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## **Clinical Spectrum of Hepatitis-Associated Cryoglobulinemia: Cross-Link between Hematological and Immunological Phenomena**

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The prevalence of cryoglobulinemia (CG) in Hepatitis C Virus (HCV) infection with and without superimposed renal failure is an acknowledged finding. This study chose to investigate some immunological and hematological features dictated by CG and End-stage Renal Disease (ESRD) onto the disease course and progression in a total of 47 HCV cases classified into a hemodialysis (HD, 25 cases) and non-HD (NHD, 22 cases) groups. Further subgrouping according to presence or absence of cryoglobulins (Cgs) was done. The battery of investigations included platelet count, estimation of prothrombin INR, HCV RNA, Cgs, Autoantibodies Namely Antinuclear (ANA), anti-smooth muscle (ASMA) and antimitochondrial (AMA), markers of complement (c) activation (C3, C4, C3d), immunoglobulins (Ig) G and M, in addition to flow cytometry for B-cell separation and detection of CD81 and CD5 positive cells. We came to the conclusion that HCV patients on HD had lower viremia levels and more impaired immune status than those with normal renal function. CG when present manipulated the immune system further and was correlated to most of the immunological (RF, autoantibodies, C4, C3d and IgM) and hematological (platelets and INR) markers. Overexpression of CD81 and CD5 was observed particularly in Cg positive cases, thus necessitating follow-up for fear of potential lymphotransformation.

**Key words:** Hepatitis C, hemodialysis, cryoglobulinemia

## INTRODUCTION

Cryoglobulinemia (CG) is an immune-complex mediated vasculitis involving mostly small but sometimes larger vessels. It can be associated with a variety of clinical conditions including myeloma, macroglobulinemia, chronic infections and inflammatory disorders (Rieu *et al.*, 2002). Deposition of immune complexes on the vessel wall activates complement and mediates damage. When no underlying disease exists, the condition is referred to as Essential Mixed Cryoglobulinemia (EMC), but the high frequency of coexisting liver affection introduced the possible role of hepatotropic viruses in the pathogenesis (Ferri *et al.*, 2002). The viral origin for CG was first alleged when cryoglobulins (Cgs) were detected in a high percentage of Human Immunodeficiency Virus (HIV) positive patients and the Cgs well correlated with both the viral titer and the accompanying symptoms (Dimitrakopoulos *et al.*, 1999). Several studies tackled the prevalence of CG in Hepatitis C Virus (HCV) infection where figures ranging between 19 to 57% were obtained (Dammacco and Sansonno, 1992; Misiani *et al.*, 1992) and figures ranging from 40-66% were obtained in chronic active hepatitis cases (Persico *et al.*, 2003). On the other hand, anti-HCV antibodies were found in 50-80% of patients with cryoglobulinemic syndrome in the Mediterranean basin (Cacoub *et al.*, 1994). HCV RNA was detected in the serum and/or peripheral mononuclear cells of almost all patients with EMC (Zuckerman and Zuckerman, 2002). Factors involved in the production of Cgs in HCV infection are not yet clear although the female gender, human leucocyte antigen, advanced age and HCV genotype may play a role (Cacoub *et al.*, 2000). It is interesting that despite the high prevalence of Cgs in HCV infection, signs of cryoglobulinemia are relatively uncommon ranging from 13 to 30% (Cicardi *et al.*, 2000). In contrast EMC is frequently accompanied by symptoms of liver disease (Lunel *et al.*, 1994), a finding causing confusion as to whether the liver affection is directly associated with a superimposed HCV infection, or is a result of liver dysfunction in the clinical setting of CG *per se*.

Another extrahepatic abnormality encountered in HCV infection and independent of a coexisting CG is the clonal expansion of peripheral B lymphocytes (Franzin *et al.*, 1995). This implies that HCV is not only hepatotropic but also lymphotropic. HCV like any RNA virus can infect and replicate within Peripheral Blood Mononuclear Cells (PBMCs) while its genomic sequence can also be recovered from lymph nodes and lymphocytes infiltrating the liver. Recent studies confirm the existence

of lymphotropic HCV strains and indicate that HCV infection of lymphoid cells may favour selection of distinctive viral variants. This peculiar lymphotropism may be responsible at least in part for the multiple immune-mediated extrahepatic manifestations of HCV infection among which CG is common. It is noteworthy to mention that 24% of HCV patients without CG still presented with clonal B cell expansion (Ferri *et al.*, 1994; Fozzato *et al.*, 1994; Tornita *et al.*, 2003).

There are several ways by which viruses can take part in uncontrolled cellular proliferation: either directly by activation of oncogenes, promotion of cellular growth, inhibition of tumor suppressor genes (like p53), or indirectly by affecting apoptotic death of infected cells (Zehender *et al.*, 1995; Zignego *et al.*, 1996). Since HCV is an RNA virus without a DNA intermediate in its replicative cycle, the genomic sequence cannot be integrated into the host genome. Therefore, the virus exerts its oncogenic potential indirectly. The HCV alone or in combination with other factors whether infectious, environmental or genetic can trigger various immunological alterations, not to ignore the fact that the nature of HCV itself contributes to its ability to escape immune surveillance and favour its persistence in the lymphoid cell. In the early phase, persistence of HCV in PBMCs leads to chronic B cell stimulation, followed by polyclonal and later monoclonal expansion of immunoglobulin (Ig) secreting cells (Shirin *et al.*, 2002).

CD81 protein was recently identified as one of the HCV receptor candidates, raising a wide spectrum of interesting issues regarding the pathogenetic link between HCV and lymphoproliferation. On B cells, CD81 is a member of a signalling complex that includes CD19 and CD21. Crosslinking these complexes using either antibodies to CD81 or CD19 was shown to lower the threshold for B cell activation and proliferation. Alternatively binding of HCV particles to a CD81 containing complex can facilitate B cell activation possibly explaining the association between HCV, B-cell activation and lymphoproliferation (Pileri *et al.*, 1998; Zuckerman *et al.*, 2002).

HCV lymphotropism is further evidenced by the expansion of the CD5<sup>+</sup> cell sub-population. This B cell subset is rare in adults, but predominant in the fetus in which they constitute a rather primitive but effective first line of defense against foreign antigens (Curry *et al.*, 2001). These cells are characterized by the production of low affinity IgM with Rheumatoid Factor (RF) activity and may represent the bridge linking innate and acquired immune responses (Sasso, 2000), hence their implication in autoimmune development is not unlikely.

Immunohistochemical techniques have identified CD<sup>5+</sup> B cells in the hepatic lymphoid follicles of HCV-infected patients where they produced both monoclonal and polyclonal IgM. It was noted that these CD<sup>5+</sup> lymphoid aggregates decrease in number with advanced disease, suggesting their role in disease progression (Pietrogrande *et al.*, 1995). Nevertheless, it should be remembered that lymphoproliferation is a complex multi step process in which HCV infection is only the initiating trigger aided by other genetic and environmental factors, i.e., several different pathogenetic mechanisms operate in the wide spectrum of HCV related lymphoproliferation.

To add to the complexity of the immunological and hematological aspects of HCV infection, comes the onset of End Stage Renal Disease (ESRD), a common complicating factor, driving 10-70% of HCV patients to maintenance hemodialysis (HD) as victims of Chronic Renal Failure (CRF) (Siagris *et al.*, 2003). Viral associated glomerulonephritis (GN) is considered a post or para-infectious autoimmune phenomenon. The disease is mediated by immune complexes containing viral antigens and in HCV infection the associated CG is said to cause membrano-proliferative GN (Tillman and Schwartz, 2003). This group of patients is exposed to manipulations of their immune system conferred by the renal disease itself or its treatment that can further compromise their immune system. Moreover, it is well known that in CRF alterations occur in monocyte, lymphocyte and neutrophil functions, with a resultant impairment of acute inflammatory responses, decreased delayed hypersensitivity and altered late immunofunctions (Rehermann, 2000; Pesanti, 2001). Patients on HD have disturbed leucocyte function because of bio-incompatibility of some dialysis membranes and activation of cytokine and complement cascades occurs when blood comes into contact with dialysis membranes. These substances in turn alter the inflammatory and immune responses of the uremic patient (Pertosa *et al.*, 2000).

To sum up, HCV infection is a clinically complex 'milieu' involving both hepatic and extrahepatic manifestations which reflect a number of immunological and hematological changes targeting the patient's immune system and peripheral blood lymphocytes. The purpose of this study is to investigate some of the factors responsible for this pathogenetic interplay namely the associated cryoglobulinemia, the viral load and its lymphotropic nature as evidenced by the B cell expansion, besides the additional threat of ESRD in some patients. Evaluation of these factors, alone and combined, is no doubt relevant to the immunoregulatory disturbances and the immunophenotypic lymphocyte

changes that can promote disease progression in HCV infected patients. With this born in mind, one can select a treatment design that does not further suppress the immune system, thus indirectly hindering disease progression.

## MATERIALS AND METHODS

The present study was conducted on a total of 47 cases suffering from HCV infection, selected from Theodor Bilharz Research Institute and Nasser Institute classified into a Haemodialysis Group (HD) and a Non-hemodialysis Group (NHD) comprising 25 and 22 patients respectively. Chronic HCV infection was defined by persistent or fluctuating increase in alanine aminotransferase levels more than 1.5 times the upper limit of normal and presence of anti-HCV antibodies for more than 6 months. Clinical nephrological diagnosis showed the reason for renal failure in the HD group to be: chronic glomerulonephritis (8), pyelonephritis (4), hypertension and nephrosclerosis (5), diabetic nephropathy (3) and unidentified (5). The duration of HD was 36±16 months. These patients received 3 hemodialysis sessions per week, each for 4 h, using a cuprophane dialyser and sodium acetate dialysate. Patients receiving interferon were not included because of its possible interference with cryoglobulin level. Autoimmune hepatitis (AIH) was also excluded using the criteria for the International Autoimmune Hepatitis Group for definite diagnosis of AIH (Alvarez *et al.*, 1999). Autoimmune systemic disease such as rheumatoid arthritis and systemic lupus erythematosus were excluded according to their criteria of definitive diagnosis.

Upon cryoglobulin estimation, each group was subdivided into a positive (+) and negative (-) group according to presence or absence of cryoglobulins.

### Study groups

- HD:** HCV patients on maintenance hemodialysis (n = 25).
- HD (+):** Cryoglobulin positive (15) patients of HD groups
- HD (-):** Cryoglobulin negative (10) patients of HD groups
- NHD:** HCV patients not on HD (22)
- NHD(+):** Cryoglobulin positive (11) patients of NHD groups
- NHD(-):** Cryoglobulin negative (11) patients of NHD groups

**Control group:** Eight healthy age and sex-matched subjects. The results obtained for this group only served to ensure that all tested parameters fell within normal range in order to validate technique and work procedure, but were not used for statistical comparison.

**Methods:** Biochemical parameters { $\gamma$ -glutamyl transferase ( $\gamma$ GT) and cholinesterase enzymes and hematological parameters (platelet count and prothrombin INR) were assessed using commercially available kits.

**Viral markers:** Anti-HCV antibodies were detected using third generation ELISA (Murex Diagnostics, UK). All patients were tested for HCV RNA by reverse transcriptase polymerase chain reaction (RT-PCR), (Ampiclor HCV PCR) and quantification of serum HCV RNA was performed by the branched DNA method (Quantiplex), the detection limit of which was  $2 \times 10^5$  genome equivalent per ml serum.

False positive results were avoided using completely controlled positive and negative reference sera in each round and by strict application of contamination prevention guidelines (Kwok and Higuchi, 1989).

**Detection of cryoglobulins (Cg):** was done according to McMurray (1998). Blood was coagulated at  $37^\circ\text{C}$  and then centrifuged to separate 5 mL serum, followed by placing it in refrigerator at  $4^\circ\text{C}$ . Serum was watched for cryoprecipitation once daily for 7 consecutive days. When cryoprecipitate (cryoppt) was detected, tubes were reincubated at  $37^\circ\text{C}$  for 30 min to verify dissolution of cryoppt. The cryocrit was determined by measuring the height of the column of precipitated protein relative to the total height of serum column using a commercial heamatocrit reader. Results were expressed as (%).

**Rheumatoid Factor (RF):** was measured using antibody-coated sheep erythrocytes where titre below 1:16 was considered negative.

**Markers of complement activation:** C3 and C4 were measured by routine nephelometric assays with a reference normal range of  $50\text{-}130 \text{ mg dL}^{-1}$  and  $14\text{-}40 \text{ mg dL}^{-1}$  respectively. C3d, a marker of intravascular complement turnover was measured by single radial immuno-diffusion in fresh EDTA plasma (normal range  $<10 \text{ mg L}^{-1}$ ).

**Auto-antibody detection:** Serum Anti-nuclear Antibodies (ANA), Anti-mitochondrial Antibodies (AMA) and Anti-smooth Muscle Antibodies (ASMA) were detected using indirect immunofluorescence, (McCarty *et al.*, 1984) and a titre of  $\geq 1:20$  was considered positive.

**Immunoglobulin (Ig) estimation:** IgG and IgM were assessed quantitatively using ELISA (Silverman *et al.*, 1986) with commercially available kits. Reference values were 850-1600 and 70-190 mg%, respectively.

**Flow cytometry:** Although samples should best be processed within 6 h of collection, lymphocytes can generally be recovered up to 24 h following sample collection with minimal effect on yield or viability. Peripheral Blood Mononuclear Cells (PBMCs) were isolated by Ficoll-Hypaque density gradient separation. Cells were stained with direct immunofluorescence using antibodies against two main pan-B cell markers namely CD19 and CD22. Criterion for immunophenotypic marker positivity was labeling of at least 20% of cells by the markers in question as observed from the relevant histogram scatter plots. Subsequent immunostaining with phyco-erythrin (PE) and fluorescein (FITC) conjugated monoclonal antibodies (Mo Ab) for CD81 and CD5 was done. Because the emission spectra of these 2 fluorochromes differ, cells labelled with FITC can be distinguished from those labelled with PE. When attached to antigen-specific antibodies, the fluorochrome can then be used to label cells that express the corresponding antigen. Antibodies specific for different antigens can be labelled with different fluorochromes allowing simultaneous multicolour flow cytometric analyses of two or more cell-associated antigens. The total population of viable cells was gated according to their typical forward and right-angle light scatter. Fluorescence of cells treated with fluorescent isotype MoAb was evaluated in each experiment to determine the level of background fluorescence of negative cells. Data were acquired on the flow cytometer and  $\text{CD}5^+$  B cells were expressed as a percentage of  $\text{CD}81^+$  cells. MFI of CD81 was expressed as Optical Density (OD) units.

**Statistical analysis:** Univariate statistical analysis was carried out with student t-test for quantitative data. Fisher's exact test was used for small number comparison. Multivariate analysis was done using logistic regression model with the step wise method applying the SPSS computer program, version 6. Significant level was set at  $p \leq 0.05$ . The Odds Ratio (OR) and Confidence Interval (CI) at 95% were calculated by Fleiss quadratic method.

## RESULTS

Results of all tested parameters in the different study groups, correlation analysis and relevant clinical data are illustrated in Table 1-7.

Table 1: Tested parameters and their results in HD and NHD groups

	HD group (n = 25)	NHD group (n = 22)	p-value
Cg cases level	n = 15/25 (60)	n = 11/22 (50)	NS
Cg level (%)	1.3±0.9	1.8±1.4	NS
HCV RNA (mEq L <sup>-1</sup> )	3.8±2.1	5.2±1.6	p<0.05
RF positivity	11 (44)	12 (54.5)	p<0.05
Auto antibodies positivity:			
ANA	4 (16)	6 (27.3)	
AMA	2 (8)	1 (4.5)	NS
ASMA	0 (0)	2 (9)	
Markers of C activation: (mg dL <sup>-1</sup> )			
C3	116±8.5	92±8.4	
C4	19.5±9.2	14.2±6.4	NS
C3d	21±2.8	23±3.8	
IgG >1600 mg	10 (40)	8 (36.5)	NS
IgM >190 mg	6 (24)	10 (45.5)	p<0.05
CD81 (OD)	228±34	248±52	NS
CD 5 (%)	26.5±8.5	32±6.4	NS

NB: Results of Cg, RF, autoantibodies and Igs are expressed as number of patients and their percentage in each group: n (%)

Table 2: Tested parameters and their results in HD (+) and HD (-) groups

	HD (+) n = 15	HD (-) n = 10	p-value
Cg (%)	2.52±1.12	0	<0.001
HCV RNA (mEq L <sup>-1</sup> )	3.2±1.5	3.8±1.8	NS
RF positivity	9/15 (60)	2/10 (20)	<0.05
Auto antibodies positivity:			
ANA	3/15 (20%)	1/10 (10)	NS
AMA	1/15 (6.6)	1/10 (10)	0 (0)
ASMA	0 (0)	0 (0)	
Markers of C activation:			
C3 (mg dL <sup>-1</sup> )	98±23.2	122±11.6	<0.05
C4 (mg dL <sup>-1</sup> )	14.8±6.6	22.5±6.2	<0.05
C3d (mg dL <sup>-1</sup> )	29±5.2	11.5±4.3	<0.01
IgG >1600 mg %	7 (46.6%)	3 (30%)	<0.05
IgM >190 mg %	5 (33.3%)	1 (10%)	<0.01
CD81 (OD)	265±68	188±85	<0.05
CD5 (%)	32±6.5	18±6.1	<0.05

NB: Results of RF, autoantibodies and Igs are expressed as number of patients and their percentage in each group: n (%)

Table 3: Tested parameters and their results in NHD (+) and NHD (-) cases

	NHD (+) n = 11	NHD (-) n = 11	p-value
Cg (%)	3.85±1.4	0	<0.01
HCV RNA (mEq L <sup>-1</sup> )	4.2±2	5.4±1.6	NS
RF positivity	8 (72.7)	3 (27.3)	<0.05
Auto antibodies positivity:			
ANA	4 (36.4)	2 (18.2)	<0.05
AMA	1 (9.1)	0 (0)	NS
ASMA	2 (18.2)	0 (0)	NS
Markers of C activation:			
C3	115±28.2	122±25.5	NS
C4	18±10.2	26±12.4	<0.05
C3d	24±8.1	9±4.8	<0.05
IgG >1600 mg %	6 (54.5)	2 (18.2)	<0.05
IgM >190 mg %	7 (63.6)	3 (27.2)	<0.05
CD81 (OD)	288±95	210±74	<0.05
CD5 (%)	34.2±12.6	21±4.5	<0.05

NB: Results of RF, autoantibodies and Igs are expressed as number of patients and their percentage in each group: n (%)

Table 4: Epidemiologic features and markers of disease severity according to presence or absence of cryoglobulins in the disease groups

Feature	Cg (+) n = 26	Cg (-) n = 21	p-value
Sex: male n (%)	10 (38.5)	14 (66.6)	<0.01
female n (%)	16 (61.5)	7 (33.3)	
Age: <50 years	12 (46.1)	9 (42.8)	NS
>50 years	14 (53.9)	11 (52.3)	
Disease Duration:			
<5 years	7 (27)	16 (76.2)	p<0.05
>5 years	19 (73)	5 (23.8)	
Markers of Disease Severity:			
Platelet count			
>150,000 uL <sup>-1</sup>	8 (30.8)	15 (71.4)	p<0.05
<150,000 uL <sup>-1</sup>	18 (69.2)	6 (28.6)	
INR			
<1.2	9 (34.6)	16 (76.2)	p<0.05
>1.2	17 (65.4)	5 (23.8)	
γ GT			
<32 U	12 (46.1)	10 (47.6)	NS
>32 U	14 (53.9)	11 (52.4)	
Cholinesterase			
>4500 U	6 (23)	15 (71.4)	p<0.05
<4500 U	20 (77)	6 (28.6)	

NB: Results are expressed as n (%) i.e., number of patients and their percentage in each group

Table 5: Correlation analysis in main groups and cg (+) subgroups

	HDN = 25	NHD n = 22	HD (+) n = 15	NHD (+) n = 11
Cg vs:				
RF	0.58*	0.68*	0.64*	0.72*
HCV RNA	0.23	0.34	-0.52*	-0.6*
C3	0.4	0.42	0.38	0.43
C4	-0.56*	-0.63*	-0.68*	-0.65*
C3d	0.45	0.48*	0.66*	0.72*
CD81	0.48*	0.42	0.64*	0.68*
CD5	0.38	0.44	0.58*	0.64*
IgG	0.42	0.46	0.55*	0.58*
IgM	0.41	0.43	0.48*	0.46*
CV RNA vs				
CD81	0.46*	0.44	0.56*	0.53*
CD5	0.45	0.45	0.58*	0.55*
Auto antibodies				
CD5	0.33	0.36	0.42	0.45

•: Highly significant (p<0.01), \*: Significant (p<0.05)

Table 6: Relation between detectability of cryoglobulins and related clinical features

	Cryoglobulin level (%)			
	HD+ (n = 15)		NHD+ (n = 11)	
	<2	>2	<2	>2
n (%)*	6 (40)	9 (60)	3 (27.3)	8 (72.7)
Reported Feature	1(16.6)	3(33.3)	0	2(25)
• Fatigue	0	3(33.3)	0	3(37.5)
• Arthralgia	0	2(22.2)	0	0
• Vasculitis	1(16.6)	4(44.4)	0	0
• Chronic GN				

\* n (%) : Number of cases and their percentage in each group, NB. The same patient may display more than one feature

Table 7: Variables independently and significantly associated with risk for cryoglobulinemia

Variable	OR	95% CI
Female vs. male	1.735	1.82-2.442
RF positivity	1.672	1.053-1.982
Cholinesterase	3.21	1.168-4.462
<4500 vs. >4500		
C4<60 vs >60	1.759	1.129-1.995
IgM>190 vs <190 mg%	2.112	1.334-2.864

OR: Odds Ratio, CI: Confidence Interval, Table reports OR with their 95% CI for higher risk classes vs ones at lower risk

### DISCUSSION

An important clinical feature of HCV infection is its tendency to chronicity which entails long-standing immune stimulation and subsequent development of a variety of auto-immune phenomena. HCV infection is therefore characterized by a wide range of immunologic markers and extrahepatic manifestations (Agarwal *et al.*, 2001; Ramos *et al.*, 2005). Furthermore, a putative role of this viral infection in the pathophysiology of lymphoproliferation has been elicited (Hausfater *et al.*, 2000; Balwani *et al.*, 2004). It was the interest of this study to explore the pathogenetic link between some of the extrahepatic events, particularly immunological and hematological findings, observed in this clinical setting. The additional association between HCV and Cg production raised the question whether CG was an intermediate step in the recorded clinical and laboratory changes arising in the course of the disease. The onset of ESRD in some HCV patients is another factor exerting its influence on both the patient's immune status and the course and prognosis of the disease (Siagris *et al.*, 2003). Thus the present study chose 2 broad groups of HCV patients, one with and one without a superimposed renal failure necessitating hemodialysis. These two groups served to define the role conferred by ESRD on the immunological and hematological findings in question and to assess whether HCV patients on maintenance HD have different immune response from those with normal renal function. Any role played by Cgs produced in the course of the disease was highlighted by subsequent classification of the 2 main groups into positive (+) and negative (-) subgroups depending on the presence or absence of Cgs.

The mechanism by which HCV promotes the formation of Cg is not fully understood. Most probably the persistence of the virus in the cells of the immune system and/or the chronic stimulation of the immune response by the virus are responsible. Genetic factors such as the human leucocyte antigen are also thought to be involved in the pathogenesis (Peano *et al.*, 1994). It is also reasonable to expect a relationship between liver injury and Cg formation. Liver cirrhosis *per se* is said to

induce Cgs. The proposed underlying mechanism is decreased hepatic perfusion and kupffer cell alterations which may delay clearance of circulating immune complexes (Liakina *et al.*, 2002).

Serum Cgs are found in a wide spectrum of disorders but are often transient and without clinical implication. Monoclonal Cgs are said to be associated with hematological disorders while mixed Cgs are more related to infections and systemic disease and a striking association with HCV infection exists (Schott *et al.*, 2001).

Previous studies on patients with chronic HCV infection revealed the presence of CG in the range of 15-54% with a tendency for higher rates in women, older patients and long disease duration (Gad *et al.*, 2003; Gaafar *et al.*, 2004). The lower incidence detected in some studies (Tanako *et al.*, 2003) could result from inclusion of interferon-treated patients in the study population. Lunel *et al.* (1994) reported that CG became undetectable in 21 out of 43 cases with HCV infection upon treatment with interferon. The positivity rate for CG among patients in our HD group was 60% while that in the NHD group was 50%, running almost parallel to earlier studies (Persico *et al.*, 2003; Okuda *et al.*, 1998). The Cg level in all patients fell within the mild to moderate range (2-5 g L<sup>-1</sup>) and the patients who complained of signs and symptoms dictated by the presence of CG, had a cryocrit >2 g L<sup>-1</sup>.

While some studies denied a correlation between Cgs, age, sex or biochemical parameters (Wu *et al.*, 2000), others suggested the reverse (Cicardi *et al.*, 2000; Liakina *et al.*, 2002; Fabris *et al.*, 2003). In this study, age did not carry statistical significance when Cg (-) and Cg (+) cases were compared while the disease duration did. An influential factor in Cg formation was gender, where females constituted 61.5% of the Cg (+) cases of our study (16/26) and only 33.3% of the Cg (-) cases (7/21). This observation was detected in many earlier reports (Gad *et al.*, 2003; Fabris *et al.*, 2003). Actually, the female predominance among Cg (+) cases is not surprising in view of the well known association between female gender and autoimmunity (Gad *et al.*, 2003).

Some hematological and biochemical markers of disease severity were used in this study, namely platelet count, INR,  $\gamma$ GT and cholinesterase. Both platelet count and INR posed a predictive threat to the development of CG in Cg (+) cases. Cholinesterase is a serum factor reflecting the synthetic ability of the liver and is said to be reliable in predicting the presence of Cgs even better than liver histology (Liakina *et al.*, 2002).  $\gamma$ GT as an indicator of liver affection was high in 70.2% of our study population, but reports of low levels in CRF also exist (Pereira and Levey, 1997), possibly due to suppressed

hepatocyte synthesis, inhibition of release into the circulation, or accelerated clearance from serum (Pereira and Levey, 1997). Our study confirmed the correlation between cholinesterase and Cgs but not with  $\gamma$ GT.

A higher prevalence of CG in patients with HCV induced cirrhosis than without cirrhosis is also reported (Weiner *et al.*, 1998; Cicardi *et al.*, 2000; Liakina *et al.*, 2002). Forty percent of patients with chronic HCV infection have detectable serum Cgs although they may not show clinical or physiological signs of CG syndrome (Kayali *et al.*, 2002). Still, Cgs are thought to be a useful prognostic index for increased risk of cirrhosis. Low antigen containing diets such as the traditional Japanese foods (boiled rice, raw fish, lack of cheese and meat) can affect not only the level of circulating Cgs but also the clinical symptoms they may provoke (Tanako *et al.*, 2003) and may explain the discrepancy noted in different geographical study populations.

It is interesting to note that the immunological abnormalities in HCV infection can be more striking than the clinical symptoms they dictate. In our study, Cg-related features were present in 9/15 of Cg (+) HD group and 5 (11) of Cg (+) NHD group (60 and 45.5%, respectively) and were usually mild in nature. They ranged from chronic GN to arthralgia, fatigue and vasculitis. Reported incidence of such features fall in the range of 13-30% (Weiner *et al.*, 1998; Liakina *et al.*, 2002). Viral associated GN is considered a para- or post-infectious autoimmune phenomenon. The disease is mediated by immune complexes containing viral antigen in case of hepatitis B or C or HIV (Almirall *et al.*, 2002; Dominguez and Sha, 2002). Hepatitis B gives rise to membranous GN, HIV results in focal sclerosing GN, while hepatitis C results in membrano-proliferative GN mainly due to Cg formation (Tillman and Schwartz, 2003). What are the factors that can lead to symptomatic CG. The level of Cg >2% was used in this study as opposed to 3% in earlier works (Weiner *et al.*, 1998; Liakina *et al.*, 2002). It is still noteworthy that 3 patients who had a Cg level >3% did not elicit any clinical feature of CG. The thermal amplitude of cryoprecipitation is no doubt a decisive factor, beside the capacity of Cg to activate complement. This latter factor was confirmed by the elevated C3d levels in symptomatic cases. Composition of the immune complexes is also likely to influence the pathological consequences.

Comparison of the viral load in the 2 main groups (HD and NHD) showed a higher and statistically significant level in the latter group. This may be partly due to destruction of the viral particles by the dialyser membrane during hemodialysis (Okudo *et al.*, 1998). Since our samples were taken before the hemodialysis session

i.e., at least 2 days after last contact with the dialyser membrane, other factors connected to the CRF itself or the treatment may be responsible for the lower HCV RNA titre in the HD group.

Subgrouping of patients into Cg (+) groups revealed that the viral load in these patients was less than that detected in Cg (-) cases. Weiner *et al.* (1998) identified HCV RNA at higher concentrations in the cryoprecipitate than in the supernatant of all samples they analysed. It is assumed that HCV can bind to Cgs at variable degrees of affinity depending on the composition of the immune complexes. Fluctuation of viraemia during the course of the disease may also affect the results and the immune response or more accurately the state of immune suppression in HCV patients can result in both normalization of liver enzymes and a surge of viraemia (Rehermann, 2000). Some risk factors that can foresee the development of CG in HCV infection were statistically evaluated as indicated in Table 7 whereby the main influential predictive parameters were female gender, RF positivity, low cholinesterase levels, low C4 and high IgM values.

RF is a consistent marker of immunological disturbance in HCV infection and plays a major pathogenetic role in the extrahepatic associated findings. Its reported prevalence is 24-76% (Dammacco *et al.*, 2000; Siagris *et al.*, 2003). The positivity rate in the HD group was 60% Cg (+) and 20% in Cg (-) cases, while the respective percentage in NHD group was 72.7 and 27.3%. This evident link between presence of Cgs and RF positivity was further reinforced by the statistical positive correlation between the two parameters.

C4 levels were higher in the HD than the NHD group, but statistical significance was obtained when the Cg (+) and Cg (-) cases were compared in each group where Cg (+) cases had lower C4 levels. This is not surprising and is probably a consequence of Ig aggregation that leads to activation of the classical complement pathway (Cicardi *et al.*, 2000). Complement activation while causing measurable depletion of plasma C4 in both HD and NHD groups, had little effect on the common pathway as shown by the negligible reduction in C3 levels. The higher C4 levels observed in the main HD group is also expected and follows the concomitant lower RF positivity and cryocrit levels as compared to the main NHD group. These findings denote a less efficient mechanism of creating immune complexes in hemodialysis patients. A more reliable marker for Cg-induced complement activation is the measurement of complement split product C3d which is known to increase in active and clinically relevant CG and is considered a marker of intravascular complement turnover. The level recorded in the HD and



NHD groups was almost the same, but the Cg (+) groups had significantly higher C3d levels, while Cg (-) cases had near normal values. Here again a clear and positive correlation was detected between Cg and C3d, in agreement with earlier works (Pietrogrande *et al.*, 1995; Rehermann, 2000).

A study on immunological features of rheumatoid arthritis (Balabanova *et al.*, 2004) in patients infected with virus B and C revealed that functional activation of complement was significantly decreased and CG was evident in cases with superimposed HCV infection. This should have its clinical relevance when choosing a drug that does not further depress the complement system. Enhanced T-cell apoptosis is reported in HCV infection and may lead to down regulation of cellular immune response, a factor that no doubt promotes persistence of infection (Toubi *et al.*, 2001).

Autoantibody positivity was more frequent in the NHD group where the recorded positivity for ANA, AMA and ASMA was 27, 4.5 and 9%, respectively as opposed to 16, 8 and 0% in the HD group. Positivity was clearly related to presence of Cgs as revealed by the higher frequency in Cg (+) groups. Studies on autoimmune markers in HCV infection showed that 72% of patients had autoimmune phenomena in the form of ASMA, ANA, RF and Cgs at rates ranging from 12-32%, while the positivity for AMA or double-stranded DNA was less evident (Agarwal *et al.*, 2001; Zabek *et al.*, 2001). Reported ASMA positivity in chronic hepatitis is 15-20% (Dammacco *et al.*, 2000) while that of ANA is 6-13% (Zahran *et al.*, 2001). ASMA was said to possess low incidence as a serological marker of autoimmunity in HCV infection, unlike RF and Cgs (Valentini *et al.*, 1999; Zuckerman *et al.*, 2002). In our study the presence of autoantibodies was not correlated with markers of B-cell expansion indicating a different stimulatory and pathogenetic mechanism inducing their production. Furthermore, autoantibody positivity was lower in the HD group which indicates that uremia and hemodialysis are coupled with immune hyporesponsiveness (Siagris *et al.*, 2003). Measurement of IgG and M revealed a statistically higher level in the Cg (+) groups, a finding also observed by Gad *et al.* (2003) and Fabris *et al.* (2003) and their levels correlated with Cg production in those groups. It was suggested that HCV infection may induce antigen-driven benign proliferation of selected B-cells producing IgM with RF activity (Sasso, 2000; Nishiyama *et al.*, 2001). Elevated plasma levels of IgG, IgM and RF were expected in patients with CG and considered a prelymphomatous phase of B-cell lymphoproliferation both peripherally and centrally in the bone marrow (Idilman *et al.*, 2004).

The exact mechanism linking HCV infection with autoimmunity and lymphoproliferation is yet unclear and some studies still deny the association of this viral infection and lymphoproliferation (Balwani *et al.*, 2004). In our study, peripheral B cells from patients infected with the virus over expressed both CD81 and CD5. The corresponding values recorded in normal controls in this study were  $145 \pm 33$  OD and  $5.19 \pm 3.7\%$  for CD81 and CD5 respectively. Research suggests (Shirin *et al.*, 2002) that a specific population of B-cells is involved in the host response to HCV infection. They are characterized by the ability to proliferate with little or no somatic mutation of Ig genes, are self replicating, are stimulated by self-antigens in a T-cell independent manner and bear the CD5 marker (Simarro *et al.*, 2002). It can thus be assumed that HCV infection can induce antigen-driven benign proliferation of selected B cells producing Igs. This proliferation is probably enhanced by properties specific to HCV, including the ability of the virus proteins to bind to CD81 on B-cell surface and to influence intracellular regulatory functions following viral entry into the cell (Zuckerman *et al.*, 2002). A positive correlation was detected in our study between CD81 over-expression and the viral load in the Cg (+) subgroups. Thus viral factors may up-regulate CD81 expression and in turn facilitate the binding of virus proteins to this receptor. In support of this postulation is the decline observed in both CD81 expression and HCV RNA upon initiation of antiviral therapy (Tornita *et al.*, 2003).

The correlation detected in this study and other studies between CD<sup>5+</sup> B-cell sub-population and the presence of RF and Cgs, indirectly implies the involvement of CD<sup>5+</sup> cells in production of RF activity, Cgs and possibly autoantibodies. In support of this hypothesis are recent data indicating that HCV infection and clonal expansion of B-cells within the liver preferentially involve RF-producing cells (Fornasieri *et al.*, 2000), or that B cell subsets expressing RF activity are the prevalent cell type targeted by HCV (Gaafar *et al.*, 2004).

Another postulation is that B cells infected by HCV can undergo bcl-2 translocation and Ig gene rearrangement ending in clonal lymphoproliferation and monoclonal IgM and RF activity (Schott *et al.*, 2001; Weng *et al.*, 2003). The expansion of CD<sup>5+</sup> B cells was found to correlate with anticardiolipin antibodies, but in our study, in parallel to earlier works (Valentini *et al.*, 1999; Gaafar *et al.*, 2004) such a correlation did not exist with ANA. This probably indicates a different stimulatory and pathogenetic mechanism for antibody production in HCV-infected subjects. A question here poses itself: Do CD<sup>5+</sup> B cells have a role in liver disease progression? This remains speculative, but the evident correlation between

CD<sup>5+</sup> B cells, RF and Cgs and the established association of the latter two with the extent of fibrosis in HCV infection (Schmidt *et al.*, 2000), may suggest that these cells play some role in liver injury. Moreover, although B cells are not usually associated with cytokine production, however CD<sup>5+</sup> B cells are known to produce the immunoregulatory cytokine, interleukin-10 (O'Garra *et al.*, 1992). This cytokine is thought to have an autocrine loop, resulting in further expansion of this B-cell sub-population (Pers *et al.*, 1999).

In conclusion, HCV patients with ESRD necessitating maintenance HD exhibited two seemingly contradictory peculiarities: a significant defect in immune response and lower levels of viremia than patients with normal renal function. These apparently conflicting features became more understandable when the interplay with the existing CG was elaborated upon subdivision of patients into Cg (+) and Cg (-) cases. Subtle differences in autoimmune profile become clear when CRF sets in, providing evidence that the in HD group the pattern of immunological reaction is modulated or rather down regulated.

The cause of the frequency of CG in chronic HCV infection remains elusive. However, the fact that the immunological disorders are a common event in this viral infection makes CG an expected part of a wider immune dysregulation enveloping this clinical setting. CG, however, is not a mere passive bystander in this milieu of immunologic aberration but it surpasses this to a more positive role to manipulate the immune system further, as indicated by the direct correlation between CG and most of the immunological (RF, C4, IgM) and hematological (INR and platelets) markers.

Predilection of the virus to infect B-cells leading to B-cell expansion with exaggerated CD81 and CD5 expression may lead to other immune mediated sequelae, namely lymphoproliferation. Patients with this inclination require close observation and regular follow-up for fear of future malignant lymphotransformation. The benefit of anti-viral therapy in down regulating CD81 and CD5 expression should interest future studies, but in general therapy should always be cautious with drugs that may compromise the already fragile immune host status, as this is probably the main reason for persistence of infection and disease progression.

#### REFERENCES

Agarwal, N., R. Handa and J.P. Wali *et al.*, 2001. A study of autoimmune markers in hepatitis C infection. *Ind. J. Med. Res.*, 113: 170-174.

- Almirall, J., M.J. Amengua and T. Lopez *et al.*, 2002. Type I essential mixed cryoglobulinemia and renal disease: HCV-association. *Nephrologia*, 22: 531-539.
- Alvarez, F., P.A. Berg and F.B. Bianchi *et al.*, 1999. International Autoimmune Hepatitis Group report: Review of criteria for diagnosis of autoimmune hepatitis. *J. Hepatol.*, 31: 929-938.
- Balabanova, R.M., E.V. Shekshina and L.V. Kozlov *et al.*, 2004. Immunological features of rheumatoid arthritis in patients infected with viruses of hepatitis B, C and in patients with cryoglobulinemia. *Ter. Arkh.*, 76: 74-77.
- Balwani, F., Y. Zaidi and G.N. Kakepoto, 2004. Prevalence of HCV in lymphoproliferative disease. *J. Pak. Med. Assoc.*, 54: 2002-2006.
- Cacoub, P., F.L. Fabiani and L. Musset *et al.*, 1994. Mixed cryoglobulinemia and hepatitis C virus. *Am. J. Med.*, 96: 124-132.
- Cacoub, P., C. Renou and E. Rosenthal *et al.*, 2000. Extrahepatic manifestations associated with HCV infection. *Medicine*, 7: 47-56.
- Cicardi, M., D. Cesana and E. Del Ninno *et al.*, 2000. Prevalence and risk factors for presence of serum cryoglobulins in patients with chronic hepatitis C. *J. Viral Hepatol.*, 7: 138-143.
- Curry, M.P., L. Golden-Mason and N. Nolan *et al.*, 2001. Expansion of peripheral blood CD<sup>5+</sup> B cells is associated with mild disease in chronic HCV infection. *J. Hepatol.*, 32: 121-125.
- Dammacco, F. and D. Sansonno, 1992. Antibodies to hepatitis C virus in essential mixed cryoglobulinemia. *Clin. Exp. Immunol.*, 87: 352-356.
- Dammacco, F., D. Sansonno and C. Piccoli *et al.*, 2000. The lymphoid system in hepatitis C infection: Autoimmunity and cryoglobulinemia and overt B cell malignancy. *Semin. Liv. Dis.*, 20: 143-157.
- Dimitrakopoulos, A.N., T. Kordossis and A. Hatzakis *et al.*, 1999. Mixed cryoglobulinemia in HIV-I infection: The role of HIV. *Ann. Intl. Med.*, 130: 226-230.
- Dominguez, J.H. and E. Sha, 2002. Apheresis in cryoglobulinemia complicating hepatitis C and other renal disease. *Ther. Apher.*, 6: 69-76.
- Fabris, P., G. Tositti and M.T. Giordani *et al.*, 2003. Prevalence and clinical significance of circulating cryoglobulins in HIV-positive patients with without co-infection with hepatitis C virus. *J. Med. Virol.*, 69: 339-343.
- Ferri, C., F. Caracciolo and A.L. Zignego *et al.*, 1994. Hepatitis C virus infection patients with non-Hodgkin's lymphoma. *Br. J. Hematol.*, 88: 392-394.
- Ferri, C., A.L. Zignego and S.A. Piler *et al.*, 2002. Cryoglobulins. *J. Clin. Pathol.*, 55: 4-13.

- Fozzato, G., C. Mazzaro and M. Crovatto *et al.*, 1994. Low-grade malignant lymphoma, hepatitis C virus infection and mixed cryoglobulinemia. *Blood*, 84: 8047-8053.
- Franzin, F., D.G. Efremov and G. Pozzato, 1995. Clonal B-cell expansion in peripheral blood of HCV infected patients. *Br. J. Haematol.*, 90: 548-552.
- Gaafar, M., S. Lashin and M. Negm, 2004. Cryoglobulinemia and lymphomagenesis in HCV-infected patients. *Egypt J. Lab. Med.*, 16: 655-668.
- Gad, A., Tanaka Eijt and A. Matsumoto *et al.*, 2003. Factors predisposing to occurrence of cryoglobulinemia in 2 cohorts of Egyptian and Japanese patients with chronic HCV infection. *J. Med. Virol.*, 70: 594-599.
- Hausfater, P., P. Cacoub and E. Rosenthal *et al.*, 2000. HCV infection and lymphoproliferative disease in France: A national study. *Am. J. Haematol.*, 64: 107-111.
- Idilman, R., A. Colantoni and N. De Maria *et al.*, 2004. Lymphoproliferative disorders in chronic hepatitis C. *J. Viral. Hep.*, 11: 302-309.
- Kayali, Z., V.E. Buckwold and B. Zimmerman *et al.*, 2002. Hepatitis C cryoglobulinemia and cirrhosis a meta analysis. *Hepatology*, 36: 978-985.
- Kwok, S. and R. Higuchi, 1989. Avoiding false positives with PCR. *Nature*, 339: 237-238.
- Liakina, V., A. Speiciene and J. Naraskeviciene *et al.*, 2002. Prevalence of cryoglobulinemia in patients with chronic HC virus infection. *Med. Sci. Monit.*, 8: CR31-36.
- Lunel, F., I. Musset and P. Cacoub *et al.*, 1994. Cryoglobulinemia in chronic liver disease: Role of hepatitis C virus and liver damage. *Gastroenterology*, 106: 1291-1300.
- McCarty, G.A., D.W. Valencia and M.J. Fritzler, 1984. Antinuclear Antibodies, Contemporary Techniques and Clinical Application to Connective Tissue Disease. New York: Oxford University Press.
- McMurray, R.W., 1998. Hepatitis C-associated autoimmune disorders. *Infect. Arthritis*, 24: 353-374.
- Misiani, R., P. Bellavita and D. Fenili *et al.*, 1992. HCV infection in patients with EMC. *Ann. Intl. Med.*, 117: 573-577.
- Nishiyama, Y., A. Shimatsu and M. Arakawa *et al.*, 2001. Characterization of cryoglobulins, M-protein, low molecular weight IgM in a patient with chronic hepatitis C and type II mixed cryoglobulinemia. *Rinsho Byori*, 49: 1139-1145.
- O'Garra, A., R. Chang and N. Go, 1992. Ly-1 B (B-1) cells are the main source of B cell-derived interleukin 10. *Eur. J. Immunol.*, 22: 711-717.
- Okuda, K., O. Yokosuka and Y. Otake *et al.*, 1998. Cryoglobulinemia among maintenance HD patients and its relation to HCV infection. *J. Gastroenterol. Hepatol.*, 13: 248-252.
- Okudo, M., H. Hayashi and K. Yokozeki *et al.*, 1996. Destruction of hepatitis C virus particles by hemodialysis. *Lancet*, 347: 909-910.
- Peano, G., G. Menardi and A. Ponzetto *et al.*, 1994. HLA-DR antigen, a genetic factor influencing outcome of HCV infection. *Arch. Intl. Med.*, 154: 2733-2736.
- Pereira, B.V.G. and A.S. Levey, 1997. HCV infection in dialysis units and renal transplantation. *Kidney Intl.*, 51: 981-999.
- Pesanti, E.L., 2001. Immunologic defects and vaccination in patients with chronic renal failure. *Infect. Dis. Clin. North Am.*, 15: 813-832.
- Pers, J.O., C. Jamin and F. Prendine-Hug *et al.*, 1999. The role of CD5-expressing B cells in health and disease [review]. *Intl. J. Mol. Med.*, 3: 239-245.
- Persico, M., F.A. De Marino, D.I. giacomo and G. Russo *et al.*, 2003. Prevalence and incidence of cryoglobulins in HCV-related chronic hepatitis patients: A prospective study. *Am. J. Gastroenterol.*, 98: 884-888.
- Pertosa, G., G. Grandaliano and L. Gesualdo *et al.*, 2000. Clinical relevance of cytokine production in hemodialysis. *Kidney Intl.*, 76: 104-111.
- Pietrogrande, M., M. Corona and S. Milani, 1995. Relationship between rheumatoid factor and the immune response against hepatitis C virus in essential mixed cryoglobulinemia. *Clin. Exp. Rheumatol.*, 13: 109-113.
- Pileri, P., Y. Uematsu and S. Campognol *et al.*, 1998. Binding of HCV to CD81. *Science*, 282: 938-41.
- Ramos Casals, M., L.J. Jara and F. Medina *et al.*, 2005. Systemic autoimmune disease coexisting with chronic hepatitis C infection: Pattern of clinical and immunological expression. *J. Intl. Med.*, 257: 549-557.
- Rehermann, B., 2000. Interaction between hepatitis C and the immune system. *Semin. Liv. Dis.*, 20: 127-141.
- Rieu, V., P. Cohen and M.H. Andre *et al.*, 2002. Characteristics and outcome of 49 patients with symptomatic cryoglobulinemia. *Rheumatology*, 41: 290-300.
- Shirin, H., Y. Awni and P. Petchenko *et al.*, 2002. Prevalence of hepatitis C infection in patients with lymphoproliferative disease. *Isr. Med. Assoc. J.*, 4: 24-27.
- Sasso, E.H., 2000. The rheumatoid factor response in the etiology of mixed cryoglobulins associatrd with hepatitis C virus infection. *Ann. Med. Intl. (Paris)*, 15: 30-40.

- Schmidt, W.N., J.T. Stapleton and D.R. LaBrecque *et al.*, 2000. Hepatitis C Virus (HCV) infection and cryoglobulinemia: Analysis of whole blood and plasma HCV-RNA concentrations and correlation with liver histology. *Hepatology*, 31:737-744.
- Schott, P., H. Hartmen and G. Ramadori *et al.*, 2001. Hepatitis C virus associated mixed cryoglobulinemia: Clinical manifestations, histopathological characterization, mechanism of cryoprecipitation and options of treatment. *Histol Histopathol.*, 16: 1275-1285.
- Siagris, D., C. Labropoulou-Karatza and M. Christofidin *et al.*, 2003. Viremia, cryoglobulins and auto-antibodies in hemodialysis patients infected with HCV. *Eur. J. Gastroenterol. Hepatol.*, 15: 133-137.
- Silverman, L.M., R.H. Christenson and G.H. Grant, 1986. Aminoacids and proteins. In: *Textbook of Clinical Chemistry*. Tietz, N.W. (Ed.). Philadelphia: WB Saunders, pp: 572-573.
- Simarro, M., C. Pelassy and J. Calvo *et al.*, 2002. The cytoplasmic domain of CD5 mediates both CD3-dependent and independent diacylglycerol production. *J. Immunol.*, 159: 4307.
- Tanako, K., T. Aiyama and J. Imai *et al.*, 2003. Serum cryoglobulin and chronic HCV disease among Japanese patients. *Am. J. Gastroenterol.*, 90: 1847.
- Tillman, H.L. and A. Schwartz, 2003. Viral associated glomerulonephritis. *Internist (Berl)*, 44: 1098-1106.
- Tornita, N., F. Kodama and M. Takabayashi *et al.*, 2003. Clinical features and outcome in HCV-positive aggressive non-Hodgkin's lymphoma. *Leukemia and Lymphoma*, 44: 1159.
- Toubi, E., A. Kessel and L. Goldstein *et al.*, 2001. Enhanced peripheral T-cell apoptosis in hepatitis C virus infection associated with liver disease severity. *J. Hepatol.*, 35: 774-780.
- Valentini, G., A. Mantelli and M. Persico *et al.*, 1999. Serological and clinical markers of autoimmune disease in HCV-infected subjects with different disease conditions. *Clin. Exp. Rheumatol.*, 17: 75-79.
- Weiner, S.M., T. Berg and H. Berthold *et al.*, 1998. A clinical and virological study of hepatitis C virus related cryoglobulinemia in Germany. *J. Hepatol.*, 29: 375-385.
- Weng, W. and Levys *et al.*, 2003. Hepatitis C virus (HCV) and lymphomagenesis. *Leukemia and Lymphoma*, 44: 1113.
- Wu, M.J., K.H. Shu and C.H. Cheng *et al.*, 2000. Prevalence of subclinical cryoglobulinemia in maintenance hemodialysis patients and kidney transplant recipients. *Am. J. Kidney Dis.*, 35: 52-57.
- Zabek, J., J. Kosowska and J. Cianciara *et al.*, 2001. Immunological disorders in patients with chronic viral hepatitis type C. *Przegl. Epidemiol.*, 55: 459-464.
- Zahran, N.M., A.M. Amer and M.A. El Mansy *et al.*, 2001. ANCA in chronic hepatitis C patients: Clinical significance, relation to other auto-antibodies, cryoglobulin and hemogram changes. *Egypt J. Hematol.*, 26: 945-962.
- Zehender, G., C. De Maddatena and G. Monti *et al.*, 1995. HCV genotypes in bone marrow and peripheral blood mononuclear cells of patients with mixed cryoglobulinemia. *Clin. Exp. Rheumatol.*, 135: S87-S90.
- Zignego, A.L., C. Ferri and C. Giannini *et al.*, 1996. Hepatitis C virus genotype analysis in patients with type II mixed cryoglobulinemia. *Ann. Intern. Med.*, 124: 31-34.
- Zuckerman, E. and T. Zuckerman, 2002. Hepatitis C and B-cell lymphoma: The hemato-hepatologist linkage. *Blood Rev.*, 16: 119-125.
- Zuckerman, E., G. Slobodin and A. Kessel *et al.*, 2002. Peripheral B-cell CD5 expansion and CD81 over-expression and their association with disease severity and autoimmune markers in chronic hepatitis C virus infection. *Clin. Exp. Immunol.*, 128: 353-358.