Cryofibrinogen—Characteristics and Association with Cryoglobulin: A Retrospective Study Out of a Series of 1,712 Samples over 7 Years

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Thromb Haemost 2023;123:669–678.

Abstract

Objective Cryofibrinogens (CFs) and cryoglobulins (CGs) are cryoproteins responsible for obstructive vasculopathy and vasculitis. The aim of this study was to compare the characteristics of CF and CG, and to define the conditions of their association.

Methods and Results This retrospective study was conducted at the Lyon University Hospitals, and included patients with at least one sample tested for CF and/or CG between September 2013 and April 2021. Serum and plasma samples were analyzed in very strict conditions of temperature. After cold precipitation, CF and CG were characterized and quantified in the cryoprecipitates. CRP and plasma fibrinogen levels were also investigated. Over this 7-year period, 1,712 samples for CF detection and 25,650 samples for CG detection were sent to the laboratory. Simultaneous testing of CF and CG was performed in 1,453/1,712 samples (85%). CF was less often positive than CG (8.3 vs. 13.5%, $p < 0.0001$). In positive CF samples, CG was associated in 28.9% of cases. In CF, fibrinogen was associated with fibronectin in 98/142 (69%) samples, especially in highly concentrated CF. CF concentration was independent of C-reactive protein and plasma fibrinogen concentrations.

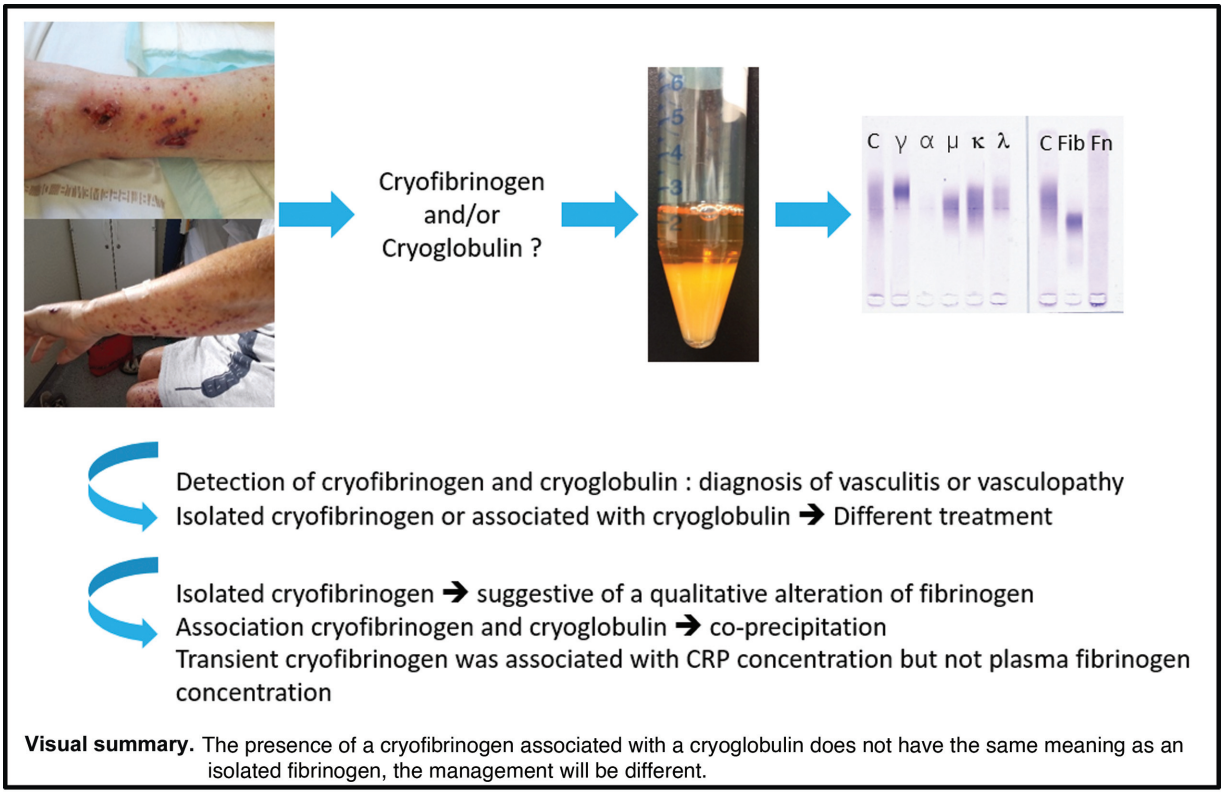
Conclusion The simultaneous detection of CF and CG is essential for the diagnosis of vasculitis or thromboembolic events and their treatment.

Keywords
- cryofibrinogens
- cryoglobulins
- inflammatory
- vasculitis

received
June 28, 2022
accepted after revision
March 1, 2023
accepted manuscript online
April 18, 2023
article published online
May 23, 2023

DOI https://doi.org/10.1055/a-2075-8204.
ISSN 0340-6245.

Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany



Visual summary. The presence of a cryofibrinogen associated with a cryoglobulin does not have the same meaning as an isolated fibrinogen, the management will be different.

Introduction

Cryofibrinogens (CFs) and cryoglobulins (CGs) are proteins that precipitate in cold temperature and dissolve when warmed at 37°C. CFs are poorly studied cryoproteins, and little is known about their physicochemical characteristics and the mechanisms of their cold deposition. These cryoproteins are less common than other cryoproteins such as CG and cold agglutinins.[1] CFs are complexes composed of fibrinogen, fibrin, fibronectin, and other proteins that precipitate in cold temperature.[2] They are essential or secondary to infections, autoimmune diseases, malignancies, drugs, or thromboembolic conditions.[3–7] They are responsible for ulceration and necrosis due to CF cold polymerization in small vessels, especially in lower limbs (malleoli and toes), sometimes leading to amputation.[4,5,8,9] Compared with CF, CGs are largely more studied, they are composed of immunoglobulins (Ig) and are classified according to their immunochemical composition.[10] Type I CGs are composed of monoclonal Ig, IgM or IgG and are secondary to lymphoproliferative diseases. Mixed CGs associate monoclonal and polyclonal Ig (type II CG) or only polyclonal Ig (type III CG), and are secondary to chronic viral infections, especially hepatitis C virus infection, and autoimmune diseases such as systemic lupus erythematosus, Sjögren syndrome, or rheumatoid arthritis.[11] CG can also be essential without underlying disease. The mechanisms of cryoprecipitation of CG are multiple and involve structural Ig anomalies (amino acids, glycosylation) responsible for noncovalent association and formation of aggregates or immune complexes that precipitate in cold temperature.[12] This phenomenon is reversible and dependent on environmental conditions (temperature, pH, ionic strength).[13,14]

The aim of this biological study was to describe and compare the characteristics of CF and CG, and to define the conditions for their association, without addressing the clinical data. This study concerned a large series of 1,712 samples for CF testing and 25,650 samples for CG testing received in the immunology laboratory of the Lyon University Hospital over a 7-year period.

Methods

Study Population and CF/CG Sampling

This retrospective study conducted at the Lyon University Hospital (France) included patients who had at least one sample for CF and/or CG detection sent to the central immunology laboratory between September 2013 and April 2021. This study concerned the biological characteristics of CF and CG, and will not address the clinical data of the patients. This study was approved by the Ethics Committee of the Hospitals of Lyon for the protection of people participating in biomedical research under the number AC-2010–1164 and AC-2016–2729.

CF must be detected in plasma and not in serum samples, from tubes containing an anticoagulant, such as citrate or ethylenediaminetetraacetic acid (EDTA). In our laboratory, blood samples for CF detection were collected in 2×4.5 mL blue top tubes with sodium citrate 9NC 0.109 M (BD Vacutainer, BD, Franklin Lakes, New Jersey, United States). CGs

were analyzed using the previously described techniques,[11,15] blood samples were collected in 3×5 mL tubes with red top without gel and with clot activator (BD Vacutainer). The next steps were similar for both CF and CG detection. Sampling and transport were performed at 37°C and precipitation at +4°C.

After sampling, tubes were maintained in an incubator at 37°C. For serum, a minimum of 2 hours of coagulation at 37°C was needed to avoid CG precipitation in the blood clot. Plasma and serum were decanted in conical bottom polystyrene tubes (15 mL Falcon polystyrene tubes, Corning Science Mexico SA, Mexico, MEX) after 15 minutes of centrifugation at 2,200 g in a thermostated centrifuge (+37°C, Centrifuge 5702RH, Eppendorf France SAS, Montesson, France). These tubes were then kept at +4°C for 7 days, vertically in a refrigerator whose temperature is controlled by a sensor. After 7 days, the presence of cryoprecipitate was evidenced by visual observation, in serum and plasma. CFs precipitate only in plasma, but CGs precipitate in serum and frequently in plasma. In rare cases, CGs precipitate in serum, but not in plasma because of lack of calcium in blood collected in tubes with anticoagulant.[16]

CF and CG Purification and Characterization

In positive samples, the cryoprecipitates corresponding to a CF in plasma and a CG in serum and possibly in plasma, were isolated by cold centrifugation (15 minutes, 2,200 g, +4°C, Centrifuge 5804R, Eppendorf France SAS) in 2 mL microtubes with conical bottom (safe-lock tubes 2 mL, Eppendorf tubes, Hamburg, Germany). Then precipitates were purified using three washes with cold phosphate-buffered saline (PBS; pH = 7.4, +4°C, BioRad, Marnes-la-Coquette, France). After each wash, the samples were centrifuged at 4,500 g for 15 minutes at +4°C. Following the last wash, 500 µL PBS or more was added to the pellet and the 2 mL microtubes were placed at 37°C for at least 2 hours, this allowed to dissolve the precipitate for further analysis. The CF and CG were then identified by electrophoresis-immunofixation (EIF; SAS, Helena Biosciences Europe, Gateshead, United Kingdom) of the dissolved cryoprecipitate with specific antisera, and quantified.

For CF characterization (adapted from Michaud et al[17]) antifibrinogen (rabbit anti-human fibrinogen Ab, Dako, Glostrup, Denmark) and antifibronectin (rabbit anti-human fibronectin Ab, Siemens Healthcare, Marburg, Germany) antisera were used on EIF. In CF-positive samples, fibrinogen was quantified by radial immunodiffusion (RID), technique adapted for low concentrations of fibrinogen[18] (NOR-Partigen, Fibrinogen, Siemens Healthcare). After 48 hours of diffusion, diameters of precipitates were measured and used with an abacus to the quantification of fibrinogen in mg/L. The presence of fibronectin associated with CF was first considered as positive or negative on EIF. Then, a semi-quantification was done according to a scale defined as a function of staining intensity on EIF: class 0 = absence of fibronectin; class 1 = presence of fibronectin +, class 2 = presence of fibronectin ++.

For CG characterization, antisera anti-γ, anti-α, anti-µ, anti-κ, and anti-λ (SAS, Helena Biosciences Europe) were used on EIF allowing their classification according to their Ig monoclonal and/or polyclonal profile.[10] In CG, the identified Ig (IgG, IgM, and/or IgA) were then quantified by immunonephelometry on BN ProSpec nephelometer (Siemens Healthcare), with Siemens Healthcare reagents for low concentrations (IgG, antiserum anti-IgG ref OSAS15 ; latex IgA, antiserum to human IgA (OSAR15) with supplementary reagent for precipitation (OUMU15); latex IgM, antiserum to human IgM (OSAT15) with supplementary reagent for precipitation (OUMU15).[15]

CF and CG concentrations were adjusted to the initial volume of plasma or serum and the results were expressed as mg/L of plasma/serum.

Associated Biological Analysis

Biological markers of inflammation, including C-reactive protein (CRP) measured by immunoturbidimetry (CRP Vario, Sentinel CH. SpA, Milan, Italy, for Abbott Architect, Abbott Rungis, France) and plasma fibrinogen measured by a Clauss-based functional method (Q.F.A. Thrombin HemosIL Werfen, Milan, Italy on ACL TOP 750, Werfen, Le Pré-Saint-Gervais, France), were collected and compared with fibrinogen concentration in CF cryoprecipitates.

Statistical Analysis

For continuous variables, the results were expressed as mean ± standard error of the mean or median [range] when the distribution was not normal. Distribution normality was tested using the D'Agostino–Pearson omnibus normality test. The Kruskal–Wallis test was used for the variance analysis and the Mann–Whitney test for the comparison of quantitative variables. The Spearman correlation test was used to analyze the association between quantitative variables. The Student's *t*-test and Wilcoxon test were used for the comparison of quantitative parameters. The chi-squared test and Fisher's exact test were used to analyze qualitative differences. $p < 0.05$ was considered statistically significant. Calculations were performed using the GraphPad Prism version 5.01 software (La Jolla, California, United States).

Results

Demographic Data

From September 2013 to April 2021, 1,712 samples for CF detection and 25,650 samples for CG detection were sent to the laboratory. Patients with CF testing were significantly younger than patients with CG testing (50.5 ± 22.7 vs. 53.1 ± 18.1 years, respectively, $p = 0.0002$). There was no difference in sex ratio, with a predominance of females in both the CF and CG groups (F/M = 1.64 vs. F/M = 1.59, respectively, $p = 0.63$; ►**Table 1**).

Positive CF were found in 142/1,712 samples and positive CGin 3,454/26,650 samples (8.3 vs. 13.5%, respectively, $p < 0.0001$). The mean age of CF+ patients was significantly lower than that of CG+ patients (48.7 ± 23.1 vs. 56.1 ± 17.4 years, respectively, $p = 0.0005$), but the sex ratio was not

Table 1 Cryoglobulin and cryofibrinogen characteristics

	CG	CF	*p*
n	25,650	1,712	
Age (y)	53.1 ± 18.1	50.5 ± 22.7	<0.001
F/M	1.59	1.64	0.63
	(15,765 F/9,885 M)	(1,063 F/669 M)	
Positive detection	3,454 (13.5%)	142 (8.3%)	<0.0001
Age (y)	56.1 ± 17.4	48.7 ± 23.1	0.0005
F/M	1.55	1.96	0.23
Positive CG (*n* = 3,454)			
Type I	364 (10.5%)		
Type II	1,301 (37.7%)		
Type III	1,789 (51.8%)		
CG concentration mg/L (mean ± SEM)			
Type I	3,953 ± 471		
Type II	644 ± 71.4		
Type III	71.7 ± 2.6		
Positive CF (*n* = 142)			
Fibrinogen +		142 (100%)	
Fibronectin +		98 (69%)	
CF concentration mg/L (mean ± SEM)		185.9 ± 38	
Fibronectin + (*n* = 98/142)		224.1 ± 54.3	0.01
Fibronectin − (*n* = 44/142)		100.9 ± 16	
Positive CF associated with CG testing (*n*)		114/142	
CF + CG+ (33/114, mg/L)	46.2 [7–2,925]	74.2 [10–781]	
CF + CG− (81/114, mg/L)		80 [10–4,706]	
Negative CF associated with CG testing		1,339/1,570	
CF–CG+ (115/1,339, mg/L)	38 [7.3–35,520]		
CF–CG− (1,224/1,339)			

Abbreviations: CG, cryoglobulin; CF, cryofibrinogen; CRP, C reactive protein.

significantly different (F/M = 1.96 vs. F/M = 1.55, respectively, $p = 0.23$; ►**Table 1**).

CF and CG Detection and Characterization

The visual observation of the plasma and serum kept for 7 days at +4°C allowed the detection of a precipitate in plasma and/or in serum. The delays for CF and CG precipitation were similar (within 7 days). It was difficult to differentiate a CF precipitate from a CG precipitate based solely on their appearance: white precipitate forming volutes or a pellet at the bottom of the tube, or gelified plasma/serum (►**Fig. 1**). Some CG, especially type I CG, formed translucent precipitates characteristic of cryoprecipitating monoclonal IgM.[15]

EIF using specific antisera was used for CF characterization (presence of fibrinogen and/or fibronectin, ►**Fig. 2A**) and for CG typing (monoclonal or polyclonal IgG, IgA, or IgM, ►**Fig. 2B**). In CF, fibronectin (Fn) was associated with fibrinogen in 98/142 samples (69%, ►**Table 1**), and the evaluation of staining intensity on the EIF allowed the semi-quantification of Fn as class 0 (absence of a colored band with anti-Fn Ab, ►**Fig. 2A**-1), class 1 (presence of a thin colored band with anti-Fn Ab, ►**Fig. 2A**-2), or class 2 (presence of a large colored band with anti-Fn Ab, ►**Fig. 2A**-3).

CF and CG Association: Type and Concentration

The simultaneous testing of CF and CG offered four possibilities: absence of precipitate for CF and CG; presence of a precipitate in serum but not in plasma for a positive CG and negative CF; presence of a precipitate in plasma but not in serum for a positive CF and negative CG; presence of a precipitate in both serum and plasma for a positive CG and a possible CF, as CG can also precipitate in plasma. The EIF with specific antisera allowed us to characterize and differentiate CG from CF. As shown in ►**Fig. 2C**, a CG (►**Fig. 2C**-1) and a CF (►**Fig. 2C**-2) could be detected in the same patient,

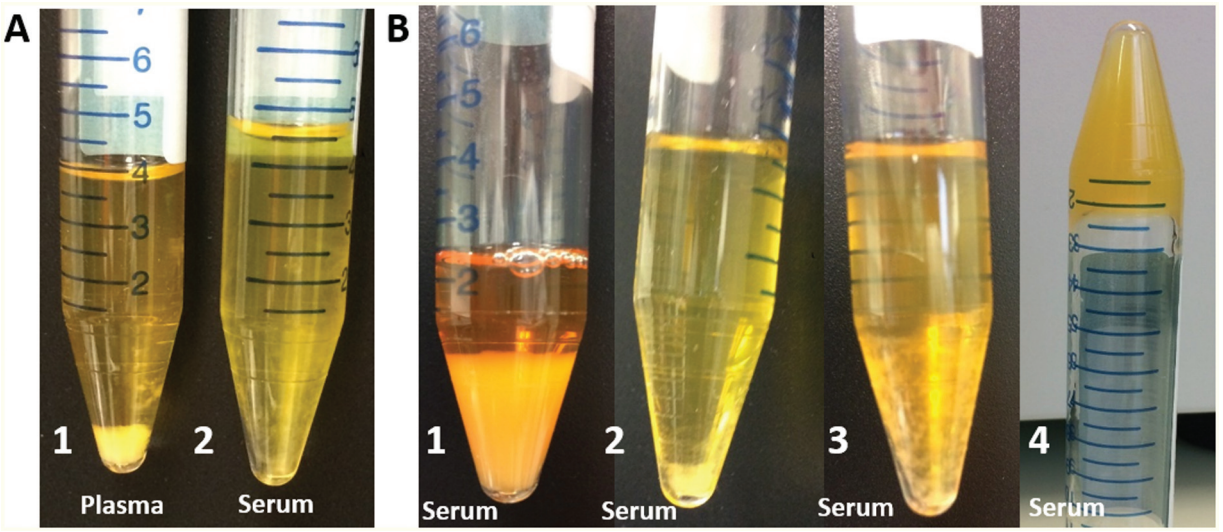


Fig. 1 Examples of appearance of cryofibrinogen (CF) and cryoglobulin (CG) precipitates in plasma and serum. (**A**) Positive CF in plasma (A1) and negative CG in serum (A2) of the same patient. In this case of positive CF, the precipitate formed a pellet at the bottom of the conical tube containing plasma (A1) and no precipitation in the serum (A2) when the samples are placed for 7 days at +4°C. (**B**) Example of aspects of CG precipitates in serum, such as a large pellet in B1, fine flakes deposited on the wall of the tube and forming a pellet in B2, thicker flakes on the wall or at the bottom of the tube (B3). Cryogel formation (B4) is more common with CG than with CF.

while in some cases, CG could be present in both serum (►**Fig. 2D**-1) and plasma of the same patient, but without CF (►**Fig. 2D**-2).

Among all the samples tested for CF in the plasma ($n = 1{,}712$), simultaneous testing for CG in the serum was done for 1,453/1,712 (85%) samples: 1,224/1,453 (84.2%) were CF − CG −, 115/1,453 (7.9%) were CF − CG +, 81/1,453 (5.6%) were CF + CG −, and 33/1,453 (2.3%) were CF + CG +. Concentration ranges and median of CF and CG in the different subgroups are reported in ►**Table 1**.

Among the CF+ samples that were also tested for CG (114/142), CFs were more frequently found alone (CF + CG −, 81/114, 71.1%) than associated with a CG (CF + CG +, 33/114, 28.9%, $p < 0.001$, ►**Table 1**).

There was no significant difference in the type of CG according to its association with CF (CF + CG +) or not (CF − CG +): type I CG (4/33 vs. 9/115), type II CG (8/33 vs. 36/115), and type III CG (21/33 vs. 70/115, $p = 0.60$; ►**Table 2**). There was no significant difference in CG concentration according to CG type in CF + CG+ and CF − CG +: for type I ($p = 0.47$), type II ($p = 1$), and type III ($p = 0.3$; ►**Table 2**).

CF concentrations were measured by RID (►**Fig. 3A**). CF concentrations were compared when CFs were associated with CG (CF + CG +) or not (CF + CG −). There was no significant difference in CF concentration between CF + CG+ and CF + CG− (122.4 ± 24.4 vs. 218.3 ± 64.7 mg/L, respectively, $p = 0.85$). There was no correlation between CF concentration and CG concentration in samples CF + CG+ ($p = 0.78$), whatever the type of CG (for type I CG $p = 0.46$, for type II CG $p = 0.91$, for type III CG $p = 0.60$).

Fibronectin in CF and CF/CG Association

Among the 142 CF+ samples, fibrinogen was associated with fibronectin in 98/142 samples (69%), and fibrinogen was found alone in 44/142 samples (31%). The CF concentration was significantly higher in fibrinogen+ fibronectin+ samples compared with fibrinogen+ fibronectin− ones (224.1 ± 54.3 vs. 100.9 ± 16 mg/L, respectively, $p = 0.01$; ►**Table 1**).

According to the semiquantitative evaluation of fibronectin (divided into class 0, 1, or 2), fibrinogen concentration in cryoprecipitate was significantly lower in fibronectin class 0 compared with fibronectin class 2 (100.9 ± 16 vs. 169 ± 27 mg/L, $p = 0.002$) but not in fibronectin class 0 compared with fibronectin class 1 (100.9 ± 16 vs. 150 ± 20.9 mg/L, $p = 0.08$) nor in fibronectin class 1 compared with class 2 ($p = 0.13$; ►**Fig. 3B**). There was no significant difference in the presence of fibronectin between CF + CG+ and CF + CG− ($p = 0.82$).

Repeated Detections of CF and CG

Among the samples tested for CF ($n = 1{,}712$), CF was tested in more than one sample for 182 patients (≥2 samples). These re-tested patients represented only 10,6% of CF (182/1,712), this is a limitation of these results. For 134/182 (73.6%) patients, CF was not detected in all the samples and for 18/182 (9.9%) patients, all the samples tested were positive. For 9/182 (5%) patients, a first sample was negative and the next was positive, and for 21/182 (11.5%) patients, CF was positive on a first sample and negative on the next samples (13 after 1–6 months, 3 after 7–12 months, 4 after >12 months), i.e., 30/182 (16%) were transient CF.

Transient or Persistent CF and Inflammation Markers

Among the 142 patients with positive CF, CRP and plasma fibrinogen were measured at the same time as CF detection in 110/142 and 66/142 samples, respectively. Among the 110 patients with CRP determination, 60 had CRP < 10 mg/L (noninflammatory group) and 50 had CRP ≥ 10 mg/L (inflammatory group). There was no significant difference in

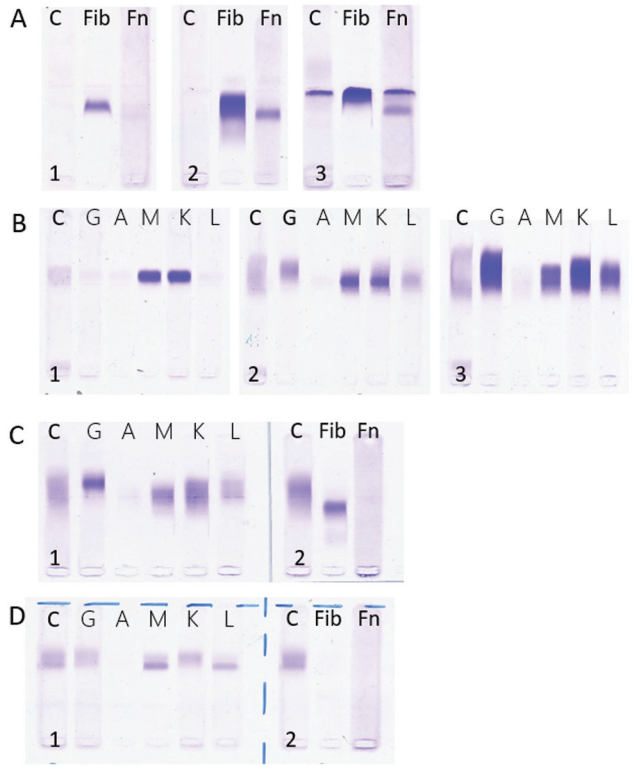


Fig. 2 Specific electrophoresis-immunofixation for the characterization of cryofibrinogen (CF) and cryoglobulin (CG) in cryoprecipitates. C: purity control (total protein antiserum); Fib: antifibrinogen antiserum; Fn: antifibronectin antiserum; G: anti-gamma chain antiserum; A: anti-α chain antiserum; M: anti-mu chain antiserum; K: anti-kappa chain antiserum; L: anti-lambda chain antiserum. Line (**A**): (1) detection of fibrinogen not associated with fibronectin (class 0); (2) detection of fibrinogen associated with fibronectin+ (class 1); (3) detection of fibrinogen associated with fibronectin++ (class 2). In this last case (A-3), the band observed in lane C corresponds to an interference linked to the high concentration of fibrinogen and fibronectin in this sample, certainly forming complexes, which become blocked in the meshes of the agarose gel.[15] Line (**B**): (1) IgM kappa type I CG; (2) IgM kappa and polyclonal IgG type II CG; (3) polyclonal IgG and IgM type III CG. Line (**C**): presence of a type III CG (1: polyclonal IgM and IgG) associated with a CF (2). CG has precipitated in the plasma and is visible on the CF gel associated with fibrinogen (2). Line (**D**): presence of a type II CG (1: IgM lambda and polyclonal IgG), without a CF (2). CG has precipitated in the plasma and is visible on the CF gel in the absence of fibrinogen and fibronectin (2).

the CF concentration of the noninflammatory group compared with the inflammatory group (164 ± 24.8 vs. 271 ± 103 mg/L, respectively, $p = 0.77$; ►**Fig. 4A**). There was no correlation between CRP concentration and CF concentration ($r = 0.13$, $p = 0.16$).

For the 66 CF+ patients with plasma fibrinogen determination, there was no significant difference in CF concentration between patients with normal plasma fibrinogen (<4.2 g/L, $n = 41$) and patients with high plasma fibrinogen (≥4.2 g/L, $n = 25$) (146 ± 28.8 vs. 343 ± 192 mg/L, respectively, $p = 0.11$; ►**Fig. 4B**). There was no significant correlation between plasma fibrinogen concentration (4.22 ± 0.22 g/L) and CF concentration (217.7 ± 71.9 mg/L; $r = 0.14$, $p = 0.27$).

Among the 30 patients with transient CF (CF+/CF− or CF−/CF+), 25/30 had a CRP assay associated with both detections of CF/CG and 8/30 had a plasma fibrinogen assay associated with both detections. A paired Wilcoxon test showed that CFs were positive in the presence of elevated CRP and became negative when CRP decreased (►**Fig. 4C**, $p = 0.02$). However, the concentration of CF was not a function of the concentration of CRP (intensity of inflammatory reaction, ►**Fig. 4A**). On the other hand, there was no relationship between the presence of CF and the concentration of plasma fibrinogen (►**Fig. 4D**).

Discussion

Out of this large number of samples, this study reports on the CF detection associated or not with CG detection. CFs were found less frequently than CG (8.3 vs 13.5%). Among the patients tested multiple times for CF detection, 16% had transient CF, the presence of CF was dependent on the concentration of CRP, but not of plasma fibrinogen. Positive CFs were associated with CG in 30% of samples, and this association was independent of CF and CG concentrations. In CF, fibrinogen was frequently associated with fibronectin, especially in highly concentrated CF, but the presence of fibronectin was not associated with co-precipitation of CF and CG. CF concentration was independent of CRP and plasma fibrinogen concentrations.

CF detection has been largely much less requested than CG detection, this was observed in this series over a long period.

Table 2 Cryoglobulin type and concentration in cryofibrinogen positive (CF+) and negative (CF−) patients

	CF+CG+	CF−CG+	*p*
n	33	115	
Type I CG (*n*, %)	4 (12.1%)	9 (7.8%)	0.60
Type II CG (*n*, %)	8 (24.2%)	36 (31.3%)	
Type III CG (*n*, %)	21 (63.6%)	70 (60.9%)	
CG concentration (mg/L)	Median [min–max]		
Type I CG	44.7 [11–2,925]	38.9 [13–35,520]	0.47
Type II CG	61.2 [18–379]	63.2 [8.8–14,342]	1
Type III CG	42.5 [7–145]	29.9 [9–506]	0.3

Abbreviations: CG, cryoglobulin; CF, cryofibrinogen.

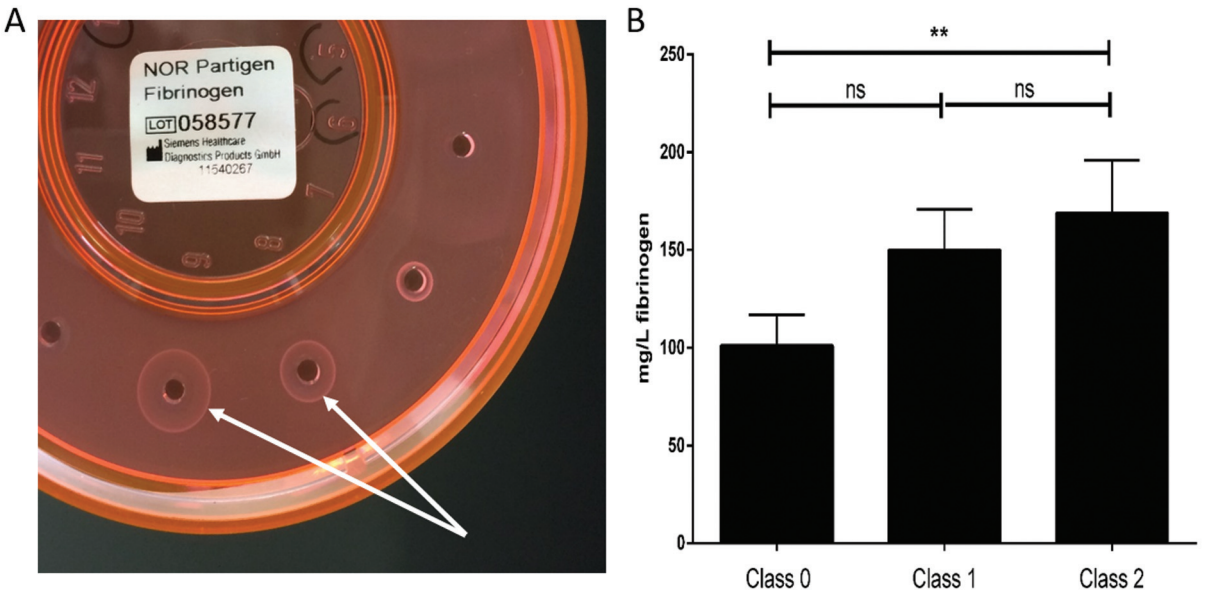


Fig. 3 Cryofibrinogen (CF) quantification method (**A**) and concentration according to the presence of fibronectin (**B**). (A) Radial immunodiffusion for fibrinogen antigenic quantification in cryoprecipitates. After 48 hours of cryoprecipitate diffusion in the gel, circles of precipitation (*white arrows*) appear and their diameters are measured to quantify fibrinogen according to an abacus. (B) CF concentration according to fibronectin semi-quantification (class 0–1–2) on electrophoresis-immunofixation (staining intensity). Class 0: fibronectin negative in the cryoprecipitate; class 1: fibronectin positive+ in the cryoprecipitate; class 2: fibronectin positive ++ in the cryoprecipitate; $^{**}p = 0.002$; ns = not significant.

This study allowed us to sensitize clinicians to a systematic detection of CF in clinical contexts suggestive of the presence of cryoproteins, especially if no CG was detected. For this, clinician must make a formal request because blood must be collected on different tubes. For CG and CF, methods for detection and quantification are absolutely critical. CF is a cryoprotein found in plasma and not in serum. Tubes containing an anticoagulant such as citrate or EDTA can be used to detect CF. Heparinized tubes cannot be used because of the spontaneous formation of cryoprecipitating heparin–fibrinogen–fibronectin complexes when the sample is placed at +4°C, which could lead to a false-positive detection.[19,20] CG precipitates in serum (tubes without anticoagulant or gel), but can also be detected in plasma. The use of a dry tube is important to detect CG because some CG cannot precipitate in the absence of calcium in tubes with a calcium-chelating anticoagulant.[16,21] The search for cryoproteins must thus be done simultaneously, on a serum sample for CG and a plasma sample for CF. Furthermore, these two types of cryoproteins cannot be differentiated based on the appearance of the precipitate, and EIF characterization using specific antiserum is therefore essential.

CF concentration can be estimated using spectrophotometry at 280 nm[5] or by assessing the difference in plasma fibrinogen concentration before and after fibrinogen cryoprecipitation.[2,22] The latter technique, however, lacks sensitivity as CF accounts for only approximately 1 to 5% of total fibrinogen. The use of RID to quantify fibrinogen in the cryoprecipitate is a more sensitive and specific method. Similarly, the quantification of CG by determination of Ig in the cryoprecipitate, as performed in the present study, is more sensitive and specific than the measurement of cryocrit or total proteins in the cryoprecipitate.[15,23–26] There is limited information on the relationship between CF concentration, its composition, and a possible link with clinical manifestations. CF concentration was reported significantly higher in patients with thrombosis, but not its composition and especially the presence of fibronectin.[4] The finding herein that fibronectin was present in the most concentrated CF might represent an important element for the diagnosis of thrombotic events associated with CF.[17,22]

Repeated detection of CF is interesting to evaluate its permanent or transitory presence. Herein in approximately 16% of samples, CF was transient, not systematically found in two or more consecutive samples with the limitation that there were only approximately 11% re-tested patients. The presence of a transient CF is related to the concentration of CRP in this study, as reported in two studies.[8,22] In an inflammatory context, a CF of variable concentration could appear in the plasma. This CF is not linked to an elevation of plasma fibrinogen, which could have been a pathophysiological hypothesis of the appearance of this abnormal cold-sensitive fibrinogen. Such a transient CF, probably associated with an inflammatory reaction, is likely to be less pathogenic than persistent CF as it disappears when the inflammation resolves.[4,8,27,28] Confirming the presence of CF on a second sample, apart from an inflammatory episode if possible, is thus necessary to differentiate a transient CF from a persistent one, possibly responsible for occlusive vasculopathy or thrombosis.[4]

The clinical outcomes of CF associated with CG or isolated CF are very different. The association of CF and CG was lower than the 88 to 100% association reported previously.[2,4] The association of these two cryoproteins is largely due to the co-

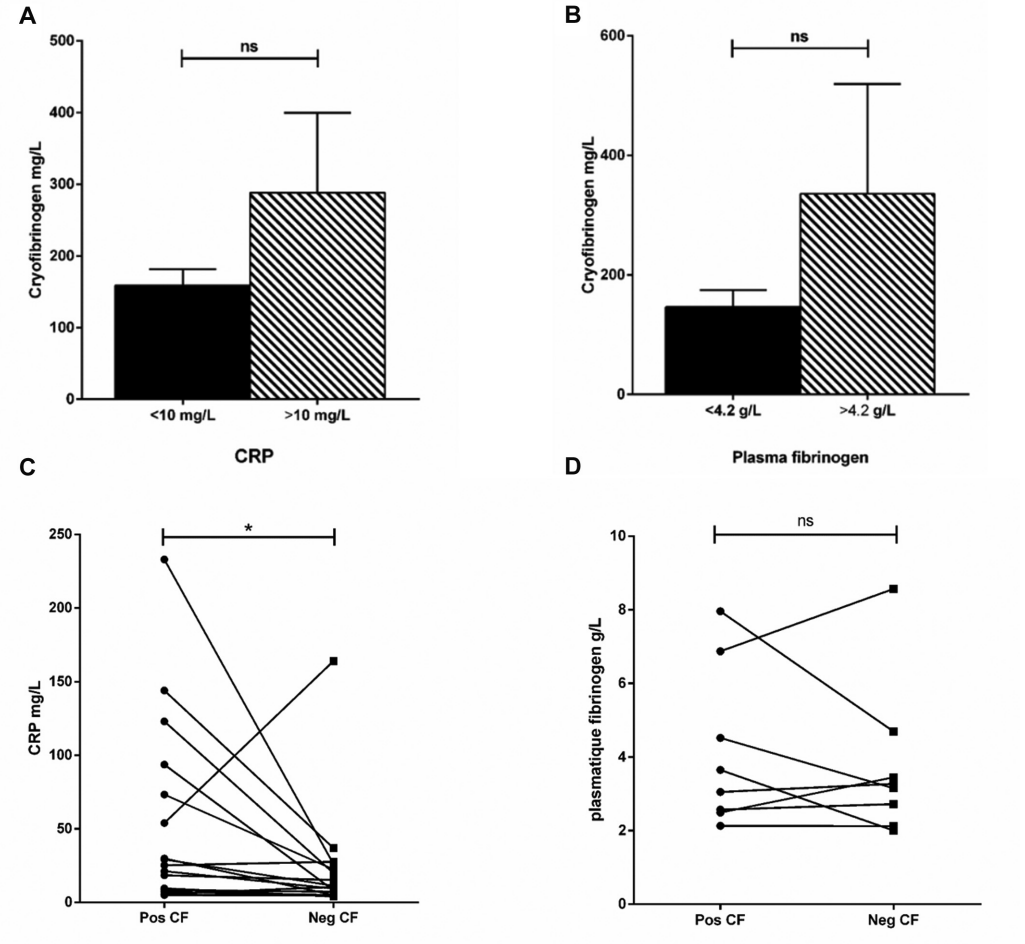


Fig. 4 Cryofibrinogen and inflammation. (**A**) CF concentration in mg/L in function of inflammatory state, noninflammatory group with CR$p < 10$ mg/L and inflammatory group with CRP ≥ 10 mg/L. (**B**) CF concentration in mg/L in function of inflammatory state, noninflammatory group with plasma fibrinogen <4.2 g/L and inflammatory group with plasma fibrinogen ≥4.2 g/L. (**C**) CRP concentration in patients with transient CF (positive CF and negative CF). (**D**) Plasma fibrinogen concentration in patients with transient CF (positive CF and negative CF). CF, cryofibrinogen; CRP, C-reactive protein; ns, not significant. $^{*}p = 0.02$.

precipitation of fibrinogen, and possibly other proteins, within the CG complex,[4,22,27] but is not linked to fibronectin, as shown in the present study. When co-precipitation occurs, the treatment of CG leads to the disappearance of CF. However, detection of a positive CF without CG is suggestive of a qualitative alteration of fibrinogen, possibly favoring its cold polymerization and the formation of complexes that can affect small vessels.[2,3,29] This could explain why patients with isolated CF can present with severe complications such as necrosis and gangrene,[8,27,29] but the pathogenic role of CF, alone or associated with a CG, remains to be demonstrated,[30] a clinical study must complete this biological study. The pathogenic mechanisms of these two cryoproteins are very different. CG involves an immune response, especially through the contribution of Ig with rheumatoid factor activity and complement activation.[31] The presence of a rheumatoid factor in a CG, often anti-IgG IgM, promotes the formation of immune complexes, which will be larger at low temperature, increasing further their precipitation.[12] The complement system is then activated at the site of precipitation and causes a local inflammatory reaction and vasculitis. CGs are responsible for vasculitis of small- and medium-size vessels, especially in skin and extremities.[32] Conversely, cold deposition of CF does not result in an inflammatory reaction, but rather in the occlusion of small- and medium-size vessels. Cold conditions are responsible for the formation of stabilized CF oligomers, fibronectin probably binds to fibrinogen, and fibrin and acts as a nucleus favoring the cold precipitation of CF that can deposit and decrease circulation in small vessels.[2] Skin biopsy usually reveals occlusive vasculopathy or microthrombi in dermis not confined exclusively to the ulceration.[9] Clinical arguments and biopsies are not sufficient to differentiate CF from CG and CG/CF association. The simultaneous detection of a CG in serum and a CF in plasma is therefore a key criterion for the differential diagnosis of the clinical manifestations linked to these two cryoproteins. This can be useful to select treatment, as immunosuppressive agents will be favored for CG or CG/CF association, and fibrinolytic agents for isolated CF.[9,27]

Conclusion

This large series of samples found that CF was less commonly detected than CG and that these two cryoproteins could be associated. Since they act through different mechanisms, the

simultaneous detection of CF and CG is essential to differentiate between immune-mediated vasculitis with CG, and more mechanical thromboembolic events with CF. Multiple testing over time of both CF and CG is important to assess their transient or persistent nature and for treatment monitoring.

What is known about this topic?

- Cryofibrinogen is a fibrinogen that precipitates in the cold, associated or not with a cryoglobulin.
- Cryofibrinogen is underestimated because it is rarely tested.
- Cryofibrinogen is essential or secondary to inflammatory or thromboembolic conditions and can be responsible for severe vasculopathies.

What does this paper add?

- Methods for detection and quantification of cryofibrinogen and cryoglobulin are critical.
- Cryofibrinogen is found less frequently than cryoglobulin; its presence can be transient or permanent.
- CF concentration was independent of inflammatory status (CRP $>$ 10 mg/L, fibrinogen $>$ 4.2 g/L), but transient CF was related to CRP concentration but not to plasma fibrinogen concentration.
- Cryofibrinogen must be tested at the same time as cryoglobulin to differentiate isolated cryofibrinogen from cryofibrinogen associated with cryoglobulin, the treatment will be different.

Authors' Contribution
Conceptualization: M.N.S.K. and P.M.; Methodology: M.N.S.K.; Validation: P.M.; Formal analysis: M.N.S.K. and P.M.; Resources: M.N.S.K.; Writing—original draft preparation: M.N.S.K.; Writing—review and editing: M.N.S.K.

Funding
None.

Conflict of Interest
None declared.

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