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Micro and nanoparticles as possible pathogenetic co-factors in mixed cryoglobulinemia

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Background	Mixed cryoglobulinemia (MC) is a rare multisystem disease whose aetiopathogenesis is not completely understood. Hepatitis C virus (HCV) infection may have a causative role, and genetic and/or environmental factors may also contribute.
Aims	To investigate the presence and possible role of environmental agents in MC.
Methods	We recruited 30 HCV-infected MC patients with different clinical manifestations and a control group of 30 healthy, sex-/age-matched volunteers. We collected serum samples from each patient and incubated at 4°C for 7 days to obtain cryoprecipitate samples. We used environmental scanning electron microscopy (ESEM) and energy dispersive X-ray spectroscopy microanalysis to verify the presence of microparticles (MPs) and nanoparticles (NPs) in serum and cryoprecipitate samples. We evaluated environmental exposure using a medical and occupational history questionnaire for each subject.
Results	MC patients had a significantly higher risk of occupational exposure (OR 5.6; 95% CI 1.84–17.50) than controls. ESEM evaluation revealed a significantly higher concentration, expressed as number of positive spots (NS), of serum inorganic particles in MC patients compared with controls (mean NS 18, SD = 16 versus NS 5.4, SD = 5.1; $P < 0.05$). Cryoprecipitate samples of MC patients showed high concentrations of inorganic particles (mean NS 49, SD = 19). We found a strong correlation between NS and cryocrit (i.e. percentage of cryoprecipitate/total serum after centrifugation at 4°C) levels ($P < 0.001$).
Conclusions	In addition to HCV infection, MPs and NPs might play an important role in the aetiopathogenesis of MC.
Key words	Aetiopathogenesis; cryoglobulinemic vasculitis; HCV; microparticles; nanoparticles.

Introduction

Mixed cryoglobulinemia (MC) is a systemic vasculitis characterized by multiple organ involvement due to the vascular deposition of circulating immune complexes, mainly mixed IgG–IgM cryoglobulins and complement [1]. Clinically, MC is considered a rare multisystem disease and represents a crossroad between classical rheumatic diseases and other autoimmune lymphoproliferative disorders. Although numerous epidemiological studies have reported the presence of circulating cryoglobulins in >50% of hepatitis C virus (HCV)-infected individuals, MC develops in only a minority of these individuals (<5%) [2,3]. Thus, it is possible that other unknown factors may contribute to the pathogenesis of MC.

The impact of environmental pollution on health has been discussed since the middle of the 20th century [4]. In the past decade, toxicological studies have demonstrated that small organic and inorganic microparticles (MPs) and nanoparticles (NPs) can pass rapidly into the circulatory system [5]. Since there are no reports in the literature focusing on particle exposure history and MC, this preliminary study aimed to evaluate the presence and possible role of environmental agents such as MPs/NPs in MC patients.

Methods

We investigated 30 consecutive HCV-infected (with detectable serum HCV-RNA) MC patients compared to 30 healthy, sex-/age-matched volunteers (Table 1). The pre-clinical study was approved by the University of Modena and Reggio Emilia Ethics Committee. All study subjects (cases and controls) were resident in the Italian province of Modena and recruited at the Policlinico Hospital of the University of Modena and Reggio Emilia. We interviewed all subjects using a structured questionnaire (Appendix, available as Supplementary data at *Occupational Medicine* Online) based on a previous model [6] and we categorized four major sources of particle pollution: occupational exposure, environmental exposure, smoking habits and prosthesis implants. After informed consent, we collected data from all subjects and fresh blood samples in silica and metal-free polystyrene tubes (Vacutest Kima, PD, Italy), which we immediately kept at 37°C for 2 h. We separated serum by centrifuging blood samples at 37°C for 10 min at 2500 rpm. Thereafter, we stored serum samples at 4°C for 7 days and we quantified the cryoprecipitates as cryocrit, i.e. percentage of cryoprecipitate/total serum after centrifugation at 4°C. We analysed serum and cryoprecipitate samples by environmental scanning electron microscopy (ESEM Quanta200; Fei, Netherlands) using the method previously described by Fassina *et al.* [7]. We scanned all samples inside a selected area of each sample at ×40. Inside this area, we analysed 64 non-continuously and non-overlapping zones at ×400. We counted the number of positive spots (NS) containing MPs or NPs in the analysed zones of each sample for MC patients and controls. We investigated particle chemical composition by

Table 1. Demographic, clinico-serological, virological features and exposure data of MC patients and controls

	MC patients	Controls
Demographic characteristics		
Number	30	30
Age, mean ± SD years (range)	72 ± 10 (42–84)	67 ± 11 (44–80)
Males/females	8/22	9/21
MC disease duration, mean ± SD years (range)	9 ± 7 (<1–32)	–
Clinical features, *n* (%)		
Purpura	29 (97)	–
Ulcers	5 (17)	–
Weakness	29 (97)	–
Arthralgia	27 (90)	2 (7)
Arthritis (non-erosive)	6 (20)	4 (13)
Fibromyalgia	4 (13)	1 (3)
Raynaud's phenomenon	11 (37)	–
Sicca syndrome	14 (47)	–
Peripheral neuropathy	22 (73)	–
Renal involvement (MPGN)	10 (33)	–
Liver involvement	19 (63)	–
B-cells non-Hodgkin's lymphoma	4 (13)	–
Thyroid involvement	19 (63)	4 (13)
Serological and virological findings		
Type II/type III MC ratio[a]	3: 1	–
Cryocrit (%), mean ± SD (range)	1.8 ± 1.5% (0.5–3.5)	–
C3 (mg/dl, normal 60–130), mean ± SD	92.3 ± 28.4	–
C4 (mg/dl, normal 20–55), mean ± SD	11.4 ± 10.4	–
HCV-RNA, *n* (%)	30 (100)	–
HBV-RNA, *n* (%)	3 (10)	–

Exposure data of MC patients and controls (*n*/total)			OR (95% CI)
Categories	MC patients	Controls	
Occupational exposure	19/30	7/30	5.6 (1.84–17.50)
Environmental exposure	14/30	8/30	2.4 (0.81–7.09)
Smoking habits	17/30	11/30	2.2 (0.80–6.36)
Prosthesis implants	15/30	9/30	2.3 (0.80–6.73)

[a]According to the presence of monoclonal (type II) or polyclonal (type III) IgM. CI, confidence interval; HBV, hepatitis B virus; MPGN, membranoproliferative glomerulonephritis; OR, odds ratio.

energy dispersive X-ray spectroscopy (EDS) microanalysis (INCA; Oxford Instruments, UK).

Results

The demographic and clinico-serological and virological features of MC patients are reported in Table 1. The type II/type III ratio of serum mixed cryoglobulins in MC patients was 3:1, while cryoglobulins were absent in all serum samples from controls. Clinical features of cryoglobulinemic patients included arthralgia, weakness, purpura, peripheral neuropathy, hypothyroidism, liver and renal involvement, confirming previously observed prevalence rates [1]. All patients were HCV infected and three of them were also hepatitis B virus infected.

MC was associated with a significantly higher prevalence of occupational exposure compared with controls (OR 5.6; 95% CI 1.84–17.50; Table 1). All 19 exposed patients were involved in occupations with intermediate or high silica exposure (ceramic, foundry and metal workers, painters and decorators).

Twenty-four of the 30 control subjects had few inorganic particles in their serum samples with mean NS of 5.4 (SD = 5.1). These levels were statistically lower compared with the mean NS of 18 (SD = 16) detected in 27/30 sera of MC patients ($P < 0.05$; Figure 1A). The cryoprecipitate samples of MC patients group had a higher NS (mean 49; SD = 19) compared with the serum samples of controls (mean 5.4; SD = 5.1; $P < 0.001$; Figure 1B). The presence of both MPs and NPs was significantly higher in MC patients cryoprecipitate samples compared with MC patients serum samples ($P < 0.001$) (Figure 1C). We found no correlation between particles presence and cryoglobulinemia types or MC duration, but there was a statistically significant correlation ($r = 0.923$; $P < 0.001$) between the NS and cryocrit levels (Figure 1D).

EDS analysis, of the elemental composition of fine particulate matter in the samples, indicated that particles had a complex composition that included several elements in minor or trace amounts: Si (50%), Fe (35%), Ti (31%), Al (29%) and Mg (19%) and a lower frequency (<1%) for Cu, Zn, Mn, Ni, Cr, Ba, Sn and Zr.

Discussion

We found high or intermediate occupational silica exposure to be significantly associated with MC. ESEM analysis confirmed the presence of MPs and NPs in cryoglobulinemic subjects (Si, Al, Fe and Ti particles).

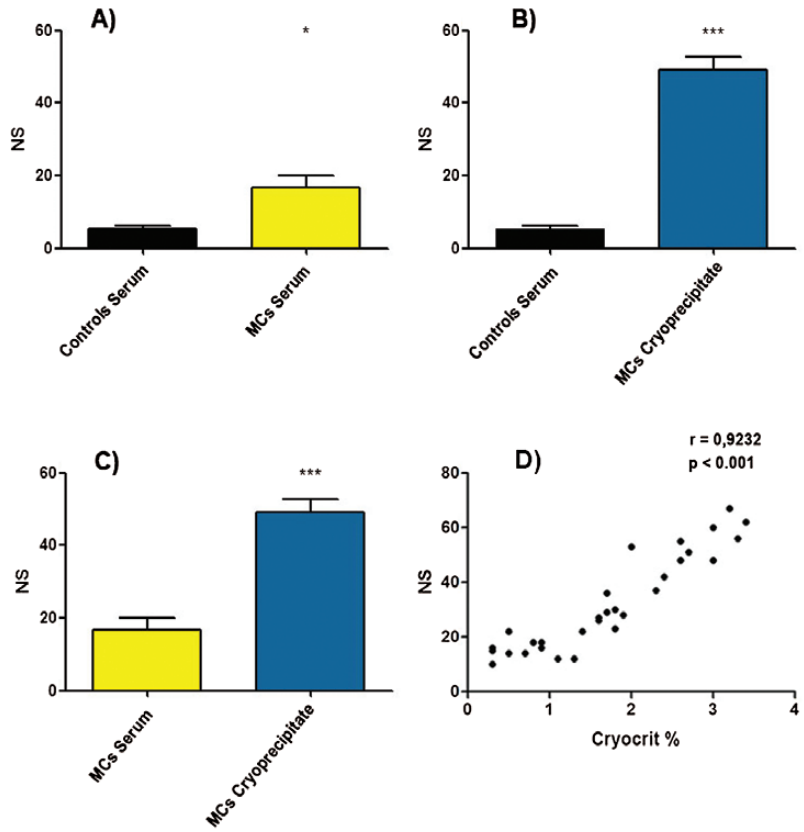

Figure 1. (A) NS containing MPs or NPs in controls serum samples versus MC serum samples (*$P < 0.05$); (B) NS in controls serum samples versus MC cryoprecipitate samples (***$P < 0.001$); (C) NS in MC serum samples versus MC cryoprecipitate samples (***$P < 0.001$). NS are reported as a mean ± SD. (D) Correlation between NS containing particles and cryocrit levels (%) of MC patients.

NS was significantly correlated to cryocrit levels, suggesting possible involvement of MPs and NPs particles in MC immune complex composition.

Although we evaluated a limited number of patients, the comparison of MC patients with healthy controls from the same residential area and the ESEM qualitative/quantitative analysis of samples represent a strength of our study.

In particular, the prevalence of Si in all MC samples suggests a possible role of silica in MC. Previous data concerning individuals with silica exposure developing anti-neutrophil cytoplasmic antibody-associated systemic vasculitis support our hypothesis [8]. Also, recent *in vitro* studies showed that silica nanoparticles can indirectly induce circulating immune complex formation by absorbing on their surface human IgM rheumatoid factor and IgG [9].

For a better understanding of the *in vivo* toxicity of particles and their actual role in the pathogenesis of MC, future research should include a biophysical approach considering the predominant elements found (Si, Al, Fe and Ti) and their possible immunological interactions. Finally, the study of MPs and NPs in a significant number of MC patients not infected with HCV, quite a rare disease variant in Italy, might further reinforce the suggested aetiopathogenetic role of these environmental co-factors.

Key points

- Environmental scanning electron microscopy revealed the significant presence of inorganic micro and nanoparticles in cryoglobulinemic subjects.
- Energy dispersive X-ray spectroscopy microanalysis indicated that particles had complex compositions and the most frequent element found was Si.
- The data revealed a strong correlation between the presence of particles and cryocrit levels, suggesting a potential direct role of micro and nanoparticles in the aetiopathogenesis of mixed cryoglobulinemia.

Acknowledgement

Pre-clinical trial registration: UFP2015, University of Modena and Reggio Emilia Ethics Committee approved.

Conflicts of interest

All the authors declare no conflicts of interest.

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