Relationship of differences in immunoglobulin heavy/light chain pairs (Hevylite™), selected laboratory parameters and stratification systems in different immunochemical types of multiple myeloma

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Background and Aims. Advances in the diagnosis and treatment of multiple myeloma (MM), place increasing demands on accurate stratification of patients as the starting point for optimal individualized therapy. The present study focused on assessing the association between HLC levels and the HLC-r to parameters of MM activity, prognosis and tumor mass volume. The objective was to assess the correlation of immunoglobulin (Ig), heavy/light chain (HLC) pairs (IgG- κ and - λ , IgA- κ and - λ HLC) and the ratio of monoclonal involved-HLC (i-HLC) to polyclonal uninvolved (u-HLC) Ig concentrations assessed by the Hevylite™ method with the free light chain κ/λ ratio (FLC-r), selected prognostic laboratory parameters i.e. Hb, platelets, albumin, β_2 -microglobulin (β_2 -M), Ca, lactate dehydrogenase (LDH), creatinine and the Durie-Salmon (D-S) and International Staging System (ISS), stages (1–3) for MM.

Methods. Hevylite™ assays were done on the sera of 132 MM patients at the time of diagnosis (IgG 94, IgA 38). HLC-r was calculated in the case of i-HLC- κ from the i-HLC- κ /u-HLC- λ ratio and for i-HLC- λ from the i-HLC- λ /u-HLC- κ ratio. D-S and ISS stages were evenly distributed.

Results. Md IgG- κ HLC-r was 64.8 (2.7–2222) and of IgG- λ HLC-r 49.6 (0.7–465.1), in the case of IgA- κ , Md HLC-r was 408.9 (3.4–3966) and for IgA- λ HLC-r the Md was 180.0 (0.1–3110). Normal levels of HLC pairs and HLC-r did not always rule out the diagnosis of MM. HLC-r correlated with FLC-r in IgG ($r = 0.244$, $P = 0.018$), but not in the IgA type. For IgG, HLC-r values were significantly different in patients with abnormal vs normal levels of Hb ($P < 0.0001$), albumin ($P < 0.043$), β_2 -M ($P < 0.0001$) and creatinine ($P = 0.034$) but not thrombocyte count, Ca or LDH. For the IgA isotype, we found a significant difference in HLC-r values only for thrombocyte count ($P = 0.026$) and β_2 -M ($P = 0.016$) but not for Hb, albumin, Ca, LDH or creatinine. For the IgG isotype there was a significant relationship of HLC-r index to stages 1–3 ($P = 0.038$) and substage A vs B ($P = 0.048$) according to D-S, and with high significance to stages 1–3 according to ISS ($P = 0.005$) and between stages 1 vs 3 ($P = 0.001$). For the IgA isotype, we found significant differences in HLC-r only between stages 1–3 ($P = 0.025$) according to D-S but not in the case of ISS. There were no significant correlations between i-HLC Ig levels and D-S or ISS stages in both IgG- κ and λ and IgA- κ and λ . Exceptions were significant differences for stages 1 vs 3 ($P = 0.012$) and 2 vs 3 ($P = 0.017$) for the IgG- λ isotype. There were no correlations of the HLC-r and u-HLC levels for either D-S or ISS stratifications in all HLC isotypes.

Conclusion. We found a significant positive contribution of HLC-r using the i-HLC/u-HLC ratio even in the case of i-HLC- λ i.e. i-HLC- λ /u-HLC- κ . Variable results for the relationship of important laboratory parameters and D-S and ISS stratifications (stage 1–3) to HLC-r values in IgG and IgA isotypes make separate interpretation of the Hevylite™ method results necessary in clinical practice.

Key words: multiple myeloma, heavy/light chain measurements, heavy/light chain ratio, free light chain ratio, prognostic parameters, multiple myeloma stratification

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INTRODUCTION

Advances in the diagnosis and treatment of multiple myeloma (MM), placed increasing demands on accurate stratification of patients as the starting point for optimal individualized therapy. A characteristic feature of MM closely related to its biological properties, is the produc-

tion of monoclonal immunoglobulin (MIg) by myeloma cells, accompanied by decreased production of polyclonal immunoglobulins (Ig), by normal residual plasma cells in bone marrow (BM). The International Myeloma Working Group (IMWG) guidelines recommend that the diagnosis and monitoring of the course of monoclonal gammopathies (MG), should be performed with standard serum

protein electrophoresis (SPE), and immunofixation electrophoresis (IFE), of serum/urinary proteins, nephelometric assays of normal (polyclonal) serum Ig, and measurements of serum levels of serum free light chains; κ and λ (sFLC), including their free light chain ratio (FLC-r), using the Freelite™ method¹⁻⁷. However, SPE is severely limited in its use, particularly at low serum MIg concentrations and in the IgA class of MIg (common migration of MIg with haptoglobin, transferrin and complement₃ component), resulting in inaccurate quantification of serum MIg and thus of the extent of MM (ref.^{2,4,8-9}). Hevylite™, a novel analytical technique introduced in 2009, allows accurate quantification of the monoclonal/malignant, i.e. involved MIg produced by myeloma plasma cells, and normal/non-malignant, i.e. uninvolved Ig produced by residual normal plasma cells¹⁰. When used in

MG, this nephelometric and turbidimetric assay is free from SPE limitations. It is also free from error in providing the actual MIg concentration due to changes in plasma volume, hematocrit and differences in the catabolic half-life of MIg in the IgG, IgA and IgM classes of MIg. Moreover, Hevylite™ using specific antibodies against junctional epitopes between the heavy and light chains of the Ig molecule allows accurate separate quantification of κ and λ Ig heavy/light chain pairs (HLC- κ and HLC- λ) (ref.^{2,7}). In addition to separate measurements of serum levels of the IgG- κ and - λ , IgA- κ and - λ , and IgM- κ and - λ pairs, Hevylite™ may be used to calculate the HLC-r, that is, the ratio of the monoclonal i-HLC/u-HLC Ig pairs (HLC- κ /HLC- λ and HLC- λ /HLC- κ) (ref.^{2,7,8,10-14}).

Given the still unresolved role of Hevylite™ in clinical practice and only sporadic studies on the association be-

Table 1. Basic characteristics of the analyzed groups of patients with multiple myeloma.

	IgG	IgA	IgG+IgA
n	94	38	132
Age	69 (29-89)	65 (49-88)	68 (29-89)
Sex : Male/Female	47/47 (1.0)	16/22 (0.73)	63/69 (0.91)
FLC $\kappa + \lambda$:	Md (range)	Md (range)	Md (range)
- FLC- κ (mg/L)	65.6 (0.4-10264)	80.5 (0.8-39721)	71.4 (0.4-39721)
- FLC- λ (mg/L)	8.8 (0.1-35038)	7.7 (0.5-16610)	8.6 (0.1-35038)
- FLC-r (κ / λ)	9.8 (0.0-5104)	21.7 (0.0-41376)	12.8 (0.0-41376)
HLC - $\kappa + \lambda$:			
- HLC- κ (g/L)	18.3 (0.1-97.8)	15.6 (0.02-89.6)	16.9 (0.018-97.8)
- HLC- λ (g/L)	0.746 (0.044-66.6)	0.347 (0.017-65.7)	0.611 (0.017-66.6)
- HLC-r(HLC- κ /HLC- λ)	54.4 (0.7-2222.3)	396.6 (0.1-3966.0)	86.1 (0.1-3966.0)
HLC- κ :	64	25	89
- „involved“ - κ (g/L)	26.6 (4.4-97.8)	24.0 (0.0-89.6)	26.5 (0.0-97.8)
- „uninvolved“ - κ (g/L)	0.36 (0.04-7.1)	0.07 (0.002-1.34)	0.3 (0.02-7.14)
- HLC-r - (HLC- κ /HLC- λ)	64.8 (2.7-2222)	408.9 (3.4-3966)	97.0 (2.7-3966)
HLC- λ :	30	13	43
- „involved“ - λ (g/L)	28.5 (2.3-66.6)	40.4 (0.7-65.7)	29.6 (0.7-66.6)
- „uninvolved“ - λ (g/L)	0.63 (0.9-5.5)	0.1 (0.02-0.6)	0.5 (0.02-5.5)
- HLC-r (HLC- κ /HLC- λ)	0.02 (0.002-1.4)	0.005 (0.00-0.9)	0.002 (0.000-1.4)
- ^x HLC-r (HLC- λ /HLC- κ)	49.6 (0.7-465.1)	180.0 (0.1-3110.0)	57.9 (0.1-3110.0)
β_2 -M (mg/L)	4.1 (1.4-39.7)	4.3 (1.6-43.4)	4.1 (1.4-43.4)
Albumin (g/L)	34.7 (14.6-48.6)	37.1 (17.7-47.6)	35.5 (14.6-48.6)
MIg (SPE, g/L)	29.7 (2.9-90.4)	22.8 (2.9-52.6)	25.9 (2.9-90.4)
Durie-Salmon			
Stage: 1	11 (12%)	5 (13%)	16 (12%)
2	31 (33%)	12 (32%)	43 (33%)
3	52 (55%)	21 (55%)	73 (55%)
Substage: B	14 (15%)	9 (24%)	23 (17%)
ISS			
Stage: 1	26 (28%)	13 (34%)	39 (30%)
2	37 (39%)	9 (24%)	46 (35%)
3	31 (33%)	16 (42%)	47 (36%)

n - number of patients, Md - median, FLC - serum immunoglobulin free light chains, HLC - immunoglobulin heavy/light chain pairs, HLC-r - HLC- κ /HLC- λ ratio, ^xHLC-r - HLC- λ /HLC- κ ratio, β_2 -M - beta₂-microglobulin, MIg - monoclonal immunoglobulin, SPE - serum protein electrophoresis, ISS - International Staging System

Table 2. Median levels and range of selected laboratory parameters in groups of IgG, IgA and IgG plus IgA patients with multiple myeloma.

	n	Hb (g/L) Md (range)	Platelets ($\times 10^9/L$) Md (range)	Albumin (g/L) Md (range)	β_2 -M (mg/L) Md (range)	Ca (mmol/L) M (range)	LDH (μ kat/L) Md (range)	Creatinine (μ mol/L) Md (range)
IgG	94	108.0 (67-153)	198.5 (45-427)	35.9 (17.4-48.6)	4.1 (1.4-39.7)	2.3 (1.7-3.3)	2.7 (1.5-10.8)	88.5 (46-1666)
IgA	38	99.0 (67-144)	195.0 (67-485)	37.6 (17.7-47.6)	4.4 (1.6-43.4)	2.4 (1.9-3.5)	2.7 (1.1-4.2)	90.0 (30-924)
IgG+IgA	132	102.0 (67-153)	198 (45-485)	36.1 (17.4-48.6)	4.1 (1.4-43.4)	2.3 (1.7-3.5)	2.7 (1.1-10.8)	90.0 (30-1666)

n – number of patients, Md- median, Hb – hemoglobin, β_2 -M – beta₂ microglobulin, Ca – calcium, LDH – lactate dehydrogenase

Table 3. Correlation analysis of the relationship between groups of normal vs abnormal values of selected laboratory parameters and ^sHLC-r values measured with Hevylite™ in patients with IgG, IgA and IgG plus IgA type of multiple myeloma.

^s HLC-r	vs: cut off:	FLC-r (0.26 - 1.65)		Hb (120g/L)	Hb (100g/L)	Platelets (150x 10 ⁹ /L)	Albumin (30 g/L)	β_2 -M (2.3 mg/L)	Ca (2.6 mmol/L)	LDH (3.6 μ kat/L)	Creatinine (104 μ mol/l)
	N	R	P	P	P	P	P	P	P	P	P
IgG	94	0.244	0.018	<0.0001	<0.0001	NS	0.043	<0.0001	NS	NS	0.034
pa (%)				(80%)	(42%)	(23%)	(23%)	(79%)	(11%)	(23%)	(37%)
IgA	38	0.017	NS	NS	NS	0.026	NS	0.016	NS	NS	NS
pa (%)				(84%)	(53%)	(34%)	(21%)	(79%)	(34%)	(22%)	(42%)
IgG+IgA	132	0.174	0.046	<0.0001	<0.0001	0.002	NS	<0.0001	0.033	NS	NS
pa (%)				(81%)	(45%)	(26%)	(22%)	(79%)	(17%)	(22%)	(39%)

HLC-r – ratio of immunoglobulin heavy/light chain pairs, i.e. HLC- κ /HLC- λ , ^sHLC-r, i.e. HLC- λ / HLC- κ assessed with Hevylite™, FLC-r - ratio of free light chains κ/λ , Hb – hemoglobin, β_2 -M – beta₂ microglobulin, Ca – calcium, LDH – lactate dehydrogenase, n – number of patients, r – correlation coefficient, pa (%) – percentage of abnormal values, NS – nonsignificant correlation

tween HLC and markers related to the biological properties and mass volume of myeloma tissue, the present study focused on assessing the association between HLC levels and the HLC-r to parameters related to MM activity, prognosis and tumor mass volume, namely FLC and FLC-r, hemoglobin (Hb), platelets, albumin, β_2 - microglobulin (β_2 M), Ca, lactate dehydrogenase (LDH) and creatinine levels and MM stages as determined by the D-S system and International Staging System^{5,15}. Given the natural biological variations between individual immunochemical classes of MM and the broad range of both positive and negative HLC-r values, we tried to assess, besides the overall analysis of the groups of patients with IgG and IgA Mlg, eventually with IgG plus IgA and the basic immunochemical types of MM (IgG- κ and - λ , IgA- κ and - λ), also the analysis of HLC-r in the case of i-HLC- λ isotype by the assessment of HLC- λ /HLC- κ ratio, i.e. i-HLC/u-HLC that enable to assess positive values of HLC-r index also in the case of i-HLC- λ .

MATERIALS AND METHODS

The prospective study analyzed 163 patients who met the IMWG diagnostic criteria^{1,5}. Excluded from the analysis were 31 individuals (19%) with the absent intact Mlg molecule (the Bence-Jones and nonsecretory MM types) and specific Hevylite™ kits (IgD and the biclonal MM type). Of the 132 patients undergoing Hevylite™ analysis at the time of MM diagnosis, 94 individuals (71.2%) were of the IgG class and 38 (28.8%) were of the IgA class; the κ/λ ratio was 1.9. The analysis was performed using serum samples frozen at -78 °C and stored in the University Hospital Olomouc serum biobank. M-protein concentrations were measured with the Sebia HYDRASYS assay (Hydragel 30 Protein kit) and the M-gradient was quantified using the Epson 1680 scanner, IFE was performed with the Hydragel 4 IF kit. Serum FLC levels were assessed with the Freelite SPA_{PLUS} κ free (normal range, 3.3–19.4 mg/L) and Freelite SPA_{PLUS} λ free (5.7–26.3 mg/L) kits, supplemented with FLC-r calculations (0.26–1.65) (ref.^{2,3}). Serum HLC concentration was measured with the SPA_{PLUS} turbidimeter (The Binding Site) and kits

Hevylite Human IgG- κ (3.84–12.07 g/L), IgG- λ (1.91–6.74 g/L), IgA- κ (0.57–2.08 g/L) and IgA- λ (0.44–2.04 g/L). HLC-r index was calculated in the case of dominant κ light chain as HLC- κ /HLC- λ , whereas in the case of dominant λ light chain as HLC- λ /HLC- κ ($^{\lambda}$ HLC-r, Table 1). Due to high HLC concentrations exceeding the measuring range of the appliance, some of the samples had to be manually prediluted. Serum β_2 -M levels were assessed with the ELISA, Enzyme-linked immunosorbent assay; method (< 2.3 mg/L). Standard procedures were used to measure Hb and platelet levels in blood and serum concentrations of albumin, Ca, LDH and creatinine. The MM patients were prognostically stratified according to the D-S and ISS systems^{5,15}. Data were analyzed using the SPSS v. 15 statistical software (SPSS Inc., Chicago, USA). The analyses were performed using the Spearman correlation, Kruskal-Wallis and Mann-Whitney U post hoc test with the Bonferroni correction of significance for multiple comparisons. All the tests were performed at a level of significance of $P < 0.05$.

RESULTS

Characteristics of the three analyzed groups, i.e. IgG, IgA and the entire IgG plus IgA set, including the median values of the analyzed laboratory markers and D-S and ISS clinical stages 1-3 are summarized in Table 1. Serum levels of the FLC including their ratio (FLC-r) and HLC pair values including monoclonal i.e. involved (i-HLC), and polyclonal i.e. uninvolved (u-HLC) and their ratio HLC-r or $^{\lambda}$ HLC-r in the IgG, IgA and IgG plus IgA groups classified according to the light chain types (LC- κ and LC- λ) are shown in Table 1. Abnormal HLC and/or HLC-r and only abnormal HLC-r values varied in the analyzed groups: IgG- κ 83% and 98%; IgG- λ 93%

and 97%; IgA- κ 84% and 100%; and IgA- λ 92% and 92%, respectively¹⁶. The median (range) levels of Hb, platelets, albumin, β_2 -M, Ca, LDH and creatinine in the groups of IgG, IgA and IgG plus IgA are shown in Table 2, whereas the percentage of abnormal values (pa) is shown in Table 3. Spearman correlation analysis revealed weak significant positive relationship between HLC-r and FLC-r index in the IgG group with missing relationship in the IgA group (Table 3). The overview summarizing the results of the analysis of correlations between the studied laboratory parameters and HLC-r values in patient subgroups with different MIg isotypes, clearly suggests that the correlations are significantly different between serum dominant FLC- λ levels and HLC-r values only in the case of IgG- κ ($r = 0.56$), IgG- λ ($r = 0.15$) and IgA- κ ($r = 0.44$). No significant association was found in the case of dominant FLC- κ in IgG- κ or- λ and IgA- κ or- λ . Similarly, for FLC-r and HLC-r, there was a positive association in patients with the IgG isotype of MM with both IgG- κ and - λ ($r = 0.27$ and $r = 0.42$) but not in patients with IgA isotype of MM.

We used the Mann-Whitney U test used to compare patients with normal and those with abnormal laboratory parameters. For the IgG type of MM, we found significantly increased serum levels of the HLC-r index related to low levels of Hb and albumin, increased levels of β_2 -M and less significantly of creatinine but not to abnormal values of thrombocytes, Ca and LDH (Table 3). In the of IgA isotype group, there were significantly higher values of HLC-r index only in the case of low thrombocyte count and increased β_2 -M. Analysis of the whole cohort, i.e. patients with IgG plus IgA MM showed significant relationship of HLC-r to Hb, thrombocytes, β_2 -M and Ca which was slightly different from individual analysis of IgG and IgA isotypes (Table 3). Positive Spearman's correlation between Hb levels and HLC-r values was found in IgG- κ ($r = 0.50$) and - λ ($r = 0.66$) MM isotypes, but not

Table 4. Correlations between $^{\lambda}$ HLC-r and multiple myeloma stages classified according to Durie-Salmon and International Staging System in patients with IgG, IgA and IgG plus IgA type of multiple myeloma.

^x HLC-r vs.	IgG (n-94)				IgA (n-38)				IgG + IgA (n-132)			
	Md	^x HLC-r (range)	<i>P</i>	(n)	Md	^x HLC-r (range)	<i>P</i>	(n)	Md	^x HLC-r (range)	<i>P</i>	
D-S												
Stage (n):												
1 (11)	17.2	(7.7-825)	0.038	(5)	87.9	(32.8-330.4)	0.025 ^x	(16)	30.1	(4.7-825)	NS	
2 (31)	27.8	(2.7-1485)		(12)	799.3	(85.8-3966)		(43)	85.8	(2.7-3966)		
3 (52)	85.5	(0.7-2222)		(21)	159.4	(0.1-3465.5)		(73)	95.5	(0.1-3465)		
Substage:												
A (80)	49.3	(0.7-2222)	0.048	(29)	408.9	(0.1-3966)	NS	(109)	68.9	(0.1-3966)	NS	
B (14)	91.7	(19.3-1485)		(9)	322.7	(3.4-2243)		(23)	99.9	(3.4-2243)		
ISS												
Stage (n):												
1 (26)	24.9	(2.7-825)	0.005	(13)	180.0	(32.8-2641)	NS	(39)	47.4	(2.7-2641)	0.015 ^x	
2 (37)	57.3	(0.7-2222)		(9)	95.5	(35.3-3465)		(46)	63.1	(0.7-3465)		
3 (31)	106.5	(19.3-884)		(16)	691.6	(0.1-3966)		(47)	135.1	(0.1-3966)		

^x Mann-Whitney U test with the Bonferroni correction of the significance

HLC-r – ratio of immunoglobulin heavy/light chain pairs, i.e. HLC- κ /HLC- λ , $^{\lambda}$ HLC-r i.e. HLC- λ /HLC- κ assessed with Hevylite™, Md – median, D-S – Durie-Salmon staging system, ISS – International Staging System, NS – nonsignificant correlation

Table 5. Correlations of serum of levels if uninvolved HLC immunoglobulins and multiple myeloma stages classified according to Durie-Salmon and International Staging System in groups with different immunochemical isotypes of immunoglobulin.

HLC uninvolved vs.	IgG-κ		IgG-λ		IgA-κ		IgA-λ	
	IgG-λ		IgG-κ		IgA-λ		IgA-κ	
	Md	P	Md	P	Md	P	Md	P
D-S								
Stage:								
1	0.89		1.81		0.16		0.23	
2	0.46	NS	0.63	NS	0.02	NS	0.02	NS
3	0.35		0.57		0.10		0.25	
Substage:								
A	0.45	NS	0.59	NS	0.70	NS	0.15	NS
B	0.21		0.70		0.14		0.13	
ISS								
Stage:								
1	0.73		1.1		0.10		0.19	
2	0.35	NS	0.57	NS	0.18	NS	0.14	NS
3	0.32		0.49		0.05		0.11	

HLC - immunoglobulin heavy/light chain pairs assessed with Hevylite™, Md - median, D-S - Durie-Salmon staging system, ISS - International Staging System, NS - nonsignificant correlation

in patients with IgA-κ and -λ type. There was a positive correlation of thrombocyte count with HLC-r only in the case of IgA-λ isotype ($r = 0.74$). Except for the patients with IgA-κ isotype, for all other immunochemical types of MM we found significant correlations between HLC-r and serum albumin levels (IgG-κ, $r = 0.35$; IgG-λ, $r = 0.44$; IgA-λ, $r = 0.59$). As for serum β_2 -M, significant correlation with HLC-r (IgG-κ, $r = 0.35$; IgG-λ, $r = 0.59$) was found, but not in IgA-κ and -λ MM isotypes. In the case of Ca, LDH and creatinine there was no positive correlation with HLC-r in individual isotypes of MM.

Using the Kruskal-Wallis test, we found significant differences in the x HLC-r index (in LC-κ as κ/λ and in LC-λ as λ/κ) for D-S stratification and stages 1-3 and especially between individual stages of ISS with clear increase in the HLC-r index with advanced stages of MM. The Mann-Whitney U post hoc analysis with Bonferroni correction showed significantly higher levels of x HLC-r in substage B vs A according to D-S and stage 3 vs 1 according to ISS (Table 4, Fig. 1). In the IgA group we found significant differences using the Kruskal-Wallis test in the HLC-r index only between stages 1-3 and not between substages A and B according to the D-S. Using the Mann-Whitney U post hoc analysis with Bonferroni correction there were significantly higher values of x HLC-r in stage 2 vs 1 (Table 4). The x HLC-r index in the IgA isotype did not behave consistently in the case of ISS stratification but the differences were not statistical significant (Table 4, Fig. 1). In the whole cohort patients with IgG plus IgA MM there was trend to increasing x HLC-r values with advanced stage of MM with a statistically significant difference in x HLC-r index only between stages 1-3 according to ISS, and using Mann-Whitney U post hoc test between higher values of x HLC-r in stage 3 vs 1 (Table 4). In a more detailed analysis aimed at separate assessment of the correlation between involved HLC levels in MM groups with IgG-κ or -λ and/or IgA-κ or -λ and 1-3 D-S stages we found no

significant results, despite an obvious moderate trend for increasing concentration of i-HLC with higher MM stage especially in the IgG-λ type of MM (Fig. 2). In the case of ISS stratification, there were significant differences between i-HLC values only between stages 3 vs 1 ($P = 0.012$) and 2 vs 3 ($P = 0.017$) in MM patients with the IgG-λ type (Fig. 3). Similar analysis of the correlation of u-HLC levels vs results of stratification according to D-S and ISS systems showed no statistical significance although for IgG-κ and -λ isotypes, unlike IgA-κ and -λ isotypes, there was an obvious trend to increase in specific suppression of serum u-HLC levels with higher D-S and ISS stages (Table 5).

DISCUSSION

Comparison of the median i-HLC levels found in the present study with those in an analogical study by Kouliris et al. showed similar results for individual isotypes: IgG-κ 26.6 vs. 25.8 (ref.¹²); IgG-λ 28.5 vs. 23.4 (ref.¹²); IgA-κ 24.0 vs. 28.9 (ref.¹²) and IgA-λ 40.4 vs. 36.4 (ref.¹²) g/L, respectively. Unlike most studies reporting abnormal HLC-r values in all MM patients^{2,3,12}, this study showed the same result in the IgA-κ type of MM only; the other results were 98% for IgG-κ, 97% for IgG-λ and 92% for IgA-λ, suggesting that detection of a normal HLC-r does not rule out the diagnosis of MM in all cases¹⁶. Correlation analysis showed only slightly significant relationship between HLC-r (Hevylite™) and FLC-r (Freelite™) indices in the IgG MM group, both in IgG-κ and -λ isotypes, but there was no significance in the IgA group. In the pilot study the analysis aimed at high values of both HLC-r and FLC-r, there were correlations for both IgG and IgA types of MM (ref.¹²). The biological variability of FLC-κ and FLC-λ demonstrates significant relationship of HLC-r index to the levels of FLC-λ, but not

Fig. 1. Comparison of HLC-r index (HLC-κ/HLC-λ) and ^xHLC-r (HLC-λ/HLC-κ) with the stage of multiple myeloma (stage 1-3 and substage A-B) assessed using Durie-Salmon (D-S) and International Staging System (ISS) in the group of IgG and IgA multiple myeloma patients.

Fig. 2. Comparison of medians of serum levels of involved HLC to stages 1-3 of multiple myeloma assessed using Durie-Salmon stratification system (D-S) with regard to κ and λ types in the group of IgG and IgA multiple myeloma patients.

Fig.3. Comparison of medians of serum levels of involved HLC to the stage 1-3 of multiple myeloma assessed using International Staging System (ISS) with regard to κ and λ types in the group of IgG and IgA multiple myeloma patients.

to FLC- κ in IgG- κ and IgG- λ types of MM. The absence of this relationship in the IgA type of MM might, among others, be due to small sample size in our study. The above data suggest that for clinical interpretation of the HLC-r and the levels of FLC and FLC-r, it is necessary to take into consideration not only the IgG and IgA types of MM but also the type of LC- κ or λ .

Univariate analysis of the correlations between the x HLC-r and diagnostic and stratification criteria of MM i.e. Hb, platelets, albumin, β_2 -M, Ca, LDH and creatinine produced rather heterogeneous results in previous studies^{2,5,15,17}. Spearman correlation analysis in our study showed significant correlation between x HLC-r values and Hb in IgG- κ and λ type, confirmed using Mann-Whitney test in the groups with Hb cut off level both < 120 and < 100 g/L only in IgG type, whereas Koulieris et al. reported different correlations of i-HLC and high HLC-r with Hb in both the IgG and IgA types of MM (ref.¹²). In agreement with our results, a significant association between HLC and severity of anemia was described in Waldenström macroglobulinemia, with the median IgM i-HLC being significantly higher in patients with Hb < 100 g/L (ref.^{18,19}). In the case of the relationship of HLC-r index to low thrombocyte count the Mann-Whitney U test showed significant difference only in IgA type but not in IgG type of MM. Spearman correlation test was positive only in the IgA- λ isotype, too. In the case of albumin, an important prognostic and stratification marker in MM, the present study found using the Spearman correlation analysis very good correlations with the x HLC-r in IgG- κ , IgG- λ and IgA- λ , but not in IgA- κ isotype. The Mann-Whitney test, comparing values of x HLC-r index between patients with an albumin cut off level 30 g/L, we found significant difference in IgG type of MM only, which is in values with the results a previous study¹². As for β_2 -M, the key prognostic and stratification marker in MM, the present analysis, comparing individually normal vs increased level found significant relationship to x HLC-r using the Mann-Whitney U test in IgG and less significantly in IgA type of MM. Spearman correlation analysis showed also significant results between x HLC-r vs β_2 -M in IgG type of MM, confirmed by significant separate correlation in IgG- κ and λ isotype with no significance for IgA- κ and λ type of MM. This finding is in agreement with Koulieris et al. demonstrating a relationship between a high HLC-r, exceeding the median of the obtained values, and β_2 -M > 3.5 mg/L (ref.¹²) in the IgG type of MM only¹². Published univariate analyse showed more significant correlations of the HLC-r (< 0.02 , > 40) (ref.^{3,20}) and β_2 -M (> 5.5 mg/L) (ref.^{3,20}) with progression-free survival (PFS) than with albumin (> 35 g/L) (ref.^{3,20}), FLC-r (< 0.03 and > 32) (ref.^{3,20}) and LDH (> 248 IU/L) (ref.^{3,20}) and even with the results of a previous study on cytogenetic analysis (del:13, t 4:14 and del:17p) (ref.³). To a certain extent, the difference in the association between the x HLC-r to β_2 -M and albumin in the IgG but not the IgA type of MM may be explained by the specific relationship of the metabolism of β_2 -M, albumin and especially IgG Ig to the neonatal Fc receptor (FcRn) molecule^{2,21-24}. While β_2 -M is one of the components of the heterodimeric FcRn light

chain, this molecule is involved in the recycling and thus the duration of the half-life of albumin and the IgG type of Ig (ref.^{2,21-23}). Multivariate analysis showed that only β_2 -M and highly abnormal values of the HLC-r (< 0.01 or > 200) (ref.^{3,25}) are independent risk factors for MM (ref.^{2,3,25-26}). Given the fact that an abnormal HLC-r is a predictor of a shorter PFS, independent of β_2 -M and albumin levels, it was selected as a criterion in a novel staging system that modifies the ISS; there, it replaced albumin^{2,25}. With respect to PFS, this stratification model is characterized by better prognostic prediction than the ISS (ref.^{3,4,25-26}). Analogically, there is a stratification model for Waldenström's macroglobulinaemia (WM) that is based on high IgM HLC-r, β_2 -M and LDH, classifying patients into 3 prognostically distinct groups with different overall survival (OS) rates²⁷. One analysis of the prognostic role of high HLC-r in the IgG and IgA types of MM showed a high risk of progression with a short PFS only in the IgG but not the IgA type of MM (ref.³). There are studies, however, that have demonstrated a correlation between the HLC-r in the IgA isotype of MM and the length of PFS (ref.²⁶). The prognostic impact of the HLC-r is, among others, caused by suppression of isotype-specific polyclonal u-HLC induced by selective involvement of the bone marrow "niche" microenvironment due to proliferation of neoplastic plasma cells, mainly of the IgG type^{3,28}. The IgG clone of myeloma plasma cells is capable of effective inhibition of the growth of normal plasma cells and thus the synthesis of the polyclonal IgG type of Ig that is more effective than that of polyclonal immunoglobulin IgA or IgM (ref.²⁸⁻²⁹). In the case of monoclonal gammopathy of undetermined significance (MGUS), suppression of isotype-specific u-HLC (e.g. IgG- λ in the IgG- κ type of MGUS) has an independent prognostic role documented by a 2-fold increase in the risk for progression in MM, supporting the hypothesis that suppression of non-clonal plasma cells is a marker of a high risk for malignant transformation in MGUS (ref.²⁸). The dissimilarity of the IgG type of MM also stems from the short half-life of the IgG type of Ig due to full saturation of IgG Fc receptors in the case of high M-protein levels, manifested by shortened half-life of both involved and uninvolved IgG (ref.²⁸). Ludwig et al. using multivariate analysis showed that an abnormal HLC-r (< 0.022 and > 45 or the presence/absence of $> 50\%$ of HLC suppression) (ref.^{4,30}) together with β_2 -M are independent risk factors for OS and therefore have criteria in two novel 3-stage stratification models for MM (ref.^{4,30}). The Mann-Whitney test showed there was no significant difference between x HLC-r values in patients with normal vs abnormal increased serum level of Ca and LDH in IgG and IgA isotypes, and in the case of creatinine in IgA MM. This finding was confirmed for all three parameters related to tumor mass and prognosis of MM by non-significant Spearman correlation analysis which was carried out separately in individual κ and λ isotypes of IgG and IgA MM. Also in our analysis, in agreement with a similar study, there was no significant relationship of x HLC-r in IgA MM to the deterioration of renal function, unlike FLC-r, most likely due to completely different renal secretion of FLC and HLC (ref.³¹).

In agreement with the initial assumption about correlations with HLC-r, expressing the ratio of concentrations of involved MIg produced by myeloma clone and uninvolved Ig produced by normal plasma cell population, there was a statistically significant association between the HLC-r and stages 1-3 and substages A vs B of IgG type of MM, assessed according to D-S and with substantially higher significance in ISS stratification with highly significant difference between stage 1 vs 3. This finding was in the case of D-S and ISS stratification was documented by obvious increase in median $^x\text{HLC-r}$ index values with advanced stage of MM. In the IgA MM group there was significant difference only in D-S staging due to significantly higher $^x\text{HLC-r}$ value in stage 2 vs 1. In IgA MM with respect to ISS, the values of the $^x\text{HLC-r}$ index did not behave consistently in individual stages of MM, leading to absence of significance, which might be explained by the above discussed biological diversity of IgG and IgA isotype of MIg. Lack of statistical significance between $^x\text{HLC-r}$ and D-S stratification in our analysis indicates that joint assessment of patients with IgG plus IgA isotype of MM is not suitable for such assessment.

Detailed analysis aimed at i-HLC especially in IgG type and more evidently in IgG- λ than IgG- κ isotype, confirmed clear, however statistically non-significant trend of the increase of i-HLC levels with advanced stage of MM assessed according to D-S. In the case of stratification according to ISS, there were significant differences between i-HLC values and stages 3 vs 1 and 3 vs 2 in patients with the IgG- λ type of MM only. There was no statistically significant difference in the degree of specific immunosuppression, that is, the depth of the decrease of u-HLC to the extent of MM, i.e. stages 1-3 according to D-S and ISS. In IgG- κ and - λ isotypes but not in IgA- κ and - λ isotypes there was a clear trend towards the decrease of u-HLC levels with the stage of MM. The above findings may be explained by natural variation of a biological character, synthesis and catabolism of the IgG and IgA types of Ig and albumin in the organism. FcRn molecules protect IgG Ig and albumin from digestion by interfering with their recycling and release from cell surfaces back to the blood, leading to prolongation of the half-life of IgG ranging from 3 to 21 days. In the absence or functional insufficiency of FcRn receptors and/or high IgG Ig concentration, the half-life of IgG and albumin is shortened to approximately 3 days. At normal concentration, however, it is 21 days as a result of full FcRn protection while at very low IgG levels, the half-life may be as long as several weeks, resulting from maximum FcRn protection (ref.^{2,21-23}). Unlike in case of the IgG type of Ig, clearance of the IgA type of Ig is not equally dependent on concentration. Thus, the half-lives of the IgA type of Ig may be prolonged from 2-3 days to 5 days, respectively^{2,3}. Due to the above reasons, MIg concentrations do not reflect the actual tumor production as high concentrations of the IgG type of M-protein underestimate tumor growth and low MIg levels underestimate the degree of therapy-induced tumor mass reduction³. In case of high MIg, serum levels of polyclonal IgG are low as FcRn receptors are fully satu-

rated; this phenomenon, however, is also contributed to by suppression of normal plasma cells leading to reduced production of normal IgG Ig (ref.³²). Given the fact that both the polyclonal and monoclonal fractions of IgG are similarly affected, the HLC-r values are not substantially influenced by these changes and express the degree of tumor production more accurately than MIg concentrations measured with SPE (ref.³). The above circumstances, that is, the variable half-life of IgG Ig and changes in both the hematocrit and blood volume, explain why intact MIg values themselves are not a good stratification and prognostic marker in individual immunochemical types of MM (ref.^{3,33}). The biological variation between the IgG and IgA types of Ig have been confirmed by results of a prognostically oriented study by Ludwig et al. showing a more significant association between suppression of i-HLC and the length of OS in the IgG type of MM as compared with the IgA type³⁴. The biological variation between the IgG and IgA types of HLC is also demonstrated by the predictive role of increasing HLC-r to malignant transformation in MGUS, mainly determined by suppression of u-HLC with the opposite isotype (e.g. suppression of IgG- λ in the IgG type of MGUS) (ref.²⁸), only valid for the IgG but not the IgA type of MGUS (ref.²⁸). The absence of this prediction potential in the IgA type of MGUS is likely to be determined by the absence of the so-called "niche" effect that is only specific to the IgG type of MGUS (ref.²⁸). There is an opinion that when assessing HevyliteTM results in individual patients with MM, individual biological variability should be also taken into an account in the context of proposed reference values based on population study results³⁵.

CONCLUSION

Normal levels of HLC pairs and HLC-r do not always rule out the diagnosis of MM. Simultaneous paired examination of monoclonal (i-HLC) and polyclonal (u-HLC) confirmed considerable biological variability between the MM isotypes IgG and IgA. In IgG isotype there exists a significant correlation between $^x\text{HLC-r}$ vs FLC-r, anemia, hypoalbuminemia, increased $\beta_2\text{-M}$ and creatinine whereas in the IgA isotype to thrombocytopenia and $\beta_2\text{-M}$ only. In IgG type of MM there was a significant increase of the HLC-r with advanced stage of MM assessed using D-S and ISS stratification, but not in the case of ISS and IgA isotype. The levels of IgG and IgA i-HLC had no significant relationship to DS and ISS stratification of MM, the degree of specific immunoparesis, i.e. the decrease of u-HLC had no significant relationship to advanced stage of MM. We confirmed the utility of our calculation of the $^x\text{HLC-r}$ index using the involved-HLC/uninvolved-HLC ratio, i.e. $\text{HLC-}\kappa/\lambda$ or $\text{HLC-}\lambda/\kappa$ in contrast to earlier recommended calculations of $\text{HLC-}\kappa/\lambda$ regardless of the isotype of involved HLC. Involved-HLC, u-HLC and $^x\text{HLC-r}$ should be examined and interpreted in IgG and IgA isotype of MM in clinical practice separately.

ABBREVIATIONS

BM, Bone marrow; β_2 -M, β_2 microglobulin; Ca, Calcium; D-S, Durie-Salmon staging system of MM; ELISA, Enzyme-linked immunosorbent assay; F, Female; FcRn, Neonatal Fc receptor; FLC, Free light chain; FLC-r, Free light chain ratio of free κ/λ light chains serum levels; Hb, Hemoglobin; HLC, Heavy/light chain pairs; HLC-r, Heavy/light chain ratio (HLC- κ /HLC- λ) or in i-HLC- λ λ -HLC-r (HLC- λ /HLC- κ); IFE, Immunofixation electrophoresis; Ig, Immunoglobulin; IgA, Immunoglobulin A; i-HLC, Involved heavy/light chain pairs; IMWG, International Myeloma Working Group; ISS, International Staging System; LC, Light chain; LDH, Lactate dehydrogenase; M, Male; Md, Median; MG, Monoclonal gammopathy; MGUS, Monoclonal gammopathy of undetermined significance; MiG, Monoclonal immunoglobulin; MM, Multiple myeloma; NS, Nonsignificant; OS, Overall survival; PFS, Progression-free survival; R, Correlation coefficient; SPE, Serum protein electrophoresis; u-HLC, Uninvolved heavy/light chain pairs; WM, Waldenström macroglobulinemia.

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