Cryoglobulinemia and cryoglobulinemic vasculitis: etiological aspects and pathophysiological associations

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The term cryoglobulinemia (CG) is used when detecting serum immunoglobulins that reversibly precipitate and form a gel at a temperature below 37 °C and dissolve when the temperature rises above 37 °C. Type I CG consists of only one isotype or a subclass of monoclonal immunoglobulins, while types II and III are classified as mixed CG (MCG) that is primarily characterized by the presence of immunoglobulins G and M. Types II and II-III MCG can result in cryoglobulinemic vasculitis (CGV) more frequently, whereas type III can lead to this condition less frequently. The presence of type I cryoglobulins is always associated with B-cell lymphoproliferative diseases. On the contrary, type II or type III MCG is more commonly associated with systemic autoimmune diseases and chronic infections. Thus, hepatitis C virus infection contributes to the development of MCG in 80–90% of cases. CGV is considered a rare disease worldwide (<5 cases per 10,000 people in the general European and North American populations). Among autoimmune diseases, primary Sjögren's syndrome (Sjögren's disease), systemic lupus erythematosus, and rheumatoid arthritis are most often associated with MCG. The pathogenetic role of cryoglobulins in inducing vasculitis is associated with both leukocyte recruitment to the vessels and deposition of immune complexes, with complement system activation and microvascular damage. The pathogenesis of MCG is associated with B-cell lymphoproliferation, autoantibody production, immunoglobulin synthesis, rheumatoid factor activity and the subsequent formation of cryoprecipitated immune complexes in conjunction with ineffective cryoglobulin clearance by monocytes and/or macrophages. This review contains updated information on the epidemiology, etiology, and pathogenesis of CG, with particular emphasis on MCG and CGV.

Keywords: *cryoglobulinemia; vasculitis; immunoglobulins; hepatitis C virus; pathogenesis; lymphoproliferative process; B cells.*
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The term "cryoglobulinemia" is used when immunoglobulins (Ig) detected in serum reversibly precipitate and form a gel at a temperature below 37 °C, and dissolve when the temperature rises above 37 °C. Three subtypes of cryoglobulins are described depending on the composition of Ig. Type I cryoglobulinemia is characterized by one type of monoclonal Ig (most often IgM, less often IgG or IgA), while type II and type III are classified as mixed cryoglobulinemia, since they include two types of Ig – usually IgG and IgM [1]. Mixed type II cryoglobulinemia includes a combination of monoclonal and polyclonal immunoglobulins (usually monoclonal IgM plus polyclonal IgG), mixed type III cryoglobulinemia encompasses polyclonal IgM and IgG (Table 1, Figure 1). Sensitive methods, such as immunoblotting, 2D polyacrylamide gel electrophoresis, or immunofixation methods can be used to detect the microheterogeneous composition of mixed type II cryoglobulins [2]. Often, oligoclonal IgM or mixed polyclonal and monoclonal IgM can be detected together with polyclonal IgG. This specific serological subset, known as type II–III mixed cryoglobulinemia, can be intermediate evolution from type III mixed cryoglobulinemia to type II and vice versa [3]. It is important to note that cryoglobulins in mixed cryoglobulinemia are autoantibodies with rheumatoid factor activity (i.e. antibodies with the ability to bind another antibody), which allows them to form immune complexes, and this ability is extremely important in the pathogenesis of cryoglobulinemic vasculitis.

The main mechanism contributing to cryoglobulinemia is the aberrant production of autoantibodies by B-cells and proliferation of B-cells [5]. Some diseases can contribute to this by altering the normal function of B cells. The presence of Type I cryoglobulins is always associated with B-cell lymphoproliferative diseases (Table 1, Figure 1). In contrast, mixed type II or type III cryoglobulinemia is more often associated with systemic autoimmune diseases, chronic infections. Thus, viral hepatitis C (HCV) contributes to the development of mixed cryoglobulinemia in 80–90% of cases [6]. Among autoimmune diseases, mixed cryoglobulinemia is more commonly associated with primary Sjogren's syndrome (PSS), systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). In the absence of a clearly defined underlying disease, which could be an etiological factor, and the detection of mixed cryoglobulinemia, the syndrome is designated as essential mixed cryoglobulinemia.

The term "cryoglobulinemia" refers only to the presence of cryoglobulins in a patient's serum; nevertheless, this term is often used in relation to a systemic inflammatory syndrome which is due to vasculitis of small and medium caliber vessels caused by immune complexes containing cryoglobulins. In our work, the term "cryoglobulinemia" will be used to designate the presence of cryoglobulins in the blood (with or without clinical symptoms), while the term "cryoglobulinemic vasculitis" – for clinically manifest disease (purpura, arthralgia and / or arthritis, weakness, skin ulcers, peripheral neuropathy, nephritis). More often, mixed cryoglobulinemia of type II and type II–III and, less often, type III can lead to cryoglobulinemic vasculitis. Of the available classifications of vasculitis, the set of criteria developed by the International Consensus Conference (Chapel Hill, 2012) [7] is currently the most widely used; it is based on the anatomical differences of the affected dominant vessels. Cryoglobulinemic vasculitis refers to small-caliber vasculitis with immunocomplex pathogenesis and, in the case of HCV-associated cryoglobuline-

Table 1. Classification of types of cryoglobulinemia and associated diseases [22]

Types of cryoglobulinemia	The composition of cryoprecipitate	Major Associated Diseases
Type I	Monoclonal IgM (rarely IgG or IgA)	Lymphoproliferative diseases, plasma cell dyscrasia, multiple myeloma, Waldenstrom macroglobulinemia, monoclonal gammopathy, chronic lymphocytic leukemia, B-cell non-Hodgkin lymphoma, hairy cell leukemia
Type II	The combination of monoclonal (usually IgM) and polyclonal Ig (IgG)	HCV (80–90% of cases) and other infections (e.g., HBV), lymphoproliferative diseases
Type III	Polyclonal IgM, Polyclonal IgG	HCV and other infections; often autoimmune diseases
Type II-III	Oligoclonal IgM, Oligoclonal IgG	HCV and other infections, autoimmune diseases or lymphoproliferative diseases

Note. Ig - immunoglobulins, HCV - hepatitis C, HBV - hepatitis B.

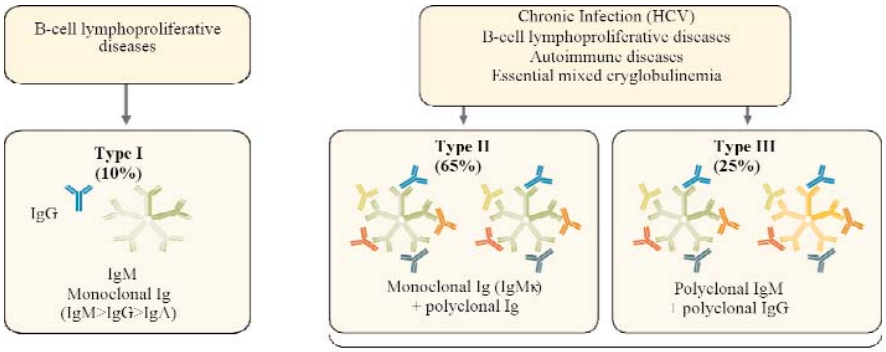

Fig. 1. *Immune typing-based classification of CG [2].*
*Type I CG is associated exclusively with B-cell proliferative diseases; the serum in type I CG exhibits monoclonal IgM and, less commonly, IgG or IgA. Type II MCG includes the serum immune complexes formed from monoclonal IgM and polyclonal IgG, and type III MCG comprises the immune complexes formed from polyclonal IgM and polyclonal IgG. Types II and type III MCG are associated with HCV infection, autoimmune diseases, or B-cell proliferative diseases. In the figure, different colored types of antibodies reflect different clones of immunoglobulins**

Table 2. Infectious triggers of mixed cryoglobulinemia

Viruses • Hepatitis C • Hepatitis B • Epstein-Barr virus • Cytomegalovirus • Hepatitis A • HIV • Adenovirus • Parvovirus B19	**Parasites** • Plasmodium species (pathogens of malaria) • Leishmania species (causative agents of leishmaniasis) • Toxoplasma species (causative agents of toxoplasmosis) • Schistosoma species (pathogens of schistosomiasis) • Echinococcus species (causative agents of echinococcosis)
Bacteria • Streptococcus • Brucella species (pathogens of brucellosis) • Coxiella burnetii (Q-fever causative agent) • Mycobacterium leprae (leprosy causative agent) • Borrelia burgdorferi (Lyme pathogen) • Treponema pallidum (causative agent of syphilis)	**Fungus** • Candida species (pathogens of candidiasis infection) • Coccidioides species (causative agents of coccidoidomycosis)

mic vasculitis it has a known etiology. Cryoglobulinemic vasculitis (CV) is not associated with antineutrophilic cytoplasmic antibodies (ANCA).

We have collected updated information on the epidemiology, etiology and pathogenesis of cryoglobulinemia with special emphasis on mixed cryoglobulinemia and cryoglobulinemic vasculitis.

Epidemiology, etiology

Data on the prevalence and incidence of cryoglobulinemia among the general population are scarce; there are only a few studies devoted to this problem. Worldwide, CV is considered a rare disease (<5 cases per 10,000 people in the general European and North American population), although its prevalence is slightly higher in the countries of the Mediterranean basin [8].

HCV-associated cryoglobulinemia

As mentioned above, the main etiological factor of mixed cryoglobulinemia type II and III is chronic HCV infection [9]. Infection with HCV is a common problem that affects more than 184 million people worldwide. The prevalence varies by geographic region: HCV infection is widespread in Central and East Asia, the Middle East and North Africa (> 3.5% of the population) [9,10]. In two large prospective studies, mixed cryoglobulinemia was detected in ~ 40–60% of patients infected with HCV, but only 5% of people with HCV infection developed CV [11].

Non-HCV Associated Cryoglobulinemia

The main causes of mixed cryoglobulinemia, not associated with chronic HCV infection, in 10–20% of cases are other infectious agents, malignant B-cell neoplasms and autoimmune diseases [12]. Thus, a link was found between cryoglobulinemia and chronic hepatitis B virus (HBV) infection [13]. However, only ~ 2% of cases of CV are associated with HBV infection [1]. There is evidence of a link between CV and HIV infection, especially in cases of co-infection with HCV [14]. Infectious mixed cryoglobulinemia not associated with HCV is mainly caused by viruses, bacterial pathogens or parasites (Table 2).

Patients with active SLE and RA may have cryoglobulins circulating in the blood: mixed cryoglobulinemia was observed in ~ 10% of patients with SLE or RA, however, CV was diagnosed in

only 2% of cases [15]. Approximately 5–20% of patients with primary SS may have type II cryoglobulinemia [16]. Mixed type II cryoglobulinemia is considered to be one of the key prognostic factors in primary SS, as it is associated with extra-glandular lesions, the development of systemic vasculitis, B-cell lymphoma and low survival rates [16].

Blood diseases

The presence of cryoglobulinemia is also associated with hematologic diseases. The overall risk of non-Hodgkin lymphoma in patients with cryoglobulinemia is ~ 35 times as high as in the general population, or 12 times higher if non-aggressive lymphomas are excluded [17]. Serum cryoglobulin levels > 0.6 g/l, the presence of CV and hypogammaglobulinemia are independent signs associated with the development of B-cell non-Hodgkin lymphoma [18]. Patients with HCV have a high risk of developing non-Hodgkin lymphoma after a long period of infection (> 15 years). At the same time, in patients with non-Hodgkin's B-cell lymphoma, the transformation into diffuse B-large cell lymphoma is significantly higher in patients infected with HCV (32%) than in patients without HCV (6%) [19].

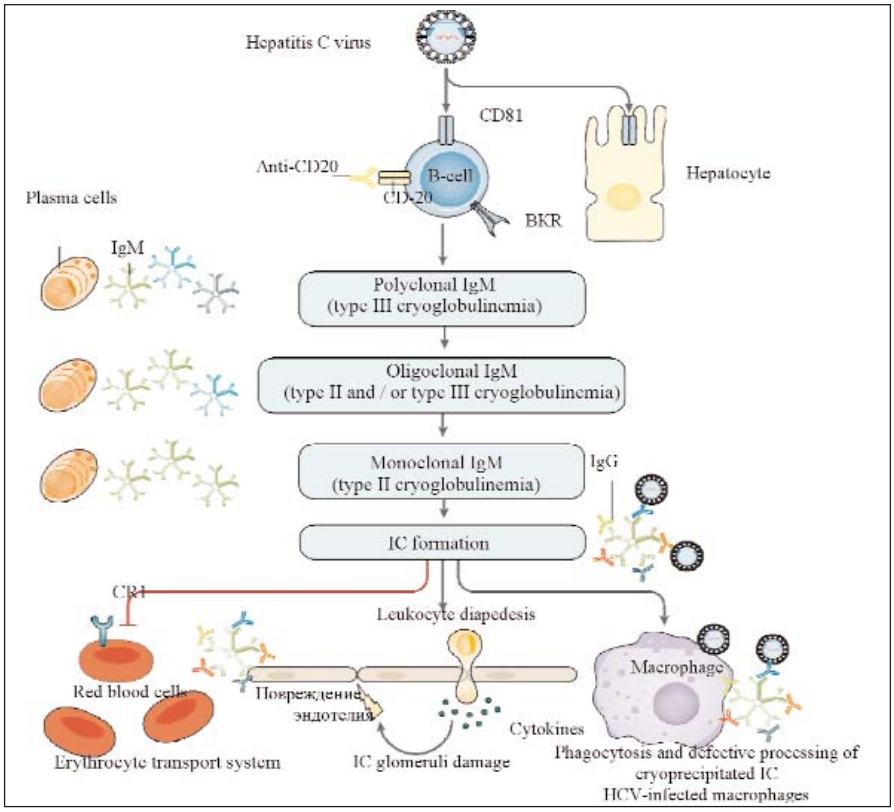

Fig. 2. *Mechanisms for the development of CGV associated with hepatitis C virus. Adapted from [2].*
Note. BCR – B-cell receptor; CR1 – complement receptor 1; IC – immune complexes

As mentioned above, type I cryoglobulinemia is almost always associated with B-cell proliferative diseases. A French study of the main lymphoproliferative disease in 36 patients with type I cryoglobulinemia showed that 13 patients were diagnosed with non-malignant monoclonal gammopathy, and 23 patients had a malignant hematological tumor (12 patients with Waldenstrom macroglobulinemia, 6 patients with a low degree of differentiation of non-Hodgkin lymphoma, 4 – with multiple myeloma and 1 with chronic lymphocytic leukemia) [20].

Pathogenesis / pathophysiology

One of the central events in the pathogenesis of cryoglobulinemia is the interaction between the predisposition of the host and environmental triggers, leading to impaired function of B cells [21]. In this model, chronic immune stimulation and lymphoproliferation are the main pathogenetic links in the development of cryoglobulinemia. This leads to the synthesis of monoclonal, oligoclonal or polyclonal cryoglobulins, the deposition of immune complexes after binding to the Ig antigen, in combination with insufficient and / or defective clearance of cryoglobulin-containing immune complexes in the affected organism [22]. For many cryoglobulins, aggregation and pathogenicity appear to depend on several factors, including pH, weak non-covalent interactions, cryoglobulin concentrations, and specific temperature conditions for antibody synthesis.

HCV-related cryoglobulinemia

The mechanism of cryoglobulin pathogenicity is best described for mixed cryoglobulinemia associated with HCV. HCV can simultaneously infect B cells and hepatocytes due to common expression of the CD81 receptor on the plasma membrane of both cell types [23] (figure 2). Active HCV replication has been demonstrated in CD19-positive B cells; HCV RNA and non-structural NS3 proteins have been found only in peripheral blood CD19-positive mononuclear cells [24]. HCV replication has also been described in monocytes, peripheral dendritic cells, and macrophages [24].

B-cell proliferation and HCV infection. B cell proliferation is an important mechanism in the pathogenesis of cryoglobulinemia and CV. The multi-stage process supports the transition from simple serological changes (cryoglobulinemia) to clinical manifestations (cryoglobulinemic vasculitis) and, ultimately, to obvious and unconditional lymphocyte proliferation of B cells (such as in non-Hodgkin's lymphoma) [25]. The discovery of the affinity of the HCV membrane for CD81 transmembrane protein has become a cornerstone in understanding the mechanisms of HCV-induced lymphoproliferation [5]. On the surface of B cells, CD81 creates a multiprotein complex with CD21 and CD19. This complex, activated by binding to HCV, regulates the polyclonal expansion of B cells. In addition, B cells are stimulated by binding of the antigen to the B cell receptor (BCR) on the surface, which also leads to polyclonal expansion [26].

B cells are targets for hepatitis C virus (HCV) due to the expression of CD81 receptor on their cell surface, which also contributes to the infection of hepatocytes. HCV-induced proliferation of B cells and a decrease in their activation threshold cause significant production of autoantibodies. HCV-dependent gene translocation, which can protect cells from apoptosis, sup-

ports oligoclonal monotypic selection resulting from mixed cryoglobulinemia. Clonal B cells produce IgM, which has rheumatoid factor activity (autoantibody activity) against anti-HCV IgG. These components bind to each other and to HCV antigens and form immune complexes (IC), which do not bind to the erythrocyte transport system, remaining freely circulating, and subsequently undergo phagocytosis [27]. HCV infection blocks lysosomal enzymes in phagocytes, which makes cells unable to "digest" cryoglobulins / IC after phagocytosis [28]. In the kidneys, due to the affinity of the mesangial matrix for the monoclonal component of IgM, cryoprecipitating ICs are deposited in the glomeruli, where the production of cytokines promotes leukocyte diapedesis and endothelial damage. The monoclonal anti-CD20 antibody acts at the initial stage of this cascade, blocking the proliferation of B cells and their production of IgM, which is critical both for the formation of cryoglobulins and for the deposition of IC in the glomeruli [22].

Although in vitro studies have shown that specific anti-HCV antibodies can stimulate BCR on B cells, the activation of CD81 by the HCV envelope protein appears to cause proliferation of naive B cells regardless of stimulation of B cell receptors. CD81-mediated activation of naive (CD27-negative) B cells, followed by differentiation into memory cells producing autoantibodies, may play a paramount role in the development of subsequent lymphoproliferative disorders. In addition, chronic antigenic stimulation can lead to over-expression of B cells (in favor of certain clones) and support immune dysregulation mechanisms that lead to the development of mixed cryoglobulinemia and, ultimately, to the malignant transformation sometimes observed in patients chronically infected with HCV [29].

B-cell transformation. In addition to inducing proliferation of B cells and lowering their activation threshold, HCV infection can lead to transformation of B cells [6]. Patients with HCV infection have clonal B-cell populations that are predominantly IgM-producing memory B-lymphocytes expressing hypermutated immunoglobulin genes [29]. Many of the idiotypes of immunoglobulins and limited rearrangements of Ig gene sequences are observed both in HCV-positive non-Hodgkin lymphoma and in CV, which indicates their common pathogenesis. In addition, new studies on the pathogenesis of HCV-associated lymphomas have reported evidence of a mutagenic potential in HCV [25]. In vitro B cells exposed to HCV had 10 times more mutations in the immunoglobulin heavy chain genes. In addition, an increase in the mutation frequency is observed in HCV-associated lymphomas compared with non-HCV-associated ones[25].

Among genetic mutations, the translocation t (14; 18) of BCL2 gene is the most common chromosomal rearrangement in a lymphoid tumor, especially follicular lymphoma, a subtype of non-Hodgkin lymphoma. About 35% of patients with chronic HCV infection have this translocation t (14; 18) in their peripheral mononuclear cells [25]. Mutations in other oncogenes, such as MYC, and in regulators of apoptosis can be a significant "missing link" in our understanding of lymphomagenesis in chronic HCV infection.

Autoantibody production. Chronic stimulation of B cells by HCV infection induces production of autoantibodies, which supports the development of a number of immune manifestations associated with HCV (besides CV) [5,30], such as autoimmune thyroiditis, "dry syndrome", thrombocytopenia, hemolytic anemia, autoimmune diabetes and pulmonary fibrosis [22].

Clonal B cell populations are present in the liver and peripheral blood of patients with chronic HCV infection. Interestingly, B cells extracted from the patients' lymph nodes with HCV-associated non-Hodgkin lymphoma show the affinity for rheumatoid factor [22]. In patients with HCV-mixed cryoglobulinemia, lymphoid infiltrates with cells expressing oligoclonal or monoclonal IgMs with rheumatoid factor activity were found in several organs, including portal tracts of the liver, spleen, and bone marrow [31]. Thus, mixed cryoglobulinemia appears to be a connecting and cross-linking element between classical autoimmune disorders and hematologic neoplasia (ie, B-cell lymphoma) [22].

Continuous stimulation of B cells with viral antigens and increased expression of genes associated with lymphomagenesis (in particular, induced by the activation of cytidine deaminase, which is critical for somatic hypermutation), lead to polyclonal and then monoclonal expansion of B cells. Indeed, among other hematological malignancies, a close relationship was found between HCV infection and B-large cell lymphoma, marginal zone lymphoma and lymphoplasmacytic lymphoma [32].

The formation of immune complexes. The clinical symptoms of cryoglobulinemic vasculitis are caused by the deposition of immune complexes in small blood vessels and the resulting damage to the endothelium. Cryoprecipitated immune complexes leave the erythrocyte transport system due to cloned IgM [33]. The presence of IgM in cryoprecipitated immune complexes makes them capable of causing activation and consumption of complement, however, they cannot activate complement fragments, including complement C3b, which promotes binding of immune complexes to the erythrocyte complement 1 receptor (CR1) [1]. These immune complexes remain freely circulating in the blood since the liver and spleen macrophages are not able to process immune complexes due to HCV-induced abnormalities in the biogenesis of lysosomal enzymes [22]. In addition, the same anomaly was detected in circulating monocytes [22]. Interestingly, histological studies of the renal parenchyma using electron microscopy revealed monocytes that contain trapped cryoglobulins [34], but the exact role of these cells is unclear.

With cryoglobulinemic nephritis, phagocyte migration to the glomeruli is observed. Phagocytes try to remove precipitated cryoglobulins, but they are not able to "digest" phagocytized cryoglobulins, which probably reflects the ineffective clearance of cryoglobulins [35]. This mechanism potentiates glomerular damage, as shown in a study of cryoglobulinemic membrane proliferative glomerulonephritis on a mouse model [35]. In this study, macrophage ablation prevents the expansion of the mesangial matrix and the accumulation of collagen in it (without affecting the level / clearance of cryoglobulins). This experimental model suggests that the recruitment of macrophages into the glomeruli plays a critical role in the progression of kidney damage. The influx of macrophages, vascular infiltration and diapedesis of leukocytes are associated with increased tissue damage after deposition of immune complexes.

Dysfunction of lysosomal monocyte enzymes (possibly associated with HCV infection), including extracellular release of procathepsin D62 and / or release of distress-associated molecular patterns (DAMP) from damaged resident cells [9], disrupt the innate function of macrophages to cleanse blood of immune complexes through gamma-receptor crystallizing fragment (Fc) of immunoglobulin. The proliferation of the mesangial matrix and glomerular cells can be maintained by additional cellular

activation of the released procathepsin D62 or pro-inflammatory cytokines released from DAMP-activated macrophages [35]. Monoclonal IgM has a strong affinity for the components of the glomerular matrix, including fibronectin, opening up the possibility for the mechanism of in situ binding of immune complexes to components of the renal parenchyma [22].

Consequently, the pathogenetic role of cryoglobulins in the induction of vasculitis is associated both with the recruitment of leukocytes into blood vessels, and with the deposition of immune complexes, with the activation of the complement system and damage to microvessels. A low level of complement C4, a diagnostic sign of CV, is associated both with activation of the complement system with binding of C4 to immune complexes, and with C4 genetic polymorphism in these patients [33].

Non-HCV Cryoglobulinemia

Compared with cryoglobulinemia associated with HCV, data on the pathogenetic mechanisms underlying the development of mixed cryoglobulinemia in the context of other disorders (infectious or autoimmune) are less studied, but similar.

Conclusions

Thus, chronic infectious B-cell stimulation, most often associated with HCV, is the most studied pathogenetic mechanism of CV. The mechanism of the development of cryoglobulinemia associated with HCV depends on the synthesis of IgM with rheumatoid factor activity and subsequent formation of cryoprecipitate immune complexes, abnormal clearance of the immune complexes with their deposition in tissues in combination with ineffective clearance of cryoglobulins by monocytes and / or macrophages that are involved, for example, in damaging the glomeruli, and the development of further lymphoproliferative disease. However, some etiopathogenetic problems have yet to be clarified. So, it is still unclear whether HCV infection acts as a simple trigger or whether it also contributes to the self-maintenance of the disease mechanisms. In addition, the natural course of mixed type II or type III cryoglobulinemia can develop with various clinical phenotypes among patients, which suggests a multi-factor and multi-stage process. Thus, the role of possible genetic and / or environmental co-factors remains a target for further research. B-cell lymphoproliferation is another important problem. In particular, in patients with mixed cryoglobulinemia associated with HCV, maintaining the proliferation of benign B-lymphocytes can explain the paradoxical effects of relapse or newly diagnosed CV observed after eradication of HCV in patients who have immunological disorders and clinical symptoms. Thus, it can be assumed that in some patients, possibly people with a longer history of HCV, changes in the immune system have passed a kind of "point of no return". Recognition of this as a biological condition may be crucial for overall management of such patients.

The mechanisms involved in the development of lymphomas associated with mixed cryoglobulinemia should be investigated more deeply. Identification of factors involved in non-HCV associated mixed cryoglobulins remains an important unresolved research challenge.

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Conflict of Interest Statement

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