

## Hepatitis C virus positive patient diagnosed after detection of atypical cryoglobulin

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### Abstract

A 60-year-old male patient presented with jaundice and dark urine for three days, icteric sclerae and skin rash on his legs for six months. Laboratory investigations revealed an atypical cryoglobulinemia with high hepatitis C virus (HCV)-RNA levels. Imaging studies showed cholestasis was accompanying HCV. Capillary zone electrophoresis using immunosubtraction method revealed a polyclonal immunoglobulin G and immunoglobulin A (IgA) monoclonal cryoglobulin and that IgA lambda was absent in immunofixation electrophoresis. After a liver biopsy, chronic hepatitis C, HCV related mixed cryoglobulinemia and cryoglobulinemic vasculitis were diagnosed and antiviral therapy was initiated. Our HCV patient presented with cryoglobulinemic symptoms with an atypical cryoglobulinemia that was detected by an alternative method: Immunosubtraction by capillary electrophoresis. Different types of cryoglobulins may therefore have a correlation with clinical symptoms and prognosis. Therefore, the accurate immunotyping of cryoglobulins with alternative methods may provide more information about cryoglobulin-generated pathology.

**Key words:** Cryoglobulinemia; Hepatitis C; Immunosu-

btraction; Immunotyping; Electrophoresis

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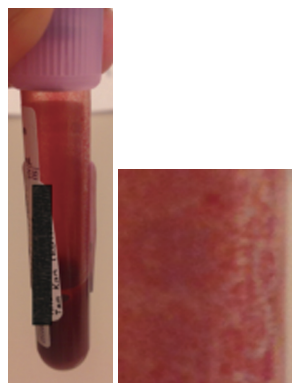
**Core tip:** We describe atypical IgA monoclonal cryoglobulinemia as the presenting symptom of chronic hepatitis C. Immunotyping of the cryoglobulin was performed with capillary zone electrophoresis with immunosubtraction method which is an alternative method to classical immunofixation electrophoresis. Accurate immunotyping of cryoglobulins with alternative method provide more information about cryoglobulin-generated pathology in atypical patients.

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## INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded RNA virus that causes chronic liver disease since, in most affected patients, the immune system cannot completely clear the virus. Patients may develop a variety of extra-hepatic manifestations including arthritis, arthralgia, fibromyalgia, lymphadenopathy and skin lesions. These various symptoms may sometimes lead to misdiagnosis and inappropriate therapies. The interaction of immune system cells and the surface proteins of HCV can cause immunological symptoms similar to those observed in autoimmune disorders. In addition, chronic immune response to HCV can produce cryoglobulins resulting in vasculitis-related skin ulcers and immune complex related nephropathy<sup>[1]</sup>.

Cryoproteins are immunoglobulins in a form that precipitates in serum and plasma<sup>[2]</sup> at low temperatures. Wintrobe and Buel first described Cryoglobulinemia in 1933, and it was clinically associated with palpable purpura, arthralgia and weakness, also known as the Meltzer's triad. Cryoproteins precipitate at temperatures below 37 °C and redissolve upon warming. They have clinical importance as they form intravascular precipitates, leading to clinical consequences such as obstruction in peripheral vessels resulting in Raynaud phenomenon, and immune-complex related vasculitis in skin, peripheral nerves and kidneys<sup>[3]</sup>. Three types of cryoglobulins have been defined depending on their immunoglobulin composition; Type I cryoglobulins are monoclonal immunoglobulins most frequently made of immunoglobulin M (IgM), followed by immunoglobulin G (IgG), and IgA. They are associated with immunoproliferative disorders like multiple myeloma and Waldenström macroglobulinemia<sup>[4]</sup>. Type II and type



**Figure 1** Agglutinations on the complete blood count tube. It was anticoagulated with K2-EDTA, analyzed at room temperature and red blood cell, Hemoglobin and Hematocrit results were discordant with each other.

III cryoglobulins are polyclonal immunoglobulins, occasionally associated with monoclonal ones, that are considered to be mixed cryoglobulins<sup>[5]</sup>. Mixed cryoglobulins are associated with infectious and chronic inflammatory diseases, and constitute 90% of all types of cryoglobulins<sup>[6]</sup>. Among patients who have mixed cryoglobulinemia, 92% have HCV, 1.8% have hepatitis B virus (HBV) infection<sup>[5,6]</sup>; whereas only 5% of the patients with HCV infection show clinical signs of cryoglobulinemia<sup>[3]</sup>. Here we present a HCV positive patient with atypical cryoglobulinemia that was suspected by the discordant complete blood count (CBC) results. Immuno-typing of the cryoglobulin was carried out with capillary zone electrophoresis-immunosubtraction method (CZE/IS).

## CASE REPORT

A sixty-year-old male patient presented with jaundice and dark urine that had started 3 d previously. He had had a rash on his legs for 6 mo and all examinations at that time were stated to be normal. Despite steroid therapy, there was no improvement in his rash. Physical examination revealed a BP of 120/70 mmHg, icteric sclerae and skin and there were diffuse rashes on arms and legs. An ultrasonographic scan of liver revealed cholestasis with minimal parenchymal hepatosteatosis. Laboratory investigations showed discordance in RBC, haemoglobin and haematocrit values in CBC and when blood sample tube was inspected agglutinations on the walls of the tube were remarkable (Figure 1). Upon suspicion of cryoglobulinemia, a second sample was obtained from the patient. The new sample was collected in a tube which was incubated at 37 °C and CBC was repeated after keeping it in the incubator for 20 min at 37 °C. This time results were concordant (Table 1) hence a sample was obtained at appropriate conditions in order to investigate cryoglobulins and a positive cryocrit was detected (Figure 2). Laboratory investigations yielded the following results: Serum protein electrophoresis was normal, cryoglobulins were positive, rheumatoid factor (RF) was positive,

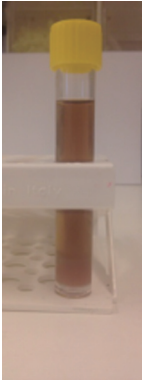


Figure 2 Cryoglobulin sediment at 4 °C.

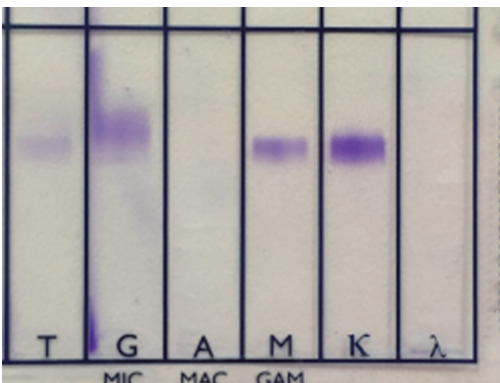


Figure 3 Cryoglobulin immunofixation electrophoresis with SAS-1 agarose gel (Helena, United Kingdom). A polyclonal IgG and monoclonal IgM kappa are detected. T lane shows total protein electrophoresis of cryoglobulin and an absent albumin band shows washing and isolating of the cryoprecipitate is successfully performed.

Complement 3 and 4 (C3, C4) levels were normal, anti-HCV antibody was positive, HCV RNA levels 6770728 IU/mL and HCV was type1b genotype. Clinical Chemistry results were as follows (reference ranges are in parenthesis): Serum Urea concentration 66 mg/dL (17-49 mg/dL); Creatinine 0.97 mg/dL, (0.8-1.3 mg/dL); Total bilirubin, 3.8 mg/dL, (0.2-1.2 mg/dL); Conjugated bilirubin, 3.39 mg/dL (< 0.2 mg/dL); Alanine aminotransferase, 548 U/L (16-63U/L), Aspartate aminotransferase, 251 U/L (15-37 U/L); Gama-glutamyl transferase, 3126 U/L (15-85U/L); Total protein, 6.32 g/dL (6.4-8.3 g/dL); Albumin, 2.76 g/dL (3.5-5.0 g/dL); Prothrombin time (PT), 10.6 (10-14 s); International normalized ratio for PT, 0.96 (0.8-1.25). Pathological examination of a liver biopsy revealed a moderate cholestatic injury accompanying chronic hepatitis, and degenerative and dysplastic changes in bile ducts. The patient was diagnosed with chronic hepatitis C, HCV related mixed cryoglobulinemia and cryoglobulinemic vasculitis. Antiviral therapy, (ledipasvir and sofosbuvir-Harvoni) was initiated.

#### Detection and characterization of cryoglobulin

In the first sample that was obtained at room temperature, the cryoglobulins were agglutinated and may have

Table 1 Complete blood count results at room temperature vs 37 °C<sup>1</sup>

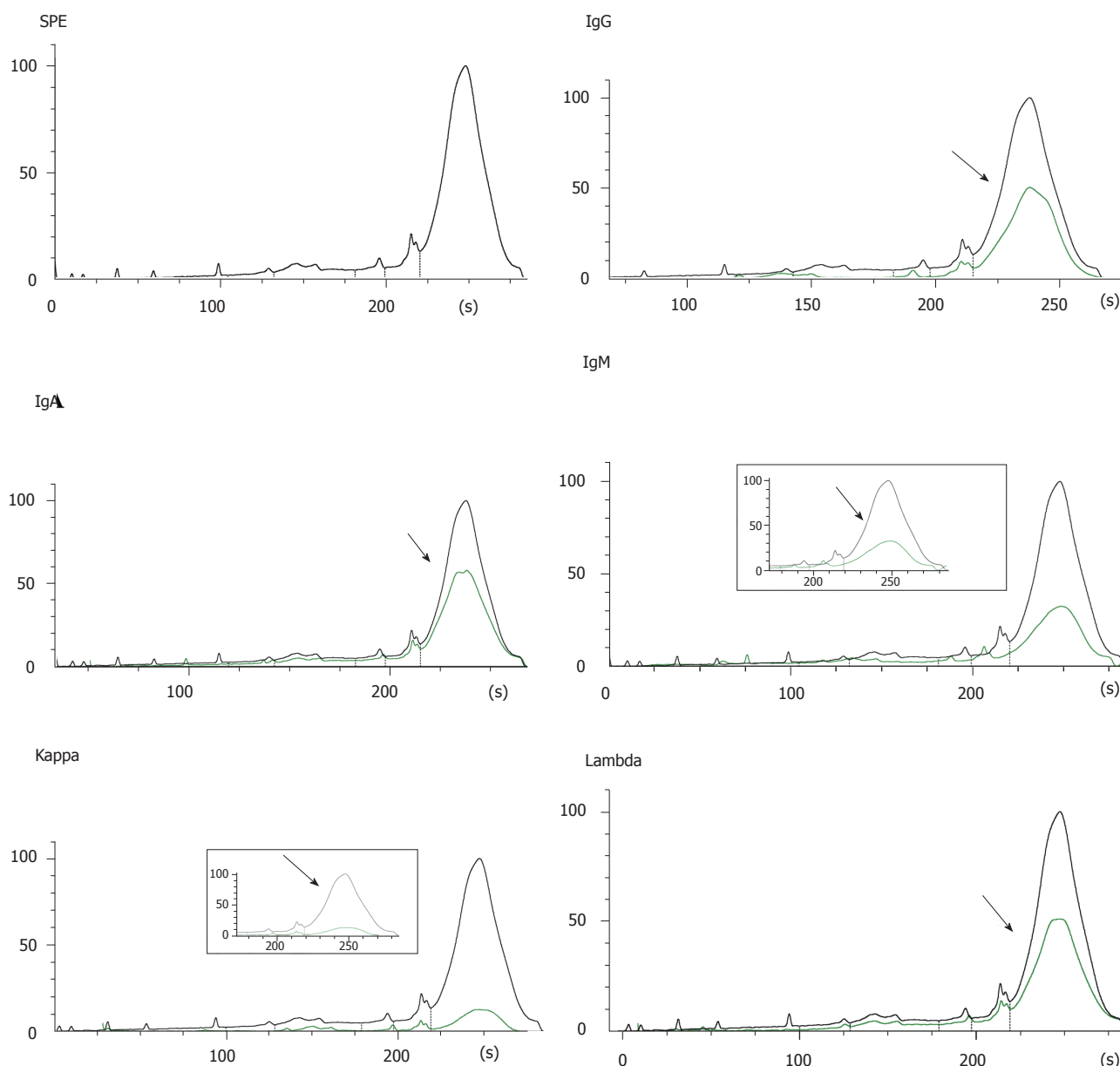
	At room temperature	At 37 °C	Reference ranges
White blood cells	13.93	15.27	$3.8-10.0 \times 10^3/\mu\text{L}$
RBC	2.0	3.1	$4.0-6.2 \times 10^6/\mu\text{L}$
Hemoglobin	12.1	12.6	13.0-17.5 g/dL
Hematocrit	22	32.5	40%-52%
Mean corpuscular volume	111.7	103.5	80-95 fL
Mean corpuscular hemoglobin	61.4	40.1	25-34 pg
Mean corpuscular hemoglobin concentration	55	38.8	31-37 g/dL
RBC distribution width	Cannot be calculated	17.1	11.2%-15%
Platelets	124	142	$150-400 \times 10^3/\mu\text{L}$

<sup>1</sup>Measurements are performed from different samples. RBC: Red blood cells.

acted as cold agglutinins, leading to the agglutination of erythrocytes, providing falsely low measurements of RBC and HCT, whereas the hemoglobin measurement was not affected since erythrocytes were hemolyzed prior to analysis.

For detection of cryoglobulins, prior to sample withdrawal, sample tube was warmed up to 37 °C, and transported to the laboratory at 37 °C. It was incubated at 37 °C until serum was separated. Separated serum was transferred to secondary tubes, and evaluation was carried out by incubating the tubes at 4 °C for seven days<sup>[4]</sup>. Tubes were inspected every day for any precipitate presence. At day 6 and 7 a precipitate was obvious and the cryocrit was measured to be 15% (Figure 2). Samples were incubated at 37 °C for 30 min and the precipitate dissolved. In order to separate cryoglobulins from other proteins in serum such as albumin, cryoprecipitate was washed with saline at 4 °C, and then it was centrifuged at 1500 rpm for five minutes at 4 °C. Supernatant was removed and saline, with the same volume of supernatant, was added. Washing was repeated for 3 times. Finally with the added saline sample it was dissolved at 37 °C<sup>[7]</sup>. Total protein and immunoglobulin concentrations in cryocrit were analyzed; immuno-typing of cryoglobulins were made using immunofixation by agarose gel electrophoresis, and CZE/IS. Absence of an albumin band in agarose gel electrophoresis indicated washing was complete. A polyclonal band at IgG heavy chain and monoclonal bands at IgM heavy chain and kappa light chain were remarkable in agarose gel electrophoresis (Figure 3). In capillary electrophoresis, albumin band was also absent, and besides polyclonal IgG and IgA gamma-globulins there was monoclonal subtraction at IgM heavy chain and kappa light chain (Figure 4). IgA lambda was absent in IFE (Figure 3). Total protein, Immunoglobulin and light chain concentrations in the cryocrit were as follows: Total protein 200 mg/dL, IgA 2.2 mg/dL, IgG

# Immunodisplacement report



**Figure 4** Cryoglobulin immunosubtraction was performed with V8 automated clinical capillary electrophoresis (Helena, United Kingdom). Arrows indicate specifically subtracted parts of immunoglobulins which mean cryoglobulin is composed of these. In this report, a mixed cryoglobulin is present: Monoclonal IgM kappa and polyclonal IgG and IgA heavy chains together with lambda light chain are detected (report shows heavy and light chains separately). Small frames indicate zoomed traces for monoclonal IgM kappa. Serum protein electrophoresis (SPE), shows total protein electrophoresis of cryoglobulin and an absent albumin band indicates washing and isolating of the cryoprecipitate is successfully performed.

28 mg/dL, IgM 108.5 mg/dL, total kappa 31.5 mg/dL, total lamda 11.8 mg/dL.

## DISCUSSION

HCV has been defined as a both heterotropic and lymphotropic virus and it may exert chronic stimulus to the immune system through different viral proteins. Chronic stimulation of the B-cells by HCV epitopes may trigger increase in some B-cell subpopulations causing the production of oligoclonal and monoclonal antibodies. Those antibodies may end up as cryoglobulins and/

or cold agglutinins<sup>[8]</sup>. Only 5% of HCV patients with cryoglobulinemia have clinical symptoms. Most patients, infected with the HCV have no obvious clinical symptoms, and generally patients do not know they are infected with the virus. This was the case with our patient, too. He had no clinical symptoms other than cryoglobulinemic symptoms until development of jaundice three days previously occurring probably with the increase in cholestasis.

Healthy individuals may have cryoglobulins at low concentrations (< 0.06 g/L), which do not cause any clinical symptoms<sup>[9]</sup>; however, cryoglobulins must be



**Table 2** Classification of cryoglobulins and laboratory findings<sup>[4,18]</sup>

Cryoglobulin type	Content	Related diseases	Laboratory findings
Type I	Monoclonal immunoglobulins (IgG, IgM or IgA) or Bence Jones protein/monoclonal free light chains	Multiple Myeloma Waldenstrom Macroglobulinemia Lymphoproliferative disease related monoclonal gammopathy Light chain disease	Precipitation within 24 h Hyperviscosity
Type II (mixed)	Monoclonal immunoglobulins (IgG, IgM or IgA) and polyclonal immunoglobulins (usually IgG)	HCV Essential cryoglobulinemia Sjogren's syndrome Rheumatoid arthritis Chronic lymphocytic leukemia	Precipitation within 7 d HCV positivity Decreased C3 Decreased C4 Decreased CH50
Type III (mixed)	Polyclonal immunoglobulins	Essential cryoglobulinemia Sjogren's syndrome Systemic lupus erythematosus Viral infections (HBV, CMV, EBV, HIV) Endocarditis Biliary cirrhosis	Increased autoantibodies such as ANA, ENA, AMA

Type I cryoglobulin concentrations are > 5 g/L, so that they tend to precipitate within 1 d; whereas in mixed cryoglobulinemia, development of precipitation can take a couple of days<sup>[2]</sup>. After the precipitation is observed samples should be incubated at 37 °C again and dissolution of the precipitates at this temperature should be confirmed. If the precipitates do not dissolve at 37 °C, test should be reported to be negative. HBV: Hepatitis B virus; CMV: Cytomegalovirus; EBV: Epstein-Barr virus; ANA: Antinuclear antibodies; ENA: Extractable nuclear antigen antibodies; AMA: Antimitochondrial antibodies; HIV: Human immunodeficiency virus.

investigated in presence of Raynaud phenomenon, peripheral cyanosis or ischemia, skin purpura, membranoproliferative glomerulonephritis, chronic HCV and HBV<sup>[10]</sup>. Circulating mixed cryoglobulins are much more common and their prevalence is stated to be 40%-50% in chronic HCV patients<sup>[11]</sup>. HCV related cryoglobulinemia is thought to be a result chronic antigenic stimulation of the humoral immune system however other clinical viral infections including HBV are not associated with the same high prevalence<sup>[11]</sup>. Biochemical grounds for why cryoglobulins precipitate at cold temperatures is not clearly understood. Protein sizes, concentration, hydrophobic content and strength of ionic bonds are thought to contribute; precipitating proteins are observed to have relatively greater ratio of hydrophobic amino acids and lower number of tyrosine and sialic acid residues<sup>[12]</sup>. IgM-RF-IgG complexes are thought to be an important factor for cryoprecipitation in mixed cryoglobulinemia<sup>[13]</sup>. Development of cryoaggregates can trigger vasculitis, whereas changes in chloride and calcium concentrations have been suggested as an influencing factor in kidneys and nerves, where cold exposure is not the case<sup>[14,15]</sup>. Our patient had recurrent lower extremity rash which is one of the vasculitic

symptoms seen in especially type II and type III cryoglobulinemia. Although there are studies indicating the presence of bile duct abnormalities in HCV patients, a direct correlation between these abnormalities and cryoglobulinemia was not shown<sup>[16,17]</sup>. Table 2 illustrates types of cryoglobulins and laboratory findings.

Sample withdrawal and transport are the most important and the critical steps for detection of cryoglobulins. Several groups have been described different analytic approaches for detection of cryoglobulins<sup>[4,10,18-20]</sup>. The main reason for false negative cryoglobulin results is incorrect withdrawal and transport procedures. United Kingdom National External Quality Assurance Scheme (UKNEQAS) organization conducted a research about detection and reporting of cryoglobulins in 137 laboratories; only in 36% of the laboratories the analysis was done without letting the temperature drop below 37 °C during serum separation, sample transport and centrifugation<sup>[7]</sup>.

After washing and isolating the cryoprecipitates, cryoglobulin quantification and characterization is essential for follow up and prognosis of the patients<sup>[21]</sup>. Total protein and immunoglobulin content can be measured by nephelometry while immunotyping can be performed by immunofixation electrophoresis (IFE). CZE/IS is an alternative method to IFE for immunotyping of monoclonal M-spike in immunoproliferative malignancies. We have used both IFE and CZE-IS methods for immunotyping of the cryoprecipitate in our patient and found out their results were slightly different. In the IFE study a polyclonal IgG and monoclonal IgM kappa bands were detected however CZE-IS study revealed a monoclonal IgM kappa and a polyclonal IgA lambda besides a polyclonal IgG content. With these findings we think we are facing an atypical mixed cryoglobulinemia, between Type II and III which may have caused cryoglobulinemic symptoms in our patient.

During IFE, specific antibodies are overlaid after electrophoresis and the corresponding immunoglobulin heavy and light chains are bound and stained. IFE is a highly sensitive and specific method to classify monoclonal immunoglobulin<sup>[22]</sup>. With the development of CZE, Immunosubtraction method is began to be used as an alternative method combined with CZE for identifying monoclonal immunoglobulins. Immunosubtraction, separates serum proteins after incubating serum with antisera for heavy and light chains, thus removing them, and detection is based on their absence when compared to serum protein electrophoresis. In CZE, the sample runs through the narrow capillary tubes and direct protein detection is performed by a measurement at 200 nm, eliminating the need for staining. We have detected especially IgA lambda by CZE/IS, which was not detected with IFE in this particular patient.

As it is previously stated only 5% of HCV patients with cryoglobulinemia have clinical symptoms<sup>[3]</sup>, however, our patient had cryoglobulinemic symptoms with an atypical cryoglobulinemia. Hence, different

types of cryoglobulins may have a correlation with the presentation of clinical symptoms. Although our theory must be confirmed with additional case reports we conclude that accurate immunotyping of cryoglobulins with alternative methods like CZE/IS may provide opportunities for proper management of these special patients.

## COMMENTS

### Case characteristics

A sixty-year old male patient presented with jaundice and dark urine for 3 d and rash on his legs for six months.

### Clinical diagnosis

Jaundice and dark urine with a rash on legs for six months.

### Differential diagnosis

All vasculitic syndromes, viral hepatitis, autoimmune diseases.

### Laboratory diagnosis

There was a discordance in red blood cells, haemoglobin and haematocrit values in complete blood count and when blood sample tube was inspected agglutinations on the walls of the tube were remarkable. A cryocrit was positive with an atypical presentation.

### Imaging diagnosis

An ultrasonographic scan of liver revealed cholestasis with minimal parenchymal hepatosteatosis.

### Pathological diagnosis

Moderate cholestatic injury accompanying with chronic hepatitis, and degenerative and dysplastic changes in bile ducts revealed in liver biopsy.

### Treatment

Ledipasvir and sofosbuvir were initiated for hepatitis C virus (HCV) therapy.

### Related reports

Chronic immune response to HCV can produce cryoglobulins resulting in vasculitis-related skin ulcers and immune complex related nephropathy. These various symptoms may sometimes lead to misdiagnosis and inappropriate therapies.

### Term explanation

Cryoglobulinemia, cryoproteins are immunoglobulins in a form that precipitates in serum and plasma at low temperatures resulting in various vasculitic symptoms.

### Experiences and lessons

Different types of cryoglobulins may have a correlation with clinical symptoms and prognosis. Accurate immunotyping of cryoglobulins with alternative methods may provide more information about cryoglobulin-generated pathology.

### Peer-review

A case report written by Ongen *et al* describes a unique case with HCV infection that was diagnosed by the presence of mixed cryoglobulinemia. They analyzed the characteristics of the methods for the detection of various cryoglobulins that are essential for the diagnosis of cryoglobulinemia. The case is interesting and their analysis regarding the methods for the detection of cryoglobulin such as capillary zone electrophoresis with immunosubtraction or agarose gel electrophoresis provides important information to the readers.

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