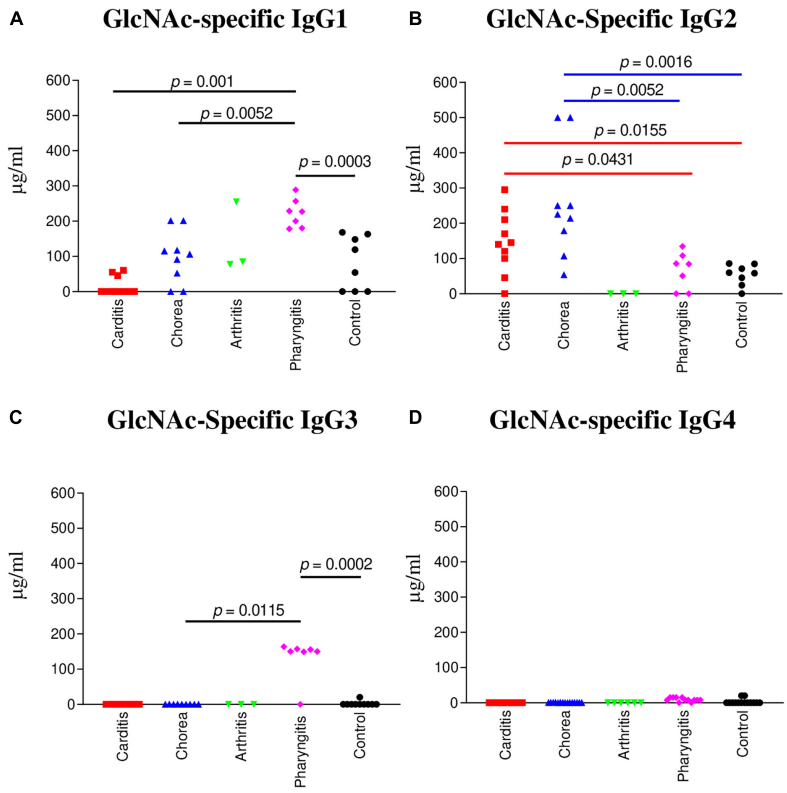

FIGURE 1
IgG subclass responses (µg/ml) to GlcNAc in ARF (RHD, SC, Arthritis) sera compared to pharyngitis and healthy control groups. **(A)** The concentration of GlcNAc-specific IgG1 was significantly elevated in uncomplicated pharyngitis sera in comparison to RHD (p = 0.001), SC (p = 0.0052), and healthy control sera (p = 0.0003), but not to rheumatic arthritis (p = 0.2667). **(B)** GlcNAc-specific IgG2 concentrations were significantly elevated in SC sera in comparison to uncomplicated pharyngitis (p = 0.0052) and healthy control (p = 0.0016) sera, but not to RHD sera (p = 0.0653). RHD sera had significant amounts of IgG2 to GlcNAc in comparison to uncomplicated pharyngitis (p = 0.0431) and healthy control sera (p = 0.0155). **(C)** GlcNAc-specific IgG3 in sera from uncomplicated pharyngitis was significantly elevated in comparison to SC (p = 0.0115) and healthy control (p = 0.002) sera. **(D)** GlcNAc-specific IgG4 concentrations were negligible for all groups. *P* values were calculated by the Mann-Whitney two-tailed *t* test for comparison of individual sera groups of carditis (n = 10) SC (n = 9), arthritis (n = 3), pharyngitis (n = 7), and healthy control (n = 8) (Red p value bars are RHD comparisons vs Blue p value bars are SC comparisons vs Black p value bars are pharyngitis comparisons). GlcNAc-specific IgG2 was the significant subclass response to GlcNAc in RHD and SC sera compared to pharyngitis and healthy controls, while GlcNAc-specific IgG1 and IgG3 were significantly elevated in pharyngitis sera. NS, not significant.

weakest as −/− or 1+ accordingly and as described in the Methods Immunochemistry section.

Indirect enzyme-linked immunosorbant assay (ELISA)

Pepsin fragment of streptococcal M5 protein (pepM5) (65, 66) and GlcNAc-BSA antigens were made as previously described (67–69). Purified human IgG1, IgG2, IgG3, and IgG4 antibodies were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Ninety-six well polyvinyl, Immunolon 4 microtiter plates (Dynatech Laboratories, Chantilly, VA, USA) were coated with 10 µg/ml of pepM5 or GlcNAc-BSA or 50 µg/ml of mouse anti-human IgG1, IgG2, IgG3, and IgG4 mAbs for standard curve overnight at 4°C. Plates were incubated overnight at 4°C with 1:10 dilution CSF, 1:1,000 dilution of sera, or 500 to 0 µg/ml concentrations of human IgG1κ, IgG2κ, IgG3κ, and IgG4κ purified monoclonal antibodies (Sigma Chemical Co.) to obtain a standard curve. Antibody binding was detected using biotin-conjugated rabbit anti-human IgG1, IgG2, IgG3, and IgG4 monoclonal antibodies (Sigma Chemical Co.) followed by alkaline phosphatase-conjugated strepavidin (Jackson ImmunoResearch, West Grove, PA, USA). Plates were developed with two mg/ml p-nitrophenyl phosphate 104 substrate (Sigma Chemical Co.) at 100µl/well and optical density quantified at 405 nm in an Opsys MR microplate reader (Dynex Technologies, Chantilly, VA, USA). IgG subclass concentrations of the sample reactivity in the ELISA was determined from the subclass standard curves and reported as µg/ml after BSA reactivity was deducted. Healthy control sera and non-SC disease CSF served as negative controls for ELISA. Reactivity of IgG subclass antibodies with GlcNAc and bovine serum albumin (BSA) were calculated separately and any reactivity with BSA alone subtracted from the overall optical density of each sample.

Statistical analyses

IgG subclass responses to GlcNAc and PepM5 in ARF (RHD, SC, Arthritis) sera (**Figures 1**, **2**) were compared to pharyngitis or healthy controls using the Mann–Whitney test. The Mann–Whitney test was chosen due to the small sample size and skewed distribution of the responses. Adjusted analysis was not deemed necessary due to small sample size and relative balance in demographic variables among

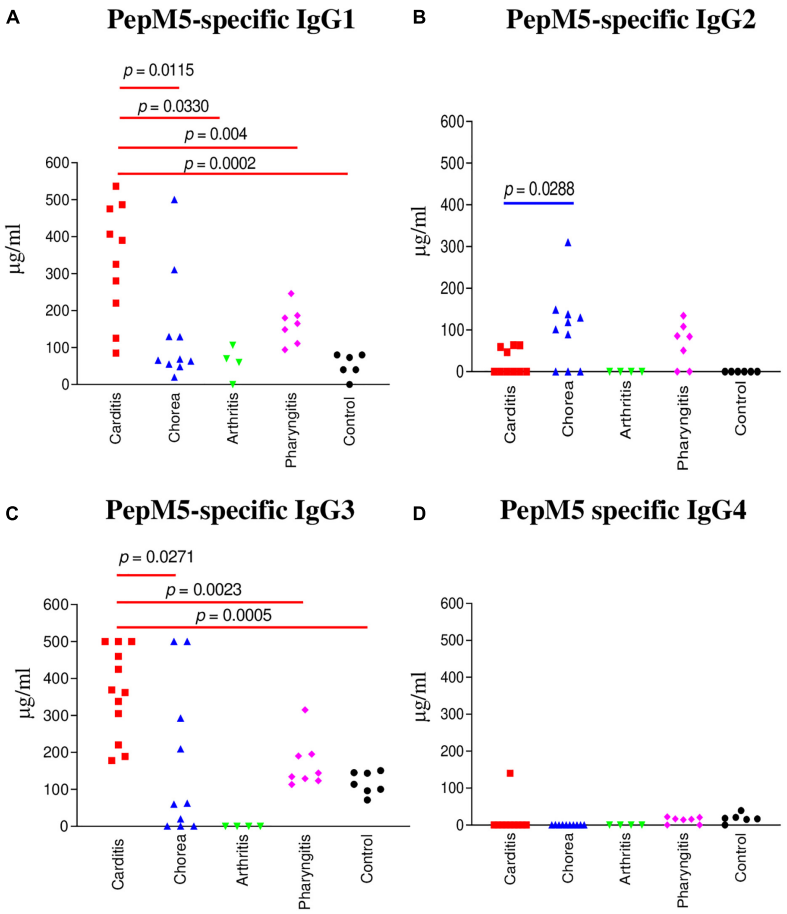

FIGURE 2
IgG subclass responses (µg/ml) to the group A streptococcal M protein type 5 (pepM5) in ARF (RHD, SC, Arthritis) sera, pharyngitis and healthy control groups. **(A)** In rheumatic carditis, IgG1 was the dominant subclass response to pepM5 in comparison to SC (p = 0.0115), rheumatic arthritis (p = 0.004), uncomplicated pharyngitis (p = 0.0330), and healthy control (p = 0.0002) sera. **(B)** PepM5-specific IgG2 was significantly elevated in SC sera in comparison to RHD samples (p = 0.0288). Neither SC nor RHD sera had significant amounts of anti-pepM5 IgG2 in comparison to uncomplicated pharyngitis (p = 0.3148 and p = 0.0688, respectively). **(C)** Significantly elevated pepM5-specific IgG3 was found in rheumatic carditis in comparison to SC (p = 0.0271), pharyngitis (p = 0.0023), and healthy control (p = 0.0005) sera. **(D)** PepM5-specific IgG4 concentrations were negligible in all samples. *P* values were calculated by the Mann–Whitney two-tailed *t* test for comparison of individual sera groups of carditis (n = 10) SC (n = 9), arthritis (n = 3), pharyngitis (n = 7), and healthy control (n = 8) (Red p value bars are RHD comparisons vs Blue p value bars are SC comparisons vs Black p value bars are pharyngitis comparisons). IgG1 and IgG3 were the significant subclass responses in sera to pepM5 in RHD while IgG2 was significant in SC. NS, not significant.

individual groups. In Figure 3, Welch's t test was used for CSF ELISAs as the Mann–Whitney was not sufficient to determine the p values for comparisons between the smaller numbers of normal CSF and SC CSF sample values. All statistical analyses were performed using the PRISM software and a p-value of <0.05 was considered significant.

Results

Serum IgG subclass response in acute rheumatic fever: IgG2 dominates the response against the group A carbohydrate epitope, N-acetyl-beta-D-glucosamine (GlcNAc) in RHD and SC

To determine which IgG subclass dominated humoral responses against the group A streptococcal carbohydrate in ARF, sera from RHD, SC, polymigrating arthritis, uncomplicated pharyngitis, and healthy controls were examined for GlcNAc immunoreactivity. In Figure 1B, both RHD and SC sera demonstrated significantly higher GlcNAc-specific IgG2 antibody responses in comparison to uncomplicated pharyngitis (RHD p = 0.0431, SC p = 0.0052) and to healthy control sera (RHD p = 0.0155, SC p = 0.0016) (Figure 1B). In contrast, pharyngitis sera demonstrated significantly elevated GlcNAc-specific IgG1 responses in comparison to healthy controls (p = 0.0003), RHD (p = 0.001), and SC (p = 0.0052) sera (Figure 1A). Of note, GlcNAc-reactive IgG3 (Figure 1C) was significantly elevated in pharyngitis sera compared to healthy control sera (p = 0.0002) and SC sera (p = 0.0115). Detectable concentrations of GlcNAc-specific IgG3 were not present in RHD or polymigrating arthritis sera. The finding was of interest as IgG1 and IgG3 typically are induced by protein antigens and are characterized by an increased ability to direct complement activation and opsonization in comparison to IgG2 (70). IgG4 was not elevated to GlcNAc in any of the sera tested. Thus, IgG2 appears to characterize RHD and SC sera as a disease-specific response against the carbohydrate epitope GlcNAc in ARF.

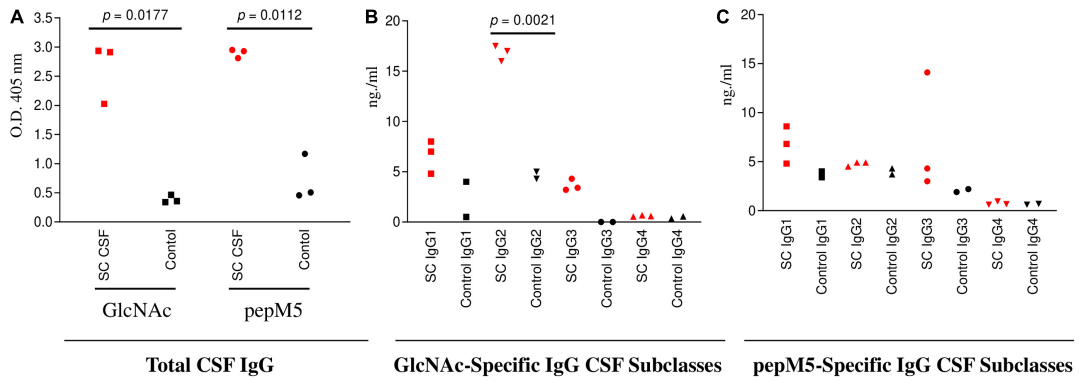

FIGURE 3
SC CSF (n = 3) IgG responses to GlcNAc and PepM5 GAS protein. **(A)** Total CSF IgG recognition of GlcNAc and PepM5 streptococcal protein. SC CSF demonstrated significantly elevated GlcNAc-specific IgG and pepM5-specific IgG in comparison to disease control CSF (p = 0.0177 and p = 0.0112, respectively). P values were calculated by the two-tailed Welch's t test for comparison of SC and control CSF groups. **(B)** GlcNAc-specific IgG2 dominates SC CSF subclass responses. SC CSF demonstrates significantly higher concentrations of GlcNAc-specific IgG2 (p = 0.0021) in comparison to disease control CSF. The concentration of SC CSF IgG2 was significantly elevated in comparison to SC CSF IgG1 (0.01) and SC CSF IgG3 (0.0002). P values were calculated by the two-tailed Welch's t test for comparison of SC and control CSF groups. **(C)** SC CSF IgG concentrations reactive to pepM5 show slightly elevated levels of IgG1 and IgG3 in comparison to disease control CSF, but were not significantly elevated (p = 0.1166 and 0.2842, respectively). P values were calculated by the two-tailed Welch's t test for comparison of SC and control CSF groups. Red dots are SC disease CSF (n = 3) vs Black dots disease control CSF (n = 3). NS, not significant.

IgG subclass specificity was also determined for the group A streptococcal M protein fragment, pepM5, the extracellular, N-terminal half of the M5 protein cleaved by pepsin at suboptimal pH (65, 66). IgG subclass responses to pepM5 protein were distinct from those against the group A carbohydrate epitope GlcNAc (**Figure 2**). In rheumatic carditis, pepM5 protein-reactive IgG1 (**Figure 2A**) responses were significantly elevated above SC (p = 0.0115), arthritis (p = 0.0330), uncomplicated pharyngitis (p = 0.004), and control sera (p = 0.0002). RHD IgG3 (**Figure 2C**) concentrations were also significantly higher (p = 0.0271) to SC, pharyngitis (p = 0.0023) and healthy controls (p = 0.0005). In contrast, in SC, IgG2 responses against pepM5 were significantly higher in comparison to RHD (p = 0.0288) (**Figure 2B**). IgG4 was not elevated to PepM5 protein in any sera tested. Evidence provided in **Figures 1**, **2** show that IgG subclass responses to streptococcal antigens distinguishes ARF from uncomplicated pharyngitis and highlights GlcNAc-reactive IgG2 as a feature of both RHD and SC, which are distinct from the anti-GlcNAc IgG1 and IgG3 responses of uncomplicated pharyngitis. Furthermore, the IgG responses to GlcNAc are distinct from those to the M protein, which is shown in **Figure 2** to be significantly elevated in carditis compared to pharyngitis. Interestingly, the IgG subclass response to M protein in SC was definitively IgG2 compared to carditis.

IgG2 from SC CSF preferentially binds to GlcNAc and targets human neuronal cells

To evaluate CSF reactivity to streptococcal antigens, total IgG specificity to GlcNAc and the pepM5 protein was tested by ELISA. Significantly elevated levels of CSF total IgG to GlcNAc (p = 0.0177) and pepM5 (p = 0.0112) were found in SC CSF (**Figure 3A**) in comparison to CSF samples from other neurological disorders (Black dots; **Figure 3**). Evaluation of IgG subclass distribution to the streptococcal antigens showed significantly increased concentrations of GlcNAc-specific IgG2 in SC CSF compared to disease control CSF (p = 0.0021) (**Figure 3B**). The concentration of anti-GlcNAc IgG2 is SC CSF was also significantly higher than the concentrations of IgG1 or IgG3 from SC CSF. In **Figure 3C**, SC CSF IgG concentrations reactive to pepM5 show slightly elevated concentrations of IgG1 and IgG3 in comparison to disease control CSF, but were none were significantly elevated (p = 0.11 and 0.28, respectively).

To demonstrate which IgG subclasses potentially target neurons, SC and disease control CSF samples were incubated with SK-N-SH human neuronal cells and probed with antibody specific to the four IgG subclasses. Autoreactive IgG2 was found to qualitatively bind to the neuronal cell surface to a much greater degree than IgG1, IgG3, and IgG4 in both SC CSF samples (**Figure 4**). IgG2 staining was largely absent from the disease control CSF (**Figure 4** control, bottom panel). Interestingly, IgG2 was the dominant subclass bound to the neuronal cell surface even though the CSF ELISA results (**Figure 4**) showed detectable concentrations of IgG1 and IgG3. IgG complement-mediated cytotoxicity against SK-N-SH cells were all below 10% in chromium release assays (data not shown). The data suggest that in SC-derived CSF IgG2 dominated the antibody response to streptococcal group A carbohydrate epitope GlcNAc, and autoreactive IgG2 qualitatively bound (red stain SC CSF 1 = 3+, SC CSF = 4+) to human neuronal cells substantially more than other subclasses in SC CSF.

Autoreactive IgG2 is the dominant subclass deposited on RHD cardiac valve tissues

To determine subclass specificities of autoreactive IgG involved in RHD, cardiac tissues from valve replacement surgery or autopsy were tested for bound IgG1, IgG2, IgG3, and IgG4. Pronounced IgG2 staining of RHD cardiac valve tissues was observed in all four RHD tissues and IgG2 was distinctly overrepresented in comparison to other IgG subclasses (**Figure 5**). A small amount of IgG3 was evident on valvular tissues compared to the IgG2, with very little

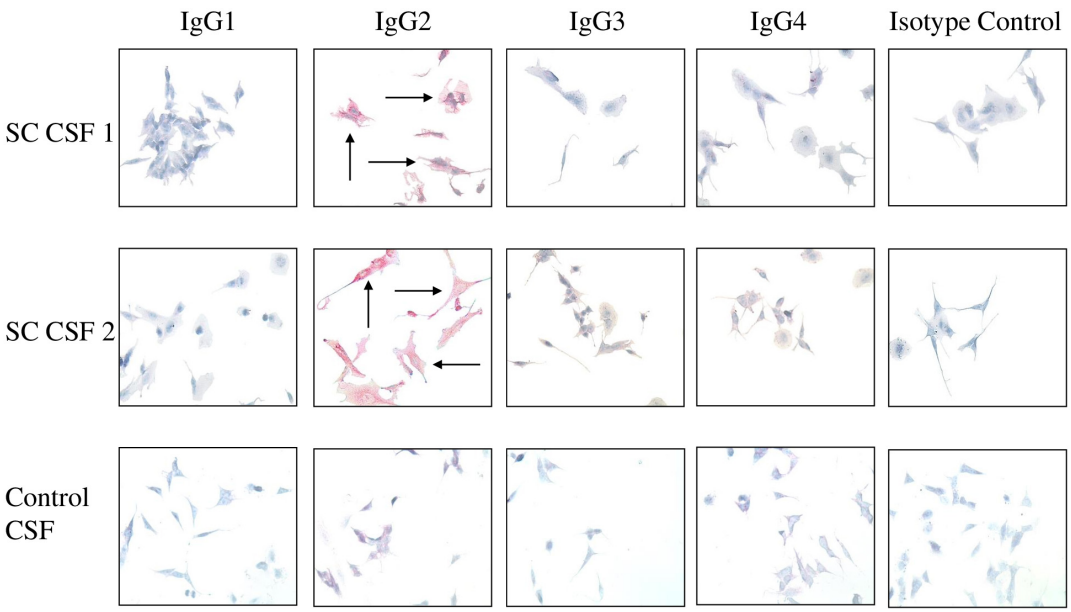

FIGURE 4
IgG2 in SC CSF targets human neuronal cells. The IgG subclasses from CSF capable of binding to surface antigen on SK-N-SH human neuronal cells were determined by Fast Red staining followed by counterstaining with hematoxylin. Red staining of cells indicates positive IgG binding. IgG2 from SC CSF predominantly targeted the extracellular surface of SK-N-SH cells (SC CSF 1 = 3 + staining and SC CSF 2 = 4 + staining) in comparison to the other subclasses (blue stained cells, 0 staining). Arrows indicate positive reactivity. Very faint IgG2 staining is shown for control CSF (0.5 + staining). In contrast, there is no IgG1, IgG3, or IgG4 binding to human neuronal cells by disease control CSF (blue stained cells, 0 staining). Magnification 400×.

IgG1 or IgG4 staining observed. No IgG subclass deposition was observed on control heart tissue. IgG2 deposits in RHD valvular tissues clearly stood out and emphasized that anti-GlcNAc IgG2, which was significantly elevated in RHD sera, may directly deposit and contribute to carditis in RHD. Although there were significantly elevated serum concentrations of IgG1 and IgG3 to pepM5 from RHD sera, only weak IgG3 staining was visible on the heart tissues. Collectively, the data suggest that autoreactive IgG2 preferentially binds to valvular heart tissues in RHD and may contribute to cellular infiltration and inflammatory valvular changes seen in rheumatic carditis. The previous findings that elevated responses to the group A carbohydrate antigen correlated with severity and poor outcomes in valve disease supports our findings (59).

Th17/Th1 cells infiltrate the heart valve and coincide with IgG2 deposition in rheumatic carditis

To determine if autoreactive IgG2 deposition occurred at the site of cellular pathology, RHD tissue containing a prominent granuloma (Ashoff nodule) was tested for IgG subclass staining. Strong IgG2 deposition was observed at the site of the granuloma with lesser amounts of IgG3 and IgG4 seen (**Figure 6**). The four RHD heart tissues with elevated IgG2 deposition all had pronounced mononuclear cell infiltrates as seen in hematoxylin and eosin-stained tissues (**Figure 7** top row), which was absent in non-RHD control heart tissue (**Figure 7** top row).

IFN-γ is associated with isotype switch events in B cells that leads to IgG2 secretion, and has been reported in RHD heart lesions and T cells or clones isolated from RHD hearts as well as from cardiac tissues from the Lewis rat experimental autoimmune valvulitis (EAV) model (29, 71–73). All four RHD tissues were found to have elevated levels of IFN-γ in comparison to control non-RHD heart tissue (**Figure 7** middle row). Th17 cells have been reported to contribute to the pathology of myocarditis and a previous report has indicated the presence of Th17 cells in RHD hearts (64, 74). To determine if Th17 cells were present in rheumatic carditis valve tissues, IL-17A was evaluated by immunohistochemistry. IL-17A was seen throughout all four RHD myocardial and valvular tissues suggesting autoreactive Th17 cells invade into valve and myocardial tissues (**Figure 7** bottom row). No IL-17A was observed in control non-RHD tissue (**Figure 7** bottom row). In this study, we demonstrate that autoreactive IgG2, IFN-γ, and IL-17A was found simultaneously in the RHD tissues evaluated. Collectively, the data suggest that autoreactive IgG2 along with cooperative Th1/Th17 responses contribute to the development and progression of RHD.

Discussion

Previously, our studies have focused on understanding the immunologic crossreactivity and molecular mimicry between GAS and heart and brain tissue antigens (14, 15, 21). In the current study, new evidence shows deposition of IgG2 on RHD cardiac tissues and suggests that antibody targeted the valves concomitant with infiltration by autoreactive Th17/Th1 T cell subsets. Additionally, in SC, GlcNAc-specific IgG2 concentrations from SC-derived CSF were significantly higher than IgG1, IgG3, and IgG4, and IgG2 was the predominant subclass that recognized cell surface antigen on human neuronal cells. Our evidence suggests IgG2 antibodies against the group A carbohydrate epitope GlcNAc as a disease specific antibody or biomarker in ARF which targets valve in RHD and human neuronal cells in SC. The elevated IgG2 response to GlcNAc

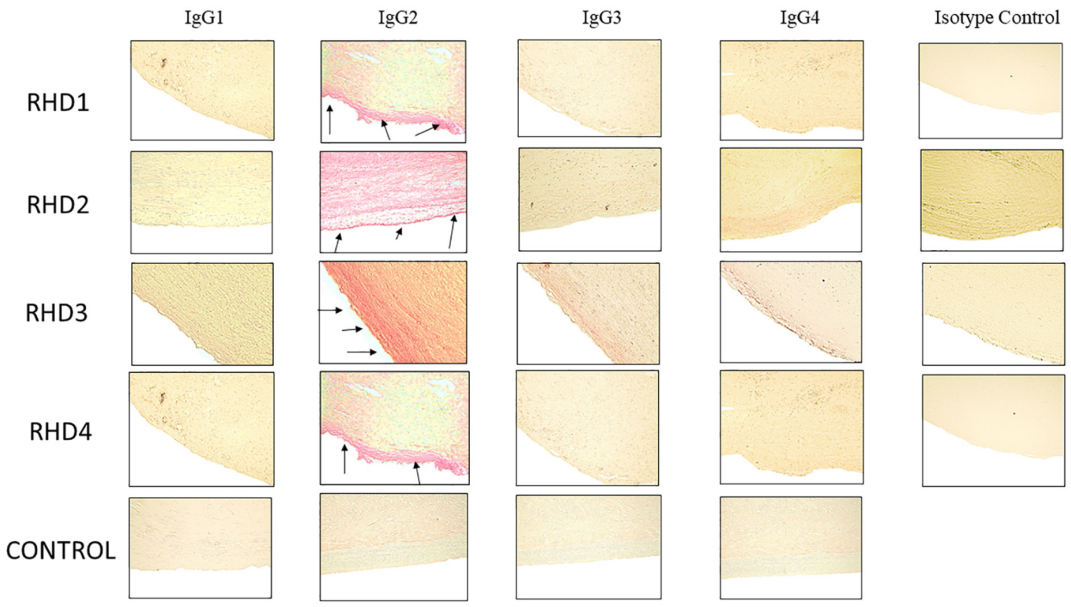

FIGURE 5
IgG2 deposits *in vivo* in RHD valvular tissues. Immunohistochemistry for IgG subclasses reveals strong human IgG2 deposition in RHD heart tissues from four different patients compared to other subclasses as seen by Fast Red stain of IgG subclass deposition. Red staining of cells indicates a positive IgG binding (see arrows). RHD 1 IgG2 staining is 4+, RHD 2 IgG2 staining is 4 +, RHD 3 IgG2 is 3+, RHD 4 IgG2 staining is 4 + . Faint staining IgG3 (RHD 3 0.5+) and IgG4 (RHD 2 1 =, RHD 3 1+) staining was present. No visible IgG1 staining was observed for any of the four RHD samples. IgG subclass deposition is absent from control, non-RHD heart tissue. Magnification 400×.

was unique to RHD and SC sera in comparison to polymigrating arthritis and uncomplicated pharyngitis. In RHD, persistently high levels of anti-GlcNAc directed against heart tissues may lead to predisposition of the valves to cellular in filtration via opsonization after which the valves have the potential to upregulate fibrosis due to the IL-17A response as well as TGF-β1 genetic variations in RHD (49). IgG2 was so strongly deposited compared to other subclasses on valvular surface, that in some cases, it could be observed on some tissues with the naked eye after immunostaining. In SC CSF, only the IgG2 subclass substantially bound to the human neuronal cells. Of interest was the significantly elevated concentrations of GlcNAc-specific IgG1 and IgG3 in uncomplicated pharyngitis sera in comparison to RHD and SC sera. Both IgG1 and IgG3 strongly direct both complement activation and opsonization-phagocytosis. It may be that the secretion of IgG1 and IgG3 leads to rapid removal of GAS during uncomplicated pharyngitis, thus decreasing the potential for the development of sequelae.

While we propose mechanisms in a hypothesis where GlcNAc-specific IgG2 could play a dominant role in RHD to direct the T cell subsets to the valve, IgG3 cannot be discounted and must be considered for its potential role in RHD where IgG3 would strongly direct complement-mediated cytotoxicity, an effector function largely absent from IgG2 (75). Previously, we demonstrated endothelial cell cytotoxicity by RHD-derived human mAbs which were strongly crossreactive with (1) GlcNAc and laminin as well as (2) a highly crossreactive laminin peptide and (3) the valve surface endothelium (15). We can speculate that early production of cardiac-specific IgG3 would initiate damage at the valve leading to pronounced inflammation with edema of the valve leaflets that in turn may be replaced by a sustained IgG2 deposition and concomitant cellular infiltration with IFN-γ and IL-17A directing inflammation that characterizes RHD. The early edematous damage to valve leaflets is demonstrated in our RHD Lewis rat autoimmune model of valvulitis (73). Additional studies of the valvulitis model will be instrumental in identifying the subclasses and T cell subsets associated with initiation and persistence of heart damage.

GlcNAc-specific IgG2 was significantly lower in pharyngitis compared to RHD and SC sera, making it a potential biomarker of disease. Elevated as well as persistent IgG2 concentrations from repeated streptococcal infections may begin the inflammatory cascade at the valve endothelial surface for further injury by inflammatory T cell subsets Th1 and Th17 and the potential for enhanced fibrosis in the presence of the TGF-β1 gene variants found in RHD (49). The surface of the valve some years ago was shown to express carbohydrate epitopes (76) similar to GlcNAc, and it is well established that laminin is a glycosylated protein.

The contributions of autoreactive IgG2 to RHD is not completely clear. IgG2 is efficient in directing opsonization and can recruit macrophages, DCs, and neutrophils through the FcγRIIa receptor (CD32a), however, it is a poor activator of the complement cascade (75). In this study, IgG2 was the dominant subclass bound to RHD tissues. Previously we have shown an upregulation of VCAM-1 on cardiac valvular tissues (24). The same heart tissues shown herein were used for the VCAM-1 and IgG2 studies, thus, it is clear that VCAM-1 upregulation occurs concomitantly with IgG2 deposition with infiltration of immune cells into the valve leading to fibrosis and valve deformity. Failure to follow preventive antibiotic treatment regimens to prevent continued repeated streptococcal infection in ARF may allow for disease to intensify in the valve when there is a subsequent GAS infection (24). Thus, IgG2 may specifically contribute to each episode of cardiac damage by directing macrophages, DCs, and neutrophils into heart tissues to promote inflammation and further valve damage with epitope spreading suggested (77). Infiltration of CD68 + macrophage and CD80 + DCs correlated with increased tenascin-C concentrations, a marker for extracellular matrix remodeling and fibrosis in RHD hearts (78, 79).

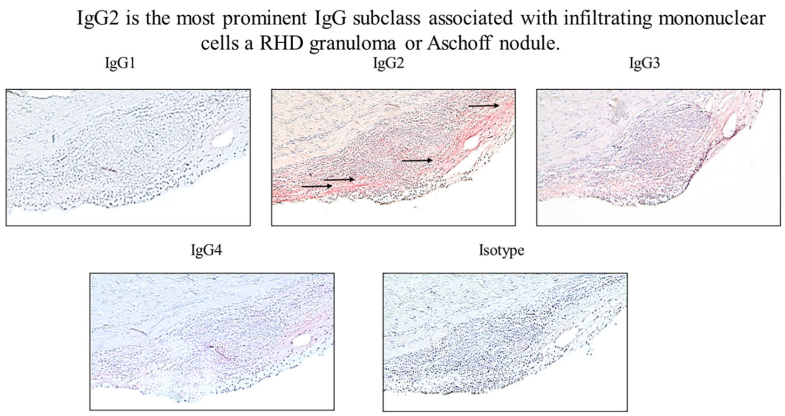

FIGURE 6
IgG2 is associated with infiltrating mononuclear cells in RHD granuloma. Elevated levels of autoreactive IgG2 were found in association with a mononuclear cell infiltrates in an RHD cardiac valvular tissue granuloma (Aschoff nodule). IgG2 deposition was qualitatively higher in comparison to other IgG subclasses at the valve surface where the granuloma was present (IgG1 0 staining, IgG2 4 + staining, IgG3 2 + staining, IgG4 1 + staining, isotype control 0 staining). Magnification 200×.

In IgG2+ RHD tissues, cellular infiltration by IFN+ and IL-17A+ cells suggested a potential pathogenesis by Th17/Th1, which may involve Th17 plasticity (80), which raises questions about the relationship between Th17 and Th1 (81, 82). In autoimmune disease, Th1 cells may evolve to become autonomous and responsive directly to antigen presentation of peptides from local tissue (83) whereas in acute RHD/ARF the Th1 cells must be responding to the streptococcal and local crossreactive antigens due to molecular mimicry and have not reached the autonomous state where they would be responsive primarily to self-antigens potentially later in disease. Unlike other autoimmune diseases which wax and wane, penicillin treatment prevents progression of severe disease in ARF in the first several years after onset by preventing repeated streptococcal infections and further autoimmunity in the susceptible host (84). However, chronic valve disease may not be as responsive to preventative antibiotic therapy (85) and prevention due to hemodynamic stress attributable to transvalvular pressure gradients (49) on already damaged valves which may be targeted by immune responses against released collagen (48).

Th17 cells have been closely associated with autoimmunity (86–90). GAS have been shown to induce a robust Th17 response in mice and humans which protects against upper respiratory tract and mucosal infections and can lead to autoimmunity (64, 74, 90–103). In our current study herein, RHD hearts were infiltrated with both IL-17A and IFN-γ producing mononuclear cells concomitant with IgG2 deposition. In an earlier study, mitral valve tissues from RHD demonstrated increased expression of IL-6, IL-17, IL-21, and IL-23, and in human autoimmune myocarditis, Th17 cells secreted both IL-17A and IFN-γ in the presence of IL-23 (64, 74).

The evidence suggests a potential role of GlcNAc specific antibodies and their association with poor prognosis of valvular heart disease and outcomes in acute RHD (59). We hypothesize that the IgG2 targeting of the valve is dependent on the carbohydrate epitope and involves mimicry between the streptococcal GlcNAc and the valve surface glycoproteins in the basement membrane and valvular endocardium. Laminin contains the alpha helical structure which crossreacts with human RHD-derived human mAbs reactive with the group A streptococcal carbohydrate epitope GlcNAc and alpha helical coiled coil streptococcal M protein (15, 76, 104–109). The IgG2 deposition on valve and heart tissues and elevated anti-GlcNAc antibodies in RHD sera combined with a lower GlcNAc-specific IgG2 response in pharyngitis suggests disease specificity and ultimately IgG2 may be responsible for directing infiltration of Th1 and Th17A cell subsets as well as potential cytotoxic IgG3 responses to the valve. We propose there may be abnormally high concentrations of GlcNAc-specific IgG2 production against the GAS in RHD and SC phenotypes similar to the very high levels of anti-GlcNAc responses reported in Balb/c mice immunized with group A carbohydrate antigen. Significantly higher immune responses were seen to GlcNAc in BALB/c mice than in lower responders C57BL/6, CBA, and DBA/2 mice (110, 111).

Interferon gamma is known to signal B cell isotype switch events to the IgG2 subclass (61). In the current study, IFN-γ was found in all RHD hearts tested, and was previously reported in the pathogenesis of RHD (29, 71, 112, 113). Human T cell clones were also found to secrete a Th1 pattern of cytokines when stimulated with group A streptococcal antigens such as the M protein (16, 25, 29, 114, 115). Further, the Lewis rat model of RHD immunized with recombinant M5 protein developed myocarditis and valvulitis characterized by secretion of high levels of IL-17A and IFN-γ suggesting that the two cytokines are together important in the pathology of RHD (116).

Transition of Th17 to non-classic Th1 cells (CD161+/CCR6+) is believed to be related to the development of the autonomous responsive state of Th1 cells, which after transitioning from Th17 to Th1, respond to self-antigens presented in the attack organ leading to tissue damage (83, 117–121). This could direct responses to become more valve specific as disease progresses (48). In our previous studies, CD4+ T lymphocytes were more abundant in RHD hearts and were in the Aschoff lesions in valves, however CD8 + lymphocytes were also present and are known to also produce IFN-γ. The specific role for CD8 + T cells in RHD has been unknown in valvular heart disease (24). In recent studies, cytotoxic CD8 + T cells isolated from chronic adult human RHD valve lesions recognized collagen and identical sequence from the collagen-like streptococcal protein. The CD8+ T cell expression of granzyme and perforin as well as estrogen receptor alpha and HLA I were promoted by prothymosin alpha, a new potential blood and tissue biomarker described in RHD (48). The study emphasized valve disease primarily in women and

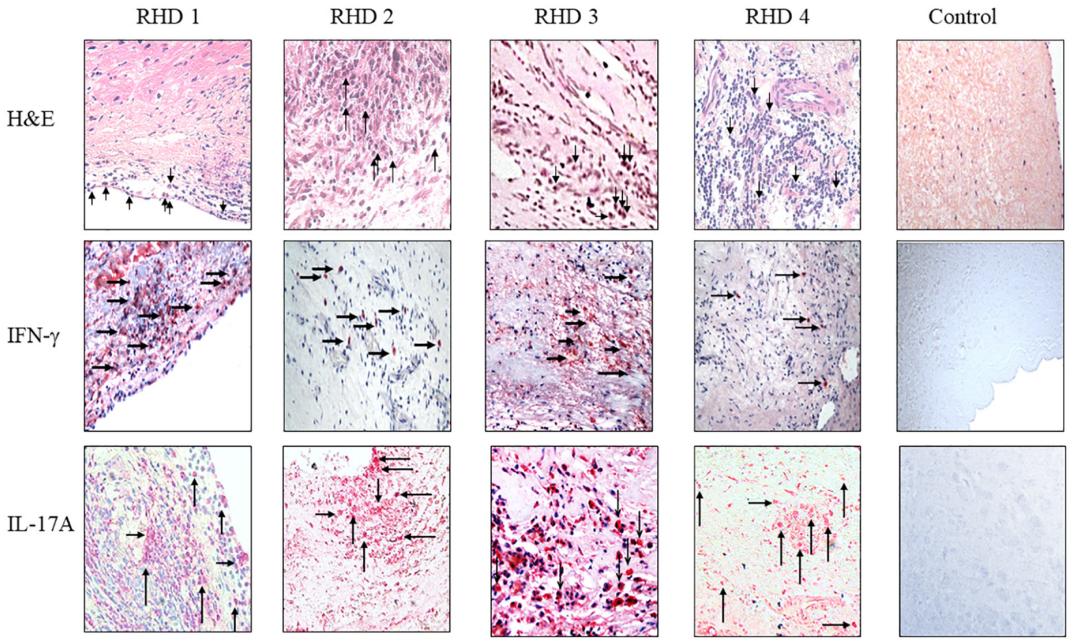

FIGURE 7
IgG2 is associated with IFN-γ and IL-17A in RHD hearts. **(Top row)** hematoxylin and eosin (H&E) staining of four RHD heart tissues showed infiltration of mononuclear cells (arrows in top row) into the heart at the valve surface endothelium (RHD 1) and within the myocardial tissues (RHD 1-4). Focal lesions (large arrow) were observed within the myocardium of RHD4. Non-RHD valvular heart tissue (control) lacked mononuclear cell infiltration and focal lesions. **(Middle row)** IFN-γ staining of RHD cardiac tissues demonstrates widespread IFN-γ staining of heart tissues was found for RHD1, and RHD3 tissues. Concentrated IFN-γ staining can be seen around invading mononuclear cells (RHD 1-RHD 4). IFN-γ is absent in the control heart tissue. Magnification 400×. **(Bottom row)** IL-17A staining of RHD cardiac tissues. Four RHD tissues shows IL-17A throughout the heart tissues as well as concentrated around mononuclear cells (arrows) (RHD 1-RHD 4). The control, non-RHD heart tissue had no IL-17A present. Magnification 400×.

the importance of mimicry with streptococcal or other bacterial collagen sequences (48). Previous studies have been primarily limited to CD4 + T cells in RHD with the acknowledgment that CD8+ T cells were present but not in the same numbers as the CD4+ T cells (24). Gender bias in chronic RHD in women is further supported by the identification of a dominant shared antibody idiotype found in RHD and shared with systemic lupus erythematosus (SLE) and Sjogrens syndrome (122), both systemic autoimmune diseases primarily in women where the heart can also be affected.

Molecular mimicry is important in the initiation of disease with the onset of autoimmunity. Following the development of autoimmunity, a more chronic disease course in the valves may develop at different rates in different individuals due to the number of repeated infections, hemodynamic sheer stress to the valves, as well as genetic predispositions to susceptibility to disease. These disease susceptibility factors may be genetic and include increased levels of TGF-β1 production (49), HLA (50–55), and exposure of other heart or tissue proteins (77) involved in epitope spreading with tissue antigen recognition by Th1 cells which may dominate in more chronic disease in the heart. If IgG2 continues to follow the course of deposition on the valves concomitant with exposure of collagen (3, 46), elevated TGF-β1 (49), and fibrosis with scarring and neovascularization from the damaged valves, further damage in later stages of chronic disease may be in part by CD8 + T cells and their recognition of collagen (37). Clearly, inflammation leads to fibrosis.

SC, the brain manifestation of ARF, is characterized as an antibody driven basal ganglia encephalitis (40, 41, 123–130), and the main pathologic finding is perivascular cuffing of vessels and deposition of antibody in the basal ganglia (129, 131). The role of Th17 cells in SC as well as other group A streptococcal autoimmune sequelae in humans remains obscure. However, in the murine model of GAS infection, Th17 cells found in the nasal-associated lymphoid tissue (NALT) migrated through the olfactory bulb into the CNS following intranasal GAS challenge (99). While no GAS cells were found in the CNS, the evidence strongly suggested that IL-17A disrupts the blood brain barrier (BBB) allowing crossreactive antibodies access to the brain. In murine GAS-induced autoimmune encephalitis, CNS-infiltrating Th17 cells secreting IL-17A and IFN-γ were required for the disruption of the BBB that allowed for the transit of antibody and microglial activation leading to neuropathological changes in experimental animals (132). Similar mechanisms may disrupt the BBB in SC allowing for passage of autoreactive antibody and T cells.

In SC and related disorders, brain imaging studies showed enlargement of the basal ganglia suggesting an inflammatory process (37, 38, 124, 125, 133, 134). Anti-neuronal autoantibodies found in SC and the related pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS) which target dopamine receptors may cause the characteristics of these disorders and serve as biomarkers of disease (3, 12, 40, 41, 135). Collectively, the pathology of SC is indicative of non-cytotoxic autoantibody-mediated basal ganglia encephalitis and supports an IgG2 etiology where the crossreactive antibodies against the group A carbohydrate antigen GlcNAc lead to aberrant signaling of the neurons rather than complement-mediated cytotoxic destruction in the basal ganglia (14).

Limitations of our study include the small number of tissue and sera samples for this pilot study, and we did not perform any studies of crossreactivity except with the bacterial carbohydrate antigens. Further, many of our previous studies of ARF-derived human mAbs have demonstrated crossreactivity between GlcNAc

and autoantigens including cardiac myosin and laminin in heart as well as lysoganglioside, tubulin, and dopamine receptors in human brain tissue or neurons (14, 15, 39, 40, 44, 73, 136). Although we do not have human mAbs of the IgG2 subclass to investigate, the human ARF derived mAbs which react with GlcNAc are the strongest evidence linking the anti-streptococcal GlcNAc antibodies (serum and CSF IgG and human mAbs) with crossreactivity to heart and brain.

In summary, our study suggests a new potential biomarker for ARF, RHD, and SC which can raise understanding of ARF pathophysiology. GlcNAc specific IgG2 was defined as the dominant IgG GlcNAc specific subclass in both RHD and SC in both tissues and blood, which adds to our basic understanding of group A streptococcal sequelae and pathogenesis. Further, GlcNAc-specific IgG2 distinguishes and separates the humoral IgG subclass responses of uncomplicated pharyngitis from ARF (RHD and SC). Th1/Th17 cells found in RHD and SC may cooperate to cause organ specific autoimmune T cell disease responses in the heart and brain and Th1 IFN-γ responses would promote switching the IgG subclass to IgG2. Disease specific IgG2 immune responses to GlcNAc in RHD and SC is supported by many of our previous studies demonstrating heart and brain crossreactive IgG autoantibodies and human mAbs from RHD and SC that recognize the GlcNAc epitope of the streptococcal group A carbohydrate. The novel discovery that increased levels of GlcNAc-specific IgG2 defines ARF (RHD and SC) raises our level of understanding of ARF pathophysiology as well as management of streptococcal vaccines and their safety, the future development of novel diagnostics and therapeutic strategies to identify risk and early disease, and to identify, manage, and protect against severe and chronic heart valve damage or neuropsychiatric disability in ARF. Future studies of patients from worldwide cohorts are needed to confirm our "GlcNAc-specific IgG2" hypothesis presented here and supported by our evidence.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

University of Oklahoma Health Sciences Center, University of Utah School of Medicine Internal Review Boards and the NIMH Internal Review Board reviewed and approved all protocols for the study of human subjects analyzed in this manuscript. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

MC and CK wrote the manuscript and designed the experiments, graphs and diagrams. AH, HC, CK, and KA performed experiments for the manuscript. MC, KA, and CK were responsible for design and production of the figures for the manuscript. HH and GV provided patient samples from ARF outbreak and provided discussions and clinical information for the study and HH assisted with the manuscript. DJ provided tissues samples from RHD patients from South Africa and assisted with the manuscript. SK performed the pathology studies and assisted with the manuscript. SS provided well characterized clinical samples of serum and cerebrospinal fluid from Sydenham chorea patients and assisted with the manuscript. YZ provided expert statistical analysis and writing of the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by grants from the National Institutes of Health (NIH) from the National Heart, Lung, and Blood Institute R37HL35280, a MERIT Award to MC, and R01HL56267 and R01HL135165 to MC. Statistical analysis was partially supported by a National Institutes of Health, National Institute of General Medical Sciences Grant 2U54GM104938, PI, Dr. Judith James.

Acknowledgments

We would like to dedicate this manuscript to the memory and work of the late Drs. Elia Ayoub and Maclyn McCarty for their support and their important contributions to the understanding of the group A carbohydrate and to the late Dr. John Barlow who connected us to Dr. Jankelow and made possible the availability of the rheumatic heart disease tissues. Dr. Ayoub discovered the correlation of elevated antibodies against the group A streptococcal carbohydrate with outcomes/prognosis of rheumatic valvular heart disease. Dr. McCarty published early work on the group A carbohydrate and was a colleague of Dr. Rebecca Lancefield who identified the streptococcal groups based on their carbohydrate antigens. Dr. Barlow was a giant in cardiology and an expert in the heart valve. Our discoveries are built on the shoulders of those who came before us in times when rheumatic fever was rampant in pediatric hospital wards everywhere in the United States. Dr. Gene Stollerman, Dr. Lewis Wannamaker, and many others of that era left legacies in translational science and medicine that impact us today. We also thank the late Drs. Edwin Beachey and Gene Stollerman, who were Dr. Cunningham's Ph.D. mentors, as well as Dr. Vince Fischetti who provided guidance and made important contributions to streptococcal vaccines and understanding the structure of the group A streptococcal M protein; and to Dr. Joseph Ferretti who made it possible for Dr. Cunningham to begin her career at the University of Oklahoma Health Sciences Center. We express our deepest appreciation to Janet Heuser and Adita Mascaro-Blanco for their years of expert technical assistance, to Dr. Chandra Menendez for review of the manuscript, and to Dr. Paul Grant and Margaret Pekar who tirelessly worked to provide clinical samples to our laboratory from the National Institute of Mental Health (NIMH).

Conflict of interest

MC, discloses her affiliation as chief scientific officer/consultant and co-founder of Moleculera Labs in Oklahoma City, OK where the company offers diagnostic testing for anti-neuronal autoantibodies in autoimmune neurologic and psychiatric disorders. SS, has no

financial conflicts of interest. Although she is a co-inventor on the anti-neuronal autoantibodies/CaMKII panel, neither she nor the NIMH receive any royalties from the patent. CK, discloses holding in part a patent for development of the anti-neuronal autoantibody assays and receives royalties from Moleculera Labs.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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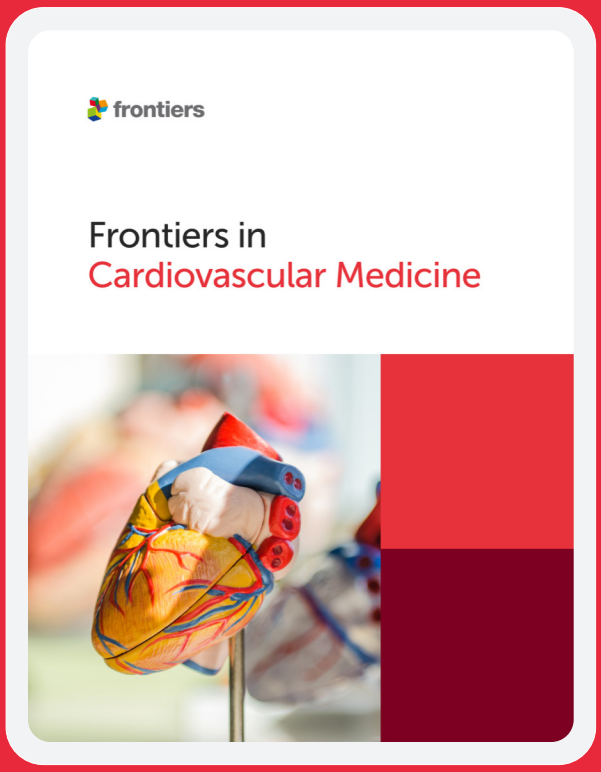

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