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A novel biomarker score for the screening and management of patients with plasma cell proliferative disorders

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Abstract. – OBJECTIVE: Monoclonal plasma cell proliferative disorders comprise a wide spectrum of diseases associated to clonal B-cell expansion. Serum protein electrophoretic profile (SPEP) and circulating free light chains (FLCs) levels are the mainstay of diseases management. Recently, soluble (s) Syndecan-1 (SDC1, CD138) produced by myeloma plasma cells has been suggested in the monitoring and follow-up of patients with myeloma. The aim of our study is to evaluate sCD138 in addition with FLCs and SPEP for the screening of patients with different evolutive disease pathways.

PATIENTS AND METHODS: Sera from 73 patients with monoclonal gammopathy of undetermined significance (MGUS), 120 smoldering and 42 multiple myeloma (SMM and MM, respectively), 70 HCV-related mixed cryoglobulinemia (MC), 35 B-cell non-Hodgkin's lymphoma (B-NHL) and sera from 50 healthy donors (HD), were tested for sCD138, FLCs (assessed by means of ELISA and turbidimetric assay, respectively) and electrophoresis pattern (performed on Capillarys system) for the generation of a novel biomarker score (BS).

RESULTS: Our results were grouped according to the two main lines of disease progression (vs. MM or B-NHL): in one group we found BS mean values of 0.2, 3.4, 5.3, 7.1 for HD, MGUS, SMM and MM, respectively; in the other group of 0.2, 4.4, 6.7 for HD, MC and B-NHL.

CONCLUSIONS: We showed that BS mean values follow the ingravescence disease status towards the two main lines of progression to cancerous conditions; it could represent an additional useful tool in the management of screening and/or follow-up.

Key Words:
Biomarker, sCD138, Free Light Chains, Monoclonal Gammopathy, HCV, Mixed Cryoglobulinemia, Multiple myeloma, B-cell non-Hodgkin's lymphoma.

Abbreviations

Serum Protein Electrophoretic Profile (SPEP); Free Light Chains (FLCs); Syndecan-1 (SDC1); Soluble CD138 (sCD138); Monoclonal Gammopathy of Undetermined

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Significance (MGUS); Smoldering Multiple Myeloma (SMM); Multiple Myeloma (MM); Mixed Cryoglobulinemia (MC); B-cell Non-Hodgkin's Lymphoma (B-NHL); Healthy Donors (HD); Biomarker Score (BS); Hepatitis C Virus (HCV); Cryoglobulins (CGs).

Introduction

Monoclonal plasma cell proliferative disorders comprise a wide spectrum of diseases ranging from monoclonal gammopathy of undetermined significance (MGUS) to multiple myeloma (MM) and light chain amyloidosis[1]. In patients with clonal plasma cell proliferation, but without any signs of end organ damage (as hypercalcemia, renal insufficiency, anemia, bone lesions, or recurrent bacterial infections), MGUS or smoldering multiple myeloma (SMM) are suspected. SMM has a much higher risk of progression to malignancy than MGUS (approximately 10% *vs.* 1% per year, respectively) in the first 5 years after diagnosis[2]. Hepatitis C virus (HCV) chronic infection is associated with a wide range of extra-hepatic complications and is increasingly recognized as a trigger of clonal B-cell expansion leading to MGUS, Mixed Cryoglobulinemia (MC) and overt B-cell non-Hodgkin's lymphoma (B-NHL)[3,4]. Mechanisms determining the evolution of HCV infection into lympho proliferative and/or autoimmune disorders are still not definitively understood, although several factors are known playing a role in the pathogenesis and progression. Above all, the chronic virus-sustained antigenic stimulation could trigger B-cell clonal expansion[5]. MC consists of a systemic vasculitis characterized by the deposition of circulating immunocomplexes composed by polyclonal Immunoglobulin G (IgG), behaving as auto-antigens, and IgM with Rheumatoid Factor (RF) activity. In type II cryoglobulinemia (CG) we found Ig complexes formed by a monoclonal component (usually IgM) and polyclonal components (usually IgG, more rarely IgA); in type III CG different Ig isotypes, including one isotype with RF activity: this pattern is a transitional state that evolves gradually into a type II CG from polyclonal B-cells to oligoclonal, and finally to a monoclonal population[6,7]. Unlike malignant lymphomas, MC can remain unchanged even for decades, followed by overt lymphoid tumors in about 10% of cases[8]; when MC is associated to HCV infection, the risk of evolution into lymphomas is 35 times higher than in the general population[9].

Serum protein electrophoretic profile (SPEP) and assessment of circulating monoclonal Ig have been considered the mainstay of diagnosis, prognosis, and management of clonal plasma cell proliferative disorders until the 2000s[10]. Nevertheless, the determination of serological free immunoglobulin light chains (FLCs) levels is considered a marker of increasing interest in clinical laboratory of autoimmune disorders and approved for diagnosis, monitoring and follow-up of monoclonal gammopathies (MG) in many clinical practice guidelines[11-13]. FLC k/λ ratio $\geq$ 100 is a predictor of disease progression in SMM or MM requiring therapy[14]. Commercially available FLC assays represent a major improvement in MG management[15,16]. However, some critical aspects of these assays still require amelioration, mainly due to the intrinsic characteristics of FLCs and their elevated variability, making FLCs available assays lacking of standardization[17].

Several studies underlined the role of soluble Syndecan-1 (sCD138) as a possible biomarker for diagnosing and monitoring of MG. It consists in a heparan sulfate proteoglycan that is highly expressed in epithelial as well as hematopoietic plasmacytoid cells. Higher levels of sCD138 have been reported in correlation with poor outcome in myeloma[18,19]. The assessment of circulating sCD138 could represent an additional valuable tool for MM patients undergoing treatments. As matter of fact, serum sCD138 is less dependent to individual variability if compared to FLCs, making it a more stable analyte to be used alongside standard markers[17]. In HCV infection, CD138 is the major receptor protein for virus attachment to the hepatocyte surface[20]. Either removal of heparan sulfate from the cell surface by heparinases or addition of heparin and its derivatives could efficiently block HCV infection[21], providing a potential molecular target for antiviral drugs against HCV. Moreover, sCD138 assessment represents a non-invasive alternative for liver biopsy to evaluate fibrosis. The main advantage is that it is not increased in other chronic inflammatory diseases, and its determination is achievable with a simple, relatively cheap and time-saving assay. sCD138 levels could select patients for treatment without liver biopsy[22].

The aim of our study was to evaluate serological sCD138 levels in the management of patients with different precancerous stages of plasma cell disorders (MGUS, HCV-related type II CG and SMM), in association with SPEP and FLCs assays

(free k and λ chains and their ratio). We integrated results of sCD138, FLCs and SPEP to generate a "Biomarker Score" (BS) and screen patients with the major probabilities of worsening progression towards MM or HCV-related B-NHL.

Patients and Methods

Patients

For this study, 340 patients (183 females and 157 males, mean age 63.2 ± 14.2 years) were retrospectively enrolled: 235 with monoclonal gammopathy (73 MGUS, 120 SMM and 42 MM) and 105 HCV-patients (70 HCV related MC with type II CG and 35 with HCV related B-NHL). Demographical data of patients' subgroups enrolled in the study are described in Table I. They were either inside-patients or outside-patients, referred to three Italian Centers: Department of Internal Medicine and Gastroenterology of the Fondazione Policlinico Universitario Agostino Gemelli in Rome; Department of Experimental and Clinical Medicine, University of Florence; National Cancer Institute "Regina Elena" in Rome. All patients were evaluated and diagnosed by hematologists and hepatologists. Furthermore, 50 healthy donors (HD, 25 females and 25 males, mean age 42.5 ± 11.8 years) were included as negative controls.

MG patients, without therapy, were included in the study according to the SLimCRAB criteria updated by the International Myeloma Working Group[23].

In HCV patients, liver disease stage was assessed by liver elastography using FibroScan (Echosens, Paris, France), and the resulting stiffness value was converted in the corresponding METAVIR score. Inclusion criteria for HCV patients with type II CG were: serum detectable HCV-RNA, positive cryocrit, no evidence of autoimmune/lymphoproliferative disorders, free antiviral therapy period of at least 6 months and the absence of HIV or HBV co-infection; liver disease stage F0. Inclusion criteria for HCV patients with new diagnosed B-NHL were: serum detectable HCV-RNA, no evidence of autoimmune disorders, free antiviral therapy period of at least 6 months and the absence of HIV or HBV co-infection; liver disease stage F2.

Patients with liver cirrhosis and with diagnosed renal failure (estimated glomerular filtration rate <60 mL/min/1.73 m^2) were excluded.

The study was conducted according to the recommendations of the Declaration of Helsinki and the Local Ethical Committee. Our Institutional Review Board approved the study and all participating subjects provided written informed consent. All samples were processed anonymously.

Table I. Demographical data of the population enrolled in the study

	N	Age (Mean±DS)	Male/Female
HD	50	43 ± 12	25 / 25
MC	70	67 ± 10	33 / 37
B-NHL	35	45 ± 8	16 / 19
MGUS	73	66 ± 30	34 / 39
SMM	120	65 ± 16	58 / 62
MM	42	65 ± 12	16 / 26

Methods

Venous blood samples obtained from patients and healthy controls were centrifuged, and serum samples were thus separated and collected into aliquots and stored at -80°C. All samples were assayed at the same time for sCD138 and FLCs after an adequate thawing process.

Serum sCD138 levels were determined using ELISA kits for human sCD138 (Diaclone Research, Besancon, France). The assay was performed according to the manufacturer's instructions: 100 µL of serum was added to pre-coated wells and incubated with anti-CD138 biotinylated antibody. Wells were washed, and horseradish peroxidase (HRP)-streptavidin conjugate was added. After washing, the substrate was added and, after stopping the reaction, the absorbance was read at 450 nm. Serum levels of sCD138, expressed as ng/mL, were measured at least twice for each patient, with reproducible results.

Serological FLCs levels were assessed by means of turbidimetric assay (Freelite ™ Human Kappa and Lambda Free Kits, The Binding Site, Birmingham, UK) and performed on the SPA plus instrument (The Binding Site, Birmingham, UK). The immunoassay consisted of two separate assessments for free k and free λ (normal range: 3.3-19.4 mg/L and 5.7-26.3 mg/L, respectively). A ratio of k/λ < 0.26 or > 1.65 is considered abnormal, according to the manufacturer's recommendations.

Serum protein electrophoresis was performed on Capillarys® systems (Sebia, Evry, France) according to the manufacturer's instructions. The instrument is an eight-channel automated system with eight fused silica capillaries (17 cm in length and 25 µm ID). Additional features include: buf-

fer (borate and additives) pH 10; detection voltage 9 kV; separation is carried out at 35°C in 2.5 min; ultraviolet detection at 200 nm is used for direct quantification of the peptide bonds. Each serum was diluted 1: 10. Samples were tested blinded according to the manufacturer's instructions.

Statistical Analysis and Generation of Biomarker Score

Statistical analysis was performed by using the Statistical Package for Social Science (SPSS Inc., Chicago, IL, USA), version 15.0. Data were analyzed for normality of distribution using the Kolmogorov-Smirnov normality test. Continuous variables were expressed as mean ± SD or median with interquartile range, categorical variables displayed as frequencies and the appropriate parametric (Student's *t*-test or ANOVA) or non-parametric test (Mann-Whitney U-test, Kruskal-Wallis test or χ^2-test) were used to assess significance of the differences between subgroups; *p*-values < 0.05 was considered statistically significant. The measurements of serum FLCs k and λ and the k/λ values were stratified into two categories depending on cut-off values, as suggested by IMWG guidelines[13]: 1) Negative = subjects with a range of 3.3-19.4 mg/L for k, 5.7-26.3 mg/L for λ, and 0.26-1.65 for k/λ ratio; 2) Positive = subjects with higher k and λ serum levels and with a k/λ ratio higher or lower than the previously reported cut-off values. A score value of 0 and 1 was assigned to negative and positive classes, respectively. Depending on the relationship between FLCs levels and their ratios, a+1 bonus point was assigned to subjects with both free k and λ chains levels included in the positive class. Serum protein electrophoresis pattern were stratified as follows: normal (score 0), qualitative/quantitative alteration of SPEP profile without evidence of spike-like peak (score 1), presence of a narrow spike in the gamma, beta, or alpha-2 regions with M-protein level lower (score 2) or greater than 3 g/dL (score 3). Median, 25° and 75° percentiles of sCD138 concentration in all 340 patients (excluding HD) were determined, thus stratifying sCD138 levels into four classes (≤ 50, 51-120, 121-250, > 250 ng/mL). A specific score value was assigned to each category (0, 1, 2 and 3, respectively).

Depending on the relationship between FLCs concentrations and their ratios, a + 1 bonus point was assigned to subjects with free k and λ chains values included in the positive class to compensate for an eventually negative ratio score. Considering the FLC profile, sCD138 and SPEP determinations, we generated a specific laboratory "Biomarker Score" (BS) to be associated with each patient. The dispersion of BS values showed a normal distribution in each group. The predictions for disease status for each patient of a specific clinical group are conducted based on the BS number using the Bayes theorem[24,25]. The datasets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Results

We enrolled 340 patients divided into 5 subgroups considering their different diagnoses: 73 MGUS patients, 120 SMM patients, 42 MM, 70 HCV-related MC patients with type II CG and 35 with B-NHL; 50 HD were included as negative control. Main demographic features of patients and HD are reported in Table I. Results were divided in two groups according to the two main lines of disease progression: in one group we compared monoclonal gammopathy of undetermined significance to smoldering or established multiple myeloma; in the other one, HCV-related Mixed Cryoglobulinemia to B-cell non-Hodgkin's lymphoma.

Considering the whole population of 340 patients, sCD138, free k and λ chains were significantly higher than in HD ($p<0.001$ for each comparison, data not shown). Similarly, significant differences were detected in k/λ ratio between patients and controls ($p= 0.001$, data not shown).

Regarding the different patients' subgroups, serum sCD138 levels were significantly different in each subgroup in the two disease lines (Table II AI, 2BI and Figure 1). Significant differences for FLCs levels were detected among almost each patients' subgroups; however, as shown in Figure 1 and in Table II AI, AII, BI, BII a high extent of overlapping and outlier data is observed for all variables. In this way our results cannot be used to stratify patients among different clinical pictures. Therefore, stemming from these data, the generation of a combined variable, the "Biomarker Score", was introduced. The probabilities of worsening prognosis for subjects with a specific score belonging to each specific subgroup is displayed in Figure 2a and 2b for the two main lines of disease progression. The mean BS values were 0.2 [range 0-2], 3.4 [range 2-7], 5.3 [2-8], 7.1 [4-10] for HD, MGUS, SMM, MM, respectively, and 0.2 [range 0-2], 4.4 [range 2-8] and

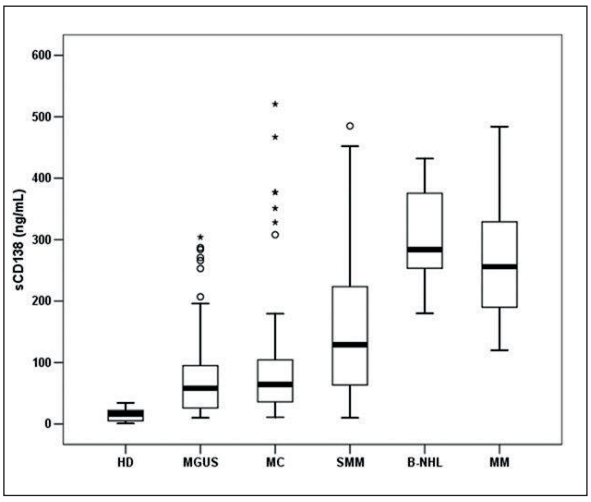

Figure 1. Box-plot of sCD138 concentrations in the different patients' subgroups (HD, healthy donors; MGUS, benign monoclonal gammopathy of undetermined significance; MC, Mixed Type II cryoglobulinemia; SMM, smoldering multiple myeloma, B-NHL, HCV positive, B-cell non-Hodgkin's lymphoma. MM: multiple myeloma. ○ are cases with values between 1.5 and 3 times the Interquartile Range. ★ are cases with values more than 3 times the Interquartile Range.

6.7 [range 4-9] for HD, MC and B-NHL, respectively. Main information from experimental data of BS, before normalization and fitting to Gaussian distribution in order to calculate the posterior probabilities for each patient belonging to a specific clinical group using the Bayes theorem (Figure 2a-b), are summarized as follows: 1) BS score 0 and 1 included 98% of HD; 2) 77% of MGUS felt in the 2-4 BS range; 3) 88% of MM displayed a BS $\geq$ 6; 4) SMM patients exhibit a spreader distribution: 11%, 38% and 52% for 2-3, 4-5, 6-7+ BS ranges, respectively; 5) 94% of HCV positive B-NHL shows a BS $\geq$ 5; 6) 57% and 36% of MC subjects felt in 2-4 and 5-6 BS range, respectively.

Discussion

The dysregulation of immune cells is involved in all types of plasma cell proliferative disorders, with a wide spectrum of signs and symptoms often overlapping. Moreover, Hepatitis C virus chronic infection is also characterized by the possible development of extrahepatic manifestations linked to clonal B-cell expansion leading to MGUS, MC and B-NHL. New biomarkers that allow to orientate diagnosis, prognosis and treatment, are widely desired[26]. In the present study, we explored the clinical value of serological sCD138 assessment as screening marker in association with SPEP and FLCs assays, for the management of plasma cell proliferative disorders. Patients from different diseases were studied: 73 MGUS, 120 SMM, 42 MM, 70 HCV related type II MC and 35 HCV related B-NHL. The control group of 50 HD was well matched by age only with B-NHL (Table I).

The FLCs biological role is still under definition; we recently investigated their potential as inflammatory agents leading to oxidative stress and chronic inflammation[27] and we previously showed a correlation between FLCs and sCD138 in MG[28]. In the present work, we investigated if this relationship may be employed for a rapid and efficient diagnosis, not only in those cases in which MG is difficult to detect, but also to elaborate different diagnostic patterns for MG. As we reported, sCD138 and FLCs levels are higher in MG patients than in HD and the assessments of both markers could be a precious diagnostic tool to ameliorate therapeutic option in a patient-personalized therapy.

An abnormal serological FLC profile together with focal lesions detected by magnetic resonance imaging have been introduced in the new definition of multiple myeloma and can be determining for clinicians to start therapy even in asymptomatic patients[23]: instead, reliable biomarkers correlated to the risk of worsening progression, are lacking[29-31]. An altered FLCs profile has been correlated with MC and/or B-NHL in HCV-positive patients[32]. The same authors also suggested that serum FLC ratio could be used as a surrogate marker for HCV-related lymphoproliferation after IFN-based antiviral therapy[33]. Moreover, the FLC ratio assessed before treatment may be useful in predicting response to Rituximab that represents an expensive treatment for patients with HCV-related MC ineligible for IFN-based therapy[34].

In the present study, subgroups of patients with different types of MG, display significantly different levels of free k and λ chains and k/λ ratio, suggesting an irregular and dysfunctional production of FLCs by B cells. These evidences strengthen the importance of FLCs in plasma cell dyscrasias as a marker of advanced disease, considering type II CG as an intermediate condition of chronic inflammation and with a higher risk of malignant transformation if compared to MGUS.

In experimental studies with hepatoma cell lines and human primary hepatocytes, it has been demonstrated that HCV infection leads to increased mRNA and surface expression of heparan sulfate proteoglycans, particularly CD138 (SDC1), which promote HCV entry and propagation[36].

Table II. AI, AII, BI, BII. Laboratory data of the population enrolled in the study. Data are expressed as median and [minimum-maximum] values. The *p*-value indicates the significance of the difference among subgroups. Apex letters locate differences between subgroups after correction for multiple comparisons (a= HD *vs.* MGUS; b= HD *vs.* SMM; c= HD *vs.* MM; d= MGUS *vs.* SMM; e= MGUS *vs.* MM; f= SMM *vs.* MM; m= HD *vs.* CGs II; n= HD *vs.* B-NHL; o= CG II *vs.* B-NHL). The intra-group frequency distributions of partial Biomarker Score subclasses of each variable are also reported (i.e. frequency of quartiles or negative/positive cases for sCD138 and FLCs, respectively). Conversely, in Table AII and BII the inter-groups frequency distribution for each partial Biomarker Score class of each variable is shown.

AI	HD	MGUS	SMM	MM	p
sCD138 (ng/mL)	16 [1-35][a,b,c,]	58 [10-304][a,d,e]	129 [10-485][b,d,f]	256 [120-484][c,e,f]	***<0.001***
Intra-group frequency distribution of partial score classes of sCD138 (mg/mL) (%)	*100/0/0/0*	*48/36/8/8*	*20/27/35/18*	*0/2/48/50*	
k-FLC (mg/L)	11 [3-23][b,c]	13 [1-115][d,e]	33 [1-38666][b]	20 [5-1695][c,d,e]	***<0.001***
Intra-group frequency distribution of partial score classes of k-FLC (mg/L) (%)	*94/6*	*70/30*	*39/61*	*48/52*	
λ-FLC (mg/L)	10 [5-26][c]	13 [1-122]	11 [1-4238]	15 [8-2100][c]	***<0.001***
Intra-group frequency distribution of partial score classes of λ-FLC (mg/L) (%)	*100/0*	*85/15*	*64/36*	*64/36*	
k/λ	1.0 [0.5-1.9][b]	1.0 [0.1-1.6][d]	2.7 [0.1-4833][b]	1.1 [0.01-218][d]	<0.001
Intra-group frequency distribution of partial score classes of k/λ (%)	*94/6*	*100/0*	*0/100*	*31/69*	

AII - To be compared to the Biomarker Score Frequency distribution among groups shown in Figure 2a	Patients Groups				
	HD	MGUS	SM	MM	p
Frequency (%) among patients' groups in respect to partial score classes of sCD138 (ng/mL) levels*:					
<50 (score 0)	46	32	22	0	***<0.001***
51-120 (score 1)	0	44	2	54	
121-250 (score 2)	0	9	29	62	
>250 (score 3)	0	12	43	45	
Frequency (%) among patients' groups in respect to partial score classes of k-FLC (mg/L) levels*:					
3.3-19.4 (score 0)	28	31	29	12	***<0.001***
<3.3 or >19.4 (score 1)	3	18	18	61	
Frequency (%) among patients' groups in respect to partial score classes of λ-FLC (mg/L) levels*:					
5.7-26.3 (score 0)	23	29	36	12	***<0.001***
<5.7 or >26.3 (score 1)	0	16	22	62	
Frequency (%) among patients' groups in respect to partial score classes of k/λ levels*:					
0.26-1.65 (score 0)	35	55	0	10	***<0.001***
<0.26 or >1.65 (score 1)	2	0	19	79	
Frequency (%) among patients' groups in respect to partial score classes of SPEP profile*:					
normal (score 0)	100	0	0	0	***<0.001***
qualitative/quantitative alteration (score 1)	7	0	0	93	
presence of a narrow spike with M-protein level lower than 3 g/dL (score 2)	0	43	0	57	
presence of a narrow spike with M-protein level greater than 3 g/dL (score 3)	0	0	100	0	

*see Methods section for details.

Continued

Table II AI, AII, BI, BII (Continued). Laboratory data of the population enrolled in the study. Data are expressed as median and [minimum-maximum] values. The *p*-value indicates the significance of the difference among subgroups. Apex letters locate differences between subgroups after correction for multiple comparisons (a= HD *vs.* MGUS; b= HD *vs.* SMM; c= HD *vs.* MM; d= MGUS *vs.* SMM; e= MGUS *vs.* MM; f= SMM *vs.* MM; m= HD *vs.* CGs II; n= HD *vs.* B-NHL; o= CG II *vs.* B-NHL). The intra-group frequency distributions of partial Biomarker Score subclasses of each variable are also reported (i.e. frequency of quartiles or negative/positive cases for sCD138 and FLCs, respectively). Conversely, in Table AII and BII the inter-groups frequency distribution for each partial Biomarker Score class of each variable is shown.

BI	HD	CG II	B-NHL	p
sCD138 (ng/mL)	16 [1-35][m,n]	65 [11-521][m,o]	284 [180-432][n,o]	***<0.001***
Intra-group frequency distribution of partial score classes of sCD138 (mg/mL) (%)	*100/0/0/0*	*37/41/11/10*	*0/0/26/74*	
k-FLC (mg/L)	11 [3-23][m,n]	26 [6-107][m,o]	178 [35-360][n,o]	***<0.001***
Intra-group frequency distribution of partial score classes of k-FLC (mg/L) (%)	*94/6*	*21/79*	*0/100*	
λ-FLC (mg/L)	10 [5-26][m,n]	21 [7-52][m,o]	33 [13-91][n,o]	***<0.001***
Intra-group frequency distribution of partial score classes of λ-FLC (mg/L) (%)	*100/0*	*71/29*	*43/57*	
k/λ	1.0 [0.5-1.9][m,n]	1.3 [0.4-4.8][m,o]	4.4 [0.8-20.4][n,o]	***<0.001***
Intra-group frequency distribution of partial score classes of k/λ (%)	*94/6*	*84/16*	*9/91*	

BII - To be compared to the Biomarker Score Frequency distribution among groups shown in Figure 2b	Patients Groups			
	HD	CG II	B-NHL	*p*
Frequency (%) among patients' groups in respect to partial score classes of sCD138 (ng/mL) levels*:				
<50 (score 0)	66	34	0	***<0.001***
51-120 (score 1)	0	100	0	
121-250 (score 2)	0	47	53	
>250 (score 3)	0	21	79	
Frequency (%) among patients' groups in respect to partial score classes of k-FLC (mg/L) levels*:				
3.3-19.4 (score 0)	76	24	0	***<0.001***
<3.3 or >19.4 (score 1)	3	59	38	
Frequency (%) among patients' groups in respect to partial score classes of λ-FLC (mg/L) levels*:				
5.7-26.3 (score 0)	43	43	14	***<0.001***
<5.7 or >26.3 (score 1)	0	50	50	
Frequency (%) among patients' groups in respect to partial score classes of k/λ levels*:				
0.26-1.65 (score 0)	43	54	3	***<0.001***
<0.26 or >1.65 (score 1)	7	24	70	
Frequency (%) among patients' groups in respect to partial score classes of SPEP profile*:				
normal (score 0)	87	0	13	***<0.001***
qualitative/quantitative alteration (score 1)	8	0	92	
presence of a narrow spike with M-protein level lower than 3 g/dL (score 2)	0	95	5	
presence of a narrow spike with M-protein level greater than 3 g/dL (score 3)	0	0	0	

*see Methods section for details.

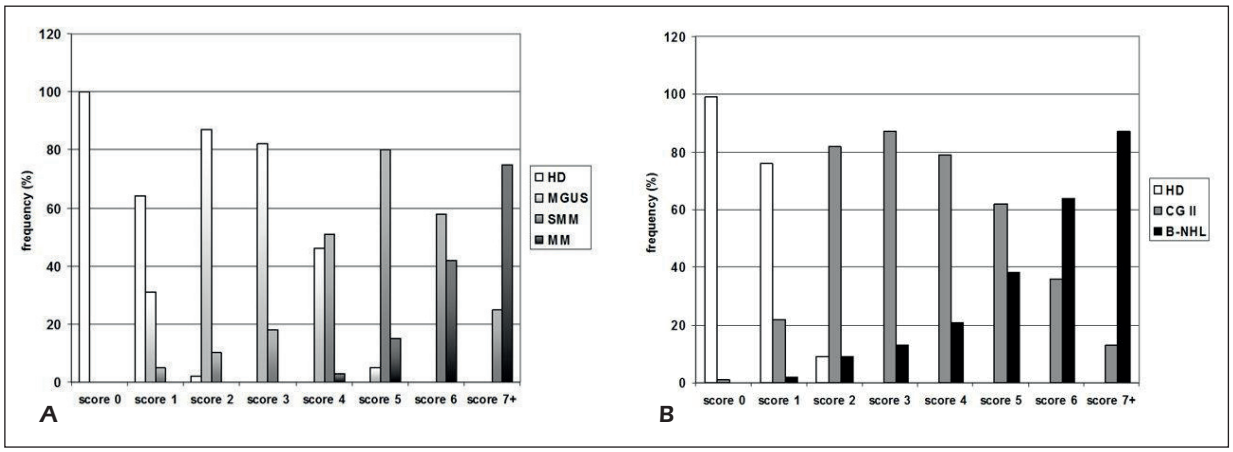

Figure 2 A-B,. Frequency distribution of clinical groups for each BS class (score 0 to 7+) for the two main lines of disease progression.

In patients with chronic HCV infection, soluble CD138 has been described as a non-invasive marker for liver fibrosis stage[22]. Liver fibrosis occurs after the activation of hepatic stellate cells in response to various liver injuries, including viral hepatitis, alcohol and drugs. Similarly, to wound healing, liver fibrosis results in the shedding of syndecans, such as soluble CD138, which can be detected in serum[22]. While sCD138 is considered an easily biomarker of overall survival in alcoholic cirrhosis with and without hepatocellular carcinoma[37], no association has been reported so far between circulating levels of CD138 and clinical or laboratory biomarkers during HCV infection (unless liver fibrosis).

In our hands, sCD138 displayed higher levels in type II CG than in MGUS probably due to B cell activation occurring from the antigenic stimulus of HCV; CD138 can be cleaved from the cell surface and released, first into the extracellular compartment and later into the intravascular compartment from the hepatocytes. In multiple myeloma surface CD138 represents the gold-standard marker for diagnosis and elevated sCD138 serum levels have been correlated with poor outcome for patients[18,19]. More in general, sCD138 levels parallel the release of inflammatory cytokines and specifically associated to malignancies[38,39].

In SMM, that can be considered an intermediate stage between MGUS and MM, sCD138 and FLCs ratio display higher levels compared to MGUS and type II CG. This data is in accordance with previous reports, which showed different risks of progression to malignancy in the first 5 years after diagnosis: 10% in SMM *vs.* 1% in MGUS[2,23] (per year). However, previous reports that correlated a single biomarker to a specific clinical status of MG failed to stratify patients with different conditions, for the high rate of outliers and for the spread distributions of values among different clinical categories. Stemming from these evidences, we tried an innovative approach based on the combination/integration of results obtained from each different biomarker. The specific laboratory patterns of positivity markers (SPEP, FLCs, and sCD138 levels), were used to generate a BS to screen MG patients according to the disease status and in correlation to the two main lines of disease progression (Figure 2a-b). The Italian epidemiological scenario of HCV infection has changed significantly over the last 20 years[40]; new reliable diagnostic tools to follow patient clinical outcome are widely desired. We retain that the employment of our BS could ameliorate clinical management: as a screening test, BS may find useful application during diagnosis and initial classification of patient; on the other hand, the evolution of the clinical picture could be monitored by the time-dependence of BS.

Conclusions

We found that BS profile seems to follow the ingravescence of precancerous stages of plasma cell disorders and their progression towards cancerous conditions (MM or B-NHL). Nevertheless, a univocal correspondence of BS with a defined clinical diagnosis cannot be observed and areas of overlapping among different diseases are still detected. The limit of this study is that our results are referred to different subjects for each clinical group. The BS should be calculated in the same patient at different time points during the natural history of disease evolution. To fill this gap, due

to the relative slow progression of these diseases, a protocol of at least 10 years of follow-up is under consideration. Furthermore, additional investigations are necessary to explore the possibility of implementing with other biomarkers the BS, so that a well-defined, specific pattern for each clinical picture could be identified. Our main goal, however, is the generation of a novel biomarker score from different specific biomarkers that could be very helpful in patients' management as a screening test, for setting diagnosis and classification of patients with plasma cell proliferative disorders; moreover, recording the BS of HCV patient at different time points, the worsening progression of disease towards extrahepatic manifestations could be monitored.

Conflict of Interests

The authors declare that they have no conflict of interest.

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