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ORIGINAL PAPER

Haematological Malignancy – Clinical

Inflammatory profile of lower risk myelodysplastic syndromes

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Summary
The precise link between inflammation and pathogenesis of myelodysplastic syndrome (MDS) is yet to be fully established. We developed a novel method to measure ASC/NLRP3 protein specks which are specific for the NLRP3 inflammasome only. We combined this with cytokine profiling to characterise various inflammatory markers in a large cohort of patients with lower risk MDS in comparison to healthy controls and patients with defined autoinflammatory disorders (AIDs). The ASC/NLRP3 specks were significantly elevated in MDS patients compared to healthy controls ($p < 0.001$) and these levels were comparable to those found in patients with AIDs. The distribution of protein specks positive only for ASC was different to ASC/NLRP3 ones suggesting that other ASC-containing inflammasome complexes might be important in the pathogenesis of MDS. Patients with MDS-SLD had the lowest levels of interleukin (IL)-1β, tumour necrosis factor (TNF), IL-23, IL-33, interferon (IFN) γ and IFN-α2, compared to other diagnostic categories. We also found that inflammatory cytokine TNF was positively associated with MDS progression to a more aggressive form of disease and IL-6 and IL-1β with time to first red blood cell transfusion. Our study shows that there is value in analysing inflammatory biomarkers in MDS, but their diagnostic and prognostic utility is yet to be fully validated.

KEYWORDS
cytokines, inflammation, MDS, NLRP3 inflammasome

INTRODUCTION

There is increasing evidence that autoinflammation plays an important role in the pathogenesis of myelodysplastic syndromes (MDS), with several studies describing dysregulation of the innate immune response, as being particularly relevant.[1] For example, Toll-like receptors (TLRs) have been shown to be over-expressed in haematopoietic stem/progenitor cells (HSPCs), compared to age-matched control subjects.[2] Furthermore, in the HSPCs of MDS patients, there is enhanced activation of the TLR4 signalling pathway through upregulation of TLR4 and overexpression of myeloid differentiation primary response 88 (MYD88), a TLR4-associated adaptor protein.[2,3] Activation of TLR4 in MDS leads to NLR family pyrin domain containing 3 (NLRP3)-inflammasome activation in CD34+ cells, and

For affiliations refer to page 9.

their subsequent pyroptotic death.[4] Alarmins released by these dying cells cause further activation of the TLR4 pathway, driving sterile inflammation.[5] In addition, certain somatic mutations such as those found in *TET2*, not only support the survival of the malignant MDS clones but are also linked to activation of the NLRP3 inflammasome complex.[6] One mechanism for activation of this complex is via the redundant sources of cytoplasmic DNA, which also activate the cytosolic cGAS/STING leading to enhanced type I IFN response.[7] The NLRP3 inflammasome activation, in addition to causing pyroptosis, catalyses the release of proinflammatory cytokines Interleukin (IL)-1β and IL-18, which together with IL-6, tumour necrosis factor-alpha (TNFα) and IL-8 have all been shown to directly support the growth of aberrant MDS stem cells in preclinical models.[8–12]

Further evidence supporting the link between the innate-immune driven inflammation and MDS is the observation that autoinflammatory conditions, which are themselves caused by aberrant activation of the innate immune response,[13] are common in patients with MDS. Historically, the range of reported autoimmune/autoinflammatory complications in patients with MDS has been highly variable, with a prevalence ranging from 7% to 30% depending on the various definitions of autoinflammatory disorder (AID).[14–16] A recent study using an expanded definition of autoinflammatory complications, found that 46% (62/134) of MDS patients had either well-defined AID (10/62) or 'undifferentiated AID' (50/62)—defined as C reactive proteine over 10.0 mg/L on five consecutive occasions, not explained by infection.[17] Significantly, transformation to acute myeloid leukaemia (AML) was more frequent in those MDS patients with autoinflammatory features, than those without (27.4% vs. 9.7%, $p = 0.008$). These findings suggest that knowing the patients' inflammatory status might be predictive of long-term outcomes.

However, it remains unclear how the activation of autoinflammatory pathways relates to the initiation and/or progression of MDS and whether measuring inflammatory biomarkers helps improve risk stratification or inform treatment decisions. This is despite several previous studies showing that patients with MDS have both, increased expression and elevated serum levels of inflammatory cytokines, including IL-1β, IL-8, TNFa and IL-6, interferons, granulocyte colony-stimulating factor and thrombopoietin.[8,18–20] There are also studies that demonstrated evidence of inflammasome activation by measuring inflammasome components in the form of ASC (apoptosis-associated speck-like protein, containing a caspase recruitment domain) protein specks in the serum of patients with MDS.[4,21,22] However, the methods used in these studies detected ASC specks only, which is not specific for NLRP3 inflammasome, since the ASC is also important adaptor for a range of other inflammasomes, including NLRP1, AIM2, Pyrin, NLRC4 and newly discovered NLRP10.[23] Therefore, it is uncertain whether other inflammasome complexes might also play a role in the pathogenies of MDS. All these unanswered questions are of particular relevance for the patients with lower risk MDS, who, even currently, have limited treatment options, and in whom therapeutically targeting selected inflammatory pathways could potentially improve blood counts and reduce the risk of disease progression.

To address some of these issues, we tested a large cohort of MDS patients with low bone marrow (BM) blasts (<5%), for whom we had available long-term outcome data. We developed a novel methodology, to specifically measure activation of the NLRP3 inflammasome and we combined this with the measurement of a range of inflammatory cytokines. We then performed statistical analysis to determine the utility of these tests in predicting the risk for transfusion dependency, progression to AML and overall survival.

METHODS

EUMDS cohort

The EUMDS Registry (https://eumds.org) is a prospective, non-interventional longitudinal registry, originally enrolling patients with lower risk MDS, within 3 months of initial diagnosis from 142 centres in 16 countries across Europe and Israel.[24] Patients, newly diagnosed with International Prognostic Scoring System (IPSS) low- or intermediate-1-risk MDS, who had serum samples available at the time of initial diagnosis from five participating countries were included; these 183 patients were diagnosed from 4 February 2008 to 19 June 2014 and were followed up till 23 March 2023. Patients for whom cytogenetic testing was not available or had failed could be included as long as the percentage of BM blasts was <5% and the cytopaenia score was zero (0/1 cytopenia). Data were collected at baseline and each outpatient routine follow-up visit for 6 months. Information was collected on: comorbidities, transfusion history, management of MDS, peripheral blood counts, conventional iron parameters, BM pathology and date of progression to higher risk (HR) MDS or AML. Patients were prospectively followed until death, loss to follow-up or withdrawal of informed consent. Considering that IPSS-R has been shown to be a more refined prognostic tool, this scoring system was used for the data analysis. Validation of the revised international prognostic scoring system in patients with lower risk myelodysplastic syndromes from the EUMDS registry has previously been described.[24] The EUMDS Registry protocol (ClinicalTrials.gov identifier NCT00600860) has been approved by each Institution's Ethics Committee, in accordance with national legislation, and written informed consent has been obtained by all the participating patients.

Healthy controls (HC) and disease controls, who were patients with AID recruited via the ImmunAID (IMMUNome project consortium for AutoInflammatory Disorders—https://www.immunaid.eu) project. This is a large multinational EU-funded study, aiming to improve the diagnosis and management of patients with autoinflammatory diseases. Detailed inclusion and exclusion criteria for the various disease categories are shown in Supporting Information. Serum

samples used for this study were collected at the initial visit, during active disease and prior to commencing treatment.

ASC/NLRP3 protein speck quantification by flow cytometry

We used in-house flow cytometry assay, for quantification of ASC/NLRP3 protein specks in sera. Briefly, 100 µL of patients' sera was incubated in Phycoerythrin conjugated anti-ASC (TMS-1) Antibody (653904; BioLegend) and APC-conjugated NLRP3 Antibody (IC7578A; BioTechne) for 1 h, at RT, with continuous shaking. A 50 µL sample volume was measured using a Cytoflex S Flow Cytometer, gating for events 1 µm in size, using Flow Cytometry Sub-micron Particle Size Reference beads (F13839; Thermo-Fisher Scientific). Although ASC/NLRP3 speck can vary in size, 1 µm is the most frequently reported diameter, thus our rationale for choosing this size reference bead gate. Further work is ongoing to validate the assay and test if the ASC/NLRP3 specks of different sizes and any degradation products should be included in the analysis. These findings will be reported in a future follow up manuscript. For the detailed gating strategy (Figure S1). Results were analysed using CytExpert software and presented as ASC/NLRP3-positive events/µL.

Cytokine profiling

The inflammatory profile was investigated using the multiplex LEGENDplex™ Multi-Analyte Flow Assay kit (Biolegend). The human inflammation panel 1 includes IL-1β, IFN-α2, IFN-γ, TNF-α, CCL2, IL-6, CXCL8, IL-10, IL-12p,70, IL-17A, IL-18, IL-23 and IL-33. Serum samples were all diluted twofold in assay buffer prior to analysis and the assay was carried out according to the manufacturer's assay instructions for analysis of plasma samples in a V-bottom 96-well plate (Biolegend). Briefly, diluted samples and standards were mixed with an equal volume of capture beads prior to incubation for 2 h at room temperature (RT), protected from light with continuous shaking at 800 rpm. Following incubation, the plate was centrifuged at 250 *g*, RT for 5 min in a swing out rotor equipped with plate adaptor. The supernatant was discarded, and wash step carried out with wash buffer, followed by shaking at 800 rpm, RT for 1 min and centrifugation at 250 *g*, RT for 5 min. After removal of supernatant the detection antibody was added and the samples incubated for 1 h at RT, protected from light with shaking at 800 rpm. Immediately thereafter, Strep-PE was added, and samples were incubated for an additional 30 min at RT, protected from light with continuous shaking at 800 rpm. Following a final wash, bead pellets were re-suspended in wash buffer for analysis. All standards and samples were run in duplicate. Data were acquired using a BD Canto II flow cytometer (BD Bioscience) and BD Diva software. Totally, 1000 events were collected per well, using a high-throughput 96-well plate autosampler. Analysis was carried out using the LEGENDplex Data Analysis Software.

Statistical analysis

Variables were described by median (interquartile range) and compared between groups by the Kruskal–Wallis test. Overall survival, time to progression and time to first transfusion were estimated from the date of initial diagnosis. Time to first transfusion was calculated only in patients, who were transfusion-free at baseline. Univariate analysis of the effect of biomarkers on times-to-events was performed with Cox regression, unadjusted and adjusted for age and sex. Multivariable time-to-event models were developed with variable selection, using the Lasso in combination with Cox regression, with models presented as the relaxed Lasso. Univariate logistic regressions were used to investigate the relationship between biomarkers and presence of autoimmune or cardiac disease at presentation. Prior to performing regressions, continuous covariates, other than age were scaled and centred. *p* Values were adjusted for multiple testing within each table using the Benjamini and Hochberg method. All analyses were performed with Stata 18.0 (StataCorp, College Station, TX) or R version 4.3.1 (https://www.R-project.org/).

RESULTS

EUMDS cohort-baseline patient characteristics

In total 183 patients from the EUMDS registry were included in the study. Their demographics and disease characteristics are shown in Table S1.

ASC/NLRP3 protein specks in relation to age, sex and MDS subtype

Using our novel method that specifically detects ASC specs derived from activation of the NLRP3 inflammasome, we measured ASC/NLRP3 double positive specks and ACS-only specks in sera of patients with MDS and compared these to HC and patients with AID, in whom NLRP3 inflammasome activation is known to occur. Overall, patients with MDS had significantly elevated levels of ASC/NLRP3 specs and ASC-only specks, compared to HC ($p < 0.001$) (Figure 1A,B). The ASC/NLRP3 specks were also elevated in MDS patients compared to patients with AID, but this did not reach statistical significance (Figure 1A,B). Within the MDS cohort, the levels of ACS/NLRP3 specks were broadly distributed, with a subgroup of patients showing particularly high levels (>1000 events/µL). Disease and demographic characteristics of this group were in general, similar to those of the overall patient cohort (data not shown). Interestingly, although overall ASC-only speck levels were also significantly elevated in

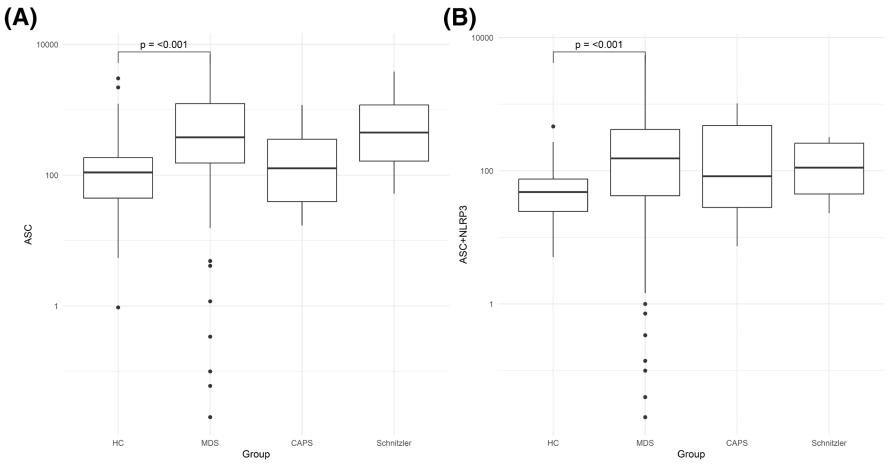


FIGURE 1 ASC + NLRP3 and ASC (only) protein speck levels in MDS and disease control cohorts. (A) ASC/NLRP3 specks and (B) ASC speck levels (events/μL) in healthy controls (HC, $n = 30$) were compared to MDS ($n = 183$), CAPS ($n = 11$) and Schnitzler ($n = 10$) cohorts. The weighted bars represent median values. CAPS, cryopyrin-associated autoinflammatory syndromes; MDS, myelodysplastic syndrome.

MDS patients compared to HC, the distribution of results in the MDS cohort did not match that of NLRP3/ASC specks. For example, MDS patients had almost statistically significantly higher ASC speck levels compared to patients with cryopyrin-associated autoinflammatory syndromes (CAPS) ($p = 0.052$). Furthermore, the median levels of ASC/NLRP3 specks and ASC alone specks were significantly different in HC, MDS and Schnitzler patients (Table S2), suggesting that some ASC specks might originate from activation of non-NLRP3 inflammasome complexes (Figure 1B).

We were also interested to know whether the levels of ASC/NLRP3 or ASC specks were associated with patients' sex, age or MDS disease subtype. This analysis was particularly important when considering the age, since the average age of HC used for this study was lower compared to the MDS cohort. When we conducted this analysis across the whole cohort, we found no significant correlation between any of these parameters and ASC/NLRP3 or ASC speck levels (Tables 1 and 2; Tables S3 and S4). The category 'other' contained six MDS patients with isolated Del(5q) and four who were unclassifiable (MDS-U).

Cytokine profile in relation to age, sex and MDS subtype

In addition to ASC/NLRP3 specks, we measured levels of IL-1β, IL-18 and IL-33 (NLRP3-related and IL-1 family of cytokines), additional inflammatory cytokines (IL-6, TNFα, IL-8, IL-12p70, IL-23 and MCP-1) and other cytokines which have previously been implicated in the pathogenesis of MDS (IL-10, IFN-α2 and IFNγ). The levels of several cytokines including IL-18, IL-33, IL-23, IL-6, IL-8, MCP-1, IFN-α2 and IFN-γ were all significantly higher in MDS patients compared to HC and comparable to the levels seen in AID controls (Figure 2A–C). As for ASC/NLRP3 specks, we analysed whether there was a relationship between the cytokine levels, patients' age, sex and MDS characteristics. We found no statistical association between the cytokine levels and age and sex of the patients (Tables S3 and S4). However, we did find that initial diagnosis was significantly associated with serum levels of several cytokines. For example, lowest levels of IL-1β were seen in patients with MDS-SLD. These patients also had the lowest levels of IL-23, IL-33, INFγ and IFN-α2 (Table 1). Similarly, several cytokines, including IL-1β, IFNγ and TNFα all showed significant association with IPSS-R classification (Table 2). Last, we were interested to find out if there was any correlation between the ASC/NLRP3 and ASC-only specks with the levels of various cytokines. For this purpose, we log-transformed the variables and performed linear regressions with ASC-only and ASC/NLRP3 specks as outcome for each cytokine, with MDS diagnosis group and an interaction term. We compared these with the model without the interaction term using likelihood ratio test—none of the p values reached significance level of 0.05. Furthermore, compared these with models without MDS diagnosis group—again none reached significance 0.05.

Prediction of disease outcomes and complications

To determine the utility of various biomarkers to predict disease outcomes, such as overall survival, time to progression to a more aggressive form of MDS or AML, or development of complications including autoimmune/inflammatory or cardiac disease, we used standard time-to-event analysis techniques. Concerning overall survival, none of the biomarkers were found to be predictive by univariate regression analysis, or when this univariate analysis was adjusted for age and sex, using Cox regression analysis (Table 3). TNFα levels were the only biomarker that was found to be predictive for the time to progression, both, by the initial univariate regression analysis ($p = 0.001$) and after adjusting for age and

TABLE 1 Association between ASC(NLRP3) specks and cytokine profile according to baseline MDS diagnosis.

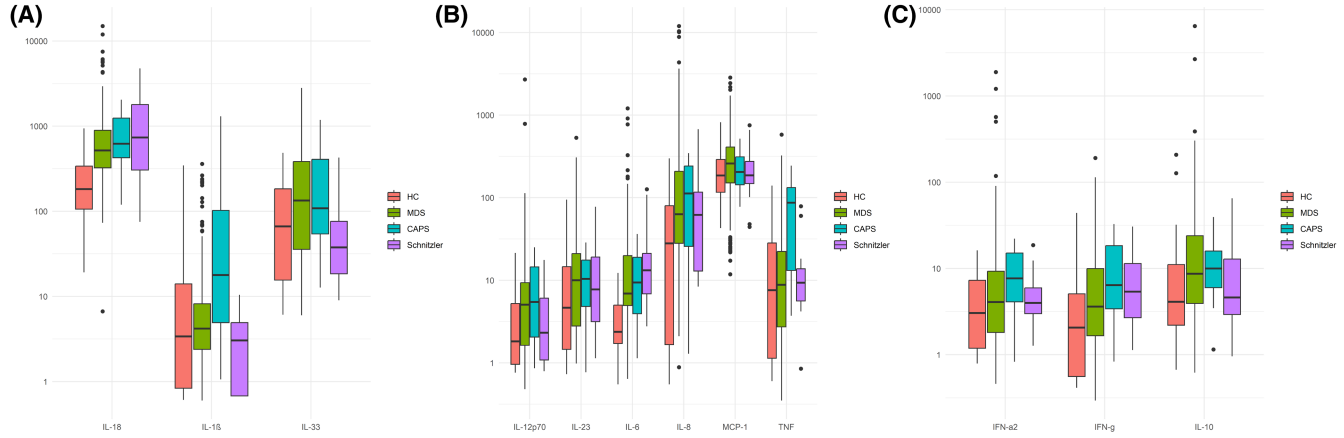
	MDS-SLD	MDS-RS	MDS-MLD	Other	Total	Raw *p* value	Adjusted *p* value
ASC + NLRP3	241.6 (26.5–456.8)	113.5 (26.9–307.4)	110.9 (35.6–376.5)	265.1 (125.9–377.2)	125.9 (35.6–383.2)	0.441	0.475
ASC	768.3 (87.3–2612.6)	339.3 (88.2–1150.6)	288.1 (135.4–891.7)	949.9 (323.4–1114.9)	347.0 (130.3–1150.6)	0.391	0.456
IL-1β	2.4 (1.0–2.4)	4.2 (2.4–12.3)	4.2 (2.8–9.6)	5.1 (2.4–8.5)	4.2 (2.4–8.2)	<0.001	0.001
IL-18	508.5 (199.0–1133.9)	462.6 (298.6–752.8)	582.1 (354.1–931.1)	461.4 (341.6–784.4)	518.8 (324.4–897.4)	0.236	0.300
IL-33	46.5 (11.7–146.9)	187.6 (34.3–400.1)	138.7 (50.1–412.7)	50.4 (12.0–357.1)	134.3 (35.7–383.6)	0.017	0.030
IL-6	5.9 (1.8–7.0)	6.9 (5.9–24.3)	8.4 (4.9–20.0)	6.9 (2.2–28.2)	6.9 (4.9–19.9)	0.020	0.031
TNF	3.8 (1.5–8.9)	8.8 (1.6–14.9)	8.9 (4.1–24.8)	8.9 (1.7–25.0)	8.8 (2.7–22.4)	0.008	0.016
IL-8	115.0 (8.3–207.7)	55.9 (27.6–237.0)	63.2 (28.0–203.5)	89.8 (28.0–427.0)	63.2 (28.0–209.9)	0.814	0.814
IL-23	2.9 (1.1–10.5)	10.1 (2.9–20.2)	11.8 (3.8–23.0)	14.8 (3.1–23.0)	10.0 (2.8–21.5)	0.007	0.016
IL-12p70	2.6 (0.7–7.5)	6.6 (2.3–9.0)	5.1 (1.6–11.1)	2.7 (1.1–7.5)	5.1 (1.6–9.5)	0.002	0.007
IFN-α2	1.5 (0.6–6.9)	5.1 (2.0–11.0)	4.8 (2.0–10.3)	4.4 (0.8–8.6)	4.1 (1.8–9.3)	0.001	0.007
IFN-γ	1.2 (0.6–6.7)	3.0 (1.5–9.4)	4.1 (2.4–12.1)	5.6 (1.0–10.6)	3.6 (1.7–10.0)	0.008	0.016
IL-10	6.5 (1.3–11.8)	8.7 (4.5–26.5)	8.7 (4.5–26.6)	5.4 (2.1–14.4)	8.7 (3.9–24.0)	0.089	0.125
MCP-1	266.6 (118.2–458.9)	328.5 (230.3–479.3)	229.0 (126.1–368.3)	295.2 (168.6–477.6)	259.7 (150.6–411.1)	0.002	0.007

Abbreviations: IFN, interferon; IL, interleukin; MDS, myelodysplastic syndrome; TNF, tumour necrosis factor.

TABLE 2 Association between ASC(NLRP3) specks and cytokine profile and IPSS-R group.

	Very low	Low	Int/high/very high	Unknown	Total	Raw *p* value	Adjusted *p* value
ASC + NLRP3	105.5 (27.1–470.8)	120.2 (26.2–362.4)	108.2 (47.6–221.5)	234.7 (72.8–657.8)	125.9 (35.6–383.2)	0.258	0.354
ASC	304.7 (89.4–1278.8)	353.1 (140.2–1179.5)	263.3 (122.8–735.9)	528.5 (234.4–2105.6)	347.0 (130.3–1150.6)	0.625	0.729
IL-1β	4.2 (2.5–9.7)	3.5 (2.4–6.1)	5.1 (4.2–20.8)	2.8 (2.4–4.2)	4.2 (2.4–8.2)	<0.001	0.004
IL-18	552.5 (337.5–892.7)	499.4 (306.9–931.8)	582.1 (368.0–1004.1)	494.8 (316.6–752.1)	518.8 (324.4–897.4)	0.858	0.858
IL-33	152.7 (54.2–374.8)	106.2 (26.2–317.2)	154.0 (50.1–458.4)	125.3 (12.0–384.0)	134.3 (35.7–383.6)	0.153	0.306
IL-6	7.0 (5.9–19.9)	6.9 (4.1–22.7)	9.5 (5.9–20.3)	5.9 (4.7–18.8)	6.9 (4.9–19.9)	0.233	0.354
TNF	8.9 (8.2–21.7)	7.8 (1.8–21.5)	10.5 (6.6–82.7)	3.8 (1.5–10.5)	8.8 (2.7–22.4)	<0.001	0.001
IL-8	71.9 (31.2–268.3)	62.3 (28.0–175.7)	72.2 (22.4–288.6)	45.8 (26.0–161.0)	63.2 (28.0–209.9)	0.738	0.795
IL-23	14.5 (3.4–22.2)	7.7 (2.2–21.8)	11.7 (5.1–22.2)	5.1 (2.6–14.7)	10.0 (2.8–21.5)	0.122	0.285
IL-12p70	6.6 (1.9–8.7)	4.3 (1.6–10.2)	7.5 (3.9–11.9)	3.9 (1.6–8.2)	5.1 (1.6–9.5)	0.218	0.354
IFN-α2	6.0 (2.0–9.3)	3.3 (1.4–8.9)	4.8 (2.1–17.7)	2.5 (0.8–7.7)	4.1 (1.8–9.3)	0.087	0.244
IFN-γ	6.3 (2.4–12.4)	3.2 (1.3–8.6)	5.5 (2.6–19.5)	2.6 (1.1–7.3)	3.6 (1.7–10.0)	0.002	0.009
IL-10	8.7 (4.5–19.4)	6.8 (3.0–24.2)	10.4 (4.5–31.1)	5.2 (2.2–12.5)	8.7 (3.9–24.0)	0.047	0.165
MCP-1	262.5 (182.2–437.7)	266.0 (126.1–431.2)	231.1 (97.4–375.5)	265.3 (146.8–384.3)	259.7 (150.6–411.1)	0.278	0.354

Abbreviations: IFN, interferon; IL, interleukin; TNF, tumour necrosis factor.



(D)

	p value	Adjusted p value
IL-18	<0.001	<0.001
IL-1ß	0600	0.600
IL-33	<0.001	<0.001
IL-12p70	0.042	0.054
IL-23	0.009	0.014
IL-6	<0.001	<0.001
IL-8	<0.001	<0.001
MCP-1	<0.001	<0.001
TNF	0.287	0.309
IFN-α2	0.014	0.020
IFN-γ	<0.001	<0.001
IL-10	0.076	0.089

FIGURE 2 Cytokine profile in MDS patients, disease and healthy controls (HC). Panels (A–C) show the serum cytokine levels (pg/mL) for 12 targets and are displayed on bar charts, comparing HC ($n = 30$) to MDS ($n = 183$), CAPS ($n = 11$) and Schnitzler ($n = 10$) cohorts. Weighted bars represent median values. Panel (D) shows significant p values after comparing HC to total MDS patient cohort. CAPS, cryopyrin-associated autoinflammatory syndromes; MDS, myelodysplastic syndrome.

sex ($p < 0.003$) (Table 4). Similarly, IL-6 levels were positively associated with time to the first blood transfusion, for patients, who were transfusion-independent when they first entered the study. This was statistically significant unadjusted ($p = 0.001$) and adjusted for age and sex ($p = 0.014$) (Table 5).

Variable selection using the Lasso was used to develop a multivariable proportional hazards model, showing IL-18, MCP-1 and TNFα as implicated in overall survival, with a concordance index of 0.617. When age and sex were included in the variable selection process, only age remained relevant with a concordance index of 0.672 (Table 6). Several cytokines were also implicated in the time to progression, but after including age and sex, again only age remained relevant ($p = 0.022$) (Table 6). Lastly, after adjusting for age and sex, IL-6 was found a significant predictor of the need for transfusions ($p < 0.001$), and IL-1β was very close to statistical significance ($p = 0.051$) (Table 6).

Finally, we performed a univariate logistic regression analysis to determine predictors for patients exhibiting an autoimmune/inflammatory or cardiac disease at the presentation. None of the inflammatory biomarkers were significantly associated with the presence of these manifestations (Table S5).

DISCUSSION

The inflammatory environment in MDS is complex and the precise link between various inflammatory signals, disease pathogenesis, progression or development of various complications is yet to be fully established. Our study tested the utility of various serum-derived inflammatory markers/cytokines to predict the disease type and course of MDS. This included a novel assay to specifically measure NLRP3 inflammasome activation, which is one of the main inflammatory pathways implicated in the MDS pathogenesis. We benefited from having access to a large well-characterised MDS patient cohort, and disease controls with defined AIDs. We found that NLRP3 inflammasome was significantly activated in the MDS cohort overall, compared to HC, and that the level of activation was comparable to several AIDs. When we measured the levels of ASC specks alone, we found that these were also significantly elevated in MDS. Similar findings were demonstrated previously by another study which showed that ASC specks were not just significantly elevated in MDS compared to HC, but also compared to non-myelodysplastic syndrome, that is, haematological cancers.[21] In addition, our study showed that the distribution of ASC specks alone was different, compared to the ASC/NLRP3 specks, suggesting that other inflammasomes which use ASC adaptor protein in their activation might be involved. Indeed, a recent study has showed increased AIM2 inflammasome activation and IL-1β production by murine macrophages carrying truncating *ASXL1* mutation that are typically associated with MDS.[25] An alternative explanation might be that, in time, the NLRP3 dissociates from the complex resulting in ASC only specks being detected. Further studies are needed to fully characterise this dynamic process.

TABLE 3 Association of ASC(NLRP3) specks and cytokines with survival.

	Univariate			Adjusted for age and sex		
	Hazard ratio (95% CI)	Raw *p*-value	Adjusted *p*-value	Hazard ratio (95% CI)	Raw *p*-value	Adjusted *p*-value
ASC + NLRP3	1.024 (0.86–1.21)	0.785	0.981	1.049 (0.89–1.23)	0.568	0.981
ASC	0.99 (0.84–1.18)	0.950	0.981	1.0029 (0.85–1.18)	0.973	0.981
IL-1β	1.22 (1.030–1.44)	0.022	0.215	1.23 (1.029–1.47)	0.023	0.215
IL-18	1.017 (0.89–1.17)	0.805	0.981	0.96 (0.83–1.12)	0.635	0.981
IL-33	0.97 (0.80–1.20)	0.780	0.981	0.92 (0.75–1.12)	0.416	0.981
IL-6	1.074 (0.92–1.25)	0.358	0.981	1.08 (0.91–1.29)	0.370	0.981
TNF	1.23 (1.034–1.47)	0.020	0.215	1.11 (0.92–1.33)	0.291	0.981
IL-8	1.075 (0.91–1.28)	0.410	0.981	1.047 (0.89–1.24)	0.586	0.981
IL-23	1.040 (0.87–1.25)	0.672	0.981	0.97 (0.80–1.16)	0.707	0.981
IL-12p70	1.023 (0.88–1.19)	0.771	0.981	1.00 (0.86–1.16)	0.981	0.981
IFN-α2	0.96 (0.84–1.11)	0.601	0.981	0.93 (0.81–1.075)	0.331	0.981
IFN-γ	0.99 (0.83–1.18)	0.907	0.981	0.95 (0.79–1.13)	0.539	0.981
IL-10	1.030 (0.87–1.19)	0.707	0.981	1.0037 (0.87–1.16)	0.961	0.981
MCP-1	0.91 (0.74–1.12)	0.374	0.981	0.98 (0.79–1.21)	0.839	0.981

Abbreviations: IFN, interferon; IL, interleukin; TNF, tumour necrosis factor.

TABLE 4 Association of ASC(NLRP3) specks and cytokines with time to progression.

	Univariate			Adjusted for age and sex		
	Hazard ratio (95% CI)	Raw *p*-value	Adjusted *p*-value	Hazard ratio (95% CI)	Raw *p*-value	Adjusted *p*-value
ASC + NLRP3	0.80 (0.48–1.33)	0.388	0.905	0.75 (0.43–1.29)	0.297	0.756
ASC	0.74 (0.46–1.20)	0.221	0.619	0.71 (0.43–1.20)	0.184	0.619
IL-1β	1.23 (0.93–1.62)	0.139	0.592	1.24 (0.94–1.63)	0.128	0.592
IL-18	1.15 (0.93–1.43)	0.199	0.619	1.16 (0.95–1.43)	0.148	0.592
IL-33	1.013 (0.71–1.45)	0.943	0.990	1.053 (0.72–1.53)	0.787	0.990
IL-6	1.18 (0.97–1.43)	0.106	0.592	1.15 (0.95–1.40)	0.140	0.592
TNF	1.51 (1.21–1.88)	<0.001	0.004	1.60 (1.27–2.011)	<0.001	0.003
IL-8	0.92 (0.56–1.50)	0.723	0.990	0.86 (0.46–1.60)	0.618	0.990
IL-23	1.068 (0.78–1.45)	0.678	0.990	1.12 (0.81–1.55)	0.483	0.966
IL-12p70	0.91 (0.53–1.56)	0.738	0.990	0.94 (0.55–1.61)	0.818	0.990
IFN-α2	1.0077 (0.77–1.31)	0.955	0.990	1.033 (0.79–1.35)	0.808	0.990
IFN-γ	1.0004 (0.70–1.42)	0.998	0.998	1.030 (0.72–1.48)	0.873	0.990
IL-10	0.95 (0.63–1.44)	0.804	0.990	0.98 (0.64–1.49)	0.907	0.990
MCP-1	0.88 (0.58–1.32)	0.532	0.990	0.86 (0.57–1.30)	0.475	0.966

Abbreviations: IFN, interferon; IL, interleukin; TNF, tumour necrosis factor.

Our study confirms previous findings which demonstrated activated NLRP3 inflammasome in MDS, but it also shows for the first time that the level of activation is comparable to what we see in classical autoinflammatory diseases and implies that other, non-NLRP3, ASC-containing inflammasomes might have a role in the pathogenesis of MDS. However, when we looked at the utility of ASC/NLRP3 or ASC specks to predict the disease subtype, its evolution, or complications, we did not find any statistically significant correlation.

In addition to NLRP3 inflammasome activation, we also found several cytokines significantly elevated in the sera of the MDS cohort, compared to HC. This included levels of IL-6 and IL-18. The latter is directly linked to NLRP3 inflammasome activation, but it is also released following NLRC4 activation, which favours IL-18 over IL-1β,[26] again suggesting that other inflammasome complexes might be relevant in MDS pathogenesis. Several cytokines were significantly associated with a specific

TABLE 5 Association of ASC(NLRP3) specks and cytokines with time needing transfusion.

	Univariate			Adjusted for age and sex		
	Hazard ratio (95% CI)	Raw *p*-value	Adjusted *p*-value	Hazard ratio (95% CI)	Raw *p*-value	Adjusted *p*-value
ASC + NLRP3	1.0019 (0.82–1.23)	0.986	0.986	1.00 (0.82–1.21)	0.972	0.986
ASC	1.015 (0.82–1.26)	0.891	0.960	1.015 (0.83–1.25)	0.891	0.960
IL-1β	1.28 (1.047–1.55)	0.016	0.108	1.26 (1.016–1.55)	0.035	0.109
IL-18	0.88 (0.66–1.19)	0.411	0.639	0.79 (0.56–1.11)	0.180	0.336
IL-33	1.27 (1.0004–1.60)	0.050	0.117	1.22 (0.97–1.54)	0.096	0.192
IL-6	1.40 (1.16–1.70)	<0.001	0.014	1.41 (1.15–1.73)	0.001	0.014
TNF	1.27 (1.028–1.57)	0.027	0.108	1.28 (1.033–1.59)	0.024	0.108
IL-8	1.10 (0.90–1.35)	0.348	0.609	1.076 (0.88–1.32)	0.479	0.671
IL-23	1.20 (1.0096–1.44)	0.039	0.109	1.16 (0.97–1.38)	0.094	0.192
IL-12p70	1.20 (1.03–1.40)	0.020	0.108	1.17 (1.0034–1.36)	0.045	0.115
IFN-α2	0.84 (0.52–1.34)	0.463	0.671	0.80 (0.49–1.33)	0.397	0.639
IFN-γ	1.038 (0.83–1.30)	0.742	0.929	1.021 (0.82–1.27)	0.850	0.960
IL-10	1.20 (1.04–1.40)	0.014	0.108	1.17 (1.012–1.36)	0.034	0.109
MCP-1	1.042 (0.80–1.36)	0.763	0.929	1.084 (0.84–1.40)	0.542	0.723

Abbreviations: IFN, interferon; IL, interleukin; TNF, tumour necrosis factor.

TABLE 6 Association of ASC(NLRP3) specks and cytokines with various MDS outcomes.

Model 1				Model 2			
ASC, ASC + NLRP3 and cytokines only				Including sex and age at diagnosis			
Covariate	Hazard ratio	Raw *p*-value	Adj. *p*-value	Covariate	Hazard ratio	Raw *p*-value	Adj. *p*-value
Overall survival							
IL-18	1.093	0.311	0.311	Age	1.052	<0.001	<0.001
MCP-1	0.744	0.057	0.102	MCP-1	0.751	0.068	0.111
TNF	1.256	0.006	0.020				
Concordance = 0.617				Concordance = 0.644			
Time to progression							
IL-18	1.133	0.191	0.229	Age	1.031	0.005	0.020
IL-1β	1.112	0.210	0.236	Female	0.774	0.236	0.249
MCP-1	0.759	0.081	0.113	MCP-1	0.759	0.082	0.113
TNF	1.200	0.023	0.059	TNF	1.187	0.040	0.080
Concordance = 0.611				Concordance = 0.629			
Time to first transfusion							
IL-1β	2.693	0.026	0.059	Age	1.027	0.131	0.169
IL-6	1.493	<0.001	<0.001	IL-1β	2.935	0.017	0.051
				IL-6	1.517	<0.001	<0.001
Concordance = 0.559				Concordance = 0.648			

Abbreviations: IL, interleukin; TNF, tumour necrosis factor.

MDS subtype and IPSS-R scores, implying a link between various inflammatory pathways and disease evolution. Furthermore, we found that several inflammatory cytokines, including TNFα, IL-6 and IL-1β were positively associated with MDS progression to a more aggressive form of disease or time to first red cell blood transfusion. This suggests that targeted cytokine blockade might be beneficial to modulate disease evolution or prevent some disease complications. This view is supported by a recent study showing that chronic inflammation can drive leukaemic evolution in certain circumstances.[27] However, a large population-based analysis of over 15 000 MDS patients

found that those with lower risk disease and pre-existing autoimmune conditions, had higher requirements for transfusions, and HR of progression to AML, but interestingly, a reduction in the overall risk of death. The reason for these conflicting results is unclear but the authors postulated that autoimmunity may promote the development of less aggressive neoplastic clones while inflammation driven clonal dynamics could favour leukaemogenesis.[28] A third possibility is that autoimmune/autoinflammatory activation may not be the dominant mechanism influencing disease progression and leukaemic transformation, which is mainly directed by genetic and other intracellular defects of the clonal cells, and therefore, revealing immune dysregulation in lower-risk MDS, although important, may not be sufficient to depict prognosis.

One important limitation of our study is the lack of molecular data on these patients, which would enable the analysis of the potential relationship between inflammatory signatures and specific MDS-associated mutations. Furthermore, the findings of our study need to be confirmed using a separate (validation) cohort. A study, using a cohort of MDS patients with a known molecular diagnosis is currently in progress to address both points. Previous studies have shown potential utility in testing ASC specks to differentiate MDS from other haematological malignancies and predict certain treatment responses.[21,22] However, the true utility of analysing various inflammatory markers, including ASC/NLRP3, to help with disease stratification, prognosis or to direct treatment decisions is yet to be fully validated and studies using large patient cohorts with complete clinical and molecular characterisation will be needed to achieve these aims. This is the subject of our ongoing work. In summary, our study shows that there is value in analysing inflammatory biomarkers in MDS patients with low-risk disease and this could provide a potential target for future therapies as demonstrated by the high number of clinical trials targeting various inflammatory pathways, designed for this patient population.[29]

AUTHOR CONTRIBUTIONS

SS, JT and AT wrote first draft of the manuscript; JT, FT and AI performed laboratory test. JT, AT SC, AS, CCa and SS analysed data. DB, CCa, EHL, HGG, AT, SL, JC, DC, JA, EN, JP, SK, TW and AS provision of patients, assembly of samples and data. DB, CCa, MM and SS contributed to experimental design of the study. SS provided funding for experimental work. All authors approved the version to be published and agreed to be accountable for all aspects of the work.

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CONFLICT OF INTEREST STATEMENT

SS received honoraria for participation in advisory board meetings and speaking engagements, travel support and research grants from SOBI and Novartis. AS received honoraria for participation in advisory boards for Bristol Myers Squibb, Abbvie, Genesis/Incyte and Sanofi. AT received fees from Bristol Myers Squibb, Sandoz, Alvogen and Abbvie. Other authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, (SS), upon reasonable request.

ETHICS STATEMENT

The EUMDS Registry protocol (ClinicalTrials.gov identifier NCT00600860) has been approved by each Institution's Ethics Committee, in accordance with national legislation, and written informed consent has been obtained by all the participating patients.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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