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ORIGINAL ARTICLE

Immunoparesis defined by heavy/light chain pair suppression in smoldering multiple myeloma shows initial isotype specificity and involves other isotypes in advanced disease

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Abstract

Smoldering multiple myeloma (SMM) is an asymptomatic and biologically heterogeneous plasma cell disorder, with a highly variable clinical course. Immunoparesis, defined by total immunoglobulin measurements, has been shown to be an independent risk factor for progression to symptomatic disease. The heavy/light chain (HLC) assay allows precise measurement of the polyclonal immunoglobulin of the same isotype, enabling the evaluation of isotype-matched immunoparesis (IMI). In this study, we prospectively characterized immunoparesis, as determined by HLC measurements, in 53 SMM patients. Severe IMI was present in 51% of patients, while severe IP of uninvolved isotypes (HLC IP) was present in 39%. Most of the patients with severe HLC IP presented with severe IMI, but not the other way around. Isotype specificity of immune suppression was suggested by lower relative values of isotype-matched HLC pairs, both for IgG and IgA SMM. Severe IMI was associated with other risk factors for progression while patients with severe IMI and severe HLC IP showed an even higher risk profile. Both severe IMI and severe IgM HLC IP showed a significantly shorter time to progression. Finally, gene expression analysis demonstrated differences in the bone marrow microenvironment between patients with IMI and IMI plus HLC IP, with an increased expression of genes associated with cytolytic cells. In conclusion, our data supports isotype specificity of early immunoglobulin suppression mechanisms. While suppression of both involved and uninvolved isotypes is associated with risk of progression, the later appears to develop with more advanced disease and could be mediated by different mechanisms.

Keywords Smoldering myeloma · Multiple myeloma · Monoclonal gammopathy of undetermined significance · Immunoparesis · Heavy/light chain pair

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Introduction

Asymptomatic monoclonal gammopathies, such as monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM), are clinical conditions that usually precede symptomatic multiple myeloma (MM). Risk stratification is crucial, particularly in the case of SMM, an entity that includes patients with a true asymptomatic malignancy destined to progress in a short period of time for whom chemoprevention trials are encouraged [1].

The Mayo Clinic and the Spanish Group have developed different risk stratification models that can identify patients with SMM and a 2-year risk of progression to MM ≥ 50% [2, 3]. The Mayo Clinic model includes the serum M-protein (≥ 3 g/dL), bone marrow plasma cell infiltration (%BMPC, ≥ 10%), and the ratio of involved to uninvolved serum free light chains (FLCr, ≥ 8), while the Spanish model uses the proportion of BMPC with aberrant phenotype by flow cytometry (≥ 95%) and the reduction in uninvolved immunoglobulins (immunoparesis) to identify high-risk patients. The International Myeloma Working Group (IMWG) has recently reported the "2/20/20" risk stratification model (based on a M-protein > 2 g/dL, the presence of BMPC > 20%, and a FLCr > 20), in which patients with 2 or 3 risk factors have a 50% risk of progression at 2 years from diagnosis [4]. Additional risk factors for progression have been described by other groups, including positive uptake on positron emission tomography-computed tomography (PET-CT) [5], type of M-protein [6], cytogenetic abnormalities [7], evolving changes in M-protein and hemoglobin [8, 9], genetic signatures [10] and Bence-Jones proteinuria [11], among others.

The recently developed heavy/light chain pair (HLC) assay allows the measurement of both pairs of a specific isotype of immunoglobulin, e.g., quantification of IgGλ and IgGκ in a patient with IgGλ monoclonal gammopathy. The introduction of the HLC assay made possible the evaluation of the suppressive effect of the malignant clone on the isotype-matched polyclonal pair, enabling the study of a new type of immune suppression: isotype-matched immunoparesis (IMI). This phenomenon has been reported as being an independent risk factor in predicting malignant MGUS transformation [12] and poor survival in patients with newly diagnosed and relapsed/refractory MM [13, 14].

Immunoparesis defined by total immunoglobulin (Ig) measurements (reduction below the lower normal limit of an Ig of a different isotype) has been shown to be and independent risk factor for progression to symptomatic disease of SMM patients [3]. However, data regarding HLC defined immunoparesis in SMM is scarce. In a prospective series of 50 patients with SMM, IMI proved to be more prevalent than suppression of Ig of different isotypes and was related to adverse biological features [15]. Using the HLC assay, suppression of HLC pairs of a different isotype (IgAλ and IgAκ, or IgMλ and IgMκ) was significantly associated with a shorter time to progression of IgG MGUS/SMM patients in a small series from a single institution [16].

The aim of this study was to prospectively characterize the prevalence and severity of immunoparesis as determined by HLC measurements, its association with other risk factors for progression and its prognostic significance, in patients with SMM diagnosed at a single institution.

Methods

The study group consisted of 53 patients with newly diagnosed SMM at our institution from January 2014 to March 2019. Serum samples collected at the time of diagnosis and at follow-up visits (when available) were obtained prospectively and frozen for later analysis. Clinical and laboratory data at diagnosis and during follow-up, related to the monoclonal gammopathy, were recorded.

Diagnostic samples were tested for serum free light chain (FLC) concentrations (κFLC and λFLC) using Freelite™ assays (The Binding Site Group Ltd., Birmingham, UK) and HLC concentrations (IgGκ, IgGλ, IgAκ, IgAλ, IgMκ, and IgMλ) using Hevylite™ assays (The Binding Site Group Ltd.) on a BN™II System nephelometer (Siemens, Munich, Germany). Total immunoglobulins (IgG, IgA, and IgM) were measured on a Dimension Vista nephelometer (Siemens, Munich, Germany). Follow-up samples were tested for FLC and HLC pair concentrations, but only of the pair corresponding to the involved isotype (e.g., IgGκ/IgGλ pair in patients with IgGk SMM). Serum protein electrophoresis (SPE) and immunofixation electrophoresis (IFE) were carried out using standard laboratory procedures.

Isotype-matched immunoparesis (IMI) was defined by the HLC assay as a concentration below the lower limit of the reference range of the polyclonal pair with the same isotype as the M-protein (e.g., IgGκ suppression in a patient with IgGλ SMM). Additionally, HLC immunoparesis (HLC IP) was defined as the suppression of any Ig with a different heavy chain (e.g., IgAλ, IgAκ, IgMλ, or IgMκ suppression in a patient with IgGλ SMM). Classical immunoparesis (CIP) was defined as either one or both of the two polyclonal total immunoglobulins being below the lower limit of the reference range (e.g., suppression of IgA and/or IgM in a patient with IgG SMM). Severe immunoparesis was defined by reduction of values by 50% or more below the lower limit of normal.

We assessed "quantitative immunoparesis" by expressing the value of each polyclonal HLC pair as the % of its respective lower limit of normal, using the following formula:

(Level of the polyclonal HLC pair (e.g., IgGλ in a patient with IgGκ SMM)/lower limit of the HLC pair reference range (e.g., 1.91 for IgGλ)) × 100.

Total immunoglobulins and FLC reference ranges used were those stablished by the Immunology Laboratory of our institution, while HLC reference ranges were provided by the manufacturer (Supplementary Table S1).

Sample collection and clinical record review were performed after informed written consent in accordance with the Declaration of Helsinki. Study protocol was approved by the Institutional Review Board at our institution. Patients were diagnosed according to standard International Myeloma Working Group (IMWG) criteria [17].

Differences in time to progression (TTP) between patient groups were analyzed using Kaplan–Meier survival curves with the log-rank test used to indicate significance. Statistical differences for numerical values were calculated using Mann–Whitney *U* test, ANOVA test or unpaired two-tailed *t*-tests. Survival graphs were generated using GraphPad Prism 9 software (Graph Pad Software Inc., La Jolla, CA, USA).

We used the nCounter platform (Nanostring Technologies) to assess immune transcriptomic profiles in the CD138 − bone marrow cell fraction of 6 patients with severe IMI and 6 patients with severe IMI + severe HLC IP. CD138-depleted BM cell fraction was isolated with anti-CD138 mAb-coated immunomagnetic beads (Miltenyi Biotec, San Diego, CA) using an AutoMacs cell sorter (Miltenyi Biotec). Total RNA from the CD138 − BM cell fraction was extracted using the TRIzol reagent (Invitrogen, Oslo, Norway). A minimum of 100 ng of total RNA was analyzed at the nCounter platform (Nanostring Technologies) using the PanCancer Immune Profiling Panel, which assesses the expression of 730 immune-associated genes and 40 housekeeping genes. Expression counts were then normalized using the nSolver 4.0 software and custom scripts in R 3.6.3. Unpaired significance analysis of microarrays (SAM), using false discovery rate (FDR), were used to identify differential gene expression across sample groups.

Results

Patient characteristics and classical immunoparesis

Baseline characteristics for the 53 patients with SMM are presented in Table 1. Nine SMM patients (17%) were classified as high risk by the Mayo Clinic model [2] and 23 (43%) were classified as high risk by the Spanish model [3]. According to the IMWG revised risk stratification model [4], 17 patients (32%) were classified as low risk, 17 patients (32%) as intermediate risk, and 19 patients (36%) as high risk. Concordance between the three risk models is represented in Supplementary Figure S1. The Mayo Clinic and the Spanish PETHEMA model concurred in the classification of 17 out of the 48 evaluable patients (35% rate of agreement), while the IMWG model and the Spanish model concurred in the classification of 23 out of 48 patients (48% rate of agreement). Total serum Ig measurements identified classical immunoparesis (CIP) of at least one isotype in 29/53 SMM patients (54%), which was severe in 19/53 (36%).

Table 1 Baseline patient characteristics

	SMM
Number of patients	53
Age (years)*	72 (60–79)
Gender, male/female	24/29
Isotype	
IgG	31 (58)
IgA	20 (38)
Biclonal	2 (4)
Serum M-protein g/L*	19.8 (13.2–26.7)
Serum FLCr*	8.3 (3.2–34.3)
BMPC (%)*	17 (12–22.5)
Normal BMPC (%)*ǂ	3 (0.4–10)

*Measurements are median (interquartile range)

ǂPercentage of bone marrow plasma cells with normal phenotype by flow cytometry

Immunoparesis determined by HLC pair measurements

HLC measurements identified isotype-matched immunoparesis (IMI) in 42/53 (79%) SMM patients and was severe in 27 (51%). HLC immunoparesis of uninvolved isotypes (HLC IP) was present in 37/51 (72%) of patients with SMM and was severe in 20 (39%). IgM HLC IP was present in 18 patients with SMM (36%) and was severe in 11 of them (22%) (Table 2, Supplementary Figure S2). All but one of the patients identified as having immunoparesis by total Ig measurements (CIP) were also identified by HLC suppression (HLC IP), but not the other way around, with discordance in the severity of the immunoparesis (Supplementary Figure S2).

Eleven SMM patients presented severe IMI without severe HLC IP. On the other hand, only two patients with severe HLC IP presented without severe IMI. In the case of the 11 patients that showed severe immunoparesis of any IgM HLC pair, they all had concomitant severe IMI and IP

Table 2 Frequency of immunoparesis

Type	SMM	
	Total	Severe
IMI	42/53 (79%)	27/53 (51%)
HLC IP	37/51 (72%)	20/51 (39%)
HLC IP IgM	18/50 (36%)	11/50 (22%)
CIP	29/53 (54%)	19/53 (36%)

Isotype-matched immunoparesis (IMI), concentration below the lower limit of the reference range of the polyclonal HLC pair with the same heavy chain as the M-protein (e.g., IgGκ suppression in a patient with IgGλ SMM); *HLC uninvolved isotype (HLC IP)*, concentration below the lower limit of the reference range of any HLC pair with a different heavy chain (e.g., IgAκ in a patient with IgGλ SMM); *HLC IgM immunoparesis (HLC IP IgM)*, concentration below the lower limit of the reference range of any HLC pair with an IgM heavy chain; *Classical immunoparesis (CIP)*, either one or both of the two polyclonal total immunoglobulins being below the lower limit of the reference range (e.g., IgA and/or IgM in a patient with IgG SMM). Severe immunoparesis was defined by values suppressed by 50% or greater below the lower limit of normal

of the other uninvolved heavy chain isotype (Supplementary Figure S2).

Isotype specificity of immune suppression was evident when we analyzed "quantitative immunoparesis" (Fig. 1). In the case of IgG SMM, levels of the non-clonal HLC pairs (expressed as a % of its respective lower limit of normal, see "Methods" section) were lower for the isotype-matched pairs (median 54% for non-clonal IgG vs. 88% for IgA vs. 177% for IgM). IgA SMM patients showed similar results (median 81% for IgG vs. 24% for non-clonal IgA vs. 100% for IgM).

Immunoparesis and risk factors for progression to symptomatic disease

Analyzing the frequency of the different types of immunoparesis according to the IMWG risk group, most of the patients with low-risk SMM ($n = 11$, 65%) had no severe immunoparesis, while most high-risk patients had severe HLC IP ($n = 10$, 59%) (Fig. 2A). The association of baseline prognostic variables with severe IMI and severe IMI + severe HLC IP is presented in Fig. 2B. Patients with severe IMI showed significantly higher serum M-protein levels and lower % of normal phenotype BMPC, with a trend towards higher FLC ratios and BMPC infiltration. Patients with severe IMI and severe HLC IP of uninvolved isotypes showed significantly lower % of normal phenotype BMPC, higher FLC ratios and %BMPC than patients with only severe IMI. Of the eight patients demonstrating an evolving behavior of the M-protein [8], seven had severe IMI at diagnosis and one developed it during follow-up.

With a median follow-up of 2.5 years, twelve patients with SMM progressed to symptomatic disease; 9 of them showed severe IMI at diagnosis and maintained it. Of the other three, one developed severe IMI during follow-up, one showed a consistent decrease of the isotype-matched Ig with borderline severe suppression, and one could not be evaluated at follow-up (Supplementary Figure S3). Severe IMI and severe suppression of any IgM HLC pair were significantly associated with a shorter time to progression (TTP) to symptomatic disease (Fig. 3) while severe HLC

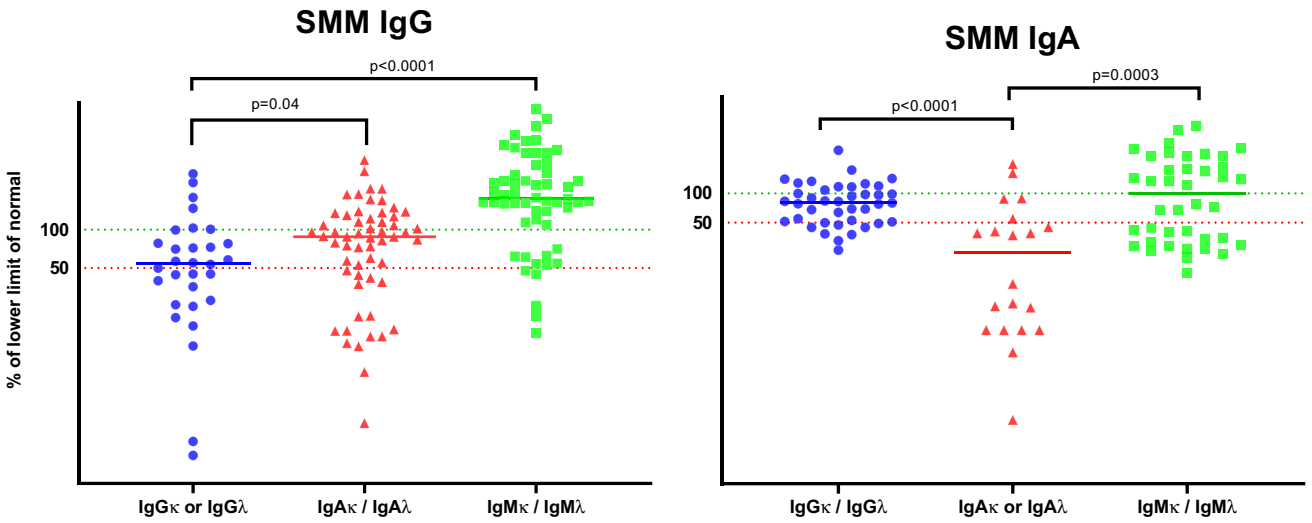

Fig. 1 Quantitative immunoparesis: levels of the HLC pairs corresponding to non-clonal isotypes expressed as % of the lower limit of normal (level of polyclonal HLC pair/lower limit of normal of the HLC pair × 100) in patients with IgG SMM (median 54% vs. 88% vs. 177%) and IgA SMM (median 81% vs. 24% vs. 100%). Horizontal lines represent median values. *p* values were calculated using the Mann–Whitney test

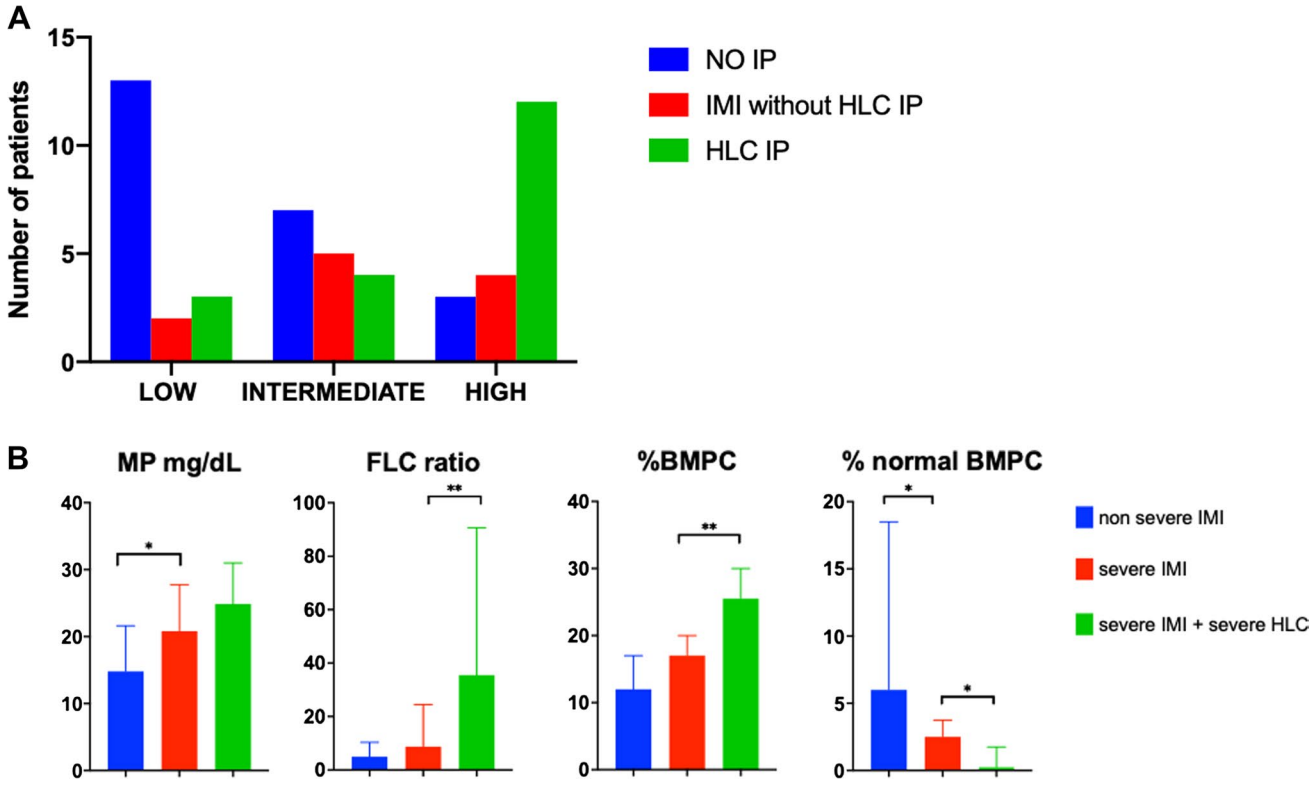

Fig. 2 **A** Immunoparesis according to SMM risk category. Risk stratification was based on the IMWG model. NO IP, absence of severe immunoparesis (IP); IMI without HLC IP, severe isotype-matched IP without severe IP of a different isotype; HLC IP, severe IP of a different isotype. **B** Comparison of serum M-protein (median mg/dL: 14.8 vs. 20.8 vs. 24.88), FLC ratio (median: 4.97 vs. 8.67 vs. 35.6), % BMPC (median: 12 vs. 17 vs.26), and % of normal phenotype BMPC (median: 6 vs. 2.5 vs. 0.25) between patients without severe isotype-matched immunoparesis (IMI), patients with severe IMI without severe immunoparesis of uninvolved isotypes (HLC IP), and patients with severe IMI + severe HLC IP. Bar graphs show median ± interquartile range. *p* values were calculated using the Mann–Whitney test (*, $p < 0.05$; **, $p < 0.01$)

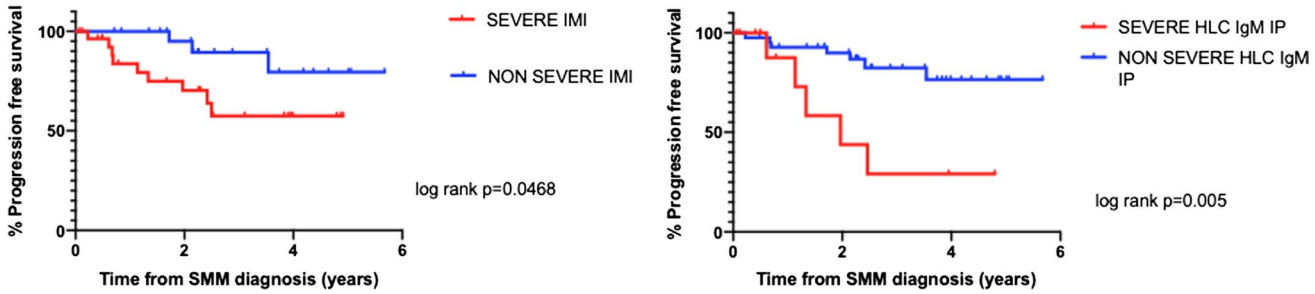

Fig. 3 Progression-free survival according to the absence or presence of severe isotype-matched immunoparesis (IMI), HR (95% CI): 3.16 (1.02–9.85). Progression-free survival according to the absence or presence of severe immunoparesis of any IgM HLC pair (HLC IgM IP), HR (95% CI): 4.52 (0.87–23.4)

IP of uninvolved isotypes was associated with a non-significant trend towards a shorter TTP [median TTP not reached vs. 2.46 years, log rank $p = 0.06$, HR (95% CI): 2.78 (0.77–10.04)]. Patients with severe IMI alone did not have a significantly longer TTP than patients with severe IMI + severe HLC IP of uninvolved isotypes [median TTP not reached vs. 2.46 years, log rank $p = 0.42$, HR (95% CI): 1.74 (0.47–6.45)] or severe IMI + severe IgM HLC suppression [median TTP not reached vs. 1.97 years, log rank $p = 0.109$, HR (95% CI): 2.79 (0.68–11.46)].

Gene expression profile of the tumor microenvironment in SMM patients with immunoparesis

In a final set of experiments, we evaluated the expression of immune-related genes in the CD138 − fraction from diagnostic bone marrow samples from 6 patients with severe isotype-matched immunoparesis (IMI) and 6 patients with severe IMI and severe immunoparesis of uninvolved isotypes (HLC IP). We studied gene expression signatures associated with specific immune cell types (Supplementary Table S2) [18]. While there were no significant differences in the expression of genes corresponding to NK cells, T CD4 + cells, Th2 cells, Tregs, mast cells, macrophages, dendritic cells, or neutrophils, patients with IMI + HLC IP showed higher expression of genes associated with T CD8 + cells and Th1 cells than patients with only severe IMI. The "cytolytic score," based on the expression of highly specific genes associated with cytolytic effector functions (*GZMA*, *GZMH*, *GZMM*, *PRF1*, and *GNLY)* [19] was also significantly higher in the IMI + HLC IP group of patients (Fig. 4). Among the upregulated genes in the IMI + HLC IP group, we also found immune checkpoints involved in T-cell regulation and linked to T-cell disfunction/exhaustion (LAG-3, CD96, TIGIT) [20–22]. TIGIT expression, in particular, has been associated with upregulation of genes involved in T-cell function and cytotoxicity in the BM of patients with MM, suggesting that it may play a role restraining immune activation involving Tregs and T cell exhaustion [21]. Upregulated genes in patients with IMI + HLC IP as compared to those with only IMI are detailed in Supplementary Figure S4.

Discussion

Our study is the first, to our knowledge, to analyze the depth of immunoparesis for different antibody isotypes using the HLC assay prospectively in patients with SMM. While our findings confirm the previously reported high prevalence of isotype-matched immunoparesis in patients with SMM [15, 16], a striking observation in this series was both the lower prevalence of severe immunoparesis of the uninvolved isotypes (almost always accompanied by severe IMI) and the lower proportional levels of Ig of the involved isotype and different light chain compared to Igs of a different isotype. The higher prevalence of IMI over classical IP has been previously described in MGUS [23, 24] and was thought to be possibly related to the higher sensitivity of the Hevylite assay. The finding in our study, however, seems to indicate isotype specificity in the early mechanisms involved in suppression of polyclonal immunoglobulin production.

The mechanisms of suppression of normal Ig in plasma cell dyscrasias remain poorly understood. The decrease in polyclonal Igs found in patients with MM and less frequently observed in MGUS/SMM has been related to the reduction in normal bone marrow plasma cells. However, it has been shown in MM that both parameters appear to be independently associated with patient outcome [25]. The depletion of normal plasma cells is suggested to result from

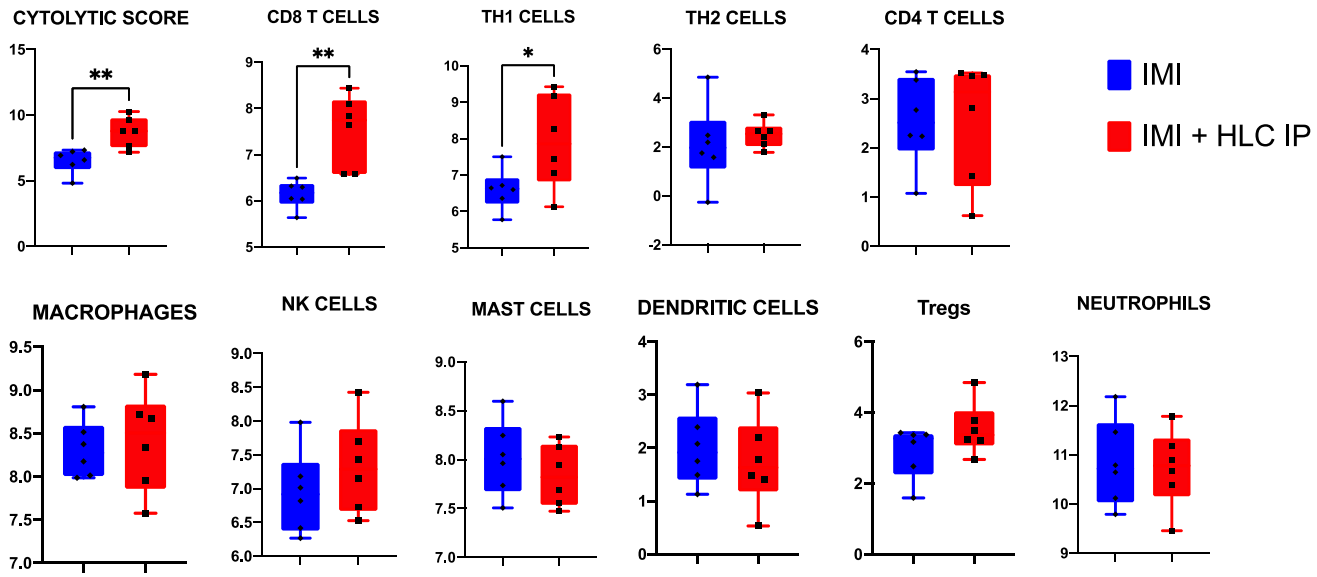

Fig. 4 Scores corresponding to gene expression signatures associated with specific immune cell types in the CD138 − cell fraction of diagnostic bone marrow samples. IMI, severe isotype-matched immunoparesis; IMI + HLC IP, severe isotype-matched + severe immunoparesis of a different isotype. *p* values were determined using unpaired two-tailed *t*-tests (*, $p < 0.05$; **, $p < 0.01$)

progressive competition and replacement by clonal plasma cells in BM niches [26]. Nonetheless, this mechanism fails to explain the apparent isotype specificity of Ig suppression noted in our series.

Wang and Young [27] were the first to report the isotype specificity of Ig suppression in IgG MGUS when they found a greater reduction of polyclonal IgG than in IgA or IgM patients. The lack of correlation between the M-protein and the polyclonal immunoglobulin led them to preclude a negative feedback mechanism solely dependent on the increase in the catabolic rate of IgG immunoglobulins. T cells bearing surface receptors of the respective monoclonal isotype have been observed in mouse models with plasmacytomas [28, 29] and Tregs have been attributed to suppress immunoglobulin production and secretion in a manner that could be isotype specific [30, 31]. The fact that severe immunoparesis is also described in patients with light chain only MM suggests the existence of suppressive mechanisms independent of the heavy chain isotype of the M-protein [32].

A striking finding of this study was that all patients with severe IgM immunoparesis had concomitant severe IMI and suppression of the other uninvolved isotype. In the case of IgM immunoparesis, the mechanisms involved would have to affect IgM plasma cells distant from the bone marrow (lymph nodes and spleen). The B-cell maturation antigen (BCMA) has been shown to sequester B-cell ligands, such as B-cell activating factor (BAFF), preventing plasma cell development and antibody production in mice [33]. An inverse relationship between serum BCMA levels and levels of uninvolved Ig has been reported in patients with MM [34].

The comparison of the tumor microenvironment between patients with isotype-matched immunoparesis and patients with IMI plus immunoparesis of a different isotype showed significant differences, with a higher expression of genes related to a cytotoxic immune response in the second group. Although these results could point to the existence of alternative suppressive mechanisms, they are based on a small number of samples and require further examination.

As previously described in MGUS [12] and in MM [13, 14], in our series severe IMI was significantly associated with adverse biological features and with a shorter time to progression to symptomatic disease, highlighting the prognostic importance of IMI throughout the disease course. Interestingly, patients with severe HLC IP of uninvolved isotypes were characterized by even more adverse prognostic features than patients with severe IMI alone. Severe suppression of IgM HLC pairs was significantly associated with shorter TTP, as previously reported in MGUS/SMM [16], a finding that has also been linked to survival outcomes in newly diagnosed MM [32]. Progression to symptomatic disease was not significantly different between patients with severe IMI alone and patients with severe IMI plus suppression of uninvolved isotypes (both isotypes or IgM), but this analysis was limited by the small number of patients and the fact that some of the patients with classical immunoparesis were enrolled in clinical trials and consequently censored for further analysis.

The observation that two of the three patients without severe IMI that progressed to symptomatic disease showed a decrease of the isotype-matched Ig closer to progression could point to the value of the evolving changes in HLC values as a prognostic marker, both for the involved and uninvolved isotypes. Larger prospective studies are needed to further clarify this matter.

In concordance with previous reports [35], our analysis revealed significant discrepancies between the clinical models currently used to assess risk of progression from SMM to symptomatic disease. This finding highlights the need for the study of new risk factors and biomarkers, such as isotype-matched immunoparesis, to more accurately characterize the risk of transformation to MM and to help us understand the different mechanisms involved in the pathogenesis of the disease.

In conclusion, the greater prevalence of IMI over suppression of uninvolved isotypes along with the lower proportional values for isotype-matched immunoglobulins supports an isotype specificity of early suppression mechanisms in the case of IgG and IgA SMM. Both IMI and IP of uninvolved isotypes are associated with other recognized risk factors for progression, but the later (especially in the case of IgM) appears to develop with more advanced disease and could correspond to different suppression mechanisms. These findings could be of interest both at the time of the initial evaluation and during follow-up of patients with SMM using the serum heavy/light chain pair assay.

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Author contribution II designed and performed the research, collected and analyzed data, and wrote the manuscript. DM, MM, FB, and EM performed the research and analyzed data. NT, LGR, AO, MCS, JY, MTC, LR, and JB collected data. AP contributed essential tools for research and analysis. CFL designed the research and analyzed the data. All authors reviewed and approved the final version of the manuscript.

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Availability of data and material The datasets generated during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Ethics approval Study protocol was approved by the Institutional Review Board at Hospital Clínic of Barcelona.

Consent to participate Sample collection and clinical record review were performed after informed written consent in accordance with the Declaration of Helsinki.

Consent for publication Sample collection and clinical record review were performed after informed written consent in accordance with the Declaration of Helsinki.

Conflict of interest C.F.L. has received research grants and consultancy by The Binding Site. All other authors declare no conflict of interest.

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