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Cryoglobulins: Identification, classification, and novel biomarkers of mysterious proteins

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Abstract

Cryoglobulins consist of serum immunoglobulins that precipitate below 37°C and resolubilize upon warming. The clinical triad of cryoglobulinemia usually includes purpura, weakness, and arthralgia. Cryoglobulinemic syndrome, clinically defined as a systemic vasculitis, is associated with chronic infection with hepatitis C virus (HCV) and autoimmune disorders and can evolve into B-cell malignancies. While the current literature about HCV-associated cryoglobulinemia is not very limited, little is known about the immunologic and serologic profiles of affected patients. Therefore, comprehension of the pathogenetic mechanisms underlying cryoprecipitation could be very helpful. Due to the persistence of viral antigenic stimulation, biomarkers to use after the worsening progression of HCV infection to lymphoproliferative and/or autoimmune diseases are widely needed. Laboratory methods used to detect and characterize low concentrations of cryoprecipitates and immunotyping patterns could improve patient management. The most critical factor affecting cryoglobulin testing is that the pre-analytical phase is not fully completed at 37°C.

Abbreviations

ANA	antinuclear antibodies
anti-LKM1	antibodies against liver-kidney microsomes
AutoAbs	autoantibodies
BCR	B-cell receptor
CD138	Syndecan-1
CGP	cryoprecipitate
CGs	cryoglobulins
COVID-19	coronavirus disease 2019
CRT	cryocrit
DAA	direct acting antivirals
FLC	free light chains
HCV	hepatic C virus
HLC	heavy/light chain
IB	immunoblotting
IC	immune complexes
IEP	immunoelectrophoresis
IFE	immunofixation electrophoresis
Igs	immunoglobulins
MC	mixed cryoglobulinemia
MM	multiple myeloma

MZ	marginal zone
NHL	non-Hodgkin lymphoma
PBS	phosphate-buffered solution
pERK	extracellular signal-regulated kinase
RA	rheumatoid arthritis
RF	rheumatoid factor
RTX	rituximab
RTX	rituximab
SjS	Sjögren's syndrome
SVR	sustained virologic response
VEGF	endothelial growth factor

Much evidence has shown that peripheral blood B cells circulating in patients with HCV-associated mixed cryoglobulinemia (MC) are profoundly abnormal. After viral eradication, these cells persist in peripheral blood, and their occurrence does not correlate with serum cryoglobulins or with the response or relapse of vasculitis. During the course of MC, these monoclonal B cells producing rheumatoid factor can be reactivated by circulating immune complexes that are mostly produced during infection or neoplasia.



1. Introduction

Cryoglobulins (CGs) represent a possible dysfunction of the immune system. Any substance that triggers a specific activation of immune system receptors is known as an antigen (well-established molecular mechanisms allow distinction between self and non-self-antigens). Based on the prevalent mechanism, immune responses are distinguished as antibody (B cell-dependent) and T-cell-mediated responses. However, antibody responses to protein antigens require T cell and B cell cooperation, which is responsible for the response against infectious pathogens.

CGs are immunoglobulins that undergo reversible precipitation or gelling when exposed to temperatures below 37°C and re-dissolve upon rewarming. Most CGs (95%) consist of immune complexes (ICs) containing rheumatoid factor (RF); they are known as “mixed” CGs to differentiate them from monoclonal CGs, which do not contain RF or antigen-antibody complexes [1].

The prevalence of cryoglobulinemia, which is significantly related to geographic location, is higher in the south of Europe than in Northern

Europe or America [2,3]. This different distribution reflects the epidemiology of HCV. While the worldwide prevalence of HCV is approximately 3%, it increases up to 11% in southern France. Conversely, allergic and autoimmune disorders are generally more prevalent in Northern European countries [4]. This evidence suggests that different risk factors, such as infections, genetics and/or environmental conditions, may contribute to the pathogenesis of the disorder. In contrast, the “hygiene hypothesis” ascribes the onset of autoimmune and atopic disorders to a lack of infections (that ultimately induce immunoregulation to keep the immune system balanced) [5]. In the Netherlands and in countries where the HCV infection load is negligible (<1%), cryoglobulinemia is defined as “essential” (~50%) and is associated with lymphoproliferative/autoimmune disorder (~50%) [6].

Although CGs were first characterized in a patient with multiple myeloma (MM) [7], the term “cryoglobulin” was coined later, referring to a phenomenon of cold-precipitable serum immunoglobulins in a clinical case of purpura [8]. Lospalluto et al. described two proteins of 19S and 7S in a cryoprecipitate associated with an antibody-antigen response [9]. The phenomenon is caused by immunoglobulin interactions that occur during precipitation rather than by the specific characteristics of the immunoglobulins. It has been proposed that cryoprecipitation occurs because of the rapid formation of cold-insoluble immune complexes formed by IgM that display RF activity and are complexed to IgG [9]. The cryoprecipitation of circulating immune complexes in serum in association with the classic “Meltzer’s triad” of purpura, weakness and arthralgias is referred to as mixed cryoglobulinemia (MC) [10]. Indeed, fatigue, myalgias, and palpable purpura due to cutaneous vasculitis and sensory changes or weakness due to peripheral neuropathy is observed in most patients [11]. MC is a multifactorial disorder, and several factors, such as genetic, environmental, and immunologic factors, are connected to the development of this disorder. The specific trigger involved in the development of MC, such as HCV, has not been conclusively identified, but MC is most often associated with chronic inflammatory disease. Patients at risk of disease flare could be monitored by CG assessment to allow early intervention and possibly reduce end-organ damage and mortality. In fact, CG concentration appears to be correlated with disease activity in different autoimmune disorders.

Although cold insolubility is not completely understood and may be due to a variety of factors, this phenomenon mainly depends on low temperatures that, through the induction of steric modifications, trigger cryoprecipitation that is reversible upon warming to 37°C [12,13]. This event represents the

pathogenic mechanism of a wide range of symptoms; systemic vasculitis affecting the skin, joints, nerves and kidneys represents the most common clinical manifestation. Interestingly, CGs are not always symptomatic; therefore, their role in the pathogenesis of vasculitis remains unclear. CG detection and quantification are, however, critical. Unfortunately, most of the methods of measurement lack standardized protocols for specimen collection, analysis and reporting.



2. Definition of cryoglobulins

In 1974, Brouet et al. [10] proposed a classification scheme that correlated immunochemical characteristics of cryoglobulinemia with clinical presentation. In this study, a total of 86 patients with cryoglobulinemia underwent comprehensive immunochemical and clinical evaluations during their disease course. Immunochemical analysis of purified CGs allowed their classification into three groups. Type I consisted of isolated monoclonal immunoglobulins ($n=21$). These include IgM (11), IgG (7), IgA (2) and Bence-Jones proteins (1). Type II consisted of mixed CGs with a monoclonal component behaving as autoantibodies toward polyclonal IgG, i.e., behaving as autoantigens ($n=22$). These include IgM-IgG (19), IgG-IgG (2) and IgA-IgG (1). Type III consisted of mixed CGs constituting one or more classes of polyclonal Igs ($n=43$) [10]. Cutaneous and vasomotor symptoms were most severe in types I and II. Clinically, types II and III were associated with purpura and Raynaud's phenomenon. Renal and neurologic involvement was more frequent in types II and III. Immunoproliferative disorders were associated with type I. The clinical features of types II and III were similar. Type I is composed of a single monoclonal immunoglobulin, mostly IgM, and is usually associated with an underlying disorder, specifically plasma cell disorders. Types II and III are classified as mixed cryoglobulinemia because they include both IgG and IgM components. In these cases, CGs are associated with abnormal immune complex formation. Different from type I, types II and III contain an IgM rheumatoid factor, which is an autoantibody. The distinction between types II and III is mostly technical, i.e., depends on the mono- or polyclonality of rheumatoid factor.

Although this taxonomic classification is still widely accepted because it is well correlated to clinical manifestations, other authors have described the presence of atypical CGs in the serum of HCV-infected patients [14,15].

In 1992, Musset et al. [16] observed microheterogeneity characterized by two or more monoclonal IgM or IgG components in patients with CGs.

This study analyzed 157 specimens by immunoblotting (IB) that was specifically modified for the detection of CGs, and the results were compared to those obtained with traditional methods, i.e., immunoelectrophoresis (IEP) and immunofixation electrophoresis (IFE). Novel microheterogeneity was observed by IB and IFE in 13% and 6% of specimens, respectively. Unfortunately, the pathophysiologic relevance of the observed microheterogeneity and its classification remained unclear. It should be noted, however, that IB is more specific and sensitive than IEP and IFE. An additional highly sensitive method employed for analysis is two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), which was used to characterize CGs in the serum of cryoglobulinemic patients [17]. By analyzing 60 MC patients with 2-D PAGE, Tissot et al. identified a new type of cryoprecipitate, named type II-III, that was characterized by polyclonal IgG mixed with polyclonal and monoclonal IgM and not associated with a clinical pattern [17].

The microheterogeneity of type II-III CGs, consisting of two or more oligoclonal bands in MC, introduces innovative taxonomic elements. This form is considered to be an intermediate between types II and III cryoglobulinemia [16–18]. In 1997, a novel immunochemical CG profile was described in a patient with Gougerot-Sjögren syndrome using IFE [19]. The main feature of CGs was a biclonal IgM κ associated with polyclonal IgG. Therefore, the authors proposed subdividing the type II CG taxonomic classification into type IIa, which would include CGs composed of one monoclonal immunoglobulin and polyclonal immunoglobulin (i.e., type II of the previous classification), and type IIb, which would include CGs composed of two (or more) monoclonal immunoglobulins (multiclonal immunoglobulins) and polyclonal immunoglobulin. The presence of CG subtypes, i.e., bi-, tri- and multiclonal immunoglobulins, would be similar to type III. This new immunochemical pattern described a new taxonomic definition of microheterogeneity formed by oligoclonal CGs (Fig. 1) [20]. HCV plays an important role both in the induction and persistence of cryoglobulinemia, as well as the transition from type III to II [21,22]. The new subtype of CGs, i.e., the transitional step between types II and III, places increased attention on immunochemical and histopathologic findings as well as the development of more accurate diagnostic and therapeutic tools. As such, the next advances in biotechnology will allow CG typing in a more accurate and precise manner (Table 1).

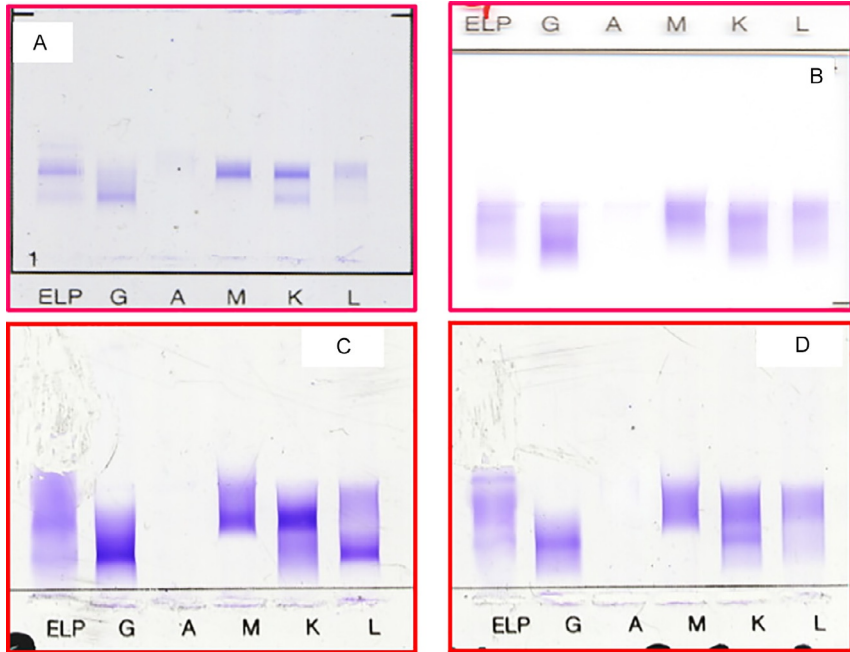


Fig. 1 Atypical cryoglobulin pattern. (A) Presence of a double monoclonal component (IgG k—IgM k). (B) Presence of classic microheterogeneity. (C) Presence of a double monoclonal component (IgG lambda—IgM k). (D) Presence of an IgG k monoclonal component and an IgG-IgM polyclonal component.



3. Molecular basis of cryoprecipitation

CG solubility depends on different physical and chemical factors, i.e., concentration, temperature, pH, ionic strength, amino acid composition, net charge, and carbohydrate content. A larger amount of hydrophobic amino acids than tyrosine and sialic residues has been implicated as a major determining factor [13]. A reduction in sialic acid and galactose residues in the Fc region potentially decrease solubility at lower temperatures. Unfortunately, a full comprehension of all biochemical mechanisms is still lacking.

Type I cryoprecipitation can be considered a classic phenomenon of solubility/insolubility derived from favorable/unfavorable interactions between the CG and solvent at decreased temperatures [23]. Aggregation

Table 1 Characteristics and classification of cryoglobulins.

Cryoglobulinemia	Type I	Type II	Type III
Immunoglobulin classes	IgM (most abundant) IgG (IgG2, IgG3) IgA (rarely) FLCs	IgM vs IgG IgG vs IgG IgA vs IgG (rarely)	IgM-IgG IgM-IgG-IgA IgG-IgA-FLCs
Clonality	Monoclonal Ig	One or more monoclonal Igs + polyclonal Igs	Polyclonal Igs Oligoclonal Igs + polyclonal Igs (microheterogeneous)
Mixed cryoglobulinemia	No	Yes	Yes
Frequency	25–30%	25%	50%
Associated diseases	MGUS SMM Waldeström macroglobulinemia MM Other lymphoproliferative diseases	SAD: Sjögren's syndrome, SLE, RA LPD: B-cell lymphoma, NHL Solid tumors Cold-agglutinin disease <i>Infectious</i> : Chronic HCV infection Other infections (HIV, HBV) <i>Essential MC (unknown cause)</i>	SAD: LES, RA Intestinal diseases Biliary cirrhosis Solid tumors <i>Infectious</i> : HBV, HIV, Epstein-Barr, cytomegalovirus Endocarditis, spirochetes Fungal infections Parasitosis <i>Essential MC (unknown cause)</i>

Clinical manifestations	<p><i>Cutaneous manifestations:</i> Purpura, acrocyanosis, skin necrosis, skin ulcers, livedo reticularis</p> <p><i>Extra cutaneous manifestations:</i> Peripheral neuropathy, renal involvement, joint involvement</p> <p><i>Systemic vasculitis</i></p>	<p><i>Cutaneous manifestations:</i> Meltzer's triad</p> <p><i>Internal organ affected:</i> Kidney: renal disease, membranoproliferative glomerulonephritis; Neuropathy severe manifestations: Intestinal ischemia, alveolar hemorrhage, involvement of CNS and myocardium</p>	<p><i>Cutaneous involvement peripheral neuropathy:</i> renal involvement, joint involvement</p>	
Biologic markers	<p>RF-IgM/IgG</p> <p>Type I: +</p> <p>FLC</p>	<p>AutoAb</p> <p>(ANA, ENA, AMA)</p> <p>Hypocomplementemia (↓ C3, ↓ C4, ↓ CH50)</p> <p>RF-IgM/IgG</p> <p>Type II: ++</p> <p>Type III: ++</p> <p>FLC: κ: ++; λ: +</p>		

MGUS, monoclonal gammopathy of undetermined significance; SMM, smoldering multiple myeloma; MM, multiple myeloma; SAD, systemic autoimmune diseases; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; LPD, lymphoproliferative disorders; NHL, non-Hodgkin lymphoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HBV, hepatitis B virus; MC, mixed cryoglobulinemia; CNS, central nervous system; RF, rheumatoid factor; ANA, antinuclear antibodies; ENA, extractable nuclear antigens; AMA, anti-mitochondrial antibodies; FLC, free light chains.

may occur for electrostatic reasons involving altered glycosylation [24]. The persistence of immune stimulation may induce the production of circulating desialylated immunoglobulins that represent a normal byproduct of the enhanced stimulation of B lymphocytes [25]. The reduction/absence of sialic acid residues in the Ig molecules generates new epitope sequences that are exposed, triggering an immune response; the resulting immune complexes may acquire the capacity to precipitate [26]. Under normal conditions, the removal of CGs is mediated by specific hepatocellular receptors for desialylated glycoproteins. For this reason, liver disorders are commonly associated with significantly increased levels of serum cryoglobulins [25].

Type I CG precipitation is characterized by specific amino acid sequences producing a structural change and auto-aggregation at the level of the quaternary structure of the protein that begins with a slow phase (lag phase) of small aggregates of monoclonal Igs, followed by rapid and extensive aggregation due to a combination of weak non-ionic and hydrophobic interactions culminating in precipitation [26]. By analyzing the structure of IgG CGs, it has been shown that amorphous, gelatinous and/or crystalline precipitates may be produced [27]. The physical and chemical factors determining monoclonal CG precipitation also occur in MC, apart from the lack of a lag phase. MC precipitation consists of a rapid and progressive increase in the size of IgG-IgM precipitates at low temperatures, showing typical biological properties of immune complexes, such as the ability to activate the complement system [28]. Type II and III CGs (occurring in MC) are composed of polyclonal IgG, autoantigens, and mono- or polyclonal IgM, respectively; IgM displays rheumatoid factor (RF) activity [29].



4. Pre-analytical procedures

The occurrence of CGs is not necessarily indicative of a disorder (transient levels of CGs may be detected during infections, and healthy individuals may present low levels of CGs), and serum concentrations do not always correlate with the severity of symptoms; some patients with apparently low levels of CGs may show severe symptoms associated with cryoglobulinemic syndrome. Appropriate laboratory testing of CGs involves simple biochemical quantification, which requires strict pre-analytical protocol adherence to maintain the specimen at a temperature of 37°C, especially during the initial steps of the test; the lack of a stable temperature from sample collection to analysis may result in misdetection of CGs, with a detriment for the patient.

This analysis is often neglected by clinicians, despite its usefulness in patient management [13,26,30]. The difficulty in maintaining appropriate pre-analytical conditions, as well as the variability during different phases of the procedure, implies heterogeneity of the results. CG detection requires global standardization, but internationally accepted standard protocols are still lacking although several approaches have been described and proposed [14,18,26].

Most authors agree about the importance of maintaining samples at 37°C from collection and transport throughout the clotting procedure until the initial centrifuging stages. During sample collection and specimen transport, the sample should be kept warm; the most common reason for CG mis-detection and false-negative results is that the temperature often drops to values below 37°C upon arrival at the destination [13,30]. Blood samples must be collected on an empty stomach, in a tube without anticoagulant that is preheated to 37°C. Tubes with separating gel are highly discouraged due to the risk of interfering substances that may be released by the gel during incubation at 37°C to enable clotting. If separating gel tubes are used, it is desirable to review the gel composition and its specific characteristics [26]. Blood samples (at least 10 mL in volume) must be immediately transported to the laboratory under conditions that maintain 37°C for at least 30 min until clotting is complete. The sample is generally centrifuged at 2000 × *g* for 10 min at 37°C [31]; however, some authors recommend 2000 × *g* for 30 min at 37°C [16] or do not specify a time or speed [10]. Then, serum is transferred to a thin tube containing a preservative such as sodium azide and stored at 4°C (Fig. 2).



5. Laboratory investigation

Laboratory procedures for the detection and typing of CGs can be divided into three phases: the pre-analytical, analytical and characterization phases.

CG analysis requires strict pre-analytical protocol adherence to maintain the sample at a stable temperature of 37°C, especially throughout the initial steps. Failure to guarantee these crucial conditions for specimen collection may result in undue execution, to the detriment of the patient. Difficulty in ensuring the appropriate pre-analytical conditions of the samples can result in heterogeneity of the results, altering the reliability of a test even if it is relatively simple [30].

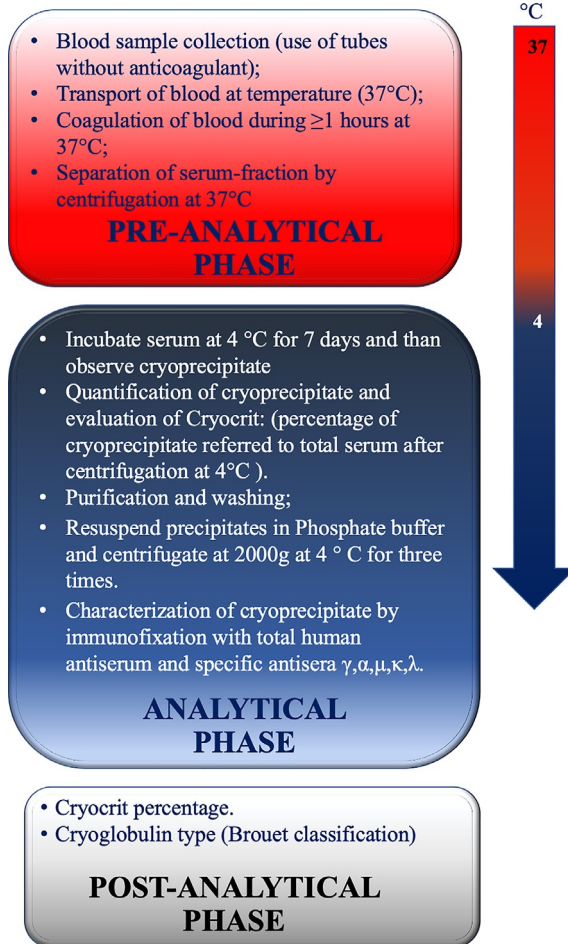


Fig. 2 Laboratory protocol for CG research and characterization.

5.1 Demonstration of cryoglobulinemia

The tube containing the serum sample is placed at 4°C and observed daily for at least 7 days. Recently, it has been proposed to extend this observation period to 15 days because of the occurrence of late-appearing cryoglobulins [32]. Often, CG cause a fine whitish or granular precipitate but can also produce a gel or crystals; redissolution is completed in a few minutes at 37°C.

5.2 Isolation and purification

After qualitative characterization, the next step is the isolation of cryoglobulins. This step is generally carried out on another blood sample (10 mL of serum may be necessary). After precipitation of the serum at 4°C, the supernatant is removed, and the cryoprecipitate is purified by means of a succession of three series of precipitation at 4°C and redissolution in washing buffer at 37°C during a time course of 3 days. Some authors emphasize that centrifugation at 37°C may be required and, alternatively, suggest performing serum removal from the clot without using a centrifuge if not preheated [30,33].

5.3 Quantification

Before measurement, cryoprecipitates may be washed with phosphate-buffered saline (PBS) or 3% polyethylene glycol 6000 in PBS. In all cases, the washing solution must be kept at 4°C, and CGs should be resuspended by shaking in a solution comparable to the supernatant volume discarded after centrifugation at 4°C. A minimum of three wash cycles is required [26,31].

Then, the cryoprecipitate should be dissolved by incubation at 37°C. CGs may also be treated with reducing solutions, such as 10% acetylcysteine, 1% β -mercaptoethanol or 0.5 mmol/L dithiothreitol [26].

The amount of CGs may be assessed and expressed in the following ways: as a cryocrit (CRT) that represents the measurement of total proteins, via immunonephelometric quantification of immunoglobulins or as the area under the curve of the gamma region over the course of electrophoresis of the resolubilized cryoprecipitate (performed at 37°C). The CRT represents a semi-quantitative parameter that is routinely used in clinical practice, as it is simple and affordable; however, different variables may discourage its use as a reliable biomarker: it consists of the measured percentage ratio between the volumes of cryoprecipitate and serum obtained by centrifugation at 4°C for 15 min at $1700 \times g$ [31]. CRT evaluation is operator dependent; therefore, there is frequently diminishing trust in data accuracy and assessment. Cryoprecipitate proteins could be quantified by a sensitive method derived from the Biuret method with reading at 485 nm; the calibration range should be performed with purified immunoglobulins. The sensitivity of this method is 20 mg/L [31]. Because it consists of the

measurement of the total amount of proteins and is not a specific immunometric assay for immunoglobulins, it could be affected by the different affinities toward the various serum proteins. However, CRT values are often reported in published clinical case reports and referred to as recommended quantitative data.

5.4 Immunochemical typing

The purity of the cryoprecipitate is verified by good-sensitivity electrophoresis that allows the detection of a monoclonal component by a narrow strip. Typing is carried out by immunofixation electrophoresis using monospecific antisera for each heavy (γ , α , μ) and light (κ , λ) chain of immunoglobulins. An automated method for immunofixation has recently become available [34]. The most sensitive method is the immunoblot (Western blot), which is limited for most specialized laboratories. All these methods make it possible to classify CGs according to the monoclonal, oligoclonal or polyclonal characteristics of the Igs that they are composed of [26].

5.5 False negatives or false positives

The pre-analytical phase can be considered crucial for the whole procedure. The lack of strict adherence to the rules of good practice for collection and transport can determine the precipitation of CGs in the clot and therefore their loss in the serum [30]. A lipid component in the serum of a patient who is not fasted or has significant dyslipidemia can yield disorder that dissolves at 37°C. The presence of a flocculate cryoprecipitate may be due to fibrinogen.



6. Biomarkers

Different combinations of serum biomarkers that are potentially able to assist in diagnosis, prognosis, and treatment have been, and still are, widely analyzed. Biomarkers are measured and evaluated as indicators of normal biological, pathogenic, or pharmacologic responses to therapeutic intervention [35,36]. The evaluation of biomarkers and clinical outcomes may be considered an endpoint to analyze using statistical methods: clinical outcomes are important for the approval of therapeutics steps, whereas biomarkers might be used as measures to predict clinical outcomes [36,37]. The most common and dangerous error in the evaluation of biomarkers consists of considering their levels in correlation with clinical status, making the biomarker a valid

surrogate. Biomarkers may effectively play this role when the measured levels are correlated with the disease state, but any variation must be monitored over the course of multiple therapies; for this reason, when its role as a surrogate is validated, that validation only pertains to the specific context of use [36,37]. Biomarker evaluation can be added to several diagnostic panels when its role has been demonstrated as reliable; although overlap sometimes occurs, different biomarkers can be related to specific characteristics of disease. Therefore, a panel that groups a selection of biochemical markers can have better predictive value for diagnosis. In the era of precision medicine, the roles of biomarkers include evaluating the risk of developing disorders and identifying multifactorial and multistep biochemical and pathogenetic processes that may evolve from mild manifestations to malignant neoplasia. A biomarker can be assessed serially over time to monitor the outcome of disease or to provide evidence of a clinical effect of an intervention, including exposure to a medical product or an environmental agent. Patients at risk of disease flare could be monitored with biomarkers to allow early intervention and possibly to reduce end-organ damage and mortality. Increased levels of biomarkers have also been described in the course of autoimmune diseases and are correlated with disease activity, facilitating the best choice of a therapeutic scheme [38,39].

To date, some predictive markers of responses to biological drug treatment have been proposed for different rheumatic diseases, including serum circulating molecules or genetic factors. The increasing employment of biological therapies has radically changed patient treatment in the last several decades, thanks to their roles in altering the pathogenic mechanisms underlying several chronic inflammatory diseases. Measurement of biomarkers seems to be a promising assessment of therapeutic responses to the B-cell-depleting agent rituximab (RTX) in patients with different chronic diseases. Different mechanisms are involved in the beneficial therapeutic response to RTX observed in the course of different antibody-dependent and cell-mediated autoimmune diseases [40]. Assessment of circulating biomarker levels represents a promising tool for a more accurate evaluation of both disease activity and response to RTX treatment [41–43]. For the best strategy of personalized medicine, therapeutic monitoring of biological drugs represents an important effort in the diagnostic-therapeutic pathway to improve overall patient management and favor an appropriate clinical approach. An increasing number of biomarker assays have been designed to elucidate the efficacy and/or safety of a specific drug or class of drugs for a targeted patient group [44]. A “predictive” biomarker predicts different responses of an

individual or group of individuals to treatment strategies that are significantly monitored by the level of the biomarker.

Safety biomarkers are useful to identify patients who are experiencing adverse effects from a drug treatment. An interesting aspect of developing safety biomarkers is the balance that should be considered between safety and the potential benefits of therapy. For many therapies, monitoring for hepatic, renal, or cardiovascular toxicity is critical to assuring that a given therapy can be safely sustained [37]. In asymptomatic subjects, biomarkers allow detection in the early stages of MC, and in patients evaluated for MC disease, biomarkers are useful to confirm malignant or benign disease stages. In the diagnosis of HCV-related non-Hodgkin lymphoma (NHL), biomarkers may have a predictive role and be useful in choosing targeted treatment strategies. A high prevalence of low cryoglobulin levels (≤ 0.05 g/L) in non-HCV patients may account for renal and neurological complications, leading to high morbidity and mortality [45]. Clinical manifestations and autoantibody presence in chronic HCV infection can easily be misdiagnosed as classic autoimmune diseases, and the possibility of early serological biomarkers of MC could be crucial for the effective control of extrahepatic manifestations [46]. Here, we review the most reliable biomarkers for CGs according to the literature (also summarized in Table 2).

6.1 IgG subclasses

Human IgGs display four subclasses, IgG1 (up to 60–65% of total IgG), IgG2 (20–25%), IgG3 (5–10%) and IgG4 (3–6%), which show different chemical and physical features and whose clinical significance is still not completely understood. Functional differences are derived from structural variations in the hinge and heavy chain constant regions [47]. Clinical studies have shown that cryoglobulins of distinct IgG subclasses may be characterized in different syndromes [48,49]. The distribution and the roles of different IgG subclasses in the pathophysiology of cryoglobulinemia have been deeply investigated since 1971 [50]. Analysis of immunoglobulin components in murine CGs revealed selective enrichment of the IgG3 subclass. In describing the relationship between serum hyper-viscosity in cryoglobulinemic syndrome and IgG subclasses, they observed that IgG3 formed concentration-dependent aggregates. The hypothesis of frequent interactions toward similar molecules, perhaps for conformational similarities, was confirmed by the prevalence of IgG3 proteins among IgG CGs. The autoreactivity of the IgG3 subclass has also been confirmed in murine and

Table 2 Associations between cryoglobulins and serological biomarkers

Biomarker	Association with the CGs	Papers
IgG subclasses	• Identification of IgG3 RF in patients with HCV could be used for the assessment for antiviral therapy eligibility	[22]
	• Increased IgG-RF levels and the presence of IgG3 could suggest a follow-up to identify the evolution of an autoimmune or lymphoproliferative disease	[22]
	• Identification of IgG3 RF in patients with HCV could be an early predictor of the development of MC	[22]
	• Selective enrichment of IgG3 subclass in murine CGs	
	• IgG3 monoclonal CGs with RF activity in murine CGs	[50]
	• Presence of IgG1 and IgG3 in the CGs of patients with HCV infection	[51]
	• IgG3 and ANA in cryoprecipitates in presence of HCV infection could stimulate the indicate the immune system activation	[52]
	• The positivity of IgG3 is indicative of an RF activity against the IgG1-HCV complex	[55]
	• Strong correlation between ANA positivity and the presence of the IgG3 subclass in CGs of patients with HCV infection	[56]
	Rheumatoid factor	• IgM component presents predominantly the RF activity in MC (Type II and Type III CG)
• Presence of RF is a serological characteristic in most patients with MC		[22]
• The RF are autoantibodies directed against the Fc portion of the IgG that enables them to form immune complexes		[29]
• B cells, isolated from liver areas of patients, produced a specific monoclonal RF bearing the WA cross-idiotype, presents in about 60% of cases of HCV-related type II MC		[47]
• 100% of serum from ANA-positive HCV patients shows RF-IgG positivity at 37°C		[55]
• Polyclonal IgM RF in Type III CG is a transitional state observed in HCV patients (type-II-type-III mixed cryoglobulinemia variant)		[57]
• WA B cells could be a marker to predict the development of cryoglobulinemic vasculitis and NHL in asymptomatic patients with HCV infection		[57]
• Type II MC is a process that occurs through transformations of RF-producing B-cells by a multiple-step process		[59]
• IgM RF is characteristic of type II MC		[60]

Continued

Table 2 Associations between cryoglobulins and serological biomarkers—cont'd

Biomarker	Association with the CGs	Papers
	<ul style="list-style-type: none"> • Type II-RF CGs quantification is higher for patients with major signs of vasculitis • Type II-RF CGs have a pathological role in the palpable purpura and membranoproliferative glomerulonephritis lesions • The associations between Type II-RF CGs and cryoglobulinemic vasculitis are most expressed in the presence of HCV infection • WA and PO are two major IgM RF cross-idiotype groups • Autoreactive variant WA is unique to HCV infection in about 80% of cases of MC • The production of WA monoclonal RF could be driven by HCV. 	<p>[64]</p> <p>[65]</p> <p>[67]</p>
Autoantibody	<ul style="list-style-type: none"> • In subjects with RA and HCV was observed ANA positivity in the cryoprecipitate (82%) and in the supernatant (48%). • AMA, ASMA, anti-parietal and anti-LKM 1 antibodies was negative in subjects with RA and HCV, but positive in patients with RA only. • Coarsely spotted pattern was completely absent among RA-HCV positive patients while the finely spotted pattern was mainly expressed. • The presence of ANA appears to be related to CG production. • Strong influence of CGs in comparison with Ro/La autoantibodies and ANA on the phenotype of primary SjS at diagnosis. • CGs correlate with positive anti-ds DNA in SLE patients. 	<p>[55]</p> <p>[55]</p> <p>[55]</p> <p>[56]</p> <p>[68]</p> <p>[69]</p>
Complement	<ul style="list-style-type: none"> • Involvement of complement in HCV manifestations, such as liver fibrosis and type II cryoglobulinemia. • Reduced values of the C4 fraction of the complement can be observed in most patients with MC. • Presence of C1q into cryoprecipitates of the patients with lupus nephritis. • C1q mRNA levels are much higher in HCV patients with MC compared to HCV patients without MC or healthy controls • Positive correlation between circulating C1q with RF activity and C1q concentrations in HCV patients with MC 	<p>[71]</p> <p>[77]</p> <p>[73]</p> <p>[74]</p> <p>[74]</p> <p>[74]</p>

Table 2 Associations between cryoglobulins and serological biomarkers—cont'd

Biomarker	Association with the CGs	Papers
	<ul style="list-style-type: none"> • Presence of C1q in the cryoprecipitates of patients with CG associated with HCV infection • C4 levels progressively decreased among HCV patients' groups: naïve > asymptomatic > symptomatic 	[86]
Free light chains	<ul style="list-style-type: none"> • FLCs could represent the signature of “dormant” B cell clones' activity indicative of possible relapse or worsening outcome in patients HCV positive with MC after rituximab treatment 	[43]
	<ul style="list-style-type: none"> • HCV-related MC is strongly associated with high levels of FLCs 	[46]
	<ul style="list-style-type: none"> • FLC levels could be used as a diagnostic marker of vasculitis and MC syndrome 	[55]
	<ul style="list-style-type: none"> • FLC levels could be used as a new tool to identify patients requiring urgent access to antiviral treatment 	[55]
	<ul style="list-style-type: none"> • k/λ ratio can be useful to monitor therapy 	[55]
	<ul style="list-style-type: none"> • Serum levels of kFLC have been used to monitor extrahepatic manifestations caused by HCV 	[77]
	<ul style="list-style-type: none"> • Relevance of serological FLC levels in the diagnosis and prognosis pathway of diseases caused by deposition of immunocomplexes and immune-proliferative disorders 	[88]
	<ul style="list-style-type: none"> • In positive HCV patients, the determination of serum FLC can be clinically useful as a predictive index of MC for the diagnostic and prognostic value of the k/λ ratio 	[88]
	<ul style="list-style-type: none"> • High concentrations of monoclonal component k sFLC have been found in HCV patients with type II MC 	[88]
	<ul style="list-style-type: none"> • The alteration of the κ/λ ratio correlates with the clinical conditions of the lymphoproliferative disorder that accompanies the HCV infection 	[88]
Heavy chain	<ul style="list-style-type: none"> • Post-treatment HLC values could be useful for recognizing those patients that could benefit from additional anti-CD20 therapy, with an improvement in patient-tailored treatments 	[43]
	<ul style="list-style-type: none"> • Serum biomarker panel with HLC identifies patients with overt B-NHL associated with MC vasculitis in chronic HCV infection 	[94]
Syndecan-1	<ul style="list-style-type: none"> • Could be used during the follow-up of HCV related MC affected subjects undergoing treatments 	[46]

CGs, cryoglobulins; RF, rheumatoid factor; HCV, hepatitis C virus; ANA, antinuclear antibody; MC, mixed cryoglobulinemia; SjS, Sjögren's syndrome; SLE, systemic lupus erythematosus; AMA, anti-mitochondrial antibodies; ASMA, anti-smooth muscle antibodies; anti-LKM1, anti-liver kidney microsome type 1; RA, rheumatoid arthritis; FLC, free light chains; B-NHL, B-cell non-Hodgkin lymphomas.

IgG3 monoclonal CGs with RF activity which induce extensive extrahepatic manifestations [51]. IgG3 displays many effector functions and is a powerful pro-inflammatory antibody. Human and murine IgG3 shows an equal tendency to self-assemble, despite its structural and functional differences, and plays a role in the molecular mechanism of cryoprecipitation [47].

Currently, little is known about the involvement of HCV infection in the evolution of lymphoproliferative and/or autoimmune diseases, although several factors seem to contribute to their pathogenesis and their progression, and specific IgG subclasses seem to play a role [22]. According to the literature regarding the distribution of IgG subclasses in patients with cryoglobulinemic syndrome and HCV infection, monoclonal IgG1 and IgG3 are often detected [52,53]. In 1998, in patients with chronic HCV infection and cryoglobulinemia who were predominantly asymptomatic, it was observed that cryoprecipitate-derived IgG was relatively enriched in CGs that displayed immunoreactivity to the HCV core protein [54]. The analysis of polyclonal IgG in the cryoprecipitate of these patients showed its non-involvement in the antibody response against HCV because of failed recognition of the amino-terminal subregion of the HCV core protein [54].

Together with the analysis of other markers, such as antinuclear antibodies (ANAs), the occurrence of the IgG3 subclass in the cryoprecipitate of HCV-positive patients was largely confirmed, and the switch of this specific subclass was evaluated on time [22,55,56]. The results showed that IgG3 in cryoprecipitates and ANA could indicate that the immune system is stimulated to a higher degree of activation and that this could represent a trigger factor in the long-term activation of the autoimmune mechanism. Furthermore, there was a strong correlation between the presence of the IgG3 subclass and the prevalence of the finely spotted model of ANA positivity in the HCV IgG3-positive subgroup [56]. Therefore, these biomarkers represent prognostic factors for the onset of autoimmune disease in HCV-positive patients [55].

An increase in IgG3 is also indicative of RF activity against the IgG1-HCV complex, and their self-reactivity can be indicative of stimulation of the immune system due to the persistence of the antigen [56]. The analysis of the role of IgG3 as an antibody subclass strongly associated with immune stimulation allowed the development of a new hypothesis for clonal expansion. In this hypothetical scenario, cryoglobulins are originally formed by two IgG subclasses. First, IgG1 RF recognizes the virus and activates IgG3, which is capable of precipitating immune complexes. The persistence

of the antigenic stimulus provokes the production of polyclonal IgM RF and causes the formation of oligoclonal IgG/polyclonal IgM. In the last stage, a monoclonal IgM RF clone that can coexist with a monoclonal IgG3 RF one is formed. Therefore, the early identification of IgG3 RF in patients with HCV could be used as an early predictor of the development of MC for the assessment for antiviral therapy eligibility and for the identification of increased IgG-RF levels, and the presence of IgG3 could suggest that careful follow-up should be performed for these patients to identify evolution toward an autoimmune or lymphoproliferative disease at an early stage [22].

6.2 Rheumatoid factors

The involvement of IgG-IgM RF CGs in the pathogenesis of vasculitis has been well recognized [11]. In type II and type III MC [10], the IgM component predominantly shows rheumatoid factor activity.

RFs are autoantibodies of different isotypes directed against the Fc portion of IgG that enable them to form immune complexes; this ability is important in the pathogenesis of cryoglobulinemic vasculitis [29]. RF is found in various diseases characterized by alterations of the immune system. Since it was discovered in 1940, RF has been extensively analyzed in association with autoimmune phenomena, as well as in the context of host responses to chronic infections [57]. In fact, the occurrence of an RF response is widely described in different infectious diseases, both as a response to immune complexes formed by the infectious microbes and antibodies and as a consequence of direct infection and stimulation of polyclonal B cells.

In the course of HCV infection, many reports have described cross-reactivity between RF and viruses (acting as antigens) and the continuous activation of RF-IgM or IgG, which is at first oligoclonal and then monoclonal, caused by the persistence of the virus in the host [58]. It is thought that the pathogenesis of type II MC may be an event that takes place following a series of modifications of RF-producing B cells and not as a consequence of a single-step process. Before the typing of IgM RF of type II MC, a transitional state has been observed in HCV patients represented by type III cryoglobulinemia with polyclonal IgM RF, which is sometimes described as a new type II-type III mixed cryoglobulinemia variant [57,59]. Progressive RF-producing B cell expansion and chronic antigen stimulation can determine the development of a neoplastic process such as NHL: after virus eradication, the clonal expansion of B cells also regresses [57,58].

De Rosa et al. observed that the quantification of type II RF allows differentiation of patients who are symptomatic from asymptomatic patients based on major signs of vasculitis. Type II RF cryoglobulins also display a clinical association with cryoglobulinemic vasculitis with a proven pathological role in the palpable purpura and membranoproliferative glomerulonephritis lesions of patients with cryoglobulinemic vasculitis [60,61].

Both the qualitative and quantitative associations of type II RF cryoglobulins with cryoglobulinemic vasculitis appear to occur mainly in HCV-infected patients rather than in non-infected individuals [60]. Stemming from these data, the detection and quantification of type II RF cryoglobulins may ameliorate the monitoring of HCV-infected patients with cryoglobulinemic vasculitis [60].

In type II cryoglobulins, the contribution of IgM and IgM RF to the total protein amount shows a spectrum of different levels and clonality, ranging from cryo-immunocomplexes with RF activity that are largely abundant in serum to an increased frequency of IgM cross-reactive idiotypes [60,62].

Human IgM RFs have been classified into major (WA) and minor (PO) idiotypically cross-reactive families based on reactivity with polyclonal anti-idiotypic antisera. WA are frequent in healthy subjects with no IgG reactivity, probably arising only as the result of somatic mutations in the setting of chronic antigen stimulation [63], and the autoreactive variant may be unique to HCV infection in approximately 80% of cases of MC [64]. Recently, Sansonno et al. reported that B cells isolated from liver areas of HCV-infected patients produced WA, which is a specific monoclonal RF bearing the WA cross-idiotypic that is present in approximately 60% of cases of HCV-related type II MC [65,66]. Antiviral therapy induces a decline in the WA B cells that produce WA monoclonal RF and cryoglobulinemia, in parallel with a decrease in viremia, leading to the hypothesis that the production of WA monoclonal RF is driven by HCV [67]. WA B cells can also be detected in asymptomatic patients with HCV infection; thus, WA monoclonal RF could be a marker to predict the development of cryoglobulinemic vasculitis and non-Hodgkin lymphomas; it may be used as a prognostic marker for this complication that could be monitored to initiate early antiviral therapy if needed [47,57].

Moreover, the occurrence of RF together with a reduction in the C4 fraction of the complement represent serological signs that can be observed in most patients with MC [22]. Furthermore, Basile et al. reported that 100% of serum from ANA-positive HCV-affected patients also shows RF-IgG positivity at 37°C [55].

6.3 Autoantibodies

In recent years, the co-occurrence of CGs and autoantibodies in the course of HCV infection and autoimmune syndromes has been a matter of intense research and debate. These immunological markers play key roles in the progression of these diseases, and the study of their correlation can help elucidate the pathological mechanisms and design specific personalized management during the follow-up of patients. The analysis of a large cohort of patients with primary Sjögren's syndrome (SjS) confirmed the key role of immunological markers such as for CGs in comparison with Ro/La autoantibodies and ANA in the definition of the phenotype of primary SjS at diagnosis [68]. Analyzing sera from systematic lupus erythematosus (SLE) patients, it has been observed that the presence of CGs is correlated with positive anti-ds DNA and low CH50 but seems not to predict the activity of the disease [69]. Detection of immunological phenomena, such as the occurrence of circulating autoantibodies in HCV-related MC patients, has been extensively performed and characterized. A considerable body of evidence suggests that HCV is involved in the breaking of tolerance to self-antigens and thus in triggering autoreactivity.

Serum autoantibodies are commonly found in approximately one-third of all HCV infection cases as a direct result of the transformation of infected B cells and as an indirect consequence of chronic stimulation of a limited pool of self-reactive B cells by the virus [29,70].

The characterization of autoantibodies in the cryoprecipitate of subjects with rheumatoid arthritis (RA), with or without HCV infection, was carried out to analyze the association between HCV and the evolution of autoimmune disease. In RA patients with HCV infection, ANAs were detected particularly in the cryoprecipitate (82%) compared to the supernatant (48%). In contrast, the detection of anti-mitochondrial, anti-smooth muscle, anti-parietal, and anti-liver-kidney microsomes (anti-LKM1) autoantibodies yielded negative results, unlike in RA patients without HCV. Analyzing the pattern of positivity, the coarsely spotted pattern was completely absent in HCV-positive patients, while the finely spotted pattern was mainly expressed. The increased frequency of a less specific pattern in HCV patients could be linked to a nonspecific antibody response, probably due to the persistent antigenic stimulus of the virus [55]. Furthermore, these data suggest that the presence of ANA is closely related to CG production [56].

HCV-related MC represents an ideal model to understand the complex interactions between autoimmunity and oncogenesis, and epidemiologic

studies of HCV-naïve patients suggest the opportunity of more accurate and frequent testing of circulating CGs in HCV-positive patients because it would be useful to detect new cases with a possible risk of evolution to more severe diseases [56].

In HCV-related MC, the antigenic stimulus represented by HCV as well as the occurrence of RFs with dual specificity for viral polypeptides and IgG3 could play a role. Nevertheless, the occurrence of oligoclonal IgM in CGs at low concentrations has been previously reported [46]. IgM oligoclonality represents a transitional stage from polyclonal to monoclonal, and its production may be induced by the persistence of IgG-HCV immune complexes. Indeed, it has been shown that immune complexes stimulate T-cell-mediated activation of B cell synthesis of rheumatoid factor, leading to affinity maturation and clonal selection, which, in time, results in oligoclonal and subsequent monoclonal expansion of an RF-synthesizing clone [54].

6.4 Complement

The complement system connects innate to adaptive immunity against microbial infections, and viral infection represents a trigger for its activation. It is also a critical effector of the clearance of immune complexes and damaged cells. In fact, the complement system produces important mediators of inflammation that play central roles in rheumatic and autoimmune diseases and are mostly associated with cryoglobulins. Clinical and experimental evidence highlight complement-mediated mechanisms in intra- and extrahepatic manifestations of HCV infection, such as liver fibrosis and type II cryoglobulinemia [71]. Viral infections can trigger dysregulation for direct effects on the complement components (immune escape) and for interactions with specific antinuclear antibody detection in cryoprecipitates, such as distinctive patterns in hepatitis C virus-infected patients' membrane receptors used for entry into cells or promoting the production of RF immunity as part of chronic stimulation [62].

The first subcomponent of the C1 complex of the classical pathway of complement activation is C1q. Several functions have been assigned to C1q, which include antibody-dependent and independent activity, and are mediated by C1q receptors on the effector cell surface [72]. C1q is part of a cascade of activation through the classic pathway, which in turn leads to the production of anaphylatoxins (C3a and C5a), chemotactic factors and inflammatory mediators. C3a and C5a are the products of the cleavage of

the corresponding complement components that increase vascular permeability and induce vasodilation through the release of histamine from mast cells. For these reasons, altered regulation is often associated with chronic autoimmune inflammatory conditions, such as systemic lupus erythematosus, cryoglobulinemia and rheumatoid arthritis. Sansonno et al. observed that circulating C1q mRNA levels are much higher in HCV-MC patients than in HCV patients without MC or healthy controls, and a positive correlation between circulating C1q with RF activity and C1q levels in HCV-MC patients was found, suggesting the involvement of C1q in the pathogenesis of HCV-induced autoimmunity.

In 1967, the occurrence of C1q in cryoprecipitates isolated in patients with lupus nephritis was demonstrated [73]. Recent studies have confirmed that the cryoprecipitates in patients with CG associated with HCV infection consist of C1q [74] as well as from antibodies to HCV antigens [75] and viral core protein [62,74].

Low levels of C4 have been observed in patients with cryoglobulinemia that, together with RF seropositivity, contribute to the diagnosis of this syndrome. This selective depression of C4 implies the activation of the classical complement pathway in the formation of cryoglobulin. This is related both to the complement system activation, with C4 binding to immune complexes, and to the genetic polymorphisms of C4 in these patients [29]. Recently, Basile et al., who studied different HCV-positive populations (naïve without CGs; MC asymptomatic patients with low levels of CGs; and MC symptomatic patients with high levels of type II CGs), observed that the C4 levels progressively decreased among patient groups (naïve > asymptomatic > symptomatic) [46]. The detection of low C4 levels together with the assessment of low levels of CGs may represent a biomarker of this activation.

6.5 Free light chains

Free light chains (FLCs) are physiologically produced in excess during the synthesis of Ig by activated B lymphocytes. Of the physiological light chains produced, only 60% are incorporated into Ig molecules, while the remaining 40% are released in the circulation as polyclonal (p) FLCs. FLCs are normally monomeric, while λ FLCs tend to be dimeric, joined by disulfide bonds; however, higher oligomeric and polymeric forms of both light chains may occur [76]. Monomeric FLCs, characteristically κ , are cleared in 2–4 h at 40% of the glomerular filtration rate. Dimeric FLCs of

approximately 50 kDa, typically λ , are cleared in 3–6 h at 20% of the glomerular filtration rate [77]. Serum concentrations of FLCs depend upon the balance between production by plasma cells and renal clearance. When there is increased polyclonal immunoglobulin production and/or renal impairment, both free κ and λ chains may increase up to 30–40-fold. However, the κ/λ ratio remained unchanged or only slightly increased [77]. Thanks to their short half-life, and in the condition of normal kidney function, their serum levels can be considered a direct marker of B lymphocyte activity, which is otherwise difficult to measure in routine clinical practice [77]. Until a few years ago, clinical interest in FLCs was limited to the measurement of monoclonal FLCs for the management of patients with plasma cell dyscrasias, and their determination was added to the criteria for diagnosis and monitoring of these dyscrasias [78].

In recent years, a deep analysis of pFLCs in the course of different diseases has been performed, and it has been widely demonstrated that during inflammatory pathologies, their circulating levels increase significantly, and they play an active role in the pathogenetic mechanisms of inflammatory processes [79]. Therefore, pFLCs are not simply considered a waste product of Ig synthesis but effective bioactive molecules that exhibit enzymatic activity and are able to bind specifically to intracellular and extracellular proteins and establish cellular interactions [80–82]. In fact, it has been shown that they actively participate in inflammatory processes thanks to their direct link to mast cells and neutrophils, triggering their activation [77,83–85]. Their contribution in the course of autoimmune diseases is also crucial, as they represent the expression of different B-cell-activated clones; for this reason, they could be used both as prognostic markers of disease severity and/or to evaluate disease recurrence and to monitor the activity of these pathologies and treatment efficacy [38,86,87]. The close dependence with B cell activity allows us to speculate on the presence of a correlation between their concentration and the presence of CGs. In the case of patients with HCV infection, abnormalities in serum FLC levels and in their ratio suggest polyclonal B-cell activation induced by the virus. Different reports and guidelines have demonstrated the relevance of serological FLC levels for the diagnosis and prognosis of diseases, caused by the deposition of immunocomplexes and immunoproliferative disorders, and for monitoring the therapeutic outcomes [77,88]. Therefore, in HCV patients, the assessment of serum FLCs can be clinically useful both for early diagnosis and monitoring of B-cell lymphoproliferative disorders and as a predictive index of MC for the diagnostic and prognostic values of the κ/λ ratio [56,88].

High concentrations of free κ chains have been found in HCV-positive patients with type II MC, which is characterized by a monoclonal component with the κ light chain [88], and their serum levels have recently been used to monitor extrahepatic manifestations caused by HCV [77]. FLC quantification could be used both as a diagnostic marker of vasculitis/MC syndrome and as a new tool to identify patients requiring urgent access to antiviral treatment [41].

The alteration of the κ/λ ratio is positively correlated with the severity of the clinical conditions associated with lymphoproliferative disorders following HCV infection [88], from vasculitis to NHL, and represents an early index of activation of the lymphoproliferative mechanism during follow-up. Moreover, the κ/λ ratio can be useful for monitoring therapeutic outcomes thanks to the predictive role of FLCs because the FLC ratio falls within the reference limits during treatment with specific antiviral drugs [41]. The analysis of sera from a larger group of HCV-MC patients in comparison to HCV-negative patients with a systemic autoimmune disorder confirmed that HCV-MC is associated with high levels of serum FLCs comparable to those in autoimmune disorders, underlying the strong correlation with B-cell hyperactivity in both diseases [89].

FLCs could represent the signature of “dormant” B-cell clone activity and be very useful for identifying the minimal residual disease indicative of a possible relapse or worsening outcome in HCV-MC patients after rituximab treatment, despite positive (complete + partial) clinical and laboratory responses of 85% and 50%, respectively, suggesting that a better clinical endpoint for drug administration could be widely useful [42].

6.6 Heavy chains

The production of antisera binding the junctional regions between κ or λ light chains and their respective heavy chain partners allows the specific measurements of serum heavy/light chain (HLC) pairs: IgG- κ and - λ , IgA- κ and - λ , and IgM- κ and - λ [90]. The generation of IgG κ /IgG λ , IgA κ /IgA λ , and IgM κ /IgM λ ratios (HLC or HLC ratios) allows the evaluation of clonality similar to FLC κ/λ ratios.

In samples with increased levels of IgM κ , a typical trend to suppress the uninvolved IgM λ isotype emerged that never occurred in cases of uninvolved IgM κ [91]. Abnormal IgM κ /IgM λ ratios have been reported to be associated with poor outcomes in patients with B-cell non-Hodgkin lymphoma [92,93]. Because of the increased risk of B-NHL in patients with

HCV-related MC, it is mandatory to determine sensitive and specific biomarkers for prediction. A serum biomarker panel with HLC can identify patients presenting with overt B-NHL associated with mixed cryoglobulinemia vasculitis in chronic HCV infection, requiring invasive explorations to demonstrate the presence of malignant lymphoma [94]. The correlation between post-treatment HLC values and clinical and laboratory responses demonstrated their reliability as markers of clonality and their utility in the evaluation of MRD and therapeutic response to RTX. Their employment could be useful for recognizing patients who could benefit from additional anti-CD20 therapy, with an improvement in patient-tailored treatments [42].

6.7 Syndecan-1

Circulating syndecans have been reported in several cancer types and may represent a new therapeutic target. Syndecan-1 (CD138) is a transmembrane heparan sulfate [95]. On the cell surface, CD138 interacts with growth factors and integrin receptors, regulating cell behaviors including adhesion, invasion and signaling [96]. CD138 can be cleaved from the surface of the membrane by proteases (heparanases), which release the bioactive extracellular portion of the molecule [96]. Cell membranes constitutively release low levels of CD138, although the process is significantly accelerated in response to growth factors, chemokines, bacterial toxins, insulin, and cellular stress [97]. The release of soluble CD138 into circulation occurs at high levels in some pathological states, such as inflammation or microbial infections, and in tumors [98]; in these conditions, CD138 amplifies the signal of cell growth factors, thus supporting tumor growth. Serum circulating CD138 levels are reliable prognostic markers for some cancers, such as lung cancer and myeloma, that display high serum levels of CD138 and are correlated with a negative outcome [99]. Increased sCD138 was associated with a poor outcome in myeloma and may be considered a reliable prognostic factor at different phases of the disease [98].

In HCV infection, sCD138 is the major receptor protein for virus attachment to the hepatocyte surface [100], and its detection could represent a precious tool to be used during the follow-up and monitoring of HCV-infected MC patients following treatments [101]. Moreover, serum CD138 represents a non-invasive alternative to biopsy to estimate liver fibrosis [102]. Chronic HCV infection is also characterized by the possible development of extrahepatic manifestations linked to clonal B-cell expansion leading to

MGUS, MC and B-NHL. A new biomarker score has been recently developed that includes FLCs, protein electrophoresis and serum CD138 and is able to follow ingravescent disease status toward the main line of progression to cancerous conditions; this score represents a valuable and useful tool in the management of screening and/or follow-up [101]. The lack of correlation between sCD138 and FLCs suggests that sCD138 reflects the clinical stage of disease and the clinical course or progression. It is conceivable that these molecules identify patients with high tumor burdens, while the FLC assay could suggest a major improvement in monoclonal gammopathy detection and monitoring [103].



7. Mixed cryoglobulinemia and B cells

HCV-induced B-cell dysregulation probably is due to an indirect process arising from chronic antigenic stimulation of a limited pool of pre-existing autoreactive B cells; the continued stimulation of HCV is necessary for abnormal B-cell lymphoproliferation, as eradication of HCV typically results in resolution of both HCV-related MC and NHL [104]. Clonal B-cell populations are present in the liver and peripheral blood of HCV-MC patients [105]. Most MC patients display clonal CD21^{low} B cells expressing a BCR encoded by the V_h1–69 and V_k3–20 heavy and light variable genes. This BCR is endowed with RF activity responsible for cold-precipitable immune complexes and is putatively directed to an HCV antigen; however, the inciting viral antigen remains elusive. In most cases, the normal B-cell pool is replaced by B cells displaying decreased expression of CD21 (CD21^{low} B cells), the CR2 complement receptor [106].

Because CD21 augments B-cell receptor (BCR)-mediated signaling as part of the B-cell co-receptor complex, its downregulation may confer a state of relative anergy to these cells, as has been demonstrated among CD21^{low} naïve B cells from patients with chronic variable immunodeficiency and rheumatoid arthritis [107].

CD21^{low} B cells occurring in MC patients resemble the CD21^{low} B cells found in other immunological and chronic infectious diseases based on a B-cell hyperactivation. The expression of CD11c, a typical marker of dendritic cells, represents the common trait. CD21^{low} CD11⁺ B cells also look like a population of murine B cells recently identified in aged mice (ABCs) that seem to play a role in autoimmunity and infections. Phenotypically, expanded CD21^{low} B cells observed in MC patients are similar to marginal zone (MZ) B cells by the co-expression of IgM and CD27 and by increased

expression of CD11 and CXCR3, indicating preferential migration of CD21^{low} B cells to inflammatory sites. Instead, other trafficking receptors such as CCR7, CD62L, CXCR4, and CXCR5 that are required for migration to lymph nodes or germinal centers are downregulated. The *in vitro* stimulation of BCR, even after co-stimulation with CD40, causes reduced activation of CD21^{low} B cells and increased apoptosis. This attenuated response might be the result of an increased expression of several inhibitory receptors such as CD22, FcγRIIB (CD32b), CD72, CD85j, CD85k, CD95, LAIR-1, Siglec-6 and Fc receptor-like 4 (FCRL4), an inhibitory receptor that was first described in a subset of human tonsillar B cells with low CD21 expression [108–110].

CD21^{low} B cells resemble a tissue-like memory B-cell subpopulation identified in the blood of HIV-viremic individuals, defined as “exhausted” rather than anergic and characterized by high expression of multiple inhibitory receptors and a low rate of proliferation in response to B-cell stimuli [111]. In addition, CD21^{low} B cells appear to be anergic as they fail to flux calcium upon B-cell receptor triggering, display high constitutive expression of the active phosphorylated form of extracellular signal-regulated kinase (pERK) and are prone to mortality by apoptosis. These functional signatures usually characterize murine B cells that are made anergic by continual BCR engagement by an antigen. In accordance, dysregulated expression of apoptosis and anergy-related genes has been found in MC clonal B cells, and high expression of DEC1/STRA13, a negative regulator of B-cell activation, seems to be a common trait.

As in murine models, the features of anergy in MC B cells may be reverted by disengagement from the antigenic stimulus. Therapeutic eradication of HCV, achieved with direct acting antivirals (DAAs), has offered the unique opportunity to analyze the functional properties of B cells without affecting the immunomodulatory circuit typical of interferon-based therapies. It was shown that the anergic features of clonal B cells rapidly reverted after disengagement from HCV as pERK overexpression and accelerated apoptosis occurred, while phenotypic and functional features of exhaustion persisted in the patients’ peripheral blood after a viral-sustained response for several months [112]. The clonal exhausted B-cell population in MC survived despite clearance of the chronic antigen stimulation provided by HCV. The persistence of B-cell clones was not correlated with serum CGs or response to therapy or relapse of vasculitis after viral eradication, suggesting that other extracellular signals might be responsible for their survival. The possibility that rheumatoid factor-bearing monoclonal B cells in

MC might be reactivated by circulating immune complexes could explain why a proportion of MC patients experience vasculitis relapse after eradication of HCV in the course of infections or tumors [112]. B-cell clones persist in MC vasculitis patients long after HCV infection has been cleared but halt the production of pathogenic antibody [113]. These “dormant” cells may be reactivated by events that perturb B-cell homeostasis and can give rise to the relapse of cryoglobulinemic vasculitis. The hypothesis of a switch to a non-pathogenic state is supported by the phenotypic and functional changes that take place in clonal B cells once HCV is cleared [113]. In fact, we provide evidence that after antiviral therapy, the predominantly CD21^{low} clonal B-cell population is gradually substituted by a clonal population mostly made up of CD21 high cells lacking the peculiar array of homing and inhibitory receptors typical of CD21^{low} B cells. These cases suggest that abundant immune complexes produced during infection might reactivate B-cell clones, leading to the relapse of MC [113]. IgG subclasses in the form of immune complexes circulating could be the autoantigenic trigger responsible for the survival and reactivation of B-cell clones beyond HCV.



8. Therapeutic strategies for mixed cryoglobulinemia

MC develops from circulating immune complexes that can deposit in small vessels, leading to complement activation and leucocyte recruitment, and, ultimately, clinical vasculitis involving the kidneys, joints, skin, and sometimes also the peripheral nerves; up to 90% of cases are associated with HCV infection. HCV binding to the surface of B cells induces clonal memory B-cell proliferation, reducing the activation threshold and causing widespread autoantibody production, mostly of IgM with RF activity toward anti-HCV IgG. Considering the pathogenesis of MC, different therapeutic strategies have been developed in recent years and are available for patients suffering from this vasculitis [114,115]. Therapeutic approaches may be etiologic (the eradication of viral infection conditioning the response of the immune system) and/or pathogenetic (reducing the abnormal response of the immune system of the host). In HCV-related MC, the main clinical goal must be early eradication of HCV to avoid organ complications and manifestations of lymphoproliferative diseases. After the introduction of the new DAAs, remarkable 90–100% eradication rates have been reported already after 2–3 weeks of treatment, regardless of HCV genotype, even if clinical improvements in vasculitis can be observed in only half of the patients [116,117]. The shift toward a therapeutic pathogenetic approach with

immunosuppression, including therapies that target B-cell expansion, may occur in patients displaying persistent B-cell proliferation and impairment or relapse of renal function also after a sustained virologic response (SVR) with DAAs [118]. The persistence of MC despite viral eradication after successful DAA treatment suggests the presence of a monoclonal, autonomous, B-cell clone. In these cases, rituximab seems to be the best option as an anti-human CD20 monoclonal antibody targeting B cells for reducing disease activity and preventing vasculitis flares. In addition, plasma exchanges to remove cryoglobulins may be indicated in severe cases [115]. The development of a dangerous immune complex-mediated glomerulonephritis represents worsening evolution of the immunologic disorder initially triggered by HCV infection [119] that could impair renal function. In this setting, the evaluation of a minimal residual disease in rituximab-treated patients with MC through the assessment of FLCs, heavy/light chains pairs or vascular endothelial growth factor (VEGF) may be crucial to improve the prognosis [42].



9. COVID-19-induced vasculitis: A model resembling HCV-related MC

Coronavirus disease 2019 (COVID-19), caused by SARS-CoV-2 infection, represents an ongoing public health emergency. COVID-19 in combination with other diseases could be dangerous for a patient's life. More knowledge about the pathophysiology of COVID-19 is considered necessary for the overall understanding of its immunopathogenic mechanisms. Given the several inflammatory and immunopathological complications in COVID-19 patients, we explore some considerations regarding the potential link between MC and COVID-19. Roncati et al. reported scientific evidence that in COVID-19, vasculitis indicates that a life-threatening escalation and switch from humoral immunity (type 2 T-helper immune response) to type 3 hypersensitivity has taken place. The deposition of immune complexes inside the vascular walls could induce a severe inflammatory state with the consequent inflammatory amplification ascribed as cytokine storm syndrome [120]. Type 3 hypersensitivity represents an immunopathogenic mechanism of disease that involves immune complexes deposition inside tissues and blood vessels, inducing a severe inflammatory state by the action of complement anaphylatoxins (C3a and C5a) that ultimately leads to tissue damage. The final effect of this process in the walls of the blood vessels is "leucocytoclastic vasculitis" or "hypersensitivity vasculitis," which is characterized by neutrophilic infiltration, kariorexis

and fibrinoid necrosis [120]. SARS-CoV-2 binds to its receptor, the angiotensin-converting enzyme 2 (ACE2) receptor. ACE2 receptors are largely expressed by endothelial cells, and direct viral infection of the endothelial cells has been demonstrated *in vivo* [121]. Moreover, post mortem histological analysis of different tissues has revealed the presence of diffuse endothelial inflammation in several organs [121]. It can therefore be assumed that vasculitis (developing from an initial endotheliitis) may be a direct consequence of COVID-19 and an indirect consequence of the altered immune response that is observed during SARS-CoV-2 infection. This pathogenetic model of virus-triggered damage (that, in the end, evolves independent of the virus itself) resembles HCV-related MC vasculitis [122]. The prognosis and worsening evolution are strong dependent on endothelial-induced damage to vital organs and on comorbidities associated with underlying diseases.



10. Conclusion

The immune system continuously faces endogenous and exogenous antigens; however, even when it is compromised, it will still endeavor to maintain tolerance to self; in this context, CG formation could represent an aberrant response of the adaptative immune system to different degrees of disorders (Fig. 3). Molecular identification of the biomarker complexes of self-directed inflammation in the MC disease process will further provide a better understanding of pathogenesis and drug treatment in the biologic drug era. In asymptomatic subjects, biomarkers allow early diagnosis of MC, and in the course of MC disease, biomarkers are useful to confirm malignant and benign disease stages. In the diagnosis of HCV-related NHL disease, biomarkers may have predictive value and be useful in choosing targeted treatment strategies.

The need to identify reliable biomarkers able to predict the progression of HCV-associated MC to NHL is an important aim. In this scenario, free light chains in HCV-associated MC, RF and IgG3 subclasses show predisposing factors involved in the development of cryoglobulinemia. Nevertheless, many aspects remain unsolved. Elucidation of the mechanisms and predisposing factors involved in the development of cryoglobulinemia remain the “dark side” of future research. The characterization of immune phenotypes associated with treatment efficacy is crucial for the perspective of personalized therapy for MC and other autoimmune disorders.

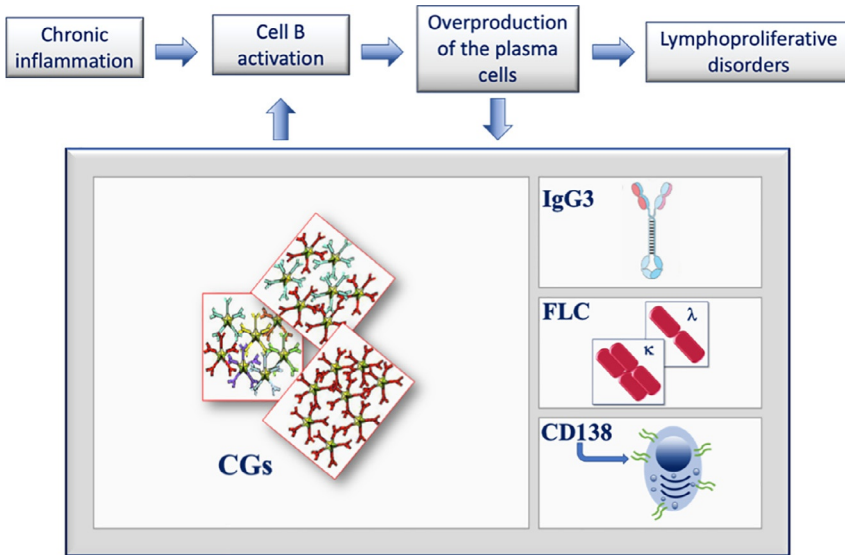


Fig. 3 Activation and consequences of adaptative immune system responses.

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Competing interests

The authors have no conflicts of interest to disclose.

Ethical approval

The ethics committee of the “Fondazione Policlinico Universitario A. Gemelli I.R.C.C.S., Roma” approved this study.

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