Introduction

Mixed cryoglobulinaemia is a systemic vasculitis predominantly mediated by cryoprecipitable immune complexes, and is usually associated with hepatitis C virus (HCV) infection [1]. The most frequent manifestations of mixed cryoglobulinaemia are cutaneous leukocytoclastic vasculitis leading to purpura and ulceration, peripheral neuropathy, glomerulonephritis and central nervous system vascular lesions that may underlie cognitive impairment [2–4]. In some patients, mixed cryoglobulinaemia has an acute onset and presents with multisystem polyarteritis nodosa-like necrotizing vasculitis with prominent cutaneous, neurological and renal involvement [5]. Skin ulcers represent the only relevant clinical feature in a proportion of patients seeking treatment [6, 7].

There is growing evidence that prothrombotic coagulation defects may facilitate vascular complications in vasculitides. In SLE, hyperhomocysteinaemia and aCLs increase the risks of thromboembolism and of coronary artery disease [8–10], especially if combined with other thrombophilic defects [11]. The prothrombin G20210A mutation [12] and the presence of aCL [13] might increase the risk of thrombosis in Behçet’s disease, although some studies [14, 15] do not support this concept. In addition, thrombophilic defects have also been described in association with necrotizing skin ulcers caused by heterogeneous vasculitic disorders [16, 17].

So far, few studies have addressed the influence of thrombophilic conditions on the vasculitic manifestations of HCV-associated mixed cryoglobulinaemia. The aCLs have been found in a high proportion of patients, but they were β2-glycoprotein I-independent and were not associated with thrombotic events [18, 19]. Anti-endothelial cell antibodies were also frequent in these patients, but their pathogenic role is unclear [20].

In this study, we investigated the contribution of inherited and acquired thrombophilic factors to the clinical manifestations of HCV-associated mixed cryoglobulinaemia.

Patients and methods

Patients and controls

In this single-centre study, patients with mixed cryoglobulinaemia secondary to chronic HCV infection were followed at the Department of Clinical Immunology of the Policlinico Umberto I in Rome. Sixty-four consecutive patients were recruited after informed consent.

The following associated vascular risk factors [21] were assessed: hypertension (systolic blood pressure >160 mmHg or diastolic pressure >90 mmHg or use of anti-hypertensive drugs); diabetes (fasting glucose level >140 mg/dl or use of anti-diabetic drugs); hypercholesterolaemia (total cholesterol >240 mg/dl); and smoking habit (any number of cigarettes). The duration of disease was defined as the onset of any symptom or sign of vasculitis, independently of the duration of HCV infection.

Control groups included patients with chronic HCV infection without cryoglobulinaemia and HCV-negative normal subjects without family or personal history of deep venous thrombosis or cardiovascular diseases. For the analysis of acquired thrombophilic defects (homocysteine, protein C and protein S levels, aCLs and LAC), we selected control groups with age distributions comparable with that of mixed cryoglobulinaemia patients: 29 HCV-positive patients without cryoglobulinaemia (nine females; median age 58 yrs, range 45–85 yrs) and 22 normal subjects (14 females; median age 58 yrs, range 48–73 yrs). For inherited thrombophilic defects larger control groups were included in the study regardless of age: 49 HCV-positive patients without cryoglobulinaemia (13 females) and 57 normal subjects (27 females).

All study subjects provided their informed consent. The study was approved by the review board of the Policlinico Umberto I.
and was conducted according to the Declaration of Helsinki and to the international standards of Good Clinical Practice.

**Classification of the clinical manifestations of cryoglobulinaemic vasculitis**

Clinical assessments were done by two expert clinicians (M.C. and Ma.C.) and by one neurologist (A.F.), blindly with respect to the results of laboratory studies.

The severity of purpura was classified according to the following scoring system. Grade 0: no purpura; Grade 1: episodic painless purpura limited to the malleolar region, usually triggered by exogenous factors (e.g. exposure to cold or prolonged standing position); Grade 2: episodic painful purpura independent on exogenous factors, responsive to low-dose steroids; Grade 3: chronic painful purpura, not responsive to steroids.

Patients were classified as having or not cryoglobulinaemic nephropathy, defined by typical histological findings on renal biopsy, and/or by creatinine higher than the upper laboratory reference value on more than two consecutive occasions, and/or by nephrotic-range proteinuria in the absence of other causes of renal damage.

A clinical diagnosis of peripheral neuropathy was made when symptoms (weakness, sensory disturbances) and signs (atrophy, reduced/absent tendon reflexes) of peripheral sensory and/or motor involvement were present. In most of the patients neuropathy was confirmed by electrophysiological studies.

**Laboratory studies**

All coagulation tests were performed in the same laboratory by investigators blinded to the clinical characteristics of the patients. The serum concentration of homocysteine (micromoles/litre) was evaluated by fluorescence polarization immunoassay (IMx Homocysteine; Abbott Laboratories, Abbott Park, IL, USA). Protein C was measured using the Sta Protein C chromogen test (Diagnostica Stago, Asnières sur Seine, France). Protein S was measured using the turbidimetric, microparticle-based, Liatest Free Protein S (Diagnostica Stago). Activated protein C (APC) resistance was measured using the Sta Staclot APCR kit (Diagnostica Stago).

Methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C mutations, factor V Leiden, factor V H1299R, prothrombin G20210A mutation and plasminogen activator inhibitor-1 (PAI-1) 4G5G polymorphisms were analysed by PCR using specific commercial kits using biotin-labelled primers (Nuclear Laser Medicine, Settala, Milan, Italy). Bound biotinilated sequences were detected using streptavidin alkaline phosphatase and colour substrate.

Detection of aCLs and determination of the IgG or IgM isotype were performed by ELISA using commercially available kits (Orgentec-Diagnostika, Mainz, Germany). Microplates were coated with highly purified cardiolipin and saturated with purified human β2 glycoprotein I. A standard curve was established using calibrators for IgG and IgM aCLs. Values of IgG phospholipid (GPL) or IgM phospholipid (MPL) >11 U/ml were considered positive. This cut-off was higher than the 99th percentile of 50 control sera for both IgG and IgM antibodies.

LAC was determined according to the criteria of the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the International Society of Thrombosis and Haemostasis [24]. Screening assays included the kaolin clotting time, dilute activated partial thromboplastin time (APTT), LAC sensitive aPTT reagent (PTT-LA) and two dilute Russell viper venom times. Positive test results were confirmed with the same reagent in the presence of excess phospholipids. At least one test system result had to be positive in all steps for a patient to be considered LA positive.

**Statistical analysis**

Differences between groups were evaluated by the Mann–Whitney U-test (tied P-values) for continuous variables and by the Fisher’s exact test for categorical data. Spearman’s correlation was used for the analysis of association of continuous variables with the discrete variable ‘severity of purpura’. Independent variables showing association at \( P < 0.15 \) with dependent covariates were included in multivariate binomial or polytomous logistic regression models. Scoring of the severity of purpura was transformed into discrete nominal variable for polytomous logistic regression.

All statistics were produced using the StatView software (SAS Institute Inc., Cary, NC, USA), with a type I error rate of 0.05 for two-sided tests of significance.

**Results**

A summary of demographic and clinical data in 64 patients with HCV-associated mixed cryoglobulinaemia is reported in Table 1. Table 2 summarizes inherited and acquired thrombophilic defects in patients with HCV-associated mixed cryoglobulinaemia and in control groups. Patients with mixed cryoglobulinaemia had significantly higher homocysteine and lower protein S concentrations than normal subjects, whereas patients with HCV infection without cryoglobulinaemia had reduced protein C levels compared both with patients with HCV-associated mixed cryoglobulinaemia and with normal subjects. The latter finding is probably accounted for by the higher prevalence of cirrhosis among our patients with hepatitis C without cryoglobulinaemia, since protein C levels are reduced in patients with advanced liver fibrosis [25]. Neither the prevalence of aCL and LA nor those of inherited thrombophilic defects were significantly different between mixed cryoglobulinaemia patients and control groups.

The MTHFR genotype did not correlate substantially with homocysteine concentration. Only in patients with cryoglobulinaemia, high homocysteine was associated, although weakly (\( P = 0.03 \)), with MTHFR C677T homozygosity (21% of patients). In normal subjects and in patients with HCV infection without cryoglobulinaemia no correlation between MTHFR C677T genotype and homocysteine concentration was observed, although

<table>
<thead>
<tr>
<th>TABLE 1. Demographic and clinical data in patients with HCV-related mixed cryoglobulinaemia. Data are expressed as the median and range or as the number of patients and percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases</td>
</tr>
<tr>
<td>Female gender</td>
</tr>
<tr>
<td>Age, median (range), yrs</td>
</tr>
<tr>
<td>Cryocrit, n (%)</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
</tr>
<tr>
<td>Hypercholesterolaemia, n (%)</td>
</tr>
<tr>
<td>Smoking habit, n (%)</td>
</tr>
<tr>
<td>Duration of disease, median (range), yrs</td>
</tr>
<tr>
<td>Cryocrit, median (range), %</td>
</tr>
<tr>
<td>Purpura grade, n (%)</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>Skin ulcers, n (%)</td>
</tr>
<tr>
<td>Nephropathy, n (%)</td>
</tr>
<tr>
<td>Peripheral neuropathy, n (%)</td>
</tr>
</tbody>
</table>
normal individuals with heterozygous MTHFR A1298C had slightly higher homocysteine that those with normal genotype ($P < 0.01$). Significant correlations between MTHFR status and homocysteine concentration were not apparent even when analyses were restricted to the younger halves of groups. The reason for these discrepancies is probably the small size of the samples.

We sought for associations between demographic, clinical and laboratory data and specific manifestations of mixed cryoglobulinaemia. The following clinical manifestations were used as outcome covariates for statistical analyses: severity of purpura, presence of skin ulcers, presence of nephropathy and presence of peripheral neuropathy.

Neither the cryocrit level nor the coexistence of cirrhosis and/or of vascular risk factors, namely diabetes, hypertension, hyperlipidaemia and smoking habit, influenced the clinical manifestations of cryoglobulinaemia vasculitis. Older age was weakly associated with the presence of skin ulcers ($P = 0.012$) and of nephropathy ($P = 0.041$). The duration of the disease was significantly ($P = 0.0004$) longer in patients with nephropathy (median 11 yrs; range 1–24 yrs) than in those without nephropathy (median 3 yrs; range 0.5–16 yrs).

Among thrombophilic defects, no significant associations were observed between clinical manifestations and the following variables: protein C concentration, protein S concentration, the presence of aCL or LA, APC resistance, MTHFR C677T, MTHFR A1298C, factor V Leiden, factor V H1299R, prothrombin G20210A and PAI-1 4G/4G/5G/5G homozygosity. In contrast, high homocysteine concentration was significantly associated with the severity of purpura ($P = 0.0006$ by Spearman’s correlation) and with the presence of skin ulcers ($P = 0.0007$). The presence of nephropathy was also associated with high homocysteine ($P < 0.0001$). However, linear regression analysis revealed a significant positive correlation ($P = 0.005; R^2 = 0.44$) between homocysteine and creatinine concentrations. Based on this observation, we assumed that hyperhomocysteinemia in this group was the consequence rather than the cause of renal damage and, therefore, we did not include homocysteine as an explanatory variable for nephropathy in further analyses. High homocysteine concentration did not correlate with the presence of peripheral neuropathy.

Independent variables showing association at $P < 0.15$ in exploratory univariate analyses were included in logistic regression analysis. In single-risk variable logistic regression models significant determinants were: for the severity of purpura (Grade 0–2 vs Grade 3) homocysteine odds ratio $[(P < 0.0001; odds ratio (OR) = 1.36; 95% CI 1.15, 1.62)$ for skin ulcers homocysteine ($P < 0.0001; OR = 1.31; 95% CI 1.12, 1.54$) and age ($P = 0.017; OR = 1.07; 95% CI 1, 1.14$) for nephropathy the duration of disease ($P = 0.0001; OR = 1.25; 95% CI 1.1, 1.42$) and age ($P = 0.016; OR = 1.07; 95% CI 1.0, 1.15$). Application of stepwise multivariate logistic regression to the candidate explanatory variables (Table 3) identified homocysteine as the only risk factor for severe purpura and skin ulcers, and the duration of disease for nephropathy.

### Discussion

We investigated the contribution of inherited and acquired thrombophilic defects to the clinical manifestations of HCV-associated mixed cryoglobulinaemia, and found that hyperhomocysteinaemia was independently associated with an elevated risk of severe cutaneous lesions. None of the other thrombophilic defects investigated in this study was associated with any specific manifestation of cryoglobulinaemic vasculitis.
The relative risk for developing skin ulcers was increased of 30% (OR, $e^{0.273} = 1.31$ by single-risk variable logistic regression) by a 1-U increase of homocysteine concentration. Thus, an increase of serum homocysteine of 5 μmol/l, corresponding to the difference between the median concentrations in patients with (median 16.1, range 8.3–28.5) or without skin ulcers (median 10.5, range 6.8–19.9), results in a 4-fold higher risk (OR, $e^{5×0.273} = 3.92$) for this complication in patients with mixed cryoglobulinaemia.

The prevalence of thrombophilic defects was similar in patients with HCV-associated mixed cryoglobulinaemia and in control groups, with the exception of homocysteine and protein S levels. Homocysteine concentration in patients with mixed cryoglobulinaemia was similar to that of patients with HCV infection without cryoglobulinaemia, and higher than that of normal subjects. Hyperhomocysteinaemia in patients with HCV infection with or without cryoglobulinaemia is seemingly secondary to multiple factors associated with their pathological conditions [26]. The low protein S concentration in mixed cryoglobulinaemia and in HCV-infected patients is also seemingly related to liver disease and other associated factors [27].

We observed an overall 10% prevalence of aCL in patients with HCV-associated cryoglobulinaemia; the presence of aCL did not influence the clinical manifestations of cryoglobulinaemia nor was associated with previous episodes of venous or arterial thromboembolism. Prior studies in patients with chronic HCV infection reported prevalences ranging from 3.3% to 44% (median 21%) [18, 19, 28–30]. The prevalence of aCL in HCV-infected patients was independent on the presence or absence of cryoglobulinaemia [18, 19]. None of those studies, except one [28], reported vascular complications associated with the presence of aCL.

We investigated the influence of several thrombophilic defects on four major clinical manifestations of cryoglobulinaemia as dependent covariates. The frequent concomitance of different clinical manifestations could confound the assessment of the impact of explanatory variables on each of them. At this regard, however, restricting the analysis to subgroups of patients with or without comorbidity supported the conclusion that homocysteine and duration of the disease were independent risk factors for cutaneous lesions and for nephropathy, respectively. In fact, patients with nephropathy and skin ulcers had significantly ($P = 0.002$) higher homocysteine concentration than patients with nephropathy only. Similarly, patients with nephropathy and skin ulcers had higher homocysteine ($P = 0.016$) than patients with nephropathy only. Conversely, patients with skin ulcers without nephropathy had a shorter duration of disease ($P = 0.03$) than patients with skin ulcers and nephropathy. The risk for renal disease was associated only with longer disease duration.

The fact that high homocysteine concentration was associated with an increased risk of severe cutaneous lesions but not of other manifestations of cryoglobulinaemic vasculitis may reflect differences in pathogenetic mechanisms. Indeed, while purpura is associated with leucocytoclastic lesions, a T cell-mediated process [31] and direct or indirect effects of HCV infection [32–34] appear to be the primary mechanisms of nerve injury. On the other hand, most evidence suggests that glomerular injury in cryoglobulinaemia results from deposition of immune complexes [35], although vasculitis of the small- and medium-sized renal vessels is rarely observed [36].

The strong impact of prothrombotic conditions on cutaneous vascular pathology is well illustrated by the case of livedoid vasculopathy. Originally described as ‘livedoid vasculitis’, this chronic cutaneous disease is characterized by focal purpura progressing to ulceration, and it is idiopathic or associated with CTDs or malignancies [17, 37]. Histopathology reveals dermal blood vessel thrombosis and deposition of complement and immunoglobulins, without inflammatory infiltrate or leucocytoclasis [17, 37, 38]. Laboratory studies reveal genetic or acquired thrombophilic defects in many patients with livedoid vasculopathy, including hyperhomocysteinaemia, factor V Leiden, aCL or LA [17]. Although literature data are scarce, the histopathology of ulcerative lesions in cryoglobulinaemia reminds that of livedoid vasculopathy since thrombosis with minimal inflammation was described at the ulcer edge in a patient with cryoglobulinaemia [39]. Thus, the similar features and the role of thrombophilic conditions suggest a common pathogenesis for necrotizing cutaneous lesions in mixed cryoglobulinaemia and livedoid vasculopathy. Vasculitic damage to the vessel wall initiates the coagulation cascade, but it is insufficient to determine tissue necrosis unless a coexisting hypercoagulable state promotes extensive microvascular thrombi formation, eventually leading to necrosis and ulceration. Homocysteine may act by further damaging the endothelium through oxidative stress and other mechanisms, and by promoting thrombosis by increasing platelet aggregation and interfering with coagulation factors [26].

Necrotizing skin ulcers can be the only manifestation of vasculitis in patients with mixed cryoglobulinaemia [6, 7, 39–41]. Skin ulcers in these patients are difficult to treat by local [40] and systemic [6, 41] therapies, and therefore novel preventive and therapeutic strategies are appealing. In this regard, our finding of a contribution of hyperhomocysteinaemia to the cutaneous manifestations of cryoglobulinaemia may have practical implications.

Supplementation with folic acid lowers homocysteine concentration and may be effective in reducing the risk of stroke in primary prevention [42]. We administered folic acid (5 mg/day) and vitamin B6 (300 mg biw) to two mixed cryoglobulinaemia patients (an 80-yr-old male and a 75-yr-old female) with high homocysteine and chronic Grade 3 purpura, with ulcers in one case. Low-dose prednisone (15–20 mg/day) was chronically given to both patients to attenuate painful purpura and to ameliorate skin ulcers. After 6 months of vitamin supplementation homocysteine concentration had decreased from 20.4 to 12 and from 20 to 10.2 μmol/l, respectively, and cutaneous manifestations had healed in both patients. Over the next 4 yrs, while on maintenance vitamin supplementation therapy, the patients had only sporadic episodes of painless purpura generally triggered by exogenous factors, and corticosteroids were no longer needed. Thus, notwithstanding the necessity for controlled studies, cost/risk/potential benefit considerations encourage the inclusion of homocysteine-lowering therapy in the management of mixed cryoglobulinaemia patients with hyperhomocysteinaemia and severe skin manifestations.

**Rheumatology key messages**

- Hyperhomocysteinaemia influences the clinical presentation of mixed cryoglobulinaemia.
- High homocysteine may be a risk factor for severe cutaneous manifestations.

**Acknowledgements**

We are grateful to the staff of the Division of Clinical Immunology for the contribution to the care of patients, and particularly to Maria Luisa Veneziano for the care of patients with chronic skin ulcers. We are extremely grateful to Carmine Tinelli for invaluable help with statistical analyses. This work was supported by grants from the Italian Ministry of Scientific Research and from the Istituto Pasteur-Fondazione Cenci-Bolognetti, Sapienza University of Rome.

**Disclosure statement:** The authors have declared no conflicts of interest.
References