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ORIGINAL RESEARCH

Assessment of some Cytokines Levels in A pauci-immune Crescentic Glomerulonephritis Patients

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ABSTRACT

Background: Glomerulonephritis is the common cause of end-stage of renal failure. Rapidly progressive glomerulonephritis (RPGN) is characterized by glomerular injuries and formation of crescents. Moreover, anti-neutrophil cytoplasmic antibodies (ANCA), and pro-inflammatory cytokines play a damaging role in a crescent glomerulonephritis.

Objectives: The main goal of the current study was aimed to determine the ANCA autoantibodies, and the serum levels of pro-inflammatory cytokines (IL-6, IL-17, IL-18, INF-γ), as well as the relationship between these cytokines and ANCA test in a pauci-immune crescentic glomerulonephritis patients.

Methodology: The study was carried out in AL-Yarmouk teaching hospital and AL-Sader teaching hospital during the period from April 2022 to April 2023. This study was involved 90 patients with a crescentic glomerulonephritis disease, the diagnosis was made by the consultant medical staff. In addition to a 60 samples of healthy individuals as control group was involved to comparison the results. ANCA test was made by Indirect immunofluorescence method for the detection of ANCA autoantibodies in serum, in addition to measure the serum level of cytokines including IL6, IL-17, IL-18, and IFN-γ by using Enzyme Linked Immune Sorbent Assay methods.

Results: This study has shown that the majority of patients were male (66.7%). Moreover, the most effected age groups within the age ≥ 61 years in percentage (65.5%) followed by the age group 51-60 years old (28.8%). There were 59 (65.5%) positive cases for ANCA from total patients and all of these cases were MPO-ANCA. The total RPGN patients were showed a highly significant increased mean serum levels of IL-18, IL-17, IL-6, and INF-γ as compared to healthy controls, as well as, the same cytokines were showed significant increased levels in myeloperoxidase (MPO)-ANCA positive serum in crescent glomerulonephritis patients as compared to ANCA –negative serum of patients. These results indicate a functional role of IL-6, IL-17, IL-18, and INF-γ in ANCA-mediated neutrophil activation by the interdependence of IL-6, IL-17, IL-18, and INF-γ priming for ANCA responses.

Conclusion: It can be drawn from the present study that, the pro-inflammatory cytokines play an integral role in determining the course of disease and it can be used as major drug targets for therapy, as well as these cytokines have the ability to priming neutrophils thus contribute in the progression of a pauci-immune crescentic glomerulonephritis disease.

Keywords: IL-6, IL-17, IL-18, INF-γ, a pauci-immune crescentic glomerulonephritis.

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INTRODUCTION

Rapidly progressive glomerulonephritis (RPGN) is the rapid loss of renal function [1, 2]. Early diagnosis and treatment is important to prevent irreversible losses of renal functions. RPGN patients have urinary protein (proteinuria) and blood in urine (hematuria) [3] and may be have high blood pressure and edema. Moreover, untreated disease may progress to decrease urinary volume (oliguria) [4], associated with poor kidney function. RPGN is a very heterogeneous disease with a diverse etiology causes injuries within glomeruli trough alteration in many inflammatory pathways [5]. It can be caused by inherited or acquired disorder, and manifest by a variety ways ranging in a severity from asymptomatic urinary abnormality to acute kidney injuries or end stage renal disease [6].

Despite the absence of clinical manifestations of systemic vasculitis, the presence of necrotic glomerulonephritis and the presence of antibodies against specific components of the neutrophilic cytoplasm, would have suggested a vasculitic process. Primary RPGN is divided into three types, based on the immunofluorescence pattern. Type I, injury was caused by antibodies directed against glomerular basement membrane, Type II was characterized by the deposition of immune complex in the glomerulus [7, 8]. The remainder of RPGN cases were type III, also called pauci-immune crescentic glomerulonephritis (PICG), which antibodies directed against neutrophils ANCA (anti-neutrophil cytoplasmic antibodies). ANCA-associated vasculitis (AAV) is a group of autoimmune disorders that predominantly affects small vessels [9]. ANCA react with antigens in the primary granule in the cytoplasm of neutrophils antiproteinase-3 (PR3) [10] and in the lysosomes of monocyte myeloperoxidase (MPO) [11,12]. Neutrophil is a type of granulocyte and a type of phagocyte, Granulocyte-macrophage colony-stimulating factor (GM-CSF), in addition to being a growth factor for granulocytes and macrophages, is an activator of cells of the monocyte/macrophage lineage and induces HLA class II expression and cytokine synthesis in these target cells [13]. ANCA demonstrates two major types of staining patterns; cytoplasmic ANCA (cANCA-PR3), that produce a cytoplasmic staining patterns with central accentuations in alcohol-fixed neutrophils, and perinuclear patterns ANCA (pANCA-MPO) which demonstrate a perinuclear staining patterns of alcohol-fixed neutrophils, which is actually an artifact of fixation process [14]. PICG involve severe injury to the kidney glomeruli [15], with many of the glomeruli containing characteristic glomerular crescents (crescent-shaped scars), because of this microscopic features, RPGN is called a crescentic glomerulonephritis [16].

IL-6 is a pleiotropic cytokine that plays a key role in regulating immune responses, inflammation, and hematopoiesis. An elevated IL-6 level also occurs in various immune diseases, such as rheumatoid arthritis and Castleman disease, and nephropathies in multiple myeloma [51]. In ANCA-positive PICGN patients, IL-6 plays an essential role in neutrophil activation, mainly due to its induction of helper T lymphocytes that can produce IL-17 [52].

Moreover, it has become clear that the Th17 pathway is important in the development of crescentic GN. In experimental MPO-AAV, mice lacking IL-17A are protected from early glomerular injury [53].

IL-18 is a pro-inflammatory cytokine that is structurally similar to IL-1β, IL-18 promotes the production of interferon gamma (IFN-γ) and strongly induces a Th1 response. In recent years, the biological and pathological roles of IL-18 have been studied in many diseases. Inflammation underlies the pathogenesis of many acute or chronic kidney diseases, and IL-18 plays an important role [54].

IFN-γ is a pleiotropic cytokine produced by activated immune cells, including NK cells [55]. The proinflammatory roles of IFN-γ in kidney disease include the macrophage activation [56], modulation of effector T-cell responses, induction of major histocompatibility complex class I and II molecules for antigen presentation, [57] and up regulation of chemokines that augment immune cell infiltration. In addition to its proinflammatory effects, IFN-γ has also been reported to limit kidney disease progression and preserve renal function [58].

AIMS OF THE STUDY

The current study was aimed to determine the ANCA autoantibodies, and measure the serum level of pro-inflammatory cytokines (IL-6, IL-17, IL-18, and INF-γ), as well as the relationship between these cytokines and ANCA test in a pauci-immune crescentic glomerulonephritis patients.

METHODOLOGY

This study was conducted from April 2022 to April 2023 at AL-Yarmouk teaching hospital and Al-Sader teaching hospital; involve (90) patients with PICG disease. The diagnosis made by the consultant medical staff, in addition to (60) samples of healthy individuals as control group to comparison the results.

Blood Collection:

five ml of peripheral blood were obtained by venipuncture from each patient using 5 ml disposable syringe, the blood was drawn in a plain tube (without anticoagulant). The tubes were centrifuged (2000 rpm for 15 minutes) after 15 minutes to collect the sera, and frozen at -20ºC until tested.

ANCA test:

Indirect immunofluorescence method for the detection of ANCA autoantibodies in serum, which is based on the reaction of ANCA autoantibodies in serum with their fixed substrate (neutrophils) on a slide. The reacted autoantibodies are then detected by a secondary antibody conjugated with fluorescin (sheep anti-human IgG). The Slide is then examined under a fluorescent microscope, in which the cells appear as apple green.

- **Kit contents:** (The Binding Site Company, United Kingdom):

  Ethanol and formalin-fixed, neutrophil substrate slides (5 or 10 well); positive control serum; negative control serum; sheep anti-human IgG conjugated with fluorescein; evans blue stain; Phosphate buffered saline (PBS) concentrate; blotters; Mounting medium (DABCO, 1,4, diazabicyclo (2.2.2) octane); cover slips (22 x 70 mm). Cat No.: 10070-L-11.

- **Cytokine Tests:**

  Serum levels of IL-6, IL-17, IL-18, and INF-γ were quantitatively determined in patients and control subjects by means of indirect sandwich ELISA test using commercially available kit.

  Kits: IL-6 ELISA kit: Bio-Source, Europe S.A. Cat No.MBS261259, IL-17 ELISA kit: BioSource, Europe S.A. Cat No. MBS764076, IL-18 ELISA kit: Bio-Source, Europe S.A. Cat No.MBS2510436, and INF-γ ELISA kit: Bio-Source, Europe S.A. Cat No.MBS161542.

Calculation of the Results:

The results of patient samples were calculated by interpolation from a standard curve using a curve fit equation for IL-18, IL-17, IL-6, and IFN-γ.

Statistical Analysis

The values of these parameters were presented as mean ± standard deviation (S.D.), and significant differences between means were assessed by using t-test, the least significant difference (LSD) or Duncan´s test by using the computer programmed social package for statistical analysis (SPSS) version 7.5 in which a probability (P) equals or less than 0.05 were considered significant. ANCA values were given as a percentage frequency, and a significant difference between these frequencies was assessed by Fisher's exact probability.

RESULTS:

Table (1): Gender distribution of PICG patients and controls

Groups	Total number	Males		Females		P value ≤
		No.	%	No.	%	
Controls	60	35	58.3	25	41.7	
Patients	90	60	66.7	30	33.3	0.005

Significant difference (P≤ 0.05) as compared to the corresponding controls.

A total of 90 patients with PICG disease were included in this study, male patients showed increased percentage frequency as compared to the female patients (66.7% vs. 33.3%), Table 1.

Table (2): Age distribution of PICG patients and controls

Age groups	Control		Total patients		P value ≤
	No.	%	No.	%	
41-50	9	15	5	5.5	0.05
51-60	16	26.7	26	28.8	
61 and >	35	58.3	59	65.5	
Total	60	100.0	90	100.0	

Significant difference (P≤ 0.05) as compared to the corresponding controls.

PICG patients were significantly increased in older aged patients within the age ≥ 61years in percentage (65.5%), Table 2.

Table (3): Frequencies distribution of ANCA positive and negative sera in PICG patients.

Groups	positive-ANCA (MPO-ANCA)		ANCA-negative sera		P value
	No.	%	No.	%	
Total patients (90)	59	65.5	31	34.4	1×10^{-5}

P: Fishers exact probability.

None of the 60 control samples was positive for ANCA; therefore, the statistical analysis was limited to the total patients (90); and the comparison was based on the observed percentage frequencies. Out of 90 PICG patients, there were 59 (65.5%) positive sera for ANCA and all of these cases was MPO-ANCA, Table 3.

Table (4): Serum level of IL-18 in PICG patients and controls

Groups	Number	Mean± S.D. (pg/ml)	P value ≤
Control	60	125.3±11.4	0.01
Total patients	90	736.5±16.8	
MPO-ANCA sera	59	880.2±19.3	0.01
ANCA-negative sera	31	470.1±13.4	

Significant difference (P≤ 0.05), Highly Significant difference (P≤ 0.01) as compared to the corresponding controls.

A highly significant (P ≤ 0.01) increased mean serum level of IL-18 in total PICG patients as compared to control subjects (736.5 vs. 125.3 pg / ml) was observed, also MPO positive patients showed a highly significant increased mean serum level of IL-18 when compared with ANCA-negative patients (880.2 vs. 470.1 pg/ml), Table 4.

Table (5): Serum level of IL-17 in PICG patients and controls

Groups	Number	Mean± S.D. (pg/ml)	P value ≤
Control	60	10.42±4.91	0.01
Total patients	90	68.45±31.53	
MPO-ANCA sera	59	91.46±37.71	0.05
ANCA-negative sera	31	40.63 ±19.16	

Significant difference (P≤ 0.05), Highly Significant difference (P≤ 0.01) as compared to the corresponding controls.

The total patients were showed a highly significant increased mean serum level of IL-17 as compared to control subjects (68.45 vs. 10.42 pg/ml), and MPO positive patients showed a significant increased mean serum level of IL-17 when compared with ANCA-negative a pauci-immune crescentic glomerulonephritis patients (91.46 vs. 40.63 pg/ml), Table 5.

Table (6): Serum level of IL-6 in PICG patients and controls

Groups	Number	Mean± S.D. (pg/ml)	P value ≤
Control	60	171.4±8.1	0.01
Total patients	90	635.7±25.3	
MPO-ANCA sera	59	754.2±32. 9	0.05
ANCA-negative sera	31	534.8±20.9	

Significant difference (P≤ 0.05), Highly Significant difference (P≤ 0.01) as compared to the corresponding controls.

The total patients were showed a highly significant increased mean serum level of IL-6 as compared to the controls (635.7 vs.171.4 pg/ml), as well as, MPO positive patients showed a significant increased mean serum level of IL-6 when compared with ANCA-negative patients (754.2 vs. 534.8 pg/ml), Table 6.

Table (7): Serum level of INF-γ in PICG patients and controls

Groups	Number	Mean± S.D. (IU/ml)	P value ≤
Control	60	11.2±5.4	
Total patients	90	65.5±18.7	0.01
MPO-ANCA sera	59	89.3±29.3	
ANCA-negative sera	31	40.6±18.6	0.05

Significant difference (P≤ 0.05), Highly Significant difference (P≤ 0.01) as compared to the corresponding controls.

PICG patients were showed a highly significant increased mean serum level of INF-γ as compared to the controls (65.5 vs. 11.2 IU/ml), as well as, MPO positive patients showed a significant increased mean serum level of INF-γ when compared with ANCA-negative patients (89.3 vs. 40.6 IU/ml), Table 7.

DISCUSSION:

Rapidly progressive glomerulonephritis (RPGN) is a syndrome characterized by a sudden loss of kidney function associated with the presence of more than 50% of glomeruli with epithelial crescents in the kidney biopsy [62, 63]. The pauci-immune crescentic glomerulonephritis (PICG) is the most common RPGN, representing more than 80% of the cases and it is defined as the extensive glomerular inflammation with few or no immune deposits, generally associated with ANCA-associated vasculitis [64, 65].

The present study demonstrated increased percentage of p-ANCA in RPGN patient, which is in agreement with the results of Berden et al., [17], who found that ANCA-associated vasculitis (AAV) is the most cause of RPGN worldwide, and it is a group of autoimmune disorders that predominantly affects

small vessels [9]. Therefore, a pauci-immune crescentic glomerulonephritis was consider the most common cause of RPGN in adult [18, 19]. This result can be explained by the link between ANCAs and the pathogenesis of ANCA-associated disease. However, it is postulated that ANCAs induce a premature degranulation and activation of neutrophils at the time of their Margi nation, leading to the release of lytic enzymes and toxic oxygen metabolites at the site of injury. There is now evidence that ANCAs are directly involved in the pathogenesis of pauci-immune small vessel vasculitis or glomerulonephritis. Typically, MPO–ANCA is observed in (80 to 90%) of patient with RPGN type III (pauci-immune), but neither MPO-ANCA nor PR3-ANCA is 100% specific for type III. Myeloperoxidase ANCA-associated vasculitis (MPO-AAV) often manifests as rapidly progressive GN (RPGN) and is much more common in China than PR3-AAV [59]. Most cases about 90% are associated with anti-neutrophil cytoplasmic antibody, or more specifically, anti-myeloperoxidase or anti-proteinase 3 antibodies [60]. In vivo and in vitro studies have shown the potential of anti-proteinase 3 and anti-myeloperoxidase antibodies to directly contribute to kidney damage in the absence of immune complex formation [61].

Crescent is characterized by extra-capillary proliferations within glomerulus partially or completely filling up Bowman's space, it is composed of proliferated parietal epithelial cells, macrophages, and fibroblasts [20]. A crescent formations stimulate by entry of fibrin and another plasma proteins from capillary lumen following ruptures of glomerular basement membrane (GBM), then followed by migration of T-cell, macrophage, and other inflammatory cell to the site of injury accelerating cytokine release and tissue injuries [17]. ANCA are specific for proteins in the cytoplasm of neutrophil and monocyte, the major target antigens in patient with glomerulonephritis are PR3 and MPO, after binding with MPO or PR3 antigens, ANCA results in the activations of monocyte and neutrophil once the cells are primed with low dose of cytokine like interleukin-1, interleukin-18, and TNF-α [21, 22].

There is priming result in the surface of MPO and PR3 allowing interaction with ANCA. ANCA associated vasculitis is autoimmune disease commonly causing kidney impairment, there is a rising interest about the role of T-lymphocyte in recent years. Polarization towards predominant Th1 and Th17 responses in acute phase of the disease [23] along with decline in the numbers of T-regulatory lymphocyte, which show functional impairment, interaction between different T-cells subset, as well as between T-cell, B-cell and neutrophils enhance the inflammatory responses forming complex network, also CD8+T cells subset can activate polymorphonuclear cells (PMN), PMN cells activation is a key events in pathogenesis of RPGN [24], that lead to exposure of MPO and PR3 on their surface allowing their recognitions by ANCA, interferon-gamma (IFN-γ) secreted by CD8+T cell [25]. during the acute phase of ANCA-associated vasculitis is the potent activators of PMN cell, thus explained the contributions of CD8+T cells in pathogenesis of this disease, this explain our results about increased serum level of IFN- γ in PICG patients [26, 45, 46, and 47] Numerous studies in various models of crescentic GN have shown that CD4 + T cells play a critical role in this disease [66].

The present study was also observed that the mean serum level of IL-18 was significantly increased in MPO-ANCA positive of PICG patients, such finding is in agreement with the results of AL-Roubaey [27], who found that IL-18 concentrations was significantly increased in serum of p-ANCA positive RPGN patients. This interleukin is play a damaging role in a murine model of crescentic glomerulonephritis. In addition, the presence of this interleukin in renal tissue of patients with ANCA disease, and the ability of this cytokine to prime neutrophils, demonstrate that Th1-mediated responses may be involved in disease progression [28, 29]. Moreover, IL-18 is capable of priming neutrophils in vitro, thus augmenting

superoxide productions by cells binding ANCA. RPGN involve severe injury to the kidney glomeruli. Recent evidence suggested, that this lesion which is the result of a T helper1 (Th1) immune responses involves the kidney. Interleukin-18 is a cytokine that is emerging as an important co-factor in the generation of Th1 responses, and this is due to its role in polarization of naive T cells to T- helper type 1 (Th1) response, and subsequent production of IFN-γ by T cells [30].

IL-17 seems to has a central role in inflammation and progression of kidney injury [31, 32]. The role of IL-17 producing cell in a murine model of crescentic glomerulonephritis proved by many authors [33, 34]. IL-17 induces renal expression of C-X-C Motif chemokine ligand 5 by tubular epithelium, that is responsible for neutrophil attractions [35]. In addition, Nogueira et al., [36], demonstrate a significant high level of IL-17 in serum of AAV patients compared with healthy controls, and higher percentage of IL-17 producing cell after stimulation with MPO or PR3. This finding is in agreement with our results which found that IL-17 concentrations were significantly increased in serum of PICG patients as compared to healthy controls. Th-17 cell subset enhances the recruitment of neutrophil to inflammation sites and contributes in organ damage in ANCA-associated vasculitis. This cross-talk is reciprocal and neutrophil also is able to induce chemotaxis of IL-17 cell, makes this cellular axis more interesting as possible targets for treatment of this disease [37, 38].

Clinical and experimental studies suggest that IL-6 contributes to renal injury in glomerulonephritis and other forms of renal disease. Mechanistically, IL-6 may contribute to renal disease by enhancing the signaling response of tubular epithelial cells to pro-fibrotic cytokines such as transforming growth factor-β (TGFβ),1L-8 [39, 40]. Renal dysfunction is commonly seen in patients with thalassemia [41, 42]. In patients with acute kidney injuries, high circulating level of IL-6 is predictive of increased mortality [43, 44, and 45]. In another study IL-6 trans-signaling in tubular epithelial cells ameliorated injury and led to preservation of renal function. This led many authors to conclude that, IL-6 simultaneously promotes an injurious inflammatory responses and, through a mechanism involving IL-6 trans-signaling, can protects the kidney from further injuries [46, 47, 48, 49, and 50].

CONCLUSIONS:

Elevating levels of pro-inflammatory cytokines may possibly contribute to the pathogenesis of this disease. Early treatment with an immunosuppressive agent may significantly alleviate the proteinuria and improve the renal function in these patients.

RECOMMENDATIONS:

Further investigations we need to study the role of pro-inflammatory cytokines, as well as their role in priming for ANCA responses are imperative for getting a full view of kidney disease and developing more effective drugs.

Ethical Clearance:

All experimental protocols were approved under the Faculty of science.

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