THE CORRELATION BETWEEN ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODY AND TWO CYTOKINES (IL-18, TNF-A) IN RAPIDLY PROGRESSIVE GLOMERULONEPHRITIS DISEASE [+]

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Abstract:

Glomerulonephritis is the commonest cause of end-stage renal failure, all types of rapidly progressive glomerulonephritis (RPGN) are characterized by glomeruluar injury and the formation of crescents, anti-neutrophil cytoplasmic antibodies(ANCA), interleukin-18 (IL-18) and tumor necrosis factor-α (TNF-α) plays a damging role in crescent glomerulonephritis.

This study was conducted to evaluate the diagnostic value of ANCA measurement, the serum levels of two cytokines(IL-18,TNF-α) and the realtionship between them in RPGN disease.

The mean serum level of IL-18 and TNF-α showed significant increased level in patients with RPGN disease as compared to healthy controls, as well as, the same cytokines showed significantly increased levels in perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA) positive serum in rapidly progressive glomerulonephritis (RPGN) patients as compared to ANCA –negative serum in RPGN patients, these results indicate a functional role of IL-18 and TNFα in ANCA-mediated neutrophil activation by the interdependence of IL-18 and TNFα priming for ANCA responses.

العلاقة بين الاضداد– ANCA والحركيان الخلويان IL-18 و TNF-α في مرض ألتهاب الكبيبات الكلوي التقدمي السريع

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المستخلص :

ألتهاب الكبيبات الكلوي هو السبب الاكثر شيوعا للفشل الكلوي النهائي وان جميع أنواع التهابات الكبيبات الكلوي التقدمي السريع تتميز بجرح glomeruluar وتشكيل الأهلة. تساهم الأضداد ANCA والحركيان الخلويان IL-18 و TNF-α بالدور التحطيمي في ألتهاب الكبيبات الكلوي الهلالي. وقد اجريت هذه الدراسة لتحديد القيمة التشخيصية للاضداد ANCA والمستوى المصلي للحركيان الخلويان IL-18 و TNF-α والعلاقة بينهم في مرض ألتهاب الكبيبات الكلوي التقدمي السريع

[+]**Received on 7/10/2012 , Accepted on 25/2/2013 .**
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اظهر معدل المستوى المصلي للحركيين الخلويين IL-18 و TNF-α ارتفاعا معنويا في المرضى المصابين بألتهاب الكبيبات الكلوي التقدمي السريع بالمقارنة مع مجموعة السيطرة , وكذلك اظهر نفس الحركيان الخلويان ارتفاعا معنويا في المرضى ذو المصل الموجب للاضداد p-ANCA بالمقارنة مع المرضى ذو المصل السالب للاضداد ANCA مما ادى الى أستنتاج الدور الوظيفي ل IL-18 و TNF-α في تنشيط ANCA-mediated-Neutrophil بالأعتماد على IL-18 و TNF-α في عمل ال priming لأستجابة الأضداد ANCA

Introduction:

Rapidly progressive glomerulonephritis (RPGN) is a syndrome of the kidney that is characterized by a rapid loss of renal function , usually 50% decline in the glomerular filtration rate (GFR) within 3 months [1]. If left untreated, it rapidly progresses into acute renal failure and death within months. Patients with RPGN have blood in the urine (hematuria), urinary protein (proteinuria), and occasionally high blood pressure (hypertension) and edema. The clinical picture is consistent with nephritic syndrome, although the degree of proteinuria may occasionally exceed 3 g/24 h, a range associated with nephrotic syndrome. Untreated disease may progress to decreased urinary volume (oliguria), which is associated with poor kidney function [2]. 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, in the form of , anti-neutrophil cytoplasmic antibodies (ANCA)-positive pauci-immune RPGN. Based on the ChapelHill Consensus Conference (CHCC) classification of vasculitis [3], our patient might be considered as having ANCA-associated small vessel vasculitis [4]. Rapidly progressive glomerulonephritis can be classified into three types, based upon the immunofluorescence patterns. Type I RPGN, which, accounts for approximately 20% of RPGN cases, injury is caused by antibodies directed against the glomerular basement membrane. Type II RPGN accounts for roughly 25% of RPGN cases and is characterized by the deposition of immune complexes in the glomerulus [5]. The remainder of RPGN cases are type III, or pauci-immune RPGN, which features antibodies directed against neutrophils (anti-neutrophil cytoplasmic antibodies, ANCA). ANCAs react with antigens in the primary granules in the cytoplasm of neutrophils (antiproteinase-3 [PR3]) and in lysosomes of monocytes myeloperoxidase (MPO) [6]. , anti-neutrophil cytoplasmic antibodies (ANCA) demonstrates 2 major types of staining patterns; cytoplasmic ANCA (cANCA) that produces a cytoplasmic staining pattern with central accentuation in alcohol-fixed neutrophils, and perinuclear pattern ANCA (pANCA) which demonstrates a perinuclear staining pattern of alcohol-fixed neutrophils, which is actually an artifact of the fixation process [7]. Rapidly progressive glomerulonephritis (RPGN) involves severe injury to the kidneys' glomeruli, with many of the glomeruli containing characteristic glomerular crescents (crescent-shaped scars). Because of this microscopic feature, RPGN is also called crescentic glomerulonephritis [8]. Recent evidence suggests that this lesion is the result of a T helper1 (Th1) immune response involving the kidney. Interleukin-18 is a recently described cytokine that is emerging as an important co-factor in the generation of Th1 responses , and this is due to IL-18's role in polarization of naive T cells to a T helper type 1 (Th1) response, and subsequent production by these T cells of IFN-ϒ [9]. This interleukin is also play a damging role in a murine model of crescentic glomerulonephritis, and, 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 [10]. Furthermore, IL-18 is capable of priming neutrophils *in vitro*, thus augmenting superoxide production by cells ANCA binding. This increased activity is similar to that seen with tumor necrosis factor-α (TNF-α) priming [11]. Tumor necrosis factor-α (TNF-α) is a cytokine involved in systemic inflammation and is a member of cytokines that stimulate the acute phase reaction, the primary role of TNF is the regulation of immune cells, and is also able to induce apoptotic cell death to induce inflammation as well as TNF-α has well-documented proinflammatory effects in tissue inflammation and autoimmunity [12].

Materials and methods:

One hundred-twenty patients admitted to AL-Yarmouk teaching hospital with RPGN disease, for the period between (6-3-2011 and 20-1-2012), and in addition to patients, a total of 40 healthy individuals who were age and sex matched with the patients and no history of RPGN disease, were included as control in this study.From each subject included in this study, 10 ml of venous blood was collected, and centrifuged on speed 450 Xg at 4°C for 10 minutes. The serum was then aspirated using a Pasteur pipette and dispensed into sterile glass tubes (1 ml in each) and stored at -20°C until used.

Indirect immunofluorescence method for the detection of ANCA autoantibodies in serum was used. This method 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 secondry antibody conjugated with 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).

Assay procedure: The PBS concentrate was diluted (1:19) with distillated water, and the obtained buffer was used as a sample diluents and a washing buffer; the serum sample was diluted by mixing 10 µl of serum with 190 µl of PBS; the substrate slide was allowed to reach room temperature (approximately 20 minutes), and then 25 µl of diluted serum, negative serum and positive serum were added separately to the respective wells in the slide, and then incubated at room temperature for 30 minutes in a humid chamber. After incubation, each well was rinsed with PBS, and then the slide was immersed in a jar containing PBS and placed on a magnetic stirrer for 10 minutes. Each well was covered with a droplet of fluorescent conjugate, and the slide was then incubated at room temperature for 30 minutes in a humid dark chamber, and then each well was rinsed with PBS, and then the slide was immersed in a jar containing 2-3 drops of 1% evan's blue stain were added to 100 ml of PBS and the jar was placed on a magnetic stirrer for 10 minutes, and then the slide was blotted, mounted and cover-slipped, and examined under fluorescent microscope.

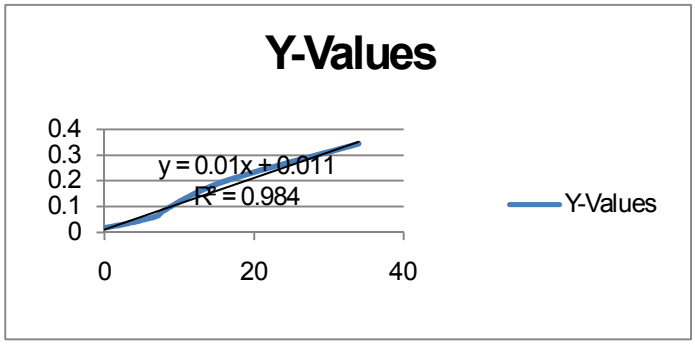
Enzyme Linked Immunosorbent Assay was used for the detection of IL-18 and TNF-α in serum: Kit contents; materials provided using with the kits: (Bio-Source, Europe S.A. for IL-18, Beckman Coulter, France for TNF-α). Including microtiter plate: 12x8 wells pre-coated with a monoclonal anti-human interleukin IL-18 or TNF-α antibody (ready-to-use); recombinant human interleukin standard IL-18 and TNF-α**;** biotin monoclonal antibody; streptavidin alkaline phosphatase or streptavidin-HRP conjugate; diluent; washing solution; substrate; and stop solution.

Assay Procedure: Before carrying out the assay procedure of an interleukin-18 (IL-18) or TNF-α determination, the kit were left at room temperature (18-25°C) for 30 minutes to equilibrate, as suggested by the manufacturer. After that, the assay was carried out following the instructions in the kit's leaflet, which are summarized in the following steps: Serial concentrations (they were dependent on the parameter investigated as suggested by the kit's manufacturer) of the standard were made using the diluents; aliquot (50 µl for IL-18 and 100 µl for TNF-α) of the standard or sample was added to the well, and the plate was incubated for two hours at room temperature with shaking. The well was washed with three cycles of washing solution, by the microtiter plate washer; an aliquot (50 µl) of biotinylated antibody was added to the well for IL-18 and the plate was incubated for 30 minutes at room temperature; and then the washing step was repeated (three cycles). An aliquot (100 µl) of streptavidin alkaline phosphatase or streptavidin-HRP (it was dependent on the parameter investigated as suggested by the kit's manufacturer) conjugate was added to the well and the plate was incubated for 30 minutes at room temperature;and then the washing step was repeated (three cycles). An aliquot (100 µl for IL-18 and 200 µl for TNF-α) of substrate was added to the well and the plate was incubated for 20 minutes for IL-18 and 30 minutes for TNF-α at room temperature with shaking; An aliquot (50 µl) of stop solution was added to the well and the absorbance was read at a wave length of 450 nm for IL-18 and 405 nm for TNF-α using ELISA reader.

Calculation of the Results: The sample results were calculated by interplotation from a standard curve that was performed in the same assay as that for the sample (Figures 1 and 2 for IL-18 and TNF-α respectively), using a curve fit equation.

(x axis for the concentration , y axis for the O.D. (Optical density))

Figure 1: Standard curve of IL-18 serum level.



(x axis for the concentration , y axis for the O.D. (Optical density))

Figure 2: Standard curve of TNF-α serum level.

Statistical Methods:

Values of IL-18 and TNF-α were presented as mean ± standard error (S.E.), and significant differences between means were assessed by ANOVA F test, Duncan's test, using the computer programme Social Package for Statistical Analysis (SPSS) version 7.5, in which a probability (P) equals or less than 0.05 was considered significant, while in ANCA parameter, the values were given as a percentage frequency, and a significant difference between these frequencies was assessed by Fisher's exact probability.

Results and discussion:

A total of 120 patients with RPGN disease were included in this study. Males patients showed increased percentage frequency as compared to the female patients (62.5% vs. 37.5%), Table1.

Table 1: Gender distribution of rapidly progressive glomerulonephritis patients and controls.

Subject groups	Total	Males		Females	
		No.	%	No.	%
Control	40	20	50	20	50
Patients	120	75	62.5	45	37.5

Most RPGN patients were within the age ≥ 60 years. Table 2,

Table 2: Age distribution of rapidly progressive glomerulonephritis patients and controls.

Subject groups	Total	Age groups					
		<50		50-59		60 and >	
		No.	%	No.	%	No.	%
Control	40	3	7.5	12	30	25	62.5
Total patients	120	6	5	42	35	72	60

None of the 40 control samples was positive for ANCA; therefore the statistical analysis was limited to the total patients; and the comparison was based on the observed percentage frequencies. Out of 120 RPGN patients, there were 65 (54.17%) positive sera for ANCA and all of these cases was P-ANCA (MPO-ANCA) Table 3.

Table 3: Frequencies distribution of ANCA positive sera in rapidly progressive glomerulonephritis patients.

Subject groups	Number	ANCA positive sera		
		No.	%	P
Total patients	120	65	54.17	1×10^{-6}

P: Fishers exact probability.

Total RPGN patients showed a significant increased mean serum level of IL-18 as compared to controls (754 *vs.* 130 pg/ml), dividing the subjects according to the ANCA positive cases revealed that the patients with p-ANCA positive serum showed a significantly higher mean serum level of IL-18 as compared to the corresponding patients with ANCA negative serum (877 *vs.* 456 pg/ml) Table 4.

Table 4: Serum level of IL-18 in rapidly progressive glomerulonephritis patients and controls.

Groups	Number	Mean ± S.E. (pg/ml)	P
Control	40	130±12.4	≤0.01
Total RPGN patients	120	754±15.8	
ANCA positive sera	65	877±19.1	≤0.01
ANCA negative sera	55	456±12.3	

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

Total RPGN patients showed also a significant increased mean serum level of TNF-α as compared to controls (430 *vs.* 101 pg/ml), as well as p-ANCA positive serum patients showed a significantly increased mean serum level of TNF-α as compared to the corresponding patients with ANCA negative serum (530 *vs.* 230 pg/ml) (table 5).

Table 5: Serum level of TNF-α in rapidly progressive glomerulonephritis patients and controls.

Groups	Number	Mean ± S.E. (pg/ml)	P
Control	40	101±23.7	≤0.01
Total RPGN patients	120	430±15.9	
ANCA positive sera	65	530±39.1	≤0.01
ANCA negative sera	55	230±18.7	

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

Rapidly progressive deterioration of renal function is a common and usually severe feature of anti-neutrophil cytoplasm autoantibody (ANCA)-associated vasculitis, which may lead to end-stage renal failure or death [13]. The present study demonstrated increased percentage of p-ANCA in RPGN patient,

which is in agreement with the results of Berden et al, [14], who found that ANCA-associated vasculitis is the most cause of rapidly progressive glomerulonephritis worldwide, and the renal biopsy is the gold standard for establishing the diagnosis, which was confirmed in this study by the consultant medical staff at the hospital. 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 margination, leading to the release of lytic enzymes and toxic oxygen metabolites at the site of injury. There is now substantial evidence that ANCAs are directly involved in the pathogenesis of pauci-immune small vessel vasculitis or glomerulonephritis. *In vitro* data demonstrated that these autoantibodies activate normal human polymorphonuclear (PMN) leukocytes. Neutrophils are an indispensable component of the immune system, circulating in an ordinarily quiescent state but rapidly transmigrating into the foci of infection in order to mediate efficient microbial killing. *In vitro*, neutrophil stimulation by ANCA requires priming [15], attention has mainly focused upon increased cell surface expression of target antigens after priming, which may influence the magnitude of ANCA responsiveness [16]. However, ANCA also engage Fcγ receptors and β_2-integrins, and it is clear that priming can operate at the level of intracellular signalling pathways that facilitate activation of the superoxide anion (SO)-generating nicotinamide adenine dinucleotide phosphate (reduced form) oxidase complex [17]. *In vivo*, the importance of neutrophil priming in associated systemic vasculitis (ASV) has been inferred from the frequent association of vasculitis activity with infection, which could provide the priming signal. Circulating neutrophils have been shown to be primed in active ASV [18]. The present study demonstrated increased percentage of p-ANCA in RPGN patients, these results strongly supported by Day et al, [19], who reported that the neutrophils are principal mediators of tissue damage when aberrantly activated, as for example, in antineutrophil cytoplasm autoantibody (ANCA)-associated systemic vasculitis (ASV), although ASV encompasses a number of related diseases, but evidence demonstrates that ANCA are pathogenic autoantibodies that stimulate pro-inflammatory neutrophil functions. In vasculitis, ANCA are directed against either proteinase 3 (PR3) or myeloperoxidase (MPO). Recently, pathogenic transfer of anti-MPO antibodies was reported in a murine model of vasculitis [20]. Additionally, degranulated PR3 and MPO may themselves exert pathogenic effects upon the endothelium [21]. Although ANCA antigens are normally found in the cytoplasm of neutrophils and monocytes, priming of these cells, as occurs following exposure to certain cytokines, results in the release of small amounts of ANCA antigens at the cell surface [22]. In this study the mean serum level of IL-18 was significantly increased in p-ANCA positive RPGN patients , such finding is in agreement with the results of Hultgren et al, [23], who found that IL-18 concentrations was significantly increased in serum of ASV patients as compared to healthy controls, these results also suppotted by Calvani et al, [24], who reported that, IL-18 is upregulated in renal biopsies from patients with ASV. Specifically, IL-18-positive cells within glomeruli were podocytes, IL-18 subsequently recognized as an important component of the innate immune system with effects upon many cell types. Reported effects of IL-18 upon neutrophils include CD11b upregulation [25], mobilization of nicotinamide adenine dinucleotide phosphate (reduced form) complex components [26], p38 mitogen-activated protein kinase (MAPK) phosphorylation [27], cytokine and leukotriene synthesis, priming of *N*-formyl-methionyl-leucyl-phenyalanine-induced superoxide production [28], and this interleukin was originally identified as interferon γ-inducing factor for T-lymphocytes [29]. The present study was also observed that the mean serum level of TNFα in ANCA positive RPGN patients was significantly higher when compared to ANCA negative patients or control group, this result was in agreement with the results of Radford et al, [30], who found that *in vitro*, neutrophil stimulation by ANCA requires priming, typically by TNFα. *In vitro*, neutrophil responsiveness to ANCA has been reported to require TNFα priming, in addtion to IL-18 priming. IL-18- and TNFα was observed to prime neutrophil responses to both PR3-ANCA and to MPO-ANCA. Both TNFα and IL-18 induced a rapid and transient phosphorylation of p38 MAPK in neutrophils, other studies suggested that they may be important in ANCA-mediated neutrophil activation [31]. Modulation of intracellular signalling events by cytokine priming, as outlined above, may also be important. *In vivo*, expression of ANCA antigens on neutrophils sequestered within the microcirculation is likely to be most important, and the relationship between antigen expression on isolated neutrophils and those in the microcirculation

remains to be determined. These observations may offer new insights into the pathogenesis of ASV. *In vitro*, IL-18 primed superoxide production by ANCA-activated neutrophils comparably to TNFα [32]. We conclude that IL-18 and TNFα are likely to be important for neutrophil recruitment and priming in ASV. Some reports indicate that anti-TNFα therapies may be beneficial in ASV, but at least some patients still incur irrecoverable organ damage, perhaps suggesting that other cytokines modulate neutrophil activity under TNFα blockade. Therapies targeting single priming agents may have limited efficacy in controlling disease [33].

References:

1-Lohr J.W. and Owens K. C**.** "Glomerulonephritis, Rapidly Progressive". *Nephrology*., 4: 1-6, 2008.

2-Cotran R. S., Kumar V., Fausto N., Nelso F., Robbins S. L. and Abbas A. K. *Robbins and Cotran pathologic basis of disease*., St. Louis, MO: Elsevier Saunders. pp. 976–8, 2005.

3-Deliyska B. and Shurliev V. " The efficacy of an individual treatment schedule in patients with vasculitis" . *Nephrol. Dial. Transplant*., 18 (suppl 5): 13-5, 2003.

4-Tesar V., Rihova Z., Jancova E., Rysava R. and Metra M. "Current treatment in ANCA-positive renal vasculitis-lessons from European randomized trials". *Nephrol. Dial. Transplant.*, 18(suppl 5): 2-4, 2003.

5-Alexopoulos E., Gionanlis L. , Papayianni E., Kokolina E., Leontsini M. and Memmos D. "Predictors of outcome in idiopathic rapidly progressive glomerulonephritis (IRPGN) ". *BMC Nephrol*., 7: 16, 2006.

6-Moore E. "ANCA and autoimmune diseases". Jefferson, NC: McFarland Publishing. 24: 1-6, 2007.

7-Cranmer L.D., Warrington K.J. and Ytterberg S.R. " 61-year-old man with dyspnea and bilateral foot drop". *Mayo. Clin. Proc.*, 77: 363-366, 2002.

8-Aasarad K., Bostad L., Hammerstrem J., Jorstad S. and Iversen BM. "Renal histopathology and clinical course in 94 patients with Wegeners granulomatosis" . *Nephrol. Dial. Transplant.*, 16: 953-60, 2001.

9-Reddy P. "Interleukin-18: recent advances". *Curr Opin Hematol*., 11: 405–410, 2004.

10-Pressler B.M., Falk R.J. and Preston C.A. "Interleukin-18, neutrophils, and ANCA". *Kidney International*., 69: 424–425, 2006.

11-Popa ER., Franssen CF. and Limburg PC. *et al*. "In vitro cytokine production and proliferation of T cells from patients with anti-proteinase 3- and antimyeloperoxidase-associated vasculitis, in response to proteinase 3 and myeloperoxidase". *Arthritis Rheum*., 46**:** 1894–1904, 2002.

12-Locksley RM., Killeen N. and Lenardo MJ. "The TNF and TNF receptor superfamilies: Integrating mammalian biology". *Cell*., 104(4): 487-501, 2001.

13-Hauer H.A., Bajema I.M., Van Houwelinge H.C., Ferrario F., Noël L.H., Waldherr R., Jayne D.RW., Rasmussen N., Bruijn J. A. and Hagen E.C. "Determinants of outcome in ANCA-associated glomerulonephritis: A prospective clinico-histopathological analysis of 96 patients". *Kidney International*., 62: 1732-1742, 2002.

14-Berden A.E., Ferrario F., Hagen E.C., Jayne D.R. , Jennette J.C., Joh K., Neumann I., Noel L.H., Pusey C.D., Waldherr R., Bruijn J.A. and Bajema I.M. "Histopathologic classification of ANCA-associated glomerulonephritis". *J. Am. Soc. Nephrol.,* 8: 1-3, 2010.

15-Harper L., Radford D. and Plant T. *et. al.* "IgG from myeloperoxidase-antineutrophil cytoplasmic antibody-positive patients stimulates greater activation of primed neutrophils than IgG from proteinase 3-antineutrophil cytosplasmic antibody-positive patients". *Arthritis Rheum*., 44**:** 921–930, 2001.

16-Schreiber A., Luft FC. and Kettritz R. "Membrane proteinase 3 expression and ANCA-induced neutrophil activation" . *Kidney Int*., 65: 2172–2183, 2004.

17-Hewins P., Williams JM. and Wakelam MJ. *et. al.* "Activation of Syk in neutrophils by antineutrophil cytoplasm antibodies occurs via Fcgamma receptors and CD18". *J. Am. Soc. Nephrol*., 15: 796–808, 2004.

18-Harper L., Cockwell P. and Adu D. *et. al.* "Neutrophil priming and apoptosis in anti-neutrophil cytoplasmic autoantibody-associated vasculitis". *Kidney Int*., 59: 1729–1738, 2001.

19-Day C.J., Hewins P. and Savage C.O. "New developments in the pathogenesis of ANCA-associated vasculitis". *Clin. Exp. Rheumatol*., 21: S35–48, 2003.

20-Xiao H., Heeringa P., Hu P. *et al.* "Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice". *J. Clin. Invest*., 110: 955–963, 2002.

21-Preston GA., Zarella CS. and Pendergraft WF. *et al.* "Novel effects of neutrophil-derived proteinase 3 and elastase on the vascular endothelium involve *in vivo* cleavage of NF-kappaB and proapoptotic changes in JNK, ERK, and p38 MAPK signaling pathways". *J. Am. Soc. Nephrol*., 13**:** 2840–2849, 2002.

22-Jennette J.C. and Falk R.J. "Antineutrophil cytoplasmic autoantibodies and associated diseases: a review". *Am. J. Kidney Dis.*, 15 (6):517-29, 1990.

23-Hultgren O., Andersson B., Hahn-Zoric M. and Almroth G. "Serum concentration of interleukin-18 is up-regulated in patients with ANCA-associated vasculitis". *Autoimmunity*., 40 (7): 529-531, 2007.

24-Calvani N., Richards HB. and Tucci M. *et. al.* "Up-regulation of IL-18 and predominance of a Th1 immune response is a hallmark of lupus nephritis". *Clin. Exp. Immunol*., 138**:** 171–178, 2004.

25-Leung BP., Culshaw S. and Gracie JA. *et. al.* "A role for IL-18 in neutrophil activation". *J. Immunol*., 167: 2879–2886, 2001.

26-Wyman TH., Dinarello CA. and Banerjee A. *et. Al .*" Physiological levels of interleukin-18 stimulate multiple neutrophil functions through p38 MAP kinase activation". *J. Leukocyte Biol*., 72**:** 401–409, 2002.

27-Kettritz R., Schreiber A. and Luft FC. *et. al.*"Role of mitogen-activated protein kinases in activation of human neutrophils by antineutrophil cytoplasmic antibodies'. *J. Am. Soc. Nephrol*., 12**:** 37–46, 2001.

28-Ward RA., Nakamura M. and McLeish KR. "Priming of the neutrophil respiratory burst involves p38 mitogen-activated protein kinase-dependent exocytosis of flavocytochrome *b*558-containing granules". *J. Biol. Chem*., 275**:** 36713–36719, 2000.

29-Gracie JA., Robertson SE. and McInnes IB. "Interleukin-18". *J. Leukocyte Biol.*, 73: 213–224, 2003.

30-Radford DJ., Lord JM. and Savage CO. "The activation of the neutrophil respiratory burst by anti-neutrophil cytoplasm autoantibody (ANCA) from patients with systemic vasculitis requires tyrosine kinases and protein kinase C activation". *Clin. Exp. Immunol*., 118: 171–179, 1999.

31-Cannetti CA., Leung BP. and Culshaw S. *et. al.* "IL-18 enhances collagen-induced arthritis by recruiting neutrophils via TNF-alpha and leukotriene B4". *J. Immunol.*, 171: 1009–1015, 2003.

32-Hewins P., Morgan M.D. and Holden N. "IL-18 is upregulated in the kidney and primes neutrophil responsiveness in ANCA-associated vasculitis". *Kidney Int*., 69: 605–615, 2006.

33-Booth A., Harper L. and Hammad T. *et. al.* "Prospective study of TNF alpha blockade with infliximab in anti-neutrophil cytoplasmic antibody-associated systemic vasculitis". *J. Am. Soc. Nephrol*., 15: 717–721, 2004.