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The kidney in auto-immune and auto-inflammatory processes: Definitions, mechanisms, and biomarkers

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The kidney in auto-immune and auto-inflammatory processes: Definitions, mechanisms, and biomarkers

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Editorial: The kidney in auto-immune and auto-inflammatory processes: Definitions, mechanisms, and biomarkers

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Editorial on the Research Topic
The kidney in auto-immune and auto-inflammatory processes: Definitions, mechanisms, and biomarkers

Autoimmune and autoinflammatory processes represent the starting mechanism of renal damage in a number of pathologies of the kidney. Autoimmune processes include diseases in which autoantibodies represent the major pathogenic mediator of lesions that, in general, involve glomeruli; auto-inflammatory conditions are, instead, characterized by the activation of an inflammatory cascade diffuse to all the renal compartments that may occur as a manifestation of genetic background or, more frequently, as the result of an inflammatory trigger.

There are some overlaps between autoimmunity and inflammation in the mechanisms that lead to renal damage: a clear distinction between the two processes does not exist and it makes sense to attempt a classification only for clinical purposes. Systemic Lupus erythematosus (SLE) with lupus nephritis (LN), anti-neutrophil cytoplasmic antibody (ANCA)-associated renal vasculitis and membranous nephropathy (MN) are representative of the autoimmune group. Inflammatory and auto-inflammatory diseases that can involve the kidney include rarer conditions such as IgG4 related pathologies, PFAPA, and hemophagocytic syndromes, which are characterized by a diffuse process in which the kidney is one of the target organs but other manifestations may occur. IgA vasculitis (formerly Henoch-Schönlein purpura) with glomerulonephritis is a condition with still unclear pathogenesis that is characterized by multisite localizations of the vasculitic process (with deposition of IgA mainly in skin and kidneys) and active inflammatory elements present at both sites (Figure 1).

This Research Topic of Frontiers has the ambitious aim to cover at least some of the above issues including either pathogenetic mechanisms and clinical aspects. The selection of articles includes (1) a larger section addressing classical autoimmune diseases (SLE/LN and ANCA associated vasculitis) with the proposal of new classification criteria for Eosinophilic Granulomatosis with Polyangiitis (EGPA), a condition between ANCA-associated vasculitis

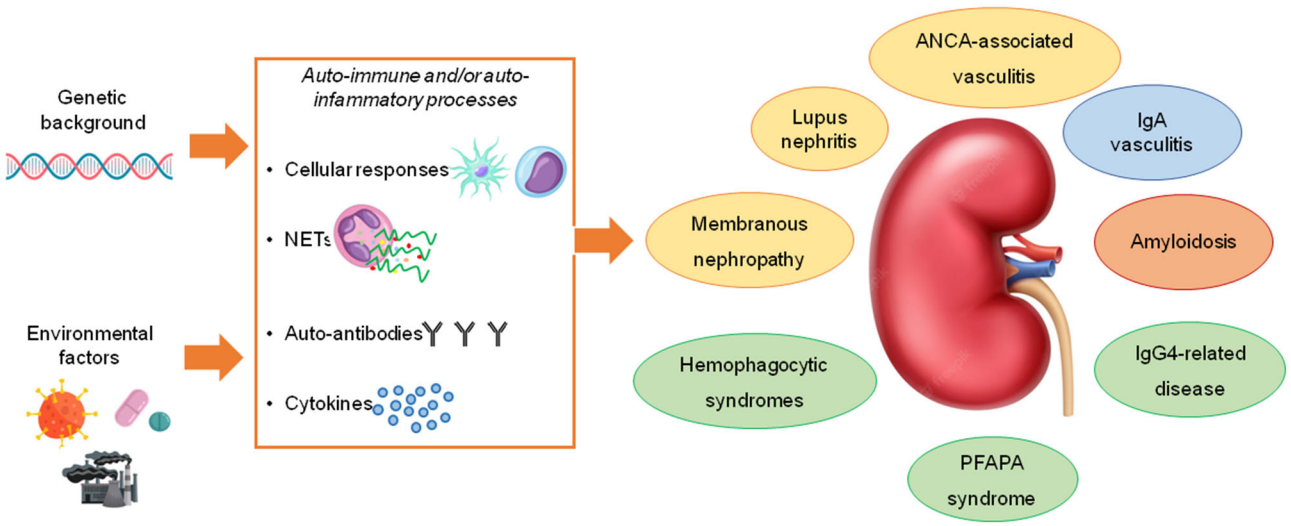

FIGURE 1
Most of the immune-mediated, systemic diseases that affect the kidney are "complex diseases" that result from the interaction between genetic susceptibility factors and environmental factors. The pathogenic mechanisms through which they cause kidney disease involve T-cell and macrophage/dendritic cell-mediated immunity, neutrophil activation and NET (neutrophil extracellular trap) formation with the exposure of auto-antigens and the consequent stimulation of autoimmune responses, the production of autoantibodies and the secretion of immuno-modulatory cytokines. The diseases included in this spectrum may have well-known pathogenic autoantibodies (e.g., lupus nephritis, ANCA-associated vasculitis, membranous nephropathy- all highlighted in yellow), pathogenic immune-complexes (IgA nephropathy, highlighted in blue), pathogenic monoclonal or polyclonal proteins able to form amyloid (amyloidosis, in orange), autoinflammatory or yet unknown pathogenic hallmarks (all the conditions highlighted in gray).

and hypereosinophilic diseases. In terms of autoimmunity, a few papers describe the cells and regulatory molecules involved in the pathogenesis of renal damage, and a special section has been devoted to neutrophil extracellular traps (NETs), a specific mechanism involved in the formation of anti-DNA and other auto-antibodies. (2) The collection includes an article focussing on the involvement of complement and bilirubin in the progression of IgA vasculitis. (3) An overview of auto-inflammatory (PFAPA) and hemophagocytic syndromes. (4) A paper devoted to IgG4 related renal disease and, finally (5) a study on renal amyloidosis.

1. Renal autoimmune conditions

1.1. Lupus nephritis

Lupus nephritis is the most typical autoimmune glomerulonephritis. It is determined by intra-glomerular deposition of antibodies and complement that bind the basement membrane and modify *per se* permeability of the glomerular barrier causing proteinuria and activating an inflammatory cascade leading to the proliferation of extra and intra-glomerular cells and the development of sclerosis. Studies on the cells and cytokines involved in the inflammatory phase of LN focused on the Th17/IL17 axis as a specific mediator associated with $CD8^+$ T cell hyperactivation. These aspects have been reviewed by Paquissi and Abensur who reported high levels (and high percentages) of IL-17 expressing cells in both the circulation and glomeruli of patients with LN that correlated with general parameters of SLE activity such as the SLEDAI index and with the specific renal parameters of renal damage such as GBM thickening. IL-17 can also be considered the determinant of a complex series of events within all the compartments of the kidney including modulation of the cytoskeleton in glomerular cells, activation of oxidative stress, and stimulation of the synthesis of other inflammatory cytokines such as IL-23 (1). All these effects can amplify the mechanisms of glomerular damage typical of LN (2). As correctly recognized by the authors, several association studies provide indirect support on the implication of the Th17/IL-17 axis in LN but not direct evidence. Assessing the efficacy of new drugs that play a specific inhibitory effect on IL-17A could provide an answer to this question. The first inhibitor, secukinumab, is currently being tested for safety and efficacy in a trial on the cutaneous manifestation of SLE (NCT03866317) that is preliminary to assessing its effects on other organs (3).

Wiechmann et al. investigated the amount of $CD8^+$ T cells in LN and their activation status as reflected by the expression of CD107a. Positivity for CD107a in CD8+ T cells is considered a marker of killing activity given by the capability of CD107a positive cells to secrete perforin and granzyme B (4). In analogy, high circulating CD107a+ $CD8^+$T-cells in patients with SLE correlated with the SLEDAI activity index. In the kidney of LN patients, CD107a+ $CD8^+$T-cells were localized almost completely in tubule-interstitial areas and correlated with proteinuria and the chronicity index. These data lead us to consider CD107a+CD^+8T cells as a major effector of cytotoxic activity in the tubule interstitium, which represents a very important functional compartment of the kidney. Targeting CD107a could be a possible evolution of new therapies.

Two papers in the Research Topic were dedicated to the mechanisms leading to the formation of autoantibodies; one specifically addressed the reasons for the IgG2 isotype switch that is typical of LN. It is well known that autoantibodies are involved in the pathogenesis of LN target, in the majority of cases, dsDNA and other intracellular proteins. In both cases, activation of the autoimmune process requires that target antigens are exposed to the microenvironment. The mechanisms for their exposition are poorly understood. NETosis is a process characterized by extracellular exposure of dsDNA from the intracellular compartment of neutrophils and is, therefore, of interest for anti-dsDNA formation. Neutrophil extracellular traps (NETs) contain, besides dsDNA, many other intracellular proteins that become potential antigens for autoimmunity in LN. In their contribution to this collection, Bruschi et al. review many aspects related to the composition and metabolism of NETs in healthy and pathological conditions. In normal conditions, the formation of NETs represents a physical barrier made of DNA, nucleosome and other intra-neutrophil proteins that is functional to protect from external infectious organisms (suicidal NETs) (5). In this context, NETosis is considered an early step of innate immunity. Another pathway for NET generation in SLE is by immunologic triggers (sterile NETs) in which case NETs are directly connected with the overall autoimmune activity. Once formed, NETs are removed by DNase1 and 13 that digest DNA and it is the imbalance between production and removal of NETs (for DNase deficit) that in SLE leads to the abnormal production of anti-DNA antibodies. *DNase13* mutations have been associated with familial LN and, more generally, the circulating inhibitors of DNases (probably antibodies) have been detected in patients with SLE in association with defective removal of DNA (6). A significant aspect of NETs is that they contain, besides DNA, other intracellular proteins, such as enolase and annexin A1, that are targets of autoantibodies whose levels are high in the circulation and micro-dissected glomeruli of patients with LN (7, 8). Bertelli et al. reported new experiments that aimed to explain why the majority of autoantibodies in SLE are of the IgG2 isotype that requires a class switch recombination step. The basic hypothesis made by the authors was that this passage implicated NETs. They show that NETs purified from SLE patients induced an important release of soluble IgG2 by the naïve B cells of SLE patients that did not take place in healthy donors, implying the existence of regulatory factors linked with SLE. In parallel, NETs stimulated *ex vivo* IgG2 isotype class switch through the induction of T-bet, a transcriptional factor that was expressed by an "atypical memory" CD19 clone highly expanded in patients with SLE. T-bet acts in association with TLR-9, with other stimulating factors such as type I IFN, and with components of the MyD88-related pathways. T-bet activity was also associated with other antagonizing factors. These data strengthen the notion that NETs are implicated in the autoimmune milieu, which characterizes LN, and indicate that the regulatory mechanisms of the IgG2 isotype class switch could become a target of specific therapies.

A unique manuscript in this issue addresses the clinical aspects of SLE. Bao et al. describe a rare association between SLE/LN and mantle cell lymphoma, which is an aggressive B-cell non-Hodgkin lymphoma that is usually unresponsive to common therapies. The association of mantle cell lymphoma with glomerular diseases (i.e., minimal change disease, membrano-proliferative, and ANCA associated glomerulonephritis) has already been reported (9) but this is the second case of an association with LN that could suggest a pathogenic link with B-cell anomalies. On a more clinical vein, it is of interest that renal symptoms are remitted after the first chemotherapy, in concomitance with the remittance of the lymphoma itself.

1.2. Membranous nephropathy

Membranous nephropathy is a primary autoimmune disease limited to the kidney and characterized by sub-epithelial deposits of autoantibodies and complement. In this view, MN shares some pathologic features with Class V LN such as the same sub-epithelial deposition of autoantibodies. MN causes nephrotic syndrome and represents a main leading factor for the evolution of chronic renal failure. In the last decade, several target antigens of the auto-antibodies responsible for MN have been characterized which represents a significant evolution in the pathogenesis of the disease. Anti-PLA2R1 is the most common (present in 65% of patients), followed by anti-TSHD7A (3%) and by several others which have a minor numerical impact being, in some cases, described in less than 5 patients (10, 11). Most of the antigens above are proteins expressed in the glomerular membrane, which explains why their deposition causes proteinuria. Antibodies targeting intracellular antigens such as anti-SOD2 have been reported and considered an adjunctive reason for the evolution of tissue lesions, given the well-known anti-oxidant power of this molecule (12). In their study, Hu et al. describe particular aspects of the reparative process related to the expression of macrophage sub-populations in renal tissue of a numerically significant cohort of 55 patients with MN. They focused on M2a, M2B, and M2c macrophages which are the major cells involved in the anti-inflammatory and reparative processes in glomeruli and tubule-interstitial areas. Correlations between a number of macrophages in each category and pathologic parameters (IgG1 and C3 staining) were reported and interpreted as proof of the involvement of these cells in post-acute damage. This observation re-opens a topic that attracted the interest of pathologists a few years ago when some studies proposed that the degree of tubulointerstitial macrophage infiltration determines the prognosis of MN (13, 14).

1.3. ANCA-associated renal vasculitis

ANCA-associated renal vasculitis is less common but usually more severe than LN and four papers in this Research Topic are dedicated to its pathogenetic and clinical aspects. Wang et al. evaluate the potential association with Annexin A1 in 66 patients with AAV, 31 in the active state of the disease, and 35 in remission. The intent was to characterize the activation state of neutrophils since Annexin A1 is a circulating protein that has a main role in the regulation of neutrophil trafficking, adhesion, and transmigration (15) and contributes, in this way, to the resolution of inflammation (16). In this study, expression of Annexin A1 was found, besides in neutrophils, also in monocytes/macrophages and T cells, as well as in several renal cell types, i.e., podocytes, mesangial cells, and tubular epithelia, widening the significance of this protein in renal

pathology. Plasma levels were high in active AAV and correlated with the proliferation of intrinsic glomerular cells (podocytes, mesangial cells) and circulating neutrophils and macrophages. The conclusions were that renal and cell Annexin A1 is stimulated by inflammation and potentially participates in its resolution based on the strong anti-inflammatory effect of this protein. The unique caveat of the study was the concomitance of steroid therapies since it is well known that Annexin A1 levels in circulating neutrophils are under the control of glucocorticoids (endogenous and exogenous) that involve the Annexin A1 receptor (ALXR), the glucocorticoid-induced leucine zipper gene (*GILZ*) (17) and probably other cytokines such as IL6 (18).

Xia et al. evaluated the accuracy of a score for predicting the evolution of ANCA-associated renal vasculitis into end-stage renal disease, also known as the ANCA Renal Risk Score (ARRS). They utilized a meta-analysis approach and found 1.568 ANCA positive patients with glomerulonephritis reported in 11 studies that utilized the ARRS for predicting the clinical outcome. Pooled ANCA-GN patients were subdivided into three different groups with low (score 0–1), medium (score 2–7), and high ARRS (score 8–11), and they found a cumulative number of patients with end-stage renal disease after 60 months of follow up of 5%, 22%, and 59% respectively. The pooled sensitivity of the test in predicting ESRD was 98% with a specificity of 30% for ARRS $>$ 2, and 58% with a specificity of 86% for ARRS $>$ 8. This led to the conclusion that the ARRS performs well in predicting poor outcomes in ANCA-GN patients.

Xu et al. described a 14-year-old girl with microscopic polyangiitis who developed intracerebral hemorrhage associated with posterior reversible encephalopathy syndrome (PRES). This girl presented abrupt seizures in concomitance with severe hypertension and underwent remission after combined therapy with methyl-prednisolone pulses, plasmapheresis, anti-seizure, and anti-hypertensive medications. The authors reviewed the literature and found 6 cases that presented the same association. The importance of this finding is that in the presence of microscopic polyangiitis, cerebrovascular complications may occur and must be correctly recognized since they can revert after appropriate therapies.

1.4. Eosinophilic granulomatosis with polyangiitis

Eosinophilic granulomatosis with polyangiitis (EGPA) is a rare multisystem disease that is considered to be between an inflammatory disorder with hyper-eosinophilia and a classical ANCA-associated vasculitis. Fagni et al. attempted a classification of EGPA based on ANCA positivity (mainly MPO) in patients with clear vasculitic manifestations vs. patients with generalized symptoms more linked with eosinophilia and ANCA-negativity. Renal involvement was also predominant in the former group. Clinically, EGPA is characterized by classical signs of eosinophil activation without inter-group difference where cardiac, neural, pulmonary, and vascular manifestations correlated with the entity of eosinophil infiltration (cardiac is the most frequent cause of death) (19). Genome-wide association studies supported the view that there is a dualism between being ANCA positive and having negative EGPA, showing an association with HLA class II variants in the former group (20), suggesting a link with $CD4^+$ T-lymphocyte activation. The predominance of IL17 and Th17 cells in ANCA positive patients would create an amplification loop promoting neutrophil tissue recruitment and activation that is the basis for anti-MPO formation (21). By contrast, ANCA negative EGPA was associated with the *IRF1/IL5* that interacts with IL-4 and IL-5 promoter regions and is directly linked with eosinophil activation and asthma (20, 22). Since a clear phenotypic differentiation is not the rule in EGPA patients, the authors concluded that the application of a strict dualism cannot yet be translated into routine clinical practice and remains a goal for future studies.

2. IgA vasculitis with glomerulonephritis

Cutaneous IgA vasculitis is a common disease in children, characterized by the formation of typical purpuric lesions of the skin that usually resolve after a few weeks. In adolescents and adults, cutaneous IgA vasculitis, although rare, is frequently associated with renal lesions initially limited to glomerular proliferation but may evolve to glomerulosclerosis and tubule-interstitial with worsening renal function. IgAV-glomerulonephritis (GN) is characterized by urinary abnormalities such as hematuria (macro and micro) and variable degrees of proteinuria (23). Clinical or laboratory features compatible with IgAV-GN mandate kidney biopsy for the confirmation and staging of tissue lesions.

Two papers address the key problem of the severity of IgAV-GN in adults and underline the need to develop predictors of clinical outcomes. Romero et al. determined the amount of C4d deposition in glomeruli of 120 adults with IgAV-Gn and found that it was correlated with either the index of renal disease activity or clinical long term outcome. C4d is generically considered an index of poor prognosis in patients with isolated IgA nephropathy (24) and it seemed important to confirm the importance of this parameter in adults with IgAV-GN. C4d is a complement fraction with no clear biological function that derives from the degradation of complement within the lectin pathway. The concomitant presence in the glomeruli of C4d and mannose modified IgA has pathogenetic significance for the activation of this alternative complement cascade (25). Glomerular C4d was found in 23% of patients with IgAV-GN in association with increased mesangial proliferation and baseline proteinuria, which were considered a predictor of poor prognosis and justified a more aggressive therapeutic approach. The renal outcome in terms of proteinuria and renal function in the two cohorts of C4d+ and C4d- patients evaluated retrospectively was comparable, limiting the interest on C4d (and more in general on compliment) as a biomarker of the progression of IgAV-Gn. The second paper published in the Research Topic by Tan et al. proposed bilirubin as a protective factor for the progression of IgAV-Gn based on the antioxidant, anti-inflammatory, and vascular protective functions of this molecule. Previous reports have already demonstrated that low bilirubin levels are associated with poor evolution of IgA GN and diabetic nephropathy and are, more in general, correlated with the progression of CKD (26, 27). The authors studied 189 young and young adults with IgAV-GN (age $>$16 years) and found, with multivariate Cox analysis, that the serum bilirubin was an independent protective factor for renal survival where the composite endpoint was defined as a 50% decline in e-GFR, end-stage renal disease or death. The result was confirmed in 89 patients who were matched for baseline clinicopathological

manifestations and treatments except for serum bilirubin. In this case, the retrospective model of the study also refrains from definite conclusions still pointing to unexpected functions of this common molecule. The protective activity of bilirubin can be explained by the known inhibitory effect that this molecule plays on complement and on cytokines.

3. Auto-inflammatory and hemophagocytic syndromes

Two papers in this section address the main issue of renal involvement in two inflammatory conditions, one with a genetic origin, i.e., Heterozygous mutations of the proline-serine-threonine phosphatase interacting protein 1 gene (*PSTPIP1*) and the other, i.e., hemophagocytic syndrome (HPS) or hemophagocytic lymphohistiocytosis.

Borgia et al. described a 22-year-old male presenting the genetic imprinting of a variant of the PSTPIP1 syndrome also known as *PSTPIP1*-associated myeloid-related proteinemia inflammatory syndrome or PAMI (p.E250K and p.E257K) (28, 29). Renal involvement is part of the clinical picture that may vary from vasculitis to more generic tubulo-interstitial involvement. In line with a few reports in the literature on renal involvement in PAMI, the young adult here described developed glomerular lesions compatible with Focal Segmental Glomerulosclerosis (FSGS) and consequent renal failure (30) and was treated with the association of Canakinumab and Tacrolimus. The effects of this association were particularly satisfactory on the general inflammatory status and on skin lesions, which reverted significantly, whereas they were only partially effective in treating proteinuria and renal function that only stabilized. The pathogenesis of FSGS is unclear (31), the participation of the IL 1 pathway has been proposed and it is indirectly supported by the positive effects produced by blockers of the IL-1β/IL-1R1 signaling pathway (32). Therefore, this case report strengthened this pathogenetic concept.

HPS is a rare syndrome characterized by fever, pancytopenia, hepatosplenomegaly, liver dysfunction, and by the presence of nonmalignant macrophages infiltrating the bone marrow. A generic renal involvement is frequently reported without a clear site-specific pathologic definition (33). Roccatello et al. attempted to widen the spectrum of HPS to renal-limited forms, describing four cases with severe renal involvement, two presenting with AKI and the other two with chronic renal failure of varying degrees, telescopic urinary sediment, with hematuria and proteinuria and the presence of $CD68^{+}$ infiltrating cells in the renal tissue as common features. Hyperferritinemia and platelet consumption were present in only one case. The renal aspects of this series of patients are in line with what is reported in wider literature on renal involvement in HPS (60% of patients presented with AKI and 40% with the nephritic syndrome) (34, 35). Other renal histology reports also indicate that inflammatory or ischemic tubule-interstitial lesions, in general, correlate with AKI, and podocytopathies with collapsing lesions and/or thrombotic microangiopathy in some patients with a chronic outcome of renal failure; macrophage infiltration, as here reported, has been only occasionally described (35). The definition of "renal limited HSP" requires confirmation in other cohorts of patients before being consolidated as a particular aspect of HSP.

4. IgG4 related disease

IgG4-related disease (IgG4-RD) is a rare inflammatory condition (1:100.000) characterized by lymphoplasmacytic infiltrate in many organs, obliterative phlebitis, and storiform fibrosis; eosinophilia and increased levels of IgG4 are also common (36, 37). Responsiveness to steroids is a major clinical characteristic of IgG4-RD (38).

Capecchi et al. described 4 cases of IgG4-RD with prevalent renal involvement that summarize the expression this syndrome may have in the kidney. Focal tubulointerstitial multifocal lymphocytic infiltrates, where IgG4 + plasma cells predominated, and tubulitis were hallmarks of the disease. Typical storiform or "cartwheel fibrosis" represents the evolution of tubulointerstitial lesions to more chronic and irreversible alterations that preceded end stage renal failure (39). Membranous glomerular lesions usually coexist with tubulointerstitial nephritis and nephrotic proteinuria. Since primary membranous nephropathy is also typically characterized by IgG4 sub-epithelial deposits the differential diagnosis between the two conditions may pose some difficulties and is merely based on the presence of TIN and the association with other organ involvement that is always absent in primary membranous nephropathy. Retroperitoneal fibrosis evolves into obstructive nephropathy that may occur in IgG4-RD, an additional possible cause of renal failure (39). In general, this condition is clinically silent and causes progressive chronic renal lesions. Acute renal obstruction is in general characterized by severe pain and may be easily detected by renal ultrasound, and ureteral stenting or percutaneous nephrostomy allows rapid resolution of the obstructive state.

5. Amyloidosis

Secondary amyloidosis (AA amyloidosis) is a common complication of autoinflammatory diseases as well as of organ and site-specific chronic conditions that occur frequently such as those involving the bowel (IBD) and joints (rheumatoid arthritis, ankylosing spondylitis)(40). Serum Amyloid A (SAA) is an acute phase protein, produced by the liver and macrophages in response to an inflammation under the transcriptional control of pro-inflammatory cytokines, particularly tumor necrosis factor (TNF) alpha, interleukin-1 (IL-1) beta and IL-6 (41, 42). SAA circulates and deposits in many organs where it forms misfolded aggregates of proteins and fibrils that are resistant to degradation. The heart, kidney, liver, gastrointestinal tract, and peripheral nerves are targets of this process, which is chronic, and sustained by the failure of the degrading systems to remove amyloid. Early therapeutic interventions with TNF-blockers and/or anti-interleukin agents seem the most promising approaches, aiming to block SAA formation before deposition (42).

Another article in this issue by Eriksson et al. describes the encouraging effects of the anti-IL6 receptor antibody tocilizumab in two patients with long-standing ankylosing spondylitis who developed proteinuria and presented renal deposits of amyloid in vessels and glomeruli. At the start of the therapy, the inflammatory parameters had a rapid drop, with normalization of SAA levels that were followed by an improvement of either proteinuria and

renal function after 18 and 54 months follow up. While tocilizumab had been already utilized in AA secondary to rheumatoid arthritis (43), these two patients were the first with ankylosing spondylitis-related AA amyloidosis to be treated with this agent and the positive effect on renal parameters supports a more diffuse use of this drug in AA secondary to ankylosing spondylitis. Some pathogenetic clues are outlined by these data, indicating that a late intervention to block SAA production may activate a beneficial reparative mechanism in the kidney. In other words, it seems that a divergent association between amyloid accumulation in the kidney, which is irreversible, and renal symptoms (i.e., proteinuria, renal function) exists, suggesting the acute toxic effect of SAA in the kidney (44, 45). The progressive nature of renal amyloidosis may be blocked at a stage in which the function of the organ is still normal.

Author contributions

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

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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.

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MINI REVIEW
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Eosinophilic Granulomatosis With Polyangiitis: Dissecting the Pathophysiology

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Eosinophilic Granulomatosis with Polyangiitis (EGPA) is a rare multisystemic disease classified both amongst hypereosinophilic disorders and ANCA-associated vasculitis. Vessel inflammation and eosinophilic proliferation are the hallmarks of the disease and main effectors of organ damage. Two distinct disease phenotypes have classically been described according to ANCA-status: the ANCA-negative subset with eosinophil-driven manifestation and the ANCA-positive one with vasculitic manifestations. An analogous dichotomization has also been backed by histological findings and a distinct genetic background. EGPA is typically consider a Th2-mediated disease and blood and tissue eosinophilia represent the cornerstone of diagnosis. Besides, ANCA are known for inducing endothelial injury and vascular inflammation by activating the circulating neutrophils. Thus, the pathogenesis of EGPA seems to be mediated by two coexisting mechanisms. However, the verbatim application of this strict dualism cannot always be translated into routine clinical practice. In the present review we describe the current knowledge on the eosinophilic and ANCA-mediated aspects of EGPA pathogenesis. Finally, we review the rationale of the currently proposed EGPA dichotomy and future research perspectives.

Keywords: Eosinophilic Granulomatosis with Polyangiitis, Churg-Strauss syndrome, eosinophils, hypereosinophilic syndromes, ANCA-associated vasculitis, neutrophils, myeloperoxidase, EGPA classification

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INTRODUCTION

Eosinophilic Granulomatosis with Polyangiitis (EGPA) is a rare disease characterized by granulomatous and eosinophil rich inflammation and systemic necrotizing vasculitis affecting small-to-medium sized vessels. EGPA occurs in patients with asthma and peripheral and tissue eosinophilia, and ∼30% of the patients present antineutrophil cytoplasm antibodies (ANCA) mainly specific for myeloperoxidase (MPO) (1). The disease is unique in its genre as it combines asthmatic manifestations with hypereosinophilic disorders and ANCA-associated vasculitis (AAV) features. Therefore, a full comprehension of its pathophysiology still lies beyond our reach.

An increasing amount of evidence indicates that EGPA's clinical phenotypes tends to segregate according to ANCA-status, as the major eosinophil-driven complications are most frequently found in the ANCA-negative subset of EGPA, namely lung infiltrates, myocardiopathy, and gastrointestinal manifestations. In contrast, MPO-ANCA-positive patients present a more "vasculitic phenotype," which comprises palpable purpura, peripheral neuropathy, rapidly

progressive glomerulonephritis and, rarely alveolar hemorrhage (2, 3) (**Table 1**). An analogous dichotomy is also supported by histological findings, as biopsy-proven vasculitis is found more frequently in ANCA-positive patients than in ANCA-negative ones, whereas eosinophilic infiltrates and granulomas are found with a similar frequency in the two groups (24).

The dualism between ANCA-positive and ANCA-negative EGPA is also supported by genetic background. A recent genome wide association study (GWAS) found differential association of genetic variants between the two serological subsets. MPO/ANCA-positive EGPA has a significant association with HLA class II DQ haplotype, which is shared with the other MPO-AAV (i.e., microscopic polyangiitis, MPA), while ANCA-negativity is associated with GP33 and IL5/IRF1 loci, indicating a possible mucosal/barrier dysfunction origin (25).

The pathogenesis of the disease also results from the complex interaction among innate and adaptive immunity, including eosinophils, neutrophils, T-helper lymphocytes, and B lymphocytes (26).

Based on these premises, the present review will focus on untangling the interactions between the two main pathogenic processes in EGPA (i.e., eosinophilic vs. vasculitic). The validity of the current two-faced model of the disease will also be examined.

EGPA AS AN EOSINOPHILIC DISORDER

Eosinophils are granulocyte innate immune cells that have classically been described in allergy, host defense against parasites, myelo- and lympho-proliferative disorders, and in autoimmune diseases. Particularly, blood and tissue eosinophilia represent the diagnostic cornerstone of EGPA, making it the prototype of eosinophilic vasculitis (5). From a pathophysiological point of view, EGPA shares intrinsic mechanisms with allergy and anti-helminthic response (27–30). It is characterized by the *en masse* polarization of T helper lymphocytes toward a Th_2 phenotype, the upregulation of eosinophil-selective eotaxin chemokines (particularly eotaxin-3), and an increased secretion of eosinophilotropic cytokines [i.e., interleukin (IL)-4, IL-5, IL-9, IL-13, and IL-25] (28, 31). So-called "allergic granulomas," consisting of palisading giant cells surrounding a core of necrotizing eosinophils, are also a distinctive histopathological feature of EGPA and are a sign of chronic eosinophilic inflammation which have also been described within persistent helminthic infections (29). Eosinophil-mediated organ damage is a shared feature of both EGPA and hypereosinophilic syndrome (HES), and clinical aspects overlap considerably (32). From a pathogenic standpoint however, a myeloid or lymphoid clonal origin can be detected in nearly half of HES (32, 33), and sensitivity to imatinib has been reported in a number of FIP1L1-PDGFRA (F/P)-negative HES patients bearing other novel fusion genes (34–36). Nonetheless, imatinib also anecdotally showed efficacy in F/P-unmutated EGPA, while a F/P-positive EGPA patient was reported, suggesting possible shared pathogenic mechanisms with HES (37, 38).

Eosinophils' Cytotoxicity

Eosinophils exhibit a wide spectrum of cytotoxicity, that is mediated by an array of enzymes stored in cytoplasmic granules, each of which associated with distinct type of clinically observable organ damage (39) (**Figure 1A**). Cardiac involvement is the major cause of mortality and morbidity in EGPA and has been widely associated to eosinophilia (40–42). *In vitro* evidence suggests that cardiotoxicity is mainly mediated by eosinophilic cationic protein (ECP) by altering the membrane sodium permeability of cardiomyocytes and inhibiting mitochondrial respiration (41). ECP also mediates fibrogenesis by inducing the release of fibrogenic cytokines transforming growth factor β (TGF- β), IL-1α, and IL-1β (43). Consistently, the presence of eosinophilic infiltrates and granule proteins has been widely documented in fibrotic tissues, including endomyocardial biopsy specimens of patients with EGPA (44, 45). The neurotoxic properties of eosinophils are clinically evident in the form of axonal neuropathy, a frequent finding in EGPA. Histologically, this relates to the presence of infiltrating eosinophils in the endoneurium and epineural vessels of EGPA patients (46). Fiber damage is probably due to the activity of eosinophilic neurotoxin (ENT) and ECP, which have been found to induce it *in vivo* (47). Interestingly, ENT acts as activating factor for myeloid dendritic cells by triggering the Toll-like receptor 2 (TLR2)–MyD88 signaling pathway, which is associated with the secretion of Th_2 type interleukins (48). Thus, ENT might have similar properties to an endogenous alarmin, alerting the immune system for preferential enhancement of antigen-specific Th_2 response (48). Airway remodeling, subepithelial fibrosis, and ciliated cells destruction have been linked to the activity of major basic protein (MBP) *in vivo* (49). MBP is an abundant granule protein that can induce histamine release from basophiles, superoxide generation by alveolar macrophages, and fibrogenesis through TGF-β signaling (39, 50). Consistently, toxic-range concentrations of MBP were found in sputum and pleural fluid from asthmatics (51). An emerging aspect in eosinophils pathophysiology is their ability to induce a prothrombotic microenvironment on the endothelium. This clinically relates to an increased risk of arterial and venous thrombosis, which can be observed in EGPA (52, 53). Eosinophils can autonomously generate thrombin and induce tissue factor exposure on endothelial cells. This leads to increase platelet adhesion to the vascular wall and thrombus growth (54). Intravascular eosinophil activation also induces the formation of eosinophil extracellular traps, which can be found in human thrombi and could have a potential role in injury-related thrombosis (55). Prothrombotic alterations are also linked to ECP- and MBP-mediated interference with the coagulation cascade and to aberrant eosinophil-derived reactive oxygen species (ROS) production (56–58). Eosinophil NADPH-oxidase works in concert with eosinophil peroxidase (EPO) to generate high levels of ROS from H_2O_2, which in turn interact with endotheliocytes' cellular signaling to upregulate genes for adhesion molecules, tissue factor, and vasoactive substances (58, 59). Most importantly, eosinophil-derived ROS promotes lipoperoxidation, thereby contributing to atheromatous plaque formation and destabilization (55).

TABLE 1 | Prevalence of EGPA clinical characteristics according to ANCA-status and biomarkers of vasculitic and eosinophilic activity*.

Main EGPA clinical manifestations	ANCA+	ANCA-
Asthma	95%	93.1%
ENT involvement	68.8%	54.5%
Lung involvement *(all kinds)*†	60.6%	84.2%
Alveolar hemorrhage	16%	3.2%
Skin involvement *(all kinds)*#	50%	40.9%
Palpable purpura	30%	17.6%
Peripheral neuropathy	69.3%	50.6%
CNS involvement	9.7%	16.6%
Renal involvement *(all kinds)*§	33.3%	13.1%
NCGN	23%	2.3%
Heart Involvement	14.5%	32.6%
Gastrointestinal involvement	26.2%	23.6%

Potential biomarkers of disease activity

Biomarkers of vasculitic activity

ANCA

Patients with persistently elevated ANCA titer, or with re-appearance of ANCA or increase in serum ANCA levels present an higher risk of vasculitis relapse (4).

Urinary-MCP-1

MCP-1 is a chemokine that attracts circulating monocytes in renal glomeruli. Urinary MCP-1 levels are elevated in patients with active renal vasculitis and correlate renal disease activity and response to therapy (6).

Urinary soluble-CD163

Urinary sCD163 is released by crescent macrophages. Its detection correlates with necrotizing crescentic glomerulonephritis and it may represent a biomarker for active renal vasculitis (9). The combination of elevated usCD163 plus either elevated uMCP-1 or new/worse proteinuria improved the positive likelihood ratio of active renal vasculitis (10).

Serum soluble CD25 and urinary soluble CD25

CD25 is a T cell activation marker. Measurement of ssCD25 and usCD25 supports usCD163 in the detection of active renal vasculitis (12).

Alternative complement pathway

Serum C3a, C5a, soluble C5b-9, and Bb fraction correlate with disease activity in MPO-ANCA positive renal vasculitis (14). Avacopan (C5aR antagonist) is being explored as a potential therapeutic target, but specific data on EGPA are lacking (15).

Markers of B cells activation

Markers of B cell activation (BAFF) and B cell repopulation after rituximab therapy (high frequencies of switched memory B cells and circulating plasmablasts—$CD27^{+}CD38^{hi}$) have been shown to correlate with AAV disease activity and relapse. However, specific data on EGPA are missing. Further studies are required to determine whether they may become potential biomarkers for EGPA vasculitic activity (18–22).

Biomarkers of eosinophilic activity

Eosinophils

Absolute eosinophil count correlates with disease activity and risk of relapse with moderate sensitivity and specificity. In untreated EGPA. However, treatment with glucocorticoids and immunosuppressants may be a source of confoundment (5).

IgG4

Th2 cytokines promote Ig class switching to IgG4. Preliminary studies suggested that IgG4 levels may reflect EGPA disease activity. However, there is conflicting evidence from longitudinal studies on serum IgG4 and IgG4/IgG ratio. Routine determination is not yet recommended (7, 8).

CCL26/Eotaxin-3

Eotaxin-3 is a highly eosinophil-specific chemoattractant. Preliminary studies suggested that serum eotaxin-3 levels may be a sensitive and specific marker for active EGPA. However, there is conflicting evidence from longitudinal studies. Routine determination is not yet recommended (7, 11).

CCL17/TARC

CCL17/TARC is a chemokine that can induce the chemoattraction of activated Th2 cells. Preliminary studies suggested that CCL17/TARC levels may reflect EGPA disease activity. However, there is conflicting evidence from longitudinal studies. Routine determination is not yet recommended (7, 13).

ECP

ECP is a cardiotoxic and neurotoxic eosinophil granule protein. It was correlated with EGPA disease activity and eosinophil count in preliminary studies. A significant independent correlation with atherothrombotic risk in EGPA was also described (16, 17).

Periostin

Periostin has been implicated in eosinophil function and recruitment. Serum periostin was modestly associated with EGPA disease activity and was higher in EGPA compared to healthy controls and asthmatics in a preliminary study (23).

**Percentages for every clinical feature were obtained by combination (weighted average) of data from Sinico et al. (2), Sablé-Fortassou et al. (3), and Comarmond et al. (24); data on NCGN were obtained by combination (weighted average) of data from Sinico et al. (2) and Sablé-Fortassou et al. (3).*

† Lung involvement (all kinds) comprises migratory lung infiltrates, lung nodules, chest pain, pleural effusion, and alveolar hemorrhage.

 # Skin involvement (all kinds) comprises urticaria, purpura, livedo, subcutaneous nodules, and ulcers.

§ Renal involvement (all kinds) comprises raise in creatinine serum levels, proteinuria > 0,4 mg/24 h, haematuria> 10 red blood cells/high power field, and NCGN.

EGPA, eosinophilic granulomatosis with polyangiitis; ANCA, antineutrophil cytoplasmic antibodies; ENT, ear-nose-throat; CNS, central nervous system; NCGN, necrotizing crescentic glomerulonephritis; u-MCP-1, urinary-monocyte chemoattractant protein 1; ECP, eosinophil cationic protein; BAFF, B cell-activating factor.

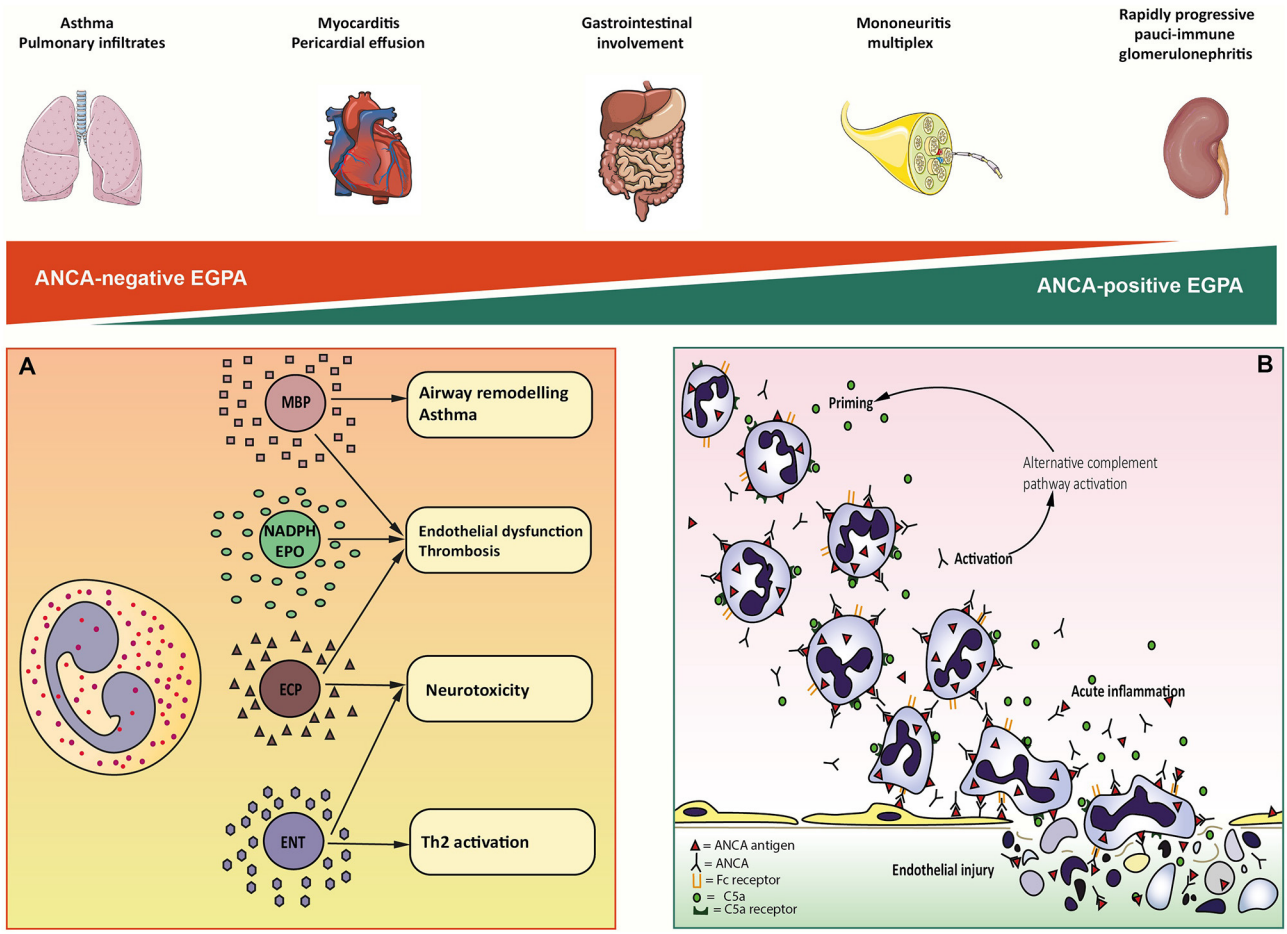

FIGURE 1 | Overview of eosinophil-mediated and ANCA-mediated mechanisms of EGPA immunopathogenesis. On top, a schematic representation of the spectral distribution of clinical features in MPO-ANCA-negative (red triangle) and MPO-ANCA-positive EGPA (green triangle). At the extreme ends of the spectrum, lung involvement and rapidly progressive pauci-immune glomerulonephritis come as the result of predominantly eosinophilic and vasculitic mechanisms of organ damage, respectively. In contrast, the other clinical manifestations of EGPA result from the combination of the two processes. **(A)** Eosinophil-mediated pathogenesis of EGPA. Eosinophil granule proteins are highly cationic compounds that act synergically to mediate the cell's cytotoxicity. Mayor Basic Protein (MBP) is associated with airway remodeling and asthma, fibrogenesis, and procoagulant activity. NADPH-oxidase (NADPH) and Eosinophil Peroxidase (EPO) generate high quantities of reactive oxygen species that contribute to endothelial dysfunction and thrombosis. Eosinophil Cationic Proteins (ECP) is implied in cardiac toxicity, procoagulant activity, and nerve fibers degeneration. Eosinophil Neurotoxin (ENT) has marked neurotoxic potential *in vivo*. **(B)** Putative sequence of neutrophil-mediated endothelial injury. Circulating neutrophils get primed for ANCA activation by inflammatory cytokines and C5a complement factor. Priming induces the exposition on neutrophils cell-surface of ANCA antigens. Circulating ANCA bind to ANCA-antigens through F(ab)2 fragment and activate neutrophils by interaction with Fc receptor. Activated neutrophils release cytotoxic enzymes and factors that activate the alternative complement pathway, producing C5a that further enhances neutrophils priming. ANCA-activated neutrophils marginate and penetrate the vessel wall, where they undergo respiratory burst, degranulation, NETosis, and necrosis causing endothelial damage.

Genetic Background of Eosinophilia

Our knowledge of eosinophils' biology allowed their identification as the sole perpetrators of non-vasculitic clinical manifestations in EGPA. However, our current understanding of the primitive pathological alterations underlying the triggers and drivers of eosinophilic inflammation in EGPA is still incomplete (25).

A predisposition based on immunogenetic factors is known. EGPA is associated to HLA alleles DRB1*04 and *07 and with HLA-DRB4, suggesting a strong link with CD4+ T-lymphocyte activation (60, 61). Furthermore, functionally relevant variations of the IL-10 gene promoter were associated with EGPA in general (62), whereas IRF1/IL5 and GPA33 genes variants were associated with MPO-ANCA-negative EGPA (25). Interestingly, IL-10 and IRF1/IL5 both relate to eosinophilic inflammation. IL-10 is pivotal for the activation of the Th_2pathway, while IRF1/IL5 can interact with the regulatory regions of IL-4 and IL-5 (25, 62). Analyzed IRF1/IL5 variants were associated with an increased risk to develop EGPA, higher eosinophils, and severe asthma (25). Intriguingly, GPA33 encodes a surface glycoprotein that contributes to intestinal and bronchial mucosal function, hinting at a role of barrier dysfunction and innate immunity in disease development (63).

Adaptive and Innate Immune Response and Eosinophils

Ultimately, a dysfunctional communication between innate and adaptive type 2 immunity seems to be at the root of eosinophilia in EGPA. EGPA is generally considered as a Th_2-response-mediated disease due to the high eosinophil activity and the characteristically elevated serum levels of Th_2cytokines (7, 28). Amongst them, IL-5 has the most relevant impact on the differentiation, proliferation, and survival of eosinophils and proved to be a promising therapeutic target (64). Indeed, the surface expression of the IL-5 receptor (CD125/CD131) is a key terminal step in eosinophil haematopoiesis and circulating IL-5 levels regulate the mobilization of eosinophils from the bone marrow (65). At a tissue level, the Th_2 differentiation marker CD294 is abundantly present in biopsies from EGPA patients (13), and a Th_2-dominant transcriptomic profile (STAT3, STAT6, GATA3, IL10, and IL4) was also described in bronchoalveolar lavage (66). Tissue and circulating eosinophils in EGPA also secrete IL-25, a potent eosinophilotropic cytokine that induces their own proliferation and Th_2-response, thereby maintaining a vicious cycle (67). Eosinophils is EGPA are also impaired qualitatively. This is revealed by an increase in surface-expressed eosinophil activation CD69 and CD25, and by evidence of dysfunctional apoptotic pathways (68, 69). EGPA is associated with variants of the apoptosis-controlling BCL2L11 and MORRBID genes (25), the latter of which is dysregulated in hypereosinophilic syndromes (70). Furthermore, several proapoptotic genes (BCL2L13, CASP2, and CARD4) were found underexpressed in the eosinophils of EGPA patients (69), and high circulating levels of soluble CD95 (an inhibitor of Fas-mediated apoptosis) were also described (71). Finally, although their role has yet to be fully characterized, it must be mentioned that IL-5-producing innate lymphoid cells type 2 (ILC2) were also found elevated in EGPA and their blood concentration correlates with eosinophil count and disease activity (72). ILC2s are strategically embedded in peripheral tissues and orchestrate the crosstalk between epithelial cells and the immune system. Their activity has been linked to the initiation of type-2 immune responses *via* IL-5 and IL-13 production, eosinophils and mast cells recruitment, and M2 macrophage polarization (73). Although their real contribution to EGPA still needs to be investigated, their privileged position as an interface between innate immunity and the adaptive Th_2 response could be promising for future research developments (74, 75).

EGPA AS A VASCULITIC DISORDER

Animal and Human Models of ANCA-Mediated Vascular Inflammation

ANCA prevalence in EGPA varies from 30 to 40%. Of the EGPA patients that test positive for ANCA, 90-to 100% have MPO-ANCA specificity. Although the pathogenic role of anti-MPO ANCA has not been overtly demonstrated in EGPA, it is presumed that similar mechanisms to the ones known in MPA occur. Several animal models have demonstrated the direct noxious role of ANCA toward endothelial cells and their key interaction with neutrophils in vasculitis pathogenesis as the cause of necrotizing-crescentic glomerulonephritis (NCGN) and pulmonary hemorrhage (76). The pathogenic potential of MPO-ANCA has been documented either by injection of anti-MPO IgG in mice (77), by injection of splenocytes containing anti-MPO positive B-cells in $Rag2^{-/-}$ mice (which lack B and T cell responses) (77) or by transplanting bone marrow that contain MPO-positive myeloid cells in irradiated $MPO^{-/-}$ mice previously immunized with MPO antigen (78).

However, the relationship between ANCA and EGPA manifestations appears more complex as autoantibody titer does not always correlate with disease severity and ANCA can persist in remission phases or re-appear without clear disease activity (79, 80). Furthermore, a percentage of patients with vasculitic manifestations test negative for ANCA (81, 82), and conversely low-titer non-pathogenic ANCA have been described in healthy individuals (83, 84). These elements suggest that, despite ANCA being directly pathogenic, not all ANCA appear effectively involved. Indeed, more than 20 MPO epitopes have been identified. Antibodies to MPO specific for disease active phases were proven to be strong ROS inducers from neutrophils, whereas antibodies in healthy individuals evoked a poor neutrophilic response (85).

Recently anti-lactoferrin antibodies have been described in EGPA patients, but not in GPA and MPA, and have been directly correlated to disease activity (86). However, anti-lactoferrin antibodies can also be found in other several autoimmune diseases. Thus, whether these autoantibodies are effectively pathogenic or represent just an epiphenomenon is still unknown. Nevertheless, their detection may suggest the presence of ANCA directed toward alternative epitopes, which we are not able to identify yet.

Studies on mouse models also showed that disease manifestations could be limited by modification of ANCA IgG glycosylation on the Fc fragment (87), affecting FcγR-antibody interaction or through inhibition of p38 mitogen-activated protein kinase (MAPK), which is thought to prime and activate neutrophils (88). The *scenario* proposed for ANCA-mediated vascular injury starts with neutrophil priming by circulating inflammatory cytokines (**Figure 1B**). Once primed, neutrophils expose a small amount of ANCA antigens (normally sequestered in the cytoplasmic granules) on cell surface. ANCA F(ab)2 fragment binds to surface antigens, while the Fc fragment interacts with FcγRIIa and FcγRIIIb, triggering the respiratory burst (89). Activated neutrophils penetrate the vessel wall and release ROS and toxic enzymes causing the necrosis of endothelial cells and adjacent matrix. In addition, monocytes can be similarly activated by ANCA. Activated monocytes contribute to further neutrophil recruitment and activation and to granulomatous lesions formation. Several studies have also suggested the participation of alternative complement pathway in ANCA-induced inflammation (90, 91). Activated neutrophils release factors which activate the alternative complement pathway, resulting in the generation of C5a fragment, which in turn attracts neutrophils at the site of inflammation and primes the incoming neutrophils for ANCA activation (92).

ANCA and Adaptive Immune Responses

Adaptive immune responses also appear to be involved. A significant contribution comes from T cells, which can be found in NCGN biopsy specimens and in EGPA granulomatous lesions. It has been shown that activated neutrophils do not only cause endothelial injury but also deposit MPO-antigen in glomeruli and in the basal membrane. Anti-MPO CD4+ T cells recognize the planted glomerular MPO and amplify the immune-mediated damage (93, 94).

Increased frequencies of Th17 lymphocytes in peripheral blood samples from EGPA patients are reported during relapse of vasculitic manifestations (95). It has been proposed that MPO-ANCA activated neutrophils, through IL-6, IL-17, and IL-23 production create a permissive environment for T cells to differentiate toward a Th17 phenotype (96, 97). This generates an amplification loop in which Th17 lymphocytes promote neutrophils recruitment and activation (98).

Origin of Pathogenic-ANCA

If it is almost ascertained that ANCA play a direct role in the pathogenesis of vasculitic manifestations, the initial events leading to tolerance breakdown and to autoantibodies production remain still enigmatic. Since GWAS studies have linked MPO production with specific HLA haplotypes, it is possible to theorize that in patients with a genetically determined predisposition in antigen recognition, ANCA are produced as an initially appropriate immune response, that would lately transform into an aberrant autoimmune process. The proposed antigens include microbial peptides (99, 100), drugs (namely hydralazine, minocycline, propylthiouracil, and levamisole-adulterated cocaine) (101) or endogenously produced antisense transcripts of MPO or PR3 genes (102, 103). However, such a process has never been proved neither for MPO or PR3 ANCA.

Another factor that may influence ANCA production is epigenetic modification of MPO expression, which appear disrupted in AAV patients, potentially contributing to overexpression of ANCA antigens on neutrophil surface (104). Central loss of tolerance toward MPO was proposed to be involved in ANCA production. MPO is expressed in an AIRE-dependent manner in the thymus and its expression is involved in the central deletion of potentially autoreactive anti-MPO CD4+ T cells (105). Moreover, a defective activity both in regulatory T cells (Tregs) and regulatory B cells (Breg) have been reported in EGPA (106–108). From a functional point of view, it appears that the depletion of Tregs and Bregs, which normally would suppress immune responses, could facilitate the production of ANCA from effector B cells. The role of Bregs and autoreactive B cells is also suggested by the efficacy of rituximab (anti-CD20 antibody) (109, 110). In patients treated with rituximab, the peripheral Breg restoration rate correlates with a more effective remission of the vasculitic process (111). Finally, recent studies have focused on the possible role of a mechanism known as NETosis in ANCA production. Neutrophil extracellular traps (NETs) are a framework of chromatin fibers and antimicrobial proteins, including MPO that are released from dying neutrophils as a defense mechanism against microbes. Even in the absence of infective stimuli, the formation of NETs is enhanced in AAV patients compared to healthy controls. NETs are thought to facilitates ANCA developing by presenting antigens to the adaptive immune system (112, 113).

Intriguingly, ANCA have been detected in sputum samples of EGPA patients, irrespective to their serum ANCA-status. Despite unknown specific targets, sputum autoantibodies induced both neutrophil and eosinophil extracellular traps *in vitro*, suggesting their possible pathogenicity (114). It is tempting to speculate that sputum-ANCA may preceded the development of serum-ANCA positivity in a subset of EGPA patients.

Despite the presented evidence, the role of ANCA in EGPA pathogenesis is still under query. ANCA-positive EGPA patients suffer more, albeit not excursively, from vasculitis symptoms, and contrarily, not all patients which developed vasculitic manifestations display ANCA positivity.

Furthermore, the clinical differences of EGPA with other MPO-positive vasculitis contribute to the puzzling picture. Indeed, in ANCA-positive EGPA the prevalence of renal involvement varies from 27 to 51% (2, 3, 24), while in MPA, NCGN involves almost all patients (70 to 90–100%) (115, 116). A similar gap may be found also for alveolar hemorrhage, whose prevalence varies from 2 to 16% in ANCA-positive EGPA patients (24, 117, 118) against 12–30% in MPA patients (116, 119). The reasons for this attenuated vasculitic phenotype in MPO-positive EGPA compared with MPA remains undercover.

WHAT IS MISSING IN EGPA DICHOTOMY?

Progressive improvements in our clinical and pathophysiological understanding of EGPA have reflected into significant advances in the early diagnosis and treatment of the disease. However, the processes through which molecular- and cellular-scale disease mechanisms translate into macroscopic clinical changes still needs to be thoroughly elucidated.

While the pathophysiology of EGPA could be dichotomised as either "eosinophil-driven" or "ANCA-driven," the same principle cannot be applied clinically. EGPA has been classically described to evolve through a prodromic allergic phase characterized by asthma and rhinosinusitis, an eosinophilic phase with blood and tissue eosinophilia, and a vasculitic phase with organ involvement secondary to small-vessel vasculitis. However, these phases partially overlap and may not appear in such a defined order.Besides, clinical features typical of vasculitis, such as glomerulonephritis or neuropathy, can be observed in both ANCA-negative and ANCA-positive EGPA regardless of the disease phase (81). Moreover, some clinical manifestations including cardiomyopathy and neuropathy could come as a result of the overlapping influence of eosinophilic infiltration and vasculitis (120, 121). These phases and the varied pathological findings suggest that the pathophysiology of the disorder might evolve over time. We could therefore speculate that EGPA may represent a spectrum of disease phenotypes that cannot be perfectly encapsulated by a single serological or clinical descriptor (**Figure 1**). For instance, a clear phenotypic subdivision is not commonly observed in the clinical practice.

Cluster analysis performed on the basis of clinical features supports this observation, as it failed in demonstrating an explicit *dichotomy* and an intermediate phenotype with frequent renal, gastrointestinal, and cardiovascular involvement emerged (122).

Furthermore, the two subgroups share clinical, pathological and genetic features. Indeed, patients display severe asthmatic manifestations, and a percentage has a history of allergy, independently from ANCA-status. Eosinophilia and granulomatous lesions are found with analogous frequencies in both subgroups and several genetic variants associated with asthma and elevated eosinophil levels are shared (25). We can therefore speculate that EGPA is primarily caused by intrinsic eosinophil dysfunction, upon which a group of genetically predisposed patients, which present HLA-DQ variant, develop anti-MPO autoantibodies in response to an unidentified stimulus.

Additionally, the clinical value of ANCA-positivity should not be overestimated. Since MPO targeted epitopes have never been characterized, it is tempting to speculate that alternative MPO epitopes, other than MPA ones, develop in ANCA-positive EGPA, thus contributing to a mitigated vasculitic phenotype.

Therefore, stronger efforts should be made to better characterize the mechanisms underlying EGPA pathogenesis. Further studies are required in order to molecularly characterize clinical phenotypes by taking into account complex -omics data (e.g., genomics, epigenomics, transcriptomics, proteomics, and metabolomics). Shifting the subject of EGPA research from clinical phenotype to molecular endotype would possibly allow to identify valuable biomarkers and therapeutic targets that could improve diagnostic precision and therapeutic outcomes.

AUTHOR CONTRIBUTIONS

FF and FB equally contributed to the conception and design of the work and drafted the article. GE critically revised the article. All authors approved the final version of the manuscript.

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Neutrophil Extracellular Traps in the Autoimmunity Context

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The formation of neutrophil extracellular traps (NETs) is a strategy utilized by neutrophils for capturing infective agents. Extracellular traps consist in a physical net made of DNA and intracellular proteins externalized from neutrophils, where bacteria and viruses are entrapped and killed by proteolysis. A complex series of events contributes to achieving NET formation: signaling from infectious triggers comes first, followed by decondensation of chromatin and extrusion of the nucleosome components (DNA, histones) from the nucleus and, after cell membrane breakdown, outside the cell. NETs are composed of either DNA or nucleosome proteins and hundreds of cytoplasm proteins, a part of which undergo post-translational modification during the steps leading to NETs. There is a thin balance between the production and the removal of circulating NETs from blood where digestion of DNA by circulating DNases 1 and IL3 has a critical role. A delay in NET removal may have consequences for autoimmunity. Recent studies have shown that circulating NET levels are increased in systemic lupus erythematosus (SLE) for a functional block of NET removal mediated by anti-DNase antibodies or, in rare cases, by DNase IL3 mutations. In SLE, the persistence in circulation of NETs signifies elevated concentrations of either free DNA/nucleosome components and oxidized proteins that, in some cases, are recognized as non-self and presented to B-cells by Toll-like receptor 9 (TLR9). In this way, it is activated as an immunologic response, leading to the formation of IgG2 auto-antibody. Monitoring serum NET levels represents a potential new way to

herald the development of renal lesions and has clinical implications. Modulating the balance between NET formation and removal is one of the objectives of basic research that are aimed to design new drugs for SLE.

Clinical Trial Registration Number: The Zeus study was registered at https://clinicaltrials.gov (study number: NCT02403115).

Keywords: *Lupus nephritis*, systemic lupu erythematosus, biomarker, anti-histone, anti-alpha enolase, anti-C1q antibodies

INTRODUCTION

The pathway(s) leading to the formation of specific autoantibodies to dsDNA in systemic lupus erythematosus (SLE) has long been investigated (1), and mechanisms causing the externalization of DNA and of nucleosome proteins have been a main focus. Exposure to the environment of otherwise hidden molecules such as DNA and nuclear proteins logically represents a possible starting step for autoimmunity.

The formation of neutrophil extracellular traps (NETs) is a special event leading to neutrophil cell death and, as a consequence, to the externalization of DNA and histones. A recent discovery is that a few NET proteins are oxidized by reactive oxygen species (ROS) and undergo a process of post-translational modification by which they are transformed in potential new antigens for autoimmunity (2). The structural characterization of those intracellular proteins that are externalized through NETs and become auto-antigens in SLE has now been completed. The formation of NETs and externalization of NET components are the main foci of research: since DNA and nucleosome are tightly linked in a compacted structure and are not prone to be externalized from the nucleus, solubilization of the nucleosome complex plays a particularly important role.

NET composition and removal are the main topics of the present review that propose NETs as an important source of auto-antigens involved in SLE and, in particular, in lupus nephritis. In this view, NETs would represent an important target for new preventive strategies aimed at blocking the autoimmune process at an early stage, before the generation of autoantibodies.

NEUTROPHIL EXTRACELLULAR TRAPS IN HEALTHY AND IN DISEASE

Pathways for NETs

Neutrophils represent the first line of defense against aggression by bacteria, virus, mumps, and other external potential infectious triggers. One of the strategies that neutrophils utilize for contrasting any external infectious attack is the release of NETs (3, 4). NETosis is a sort of premature cell death that leads to the formation of a physical net where pathogens are entrapped and killed by elastase, defensin, myeloperoxidase, etc. (3, 5, 6). It is a suicidal procedure during which neutrophils die but play their defensive function by also capturing and killing bacteria after their death. Therefore, activation and production of NETs is an important step of innate immunity, and in rare cases, the formation of NETs is reduced for genetic reasons such as NADPH oxidase mutations (see the discussion below) and patients suffering from recurrent and severe infections (7).

The formation of NETs is stimulated by signals that come from outside the cells and then continues with mobilization of granule proteins to the nucleus, decondensation of nuclear chromatin, nuclear membrane dissolution, and then NET externalization. There are several stimuli that can induce the formation of NETs and that can be divided in two major families depending on the participation of infectious or suicidal NETosis or of immunologic triggers, in which cases NETosis is defined as sterile (5, 8). Generation of ROS in the mitochondria has a key role in initiating suicidal NETosis. Ionophores produced by Gram-positive bacteria directly bring calcium inside the mitochondria and induce this process. Specific bacterial toxins such as lipopolysaccharide (LPS) lead to ROS formation *via* an alternative pathway that involves TLR4-dependent NADPH-oxidase activation and suppression of anti-oxidative enzymes (9, 10). ROS generation is, in turn, followed by the activation of several kinases downstream of PKC (i.e., c-Raf, MEK, Akt, ERK) (11–13). Triggers of sterile NETosis include antibodies, cytokines, and inflammation *per se* that activate neutrophil PKC *via* phosphorylation of NADPH-oxidase (14–16).

The second key event is the release of NETs that takes place after activation of neutrophil elastase that dissembles F-actin and moves to the nucleus where it catalyzes the cleavage of histone 1 and de-condenses chromatin; neutrophil elastase also destroys the membrane, allowing DNA to be released outside the cell (17, 18). Enzymatic deimination of arginine residues of histone 1 by peptidylarginine deaminases (especially PAD4) may take place in this phase and play an addictive role in weakening the chromatin backbone (19, 20). Therefore, decondensation and loss of chromatin stability induced by neutrophil elastase, with the contribution of PAD4, is extremely important to modify the rigid structure of chromatin into a fluid compost that is functional for DNA release from the cell.

A less common type of DNA externalization that does not require lysis of neutrophil is "vesicular NETosis," in which case NETs are released *via* budding from the nucleus and in a vesicular form from the cell. This particular mechanism of NETosis does require modification of cell membrane, maintains neutrophils with vital function, and generates a DNA that is entrapped in micro-vesicles and that requires specific mechanisms of digestion (see below).

NETs Is a Source of Bio-Available DNA

The physical form of extracellular DNA influences the dynamics and mechanisms of anti-DNA antibody generation, and it is probably critical for DNA removal. In many cases, DNA in NETs or in microvesicles is presented as a DNA–protein complex, including nucleosome proteins, and ideally represents an antigenic source where proteins function as epitopes for B cell presentation (21). In NETs, DNA co-localizes with other non-nucleosome proteins that may, on their own, function as epitopes for auto-antibody formation (22, 23). This part will be discussed below.

The removal of DNA in NET filaments plays a role in antibody generation; since more DNA is digested by DNases, less is the probability that formation of anti-DNA antibodies will be carried out. DNase1 and DNase1L3 are two homologous extracellular enzymes deputed to the removal of circulating bio-available DNA (24). The former enzyme usually digests protein-free DNA; DNases1L3 instead has more powerful functions to digest DNA packed in chromatin and in microvesicles (24–26). Therefore, the bio-availability of extracellular DNA is dependent mainly on the activity of these two enzymes whose importance is strengthened by the finding that genetic conditions carrying molecular defects in *DNASE1 or DNASE1L3* genes are associated with severe forms of pediatric SLE or with other forms of autoimmune disease such as rheumatoid arthritis and sclerodermia (27).

NET Protein Composition

The literature on the composition and structure of NET proteins is scanty, and only few studies allow the comparison among different auto-immune conditions. The report by Urban et al. (28) is the first complete analysis of NETs deriving from normal neutrophils: these authors utilized western blot and reported a list of 25 proteins that included histones, proteins of granules, cytoplasm, cytoskeleton, and glycolytic enzymes. Petretto et al. (29) analyzed the protein composition of NETs produced spontaneously *ex vivo* by normal neutrophils or after stimuli that mimic an infectious trigger (LPS, calcium ionophores) or sterile triggers (PMA). They found many more proteins in NETs after stimulation of neutrophils and characterized 330 proteins overall. Bruschi et al. (2) analyzed NET proteins produced by neutrophils derived from 33 SLE patients (18 with lupus nephritis) and 21 normal people and reported the presence of NETs in more than 800 proteins overall, mostly belonging to proteins described in the area of autoimmunity, inflammation, and lupus. Many, if not all the proteins found in NETs, presented one or more post-translational modifications (i.e., methionine sulfoxide, thiol oxidation, deamination, phosphorylation) that coexisted in some cases. This is the direct demonstration that post-translational modifications of NET proteins take place *in vivo*. Fifteen proteins maximized the discrimination between SLE and LN (**Table 1**): four proteins were high in SLE (i.e., VGLL3, MAGOHB, GSTO1, CADPS). The other 11 were instead high in NETs produced by LN patients (GLOD4, MYCBP2, WDR1, ANXA1, ENO1, MPB-ENO1, ESD, NUTF2, DSG1, SYTL3, RAB11FIP1). Two of them, i.e., annexin A1 (ANXA1) and αenolase, were modified for deamination (the former) and for oxidation (the second); αenolase was modified for the presence of methionine sulfoxide in place of methionine 93 (2). Overall, these data give an impressive view of the complex composition of NETs that would be propedeutical to studies on mechanisms.

TABLE 1 | List of the fifteen NETs proteins that maximize the discrimination between SLE and LN.

Protein	Gene	SLE/LN	Modification
1- Transcription cofactor vestigial-like protein 3	VGLL3	+/–	Nd
2- Protein mago nashi homolog	MAGOHB	+/–	Nd
3- Glyoxalase domain-containing protein 4	GLOD4	–/+	Nd
4- E3 ubiquitin-protein ligase	MYCBP2	–/+	Nd
5- WD repeat-containing protein 1	WDR1	–/+	Nd
6- Annexin A1	ANXA1	–/+	Deamination
7- Alpha-enolase	ENO1	–/+	Oxidation
8- Alpha-enolase MBP-1	ENO1	–/+	Nd
9- S-formylglutathione hydrolase	ESD	–/+	Nd
10- Nuclear transport factor 2	NUTF2	–/+	Nd
11- Glutathione S-transferase omega-1	GSTO1	+/–	Nd
12- Desmoglein-1	DSG1	–/+	Nd
13- Synaptotagmin-like protein 3	SYTL3	–/+	Nd
14- Rab11 family-interacting protein 1	RAB11FIP1	–/+	Nd
15- Calcium-dependent secretion activator 1	CADPS	+/–	Nd

Four proteins were higher in SLE (VGLL3, MAGOHB, GSTO1, CADPS) the other 11 were higher in LN.
Nd, not detected. +/– indicates that the protein is specific for NETs produced by patients with SLE, –/+ means the opposite.

NETs in the Immunologic Context

The implication of NETs in autoimmunity is now a topic of intense discussion (30–32), and SLE is a main focus (33). The interest is about the possibility that NETs are a source of antigens for autoantibodies. A first point concerns DNA since the formation of NETs represents a cell death mechanism according to which DNA is externalized. A second key aspect is the protein composition of NETs and the potential implication of NETs as a source, in addition to DNA, of post-translational modified proteins. One example is αenolase that, in NETs, co-localizes with DNA and is modified by oxidation (**Figure 1**). Actually, anti-αenolase antibodies of IgG2 isotype represent the major nephritogenic autoantibodies purified in circulation and in the glomeruli of patients with lupus nephritis (22, 23, 34), and studies are now focalizing on the significance of this new class of autoantibodies that identifies particular classes of patients with lupus nephritis (35, 36). There is now consensus in considering IgG2 as the major isotype of autoantibodies in SLE and in LN, and the interest is that IgG2s are secreted upon stimulation of TLR9s that is the class of Toll-like receptors deputed to produce

an isotype switching. TLR9s are also the Toll-like receptors that bind DNA. A possibility is that the complex DNA–αenolase in NET filaments is recognized by TLR9s that stimulate B cells to produce anti-DNA and anti-αenolase IgG2 (see the scheme in **Figure 2**).

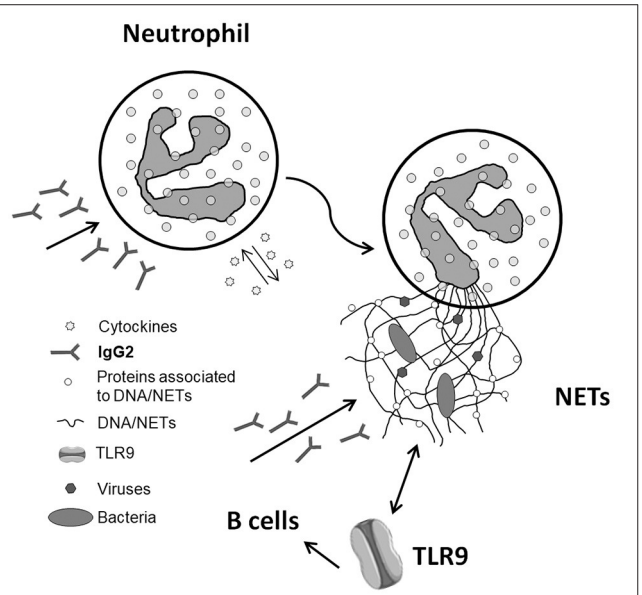

FIGURE 2 | Schematic representation of the pathway that potentially connects neutrophil extracellular traps (NETs) with the formation of antibodies of IgG2 isotype in systemic lupus erythematosus. The various steps are detailed in the text. As for the last step that involves TLR9, the data supporting a direct link between NETs and IgG2 *via* TLR9 are presented in this special issue of *Frontiers* (The kidney in inflammatory and immune-mediated diseases) (see Bertelli et al.).

Circulating NET Balance in Healthy and in Disease

Removal of NETs from the circulation is a key event that requires availability of DNases; failure or impairment of this process is a potential starting event leading to autoimmunity. In fact, for the reasons presented above, the persistence of NETs in the circulation would increase the time of exposure of potential antigens to TLR9s and potentiate the autoimmune response (**Figure 2**).

Circulating NETs can be detected by ELISAs that target the complex of DNA–MPO and/or DNA–elastase that are reported as NET remnants (the second ELISA is more specific for NETs derived from neutrophils and exclude NETs from monocytes); NET remnants are released from the NET complex and are present in free form in serum and biological fluids (37). The evidence that circulating NETs are increased in SLE and in LN and more, in general, in autoimmune conditions has been accumulating in the recent years (38–40). Kessenbrock et al. (41) and Soderberg et al. (42) reported high NET remnant levels, respectively, in serum and plasma of patients with small vessel vasculitis. Knight et al. (43) demonstrate enhanced NET formation in the New Zealand mixed 2328 (NZM) model of murine lupus. Studies in humans confirmed high circulating levels also in SLE and supported their inflammatory potential. Zhang et al. (44) reported high plasma levels of circulating free DNA in patients with SLE that, in part, derived, but not solely, from NETs. Lood showed that SLE individuals had increased plasma levels of myeloperoxidase (MPO)–DNA, and human neutrophil elastase–DNA compared to controls (45). More recently, Bruschi et al. (46) studied 216 patients with SLE and with lupus nephritis, utilizing the assay that immobilizes the MPO tail of the MPO–DNA complex (37). This study showed an increase in circulating NET levels in patients with lupus nephritis and its association with parameters of disease activity

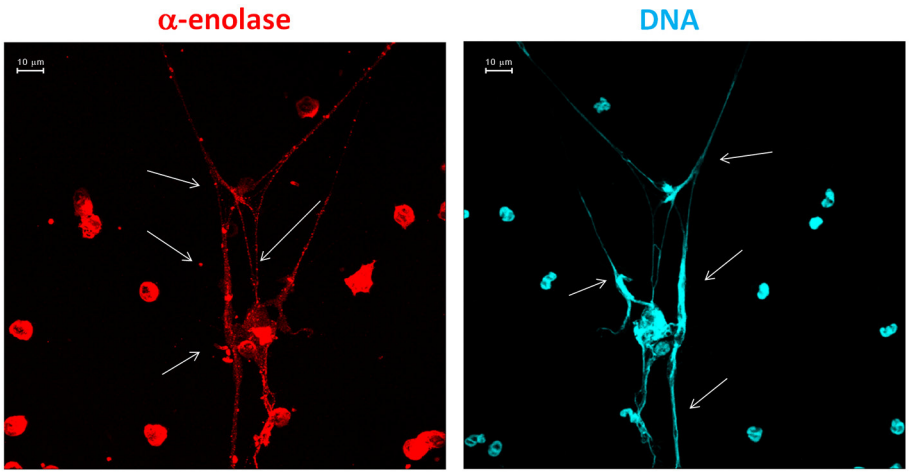

FIGURE 1 | Traditional immunofluorescence microscopy analysis of neutrophil extracellular trap (NET) filaments showing that αenolase and DNA are intensely present in NET filaments and co-localized in large segments. The images were acquired using LSM 510 Meta confocal system scan.

such as complement consumption, ESV, RCP, and proteinuria. It is noteworthy that serum NETs were also strongly correlated with anti-dsDNA and anti-C1q antibody levels. On a more clinical vein, determining serum NET remnants represents a potential new biomarker of autoimmunity activity that may occur in concomitance with or may even precede the formation of autoantibodies. The increased level of circulating NETs in lupus nephritis also suggest that the formation of NETs is, in some ways, correlated with the formation of antibody specific for the kidney.

The study by Bruschi et al. (46) also addressed the question of removal of NET remnants by DNASE1 and DNASE1L3 (26). They found normal serum levels of both enzymes in spite of reduced DNase activity; when the same sera were pre-treated with Protein-A in order to remove antibodies, DNase activity was restored. Other authors (40, 47) have already shown the existence, in circulation, of inhibitors of NET removal in patients with SLE. In particular, Hakkim et al. (40) showed that a subset of SLE patients present an impaired degradation of NETs due to the presence of anti-NET antibodies that inhibit DNASE1. These patients, defined as non-degraders, were prone to develop lupus nephritis, suggesting that impaired NET degradation is linked with renal manifestation of lupus. The results of Soderberg et al. (42) reinforce the concept discussed above.

Several data of the literature in humans carrying mutations in DNases (48–51) and results deriving from experimental models (25, 26, 52, 53) indicate a clear association of reduced DNase activity with autoimmune renal lesions. Overall, the data presented above (46) strengthen the concept that circulating inhibitors of DNase activity reduce the removal of NETs and represent a key factor for increasing the exposure of antigens (i.e., DNA and other soluble proteins) to the environment as triggers of autoimmunity.

NETs–Macrophages Interaction

The interaction of macrophages with NETs is a final aspect that governs the thin balance between a physiological antimicrobial function and autoimmunity. M1 and M2 macrophages act synchronically in this context and are synergic with DNase for removing DNA from NETs. After interacting with NETs, M2 macrophages produce chemotactic substances such as MIF, CCL2/MCP- 1, CCL3/MIP-1a, and CCL4/MIP 1b that recruit monocytes and M1 macrophages that contribute to neutrophil depth and increase, in this way, the quota of extracellular DNA that derives from neutrophils (54). Dead neutrophils are then trapped and cleared by M2 macrophages. In this sense, autoimmunity may derive from an imbalance of the macrophage cycle, resulting in the accumulation of DNA. Therefore, NETs–macrophages interaction is key to the maintenance of the equilibrium between DNA antibacterial functions and autoimmunity and is addictive to DNases to govern the balance between the two. How macrophages and DNases are regulated, whether there is a cross-talk between the two, and which DNA type they process (i.e., chromatin DNA, NET DNA, or microparticle DNA) should be better defined in the future (26).

PHARMACOLOGICAL MODULATION OF NET LEVELS

Modulation of NETs could be functional to reduce the quota of antigens presented to TLRs. Drugs that inhibit NET generation are already available; however, whether or not they offer a real opportunity or play deleterious effects on host defense is not clear and needs new evidence. One possibility is to achieve a correct balance between NET production and removal, and in this sense, modulation of removal after the protective functions of NETs have been obtained could make sense. In the sections below, the possibility to either inhibit formation or enhance removal is briefly outlined.

Modulation of NET Production With Traditional Drugs

Blocking the first steps of NETosis by means of scavengers of ROS such as N-acetyl-cysteine and/or by inhibitors of NADPH has been already utilized with modest clinical effects in patients with SLE (55). Inhibitors of myeloperoxidase such as 4-aminobenzoic acid hydrazide have been used instead in mouse models of SLE and in vasculitis complicated by glomerulonephritis: the main finding was that they limited the accumulation of neutrophils in the glomeruli and also reduced proteinuria.

One way to reduce NETs is by the inhibition of PAD4. This has been investigated in different animal models, one utilizing mice with constitutional PAD4 deficiency (*PAD4−/−)* and the other with chemical inhibition of PAD4 by Cl-amidine. In the first case, PAD4-deficient mice were exposed to selected organ pathologies such as pulmonary inflammation, causing acute respiratory distress syndrome. *PAD4−/−* mice presented reduced NETosis and a decrease in neutrophil influx into the lung that were accompanied by improved survival compared with wild-type mice (56). In the second case, lupus-prone New Zealand mixed 2328 (NZM) mice, a model of lupus driven by type I IFNs, were treated with Cl-amidine, showing a reduction of NET formation *in vivo* and a significant modification of circulating autoantibody profiles and complement levels followed by reduced glomerular IgG deposition (43).

More recently, we screened a library of biologically active substances utilizing an assay based on high-content imaging and identified vanilloids as a novel class of chemical compounds able to hinder PMA and ionomycin-induced NET release (57). A parallel effect of vanilloids was to decrease cytosolic ROS production, which makes sense in view of the well-known relationship between ROS and NETosis. The identification of a novel class of ROS and NET inhibitors able to stop excessive or aberrant NET production should be considered as an option for treating those disorders associated with NET overproduction.

There are further potential targets for reducing NETosis; a brief list includes the actin cytoskeleton (58), CXCL5, integrins, and TNF (59), all of which may offer interesting therapeutic options.

Modulation of DNA Removal From NETs

For more than 20 years, inhaled recombinant DNase has been utilized in patients with cystic fibrosis and with other

inflammatory lung diseases based on the consolidated finding that this approach plays some positive effects without side effects (60, 61). Treatment with DNase I has also been proposed in neurodegenerative diseases, for example, in patients with dementia in the end-stage of Alzheimer's disease (62), but further and larger interventional studies are required in order to evaluate a positive effect.

It is clear that accelerating the removal of NETs with DNases is an option that would reduce the exposure of bio-available DNA for autoimmunity. Only few studies addressed the potential use of DNases in animal models of SLE and provided contradictory results. In one study (63), recombinant DNase 1 has been found to reduce the generation of autoantibodies and also to improve the outcome of proteinuria and kidney damage in a lupus-prone murine model, but the same findings were not confirmed considering survival as a hard endpoint (64). Recombinant DNase has also been utilized in patients with SLE (65) who tolerated the drug, but clinical results on improving the outcome of the disease are not available and need to be tested in phase II studies. The existence of anti-DNase antibodies in the serum of SLE patients may limit the efficacy and prompt new designs that also consider the combined block of anti-DNase antibodies.

CONCLUSIONS

Data in the literature provide crucial elements that implement the basic findings on NETosis and outline the correlation with autoimmunity (**Figure 2**). Determining serum NET levels could represent an informative way to herald the development of renal lesions and have a clinical implication. New findings on DNase activity in patients with lupus nephritis also support the idea that NETs accumulate in the serum for a defective removal; an ancillary result is that, in SLE patients, the serum levels of DNaseI and DNaseIL3 are normal, suggesting that DNase inhibition is a potential mechanism for the DNase functional defect. The second main conclusion is that NET composition is highly specific for SLE and lupus nephritis and also includes, beyond DNA/histones, modified proteins (i.e., αenolase with methyl sulfoxide methionine 93 among others). Therefore, NET remnant levels, their kinetics of production and removal, and their composition represent a further advancement with new diagnostic and therapeutic potential implications.

AUTHOR CONTRIBUTIONS

GG and AR were the principal investigators of the study and were involved in study design and coordination, patients' recruitment, data managing and supervision, manuscript writing, and discussion. MB had a key role in lab analysis, proteomics, supervision, statistics, and data managing. GC, AP, and MP were involved in lab analysis. GM, FF, MF, AV, LC, FP, PM, MB, AM, GR, PE, SN, LC, BT, GE, GG, DS, FS, SV, MM, and AT were involved in the Zeus study and manuscript discussion. All authors contributed to the article and approved the submitted version.

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CD107a$^+$ (LAMP-1) Cytotoxic CD8$^+$ T-Cells in Lupus Nephritis Patients

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Cytotoxic CD8$^+$ T-cells play a pivotal role in the pathogenesis of systemic lupus erythematosus (SLE). The aim of this study was to investigate the role of CD107a (LAMP-1) on cytotoxic CD8$^+$ T-cells in SLE-patients in particular with lupus nephritis. Peripheral blood of SLE-patients ($n = 31$) and healthy controls ($n = 21$) was analyzed for the expression of CD314 and CD107a by flow cytometry. Kidney biopsies of lupus nephritis patients were investigated for the presence of CD8$^+$ and C107a$^+$ cells by immunohistochemistry and immunofluorescence staining. The percentages of CD107a$^+$ on CD8$^+$ T-cells were significantly decreased in SLE-patients as compared to healthy controls (40.2 ± 18.5% vs. 47.9 ± 15.0%, $p = 0.02$). This was even more significant in SLE-patients with inactive disease. There was a significant correlation between the percentages of CD107a$^+$CD8$^+$ T-cells and SLEDAI. The evaluation of lupus nephritis biopsies showed a significant number of CD107a$^+$CD8$^+$ T-cells mainly located in the peritubular infiltrates. The intrarenal expression of CD107a$^+$ was significantly correlated with proteinuria. These results demonstrate that CD8$^+$ T-cells of patients with systemic lupus erythematosus have an altered expression of CD107a which seems to be associated with disease activity. The proof of intrarenal CD107a$^+$CD8$^+$ suggests a role in the pathogenesis of lupus nephritis.

Keywords: cytotoxic T-cells, CD107a, LAMP-1, lupus nephritis, SLE

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INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by various organ manifestations. Inflammation of the kidney, in particular, is associated with an unfavorable prognosis (1). Although the precise pathogenesis of lupus nephritis (LN) has not been elucidated, disturbances in regulatory and effector T-cell balance seem to contribute to the development of LN (2). Despite an increasing body of evidence reporting CD4$^+$ T-cell abnormalities, the role of cytotoxic CD8$^+$ T-cells is less well-understood.

CD8$^+$ T-cells can contribute to autoimmunity by chemokine secretion which is capable to attract other immune cells, recruitment of autoreactive CD8$^+$ T-cells and killing of target cells. In SLE an increase of activated CD8$^+$ T-cells expressing perforin and granzyme B has been reported (3). Interestingly, the authors found intrarenal CD8$^+$ T-cells in lupus nephritis biopsies. The

amount of periglomerular $CD8^+$ T-cells was associated with a poor prognosis (4). This finding confirmed previous histopathological study by D'Agati et al. who reported a predominant $CD8^+$ T-cell infiltrate in human lupus nephritis biopsies (5). In previous studies we proofed the hypothesis that effector T-cells migrate from peripheral blood into the kidney during active lupus nephritis and can be detected in the urine (6). The predominantly detected T-cells were $CD8^+$ T-cells. The absolute cell count of urinary $CD8^+$ T-cells was an excellent parameter to discriminate active from inactive lupus nephritis (7). This finding has been consistently found by Klocke et al. (8).

Despite the increasing body of evidence demonstrating the presence of kidney infiltrating cytotoxic $CD8^+$ T-cells suggesting a crucial role for the renal inflammation the precise mechanism of action remain to be elucidated. CD314 (NKG2D) and CD107a (LAMP-1) are molecules expressed on activated natural killer (NK)-cells as well as on $CD8^+$ T-cells. CD107a (LAMP-1) belongs to a family of highly glycosylated transmembrane proteins on human peripheral blood mononuclear cells which mediate cell adhesion to vascular endothelium which potentially enables T-cell migration into kidney (9). LAMPs may be shuttled and expressed at the cell surface after cell activation (10). Functional CD107a is required for efficient perforin delivery to lytic granules and NK-cell cytotoxicity (11).

In the present study we hypothesized that peripheral circulating cytotoxic $CD8^+$ T-cells in patients with SLE have an altered CD107a expression pattern. Moreover, we aimed to analyze the cytotoxic activity of renal infiltrating T-cells reflected by CD107a expression.

PATIENTS AND METHODS

In this study 31 patients with systemic lupus erythematosus fulfilling at least 4 ACR criteria and 21 healthy controls were enrolled (12). The mean age of SLE patients was 42.5 ± 13.7 years. The mean age of healthy controls was 38.2 ± 14.4 years. Disease activity was assessed by systemic lupus erythematosus disease activity index (SLEDAI). Active disease activity was defined as SLEDAI >4, inactive disease activity was defined as SLEDAI $\leq$4. According to this definition 11 active and 20 inactive patients were included.

Renal involvement was defined as biopsy proven lupus nephritis. Twenty-one patients had a biopsy proven lupus nephritis which were classified according to the ISN/RPS classification from 2003. The lupus nephritis classes were class II ($n = 3$), class III ($n = 1$), class IV ($n = 14$) and class V ($n = 3$) (**Table 1**). All biopsies were reviewed and classified by an experienced nephropathologist (K.A.) according to the revised criteria for LN. The activity index (AI) and chronicity index (CI) were calculated for each specimen with maximum scores of 24 for the AI and 12 for the CI (13). The assessment was completed by determining the ISN/RPS 2003 classification and activity and chronicity indices for LN. For these aspects of the assessment, the definitions of the classification systems and the activity and chronicity indices were used (14).

Twenty-nine patients received immunosuppressive treatment. Twenty-five patients were treated with prednisone [median (range), 5 mg/d, (1–60 mg/d)]. Twenty patients received a combination of prednisone and hydroxychloroquinsulfate ($n = 14$), mycophenolate mofetil ($n = 13$), azathioprine ($n = 4$) or cyclosporine ($n = 1$). A minority was treated solely with prednisone (n =5) or hydroxychloroquinsulfate ($n = 1$). The study protocol was approved by the institutional review board (15-6323-BO). All patients gave informed consent for participation in this study.

Flow Cytometry

Immunophenotyping was performed as described before (15). Briefly, 100 µl heparinized blood were mixed with antibodies: Krome Orange-conjugated anti-CD3 (clone UCHT, Beckman Coulter, Brea, USA), Pacific Blue-conjugated anti-CD8 (B9.11, Beckman Coulter, Brea, USA), Allophycocyanin (APC)-conjugated anti-CD107a (clone H4A3, Beckman Coulter, Brea, USA) and Allophycocyanin (APC)-conjugated anti-CD314 (ON72, Beckman Coulter, Brea, USA). Appropriate isotype controls were used. After vortex, all tubes were incubated for 20 min in the dark at room temperature. Next 3 ml of VersaLyse™ were added in each tube and the suspension

TABLE 1 | Laboratory and histological data of 10 SLE-patients with active renal disease are given.

#	ISN/RPS-class¶	AI[1]	CI[2]	Haematuria	Proteinuria (g/24 h)	S-crea (mg/dl)	Anti-DNA-Ab (IU/ml)
1	IV-G	10	3	+	4.2	1.07	>200
2	IV-G	7	5	++	0.6	2.31	135
3	II	1	2	+	0.6	1.30	189
4	IV-G	20	2	+++	7.0	1.35	>200
5	IV-G	8	2	-	3.2	1.67	>200
6	V	4	3	-	11.0	1.79	3.7
7	IV-G	11	1	+++	2.2	1.41	132.6
8	II	3	2	++	2.7	0.45	15
9	IV/V	16	3	+++	12.0	0.95	>200
10	n.c.	-	-	++	1.5	3.15	34

Laboratory and histological data of 10 patients with active renal disease are shown.
¶Histological ISN/RPS classification, nc, not classified.
[1]activity index (AI), [2]chronicity index (CI).

was mixed gently with vortex. Then the tubes were incubated for 12 more minutes in the dark. Thereafter the tubes were centrifugated and the supernatant was aspirated. The cell pellet was washed with 3 ml of phosphate buffered saline (PBS). This washing step was repeated and finally 300 µl PBS were added before cells were immediately analyzed with a fluorescence activated cell sorter (FACS) NAVIOS^{TM} from Beckman Coulter. Kaluza Analysis Software (Version 1.5, Beckman Coulter) was used for analysis of flow cytometric data.

Analysis and Scoring of Renal Biopsies

Immunohistochemistry

All specimens were fixed in 10% neutral buffered formalin and paraffin embedded. Five-micrometer-thick sections were deparaffinized in xylene and rehydrated in a series of different concentrations of ethanol (100, 95, 70, and 50%) (16). Tris-HCL buffer, pH 9.0, for heat-induced epitope retrieval was applied for 1 h, followed by neutralization of endogenous peroxidase with 0.3% H_2O_2. For CD107a staining epitope retrieval was performed with citrate buffer pH 6.0 applied for 40 min at 90°C. Protein block with 5% rabbit or goat serum in PBS for 30 min was performed. Incubation with a monoclonal mouse anti-human CD8 (clone C8/144, DAKO, Carpinteria, USA) or polyclonal rabbit anti-human CD107a (polyclonal, Bio-Rad, Munich, Germany) was performed for 60 min at room temperature. Next, sections were washed and incubated with a HRP-conjugated secondary antibody (Envison^{TM}, DAKO, Carpinteria, USA) for 30 min. at room temperature. A DAB substrate (Envison^{TM}, DAKO, Carpinteria, USA) was used for visualization. Washing with PBS was performed after each incubation step. Finally, the slides were counterstained with haematoxylin and mounted with Vitro-Clud® (R. Langenbrinck, Emmendingen, Germany).

Only cells with a distinctly brown and continuously stained plasma membrane were counted. Positive cells were separately counted within the interstitium and in the glomeruli. Cells with positive staining for CD8 were counted per high powerfield (40 × magnification). The average value was calculated for each biopsy.

Immunofluorescence Double Staining

Tissues were fixed, embedded in paraffin and sectioned as indicated above. Epitope retrieval was performed with citrate buffer pH 6.0 (Zytomed) at 90°C followed by a protein block with 5% rabbit and goat sera in PBS for 30 min at room temperature. Primary antibodies against CD107a (polyclonal rabbit, Bio-Rad) was used and incubated for 60 min followed by an incubation with a secondary antibody conjugated to FITC (Jackson Immuno). Next, primary antibody against CD8 (mouse IgG1, DAKO) was used and incubated for 60 min followed by an incubation with a secondary antibody conjugated to Cy3 (Jackson Immuno) and DAPI. Finally, the slides were mounted with ProLong® Gold antifade (Life Technologies). Tonsil sections served as positive control samples. Isotype controls for primary antibodies were used as negative controls.

Statistical Analysis

All values are expressed as mean ± standard deviation (SD). The significance for the differences between groups was determined by the Mann-Whitney U-test. Spearman's rank correlation was applied to detect correlations between different study parameters. Differences were considered statistically significant at a p-value < 0.05. GraphPad Prism 8.0 (GraphPad Software, Inc., California, USA) was used for statistical analysis.

RESULTS

Expression of CD314 on Peripheral Circulating Cytotoxic CD8$^+$ T-Cells

The activation marker CD314 was analyzed on cytotoxic CD8$^+$ T-cells. There was no significant difference between the percentages of CD8$^+$CD314$^+$ T-cells in healthy controls and SLE-patients (98.7 ± 0.6% vs. 98.7 ± 1.3%, n.s.). There was also no significant difference between the percentages of CD8$^+$CD314$^+$ T-cells in SLE-patients with and without lupus nephritis and healthy controls, respectively (98.8 ± 1.1% vs. 98.4 ± 1.5% vs. 98.7 ± 1.3%, n.s.). Moreover, there was no significant difference between the percentages of CD8$^+$CD314$^+$ T-cells comparing active vs. inactive patients vs. healthy controls, respectively (98.3 ± 1.7% vs. 98.9 ± 0.9% vs. 98.7 ± 1.3%, n.s.).

Decreased Percentages of CD8$^+$CD107a$^+$ T-Cells in SLE-Patients

The analysis of the cytotoxicity marker CD107a on CD8$^+$ T-cells revealed a significant different expression (**Figures 1A,E**). The percentages were significantly decreased in SLE-patients ($n = 30$) as compared to healthy controls ($n = 18$) (40.2 ±18.5% vs. 47.9 ± 14.9%, $p = 0.02$). Next, the percentages were analyzed in SLE-patients according to renal involvement (**Figure 1B**). The percentages of CD8$^+$CD107a$^+$ T-cells were not different in SLE-patients without lupus nephritis as compared to lupus nephritis patients (33.0 ±10.1% vs. 43.8 ± 20.8%, n.s.). Interestingly, the percentages of CD8$^+$CD107a$^+$ T-cells were significantly decreased in SLE-patients without lupus nephritis as compared to healthy controls (33.0 ± 10.1% vs. 47.9 ±14.9%, $p = 0.01$).

Amount of CD8$^+$CD107a$^+$ T-Cells Are Associated With Disease Activity in SLE

Percentages of peripheral circulating CD8$^+$CD107a$^+$ T-cells were analyzed in active and inactive SLE-patients (**Figure 1C**). Active SLE-patients had significantly increased percentages of CD107a$^+$ cytotoxic T-cells as compared to inactive SLE-patients (49.8 ± 20.5% vs. 34.6 ± 15.1%, $p = 0.02$). There was also a significant difference between healthy controls and inactive SLE-patients (47.9 ± 14.9% vs. 34.6 ± 15.1%, $p = 0.003$).

There was a significant correlation between the percentages of peripheral circulating CD8$^+$CD107a$^+$ T-cells and disease activity assessed by SLEDAI ($r = 0.5$, $p < 0.005$, **Figure 1D**).

Decreased Expression of CD8$^+$CD107a$^+$ T-Cells Is Associated With Immunosuppressive Treatment

To assess the influence of immunosuppressive medication on the expression of CD107a$^+$ we subgrouped patients in (i) no treatment or prednisone alone (ii) prednisone and mycophenolate mofetil, azathioprine or cyclosporine

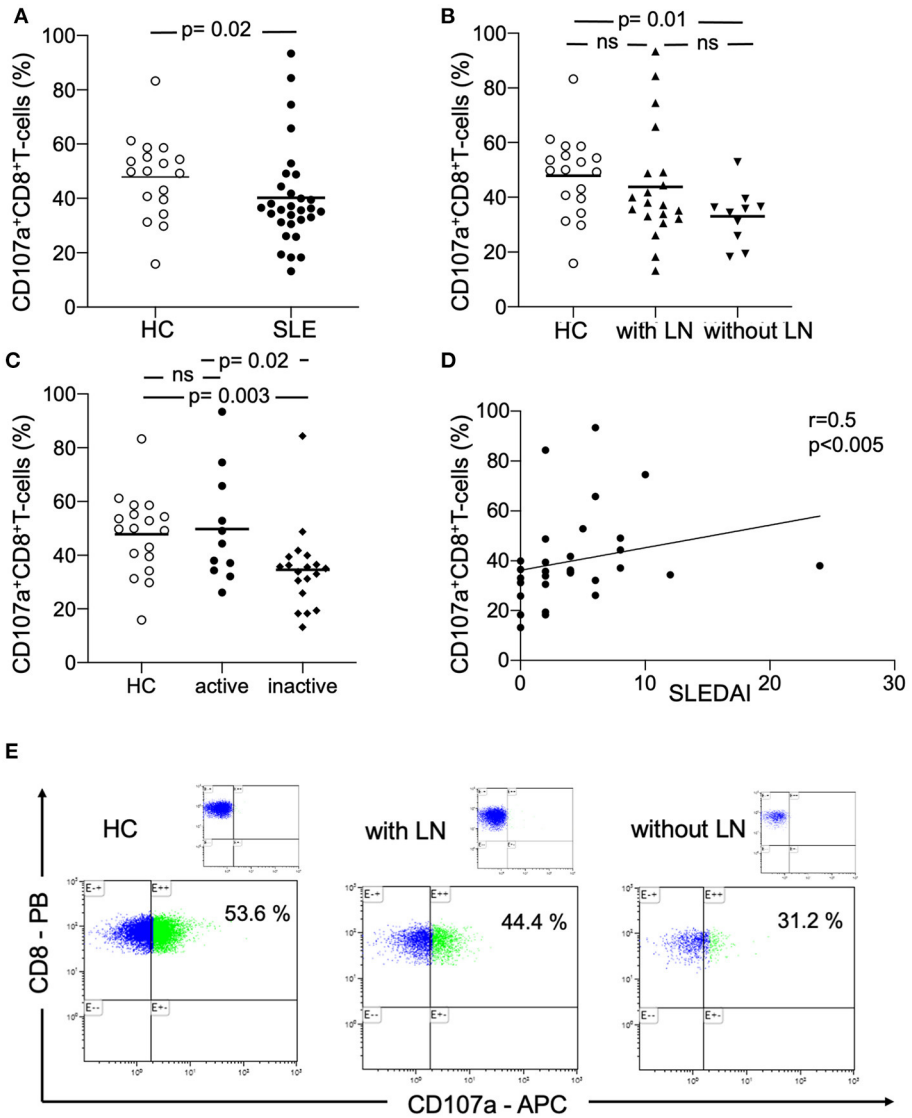

FIGURE 1 | Peripheral circulating CD107a$^+$CD8$^+$ T-cells. **(A)** The percentages of CD107a$^+$CD8$^+$ T-cells in healthy controls (HC), SLE-patients (SLE), **(B)** patients with lupus nephritis (with LN) and without lupus nephritis (without LN) are shown. **(C)** The percentages of CD107a$^+$CD8$^+$ T-cells in healthy controls (HC), active SLE-patients (SLE) and inactive patients are shown. Frequencies of these T-cells are shown. Horizontal lines represent the mean. *P*-values were calculated using the non-parametric Mann-Whitney U-test. **(D)** Correlation between percentages of CD107a$^+$CD8$^+$ T-cells and disease activity ($n = 30$) as assessed by the systemic lupus erythematosus disease activity index (SLEDAI). Spearman analysis was performed to calculate the correlation. A p-value <0.05 was considered significant. **(E)** A representative dot plot of the flow-cytometry staining is shown for a healthy control (HC), a patient with lupus nephritis (LN) and without LN. The corresponding isotype control is illustrated.

(iii) prednisone and hydroxychloroquine, respectively (iv) prednisone and hydroxychloroquine combined with mycophenolate mofetil, azathioprine or cyclosporine (**Figure 2A**). The analysis of CD107a on CD8$^+$ T-cells showed a significant different expression in patients who received a combined treatment of prednisone and hydroxychloroquine as compared to prednisone alone or no treatment, respectively (30.6 ± 10.5% vs. 56.2 ± 24.4%, $p = 0.02$). The expression was almost similar in patients in group iv who received additionally mycophenolate mofetil, azathioprine or cyclosporine as immunosuppressive treatment (29.7 ± 8.0% vs. 56.2 ± 24.4%, $p = 0.02$).

There was no significant correlation between the daily dose of prednisone (mg/d) and the expression of CD107a on CD8$^+$ T-cells. Remarkably, there was a significant negative correlation between the daily dose of hydroxychloroquine (mg/d) and the expression of CD107a on CD8$^+$ T-cells ($r = -0.5$, $p = 0.005$, **Figure 2B**). Eleven patients were active on immunosuppressive treatment, six patients in group (i) were assessed with active disease.

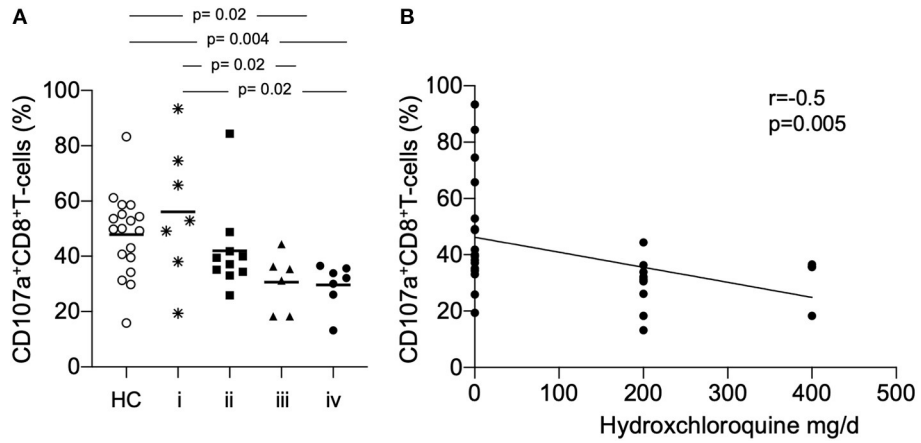

FIGURE 2 | (A) The proportion of CD107a$^+$ CD8$^+$ T-cells we were analyzed according to the immunosuppressive treatment. Patients were subgrouped in (i) no treatment or prednisone alone (ii) prednisone and mycophenolate mofetil, azathioprine or cyclosporine (iii) prednisone and hydroxychloroquine (iv) prednisone and hydroxychloroquine combined with mycophenolate mofetil, azathioprine or cyclosporine. Patients were compared to healthy controls (HC). *P*-values were calculated using the non-parametric Mann-Whitney U-test. **(B)** Correlation between percentages of CD107a$^+$CD8$^+$ T-cells for all samples taken ($n = 30$) daily dose of hydroxychloroquine is shown. Spearman analysis was performed to calculate the correlation. A p-value < 0.05 was considered significant.

Renal Expression of CD107a Is Associated With Proteinuria

Immunohistochemical CD107a staining of renal biopsies of SLE-patients showed a mean count of 59 ± 22.6 CD8$^+$-cells/mm^2 (**Figure 3**). The highest amount of 30.9 ± 20.9 CD8$^+$-cells/mm^2 where present in the extraglomerular compartment and only very few cells 0.1 ± 0.2 CD8$^+$-cells/mm^2 could be detected intraglomerular. CD107a expression could be detected on 3.7 ± 2.7 CD8$^+$-cells/mm^2. A cell count of 2.0 ± 2.4 CD107a$^+$-cell/mm^2 was extraglomerular and 0.3 ± 0.4 CD107a$^+$-cells/mm^2 intraglomerular. Double-positive cell were found in extraglomerular infiltrates (**Figure 4**). The intrarenal cell count of CD8$^+$-cells and CD107a$^+$-cells correlated with the activity and chronicity index (**Figure 5**). There was no significant correlation between cell counts and the activity or chronicity indices. The degree of the proteinuria was significantly correlated with intrarenal cell count of CD107a$^+$-cells ($r = 0.87$, $p < 0.05$).

DISCUSSION

Activated cytotoxic T-cells exert their effector function mainly by release of granzyme B and perforin. This release is dependent on cell-cell interaction. Ligation of CD107a (LAMP-1) has been described as a pivotal axis which leads to CD8$^+$ T-cell activation. CD107a is a marker of degranulation of cytotoxic NK and CD8$^+$ T-cells (17).

The present study demonstrates a decreased expression of CD107a on CD8$^+$ T-cells SLE-patients as compared to HC. The decreased proportion of CD107a$^+$CD8$^+$ T-cells was especially found in SLE-patients without lupus nephritis. This might be explained by a lower disease activity in this group because correlation with SLEDAI showed a significant correlation. This significant finding was confirmed in a previous observation by Holcombe et al. The authors reported a significant correlation between LAMP-1 expression on peripheral mononuclear blood cells of SLE-patients with disease activity assessed by Systemic Lupus Activity Measure (SLAM) but not with SLEDAI (10). The different results regarding the activity scores are most likely due to the different patients cohorts included. In our study predominantly patients with lupus nephritis were included. These patients have frequently higher activity scores in comparison to patients without lupus nephritis. Moreover, renal disease activity is considered with more items in the SLEDAI than in the SLAM resulting in different correlations (18). Besides, the study by Holcombe et al. recruited 10 of 46 patients without immunosuppressive medication and even more important for the data interpretation LAMP-1 expression was determined on PBMCs in contrast to specific subsets such as CD8$^+$ T-cells. This resulted in very low expression levels of 1.33 ± 0.25%).

Ex vivo experiments have shown that isolated PBMCs had an increase of CD107a expression in the presence of phytohemagglutinin (PHA) in a dose dependent manner. The induction peaked 30 min. after stimulation suggesting that rapid cell surface expression is due to translocation from intracellular vesiculars which are a major reservoir of LAMP proteins (9). Stimulation with IL-2 has been reported to be also a potent stimulus to trigger CD107a expression on NK and CD8$^+$ T-cells which was associated with increased cytotoxicity (17). Another study demonstrated that the surface molecule signaling lymphocytic activation molecule family member 4 (SLAMF4; CD244) is pivotal for the cytotoxic activity of CD8$^+$ T-cells assessed by CD107a in SLE-patients (19). CD8$^+$ T-cells of SLE-patients with selective loss of SLAMF4 showed a decreased CD107a expression upon stimulation with an anti-CD3 antibody for 2 h.

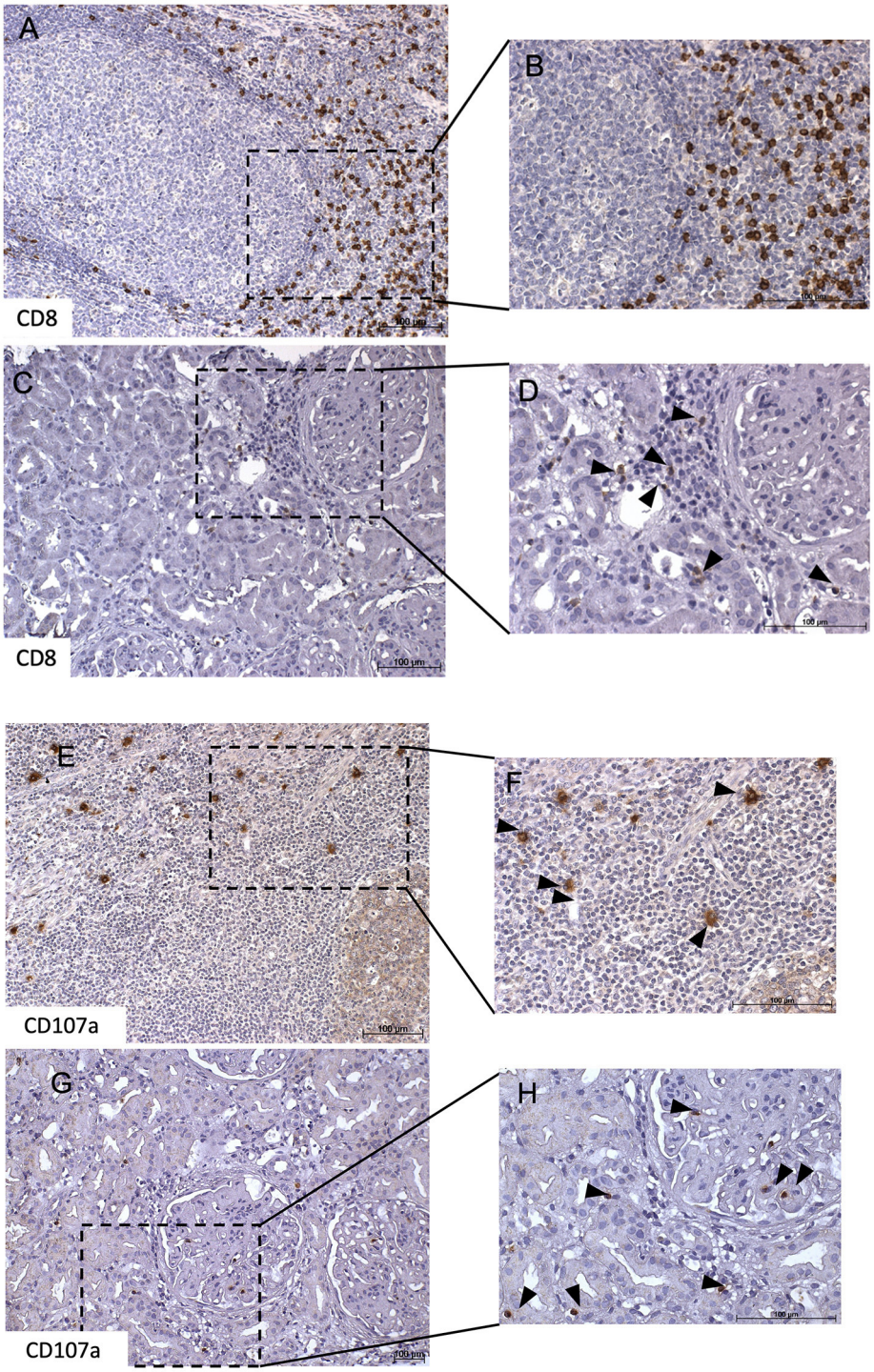

FIGURE 3 | $CD8^+$ and $CD107a^+$ T-cell infiltrates in lupus nephritis. This figure shows representative immunohistochemical staining with anti-CD8 of a tonsil which served as positive control **(A,B)**. Immunhistochemical staining of a lupus nephritis renal biopsy shows an overview **(C)** with one glomerulum and interstitial lymphocytes. Several of these lymphocytes express CD8 as demonstrated in **(D)**. Next, representative immunohistochemical staining with anti-CD107a of a tonsil which served as positive control **(E,F)**. Immunohistochemical staining lupus nephritis renal biopsy shows an overview **(G)** with one glomerulum and interstitial lymphocytes. Several of these lymphocytes express CD107a as demonstrated in **(H)**.

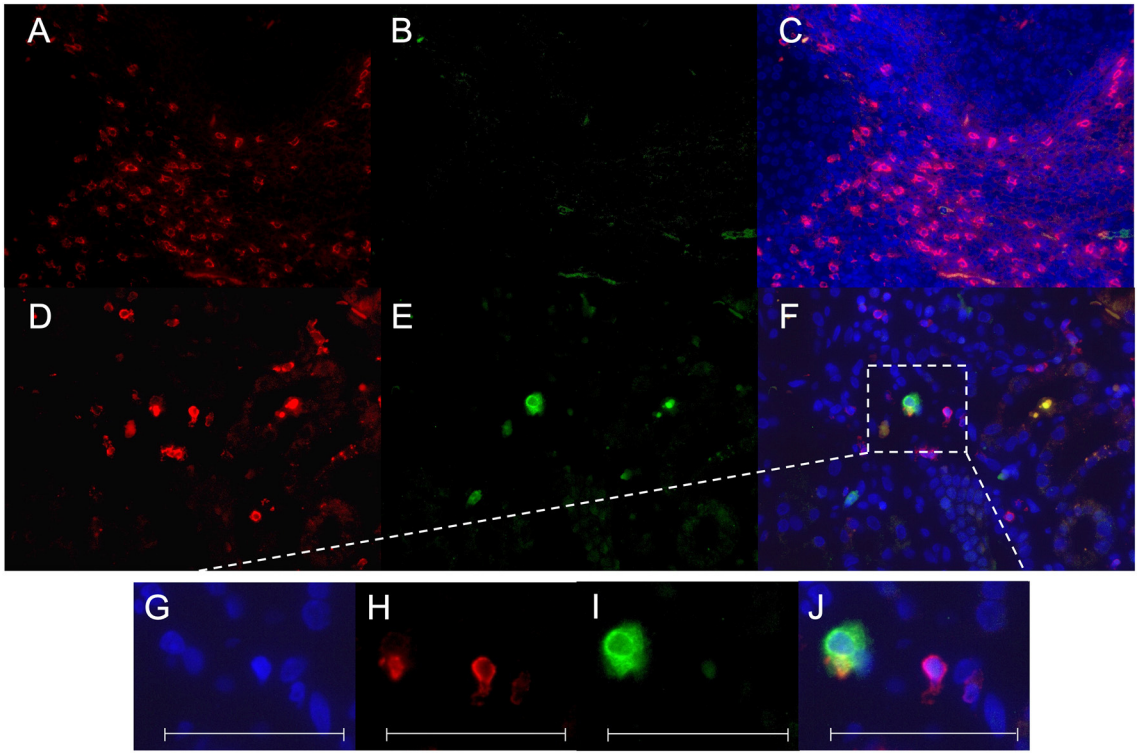

FIGURE 4 | $CD8^+$ $CD107a^+$ immunofluorescence staining. A staining for CD8 Cy3 (red), CD107a FITC (green) and colocalization of CD8/CD107a/DAPI was performed in a tonsil as positive control **(A–C)** and a representative renal biopsy of an SLE patient with lupus nephritis (WHO class IV) **(D–F)**. A magnification of a double-positive $CD8^+CD107a^+$ kidney infiltrating cell is shown in **(G–J)**. All scales represent 100 μm.

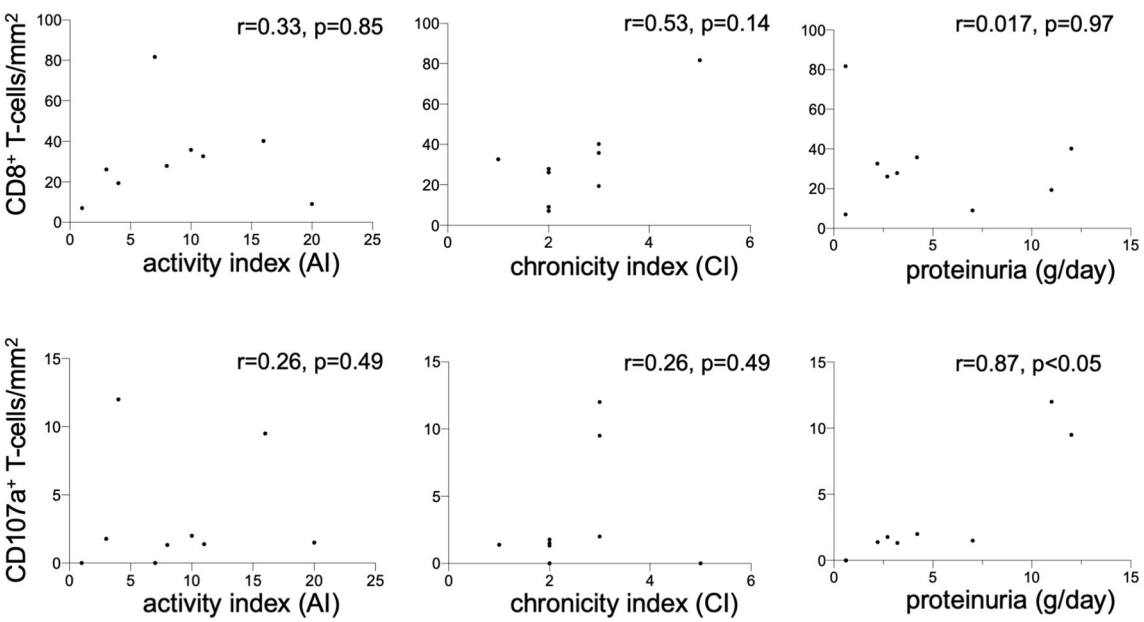

FIGURE 5 | Renal $CD8^+$ and $CD107a^+$ T-cells. Correlation between the $CD8^+$ T-cell count (cells/mm^2) in renal biopsies of nine lupus nephritis patients and renal histopathology parameters activity index (AI), chronicity index (CI) and proteinuria (g/d). The same correlation was performed for $CD107a^+$ T-cell count (cells/mm^2) in renal biopsies. Spearman analysis was performed to calculate the correlation. A p-value < 0.05 was considered significant.

In patients with Churg-Strauss-syndrome the expression of CD107a was significantly increased after polyclonal stimulation with anti-CD3 (20).

Remarkably, the expression CD107a on unstimulated $CD8^+$ T-cells was relatively low in the study by Aktas et al. In contrast to our study isolated peripheral mononuclear blood cells were used which could influence the expression. In the presence of IL-10 there was no significant increase in CD107a expression on $CD8^+$ T-cells (17). Thus, CD107a expression seems to be dependent on the surrounding cytokine milieu which might be *in vivo* variable during the course of disease in SLE-patients.

These studies support the idea that CD107a indicates T-cell activation. Besides, the lysosomal-associated membrane proteins (LAMPs) appear on the cell surface after exocytosis of cytotoxic granules. Thus Cohen et al. hypothesized that CD107a is transiently protecting cytotoxic lymphocytes from self-destruction (21).

The migration of cytotoxic T-cells to the kidneys and target organs during inflammation is a frequently reported observation. In this light the present finding of $CD8^+$ cells in renal biopsies of SLE-patients are confirmative. However, data on cytotoxic activity of $CD8^+$ T-cells in these biopsies are scarce. Thus, we stained CD107a cells in kidney biopsies. The presence of intrarenal CD107a cells was significantly correlated with proteinuria. This might indicate that these cells were activated and recently degranulated. A Denys-Drash murine model of nephrotic syndrome established to determine if lysosome activity in proteinuric mice demonstrated increased glomerular staining of LAMP-1 as compared to wild type mice (22). In accordance with our observation Carson and coauthors reported an association between LAMP-1 expression and degree of proteinuria. The present study provides evidence that CD107a is lower expressed on T-cells in SLE or downregulated by immunomodulating therapy, respectively. Interestingly, the effect diminishes during active disease. This might be explained by general disease activity or more likely by renal activity as well. The observation of relatively high CD107a expression in healthy controls in our cohort is not fully elucidated. Interestingly, the expression in our healthy control group was comparable with healthy non-pregnant women in a recent report (23). Exhaustion of cytotoxic T-cells in SLE-patients could be a possible explanation.

Moreover, CD107a has been described to mediate cell adhesion to vascular endothelium which potentially enables T-cell migration into kidney during active lupus nephritis (9). In *in vitro* experiments with stimulated NK cells from patients with granulomatosis with polyangiitis (GPA) demonstrated directly killing capacities of $CD107a^+$ NK cells of renal microvascular endothelial cells (24).

Immunosuppressive treatment might have an additional influence on cytotoxic activity and degranulation. Our data suggest that common immunosuppressive treatment could inhibit cytotoxic activity of $CD8^+$ T-cells by decreasing $CD107a^+$ expression. Interestingly, hydroxychloroquine is the agent which is most likely responsible since a strong negative correlation with the daily dose was demonstrated. A possible explanation could be the disruption of lysosomes by hydroxychloroquine which has been indicated by *in vitro* exposure of rat hepatocytes in co-culture experiments (25).

In conclusion, the present data suggest a critical role of CD107a for $CD8^+$ T-cell activation in particular in active disease. The detection of $CD107a^+CD8^+$ T-cells in lupus nephritis biopsies highlights the most likely effector cell function in renal involvement. The lack of functional experiments with renal infiltrating T-cells which remains technically very difficult is a limitation of this study. Nevertheless, there is a growing body of evidence that $CD107a^+$ might be a future therapeutic target to address cytotoxic T-cells.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Board University Hospital Essen. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AW collected the samples, performed the experiments and the statistical analysis. BW participated in research design, participated in the acquisition and analysis of the data and in the writing of the manuscript. BT and KL participated in the acquisition and analysis of the data. KA participated in the acquisition and analysis of the data and in the writing of the manuscript. AK drafted the manuscript. WA participated in the analysis of the data and drafted the manuscript. OW participated in the performance of the research, in research design and in the writing of the paper. SD designed the study, collected clinical data, analyzed the data and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Renal Involvement in IgG4-Related Disease: From Sunlight to Twilight

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IgG4-Related Disease (IgG4-RD) is a fibroinflammatory condition characterized by a typical histopathological pattern (dense lymphoplasmacytic infiltrate with prevalent IgG4+ plasma cells and storiform fibrosis), which may involve the kidney both directly (IgG4-related kidney disease, IgG4-RKD) or indirectly, as a consequence of post-renal ureteral obstruction due to retroperitoneal fibrosis (IgG4-RD RF). The most frequent presentation of IgG4-RKD is IgG4-related tubulointerstitial nephritis (TIN), but a glomerular disease can be present, in most of the cases a membranous nephropathy. Albeit steroid-responsive, in some cases renal manifestations may lead to progressive and permanent organ damage. In this review we describe four clinical cases representative of typical and less typical renal manifestations of IgG4-RD, emphasizing a potential, subclinical, early involvement of the kidney in the disease.

Keywords: IgG4-related disease, tubulointerstitial nephritis (TIN), retroperitoneal fibrosis, membranous nephropathy, ANCA - associated vasculitis

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INTRODUCTION

IgG4-RD is a rare fibroinflammatory disorder that can affect almost any organ, characterized by lymphoplasmocytoid infiltrate, obliterative phlebitis, and storiform fibrosis often associated with eosinophilia and increased levels of IgG4 (1). Rigorous epidemiological studies on IgG4-RD have not yet been conducted. The estimated frequency of autoimmune pancreatitis in Japan varies between 0.28 and 1.08 per 100,000 people with 336–1,300 new cases per year (2), but these data can underestimate the prevalence of the disease, especially when other organs are involved. Males are more frequently affected, with a peak of incidence around the age of 60.

The pathogenesis of IgG4-RD is not clear: according to a generally accepted view, a persistent antigenic stimulus, perhaps from chronic infection, induces an increased polyclonal expansion of B cells under the influence of M2 macrophages, CD4+ (mainly cytotoxic) T cells and Tfh, in a milieu of IL-4, IL-10, IL-1beta, and TGF-beta cytokines. These signals promote IgG4 class switching, somatic mutation, and plasma cells expansion, as well as a local fibrotic response (3).

Clinical manifestations are various and can affect almost any organ, with pancreas, retroperitoneum, lymph nodes, and salivary glands as the most frequently involved ones. Disease presentation is usually chronic, sometimes paucisymptomatic and insidious, without high inflammatory manifestations, and characterized mainly by the development of mass lesions that exhibit the same histological pattern in all the organs affected. The rich infiltrate of IgG4+ plasma cells and CD4+ T cells is embedded in extensive fibrosis that assume the typical, distinctive whirled

arrangement called "storiform." Obliterative phlebitis is another feature of the disease. Eosinophils are frequently detected, while necrosis is rare; in fact, the presence of necrosis or granuloma or giant cells should rather suggest a different diagnosis (4). In peripheral blood, IgG4 levels are usually elevated, as well as circulating plasmablasts. On the basis of these features, IgG4-RD diagnostic criteria were proposed (5), by which a patient can be stratified as "probable," "possible," or "definite" IgG4-RD if serum IgG4 levels above 135 mg/dL are (or not) associated to a number of IgG4+ plasma cells > 10/HPF and a IgG4+/IgG+ ratio >40% for most tissues in the context of a tumefactive lesion. Exceptions to this rule were proposed for some organ-specific manifestations, such as Mikulicz disease or Autoimmune Pancreatitis (AIP), for whom specific diagnostic criteria were already present, with the possibility for AIP to make a definite diagnosis without histological specimen if the pancreas presented suggestive radiological aspects in association with increased level of IgG4 (5).

Recently, ACR/EULAR classification criteria were proposed (6). Developed and validated in a large international cohort of patients, these criteria had an excellent performance in discriminating IgG4-RD from disease mimics. These classification criteria contain entry, exclusion, and inclusion criteria; these latter allow the diagnosis of IgG4-RD when a score of 20 points is reached. One of the exclusion criteria proposed by ACR/EULAR classification is the scarce response to steroid treatment. In fact, responsiveness to glucocorticoids is a main clinical characteristic of IgG4-RD, even if relapses are common after steroid discontinuation (3). DMARDs such as methotrexate, azathioprine, hydroxychloroquine, and tacrolimus are used, but their efficacy is reported in limited studies. Mycophenolate and cyclophosphamide were evaluated in two trials and both drugs reduced, but not eliminated, relapses. Rituximab is an anti CD20 monoclonal treatment that demonstrates a dramatic efficacy in IgG4-RD, thanks to depletion of B cells and reduction of inflammatory infiltrate: treatment with Rituximab in the early stages of the disease can reverse fibrosis. Even in this case, clinical remission may last 6–18 months. Recently, a long-term efficacy of rituximab was evaluated when used every 6 months for maintenance therapy, an approach already used in ANCA-associated vasculitis (7).

As soon as the multisystem involvement of IgG4-RD became clear, it was appreciated that kidney was a prominent target organ. Reported frequency of renal involvement is in fact comprised between 10 and 27% of the cases (8, 9).

We report 4 cases of IgG4-RD with renal involvement that exemplify the different pattern of renal disease that can be observed in this disorder, outlining the clinical course and therapeutic approach.

Informed consent was obtained from each patient, for every procedure performed.

CASE PRESENTATIONS

Case 1

A 39 years old clergyman with a history of asthma, arterial hypertension and gastroesophageal reflux came to our attention for exophthalmos, previously treated with steroid boluses, and submandibular tumefactive lesions. His right parotid had echographic signs of parenchymal subversion and a Positron emission tomography/computed tomography (PET/CT), performed 3 years before admission, showed enlargement and high uptake of multiple lymph nodes in neck, mediastinus, tracheal, periaortic, and interaortocaval regions (SUVmax 7.3). To exclude hematologic malignancy, a lymph node needle biopsy was performed and showed hyperplasia with reactive plasmocytosis (IgG4 staining not performed). Blood tests showed hypergammaglobulinemia (19.6%), rheumatoid factor positivity (72.7 <30) and hypocomplementemia (C3 76 mg/dL, C4 6 mg/dL). Creatinine levels progressively increased from 1.19 to 1.92 mg/dL. At hospital admission the patient presented left eye exophthalmos and swelling of neck lymph nodes in the right side. Serum IgG4 were 1,300 mg/dL (49-66), IgG4/IgG ratio was 44.45% and circulating plasmablasts (CD19+ CD20– CD27+ $CD38^{bright}$ cells) were highly increased (5,520 cells/mL; normal <635 cells/mL). An abdominal echography showed a hypoechogenic aspect of renal cortex. Urinary Retinal Binding Protein (RBP) and beta2microglobulin were increased (0.3 mg/dL with normal <0.1 and 337 mcg/L with normal <154 mcg/L, respectively), urinary light chain were present and urinary electrophoresis showed a mixed proteinuria (glomerular and tubular). Daily proteinuria was above 700 mg/24 h. Urinary sodium excretion, as well as potassium, calcium, and phosporous, were slightly reduced.

A renal biopsy was performed. Examination of renal parenchyma showed a diffuse and intense fibrosis, with abundant lymphomonocytic and IgG4+ plasmacellular infiltrates (IgG4+/IgG+ ratio >40%). Immunohistochemistry for IgG4 demonstrated 22 IgG4 ± plasmacells/HPF. Only 2 out of 9 glomeruli were sclerotic, while tubuli were diffusely reduced and atrophic. Small and medium vessels showed mild thickening. No complement deposits were detected by immunofluorescence (**Figure 1**).

Histopathology of a neck lymph node had similar histological characteristics, with fibrotic areas enriched in plasmocytes (IgG4+/IgG+ ratio > 40%). The diagnosis of IgG4-Related Disease was made and treatment with monoclonal anti CD20 was started, with a progressive reduction of lymph node swelling and exophthalmos and a mild creatinine reduction, up to 1.4 mg/dL in 3 months.

Case 2

A 47 years old woman was evaluated in our outpatient clinics for AIP diagnosed 3 years before in another hospital (abdominal pain, hyperamilasemia; "Salt and pepper," non-homogeneous pancreatic parenchyma at echography; serum IgG4 256 mg/dL). She had been treated with budesonide 9 mg/die and was under treatment with anti-IL-5 (Mepolizumab) for severe asthma. Blood tests showed increased ESR (108 mm/h, n.v. <25), fibrinogen (623 mg/dL, n.v. 200–450) and pancreatic amylase 150 U/L (n.v. 15–53); urinalysis showed microalbuminuria and increased number of white and red cells in the sediment. Urinary electrophoresis showed mixed proteinuria, mainly tubular. A PET/CT scan detected only low uptake on aortic arc, while

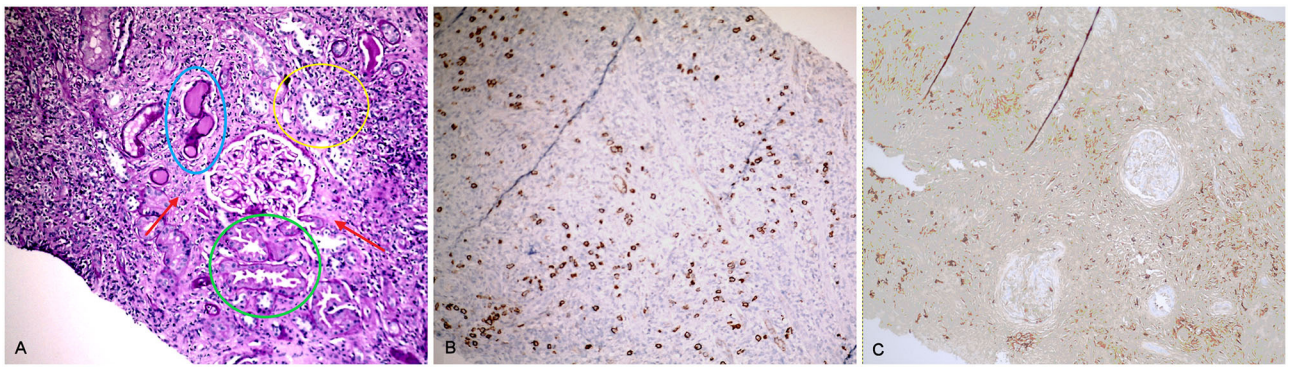

FIGURE 1 | Histopathology of renal biopsy (case 1), showing IgG4-RD TIN. **(A)**: Diffuse tubulo-interstitial infiltrate associated with various degrees of tubular damage: initial thinning of tubular cells with cytoplasmic material in the tubular lumen (green circle); marked de-epithelialization of the tubular epithelium with inflammatory cells and tubulitis (yellow circle); tubular thyroidization due to disappearance of epithelial cells (blue circle); interstitial fibrosis is also evident (red arrow) (20x). **(B)**: CD138 staining, showing plasma cells in the inflammatory infiltrate (20x). **(C)**: immunohistochemistry for IgG4, showing positivity in the interstitium (20x).

abdominal RM did not show parenchymal involvement. Anti IL-5 therapy was stopped. Slowly, within 4 months, she developed lower limbs edema, and a rapid decay of her health status. At hospital admission she had proteinuria in the nephrotic range (6 g/L), increased ESR and fibrinogen, hyperamylasemia, ANA 1:160 speckled, normal creatinine level, normal C3 and C4, total IgG 301 with IgG4 81 mg/dL. Anti PLA2R and ANCA were negative and urinary electrophoresis showed mixed proteinuria. An echography showed globular aspect of pancreas, hyperechogenicity of renal cortical and medullary columns, linear hyperechogenic bands in salivary glands. A renal biopsy was performed, showing mild thickening of glomerular basal membrane and focal mesangial expansion; minimal interstitial fibrosis and IgG4-negative lymphomonocytic infiltrate; minimal tubular atrophy (5% of tubuli) in the absence of vascular abnormalities. IgG4 staining showed focal IgG4 on glomerular basal membrane, while by immunofluorescence deposits of IgG and C3 were detected on basal membrane (**Figure 2**), with very few IgG1 positive cells but no IgG2 or IgG3 positive cells (see **Supplementary Figure 1**). Membranous glomerulonephritis was diagnosed (Ehrenreich and Churg Stage I) and the patient was treated with steroid boluses (methylprednisolone 500 mg ev/die for 3 days) and Rituximab (1 gram at time 0 and after 7 days). Remission of nephrotic syndrome was obtained after 2 months.

Case 3

A 74 year-old man was admitted to our hospital because of chronic cough, dysphonia, dysphagia, hypoacusia, fever, and weight loss. He presented chronic arterial hypertension, atrial fibrillation, and no history of allergy. At admission ESR and CRP were elevated (78 mm/H and 21.6 mg/dL, respectively; upper limits <20 and <0.5); mild anemia, neutrophilic leucocytosis, and hypergammaglobulinemia were also present. Initially, a diagnosis of cryptogenetic bronchiolitis was made on the basis of CT scan findings (pulmonary involvement with thickening of bronchial walls and a "tree-in-bud" sign) and the patient was treated with broad-spectrum antibiotics and low dose steroids. For the recurrence of fever with an increase of acute phase reactants and a progressive weakness, further examinations were performed. Autoantibody evaluation demonstrated ANA 1:80 (speckled pattern) and ANCA-MPO highly positive (84% AU, normal <18%). The rapid decline of renal function with an increase of creatinine levels from 0.81 to 2.69 mg/dL, albuminuria (100 mg/dL), micropyuria, and microhematuria strongly suggested the diagnosis of ANCA-associated vasculitis (microscopic polyangiitis, MPA). The patient was referred to our Unit in order to complete diagnostic workout and start adequate therapy.

Urinary RBP and beta2microglobulin were increased (1.9 mg/dL n.v. <0.1 and 289 mcg/L n.v. <154 mcg/L, respectively) and urinary electrophoresis showed a mixed proteinuria (glomerular and tubular) with epithelial, granular, and erythrocytic cylinders. A PET/CT scan showed a moderate 18F-fluorodeoxyglucose (18F-FDG) uptake of mediastinic, parathracheal, ilar, and axillary lymph nodes. Electroneurography and electromyography showed neurogenic damage with signs of denervation. Gadolinium-enhanced magnetic resonance imaging (MRI) of the brain disclosed a mastoiditis without other significant lesions.

Interestingly, serum IgG4 level was 529 mg/dL (normal <135 mg/dL) with an IgG4/IgG ratio of 27% and circulating plasmablasts (CD19+ CD20− CD27+ $CD38^{bright}$ cells) were slightly increased (1,402 cells/mL; normal <635 cells/mL). We then revised the histology of a "benign lesion" of the gallbladder removed 2 years before, that was 9 cm long and induced a duodenal stenosis and a cystocolic fistula. Histologically, lymphoplasmacytic infiltrate rich of eosinophils with areas of fibrosis was detected. Immunohistochemistry for IgG4 showed abundant IgG4-positive plasma cells (IgG4+/IgG+ plasma cells ratio > 40%, 45 IgG4 ± plasmacells/HPF), consistent with IgG4-RD.

Finally, a renal biopsy was performed. Examination of renal parenchyma demonstrated an intense lymphomonocytic infiltrate in interstitial space, with IgG4+ plasmacells and eosinophils. In two glomeruli there was extracapillary proliferation that induced a complete rupture of Bowman

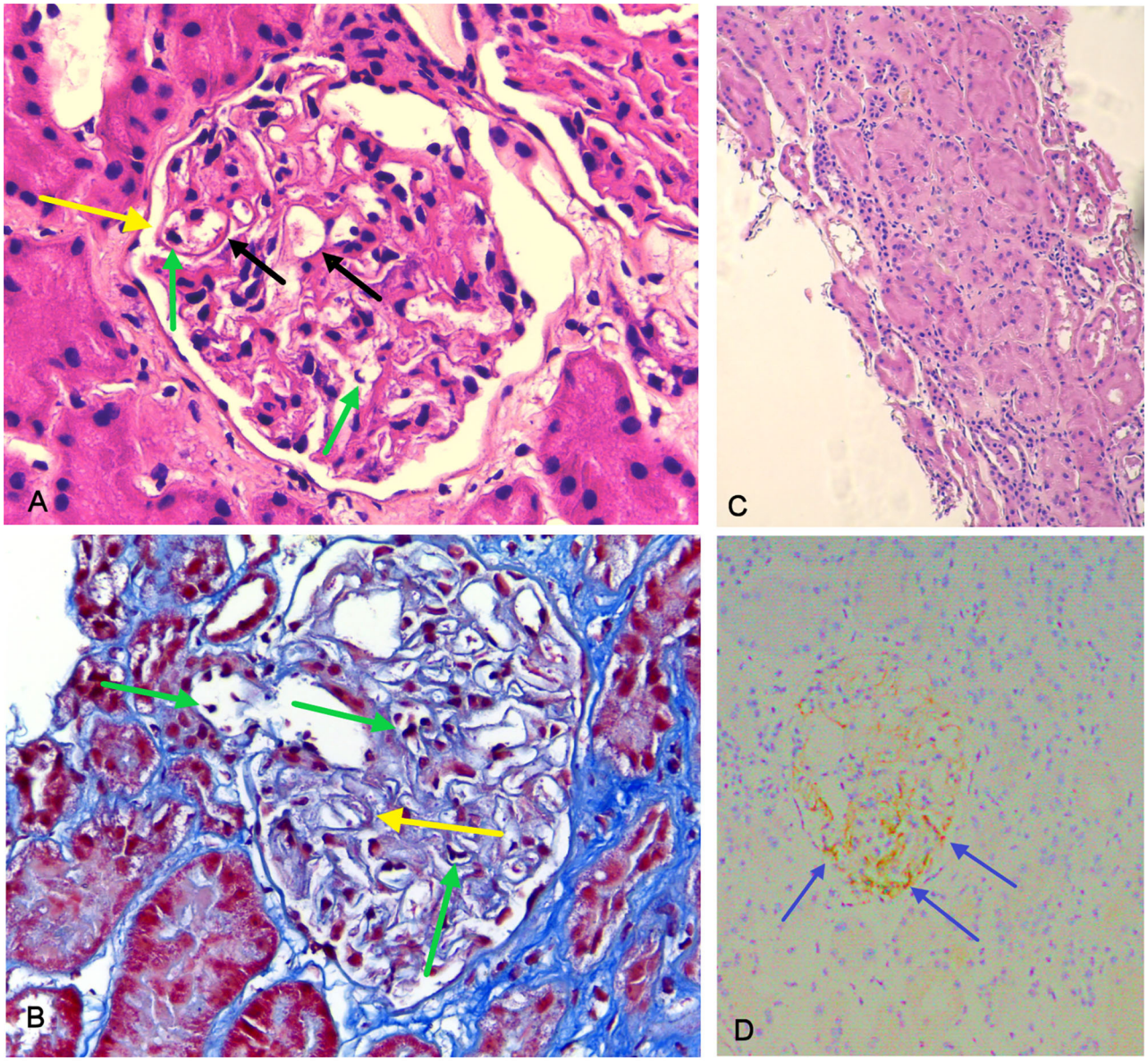

FIGURE 2 | Histopathology of renal biopsy in case 2, indicative of membranous glomerulonephritis. Membrane (yellow arrow) and capillary loops (black arrow) thickening demonstrated by hematoxylin and eosin stain (**A**, 40x) and by Masson's trichrome stain (**B**, 10x). Some inflammatory cells (granulocytes) are present in the capillary lumens (green arrow), confirming the hypothesis of a secondary membranous glomerulonephritis. The interstitium, lacking infiltrates or fibrosis, and the tubules, arranged in a palisade, are not affected by the disease (**C**, 40x). IgG4 immunohistochemistry demonstrates positivity in the glomerular basal membrane (blue arrow) (**D**, 20x).

capsulae. In 10% of tubuli, atrophy was detected, and 2 out of 9 glomeruli presented sclero-ialinosis. Some tubular segment, however, demonstrated signs of tubulitis with focal neutrophils in tubular epithelium and fibrinoid necrosis in medium-large vessels (**Figure 3**). Thus, a diagnosis of AAV-IgG4RD overlap was made. The patient underwent therapy with iv prednisone (500 mg/day for 3 days) followed by iv Cyclophosphamide (1 g/month, 7 g total dose). Six months later, creatinine level was 1.4 mg/dL; inflammatory markers (ESR 14 mm/Hg) and serum IgG4 (104 mg/dL) were normal, while ANCA-MPO antibodies, still present, were reduced in titer. Urinalysis showed absence of white and red cells and proteinuria was 250 mg/24 h.

Case 4

A 70 years old man with multiple comorbidities (COPD, arterial hypertension, atherosclerosis with carotid, and iliac stenosis) and a previous pulmonary lobectomy with lymphadenectomy for a giant cell carcinoma, performed a total body CT scan during his oncologic follow-up. A nodular lesion (19 mm) was detected between pancreas and duodenum with contrast

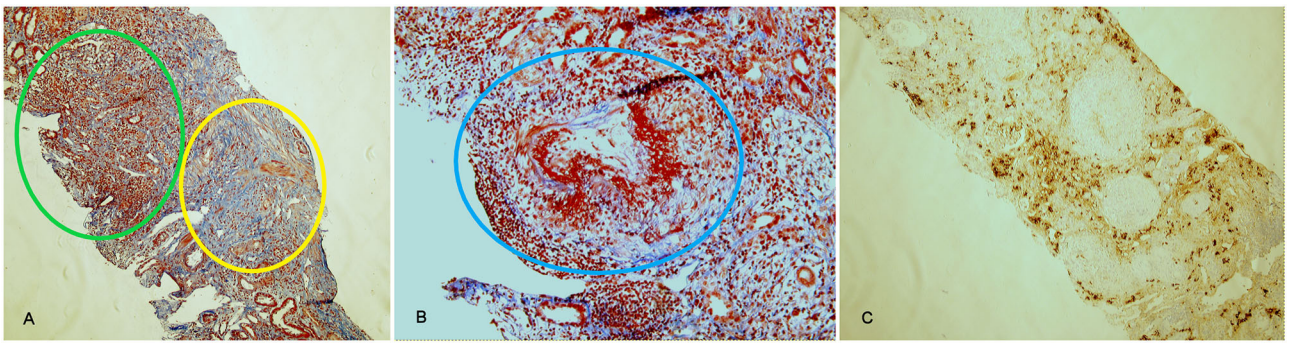

FIGURE 3 | Histological findings in AAV-IgG4-RD overlap (case 3). Acute diffuse tubulointerstitial infiltrate (green circle) associated with fibrotic involvement (yellow circle) and with destruction of renal structures, highlighted by the Masson trichrome stain (**A**, 10x). Necrotizing arteritis (blue circle) (**B**, 20x). CD138 staining, highlighting plasma cells in the infiltrate (**C**, 10x). Lesions in **(A)** are suggestive of IgG4-RD, lesions in **(B)** are typical of AAV.

enhancement, and a focal pyeloureteral hypercaptation compatible with urothelial carcinoma. An abdominal MRI demonstrated 2 pancreatic lesions of the pancreas head, suspicious for malignancy. Due to the oncologic history, the patient underwent a Whipple's pancreaticoduodenectomy and left nephroureterectomy. Surgical resection was complicated by sepsis with pneumonia and a pancreatic fistula with ascites, positive for Pseudomonas Aeruginosa, that subsequently underwent surgical treatment. He then developed portal thrombosis, heparine-induced thrombocytopenia and, after 2 months, chilotorax, intraepathic aerobily, and abdominal abscess.

The histologic analysis of surgical specimens demonstrated a low-grade urothelial carcinoma, while pancreatic parenchyma showed fibrosis areas with lymphoplasmocytic infiltrates, periduttal infiltration, follicular-like aggregates, venulitis, acinar atrophy. Immunohistochemistry for IgG4 demonstrated >10 IgG4+ plasmacells/HPF and IgG4/IgG ratio >40%.

Given the aforementioned histological findings, the patient was admitted to our inpatient clinic with the suspect of IgG4-RD. Clinical and laboratory signs of sepsis were present and broad-spectrum antibiotic treatment was started, with slow improvement. During the recovery, serum IgG4 were increased (225 mg/dL, n.v. 49–66, IgG4/IgG ratio 17.78%). Interestingly, daily proteinuria was low (<100 mg/24 h) but urinary electrophoresis showed a tubular proteinuria with a small increase in urinary RBP (0.3 mg/dL, nv 0.1 mg/dL). Histological re-examination of resected kidney revealed thyroidisation of tubules, sclerotic glomeruli, severe, fibrosis of arterial walls, and interstitial lymphocytic infiltrate. An immunostaining for IgG4 showed >10 plasma cells IgG4+/HPF and a IgG4/IgG ratio >40%. Due to the frailty of the patient, a steroid therapy regimen was started in association with antibiotics. After surgical drainage of abdominal abscess, the patient's conditions improved and no relapse of IgG4-RD in any localization was observed in follow up.

DISCUSSION

Tubulointerstitial Involvement

TIN is the most frequent renal manifestation of IgG4-RD and is diagnosed according to the criteria developed by the Mayo Clinic group and the Japanese Society of Nephrology (10, 11). At variance with other forms of TIN (drug-induced or associated with systemic autoimmune disorders as Sjogren's syndrome), the involvement of renal parenchyma is not diffuse but rather characterized by focal lesions surrounded by normal tissue.

Histologically, the hallmark is a diffuse or multifocal lymphocytic infiltrate with a predominance of IgG4+ plasma cells, an IgG4/IgG-positive plasma cell ratio >40% and >10 IgG4-positive plasma cell per high-power field (12). However, as the disease is zonal, the absence of of IgG4-rich plasma cell infiltrate in a kidney biopsy cannot exclude IgG4-RD. Eosinophils are frequently detected, while obliterative phlebitis is rarely seen (9). The infiltrate may affect well-demarcated areas of renal parenchyma, or rather extend into kidney capsule (13). Tubulitis is also present, with mononuclear cells, plasma cells and eosinophils (13), in the absence of cell necrosis. The fibrotic interstitium shows the typical storiform fibrosis but sometimes a different pattern can be detected, characterized by irregular fibers surrounding inflammatory cell clusters, defined "bird's eye fibrosis" (8, 14). According to the degree of interstitial inflammation and fibrosis, 3 different patterns of IgG4-TIN were described: acute TIN with minimal interstitial fibrosis; chronic TIN with expansile interstitial fibrosis; and advanced sclerosing pattern, with fewer inflammatory cells (15).

By immunofluorescence, granular deposits of IgG and C3 can be detected on tubular basal membrane; C1q can occasionally be present and electron microscopy shows electron dense deposits in the membrane.

Serologically, patients affected by TIN have no distinguishing feature except for hypocomplementemia, present in 60% of the patients, with decreased levels of C3 and/or C4, and higher serum levels of IgG and IgG4. CRP is rarely elevated, at variance with other forms of TIN (16). Acute or chronic renal insufficiency can be present, associated with a variable degree of hematuria and proteinuria. In contrast with drug-related TIN, urinary WBCs or casts are not a constant finding (16).

Radiographic lesions of IgG4-RD TIN are best visualized by contrast-enhanced computer tomography scan. Multiple low-density lesions with mild enhancement in delayed phase on enhanced CT are the most common radiological findings

(11, 16). By MRI, renal lesions appear iso- or hypointense in comparison with normal renal parenchyma on T1-weighted images, hypointense on T2-weighted images.

PET/CT, useful to evaluate retroperitoneal involvement, is not advised to study renal disease, because of the interference due to kidney excretion of radio-labeled drug.

Multiple and bilateral lesions are usually detected, predominantly involving the renal cortex. Four patterns of parenchymal lesions are described: small, sub-centimetrical peripheral cortical nodules, round, or wedge- shaped lesions, diffuse patchy involvement, or (more rarely) a solitary mass (17). Nephromegaly (>14.5 cm), sometime reversible after therapy, and renal pelvis involvement were also reported (10). Lymphoma, vasculitis, pyelonephritis, and metastatic cancer should be considered in the radiographic differential diagnosis of renal parenchymal lesions.

Glomerular Involvement

Different types of glomerular involvement are described in IgG4-RD, but membranous glomerulonephritis, occurring in roughly 7% of the patients, is the most frequent. In fact, only anecdotical reports of IgA nephropathy, endocapillary proliferative glomerulonephritis and membranoproliferative glomerulonephritis have been published (18–22).

In most cases, glomerular involvement coexists with TIN but occasionally it occurs in the absence of tubular involvement. Proteinuria in the nephrotic range is the usual manifestation of the disease. IgG and C3 deposits are detected in glomeruli by immunofluorescence and abundant electron dense deposits are shown in subepithelial space by electron microscopy (23). In a low percentage of cases, biopsy samples exhibit also mesangial or subendothelial deposits (10). The specificity of autoantibodies involved in the formation of these deposits is not known, but it is of interest the detection of antibodies against carbonic anhydrase II, a podocyte antigen, in IgG4-RD membranous nephritis (24).

Differential diagnosis from primary membranous nephropathy is based on the simultaneous presence of TIN, on the detection of other localizations of the disease (the kidney is very rarely the only organ affected), on the absence of antibodies specific for phospholipase A2 receptor. However, it is important to take into account that this autoantibody, marker of primary membranous nephropathy, is present in only 70% of the patients (21). The recent ACR/EULAR IgG4-RD classification criteria consider anti-PLA2R as an exclusion criterion for the diagnosis of IgG4-RD (6).

Recently, the coexistence of IgG4-RD and primary membranous nephropathy has been described in a patient positive for antibodies to the phospholipase A2 receptor (25).

Retroperitoneal Fibrosis

Idiopathic retroperitoneal fibrosis (IRF) is a rare fibro-inflammatory disease of unknown etiology characterized by periaortic and peri-iliac fibrosis. It was described for the first time in 1905 and classified in 1948 by Ormond (26). It was estimated that IRF is associated to IgG4-RD in more than half of the cases, sometimes representing the only manifestation of the disease (27, 28).

The expansion of fibrosis can entrap any of the structures in the retroperitoneum, especially ureters, inducing obstructive nephropathy. Clinical features are often non-specific: lower back and flank dull pain in over 90% of patients, anorexia, weight loss, fatigue. Legs edema associated to deep venous thrombosis can be detected when vena cava obstruction is present.

Recently, it has been reported that IgG4-RD RF is characterized by lower serum IgG4 levels and a lower IgG4-RD Responder Index at disease onset if compared with other clinical phenotypes of IgG4-RD (29, 30). Histologically, the counterpart of the RF phenotype is a lower number of plasma cells in comparison with other involved organs. However, this finding could simply depend on samples obtained in more advanced stages of the disease, when the fibrotic component is prevalent.

To evaluate retroperitoneal lesions, enhanced CT scan, MRI, or PET/CT are the preferred tools; hydronephrosis can easily be detected by ultrasound. Other localizations of the disease can be more easily biopsied and can suggest the correct diagnosis.

When acute ureteral obstruction occurs, ureteral stent, percutaneous nephrostomy, or ureteral stricture surgery are necessary to overcome hydronephrosis and progression to end-stage renal disease. However, many patients can still suffer from chronic kidney disease, because of late or ineffective medical treatment (31).

Overlap With ANCA-Associated Vasculitis (AAV)

The coexistence of IgG4-RD and AAV has been reported and a biopsy-proven overlap was described in several cases.

Chang et al. (32, 33) described eight cases out of a cohort of 43 GPA patients characterized by a high number of IgG4+ plasma cells infiltrating the kidneys, but they considered these cases as diagnostic pitfalls rather than AAV-IgG4-RD overlap. Thus, the exact incidence of AAV-IgG4-RD overlap is presently unknown, since many cases were diagnosed previously as AAV. Danlos (34) in a multicentric European retrospective study identified 10 biopsy proven overlap, but the total number of AAV patients evaluated in the study is not reported. A recent retrospective Mexican study on association between AAV and other autoimmune disease identifies only one patient out of 147 with AAV-IgG4-RD overlap (35), while another study with a more limited number of patients failed to identify any overlap with IgG4-RD (36). Thus, the incidence of AAV-IgG4-RD overlap might be estimated in 1:150 AAV, or lower.

In AAV-IgG4-RD overlap, kidney lesions attributable to both disorders can coexist and cooperate to induce anatomical and functional damage. Under this respect, the case we describe in this report is of particular relevance, because renal histology shows a glomerulonephritis compatible with AAV, with rupture of Bowman capsulae and fibrinoid necrosis, together with a tubulointerstitial involvement and IgG4+plasmacells, more typical of IgG4-RD. The coexistence of TIN with eosinophilic infiltrate has been previously described in AAV, before the identification of IgG4-RD as distinct nosological entity (37). In the light of more recent findings, some of these cases might be more correctly diagnosed as AAV-IgG4-RD overlap.

ANCA are critical for the differential diagnosis between AAV and IgG4-RD, but low titer ANCA are present in several autoimmune disorders including IgG4-RD. Thus, the detection of IgG4 ANCA has been taken into account as possible tool for diagnosis.

ANCA positivity is one of the exclusion criteria proposed for the diagnosis of IgG4-RD in ACR/EULAR IgG4-RD classification criteria (6).

However, IgG1 and IgG4 ANCA are reported by Della-Torre et al. (38) in a case of AAV-IgG4-RD overlap, and also by Su et al. (39). Similarly, Abbass et al. (40) claim that in their clinical case ANCA were predominantly IgG4. However, data on IgG2 and IgG3 ANCA in these patients are not available.

Comparing the subclass distribution of ANCA MPO in AAV and IgG4-RD, we found that ANCA MPO of any subclass, and not only IgG4, can be detected in IgG4-RD sera. However, the titer of these antibodies is very low in comparison with AAV; moreover, antibody titer and the number of ANCA subclasses are not correlated to clinical activity, at variance with the results obtained in AAV. Thus, ANCA titer seems more relevant than subclass distribution for differential diagnosis and the detection of ANCA-MPO at high titer, without analyzing ANCA subclasses, is sufficient to corroborate serologically the clinical suspect of an AAV-IgG4-RD overlap (41).

Treatment and Follow-Up

Due to the rarity of the disease, no randomized trial specifically addressing IgG4-RKD has been conducted so far. Only single case reports have been published, and the renal involvement in IgG4-RD is treated according to the therapeutic approach used for the other localizations of the disease. Since the kidney is a critical organ, early therapy is recommended to prevent long-term fibrotic damage.

First-line treatment is still represented by steroids, according to a 2015 consensus statement from an international expert panel on the treatment of IgG4-RD (42). Patients with early diagnosed IgG4-RD TIN present higher values of eGFR than patients with a late diagnosis of renal involvement, probably because of a prompt glucocorticoid therapy (43). Patients with eGFR <60 ml/min/1.73 m^2, as well as advanced histological stage, still present an improvement after steroid treatment, albeit limited (43–45), but are at major risk to develop renal cortical atrophy (46). Repeated renal biopsy in IgG4-RD TIN patients during steroid treatment shows in fact progressive fibrosis of the interstitium, despite a reduction of inflammatory infiltrate (47, 48). Despite a good response to steroids in almost all IgG4-RD patients, the relapse rate is high at tapering, and nearly 40% of patients fail to achieve remission after 1 year (49, 50). These data suggest that different strategies should be used to properly control the disease.

Many DMARDs have been employed in IgG4-RD, such as methotrexate, azathioprine, tacrolimus, but their efficacy in maintaining a relapse-free condition, with or without a low-dose steroid, has not yet been verified. Up to now, only two trials were conducted on classical DMARDs, Mycophenolate Mofetil (MMF) and oral Cyclophosphamide (CFX) (51, 52). Both drugs are effective on relapse rate in comparison with steroid-only regimen: after 1 year of therapy, relapses are observed in 20% of patients treated with MMF and in 12% treated with oral CFX, respectively.

B cell-targeted therapy with Rituximab (RTX), a monoclonal antibody anti CD-20, leads to an excellent response, with a remarkable reduction of mass lesions and a variable regression of fibrosis even in a steroid-free regimen (53). RTX administration reduces serum IgG4 level and circulating plasmablasts (54). In a case report, RTX alone substantially improved renal function in a 58-year-old man with IgG4-RD TIN (55). RTX, that depletes mature B cells but spares plasma cells, has a better safety profile than CFX, less toxicity and fewer infectious events. Albeit widely used, a specific evaluation of rituximab efficacy in renal involvement is still lacking. It has to be taken into account that RTX modulation of disease activity occurs 4–8 weeks after the infusion. Thus, Quattrocchio et al. proposed a treatment protocol for IgG4-TIN that includes steroid boluses, 2 pulses of iv CFX and 4 weekly doses of RXT; this protocol showed a sustained efficacy, both clinically and histologically, over a 4-year follow-up (56, 57).

In contrast, there is no clear demonstration that an exclusive steroid regimen is an effective approach for IgG4-membranous nephropathy (58). Patients are managed as primary membranous nephropathy with Rituximab, cyclophosphamide, or cyclosporine.

Notably, the disease can recur in the transplanted organ, as reported by Chibbar et al. Despite therapy with prednisone (5 mg daily), tacrolimus 6–8 mcg/L, and mycophenolate mofetil 2 g daily, a 25 year old patient had relapse of IgG4-TIN 5 years after renal transplant, concomitant with chronic active antibody mediated rejection (59).

IgG4-RD RF benefits of the same treatments already described. Notably, recent reports of MMF and RTX treatment in RF show efficacy whether or not it is a manifestation of IgG4-RD (60, 61). Some conditions, such as smoking habit, AKI at diagnosis, ANA positivity and lumbar pain, are associated to relapse, and all these factors represent a negative prognostic combination for renal outcome (62).

CONCLUSIONS

Renal involvement in IgG4-RD is a dangerous condition that can lead to a chronic organ damage, potentially life-threatening. Multiple reports suggest the need of an aggressive therapy in order to avoid a fibrotic remodeling and a functional damage.

Describing four clinical cases, we moved from sunlight to twilight, going from the classical pattern of renal involvement of case 1 and 2 to the more complex picture of case 3 and finally to the subclinical involvement of case 4.

These four patients enlight the heterogeneity of clinical manifestations as distinguishing feature of IgG4-RD also when affecting the kidney.

Thus, a careful periodic assessment of kidney function with evaluation of filtration rate and urinary sediment, in association with adequate imaging, is mandatory for each IgG4-RD patient.

Presently, urinary markers that allow an early diagnosis and follow up of renal involvement in IgG4-RD are not available.

More investigations are needed to define biomarkers (urinary cells, urinary cytokines/mediators) able to correctly profile the type and extent of renal involvement in IgG4-RD.

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. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RC, DG, and PM conceived the work and wrote the paper. DG, DM, and ME were in charge of nephrological evaluation of the patients. DG performed renal biopsies. AB performed biopsies examination. CC and FP performed immunohistochemical and cytofluorimetric evaluations. RC, IP, and AT were in charge of the patients and collected clinical 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/fmed.2021.635706/full#supplementary-material

Supplementary Figure 1 | Immunofluorescence staining of Case # 2 kidney biopsy: few igG1+ cells were observed but no IgG2+ or IgG3+ cells.

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BRIEF RESEARCH REPORT
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Neutrophil Extracellular Traps in Systemic Lupus Erythematosus Stimulate IgG2 Production From B Lymphocytes

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Circulating autoantibodies of IgG2 isotype predominate in Systemic Lupus Erythematosus (SLE) and concur to the development of the renal lesions characteristic of Lupus Nephritis (LN). Anti-dsDNA and anti-histones IgG2, together with anti-podocyte proteins (i.e., α-enolase) are the major autoantibodies in serum and renal glomeruli of LN patients. The mechanisms underlying autoantibody formation and isotype switching in SLE and LN are unknown. A major issue is how DNA/histones are externalized from cell nucleus, driving the autoimmune response. Neutrophil Extracellular Traps (NETs) have been recently identified as crucial players in this context, representing the main source of DNA and nucleosome proteins. A second key point is what regulates IgG2 isotype switching: in mouse models, T-bet transcription factor has been described as essential for IgG2a class switch. We hypothesized that, in SLE, NET formation is the key mechanism responsible for externalization of autoantigens (i.e., dsDNA, histones 2,3, and α-enolase) and that T-bet is upregulated by NETs, driving, in this way, immunoglobulin class switch recombination (CSR), with production of IgG2 autoantibodies. The data here presented show that NETs, purified from SLE patients, stimulate *ex vivo* IgG2 isotype class switch possibly through the induction of T-bet. Of note, we observed a prominent effect of NETs on the release of soluble IgG2 in SLE patients', but not in healthy donors' B cells. Our results add important knowledge on the mechanisms of IgG2 class switch in SLE and contribute to further elucidate the role of NETs in LN pathogenesis.

Keywords: lupus nephritis, autoimmunity, NETosis, T-bet, IgG2, naïve B cells

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INTRODUCTION

Systemic Lupus Erythematosus (SLE) is an autoimmune disease characterized by heterogeneous clinical manifestations, varying from minimal symptoms, such as fever and small joint pain, to severe organ lesions (1). Lupus Nephritis (LN) is the most frequent and severe complication of SLE, occurring in almost 50% of cases and frequently leading to renal failure (2). It is characterized by antibody deposition in glomeruli, with typical patterns that vary from modest localization in mesangium to diffuse sub-epithelium deposition, with subsequent activation of a complement-mediated inflammatory cascade (3). The clinical association of different types of autoantibodies with LN has been extensively studied in the past (4); investigators have, in particular, focused on the correlation of renal lesions with specific autoantibodies, mainly anti-dsDNA, anti-nucleosome, and anti-histones (anti-H1, anti-H2A, and anti-H3) (5–10). More recently, the analysis of potentially pathogenic antibodies, micro-eluted from glomeruli, has been completed; beside confirming the glomerular localization of anti-dsDNA, anti-histones (H2A, H3, H4) and anti-C1q antibodies (11), the presence of antibodies against two cytoplasmic proteins, i.e., anti-α enolase and anti-AnnexinA1 (12), characterized by the prevalence of IgG2 isotype (13, 14), was also shown. For their predominance, IgG2 autoantibodies have been defined as "nephritogenic".

Two main issues involved in the generation of autoantibodies vs. intracellular/intranuclear antigens remain unclear: one concerns the way how antigens are externalized so as to trigger the autoimmune cascade, the second question concerns what regulates, in SLE patients, the production of IgG2, that usually is not the dominant subclass in the immune response. For several reasons, Neutrophil Extracellular Traps (NETs) have attracted the interest of researchers, since they may explain a part of these unresolved issues. NETosis is a sort of cellular death, in which DNA and histones are externalized from neutrophils, and form a sort of net where pathogens are entrapped and killed (15): a significant feature of NETs is the massive presence, beside DNA and histones, of α-enolase, the major autoantigen in SLE (16). Moreover, NETs are potent inducers of autoimmunity (17, 18) and stimulate both memory B cells to produce IgG autoantibodies and plasmacytoid dendritic cells (pDCs) to produce type 1 Interferon (19, 20). An autoreactive B cell population, overexpressing CD11c (an integrin involved in antigen presentation by B cells), is highly expanded in SLE patients and has been associated with renal disease severity (21, 22). Human $CD11c^{high}$ cells are able to differentiate *in vitro* into antibody secreting cells (ASC) and produce IgG upon stimulation with inflammatory cytokines (23). In murine models of Lupus, the transcription factor T-bet has been shown to be overexpressed in this B cell subset and it is essential for the production of pathogenic IgG2a (24, 25), associated with the development of lupus-like disease (26).

On the basis of the above findings and given the well-established role of IgG2 autoantibodies in mice and their exclusive presence in SLE and LN patients, we hypothesized that, also in humans, NETs might act directly on the differentiation of autoreactive B cells into IgG2 secreting cells. Considering the crucial role of T-bet in the early phases of autoimmunity, we also attempted to determine whether NETs could directly induce T-bet expression in human naïve B cells. In particular, we focused on the evaluation of the immunogenic activity of NETs isolated from LN patients, because of their unique proteomic composition.

MATERIALS AND METHODS

Blood Samples and Patients

Blood samples were obtained from 14 healthy donors (HDs) and 13 SLE patients. Patients 1–10 developed SLE at pediatric age and were on long term follow-up within Rheumatology Unit of Giannina Gaslini Institute, Genoa; patients 11–13 received SLE diagnosis at adult age and were on follow-up at S. Martino Hospital, Genoa. In both cases disease diagnosis was made according to the American College Rheumatology Systemic Lupus classification criteria as revised by the Systemic Lupus International Collaborating Clinics (SLICC).

Informed written consent was obtained from each individual before participation, upon approval of the local Ethic Committee. Clinical features are shown in **Supplementary Table 1**.

LN-NETs Preparation

Polymorphonuclear cells (PMNs) were isolated from fresh heparinized blood samples of patients with LN, using the Ficoll/Dextran separation method (14) and plated onto 24-well plastic dishes at the concentration of 3×10^6 cells/well. NETosis was induced by 30 nM Phorbol Myristate Acetate (PMA) stimulation, as previously described (27). Cells were then incubated with 15 U/ml Micrococcal DNase (Cayman, Ann Arbor, MI, USA) for 30 min at 37°C and the reaction was stopped by the addition of 5 mM EDTA. Samples were cleared from cellular debris by 13,000 rpm centrifugation. The molecular size of DNA fragments, obtained by DNase digestion, was assessed by 2% agarose gel electrophoresis. DNA and protein contents of NET extracts were determined by means of Nanodrop measurements and Bradford assay, respectively. Samples were then stored in aliquots at −80°C.

Purification of B Cells and NET Uptake Assessment

Peripheral blood mononuclear cells (PBMCs) were obtained from HDs buffy coats or from heparinized blood samples of Lupus patients, through Lymphoprep (Sentinel Diagnostic, Milan, Italy) density gradient centrifugation. B cells were purified from PBMCs by positive selection with CD19+ Microbead Isolation Kit (Miltenyi Biotec Bergish Gladbach, Germany). CD19+ cells were plated onto 48-well plastic dishes, at the concentration of 5×10^6 cells/well in RPMI 1640 medium (BioWhittaker, Lonza, Belgium), containing 10% Bovine Serum (FCS, Defined, Hyclone, Euroclone, Milan, Italy), 2 mM l-Glutamine, 10 mg/ml non-essential amino acids, 1 mM pyruvate, 50 U/ml penicillin, 50 U/ml streptomycin (Gibco, Grand Island, NY, USA), 5×10^{-2} M 2-mercaptoethanol (Sigma-Aldrich, St. Louis, USA) and supplemented with 4,000 U/ml Interleukin 2 (Proleukin, Chiron Corp., Emeryville, CA, USA),

2.5 μg/ml CpG2006 (5′-tcgtcgttttgtcgttttgtcgtt-3′; TIB Molbiol, Genoa, Italy) and 1.5 μg/ml F(ab')$_2$ anti human IgM, IgA, IgG (Jackson Immunoresearch Europe, West Grove, PA, USA). Cells were cultured overnight; NET extracts (3 μg/ml protein concentration) were added after 16 h and were incubated for 2 h at 37°C. Following 2 washes in PBS, cells were stained with FITC-conjugated anti-human CD20 monoclonal antibody (Miltenyi Biotec) and plated onto polylysine coated high-quality 96-well clear bottom black plates, suitable for confocal microscopy. After fixation and permeabilization with BD Cytofix Cytoperm Buffers (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions, cells were further stained with an Alexa 647-conjugated anti-human Myeloperoxidase (MPO) monoclonal antibody (Dako, Agilents Technologies, Santa Clara, CA, USA) that specifically evidenced the cellular binding and localization of NET fragments (25). Cells were then washed, and cell nuclei were counterstained with Hoechst 33342. Imaging was performed using Opera Phenix (PerkinElmer, Waltham, MA, USA) high-content screening system. Wells were imaged in confocal mode, using a 40X water-immersion objective. Alexa 647 signal was laser-excited at 640 nm and the emission wavelengths were collected between 650 and 760 nm. Excitation and emission wavelengths for visualization of Hoechst 33342 signal were 405 and between 435 and 480 nm, respectively.

In vitro Proliferation and Differentiation of Naïve B Cells in the Presence of NET

Naïve B cells were enriched by cell sorting (BD-FACSAria instrument, BD Biosciences, San Jose, CA, USA), following the staining with Pe-Cy7-conjugated anti-CD19, FITC-conjugated anti-CD24 and APC-conjugated anti-CD38 monoclonal antibodies (Biolegend, San Diego, CA, USA). To identify the population of interest, we used the gating strategy described in **Supplementary Figure 1**. The sorted naïve B cell population had a purity of around 99.5%. IgD and CD27 expression on our gated CD19+ CD24- CD38^{low} evidenced the presence of a minimal contamination of memory B cells (CD27+ IgD-) that were ranging from 6.6 to 11.4% (data not shown).

Enriched naïve B cells, from SLE patients and HDs, were labeled with 0.5 μM 5-(and-6-)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR,) for 8 min at room temperature, plated onto 96 multi-well plates and activated with the following stimuli: 2.5 μg/ml CpG2006, 5 μg/ml, F(ab')$_2$ anti human IgM, IgA, IgG (Jackson Immunoresearch Europe, West Grove, PA, USA) and 4,000 U/ml IL-2 (Proleukin, Chiron Corp., Emeryville, CA). LN-NET extracts were added to cultures on day 5, at the concentration of 0,5 μg/ml protein.

At day 7 cells were harvested, washed in PBS and labeled with CD19 PE-Cy7, CD27 APC-Cy7, and CD38 APC for 30 min at +4°C; proliferation rate (analyzed by CFSE diluition assay) and plasmablasts (CD27+, CD38^{high}) percentages were determined in viable (propidium iodide negative) CD19^{+} cells, using a BD-FACSCanto Flow Cytometer and Kaluza Software (Beckman Coulter Life Sciences, IN, USA).

In vitro Culture of Naïve B Cells and Determination of Soluble IgG Subclasses

Soluble IgG levels were determined, by ELISA assay (28), in culture supernatants collected at day 7 from enriched naïve B cells, in the same culture conditions described above. Briefly, Dynatech M129A ELISA plates (Corning Costar, Glendale, AZ, USA) were coated with 10 μg/ml goat anti-human IgG or anti-human IgG2 antibodies (Southern Biotech, Birmingham, AL, USA), diluted in 0.1 M Phosphate Buffer (pH 9.6), and left overnight at 4°C. Plates were then washed with PBS + 0.05% Tween and saturated for 2 h with PBS + 10% FCS at room temperature (RT). Serial dilutions of cell supernatants and of IgG isotype standards were incubated for 2 h at RT. Plates were washed four times with PBS + 0.05% Tween and incubated with 0.5 μg/ml sheep HRP-conjugated anti-human IgG1 or anti-IgG2 secondary antibodies for 2 h at RT. After 4 washes with PBS + 0.05% Tween, the reaction was developed with 75 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, St. Louis, MO, USA) and it was stopped after ∼5 min by 2 N Sulfuric Acid. Absorbance values were read at 450 nm using a microplate reader (Eppendorf, Milan, Italy).

Analysis of Intracellular T-Bet Expression

Ex vivo T-bet expression was evaluated on CD19+ cells, obtained from previously isolated frozen PBMCs of 4 healthy controls and 3 patients with LN. Cells were stained with FITC-conjugated Live/Dead Fixable Dye (Molecular Probes, Eugene, OR, USA) for 15 min at 4°C in the dark, and subsequently labeled with PE-Cy7-conjugated anti-human CD19 Antibody (Biolegend). After fixation and permeabilization, cells were stained with PerCP-Cy5.5-conjugated anti-human T-bet (Thermofisher, MA, USA) for 30 min at RT. Intracellular T-bet expression was measured in alive cells (detected as FITC^{dim}) by flow cytometry, using a BD-FACS Canto instrument, and it was analyzed with Kaluza. The effect of LN-NETs on T-bet expression was evaluated in enriched naïve B lymphocytes, derived from HDs. Cells were cultured in complete medium and stimulated with 2.5 μg/ml CpG2006 and 1.5 μg/ml F(ab')$_2$ anti human IgM, IgA, IgG, or only with NET extracts, for up to 48 h, T-bet expression was detected after 48 h stimulation (T48) and compared with the baseline (T0), prior the addition of stimuli. Negative isotype controls were also included in each experiment.

Software and Statistics

For statistical analysis, GraphPad Prism (Graph Pad software, La Jolla, CA, USA.) software was used. Mann-Whitney U test was used for comparing groups. Statistical significance was defined as P-value < 0.05.

RESULTS

Among 13 SLE patients, enrolled in this study, all except one developed LN during their clinical course. Patients treated with

B cell depletion therapy were excluded. At the time of their recruitment 3 patients showed no disease activity (SLEDAI = 0) on low dose glucocorticoids, hydroxychloroquine and mofetil mycophenolate, 6 showed only minimal activity (SLEDAI = 1–5) and the remaining 4 presented a moderate to very high disease activity.

Uptake of LN-NET by Human B Lymphocytes

In order to assess if NETs are internalized by B lymphocytes, that is a prerequisite for activating intracellular signaling pathways, CD20+ B cells, cultured in the presence of anti-Ig and CpG2006 were exposed to NET fragments, obtained by PMA-stimulated neutrophils. As assessed by agarose gel electrophoresis, the molecular size of NET fragments ranged from 100 to 200 kB (**Supplementary Figure 2**), accordingly to previous observations (20). Myeloperoxidase staining was used to visualize NETs (28). As shown in **Figure 1**, NETs uptake was evidenced by the presence of intracellular small granules, detected only in viable CD20+ B cells: this pattern was not observed in CD20+ B cells, that were not exposed to NETs.

LN-NET Does Not Alter "*in vitro*" Proliferation Nor Differentiation of Naïve B Cells

In a preliminary context, we tested the ability of our enriched HD and SLE naïve B cells to proliferate and differentiate into plasmablasts, in response to canonical agents, that are known to trigger class switch recombination (CSR); in parallel, we evaluated the influence of LN-NET exposition on naïve B cells activation. Flow cytometric analysis revealed that, following stimuli, enriched naïve B cells significantly proliferate and differentiate into CD27+ $CD38^{high}$ plasmablasts; the rate of cell proliferation and the percentage of CD27+ $CD38^{high}$ cells were not modified by NET addition (**Supplementary Figure 3**).

LN-NET Stimulates IgG2 Production in SLE Naïve B Cells

We analyzed the effect of LN-NET on different immunoglobulin production from enriched, stimulated naïve B cells. We found that soluble IgG2, after LN-NET addition, were significantly increased in SLE naïve B cells supernatants; conversely, this increment was not observed for IgG1 and IgG3 (**Figure 2A**), as well as IgM and IgA levels (**Figure 2B**), neither in SLE, nor in normal B cells. We also did not detect a significant increase in IgG2 production by normal naïve B cells, following NET exposition: therefore, the ability of LN-NET to raise soluble IgG2 levels appears to be specific for SLE B cells, By analyzing the responsiveness of individual patients, we did not observe a significant correlation with disease severity (evaluated by SLEDAI score) on IgG2 induction by LN-NET (**Supplementary Table 1**): for this reason, it is conceivable that LN-NETs exert a pathogenic function in the early phases of autoimmunity, irrespectively of the disease evolution.

T-Bet Expression Is Stimulated by LN-NET

Since T-bet is a master regulator of autoreactive B cell differentiation in murine models and is able to stimulate IgG2 class switch (24), we investigated whether its constitutive expression is altered in SLE patients. We analyzed the amount of CD19+ B cells expressing T-bet, that reached a maximum level in SLE patients (>90% of T-bet expressing cells) and was higher than normal controls (**Figures 3A,B**). We postulated that the elevated T-bet levels, observed in SLE patients, could result from the *in vivo* chronic stimulation of NET and hence we investigated if normal naïve B cells might express T-bet protein to the same extent as SLE B cells, when exposed to LN-NET. We therefore tested the expression of T-bet, before (T0) and after 48 h (T48) of NET stimulation; as a positive control, cells were stimulated with CpG2006 and anti-Ig, powerful inducers of T-bet expression. The percentage of T-bet expressing cells was not elevated in normal controls (**Figure 3C**; mean = 31.18%); as expected, CpG2006 and anti-Ig induced a robust proliferation coupled with high increase in T-bet expression (mean: 89.9%; $p = 0.0076$). Notably, the addition of NETs from LN patients was sufficient to markedly increase the number of T-bet expressing cells (mean = 73.4%; $p = 0.08$), despite a small effect on cellular proliferation (**Figures 3C,D**). Consistently, the amount of T-bet protein, measured by Mean Fluorescence Intensity (MFI) detection, was significantly ($p = 0.0002$) induced after stimulation with LN-NET and showed a 30% increase compared to its baseline (T0) expression (**Figures 3E,F**).

Taken together, these results show that NET from LN patients exerts a direct and specific effect on IgG2 isotype switch in SLE naïve B cells, and also point to T-bet as a putative mediator in this mechanism.

DISCUSSION

The first finding of this study is the significant increase in IgG2 production, observed in enriched naïve B cells, stimulated with NETs: this effect is selective for IgG2, since other immunoglobulin isotypes are not significantly altered in the same experimental conditions; it is also specific for SLE, as B cells, isolated from normal donors, are unresponsive to NET stimulation. These observations extend the previous findings by Gestermann et al. (20), who showed upregulation of total IgG by memory B cells, after exposure to NETs.

This finding contributes to explain the IgG2 isotype prevalence in autoantibodies of SLE and, in particular, of Lupus Nephritis patients, and propose NET as a culprit in these conditions.

The exclusive responsiveness of SLE patients also suggests that a pre-existing condition predisposing to autoimmunity is necessary to amplify the effects of NET on IgG2 production: in this regard, recent investigations highlight an epigenetic priming in naïve B cells of SLE patients, with respect to their counterparts in healthy donors (29).

Our results, on the other hand, indicate that NET does not come into play in the first phases of class switch recombination (CSR), characterized by cellular proliferation and generation of

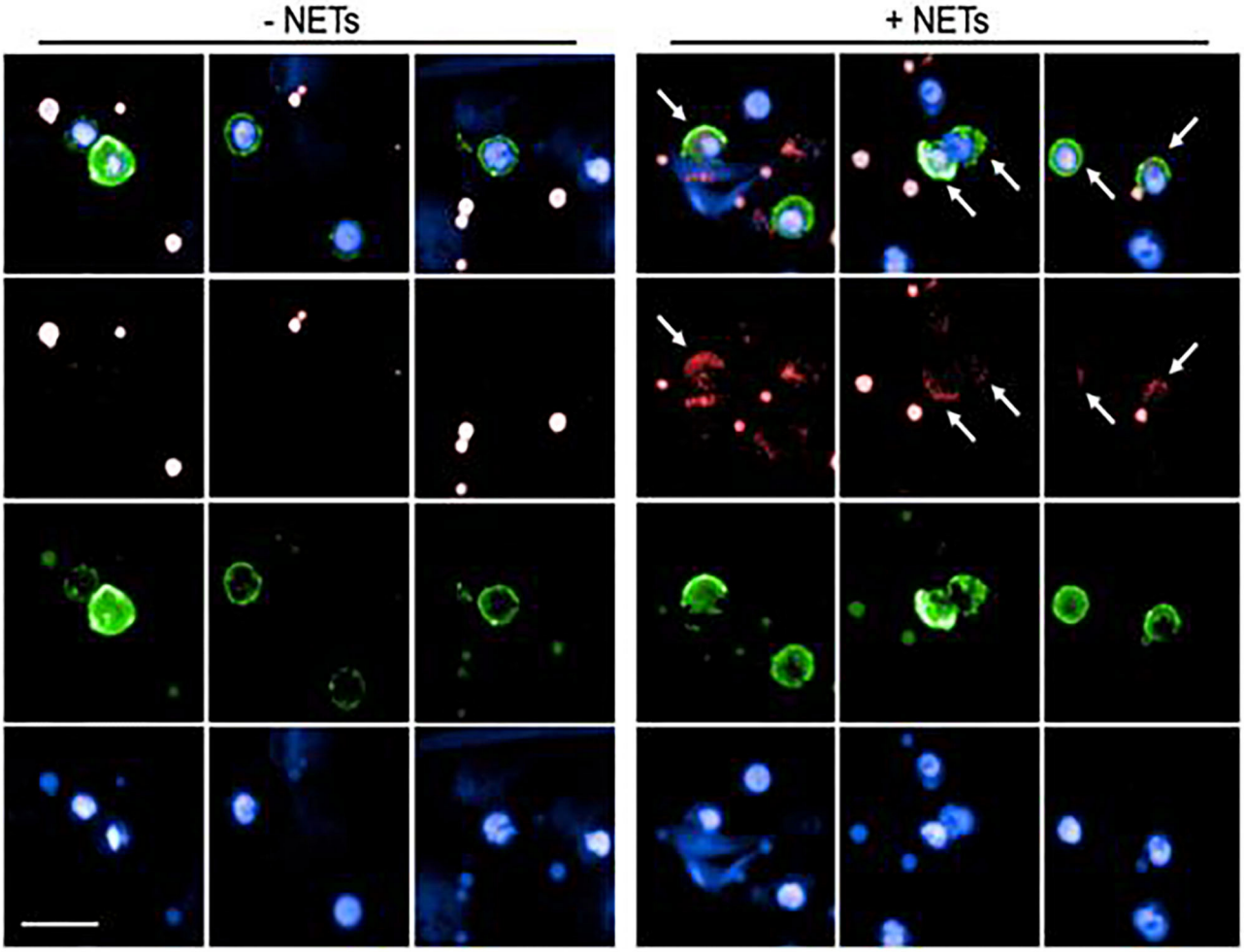

FIGURE 1 | Analysis of NET uptake by B lymphocytes. Representative images of B lymphocytes in the presence (right panel) or absence (left panel) of NET extracts. Surface green staining identifies viable CD20+ B cells. Nuclei were counterstained with Hoechst 33342 (blue staining). NETs were labeled with Alexa 647 conjugated anti-Myeloperoxidase (red staining). Both in the left (untreated cells) and in the right (NET-treated cells) panels, red dots are visible, due to aspecific anti-MPO staining in dead cells. Viable cells, characterized by the integrity of cell membrane (green stain) were analyzed for the presence of small red spots (detectable only in NET-treated cells), indicating specific MPO-staining, and demonstrating the intracellular presence of NET fragments. Arrows indicate the presence of internalized NET fragments.

plasmablasts, and it is rather conceivable that NETs activate subsequent steps that are crucial for the preferential skewing of defined IgG subclasses: in this respect NETs would interact and cooperate with specific cytokines and transcriptional factors, able to tailor a context-specific immune response (30).

We therefore focused on T-bet transcription factor, as a potential key mediator in the IgG2 induction, elicited by NETs. Experimental murine models previously clarified the role of T-bet in IgG2a production (31, 32); moreover, T-bet is the hallmark of the potentially autoreactive B lymphocyte compartment, referred as "atypical memory" (22) or "naïve activated" (33) cells, highly expanded in active SLE patients with LN. In this cell subset, T-bet expression is induced by inflammatory Th1 cytokines and, interestingly, this occurs prior to their differentiation into Antibody-Secreting Cells (22). We postulated that NETs accumulation in SLE (34) could create an inflammatory milieu (35), that would drive the formation of an autoreactive, T-bet^{high} B cell pool, prone to generate IgG2 autoantibodies: this idea seems to find a confirmation in the overexpression of T-bet, that we observed in B lymphocytes of SLE patients. A further evidence about a link between NET and T-bet derives from our results from normal enriched naïve B cells, that constitutively express T-bet from low to moderate levels: indeed, we demonstrated that, in the absence of canonical inducing factors, separately evaluated as positive controls, lupus NET fosters T-bet expression.

This is the first observation concerning the direct ability of NETs to upregulate T-bet in human naïve B cells. Given the multiple functions orchestrated by T-bet in the immune response (36), this finding strengthens the concept of NET as a crucial player in the development of SLE auto-reactivity. T-bet is indeed expressed by a wide number of immune cells and is critical for the differentiation of professional, tissue-resident memory

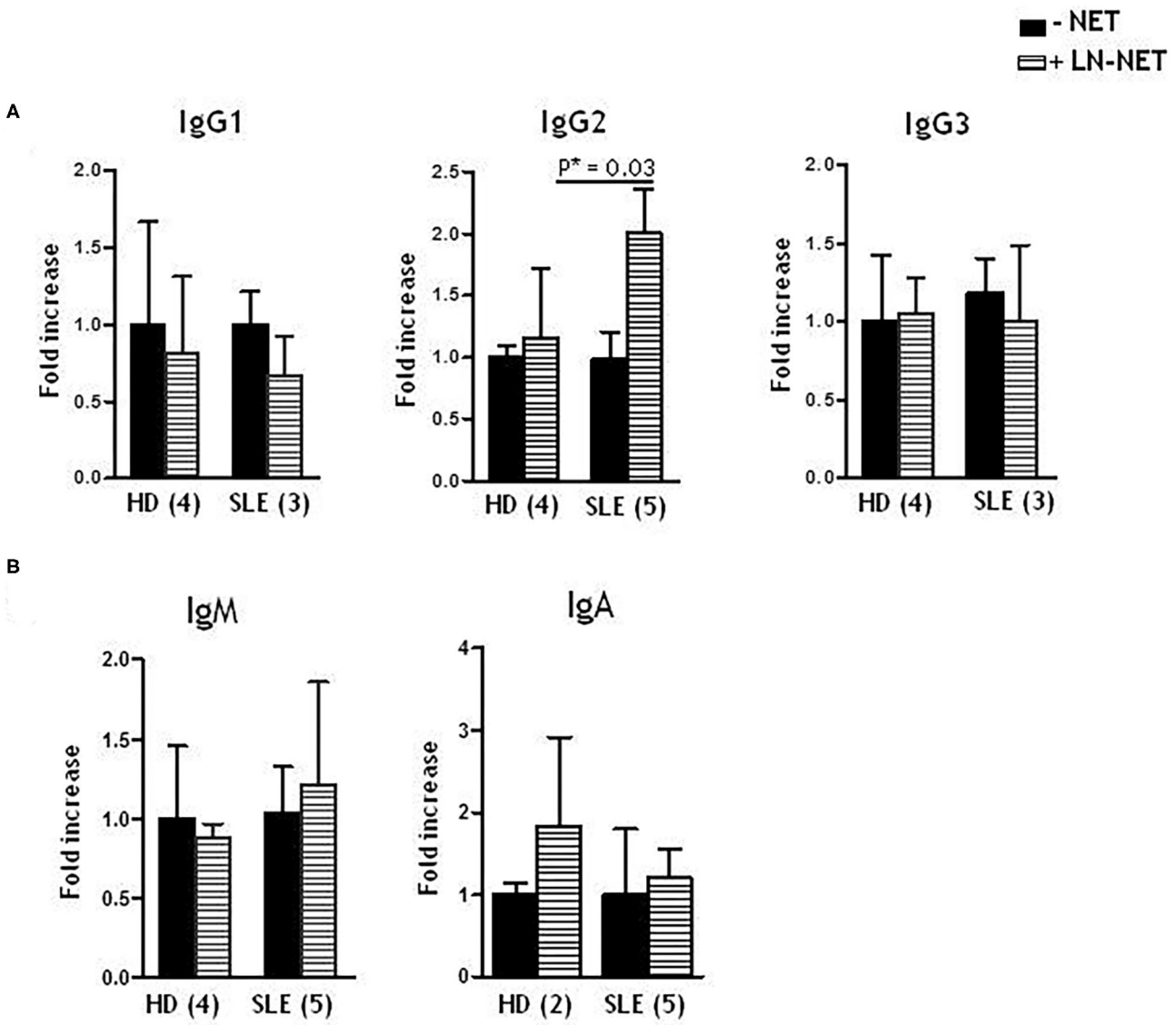

FIGURE 2 | Effect of NET on soluble IgG isotypes, IgM and IgA production. Naïve B cells, derived from healthy donors (HD) or SLE patients, were cultured as described in Materials and Methods. Where indicated (dashed bars), NET extracts were added after 5 days of culture. Immunoglobulins levels were determined by ELISA assay on cell supernatants, collected after 7 days of culture. Values indicate the fold increment of IgG1, IgG2, IgG3 **(A)**, IgM, IgA **(B)**, elicited by NET treatment, vs. untreated cells, and represent the mean of independent experiments indicated in figure.

T cells, as well as T follicular helper (Tfh) and Th17 cells. In B lymphocytes, the cooperation of T-bet with an additional transcription factor, Bcl-6, enables the formation and survival of autoreactive germinal centers (GCs), that are specialized sites for the selection of high affinity-responsive B cells (26, 31). Therefore, T-bet could play an important role in the relocation of cells from secondary lymphoid organs to ectopic GCs, able to generate autoreactive plasmablasts.

Besides these direct effects, T-bet mediates the expression of CXCR3, a specific homing chemokine receptor responsible for the migration to inflamed tissues of T and B lymphocytes, and thus promotes the co-localization of multiple cell types, with different effector functions, in target organs (37). Particularly, in the kidney, during LN development, the migration of CD4+, CD8+, and IL17-producing T cells drives the formation of an autocrine, inflammatory compartment. Notably, polymorphonuclear cells may also be recruited in the inflamed kidney and pushed to undergo NETosis: this creates a vicious cycle, responsible for the uncontrolled amplification of the autoimmune response (38).

Further investigations are required to identify the mechanisms triggered by NET and involved in T-bet induction. T-bet is known to be upregulated in mice by hypomethylated CpG through TLR9 ligation (39): nonetheless, owing to the low expression of this receptor in naïve B cells (40), we can hypothesize that BCR could exert a predominant function in

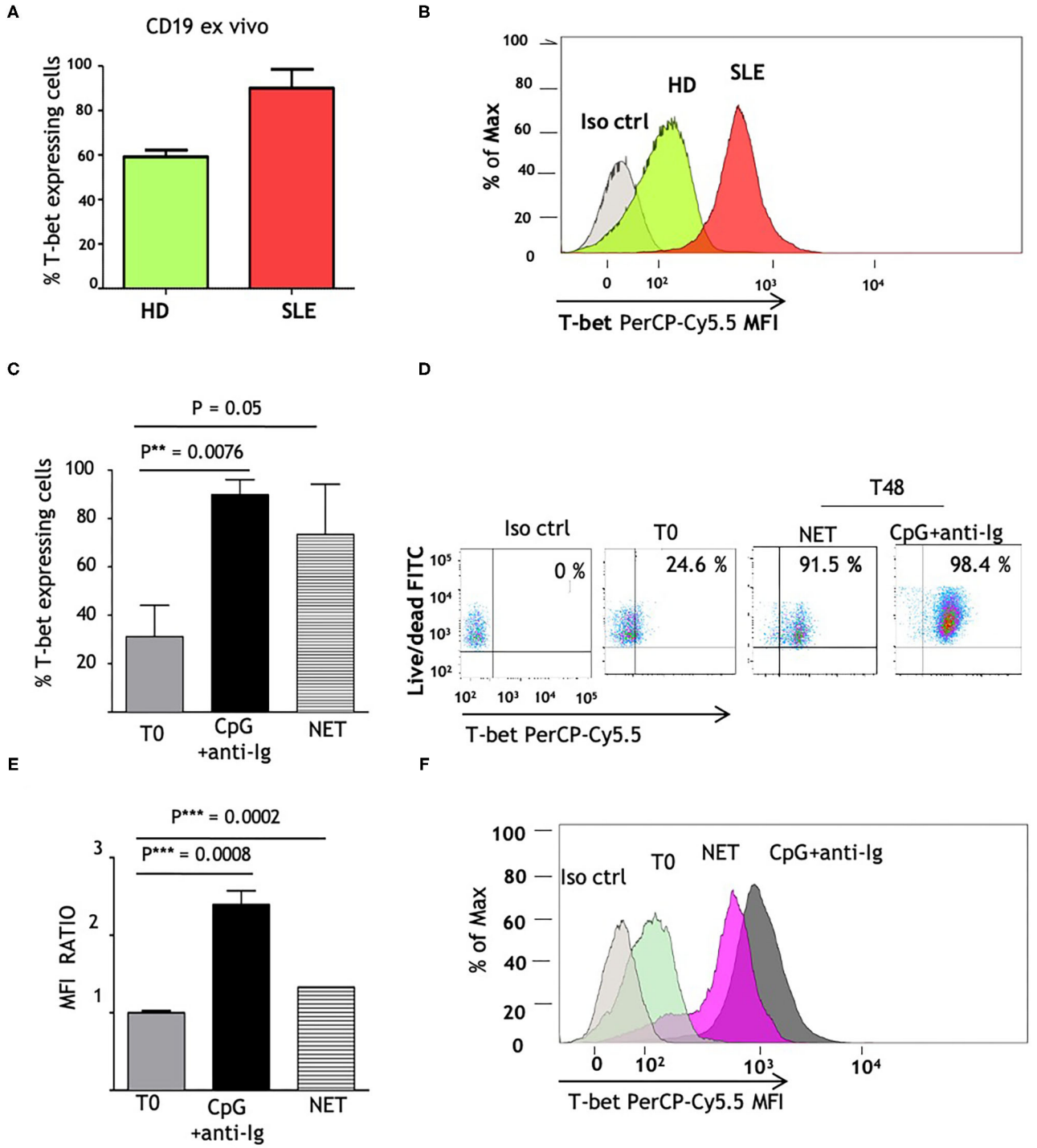

FIGURE 3 | NET induces T-bet expression in human naïve B cells. **(A)** Comparison of *ex vivo* T-bet expression in B lymphocytes, isolated from normal (HD) vs. SLE patients. CD19+ cells were purified from frozen PBMCs samples, as described in section Materials and Methods. Intracellular PerCp Cy5.5 fluorescence (T-bet expression) was assessed by flow cytometry. Values indicate the percentages of PerCp Cy5.5 positive (T-bet expressing) cells, determined in a in gated viable ($FITC^{dim}$) cell population, and represent the mean of three independent experiments. **(B)** Representative overlay histogram depicting PerCp-Cy5.5 Geometric MFI in T-bet expressing cells, detected in one normal control and in one patient with SLE. **(C)** Percentages of T-bet expression in freshly isolated naïve B cells, obtained from healthy donors as described in Materials and Methods, before (T0) and after 48 h stimulation (T48) with CpG+anti-Ig (solid bar), or with LN-NET (dashed bar). NET extracts were added at the concentration of 0,5 μg/ml protein T-bet expressing cells percentages were determined by flow cytometry as described above. The values represent the mean of three independent experiments.

(Continued)

FIGURE 3 | (D) Representative dot plot of T-bet expression in viable naïve B cells from a healthy donor, in the absence (T0) or presence of different stimuli (as indicated). Upper right quadrants in the graph display T-bet expressing cells. **(E)** Determination of T-bet expression, measured by Geometric Mean Fluorescence Intensity (MFI). Values indicate the fold increment of fluorescence in normal naïve B cells, stimulated for 48 h (T48) with CpG+anti-Ig (solid bar), or with LN-NET (dashed bar), vs. unstimulated (T0) cells, and represent the mean of three independent experiments. **(F)** Representative overlay histogram depicting T-bet expression in B lymphocytes cultured in absence (T0) or presence of indicated stimuli.

NET signaling. BCR engagement acts in synergism with TLR9 (41, 42) and, intriguingly, its ligation increases TLR9 expression in naïve B cells (43). More studies are necessary to clarify this point. Additional factors, including type I IFN, IRF5 (44, 45), or MyD88-related pathways (46), that could cooperate with NET in T-bet induction, should also be considered. In particular, the identification of signals that might be specifically activated by the peculiar protein components of LN-NETs is still lacking and represents an exciting challenge.

Moreover, the mechanisms underlying the hyperresponsiveness of SLE naïve B cells to NET need to be examined in depth. In this regard, it is known that, besides T-bet, other transcription factors are implicated in ASC differentiation (47), and their relative concentration is thought to influence significantly B cell fate in different physiological or pathological conditions. Regulatory factors antagonizing T-bet, such as the zinc finger transcription factor Ikaros (48) and c-Myb (49), could have a substantial function in healthy subjects and might justify the negative effect of NETs in normal B cells.

In conclusion, further research is needed to better define the role of T-bet in the complex network that tightly regulates CSR and IgG isotype switch (42). Our work reinforces the key role of NET in autoimmunity and propose NETosis as a target for new therapeutic protocols (50), in the treatment of SLE and Lupus Nephritis.

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 Internal Ethic Committee, IRCCS Giannina Gaslini Institute. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RB: design of the study, most of experiments and analysis, data interpretation, manuscript writing, and final approval. FS: experiments and data analysis, data interpretation, manuscript writing, and final approval. SS: coordination of patients' samples collection, clinical data collection, and elaboration of manuscript. NP: confocal analysis. FA and DR: cell sorting experiments. AC, SN, and FP: coordination of patients' samples collection, provision of patients' material, and clinical data collection. MG: provision of patients' material, financial support, elaboration of the manuscript, and final approval. SV: design of the study, data interpretation, financial support, critical revision of the manuscript, and final approval of manuscript. GMG: design of the study, data interpretation, financial support, critical revision of the manuscript, and final approval of manuscript.

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SUPPLEMENTARY MATERIAL

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frontiers in Medicine

MINI REVIEW
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Successful Treatment of AA Amyloidosis in Ankylosing Spondylitis Using Tocilizumab: Report of Two Cases and Review of the Literature

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Historically, secondary amyloidosis has been a feared complication of chronic inflammatory conditions. The fibril protein AA derives from the acute phase reactant serum amyloid A (SAA). Long-term elevation of SAA levels remains a major risk factor for the development of AA amyloidosis in rheumatic diseases, and the prognosis may be unpredictable. Nowadays, with increased availability of effective biological agents, the incidence of AA amyloidosis seems to be declining. Still, genetically predisposed subjects with slowly progressive disease and mild symptoms combined with ongoing systemic inflammation may be at risk. Interleukin-6 (IL-6) is one of the drivers of SAA release and effectiveness of the humanized anti-IL-6 receptor antibody tocilizumab (TCZ) for the treatment of AA amyloidosis has been observed in some rheumatic conditions. Herein, we report two male subjects with longstanding ankylosing spondylitis (AS) complicated by renal amyloidosis who received TCZ with rapid and beneficial effects regarding inflammation and proteinuria. To the best of our knowledge, the use of TCZ in AS patients with this extra-articular manifestation has not previously been described. The paper includes histopathology, clinical follow-up, and longitudinal data of the two cases along with a comprehensive review of relevant literature. Mechanisms behind amyloid-mediated tissue damage and organ dysfunction are discussed. Altogether, our data highlight that blocking IL-6 signaling may represent a promising therapeutic option in patients with renal AA amyloidosis.

Keywords: amyloidosis—diagnosis, ankylosing spondylitis, interleukin-6, nephropathy, proteinuria—nephrotic syndrome, tocilizumab

INTRODUCTION

Systemic amyloidosis secondary to chronic inflammatory diseases (AA amyloidosis) was once a feared and common complication in rheumatoid arthritis (RA), ankylosing spondylitis (AS), inflammatory bowel disease (IBD), and autoinflammatory conditions (1). A large number of different types of amyloidosis exists, but the main subtypes are primary AL amyloidosis (light chains), secondary amyloid A (AA) amyloidosis, familial amyloidosis, and

β_2-microglobulin-related amyloidosis. The diagnosis of amyloidosis is based on clinical organ involvement and histological evidence of target organ showing deposition of abnormally folded proteins leading to organ dysfunction. Amyloid deposits are formed from globular, soluble proteins, which undergo misfolding and, subsequently, aggregate into insoluble fibrils or proteins may also have an intrinsic tendency to form amyloid in the absence of misfolding (2). Resistance to catabolism results in progressive tissue amyloid accumulation. Congo red is considered the gold standard dye owing to its higher sensitivity and specificity when differentiating amyloid from other protein deposits (3).

AA amyloidosis is characterized by the extracellular tissue deposition of fibrils that are composed of fragments of and/or intact serum amyloid A protein (SAA), a hepatic acute phase reactant (4, 5). Apart from the kidneys, which is the organ most commonly affected by systemic amyloidosis, involvement of the heart, the liver, the gastrointestinal tract and the peripheral nervous system should be considered. Thus, presence of proteinuria but also malabsorption, intestinal pseudo-obstruction, hepatomegaly, polyneuropathy, and restrictive myocarditis should raise the suspicion of AA amyloidosis in subjects with chronic inflammatory diseases (2).

The optimal treatment strategy of AA amyloidosis includes control of the underlying inflammatory disease and complete suppression of SAA production (5). Similarly to C-reactive protein (CRP), SAA constitutes an acute-phase reactant synthesized by hepatocytes but also by other cells, including

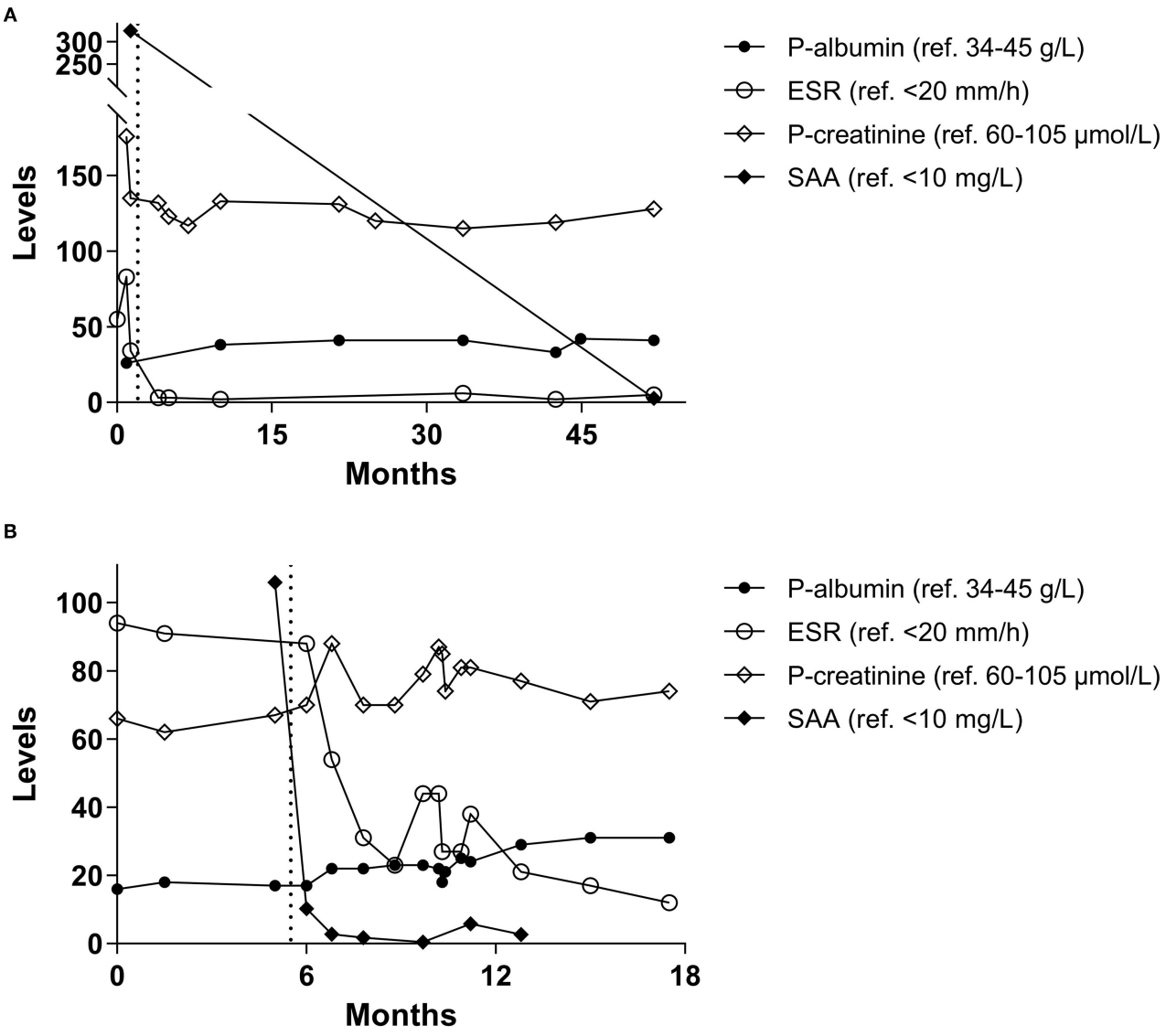

FIGURE 1 | (A) Longitudinal laboratory data from the first clinical case with albumin and creatinine in plasma (P–), erythrocyte sedimentation rate (ESR), and serum amyloid A (SAA). **(B)** Longitudinal laboratory data from the second clinical case. Months indicate time since onset of proteinuria and the dotted line illustrates time-point of first tocilizumab administration.

macrophages, endothelial cells, and smooth muscle cells, under the transcriptional regulation of proinflammatory cytokines, particularly tumor necrosis factor (TNF) alpha, interleukin-1 (IL-1) beta, and IL-6 (4, 6, 7). Before the era of anti-cytokine targeted therapies, conventional synthetic Disease Modifying Anti-Rheumatic Drugs (DMARDs) such as azathioprine, chlorambucil, cyclophosphamide, and methotrexate were frequently used to avoid heavy proteinuria and subsequent renal failure in patients with AA amyloidosis (1). In recent years, TNF-blocking agents have been shown to reduce the risk of development of AA amyloidosis, as well as to improve the renal outcome of AA amyloidosis, in patients with inflammatory arthritides (8–11).

Although disruption of the tissue architecture is an established signum of amyloidsis, the histological and biochemical pathways leading to renal damage are less well-understood. Some observations indicate that amyloidogenic precursor proteins, folding intermediates and protofilaments possess toxicities that are independent of the amyloid deposits and that these toxicities contribute to organ damage (12). Furthermore, the amyloidogenic precursors appear to be toxic to cultured cells and tissues (13). In line with this, there is a lack of correlation between the quantity of amyloid in tissue and organ dysfunction (14, 15). However, divergent associations between the amount of amyloid deposits in kidney biopsy specimens and renal function have been reported (16–18).

Improved understanding of the mechanisms underlying amyloid deposition has enabled the development of new treatment strategies, specifically those targeting formation of amyloid proteins. As IL-6 is one of the important drivers of SAA release, it is conceivable to consider blocking of IL-6 signaling in AA amyloidosis (19). Indeed, effectiveness of the humanized anti-IL-6 receptor antibody (tocilizumab) for the treatment of AA amyloidosis in RA and juvenile idiopathic arthritis has been reported (20). However, to our knowledge, reports on IL-6 receptor blockade in patients with AS and secondary amyloidosis are scarce (21). Herein, we report two cases with longstanding AS who eventually developed AA amyloidosis and had a rapid renal improvement as a response to tocilizumab (TCZ). Informed consent was obtained from both patients.

CLINICAL CASES

First Clinical Case

The first subject is a male Kurdish–Iranian non-smoking patient who was diagnosed with AS in Tehran 1983 based on radiological findings and inflammatory back pain at the age of 28. After emigration to Sweden, he has been monitored at our unit since the age of 34. Despite slightly elevated levels of CRP, the AS gave only mild axial symptoms that only required continuous use of non-steroidal anti-inflammatory drugs (NSAIDs) and physiotherapy for many years combined with occasional use of glucocorticoids and analgesics during short periods. He acquired hypertension in his 50's, but the blood pressure is now well-controlled with metoprolol. At the age of 60, he was referred from his general practitioner to the hospital due to raised CRP and plasma creatinine (176 μmol/L), combined with low plasma albumin (22 g/L) and significant proteinuria (24-h protein excretion 0.52 g).

TABLE 1 | Histopathological analysis with scoring of amyloid deposition according to Hopfer et al. (22).

	Case 1	Case 2
Age	61	68
Glomeruli	5	14
• Global sclerosis (%)	2 (40)	3 (21)
• Amyloid (%)	5	10–40
• Stage	1	2
Interstitium		
• Interstitial amyloid (%)	40 (mainly medulla)	<1
• Fibrosis (%)	30	10
Vasculature	Transmural	Focal
Dominant pattern	Vascular and interstitial	Glomerular
Type	AA	AA

Renal biopsy was performed. The histopathology was compatible with minimal glomerular amyloidosis stage 1A and interstitial amyloidosis, mainly in the medulla. Circulating levels of SAA were impressively high (325 mg/L). Infliximab (300 mg per infusion) was initiated without DMARD background, but the patient experienced an allergic reaction during the second infusion at our day care unit. Thereafter, monthly infusions of TCZ (480 mg per infusion, corresponding to 8 mg/kg) was started in monotherapy. After 3½ years, the TCZ infusions were replaced by weekly subcutaneous TCZ injections (162 mg) which are still ongoing without any reported side effects. Nevertheless, NSAIDs and analgesics are frequently required to manage the musculoskeletal symptoms. **Figure 1A** includes laboratory data of the first 52 months since the onset of proteinuria.

Second Clinical Case

The second subject is a male Caucasian tobacco-smoking patient who was diagnosed with AS in 1984 at our unit. The diagnosis was based on radiological findings, although the inflammatory back pain had started already 1975 at the age of 34. During the 90's, he developed uveitis and Crohn's disease. The AS gave rather mild axial symptoms, but erythrocyte sedimentation rate (ESR) and CRP levels were constantly elevated. Whether the laboratory findings were attributed to the gut or the spine remained unclear. Sulfasalazine was tested during a short period but ceased early due to nausea. His IBD was treated with mesalazine and glucocorticoids. As he developed more symptoms from the spine combined with polymyalgia, subcutaneous injections with adalimumab 40 mg twice a month was added during a 6-month period but unfortunately without any relieve of musculoskeletal symptoms.

At the annual visit to rheumatologist in 2019, the patient presented with nephrotic syndrome based on proteinuria (urine albumin-to-creatinine ratio 913 g/mol; reference <3.0 g/mol) and low plasma albumin (16 g/L) whereas renal function apparently was preserved (estimated glomerular filtration rate based on plasma creatinine according to MDRD: >90

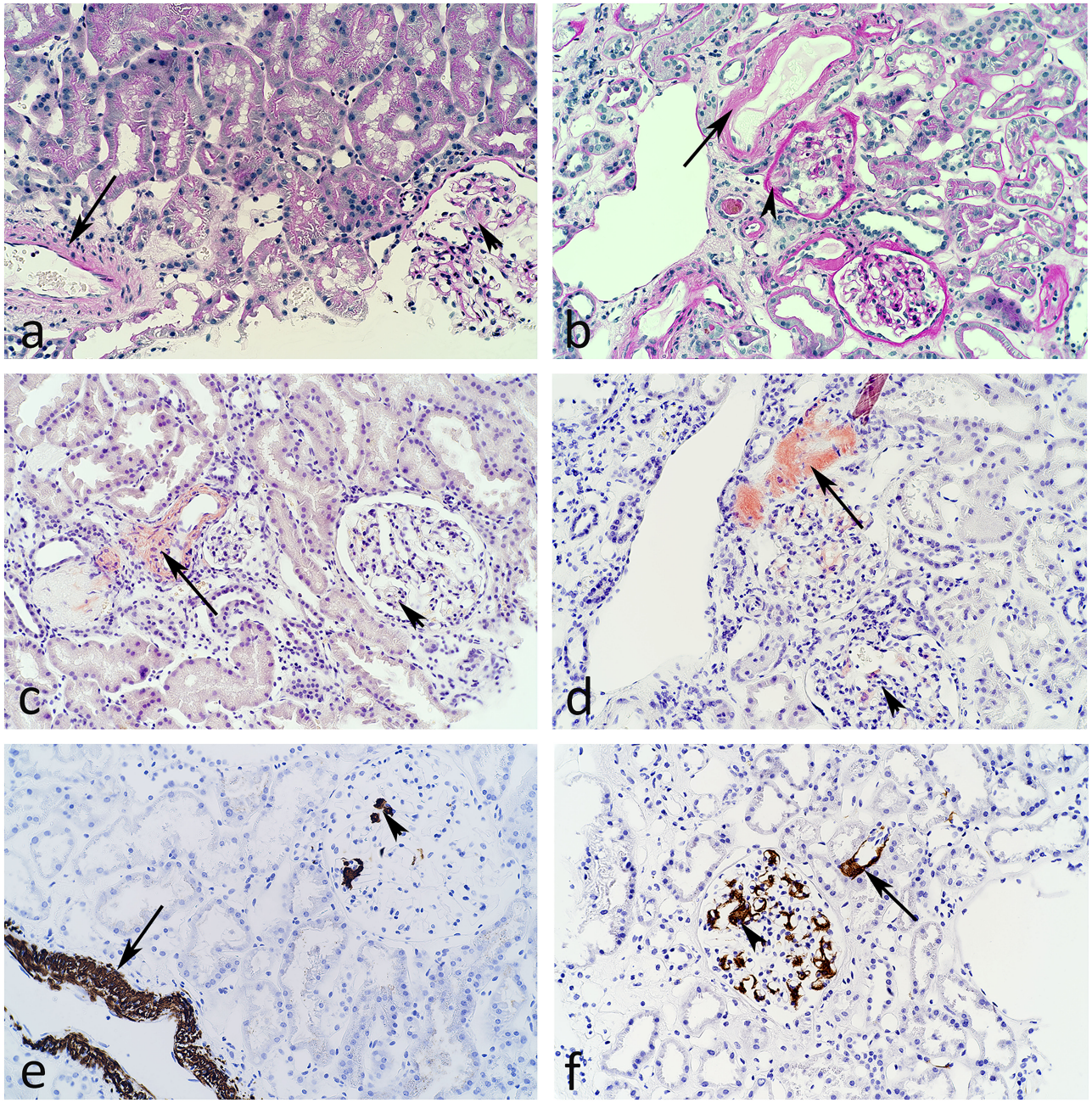

FIGURE 2 | Light microscopic images from the first **(a,c,e)** and the second **(b,d,f)** clinical case described. PAS stain **(a,b)** indicates deposition of minimal amorphous material in arteries (arrows) and a glomerulus (arrowhead) in panel **(b)**. Congo red stain **(c,d)** reveal amyloid in arteries (arrows) and focally in glomeruli (arrowhead). In addition, polarized light showed birefringence. Using antibodies against serum amyloid A (SAA) and immunoperoxidase staining **(e,f)**, the amyloid is identified as of SAA-type in glomeruli, arterioli, arteries, and interstitium.

mL/min/1.73 m^2). Renal histopathology was compatible with glomerular amyloidosis stage 1A and circulating levels of SAA were clearly elevated (106 mg/L).

Encouraged by our previous positive experience with IL-6 receptor blockade in AA amyloidosis, the patient was prescribed subcutaneous injections with TCZ (162 mg per week). Apart from an episode of fungal infection with esophagitis, which required hospitalization, TCZ has been well tolerated. The IBD had been quiescent for many years before the introduction of TCZ, precluding the possibility to judge any effect of TCZ on the intestine. However, the polymyalgia has almost disappeared according to the patient and no additional episodes of uveitis have been recorded. The patient is still prescribed TCZ weekly. **Figure 1B** includes

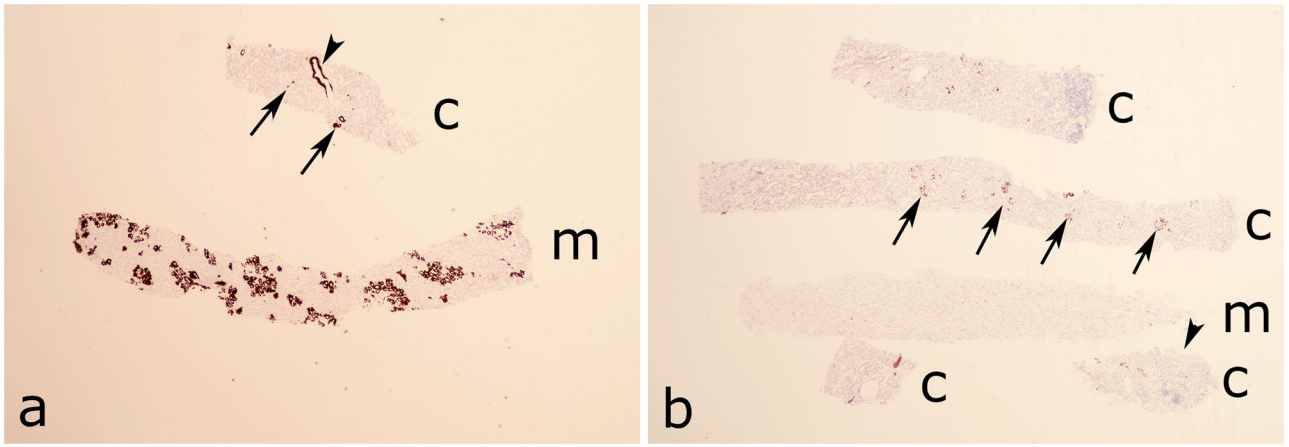

FIGURE 3 | Overview micrograph with serum amyloid A (SAA) staining of the two biopsies. **(a)** In case 1 the amyloid is mainly localized in the medulla and focally in glomeruli and vessels. Cortex is sparse. **(b)** In case 2 the amyloid is focally distributed in glomeruli and negative in an artery and the interstitium. Arrows indicates glomeruli, arrowheads arteries. c, cortex; m, medulla.

laboratory data of the first 18 months since the onset of proteinuria.

Histopathology

Renal biopsies were fixed in buffered paraformaldehyde, embedded in paraffin, sectioned, and stained with PAS, trichrome, htx-eosin, Ag-Jones, elastin/Van Gieson, and Congo red. Routine immunoperoxidase staining for IgG, IgA, IgM, C1q, C3c, C5b−9, and light chains were negative. Due to Congo red positivity, additional staining for SAA was performed. **Table 1** summarizes and classifies the morphological findings and amyloid deposits according to Hopfer et al. (22).

The biopsy from case 1 was sparse (11 mm) and contained mainly medulla. Two of five glomeruli were globally sclerosed and there were sparse, focal deposits of amyloid, mainly in the mesangium (**Figure 2a**). In the vessels, amyloid was found in both arteries and arterioli (**Figure 2c**). No amyloid was found in the cortex while large, focal amount of amyloid was found in the medulla (**Figure 2a**). SAA was positive in all Congo red deposits (**Figure 2e**).

The biopsy from case 2 was larger (total 23 mm) and contained mainly cortex. Three of fourteen glomeruli were globally sclerosed and glomeruli contained several foci of amyloid, mainly in the mesangium (**Figure 2b**). In the vessels, amyloid was detected in some but not all arterioles and arteries (**Figure 2d**). Limited amount of amyloid was observed in the interstitium.

SAA was positive in all Congo red deposits (**Figure 2f**) and an overview of the SAA positive staining is illustrated in **Figure 3**.

DISCUSSION

The last decade's widespread availability of highly efficacious biological agents for the treatment of rheumatological and other inflammatory disorders has not only resulted in fewer patients developing amyloidosis but also in improved overall survival (23, 24). Based on data from the Swedish Hospital Discharge Register and the Outpatients Register 2001–2008, AA amyloidosis among patients with RA had an estimated annual incidence of 2 per million (25). Yet, European data from the last two decades show that substantially younger individuals are nowadays diagnosed with AA amyloidosis, possibly indicating an increased awareness among clinicians (26, 27).

A nationwide, register-based study from Sweden demonstrated a prevalence of clinically diagnosed AS of 0.18% in 2009 with some phenotypical and treatment-related sex and socio-economic differences in disease prevalence (28). Reliable epidemiological data on AA amyloidosis in AS are more rare. A well-conducted study form Québec, Canada, concluded that the occurrence of renal amyloidosis in patients with AS was increased compared to the general population with a standardized prevalence ratio of 6.0 (95% confidence interval: 2.0–18.0). The data were particularly significant among men above the age of 60 (29).

A study from Turkey reported AA amyloidosis among ∼1% of AS patients at a single referral center (30). Higher disease activity captured by the gold standard, self-reported, and validated instrument "Bath Ankylosing Spondylitis Disease Activity Index" (BASDAI) (31) in addition to higher age, longer duration of AS, elevated ESR, and the presence of peripheral arthritis were all associated with amyloidosis but only the initial BASDAI score remained as an independent predictor for the development of secondary amyloidosis in the multivariate analysis (30). As an historical illustration of the severity of this complication, data from Finland reveal that AA amyloidosis was an important cause of death in patients with AS before the era of biologics (32). Secondary amyloidosis was the immediate cause of death in 13% of all deaths among 398 cases with AS followed over almost 30 years at the Rheumatism Foundation Hospital in Heinola (32).

The two cases described herein fulfilled several of the risk factors identified in the Turkish study (30). At onset of proteinuria, they were above the age of 60 and had a long duration of AS. The disease had been slowly progressive and

gave rather mild symptoms, which contrasted to the laboratory findings of systemic inflammation showing constantly elevated ESR and CRP levels. Their musculoskeletal symptoms had not been neglected at the annual visits to the rheumatology clinic, but a gradual adaptation of the patients to a decreased axial mobility cannot be excluded. Both patients had a short experience of TNF-blocking agents with inconclusive effects. Unfortunately, we were not able to retrieve initial BASDAI scores from any of them.

With increased availability of TNF inhibitors, the incidence of AA amyloidosis appears to be declining (4). As trials for IL-6 blocking agents like TCZ and sarilumab did not meet their primary endpoints in AS (33), the approach of initially adding a TNF-blocking agent in those AS patients who develop AA amyloidosis seems rational. Nevertheless, TCZ has been used with success in AA amyloidosis secondary to RA and familial Mediterranean fever (20, 34) and, to our knowledge, the two cases herein who received TCZ for AA amyloidosis secondary to AS are the first ones described. Neither did we find any report on the use of sarilumab in secondary amyloidosis related to any inflammatory disease. Besides directly IL-6 targeting agents, Janus kinase inhibitors reduce IL-6 signaling, and they may have impressive SAA-reducing effects in RA but none of the available drugs (tofacitinib, baricitinib, upadacitinib, and filgotinib) have consistently been evaluated in renal amyloidosis (35). However, of notice, upadacitinib is now licensed for the use in AS and renal impairment has a limited effect on upadacitinib pharmacokinetics (36, 37).

As demonstrated in **Figure 1**, both our patients had a rapid suppression of systemic inflammation combined with a reduction of proteinuria as a response to TCZ in monotherapy. None of the patients underwent a re-biopsy. Despite well-controlled systemic inflammation during TCZ therapy, the first clinical case witnessed increased axial symptoms that initially required addition of tenoxicam, and later etoricoxib. The second clinical case experienced relieve of musculoskeletal symptoms during TCZ and observed no deterioration of his IBD.

In conclusion, we report rapid and successful effects of TCZ in two male patients with longstanding AS complicated by renal amyloidosis. Inhibiting IL-6 is reasonable in AA amyloidosis but, to the best of our knowledge, experience of TCZ has not previously been published in patients with AS. These two clinical cases call for vigilance with regard to evolving proteinuria in AS patients with raised systemic inflammation, regardless of radiology and patient-reported symptoms.

AUTHOR CONTRIBUTIONS

CS had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. PE, JM, LW, and CS: study conception and design and analysis and interpretation of data. PE, JM, and CS: acquisition of data. All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published.

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M2 Macrophage Subpopulations in Glomeruli Are Associated With the Deposition of IgG Subclasses and Complements in Primary Membranous Nephropathy

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Objectives: The role of M2 macrophages in the pathogenesis and progression of primary membranous nephropathy (PMN) remains unknown. In this study, we aimed to investigate the relationship between M2 subsets and clinicopathological features of patients with PMN.

Methods: A total of 55 patients with PMN confirmed by biopsy were recruited. The clinical and pathological data were recorded, respectively. Immunohistochemistry was used to detect the markers of M2 macrophages, including total macrophages (CD68+), M2a (CD206+), M2b (CD86+) and M2c (CD163+).

Results: The numbers of glomerular macrophages, M2a, M2b, and M2c macrophages were 1.83 (1.00, 2.67), 0.65 (0.15, 1.15), 0.67 (0.33, 1.50), and 0.80 (0.05, 2.30) per glomerulus, respectively. Higher number of glomerular macrophages was found in stage II compared with stage III (2.08 vs. 1.16, $P = 0.008$). These macrophages also were negatively correlated with serum albumin level ($r = -0.331$, $P = 0.014$), while positively associated with complement 3 (C3) deposition ($r = 0.300$, $P = 0.026$) and the severity of glomerulosclerosis ($r = 0.276$, $P = 0.041$). Moreover, glomerular M2a macrophages were significantly correlated with the deposition of C3 ($r = 0.300$, $P = 0.026$), immunoglobulin G1 (IgG1) ($r = 0.339$, $P = 0.011$), immunoglobulin G2 (IgG2) ($r = 0.270$, $P = 0.046$) and immunoglobulin G3 (IgG3) ($r = 0.330$, $P = 0.014$) in glomerular basement membrane (GBM). In addition, M2b macrophages were positively associated with IgG1 ($r = 0.295$, $P = 0.029$) and IgG2 ($r = 0.393$, $P = 0.003$), while M2c macrophages were negatively correlated with complement 4d (C4d) ($r = -0.347$, $P = 0.009$) in GBM.

Conclusions: Our results showed that M2 macrophage subpopulations in glomeruli are associated with the deposition of IgG subclasses and complements in renal tissue of PMN, which indicate that M2 macrophages may be involved in the pathogenesis and progression of PMN. Moreover, M2a and M2c macrophages might show different tendencies in the pathogenesis of PMN.

Keywords: M2 macrophages, M2 subpopulations, primary membranous nephropathy, complement, immunoglobulin

INTRODUCTION

Membranous nephropathy (MN) is the most common cause of nephrotic syndrome in adults. Renal biopsy typically reveals diffuse glomerular capillary wall thickening and subepithelial and/or intramembranous immune deposits. About 80% of cases are renal limited (primary MN, PMN) and 20% are associated with other systemic diseases or exposures (secondary MN). PMN is characterized by the "rule of third." One-third of patients enter a state of spontaneous remission, and the remaining patients continue to relapse or gradually progress to renal failure (1–4). However, the mechanism of PMN remains unknown. Some studies have shown that cell-mediated immune mechanism may be actively involved in the pathogenesis of PMN (5). And macrophages play an important role in the immune mechanism. The interstitial infiltration of macrophages may indicate the outcome of PMN (6).

Macrophages can be divided into two subtypes: pro-inflammatory phenotypes, called M1 or classical activated macrophages, which can be activated by LPS or interferon. Anti-inflammatory phenotypes, namely M2 or alternative activated macrophages, which can secrete cytokines involved in tissue remodeling and fibrosis (7, 8). Recent studies have shown that M2 macrophages can be further subdivided into three subsets (9, 10). M2a macrophages (also known as wound-healing macrophages) characterized by the expression of CD206 receptors on the cell surface can be induced by IL-4 and IL-13 and are associated with tissue repair and fibrosis (11). M2b macrophages characterized by the expression of CD86 receptors on the cell surface can be induced by exposure to IC and agonists of Toll-like receptor (TLRs) or IL-1 and can regulate immune response (12). M2c macrophages characterized by the expression of CD163 receptors on the cell surface can be induced by IL10 and transforming growth factor β and exert regulatory, anti-inflammatory and pro-fibrotic functions (13).

Some studies have demonstrated that the degree of tubulointerstitial macrophage infiltration determines the prognosis of PMN (5, 14, 15). Ferrario et al. reported that macrophages infiltrated in proliferative lesions and immune-mediated human glomerulonephritis and they concluded that macrophages were involved in glomerular injury (16). However, researches on the relationship between macrophage subpopulations in glomeruli and PMN are very limited. In this study, we analyzed the relationship between M2 macrophage subpopulations in glomeruli and clinical and pathological parameters in patients with PMN.

MATERIALS AND METHODS

Patients

A total of 55 patients with membranous nephropathy aged 18 and over were recruited in Guangdong Provincial People's Hospital from January 2018 to July 2018. Exclusion criteria: (1) Secondary membranous nephropathy, such as diabetic nephropathy, lymphoma, renal amyloidosis and so on. (2) Patients with severe cardiovascular and cerebrovascular diseases. (3) Patients with kidney transplant. (4) Patients who lack laboratory or clinical data. For all patients, renal samples were collected by biopsy and clinical data were recorded, including sex, age, proteinuria, serum creatinine (SCr), cystatin C, blood urea nitrogen (BUN), albumin and estimated glomerular filtration rate (eGFR) calculated using the Chronic Kidney Disease Epidemiology collaboration equation (CKD-EPI) at the time of biopsy. The study involving human participants was approved by the Ethical Committee of Guangdong Provincial People's Hospital. Written informed consent was obtained from the patients before the enrollment.

Renal Biopsies

All renal samples were obtained by percutaneous biopsy. The diagnosis of PMN is based on routine light (LM) and immumofluorescence (IF) microscopy examination. Histological staging of MN adopts the criteria proposed by Ehrenreich and Churg (17). The severity of tubular atrophy and interstitial fibrosis was rated on a scale of 0, 1 or 2 based on the percentage of affected tubules (<25, 25–50, >50%). The extent of glomerular sclerosis and the intensity of IF findings were evaluated semi-quantitatively on a scale of − (absent), + (mild), ++ (moderate), and + + + (severe).

Immunofluorescence

The sections were deparaffinized with xylene and ethyl alcohol. Antigen repair was performed using EDTA (PH: 8.0). The sections were washed with PBS. The samples were permeabilized with 0.5% Triton-X 100 (10 min) and blocked with 5% bovine serum albumin (BSA) for 30 min at room temperature, and then incubated with the following primary antibodies diluted in 5% BSA at 4°C over-night: IgG (1:100, DAKO), IgM (1:100, Abcam), IgA (1:100, Abcam), C3 (1:200, Abcam), C1q (1:100, Abcam), C4d (1:200, Abcam), IgG1 (1:50, sigma), IgG2 (1:50, Sigma), IgG3 (1:50, Sigma), IgG4 (1:50, sigma), PLA2R (1:400, Abcam). The secondary antibody was Alexa Fluor 488 (1:1,000, Abcam) for 1 h. DAPI was used to stain the cell nuclei. The sections were observed under fluorescence microscope (Nikon 80i; Nikon, Tokyo, Japan).

Immunohistochemistry

In this study, CD68 positive cells were identified as total macrophages, CD206 positive cells were identified as M2a, CD86 positive cells were identified as M2b and CD163 positive cells were identified as M2c. The sections were deparaffinized with xylene and ethyl alcohol. Antigen repair was performed using EDTA (PH:8.0). Endogenous peroxidase was blocked by 3% H_2O_2. The sections were washed with PBS. The sections were preincubated with 5% bovine serum albumin (BSA) for 30 min at room temperature, and then incubated with the following primary antibodies diluted in 5% BSA at 4°C over-night: rabbit anti-human CD68 monoclonal antibody (1:400, 76437S, CST), rabbit anti-human CD206 monoclonal antibody (1:400, 91992S, CST), rabbit anti-human CD86 monoclonal antibody (1:150, 91882S, CST) and rabbit anti-human CD163 monoclonal antibody (1:500, 93498S, CST). The secondary antibody was goat anti-rabbit IgG (1:1,000, ab6721) for 45 min. Peroxidase activities were applied by diaminobenzidine (DAB) and the sections were

TABLE 1 | Patients' characteristics.

Characteristics	Parameter
Number of patients	55
Gender	
Male (*n*, %)	33 (60)
Female (*n*, %)	22 (40)
Hypertensive patients (*n*, %)	31 (56.4)
Diabetic patients (*n*, %)	8 (14.5)
Age (years, mean ± SD)	54.64 ± 14.41
$eGFR_{CKD-EPI}$ (ml/min/1.73 m^2, mean ± SD)	67.81 ± 27.68
Proteinuria [mg/day, M (1/4, 3/4)]	5345.20 (3297.60, 10204.90)
SCr [μmol/L, M (1/4, 3/4)]	83.04 (70.47, 109.46)
BUN [mmol/L, M (1/4, 3/4)]	5.88 (4.77, 7.52)
Cystatin-C [mg/L, M (1/4, 3/4)]	1.07 (0.85, 1.38)
Albumin [g/L, M (1/4, 3/4)]	23.70 (18.70, 28.50)

eGFR, estimating glomerular filter rate; SCr, serum creatinine; BUN, blood urea nitrogen.

counterstained with hematoxylin. The sections were observed under microscope (Nikon 80i; Nikon, Tokyo, Japan).

Qualitative Evaluation of Macrophages

A qualitative study was conducted in the absence of any clinical data. Under x40 microscope, CD68, CD206, CD86, CD163 positive cells were identified in 6 intact glomeruli per section. The average number of glomerular positive cells represents the number of macrophages per glomerulus.

Statistical Analysis

Statistical analysis was performed using SPSS (version 25.0; SPSS Inc., Chicago, IL, USA), GraphPad Prism (version 8.0; GraphPad Software, Inc., La Jolla, CA, USA). *ANOVA-test* and *Mann-Whitney U-test* were used to compare continuous variables. *Spearman's-test* was used for the correlation between macrophage subpopulations and clinical and pathologic data. P-value < 0.05 was statistically significant.

RESULTS

Baseline of Patients With PMN

A total of 55 patients were recruited, including 33 (60%) males and 22 (40%) females. The mean age was 54.64 (22–80) years old. The average level of eGFR was 67.81 ± 27.68 ml/min/1.73 m^2. The levels of proteinuria, SCr, BUN, cystatin C, and albumin were 5345.20 (3297.6, 10204.90) mg/day, 83.04 (70.47, 109.46) μmol/L, 5.88 (4.77, 7.52) mmol/L, 1.07 (0.85, 1.38) mg/L, and 23.70 (18.70, 28.50) g/L, respectively. Moreover, age was positively associated with SCr ($r = 0.405$, $P = 0.002$), cystatin C ($r = 0.637$, $P = 0.000$) and BUN ($r = 0.489$, $P = 0.000$), but negatively with eGFR ($r = -0.796$, $P = 0.000$). The patients' baseline is shown in **Table 1**.

All patients were in stage II or III of membranous nephropathy, and stage II was evident in 40 (80%) patients. Thirty-six (65.5%) patients had no glomerular sclerosis. Chronic

TABLE 2 | The pathological data.

Variables	*n* (%)
Glomerular sclerosis	
–	36 (65.5)
+	16 (29.1)
++	3 (5.5)
Score of renal tubular/interstitial injury	
0	53 (96.5)
1	0 (0.0)
2	2 (3.5)
Stage of membranous nephropathy	
II	44 (80)
III	11 (20)
IgG	
++	4 (7.3)
+++	51 (92.7)
IgM	
–	15 (27.3)
+	39 (70.9)
IgA	
–	50 (90.9)
+	5 (9.1)
C3	
–	6 (10.9)
+	8 (14.5)
++	37 (67.3)
+++	4 (7.3)
C1q	
–	55 (100)
IgG1	
–	21 (38.2)
+	16 (29.1)
++	17 (30.9)
+++	1 (1.8)
IgG2	
–	34 (61.8)
+	19 (34.5)
++	2 (3.6)
IgG3	
–	27 (49.1)
+	21 (38.2)
++	6 (10.9)
+++	1 (1.8)
IgG4	
–	3 (5.5)
+	3 (5.5)
++	10 (18.2)
+++	39 (70.9)
PLA2R	
–	9 (16.4)
+	46 (83.6)
C4d	
–	6 (10.9)
+	49 (89.1)

–, absent; +, mild; ++, moderate; + + +, severe; IgG, immunoglobulin G; IgM, immunoglobulin M; IgA, immunoglobulin A; C3, complement 3; C1q, complement 1q; IgG1, immunoglobulin G1; IgG2, immunoglobulin G2; IgG3, immunoglobulin G3; IgG4, immunoglobulin G4; PLA2R, M-type phospholipase A2 receptor; C4d, complement 4d.

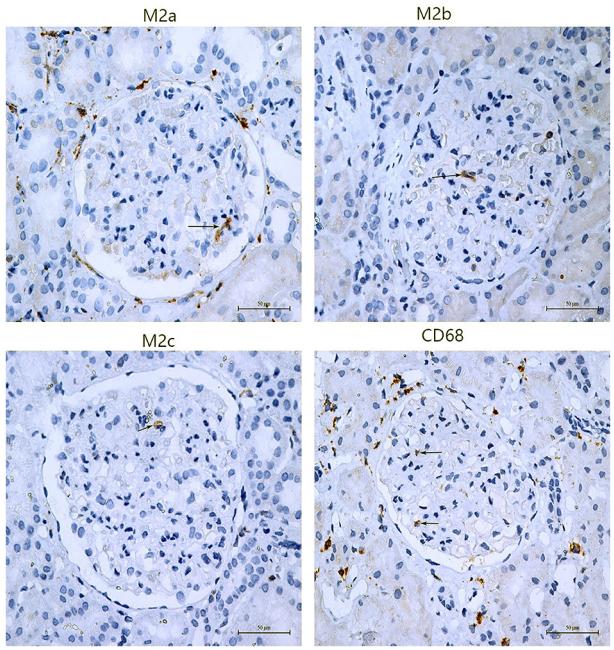

FIGURE 1 | Subpopulations of M2 macrophage in glomeruli of PMN patients. M2a macrophages were positive for CD206 by immunochemistry, M2b macrophages were positive for CD86, and M2c macrophages were positive for CD163. The arrows point to positive cells.

renal tubular atrophy/interstitial fibrosis (affected area >25%) was found in only 2 cases (3.5%). In IF, granular IgG deposition was present in all patients. Most of them were IgG4 and IgG1, while IgG2 and IgG3 were also common. A weaker IgM and IgA were observed in 39 (70.9%) and 5 (9.1%) patients, respectively. Complement C3 and C4d were found in 49 patients (89.1%), and no C1q deposition was found in glomeruli. Only 9 (16.4%) patients did not express M-type phospholipase A2 receptors. The pathological parameters are listed in **Table 2**.

M2 Macrophage Subpopulations in Glomeruli of PMN

Macrophages and M2 macrophage subpopulations were identified by immunohistochemical staining of macrophage markers CD68, CD206, CD86, and CD163. The numbers of glomerular macrophages, M2a, M2b, and M2c macrophages were 1.83 (1.00, 2.67), 0.65 (0.15, 1.15), 0.67 (0.33, 1.50), and 0.80 (0.05, 2.30) per glomerulus, respectively. The number of M2 macrophage subpopulations in glomeruli is shown in **Figure 1** and **Table 3**. The number of macrophages in stage II of MN was more than that in stage III [2.08 (1.04, 3.12) vs. 1.16 (0.50, 1.50), $P = 0.008$]. However, the number of M2 macrophage subpopulations was not related to the pathological staging of MN. The comparison of M2 macrophage subpopulations in glomeruli between stage II and stage III is shown in **Figure 2**.

TABLE 3 | The numbers of M2 macrophage subpopulations.

Variables	*M* (1/4, 3/4)
M2a	0.65 (0.15, 1.15)
M2b	0.67 (0.33, 1.50)
M2c	0.80 (0.05, 2.30)
Macrophage	1.83 (1.00, 2.67)

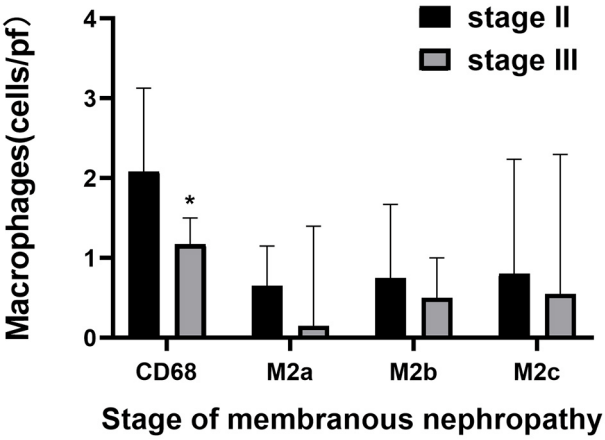

FIGURE 2 | The comparison of M2 macrophage subpopulations count in glomeruli between stage II and stage III of membranous nephropathy. Quantitative analysis of CD68, M2a, M2b, and M2c in membranous nephropathy stage II and stage III, Significant correlation $^*p < 0.05$.

The Correlation Between M2 Macrophage Subpopulations and Clinicopathological Features

We further analyzed the correlation between macrophages, M2 subpopulations and clinical data, and found that the number of macrophages was negatively associated with the level of albumin ($r = -0.331$, $P = 0.014$). However, the number of M2 macrophage subpopulations was not related to the severity of proteinuria and renal function indexes such as SCr, eGFR and cystatin C.

In terms of pathological features, macrophages count was positively correlated with C3 ($r = 0.300$, $P = 0.026$) and glomerular sclerosis ($r = 0.276$, $P = 0.041$). Moreover, M2a count was positively correlated with C3 ($r = 0.300$, $P = 0.026$), IgG1 ($r = 0.339$, $P = 0.011$), IgG2 ($r = 0.270$, $P = 0.046$), IgG3 ($r = 0.330$, $P = 0.014$), but not with IgG4 ($r = 0.218$, $P = 0.110$). M2b count was positively correlated with IgG1 ($r = 0.295$, $P = 0.029$) and IgG2 ($r = 0.393$, $P = 0.003$). For M2c, there was a negative correlation with C4d ($r = -0.347$, $P = 0.009$). The correlation between M2 macrophage subpopulations and clinicopathological data is shown in **Table 4**.

TABLE 4 | Correlation between M2 macrophage subpopulations and clinical and pathologic data.

	CD68	M2a	M2b	M2c
$eGFR_{CKD-EPI}$ (ml/min/1.73 m^2)	$r = -0.095$	$r = -0.135$	$r = -0.041$	$r = 0.060$
	$P = 0.492$	$P = 0.326$	$P = 0.767$	$P = 0.664$
Proteinuria (mg/day)	$r = 0.087$	$r = 0.108$	$r = 0.035$	$r = 0.095$
	$P = 0.525$	$P = 0.432$	$P = 0.798$	$P = 0.490$
SCr (μmol/L)	$r = 0.198$	$r = 0.064$	$r = 0.040$	$r = 0.011$
	$P = 0.147$	$P = 0.645$	$P = 0.773$	$P = 0.936$
BUN (mmol/L)	$r = 0.197$	$r = 0.063$	$r = 0.021$	$r = 0.154$
	$P = 0.149$	$P = 0.649$	$P = 0.880$	$P = 0.261$
Cystatin-C (mg/L)	$r = 0.069$	$r = 0.099$	$r = 0.021$	$r = -0.065$
	$P = 0.618$	$P = 0.473$	$P = 0.879$	$P = 0.636$
Albumin	$r = -0.331$	$r = -0.155$	$r = -0.167$	$r = -0.231$
	$P = 0.014^*$	$P = 0.257$	$P = 0.224$	$P = 0.090$
Glomerular sclerosis	$r = 0.276$	$r = -0.013$	$r = 0.113$	$r = -0.134$
	$P = 0.041^*$	$P = 0.925$	$P = 0.412$	$P = 0.329$
IgG	$r = -0.020$	$r = 0.002$	$r = -0.204$	$r = -0.038$
	$P = 0.885$	$P = 0.987$	$P = 0.135$	$P = 0.785$
IgM	$r = -0.247$	$r = -0.151$	$r = -0.218$	$r = -0.003$
	$P = 0.072$	$P = 0.275$	$P = 0.114$	$P = 0.985$
IgA	$r = -0.186$	$r = -0.006$	$r = 0.030$	$r = 0.142$
	$P = 0.175$	$P = 0.965$	$P = 0.828$	$P = 0.301$
C3	$r = 0.300$	$r = 0.300$	$r = 0.056$	$r = 0.263$
	$P = 0.026^*$	$P = 0.026^*$	$P = 0.686$	$P = 0.052$
IgG1	$r = 0.142$	$r = 0.339$	$r = 0.295$	$r = 0.262$
	$P = 0.300$	$P = 0.011^*$	$P = 0.029^*$	$P = 0.054$
IgG2	$r = 0.202$	$r = 0.270$	$r = 0.393$	$r = 0.136$
	$P = 0.138$	$P = 0.046^*$	$P = 0.003^*$	$P = 0.321$
IgG3	$r = 0.077$	$r = 0.330$	$r = 0.165$	$r = 0.085$
	$P = 0.575$	$P = 0.014^*$	$P = 0.229$	$P = 0.536$
IgG4	$r = 0.028$	$r = 0.218$	$r = -0.045$	$r = 0.087$
	$P = 0.837$	$P = 0.110$	$P = 0.747$	$P = 0.528$
PLA2R	$r = -0.047$	$r = -0.058$	$r = 0.075$	$r = 0.061$
	$P = 0.736$	$P = 0.675$	$P = 0.588$	$P = 0.660$
C4d	$r = 0.081$	$r = -0.137$	$r = -0.129$	$r = -0.347$
	$P = 0.557$	$P = 0.318$	$P = 0.347$	$P = 0.009^*$

Significant correlation $^*p < 0.05$.

The Correlation Between Clinical Data and Pathologic Data

Our data showed patients with glomerular sclerosis had poor renal function. The level of eGFR in patients with glomerular sclerosis was lower than that in patients without glomerular sclerosis (54.92 ± 26.50 vs. 74.61 ± 26.13) ($P = 0.011$). The levels of SCr and cystatin C in patients with glomerular sclerosis were higher than those in patients without glomerular sclerosis, which were 99.80 (86.47, 148.60) vs. 75.53 (67.19, 89.83) ($P = 0.005$), 1.34 (0.89, 1.86) vs. 0.99 (0.80, 1.20) ($P = 0.014$), respectively. Furthermore, the level of proteinuria in patients with glomerular sclerosis was higher than that in patients without glomerular sclerosis, which was 7191.00 (4832.00, 12292.00) vs. 3796.70 (2442.08, 6422.69) ($P = 0.001$). The correlation between clinical data and pathologic data is shown in **Figure 3**.

DISCUSSION

In this study, we identified total macrophages and M2 macrophage subpopulations in the glomeruli of patients with PMN. Correlation analysis showed that the total macrophage count was negatively associated with serum albumin level, while positively associated with C3 deposition and the severity of glomerular sclerosis. M2 subpopulations were found in glomerulus. M2a macrophages in glomeruli were significantly correlated with the deposition of C3, IgG1, IgG2, and IgG3 in glomerular basement membrane (GBM) and M2b macrophages were positively associated with the deposition of IgG1 and IgG2. M2c macrophages were negatively correlated with complement 4d (C4d).

PMN is now considered to be a renal limited autoimmune disease, with antibodies against M-type phospholipase A2 receptor (PLA2R) identified in 70–80% patients (18). In our study, 46 (83.6%) patients were identified expressing PLA2R in renal tissue, which is in accord with the previous studies. Moreover, MN is also considered to be a IgG4 dominant disease and universal presence of C3 in the subepithelial deposits (19, 20). Our results showed that IgG4 was dominant in immune complexes and complement C3 and C4d were prevalence in 89.1% of these patients. The data above indicate that our study population has representative pathological features of PMN.

Tissue macrophages are crucial players in inflammation and immunity (21). In our study, a small number of macrophages (1.83 per glomeruli) was found in glomeruli and positively correlated with complement C3 that is the main deposits of MN. Papagianni et al. showed that macrophages released cytokines, which induced consecutive complement activation leading to tubular damage in patients with membranous nephropathy. They also reported that SCr was highly correlated with the number of interstitial macrophages (22). The aforementioned evidence indicates that macrophages may be involved in the progression of MN through the mechanism of complement activation. Moreover, our results showed that the number of macrophages was positively correlated with the extent of glomerular sclerosis and negatively correlated with serum albumin. Saito and Atkins pointed out that macrophages increased in experimental and clinical focal segmental glomerular sclerosis (FSGS) (23). Morita et al. demonstrated that the level of eGFR in MN patients with FSGS was lower than those without FSGS (24). Therefore, macrophages may participate in the progress of FSGS in PMN patients.

PMN has four evolutionary stages according to the criteria proposed by Ehrenreich and Churg. In our study, all patients were in membranous nephropathy stage II or III. Papagianni et al. showed that the stage of the PMN was not related to the degree of glomerular infiltration by inflammatory cell (22). However,

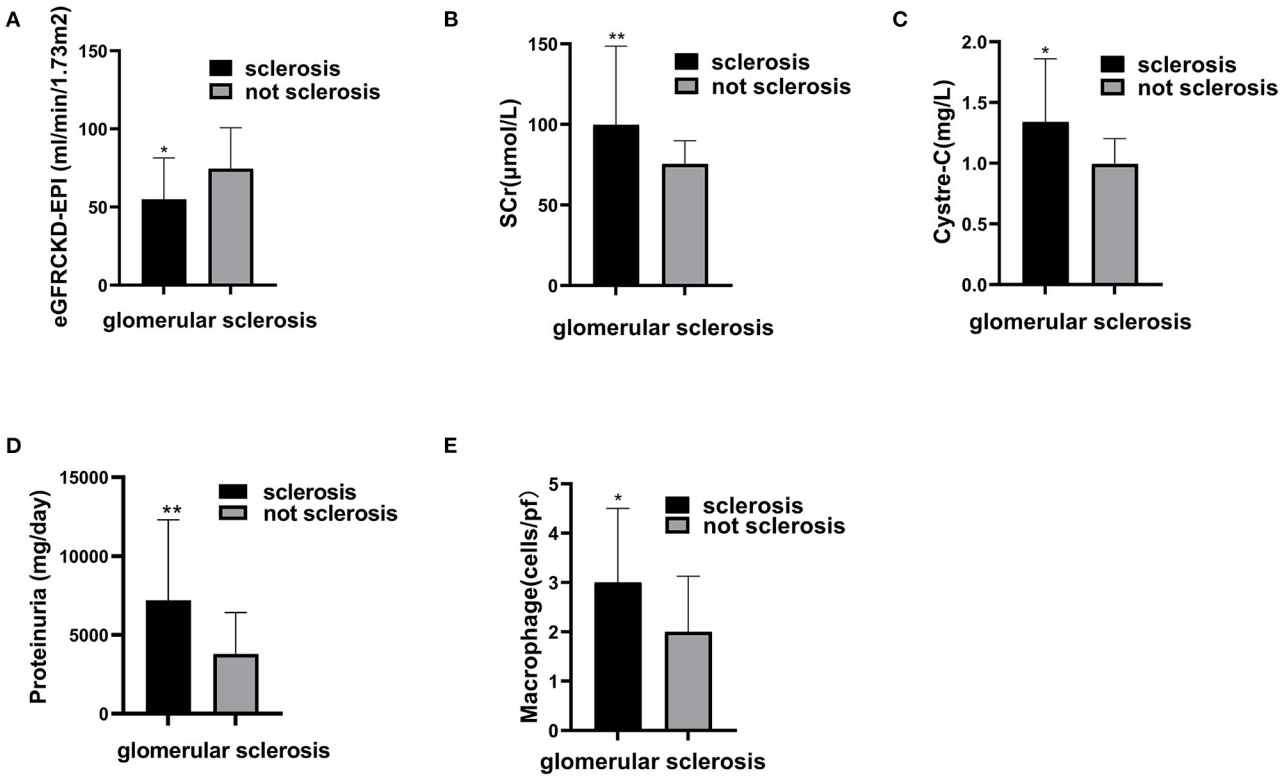

FIGURE 3 | The correlation between glomerular sclerosis and clinical features and macrophages in glomeruli. Compare estimating glomerular filter rate **(A)**, serum creatinine **(B)**, Cystre-C **(C)**, Proteinuria **(D)**, and macrophage **(E)** between patients with glomerular sclerosis and patients without glomerular sclerosis. Significant correlation $^{*}p < 0.05$, $^{**}p < 0.01$.

our results showed that more macrophages in glomeruli were found in stage II than in stage III, which may be associated with earlier immune response in stage II. But the number of M2 macrophage subpopulations was not related to the stage of membranous nephropathy. Therefore, larger samples and further studies are needed to analyze the role of macrophages in different stages of PMN.

M2 macrophages are composed of a heterogeneous subpopulation of cells with different functions and phenotypic plasticity. M2a macrophages, characterized by the expression of transmembrane marker CD206, are known to be involved in the progression of kidney disease (25). In our study, we found that M2a macrophages were positively associated with IgG1, IgG2, and IgG3. As we known, IgG4 is the predominant IgG subclass in PMN, but IgG1, IgG2, and IgG3 are also common. Recent studies suggested that there was a programmed order of immune response, starting from IgM producing B cells followed by class switching to IgG producing B cells, with production of IgG subclass antibodies in a fixed order of IgG3 > IgG1> IgG2 > IgG4 (26). Therefore, M2a macrophages may be associated with the early phase of IgG production. This hypothesis needs to be validated by further basic and clinical studies. Moreover, our data also showed that M2a macrophages were associated with C3. Previous study proved that C3 in subepithelial deposits is very common in MN (27), and IgG1 and IgG3 were associated with complement activation and fixing. These results show that M2a may be involved in the progression of PMN through promoting the deposition of IgG subtypes and activating complements.

M2b macrophages regulate immune response and are induced by immune complexes (IC). And PMN is characterized by IC. It has been reported that M2b macrophages may be a crucial mediator for the initiation and progression of autoimmune diseases (28). Our study showed that M2b macrophages were observed in PMN and were positively associated with IgG1 and IgG2. Orme and Mohan demonstrated that M2b macrophages actually play a direct role in causing SLE (29). M2b macrophages, as antigen-presenting cells, are involved in innate and adaptive immunity, and immunoglobulin is an important part of adaptive immunity, suggesting that M2b may promote the production of IgG by presenting antigen. These data above indicate that M2b might be involved in the progressionpbr of PMN.

M2c characterized by the expression of CD163 receptor on cell surface can exert regulatory, anti-inflammatory and pro-fibrotic functions. Several studies have shown that M2c macrophages have protective effects. Tseng et al. showed that the upregulation of M2c macrophages could alleviate renal fibrosis in obstructed kidney (30). Lu et al. transferred M2c macrophages into mice on day 5 after adriamycin administration, and the

results showed that M2c effectively reduced glomerulosclerosis, tubular atrophy, interstitial expansion, and proteinuria, and they concluded that M2c might protect the kidney from injury (31). In addition, Tang et al. indicated that M2c macrophages could ameliorate inflammation and fibroproliferation in acute lung injury through interleukin 10 pathway (32). These results were accordant with ours. In our study, we found that C4d was associated with M2c negatively. Positive staining with C4d was present in immune-complex related glomerulonephritis, including membranous glomerulonephritis and lupus nephritis, which revealed that C4d was involved in immune reaction in PMN (33). Therefore, we speculate that M2c may play a protective role in PMN through reducing the production of C4d.

In this study, we described the association between M2 macrophage subpopulations in glomeruli and pathological features in PMN with novelty. However, our study has some limitations. Renal biopsies from human samples can only represent a snapshot of the current state of the disease. Therefore, we have no dynamic information about the early stages of the disease and the progression of macrophage subtypes. In addition, we only analyze the surface markers of M2 subtypes and other markers of M2 subtypes (such as functional cytokines) need to be analyzed to support our conclusions. We will detect more markers and follow up the patients to investigate the role of M2 subpopulations in PMN.

CONCLUSIONS

In conclusion, our study showed that M2a and M2b macrophages were positively correlated with tissue IgG subclasses of early stage and C3, while M2c macrophages were negatively correlated with C4d. These results indicate that M2 macrophage subpopulations are involved in the progression of PMN by the deposition of IgG subclasses and complements. And M2a and M2c macrophages might show different properties in the pathogenesis of PMN.

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 authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethical Committee of Guangdong Provincial People's Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WHu: conceptualization, methodology, formal analysis, and writing-original draft. GL: conceptualization, data curation, investigation, and writing-reviewing and editing. JL and WL: data curation, visualization, investigation, and software. WD and YW: software, formal analysis, data curation, and investigation. FY: software, formal analysis, and investigation. WHa: conceptualization, supervision, writing-reviewing and editing, and validation. XL: conceptualization, resources, supervision, writing-reviewing and editing, and validation. 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.

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Serum Bilirubin Is Correlated With the Progression of IgA Vasculitis With Nephritis

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Background: Bilirubin has been identified as an endogenous antioxidant and cellular protectant. The present study was performed to clarify the potential influence of serum bilirubin on IgA vasculitis with nephritis (IgAV-N).

Methods: One hundred and eighty-nine IgAV-N patients over 14 years old were enrolled. The patients were divided into two groups by the optimum cut-off value calculated by ROC curve. The composite endpoints were defined as a 60% decline in estimate glomerular filtration rate (e-GFR), end-stage renal disease (ESRD) and/or death. Kaplan-Meier (K-M) analysis and multivariate Cox analysis were carried out to determine the predictors for renal outcomes. In order to eliminate the influence of different baseline data, a 1:2 propensity score (PS) match was performed to make the results comparable and convictive.

Results: The baseline data suggested that patients in low serum bilirubin group had significantly higher levels of systolic blood pressure, proteinuria, serum creatinine and crescent formation ratio and lower levels of serum albumin and hemoglobin. Renal survival analysis indicated that lower serum bilirubin levels were significantly correlated with a poorer prognosis. Multivariate Cox analysis demonstrated that the higher level of serum bilirubin was an independent protective factor for renal survival (HR, 0.172; 95% CI, 0.030–0.991; $P = 0.049$). After PS matching, the baseline characters of two groups had no statistical differences. Similar outcomes were demonstrated in K-M curve and the multivariate Cox analysis.

Conclusion: Elevated bilirubin levels might be related to the favorable renal outcomes.

Keywords: IgA vasculitis with nephritis, bilirubin, cohort study, prognosis, biomarker

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INTRODUCTION

IgA vasculitis with nephritis (IgAV-N) is one of the most severe complications of IgA vasculitis (IgAV) and the long-term prognosis of IgAV are mostly determined by the severity of renal lesions, owing that IgAV-N can cause end-stage renal disease (ESRD) and even death (1). The pathophysiological processes of IgAV-N have not been fully elucidated yet, however, it is widely acknowledged that the deposits of immune complexes (IC) in glomeruli might contribute to the progression of IgAV-N. Deposited IC may further cause oxidant injury, inflammation reaction and

endothelial damage, all of which play a pivotal part in the renal impairment (2). The pathogenesis is much similar to primary IgA nephropathy (IgAN), therefore IgAV-N is also considered as secondary IgAN (1).

Bilirubin has been identified as an endogenous antioxidant and cellular protectant, with potent abilities of complement inhibition and anti-inflammation (3). Previous literatures have demonstrated that low serum bilirubin concentration is related to poor outcomes of diabetic nephropathy, IgAN and other chronic kidney diseases (CKD) (4–6). Additionally, animal experiments have reported that bilirubin might protect against diabetic nephropathy (7). Given the similarities in IgAV-N and IgAN, serum bilirubin may have a likely impact on IgAV-N, but no study has proved it. Therefore, the present study was conducted to evaluate whether serum bilirubin levels could serve as an independent predictor of IgAV-N prognosis and to clarify the potential influence of serum bilirubin on the progression of IgAV-N.

METHODS

This study was approved by the Ethics Committee of the West China Hospital of Sichuan University and retrospectively registered in Thai Clinical Trials Registry. The registration number is TCTR20180313004.

Subjects

One hundred and eighty-nine adolescent and adult patients with IgAV-N proven by renal biopsy in West China Hospital of Sichuan University between October 2010 and June 2017 were enrolled prospectively in this study. Diagnostic criteria of IgAV was based on guidelines from the American College

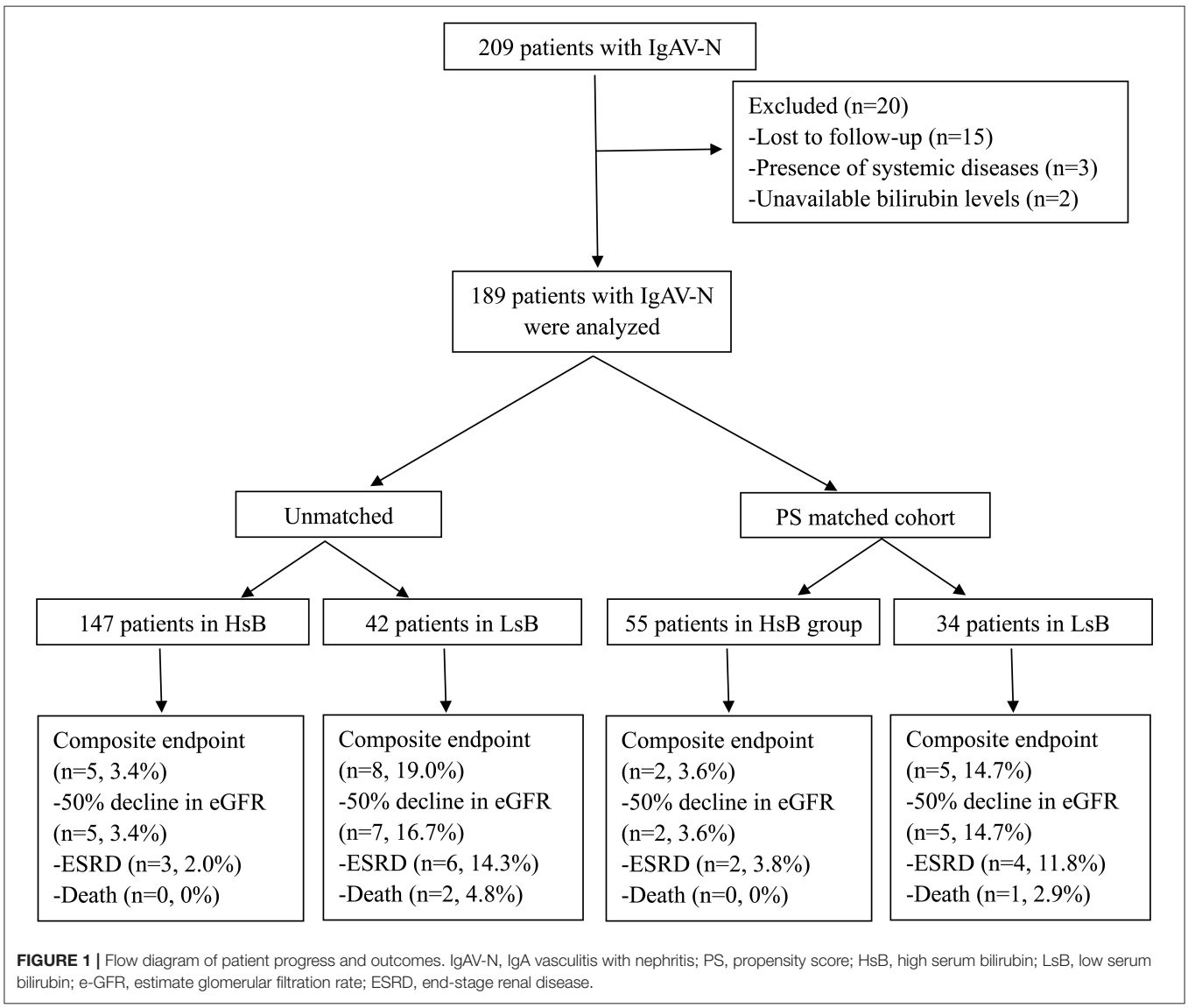

FIGURE 1 | Flow diagram of patient progress and outcomes. IgAV-N, IgA vasculitis with nephritis; PS, propensity score; HsB, high serum bilirubin; LsB, low serum bilirubin; e-GFR, estimate glomerular filtration rate; ESRD, end-stage renal disease.

TABLE 1 | Baseline characteristics of patients categorized according to serum bilirubin levels.

Characteristics	Unmatched cohort		*P*-values	Matched cohort		*p*-values
	HsB group (≥6.35 umol/L) (*n* = 147)	LsB group (<6.35 umol/L) (*n* = 42)		HsB group (≥6.35 umol/L) (*n* = 55)	LsB group (<6.35 umol/L) (*n* = 34)	
Age	31.3 ± 15	29.0 ± 16.1	0.381	33.1 ± 17.8	26.1 ± 14.2	0.080
Male (%)	66 (44.9)	21 (50.0)	0.559	36 (65.5)	17 (50.0)	0.149
Follow-up (m)	27.4 ± 19.7	26.8 ± 21.8	0.858	27.8 ± 23.2	26.5 ± 22.4	0.860
SBP	121.97 ± 18.42	129.31 ± 18.73	0.024*	125.45 ± 17.47	127.09 ± 18.53	0.658
DBP	78.81 ± 11.55	80.95 ± 13.19	0.306	81.60 ± 10.69	80.41 ± 14.22	0.655
HTN (%)	34 (23.1)	12 (28.6)	0.469	15 (27.3)	8 (23.5)	0.695
Proteinuria	1.77 (0.81–2.95)	4.62 (2.92–8.38)	0**	3.78 ± 3.44	4.34 ± 3.03	0.442
u-RBC	166.62 ± 432.336	222.07 ± 462.24	0.471	298.38 ± 660.77	116.56 ± 205.16	0.123
ALB	36.89 ± 7.02	29.07 ± 8.71	0**	33.71 ± 7.29	30.51 ± 8.70	0.080
SCr	70.11 (58.01–86.25)	69.65 (48.25–117.35)	0.056	79.54 ± 45.64	89.66 ± 55.36	0.352
UA	335.81 ± 101.71	364.22 ± 108.09	0.117	339.04 ± 110.76	356.04 ± 103.42	0.473
e-GFR	110.1 (90.1–128.9)	125.1 (69.7–136.7)	0.219	104.91 ± 37.37	104.46 ± 39.43	0.957
HGB	138.48 ± 18.23	121.40 ± 20.76	0**	130.36 ± 17.89	125.65 ± 19.52	0.246
TG	1.74 ± 0.99	1.99 ± 0.98	0.141	1.79 ± 0.84	2.07 ± 1.07	0.178
CHOL	5.15 ± 1.66	5.58 ± 1.94	0.149	5.66 ± 1.78	5.66 ± 1.90	0.993
T-BIL	11.3 (8.5–14.6)	4.8 (3.9–5.7)	0**	10.5 (8.4–14.2)	4.5 (3.9–5.7)	0**
D-BIL	3.1 (2.45–4.1)	1.4 (1.1–1.6)	0**	3.1 (2.1–4.1)	1.4 (1.0–1.6)	0**
I-BIL	7.9 (6.4–10.8)	3.2 (2.7–4.1)	0**	7.5 (6.1–10.2)	3.1 (2.7–4.1)	0**
M0/M1 (%)	21/126 (14.3/86.7)	7/35 (16.7/83.3)	0.702	4/51 (7.3/92.7)	6/28 (17.6/82.4)	0.132
E0/E1 (%)	122/25 (83/17)	35/7 (83.3/16.7)	0.959	45/10 (81.8/18.2)	30/4 (88.2/11.8)	0.419
S0/S1 (%)	96/51 (65.3/34.7)	24/18 (57.1/42.9)	0.333	39/16 (70.9/29.1)	20/14 (58.8/41.2)	0.284
C0/C1 (%)	96/51 (65.3/34.7)	20/22 (46.7/52.4)	0.038*	27/28 (49.1/50.9)	17/17 (50.0/50.0)	0.934
T0/T1 (%)	86/61 (58.5/41.5)	26/16 (61.9/38.1)	0.692	29/26 (52.7/47.3)	20/14 (58.8/41.2)	0.574

SBP, systolic blood pressure; DBP, diastolic blood pressure; HTN, hypertension; u-RBC, uric red blood cell; ALB, albumin; SCr, serum creatinine; UA, uric acid; e-GFR, estimated glomerular filtration rate; HGB, hemoglobin; TG, triglyceride; CHOL, cholesterol; T-BIL, total bilirubin; D-BIL, direct bilirubin; I-BIL, indirect bilirubin; M, mesangial proliferation; E, endocapillary proliferation; S, segmental glomerulosclerosis; C, crescents; T, tubular atrophy/interstitial fibrosis.

** Stands for $p < 0.05$.*

*** Stands for $p \leq 0.01$.*

of Rheumatology (ACR) database and methodology (8). Renal biopsy was performed in patients who had hematuria, proteinuria and/or renal dysfunction. Notably, all subjects enrolled in our cohort were over 14 years old. Individuals with systemic diseases including systemic lupus erythematosus, autoimmune hemolytic anemia, malignant neoplasm, acute/chronic liver disease, congenital kidney disease, and inherited kidney disease were excluded. Written informed consents were obtained.

Grouping and Matching

Total bilirubin levels were detected at baseline and IgAV-N patients were divided into high serum bilirubin level group (HsB) and low serum bilirubin level (LsB) group based on the optima cutoff value calculated by receiver operating characteristic (ROC) curve, which was frequently used in the medical statistics (9). In order to eliminate the influence of differences at baseline on the renal outcome, we performed a 1:2 propensity score (PS) match based on the greedy matching algorithm (10, 11). Covariates including age, gender, systolic blood pressure (SBP), diastolic blood pressure (DBP), proteinuria, urinary red blood cell (u-RBC), albumin, serum creatinine, hemoglobin and pathological findings, were considered in this multi-logit regression model, in order to make the results comparable. The caliper used for PS matching was within 0.2 SD of logit PS.

Measurement

All patients enrolled in this study were followed up regularly for at least 6 months or shorter if they had reached the endpoints of observation in our medical center. Their clinical histories and out-patient records were assessed. Venous blood samples were taken from IgAV-N patients fasting overnight in the early morning at each time of follow up for measurement of serum total bilirubin, albumin, creatinine, uric acid, triglyceride and cholesterol. Serum total bilirubin was measured by vanadate oxidizing method and the normal reference range was 5.0–28.0 umol/L. Estimate glomerular filtration rate (e-GFR) was calculated using chronic kidney disease epidemiology collaboration (CKD-EPI) formula. Besides, proteinuria levels and hematuria levels (u-RBC) were also recorded. Anemia was diagnosed by a hemoglobin of <120 g/L in men while in women, it was <110 g/L. Considering that there was no universally

TABLE 2 | Principal treatments of patients with HSPN.

Characteristics	Unmatched cohort		*P*-values	Matched cohort		*P*-values
	HsB group (≥6.35 umol/L) (*n* = 147)	LsB group (<6.35 umol/L) (*n* = 42)		HsB group (≥6.35 umol/L) (*n* = 55)	LsB group (<6.35 umol/L) (*n* = 34)	
ACEI/ARB (%)	16 (10.9)	1 (2.4)	0.126	3 (5.4)	1 (2.9)	1.000
Glucocorticoids (%)	61 (41.5)	14 (33.3)	0.340	22 (40.0)	14 (41.2)	0.913
Glucocorticoids + Immunosuppressants (%)	52 (36.4)	17 (40.5)	0.545	20 (36.4)	10 (29.4)	0.5000
Pulse therapy with methylprednisolone and cyclophosphamide (%)	18 (12.2)	10 (23.8)	0.063	10 (18.2)	9 (26.5)	0.354

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker.

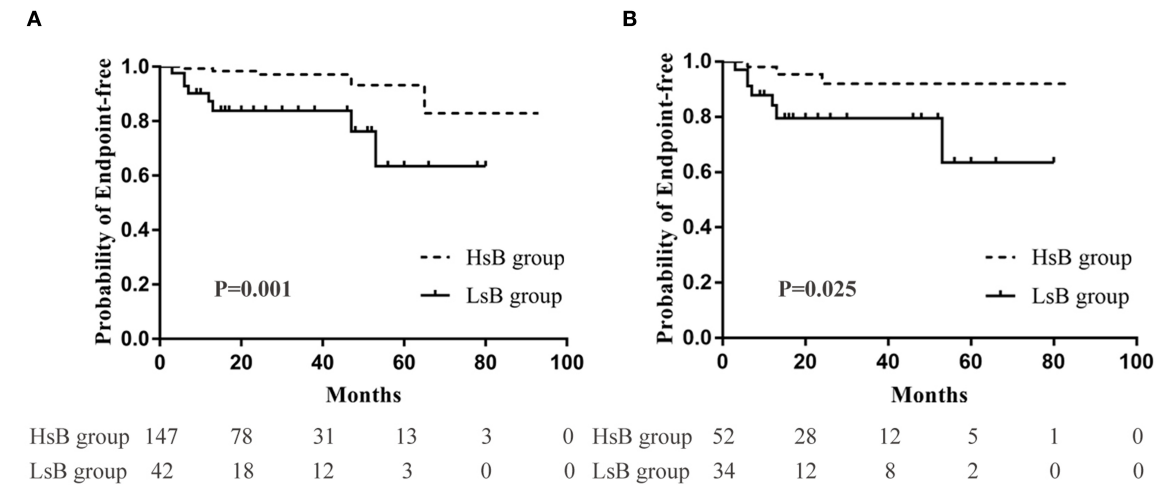

FIGURE 2 | Renal outcomes between high serum bilirubin (HsB) group and low serum bilirubin (LsB) group. **(A)** Kaplan-Meier curve for outcomes in the unmatched and **(B)** the matched cohort.

accepted pathological classification of adult-onset HPSN, the pathology results in this study were largely based on Oxford classification of IgAN. Renal pathological lesions were classified as mesangial proliferation (M), endocapillary proliferation (E), segmental glomerulosclerosis (S), tubular atrophy/interstitial fibrosis (T) and fibrocellular/cellular crescents (C) (12).

Treatment and Definitions of Renal Outcome

Four treatment regimens were used based on the KDIGO guidelines or experience of doctors, including angiotensin receptor blocker (ARB)/angiotensin-converting enzyme inhibitor (ACEI), glucocorticoids, immunosuppressants combined with glucocorticoids, and methylprednisolone + cyclophosphamide pulse therapy. The composite endpoint in our study was defined as a 50% decline in e-GFR, ESRD and/or death. The ESRD was defined as e-GFR <15 mL/min/1.73 m^2 or the initiation of maintenance renal replacement therapy (haemodialysis, peritoneal dialysis, or renal transplant). The definition of renal insufficiency was e-GFR <60 ml/min/1.73 m^2. The definition of hypoalbuminemia was that the levels of serum albumin <40 g/L. The definition of hematuria was u-RBC >5 per high power view.

Statistical Analysis

SPSS package (version 23.0; SPSS Inc., Chicago, IL) and SAS software package version 9.2 (SAS Institute, Cary, NC, USA) were used to perform the statistical analysis. For continuous data, results were presented as mean ± standard deviation or median (interquartile range) based on the covariate distribution whereas categorical variables were expressed in numbers (percentages). Patients were divided into two groups by the optima cut-off value which was calculated by carrying out the ROC curve. Baseline data between groups was assessed using Student's *t*-test, Wilcoxon-test, Chi-square-test or Fisher's exact-test. Kaplan–Meier estimates was used to compute the proportions of endpoint in both matched and unmatched cohorts. Similarly, a Cox regression analysis was adjusted to evaluated the influence of the clinicopathological manifestations on the renal outcome in both cohorts. Subgroup analysis was also carried out, whose heterogeneity was tested by addition of a multiplicative interaction term to the correlative Cox model. ROC was used

TABLE 3 | Risk factors for renal outcomes by a cox-regression analysis categorized according to serum bilirubin levels.

	Unmatched cohort		Matched cohort	
	HR (95%CI)	***p***	**HR (95%CI)**	***p***
BIL (high/low)	0.17 (0.03–0.99)	0.049*	0.05 (0.00–1.03)	0.050*
HTN	1.58 (0.27–9.37)	0.613	20.25 (0.23–1758.10)	0.187
Proteinuria (g/d)	1.20 (0.91–1.58)	0.197	1.60 (0.83–3.10)	0.159
e-GFR (ml/min/1.73 m^2)	1.00 (0.98–1.02)	0.924	1.00 (0.98–1.03)	0.831
u-RBC (/HP)	1.00 (0.99–1.00)	0.292	1.00 (0.99–1.00)	0.655
ALB (g/L)	0.95 (0.84–1.08)	0.468	0.92 (0.70–1.20)	0.521
CHOL (mmol/L)	1.34 (0.92–1.97)	0.131	1.10 (0.74–1.63)	0.641
Anemia	4.83 (0.81–28.97)	0.085	2.09 (0.06–73.04)	0.685
M (M0/M1)	0.24 (0.01–4.00)	0.318	0.05 (0.00–7.07)	0.237
E (E0/E1)	0.96 (0.12–8.05)	0.960	2.1E7 (0.00–5.8E7)	0.981
S (S0/S1)	0.47 (0.11–2.05)	0.132	0.20 (0.02–1.99)	0.171
C (C0/C1-2)	5.44 (0.81–36.65)	0.082	0.65 (0.02–27.34)	0.823
T (T0/T0-1)	0.01 (0.00–0.10)	0.000**	0.00 (0.00–0.29)	0.013*

BIL, the high serum bilirubin group/the low serum bilirubin group; HTN, hypertension; e-GFR, estimated glomerular filtration rate; u-RBC, the count of uric red blood cell; ALB, albumin; CHOL, cholesterol; M, mesangial proliferation; E, endocapillary proliferation; S, segmental glomerulosclerosis; C, crescents; T, tubular atrophy/interstitial fibrosis.
**Stands for $p < 0.05$.*
***Stands for $p \leq 0.01$.*

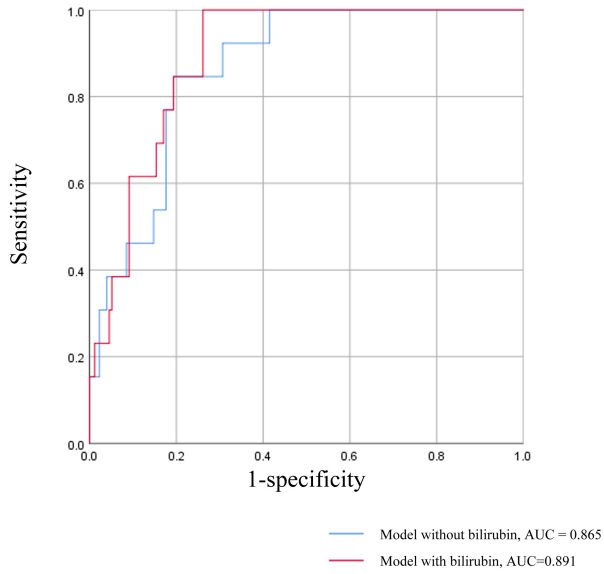

FIGURE 3 | ROC curves for the survival model in prediction of the poor renal prognosis. The survival model comprising the variables of serum bilirubin, age at biopsy, blood pressure, proteinuria, albumin, e-GFR, MEST score, and treatment.

to verify the prognostic role of serum bilirubin on renal outcomes. All tests were two-sided and P<0.05 was deemed statistically significant.

RESULTS

Selection and the Optimal Cutoff Value

According to the inclusive and exclusive criteria, 189 patients with IgAV-N (87 men and 102 women) were enrolled in our study (**Figure 1**). The bilirubin levels of patients in our cohort were ranged from 1.9 to 44.6 umol/L. Only 4 patients had slightly increased bilirubin levels (>28.0 umol/L) while the vast majority of IgAV-N patients were within normal range. The optimal cutoff value of serum total bilirubin calculated by ROC curve was 6.35 umol/L.

Actually, there were many group methods of data handling in statistics, such as ROC and terstiles. In our study, patients were divided into two groups by the optima cut-off value calculated by ROC curve. Many studies have adopted the similar methods (13, 14). Additionally, the terstiles is also a reasonable method to stratify patients. We also performed the analysis based on the interquartile range and the results were similar (**Supplementary Information**). Notably, the optimal cutoff value of serum total bilirubin calculated by ROC curve was 6.35 umol/L, which was very closed to the first quarter 6.60 umol/L. Due to the lower incidence in adult patients with IgAV-N, only 189 patients were enrolled in our study. It is difficult to make the further statistical analysis according to quartile. Hence, we used the former method.

Characteristics in Baseline

IgAV-N patients were divided into HsB group (147, 77.8%) and LsB group (42, 22.2%) based on the cutoff value (**Table 1**). The results suggested that patients in LsB group tended to have higher levels of SBP, proteinuria, glomeruli crescent rates, and serum creatinine and lower levels of serum albumin and hemoglobin. However, other variables were not significantly different ($P > 0.1$). **Table 2** demonstrated the treatment options in different groups. No obvious differences were found in both groups except that patients in LsB group tended to have higher rates of pulse therapy (23.8 vs. 12.2%, $P = 0.063$).

PS matches was performed to balance the baseline covariates, which might potentially influence the outcomes. Thirty-four patients (17 men and 17 women) were assigned to the LsB group while 55 patients (36 men and 19 women) were categorized into the HsB group. PS was derived from a multi-logit regression model with parameters including SBP, excretion levels of 24-h urinary protein, serum albumin, creatine, hemoglobin, presentation of crescents and so on, then there were no differences in clinicopathological manifestations and treatments except serum bilirubin levels between groups (**Tables 1**, **2**).

Outcomes of Renal Survival

In the unmatched cohort, Kaplan-Meier analysis demonstrated that 3.4% (5 out of 147) and 19.0% (8 out of 42) patients reached the composite endpoints in the HsB group and the LsB group, respectively (**Figure 2A**). This result suggested that patients with lower levels of serum bilirubin seemed to have a worse outcome ($P = 0.001$). Similar result was observed in the matched cohort,

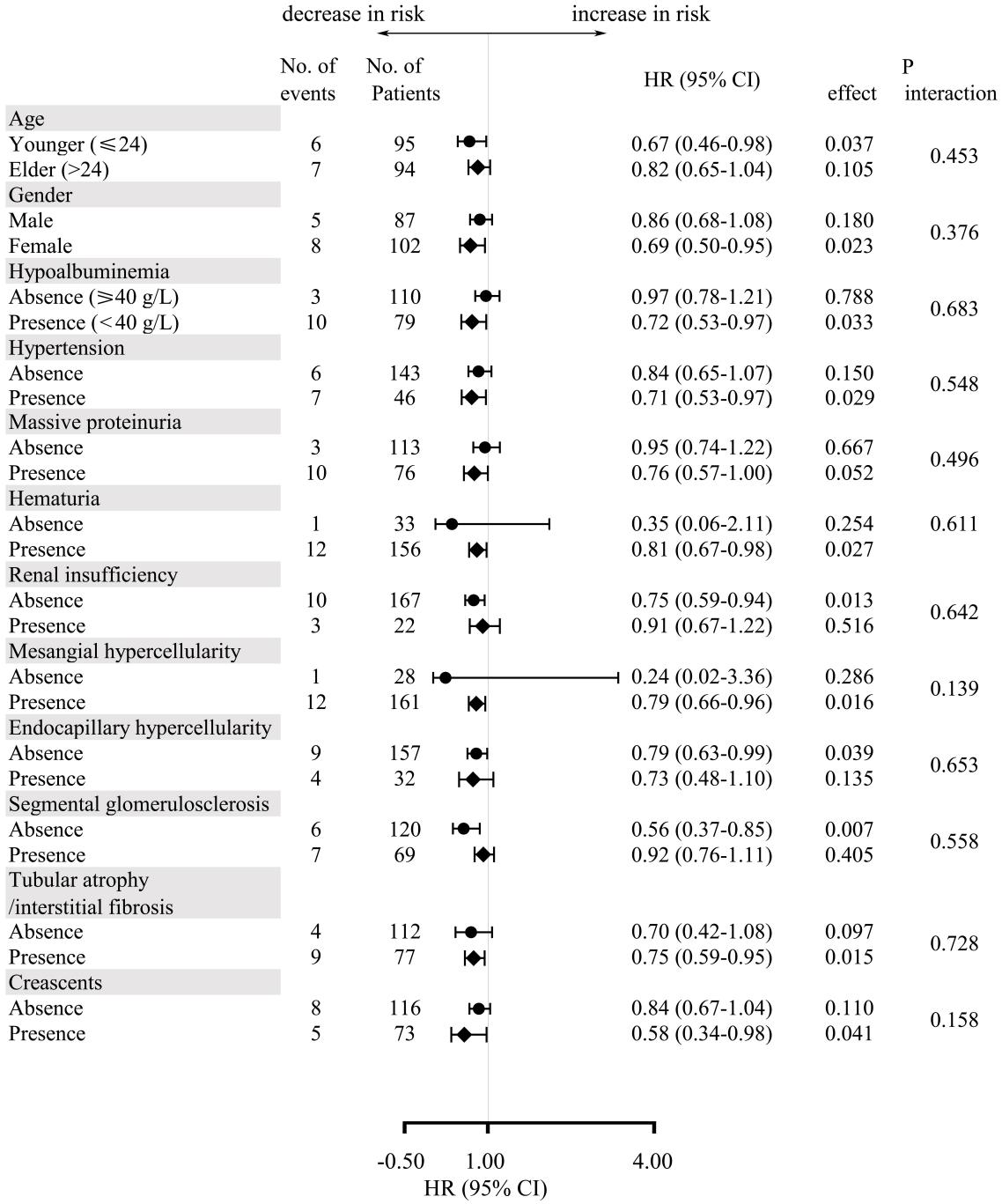

FIGURE 4 | Multivariate-adjusted hazard ratios and 95% confidence intervals for the development of composite endpoints for every 1 umol/L increase in serum bilirubin according to subgroups of baseline characteristics.

3.6% (2 out of 55) and 14.7% (5 out of 34) reached the composite endpoints ($P = 0.050$, **Figure 2B**).

Prognostic Role of Serum Bilirubin

Multivariate Cox analysis adjusted for clinicopathological confounding factors such as SBP, diastolic blood pressure (DBP), serum albumin, e-GFR, cholesterol, proteinuria, u-RBC, anemia and pathological changes (MESTC), strongly illustrated that serum bilirubin was an independent protective factor of prognosis in both unmatched cohort (HR = 0.17, 95% CI, 0.03–0.99; $P < 0.05$) and matched cohort (HR = 0.05, 95% CI, 0.00–1.03; $P = 0.050$) (**Table 3**).

A survival model comprising the variables of serum bilirubin, age at biopsy, blood pressure, proteinuria, albumin, e-GFR,

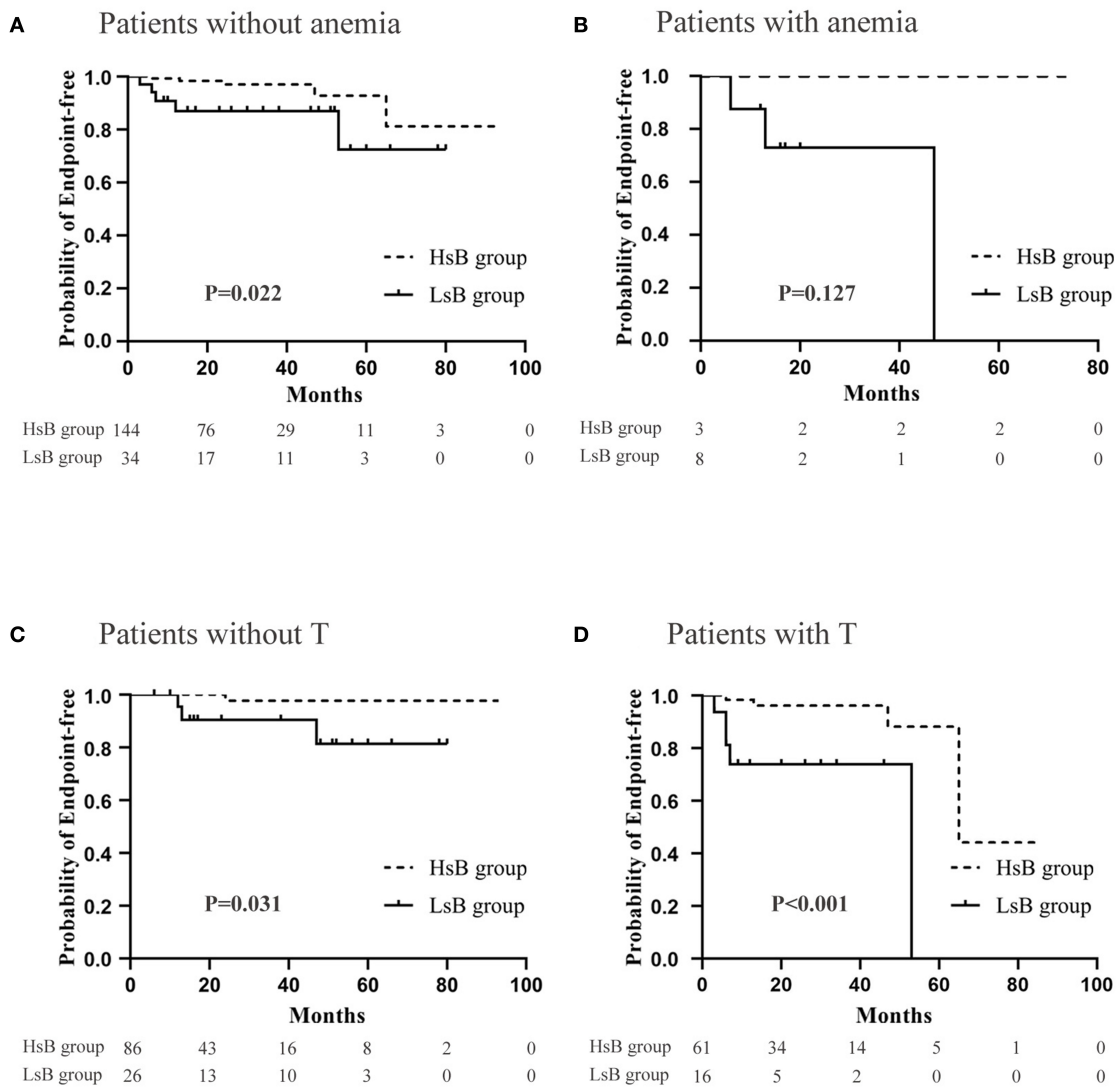

FIGURE 5 | Renal outcomes between high serum bilirubin (HsB) group and low serum bilirubin (LsB) group adjusted for anemia and tubular atrophy/interstitial fibrosis. **(A)** Patients without anemia. **(B)** Patients with anemia. **(C)** Patients without T. **(D)** Patients with T.

MEST score, and treatment was established to illustrate the predictive power of serum bilirubin, which was measured by ROC curves (**Figure 3**). The AUC value of the survival model was 0.891. But when serum bilirubin was removed, the AUC value dropped to 0.865. Accordingly, the discrimination of poor prognosis could be improved by adding the clinical indicators of serum bilirubin.

Subgroup analysis stratified by potential confounders in unmatched cohort was carried out to verify the consistency of the correlation between serum bilirubin and the renal outcomes (**Figure 4**). The multivariate-adjusted hazard ratios demonstrated that there was a trend that every 1 umol/L increase would protect IgAV-N patients from the composite endpoints. Moreover, baseline characteristics including age, gender, hypoalbuminemia, hypertension, massive proteinuria, hematuria, renal insufficiency, M, E, S, T, and C, had no multiplicative interaction with serum bilirubin ($P > 0.10$). Similar results had also been found in the matched cohort.

Renal Survival Adjusted for Anemia and T Injury

It has been well-acknowledged that anemia and tubular atrophy/interstitial fibrosis might result in an adverse prognosis. And the predictive role of bilirubin, a degradation product of heme, might be also influenced by anemia and tubular atrophy/interstitial fibrosis. Therefore, subgroup analysis was carried out in order to eliminate their potential impacts. It was easily found that patients in HsB group had a significantly better prognosis (**Figure 5**). Notably, the difference of prognosis between HsB group and LsB group in patients with anemia

was not statistically significant ($P = 0.127$). These resulted from the small sample size but we could already see the trend that patients with lower level of serum bilirubin had more unfavorable outcomes. Hence, it was quite reasonable to arrive at the conclusion that serum bilirubin could serve as an independent predictor in IgAV-N regardless of anemia and tubular atrophy/interstitial fibrosis.

DISCUSSION

IgAV-N is a common vasculitis in children, whose morbidity is about 10/100,000 every year. There are few high-quality researches of adolescent and adult IgAV-N due to a lower incidence in them. So, the present study was conducted to find out an effective prognostic marker to help clinicians have a better understanding of adolescent-onset and adult-onset IgAV-N. To our knowledge, this study is the first one to demonstrate that serum bilirubin levels could serve as an independent protective factor of IgAV-N progression. The bilirubin levels of the vast majority of IgAV-N patients in our cohort were within normal range. In this study, we found that patients with lower levels of serum bilirubin often presented with more severe clinical manifestations such as hypoproteinemia, hypertension, large amount of proteinuria, and renal dysfunction. Conversely, patients with higher serum bilirubin level tended to have a better prognosis, with only 3.4% patients reaching the composite endpoints. Moreover, multivariate Cox regression analysis also proved a strong protective effect of serum bilirubin on renal survival (HR = 0.198, 95% CI, 0.041–0.955; $P < 0.05$), adjusted for confounding factors including SBP, DBP, serum albumin, e-GFR, cholesterol, proteinuria levels, u-RBC, and renal pathological changes. Consistent with our findings, similar relationship between bilirubin and e-GFR were found previously in diabetes patients (15, 16). Considering that different baseline data might contribute to discrepancies, PS matches were applied to make the indicators more comparable. After that, similarly, 20.6% patients in the LsB group had remarkable deterioration of renal function while only 5.8% in the HsB group, with confirmed the renal protection effect of higher serum bilirubin levels.

Several literatures have reported that higher serum bilirubin level could prevent adverse renal outcomes in CKD (5, 17, 18). A meta-analysis also illustrated that the risk of progressing to CKD might get decreased due to the elevated bilirubin levels (6). In addition, Tanaka et al. found that serum bilirubin levels were negatively correlated to pathological findings based on Oxford classification of IgAN (4). In our study, the proportion of presentation of glomeruli cellular/fibrocelluar crescents in patients of LsB group was distinctly higher (52.4 vs. 34.7%, $P = 0.038$), which might lead to the poor renal outcome.

Considering the multiple function of bilirubin, it is hypothesized that bilirubin might affect the renal prognosis of IgAV-N through three main effects, which are antioxidant effects, anti-inflammatory effects, and endothelial protection. Firstly, it has been proved that increased oxidative stress contributes significantly to the development of CKD and reactive oxygen species (ROS) is permeated in the whole process of tissue damage in IgAV-N (19, 20). Bilirubin is a potent endogenous antioxidant and cellular protectant, which is capable of scavenging free radical production and prevent oxidation reactions observed in glomerulonephritis such as IgAN (21). Secondly, serum bilirubin is shown to possess potent complement inhibitory and anti-inflammatory effects. Complement activation play a crucial part in the pathogenesis of IgAV-N. Higher serum bilirubin could inhibit the complement activation and inflammatory cells infiltration. The anti-inflammation effect of bilirubin can be explained by the decreased chemoattractant and cytokine levels, the inhibition of complement and other potential mechanisms, which substantially diminish the infiltration of inflammatory cells like neutrophils (22). Thirdly, it is known that IgAV-N is a common vasculitis with prominent vascular damage. Further evidences have proved a positive correlation between ESRD and endothelial dysfunction, which can potentially give rise to atherosclerosis, vascular stenosis and hypertension (23, 24). It was reported that moderate higher level of bilirubin has a great advantage on endothelial protection and antihypertensive effects (25). Therefore, IgAV-N patients might benefit from the relatively elevated bilirubin concentration.

In theory, adding bilirubin appropriately has renoprotective effects on IgAV-N patients, which might be a new therapeutic method. Phycocyanobilin, an organic matter obtained from phycocyanin, could combine with biliverdin reductase to give rise to an analog of bilirubin. An animal experiment carried out by Zheng et al. suggested that oral administration of phycocyanobilin could avoid diabetic mice suffering from diabetic nephropathy (7). Unfortunately, the evidence that IgAV-N patients could get benefit from it is still lacking. Consequently, further studies are required to clarify whether low serum bilirubin could serve as a future therapeutic target.

There are several limitations in our study. Firstly, individuals were recruited in a single medical center with a relatively small sample size and all the subjects were Han Chinese. Hence, it might not be perfectly safe to apply our findings to other ethnic groups. Secondly, our study was based on clinical observation, intervene experiments may be needed to further verify the hypothesis. Finally, potential confounders adjusted in this study were limited. Other unmeasured confounders like insulin resistance, treatment regimens and smoking status could not be eliminated in the present study.

CONCLUSION

To sum up, elevated bilirubin levels are correlated to the favorable renal outcomes. Further studies are required to clarify whether IgAV-N patients could get benefit from administration of bilirubin.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**supplementary material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the West China Hospital of Sichuan University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

GP and JT: conception and design of the study, collection and analysis of data, drafting of the article, critical revision for important intellectual content, and final approval of the version to be published. YT, YX, and TH: collecting the follow-up data, analysis and interpretation of statistics, critical revision for important intellectual content, and final approval of the version to be published. LT, ZZ, and PT: analysis and interpretation of data, critical revision for important intellectual content and final approval of the version to be published. WQ and YT: the presenter of this project, who put forward the concept and designed the study, revised the important intellectual content critically and agrees to publish the final version. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.596151/full#supplementary-material

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REVIEW
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The Th17/IL-17 Axis and Kidney Diseases, With Focus on Lupus Nephritis

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Systemic lupus erythematosus (SLE) is a disease characterized by dysregulation and hyperreactivity of the immune response at various levels, including hyperactivation of effector cell subtypes, autoantibodies production, immune complex formation, and deposition in tissues. The consequences of hyperreactivity to the self are systemic and local inflammation and tissue damage in multiple organs. Lupus nephritis (LN) is one of the most worrying manifestations of SLE, and most patients have this involvement at some point in the course of the disease. Among the effector cells involved, the Th17, a subtype of T helper cells (CD4+), has shown significant hyperactivation and participates in kidney damage and many other organs. Th17 cells have IL-17A and IL-17F as main cytokines with receptors expressed in most renal cells, being involved in the activation of many proinflammatory and profibrotic pathways. The Th17/IL-17 axis promotes and maintains repetitive tissue damage and maladaptive repair; leading to fibrosis, loss of organ architecture and function. In the podocytes, the Th17/IL-17 axis effects include changes of the cytoskeleton with increased motility, decreased expression of health proteins, increased oxidative stress, and activation of the inflammasome and caspases resulting in podocytes apoptosis. In renal tubular epithelial cells, the Th17/IL-17 axis promotes the activation of profibrotic pathways such as increased TGF-β expression and epithelial-mesenchymal transition (EMT) with consequent increase of extracellular matrix proteins. In addition, the IL-17 promotes a proinflammatory environment by stimulating the synthesis of inflammatory cytokines by intrinsic renal cells and immune cells, and the synthesis of growth factors and chemokines, which together result in granulopoiesis/myelopoiesis, and further recruitment of immune cells to the kidney. The purpose of this work is to present the prognostic and immunopathologic role of the Th17/IL-17 axis in Kidney diseases, with a special focus on LN, including its exploration as a potential immunotherapeutic target in this complication.

Keywords: lupus nephritis, Th17 cells, inflammation, fibrosis, interleukin-17

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a disease characterized by hyperreactivity to the self, with the polarization of the immune response to a proinflammatory profile (1, 2), autoantibodies production (3), immune complex formation (4) and deposition in tissues (5). It also occurs with local production of inflammatory mediators, and additional recruitment of inflammatory cells,

resulting in tissue damage in various organs (6). These events together express the dysregulation of the local and systemic immune response, which characterize the disease (1, 7, 8). Lupus nephritis is one of the most worrying organic affectations of lupus, one of the strongest predictors of a poor outcome in SLE, being responsible for the greater burden attributable to the disease, mainly in the low-income populations (9, 10).

Several immunological pathways are involved in the pathogenesis of SLE (11), which calls us to go deeper in the knowledge about the immunopathologic complexity of this disease, aiming to explore new opportunities for targeted therapies (12). Among the effector cells, the Th17, a subtype of T helper cells (CD4+), is one of those that has shown significant hyperactivation (1, 13–15); correlates with disease activity (1, 16, 17), being involved in many manifestations of SLE as neuropsychiatric (18, 19), cutaneous (20, 21), and lupus nephritis (1, 22, 23); and correlates with the fatality of the disease (24).

Th17 cells have IL-17A and IL-17F as major cytokines, and the Th17/IL-17 axis has dominantly an effector and proinflammatory functional profile (25, 26), being involved in the pathogenesis of many immune-mediated diseases (27–30). The receptors for the IL-17 family (IL-17RA, IL-17RC, IL-17RE) are expressed in most intrinsic kidney cells (podocytes, tubular epithelial, mesangial, and renal endothelial cells) (31–35), and are involved in the promotion of a proinflammatory environment, disruption in the morphology and function of nephron elements (36, 37), in the activation of many profibrotic pathways (36, 38), which results in fibrosis, loss of architecture (37, 39), and consequent loss of organ function (23, 40).

Other studies highlight the predominant role of the Th17/IL-17 axis in LN that even in models deficient in TNF receptors (another potent proinflammatory cytokine), Th17-associated pathways were sufficient to cause the clinical and pathological changes of lupus nephritis (41). In addition, other Th17-related elements of the immune response participate in this process (16, 42, 43). Thus, IL-23 (involved in the differentiation and maintenance of Th17 by an autocrine mechanism) is also increased in lupus and correlates with disease activity (44, 45). In experimental models, the RORγt (the Th17-defining transcription factor) promoted itself glomerulonephritis; and RORγt ablation or deficiency (RORγt−/−) conferred protection to experimentally induced glomerulonephritis (46, 47). This review focuses on the role of the Th17/IL-17 axis in the immunopathology and prognosis of lupus nephritis and its exploration as a potential immunotherapeutic target in this complication.

ASSOCIATION BETWEEN TH17/IL-17 AXIS AND LUPUS NEPHRITIS PROGNOSTIC FACTORS

Proteinuria, Hematuria, and Anemia

Serum IL-17 levels are significantly associated with proteinuria (48, 49); and its concentration at the baseline keeps a positive correlation with the severity of proteinuria (50). In a study involving 15 patients (who underwent kidney biopsy), using the laser microdissection technique, the percentage of IL-17+ TCR+ among kidney-infiltrating cells correlated positively with hematuria in LN (51). In another study, elevated serum levels of IL-17 and IL-6 were associated with anemia (52).

Severity Scores and Histological Activity

Th17 cell frequencies significantly correlated with SLEDAI and inversely with C3 (53) and the concentration of IL-17 at the baseline kept a positive correlation with other parameters of severity (ESR, SLEDAI scores, and ANA titers) (50). In relation to histological activity, Th17 cell frequencies in peripheral blood and IL-17 levels in serum correlated significantly with renal biopsy classification for LN (43, 49, 53). A significant positive association has been found between serum IL-17 (and TWEAK) levels and nephritis activity index (54). In another study, the frequencies of circulating Th17-cells correlated positively with histological activity index, cellular crescent, and endocapillary proliferation. Additionally, intraglomerular levels of IL-17 and IL-23 were significantly higher in class IV LN than in MCN patients or HC (43). In another study, with measurement of urinary IL-17 (uIL-17), the levels of uIL-17 were significantly higher in the severe LN than in the control group ($P < 0.05$); and increased with disease severity seen in biopsy (mean ± SD: 43.96 ± 24.04, 55.69 ± 33.21, and 124.02 ± 256.74 pg/ml; for HC, class I-II, and class III-IV LN, respectively) (55). Another study observed that serum levels of IL-17A were significantly elevated in proliferative forms compared to non-proliferative LN (56).

Requirement for Pulse Steroids and Response to Treatment

A study found that the presence of IL-17 in renal tissue correlated with the requirement for pulse steroids ($p < 0.05$) (49). In relation to response to treatment, in a study involving 52 patients with active LN (who underwent kidney biopsy at baseline and after immunosuppressive therapy), higher IL-17 levels at baseline were associated with persisting active nephritis after treatment (WHO III, IV, V) (42). At follow-up, non-responders had higher IL-17 (and IL-23) expression by inflammatory cells infiltrating renal tissue than responders (42). On the other hand, IL-17 and IL-23 decreased significantly in patients with active LN after 6 months of therapy ($P < 0.001$) (45). Another study showed that despite a progressive decrease in serum concentrations of IL-17A and IL-21 during induction therapy, the concentration of these cytokines remained higher in the non-remission than in the remission group (50).

Renal Function, ESRD, and Mortality

Th17 cell frequencies significantly correlated with serum creatinine (53) and IL-17 was an independent risk factor for poor prognosis of LN (48). In another study, IL-17 immunostaining in biopsy correlated negatively with GFR (49). **Table 1** summarizes the clinical studies that have assessed the role of the Th17/IL-17 axis in lupus nephritis.

TABLE 1 | Clinical Studies on the role of the Th17/IL-17 axis and associated Imbalances in lupus nephritis.

References	Year	Biomarker	Patients	Main results
Xing et al. (57)	2012	Th17, IL-17, and IL-22	60 SLE patients and 28 healthy controls (HCs)	Patients with LN had a significant increase in the frequency of Th17 cells in peripheral blood, accompanied by FoxP3+ Treg cells decrease. So, the Th17/Treg ratio was significantly increased along with increased SLEDAI scores. The expression of IL-17 levels in LN patients exhibited a significant increase compared with patients without nephritis and healthy controls.
Dong et al. (58)	2003	IL-17	50 consecutively hospitalized LN patients and 15 adults HC who underwent blood samples to analyze the roles of IL-17 stimulation on the autoantibody's overproduction and IL-6 overexpression in PBMC.	In LN patients, the levels of IgG, anti-dsDNA, and IL-6 were higher in PBMC supernatants under IL-17 stimulation than in a normal culture medium. The increase in IgG, anti-dsDNA, and IL-6 levels, induced by IL-17, was dose-dependent and could be completely blocked by IL-17 monoclonal antibody and partially blocked by dexamethasone. During stimulation with IL-17, IL-6 mRNA levels were higher in LN patients than in HC (mean ± SD: 3.21 ± 0.24 vs. 1.30 ± 0.14, $P < 0.05$).
Cavalcanti et al. (59)	2017	IL-17 and IL-6	51 childhood-onset SLE patients (11 with LN) and 47 HC.	The levels of serum cytokines were significantly higher during active than inactive disease (mean ± SD: 6.14 ± 6.70 vs. 0.46 ± 1.47 pg/ml; P=0.041; and 13.64 ± 17.13 vs. 1.33 ± 0.86 pg/ml, $P = 0.02$; for IL-17, and IL-6 respectively).
Chen et al. (43)	2012	Th17 Cells, Serum and Glomerular IL-17 and IL-23 expression	24 LN patients (17 with class IV and 7 with class V), 12 HC, and 4 patients with MCD	The median frequency of circulating Th17 cells was significantly higher in LN patients than in HC [median (IQR): 0.68% (0.39–1.10%) vs. 0.12% (0.05–0.18%), $p < 0.001$]. Serum cytokine levels were significantly higher in LN patients than in HC (median: 7.26 vs. 0.82; 232.60 vs. 34.60; and 37.01 vs. 7.42 pg/ml, for IL-17, IL-6, and IL-23, respectively). The frequencies of circulating Th17-cells correlated positively with poor prognostic factors (SLEDAI, renal SLEDAI, histological activity index, cellular crescent, and endocapillary proliferation). Intraglomerular levels of IL-17 and IL-23 were significantly higher in class IV LN than in MCN patients or HC. Glomerular IL-17 and IL-23 expression levels were positively correlated with renal SLEDAI and histological activity index for LN patients.
Galil et al. (52)	2015	IL-17 and IL-6	72 SLE patients (30 with recent-onset active LN and 42 without renal disease) and 70 sex- and age-matched HC.	SLE patients were found to have significantly higher levels of IL-17 ($p < 0.001$) and IL-6 ($p < 0.001$) in relation to HC. Patients with LN had lower levels of both cytokines during periods of remission than in active disease (mean ± SD: 10.78 ± 2.38 vs. 19.54 ± 7.41 and 13.18 ± 2.73 vs. 28.46 ± 8.16, for IL-6 and IL-17, respectively, $P < 0.001$ for all). Elevated serum levels of both cytokines were associated with active LN, and anemia, and positively correlated with SLEDAI-2k scores ($P = 0.025$ for IL-17, and $P < 0.001$ for IL-6). There was a significant positive correlation between IL-6 and IL-17 serum concentrations during disease activity ($r = 0.497$, $P = 0.005$), as well as periods of remission of LN ($r = 0.662$, $P < 0.001$).
Zickert et al. (42)	2015	IL-17, IL-23, and other cytokines	52 patients with active LN who underwent kidney biopsy at baseline and after immunosuppressive treatment and 13 HC.	Baseline levels of IL-6, IL-10, IL-17, IL-23 were increased in patients vs. controls ($p < 0.001$ for all), as was IFN-γ ($p = 0.03$). Patients with persisting active nephritis after treatment (WHO III, IV, V) presented higher IL-17 levels at baseline than those who progressed without active nephritis (WHO I-II) ($p < 0.03$). At follow-up, BILAG-non-responders had higher IL-23 than responders ($p < 0.05$). This indicates that a subset of LN-patients has a Th17 phenotype that may influence response to treatment. Immunostaining of renal tissue revealed IL-17 expression in inflammatory infiltrates.
Susianti et al. (55)	2015	Urinary IL-17 (uIL-17)	50 participants with LN (38 with class III-IV, and 12 with class I-II) and 20 HC	The level of uIL-17 was significantly higher in the severe LN group than in the control group ($P < 0.05$); and increased with disease severity (mean ± SD: 43.96 ± 24.04, 55.69 ± 33.21, and 124.02 ± 256.74 pg/ml; for HC, class I-II, and class III-IV, respectively).

(Continued)

TABLE 1 | Continued

References	Year	Biomarker	Patients	Main results
Yazici et al. (49)	2014	IL-17 and FOXP3	Renal tissue samples of 39 LN patients, and normal renal tissue as control (from 20 patients with Wilms' tumor who underwent nephrectomy).	Both IFN-γ (+) and IL-17+ cells were statistically higher in LN tissues when compared with controls ($p < 0.01$). The cells in the tubulointerstitium were CD3 + CD4+, displaying a Th1 and Th17 phenotype. IL-17 immunostaining correlated with proteinuria, the requirement for pulse steroids, and SLEDAI renal score; and correlated negatively with GFR. Furthermore, glomerular and interstitial IL-17 and IFN-γ stainings were significantly associated with various parameters of histological activity ($p < 0.05$).
Kshirsagar et al. (60)	2014	Peripheral Th17 cells, IL-17, and STAT3.	17 pediatric patients with LN, 5 patients with NS, and 24 age-matched HC	Compared to controls, LN children had a higher frequency of effector IL-17 producing cells in PBMCs, added to enhanced activity of Stat3 in these cells. The mRNA expression of IL-17 and retinoic acid-related orphan receptors was also higher in LN children than in controls. Additionally, Th17 cells from children with LN exhibit enhanced migratory capacity through high Akt activity.
Sigdel et al. (56)	2016	IL-A7, Th17 cells; and Th1 cytokines	49 patients with newly diagnosed LN (12 with LN-III; 32 with LN-IV; and 5 LN-V) and 24 HC.	Serum levels of IL-17A were significantly elevated in class IV LN compared to LN-V ($p = 0.003$) or HC ($p = 0.001$). IL-6 was increased in LN-IV when compared to LN-III and HC. Th1 cytokines (IFN-γ, IL-18) were also considerably expressed in LN IV patients' serum compared to HC. Additionally, the Th17/Th2 cell cytokines IL-17A/IL-4 ratio was significantly higher in LN-IV when compared with LN-III ($p = 0.04$), LN-V ($p = 0.01$), and HC ($p < 0.0001$).
Peliçari et al. (61)	2015	IL-17 levels	67 consecutive childhood-onset SLE patients, 55 first-degree relatives, and 47 age- and sex-matched healthy controls.	The serum IL-17 level was significantly higher in SLE patients than in HC [median (IQR): 36.3 (17.36–105.92) vs. 29.47 (15.16–62.17) pg/mL, $p = 0.009$]. There was an association between serum IL-17 levels and active nephritis ($p = 0.01$). Serum IL-17 levels were not associated with disease activity ($p = 0.32$), cumulative damage ($p = 0.34$), or medication use ($p = 0.63$).
Saber et al. (53)	2017	Peripheral Th17 cells and urinary IL-17	45 patients with SLE and 20 matching HC.	Th17 frequency and urinary level of IL-17 were significantly higher in patients than controls. Th17 cell frequencies and uIL-17 levels significantly correlated with renal biopsy classification for LN. Th17 cell frequencies significantly correlated with serum creatinine and SLEDAI; and inversely with C3 ($p = 0.003$), while uIL-17 significantly correlated with proteinuria and erythrocyte sedimentation rate.
AlFadhli et al. (62)	2016	Th17-related genes, IL-17A, and IL-17F	66 SLE patients (14 with LN) and 30 matched HC	Patients with LN had significantly higher serum concentrations of IL-17A ($P = 0.002$) and IL-17F ($P = 0.002$) than those without LN. Compared to HC, patients with SLE presented a difference in the expression of 14 Th17- related genes, including IL-17A and IL-17F.
Jakiela et al. (63)	2018	Th17 and Treg	33 LN patients and 19 HC.	The percentage of circulating Th17 among CD4+ cells was increased in LN compared to HC [median (IQR): = 1.2 (0.5–1.8) vs. 0.6% (0.32–0.95), $P < 0.01$]; without significant difference on Treg. Th17 expansion in the patient group was associated with a higher cumulative dose of cyclophosphamide but was not related to LN activity, renal histology, or blood and urine inflammatory biomarkers.
Wang et al. (50)	2018	Th17 cytokines (IL-17A and IL-21)	28 LN patients on induction therapy were assessed for serological data at weeks 0, 12, and 24.	There was a progressive decrease in serum concentrations of IL-17A and IL-21 ($P < 0.01$, $P = 0.001$, respectively) during induction therapy. The concentration of these cytokines remained higher in the non-remission than in the remission group. Additionally, the concentration of these cytokines at the baseline kept a positive correlation with the severity of proteinuria, ESR, SLEDAI scores, and ANA titers.

(Continued)

TABLE 1 | Continued

References	Year	Biomarker	Patients	Main results
Edelbauer et al. (64)	2012	Th17, IL-17, and IL-23	23 patients with definite LN, 12 patients with frequently relapsing NS, and 20 age-matched HC.	There was a significant expansion of Th17 and Th1/Th17 cells in children with LN greater than in HC. Serum IL-17 and IL-23 levels correlated positively with the renal SLEDAI ($r = 0.5516$, $p = 0.0029$, and $r = 0.6116$, $p = 0.0007$, respectively).
Cheng et al. (48)	2019	IL-17	45 LN patients and 50 HC.	The IL-17 serum levels were significantly higher in LN patients than in the control group ($P < 0.001$). Serum IL-17 in LN patients was positively correlated with urinary protein ($r = 0.436$, $P < 0.05$). IL-17 was an independent risk factor for poor prognosis of LN ($P < 0.05$)
Dedong et al. (45)	2019	IL-17 and IL-23	80 patients with LN (37 of them accepted immunosuppressive therapy and followed up for 6 months) and 20 HC who underwent blood samples to analyze the roles of IL-17 and IL-23 in monitoring activity and predicting response to treatment in LN.	Baseline IL-17 and IL-23 were higher in patients with active LN than in those with inactive LN or controls ($P < 0.001$). IL-17 kept an inverse correlation with C3 ($r = -0.44$, $P < 0.001$). IL-17 and IL-23 decreased significantly in active LN patients after 6 months of therapy ($P < 0.001$). The baseline level of IL-23 was a predictor of response to the immunosuppressive treatment in patients with active LN, being lower in the complete response than in the partial response group ($P = 0.0015$) or non-response group ($P = 0.013$). IL-17 and IL-23 correlated with SLEDAI ($P < 0.001$).
Nakhjavani et al. (54)	2019	Serum IL-17 and TWEAK	50 lupus patients (25 with LN and 25 without) and 39 HC, who underwent blood samples to evaluate serum IL-17 and TWEAK as biomarkers to detect renal damage.	Increased levels of IL-17 and sTWEAK were observed in SLE patients compared to HC, and in LN compared to non-LN groups. There was a significant positive association between serum IL-17 and TWEAK levels and SLEDAI, proteinuria, nephritis activity index, and other clinical manifestations ($P < 0.05$).
Elkoumi et al. (65)	2012	IL-17A gene polymorphisms for three SNPs (rs2275913, rs8193036, and rs3748067).	320 Egyptian children and adolescents, diagnosed with JSLE (217 with and 103 without LN) and 320 matched HC.	The SNPs of IL-17 rs2275913 were significantly more frequent among JSLE patients than HC (21 vs. 7%, OR: 3.8; and 37 vs. 29%, OR: 1.4, for A/A genotype and A allele, respectively; $p < 0.003$ for both). No significant difference was found for other SNPs. Patients carrying the IL-17 SNPs rs2275913 were more likely to develop LN (OR: 5.64 and OR = 2.73, for A/A genotype and A allele, respectively).
Rastin et al. (66)	2016	IL-17, IL-6, IFN-γ, and Foxp3 genes.	20 patients with LN class IV, 20 sex- and age-matched SLE patients without LN as control who underwent blood samples.	The levels of IL-6, IL-17, IFN-γ, were significantly increased in patients with LN class IV than in those SLE patients without LN. The expression of Foxp3 genes was also significantly increased among class IV LN compared to those without; however, no significant difference was found in TGF-β expression between groups, suggesting the insufficient capacity of Treg to control the pathogenic role of IL-17-producing cells.

BILAG, British Isles Lupus Assessment Group; ESR, erythrocyte sedimentation rate; HC, healthy controls; IHC, Immunohistochemistry; IQR, interquartile range; JSLE, juvenile systemic lupus erythematosus; LN, lupus nephritis; MCD, minimal change disease; NS, nephrotic syndrome; OR, odds ratio; PBMC, peripheral blood mononuclear cells; SLE, Systemic lupus erythematosus; SLEDAI, Systemic lupus erythematosus disease activity index; SNPs, single-nucleotide polymorphisms; STAT3, Signal transducer and activator of transcription 3; TWEAK, Tumor necrosis factor-like weak inducer of apoptosis; uIL-17, levels of urine interleukin-17; WHO, world health organization.

THE TRACK AND FOOTPRINTS OF TH17/IL-17 AXIS HYPERACTIVITY IN LUPUS

From Extracellular Chromatin to APCs Maturation

An early event in the classical immunopathogenesis of SLE is the easy release of intracellular content into the extracellular space, the breakdown of immune tolerance to self, and autoantibodies production (4, 67). Components released into extracellular space function as danger-associated molecular patterns (DAMPs) (4, 68) and are recognized by dendritic cells, and other antigen-presenting cells (APCs), through toll-like receptors (TLR4) present in their plasma membrane (69–72). On the other hand, autoreactive B cells respond to immunogenic DNA with autoantibodies production (67, 71, 73), which APCs also internalizes (through FCγRII) as DNA-containing immune complexes (68) and then recognized by TLR7 and TLR9 present in endosome (69, 74, 75). The binding of DAMPs to TLRs in APCs induces their maturation (76, 77). Mature APCs, in turn, drive lymphocyte activation (78).

Activation and Differentiation of Th17 Cells

Th17 cells differentiate from naive T auxiliary cells, according to microenvironmental factors, in the presence of IL-1β, IL-6, IL-23, and TGF-β, which are the key cytokines for its differentiation

(78–80) and requires the lineage-specific transcription factor retinoid-related orphan receptor-gamma (RORγt) (80, 81). As described above, Mature APCs (after the binding of DAMPs to TLRs) trigger lymphocyte activation by the interaction of MHC II with TCR and several co-stimulatory molecules (15, 78, 80, 82). In the context of this interaction, mature APCs produce the key cytokines for Th17 differentiation (78, 80), using nuclear factor kappa B (NF-κB) and/or mitogen-activated protein kinase (MAPK) as signaling pathways (72, 77, 83). These cytokines bind to their respective receptors in naive CD4+ T Cells and trigger a chain of events downstream involving the Signal transducer and activator of transcription 3 (STAT3), which stimulates the synthesis of IL-17 and IL-21 either by binding directly to their genes or by activating RORγt (80, 84). Interestingly, T cells from SLE patients presented enhanced Stat3 activity added to higher RORγt expression (85). Once differentiated, Th17 cells secrete their cytokines (IL-17A, IL-17F, IL-17C, IL-21, and IL-22), most of them with a pathogenic role in the kidney (32, 86–88), in addition to its systemic effects (1, 15).

Regarding Th17 cells differentiation, it is also worth mentioning that podocytes, mesangial cells, and renal tubular epithelial cells can behave as antigen-presenting cells (89–92). So, these intrinsic renal cells can alone trigger the local activation of Th17 cells after recognizing, processing, presenting eventual DAMPs that cross the glomerular filtration barrier, as seen in other kidney disease models (89, 93, 94). Interestingly, a study showed that IL-17 (and IFNγ) upregulated the expression of MHC-I, MHC-II, and co-stimulatory molecules (CD80 and CD86) on the podocyte surface. Moreover, under IL-17 stimulation, podocytes increased the uptake and processing of antigen, resulting in the presentation of its peptide on the cell surface (93). This fact brings robustness to the idea that, in part, naïve T cells can enter the kidney and continue, under local factors, in the path of differentiation and activation to Th17 (95, 96). A recent study reinforces this thesis by demonstrating that, in kidneys of patients with ANCA-associated glomerulonephritis, Th17 cells develop from CD4+ tissue-resident memory T cells and exacerbate renal pathology by secreting IL-17A (97, 98).

Th17 Polarization in SLE and Related Immunes Imbalances

Although the same general mechanisms regulate the activation of all T-cell subtypes (effector and regulatory) it is worth emphasizing that in the lupus autoimmunity environment, there is favoritism of self-reactive effector (4, 73, 99); with the detriment of regulatory cells (17, 97, 100). One of the bases of this polarization lies in the fact that there are plasticity and reciprocity between Th17 and Treg (100–102), a balance influenced by various factors (103), and the inflammatory and autoimmunity environment of lupus favors to the side of the Th17 cells (2, 79, 101). Added to Th17/IL-17 axis overactivity, LN is characterized by decrease, suppression, or dysfunction of Treg cells (57, 104) and impairment of other protective factors like IL-2 (97) and IL-10 (105, 106).

Several aspects present in SLE favor the polarization of CD4+ cells to a proinflammatory profile (Th1 and Th17) (2, 107). This range from phenotypic and functional aberrations of APCs (2, 108, 109) to T cells specific aspects, like changes in immunometabolism (marked glycolysis, lipid synthesis, glutaminolysis, and hyperactivation of the mTOR pathway) (110, 111), and abnormalities in signaling pathways (112, 113). In addition, epigenetic changes, such as histone hypomethylation at naive CD4+ T Cells level, have also been described to favor Th17 polarization (114, 115) and were early events before lupus flares (114). Additionally, dysbiosis, a characteristic also present in lupus (116, 117), is a potentiating factor for Th17/IL-17 polarization (118); and a study has even shown that autoimmune kidney disease is exacerbated by the migration of pathogenic Th17 cells from the intestine to the kidney (119).

In the lupus autoimmunity environment, other elements of the immune response participate in Th17 polarization, as shown in an experimental study with lupus-prone mice, in which dendritic and B cells increased Th17 expansion, associated with limited Treg expansion, and increased renal infiltration by Th1 and Th17 cells (2). In another study, basophils obtained from patients with SLE promoted Th17 differentiation from SLE naïve CD4+ T cells *in vitro* coculture (120). Even cells with a dominant protective role, like Treg cells, in the lupus nephritis background, have been shown to facilitate the proliferation of Th17 lymphocytes and are less suppressive (47, 63). Several other elements of the immune response favor polarization to Th17 in the context of lupus (121).

MECHANISMS UNDERLYING THE EFFECTS OF TH17/IL-17 AXIS IN THE KIDNEY

Recruitment of Th17 Cells to the Kidney

Th17 cells are attracted to the kidney by chemokines CCL20, CXCL9, and CXCL10 (33, 122, 123) through binding to their receptors (CXCR3 and CCR6) expressed on the surface of these cells (124–126). Recruitment is facilitated by the enhanced migratory capacity of Th17 cells from SLE patients, through high Akt activity (60) and involvement of calcium/calmodulin-dependent kinase IV (CaMK4) (96, 127). Most intrinsic kidney cells (podocytes, mesangial, and kidney tubular cells) secrete CXCL9, CXCL10, and CCL20 in response to injury (23, 123, 128). In an experimental study, stimulation of mesangial cells with nucleosome-containing immune complexes resulted in their activation and expression of CCL20 (129). More recently, it has been shown that components of the extracellular matrix, produced by injured cells, stimulate resident macrophages to produce CCL20, CXCL9, and CXCL10, cooperating in this way in the recruitment of Th17 cells (130).

Once in the kidney, Th17 cells maintain the phenotypic and functional features through several other factors, as demonstrated in models where local T cells had elevated expression of inducible T cell costimulator (ICOS) coreceptor and were protected from apoptosis by elevating the activity of the PI3K-Akt signaling pathway. These features together result in facilitating the accumulation of active T cells in the kidney (131, 132). Additionally, a clinical study revealed that LES patients

had elevated serum autoantibodies against co-inhibitory PD-1, facilitating T cell proliferation and maintaining the hyperactive phenotype; and this kept a close association with disease activity, particularly renal involvement (133).

Effects of Th17/IL-17 Axis in the Kidney

In the kidney, the Th17/IL-17 axis participates in several points of the damage chain. In summary, this involves changes in the structure and functioning of intrinsic specialized renal cells, promoting and maintaining an inflammatory environment, participating in repetitive tissue damage and maladaptive repair, leading to renal fibrosis and loss of function (23, 25). Thus, the Th17/IL-17 axis behaves as a true chief orchestrator of immunity (134, 135). The available evidence on the Th17/IL-17 axis effects on specific renal cells or compartments is described below in this review.

Glomerular Compartment

Regarding the effect of the Th17/IL-17 axis on the filtration barrier elements, there is growing evidence about the harmful effect of IL-17 on cellular elements of this barrier; however, it remains a field in need of an extensive investigation.

Podocytes

On podocytes, an experimental study raised the possibility that Th17 cells would produce a soluble mediator that enhances podocyte motility, causing rearrangement of the actin cytoskeleton and increased permeability (136). This finding may be the basis of the correlation found between IL-17 levels and proteinuria and its severity (50, 53). According to the study, this soluble factor mimics the protease-activated receptors-1 (PAR-1) activation signaling pathways (136).

Still focusing on the potential impact on the cytoskeleton of podocytes, the exposure of mice podocytes to recombinant IL-17 induced overproduction of Cmaf-inducing protein(c-mip), with consequent induction of cytoskeletal disorganization and apoptosis in adriamycin-induced nephropathy model (137). Interestingly, silencing c-mip prevented IL-17 related podocyte apoptosis by promoting persistent activation of NF-κB and upregulation of anti-apoptotic protein Bcl-2 (137). C-mip is a protein whose expression is suppressed in healthy glomeruli (138) and increased in pathological conditions and has been associated with cytoskeletal disorganization in podocytes and proteinuria (139, 140).

In another experimental study with mouse podocytes, IL-17A stimulation disrupted the podocyte morphology by decreasing podocin expression and increasing desmin expression. In this study, podocytes expressed IL-17RA, and stimulation with IL-17A induced changes associated with activation of the NLRP3 inflammasome-caspase-1 pathway, production of intracellular reactive oxygen species (ROS), and increased IL-1β secretion. Interestingly, the blockade of these downstream signaling pathways restored the podocyte morphology (141).

Additional evidence about the harmful effect of IL-17 on podocytes comes from studies involving patients with primary nephrotic syndrome (PNS) (142). In these patients, IL-17 was highly expressed in renal tissue, being higher in patients with focal segmental glomerulosclerosis (FSGS), the glomerular disease with greater fibrosing behavior. As increased the expression of IL-17 Messenger RNA (mRNA) in the tissue, decreased the expression of podocalyxin (PCX) mRNA; and the IL-17 mRNA correlated directly with the number of podocytes lost in the urine. In the complement, with *in-vitro* experiment, IL-17 induced podocytes apoptosis and reduced podocyte health proteins such as nephrin, synaptopodin, and PCX. At the same time, IL-17 induced the expression of proteins like Fas, Fas ligand (FasL), active-caspase-3, active-caspase-8, and phosphorylated-p65. These effects occurred with the involvement of NF-κB pathways, and its inhibition attenuated the IL-17-induced podocyte apoptosis, decreasing or suppressing the molecular pathways described above (142). In another study, exposure of murine podocytes to recombinant IL-17 also induced apoptosis, increased the expression of caspase-3, caspase-8, and Fas; associated with decreased PCX expression, in a dose- and time-dependent manner (143).

Mesangial Cells

In mesangial cells, stimulation with IL-17A or IL-17F induces the production and release of chemokines CCL2 and CXCL2 in a MAPK-dependent manner. Both IL-17RA and IL-17RC are expressed in these cells, and the production of the chemokines was in a dose- and time-dependent manner (32).

Glomerular Endothelial Cells

Concerning glomerular endothelial cells (GEC), there are no specific studies in these cells. What is known are the effects of the axis on the endothelium from other vascular beds (see description in the section hypertension and thrombosis). However, these effects we believe to be applicable (in whole or part) to GEC. Specific studies are needed to assess potential local-specific effects.

Glomerular Basement Membrane

Although little is known about the potential effect of IL-17 on glomerular basement membrane (GBM), the IL 17 presence was associated with GBM thickening in a model of accelerated diabetic nephropathy; while IL-17A blockade with antibody reduced this effect (144). Additionally, in a model of anti-glomerular basement membrane glomerulonephritis (anti-GBM GN), the Th17/IL-17 pathways were drivers of inflammation and autoantibody-induced renal injury; and the knockout or inhibition of IL-17 ameliorated these effects associated with decreased proinflammatory cytokines (145, 146).

Tubulointerstitial Compartment: Tubular Epithelial Cells, Fibrosis, and Epithelial-Mesenchymal Transition

Renal Tubular Epithelial Cells and Inflammation

The Exposure of RTEC to IL-17 induces the production of various mediators, from cytokines, chemokines, and growth factors as shown in several studies and experimental models, and both receptors (IL-17RA and IL-17RC) are expressed in these cells. Stimulation of RTEC with IL-17 increases the expression of various cytokines like IL-6, IL-1β, TNF-α (31, 147).

In a model of crescent glomerulonephritis by lupus, RTEC stimulated with IL-17 and IFN-α significantly increased the expression of CCL2, which is chemotactic for dendritic cells and macrophages. In mice lacking IL-17RA, renal infiltration by macrophages was severely impaired, despite unchanged systemic response (147). In addition, stimulation of tubular epithelial cells with IL-17 increased mRNA expression of other chemokines like Cxcl1, Cxcl2, and Cxcl8, which are chemotactic for monocytes and neutrophils, as found in models of autoimmune glomerulonephritis. These effects were synergically potentiated by TNF-α (148). Thus, under IL-17 stimulation, RTEC produces mediators that recruit dendritic cells and macrophages that are important sources of TGF-β to promote renal fibrosis (149) putting IL-17 as an important driver of RTEC-mediated immunopathogenesis in LN.

The stimulation of RTEC with IL-17A impacts neutrophil kinetics, leading to the synthesis of the granulocyte colony-stimulating factor (G-CSF) in a dose- and time-dependent manner and this effect occurred in synergy with TNF-α- or IL-1β. The downstream signaling pathways of this effect involved MAPK activation (31). Added to G-CSF secretion, the stimulation of RTEC with IL-17A induced the expression of chemokines CXCL1 and CXCL5 that are responsive for massive neutrophil recruitment and consequent renal tissue injury (86, 150). Taken together, IL-17 is a potent orchestrator of neutrophil-mediated damage, promoting both differentiation and the recruitment of neutrophils to the kidney (31, 150).

IL-17, Renal Tubular Epithelial Cells, and Fibrosis

The Th17/IL-17 axis is a potent promoter of renal fibrosis (39, 151) as found in an experimental model of unilateral ureter obstruction (UUO), where TGF-β1 expression (mRNA and protein) were increased in the obstructed kidney (39). In the complement of the study, the addition of IL-17A to cultured renal proximal tubular epithelial cells or renal fibroblasts increased the production of fibronectin using the TGF-β/Smad signaling pathway; associated with increased expression of TGF-β1 mRNA and protein (39). Interestingly, the IL-17A-mediated fibronectin production was abrogated neutralizing TGF-β1 pathways, either by administering an anti-TGF-β1 antibody or TGF-β1 receptor I inhibitor (39).

In another study, IL-17A promoted myofibroblast activation and extracellular matrix deposition, and IL-17 deficient mice were protected from fibrosis secondary to obstruction (36). In an experimental model of hypertension and angiotensin II-induced fibrosis, the IL-17A or IL-17RA blockade with specific antibodies significantly reduced the fibrosis marker TGF-β1 (152). On the other hand, the antifibrotic effect of many agents in the kidney has been associated with reducing IL-17 (153–156).

IL-17 and Epithelial-Mesenchymal Transition

Some scant literature shows that the Th17/IL-17 axis induces Epithelial-Mesenchymal Transition (EMT) on tubular epithelial cells (157, 158). In one of these studies, with cultured cells, IL-17A promoted the cellular proliferation and secretion of extracellular matrix and induced inversion from epithelial to mesenchymal phenotype in a TGF-β1-dependent pathway (157). Despite few studies in the kidney, the effect of the axis on EMT is well-known in many organs such as bronchoalveolar epithelium (159, 160); epithelial cells of the salivary glands in Sjögren's syndrome (161, 162); biliary epithelial cells (163, 164); and peritoneal mesothelial cells (165). Concerning the promotion of the same effect on other intrinsic cells (mesangial and glomerular endothelial cells), it is an open gap to be elucidated in future investigations.

Vascular Compartment: Thrombotic Microangiopathy and Hypertension

One of the factors of poor prognosis in renal biopsy in lupus is thrombotic microangiopathy (which is the combination of endothelial injury and thrombosis). No primary studies evaluated the role of the Th17/IL-17 axis in thrombotic events in patients with lupus. However, IL-17A, IL-17RA, or IL-23 probably participate in this process because they are described as mediators of endothelial dysfunction (166, 167); and have been associated with the occurrence of arterial thrombosis (167, 168). In experimental studies, with psoriasis models, IL-17A shown to be a mediator of thrombotic events and vascular dysfunction (169, 170). In another study with endothelial cells from patients with rheumatoid arthritis (RA), IL-17 (in combination with TNF-α) induced a procoagulant and prothrombotic phenotype (beyond the inflammatory state). Mechanistically, this occurred due to the strong inhibition of the expression of CD39/ATPDase (an inhibitor of platelet activation), enhancement of tissue factor (the cellular receptor for FVII and FVII), combined to decreased thrombomodulin (167). Additionally, studies have found an increase in Th17/IL-17 axis activity in primary antiphospholipid syndrome (171, 172).

Hypertension is one of the manifestations of kidney involvement in lupus, and its presence is one of the factors of poor prognosis in LN (173, 174). There is a lack of primary studies evaluating the direct effect of the Th17/IL-17 axis in this event in LN. However, there is evidence associating the Th17/IL-17 axis with primary hypertension and renal inflammation in both experimental and human studies (175, 176). Additionally, basic research with angiotensin II-induced hypertension models shows that IL-17A deficiency or the blockade of IL-17A or IL-17RA with specific antibodies significantly reduces the pressure and inflammation in target organs (152, 177). In another experimental study, IL-17A appeared to be a key mediator of vascular remodeling of the small arteries. Increased IL-17A levels increased blood pressure by induction of arterial remodeling and stiffness. In addition, treatment with antihypertensive drugs lowered blood pressure without modifying structural changes. Conversely, blocking the IL-17A with antibodies decreased blood pressure and vascular remodeling, suggesting that it has a sustained effect on vascular structure, more than merely hemodynamic (178). So, despite the lack of primary studies focusing IL-17 and hypertension on NL, it is believed that there is a participation of the axis in this outcome since lupus is a disease that occurs with significant hyperactivity of the Th17/IL-17 axis.

The Th17/ IL-17 Axis and Local Immune Response Orchestration

Chemokines Production, Recruitment of More Immune Cells, Tertiary Lymphoid Structures Formation

As already described, the Th17/IL-17 axis induces the expression of chemokines like CXCL5, CXCL2, and CXCL8 for the recruitment of neutrophils (by binding to the receptors CXCR1 and CXCR2) (23, 32, 150), CCL2, CCL5 to attract monocytes and macrophages (by binding to receptors CCR1, CCR2, CCR5) (32, 36). In addition to IL-17-induced chemokine production, several other chemokines like CXCL13 (179, 180), CCL2, CCL7, CXCL1, CXCL2, and CXCL5 are produced by injured cells and resident macrophages, promoting the infiltration of B cells, dendritic cells, NK, Th1 cells (181–183) increasing the recruitment of more immune cells to the organ (184). The Sustained recruitment of immune cells can lead to the formation of kidney tertiary lymphoid structures (TLS), with some autonomy, in local activation of effector T cells, and *in situ* production of autoantibodies and components of the complement system (185, 186), sustaining by itself the inflammatory flame at the local level (95, 187, 188). A recent experimental study showed that IL-17A is an orchestrator of TLS formation in the kidney, and this formation is associated with intrarenal inflammation, fibrosis, and progression of kidney damage (189). Interestingly, genetic depletion of IL-17A or blockade with anti-IL-17A antibody significantly reduced TLS formation, associated with attenuation of renal inflammation and fibrosis (189).

Production of Autoantibodies in situ, Complement Activation, Immunocomplex Formation, and Tissue Deposition

The IL-17 seems to participate in the production of autoantibodies, *in situ*, probably involving tertiary lymphoid structures, as found the correlation between IL-17 and increased anti-double-stranded DNA (dsDNA) production in an experimental study with kidney biopsy (185). IL-17 also seems to participate in other *in situ* events, including complement activation, immunocomplex formation, and tissue deposition, as found in the association of its expression level with these critical events in LN (185). In another study, it was evidenced that in IL-17A–/– mice, there was a decreased glomerular IgG and complement deposition and decreased intrarenal expression of Th1-associated proinflammatory mediators (190).

Crosstalk Between Th17; Intrinsic Renal Cells and Resident Immune Cells in Kidney Diseases

There is a Crosstalk between the kidney cells and Th17/IL-17 axis since the intrinsic cells of the kidney can induce the polarization of the lymphocytic response to the Th17 profile; as shown in an experimental study, in which stimulation of podocytes with bacterial products Polarized Naive CD4+ T Cells into Th17 cells (94). In addition, there is a crosstalk between Th17/IL-17 and intrarenal immune cells as shown that resident dendritic cells and infiltrating monocytes secret IL-1β that activated intrarenal Th17 cells and enhanced the IL-17 secretion (95). Elevated levels of IL-17, in turn, stimulate intrinsic kidney cells to produce chemokines and G-CSF/GM-CSF, inducing the differentiation of neutrophils and macrophages from bone marrow and recruitment to the kidney (31, 191, 192). Together, these aspects show that IL-17 participates in the cross-talk between Th17, neutrophils, monocytes, and intrinsic kidney cells (32).

In this orchestrator role of local immune response, it was demonstrated, in a model of obstructive nephropathy, that monocytes and macrophages express the IL-17RA receptor, and the absence of this receptor in all myeloid cells resulted in a reduction in macrophage accumulation in the kidney and significant attenuation of fibrosis (192). IL-17 participates as a mediator or potentiator of renal damage caused by several other cells and molecules of the immune response. For example, in a model of obstructive nephropathy, the C3 component produced locally by macrophages promoted renal fibrosis through increasing T-cell proliferation and IL-17A expression. Furthermore, the blockade of C3a reduced IL-17A expression and tubulointerstitial fibrosis (38). The Th17/IL-17 axis even seems to be able to initiate the chain of kidney damage by itself as it possesses the property of activating the inflammasomes and the toll-like receptors (193). **Figure 1** is a schematic representation of the chain of events from hyperreactivity to self-DNA, activation and polarization of the Th17/IL-17 axis, to kidney damage and ESRD.

The Association Between Th17/IL-17 Axis Hyperactivity and Several Kidney Diseases (Other Than Lupus Nephritis)

The Th17/IL-17 axis role as a mediator of kidney damage and fibrosis has been found in various other renal diseases (in both patients and animal models) (198–200). These include primary glomerular diseases (198, 201), diabetic nephropathy (199, 202, 203), hypertensive nephropathy (175) ischemia-reperfusion models (37, 87, 200), renin-angiotensin-aldosterone system-mediated damage (204, 205), unilateral ureteral obstruction associated damage (36, 95, 206), and in ablation or unilateral nephrectomy associated damage (207).

Th17/IL-17 axis seems to increase the risk of CKD itself, as seen in a genetic study with 650 elderly, where single nucleotide polymorphism (SNP) of IL17RA (rs4819554 AA homozygotes) was significantly more frequent among individuals with eGFR $<$ 60 ml/min/1.73 m^2; and was associated to the risk of developing ESRD (40). Another study including 290 non-diabetic ESRD patients and 289 normal controls found that patients had a significantly higher frequency of IL17E rs10137082*C and IL17RA rs4819554*A alleles compared to control. At the same time, the genotyping analysis found that SNPs for IL17E (rs10137082) and IL17RA (rs4819554) were significantly more frequent among patients than in controls, after adjusting for confounders (208). It is important to highlight that this is an association and not necessarily a causal relationship because SNPs are hardly a causal factor alone. This is even less likely in a disease like lupus, with heterogeneous and multifactorial etiology (5, 209). However, associated SNPs may be players with additive

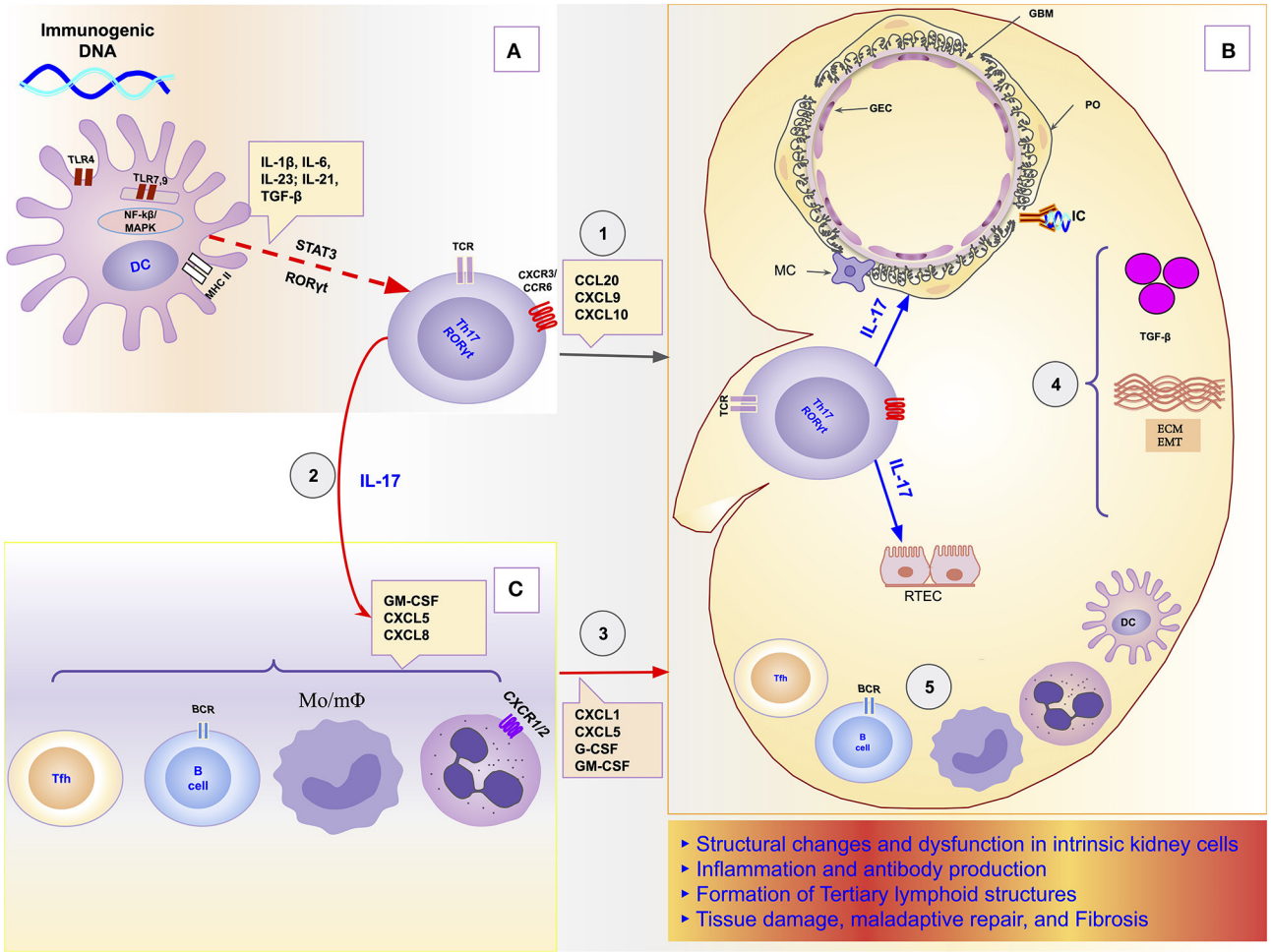

FIGURE 1 | Schematic representation of the role of the Th17/IL-17 axis in the chain of events to kidney damage and ESRD in Lupus Nephritis. **(A)** Dendritic cells sense extracellular DNA through TLR4 present in their plasma membrane (69, 71, 72) or sense phagocyted DNA-containing immune complexes by TLR7 and TLR9 in endosome (69, 74, 75). The binding of DAMPs to TLRs in APCs induces their maturation (76, 77). Part of dendritic cells migrates to draining lymph nodes to present the processed antigen to T cells and induce their activation and differentiation. The differentiation of Th17 cells is promoted by proinflammatories cytokines (IL-1β, IL-6, IL-21, IL-23) that dendritic cells secrete using the NF-κB and MAPK signaling pathways (77, 83). Dendritic cells that remained in the tissue secrete various chemokines like CXCL9, CXCL10, and CCL20 that drive the recruitment of Th17 cells to the kidney through binding to receptors (CXCR3 and CCR6) (96, 125, 126). **(B)** In the Kidney, Th17 releases its cytokines (IL-17A, IL-17F, IL-17C, IL-21, and IL-22) that act directly on intrinsic kidney cells (mesangial cells, podocytes; glomerular endothelial cells, renal tubular epithelial cells). IL-17 family cytokines are responsible for changes in the cytoskeleton of the podocytes, activation of inflammasome and caspases, and induction of oxidative stress and podocytes apoptosis. In addition, in tubular epithelial cells, IL-17 promotes the activation of the profibrotic pathways with the increase of the expression of TGF-β (36, 87), promotion of EMT (158) with consequent increase of extracellular matrix proteins and fibrosis (87). **(C)** Besides local effects, IL-17 amplifies the systemic inflammatory response by stimulating the synthesis of inflammatory cytokines, growth factors, and chemokines, resulting in granulopoiesis/myelopoiesis and recruitment of more immune cells to the kidney (31, 32, 194). In addition, it promotes autoantibody production by its effects on Tfh and GC (195, 196), and plasma cells (197). DC, dendritic cells; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; GBM, glomerular basement membrane; GEC, glomerular endothelial cells; IC, immune complex; IL-1, Interleukin-1; IL-17, Interleukin-17; IL-21, Interleukin-21; IL-23, Interleukin-23; IL-6, Interleukin-6; MAPK, Mitogen-activated protein kinase; MC, mesangial cell; *Mo/mΦ*, Monocytes/macrophages; NF-κB, Nuclear factor-κ B; PO, podocyte; RTEC, renal tubular epithelial cells; Tfh, follicular helper T cells; TGF-β, transforming growth factor-beta; Th17, T helper lymphocytes, subtype 17; TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4.

or synergistic effects at the confluence of the multi-players that characterize the disease (11, 111).

Additional evidence about the role of the Th17/IL-17 axis on Kidney diseases comes from the observation of the increased risk of CKD associated with renal inflammation in human diseases that occur with the hyperactivation of the Th17/IL-17 axis, like psoriasis (210–212), rheumatoid arthritis (213, 214), and ankylosing spondylitis (215–217). In two studies (in the United Kingdom and Taiwan), psoriasis was associated with an increased risk of chronic kidney disease independent of traditional risk factors (211, 212). The myriad of exposed situations suggests that Th17/IL-17 is a permanent participant, or at least as a pivotal element, in the pathogenesis of many kidney diseases independent of the initial insult (218); and its role

extends from initial mechanisms, ESRD, to consequences of CKD and dialysis (88, 205, 207, 219).

The role of the Th17/IL-17 axis has been found in other organic diseases that combine both inflammatory and fibrosing courses (219–221). Thus, the axis is involved in intestinal fibrosis in inflammatory bowel disease (222, 223), in pulmonary fibrosis in systemic sclerosis and cystic fibrosis (220, 224), liver cirrhosis (221, 225) and peritoneal fibrosis (219, 226). Its blockage and/or suppression has emerged as promising to prevent/mitigate the inflammatory and/or fibrosing behaviors in such conditions (226–228). **Figure 2** summarizes the Th17/IL-17 axis effects on intrinsic renal cells and immune cells with potential implications in kidney damage, particularly in lupus nephritis.

SYSTEMIC EFFECTS OF TH17/IL-17 AXIS WITH REPERCUSSIONS IN THE KIDNEY

Production of Autoantibodies

The Th17/IL-17 axis participates in the production of autoantibodies by B cells, as demonstrated in studies with autoimmune models in which the IL-17 drives the development of autoreactive germinal center (GC); and mice lacking the IL-17 receptor have reduced B cell development and humoral responses (229, 230). In another study with an autoimmune disease model, the blockade of IL-17 signaling was associated with a significant reduction in both the number and size of germinal centers (231).

The IL-17RA receptor is essential for the optimal location of follicular helper T cells (Tfh) in the light zone (LZ) of the GC to promote the production of autoantibodies by B cells (195). Additionally, the production of IL-17 initially correlates with a reduced migratory response of B cells to chemotactic like CXCL12, suggesting that IL-17 not only facilitates the interaction between Tfh and responder B cells but also prolongs this interaction by increasing the time of permanence of B cells in GC (229).

In relation to the structure and functioning of the germinal center, a lymph node study showed that IL-17 is a critical requirement for the proliferation of lymph node and splenic stromal cells, particularly fibroblastic reticular cells (FRCs), during experimental autoimmunity. Without IL-17 signaling, there was a failure in FRC proliferation (nutrient stress, arrested cell cycle, and apoptosis), resulting in the impaired germinal center formation and antigen-specific antibody production (196).

The IL-17 importance in the production of autoantibodies was also evidenced in another study in which the PBMC supernatants from LN patients expressed higher levels of IgG, anti-dsDNA under IL-17 stimulation than in a normal culture medium. This effect occurred in a dose-dependent manner, and could be blocked completely by IL-17 monoclonal antibodies or partially by dexamethasone (58). Another experimental study showed that IL-17 increased anti-double-stranded DNA antibody production, and this was the link in the correlation between cytokine levels and disease severity (185). A recent study has ratified the crucial role of IL-17 by demonstrating that IL-17 promotes autoantibody production and increases plasma cell survival. In this study, the subset of plasmocytes expressing the IL-17RC receptor had an exponential increase in the production of anti-dsDNA IgG upon IL-17A stimulation in both patients and mice. Additionally, the transfer of Th17 depleted PBMC resulted in a significant reduction of autoantibody production and attenuation of renal damage. This attenuating effect was also observed in IL-17 or IL-17RC deficient mice, while the adoptive transfer of Th17 to IL-17-deficient mice restored the plasma cell response and renal lupus damage. The most important is that IL-17 significantly promoted plasma cell survival, through phosphorylation of p38, stabilizing Bcl2l1 mRNA, which encodes the anti-apoptotic protein Bcl-xL (197).

Amplification of Systemic Inflammatory Response

Amplification of the inflammatory response is among the systemic effects of IL-17. This includes granulopoiesis and myelopoiesis by stimulating the synthesis of GM-CSF and G-CSF, increased production of chemokines, and inflammatory cytokines (31, 192); in addition to associated paralysis or impairment of anti-inflammatory pathways (105). In these effects, IL-17 makes synergy with several other inflammatory mediators such as IFN-γ, TNF-α, and IL-23 (42, 49, 232, 233). In a PBMC culture medium, stimulation with IL-17 induced significant IL-6 mRNA transcription in PBMC from LN patients than from HC (58). This study also brings the notion that, compared to controls, cells from lupus patients are hyperresponsive with higher production of inflammatory mediators under the same stimulus conditions (58).

In neutrophil kinetics, especially, IL-17 participates in various points of the chain, from differentiation through the synthesis of colony-stimulating factors (31); recruitment of neutrophils to the target organs through endothelial cell activation in a STAT3 and/or MAPK-dependent manner (34, 234) and by the synthesis of attracting chemokines like CXCL5, CXCL1; and CXCL8/IL-8 (86, 235). In relation to CXCL1, a potent chemoattractant for neutrophils, it is worth mentioning that IL-17 participates in regulating its production and in the stability of its mRNA (232, 235) and increases its biological half-life (236).

The Th17/IL-17 axis seems to have a cooperative relationship with other pathways whose importance is highlighted in the pathogenesis of SLE, like Type I interferons (6, 11, 237). This was evidenced by studies that found increased IL-17A and IL-17A-producing cells in IFN+ than in IFN- patients and HCs (238, 239). In one of these studies involving 31 patients with SLE, patients displaying high IFN-α bioactivity (58.1% of them) had a higher frequency of Th17 cells in peripheral blood than those with low IFN-α bioactivity (mean ± SD 1.9 ± 1.0 vs. 1.2 ± 0.9). Additionally, subjects with high IFN-α bioactivity and elevated Th17 cells had significantly higher disease activity and serum IL-6 levels than those with low IFN-α and Th17 cells. Suggesting that IFN-α and Th17 cell pathways co-exist and co-regulate the disease pathogenesis (238). Other studies found a significant correlation between the Th17/IL-17 axis and B-lymphocyte stimulator (BLyS/BAFF), a factor strongly correlated with IFN type I (239, 240). In another study involving 33 patients

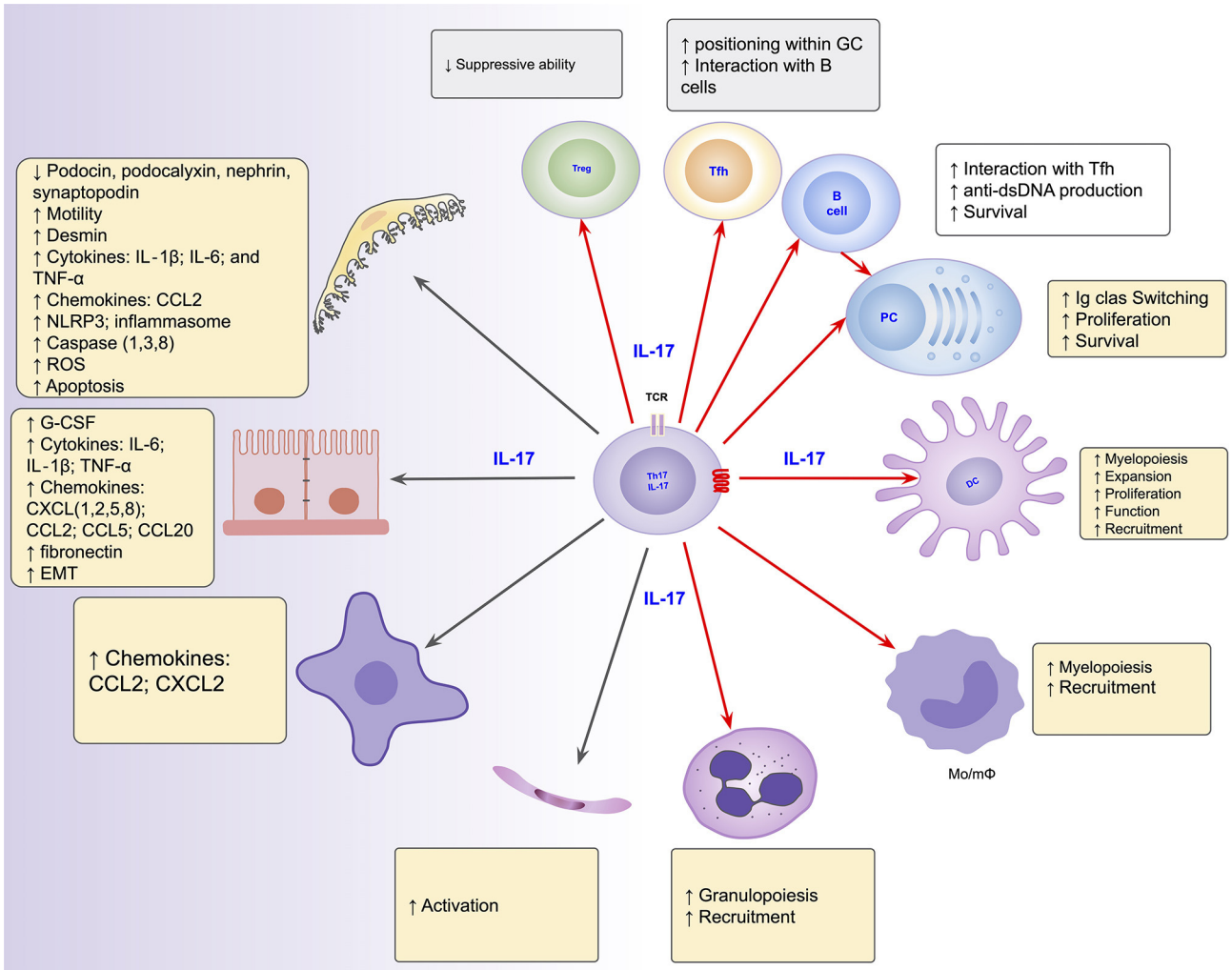

FIGURE 2 | Summarized effects of the Th17/IL-17 axis on intrinsic renal cells (black arrows), as found in several kidney disease models, and on immune cells (red arrows) potentially implicated in the induction of kidney damage in lupus nephritis. DC, dendritic cells; EMT, epithelial-mesenchymal transition; IL-1, Interleukin-1; IL-17, Interleukin-17; IL-6, Interleukin-6; Mo/mΦ, Monocytes/macrophages; PC, Plasma Cells; Tfh, follicular helper T cells; Th17, T helper lymphocytes, subtype 17; Treg, Regulatory T Cell.

with cutaneous lupus erythematosus who underwent biopsy, the level of IL-17A in tissue correlated positively with the IFN-α expression (Spearman's $\rho = 0.56$) (20).

The repercussion of this relationship between Type I IFN and the Th17/IL-17 axis in kidney damage was evident in an experimental model in which mice deficient in IL-17RA were protected from Type I Interferon-dependent crescentic glomerulonephritis. This effect was associated with impaired renal infiltration by activated macrophages, despite unaffected systemic response (147). As the underlying mechanism, the authors have shown that IL-17 in association with IFN-I differentially regulates the expression of macrophage chemoattractants genes, including Ccl2 (encoding CCL2) in RTEC (147). However, no primary study evaluated this relationship in LN in humans. A gap in knowledge to be filled in next studies, and combining genetic studies with integrative Bayesian network approaches may bring additional information to current knowledge in this disease characterized by heterogeneity (209).

The Th17/IL-17 axis may be the bridge (or part of it) between LES and other organs comorbidities and outcomes (241, 242), such as cardiovascular disease because it is known to promote endothelial activation (233, 234), prothrombotic states (167, 168), hypertension (166, 177), and atherogenesis (207, 243, 244); and osteoporosis because it is known to increase bone catabolic activity (245).

THE POTENTIAL OF TARGETING THE TH17/IL-17 AXIS AND RELATED PATHWAYS ON NEPHROPROTECTION

As described above, the Th17/IL-17 axis is involved in several points in the renal damage chain. Its effects include the induction of changes in the cytoskeleton of the podocytes

with increased motility, decreased expression of the podocyte health proteins, increased oxidative stress, activation of inflammasome and caspases, and induction of podocytes apoptosis. The axis also promotes the activation of the profibrotic pathways, such as increasing the expression of TGF-β and the promotion of EMT with consequent increase of extracellular matrix proteins. In addition, it stimulates the synthesis of inflammatory cytokines by intrinsic and immune cells, synthesis of growth factors and chemokines which together result in granulopoiesis/myelopoiesis, and recruitment of more inflammatory cells. Therefore, inhibition of the Th17/IL-17 axis (and its signaling pathways) represents a promising strategy in treating lupus nephritis in an early view.

Agents Targeting Directly the Th17/IL-17 Axis

Several agents directly interfere with the axis and are approved to treat diseases in which the Th17/IL-17 axis clearly drives the inflammation. So, secukinumab and ixekizumab are agents targeting IL17A, both approved for ankylosing spondylitis, plaque psoriasis, and psoriatic arthritis (246); Bimekizumab that neutralizes both IL-17A and IL-17F is in preclinical phases for psoriatic arthritis and ankylosing spondylitis (247) and brodalumab an anti-IL17R, is approved for plaque psoriasis (248). Regarding the use of these agents in lupus, there are no studies completed so far; however, there are two ongoing trials to assess the safety, efficacy, and tolerability of secukinumab in patients with active lupus nephritis (NCT04181762); and the safety and efficacy of secukinumab in cutaneous manifestation of lupus (NCT03866317). In a case report involving a patient with lupus nephritis complicated by psoriasis vulgaris, the use of secukinumab was reported to be effective for both conditions with improvement in clinical and laboratory parameters (249).

Agents Targeting Indirectly the Th17/IL-17 Axis and Related Pathways

The Th17/IL-17 axis can be targeted indirectly in several ways, from interfering in the differentiating pathways, inhibition of migratory capacity, acting on mechanisms that favor polarization, including immunometabolism.

The differentiating pathways of Th17 cells are also a therapeutic target to be explored to prevent the prosperity of the axis. In fact, in a clinical trial, the addition of ustekinumab, a human monoclonal antibody against IL-12 and IL-23, to standard care resulted in better efficacy in clinical and laboratory parameters (250). It is noteworthy that il-23 not only drives the expansion, survival of pathogenic Th17 and other IL-17-producing cells (84) but also decreases Treg by decreasing the production of IL-2 (the positive regulator of Treg) (251). Thus, the beneficial effect of its inhibition should involve as mechanisms the decrease of Th17 and the increase of Treg, the impairement of the IL-23/IL-17 synergisms, among other potential mechanisms. Still, on the path of differentiation, the inhibition of STAT3, the main Signal transducer in Th17 differentiation, delayed/limited the installation of lupus nephritis in experimental models (252–254). In another experimental model of LN, renal pathological damage was attenuated with the use of α-mangostin and 3β-acetyloxy-oleanolic, compounds with inhibitory activity on retinoic acid receptor-related orphan receptor gamma t (RORγt), the transcription factor for Th17 differentiation. These compounds significantly decreased serum anti-dsDNA antibody levels, IL-17A, and IFN-γ expression (255, 256).

Since metabolic changes at the level of T cells are important in Th17 polarization and immune hyperreactivity, targeting the immunometabolism is another potentially promissory indirect strategy in SLE (110, 111). In experimental studies, metformin, which inhibits oxygen consumption and glucose oxidation, inhibited the activation of T cells, with a consequent decrease in the production of IFN-γ and IL-17 (257, 258). In another study with glucose transport inhibitors (CG-5), there was a decrease in Th1 and Th17 polarization by inhibiting their differentiation, accompanied by induction of regulatory T (Treg) (259). In addition to the effect on T cells, CG-5 treatment reduced the expansion of B cells in GC and autoantibodies' production (259). However, in a clinical trial, the addition of metformin to standard care could not demonstrate an additional benefit in reducing SLE recurrence (260). The hyperactivation of the mTOR pathway, a feature that favors Th17 polarization, is another potential target. In two studies with SLE patients, the use of rapamycin (an inhibitor of mTOR pathway) in combination with IL-2 or all-trans retinoic acid (ATRA) showed clinical efficacy decreasing the disease activity, associated with reduced Th17 cells, and restoration and long-term maintenance of Treg/Th17 ratio balance (261, 262). Aligned with this data, in a 12-months prospective open-label study, rapamycin significantly reduced the disease activity scores (SLEDAI and BILAG), associated with a reduction in IL-17 production (either by Th17 cells or double-negative T cells) (263). A trial is registered to assess the efficacy and safety of rapamycin in patients with active SLE (NCT04582136).

Several other agents have shown their potential in improving SLE interfering with the axis. Thus, the immunomodulatory efficacy of stem cell therapies (either Umbilical cord, Bone Marrow or adipose-derived) involves the suppression of the axis or restoration of the Treg/Th17 balance (12, 154); and defects in the functioning of stem cells trigger the disease (121). The beneficial effect of specific MicroRNA as miR-125a-3p and MicroRNA-10a-3p also involve interference on the axis (153, 264). In an experimental study, punicalagin (a bioactive antagonist of PAR2) ameliorated lupus nephritis, in association with a significant reduction in splenic Th17 populations compared to the vehicle controls (265). Remembering that PARs are involved in Th17-induced rearrangement in the cytoskeleton and increased permeability (136). In an experimental study with MRL/lpr mice, a traditional Chinese medicinal formula suppressed the IL-17 production and Th17 activity by inhibiting the expression of CaMK4, which was associated with a decrease in renal hypercellularity and infiltration by neutrophils (266). It is worth remembering that CaMK4 is involved in Th17 activity and enhances its migratory capacity (96).

The Effect of Lupus Current Medicines on Th17/IL-17 Axis and Related Pathways

Many of the drugs with just known efficacy in the treatment of lupus, and which act on other biological targets, have a parallel effect on the Th17/IL-17 axis. Thus, for example, the effect of methylprednisolone on improving lupus nephritis was also associated with the rebalancing of Splenic CD4+ cells with a significant reduction in Th17 populations compared to controls in an experimental study (265). This corroborates the clinical observation that induction treatment was associated with the progressive reduction of IL-17A, IL-6, and IL-21 (50). Hydroxychloroquine, an immunomodulator in lupus, inhibited Th17 differentiation (267), and reduced Th17-related cytokines in patients (268). Mycophenolic acid, used in an experimental study, inhibited the production of IL-17A, which occurred with the reduction of granulopoiesis; and this effect was completely abolished in mice lacking the IL-17 receptor (269). The same drug showed an effect of reducing STAT3 phosphorylation in patients with SLE (270), which is crucial in synthesizing IL-17 and IL-21 (84). Even Belimumab, a recombinant human IgG-1λ monoclonal antibody that inhibits B-cell activating factor (BLyS/BAFF), effective in lupus nephritis (271), shown to occur, in its effectiveness, with the restoration of the Treg/Th17 balance (272).

CONCLUSIONS AND FUTURE DIRECTIONS

Dysregulated immunity at the Th17/IL-17 axis level plays a significant role in lupus nephritis pathogenesis and ongoing damage, following the initial activation of APC by immunogenic DNA or DNA-containing immune complexes. The Th17/IL-17 axis orchestrates a chain of events that promote a proinflammatory and profibrotic environment stimulating intrinsic renal and resident immune cells to synthesize inflammatory cytokines and chemokines, promoting further recruitment of immune cells into the kidney. The Th17/IL-17 axis also exercises this driver and amplifier role systemically. All resident kidney cells express receptors for IL-17 and respond to IL-17 exposure in many ways, including changes on the cytoskeleton with increased motility, decreased expression of health proteins, increased oxidative stress, and activation of the inflammasome and caspases resulting in podocytes apoptosis. In renal tubular epithelial cells, IL-17 increases the expression of profibrotic and proinflammatory factors, such as TGF-β and fibronectin; and probably induces EMT of RTEC, promoting the further synthesis of the extracellular matrix, with all consequent changes in microstructure and renal functioning.

Despite considerable evidence on the contribution of the Th17/IL-17 axis in the pathogenesis of NL, studies directed to the Th17/IL-17 axis as a therapeutic target did not change the course of the disease as expected- a real gap in translation from bench to bedside. More works are needed to dissect the role of the Th17/IL-17 axis in the pathogenesis of the disease, and the underlying signaling pathways, to open the opportunity to target it effectively, preferably in a multitarget instead of single-cell based approach. In addition, clinical trials with the best designs are necessary, taking into account the clinical and immunological heterogeneity that characterize lupus.

AUTHOR CONTRIBUTIONS

FP wrote the manuscript and prepared figures. HA provided critical comments and revised the text. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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Kidney Involvement in PSTPIP1 Associated Inflammatory Diseases (PAID): A Case Report and Review of the Literature

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Pyogenic arthritis, pyoderma gangrenosum and acne (PAPA) syndrome, and the proline-serine-threonine phosphatase-interacting protein 1 (PSTPIP1)-associated myeloid-related proteinemia inflammatory (PAMI) syndrome are two distinct clinical conditions caused by heterozygous mutations of the *PSTPIP1* gene. While skin and joint involvements are shared by both conditions, PAMI is characterized by hepatosplenomegaly, pancytopenia, and growth failure. Kidney involvement is exceptional in PSTPIP1-mediated disorders. The two missense *PSTPIP1* variants associated with PAMI syndrome are p.E250K and p.E257K. Long-term treatment with interleukin (IL)-1 inhibitors is effective to control inflammatory manifestations and is usually well-tolerated. We report a case of a patient carrying the *PSTPIP1* p.E250K mutation who developed a late-onset kidney involvement despite a long treatment with canakinumab and anakinra. Kidney biopsy showed focal segmental glomerulosclerosis that was treated with tacrolimus (0.1 mg/kg/day in two doses). A literature revision with the aim to assess the proportion and type of kidney involvement in PAMI syndrome revealed that heterogeneous nephropathies may be part of the clinical spectrum. Our study supports the importance of a periodic diagnostic work-up, including kidney laboratory tests and kidney biopsy, in individuals affected with PAMI syndrome. Kidney and liver functions may be impaired regardless of anti-cytokines treatments and additional therapy approaches (i.e., multi-drugs, hematopoietic stem cell transplantation) should be carefully considered.

Keywords: PSTPIP1, PAPA syndrome, PAMI syndrome, kidney, IL-1 inhibitors

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INTRODUCTION

Proline-serine-threonine phosphatase-interacting protein 1 (PSTPIP1) is a cytoskeleton adaptor protein mainly expressed in hematopoietic cells. Mutations of the *PSTPIP1 gene* were associated with a large group of inflammatory disorders collected under the term PSTPIP1-associated inflammatory diseases (PAID) (1, 2). The clinical spectrum of PAID ranges from a prevalent skin and joint involvement in case of pyogenic arthritis, pyoderma gangrenosum, and acne (pyoderma gangrenosum, acne, pyogenic arthritis, PAPA) syndrome, to more complex phenotypes involving several organs in case of the PSTPIP1-associated myeloid-related proteinemia inflammatory (PAMI) syndrome (3, 4).

The PAMI syndrome is due to the p.E250K and p.E257K missense mutations of the *PSTPIP1* gene. Clinical manifestations may include cytopenia, recurrent infections, vasculitis-associated skin ulcers, hepatomegaly, splenomegaly, lymphadenopathy, and growth failure. High serum calprotectin and zinc concentration is the laboratory hallmark of the disease.

Steroid administration represents the cornerstone of treatment, resulting in effectiveness in about 55% of cases (5). In remaining cases, also the steroid-sparing drugs, such as the anti-cytokines biologics, may result not be completely effective in preventing the long-term complications. Hematopoietic stem cell transplantation has been recently proposed for the management of severe hematological manifestations (i.e., cytopenia) in complicated forms not responsive to conventional anti-cytokine treatments (6).

Here, we report a 22-year-old male previously included in a PAMI cohort (1), receiving chronic treatment with interleukin (IL)-1 inhibitor and presenting kidney involvement resulting in chronic kidney disease. We also present a literature review of this rare complication.

CASE PRESENTATION

A 4-year-old Caucasian boy was referred to our unit for recurrent episodes of asymmetrical polyarthritis at the large joints of the lower limbs (**Table 1**). Family history was negative. Since he was 6 months, physical examination revealed mild hepatomegaly and splenomegaly. Laboratory tests showed microcytic anemia (hemoglobin 11 g/dL, mean corpuscular volume 70 fL), neutropenia (780 neutrophils/mm^3), and the bone marrow analysis revealed dyserythropoiesis. Joint aspirations showed sterile but purulent fluid. Partial control of the symptoms was achieved with multiple intra-articular steroid injections.

Since 8 years of age, severe pyoderma gangrenosum started at the periorbital and periungual area [refer to Figure 1B of reference (1)]. Local treatments with antibiotics and steroids were ineffective, and surgical exportation was needed. Due to the very early onset of atypical inflammatory skin and joint involvement and the evidence of high serum levels of zinc and calprotectin (113 μmol/L, normal <50, and 14 μg/mL, normal <0.5, respectively), the Sanger sequencing analysis of the *PSTPIP1* gene was performed, revealing the heterozygous c.748G>A, p.E250K mutation. Therefore, the diagnosis of PAMI syndrome was confirmed.

Since 10 years of age, only the inflammatory skin manifestations persisted: biweekly infusion of human monoclonal anti-tumor necrosis factor (TNF)α antibody adalimumab resulted ineffectively. Since 13 years of age, severe nodulocystic acne has also developed in the face. Oral colchicine was administered but early stopped after 2 weeks due to abdominal pain. IL-1 receptor 1 (IL1R1) antagonist (anakinra) was then attempted, resulting in a prompt clinical improvement of skin lesions. Due to poor patient's compliance, after 2 years,

TABLE 1 | Characteristics of the patient.

Age (years)	4	8	12	15	19	20	21	22
Clinical manifestations								
Sterile pyogenic arthritis	+	−	−	−	−	−	−	−
Pyoderma gangrenosum	−	+	+	−	−	−	−	−
Cystic acne	−	−	+	−	−	+	−	−
Hepato splenomegaly	+	+	+	+	+	+	+	+
Growth failure	−	−	−	+	+	+	+	+
Laboratory tests								
Hemoglobin (g/dL)	11	10	11	11	11	11	11	11
While blood cells (number/mmc)	3,000	3,760	3,000	3,260	7,270	10,630	4,900	4,000
C-reactive protein (mg/dL)	ND	ND	5.32	1.64	1.3	11.8	0.86	0.6
Serum amyloid A (mg/L)	ND	ND	73	14.8	42.6	213	3.7	4
Creatininemia (mg/dL)	ND	ND	0.54	0.81	0.79	1.55	1.57	1.7
Proteinuria (g/24 h)	Absent	Absent	Absent	Absent	4.49	4.28	3.18	3.2
Treatments								
Intra-articular steroid injections	+	−	−	−	−	−	−	−
Topical steroids and antibiotics	−	+	+	−	−	−	−	−
Oral prednisone	−	−	−	−	−	+	+	−
Canakinumab	−	−	−	+	+	−	+	+
Tacrolimus	−	−	−	−	−	+	−	−
Allopurinol	−	−	−	−	−	+	+	+
ACE inhibitor	−	−	−	−	−	−	+	+

ACE, angiotensin-converting enzyme; ND, not determined.

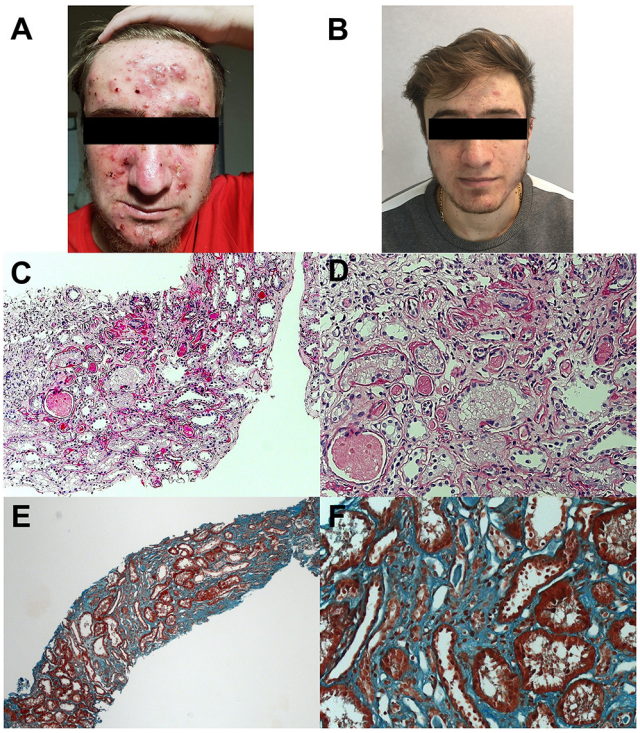

FIGURE 1 | Skin manifestations and kidney histology of the patient. Photos show cystic acne before **(A)** and after **(B)** rechallenge of canakinumab. Histology analysis shows interstitial fibrosis with interstitial infiltration by mononuclear cells and tubular atrophy, classical and with aspects of thyroidization; tubular lumens are dilated and with flaking material; epithelium of some tubules appears vacuolated; segmental hyalinosis of arteriole is also present (**C,D**; periodic acid–Schiff staining; original magnification ×50 and ×200, respectively). Diffuse interstitial fibrosis (**E,F**; trichrome staining; original magnification at ×50 and ×200, respectively).

anakinra was replaced with a monthly infusion of the fully human monoclonal anti-IL-1beta antibody canakinumab (7).

At 15 years of age, the weight and height were 48 kg and 147 cm, respectively, revealing a growth failure (10th and <3rd percentile based on Tanner's growth charts). The low serum IGF-1 levels and the abnormal provocative test with arginine confirmed a growth hormone deficiency: the recombinant growth hormone therapy was proposed but refused by the family. After that, the patient performed a blood and urine test every 6 months. At the age of 18, the patient was transferred to a local adult rheumatology unit. Due to administrative issues, the supply of IL-1 monoclonal antibody by the local health system was not regular, leading to transient flares of the skin manifestations, treated with an oral steroid.

At the age of 19 years, the patient was seen for a long-term follow-up visit in our center. Laboratory tests showed new onset of proteinuria in the nephrotic range (4.5 g/daily), microhematuria, and hyperuricemia (9 mg/dL), with normal kidney function (creatininemia 0.8 mg/dl, estimated glomerular filtration rate 129 ml/min/1.73 m^2). Mild hypoalbuminemia (3,340 mg/dl) and slightly elevated acute phase reactants (C-reactive protein 1.30 mg/dl, serum amyloid A 42.6 mg/L) were also reported. The patient was referred to a local adult nephrologist for competence. Needle kidney biopsy revealed focal segmental glomerulosclerosis (FSGS) without evidence of amyloid deposition. Canakinumab was discontinued, and prednisone (30 mg daily) was started. After 5 months, proteinuria was still in the nephrotic range (4.3 g/daily) with worsening of renal function (creatininemia 1.5 mg/dL, eGFR 63 ml/min/1.73 m^2). Tacrolimus (maintaining serum range of 4–6 ng/dl) was started, and canakinumab restarted due to worsening of the skin inflammatory manifestations (**Figure 1A**), resulting in a prompt clinical improvement of skin lesions (**Figure 1B**). One year later, due to the persistence of proteinuria in the nephrotic range (3.2 g/daily) with stable renal function (creatininemia 1.7 mg/dL, eGFR 56 ml/min/1.73 m^2), a second kidney biopsy was performed in our center, showing interstitial fibrosis (**Figures 1C–F**). Thus, tacrolimus was discontinued, prednisone gradually tapered, and he is still receiving canakinumab at the dose of 150 mg monthly.

The acute phase reactants were almost completely normalized (**Table 1**). Of note, mild anemia and neutropenia persist together with hepatosplenomegaly.

DISCUSSION

In the context of the clinical spectrum of PSTPIP1-related disorders, renal involvement is reported only in PAMI syndrome. All the English articles found in the PubMed database by querying MEDLINE with the keywords "PSTPIP1" or "PAPA" or "PAMI" were revised. We found 179 articles, among them, only seven articles reported patients with p.E250K or p.E257K mutation, of which four did not report a kidney involvement. In total, only five subjects with the PAMI-associated PSTPIP1 mutations displayed a renal involvement with at least three different phenotypes: glomerular vasculitis in three subjects, tubule-interstitial infiltration, probably related to a systemic inflammatory state, in one subject, and glomerular calprotectin deposition in the remaining one. No evidence of amyloid or immune complex deposition has been reported. Thus, kidney involvement in PAMI seems to present with heterogeneous manifestations and is linked with specific variants of the *PSTPIP1* gene. Of note, 2/6 (33%) patients in **Table 2** developed end-stage liver cirrhosis, leading them to liver transplant.

The triad of vasculitis, cytopenia, and lymphoproliferation described in PAMI can be related to specific ligands of the PSTPIP1 protein. In fact, PSTPIP1 is not expressed primitively in the kidney, whereas it is highly expressed in hematopoietic cells. The protein is able to bind with immune-related proteins, like the cytosolic protein tyrosine phosphatase (PTP-PEST), the Wiskott–Aldrich syndrome protein (WASP), the c-Abl kinase (ABL), the CD2, and the Fas ligand, probably with the aim to counteract the cytotoxic cell functioning (10) (**Figure 2**).

Mutations associated with PAID decrease the binding activity of the protein to PTP-PEST (11), suggesting that gain and loss of function should be considered. Furthermore, despite T cells

TABLE 2 | Reported cases of kidney involvement in PSTPIP1-associated inflammatory diseases.

Patient number	**1**	**2**	**3**	**4**	**5**	**6**
Study	Holzinger et al. (1)	Holzinger et al. (1)	Holzinger et al. (1)	Lindwall et al. (8)	Dai et al. (9)	Present case
Gender	Male	Female	Male	Male	Female	Male
Age (years)	35*	9	16	25	56	22
Age at onset (years)	6	0	1	4	18	4
Disease duration (years)	29	9	15	19	38	18
PSTPIP1 p.E250K	Y	Y	Y	Y	Y	Y
Clinical manifestations						
Arthritis	Y	N	Y	Y	Y	Y
Pyoderma gangrenosum	Y	N	Y	Y	N	Y
Cystic acne	N	Y	Y	Y	N	Y
Skin ulcers	Y	N	N	Y	N	N
Poor wound healing	Y	N	N	N	Y	Y
Hepatomegaly	Y	Y	Y	Y	Y	Y
Splenomegaly	Y	Y	Y	N	Y	Y
Growth failure	N	Y	Y	N	N	Y
Kidney involvement	Minimal-change glomerulonephritis	IgA nephropathy	Glomerulonephritis	Acute kidney failure	Podocyte effacement and glomerular calprotectin dense deposits	Focal segmental glomerulosclerosis
Others	Liver cirrhosis, post liver transplant complications	Mild lymphadenopathy, arthralgia, gastrostomy tube feeding, familiarity for early gout	Aseptic necrosis of femoral head	Osteomyelitis, acute cholecystitis, sepsis, colitis, cellulitis, acute respiratory failure, epistaxis, joint and skin laxity, familiarity for psoriatic arthritis	Recurrent pneumonia, lymphadenopathy, macronodular cirrhosis with mild portal hypertension	Growth hormone deficiency
Laboratory tests						
Anemia	Y	Y	Y	Y	Y	Y
Neutropenia	Y	Y	Y	Y	Y	Y
Others	N	Thrombocytopenia, *MEFV* p.E148Q carrier	N	N	Von Willebrand's factor deficiency, high agammaglobulinemia	Dyserythropoiesis at the bone marrow biopsy
Treatments						
Steroid-sparing drugs (duration; clinical response)	Anakinra (2 months; partial), infliximab (3 months; partial)	Cyclosporin (ND; partial), colchicine (NT) anakinra (ND; complete)	Cyclosporin (ND; partial), mycophenolate mofetil (ND; none), rituximab (NT), tocilizumab (ND; partial)	Infliximab (ND; none)	Sulfasalazine (ND; none), methotrexate (ND; none), colchicine (ND; complete)	Colchicine (NT), adalimumab (3 months; none), anakinra (ND; complete), canakinumab (ND; complete), tacrolimus (ND; partial)

**Age of death; ND, not determined; NT, not tolerated; PSTPIP1, proline-serine-threonine phosphatase-interacting protein 1.*

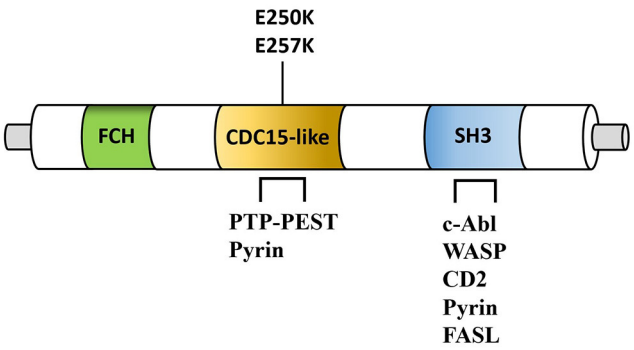

FIGURE 2 | Simplified structure of PSTPIP1 protein. Fes/CIP4 homology domain (FCH), CDC15-like adaptor protein (CDC15-like), SRC Homology 3 Domain (SH3) are shown. Note the position of the PAMI-associated mutations (E250K and E257K). PSTPIP1 interacting proteins include the protein-tyrosine phosphatase (PTP-PEST), pyrin, the Wiskott–Aldrich syndrome protein (WASP), the c-Abl kinase, CD2, and the Fas ligand (FASL). PSTPIP1, proline-serine-threonine phosphatase-interacting protein 1.

do not show a prevalent involvement in PAID lesions, the mild lymphoproliferation described in some patients with PAMI may be related to a T-cell activation defect caused by abnormal CD2 binding activity or absence of the Fas ligand, may be due to its sequestration into lysosomes. Moreover, WASP and ABL are actin-related proteins and the cytopenia and vasculitis of PAMI patients may be secondary to their dysfunction, as reported in patients with actin remodeling defects (12, 13). Thus, PAMI syndrome represents the most severe form of the PAID spectrum, and its clinical manifestations may be secondary also to actin remodeling defect and lymphocytes hyperactivation, other than the high IL-1 production as for others PAID.

As reported, kidney biopsy was negative for amyloid deposition (14), in line with previous reports (**Table 2**), and the histological findings do not support calprotectin deposition as recently described (9). Therefore, other pathogenic mechanisms causing kidney lesions should be investigated. In the first kidney biopsy, a diagnosis of FSGS was reported. The pathogenic mechanism of FSGS remains still poorly understood (15). Recent findings demonstrated the role of the IL-1 pathway as a possible pathogenic mechanism in proteinuric disease, supporting therefore the administration of treatments blocking the IL-1β/IL-1R1 signaling to delay the development of sclerotic lesions (16). In the second kidney biopsy, performed despite stable renal function and proteinuria, a diffused tubule-interstitial fibrosis was revealed, probably due to tacrolimus administration, thus discontinued. Notably, our patient developed proteinuria after irregular administration of IL-1 inhibitors: scarce adherence to IL-1 receptor antagonist (anakinra) was admitted, as above reported and, due to local administrative issues, the supplying of canakinumab was rather inconstant. At this stage, it is difficult to determine the real IL-1 dependence of the renal manifestation.

As reported in **Table 2**, in patient 4, colchicine was effective in reversing proteinuria. A multi-drug approach may control various manifestations of PAMI by silencing the concomitant defects of different components of the immune system. Furthermore, it remains a matter of speculation that the kidney involvement of other pyrin-related autoinflammatory disorders may not be due only to amyloid deposition. More studies are required to investigate the mechanisms of renal involvement and the possible role of anti-IL drugs against these manifestations.

In conclusion, PAMI syndrome is a rare inflammatory disorder and the most severe phenotype among PAID, characterized by alterations of various immune system agents. The severe kidney involvement of our patient is, according to our best knowledge, the first subjected to a documented histological modification after anti-IL-1 treatment.

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 Ethics Committee Genoa, Italy. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

PB, RP, AA, GG, and MG conceptualized the manuscript. PB and RP conducted the literature review and drafted the manuscript. MD'A, RC, and GP were involved in the clinical care of the patient. IC performed the genetic analysis. All authors read and approved the final version of the submitted manuscript.

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Prognostic Value of C4d Immunolabelling in Adult Patients With IgA Vasculitis

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Background and Objectives: Glomerular C4d deposits are associated the severity and outcomes of IgA nephropathy. Whether this holds true in immunoglobulin A vasculitis (IgAV) is not known. The main objective of the study was to analyze the prognostic value of glomerular C4d immunolabelling on kidney impairment in adults with IgAV.

Design, Setting, Participants, Measurements: This retrospective cohort study included 120 adults with IgAV and a kidney biopsy performed between 1995 and 2018 in two French university hospital centers. All paraffin-embedded biopsies were reassessed according to Oxford classification. Immunofluorescence for C4d was performed in all cases. For analysis, patients were grouped according to positivity for C4d in the glomerular area. The main outcome was a composite endpoint of 50% increase in 24 h-proteinuria, or eGFR decrease by 50%, or kidney replacement therapy.

Results: The median follow-up was 28.3 months. Twenty-three patients met the composite endpoint, 12 for kidney replacement therapy, 6 for an eGFR decrease >50% and 5 for a >50% increase in proteinuria. At time of biopsy, the median proteinuria was 1.9 g/24 h and the median eGFR 73.5 mL/min/1.73 m^2. Among the 102 patients evaluable for C4d, 24 were positive on >30% glomeruli, mainly with a parieto-mesangial pattern. In this group, the initial proteinuria was more frequently nephrotic than in the C4d– group (60% vs. 33%, $P = 0.039$). Mesangial hypercellularity was more frequent in the C4d+ group (42% vs. 13%; $P = 0.006$) whereas macroscopic hematuria was more frequent in the C4d– group (18% vs. 0%; $P = 0.03$). After a median follow-up of 28 months, kidney survival did not differ according to C4d status.

Conclusion: In a population of adult IgAV patients, glomerular positivity for C4d was associated with the severity of the kidney disease at presentation, but not with subsequent renal function deterioration.

Keywords: immunoglobulin A (IgA), vasculitis, glomerulonephritis, Henoch-Schönlein purpura, immunohistochemistry, proteinuria (MeSH: D011507), complement

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INTRODUCTION

Immunoglobulin A vasculitis (IgAV), formerly called Henoch-Schönlein purpura, is an immune complex vasculitis predominantly affecting small vessels with dominant IgA deposits. It can affect the skin, gut, joints and kidney to varying degrees. IgA vasculitis is considered to be a systemic form of IgA nephropathy and it has been suggested that the two conditions are different manifestations of a single disease process (1). A kidney involvement is more frequent and severe in adults than in children. As outlined by Audemard-Verger et al. (2), a kidney involvement occurs in 45–85% of cases, but IgA vasculitis is responsible for only 0.6–2% of adult nephropathies.

In addition to IgA deposits, glomerular deposition of complement factors including C3, mannan-binding lectin, L-ficolin, mannan-associated serine protease, and C4d has been observed in most patients with IgA vasculitis. These findings, together with the absence of C1q deposits, support the hypothesis of prevalent activation of the lectin pathway. The presence of complement deposits has been shown to be associated with a higher degree of proteinuria and hematuria as well as with more severe histological lesions (3).

C4d is a biological degradation product of the C4 fraction of complement after activation of the conventional or the lectin pathway, with no known biological function or receptor. The presence of C4d deposits has been reported in IgA nephropathy in association with mannose binding lectin deposits, indicating, in the absence of C1q, an activation of the lectin pathway (4, 5). Several publications have shown that the presence of mesangial glomerular deposits of C4d in IgA nephropathy is associated with a more severe form of the disease and an adverse prognosis (6–8).

The involvement of the lectin pathway has also been shown in IgA vasculitis, associated with an increase in serum C4d (5), but the prognostic interest of C4d immunostaining in the kidney has not yet been studied.

The objectives of the study were to analyze the presence and prognostic value of C4d deposits in the kidney in adult patients with IgA vasculitis.

MATERIALS AND METHODS

This retrospective cohort study was conducted in two French University Hospitals (Rouen and Amiens). In French hospital settings, patients are informed that their data can be used for research purposes if they have no objection. The data used in this study are derived from de-identified files, and thus, this study was exempt from Ethics Committee approval.

We retrospectively identified all persons 18-year old or older who did not object to the use of their data, who underwent a kidney biopsy between January 1995 and January 2018, were found to have glomerular IgA deposition on histopathological examination and had at least one extrarenal sign compatible with IgAV. Patients with liver disease, digestive or articular chronic inflammatory disease, primary IgA nephropathy, lack of dominant IgA mesangial deposits or lack of material were excluded.

Patients Follow-Up

Clinical and biological data were extracted from the pathology laboratories information system and electronic medical records. Patients' follow-up ran from the date of biopsy to the date of last visit, death or kidney replacement therapy.

Demographic data, hypertension status, extrarenal signs (cutaneous purpura, arthralgia, abdominal pain, digestive hemorrhage), macroscopic or microscopic hematuria, plasma creatinine, glomerular filtration rate estimated by the MDRD formula (eGFR), urinary protein excretion, and treatment with Angiotensin-Converting Enzyme Inhibitors (ACEi) or Angiotensin II Receptor Blockers (ARB) were recorded at kidney biopsy. Follow-up data included plasma creatinine and proteinuria after 1, 2 and 5 years and at last visit, treatment with immunosuppressive, corticosteroid or ACEi/ARB agents, and death or relapse defined by purpura resurgence or proteinuria >0.5 g/24 h.

The main outcome was a composite endpoint of 50% increase in 24 h-proteinuria, or eGFR decrease by 50%, or kidney replacement therapy.

Histopathology of Kidney Biopsies and Immunostaining

All biopsies were evaluated by an independent pathologist blinded to clinical outcomes. Paraffin-embedded kidney tissues (2-μm sections) were stained with Hemalun Eosine Safran, Masson trichrome, Jones methenamine silver and sometimes periodic acid–Schiff and Congo red. To be analyzed, biopsies had to contain at least five glomeruli. They were classified according to the Oxford criteria (9). Additionally, presence of extra-capillary proliferation, fibrinoid necrosis and moderate to severe arteriosclerosis were evaluated.

C4d immunohistochemical staining was performed on 2-μm sections of renal tissue using rabbit polyclonal anti-human C4d (clone A24-T, DB Biotech, Kosice, Slovakia) according to Ventana protocol. Only biopsies with at least four permeable glomeruli were analyzed. The positivity for C4d staining was defined as more than 30% of non-sclerotic glomeruli with a moderate or strong staining. The staining was global if involving more than 50% of the glomerular tuft.

Statistical Analysis

The number of cases retrieved determined the study size. Statistics were performed using R software (10). Quantitative variables were expressed as median with their interquartile range (IQR) and compared between C4d+ and C4d– groups with the Mann-Whitney U test; Categorical variables were described as absolute numbers and percentages and compared between C4d+ and C4d– groups using the Fisher's exact test. Kidney survival was analyzed using Kaplan-Meier estimates and comparisons between C4d+ and C4d groups used a log-rank test. Missing data were not replaced.

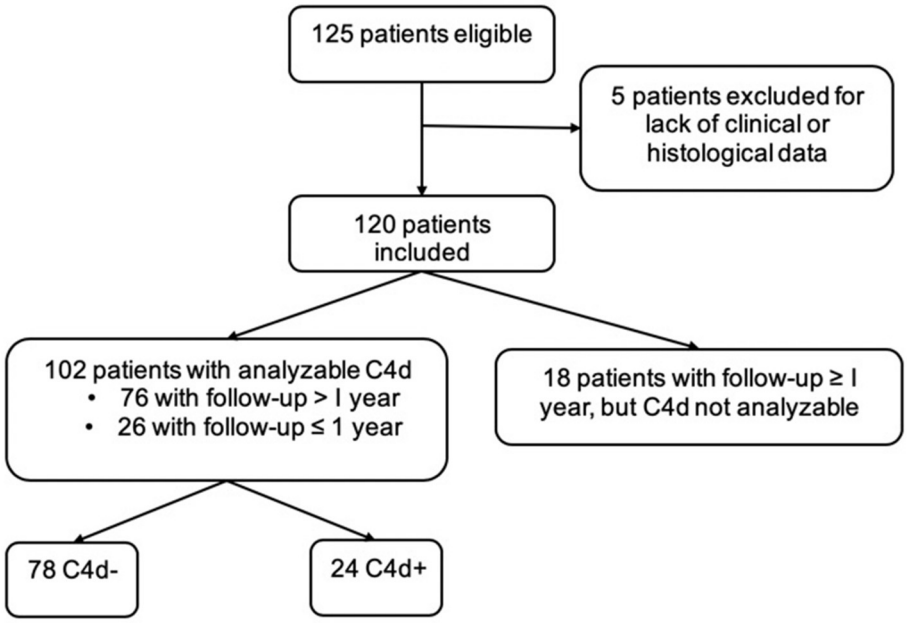

FIGURE 1 | Patient flow-chart.

RESULTS

This retrospective cohort included 120 patients biopsied and diagnosed with IgA vasculitis. A flow diagram describing the patient samples and exclusions is shown in **Figure 1**.

Baseline Characteristics and Follow-Up of the Overall Population

Table 1 shows the clinical and histopathologic characteristics at the time of diagnosis in the whole group and stratified by the presence/absence of glomerular C4d deposits on the kidney biopsy. Three patients were already on kidney replacement therapy at time of diagnosis, none had undergone a kidney transplantation. The activity of the disease was demonstrated by endocapillary inflammation E1 (73.3%), extra-capillary proliferation (66.7%) and fibrinoid necrosis (54.2%), whereas mesangial proliferation M1 was found in 21% of the patients only. At time of diagnosis, chronic lesions were already frequent, as shown by mesangial sclerosis S1 (57.5%), interstitial fibrosis and tubular atrophy T1-T2 (26.7%) and moderate to severe arteriosclerosis (61%). Immunofluorescence was available for 114 patients; all biopsies showed IgA deposits, mesangial in 50% of the cases and parieto-mesangial in 35%, and C3 deposits were found in 83% biopsies. C1q staining was negative in all patients, IgG and IgM staining were positive in respectively 23% and 41% patients. Deposits of C4d were observed in 24 (24%) patients among the 102 biopsies analyzable. In these patients, C4d staining was mainly parieto-mesangial (67%), mostly of moderate intensity (92%) (**Figure 2**).

The median follow-up was 28.3 months (IQR 13.8–70). Twenty-three patients (19%) met the composite endpoint, 12 for kidney replacement therapy, 6 for an eGFR decrease >50% and 5 for proteinuria increase >50% (**Tables 2**, **3**; **Figure 3**).

Differences Between C4d+ and C4d– Patients

A hundred and two patients were analyzable for C4d. Comparisons of clinical and histopathologic characteristics at the time of diagnosis are displayed in **Table 1**. Men were more represented and macroscopic hematuria significantly more frequent in the C4d– group. Sixty per cent patients in the C4d+ group had a nephrotic-range proteinuria (>3 g/24 h), twice more than in C4d–, although the median proteinuria did not differ significantly between groups. Treatment by an immunosuppressive therapy was more prevalent in C4d+ patients (31.8%) compared to C4d– patients (12.8%). Mesangial hypercellularity affected 42% of C4d+ patients vs. 13% of C4d– patients, while the other histopathologic findings were similar in the two groups. Immunofluorescence was available for 98 patients; all biopsies showed deposits of IgA, exclusively mesangial in 39% of C4d+ and 53% C4d– cases, parieto-mesangial in respectively 39% and 35% of the C4d+ and C4d– cases. C1q staining was negative in all patients and C3 deposits were found in respectively 91% and 85% of the C4d+ and C4d– biopsies. IgG staining was positive in respectively 41% and 19% of the C4d+ and C4d– patients and IgM in 39% and 40%. C4d immunostaining distribution is summarized in **Table 1**. The rate of affected glomeruli was significantly higher in the C4d+ group, with a diffuse pattern, whereas deposits were focal in the C4d–. The C4d pattern was significantly different between the two groups, mainly due to lack of exclusive mesangial localization in the C4d+ patients.

TABLE 1 | Clinical and histopathological characteristics at diagnosis, overall and according to the presence or absence of C4d glomerular deposits.

	All (*N* = 102)	C4d+ (*N* = 24)	C4d– (*N* = 78)	*P*-value
Age (years); median (IQ)	53 (32–68)	41 (20–62)	54 (35–69)	0.07
Men; n/N (%)*	78/120 (65%)	11/24 (45.8)	52/78 (66.7)	0.09
Extra-renal signs; n/N (%)				
Cutaneous	114/118 (96.6)	21/22 (95.5)	76/78 (97.4)	0.56
Abdominal pain	50/114 (43.9)	12/22 (54.5)	32/74 (43.2)	0.47
Gastrointestinal hemorrhage	18/112 (16.0)	3/21 (14.3)	11/73 (15.1)	1
Arthralgia	63/113 (55.8)	15/22 (68.2)	42/73 (57.5)	0.46
Hypertension; n/N (%)	56/115 (48.9)	7/21 (33.3)	36/76 (47.4)	0.32
eGFR (mL/min/1.73 m^2); median (IQR)	73 (37–98)	87 (43–113)	69 (29–92)	0.09
Creatininemia (μmol/L); median (IQR)	88 (70–152)	77 (67–120)	91 (70–200)	0.17
Proteinuria (g/24 h)	1.9 (1–3.97)	3.7 (1.06–7.03)	2.0 (1.05–3.8)	0.16
Proteinuria > 3 g/24 h; n (%)*	40/108 (37)	12/20 (60.0)	23/71 (32.3)	0.04
Hematuria; n/N (%)*				0.0024
Macroscopic	16/120 (13.3)	0	14/78 (18.2)	
Microscopic	96/120 (82.8)	19/22 (86.4)	62/78 (80.5)	
Treatment				
ACEi/ARB treatment; n/N (%)	0.79	16/22 (72.7)	47/69 (68.1)	0.79
Corticosteroid oral; n/N (%)	0.35	20/22 (90.9)	56/69 (81.1)	0.35
Corticosteroid IV; n/N (%)	1	11/22 (50.0)	45/91 (49.4)	1
Immunosuppressive; n/N (%)	0.054	7/22 (31.8)	9/70 (12.8)	0.054
Histopathologic Oxford classification; n (%)				
M1*	20 (19.6)	10 (41.6)	10 (12.8)	0.006
S1	60 (58.8)	14 (58.3)	46 (58.9)	1
E1	77 (75.5)	19 (79.2)	58 (74.4)	0.79
T1-2	24 (23.5)	8 (33.0)	16 (20.5)	0.27
C1-2	72 (70.5)	17 (70.8)	55 (70.5)	0.95
Other histopathologic findings; n (%)				
Fibrinoid necrosis	54 (52.9)	16 (66.7)	38 (51.3)	0.24
Moderate/severe arteriosclerosis	60 (58.8)	12 (50.0)	48 (61.5)	0.29
C4d distribution				
Number of glomeruli; median (IQR)	11.5 (8–19)	14.5 (10–19.5)	11.0 (7.25–18.5)	0.11
% of affected glomeruli; median (IQR)	52.5 (25–80)	83.5 (60–100)	37.0 (20–67.8)	<0.001
C4d repartition*				0.002
Parietal; n (%)	18 (17.6)	7 (29.2)	11 (17.7)	
Mesangial; n (%)	21 (20.6)	1 (4.2)	20 (32.2)	
Parieto-mesangial; n (%)	47 (46)	16 (66.7)	31 (50)	

IQR, interquartile range; ACEi, Angiotensin-converting enzyme inhibitors; ARB, Angiotensin II receptor blockers; IV, intravenous; M, mesangial cellularity; S, segmental sclerosis; E, endocapillary hypercellularity; T, interstitial fibrosis and tubular atrophy; T1 > 25% and T2 > 50%.
**Statistically significant difference between C4d+ and C4d– groups.*

Follow-up comparisons are summarized in **Table 2**. The follow-up length was similar for the two groups, and >1 year for three quarters of the patients. The rate of patients meeting the composite endpoint was not different between groups, as for the number of relapses or deaths. The renal survival did not differ significantly between groups (**Figure 3**). In the subgroup of patients with available eGFR at 2 years (**Table 3**), only the baseline values of proteinuria differed between C4d+ and C4d–, changes over time were similar.

DISCUSSION

In our study, the presence of C4d deposits in the kidney biopsy of adult patients with IgA vasculitis renal impairment was associated with a more severe initial renal involvement: a nephrotic proteinuria >3 g/24 h was reported for 60% patients and mesangial hypercellularity was more frequent, but our results did not show an association between C4d deposits and renal survival. Interestingly, there was a higher proportion of immunosuppressive treatment in the C4d+ group (31.8%)

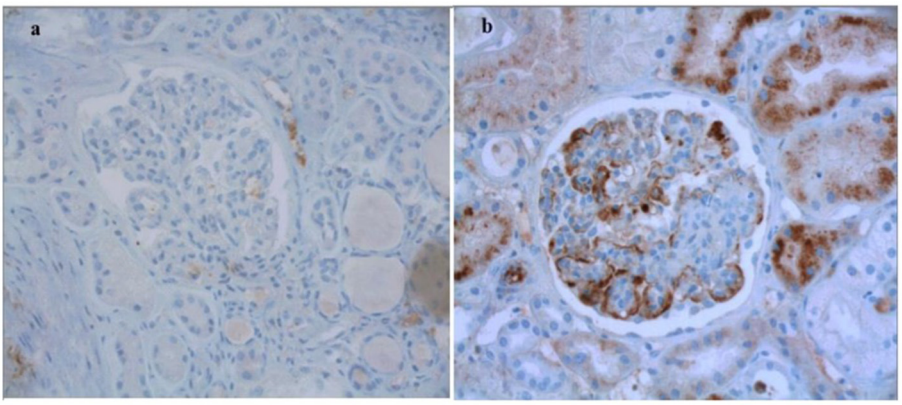

FIGURE 2 | Representative views of C4d immunohistochemistry on kidney biospies of patients with IgAV. **(a)** Absence of staining. **(b)** Positive parietal staining. Original magnification x400.

TABLE 2 | Follow-up according to the presence or absence of C4d glomerular deposits.

	C4d+ (*N* = 24)	C4d– (*N* = 78)	*P*-value
Follow-up duration (months); median (IQR)	22 (12–65)	29 (10–81)	0.54
Follow-up duration in classes; n (%)			
<1 year	5/24 (20.8)	15/65 (23)	1
[1–2 years[	7/23 (30.4)	9/66 (13.6)	0.11
[2–5 years[	6/23 (26.1)	19/66 (28.8)	1
≥5 years	5/24 (20.8)	23/66 (34.8)	0.3
Composite endpoint met			
Missing	5	20	
n (%)	6 (32)	14 (25)	0.56
Kidney replacement therapy	3	7	0.7
eGFR decrease >50%	1	4	1
Proteinuria increase >50%	2	3	0.59
Time to kidney replacement therapy (months); median (IQR)	15 (12–18)	4 (1–7)	0.13
Relapse			
Missing	1	11	0.51
n (%)	6 (26)	13 (19)	
Death			
Missing	1	20	0.67
n (%)	1 (4)	7 (12)	
5-year survival	14/19 (73.7)	34/57 (59.6)	0.41

IQR, interquartile range; Relapse = purpura resurgence or proteinuria > 0.5 g/24 h.

TABLE 3 | Follow-up in a sub-group of patients with 2-year available eGFR data according to the presence or absence of C4d glomerular deposits.

	C4d+ (*N* = 11)	C4d– (*N* = 34)	*P*-value
Follow-up duration (months); median (IQR)	45 (28–55)	33 (24–89)	0.92
Kidney replacement therapy; n (%)	1 (9)	6 (18)	0.49
Death	0 (0)	5 (15)	0.17
Changes in eGFR (ml/min/1.73 m^2); median (IQR)			
Baseline	76 (48–120)	56 (23–82)	0.08
1 year	76 (55–92)	68 (38–81)	0.15
2 years	72 (47–81)	68 (35–82)	0.57
Final	74 (57–81)	62 (30–83)	0.26
Changes in proteinuria (g/24 h); median (IQR)			
Baseline	6.8 (3.1–7.6)	2.0(1.0–3.4)	0.03
1 year	0.2 (0.1–1)	0.4 (0–0.7)	0.15
2 years	0.07 (0–0.4)	0.34 (0–0.6)	0.33
Final	0.05 (0–0.4)	0.25 (0.07–0.4)	0.35

IQR, Interquartile range.

compared to the C4d– group (12.8%), suggesting a more aggressive therapeutical approach in C4d+ patients.

Various clinical, biological and histological factors, as hypertension, high proteinuria and/or plasma creatinine, glomerulosclerosis or interstitial fibrosis, are recognized predictors of adverse kidney outcomes (11, 12). However, progression to kidney failure can occur even in the absence of these poor prognosis factors, underlining the necessity of new prognostic markers.

C4d deposits, generated by the activation of the lectin pathway, appear to be associated with a worse renal prognosis, regardless of the nephropathy studied. Xing et al. (13) were the first to suggest that a positive staining of C4d and mannan-binding lectin might be associated with poor renal outcome. The prognostic interest of C4d immunostaining has been described in IgA nephropathy and identified as an independent risk factor for progression to end-stage renal failure (6, 14) but data on C4d and IgA vasculitis are scarce. In their series of 59 patients, Espinosa et al. (6) included 8 patients with IgA vasculitis, of which 2 were positive for C4d. The frequency of C4d deposition was the same

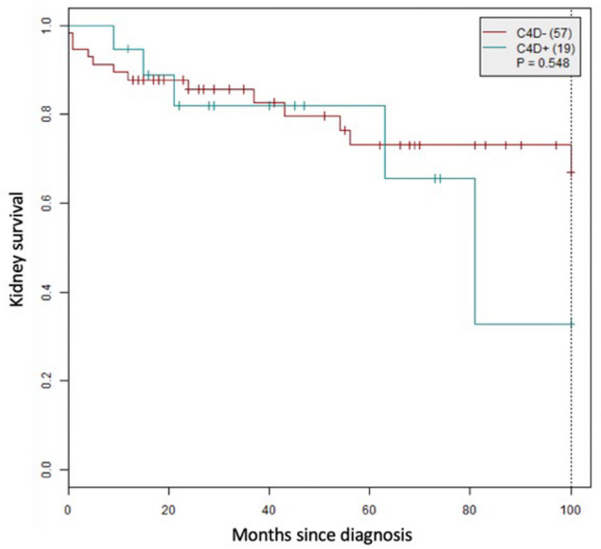

FIGURE 3 | Kidney survival according to glomerular C4d status.

between patients with vasculitis and those with nephropathy. As a possible prognostic factor, C4d could be assessed routinely in kidney biopsies in the same way as C3 or C1q.

In our cohort, C4d positivity was not associated with a more severe extra-renal picture. However, kidney involvement was more severe in the C4d+ group, as shown by worse plasma creatinine and proteinuria, especially nephrotic proteinuria. We did not show a difference in renal survival between the two groups, maybe because of a too short follow-up and a more aggressive treatment in C4d+ patients. In studies on the prognostic value of C4d deposits in IgA nephropathy, the survival curves separate after 5 years of follow-up (6, 7, 15).

We used the Oxford classification to characterize our biopsies: these criteria are prognostic factors for both IgA nephritis and vasculitis, and they are easily reproducible as most often binary. Xu et al. (16) showed the relevance of this classification for predicting long-term outcomes of IgA vasculitis. We found a more frequent mesangial proliferation in the C4d+ group (42% vs. 13% in the C4d– group). Our findings of more frequent nephrotic syndrome in the C4d+ group is in line with the findings of Xu et al. (16), who showed that mesangial proliferation was associated with a worse proteinuria.

Our study has several limitations: the retrospective design is a critical limitation, as all confounding factors might not be identified and distributed equally, which may lead to biased results. Our median follow-up was 28 months, shorter than other studies (11, 12, 17). Given the absence of correlation between initial presentation and long-term renal outcome, with possible spontaneous remission in patients with severe presentation, or possible evolution to end-stage renal disease in patients with mild symptoms (18), we cannot exclude that a longer period of observation may have revealed a discriminant value of C4d deposits.

In conclusion this study, which analyzes the largest cohort of IgAV patients in this setting, demonstrates an association between glomerular positivity for C4d and the severity of the kidney disease at presentation, without showing prognostic value on kidney outcomes after a median follow-up of 28 months.

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 for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AR collected data, performed pathology analysis, analyzed results, performed statistical analyses, wrote the draft, and reviewed the manuscript. FD performed pathology analysis, analyzed results, and reviewed the manuscript. AF and AD performed pathology analysis and reviewed the manuscript. XX and DT-B collected data and reviewed the manuscript. DB analyzed results, performed statistical analyses, and reviewed the manuscript. DG initiated the study, analyzed results, wrote the draft, and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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Case Report: A Rare Case of Lupus Nephritis Associated With Mantle Cell Lymphoma

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In this research, we described a very rare case of secondary lupus nephritis associated with B-cell lymphoma. An 84-year-old man was hospitalized at our institute for lower extremity edema persisting for over 2 months. He was diagnosed with systemic lupus erythematosus based on clinical and laboratory criteria, which showed impaired renal function and nephrotic syndrome with predominant hematuria. Renal biopsy showed IV+V lupus nephritis with highly infiltrated lymphoid cells in the kidney. Secondary lupus nephritis was suspected based on the possible pathogenesis of glomerular injury due to mantle cell lymphoma. Low-dose dexamethasone, rituximab, and lenalidomide were immediately started on the patient, and his renal function was improved after the first cycle of chemotherapy.

Keywords: lupus nephritis, mantle cell lymphoma, secondary SLE, non-Hodgkin lymphoma, acute kidney injury

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BACKGROUND

Lupus nephritis is a form of glomerulonephritis that constitutes one of the most severe organ manifestations of the autoimmune disease, systemic lupus erythematosus (SLE). In many cases, lupus nephritis is the presenting manifestation that results in the diagnosis of SLE (1). Previous literature indicated that lupus-like syndrome could be caused by infections, hematological malignancies, solid tumors, and so forth (2). However, renal involvement presented as lupus nephritis is rare (3–7). In this research, we describe a rare case of mixed proliferative and membranous lupus nephritis, secondary to mantle cell lymphoma, and partially recovered after chemotherapy.

CASE PRESENTATION

An 84-year-old Chinese man was presented to our department with both lower extremity edema for over 2 months. Initial laboratory investigations demonstrated an elevated serum creatinine of 1.64 mg/dl (normal range: 0.50–1.50 mg/dl; 144.6 μmol/L, corresponding to estimated glomerular filtration rate of 37.95 ml/min/1.73 m^2 as calculated by the CKD-EPI equation) and decreased albumin of 22.9 g/L (normal range: 40–55 g/L), with proteinuria of 24 g/day (total volume 850 ml). Urinary microscopic examination showed microscopic hematuria (80–100 cells/HPF) and pyuria

(full-field of view). Ultrasound examination of the kidneys revealed normal-sized kidneys (left 11.8 cm; right 11.7 cm). Blood leucocyte count was 7,100/μl, hemoglobin was 13.2 g/dl and platelet count was 153,000/μl. The antinuclear antibody (ANA) was positive (1:1,000 titer, homogenous) and the anti-double stranded DNA (anti-dsDNA) antibody was positive with the titer of 1:10. Complement 3 (C3) and 4 (C4) were both reduced to 0.423 g/L (normal range: 0.60–1.50 g/L) and 0.027 g/L (normal range: 0.12– 0.36 g/L), respectively. Anti-glomerular basement membrane (anti-GBM) antibody, anti-neutrophil cytoplasmic antibody (ANCA), Coombs' test, antiphospholipid antibody, serum cryoglobulins, serum and urine immunofixation electrophoresis, and anti-PLA2R antibody were all negative.

The patient had mantle cell lymphoma (MCL) 11 months ago, which was diagnosed through a biopsy of both bowel polyp and axillary lymph nodes and classified as Stage III Group A with low-intermediate risk (lymph nodes and gastrointestinal involvement). There was no further chemical treatment for lymphoma because of its indolence and low risk. Besides, he had a series of metabolic disorders, including diabetes, hypertension, and coronary heart disease, with satisfying controlment and no diabetic retinopathy. He was also diagnosed with myasthenia gravis (MG) IIb type (Ossermann Classification) for 5 years. At the time of diagnosis, the patient was on acetylcholinesterase inhibitor for his MG, which significantly improved his muscle weakness. He was treated with tacrolimus at 0.5 mg per day regularly with remission. The blood drug level of FK-506 was 0 ng/ml.

Upon admission, physical examination revealed blood pressure of 160/90 mmHg, a pulse of 99/min, a temperature of 36.3°C, and a respiratory rate of 18/min. Physical examination showed an obvious vicia-sized lymph node at the right axilla, shifting dullness, and severe edema of both lower limbs.

Bone marrow biopsy and (18)F-FDG Positron Emission Tomography/Computed Tomography (PET/CT) were performed to evaluate lymph nodes and organ involvement. Abnormal clones of B cells accounted for 5.6% of bone marrow cells as determined by bone marrow flow cytometry. On fluorescent *in situ* hybridization (FISH), 43 IGH/CCND1 fusion signals can be seen in 200 interphase cells. (18)F-FDG PET/CT revealed systemic lymphadenopathy without extranodal involvement.

To clarify the pathological changes of his kidney histology, a renal biopsy was performed. Direct immunofluorescence showed full-house staining along mesangium and capillary loops [IgG (3±), IgA (±), IgM (1±), C3 (2±), C1q (1±), fibrinogen (2±), albumin (–), kappa (2±), lambda (3±), IgG1 (1±), IgG2 (1±), IgG3 (1±), and IgG4 (–)]. Light microscopic examination showed that 5/28 glomeruli were ischemic and sclerotic and 1/28 were segmentally sclerotic. The rest of the glomeruli showed mild proliferation of mesangial cells and stroma, accompanied by segmental endothelial cell proliferation and infiltration of neutrophils. The glomerular basement membrane thickened diffusely, with the formation of segmental spikes. The tubules displayed acute injury with epithelial simplification and small focal atrophy. There were scattered proteinaceous and RBC casts. There was intimal fibrous proliferation and sclerosis of small arteries. Congo red stain for amyloid was negative. Renal interstitium was infiltrated by many lymphoma cells with variable sizes, large-oval or irregular nuclei, delicate chromatin pattern, high nuclear-cytoplasm ratio, and prominent nucleoli. Immunohistochemistry showed CD20 (2+), CD3 (1+), CD5 (2+), CyclinD1 (1+), SOX11 (1+), BCL2 (2+), Ki-67 (5%), which confirmed infiltration of mantle cell lymphoma cells in the renal interstitium. Electron microscopy showed that the basement membrane was thickened and diffusely accompanied by sub-epithelial and segmental mesangial electron-dense deposits. Severe foot process effacement was also discovered (**Figure 1**).

Chemotherapy treatment with rituximab at 375 mg/m^2 body surface area (BSA), lenalidomide 25 mg/day for 21 consecutive days, and 10 mg dexamethasone was initiated considering the advanced age and multiple combined disease. The patient developed hospital-acquired pneumonia during the chemotherapy and the renal function rapidly deteriorated as serum creatinine elevated from 1.76 mg/dl (156 μmol/L) to a peak of 2.69 mg/dl (238 μmol/L). After the successful anti-infection therapy was performed, his renal function was improved and the serum creatinine fell back to baseline at 1.32 mg/dl (117 μmol/L). The patient's ANA was 1:100, anti-dsDNA levels negative, and C3 and C4 immediately returned to normal level after just one round of chemotherapy (**Figure 2**).

DISCUSSION

Systematic lupus erythematosus (SLE) is an autoimmune disease characterized by multisystem organ involvement, heterogeneity of clinical features, and a variety of degrees of severity, of which lupus nephritis is constituted with one of the most common causes of morbidity and mortality. However, previous studies showed that SLE could be secondary to infections, hematological malignancies, solid tumors, drugs, and so on (2), with renal involvement (3–7).

Herein, we present a case of an 84-year-old man with a history of mantle cell lymphoma for 11 months and was also diagnosed as lupus nephritis confirmed by renal biopsy. The main differential diagnoses considered in our patient were whether the described associated glomerular disease is the paraneoplastic syndrome of lymphoma or that of concurrent primary lupus nephritis. However, new-onset SLE is uncommon in elderly male patients (8), and this patient notably has infiltration of mantle cell lymphoma cells in the renal interstitium and bone marrow. Besides, our patient achieved rapid remission of the glomerular disease and immune system remission after the first chemotherapy for MCL. The paraneoplastic nature of renal lesions made us consider that lupus nephritis was secondary to MCL.

Mantle cell lymphoma (MCL) is a unique type of B-cell non-Hodgkin lymphoma (NHL), which is considered aggressive. Several case reports have been published on the glomerular involvement of MCL. Some of the histopathological findings include minimal change disease (MCD) (9), membranoproliferative glomerulonephritis (MPGN) (10),

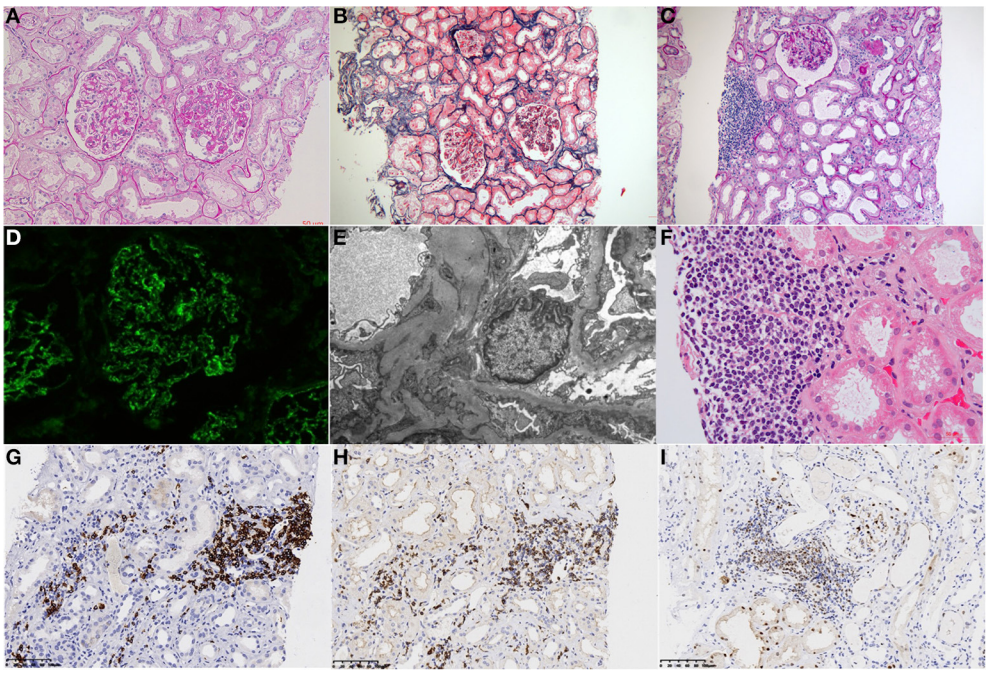

FIGURE 1 | Histological findings on renal biopsy. **(A)** Light microscopy study of the renal biopsy specimen revealed segmental endocapillary proliferation with neutrophil infiltration. (PAS, ×400). **(B)** The tubules displayed acute injury with epithelial simplification and small focal atrophy (Masson trichrome staining, ×200). **(C)** Renal interstitial was infiltrated by focal lymphocytes (×100). **(D)** Immunofluorescence analysis revealed positive granular staining of IgG in the mesangium and capillary wall. **(E)** Electron microscopy showing electron-dense deposits in the sub-epithelial and segmental mesangial. **(F)** Interstitial infiltration of a nodular mass of medium-sized lymphoid cells with irregular nuclei. (PAS, ×400). **(G–I)** Immunohistochemical analysis revealed lymphoblasts were strongly positive for CD 20 **(G)**, CD5 **(H)**, and Cyclin D1 **(I)** (Panels were indicated from left to right with letters **A–I**).

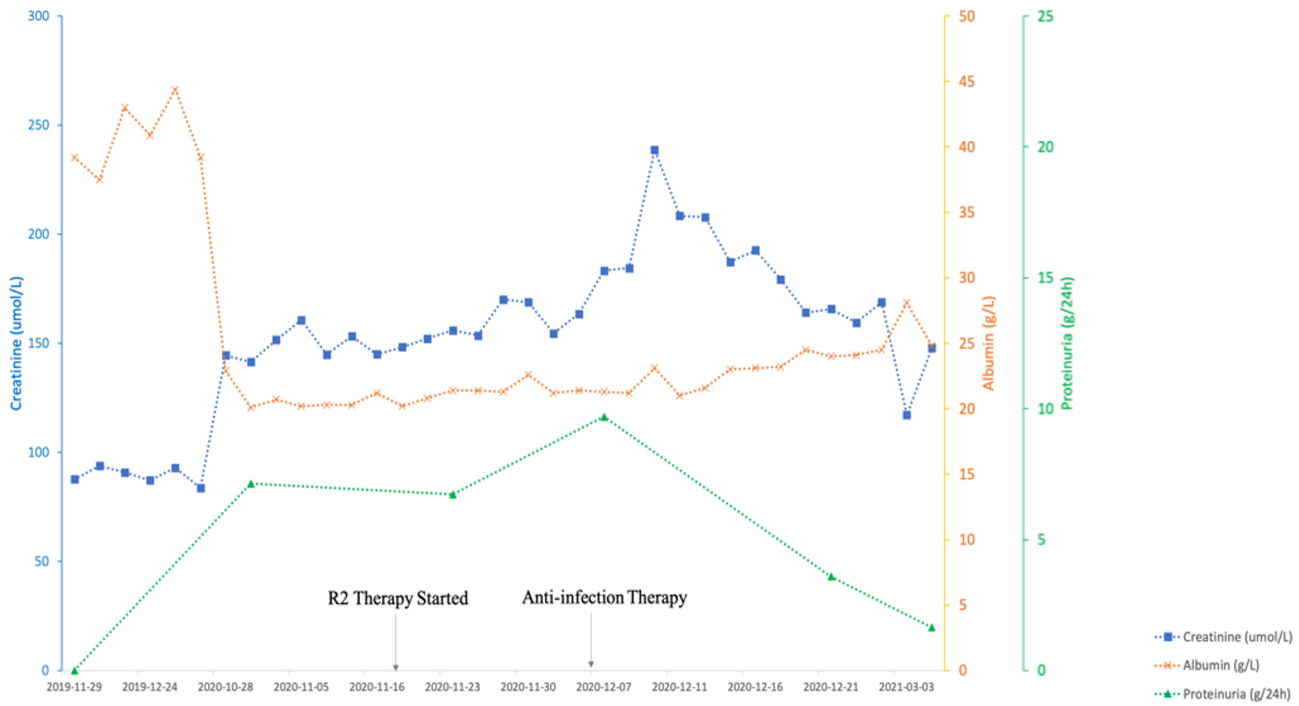

FIGURE 2 | The clinical course of the present case. R2 Therapy indicates Rituximab and lenalidomide.

proliferative glomerulonephritis (11), and ANCA-associated pauci-immune crescentic glomerulonephritis (12).

Lupus nephritis secondary to MCL is indeed rare. As far as we know, the first and the only case of lupus nephritis associated with MCL was reported in 2018 (5). No study, to date, has reported a specific mechanism and relationship between SLE and MCL. Wang et al. (13) performed a nation-wide observational study in Taiwan, evaluating if there was a bidirectional relationship between SLE and NHL. They found that the patients with NHL had a higher risk of SLE. Morth et al. (14) illustrated that the most common specific autoimmune diseases, which were categorized as primarily B-cell mediated, were rheumatoid arthritis, SLE, and primary Sjögren's syndrome in patients with diffuse large B-cell lymphoma. The suspected pathogenesis shared by some autoimmune diseases and NHL might include similar genetic risk factors or trigger factors (13). A previous study concluded that both T cells and B cells have important roles in SLE pathogenesis. Especially, B cells produce the hallmark autoantibodies like anti-DNA antibodies and antinuclear antibodies (1). T follicular helper cells, which were recognized as a novel subpopulation of helper T cells (7), activate germinal center B cells to produce autoantibodies. The expansion of lymphoma cells has efficient ways to influence the immune system toward dysregulation or chronic stimulation, which may foster SLE development in individuals who are also prone to autoimmune diseases (15).

CONCLUSION

In summary, we reported an old man who presented with nephrotic syndrome and acute kidney disease attributable to lupus nephritis. Renal biopsy showed mixed proliferative and membranous lupus nephritis with concomitant interstitial infiltration from lymphoid cells. The patient was considered to be a case of lupus nephritis secondary to mantle cell lymphoma. However, the underlying pathogenesis in lymphoma-associated lupus nephritis still needs to be addressed.

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 Peking University First Hospital, approval number: 2017[1333]. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

DB, YT, and BW analyzed and interpreted the patient data and were major contributors in writing the manuscript. XY and HW performed interpretation of pathological data. RX, FZ, and MZ performed interpretation of the clinical data and substantively revised it. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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Posterior Reversible Encephalopathy Syndrome in a Patient With Microscopic Polyangiitis: A Case Report and Literature Review

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Central nervous system (CNS) is rarely involved in microscopic polyangiitis (MPA). Here, we report a 14-year-old girl with MPA who developed new-onset seizures with deterioration of renal function. Her brain CT scan and MRI showed concurrent complications of intracerebral hemorrhage and posterior reversible encephalopathy syndrome (PRES). She got remission with combinations of methylprednisolone pulse, plasma exchange, regular hemodialysis, antiseizure and antihypertension medications. Furthermore, it is crucial to exclude the adverse effect of medications such as corticosteroid and biological therapy. We searched the literatures, retrieved 6 cases of MPA with PRES and summarized their clinical characteristics.

Keywords: microscopic polyangiitis, central nervous system, intracerebral hemorrhage, posterior reversible encephalopathy syndrome, case report

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INTRODUCTION

Microscopic polyangiitis (MPA) is one classical type of antineutrophil cytoplasmic antibody (ANCA)-associated systemic vasculitis which mainly affects small vessels like arterioles, capillaries or venules. Unlike peripheral vasculitic neuropathy common in patients with ANCA-associated vasculitis (1), central nervous system (CNS) involvement was infrequent in MPA (2). Even so, it may lead to complications like ischemic infarction (3–5), intracerebral hemorrhage (6, 7), subarachnoid hemorrhage (8), ventricular hemorrhage (9), as well as posterior reversible encephalopathy syndrome (PRES) (10–15).

At the same time, treatment with high dose of corticosteroids and biological agents like rituximab, could also lead to CNS complications including PRES (16–19).

Herein, we reported a rare case of MPA who developed concurrent intracerebral hemorrhage and PRES.

CASE PRESENTATION

A 14-year-old girl with no significant past medical history was admitted to the local hospital in January 2018 due to lower extremities edema. Her urine examination showed proteinuria (4.95 g/24 h) and microscopic hematuria, with serum creatinine value elevated to 2.29 mg/dl. She had positive result of perinuclear antineutrophilic cytoplasmic antibody (p-ANCA) (1:32)

and the myeloperoxidase-antineutrophil cytoplasmic antibody (MPO-ANCA) was 81.7 RU/ml (Enzyme linked immunosorbent assay, normal range <10 RU/ml). Her anti-glomerular basement membrane (GBM) antibody was negative. Renal biopsy was performed and reported necrotizing crescentic glomerulonephritis with pauci-immune deposits. Imaging examinations, including computed tomography (CT) scans of head and chest, showed no abnormality. She was diagnosed as MPA and was then treated with three courses of plasma exchange, methylprednisolone pulse therapy (15 mg/kg/day for 3 days), followed by oral prednisone 1 mg/kg/day (tapered gradually after 6 weeks) and intravenous cyclophosphamide (500 mg/1.73 m^2, once a month). Her serum creatinine value gradually decreased to 1.58 mg/dl and anti-MPO antibody turned to negative. She presented with a relapse six months later with her serum creatinine value increased to 3.98 mg/dl. She was admitted to our hospital on June 27, 2018. Physical examinations showed that blood pressure was 120/80 mmHg and there were no other remarkable abnormal findings on admission. She maintained with oral prednisone of 60 mg, received another 10 rounds of plasma exchange and two doses of rituximab (100 and 500 mg respectively, with an interval of one week). Three days after the second infusion of 500 mg rituximab, she was complicated with bacterial pneumonia and a further deterioration of renal function (serum creatinine level increased to 4.69 mg/dl. Ceftriaxone (1 g/day) and meropenem (0.5 g/day) were then used. Seventeen days later, she suddenly developed disorder of thought and auditory hallucination followed 10 min later by tonic-clonic seizures. Her blood pressure was 150/100 mmHg and the neurological examinations had no abnormality at that time. Her head CT scan showed a small amount of hemorrhage in the left temporal lobe (**Figure 1**). Brain magnetic resonance image (MRI) revealed multiple foci lesions under bilateral cortex of the superior frontal sulcus, parieto-occipital and watershed area was dominant, which suggested typical PRES features (**Figures 2A1–4**). The brain magnetic resonance angiography (MRA) was normal. The clinical findings timeline was presented in **Figure 3**.

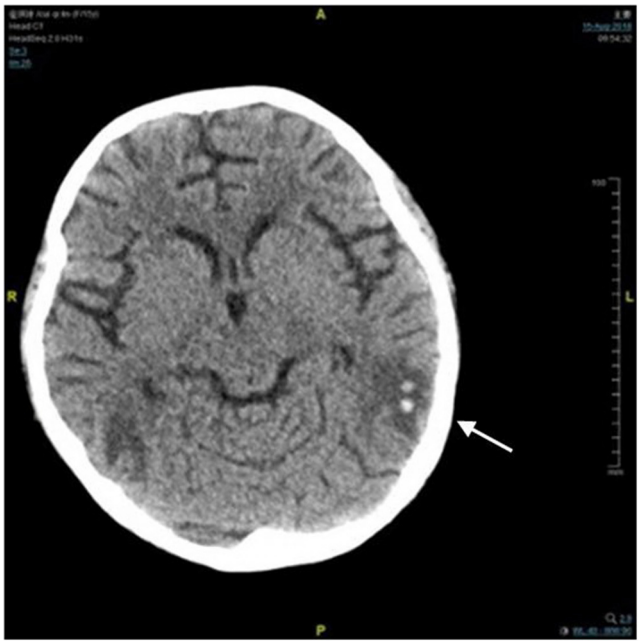


FIGURE 1 | Computed tomography image of brain. There was a small amount of hemorrhage in the left temporal lobe (arrow).

Diagnostic Assessment and Therapeutic Interventions

In our case, the head CT was normal before admission and she didn't have any other risk factors for cerebrovascular disease. Her new-onset of consciousness changing and seizures, accompanied by brain MRI showing vasogenic edema in bilateral cerebral hemispheres, indicating the classic characteristics of PRES. Based on the progressive advanced renal failure with severe microscopic hematuria, elevated erythrocyte sedimentation rate (ESR) and C-reactive protein level, her Birmingham Vasculitis Activity Score (BVAS) at the time of PRES was 21, which indicated high disease activity. Furthermore, receiving high dose corticosteroid, and cytotoxic agents such as rituximab, might contribute to hypervolemia and vasospasm, which promoted the development of PRES (19–22). But the interval between the rituximab regimen against MPA and the onset of PRES was more than two weeks, much longer than the interval reported by previous literature varying between 6 h to 8 days (19–22), which excluded its contribution. The pneumonia and blood pressure were under well-control and her hemodialysis was regular and efficient, which did not support the participation of infection and hypertension (23, 24). Thus, PRES in our patient might mainly attributed to MPA. As the intracerebral hemorrhage could happen in 15% of patients with PRES (17), we proposed that the PRES contributed to the intracranial hemorrhage. Therefore, treatment was aimed at MPA with administration of 200 mg methylprednisolone and initiation of another seven courses of plasma exchange, in combination with regular hemodialysis (three times per week), anti-seizure medication and well blood pressure control. The patient's mental symptoms resolved within 24 h.

Follow-Up and Outcomes

The follow-up brain MRI after 17 days showed that the degree of gyrus swelling was reduced, most of the abnormal high signal shadows disappeared and multiple small ischemic lesions were found under bilateral frontal-parietal cortex (**Figures 2B1,2**). Unfortunately, her renal function did not recover with maintenance hemodialysis and oral prednisone gradually tapered after 6 weeks. Her peripheral-blood CD19+ B-cell counts decreased to 1/μl after two times of rituximab infusion and increased to 5/μl six months later when she received another time of rituximab infusion (100 mg) as part of maintenance therapy. She was content with her treatment since the disease was controlled and stabilized. No PRES relapsed

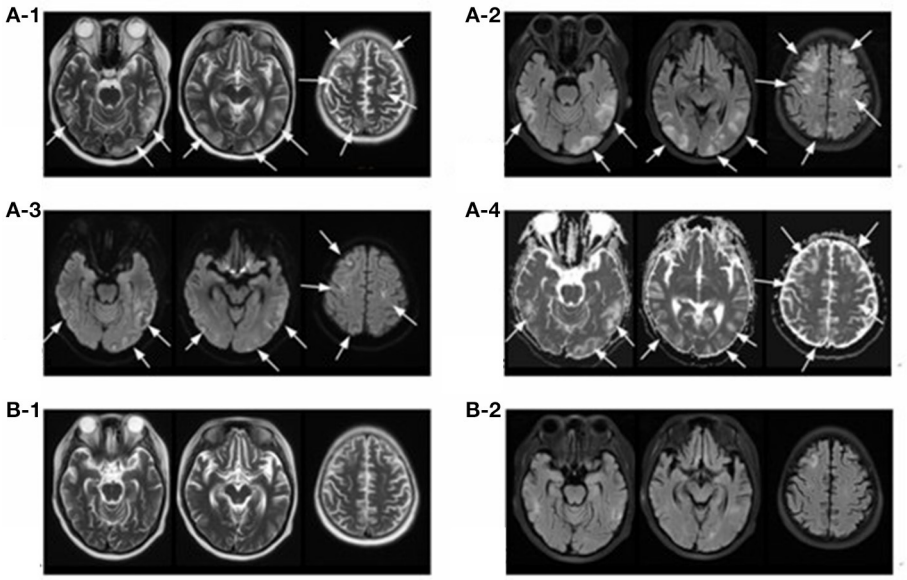
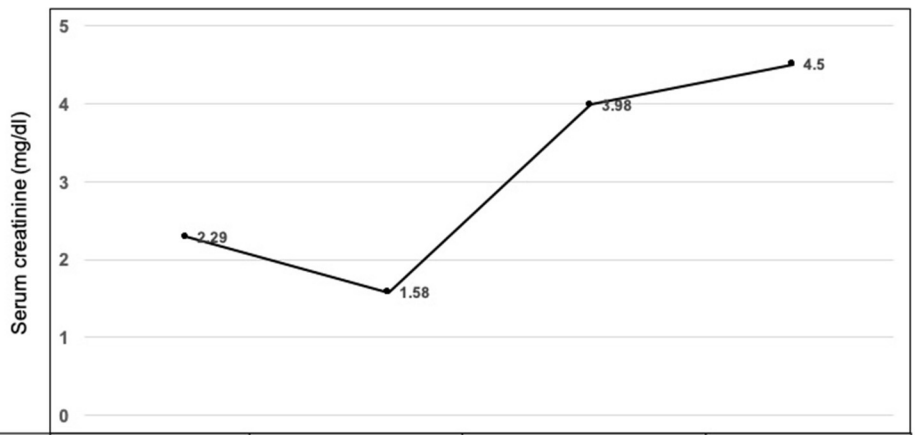


FIGURE 2 | Magnetic resonance imaging (MRI) of brain. T2-weighted **(A-1)** and FLAIR **(A-2)** sequences depict hyper-intense lesions involving the bilateral cerebral hemispheres (occipital lobe, parietal lobe, temporal lobe and frontal lobe) and cerebral cortex and subcortex (arrows), most of which disappeared 17 days later **(B-1,B-2)**. Diffusion-weighted magnetic resonance imaging **(A-3)** and apparent diffusion coefficient **(A-4)** sequences of the involved regions showed isointense or hyperintensity lesions (arrows).



Time point	Onset of the MPA	Follow-up	6 months later from the MPA onset	17 days from deterioration of renal function
Events	/	/	Deterioration of RF, followed by pneumonia and further increase of Scr	Intracerebral haemorrhage and PRES
Treatment	PE+ pulse GC	Tapering GC+ CTX monthly	PE+GC+RTX	PE+ pulse GC+HD+anti-seizure

FIGURE 3 | Clinical findings timeline. MPA, microscopic polyangiitis; GC, glucocorticoid; CTX, cyclophosphamide; PE, plasmapheresis; RTX, rituximab; RF, renal function; PRES, posterior reversible encephalopathy syndrome; HD, hemodialysis.

and she received a deceased donor kidney transplantation in December 2019. She observed the strict and routine follow-up with treatments to prevent graft rejection, and her renal function and central nervous system condition kept stable until now.

DISCUSSION

We herein report a teenage girl with active MPA and advanced renal injury who was treated with corticosteroid and rituximab, then developed PRES with intracerebral hemorrhage presenting

TABLE 1 | Cases describing microscopic polyangiitis with posterior reversible encephalopathy syndrome.

No	References	Case presentation	Imaging of the brain
1	Tajima et al. (10)	A 76-year-old woman who was diagnosed as isolated oculomotor neuropathy associated with MPA and the administration of oral prednisolone was started. Seven days later however, she suddenly began to complain of a headache and her consciousness was disturbed. Her blood pressure was 136/86 mm Hg. PRES associated with p-ANCA positive vasculitis was suspected and one gram of methylprednisolone was administered. Though her consciousness returned to normal, the patient developed pulmonary hemorrhage which did not respond to any medication. She eventually died of multiple organ failure. Her renal function estimated by creatinine was 0.8–1.2 mg/dl and was well-controlled until the end of her life.	Brain MRI examinations revealed high FLAIR and T2 signal intensities in the bilateral parietal, occipital and right frontal lobes. There were no significant signal alterations in the DWI and no apparent changes in the ADC map. With further steroid treatment, after three weeks, the previously observed high FLAIR and T2 signal intensity lesions had disappeared.
2	Wacker et al. (11)	A 51-year-old woman with pulmonary–renal syndrome was diagnosed as MPA with renal failure (creatinine 3.2 mg/dl). Despite the addition of several antihypertensive drugs, her blood pressure was rising up to 180/110 mm Hg. Twenty days after immunosuppressive treatment was started, the patient suffered from severe headache, a generalized seizure and complete visual loss. Methylprednisolone pulse therapy was commenced and anti-convulsant therapy started. Over the next days, seizures subsided and the patient completely regained vision. She remained in complete remission thereafter.	MRI images showed symmetrical subcortical edema. Diffusion-weighted sequences suggested cerebral ischemia. MRA showed severe bilateral narrowing of M1 and M2 cerebrovascular segments. Repeated cerebral MRA demonstrated full resolution of previously narrowed vessels and subcortical bilateral lesions within 6 weeks.
3	Fuentes et al. (12)	A 72-year-old female presented with chest discomfort, lower extremity edema, and fatigue for 2 days. The initial physical exam revealed a blood pressure at 139/80 mm Hg. A strongly positive MPO-ANCA together with the presence of crescentic glomerulonephritis on kidney biopsy were consistent with the diagnosis of MPA. The patient was then on immunosuppressive treatment with high-dose steroids. She suddenly developed severe headache followed hours later by tonic-clonic seizures. Her blood pressure at that time was 153/101 mm Hg. She responded well to the combination of hemodialysis, antiseizure medication, and blood pressure control. Immunosuppressive therapy with high-dose steroids, was continued throughout this episode. She regained consciousness and was in her baseline neurologic state in ∼1 week.	Brain MRI examinations revealed high Flair and T2 signal intensities in the bilateral occipital lobes.
4	Patel et al. (13)	A 40-year-old man developed sudden blackout in front of his eyes followed by involuntary left-facial twitching and a brief episode of unresponsiveness. He had such three episodes in 1 day and was found to have a blood pressure of 160/90 mm Hg on admission. Renal failure was revealed by raised serum creatinine (24.48 mg/dl) with a positive p-ANCA and pauci-immune necrotizing crescentic glomerulonephritis. MRI of the brain revealed features of PRES. He was given intravenous pulse methylprednisolone for 3 days and recovered quickly. Two months later, the patient presented with recurrent seizures with repeat MRI of the brain revealing features of PRES. The reason behind the event was related to two missed sessions of hemodialysis and the steroid dose being tapered. Symptoms resolved with intensive hemodialysis sessions and up-titration in the dose of steroids. He underwent renal allograft transplantation, after which he showed good clinical recovery.	MRI of the brain revealed areas of altered signal intensity in right-posterior temporal, bilateral medial basal ganglionic area, inferior and parasagittal bilateral occipital regions in the subcortical region showing hyper-intensity in T2-weighted and inversion recovery images, isointense on T1-weighted and no restricted diffusion, suggestive of PRES. MRA of the brain was normal.
5	Wang et al. (14)	A 10-year-old girl with pauci-immune glomerulonephritis, and a positive p-ANCA was diagnosis with MPA. She had worsening renal function with creatinine increasing to 6.3 mg/dl. She was treated with pulse methylprednisolone, intravenous cyclophosphamide and plasmapheresis. Four days later, she developed new-onset seizure activity. Her blood pressure, which was previously in the reference range at 128/82 mm Hg, was elevated at 170/100 mm Hg. MRI of the brain revealed findings consistent with PRES. she was started on increased dose of lisinopril and amlodipine for hypertension and ziprasidone for agitation and hallucinations. She recovered within 2 days. Two weeks after discharge, she presented with recurrence of generalized seizures. Repeat MRI of the brain showed recurrence of PRES. Rituximab (400 mg/m^2 weekly for 4 weeks) was administered for her underlying vasculitis. Over the ensuing months, her condition had remained stable.	MRI of the brain showed an area of FLAIR hyper-intensity involving the cortex and subcortical white matter in left superior parietal parasagittal gyrus, consistent with PRES. Repeat MRI showed multiple hyper-intensities involving frontal, parietal, and temporo-occipital regions, more severe on the right, consistent with recurrence of PRES.

(Continued)

TABLE 1 | Continued

No	References	Case presentation	Imaging of the brain
6	Bhadu et al. (15)	A 14-year-old Indian female child was diagnosed as MPO-ANCA associated vasculitis (MPA) based on clinical, immunological, histological and radiological findings. Cyclophosphamide pulse was initiated and oral prednisolone was administered. She presented 12 days later with an episode of seizure not associated with any focal neurological deficit. MRI of the brain revealed findings consistent with PRES. She was managed with antiepileptic medication, pulse methylprednisolone and cyclophosphamide. She responded well to the treatments and recovered completely.	FLAIR and T2-weighted sequences of MRI (Brain) depicted hyper-intense lesions involving the frontoparietal cortices, which showed complete resolution at 6 months.

MPA, microscopic polyangiitis; PRES, posterior reversible encephalopathy syndrome; p-ANCA, perinuclear antineutrophilic cytoplasmic antibody; MRI, magnetic resonance image; FLAIR, fluid attenuated inversion recovery; DWI, diffusion-weighted image; ADC, apparent diffusion coefficient; MRA, magnetic resonance image; MPO-ANCA, myeloperoxidase-antineutrophil cytoplasmic antibody.

with consciousness disturbance and seizure. In most MPA cases, it was characterized by rapidly progressive glomerulonephritis, lung hemorrhage and interstitial pneumonitis, while the CNS involvement was reported less frequently (18).

Besides MPA, there were some other predisposed factors related to PRES should be distinguished in our patient: (1) The adverse effect of rituximab: The time interval between the infusion of rituximab and PRES in our patients was much longer than reported in previous literature which has been stated in the previous paragraph (19–22). Besides, there was no PRES recurrence during the re-administration of rituximab to the patient which helped to rule out the participation of RTX (19–22). (2) Renal failure: Renal failure could occur in up to 55–57% of the PRES cases reported in the literatures (25), in which the uremic toxins accumulation could lead to endothelial dysfunction and account for the development of PRES. Thus, renal failure may act as the "second-hit" in our case. Taken together, we proposed that PRES might be mainly attributed to MPA with participation of its associated renal dysfunction in the patient.

Furthermore, although the differentiation of which therapy actually helped the case was difficult, it was noticed that our treatment project to the patient was mainly targeted on MPA *per se*. The PRES was alleviated and did not relapse with the MPA stabilization which also supported our previous hypothesis that the patient's PRES might be the neurologic involvement of ANCA associated vasculitis.

Although not fully understood, endothelial dysfunction and abnormal cerebral blood flow were key factors in the pathophysiological changes underlying PRES (16, 24–26). MPA could induce inflammation within the vasculature of the CNS and result in ischemic or hemorrhagic damage to the brain parenchyma with resultant focal or generalized neurologic deficits (3–9). In PubMed, only 6 cases of MPA with PRES were reported (10–15) (More details in **Table 1**). In conclusion, five of them were females and one was male, with a mean age of 43.8 years (range 10–76 years). The duration from MPA onset to PRES varied from 2 days to 3 years. Two cases had hypertension at baseline and renal failure was found in all patients (serum creatinine value ranging from 1.2 to 24.48 mg/dL). All cases received immunosuppressive therapy such as high-dose steroids, plasma exchanges, etc. They responded well to the supportive therapy combining with hemodialysis, anti-seizure medication and antihypertensive drugs. During the follow-up, two cases presented with PRES recurrence, which might be attributed to quickly tapering of steroids, irregular hemodialysis, or relapse of vasculitis.

The limitation of our case was that it was a pure clinical report and the causal relationship between MPA and PRES accompanied by intracerebral hemorrhage was not fully elucidated. Further laboratory studies like *in vitro* experiments or animal models, are needed.

In summary, this was a case which presented a MPA patient complicated with PRES. Through careful differential diagnosis. We excluded rituximab as the cause of PRES and the patient was well followed-up.

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

Peking University International Hospital, Beijing, China, does not require ethical approval for reporting individual cases. Written informed consent for patient information and images to be published was provided by the patient's legally authorized representative.

AUTHOR CONTRIBUTIONS

ZQ and FY: conceptualization, supervision, and writing—review and editing. JX, YD, ZQ, and FY: data curation and investigation. JX and YD: writing—original draft. All authors contributed to the article and approved the submitted version.

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SYSTEMATIC REVIEW
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Meta-Analytical Accuracy of ANCA Renal Risk Score for Prediction of Renal Outcome in Patients With ANCA-Associated Glomerulonephritis

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Background: To evaluate the diagnostic accuracy of antineutrophil cytoplasmic antibody (ANCA) renal risk score (ARRS) for prediction of renal outcome in patients with ANCA-associated glomerulonephritis (ANCA-GN).

Methods: We searched PubMed, EMBASE, Ovid, Web of Science, the Cochrane Library, and ClinicalTrials.gov for studies, which used ARRS to predict end-stage renal disease (ESRD) in patients with ANCA-GN. Two reviewers independently screened articles for inclusion, assessed the quality of studies with both an adapted Quality Assessment of Diagnostic Accuracy Studies 2 (QUADAS-2) tool. We calculated the combined patients with ESRD in the ARRS categories and presented the summary and individual estimates based on the ARRS categories. Then, the sensitivity, specificity, diagnostic odds ratio (DOR), positive/negative likelihood ratio, and the area under the receiver operating characteristic (AUROC) curves of the pooled data for ARRS were used to assess the accuracy of the "above the low-risk threshold" (ARRS $\geq$ 2) and "high-risk grade" (ARRS $\geq$ 8) for renal outcome of patients with ANCA-GN. The hierarchical summary ROC (HSROC) was used to verify the accuracy value. The clinical utility of ARRS was evaluated by the Fagan plot. Heterogeneity was explored using meta-regression and subgroup analysis.

Results: A total of 12 distinct cohorts from 11 articles involving 1,568 patients with ANCA-GN were analyzed. The cumulative patients with ESRD at the maximum follow-up of 60 months was 5% (95% CI: 0.02–0.07; $p < 0.001$) for ANCA-GN with low ARRS (0–1 points) and significantly increased to 22% (95% CI: 0.15–0.29; $p < 0.001$) medium ARRS (2–7 points). The combined cumulative patients with ESRD was 59% (95% CI: 0.49–0.69; $p < 0.001$) high ARRS (8–11 points). The pooled sensitivity of ARRS $\geq$ 2 in predicting ESRD was 98% with a specificity of 30% and a DOR of 15.08 and the mean AUROC value was 0.82. The pooled sensitivity of ARRS $\geq$ 8 in predicting ESRD was 58%

with a specificity of 86% and a DOR of 7.59. The meta-regression and subgroup analysis indicated that variation in the geographic regions, study design, index risk, follow-up time, age of patient, publication year, and number of patient could be the potential sources of heterogeneity in the diagnosis of ARRS $\geq$ 8.

Conclusion: This meta-analysis emphasized the good performance of the ARRS score in predicting the renal outcome in patients with ANCA-GN. However, these findings should be verified by future large-scale prospective studies.

Keywords: ANCA-GN, end-stage renal disease (ESRD), renal risk score, meta-analysis, predictive value

INTRODUCTION

Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a group of life-threatening systemic autoimmune diseases characterized by inflammation, which included granulomatous polyangiitis (GPA), microscopic polyangiitis (MPA), eosinophilic granulomatous polyangiitis (EGPA), and renal-limited vasculitis (RLV) (1). Renal involvement of AAV, which is called ANCA-associated glomerulonephritis (ANCA-GN), occurs in more than 75% of patients with AAV (2, 3), presenting as a pauci-immune necrotizing crescentic ANCA-GN on renal biopsy sample histology and likely to be further developed as end-stage renal disease (ESRD) (4). Renal survival is closely related to prognosis and outcome of patient (5), so identification of the predictive factors for renal survival and outcome in patients with ANCA-GN is very important.

Renal biopsy is a well-established diagnostic modality for the diagnosis of kidney diseases and assessment of activity in ANCA-GN, but the predictive value of renal outcome of ANCA-GN is still controversial (6). In 2010, an international working group of renal pathologists published a histological classification for ANCA-GN based on kidney biopsy. This classification divided patients to four subgroups: focal (>50% normal glomeruli), crescentic (>50% cellular crescents), sclerotic (>50% sclerotic glomeruli), and mixed (any other combination) and the probability of progressing to ESRD increased in ascending order of focal, crescentic, mixed, and sclerotic (7). Some studies have validated that this classification system can reflect the severity of the initial kidney involvement and independently predict the renal outcome (8–10), but lack the influence of clinical factors and interstitial fibrosis (IF) on the prognosis of renal survival.

In the recent years, Brix et al. proposed a validated and predictive tool for ANCA-associated renal vasculitis to estimate the renal survival at baseline, called ANCA renal risk score (ARRS) (11). It is a scoring system that consists of histopathological findings (including the percentage of normal glomeruli, tubular atrophy/interstitial fibrosis) and baseline estimated glomerular filtration rate (eGFR), which ranges from 0 to 11 and three risk groups, from low (0–1 points), medium (2–7 points), and high (8–11 points) probability of ESRD.

The RRS has been validated in several studies among patients with ANCA-GN, though its comprehensive predictive value needs to be confirmed. Therefore, the objective of this systematic review and meta-analysis was to identify and determine the accuracy of ARRS to predict ESRD with patients with ANCA-GN in the baseline.

METHODS

Search Strategy

The structure of this systematic review conformed to the recommendations from the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (www.prisma-statement.org) (12) and the "Cochrane Handbook for Systematic Reviews of Diagnostic Test Accuracy" was for reviews of diagnostic accuracy (13). The protocol for this systematic review was registered in the International Prospective Register of Systematic Reviews (PROSPERO) database, (Registered No. CRD42021254072).

We performed a systematic search in PubMed, EMBASE, Ovid, Web of Science, the Cochrane Library, and ClinicalTrials.gov from their inception to June 30, 2021. The term was used for ("antineutrophil cytoplasmic antibody" or "ANCA") and ("ARRS" or "RRS" or "ANCA renal risk score" or "renal risk score"). We did not impose any filter with respect to text availability and there is no restriction placed on language or publication status.

Eligibility Criteria

The criteria for inclusion of a study in the meta-analysis were as follows: (1) Full-text articles, which investigated the predictive value of ARRS for ESRD in patients with ANCA-GN; (2) original research articles that were written in English; (3) prospective or retrospective studies; and (4) provided sufficient data to calculate the true positive (TP), false positive (FP), true negative (TN), and false negative (FN). We excluded the following studies: (1) meeting abstracts and review articles; (2) case series, case reports, editorials, or letters to the editor that did not include complete data; and (3) lack of adequate information to accurately calculate the test estimates. If there were duplicate publications, we included the most complete version or the article with the highest number of subjects.

Data Extraction

The included articles will be selected by two independent reviewers (M Xia and D Chen). First, both will review titles and abstracts; second, they will cross-check all the information

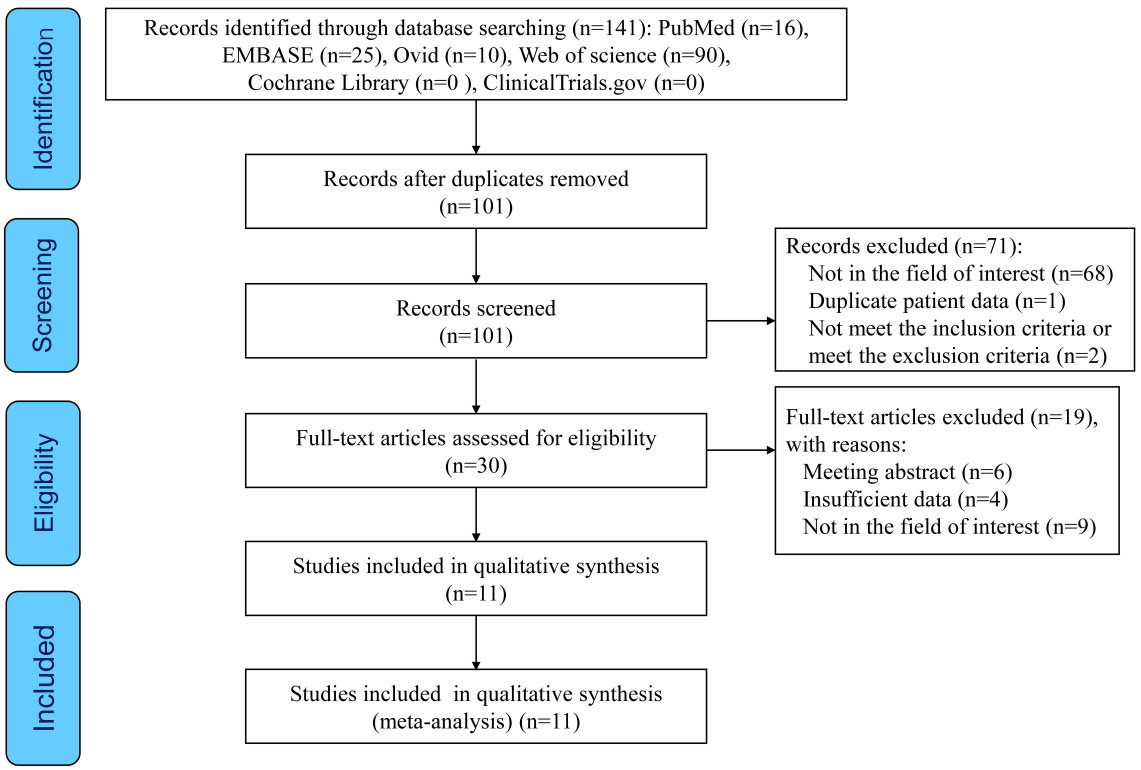

FIGURE 1 | The preferred reporting items for systematic reviews and meta-analyses (PRISMA) flow diagram of this study.

and disagreements were resolved through consensus. All the extracted data were independently verified by a third investigator (D Chen). From each included study report, we identified the first author, publication year, country, study design, sample size, percentage of male fetuses, follow-up duration, characteristics of included patients (age, histologic class, clinical diagnosis, and antibody subtype), and the number of patients with ANCA-GN who became ESRD. We also extracted data on the index test (including TP, FP, TN, and FN results), accuracy estimates, and data for 2 × 2 tables.

Quality Assessment

Risk of bias and concerns about applicability was assessed by two authors (M Xia and R Yu) with the Quality Assessment of Diagnostic Accuracy Studies 2 (QUADAS-2) tool (14).

Data Synthesis

The primary outcome of this systematic review was renal outcome, which we defined as the number of patients with ANCA-GN who became ESRD. The ARRS score was stratified into three categories and we calculated the combined patients with ESRD in the ARRS categories. Summary and individual estimates (proportion of patients with ESRD) were presented graphically with the 95% CIs by a forest plot based on the ARRS categories. We also conducted a diagnostic meta-analysis of the studies that met the criteria and had been screened. Calculate the Spearman's correlation coefficient p between the TP rate and the FP rate and analyze whether there is a threshold effect. If there was no significant threshold effect, the diagnostic accuracy was estimated by pooled statistics. We used summary receiver operating characteristic (SROC) plots to present the results of each study in ROC space, with each study plotted as a single sensitivity specificity point. This produced an SROC curve, with a summary operating point (showing summary sensitivity and specificity values), a summary area under the curve (AUC) value, 95% confidence region, and 95% prediction region. We obtained summary accuracy estimates for the sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), diagnostic odds ratio (DOR), and the AUC value and used hierarchical summary ROCs (HSROC) to verify the accuracy value. Using Fagan plot analysis, the post-test probability was calculated under the assumption that the pretest probability was 25, 50, and 75%, respectively. PLR is the ratio of the likelihood of ESRD in those with a positive test vs. those with a negative test. A PLR above 1 indicates increased evidence of ESRD; the farther higher from 1, the more chance of ESRD. NLR is the ratio of the likelihood of ESRD in those with a negative test vs. those with a positive test. NLR below 0.1 is very strong evidence to rule out an ESRD. DOR is the quotient between PLR and NLR. DOR can be calculated as the ratio of the odds of positivity in an ESRD relative to the odds of positivity in the non-ESRD, with higher values, indicating better discriminatory test performance.

TABLE 1 | Base characteristics of included studies.

Study	Region	Study design	Sample (low/ medium/ high) †	Male (%)	Age (year)*	eGFR (ml/min per 1.73 m^2) *	Follow-up (month)*	Definition of ESRD	Histologic class (%)	Clinical diagnosis	Antibody subtype (%)
Brix et al. (11) (validation)	Germany	R	90 (26/47/17)	65.6	67.5 (55.3–74)	29.5 (20–44)	31 (20.3–54)	Dialysis or kidney transplantation	F 41.1%, C 27.1%, M/S 37.8%	NR	PR3 (+) 47.8%, MPO (+) 52.2%
Brix et al. (11) (training)	Germany	P	115 (30/64/21)	73	66 (54.5–72)	27.5 (18–47)	34 (22–57)	Dialysis or kidney transplantation	F 33.9%, C 34.8%, M/S 31.3%	NR	PR3 (+) 50.4%, MPO (+) 49.6%
Li et al. (28)	United Kingdom	R	105 (36/51/18)	51.4	66 (57–73)	18 (11–28.5)	42 (26–69)	NR	NR	NR	PR3 (+) 42.9%, MPO (+) 49.5%, Both (-) 7.6%
Gercik et al. (26)	Turkey	R	106 (15/67/24)	57	55 (36–74)	NR	39.6 (24–65)	Permanent dialysis	F 17.0%, C 39.0%, M 31.0%, S 13.0%	MPA 23%, GPA 54%, RLV 18%, EGPA 5%	NR
Jebali et al. (25)	Tunis	R	37 (5/17/15)	48.6	54 (17–80)	16.7 (3–93)	33.15 (1–145)	RRT	F 2.7%, C 24.3%, M 24.3%, S 48.6%	MPA 59.5%, GPA 40.5%	PR3 (+) 40.6%, MPO (+) 59.4%
Daalen et al. (27)	World Wide	R	145 (6/91/48)	NR	63 (55–70)	23 (12–46)	71 (52–126)	NR	F 36%, C 25%, M 27%, S 12%	NR	NR
An et al. (23)	China	R	252 (68/86/98)	44.8	57.5±14.2	20.3 (9.2–45.3)	63.9 ± 49.5	RRT	NR	MPA 84.1%, GPA 4.8%, EGPA 1.6%, RLV 9.5%	MPO (+) 88.1%
Vilet et al. (24)	Mexico	R	72 (13/34/25)	33	53 (35–61)	21 (10–35)	69 (45–98)	RRT or kidney transplantation	F 8%, C 6%, M 35%, S 51%	GPA 56.9%, MPA 25%, RLV 18.1%	PR3 (+) 51%, MPO (+) 25%, Both (+) 6%, Both (-) 18%
Villacorta et al. (21)	Spain	R	147 (32/77/38)	57.8	60.2±16	14.7 (8–27.1)	41 (9.6–104)	Dialysis or renal transplantation	F 19.7%, M 23.8%, C 42.2%, S 14.3%	MPA 38.8%, GPA 6.8%, RLV 54.4%	PR3 (+) 11.6%, MPO (+) 63.9%, Both (+) 0.7%, Both (-) 23.8%
Tan et al. (22)	United Kingdom	R	178 (64/76/38)	NR	NR	NR	NR	NR	NR	NR	NR
You et al. (20)	China	R	70 (12/40/18)	51.4	61.9±10.3	19 ± 8.1	45.9 (20–96)	RRT or kidney transplantation	C 42.9%, M 57.1%	NR	PR3 (+) 5.7%, MPO (+) 88.6%, Both (-) 5.7%

(Continued)

TABLE 1 | Continued

Study	Region	Study design	Sample (low/ medium/ high) †	Male (%)	Age (year)*	eGFR (ml/min per 1.73 m^2) *	Follow-up (month)*	Definition of ESRD	Histologic class (%)	Clinical diagnosis	Antibody subtype (%)
Boudhabhay et al. (19)	French	R	251 (101/76/74)	49.4	63 (52–73)	24 (11–46)	42 (12–89)	Dialysis or kidney transplantation	F 33.9%, C 19.5%, M 23.9%, S 22.7%	RLV 21.1%	PR3 (+) 30.7%, MPO (+) 65.3%, Both (-) 4.4%

NR, no reported; R, retrospective; P, prospective; RRT, renal replacement therapy; F, focal; C, crescentic; S, sclerotic; M, mixed; GPA, granulomatosis with polyangiitis; MPA, microscopic polyangiitis; EGPA, eosinophilic granulomatosis with polyangiitis; RLV, renal limited vasculitis; PR3, proteinase 3; MPO, myeloperoxidase.

†Low denotes ARRS 0–1 points; Medium denotes ARRS 2–7; High denotes ARRS ≥ 8.

**Mean ± SD or median (interquartile range).*

Investigation of Heterogeneity

Heterogeneity among included studies was evaluated using the I^2 and Q-statistic and $p < 0.10$ was considered to show significant heterogeneity, I^2 values of 0–40, 40–70, and 70–100% indicate low, moderate, and high heterogeneity, respectively (15). A fixed-effects model was applied when $I^2 < 50\%$, while a random-effects model was applied when $I^2 > 50\%$ (16, 17). In the diagnostic meta-analysis, if $I^2 > 50\%$ and/or $p < 0.05$ was found, considerable heterogeneity was considered, and in this case, sources of heterogeneity were explored by a subsequent subgroup analysis to identify the potential covariates. Deeks' funnel plot was applied to examine the potential publication bias caused by the asymmetry of the tests. Meta-regression analysis was performed for studies included in the meta-analysis and explored possible sources of heterogeneity (18). We planned to investigate any significant findings on meta-regression using subgroup analyses. Sensitivity analysis was performed to identify the influence of an individual study on pooled estimates by removing one study at a time (17).

Statistical Analysis

Risk of bias was assessed using the Review Manager version 5.3 (RevMan version 5.3, Copenhagen; The Nordic Cochrane Center, The Cochrane Collaboration, 2014), threshold effect was tested by the Meta-Disc software (version 1.4, Clinical Biostatistics, Ramony Cajal Hospital, Madrid, Spain), and other analysis was conducted on the Stata software (version 14.0, StataCorp, College Station TX).

RESULTS

Search Results

Selection process is given in **Figure 1**. Of the 141 articles searched, 111 articles were excluded due to duplication ($n = 40$) and irrelevance ($n = 71$) following title and abstract screening. The remaining 30 potentially eligible reports were further evaluated. After excluding the articles with irrelevant contents and articles with no full-text and insufficient data, we included 12 distinct cohorts from 11 articles (11, 19–28) in the meta-analysis.

Study Characteristics

These 12 studies involving 1,568 patients with ANCA-GN were performed in different geographic regions including Europe ($n = 6$), Asia ($n = 3$), North America ($n = 1$), Africa ($n = 1$), and multicenter ($n = 1$). There was only one prospective cohort and the rest were retrospective cohorts. All the 12 cohort studies of 11 articles evaluated the ARRS score for more than 3 years incidence of ESRD in patients with ANCA-GN. In 12 studies, nine studies described the histologic class of patients. Six studies classified clinical diagnostic subtypes and nine studies detailed antibody subtypes. In these studies, the grading for renal risk was assessed by the ARRS based on the data obtained from baseline estimated glomerular filtration rate (eGFR), the percentage of normal glomeruli, and tubular atrophy/interstitial fibrosis of renal biopsy, as shown in **Table 1**.

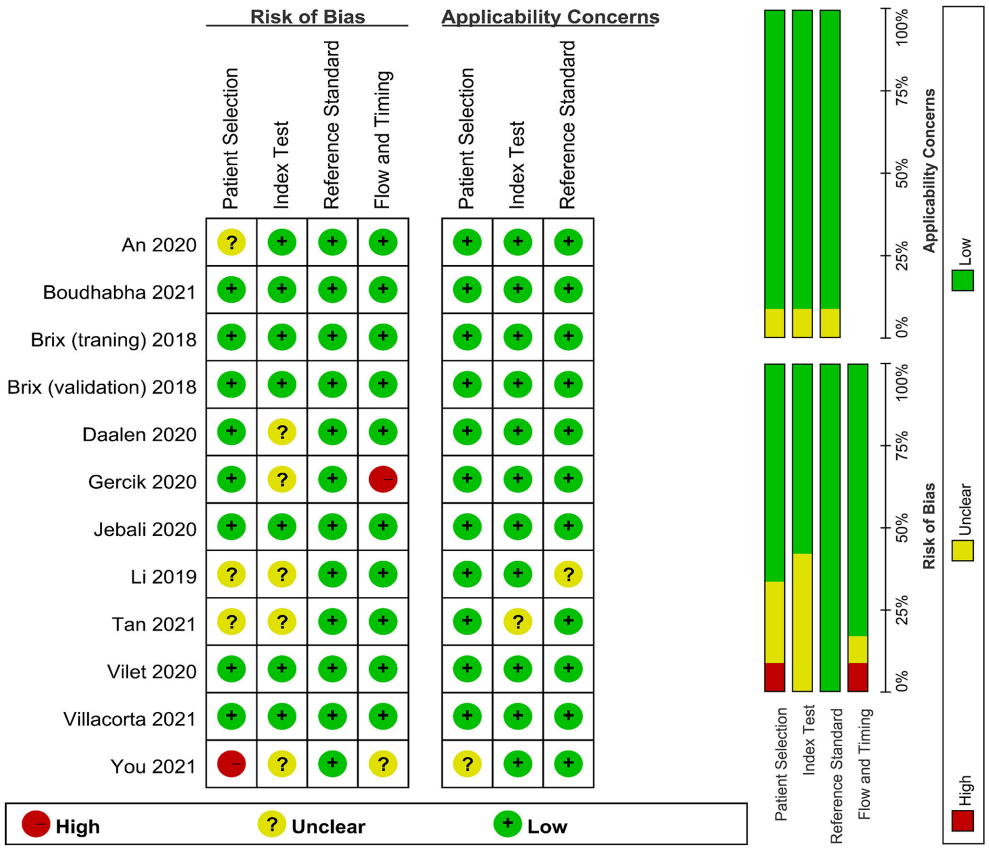

FIGURE 2 | Quality assessment of the included studies.

Quality Assessment

Quality assessments using the QUADAS-2 criteria are shown in **Figure 2**. One study was assessed as "high risk" for index test and one study was assessed for flow and timing in the risk of bias. Some studies were estimated as "suboptimal" for unclear risk in the following domains: selection of patient, index test, flow and timing in the risk of bias and selection of patient, index test, and reference standard in the applicability concerns. Most of the studies were identified as having low risk of bias for reference standard in the risk of bias.

Cumulative ESRD on ARRS Classification

We identified 12 published cohort studies that reported the cumulative patients with ESRD in three ARRS grades. The cumulative patients with ESRD at the maximum follow-up of 60 months was 5% (95% CI: 0.02–0.07; $p < 0.001$) with low heterogeneity ($I^2 = 0\%$, $p = 0.958$) for ANCA-GN with low ARRS (0–1 points) and significantly increased to 22% (95% CI: 0.15–0.29; $p < 0.001$) with high heterogeneity ($I^2 = 85.7\%$, $p < 0.001$) for ANCA-GN with medium ARRS (2–7 points) and the combined cumulative patients with ESRD was 59% (95% CI: 0.49–0.69; $p < 0.001$) with high heterogeneity ($I^2 = 77.4\%$, $p < 0.001$) for ANCA-GN with high ARRS (8–11 points), as shown in **Figure 3**.

Overall Predictive Accuracy of ARRS for ESRD

Table 2 shows that the pooled sensitivity of ARRS score ≥ 2 (above the low-risk threshold) across all the included studies was 0.98 (95% CI: 0.94–0.99, **Figure 4A**) and specificity was 0.30 (95% CI: 0.22–0.39, **Figure 4A**). The DOR for positive ESRD was 15.08 (95% CI: 8.87–25.63, **Figure 5A**), the pooled PLR was 1.42 (95% CI: 1.26–1.61, **Figure 6A**), the NLR was 0.13 (95% CI: 0.08–0.20, **Figure 6B**), and the AUC was 0.82 (95% CI: 0.78–0.85, **Figure 7A**).

The sensitivity of high-risk ARRS score ≥ 8 for prediction ESRD was 0.58 (95% CI: 0.51–0.65, **Figure 4B**) and the specificity was 0.86 (95% CI: 0.81–0.89, **Figure 4B**). The DOR for positive ESRD was 7.59 (95% CI: 5.82–9.90, **Figure 5B**), the pooled PLR was 3.81 (95% CI: 2.88–5.05, **Figure 6C**), the NLR was 0.51 (95% CI: 0.43–0.61, **Figure 6D**), and the AUC was 0.77 (95% CI: 0.73–0.80, **Figure 7B**). However, these estimates should be interpreted with caution, since considerable heterogeneity was observed in some results.

Corresponding curves from the HSROC model are given in **Figure 7**. The estimated value of *beta* was 0.41 (95% CI: −0.54 to 1.45), Z was 0.85, and $p > 0.01$ for

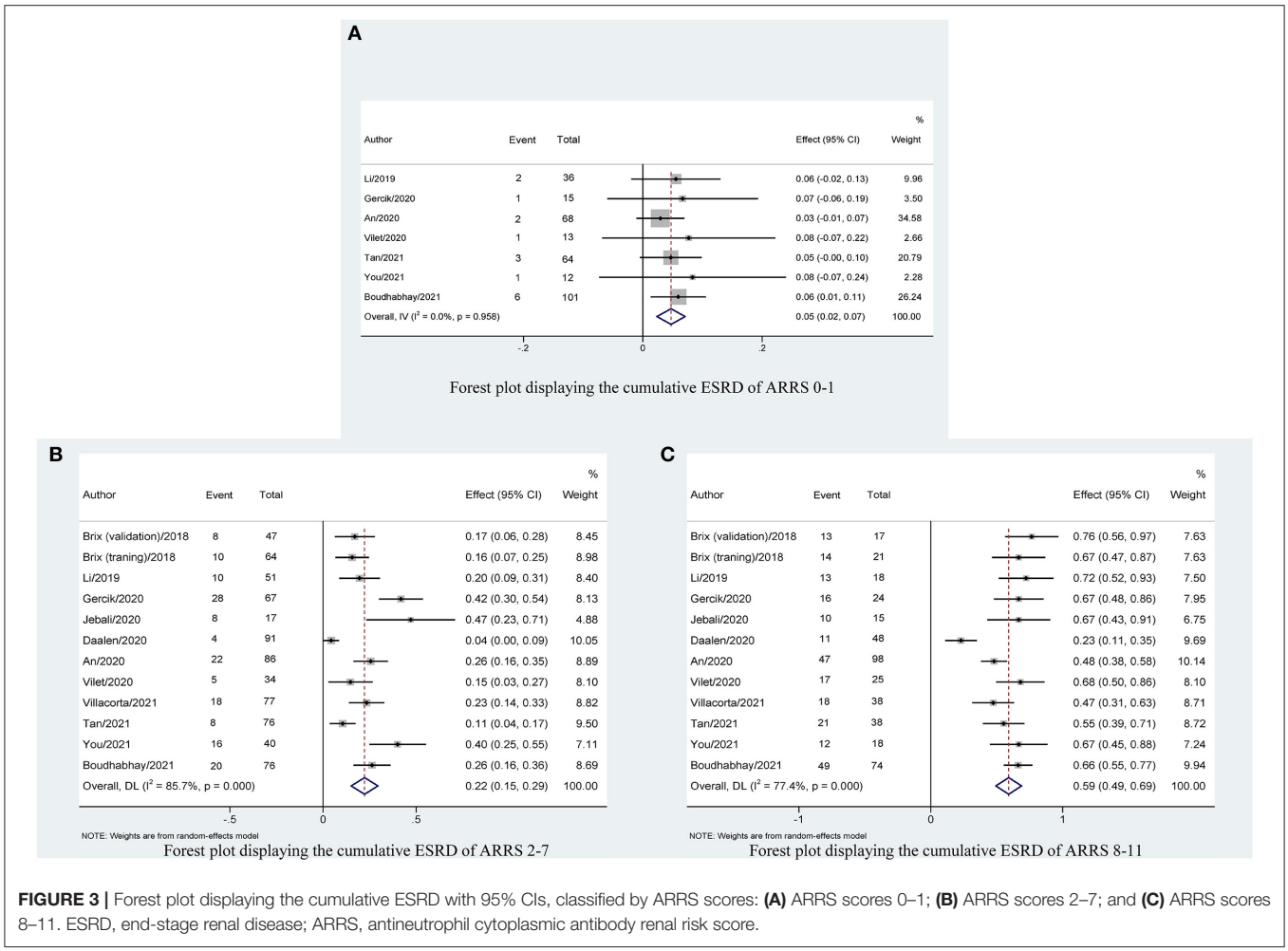

FIGURE 3 | Forest plot displaying the cumulative ESRD with 95% CIs, classified by ARRS scores: **(A)** ARRS scores 0–1; **(B)** ARRS scores 2–7; and **(C)** ARRS scores 8–11. ESRD, end-stage renal disease; ARRS, antineutrophil cytoplasmic antibody renal risk score.

TABLE 2 | Predictive accuracies of ARRS.

Category	ARRS≥2			ARRS≥8		
	Data (95% CI)	I^2(%) or Z^*	*p*	Data (95% CI)	I^2(%) or Z^*	*p*
SE %	0.98 (0.94–0.99)	10.1	0.35	0.58 (0.51–0.65)	51.15	0.020
SP %	0.30 (0.22–0.39)	89.64	<0.001	0.86 (0.81–0.89)	79.9	<0.001
PLR	1.42 (1.26–1.61)	86.3	<0.001	3.81 (2.88–5.05)	63.6	0.001
NLR	0.13 (0.08–0.20)	0	0.952	0.51 (0.43–0.61)	51.7	0.019
DOR	15.08 (8.87–25.63)	0	0.897	7.59 (5.82–9.90)	43.7	0.052
AUROC	0.82 (0.78–0.85)	–	–	0.77 (0.73–0.80)	–	–
Beta[#]	−0.28 (−1.19–0.64)	−0.6	0.552	0.41 (−0.54–1.37)	0.85	0.394
Lambda[#]	2.23 (0.76–3.71)	–	–	1.84 (1.22–2.45)	–	–

ARRS, ANCA renal risk score; SE, sensitivity; SP, specificity; PLR, positive likelihood ratio; NLR, negative likelihood ratio; DOR, diagnostic odds ratio; AUROC, area under the receiver operating characteristic curve; CI, confidence interval.

[#]From hierarchical summary receiver operating curves (HSROC) model.

[*]Z value only for Beta row, I^2 value for rest.

ARRS score ≥ 8, which indicated the symmetrical of SROC curve. 1.84 (95% CI: 1.22–2.45) of lambda verified that the high-risk ARRS score has a high accuracy for predicting ESRD.

Clinical Utility of ARRS for ESRD

In suspected patients with ESRD with score ≥ 2, the Fagan plot analysis revealed the PLR and NLR of 1 and 0.08, respectively. Thus, in this group of patients with 25% pretest probability

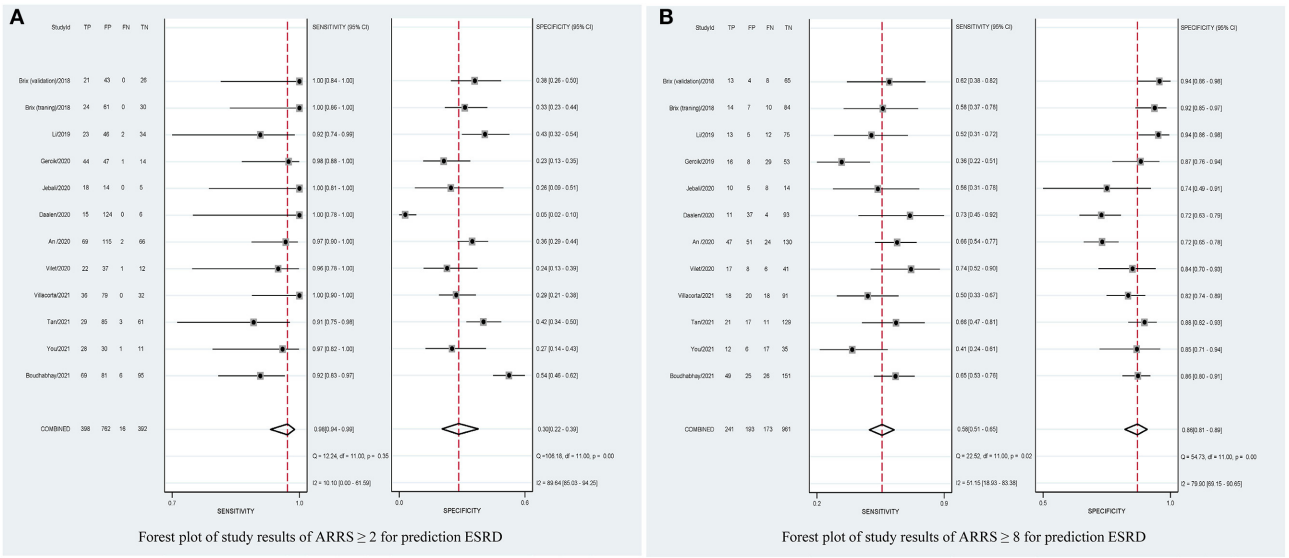

FIGURE 4 | Forest plots of pooled sensitivity and specificity of ARRS ≥ 2 **(A)** and ARRS ≥ 8 and **(B)** for prediction ESRD. ESRD, end-stage renal disease; ARRS, antineutrophil cytoplasmic antibody renal risk score; FN, false negative; TN, true negative; FP, false positive; TP, true positive.

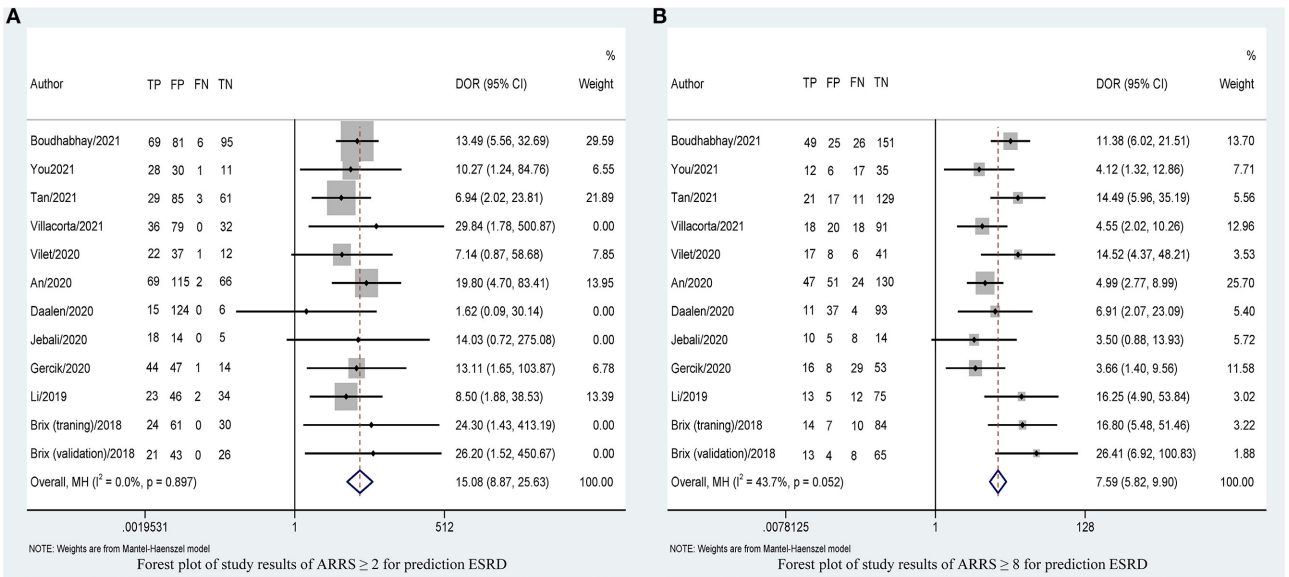

FIGURE 5 | Forest plots of DOR of ARRS ≥ 2 **(A)** and ARRS ≥ 8 and **(B)** for prediction ESRD. ESRD, end-stage renal disease; ARRS, antineutrophil cytoplasmic antibody renal risk score; DOR, diagnostic odds ratio; FN, false negative; TN, true negative; FP, false positive; TP, true positive.

(based on clinical suspicion), a positive ESRD value revealed a 32% probability of correct diagnosis and a negative ESRD value revealed a 3% probability of wrong diagnosis (**Figure 8A**). When the pretest probability (based on clinical suspicion) was set to 50%, a positive ESRD value yielded 58% probability of correct diagnosis and a negative ESRD value yielded 8% probability of wrong diagnosis (**Figure 8B**). When the pretest probability (based on clinical suspicion) was set to 75%, a positive ESRD value showed 81% probability of correct diagnosis and a negative ESRD value showed 20% probability of wrong diagnosis (**Figure 8C**).

In suspected patients with ESRD with scores 8–11, the Fagan plot analysis revealed the PLR and NLR of 4 and 0.49, respectively. Thus, in this subset of patients with 25% pretest probability (based on clinical suspicion), a positive ESRD value represented a 57% probability of correct diagnosis and a negative ESRD value indicated a 14% probability of wrong diagnosis (**Figure 8D**). When the pretest probability (based on clinical

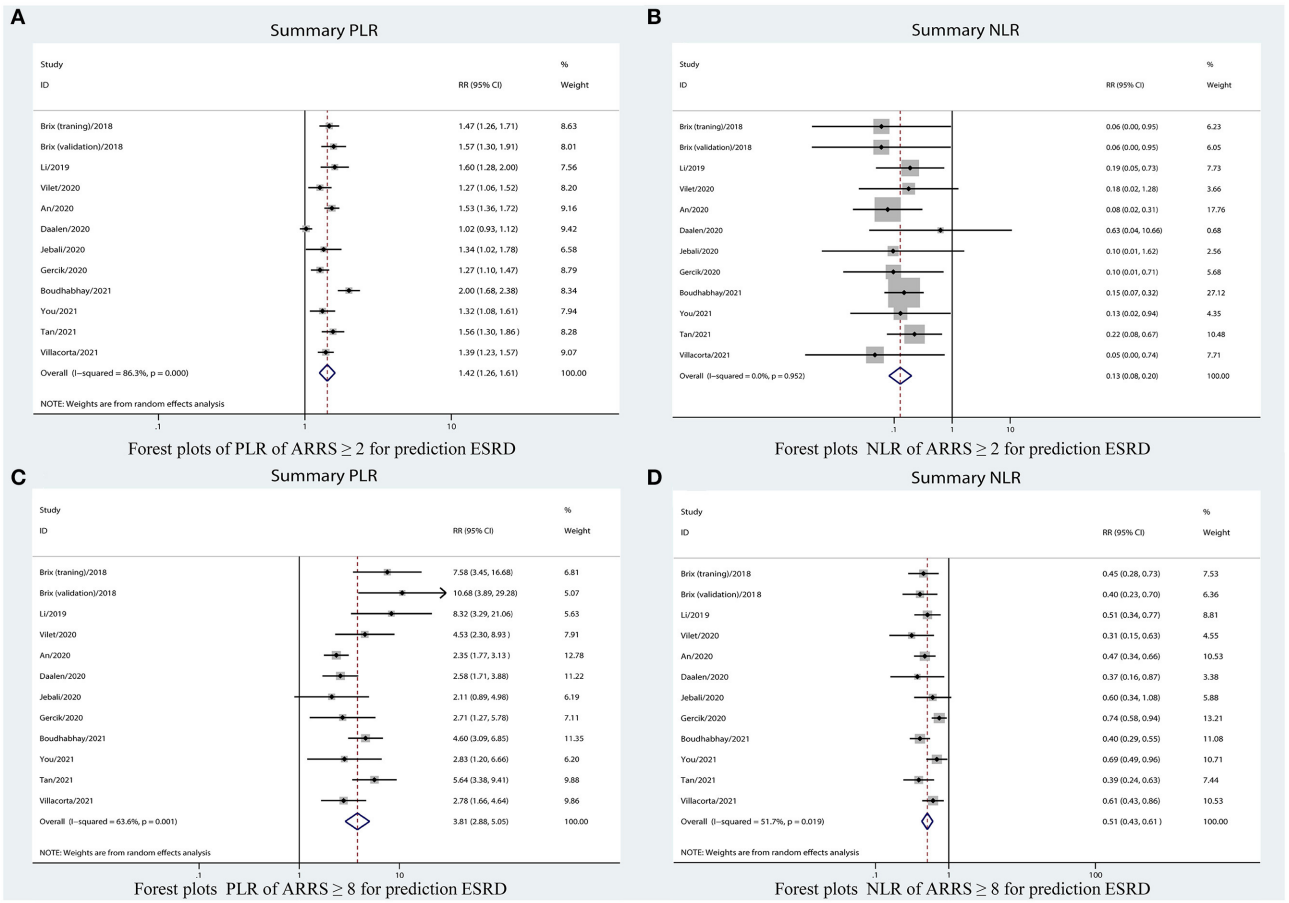

FIGURE 6 | Forest plots of LR of ARRS for prediction ESRD. **(A)** PLR for ARRS ≥ 2; **(B)** NLR for ARRS ≥ 2; **(C)** PLR for ARRS ≥ 8; and **(D)** NLR for ARRS ≥ 8. ESRD, end-stage renal disease; ARRS, antineutrophil cytoplasmic antibody renal risk score; PLR, positive likelihood ratio; NLR, negative likelihood ratio.

suspicion) was set to 50%, a positive ESRD value showed 80% probability of correct diagnosis and a negative ESRD value showed 33% probability of wrong diagnosis (**Figure 8E**). When the pretest probability (based on clinical suspicion) was set to 75%, a positive ESRD value showed 92% probability of correct diagnosis and a negative ESRD value showed 60% probability of wrong diagnosis (**Figure 8F**).

Threshold Effect

Calculate the Spearman's correlation coefficient p between the sensitivity logarithm and the (1-specificity) logarithm and the p of score ≥ 2 and score ≥ 8, the values were 0.389 ($p = 0.212$) and 0.308 ($p = 0.331$), respectively. $p > 0.05$ indicated that there was no threshold effect.

Meta-Regression and Subgroup Analysis

Overall, result of specificity showed significant heterogeneity; we explored the potential sources of heterogeneity from the region (Europe vs. non-Europe), study design (prospective vs. retrospective), index risk (low risk vs. non-low risk), follow-up (<36 vs. ≥36 months), age of patient (<65 vs. ≥65 years), publication year (<2021 vs. ≥2021), and number of patient (<100 vs. ≥100). The meta-regression analyses showed that all of these values were associated with greater heterogeneity in specificity among ARRS score ≥8 (except for age of patient), as shown in **Figure 9**.

Table 3 also shows the results of the subgroup analyses. In subgroup analyses, the predictive performance of ARRS was approximately consistent across the multiple subgroups.

Sensitivity Analysis

The sensitivity analysis was performed by reducing one article each time to evaluate the impact of a single study on this meta-analysis. Sensitivity analyses for the proportion of patients with ESRD and the result showed that removing single studies that did not have any significant impact on the final outcome. The combined DOR after each elimination had not changed significantly, showing that the results of this analysis are not excessively dependent on a certain study and the conclusion is stable.

Publication Bias

Based on the Deeks' Funnel plot, publication bias was also not detected in the studies where ESRD was used to detect

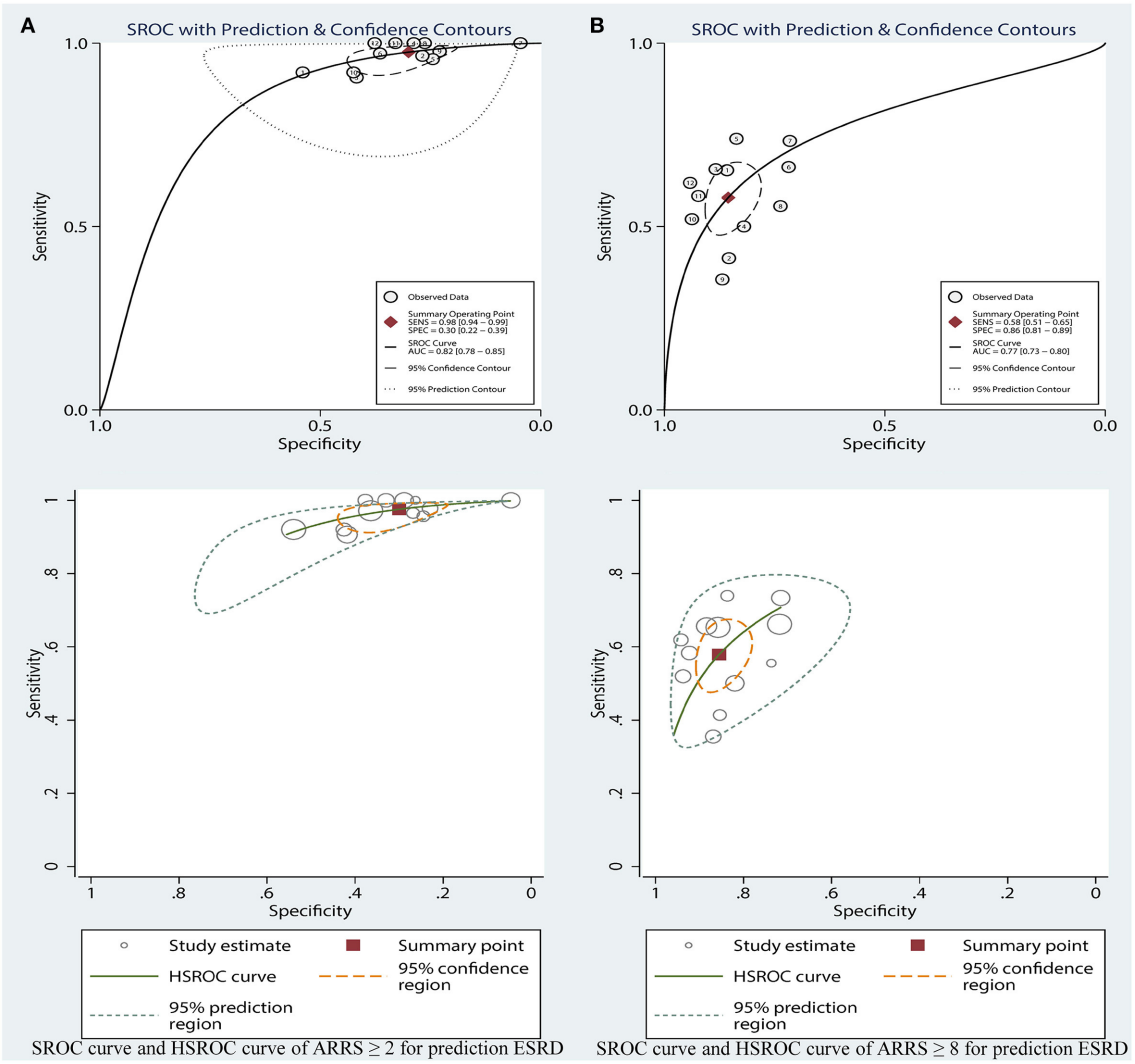

FIGURE 7 | SROC curve and HSROC curve of ARRS ≥ 2 **(A)** and ARRS ≥ 8 and **(B)** for prediction ESRD. ESRD, end-stage renal disease; ARRS, antineutrophil cytoplasmic antibody renal risk score; SROC, summary receiver operating characteristic; HSROC, hierarchical summary receiver operating characteristic.

ARRS score ≥ 2 ($p = 0.25$, **Figure 10A**) and ARRS score ≥ 8 ($p = 0.78$, **Figure 10B**).

DISCUSSION

Associated vasculitis has a high proportion of kidney injury (29). Despite current therapy improving the prognosis of patients, ANCA-GN still results in a rapid or gradual deterioration in renal function (30). ESRD caused by kidney involvement is an important adverse prognostic factor and is associated with high mortality (1, 31). In the past few decades, scholars have tried to specify the characteristics of renal involvement to determine the factors that affect the prognosis of the kidney (32, 33) and have recently developed an ARRS scoring system for predicting renal outcome.

To the best of our knowledge, this is the first systematic review and meta-analysis to report the renal outcome prediction in patients with ANCA-GN using ARRS. Our results confirmed that the pooled incidence rate of ESRD was 4, 22, and 58% in the low-, medium-, and high-risk groups, respectively. It means that patients with the higher ARRS score had a higher probability of suffering ESRD in the next 3–5 years and who had low-risk grade (ARRS score of 0) should be informed about the low possibility of suffering ESRD; this was consistent with previous validation studies. In the meta-analysis of diagnostic tests part, the diagnostic performance of ARRS in different risk grades of ESRD was evaluated in patients with ANCA-GN. Our data revealed that the AUROC of ARRS exhibited a fair diagnostic value for predicting ESRD in ARRS both for ≥ 2 and ≥ 8. Through pooled sensitivity and specificity, it was revealed that ARRS ≥ 2 had a high sensitivity of identifying the potential

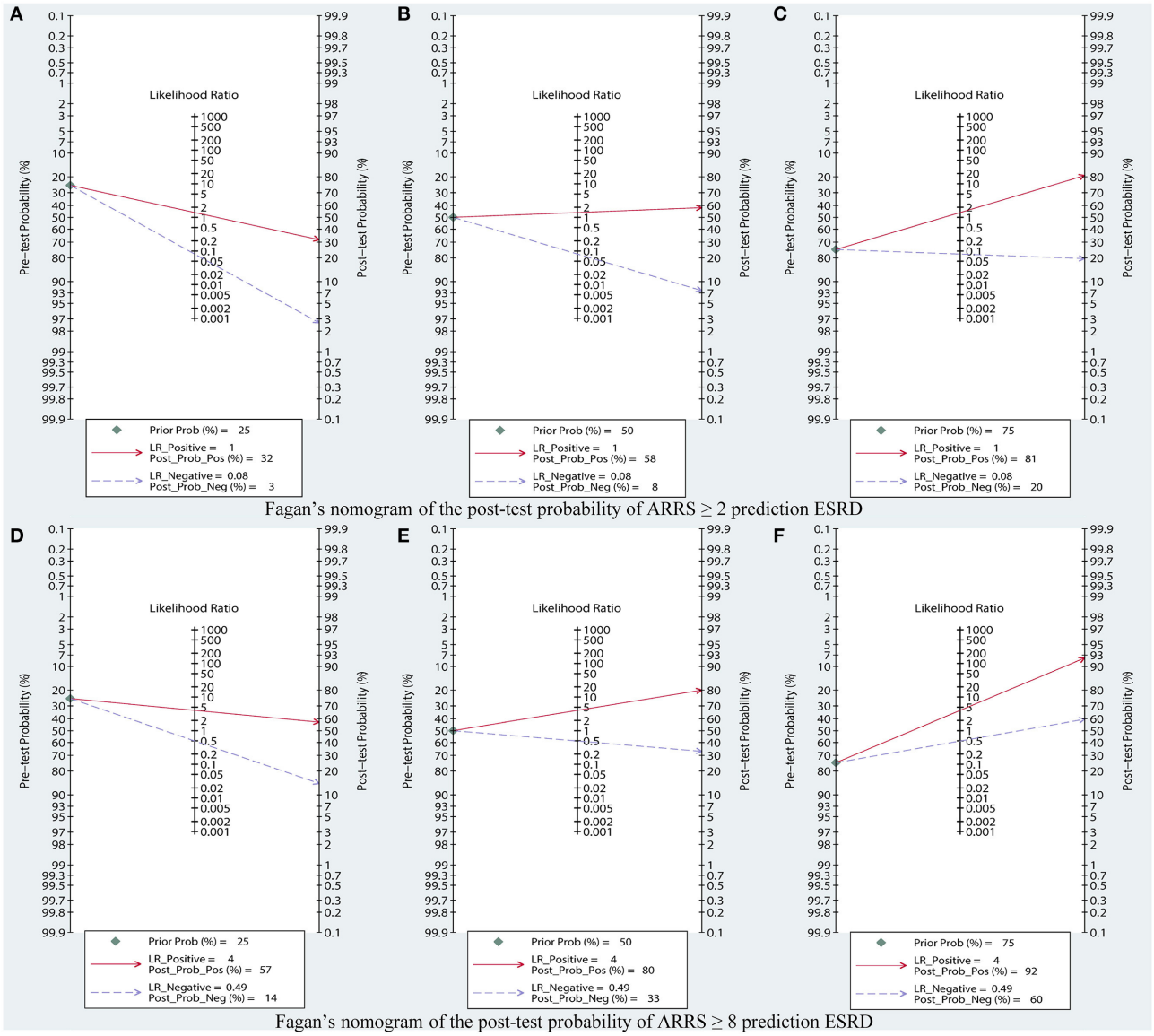

FIGURE 8 | Fagan's nomogram of the post-test probability of ARRS prediction ESRD, based on **(A)** pretest probability = 25%; **(B)** pretest probability = 50%; **(C)** pretest probability = 75% in ARRS ≥ 2; **(D)** pretest probability = 25%; **(E)** pretest probability = 50%; **(F)** pretest probability = 75% in ARRS ≥ 8. ESRD, end-stage renal disease; ARRS, antineutrophil cytoplasmic antibody renal risk score.

ESRD and ARRS ≥ 8 had a high accurate predictability of ESRD in patients with ANCA-GN. Fagan plot analysis showed that ARRS ≥ 2 had a low negative LR and wrong diagnosis rate when predicting ESRD, thus reflecting that when ARRS < 2, few developed ESRD, laterally reflecting the good performance of ARRS < 2 (low-risk class) in detecting renal survival. This analysis also set the good predictive value of ARRS ≥ 8 (high-risk grade) for ESRD.

The present scoring system has an advantage of high sensitivity to potential patients with ESRD above the low-risk threshold and high positive predictive value in the high-risk grade of ESRD and these advantages were thought to come from the characteristics of the composition. Kidney biopsy, the "gold standard" in the diagnosis of kidney disease, was reported as a predictor of kidney prognosis in patients with ANCA-GN in 1999. Bajema et al. (34) performed an observational cohort study in biopsies from 157 patients with clinically and histologically confirmed ANCA-GN. Results indicated that the proportion of normal glomeruli in initial renal biopsy is an excellent predictor of renal function in patients with ANCA-GN. They also proposed that the only active lesion that predicts renal function is from the interstitium. The number of diffuse interstitial infiltrates correlated with serum creatinine values at enrollment and during follow-up. Subsequently. Berden et al.

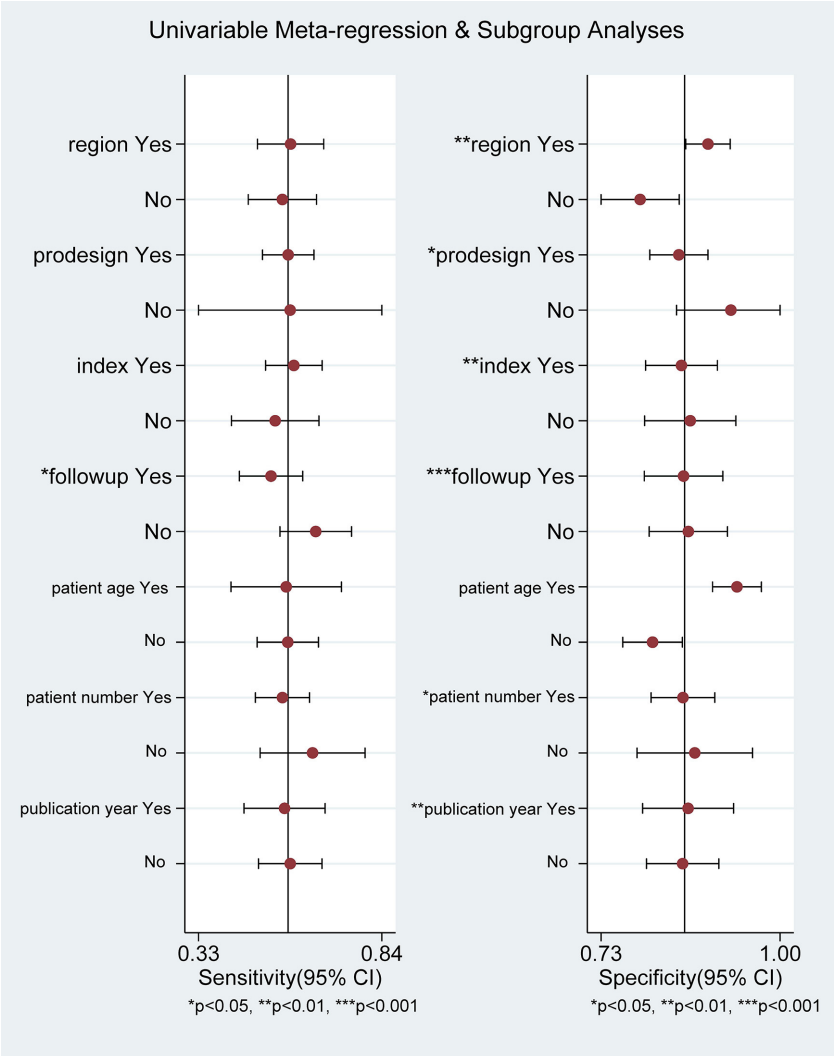

FIGURE 9 | Meta-regression analysis examining heterogeneity.

(7) proposed a classical histopathological classification based on renal biopsy. They studied 100 biopsies with ANCA-GN, lesions were classified according to their predominant glomerular state, and diagnosed from March, 1995 to September, 2002. This validation study showed focal type, with more than 50% of common glomeruli, has relatively the most intact renal function with better renal outcome. The sclerosing type, with more than 50% sclerosing glomeruli, has a high risk of irreversible severe renal impairment and death at the 1-year follow-up period. In the meantime, the predictive role of the renal interstitium for renal outcome has been repeatedly mentioned. Studies have revealed that the presence of diffuse interstitial fibrosis and high tubular atrophy predicted impaired renal function for patients with ANCA-GN during follow-up and tubular atrophy was important predictors of recovery of renal function and renal outcome, independent of initial renal function (35, 36). The third component of ARRS is the initial eGFR. A European multicenter prospective study confirmed that patients with ANCA-GN with GFR < 50 ml/min/1.73 m^2 had a 50% chance of developing ESRD (37) and other data from China also suggested that patients with ANCA-GN with serum creatinine (SCr) levels ≥ 4 mg/dl have a nearly three-fold increased risk of ESRD (38). In addition, severe insufficiency of baseline renal function affects the outcome of subsequent AAV treatment. It has been established that patients with severe renal dysfunction were less likely to respond to treatment, have an increased risk of adverse immunotherapy reactions, and an increased risk of ESRD compared to those with preserved renal function (39).

In the diagnostic meta-analysis section, we observed that the heterogeneity between studies was more pronounced in pooled specificity. Meta-regression analyses indicated that region, study design, index risk, age of patient, publication year, number of patient, and subject risk could be the source of heterogeneity. First of all, the ARRS scoring system was

TABLE 3 | Analysis of subgroups.

Subgroups	Case (*n*)	ARRS ≥ 2				ARRS ≥ 8			
		SE (95% CI)	SP (95% CI)	DOR (95% CI)	AUROC (95% CI)	SE (95% CI)	SP (95% CI)	DOR (95% CI)	AUROC (95% CI)
Region									
Europe	6	0.96 (0.92–0.99)	0.39 (0.29–0.50)	12.78 (6.97–23.44)	0.71 (0.67–0.75)	0.59 (0.49–0.68)	0.89 (0.86–0.92)	11.79 (7.36–18.89)	0.65 (0.61–0.69)
Except Europe	6	0.98 (0.97–0.99)	0.21 (0.13–0.30)	12.12 (5.22–28.13)	0.94 (0.91–0.96)	0.56 (0.47–0.66)	0.79 (0.73–0.85)	5.21 (3.54–7.69)	0.77 (0.73–0.80)
Study design									
Retrospective	11	0.97 (0.94–0.99)	0.3 (0.21–0.40)	12.13 (7.36–20.01)	0.83 (0.79–0.86)	0.58 (0.51–0.65)	0.85 (0.80–0.89)	7.29 (5.55–9.58)	0.77 (0.73–0.81)
Prospective	1	–	–	–	–	–	–	–	–
Index									
Yes	8	0.98 (0.96–0.99)	0.33 (0.23–0.44)	15.9 (8.59–29.41)	0.78 (0.74–0.81)	0.59 (0.52–0.67)	0.85 (0.80–0.91)	7.39 (5.43–10.07)	0.7 (0.66–0.74)
No	4	0.97 (0.93–1.00)	0.24 (0.12–0.36)	7.52 (3.28–17.26)	0.75 (0.71–0.79)	0.54 (0.42–0.66)	0.86 (0.79–0.93)	8.19 (4.88–13.75)	0.8 (0.76–0.83)
Follow-up									
≤36 months	6	0.97 (0.94–1.00)	0.35 (0.23–0.48)	9.65 (4.13–22.45)	0.71 (0.67–0.75)	0.66 (0.56–0.76)	0.86 (0.80–0.92)	11.57 (7.22–18.55)	0.73 (0.69–0.76)
>36 months	6	0.98 (0.96–0.99)	0.25 (0.15–0.36)	14.35 (7.85–26.25)	0.81 (0.78–0.84)	0.53 (0.44–0.62)	0.85 (0.79–0.91)	6.25 (4.52–8.63)	0.77 (0.73–0.80)
Patients age									
≥65	3	0.97 (0.93–1.00)	0.37 (0.19–0.56)	12.6 (3.77–42.14)	– –	0.57 (0.42–0.73)	0.93 (0.90–0.97)	18.78 (9.34–37.75)	– –
< 65	8	0.98 (0.96–1.00)	0.26 (0.16–0.36)	11.32 (6.61–19.38)	0.83 (0.80–0.86)	0.58 (0.49–0.66)	0.81 (0.76–0.85)	6.06 (4.23–8.67)	0.78 (0.75–0.82)
Patient number									
≥100	9	0.97 (0.95–1.00)	0.3 (0.20–0.40)	11.47 (6.81–19.32)	0.81 (0.78–0.84)	0.56 (0.49–0.64)	0.85 (0.80–0.90)	7.56 (5.09–11.21)	0.77 (0.73–0.80)
< 100	3	0.99 (0.95–1.00)	0.29 (0.11–0.47)	11.92 (2.74–51.95)	– –	0.65 (0.50–0.79)	0.87 (0.78–0.96)	11.25 (3.64–34.71)	– –
Publication year									
≥2021	4	0.96 (0.91–1.00)	0.38 (0.23–0.53)	11.33 (5.84–21.96)	0.81 (0.77–0.84)	0.57 (0.46–0.68)	0.86 (0.79–0.93)	7.92 (4.35–14.39)	0.85 (0.81–0.88)
< 2021	8	0.98 (0.96–1.00)	0.26 (0.17–0.36)	11.76 (5.65–24.49)	0.88 (0.85–0.91)	0.58 (0.50–0.67)	0.85 (0.80–0.91)	8.43 (4.99–14.22)	0.76 (0.72–0.79)

AUROC, area under the receiver operating characteristic curve; SE, sensitivity; SP, specificity; DOR, diagnostic odds ratio; ARRS, ANCA renal risk score; Index: Index text is low risk.

proposed in the recent years and the studies validated so far were more scattered and influenced by multiple variables such as geography and population. It can be found in subgroup analysis that heterogeneity can be reduced, but not completely eliminated when analyzed by a single variable. Furthermore, the composition of the scoring system itself may affect the results. It is known that renal outcomes are influenced not only by pathology and initial renal function, but also by multiple factors such as age, genetics, baseline proteinuria, ANCA serology, ANCA antibody subtypes, and treatment (6, 40–43); although some studies have

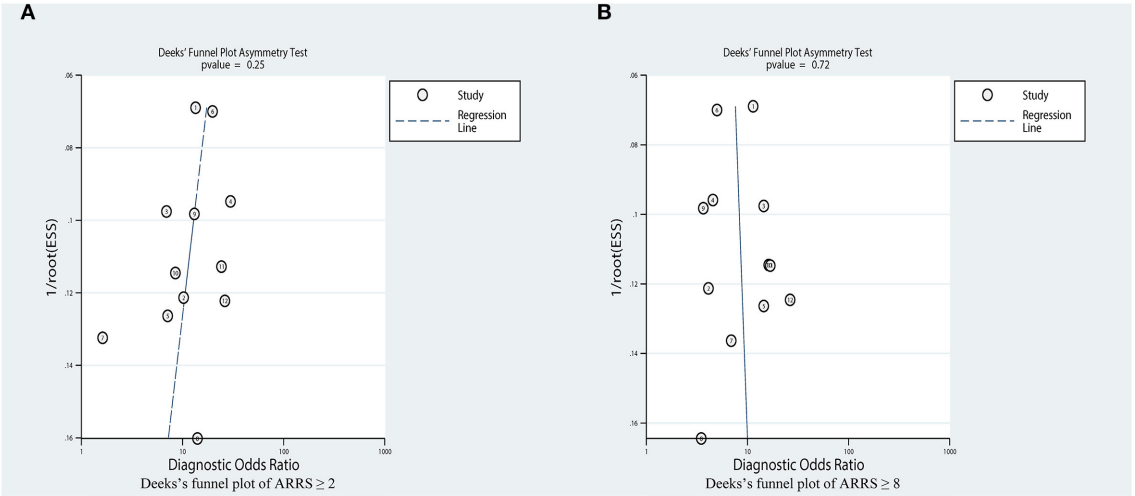

FIGURE 10 | Estimation of the publication bias by Deeks' funnel plots. **(A)** Analysis on the publications concerning ARRS ≥ 2 and **(B)** Analysis on the publications concerning ARRS ≥ 8. ARRS, antineutrophil cytoplasmic antibody renal risk score; ESS, effective sample size.

described a possible correlation between these factors and renal pathology histology and baseline renal function (33, 44, 45), the potential influence of these factors may also account for heterogeneity in including studies.

LIMITATIONS AND FUTURE DIRECTIONS

Several limitations of this study should be noted. First, there was significant heterogeneity in the pooled specificity, which may affect its accuracy. We were able to identify some sources of heterogeneity through subgroup analysis and meta-regression analysis. Second, we were unable to adequately assess the associations between subgroups and other factors due to inadequate descriptions of some of the included studies. Third, the limited sample size of the included studies may also affect data interpretation. Therefore, the generalizability of our findings needs further confirmation. Fourth, several included studies were not provided the description of remission induction and maintenance regimens. We, therefore, did not explore the potential influence of remission induction and maintenance regimens on the outcomes of ESRD; however, future studies should explore this issue.

CONCLUSION

In summary, this study emphasized the merits of ARRS having a predictive ability for ESRD events in ANCA-GN. This new grading system is associated with moderate to good diagnostic value for predicting ESRD in ANCA-GN and also predicts ESRD rates in different risk patients. Future large-scale prospective studies should be conducted to verify the accurate assessment of the diagnostic value of ARRS for predicting ESRD events that could result in screening high-risk individuals for preparing the renal replacement therapy in advance and assessing the survival prognosis.

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 authors.

AUTHOR CONTRIBUTIONS

MX, RY, and DC developed the study question. MX and DC wrote the first draft of the manuscript. DC and XX critically read and revised the final version of the manuscript before submission. All the authors contributed to the development of the review protocol, search strategies, refining of the manuscript, and approved the final manuscript.

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ORIGINAL RESEARCH
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Renal Expression of Annexin A1 Is Associated With the Severity of Renal Injury in Antineutrophil Cytoplasmic Autoantibody-Associated Vasculitis

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Background: Increasing studies demonstrated the importance of activation of neutrophils in the pathogenesis of antineutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV). Previous studies showed that annexin A1 (ANXA1) inhibited the recruitment, transendothelial migration and respiratory burst of neutrophils and induced apoptosis of neutrophils. The current study aimed to investigate the plasma and renal levels of ANXA1 as well as their association with the disease severity in AAV patients.

Methods: Thirty-one AAV patients in active stage and 35 AAV patients in remission stage were recruited. The expression of ANXA1 in renal specimens was assessed by immunohistochemistry. The co-localization of ANXA1 with renal intrinsic and infiltrating cells was detected by double immunofluorescence. The plasma levels of ANXA1 were determined by ELISA. The association of plasma and renal levels of ANXA1 with clinicopathological parameters was further analyzed.

Results: Plasma levels of ANXA1 were significantly higher in active AAV patients than those in AAV patients in remission as well as healthy controls. The renal expression of ANXA1 was significantly higher in active AAV patients than in healthy controls and disease controls. Double immunofluorescence assay showed that ANXA1 was expressed in glomerular endothelial cells, mesangial cells, podocytes, proximal tubular epithelial cells, neutrophils, monocytes/macrophages and T cells in AAV patients. The mean optical density of ANXA1 in glomeruli was correlated with serum creatinine levels ($r = -0.491$, $P = 0.005$) and eGFR ($r = 0.492$, $P = 0.005$) at renal biopsy and the proportion of crescents ($r = -0.423$, $P = 0.018$) in renal specimens of AAV patients. The expression of ANXA1 in glomeruli of AAV patients achieving complete renal recovery was significantly higher than those achieving partial renal recovery.

Conclusion: In AAV patients, the renal expression of ANXA1 was associated with the severity of renal injury.

Keywords: annexin A1, ANCA, vasculitis, renal injury, disease severity

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INTRODUCTION

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) comprises microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA) and eosinophilic granulomatosis with polyangiitis (EGPA), characterized by necrotizing inflammation of the small blood vessels (1). ANCAs against myeloperoxidase (MPO) or proteinase 3 (PR3) are the serological markers of AAV (2, 3). Glucocorticoids combined with cyclophosphamide (CTX) or rituximab in induction therapy and azathioprine (AZA) or rituximab in maintenance therapy was the most commonly used regimen for the treatment of AAV (4, 5).

Although the pathogenesis of AAV has not been fully elucidated, increasing studies have emphasized the importance of ANCA, neutrophil activation and the complement system [reviewed in (6)]. ANCA could induce neutrophils to undergo respiratory burst and degranulation, which resulted in release of free oxygen radicals and various proteases and caused further lesions of vasculitis (2, 7–9).

As a member of the annexin superfamily, annexin A1 (ANXA1) is involved in a variety of cellular biology processes, including cell proliferation, differentiation and apoptosis [reviewed in (10)]. Specifically, ANXA1 peptide can inhibit the recruitment, transendothelial migration and respiratory burst of neutrophils (11–13). ANXA1 can induce neutrophils apoptosis *in vitro* and *in vivo* (14, 15). On the other hand, as a downstream effector of glucocorticoids, ANXA1 contributes to the anti-inflammatory effect of glucocorticoids on the innate immune system (16).

It was demonstrated in animal studies that ANXA1 could alleviate the disease severity of various autoimmune diseases, including inflammatory bowel disease (IBD) and rheumatoid arthritis (RA) (17, 18). Besides, circulating levels of ANXA1 in patients with relapsing/remitting multiple sclerosis inversely correlated with the disease severity (19). A recent proteomic analysis of neutrophils in GPA patients revealed that a group of dysregulated proteins, including ANXA1, were associated with apoptosis (20). However, clinical and pathological association of ANXA1 in AAV remains unclear. In the current study, we measured the plasma levels and renal expression of ANXA1, and further analyzed their association with clinical and pathological parameters in AAV patients.

MATERIALS AND METHODS

Patients and Samples

Thirty-one patients with active AAV receiving renal biopsy diagnosed in Peking University First Hospital from 2016 to 2018 were recruited in the current study. All patients met the Chapel Hill Consensus Conference (CHCC) nomenclature for AAV (21) and had complete clinicopathological data. Patients with secondary vasculitis or coexisting renal diseases, for example, lupus nephritis (LN), IgA nephropathy or anti-glomerular basement membrane disease were excluded. Renal specimens from 10 patients with minimal change disease (MCD) and 9 patients with LN were collected as disease controls. Ten renal tissues from the normal part of nephrectomized kidneys were defined as normal on the basis of light microscopy, immunofluorescence and electron microscopy and then were used as normal controls.

Among the 31 above mentioned active AAV patients, plasma samples were collected in 26 patients for measurement of plasma levels of ANXA1. Plasma samples of 35 patients with AAV in remission were also collected at their regular ambulatory follow-up. Fifteen out of these 35 patients were also among the above mentioned 31 patients who also had plasma samples of active stage of AAV. The details were depicted in **Figure 1A**. Plasma samples of 16 healthy blood donors were collected as normal controls. These blood samples were centrifuged at 2,000 *g* for 15 min at 4°C within 30 min after collection. Then the plasma samples were stored at −80°C until use. Repeated freeze/thaw cycles were avoided.

The disease activity of AAV patients was assessed according to the Birmingham vasculitis activity score (BVAS) (22). As previously described, remission was defined as "absence of disease activity attributable to active disease qualified by the need for ongoing stable maintenance immunosuppressive therapy" (complete remission), or "at least 50% reduction of disease activity score and absence of new manifestations" (partial remission) (23). The renal response to treatment was evaluated at 6 months after initiation of immunosuppressive therapy, according to the following criteria described previously (24–26): (1) complete recovery of renal function was indicated by normalization of renal function and resolution of hematuria; (2) partial recovery of renal function was indicated by stabilization or improvement of renal function, with serum creatinine (Scr) $\geq$133 μmol/l but dialysis independent; and (3) treatment failure was indicated by progressive decline in kidney function with persistence of active urinary sediment despite immunosuppressive therapy.

Baseline data at diagnosis as well as follow-up information of patients were collected from electronic medical records of our hospital. Each participant signed the informed consent. This research was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Peking University First Hospital.

Detection of Plasma ANXA1

Plasma ANXA1 was detected by enzyme-linked immunosorbent assay (ELISA) using the commercial kit (ab222868, Abcam, Cambridge, UK). The detection was carried out according to the manufacturer's guidance. In brief, the 96-well plate was pre-coated with polyclonal antibody specific for human ANXA1. The diluted ANXA1 standards and plasma samples were added to appropriate wells. After 2-h incubation at room temperature and five washes, biotinylated antibody was added to each well. Following 1-h incubation and five washes, the streptavidin-peroxidase conjugate was added to each well. Then the plate was incubated at room temperature for another 30 min. After five washes, chromogen substrate was added in each well and then the plate was incubated in ambient light for 15 min. The reaction was stopped by adding stop solution. The absorbance was measured

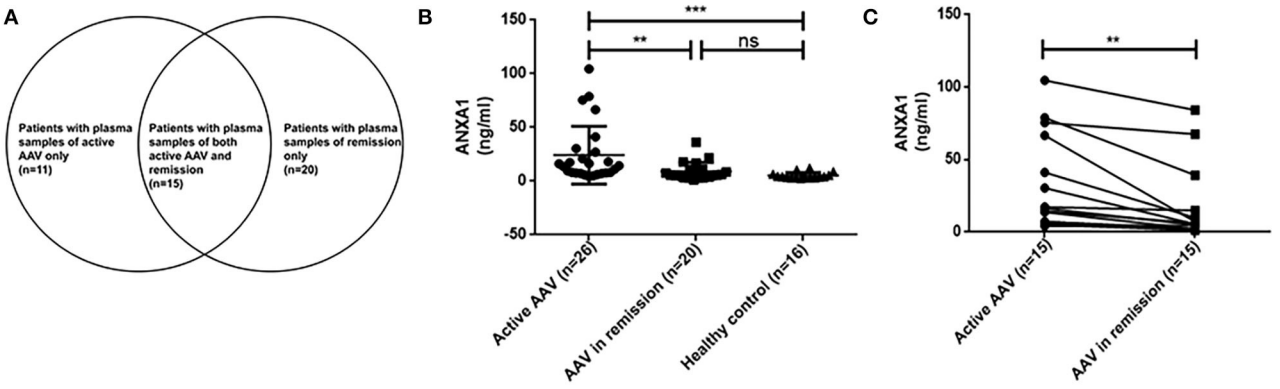

FIGURE 1 | Details of plasma samples of AAV patients **(A)**. Plasma levels of ANXA1 in active AAV patients ($n = 26$) and controls (AAV in remission, $n = 20$; healthy control, $n = 16$) **(B)**. Comparisons of ANXA1 levels in 15 AAV patients with sequential plasma samples (pairs = 15) **(C)**. AAV, anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis; ANXA1, annexin A1; ns, no significance ($P > 0.05$). $^{**}P < 0.01$, $^{***}P < 0.001$.

at 450 nm within 15 min. The concentration of ANXA1 in plasma samples was determined in accordance with the standard curve.

Renal Histopathology

Renal pathology of AAV patients was evaluated according to the standardized protocol described previously (27–29). The glomerular lesions, including crescents, fibrinoid necrosis and glomerulosclerosis, were expressed as the proportion of affected glomeruli in total glomeruli in the specimens. In terms of the area of the tubulointerstitial compartment affected, tubular and interstitial lesions were semi-quantitatively scored as followed: interstitial fibrosis (- for 0%, + for 0–50% and ++ for >50%), interstitial infiltrate (– for 0%, + for 0–20%, ++ for 20%-50% and +++ for >50%) and tubular atrophy (- for 0%, + for 0–50% and ++ for >50%).

Immunohistochemistry Staining of ANXA1 in Renal Biopsies

Slides with formaldehyde-fixed renal tissues were deparaffinized in xylene and rehydrated in graded ethanol. After washed with phosphate buffer saline (PBS), slides were immersed in 3% hydrogen peroxide for 30 min at room temperature. After another three washes with PBS, antigen retrieval was then performed through heating the slides in Tris-ethylene diamine tetraacetic acid (EDTA) buffer (pH 9.0) in an 800 W microwave oven for 2 min and then at 200 W one for 8 min. Then the slides were cooled to room temperature and washed with PBS, followed by blockage of non-specific staining with 3% bovine serum albumin (BSA) in PBS at 37°C for 1 h. After removing BSA without washing, the primary antibody, rabbit anti-human ANXA1 (1:4000, ab214486; Abcam, Cambridge, UK) was added and then incubated overnight at 4°C. Slides of the negative control were incubated with 3% BSA instead of primary antibody. After 5 washes in PBS, slides were incubated with the secondary antibody (PV9001, ZSGB-Bio, Beijing, China) at room temperature for 20 min. Then the slides were developed in fresh hydrogen peroxide with 3-3'-diaminobenzidine tetrahydrochloride solution for 45 s. Finally, the slides were incubated with haematoxylin for 8 min to attain nuclear staining and then dehydrated by graded alcohol and xylene. For quantitative analyses of immunohistochemical staining, all the glomeruli and at least 10 fields of tubulointerstitium per kidney slide were observed blindly at ×400.

The Image-Pro Plus analysis software (version 6.0; Media Cybernetics, Dallas, TX, USA) was used to assess the renal staining results of ANXA1 quantitatively. The optical intensity threshold was corrected to 0–250 in the process of analyses. Positive signals were shown as the mean optical density (integrated option density/area).

Co-localization of ANXA1 With Markers of Different Cell Types

To investigate concrete locations of ANXA1 in the kidney of AAV patients, double immunofluorescence of ANXA1 and specific markers of different cell types including renal intrinsic cells as well as inflammatory cells was performed. Glomerular endothelial cells, mesangial cells and podocytes were marked with mouse anti-human CD31 (1:50, sc-376764; Santa Cruz Biotechnology, CA), mouse anti-human integrin-α8 (1:50, sc-365798; Santa Cruz Biotechnology, CA) and mouse anti-human synaptopodin (1:50, MAB8977; R&D Systems, Minneapolis, MN, USA), respectively. Proximal tubular epithelial cells were identified with lotus tetragonolobus lectin (LTL, 1:200, FL-1321; Vector Labs, USA). Distal tubules and collecting ducts were marked with mouse anti-human Calbindin D28K (1:200, sc-365360; Santa Cruz Biotechnology, CA). Neutrophils and monocytes/macrophages were identified with rabbit anti-human neutrophil elastase (NE; 1:50, ab131260; Abcam, Cambridge, UK) and mouse anti-human CD68 (1:50, ab955; Abcam, Cambridge, UK), respectively. CD3+, CD4+ and CD8+ T cells were marked with rat anti-human CD3 (1:250, ab11089; Abcam, Cambridge, UK), rabbit anti-human CD4 (1:50, ab133616; Abcam, Cambridge, UK) and mouse anti-human CD8 (1:50, ab17147; Abcam, Cambridge, UK), respectively. In the co-localization of ANXA1 with glomerular endothelial cells, mesangial cells, podocytes, distal tubules and collecting ducts,

monocyte/macrophages and CD8+ T cells, rabbit anti-human ANXA1 (1:100, ab214486; Abcam, Cambridge, UK) was used as primary antibody. In the co-localization of ANXA1 with neutrophils, proximal tubular epithelial cells and CD4+ T cells, mouse anti-human ANXA1 (1:100, sc-12740; Santa Cruz Biotechnology, CA) was used as primary antibody. After deparaffinization, antigen retrieval and blockage of non-specific staining according to the above mentioned methods, the specimens were incubated with mixture of primary antibodies overnight at 4°C. After 5 washes with PBS, the specimens were incubated with secondary antibodies mixture of Alexa Fluor 488 (AF488) -labeled goat anti-rabbit IgG (1:200, A-32731; Invitrogen, USA) and Alexa Fluor 555 (AF555) -labeled goat anti-mouse IgG (1:200, A-32727; Invitrogen, USA) at 37°C for 1 h. Notably, in the co-localization of ANXA1 with proximal tubular epithelial cells, LTL was mixed with secondary antibodies. In the co-localization of ANXA1 and CD3, AF555-labeled goat anti-rat IgG (1:200, 4417; Cell Signaling Technology, USA) was used as secondary antibody. After washed with PBS five times, the specimens were stained with 4,6-diamidino-2-phenylindole (DAPI) and finally mounted with Mowiol. For the negative controls, the mixture of primary antibodies was replaced with 3% BSA. Zeiss LSM 710 confocal microscope (Zeiss, Jena, Germany) and fluorescence microscope (90i, Nikon, Japan) were used to capture confocal images. Then these images were exported from the ZEN 2012 (blue edition) microscopy software.

Statistical Analysis

Data were expressed as median and interquartile range (IQR; for data that were in skewed distribution) or mean ± SD (for data that were normally distributed) for continuous variables, or number (%) for qualitative variables. Differences between groups were analyzed by *t*-test for normally-distributed data or by non-parametric test for non-normally distributed data. Pearson's correlation or Spearman's rank correlation was performed to assess the correlation between two variables as appropriate. $P < 0.05$ was considered statistically significant. Data analyses were performed with SPSS 22.0 (IBM Corp., Armonk, NY).

RESULTS

General Data of the Patients

Among the 31 patients with active AAV, 13 (41.9%) were male and 18 (58.1%) were female, with a median age of 65 (IQR 60–69) years at diagnosis. All the patients were MPO-ANCA positive. The median level of initial serum creatinine was 253.5 (IQR 195.3–371.0) μmol/L. The median BVAS of these 31 patients was 15 (IQR 15–20). Among the 35 AAV patients in remission, the median serum creatinine level was 147.0 (IQR 108.0–186.0) μmol/L, and the BVAS was 0 for each of these patients. General data of the patients were listed in **Table 1**.

Among the 31 AAV patients, only three patients did not receive any immunosuppressive treatment before renal biopsy. Twenty-eight patients received oral glucocorticoids before renal biopsy. No patients received high-dose methylprednisolone pulse therapy, plasma exchange, cyclophosphamide or rituximab before renal biopsy. Among the 10 MCD patients, no patient received glucocorticoids treatment before renal biopsy. Among the 9 LN patients, 3 patients received oral glucocorticoids treatment before renal biopsy.

TABLE 1 | General data of patients with AAV.

Parameters	Active stage	Remission stage
Demographic data		
Subjects, *n*	31	35
Male/female	13/18	18/17
Age at diagnosis, median (IQR)	65 (60–69)	
Clinical manifestation		
Skin rash, *n* (%)	4 (12.9)	
Arthralgia, *n* (%)	1 (3.2)	
Muscle pain, *n* (%)	6 (19.4)	
Pulmonary, *n* (%)	15 (48.4)	
ENT, *n* (%)	7 (22.6)	
Ophthalmic, *n* (%)	2 (6)	
Gastrointestinal, *n* (%)	0 (0)	
Nervous system, *n* (%)	4 (12.9)	
BVAS		
Median (IQR)	15 (15–20)	0(0-0)
Range	12–29	0-0
Laboratory data		
MPO-ANCA (%)	31 (100)	
Serum creatinine, μmol/L		
Median (IQR)	253.5 (195.3–371.0)	147.0 (108.0–186.0)
Range	59.4–921.5	63.0–823.0
Urinary protein (g/24h)		
Median (IQR)	1.03 (0.36–2.86)	
Range	0.02–4.71	
Hematuria (%)	30 (96.8)	
Pathological data		
Total glomeruli		
Median (IQR)	28 (20–40)	
Range	15–76	
Glomerular lesions (%)		
Total crescents	48.9 ± 20.0	
Cellular crescents	33.6 ± 17.0	
Fibrous crescents	12.0 (0.0–22.5)	
Glomerulosclerosis	7.4 (4.4–22.5)	
Tubulointerstitial lesions, *n*		
Interstitial infiltration (-/+/++/+ + +)	0/5/19/7	
Interstitial fibrosis (-/+/++)	0/23/8	
Tubular atrophy (-/+/++)	1/24/6	

BVAS, Birmingham Vasculitis Activity Score; ENT, ear, nose and throat.

Plasma Levels of ANXA1

As above mentioned, we tested plasma samples from 26 out of the above mentioned 31 patients with active AAV, 35 AAV patients in remission, including 15 paired samples of both active stage and remission stage of AAV, as well as 16 healthy blood donors. The

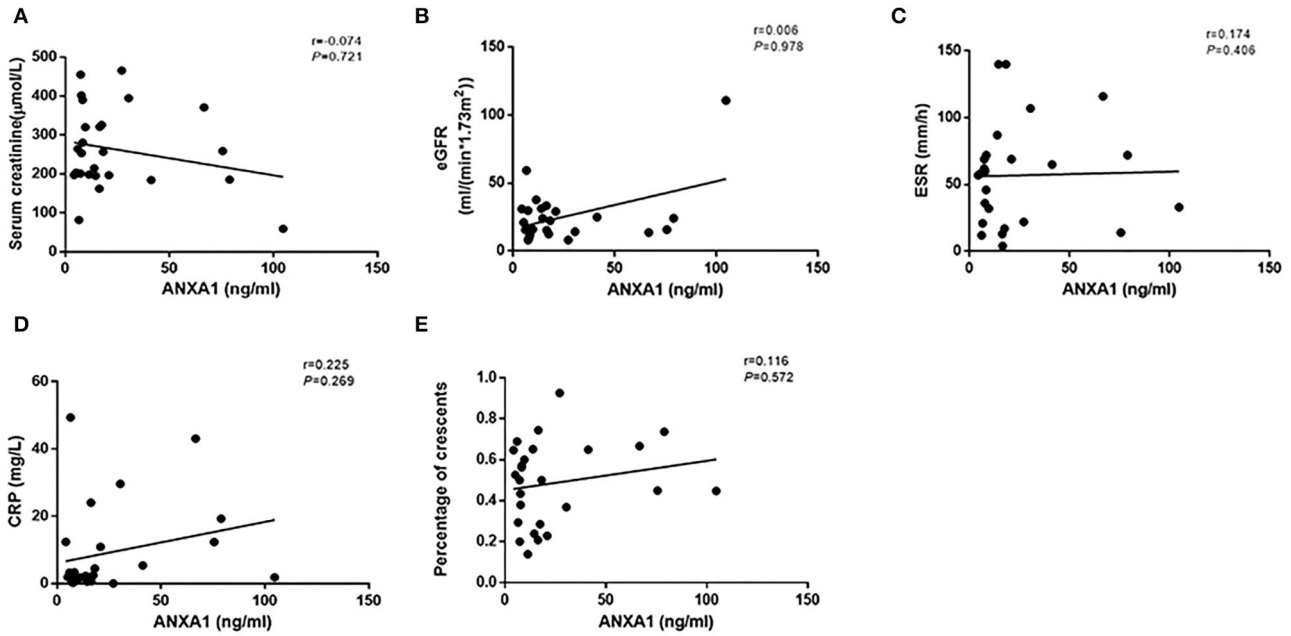

FIGURE 2 | Correlation analyses of ANXA1 levels in plasma of active AAV patients and serum creatinine **(A)**, eGFR **(B)**, ESR **(C)**, CRP **(D)** and the proportion of crescents **(E)**, respectively. ANXA1, annexin A1; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

plasma levels of ANXA1 in active AAV patients were significantly higher than those of AAV patients in remission as well as those of healthy controls [14.1 (IQR 7.6–27.9) vs. 5.6 (IQR 3.6–11.3] ng/ml, $P = 0.003$; 14.1 (IQR 7.6–27.9) vs. 4.1 (IQR 3.1–6.4) ng/ml, $P < 0.001$, respectively) (**Figure 1B**).

Then we compared plasma levels of ANXA1 in 15 AAV patients with sequential plasma samples of both active stage and remission. The plasma levels of ANXA1 were significantly higher in patients with active AAV than in their remission stage [16.4 (IQR 7.2–66.6) vs. 5.5 (IQR 2.2–14.9) ng/ml, $P = 0.001$], which was consistent with the above mentioned results. In detail, there were 14 patients in active stage with higher plasma levels of ANXA1 than those in remission, while there was only one patient exhibiting a slight increase in the plasma in remission compared with that in active stage (**Figure 1C**). However, correlation analyses did not show any significant correlation between plasma levels of ANXA1 with clinicopathological parameters in AAV patients. The details were shown in **Figure 2**.

Immunohistochemistry Analysis of ANXA1 in Renal Biopsies

Immunohistochemistry of renal specimens were performed to evaluate the renal expression of ANXA1 in 31 AAV patients in active stage. The results demonstrated that ANXA1 was widely expressed in tubulointerstitium and glomeruli (**Figure 3**). Compared with normal controls, the mean optical densities of ANXA1 in both glomeruli and tubulointerstitium of AAV patients were significantly higher (0.178 ± 0.056 vs. 0.108 ± 0.068, $P = 0.002$; 0.108 ± 0.038 vs. 0.009 ± 0.007, $P = 0.001$, respectively) (**Figures 4A,B**). Moreover, patients with LN and MCD exhibited significantly weaker expression of ANXA1 in glomeruli and tubulointerstitium than AAV patients (0.031 ± 0.028 vs. 0.178 ± 0.056, $P < 0.001$; 0.049 ± 0.021 vs. 0.178 ± 0.056, $P < 0.001$; 0.002 (IQR 0.001–0.008) vs. 0.111 (IQR 0.081–0.137), $P < 0.001$; 0.002 (IQR 0.001–0.007) vs. 0.111 (IQR 0.081–0.137), $P < 0.001$, respectively) (**Figures 4A,B**).

Co-localization of ANXA1 With Various Cell Types

According to the results of immunohistochemical assay, we found that ANXA1 was highly expressed in glomeruli and tubulointerstitium of AAV patients. Therefore, the double immunofluorescence staining for ANXA1 and markers of various cell types was performed to further investigate the specific cell types with increased ANXA1 expression. The partial merge of ANXA1 with CD31, integrin-α8, synaptopodin, LTL, NE and CD68 was observed in renal specimens of AAV patients, which suggested that ANXA1 expressed in glomerular endothelial cells, mesangial cells, podocytes, proximal tubular epithelial cells, neutrophils and monocytes/macrophages (**Figures 5A–D,F,G**). ANXA1 was expressed by CD3+, CD4+ and CD8+ T cells in both AAV patients and healthy controls (**Figures 5H–J**, **6H–J**). The partial merge of ANXA1 with glomerular endothelial cells, mesangial cells, podocytes and monocytes/macrophages was also observed in healthy controls (**Figures 6A–C,G**). The merge of ANXA1 with NE and LTL was not observed in healthy controls (**Figures 6D,F**). No obvious co-localization of ANXA1 with distal tubules or collecting ducts was observed in AAV patients or healthy controls (**Figures 5E**, **6E**).

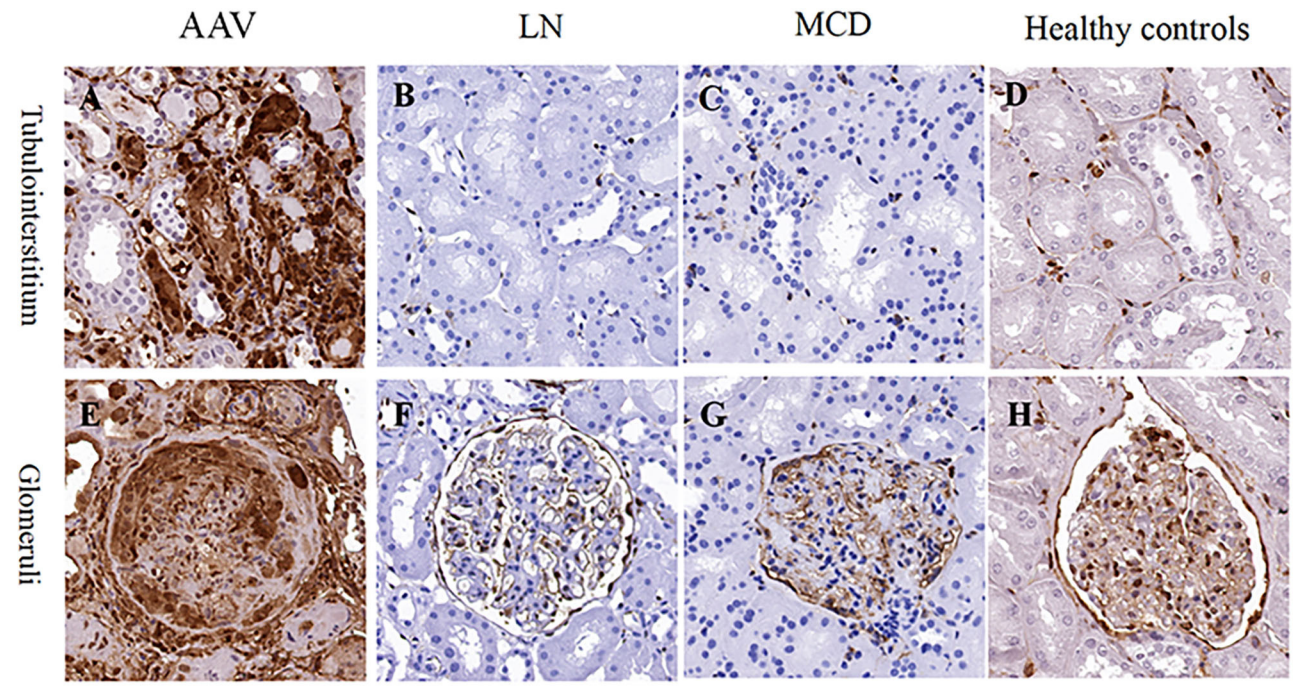

FIGURE 3 | Immunohistochemistry staining for ANXA1 in tubulointerstitium **(A–D)** and glomeruli **(E–H)** in patients with AAV **(A,E)**, LN **(B,F)** and MCD **(C,G)** as well as healthy controls **(D,H)**, respectively. AAV, anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis; ANXA1, annexin A1; LN, lupus nephritis; MCD, minimal change disease.

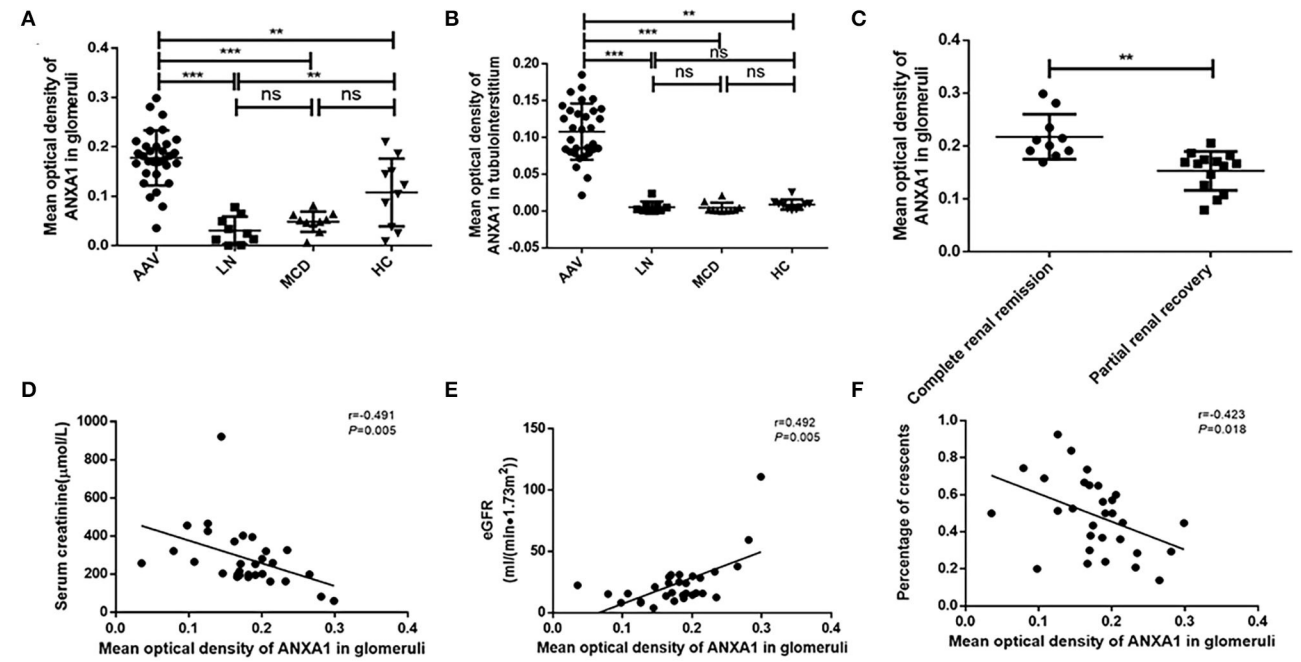

FIGURE 4 | (A) Mean optical density of ANXA1 in glomeruli of AAV patients, LN, MCD and healthy controls respectively. **(B)** Mean optical density of ANXA1 in tubulointerstitium of AAV patients, LN, MCD and healthy controls respectively. **(C)** Comparison of ANXA1 expression of patients achieving complete renal recovery with those achieving partial renal recovery. **(D–F)** Correlation analyses of ANXA1 expression in glomeruli of AAV patients with serum creatinine **(D)**, eGFR **(E)** and the proportion of crescents **(F)**, respectively. AAV, anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis; ANXA1, annexin A1; eGFR, estimated glomerular filtration rate; HC, healthy control; LN, lupus nephritis; MCD, minimal change disease; ns, no significance ($P > 0.05$). $^{**}P < 0.01$, $^{***}P < 0.001$.

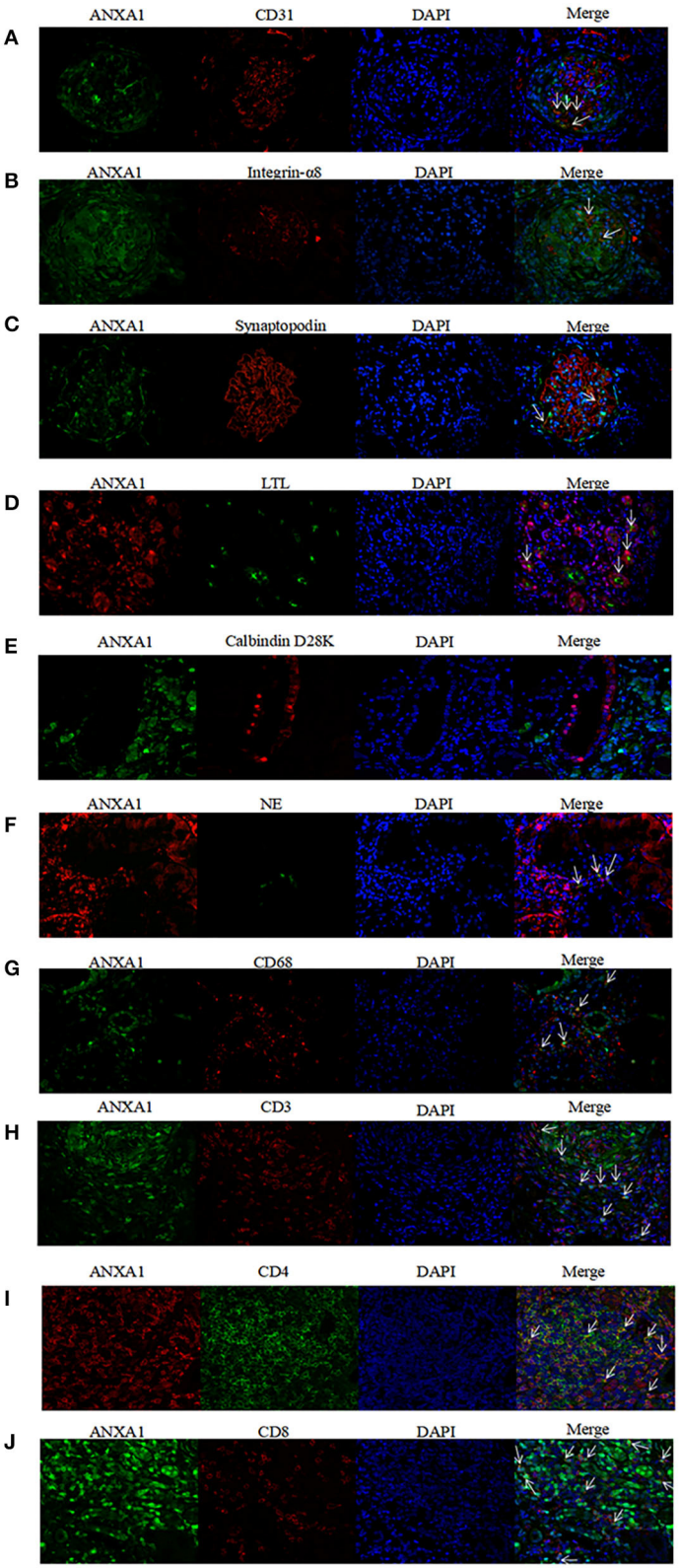

FIGURE 5 | Co-localization of ANXA1 and CD31 **(A)**, integrin-α8 **(B)**, synaptopodin **(C)**, LTL **(D)**, Calbindin D28K **(E)** and NE **(F)**, CD68 **(G)**, CD3 **(H)**, CD4 **(I)** and CD8 **(J)** in renal specimens of AAV patients respectively. AAV, anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis; ANXA1, annexin A1; CD3, cluster of differentiation 3; CD4, cluster of differentiation 4; CD8, cluster of differentiation 8; CD31, cluster of differentiation 31; CD68, cluster of differentiation 68; LTL, lotus tetragonolobus lectin; NE, neutrophil elastase.

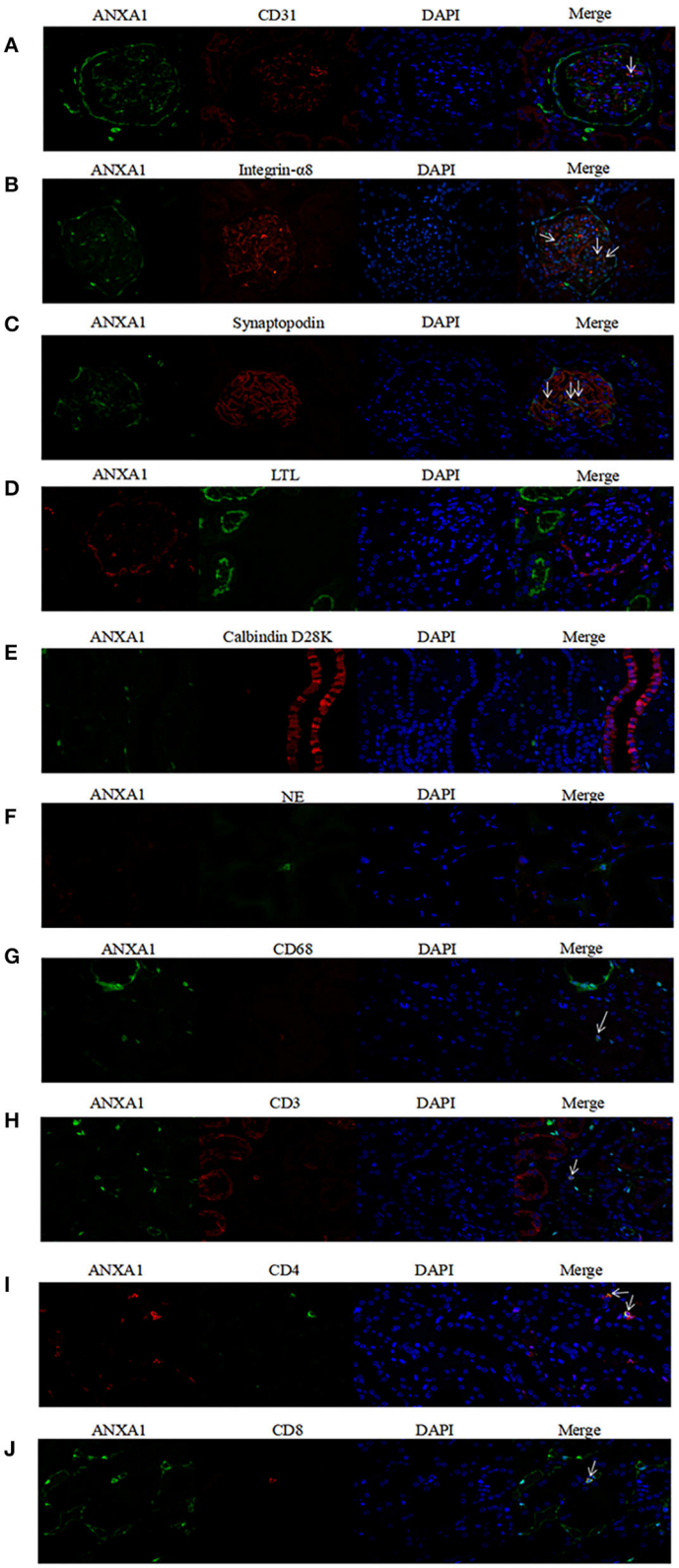

FIGURE 6 | Co-localization of ANXA1 and CD31 **(A)**, integrin-α8 **(B)**, synaptopodin **(C)**, LTL **(D)**, Calbindin D28K **(E)** and NE **(F)**, CD68 **(G)**, CD3 **(H)**, CD4 **(I)** and CD8 **(J)** in renal specimens of healthy controls, respectively. AAV, anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis; ANXA1, annexin A1; CD3, cluster of differentiation 3; CD4, cluster of differentiation 4; CD8, cluster of differentiation 8; CD31, cluster of differentiation 31; CD68, cluster of differentiation 68; LTL, lotus tetragonolobus lectin; NE, neutrophil elastase.

Associations Between Renal ANXA1 Expression and Clinicopathological Parameters of Patients With Active AAV

We further investigated associations between ANXA1 expression in renal specimens and clinicopathological parameters of AAV patients. The correlation analyses among the 31 AAV patients showed that the mean optical density of ANXA1 in glomeruli correlated with the levels of serum creatinine and eGFR at renal biopsy ($r = -0.491$, $P = 0.005$; $r = 0.492$, $P = 0.005$, respectively) (**Figures 4D,E**). Besides, the expression of ANXA1 in glomeruli correlated with the proportion of crescents ($r = -0.423$, $P = 0.018$) (**Figure 4F**). Among the 25 active AAV patients with follow-up data, complete renal recovery occurred in 10 patients (40%), with 14 patients (56%) achieving partial renal recovery and 1 patient (4%) experiencing treatment failure. The baseline expression of ANXA1 in glomeruli of patients achieving complete renal recovery was significantly higher than those achieving partial renal recovery [0.206 (IQR 0.189–0.246) vs. 0.167 (IQR 0.122–0.176), $P = 0.001$] (**Figure 4C**). The levels of ANXA1 were significantly different among AAV patients with various degree of interstitial infiltration ($P = 0.010$), i.e., patients with severer interstitial infiltration had higher levels of ANXA1. No significant correlation was observed between the levels of ANXA1 in tubulointerstitium and interstitial fibrosis or tubular atrophy ($P = 0.176$ and $P = 0.846$, respectively).

DISCUSSION

ANXA1 is a 37-kDa protein and a member of annexin superfamily binding phospholipid dependent on calcium (30). ANXA1 is also a glucocorticoid-regulated protein and has anti-inflammatory and pro-resolving effects, including blockage of leukocytes migration and acceleration of neutrophils apoptosis (31). ANXA1 inhibits neutrophils adhesion and recruitment and therefore promotes resolution of inflammation (13). ANXA1 mimetic peptide (Ac2-26) augments neutrophils apoptosis (15). ANXA1 promotes macrophages skewing toward M2 phenotype, which contributes to the resolution of inflammation and tissue repair (32). In addition, ANXA1 shows protective roles in various renal diseases. In mice with diabetic nephropathy, ANXA1 ameliorates kidney injuries through facilitating the resolution of inflammation (33). ANXA1 mimetic peptide (Ac2-26) protects against renal ischemia/reperfusion injury by abortion of neutrophil extravasation and attenuation of macrophage infiltration (34).

Our previous study found that ANXA1 was upregulated in kidney of patients with diabetic nephropathy and was correlated with renal function and renal outcomes (33). Ka et al. (35) demonstrated that urinary levels of ANXA1 were significant higher in secondary glomerular diseases (such as diabetic nephropathy and LN) than in primary glomerular disorders and normal controls. However, the significance of ANXA1 in AAV remains unknown. Thus, the present study investigated the association between ANXA1 with clinical and pathological parameters of AAV.

In the current study, we found that compared with healthy controls, the levels of ANXA1 were upregulated in kidneys of patients with active AAV and were associated with renal disease severity of AAV, including eGFR, serum creatinine and proportion of crescent formation, and to some extent, interstitial infiltration, in renal specimens. Moreover, the levels of renal expression of ANXA1 were associated with renal response to treatment. Plasma levels of ANXA1 were significantly higher in active AAV patients than in AAV patients in remission as well as healthy controls. However, further assessment did not show any significant correlation between ANXA1 levels in plasma and clinicopathological parameters of AAV. We speculated that in the context of active AAV, circulating ANXA1 translocated to local sites of inflammation, e.g., kidneys, and exerted its pro-resolving effects. As mentioned above, ANXA1 could act on various inflammatory cells and further promote resolution of inflammation, such as inhibition of neutrophil and macrophage infiltration, augmentation of neutrophil apoptosis and skewing of macrophage toward M2 phenotype, which might explain the association of ANXA1 with disease severity in AAV. Besides, ANXA1 could promote mucosal wound repair, muscle injury regeneration and cardiac repair (32, 36, 37), which might explain the association of renal ANXA1 expression with renal responses to treatment. In addition, we found that ANXA1 was expressed by neutrophils, monocytes/macrophages, T cells, mesangial cells and epithelial cells, which were consistent with the results of previous studies (38–40).

There are some limitations of the current study. First, the sample size was relatively small, and all the patients recruited were MPO-ANCA positive, since MPO rather than PR3 is the main target antigen of ANCA in China (4, 41). Second, glucocorticoids may affect the expression of ANXA1, but a majority of AAV patients recruited in the current received glucocorticoids before renal biopsy. In clinical practice, physicians usually give patients immunosuppressive therapy once the diagnosis of AAV established, even before renal biopsy. Therefore, it is very difficult to get renal specimens of AAV patients before using glucocorticoids. Third, since this was a retrospective study, all the samples were obtained from our biobank, and we did not have the samples of peripheral blood mononuclear cells (PBMCs) of these patients in our biobank. Therefore, we are not able to measure ANXA1 expression in PBMCs of these patients.

In conclusion, the renal expression of ANXA1 was upregulated in AAV patients and was correlated with the severity of renal injury.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Peking University

First Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

R-XW finished the whole experiment, analyzed statistics, and drafted the manuscript. LW, S-FC, Z-YL, MC, and M-HZ designed the study, participated interpretation of data, and revised the manuscript. Z-YL had full access to all of the data, provided final approval of the submitted manuscript, guarantor of this work, and took responsibility for the integrity of the data and the accuracy of the data analysis. All authors read and approved the manuscript.

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Renal involvement as a unique manifestation of hemophagocytic syndrome

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Renal-limited hemophagocytic syndrome (HPS) is a rare clinical setting characterized by abnormal activation of the immune system. Fever associated with pancytopenia, hepatosplenomegaly with liver dysfunction, and hypofibrinogenemia are usually observed in HPS. From a histological level, the presence of non-malignant macrophages infiltrating bone marrow and organs represents the hallmark of this condition. Non-malignant macrophages are associated with phagocytizing activities involving other blood cells. While primary HPS is usually associated with inherited dysregulation of the immune system, secondary HPS usually occurs in the context of infection or is linked to a neoplastic process. Clinical presentation varies and can potentially lead to life-threatening settings. While renal involvement has frequently been reported, however, detailed descriptions of the kidney manifestations of HPS are lacking. More critically, the diagnosis of HPS is rarely supported by renal biopsy specimens. We report four rare cases of biopsy-proven renal-limited HPS in patients presenting with acute kidney injury (AKI). The available evidence on this topic is critically discussed in light of the possible emergence of an autonomous entity characterized by an isolated kidney involvement.

KEYWORDS

acute kidney injury, hemophagocytic syndrome, macrophage activation syndrome, autoinflammatory diseases, hemophagocytic lymphohistiocytosis

Introduction

Hemophagocytic syndrome (HPS, additionally known as hemophagocytic lymphohistiocytosis) is a disorder characterized by an aberrant lytic response of natural killer cells (NKs) and cytotoxic CD8+ T lymphocytes (CTLs) (1–4), leading to an overactive yet less efficient immune reaction to an antigenic response. HPS can manifest as a primary (associated with an inherited dysregulation of the immune system) or acquired (usually associated with rheumatologic, infections, or oncologic processes) form. In both primary and secondary forms, the aberrant response to the target cells induces CTL proliferation, leading to an increase in interferon-γ (INF-γ) levels, thereby supporting the abnormal proliferation of benign histiocytes (macrophages). Those

cells have the ability to infiltrate different tissues and organs (such as the spleen, liver, and lymph nodes). This infiltrative process is further associated with an increase of inflammatory cytokines [such as interleukin (IL)-1, IL-6, IL-18, INF-γ, tumor necrosis factor-alpha (TNF-α)] amplifying the aberrant immune response (5), leading to the *so-called cytokine storm*, clinically characterized by severe systemic inflammatory response syndrome (SIRS) and multiorgan dysfunction syndrome (MODS). Both these clinical settings are associated with a high rate of morbidity and mortality. The resulting effect is the proliferation of activated histiocytes which promotes an abnormal immune response characterized by the engulfment of circulating blood elements, such as erythrocytes, leucocytes, and platelets. Fever associated with pancytopenia, hepatosplenomegaly with liver dysfunction, and hypofibrinogenemia, is usually observed in HPS (1–4). In addition to decreased levels of circulating erythrocytes, leucocytes, and platelets, abnormal liver function tests (LFTs), hyperferritinemia, increased lactate dehydrogenase (LDH), hypertriglyceridemia, and hypofibrinogenemia are frequently seen. More recent evidence supports the presence of increased soluble CD25 levels and decreased NK cell activity. Coagulopathy can occur (4). Findings consistent with HPCs can be observed at bone marrow aspiration, albeit they are not pathognomonic for the diagnosis as these may be observed in other conditions (6, 7). In addition, infiltrates suggest that HPCs can be found in other organs, such as the spleen or liver.

The kidney can be a target organ in HPS, oftentimes clinically presenting as acute kidney injury (AKI) (4, 8). Despite a growing number of case reports confirming the potential renal involvement in HPS, evidence is still scattered and precise descriptions of the renal manifestations of HPS are still lacking. More critically, the diagnosis of HPS is rarely supported by renal biopsy specimens.

We report four rare cases of biopsy-proven renal-limited HPS in patients presenting with acute kidney injury and critically discuss the available evidence on this topic.

Clinical, laboratory, and histological evidence were collected through a chart-review analysis.

Case 1

An 83-year-old man was transferred to our division due to a sudden worsening of renal function. At admission, he presented with confusion with spatial disorientation and anuric AKI. He had an unremarkable medical history except for alcohol abuse. He was taking antibiotics for community-acquired pneumonia associated with bilateral pleural effusion for 1 week before presentation at our center. Systemic edema was noticeable and associated with a 7-kg gain in body weight. The presence of peripheral lymphadenopathy was detectable. The main clinical and laboratory parameters are shown in Tables 1, 2. After admission, dialysis was started. Diagnostic investigations included a bone marrow biopsy whose results were unremarkable. Consequently, a kidney biopsy was performed, and histological findings are illustrated in Figure 1 and Table 3. Briefly, light microscopy showed a picture of diffuse endocapillary proliferation and focal aspects of extracapillary proliferation in the kidney with moderate vascular damage, mainly atherosclerotic. The immunofluorescence (IF) was predominantly positive for C3 (+ + +).

These features were compatible with glomerulonephritis with dominant deposits of C3. However, numerous CD68-positive cells were observed at immunohistochemistry, while electron microscopy examination confirmed the presence of histiocytes and a characteristic feature of hemophagocytosis. These findings were strongly evocative of HPS.

However, it is worth mentioning that, despite the histological features, our case did not fully meet the current classification criteria for HPS.

Case 2

A 79-year-old man was transferred to our division because of a severe worsening of his kidney. He presented with AKI with a serum creatinine (sCr) level of 4.65 mg/dl, hyperazotemia (487 mg/dl), thrombocytopenia (platelet count, $10.5 \times 10^4/\mu l$), and ferritin level was 2,500 ng/ml. The blood pressure was 80/50 mm Hg. Furthermore, in this case, the bone marrow biopsy was unremarkable (Tables 1, 2).

To determine the severity of the glomerulopathy of the patient, a kidney biopsy was performed (Figure 1, Table 3).

Case 3

A 69-year-old man was admitted for evaluation of recurrent AKI episodes of unknown origin. His previous medical history was characterized by acute coronary events and chronic obstructive pulmonary disease. AKI episodes were referred to as being anticipated by massive proteinuria (up to 20–25 g/day). At the first evaluation at our center, he presented with a new onset of AKI and fever (38.2°C) 10 days after an episode of upper airway infection. The main clinical and laboratory parameters are shown in Table 1. A kidney biopsy was performed (Figure 1). Immunohistochemistry showed mononuclear cells to be CD68-positive, consistent with monocyte/macrophages. Capillary lumina are occluded by vacuolized mononuclear cells.

Case 4

A 70-year-old woman was admitted for evaluation because of the onset of fever (38.2°C), edema in the lower extremities, and deterioration of renal function (sCr 2 mg/dl). Laboratory

TABLE 1 Main clinical characteristics of patients with renal-limited hemophagocytic syndrome.

Case	Clinical Presentation	Possible trigger	Laboratory and instrumental findings	Therapy	Outcome
Case 1	Anuric AKI	Community-acquired pneumonia	• Bone Marrow Biopsy: unremarkable • Hb: 6.8 g/dL • WBC 1690 /μL • Plts 56 × 10^3/μL. • sCr: 10.1 mg/dL in ESR: 71 mm/h, • CRP: 31.7 mg/dL • Ferritin: 3,520 ng/mL • C3/C4: within normal range • ANA, ANCA: negative	Steroids Pulses	Amelioration of kidney function with normalization of sCr. Resolution of the flogistic status.
Case 2	Anuric AKI	Airways infection	• Bone Marrow Biopsy: unremarkable • PET: unremarkable • CT-scan thorax and abdomen: pleural effusions and lobar pneumonia • Hb: 9.5 g/dL • WBC 2270 /μL • Plts 105 × 10^3/μL. • sCr: 4.65 mg/dL • ESR: 45 mm/h, • CRP: 16.4 mg/dL • Ferritin: 2,500 ng/mL • C3/C4 within normal range • ANA, ANCA: negative • Bronchoscopy and Bronchoalveolar Lavage: positive for Pneumocysistis Carinii e per Herpes Simplex	Steroids Pulses, anti-viral (acyclovir) and trimethoprim-sulfamethoxazole.	Amelioration of kidney function with normalization of sCr. Resolution of the flogistic status.
Case 3	Recurrent AKI episodes	Upper airways infection	• Bone Marrow Biopsy: unremarkable • Hb: 9.7 g/dL • WBC 5,930/μL • Plts 153 × 10^3/μL. • Proteinuria: 5 g/25 h • sCr: 3.7 mg/dL in • ESR: 63 mm/h, • CRP: 13,2 mg/dL	Canakinumab	Stabilization of kidney function. No further episode of AKI
Case 4	Deterioration of renal function in patient in F/U for previous renal carcinoma	Community-acquired pneumonia	• Bone Marrow Biopsy: unremarkable • PET: unremarkable • Hb: 8.5 g/dL • WBC 2,190 /μL • Plts 96 × 10^3/μL. • sCr: 2.0 mg/dL • ESR: 41 mm/h, • Proteinuria: 2.5 g/25 h • CRP: 18.5 mg/dL • Ferritin: 284 ng/mL • C3: within normal range • C4: 5 mg/dl • Total cholesterol: 249 mg/dl • ANA, ANCA: negative	Anakinra	Stabilization of kidney function.

TABLE 2 Features suggestive of reactive hemophagocytic syndrome included in the HScore.

Case	HScore items#
Case 1	3 lineages of cytopenias and elevated ferritin
Case 3	3 lineages of cytopenias and elevated ferritin
Case 3	1 lineage cytopenia, high temperature
Case 4	3 lineages of cytopenias and elevated ferritin

According to # Fardet et al. (23).

findings showed non-nephrotic range proteinuria (2.5 g/24 h) (Table 1). Her previous medical history showed MGUS and well-controlled arterial hypertension. Nephrectomy due to renal cell carcinoma was conducted 10 years before admission, leading to a moderate loss in renal function (sCr 1.4 mg/dl). With the deteriorating conditions of the patient, a kidney biopsy was carried out. Immunohistochemistry shows that CD68 is present in monocyte/macrophages.

Renal involvement in HPS

A spectrum of clinical presentations and histological findings has been associated with renal involvement in HPS over the years.

Clinical features

Acute kidney injury is the most frequently reported renal complication in HPS. Out of 95 patients with secondary HPS admitted to the intensive care unit (ICU), Aulagnon et al. (9) found a rate of AKI as high as 62%, with most of them in stage 2 or 3 AKI. Dialysis was initiated in almost 60% of the included patients. Hypoperfusion and acute tubular necrosis were identified as the main causes of AKI, albeit tumor lysis and glomerulopathy were similarly described in the cohort. However, histological renal confirmation was obtained in only 1 case. About 12% of the cohort had nephrotic syndrome (NS) which was associated with AKI in two-thirds of the cases. Among the survivors, 23% of patients had CKD when evaluated 6 months after the diagnosis of HPS. A recent retrospective study by Kapoor et al. (10), when analyzing the intensive care unit complications and outcomes of adult patients with HPS, showed that septic shock was the most common ICU complication seen in 14 (88%) patients, followed by AKI (81%) with the majority of the patients requiring renal replacement therapy.

In addition, when analyzing available literature, it is worth noting that most of the reported cases of HPS with renal involvement are described in the context of renal transplant (11–13).

Risdall et al. (14) reported a series of 19 subjects (68% renal transplant recipients) in whom HPS was triggered by infection, mainly cytomegalovirus (CMV). In a larger cohort analysis, Karras et al. (15) found a prevalence of 0.4% for HPS among 4,230 renal transplant recipients when investigating 17 HPS subjects (82% of those with confirmed viral infection). HPS occurred a median of 52 days after transplantation. Asci et al. (16) identified 13 patients with HPS out of more than 400 renal transplant recipients (a prevalence of 3.2%). HPS was observed for an average of 15 months after transplantation. In line with the other reports, an infectious trigger was found in the majority of the cases. Notably, HCV infection was reported in more than 50% of the cases. A larger cohort including more than 70 cases of HPS identified among kidney transplant recipients was described by Ponticelli et al. (17). While in most of the subjects, HPS was triggered by viral infections, bacterial and protozoal infections were also described. The observed overall mortality rate was above 50%. Since these first descriptions, a constellation of further cases of HPS associated with different infective diseases has been described in patients with kidney transplant (18–20).

Very recently, Wang et al. (21) analyzed 600 patients with adult secondary HPS attempting to identify risk factors associated with AKI occurrence in this setting. They found that several clinical (heart failure, gastrointestinal symptoms, disseminated intravascular coagulation, high heart rate at admission, and need for vasopressors) and laboratory parameters (increased serum phosphorus, total bilirubin, and low albumin levels) were independently associated with an increased risk of developing AKI. Similarly, they observed that the administration of vasopressors, AKI stage III, baseline Cystatin-C, total bilirubin, number of days of glucocorticoid therapy, fibrinogen level, and the presence of multi-organ failure were found to be independent risk factors for in-hospital mortality (21).

Renal histological findings in HPS

While the abovementioned studies provided clinical insights to improve the understanding of renal involvement in HPS, they only marginally contributed to the fine characterization of histological injuries.

First, it is worth mentioning that the clinical entity hemophagocytic lymphohystiocytosis should not be confused with the related histological findings (hemophagocytosis).

Second, from a pathogenic perspective, AKI in HPS seems to be supported by the interstitial infiltration of CTLs and activated macrophages (12).

Acute kidney injury in HPS results from inflammatory or ischemic lesions of the renal tubules. Acute tubular necrosis is the most frequent feature of renal damage in HPS. Fitzgerald et al. (22) reported the prevalence of this finding in up to

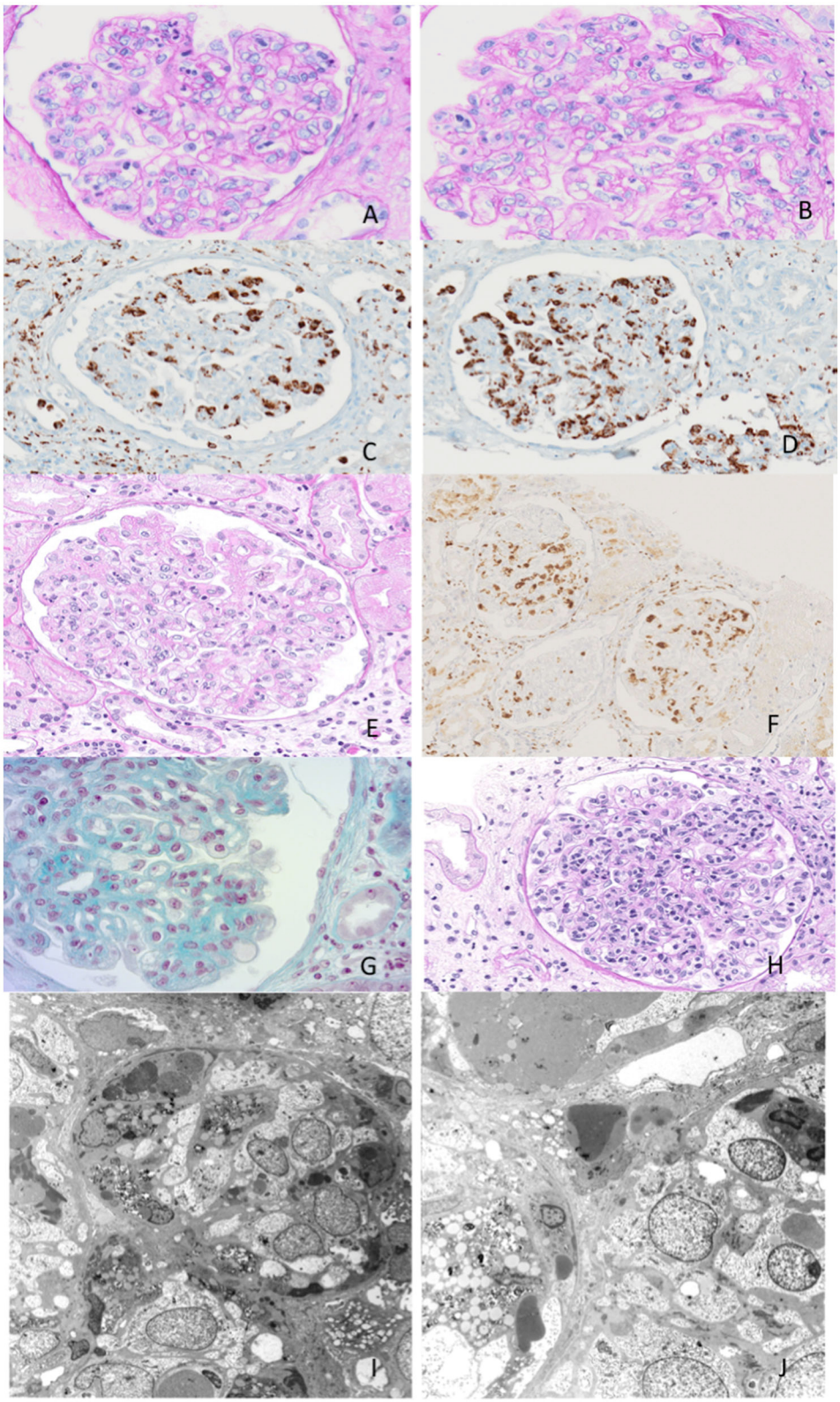

FIGURE 1
Exemplary features of renal biopsy samples from patients with hemophagocytic syndrome (HPS). **(A,B)** Diffuse thickening of the glomerular basement membrane (GBM) with double contours and capillary lumina occluded by mononuclear cells with foamy cytoplasm. **(C,D)** Immunohistochemistry shows mononuclear cells to be CD68-positive, consistent with monocyte/macrophages. **(E)** Capillary lumina occluded by vacuolized mononuclear cells. **(F)** Immunohistochemistry CD68-positive for monocyte/macrophages. **(G,H)** Capillary lumina occluded by monocyte/macrophages. **(I,J)** Features of hemophagocytosis at electron microscopy.

45% of patients with HPS in an autopsy series. In more than half of these cases, the presence of acute tubular necrosis was associated with interstitial inflammation (22). Tubulointerstitial lesions might be related to both hemodynamic alteration or coagulation disorders (e.g., disseminated intravascular coagulopathy) conditions that can occur during the acute phase of severe sepsis.

Glomerular involvement is uncommon and usually manifests as either podocytopathy with collapsing glomerulopathy or thrombotic microangiopathy. In our cohort, two patients presented with proteinuria, one in the nephrotic range and one in the sub-nephrotic range. The injuries leading to NS and glomerular damage are not fully defined and seem to be associable with primary podocyte involvement. Thaunat et al. (12) described a cohort of nine biopsy-proven patients with HPS presenting with NS and compared their experience with available cases in the literature. Histological patterns of renal injuries associated with HPS were as follows: collapsing focal segmental glomerulosclerosis (mainly seen in subjects of African descent), minimal change disease [all in White subjects, as also described in a previous report (13)], and thrombotic microangiopathies features seen in the remaining two cases.

More recently, a case of HPS with histiocytic glomerulopathy and intraglomerular hemophagocytosis has also been reported. Histological features showed massive glomerular infiltration by macrophages resembling proliferative glomerulonephritis accompanied by intraglomerular hemophagocytosis and mild features of glomerular thrombotic microangiopathy (24).

Therapeutic options

To date, the management of renal limited HPS has been based on anecdotal experience.

The grade of hyperinflammation, triggering condition, and eventual concomitant diseases are among the factors to consider when choosing the therapeutic options for renal involvement in HPS. Similarly, the presence of underlying genetic abnormalities should be investigated when possible. To date, no therapy has been trialed in a randomized fashion for this condition, and available experience mainly relies on observational studies or anecdotal case reports. Inherited forms range in severity, with familial hemophagocytic lymphohistiocytosis being associated with <5% survival rate at 12 months (19). The so-called HPS-94 protocol (based on the Histiocyte Society initiated the prospective international collaborative therapeutic study, launched in 1994) includes the use of 8 weeks of dexamethasone and etoposide with intrathecal methotrexate and has been associated with positive outcomes in selected cases. In some subjects with familial or relapsing forms, a scheme with daily cyclosporine associated with dexamethasone pulses and intermittent etoposide was also considered (24). This approach was shown to improve the estimated 5-year survival by up to 54% in a multinational study including more than 240 pediatric patients. Survival rates reached 66% in the 124 patients who underwent hematopoietic stem cell transplantation (24). In total, 49 subjects were alive after 12 months of observation time after the therapy, posing the question of whether the observed heterogeneity in the outcomes could have also been explained by a certain prevalence of secondary HPS included in the study cohort. Variations to the HPS-94 scheme have been proposed over the years, including the HPS-2004 protocol (adding cyclosporine during the 8-week induction phase) (25). The addition of anti-thymocyte globulin (ATG) has also been described, with an observed improvement in the complete response rate. The use of intermittent intravenous immunoglobulin or cyclosporine has been proposed as a maintenance therapy (26). Hematopoietic stem cell transplantation is usually considered in the most severe cases (familial, relapsing, or refractory forms) or in cases of documented genetic mutations. Reduced-intensity conditioning seemed to have a better tolerability profile compared with myeloablative conditioning in this setting.

As in primary HPS, the treatment of secondary HPS in adults is still under debate. The main challenge in the management of this condition is to control the hyperinflammatory state. However, one should also acknowledge that anecdotal experiences report positive outcomes with only supportive therapy and adequate treatment of the trigger. Among others, it seems that the use of amphotericin as an antimicrobial agent may be sufficient for leishmania-triggered HPS.

High-dose corticosteroids are usually suggested for patients requiring urgent care. The HPS-2004 scheme should be considered in the most severe cases (familial or relapsing forms). Due to its selective activity on CD8 + CTLs as proven in lymphocytic choriomeningitis virus-infected Prf–/–mice, etoposide can be considered a logical candidate agent for HPS (27). Similar effects have also been described for methotrexate and cyclophosphamide. A significant improvement in 1-month survival was associated with the first-line use of etoposide after multivariable analysis in a retrospective study involving 162 adults with sHPS (28). The management of the hyperinflammatory state should be paralleled by the identification of the eventual infectious trigger and consequent therapy should be arranged. In cases triggered by EBV, observational evidence supports the efficacy of early use of etoposide (within 1 month from symptoms onset) in the management of EBV-triggered HPS, in both pediatric (29) and adult (30) populations. The rationale for the use of etoposide relies on data demonstrating EBV infection of CD8 + CTLs in EBV-triggered HPS (31, 32). Additionally, direct antiviral effects have been speculated *in vitro* for etoposide due to its ability to inhibit EBNA synthesis and reduce the EBV-induced transformation of mononuclear cells (33). Hematopoietic stem

TABLE 3 Histological findings at kidney biopsy.

Patient	*n*. glomeruli	IF	Histological description	IFTA	EM	Findings in line with HPS
#1	21	C3+	Diffuse endocapillary proliferation and focal aspects of extracapillary proliferation in a kidney with moderate vascular damage, mainly atherosclerotic.	-	Presence of histiocytes and a characteristic feature of hemophagocytosis	CD68 positive cells were observed at immunohistochemistry; feature of hemophagocytosis at EM
#2	28	C3+	Focal segmental glomerulosclerosis with areas of interstitial fibrosis with tubular atrophy. Capillary lumina were occluded by vacuolized mononuclear cells.	Areas of interstitial fibrosis with tubular atrophy.	Presence of histiocytes and a characteristic feature of hemophagocytosis	CD68 positive cells were observed at immunohistochemistry; feature of hemophagocytosis at EM
#3	14	-	Capillary lumina were occluded by vacuolized mononuclear cells, giving rise to foamy-appearing glomeruli.	-	Presence of histiocytes and a characteristic feature of hemophagocytosis	CD68 positive cells were observed at immunohistochemistry; feature of hemophagocytosis at EM
#4	13	-	Capillary lumina occluded by vacuolized mononuclear cells, giving rise to foamy-appearing glomeruli (PAS++). Mild thickening of the basal membrane.	Diffuse interstitial fibrosis with tubular atrophy.	Feature of hemophagocytosis	CD68 positive cells were observed at immunohistochemistry; feature of hemophagocytosis at EM

cell transplantation has been proposed for the management of EBV-HPS (34). As B-lymphocytes might also play a role in EBV-triggered HPS (35), the use of Rituximab as add-on therapy has been investigated, showing the ability to reduce ferritin levels and EBV titers (36).

Some additional considerations are worth mentioning when discussing hyperferritinemia status in the context of multiple organ dysfunction syndromes, a condition occurring in a not negligible proportion of patients in specific settings, such as intensive care units. Malignancies, active rheumatologic conditions, coagulopathies, or trauma have been identified as possible triggers for HPS in addition to infections (37, 38). When managing the trigger event, the presence/suspicion of a septic status poses some challenges for the use of chemo-immunotherapy. Conversely, a family history of HPS or evidence of genetic abnormalities obtained by a rapid flow cytometric screening could support the use of intensified schemes, such as the HPS-2004 protocol (39). Two recent series describe mortality and treatment of the adult HPS cases admitted to the ICU, one based on HPS-2004 criteria (37) and one based on the HScore (38). According to two recent studies, ICU hospital mortality rates ranged from 52 to 68% (37, 38). The studies differ based on the criteria used [HPS-2004 criteria (37) or HScore (38)], steroids administration rate (55 vs. 66%), etoposide (80 vs. 40%), and intravenous immunoglobulin (5 vs. 27%) use. In addition, in the ICU settings, some authors preferred methylprednisolone over dexamethasone (40). The use of plasma exchange (PE), with or without additional therapies, including biological agents has also been investigated. In total, 23 pediatric patients with hyperferritinemia suspected of sHPS identified in a retrospective analysis were treated with PE and either IVIG or methylprednisolone. Outcomes were compared with PE and IVIG with dexamethasone, cyclosporine, or etoposide. Although a high rate of infections was observed, only three patients died, all were among those who received the HPS-204 like regimen (40). The combination of PE and IVIG has been further supported in other series (41, 42).

As previously mentioned, active malignancy, especially lymphomas, can act as a trigger for HPS. Indeed, HPS can be directly associated with the active phase of the oncologic condition or can develop as a result of immunosuppression, where it is usually triggered by an infection (4, 43). In some cases, the two conditions can co-exist with EBV being the most frequently reported infection in this setting (4). In such cases, anti-B cell therapy may be recommended in addition (43). With active neoplasm, it remains uncertain whether the first-line management should be primarily HPS-targeted (e.g., HPS-2004 scheme) or if the priority should be given to targeting the specific malignancy. Up to half of the cases of HIV-related HPS cases are associated with active malignancy, mainly lymphoma (44).

Stopping (or at least reducing) the ongoing chemotherapy should be pondered in the case of chemotherapy-related HPS resulting from infection (43).

When compared with familial hemophagocytic lymphohistiocytosis or other forms of HPS, the prognosis seems more benign in cases triggered by autoinflammatory/autoimmune conditions. Among others, for example, the observed mortality rate was <10% in the HPS cohort of patients with systemic juvenile idiopathic arthritis (45). In these settings, initial management is usually less aggressive than in the HPS-2004 scheme and avoids the use of etoposide. In the previously mentioned study including patients with systemic juvenile idiopathic arthritis (45), nearly all patients were given steroids, more than half received cyclosporine, while a third was managed with IVIG. Similar therapeutic approaches have been reported in a series of 39 juvenile lupus-associated HPS, with an overall reported mortality of approximately 10% (46). These figures are also mirrored in one of the largest series available including 116 adult patients with HPS-related autoimmune/autoinflammatory conditions in whom the coexisting of active infection or malignancy was excluded. With an overall mortality rate below 15%, the use of steroids seemed to be associated with a favorable prognosis, even when given in monotherapy (about half of the cases) (4, 47). Steroids were often associated with IVIG, cyclosporine, or, less frequently, IV cyclophosphamide. Only a strict minority of the cases were exposed to etoposide.

The management of HPS in kidney transplant recipients is based on anecdotal reports. Available data support the concept that the majority of cases are usually associated with infections, making the use of antimicrobial therapy a priority. Calcineurin inhibitor therapy could not be discounted, also taking into account the data coming from studies investigating the efficacy and safety of the HPS-2004 protocol. High-dose steroids are often preferred, while the concomitant use of anti-metabolite agents is discouraged to avoid over-immunosuppression. IVIG could be considered in the case of rejection, with the use of PE to be preferred if antibody-mediated. The combined use of high-dose steroids, etoposide, and rituximab seems reasonable in the case of EBV infection.

With the current increase in the options available for the management of autoimmune conditions, biologic agents have been applied in the treatment of HPS, with anakinra the most commonly used. Anakinra was initially proposed as a promising effective option for the management of sHPS in a retrospective case series of eight critically ill pediatric patients (46). While the therapy was well tolerated, one should also acknowledge that patients were also receiving high-dose steroids and IVIG. On the other hand, its use is safe in patients with severe sepsis (47). Other biological agents, such as anti-TNFα drugs and anti-IL-6 (tocilizumab), have been used for rheumatoid arthritis and adult Still's induced HPS] (4). More recently, canakinumab has been proposed in selected refractory cases of Still's induced HPS (48). Notably, HPS can also occur in patients with inflammatory arthritis receiving biological agents, such as tocilizumab (4, 49, 50), canakinumab (51), and anakinra (52). Anakinra dose escalation has been reported in such cases. B-cell target therapies (e.g., rituximab, or more recently, belimumab) have been proposed for SLE, and EBV-associated HPS with or without associated lymphoma (4).

Renal involvement as a unique manifestation of hemophagocytic syndrome

To date, there is no unanimous agreement on the nomenclature for HLH- and MAS-related diseases (53). While we acknowledge that the reported cases might not fully match the classification proposed for HPS, the clinical onset, the laboratory profiles, and the histological findings (albeit not pathognomonic) might be in line with a form of acquired HLH with the kidney as the main organ involvement. In detail, our patients meet some of the HLH-2004 criteria (Tables 1, 2) (54). However, the clinical applicability of those criteria is limited as they include some specialized diagnostic tests, such as soluble IL-2 receptor alpha levels (sCD25), NK-cell functional assays, and genetic testing that might not be routinely available. More critically, some of the HLH-2004 items are not pathognomonic for HLH either. For instance, hemophagocytosis is not always found on bone marrow biopsy in patients with HLH, particularly in the early phase of the disease (54–56).

When referring to the HScore (23), a score of ≥250 has been associated with a high probability of acquired HLH (>99%), however, its use has never been investigated in forms of acquired HLH with mainly organ-limited manifestations. Our pilot observation might support the concept that in selected cases presenting with severe renal involvement and some signs/symptoms in line with HLH (once excluded concomitant possible differential diagnoses), a form of renal involvement as a unique manifestation of the hemophagocytic syndrome could be considered, especially when histological evidence of CD68 histiocytes is present.

One might speculate that our cases could represent a subgroup of secondary HLH or, when specifically referring to cases #1, #2, and #4, a novel hyperferritinemia syndrome identifiable in adults with a florid inflammation, increased ferritin, and AKI with macrophages at kidney biopsy. All in all, our report describes a potentially new condition (which shares some links with HLH) with renal involvement characterized by extensive activated macrophage infiltration of the glomeruli and moderate signs of systemic inflammation. This condition can be identified only by a renal biopsy.

Conclusion

The main aim of our paper was to increase the awareness of HPS as a potential cause of AKI, with or without multiorgan failure, among nephrologists. While renal involvement has frequently been reported in patients with overt cases of HPS, one should bear in mind that occasionally HPS cases can be diagnosed solely based on the findings of renal biopsy. When AKI can develop in the context of critically ill patients (9), distinguishing HPS from severe SIRS/MODS secondary to sepsis, trauma, or autoimmune/autoinflammatory diseases can be challenging. However, renal histological discrimination is crucial, as immunomodulatory therapy may be needed to control the hyperinflammatory state once HPS has occurred, but may be harmful otherwise. However, clinicians should be aware that the availability of electron microscopy is requested to identify such cases.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, upon reasonable request.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

Author contributions

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

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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