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Abstract:

The term "Cryoglobulinemia" refers to the presence of cryoglobulins (immunoglobulins that precipitate at variable temperatures < 37° C [98.6 degrees F]) in serum. Type I cryoglobulinaemia consists of only one isotype or subclass of immunoglobulin. Types II and III are classified as mixed cryoglobulinaemia (MC) because they include both IgG and IgM components. Type II MC, that consists of a polyclonal IgG and a monoclonal IgM, is the form most frequently associated with vasculitis.

MC vasculitis is multi-organic disease involving kidneys, joints, skin, and peripheral nerves. While many lymphoproliferative and autoimmune conditions have been associated with this disorder, hepatitis C virus (HCV) is known to be the etiologic agent in the majority of patients.

The course varies widely and prognosis is influenced by both MC-induced damage to vital organs and co-morbidities associated with underlying diseases. Treatment should be modulated according to the underlying associated disease (chronic viral infections, autoimmune diseases, or cancer) and the severity of internal organ involvement and mainly relies on three main broad treatment strategies: antiviral treatment (including direct-acting antiviral agents), conventional immunosuppression, and biologic, especially anti-CD20, therapy.

The most recent studies on MC vasculitis are promoting combination/sequential regimens, including treatments targeting both HCV and HCV- induced autoimmune disease, with the aim of blocking the various etiopathogenic pathways involved. Some approaches, such as regimens including corticosteroids and Rituximab, have been successfully used in all types of cryoglobulinemia.

Introduction

Cryoglobulinaemia is defined as the presence of immunoglobulins in the serum which reversibly precipitate and form a gel when the temperature drops below 37°C and redissolve upon re-warming. Classification includes three subgroups based on Ig composition. Type I cryoglobulinaemia consists of only one isotype or subclass of immunoglobulin. Types II and III are classified as mixed cryoglobulinaemia (MC) because they include both IgG and IgM components. Types II and III cryoglobulins are immune complexes (ICs) composed of polyclonal IgGs, i.e., autoantigens, and mono- or polyclonal IgMs, respectively. IgMs are the corresponding autoantibodies having rheumatoid factor (RF) activity. More sensitive methodologies, such as immunoblotting or two-dimensional polyacrylamide gel electrophoresis, or advanced techniques of immunofixation, often reveal the micro heterogeneous make-up of type II mixed cryoglobulins. Oligoclonal IgM or mixed polyclonal and monoclonal IgM can be detected. This particular serological subset, known as type II–III mixed cryoglobulinaemia, may actually be an intermediate evolution from type III to type II MC [1]. Type III mixed cryoglobulin is often detected in a high number of infectious or autoimmune disorders. Type III MC in primary Sjögren's syndrome (pSS) is associated with extraglandular involvement, greater risk of B-cell lymphoma, and poor survival. Type III MC is also observed in about 10% of patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), although cryocrit levels are usually lower than in Sjögren's syndrome, and clinical manifestations of cryoglobulinaemic vasculitis (CV) are less common [2–5]. Type II and type II–III, and less often, type III mixed cryoglobulins, may lead to a distinct disorder that can be classified in the group of systemic vasculitides affecting small and sometimes medium-sized vessels. In most cases they are related to a hepatitis C virus (HCV) infection [6] and, in the type II variety, they can be related to low-grade proliferative B-cell lymphomas [6,7].

Epidemiology

Incidence and prevalence: available data

Data regarding the prevalence and incidence of cryoglobulinaemia in the general population are scarce since only few studies have addressed this issue. Overall, cryoglobulinaemia is considered a rare disease (<5/10,000 in the general European and North American population), although prevalence is likely to be higher in some areas such as the Mediterranean Basin [8].

HCV epidemiology and HCV-related cryoglobulinaemia

Hepatitis C virus infection is considered the main cause of MC, especially type II [9–13]. HCV infection is a common problem worldwide affecting more than 184,000,000 people. Its prevalence varies depending on the geographic areas. HCV infection is highly prevalent in Central Asia, Eastern Asia, and Middle East-North Africa, involving >3.5% of each region's population. Areas like South East Asia, the Andes, Central and Southern Latin America, Australia, the Caribbean, Oceania and sub-Saharan Africa show moderate prevalence (1.5–3.5%), while in Europe, prevalence varies from <1% in several Western European countries (for example, the UK, Denmark, France, Germany, Sweden and Switzerland) to 1-2% in the Mediterranean Basin and 2.5-3% in Eastern Europe[14]. Asia–Pacific, tropical Latin America and North America are the regions with the lowest prevalence of HCV (<1.5%)[14]

Although only few HCV-infected patients experience clinical symptoms related to circulating cryoglobulins, various studies have reported between 10% and 60% of HCV-infected patients with cryoglobulinaemia which is asymptomatic in the vast majority of cases. This is mainly due to population selection and lead time biases, as well as to the fact that clinical assessment is not standardized, and the laboratory test for MC is prone to false negative results[15,16]. However, cryoglobulinaemia would appear to be more prevalent in Southern Europe than in Northern Europe and North America, with a study showing an almost 60% prevalence in HCV-infected individuals [17]. Lastly, cryoglobulinaemia seems to correlate with the duration of HCV-infection since it lasts twice as long in HCV+ve patients with cryoglobulinaemia [15].

Patients with active SLE and RA may present with serum cryoglobulins, although not usually with the same clinical course as subjects with

cryoglobulinaemic vasculitis[3]. Five to 20% of patients with primary pSS may present type II cryoglobulinaemia [18]. These patients may have cryoglobulinaemic vasculitis, but most importantly, they may develop B-cell non Hodgkin's lymphoma (NHL) in the future [19,20].

Mechanism/Pathophysiology

Cryoglobulinaemia detection and typing

Testing methods for cryoglobulinaemia detection have some limitations and may be influenced by artifacts arising from *ex vivo* cryoprecipitation after drawing blood. Cryoglobulins are characterised by high thermal instability. Correct evaluation of serum cryoglobulins requires avoiding false-negative results due to immunoglobulin cold precipitation which may also occur at room temperature. Therefore, blood sampling for cryoglobulin detection should either be carried out immediately after drawing blood, or the blood sample should be rapidly transported to the laboratory using a thermostable device. When MC is suspected, the serum should be kept warm and tests should be carried out at 37°C [21–24]. Tests for cryoglobulin detection should be repeated if the first tests are negative and the clinical features are suggestive of cryoglobulinaemic vasculitis.

Other laboratory surrogate markers, which are easier to detect than cryoglobulins, may provide indirect evidence of the presence of cryoglobulinaemia. Decreased serum levels of early components (C1q, C2, and mainly C4) and CH50 are frequent, with C3 concentrations usually being normal. The diagnosis of mixed cryoglobulinaemic vasculitis is usually based on the association of suggestive clinical vasculitis symptoms, cryoglobulin detection and decreased C4 serum level. Moreover, rheumatoid factor activity is found in MC but not in type I cryoglobulinaemia [21,22]. Electrophoresis and immunoelectrophoresis reveal polyclonal hypergammaglobulinaemia with a monoclonal component in type II MC. Cryoglobulins may also interfere with a variety of laboratory tests and have been associated with spurious quantitation

of plasma proteins and erythrocyte sedimentation rate, pseudo-leukocytosis, pseudo-thrombocytosis or pseudo-macrocytosis.

Etiologies of cryoglobulinaemia based on immune-typing classification

The production of cryoglobulins is most often the consequence of an underlying disorder that needs an etiological check-up depending on the immunochemical determination of the cryoglobulin components (**Figure**).

In type I cryoglobulinaemic vasculitis, it is mandatory to search for an underlying B-cell lymphoproliferative disorder, mainly Waldenström macroglobulinaemia, multiple myeloma or monoclonal gammopathy of unknown significance (MGUS) [25]. More rarely, other B-cell diseases may be found such as chronic lymphocytic leukaemia, hairy cell leukaemia, or B-cell non Hodgkin's lymphoma. A cryoglobulin composed of IgM is suggestive of Waldenström disease, whereas cryoglobulinaemia composed of IgG is more often found in multiple myeloma or MGUS.

The main etiology of MC (type II and type III) is chronic HCV infection (70% to 90% of MC) [26,27] In large prospective series, the presence of MC is found in about 50% of HCV infected patients. Only a small proportion of these cryoglobulin-positive HCV-infected patients will develop symptomatic vasculitis. In case of persistent MC despite sustained HCV clearance, the presence of a B-cell lymphoma should be considered.

In up to 10% of MC that is not associated with chronic active HCV infection, the main causes include other infectious diseases (HBV, HIV), B cell malignancies (B-cell non-Hodgkin's lymphoma or chronic lymphocytic leukaemia, and autoimmune diseases (notably Systemic Lupus Erythematosus and Sjögren syndrome)[28]. Non HCV-related infectious MC is mainly caused by viruses (HBV, CMV and HIV), bacterial pathogens (endocarditis, Streptococcus, Brucella) or parasites (Leishmaniasis)(**Table**). In a recent study including 242 patients with non-infectious MC, 30% had autoimmune disorders (Sjögren syndrome, lupus, and scleroderma), 22% had a haemopathy (marginal zone

lymphoma, non-Hodgkin's B cell lymphoma, lymphoplasmocytic lymphoma), while no cause was found in 48% of patents [29].

Ethiopathogenesis

The mechanism of cryoglobulin pathogenicity is best described for HCV-associated cryoglobulinaemia. It is presently believed that HCV infects B lymphocytes while infecting hepatocytes due to the common expression of the CD81 receptors [26,30–32]. More specifically, active HCV replication has been demonstrated in CD19-positive B cells[33]. Indeed, it has recently been shown that HCV-RNA and HCV core and NS3 proteins can be detected in CD19-positive, but not CD19-negative peripheral blood mononuclear cells [34]. Of note, HCV replicates in other mononuclear cell types, including peripheral dendritic cells, monocytes, and macrophages [35,36]. Lymphocytes that are chronically stimulated by HCV are assigned to widespread autoantibody production related to HCV-induced lowering of the cell activation threshold. This favours the development of a number of immune manifestations associated with HCV infection, which are variably assembled in clinical pictures that have been collectively called “HCV syndrome” [37]. HCV syndrome includes manifestations apparently distal to the characteristic picture of MC, such as autoimmune thyroiditis, sicca syndrome, thrombocytopenia, haemolytic anaemia, autoimmune diabetes, and pulmonary fibrosis.

B cells, which are protected from apoptosis by an HCV-dependent gene translocation, develop oligoclonal monotypic lymphoproliferation [38]. Distinct lymphoid infiltrates with cells expressing oligo- or monoclonal rheumatoid factor in the portal tracts, spleen, and bone marrow (on occasion evolving towards an overt B-cell non-Hodgkin's lymphoma) can be detected in these patients. Thus, MC would appear to be a cross between classic autoimmune disorders and malignancies (i.e., B-cell lymphoma). The persistent stimulation of B cells by viral antigens and the enhanced expression of lymphomagenesis-related genes (particularly activation-induced cytidine deaminase which is critical for somatic hypermutation [39]) could lead to polyclonal and later monoclonal expansion of B cells and ultimately induce a lymphoproliferative

disorder that may eventually evolve to B-cell non-Hodgkin's lymphoma. A strong association has been found with some B-cell non-Hodgkin's lymphoma subtypes, including diffuse large B-cell lymphoma, marginal zone lymphoma, and lymphoplasmacytic lymphoma [40]. The pathogenetic implication of HCV in the formation, transport, and removal from circulation of cryoprecipitable immune complexes in MC has been studied extensively in recent years. Under the trigger effects of chronic HCV infection, oligo or monoclonal IgM that shares rheumatoid activity is produced by a permanent clone of B cells thus favouring the appearance of ICs formed by circulating HCV, anti-HCV polyclonal IgG, and the monoclonal IgM itself. Due to the clonally restricted IgM, these cryoprecipitable ICs also escape the erythrocyte transport system[41] and directly impact hepatic and splenic macrophages, which are unable to process them due to abnormalities in the biogenesis of lysosomal enzymes [41]. Possibly due to the HCV infection of phagocytic cells, the same abnormality is likely to occur in circulating monocytes which are found to be engulfed with cryoglobulins when examined by electron microscopy in tissue specimens [42], but that are unable to digest phagocytosed immune material [43]. The exact role of monocytes/macrophages, i.e., whether the phagocyte influx to the glomerulus that characterizes cryoglobulinaemic nephritis is beneficial (favouring cryoglobulin removal) or deleterious has been a controversial issue for several years. A previous hypothesis based on *in vitro* studies [43], stating that monocyte/macrophage entrapment of cryoglobulins reflects ineffective cryoglobulin clearance and could be associated with perpetuating glomerular damage has been supported by a study on a murine model of cryoglobulinaemic membranoproliferative glomerulonephritis[44] in which macrophage ablation conferred protection from mesangial expansion and collagen accumulation. In that model macrophages did not affect cryoglobulin removal since macrophage ablation had no effect on serum levels of cryoglobulins. Therefore, we can assume that macrophage influx to the glomeruli may be due to events related to amplification of injury following immune complex deposition rather than an attempt to clear cryoglobulins [44]. Moreover, these cells played a detrimental role in the progression of kidney injury in this experimental model[44]. Defective maturation of lysosomal

enzymes, specifically pro-cathepsin D [43], and/or danger-associated molecular patterns (DAMPs) released from injured resident cells [44] could attenuate macrophage innate function to clear immune complexes via Fc gamma receptors. The extracellular activation of released pro-cathepsin D[43] and/or the release of proinflammatory cytokines from DAMPs-activated macrophages[44] could drive mesangial expansion and activation.

In addition to the classical pathway of immune complex deposition, clonally restricted IgM was shown to share strong affinity for the glomerular matrix components, especially fibronectin, and was believed depositein the glomerulus together with the IgG anti-HCV that was previously bound in circulation or subsequently fixed through an "*in situ*" binding mechanism[45].

In conclusion, the pathogenetic scenario of MC is dominated by viral (most often HCV-related) chronic stimulation sustaining; i. the synthesis of IgM rheumatoid factor and consequently cryoprecipitable ICs, ii. abnormal kinetics with tissue deposition of these ICs combined with an ineffective cryoglobulin clearance by monocytes/macrophages (which are implicated in perpetuating glomerular damage) and iii. a subclinical, smoldering lymphoproliferative disorder.

Diagnosis, screening and prevention

Diagnosis and screening

Laboratory detection of circulating cryoglobulins is always required for the correct classification/diagnosis of cryoglobulinaemia and in particular of MC syndrome [46,47]. Although internationally accepted methodologies for cryoglobulin measurement are still lacking, simple standardized indications are sufficient for testing serum cryoglobulins [46]. Given their high thermal instability, it is necessary to avoid false-negative results caused by cold precipitation, even at room temperature. The first steps (blood sampling, clotting, and serum separation by centrifugation) must be carried out shortly after the blood is sampled, always at 37°C. Cryocrit determination, separation and washing of the cryoprecipitate and cryoglobulin characterization must be

performed at 4°C, after 7 days of cold serum storage. The serum with cryoglobulins should be tested for reversibility of the cryoprecipitate by re-warming an aliquot at 37°C for 24 hours. Cryocrit determinations, i.e., the percentage of packed cryoglobulins referred to total serum after centrifugation at +4°C, should be done on blood samples without anticoagulation to avoid false-positive results caused by cryofibrinogen or heparin-precipitable proteins. The immunoglobulin components of cryoprecipitate can be identified by immunoelectrophoresis or immunofixation at 37°C to avoid possible precipitation/loss of cryoglobulins. Alternatively, immunoblotting or two-dimensional polyacrylamide gel electrophoresis can be employed. The characterization of serum cryoglobulins is mandatory for a definitive diagnosis of both cryoglobulienaemia and cryoglobulinaemic syndrome [46,48]. Cryoglobulin levels, i.e., cryocrit, usually do not usually correlate with the severity/activity of the cryoglobulinaemic syndrome and the clinical symptoms [46,49]. An exception is the classical hyperviscosity syndrome that may appear in the presence of very high cryocrit levels[46].

Fig…. (Ferri 1) summarizes the possible clinical presentation of patients with cryoglobulinaemia with/without overt cryoglobulinaemic syndrome. While type I cryoglobulinaemia is a serological finding without specific clinical relevance that is detectable during the course of various haematological disorders [46], the detection of type II/III MC represents the main laboratory hallmark of cryoglobulinaemic syndrome. However, in clinical practice mixed cryoglobulins, especially at low amounts, may frequently be recorded as an isolated laboratory finding in subjects without any clinical symptoms of cryoglobulinaemic syndrome. Instead, the diagnosis of cryoglobulinaemic syndrome or cryoglobulinaemic vasculitis must be based on serological findings combined with multiple organ involvement. Isolated cryoglobulins may be detected in the early stages of the disease or during clinical remission of the cryoglobulinaemic syndrome. On the other hand, clinically overt cryoglobulinaemic syndrome without detectable cryoglobulinaemia may be also observed. This condition sometimes occurs transiently during the natural history of the disease and is generally correlated to the great variability in the

percentage of cryoprecipitable ICs [46]. In clinical practice, cryoglobulin detection should be carried out in patients with certain haematological, hepatic, renal, and/or infectious diseases, mainly HCV or HBV infection, and/or one of the typical manifestations of cryoglobulinaemic syndrome: orthostatic purpura, arthralgias/arthritis, weakness, peripheral neuropathy, sicca syndrome, urinary abnormalities, rheumatoid factor seropositivity, complement consumption. If the diseases is strongly suspected and the cryoglobulins cannot be detected by routinely methods, a precipitation technique in hypoionic medium can be employed in order to detected the so-called hypo-cryoglobulins [50].

Differential diagnosis

Differential diagnosis between cryoglobulinaemic syndrome and non-organ specific autoimmune disorders is often necessary for the correct management of patients with either “essential” or HCV-related cryoglobulinaemic syndrome. Both conditions show frequent clinico-pathological overlapping with a number of other autoimmune/neoplastic disorders, particularly Sjögren's syndrome, rheumatoid arthritis, glomerulonephritis, thyroiditis, and B-cell lymphomas [49,51,52]. In this context, careful patient evaluation is necessary for the correct classification/diagnosis of the individual clinical entity, namely MC, HCV-associated sicca syndrome or polyarthritis, rheumatoid arthritis, and primary Sjögren's syndrome (Ferri Fig. 2 …….).

The presence of urinary abnormalities may be helpful for the diagnosis of cryoglobulinemic vasculitis but should be supported by renal biopsy [53].

Patients with cryoglobulinaemic syndrome usually show mild, generally non-erosive arthritis [49,51,52]. However, some patients with HCV-associated cryoglobulinaemic syndrome may develop symmetrical, erosive polyarthritis suggesting an overlapping cryoglobulinaemic syndrome/RA syndrome. In these cases, the detection of serum anticyclic citrullinated peptide antibodies, i.e., markers of classical seropositive RA, can be employed for the differential diagnosis [31].

The relationship between sicca syndrome, pSS, and cryoglobulinaemic syndrome, especially in patients with HCV infection is another intriguing issue [49,51,52]. Almost half of the MC patients complain of sicca syndrome. However, only few cases meet the current criteria for the classification of pSS. On the other hand, MC and pSS may share several symptoms: xerostomia and/or xerophthalmia, arthralgias, purpura, serum rheumatoid factor and cryoglobulins, as well as an increased risk of developing a B-cell lymphoma [49,51,52]. In these cases, careful patient clinico-laboratory assessment may be needed in order to make a correct diagnosis. Specific histopathological alterations of the salivary glands and of the autoantibody pattern (anti-RoSSA/LaSSB) are hallmarks of pSS. Cryoglobulinaemic syndrome with or without isolated sicca syndrome is characterized by HCV infection in over 80% of patients, by cutaneous leukocytoclastic vasculitis, and type of organ involvement (hepatitis, membranoproliferative glomerulonephritis) which are less frequently recorded in pSS (Fig.2). In this respect, the presence of HCV infection *per se* should be considered an exclusion criteria for the classification of pSS [18,47]. Differential diagnosis may prove to be difficult in some HCV-negative individuals with symptoms of both pSS and cryoglobulinaemic syndrome. They would be best classified as having an overlap syndrome [49,51,52]. This latter condition is often characterized by a low rate of anti-RoSSA/LaSSB along with a high prevalence of mixed cryoglobulinaemia, hypocomplementaemia, systemic autoimmune manifestations, and complicating lymphomas which are responsible for more severe outcomes [49,51,52]. In clinical practice, the cryoglobulinaemic syndrome/pSS overlap syndrome could be considered a severe clinico-prognostic condition with relevant implications on patient monitoring and treatment [49,51,52].

Prevention and Prognosis

Survival and morbidity *in* various scenarios: from asymptomatic carriers to cryoglobulinaemic vasculitis

Asymptomatic cryoglobulinaemia is observed in up to 40% of chronically infected HCV patients but only 5-10% will develop cryoglobulinaemic vasculitis

[27]. Asymptomatic cryoglobulinaemia carriers do not seem to run a different course than their cryoglobulinaemia-negative counterparts. However, patients with chronic hepatitis C and cryoglobulins reportedly have higher incidences of cirrhosis and increased fibrosis scores after adjusting for age, gender and length of infection than those without cryoglobulin[17,54]. No association was found with advanced fibrosis on the basis of type and level of cryoglobulins.

Despite the success that has been achieved with combination of antiviral treatment and/or Rituximab (RTX), cryoglobulinaemic syndrome remains a severe disease. Most series reporting on the effects of treatment showed mortality rates of 8-15%[55,56]. In a cohort of 151 HCV-associated CV, the 1-, 3-, 5- and 10-year survival rates were 96%, 86%, 75% and 63%, respectively [57]. The most common causes of death in cryoglobulinaemic syndrome are infection, end-stage liver disease, cardiovascular disease and more rarely vasculitis and lymphoma/neoplasia. Baseline factors associated with poor prognosis are the presence of severe liver fibrosis, involvement of the central nervous system, of the kidney and the heart. In multivariate analysis, both severe fibrosis (HR 10.8) and the severity of vasculitis (HR 2.49) were significantly associated with poor prognosis [57]. In a series of non HCV-cryoglobulinaemia patients, 18/133 (13.5%) died primarily due to sepsis. In multivariate analysis, age above 60 years (OR: 1.06) and renal involvement (OR: 5.20) were independently associated with death[58]. The natural history of cryoglobulinaemic syndrome is not predictable and strongly depends on concomitant diseases and complications, and response to treatment. Death usually occurs after a prolonged course of vasculitis. Morbidity due specifically to cryoglobulinaemia may also be significant. Use of antiviral agents (especially Sofosbuvir-based therapy) is associated with better prognosis [59]. Careful monitoring of life-threatening complications (mainly nephropathy, widespread vasculitis and B cell lymphoma or other malignancies) should be carried out in all patients with cryoglobulinaemic syndrome.

Impact of renal involvement on survival

When kidney involvement is suspected (e.g. urinary abnormalities with/without decreased glomerular filtration rate), renal biopsy is recommended [53]. Kidney involvement in cryoglobulinaemic syndrome occurs in 8% to 58 % of patients [60,53,61]. Renal involvement is one of the most harmful complications of HCV-associated CV, and may severely affect the patient's clinical outcome. In 5-10% of patients, acute oliguric kidney failure is the first indicator of kidney disease. Hypertension is common (affecting more than 50% of subjects) and may be severe. The severity of hypertension may mirror the severity of the kidney disease[60]. In many patients, kidney disease shows an indolent course, and kidney failure requiring dialysis is rare (<10%). Patients with cryoglobulinaemic nephritis have poor prognosis mainly because of a high incidence of infectious, end-stage liver and cardiovascular diseases. The overall 10-year survival after a diagnosis of cryoglobulinaemic syndrome ranges from 50% to 90% in case of renal involvement [55,60]. In a cohort of 146 patients with cryoglobulinaemic nephritis cardiovascular disease was the cause of death in more than 60% of patients[53]. Older age, higher serum creatinine levels, and greater proteinuria at diagnosis are associated with the development of kidney failure and death [53].

Impact of lymphoproliferative disorders on survival

The overall risk of non-Hodgkin's lymphoma in patients with CV is about 35 times higher than in the general population (12 times higher if non-aggressive lymphomas are excluded) [62]. B-cell NHL includes marginal zone lymphoma (MZL), *de novo* or transformed diffuse large B-cell lymphoma (DLBCL) and to a lesser extent, follicular lymphoma [15]. Cryoglobulin levels above 0.6g/l, the presence of cryoglobulinaemic syndrome, and hypogammaglobulinaemia are independent variables associated with B-cell NHL[63]. HCV-positive NHL usually occurs following a long period of infection (>15 years). The proportion of transformed DLBCL is significantly higher in HCV-positive patients (32%) compared to HCV-negative patients (6%)[64]. The efficacy of direct acting antiviral (DAA) drugs was recently evaluated in 46 patients with HCV-positive indolent B-cell NHL, consisting mostly of MZL (n = 37)[65]. Sustained virological

and haematological responses were obtained in 98% and 67%, respectively. Estimated 1-year progression-free and overall survival was 75% and 98%, respectively. HCV-positive DLBCL patients usually have a high international prognostic index[66] and treatment, like for their HCV-negative counterparts, consists of anthracycline-based chemotherapy coupled with Rituximab. However, sustained viral response (SVR) after antiviral therapy has been associated with better overall survival in HCV-related MZL [67], but also in DLBCL patients.

Management

Renal Manifestations

In brief, patients with renal involvement should be treated according to two broad principles:

a) In patients with mild to moderate clinical manifestations, therapy is directed at the underlying etiology. However, immunosuppressive therapy should be provided as initial therapy in those patients identified as having a rapidly progressive, organ-threatening, or life-threatening course, regardless of the etiology of the mixed cryoglobulinemia. This is usually involves a short course of glucocorticoids[68–70] with a preference for rituximab[71–78] over cyclophosphamide and, in some patients, especially when associated with hyperviscosity syndrome, plasmapheresis[79–81].

Patients with rapidly progressive glomerulonephritis should receive one to three doses of intravenous methylprednisolone, 7.5 to 15 mg/kg per day followed by oral prednisone, 1 mg/kg per day (maximum dose of 80 mg/day) for two to four weeks, then 40 mg/day for two weeks, and then 20 mg/day for another two to four weeks. The dose is then tapered by 5 mg per week until it is discontinued. This rapid taper is an attempt to reduce infection complications [68–70].

The most common rituximab regimes are two doses of 1000 mg separated by a two-week interval[82], 375 mg/m^2 given weekly for one month, or 375 mg/m^2 given weekly for one month [83], followed by 2 more doses 1 and 2 months later, the so-called “4 plus 2 *improved* protocol [84]. Lower doses of rituximab appear less effective [76]. In cases where rituximab therapy is unavailable, fails to produce a clinical response, or is not tolerated, cyclophosphamide therapy

can be used as an alternative. The usual dose of cyclophosphamide is 2 mg/kg per day orally for two to four months, but dose should be adjusted according to the age and degree of kidney function[71–78,82].

If plasma exchange is considered, should be used early in the course and performed daily for 10 to 14 sessions or three [85,86] exchanges per week for two to three weeks. Replacement fluid can be 5 percent albumin solution, which should be warmed to prevent precipitation of circulating cryoglobulins, or with fresh frozen plasma in patients at risk of bleeding, e.g. post kidney biopsy. Plasma exchange does not prevent the formation of new cryoglobulins, and as such it should be combined with anti B-cell clones that produce cryoglobulins[79–81].

b) All patients should receive therapy directed against the underlying etiology of the mixed cryoglobulinemia. Patients with HCV should receive antiviral therapy, while patients with an underlying lymphoproliferative disorder should receive appropriate disease-specific therapy. In patients with HCV, the choice of specific antiviral drugs will depend upon the HCV genotype and the degree of kidney function. Interferon-free regimens are now the therapy of choice based on the improved safety and efficacy of these regimens, and high rates of clinical response. Duration of antiviral therapy will depend upon the HCV genotype, fibrosis stage/hepatic function, prior treatment response, viral load (in some cases), and tolerance of treatment.

Antiviral therapy can be delayed for one to four months in patients with severe disease who require initial immunosuppressive therapy. However, in patients with mixed cryoglobulinemia due to HIV or HBV infections, antiviral therapy should always be initiated before or at the same time as immunosuppressive therapy since such patients are at high risk for enhanced viral replication as a result of rituximab or cyclophosphamide therapy[87–89]. In particular, rituximab should not be used in patients with active hepatitis flares, as fatal hepatitis has been reported[90,91].

The frequency of clinical and laboratory monitoring will depend of the cryoglobulinemia disease activity, treatment regimen, comorbidities, and tolerance of therapy. In general, patients should have a complete blood count, electrolyte panel, serum creatinine, liver function testing, and blood glucose

weekly during the first two weeks of antiviral therapy, and then monthly thereafter. However, patients with cirrhosis and portal hypertension or poor degree of kidney function require more frequent monitoring due to higher risk of side effects.

All patients with mixed cryoglobulinemia who are receiving immunosuppressive therapy should receive prophylaxis against opportunistic infections, such as *Pneumocystis pneumonia*, as well as age-appropriate immunizations, ideally several weeks before the initiation of immunosuppression.

Occasional patients with mixed cryoglobulinemia may have no identifiable underlying disorder. Patients with severe renal manifestations e.g. glomerulonephritis, should be treated with immunosuppression as outlined above. Similarly, some patients may require alternate immunosuppressive regimens tailored to treat the underlying cause, e.g. lymphoproliferative disorders. Some patients with history of HCV successfully treated with anti-viral therapy and undetectable viral load may present with clinical manifestations of cryoglobulinemia. These cases are likely the result of B-cell clones that have developed as result of the HCV infection. Treatment of these patients should be with rituximab.

Management of Extra Renal Manifestations

Treatment should be modulated according to the predominant etiopathogenic mechanism (vasculitis or hyperviscosity) and disease severity [92]. The best example of how therapeutic approaches should be etiologically-driven is the essential role that the new anti-HCV therapies currently play in the treatment of extra-hepatic disease (EHD) [93].

a) Haematological cryoglobulinaemias

The main therapeutic goal must be the cure of the underlying haematological disease (overwhelmingly B-cell neoplasms). The most common neoplasias are multiple myeloma (predominantly associated with type I cryoglobulinaemia and hyperviscosity) and B-cell lymphoma (more frequently related to MC and

vasculitis). Although patients with type I cryoglobulinaemic vasculitis are mainlytreated with glucocorticosteroids (GCs), in association with immunosuppressant agents or Rituximab [25]. Treating the underlying monoclonal disorder has been associated with improvement/stabilization of cryoglobulinaemic symptoms in most patients with type I cryoglobulinaemia [94], although negativization of serum cryoglobulins was achieved in only half the patients. Alkylating agents, bortezomib and Rituximab-based regimens are the main therapeutic options [25,94,95]. Patients presenting with symptomatic hyperviscosity require urgent therapeutic intervention using plasma exchange or plasmapheresis to remove cryoglobulins from the circulation.

b) Infectious cryoglobulinaemias

b1. HCV-related cryoglobulinaemia*.* In HCV patients, pathogenic damage is related to vasculitis. A case-by-case stratification according to the severity of vasculitic involvement (mild, moderate, severe or life-threatening presentations) is recommended [96]. Patients with mild vasculitic involvement may be treated with the new antiviral agents alone, while those with moderate to severe vasculitic features may require a short-term GC course (<0.5 mg/kg/d) in order to subdue vasculitic-induced damage quickly,as a bridge to antiviral therapies [93,97]. Intravenous pulses of methylprednisolone (0.5-1.0 g/day for 3-5 days) and immunosuppressants should be used in life-threatening presentations, including multiple/extensive cutaneous ulcers, severe neuropathy, CNS involvement, alveolar haemorrhage or gastrointestinal ischaemia, in combination with plasma exchange [98]. The most promising GC-free, non-antiviral therapeutic approach to HCV-related cryoglobulinaemia, is based on the use of B-cell depleting agents [99]. There is far more evidence compared to other options, not only regarding the number of reported patients (more than 500), but also regarding the excellent results of small controlled trials, even in cirrhotic patients [92]. Specific organ-by-organ data confirm the efficacy of Rituximab in 85% of patients with cutaneous ulcers, in 79% of those having arthralgias, in 77% of those with weakness and in 67% of those with peripheral neuropathy [96]. Caution is needed regarding the potential

exacerbation of vasculitis due to the formation of immune complexes between monoclonal cryoglobulinaemic IgM and rituximab, which is a chimeric monoclonal antibody [97].

b2. Non-HCV infectious cryoglobulinaemic vasculitis.

Non-HCV symptomatic infectious cryoglobulinaemia is a rare clinical condition reported in nationwide series including less than 20 cases per study [28,100,101]. Patients are treated with the most appropriate specific anti-infectious therapy as first-line therapy, in association with short-term GC regimens, plasma exchange and/or Rituximab in the most severe vasculitic presentations. Refractory or relapsing patients are mainly affected by an underlying HBV infection, i.e., the main non-HCV infectious etiology of cryoglobulinaemia [28]. The therapeutic goal for HBV patients should be eradication of HBV-DNA using antiviral agents (entecavir, alpha-IFN, adefovir or lamivudine) [101]. The successful use of combined Rituximab has also been reported [28]. However, relapses are frequent, with a reported mortality rate of about 25% [101].

c) Autoimmune cryoglobulinaemias

Cryoglobulinaemias unrelated to underlying haematological/infectious processes should be considered primary vasculitic diseases, associated in some cases with other systemic autoimmune diseases (mainly pSS) [100,102]. The therapeutic approach is mostly based on the use of conventional immunosuppression and/or B-cell depletion in an effort to suppress the clonal B cell expansion that is responsible for cryoglobulinaemic synthesis. Traditional approaches for inducing clinical remission, based on high-dose GC and cyclophosphamide, are derived from therapeutic strategies employed in other systemic vasculitides. Azathioprine and mycophenolate mofetil are mainly used as remission maintenance agents. However, according to recent data [103,104], the use of GC and Rituximab (instead of cyclophosphamide) may now be considered the first-line therapeutic approach in patients with severe non-infectious MC.

HCV-related manifestations and Direct Antiviral Agents

Interferon(IFN)-based antiviral therapy (AVT) showed positive effects on several HCV extrahepatic manifestations (HCV- EHDs) [105–108]. However, it had side effects and limited efficacy [109]. The introduction of DAAs directly targeting non-structural proteins involved in HCV replication led to a breakthrough in the therapeutic approach. HCV NS3 protease inhibitors were introduced in 2011. These molecules block the catalytic site of NS3, preventing poly-protein cleavage and HCV replication. After the first generation DAAs (telaprevir and boceprevir), that were administered with IFN and Ribavirin (RBV), new generations of DAAs were introduced, including NS5A and NS5B-inhibitors thus allowing IFN-free treatment (Figures X). The NS5A-inhibitors block the membranous genesis stage. The NS5B polymerase-inhibitors act as chain terminators within the polymerase catalytic site (nucleos(t)ide analogues: sofosbuvir) or cause conformational changes making the polymerase ineffective (non-nucleotide inhibitors, dasabuvir). Current therapy is based on various IFN-free DAA combinations with short therapy duration (usually 8 to 24 weeks), minimal side-effects [110], and antiviral efficacy approaching 100% [110].

First-generation DAAs increased SVR rates, but also side-effects [111–114]. By contrast, IFN-free treatments were characterized by low rates of adverse events and a high clinical response rate that increased together with an improvement of antiviral effectiveness [115–121] (Table x). Despite the anti-proliferative effect of IFN, a recent analysis showed that IFN-free AVT resulted in higher clinical and immunological response rates than IFN-based protocols, thus confirming the primary role played by HCV eradication [122]. The clinical improvement rate gradually increased during the follow-up of treated patients [117,118,123]. An improvement in renal function with or without immunosuppressants was also observed [124]. IFN-based AVT showed positive effects on some kidney manifestations and mortality rates [125][126][77]. However, both IFN and RBV have limitations in patients with renal failure, and the need for first-line non etiologic treatment in rapidly evolving forms was emphasized. The availability of both IFN-free and RBV-free therapies allows the

early, possibly combined use of AVT in these conditions (despite some concerns regarding pharmacokinetics and safety in patients with severe renal failure) [127]. Only limited, though positive data exist on the effect of IFN-free regimens [108,116,117,124].

In patients with lymphoma, evidence demonstrated that IFN-based AVT induces haematological response in patients with low grade forms (especially MZL[128–130]. Recent data suggest an anti-lymphoma activity of IFN-free regimens [131]. AVT should be considered the first-line approach in HCV-associated low grade lymphomas [132], (ESMO [133] and NCCN [134]) (EASL [135]) guidelines]. For HCV-associated aggressive lymphomas, like DLBCL [129] immediate conventional therapy is required [136], whereas when IFN-based AVT is administered in combination with chemotherapy, it may significantly increase haematologic toxicity [137]. Recent data suggest that IFN-free AVT should be performed after completion of, or in combination with, immune-chemotherapy following close monitoring for potential drug-drug interactions [138–140]. Further prospective studies are needed, however, no particular overlapping toxicities are expected.

Other categories of HCV EHDs that are expected to be especially favoured by IFN-free AVT include neuropsychiatric disorders and reduced health-related quality of life (HRQoL). In fact, their relationship with the use of IFN is well known [141–144]. By contrast, IFN-free AVT [144,145] was not associated with HRQoL impairment, and SVR correlated with HRQoL improvement [154]. The use of RBV still seems to poorly influence HRQoL, and patient-reported outcomes (PRO's) suggest the use of both IFN- and RBV-free AVT[145] .

Quality of life

Only one small study specifically analysed HRQoL in cryoglobulinaemic patients. Quartuccio et al [146] evaluated 15 patients with severe HCV-related cryoglobulinaemia using the Medical Outcomes Study Short Form 36 (SF-36). Lower scores were observed for all the physical and mental domains in this group as compared to the control group. However, these scores significantly

improved following treatment with Rituximab. Presently, no studies have been carried out in non-HCV cryoglobulinaemic patients, and the main HRQoL studies involved unselected patients with chronic HCV infection. A recent meta-analysis of the prevalence, quality of life and financial burden of chronic HCV infection in the US [147] clearly supports the hypothesis that HCV infection has negative effects on the overall physical and mental health of affected patients. A large number of patients with chronic HCV infection present with chronic pain (including fibromyalgia) and/or chronic fatigue, which must be considered the main culprits of the reduced HRQoL reported in HCV patients [148,149]. These chronic symptoms are closely related to the onset of sleep disorders, depression and other neurocognitive alterations, which are independent of the severity of the liver disease, the viral genotypes or the HCV replication rates [150]. These manifestations typically occur in the absence of signs of structural brain damage on neurological imaging techniques, although a few studies have reported some metabolic or microstructural changes [150]. Lastly, other features may worsen the psychological status of HCV patients, including low socioeconomic status, HBV or HIV co-infections, discrimination and/or limited access to adequate health care [52]. The use of the new interferon- and RBV-free regimens for HCV have resulted in a better experience for the patients and increased work productivity following treatment [151]. Personalized environmental and individual psychological assessment of HCV patients is mandatory in order to establish a comprehensive counselling approach to, and management of, the main HRQoL items **[52]**.

Outlook

Current development

The identification of MC as a distinct disorder affecting multiple organs was first described in 1966 [152]. The discovery of its striking association with an HCV infection 25 years later [154,155] profoundly affected both our understanding of its pathogenesis and the therapeutic strategies, albeit a minority of patients were still classified as having HBV-related or ‘essential’ MC. Identifying the main triggering factor would represent a definite advancement. However,

several etiopathogenetic issues remain to be clarified. Firstly, it is still unclear whether HCV represents a simple triggering factor or if it also contributes to the self-perpetuating mechanism of the disease. Moreover, the natural course of the disease may develop with different clinical phenotypes among patients and in the same patient during follow-up, thus suggesting a multifactorial and multistep process. Therefore, the role of possible genetic and/or environmental co-factors remains under investigation.

The actual role of B-cell lymphoproliferation that represents the underlying disorder of the disease is another important issue. In particular, in patients with HCV-related disease the persistence of ‘benign’ B-lymphocyte proliferation may explain the paradoxical effects observed after HCV eradication in some individuals with unremitting immunological abnormalities and clinical symptoms. Therefore, we may assume that in some individuals, possibly in those with longer disease duration, the immune-system alterations have passed a ‘point of no return’. Recognizing this biological condition may be decisive for the overall management of the patients, especially with regard to the expectations of antiviral treatment.

Cryoglobulinaemic patients with no evidence of causative factors are still classified as having ‘essential’ MC, which shows a variable prevalence among patient populations in different countries. Essential MC encompasses a heterogeneous group of individuals and represents a very challenging disease variant with regard to both etiopathogenetic and therapeutic concerns [7,49,52]

Ongoing research and 5-year future perspectives and unmet needs

The mechanisms involved in the development of MC-related lymphomas should be more deeply investigated. Shedding light on the general mechanisms of lymphomagenesis could be of special interest for haematologists as well.

Identifying the factors involved in non HCV-associated MC remains a research challenge. Presently, the similarities between the clinical presentation of non-

HCV and HCV-related cryoglobulinaemic vasculitis [21,155] suggest that these alternative triggers might also be viral in nature.

IFN-free, DAA-based AVT represented an epochal evolution, especially in the field of HCV-EHDs. Broadening the use of AVT without immunomodulatory/antiproliferative properties will allow for the definitive evaluation of the role played by HCV eradication, which was in the past confounded by the immunoregulatory actions of IFN [156,157]. DAA-based AVT likely does not interfere with the pathogenesis of cryoglobulinaemic vasculitis nor does it actually impact on the development of the immune-mediated injury once the immune disorder is established [158]. The use of DAAs alone as a therapeutic tool to treat cryoglobulinaemic vasculitis requires further validation studies. Furthermore, the uncertainty of their pharmacokinetics and safety in the presence of renal impairment implies caution when using them in nephritic patients[159]. However, In the absence of the IFN-related risk of triggering autoimmune-related side effects, DAA-based AVT, even more so than in the past, can be recommended to eradicate a potentially lymphomagenic factor known to be a B-cell activator.

Despite the potential risk of exacerbation of the infection, immunosuppression was traditionally regarded as the first-line intervention in severe cryoglobulinaemic vasculitis, especially if kidney is involved. Biologic agents have raised hopes for more manageable therapeutic approaches, and Rituximab is the most widely used biologic drug. It has proven to be safer than conventional immunosuppressants [160]. Rituximab substantially changed the natural history of HCV-associated cryoglobulinaemic vasculitis by providing long-term remission, especially with intensive regimens (the so-called 4+2 regimen[37,158,161]). Besides its immunomodulatory effect, Rituximab also plays an important role by depleting CD19 positive-B cells, known to be HCV reservoirs [33].

Improved effects of a combination of DAAs and Rituximab can be envisaged **in** the treatment cryoglobulinaemic vasculitis and HCV eradication. The optimal combination protocol of RTX plus new IFN-free anti-HCV treatments need to be defined. Waiting for conclusive data, severe cryoglobulinaemic vasculitis,

especially with renal involvement, should first be treated with a Rituximab-based protocol, and DAA administration should be postponed, especially in the absence of a very high viral load [158] . DAAs alone could be effective *per se* in less severe cases of cryoglobulinaemic vasculitis[59].

Finally, studies aimed to identify the rescue treatments of Rituximab-refractory cryoglobulinaemic vasculitis need to be carried out.Refractory cryoglobulinaemic vasculitis can be defined as a clinical picture that does not improve within 4-6 weeks after the initial treatment, or that provides a less than 50% improvement after 12 weeks, or that shows chronic persistent manifestations after more than 12 weeks. First, cryoglobulinaemic vasculitis mimic conditions should be ruled out. Second, patient's adherence to medical prescriptions and adequacy of administered therapy should be critically considered. Finally, an underlying infection- or cancer-related comorbidity should be searched. Some alternative approaches have been proposed in this setting, including abatacept [162] or tocilizumab [163]. B-cell activating factor (BAFF) blocking agents [164] and interleukin-2 agonists [165] also seem to be promising. The role of plasma exchange, more specifically the double filtration cascade, can still be taken into consideration in the most severe or refractory cases [48], especially in the presence of high cryocrit before Rituximab administration [166]. Again, the combination of these agents with DAAs could lead to parallel control of the vasculitic process and eradication of the viral trigger.

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