Mediterranean Journal of Hematology and Infectious Diseases

Review article

Cryoglobulin Test and Cryoglobulinemia Hepatitis C-Virus Related

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Competing interests: The authors have declared that no competing interests exist.

Abstract. Cryoglobulins are immunoglobulins that precipitate in serum at temperatures below 37°C and resolubilize upon warming. The clinical syndrome of cryoglobulinemia usually includes purpura, weakness, and arthralgia, but the underlying disease may also contribute other symptoms. Blood samples for cryoglobulin are collected, transported, clotted and spun at 37°C, before the precipitate is allowed to form when serum is stored at 4°C in a Wintrobe tube for at least seven days. The most critical and confounding factor affecting the cryoglobulin test is when the preanalytical phase is not fully completed at 37°C. The easiest way to quantify cryoglobulins is the cryocrit estimate. However, this approach has low accuracy and sensitivity. Furthermore, the precipitate should be resolubilized by warming to confirm that it is truly formed of cryoglobulins. The characterization of cryoglobulins requires the precipitate is several times washed, before performing immunofixation, a technique by which cryoglobulins can be classified depending on the characteristics of the detected immunoglobulins. These features imply a pathogenic role of these molecules which are consequently associated with a wide range of symptoms and manifestations. According to the Brouet classification, Cryoglobulins are grouped into three types by the immunochemical properties of immunoglobulins in the cryoprecipitate. The aim of this paper is to review the major aspects of cryoglobulinemia and the laboratory techniques used to detect and characterize cryoglobulins, taking into consideration the presence and consequences of cryoglobulinemia in Hepatitis C Virus (HCV) infection.

Keywords: Cryoglobulin, HCV, Cryoprecipitate.

Citation: Gulli F., Santini S.A., Napodano C., Bottoni P., Pocino K., Rapaccini G.L., Basile U. Cryoglobulin test and cryoglobulinemia Hepatitis C-Virus related. Mediterr J Hematol Infect Dis 2017, 9(1): e2017007, DOI: http://dx.doi.org/10.4084/MJHID.2017.007

Published: January 1, 2017 **Received: October 1, 2016** **Accepted: December 12, 2016**

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Easy Definition and Classification. Cryoglobulins (CGs) are immunoglobulins (Igs) which undergo reversible precipitation or gelling when exposed to temperatures below 37°C and re-dissolve upon re-warming.

They were first described in a patient with Multiple Myeloma (MM),[1] but the term "cryoglobulin" was coined later on to describe the phenomenon of cold-precipitable serum Igs.[2] CGs remain soluble when specific conditions are applied, mostly dependent on temperature. The specific reason for their cold-insolubility is still unclear and may depend on a variety of factors, although low temperatures seemingly trigger reversible cryoprecipitation, possibly by inducing steric modifications in the molecules which can

return to the initial conformation at 37°C.[3,4] These features imply a pathogenic role of these molecules which are consequently associated with a wide range of symptoms and manifestations.[5,6,7]

In 1974 Brouet *et al*. proposed a classification which correlated the immunochemical characteristics of cryoglobulinemias with clinical features of patients.[5]

Type I monoclonal CGs are due to a lymphoproliferative process and almost exclusively found in the context of malignancies such as multiple myeloma and Waldenström macroglobulinemia. Their precipitation at low temperatures may be due to variations in amino acid composition or carbohydrate content of involved monoclonal Igs.

Type I cryoglobulinemia patients present a prevalence of clinical signs typical of the underlying lymphoproliferative disease, so cryoglobulinemia is often a casual encounter; nevertheless, vascular occlusion in association with hyperviscosity syndrome and purpuric/dystrophic lesions of the skin (usually affecting the lower limbs) are not uncommon findings.

Type II and type III have generally mixed CGs (MCs), the precipitation phenomenon being due to interactions occurring between involved Igs other than to specific characteristics of single Igs themselves. The IgM component of MCs has rheumatoid factor (RF) activity.[8] This system is still widely accepted since it offers good correlations between the associated disease state and clinical manifestations, although other authors have described the presence of atypical CGs, also in the serum of HCV infected patients.[8,9]

The employment of more sensitive methodologies such as immunofixation, immunoblotting, and two-dimensional polyacrylamide gel electrophoresis have enabled the identification of patients affected by microheterogeneous CGs. The concept of microheterogeneity is an innovative taxonomic element that consists in the presence of two or more oligoclonal bands in MCs. This form is considered as an intermediate stage between type II and type III cryoglobulinemia.[10,11,12]

In 1997, a novel immunochemical profile was described. It was observed in a patient affected by Gougerot-Sjogren syndrome, which consisted of a biclonal IgM component and a polyclonal IgG.[13]

The authors, therefore, suggested a further division of the type II group of CGs into the following two subgroups:

- Type IIa: characterized by the presence of only one monoclonal component
- Type IIb: characterized by the presence of several monoclonal components

In another study, the definition of class IIb CGs to oligoclonal CGs previously described was extended.[14] As a whole, technological progress has allowed CGs typing in a more specific and sensitive manner: as a result, Brouet's classification has been completed although without substantial modifications (**Table 1**).[15,16]

- Type III comprises immune complexes containing polyclonal rheumatoid factor (RF), but does not show a monoclonal component.

HCV could play an important role both in the induction and persistence of cryoglobulinemia, as well as affecting the evolution of such a condition from type III CG to type II CG as has been confirmed by other authors as well.[7,17,18]

The inclusion of new subtype of CG, transitional step between type II CG and type III

Table 1. Brouet reclassification**.**

Type	I	II	III
Frequency	25-30%	25%	50%
Clonality	Monoclonal Ig	One or more monoclonal Igs + polyclonal Igs	Polyclonal Igs Oligoclonal Igs + polyclonal Igs (microheterogeneous)
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

Modified from Passerini G. et al.[16] Abbreviation: Ig: Immunoglobulin; FLCs: Free Light Chains.

CG, represents progress towards greater attention on clinical, histopathological and follow-up aspects as well as on a more adequate diagnostic and therapeutic indications, which differ along each and every moment of the evolutionary pathway of the disease. For this reason, and in light of current knowledge, the CGs classification may be integrated into the Brouet classification.[16]

Molecular Basis of Cryoprecipitation. The solubility of a protein depends on numerous factors such as the concentration, the temperature, the pH, the ionic strength of the solution and the net charge that depends on the amino acids and residues from the carbohydrate content. However, the biochemical mechanisms at the basis of this process are not fully understood.[4]

Cryoprecipitation in type I CG can be considered a simple phenomenon of solubility that is derived from the unfavorable interaction between CGs and solvent at low temperatures.[19] The aggregation is often the result of electrostatic interactions, which in turn depend on the structural characteristics of CGs as an altered glycosylation with reduction in sialic acid content.[4,20] Levo assumed that the existence of an impoverishment of sialic acid would make immunogenic Igs favor cryoprecipitation, especially during persistence and intense immune stimulation. This stimulation would lead to the manifestation of a secretory defect with production of Ig without sialic acid.[21] The absence of sialic acid in the structure of Ig generates an immune response to the epitope exposure before being masked; the immune complex thus formed would acquire the capacity to precipitate. The author extended his theory by assuming that hepatocellular damage favoring the permanence of Ig without sialic acid reduced the capacity of hepatocyte deputies to remove them.[21]

Cryoprecipitation in monoclonal CGs appears to be characterized by particular amino acid sequences that may create a structural change at the level of the quaternary structure of the protein, causing an autoaggregation. Thi phenomenon starts with a slow phase (lag phase) and the formation of small aggregates of monoclonal Igs followed by rapid and extensive aggregation, due to a combination of weak non-ionic and hydrophobic interactions which culminate in the precipitation.[22] A study of IgG CGs structure showed that it can produce amorphous, gelatinous and or crystalline precipitates.[23] Most of the factors that influence the cryoprecipitation of monoclonal CGs are also present in MCs, where, however, the lag phase is absent. MC precipitation is the consequence of the rapid and progressive increase in the size of IgG-IgM immune complexes at low temperatures also in the absence of lag phase. MCs also show typical biological properties of immune complexes, such as the ability to activate complement.[24]

Rheumatoid Factor. MCs are immune complexes that contain the rheumatoid factor (RF), although the cause that induces a shift to abnormal proliferation of a single clone of B cells that produces monoclonal IgM-κ RF is not clearly understood. The RF is a monoclonal or polyclonal IgM, although other Ig may be found.[10] There is a higher prevalence of Immunoglobulin G3 (IgG3) responses to HCV antigens in those patients who are HCV- and MC-positive rather than in those who are HCV-positive and MC-negative.[41] IgG3 fixes complement most efficiently among the subclasses, thereby leading to activation of the classical pathway.[41] The presence of IgG3 in cryoprecipitates of HCV- and ANA-positive patients constitutes the decisive factor for the possible activation of autoimmune mechanisms over the long term.[30] In addition, IgG3 positive patients are also positive for IgG-RF [30], known to be autoreactive clones and their capacity to activate several cell clones is confirmed by many clinical studies.[42,43] Thus, the presence of IgG3 in cryoprecipitates may suggest a more highly activated immune system, which is then more exposed to the mechanisms of autoimmune diseases. These markers may constitute a prognostic factor for autoimmune diseases in HCV-affected individuals, as opposed to ANA-negative and IgG3- and IgG RF- negative subjects.[30] In naïve, asymptomatic CG- and HCV-positive patients, the presence of IgG RF and serum free light chains suggests their use as biomarkers, in order to identify the transition between a silent state of probable autoimmune lymphoproliferative disease and frank illness. The possibility of identifying subpopulations among HCV-positive patients may open new scenarios to targeted treatment strategies in extremely early phases (sub-clinical).[44]

Laboratory Testing for the Detection and Typing of CGs. The laboratory workup for

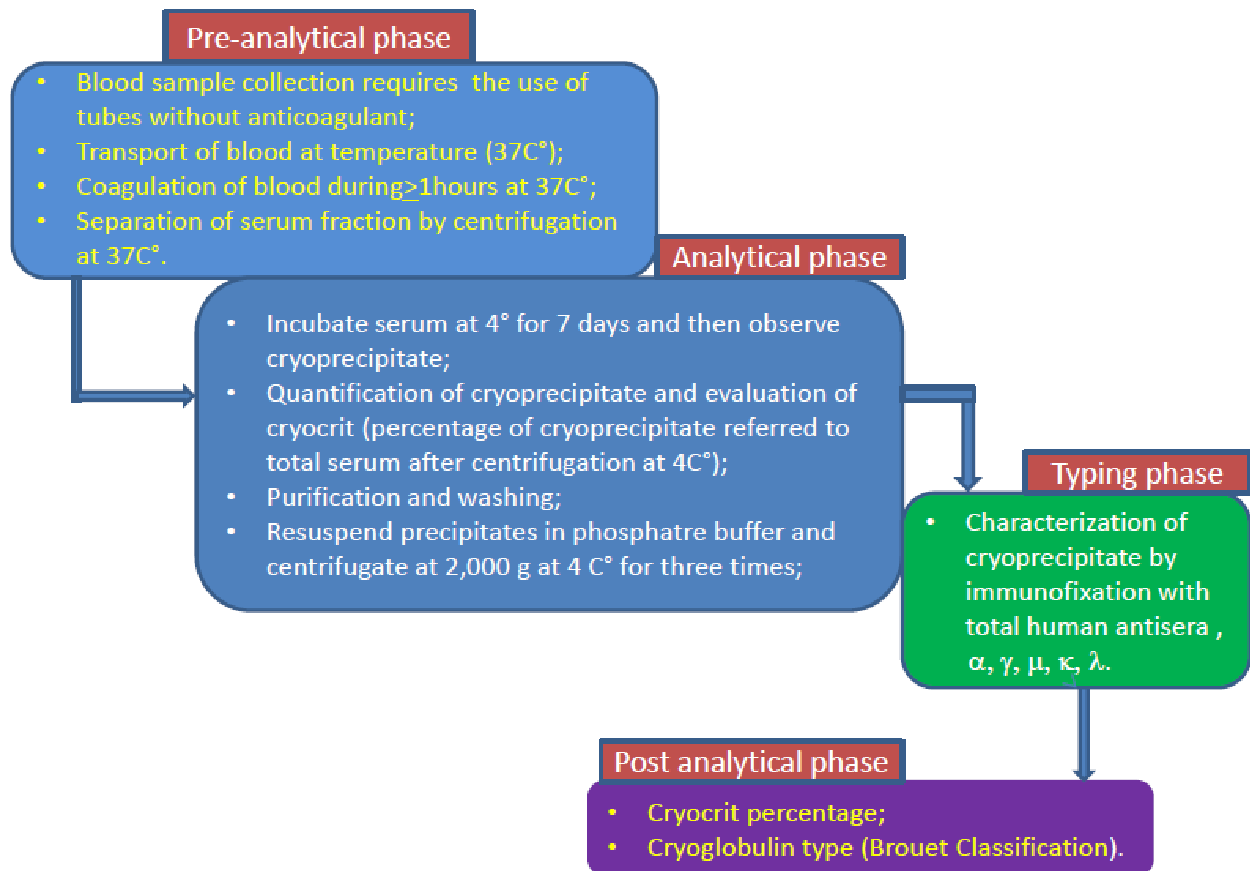

Figure 1. Protocol defines strict requirements for the pre-analytical phase of blood collection, analytical phase, typing phase and post-analytical phase.

detection and typing of CGs can be divided into 3 phases: the preanalytical phase, the analytical phase, and characterization of the cryoprecipitate phase (**Figure 1**).

Several methods have been proposed to reduce precipitation time.[45,46] One of these is based on a quick spectrophotometric measurement, at 350 nm, of serum immunocomplex aggregates that form at 10°C. A critical evaluation of such results has allowed us to propose a "functional" classification: "fast" CGs: positive results obtained with the rapid test, as well as with the traditional test; "slow" CGs: only the traditional assay shows a positive result.[46]

Detection by flow cytometry of CGs is sensitive, specific and fast. Detected CGs in positive serum by the conventional method were derived from patients suffering from autoimmune diseases potentially associated with low level of CGs.[45]

The determination of circulating CGs has been performed with these two methods, and the utility of the rapid test might be considered in the therapeutic management of cryoglobulinemic patients.

Preanalytical Phase: Several recent reports have stressed the need for international guidelines concerning CGs detection, particularly in sample handling and transport procedures, and exhorted the necessity of standardized protocols for global harmonization.[4,6,7,10,21,47] This potentially life-threatening condition requires appropriate laboratory testing, especially for those patients showing clinical symptoms associated with such a condition. The required tests, while feasible with a simple biochemical quantification, need strict pre-analytical protocol adhesion to maintain the sample at a stable temperature of 37°C, especially throughout the initial steps. Failure to ensure these critical conditions from sample collection may result in cryoglobulin misdetection, to the detriment of the patient. The difficulty in ensuring the appropriate preanalytical condition and the variability in following the procedure can entail a heterogeneity of results, thus substantiating the perceived difficulty of performing this relatively

simple test. As a result, this analysis is often neglected by clinicians, despite its usefulness in patient management.[4,47] However, CGs detection still requires global harmonization, and there are yet no internationally accepted standard protocols, although several approaches have been described and proposed.[10,16] Most authors agree about the necessity of keeping the sample at 37°C following collection and transportation, as well as throughout clotting and during the initial centrifuging stages. Sample collection is the most critical phase, and improper collection and transport accounts for the most common reasons for undetected cryoglobulins and false-negatives.[10,48] Samples should be kept warm from the moment of collection, but not kept in hand, to prevent contamination. Up to now, a cost-effective transportation means has not yet been validated, although a variety of systems has been proposed. There is also a great need to attain strict hygienic procedures (roughly 80% of Mixed Cryoglobulinemia (MC) affected patients are HCV-positive) so a disposable system would offer an ideal compromise.[48]

Sample Collection: Blood sample collection requires the use of tubes without anticoagulant, which should be kept at 37°C both before and after sample collection for at least 30 min until complete clotting.[49,50] Tubes with separating gel are highly discouraged due to the risk of interfering substances which may be released by the gel during incubation at 37°C to enable clotting. Should non-gel tubes be unavailable, it is desirable to ask the manufacturer for information concerning the gel composition and its specific characteristics. Blood samples should reach 10 mL.[50] A smaller volume might mean missing detection of low concentration CGs that could potentially be associated with severe pathologies.[8] Some authors emphasize the necessity of spinning the blood sample at 37°C, and alternatively suggest to separate the serum from the clot without using a centrifuge if this is not preheated.[48,49] Other authors assert that blood should be kept at 37°C until serum separation only for patients with type I CG, whereas samples from patients affected by MC may be handled at room temperature.[47] Kallemuchikkal suggests that clotted blood should be spun at 2000*x* g for 10 min at 37°C.[50] Musset suggests 2000*x* g for 30 min at 37°C;[11] Brouet and Dammacco recommend spinning at 37°C but do not specify either time or speed.[5,51] Since these are not critical factors for analysis they could be performed downstream

Analytical Phase:

- Sample observation. Following centrifugation, the supernatant serum sample should be transferred into Wintrobe tubes and incubated at 4°C. The precipitation process manifests itself in a variety of ways in samples, depending on the concentration of CGs present, and may require either a few hours (when CGs-often type I-become insoluble at room temperature) or longer periods of time, particularly in the case of low concentrations of type III CGs. Observation at 4°C is a critical parameter in this analysis and should be established in an adequate manner to achieve detection of even extremely low levels of CGs in the serum.[50,47] For a correct performance of the CGs search analysis, the serum sample should be kept at 4°C for at least seven days: during this time, the sample should never be frozen or warmed to avoid significant variations in immunoglobulin solubility.[11,50]
- Artefact verification. By definition, CGs precipitate or gel in a reversible manner at temperatures below 37°C. Therefore, after the quantification, it is necessary to confirm thermo-reversibility of the precipitate by re-dissolving it at 37°C for one hour or by setting aside an aliquot of serum to be stored at 37°C for the same amount of time (7 days).[5,11,50] In particular, patients undergoing anticoagulant therapy may present with cryoprecipitates composed of heparin-fibronectin complexes or by fibrinogen-fibrin which are morphologically similar to CGs. In such cases, immunotyping of cryoprecipitate is necessary to confirm the presence of immunoglobulins and exclude false-positivity due to artifacts.[50,52]
- Cryoprecipitate quantification. CGs quantification may be expressed in the following ways: as cryocrit (CRT), as a measurement of total proteins, as an immunonephelometric quantification of immunoglobulins or as the area under the curve in the gamma region following electrophoresis of resolubilized cryoprecipitate (performed at 37°C). The CRT is a semi-quantitative parameter in common use, as it is simple and cheap, although it is affected by a great number

of variables which discourage its use as a comparable parameter among patients, or as an indicator of associated pathologies. The CRT expresses the percentage ratio between cryoprecipitate volume and serum volume obtained by centrifugation at 4°C for 15 min at 1700*x* g.[50] The evaluation is performed by the operator; that implies CGs quantification is fairly inaccurate, unspecific and rather insensitive. The limiting factors include the test tube type used for measurement, the spinning conditions employed for centrifugation, the necessity of incubating large volumes of serum to achieve reliable data and the assumption of an underlying correlation between CRT protein concentration and the sedimented volume of material. Moreover, as CRT measurement is not performed on washed CG material, CRT values may be strongly affected, to a certain extent, by the presence of serum proteins trapped within the cryoprecipitate.[8,50] Nevertheless, CRT values are often used in published clinical case reports and persist as recommended quantitative data used in the literature.

- Cryoprecipitate washing techniques. Removal of serum proteins previous to cryoprecipitate characterization is a fundamental step which guarantees a correct interpretation of the immunoelectrophoretic profile. Cryoprecipitates may be washed with a physiological saline solution, with PBS (phosphate buffered solution), or with polyethylene glycol 6000 3% in PBS.[5,11,50] In all cases, the washing solution must be kept at 4°C, and CGs should be resuspended by agitation in a volume of solution corresponding to the amount of supernatant discarded after spinning of the sample at 4°C. Washed cryoprecipitates should then be centrifuged (at 4°C) to separate once again the precipitate from the washing solution/buffer. Spinning conditions and washing cycles vary in the literature. Consensus can be reached by establishing a minimum of 3 wash cycles, which should be increased for CRT values >4%. When cryoprecipitates are low (<1%) it is advisable to recuperate samples after each wash by incubating the sample at 4°C for 72h, before moving on to the next washing step. When CGs do not dissolve in the wash solution by agitation, they should be resuspended by incubating them at 37°C until complete resuspension. The sample should then be recuperated by following 72h of incubation at 4°C before carrying on with the washes.[10] In rare cases, the common wash solutions may dissolve the cryoprecipitate in an irreversible fashion so CGs cannot be recuperated to perform their characterization.[53] Washed cryoprecipitate should be dissolved by incubation at 37°C. CGs may also be treated with reducing solutions, such as 10% acetylcysteine, or 1% β-mercaptoethanol or 0.5 mmol dithiothreitol.[4]

Cryoprecipitate Typing. Immunocharacterization of cryoprecipitates, initially performed by Brouet using immunoelectrophoresis, is now carried out with the use of more sensitive methods such as agarose gel immunofixation techniques (considered the "gold standard"), immuno-subtraction by capillary electrophoresis, immunoblotting and two-dimensional polyacrylamide gel electrophoresis.[12] These procedures not only confirm the presence of immunoglobulins but also enables classification into types I-III. As mentioned, typing of the cryoglobulin provides direction toward identification of a possible underlying disease. The rather subjective reading of the results means that two independent specialist laboratories should be used. A minimum competency-based standard is required for those who review and interpret CG patterns. Protein Laboratories are encouraged to have an educational module suitable for continuing professional development.[54]

Other Quantification Methods. Total protein quantification is a method alternative to CRT measurement, although it is still awaiting validation. It permits evaluation of CGs concentration, although it is strongly affected by the presence of other proteins contained in cryoprecipitates such as albumin, fibronectin, C1q and other complement factors. Total protein quantification requires accurate washing of cryoprecipitates as well as complete re-suspension of CGs. It offers the advantage of greater sensitivity as opposed to CRT since it evaluates cryoprecipitates that adhere to the bottom of Wintrobe tubes and may, therefore, escape visual inspection. Musset *et al.* quantify total proteins in cryoprecipitates by spectrophotometric analysis at 280nm following CGs solubilization in 0.1nmol/L

NaOH.[11] Brouet *et al*. re-suspend CGs in 0.1mol/L of acetic acid and perform a colorimetric quantification of cryoprecipitate content of total proteins using either Pyrogallol Red or Coomassie Blue staining:[5] 1mL of serum is stored at 4°C for 3 days and subsequently centrifuged at 5000 rpm for 5 min at 4°C. CGs are separated from supernatant serum, washed three times with 3mL of cold water and re-dissolved physiological solution at 37°C. Nephelometric quantification of albumin may detect contamination from residual serum proteins. Literature reports indicate that the reference serum cryoprecipitate total protein content values should be <20 mg/L.[47] Other experimental quantification data may be obtained by calculating the difference between the nephelometric measurement of the total serum immunoglobulin concentration at 37°C and supernatant immunoglobulin concentration at room temperature following precipitation.[50] An electrophoretic run of re-solubilized cryoprecipitate performed at 37°C, either using capillary electrophoresis or by agarose gel electrophoresis, provides accurate CGs quantification. It is achieved by calculating the area under the curve in the gamma region of the electropherogram profile and by subtracting the equivalent amount of co-precipitating serum globulins from this value on the basis of the amount of residual albumin. The latter is therefore used as an internal standard correction factor for cryoprecipitate measurement, by performing the following calculations: γ-globulin/albumin ratio of cryoprecipitate versus γ-globulin/albumin ratio of native serum.[55]

Cryoglobulinemia and HCV. Cryoglobulinemia is considered to be a rare disorder, but its occurrence is strongly linked to the prevalence of HCV infection in the general population.[25] Other viral infections, as Hepatitis B Virus, Epstein Barr Virus , HIV can induce, even if with but with minor frequency, mixed crioglobulinema, that is almost always type III.[9,18,47]

The prevalence of type MC in HCV infection depends on the stage of the disease and the sensitivity of the analytical method. In patients with HCV cryoglobulins of type II and III can be present at different times in relationship with the presence of antibodies and the virus of HCV and the emergence of clonal lymphocyte proliferation,[18] in any case, however, the major complication, renal involvement, is strongly associated cryoglobulinemia type II MC, mostly in presence of IgM kappa.[32]

Chronic HCV infections are an issue of primary interest since, according to global WHO estimates, 3% of the total world population is infected by the virus.[26] For this reason, the development of efficacious prevention strategies and innovative therapeutic approaches that enable a major improvement from currently available treatments are of great importance.

The peculiar biological characteristics of the HCV, a hepatotropic and lymphotropic virus, may partially explain the immune and pathologic alterations responsible for HCV-correlated disorders.

HCV-infected patients are known to be at risk of developing liver complications. The risks of morbidity and mortality are frequently underestimated because they do not take into account non-liver consequences of chronic HCV infection. Numerous extrahepatic manifestations have been reported in up to 74% of patients, from perceived to disabling conditions. The majority of data concern HCV-related autoimmune and/or lymphoproliferative disorders, from mixed cryoglobulinemia vasculitis to frank lymphomas.[32]

In particular, chronic infection of immunocompetent cells (T and B lymphocytes, macrophages) may be responsible for the proliferation of B lymphocytes which trigger production of circulating immune complexes composed of CGs and autoantibodies. To date, HCV infection is known to cause deep changes in the immune response of the host, including the triggering of autoimmune diseases.[27] Autoantibodies have been detected in about 40% of HCV-positive patients, and their presence was associated with several extrahepatic complications as well as MC.[27,28] In the MC setting, a monotypic lymphoproliferation may often appear, and be clinically indolent, whereas frank B-cell Non-Hodgkin's Lymphoma (B-NHL) may be a late complication in 10% of patients. On the other hand, HCV may account for approximately one-third of "primitive" B-NHL.[29]

MCs are immunocomplexes in which the antigen is usually an IgG, and the antibody (which shows anti-IgG rheumatoid factor activity) is either a polyclonal or monoclonal IgM.[8] In HCV-related MC, the cold-dependent insolubility requires the presence of IgM-RF, IgG that targets HCV core protein and the protein itself. The

addition of an irrelevant IgG to a mixture of IgM-RF and core protein was unable to cause cryoprecipitation.[24] For the first time, cryoglobulinemia with an IgG RF has been discovered and since then, growing evidence has suggested that IgG subclasses could be involved in the development of cryoglobulinemia.[30]

The search for CGs should only be performed in subjects with suggestive clinical symptoms (asthenia, arthralgia and purpura) or clear laboratory data (Anti-nuclear antibodies, Anti-mitochondrial antibodies, Anti-smooth muscle antibodies, Anti-extractable nuclear antigen antibodies, Low level of C4, Anti-HCV antibodies ± HCV RNA), since the transient or asymptomatic observation of CGs is often associated with a variety of pathologies that set-off a hyper-stimulation of B-cells, such as inflammatory, neoplastic or infectious diseases of various etiology.[31]

The presence of cryoglobulinemia is not necessarily indicative of a disease state (transient levels of CGs may be detected during infections, and healthy individuals may present low levels of cryoglobulinemia), and serum concentrations do not always correlate with the severity of symptoms. So, some patients with apparently low levels of CGs may show severe symptoms associated with cryoglobulinemic syndrome.[32] This potentially life-threatening condition requires appropriate laboratory testing, especially for those patients showing clinical symptoms associated with such a condition.

The first classification criteria for MCs were proposed by the Italian Group for the Study of Cryoglobulinemias in 1989. In 2002 they were revised by the inclusion of pathological and virological findings.[8] The classification criteria included major and minor criteria. Major Serological criteria include the type of MC, low level of serum C4; the minors include the presence of IgM-RF and viral diseases HCV, HBV. Major and minor pathological criteria include respectively leukocytoclastic vasculitis, and clonal B cell infiltrates in the liver and/or bone marrow. Major and minor clinical manifestations include purpura and chronic hepatitis, membranoproliferative glomerulonephritis, peripheral neuropathy, skin ulcers respectively. MC syndrome was defined by the presence of typical triad (first described by Meltzer and hence known as Meltzer's triad),[33] including low level of C4, purpura, and leukocytoclastic vasculitis or the presence of MC (low C4 plus two minor clinical symptoms plus two minor serological/pathological findings).

The classification criteria have been used for epidemiological studies in patients with MC syndrome, but they have not been validated in clinically well-defined patient cohorts and therefore lack appropriate statistical support.[34]

Gene cluster variants of Human leukocyte antigen (HLA) in specific alleles could be a condition determining susceptibility to the development of MC and NHL during chronic HCV infection.[6,35,36] The Multicenter Genome-Wide Association Study (GWAS) reported an association between two particular polymorphisms on chromosome 6 and HCV-related MC vasculitis compared to HCV controls without evidence of lymphoproliferative disorders.[37] The first one is a single nucleotide polymorphism (SNP) (rs2071286) located in an intronic region of the NOTCH4 gene; the second one is a SNP (rs9461776) located between HLA-DRB1 and HLA-DQA1 gene segments of the major histocompatibility complex (MHC). Although the biological and functional meaning of these associations is unknown, a wide cohort of HCV patients with MC vasculitis present a genetic background predisposing to this kind of disorder.[38]

Genetic factors and impairment of the epigenetic regulation could make an extremely important contribution to the pathogenesis of HCV-related lymphoproliferative disorders.

The role of small, non-coding RNA, called microRNAs (miRNA), acting as post-transcriptional epigenetic regulators, has been suggested. miRNAs can modulate a wide variety of genes by either preventing the translation or inducing the cleavage of complementary mRNAs.

Deregulation of specific miRNAs seems to be involved in the pathogenesis of lymphoma, including some types typically found to be associated with HCV infection.[38,39,40]

HCV infection can represent the cause of MC in 80% of cases in regions with high incidence of HCV.[25] On the other hand low levels of circulating mixed cryoglobulins can be detected in over 50% of HCV infected individuals, while overt cryoglobulinemic syndrome develops in about 5%.[18] The diffusion of HCV infection is variable in the world, a high incidence of HCV-related MC is found in Mediterranea basin and ever a higher

incidence can be expected in low income countries where HCV in the general population is rather prevalent, and in immigrant in Europe from Africa and Asia.[25,56,57] The disease expression is variable, and the different symptoms arise from the involvement of various organs and systems, namely skin, joints, kidney, nervous system, salivary and lachrymal glands. Hence, the symptoms defining a full-blown MC can be so multiple and severe to determine a very poor quality of life for the patient.[32] HCV has a tremendous impact on patient-reported outcomes, such as health-related quality of life and fatigue. These HCV-related complications are responsible for a significant economic burden through direct medical costs associated with managing the liver disease, as well as the indirect costs associated with decreased work productivity. Antiviral therapy has been indicated as first-line therapy in patients with mild-to-moderate HCV-related MC vasculitis.[32] The importance of this extrahepatic manifestations of HCV is nowadays officially recognized and the latest AASLD guidelines for the new direct acting antivirals (DAA) indicated the MC among the highest priority conditions to treat because of the risk for severe complications.[58,59] In severe cases, or in patients intolerant/ineligible to antiviral therapy, anti-CD20 monoclonal antibody rituximab should be considered.[58,60]

Conclusion. The possibility of detecting even very limited amounts of CGs may offer an invaluable resource to clinicians operating in this field. There is also a growing demand for more efficient and rapid tests for detection of their presence. Since even limited amounts of cryoglobulins may be both pathogenic and significant in certain clinical contexts, their detection at low levels may be critical for diagnosis and especially for those patients requiring plasmapheresis. A high prevalence of cryoglobulin $\leq$0.05 g/L in clinical practice may be responsible for severe renal and neurological complications, leading to high morbidity and mortality in these patients. Therefore both appropriate therapy and careful follow-up is required to improve such patients' outcome.[58,61]

The diagnosis of cryoglobulinemia syndrome is predominantly based on the laboratory demonstration of serum CGs, with or without associated characteristic clinical signs and symptoms. Diminished serum complement components may reflect ongoing consumption by CG immune complexes.

Appropriate phases of CG research are fundamental for a correct diagnosis and adequate treatment of the associated diseases. Given the variability of testing conditions used in different laboratories and the lack of test standards and reference values, further investigation into standardization of CG testing should be performed in the future. The biological importance and activity of CGs, such as their ability to activate proinflammatory complement proteins, needs to be defined as well.

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