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H-ferritin and proinflammatory cytokines are increased in the bone marrow of patients affected by macrophage activation syndrome

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Summary

Macrophage activation syndrome (MAS) is hyperinflammatory life-threatening syndrome, associated typically with high levels of serum ferritin. This is an iron storage protein including heavy (H) and light (L) subunits, categorized on their molecular weight. The H-/L subunits ratio may be different in tissues, depending on the specific tissue and pathophysiological status. In this study, we analysed the bone marrow (BM) biopsies of adult MAS patients to assess the presence of: (i) H-ferritin and L-ferritin; (ii) $CD68^+$/H-ferritin$^+$ and $CD68^+$/L-ferritin$^+$; and (iii) interleukin (IL)-1β, tumour necrosis factor (TNF) and interferon (IFN)-γ. We also explored possible correlations of these results with clinical data. H-ferritin, IL-1β, TNF and IFN-γ were increased significantly in MAS. Furthermore, an increased number of $CD68^+$/H-ferritin$^+$ cells and an infiltrate of cells co-expressing H-ferritin and IL-12, suggesting an infiltrate of M1 macrophages, were observed. H-ferritin levels and $CD68^+$/H-ferritin$^+$ cells were correlated with haematological involvement of the disease, serum ferritin and C-reactive protein. L-ferritin and $CD68^+$/L-ferritin$^+$ cells did not correlate with these parameters. In conclusion, during MAS, H-ferritin, $CD68^+$/H-ferritin$^+$ cells and proinflammatory cytokines were increased significantly in the BM inflammatory infiltrate, pointing out a possible vicious pathogenic loop. To date, H-ferritin and $CD68^+$/H-ferritin$^+$ were associated significantly with haematological involvement of the disease, suggesting biomarkers assessing severity of clinical picture.

Keywords: cytokine, ferritin, hyperferritinaemic syndrome, macrophage, macrophage activation syndrome

Introduction

Macrophage activation syndrome (MAS) is a hyperinflammatory syndrome, associated usually with systemic juvenile idiopathic arthritis and adult-onset Still's disease (AOSD) [1–4]. Due to this close association, it has been suggested that MAS and AOSD may represent one part of the same disease spectrum, of which AOSD may be considered the milder form [5]. In this context, it has been shown that MAS occurrence may be misdiagnosed due to immunosuppressive drugs used to treat AOSD flare, thus reducing the reported prevalence of this syndrome [6,7]. Furthermore, patients affected by systemic lupus erythematosus (SLE) may also experience MAS [8]. Continuous high fever, hepatosplenomegaly, severe peripheral blood cytopenia and haemophagocytosis by activated macrophages in bone marrow (BM) are typical features of these patients [1–8]. In contrast to paediatric patients, the therapeutic strategies of adults derive mainly from case–series and retrospective experiences [9–11]. In this context, the clearance of possible triggers, immunosuppressive therapeutic strategies and supportive care are considered the main therapeutic strategies [10]. Despite aggressive therapeutic strategies, MAS is one of the most critical clinical disorders in adults, evolving to multiple organ failure and unfavourable outcome [1,8]. This result may be possibly related, in adult age, with an increased presence of co-morbidities, the latter known to be predictive of a more severe outcome in MAS patients as observed in other rheumatic diseases [3,8,12].

Recently, a multi-layer MAS pathogenic model during inflammatory rheumatic diseases has been proposed [2]. Genetic factors, proinflammatory milieu associated with the underlying rheumatic disease, trigger factors and the uncontrolled activation of macrophages and T cells may lead to the development of cytokine storm and MAS [13,14]. Activation of cytotoxic CD8 T lymphocytes with production of macrophage-activating cytokines, such as interferon (IFN)-γ, is an early event in MAS pathogenesis. Defects in granulocyte-mediated cytotoxicity, enhanced antigen presentation and repeated stimulation of Toll-like receptors determine extensive production/release of tumour necrosis factor (TNF) and interleukin (IL)-1β [13,14]. The consequent activation and expansion of monocytes and macrophages lead to pathological haematophagocytosis [1,2,13,14]. In this context, it has been considered that macrophages are categorized in M1 (classically activated), releasing proinflammatory mediators, and M2 (alternatively activated), modulating the inflammatory response [15].

During MAS, a possible pathogenic role of ferritin has been suggested and has been included in the so-called 'hyperferritinaemic syndrome' [16,17]. Ferritin is an intracellular iron storage protein including 24 subunits. These are categorized, according to molecular weight, as heavy (H) subunits and light (L) subunits. Ferritin enriched in L subunits (L-ferritin) has been found in liver and in spleen, whereas ferritin enriched in H subunits (H-ferritin) may be observed mainly in heart and kidneys [17]. Possible causes of hyperferritinaemia in these patients include increased production to sequestrate free iron of released haemoglobin due to erythrophagocytosis, reduced tissue clearance and enhanced production by macrophages [16,17].

In this study, we aimed to investigate H-and L-ferritin in inflammatory BM infiltrate of MAS patients during full-blown syndrome. Macrophage subsets expressing H-and L-ferritin were also assessed. Furthermore, we evaluated IL-1β, TNF, IFN-γ and their possible co-localization with ferritin within inflammatory cells. Finally, any possible clinical correlation of these data with the severity of the disease was analysed.

Patients and methods

A retrospective evaluation of BM biopsies obtained from adult MAS patients admitted to the Rheumatology Clinic of L'Aquila University and Rheumatology Clinic of Palermo University during the last 10 years was performed. MAS patients were diagnosed according to 2004 haemophagocytic lymphohistiocytosis (HLH) diagnostic guidelines criteria [18]. In this study, we also evaluated 10 biopsies derived from BM-donors, used as healthy controls (HCs).

For each patient we analysed age, gender, values of white blood cell count (WBC), red blood cells (RBC), haemoglobin (HB), platelet count (PLT), serum ferritin, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) during the full-blown syndrome.

The local ethics committee approved the study (ASL1 Avezzano-Sulmona-L'Aquila, L'Aquila, Italy, protocol number 0122353/17) that was performed according to Good Clinical Practice guidelines and Declaration of Helsinki.

Histological analysis of biopsies

Samples were stained with anti-H-ferritin, anti-L-ferritin, anti-CD68, anti-CD163, anti-IL-1β, anti-IL-12, anti-IFN-γ and anti-TNF antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), as reported previously [16]. Samples were acquired using a light microscope (Olympus BX53) and immunofluorescence images by CellSens software (Olympus America Inc., Center Valley, PA, USA). Cells double-positive for both CD68/H-ferritin and CD68/L-ferritin were counted in sections from randomly selected areas of the BM, counting at least 500 nucleated cells (×20 magnification) by using NIHimageJ version 1.43 (http://rsbweb.nih.gov/ij/) freeware.

Statistical analysis

To compare the results of MAS patients and HCs, the Mann–Whitney *U*-test was considered appropriate due to the non-parametric distribution of our data. To assess correlations among tissue H- and L-ferritin, the numbers of double-positive cells for CD68 and H-ferritin ($CD68^+$/H-$ferritin^+$ or double-positive cells for CD68 and L-ferritin ($CD68^+$/L-$ferritin^+$) and clinical features, Spearman's correlation analysis and linear regression were used. The same analysis was performed to evaluate a possible correlation among H-ferritin and IL-1β, TNF and IFN-γ. A *P*-value $< 0{\cdot}05$ expressed a statistically significant result. We used GraphPad Prism version 5.0 for all statistical analyses.

Results

Demographic and clinical features

In this study, we evaluated BM biopsies of 10 patients affected by MAS collected during the full-blown syndrome. All these adult patients were affected by proinflammatory rheumatic disease (eight patients by AOSD, two patients by SLE), and MAS occurred during disease flare or severe infection. We observed that two patients experienced MAS after severe Epstein–Barr virus (EBV) infection and two patients during Gram-negative sepsis, due to immunosuppressive treatments administered for underlying rheumatic disease. All these patients showed peripheral blood cytopenia [WBC, mean ± standard deviation (s.d.) $3{\cdot}23 \pm 1{\cdot}44$, 10^3/ml; RBC = $3{\cdot}22 \pm 0{\cdot}70$, 10^3/ml; PLT = $45{\cdot}52 \pm 26{\cdot}60$, 10^3/ml], increased serum levels of ferritin ($2842{\cdot}70 \pm 569{\cdot}70$ ng/ml), marked increase of inflammatory markers (ESR = $63{\cdot}87 \pm 30{\cdot}19$, mm/h; CRP = $51{\cdot}59 \pm 46{\cdot}92$, mg/day). The clinical data are shown in Table 1.

Table 1. Demographic and clinical features of evaluated macrophage activation syndrome (MAS) patients

Clinical data	Patients
Women (men)	4 (6)
Age (years ± s.d.)	58·20 ± 13·10
Underlying disease, number (%)	10 (100%)
AOSD	8 (80%)
SLE	2 (20%)
Trigger factor, number (%)	
Flare of the disease	6 (60%)
Severe infection	4 (40%)
Comorbidities, number (%)	4 (40%)
Time of follow-up, months mean ± s.d.	6·96 ± 2·82
MAS-related death, number (%)	5 (50%)
Laboratory parameters	
WBC (10^3/ml), mean ± s.d.	3·23 ± 1·44
RBC (10^3/ml), mean ± s.d.	3·22 ± 0·70
HB (g/dl), mean ± s.d.	8·79 ± 1·55
PLT (10^3/ml), mean ± s.d.	45·52 ± 26·60
Serum ferritin (ng/ml), mean ± s.d.	2842·70 ± 569·70
ESR (mm/h), mean ± s.d.	63·87 ± 30·19
CRP (mg/l), mean ± s.d.	51·59 ± 46·92
Triglycerides (mg/dl), mean ± s.d.	220·02 ± 71·25
ASAT (IU/l), mean ± s.d.	84·23 ± 51·97
ALAT (IU/l), mean ± s.d.	145·28 ± 94·23
Therapies	
High-dosage steroids pulses, number (%)	10 (100%)
Methylprednisolone pulses 1000 mg/die	7 (70%)
Methylprednisolone pulses 500 mg/die	3 (30%)
Immunosuppressive drugs, number (%)	5 (50%)
Cyclosporin A	5 (50%)

ALAT = alanine aminotransferase; AOSD = adult-onset Still's disease; ASAT = aspartate aminotransferase; CRP = C-reactive protein; ESR = serum ferritin, erythrocyte sedimentation rate; HB = haemoglobin; MAS = macrophage activation syndrome; PLT = platelet count; RBC = red blood cells; SLE = systemic lupus erythematosus; WBC = white blood cell count.

H-ferritin is increased in BM and correlates with clinical features

We observed a granular morphological pattern of H-ferritin and L-ferritin immunoreactivity, including cytoplasmatic and extracellular localization, using immunofluorescence. As shown in Fig. 1, H-ferritin was increased in BM samples of MAS patients when compared with both L-ferritin and HCs ($P < 0{\cdot}0001$ for each comparison), analysing the optical density of immunofluorescence. Conversely, L-ferritin was not increased when compared to HCs. Subsequently, clinical correlations among these histological data and severity of the clinical picture were evaluated. Our analyses showed that the peripheral blood cytopenia of these patients were correlated inversely with the levels of H-ferritin in the BM of MAS. Increased values of H-ferritin levels correlated with decreased WBC and PLT counts ($P = 0{\cdot}01$, $P = 0{\cdot}0001$; respectively). A further correlation was observed among tissue H-ferritin and the inflammatory markers. H-ferritin was correlated statistically with both serum ferritin and CRP levels ($P = 0{\cdot}012$; $P = 0{\cdot}0058$, respectively), as shown in Fig. 2. No correlation was observed among RBC count, levels of HB, ESR, ASAT, ALAT and H-ferritin. The correlations among L-ferritin and clinical data did not show significant results.

IL-1β, TNF, IFN-γ and H-ferritin

Proinflammatory cytokines were increased significantly in BM samples of MAS patients when compared with HCs analysing the immunofluorescence of our samples. Specifically, a marked increase of IL-1β was observed in BM of MAS patients ($P = 0{\cdot}0002$). In addition, the analyses showed a significant correlation between the tissue expression of H-ferritin and IL-1β ($P = 0{\cdot}006$). Interestingly, H-ferritin and IL-1β co-localized in the BM samples of our patients, as shown in Fig. 3.

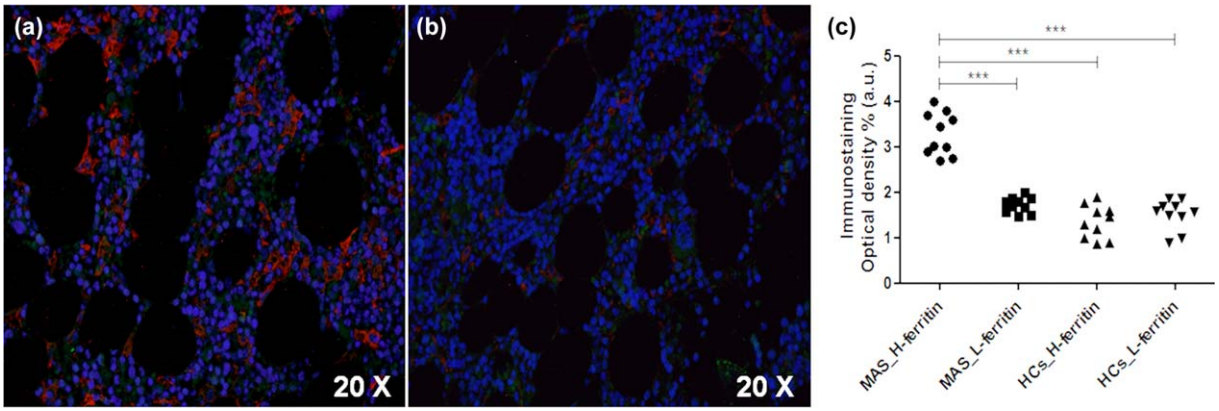

Fig. 1. Expression of H-ferritin in bone marrow samples of macrophage activation syndrome (MAS) patients. (a) high H-ferritin (red) and low tissue expression of L-ferritin (green) may be observed, respectively; (b) low H-ferritin expression may be reported in healthy controls (HCs); (c) H-ferritin was increased significantly when compared with L-ferritin increased and HCs, analysing immunostaining optical density (***$P < 0{\cdot}0001$).

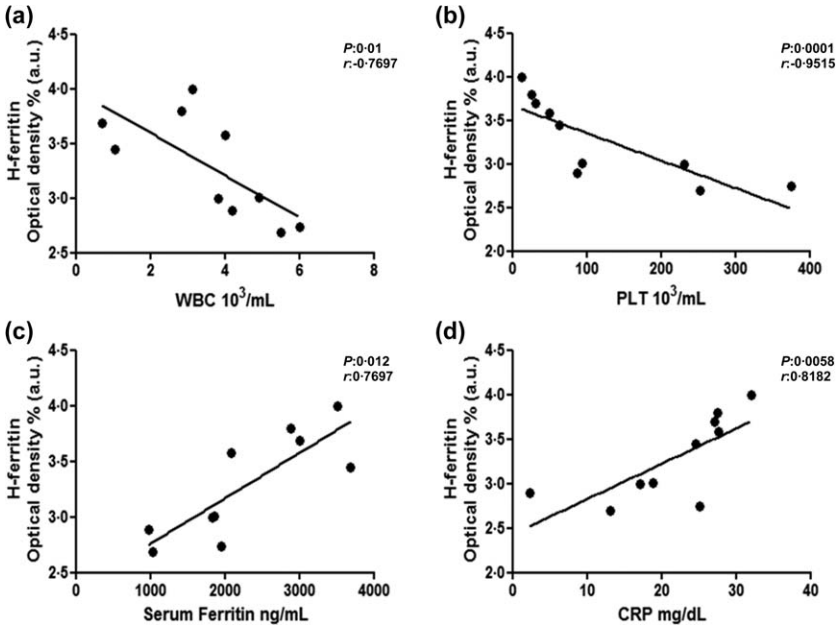

Fig. 2. Correlation of H-ferritin with clinical features. (a,b) Increased values of H-ferritin were correlated with decreased white blood cells (WBC) and platelet (PLT) counts, respectively; (c) significantly and (d) increased values of H-ferritin tissue were correlated significantly with increased values of serum ferritin and C-reactive protein (CRP), respectively.

Furthermore, we showed a significant increase of TNF in the BM of evaluated patients when compared with HCs ($P = 0{\cdot}002$) (Fig. 4). Similarly, a marked increase of IFN-γ was observed when compared with HCs ($P < 0{\cdot}0001$). However, we did not report correlation or co-localization among these proinflammatory cytokines and H-ferritin.

CD68$^+$/H-ferritin$^+$ macrophages are increased and correlate with clinical features

In our MAS patients, we reported that H-ferritin co-localized with CD68, as shown in Fig. 5. Furthermore, macrophages expressing H-ferritin in the inflammatory infiltrate were increased significantly when compared with HCs

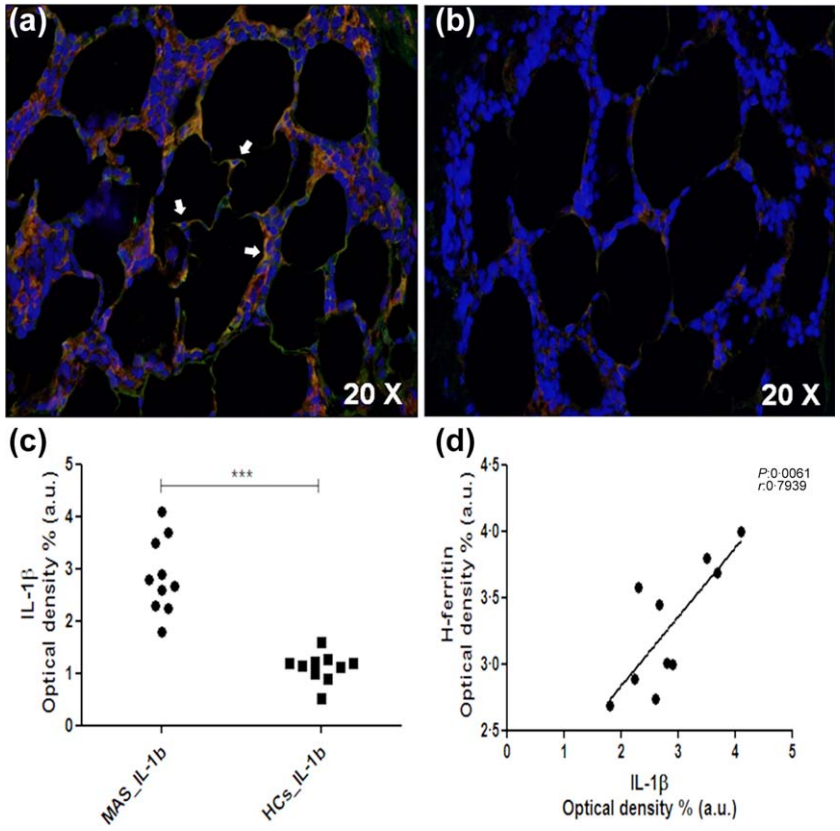

Fig. 3. Expression interleukin (IL)-1β in bone marrow of macrophage activation syndrome (MAS) patients and its correlation with H-ferritin. (a) Increased IL-1β (green) levels and co-localization (arrows) between H-ferritin (red), and this cytokine may be observed in the inflammatory bone marrow infiltrate; (b) reduced IL-1β expression in healthy controls (HCs); (c) in MAS patients, IL-1β was increased significantly when compared with HCs (***$P < 0{\cdot}001$); (d) IL-1β was correlated significantly with H-ferritin.

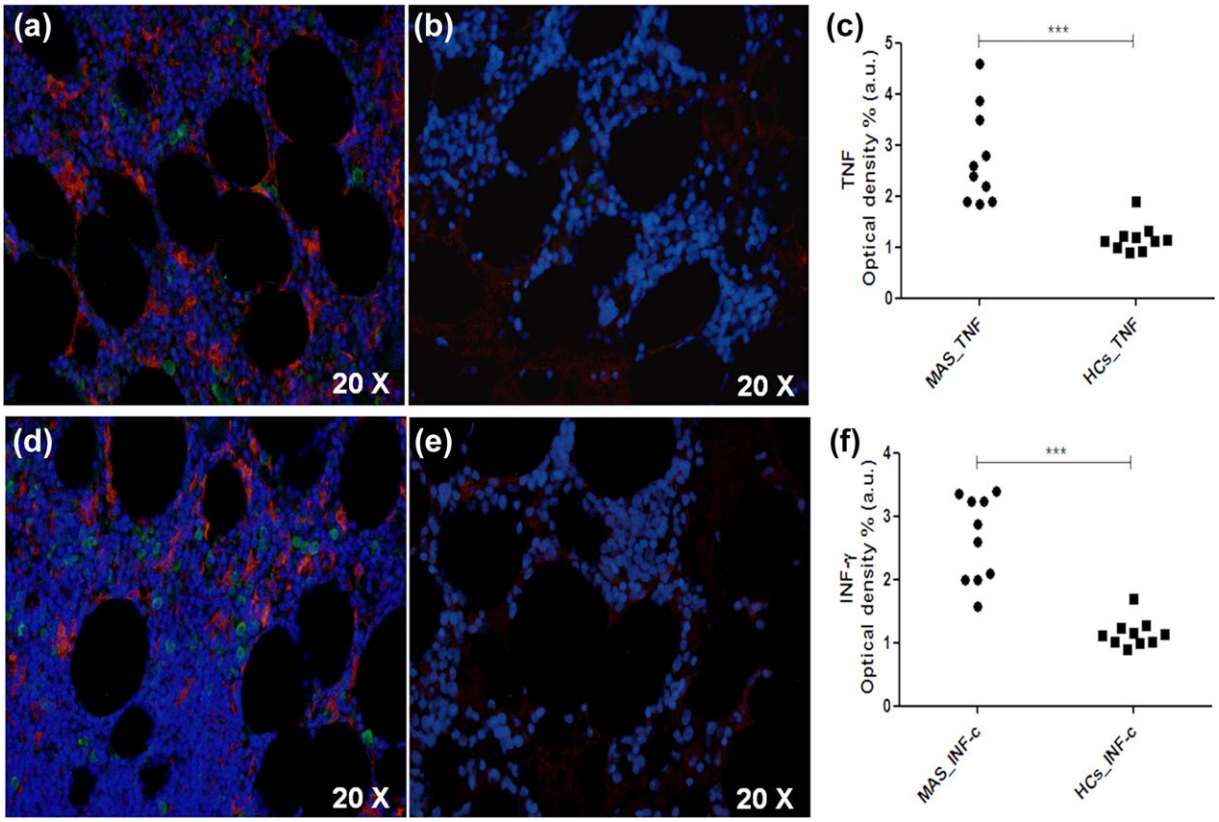

Fig. 4. Increased expression of tumour necrosis factor (TNF) and interferon (IFN)-γ in bone marrow of macrophage activation syndrome (MAS) patients. (a) Increased IFN-γ (green) levels and H-ferritin (red) may be described in MAS; (b) reduced IFN-γ levels in healthy controls (HCs); (c) IFN-γ was increased significantly when MAS patients were compared with HCs (***$P < 0{\cdot}001$); (d) increased TNF (green) levels and H-ferritin (red) may be observed in MAS; (e) reduced TNF levels in HCs; (f) TNF was increased significantly when MAS patients were compared with HCs (***$P < 0{\cdot}001$).

($P = 0{\cdot}006$) (Fig. 5). In the same samples, although we observed a weak co-localization between CD68 and L-ferritin, these cells did not differ significantly when compared with HCs. We analysed and compared the number of CD68$^+$/H-ferritin$^+$ cells with the number of CD68-/H-ferritin$^+$ cells, pointing out that a large percentage of H-ferritin$^+$ cells expressed CD68 (CD68$^+$/H-ferritin$^+$ cells: 5 median, range = 2·7; 9·0 *versus* CD68-/H-ferritin$^+$ cells 1·7 median, range = 1·5; 2·2; $P < 0{\cdot}0001$). In addition, we analysed possible co-localizations among the H-ferritin and IL-12, which is considered an M1 macrophage marker, and CD163, which is considered an M2 macrophage marker, as shown previously [19,20]. As shown in Fig. 5, a co-localization between H-ferritin and IL-12 was observed. Conversely, a weaker co-expression between H-ferritin and CD163 was shown. We analysed and compared the number of IL-12$^+$/H-ferritin$^+$ cells with the number of CD163/H-ferritin$^+$ cells, indicating that a large percentage of H-ferritin$^+$ cells expressed IL-12 (IL-12$^+$/H-ferritin$^+$ cells: 3·2 median, range = 1·6; 6·1) *versus* CD163$^+$/H-ferritin$^+$ cells 1·6 median, range = 1·3; 2·7; $P = 0{\cdot}0002$) and suggesting an increased percentage of M1 macrophages in BM inflammatory infiltrate during MAS full-blown syndrome.

In addition, clinical correlations among CD68$^+$/ferritin$^+$ cells and severity of the clinical picture were assessed. Our analyses showed that the peripheral blood cytopenia of these patients were correlated inversely with the number of BM CD68$^+$/H-ferritin$^+$ cells in the inflammatory BM infiltrate. Increased values of CD68$^+$/H-ferritin$^+$ cells in the BM inflammatory infiltrate of our patients correlated with reduced WBC and the PLT counts ($P = 0{\cdot}03$, $P = 0{\cdot}0007$, respectively). Furthermore, CD68$^+$/H-ferritin$^+$ cells were correlated significantly with both serum ferritin and CRP levels ($P = 0{\cdot}0088$; $P = 0{\cdot}049$; respectively). Figure 6 shows these statistical analyses. ESR, RBC count and HB levels did not correlate with CD68$^+$/H-ferritin$^+$ cells. pCD68$^+$/L-ferritin$^+$ cells in the BM inflammatory infiltrate of MAS patients did not correlate with these clinical parameters.

Discussion

In our study, H-ferritin, CD68$^+$/H-ferritin$^+$ cells and proinflammatory cytokines, IL-1β, TNF, IFN-γ, were increased markedly in BM inflammatory infiltrate of MAS patients. Of note, H-ferritin and CD68$^+$/H-ferritin$^+$ cells

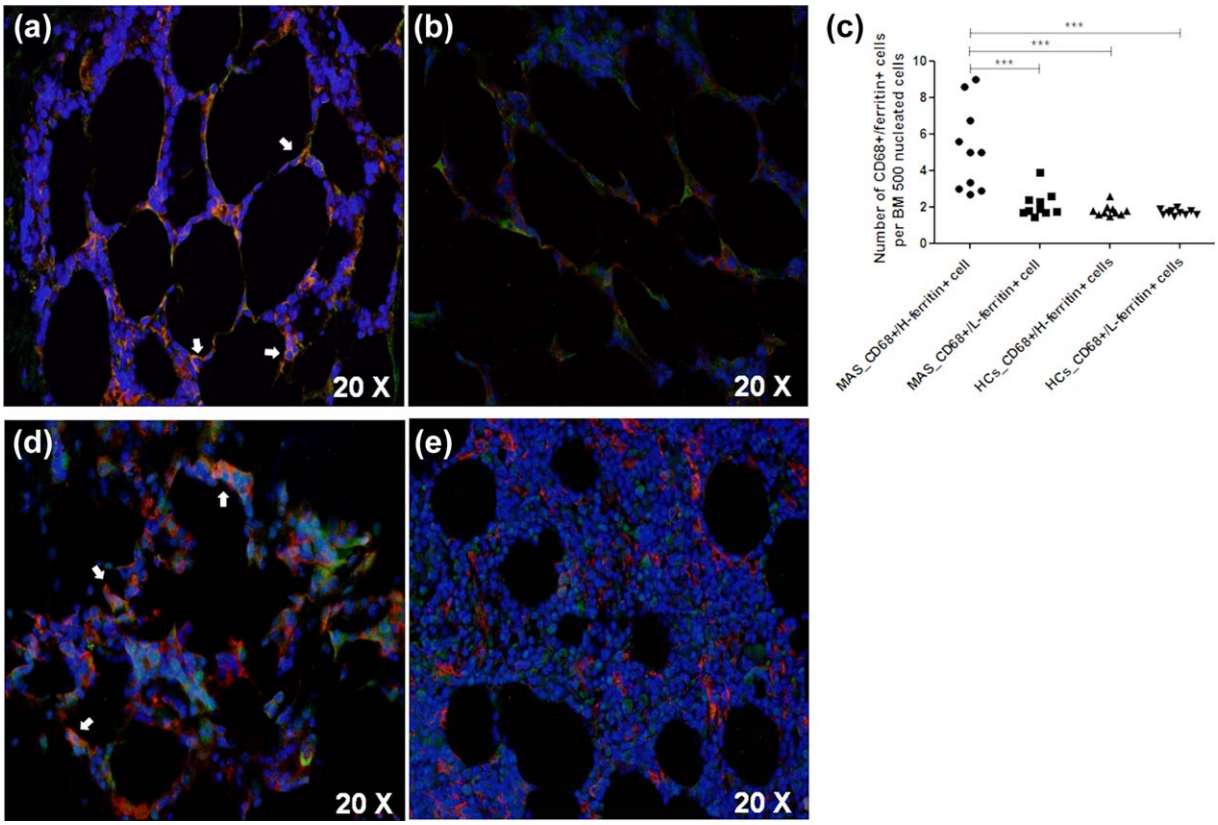

Fig. 5. CD68 macrophages expressing H-ferritin in bone marrow infiltrate of macrophage activation syndrome (MAS) patients. (a) $CD68^+$/H-ferritin$^+$ cells (arrows) may be described in the bone marrow (BM) of MAS patients; (b) reduced $CD68^+$/H-ferritin$^+$ cells in BM of healthy controls (HCs); (c) $CD68^+$/H-ferritin$^+$ cells was increased significantly in MAS when compared with HCs (***$P < 0{\cdot}001$); (d) co-localization of H-ferritin (red) and interleukin (IL)-12 (green) may be observed, suggesting M1 macrophages; (e) $CD163^+$ cells (green) showed weaker co-localization with H-ferritin (red).

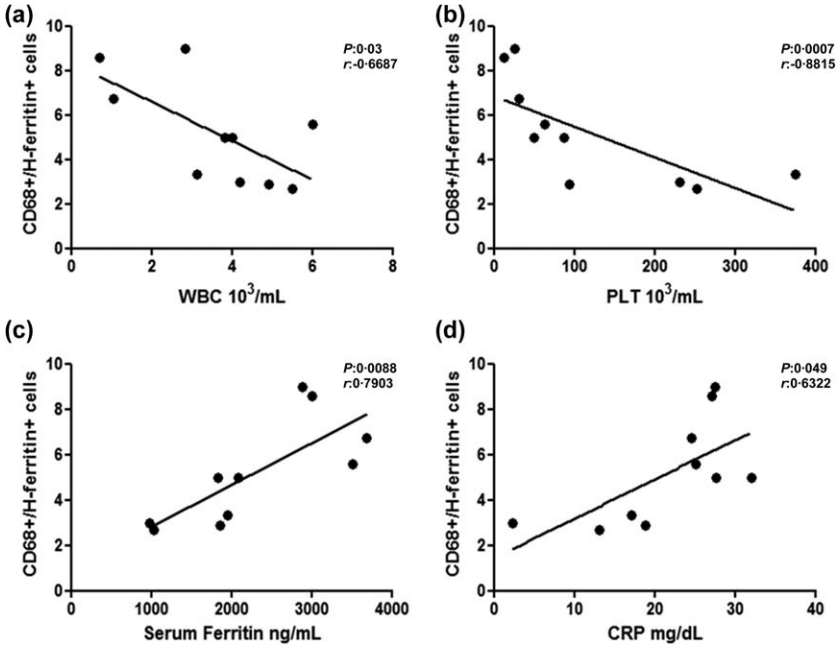

Fig. 6. Correlation between $CD68^+$/H-ferritin$^+$ cells, haematological involvement and inflammatory markers. (a,b) Increased $CD68^+$/H-ferritin$^+$ was correlated with decreased white blood cell (WBC) and decreased platelet (PLT) counts, respectively; (c,d) $CD68^+$/H-ferritin$^+$ was correlated significantly with serum ferritin and C-reactive protein (CRP).

were correlated significantly with the haematological involvement of our patients, suggesting possible biomarkers of severity.

During MAS, the massive release/production of proinflammatory cytokines may induce H-ferritin expression by activation of specific transcription factors, such as ferritin 2 (FER2), which may in turn activate production of further proinflammatory cytokines, triggering a possible vicious loop [21–26]. In fact, we observed high H-ferritin in the BM of MAS patients and co-localization as well as a correlation between H-ferritin and IL-1β, a possibly therapeutic target in MAS and rheumatic diseases [27–31]. Furthermore, a specific receptor for H-ferritin on immune cells has also been reported [32]. The specific binding between H-ferritin and the T cell immunoglobulin and mucin domain-containing protein-2 (TIM-2) receptor may modulate the inflammatory process. This is a member of the TIM gene family and is expressed by T helper type 2 (Th2) cells and macrophages in the context of the proinflammatory milieu [32,33]. The binding H-ferritin/TIM-2 may activate these immune cells, leading to subsequent pathogenic proinflammatory process [12,18–21]. Taking together all these mechanisms, it is possible to speculate that the enhanced tissue expression of H-ferritin, via a vicious loop, may perpetuate the production of proinflammatory cytokines, possible therapeutic targets in MAS [16,17,22,23,27,28].

In our samples, we described macrophages co-expressing CD68 and H-ferritin that were increased in the BM infiltrate of MAS. These data may confirm that, after proinflammatory stimuli, macrophages may release H-ferritin, confirming their role in ferritin production [34]. Interestingly, H-ferritin co-localized with IL-12, a marker of M1-macrophages, associated with increased production of proinflammatory molecules. Macrophages are polarized by environment, mainly the cytokine milieu, towards distinct functional programmes, categorized as M1 and M2 pathways. Massive production of IL-1β, INF-γ and TNF may promote the recruitment and proliferation of M1 macrophages; persistent and uncontrolled expansion of these proinflammatory macrophages is a pivotal mechanism in MAS development [35,36].

To date, H-ferritin and $CD68^+$/H-$ferritin^+$ cells were correlated significantly with haematological involvement, serum ferritin and CRP of our MAS patients. These results suggest potentially new biomarkers evaluating severity of the MAS clinical picture, possibly improving outcome in these patients [37,38]. In fact, diagnosis and appropriate treatments may be delayed, due to non-specific findings in the early phases of disease, thus new biomarkers may improve the management of this life-threating syndrome [39–42]. Although a rigorous process of validation is needed, the use of novel biomarkers in these patients may help physicians, thus improving diagnosis with early recognition and prompt therapy as well as predicting response to treatment and outcome, as suggested in other rheumatic diseases [43–47].

Our retrospective study shows different limitations. We evaluated a low number of patients, thus our findings should be confirmed further. However, it must be pointed out that MAS is a very rare disease and, as observed for other infrequent complications during rheumatic diseases, organizing specifically designed studies may be a challenge [48–50]. Furthermore, our study did not allow us to indicate if the $CD68^+$ macrophages are actively producing ferritin or phagocyting cells and future specifically designed studies are needed to elucidate these pathogenic steps entirely during MAS.

In conclusion, H-ferritin, $CD68^+$/H-$ferritin^+$ cells IL-1β, TNF, IFN-γ, were increased markedly in BM inflammatory infiltrate of MAS patients, possibly involved in a proinflammatory pathogenic vicious loop. Although future studies are needed to clarify entirely the possible role of these features, our results may allow us to hypothesize that ferritin and $CD68^+$/H-$ferritin^+$ macrophages may not only be considered a consequence of the inflammation but are involved directly in MAS pathogenesis. Furthermore, H-ferritin and $CD68^+$/H-$ferritin^+$ cells are correlated significantly with haematological involvement of our patients, suggesting possible biomarkers of severity in order to improve the management of patients, allowing physicians an early recognition and prompt therapy for this life-threating syndrome.

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Disclosure

The authors declare there are no competing interests.

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