

Prognostic Contribution of the New Immunoglobulin (Ig) Biomarkers (Freelite™ and Hevylite™) in Waldenstrom's Macroglobulinemia (WM)

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Abstract Clinical utility of serum free light chains (sFLC) and heavy chain (HLC) IgM were assessed, using Freelite and Hevylite assays in 70 WM patients. The result showed that median involved (i) sFLC -kappa and -lambda were 45.6 and 78.3mg/L respectively; median sFLC (kappa+lambda) was 67.4 mg/L. While, median FLCR (involved/uninvolved FLC ratios) were 2.7 and 6.9 in ikappa- and ilambda -restricted patients respectively. Moreover, increased isFLC, sFLC (kappa+lambda) and FLCR were correlated significantly with shorter time to first treatment (TFT) and adverse survival (OVS). Median iIgM-kappa and iIgM -lambda were 25.4 and 34.8g/L respectively; median HLCR (involved/uninvolved HLC ratios) were 185.5 and 101.9 in kappa- and lambda- restricted patients respectively. In addition, the increased iHLC and HLCR correlated significantly with shorter TFT. Hence, sFLC and HLC measurements appeared to be both prognostic in WM.

Keywords Waldenstrom's Macroglobulinemia, IgM Quantification, Freelite™, Hevylite™, Monitoring, Prognosis

1. Introduction

Waldenstrom's macroglobulinemia (WM) is an indolent B cell lymphoproliferative disease, characterized by lymphoplasmacytic infiltration of the bone marrow (BM), lymph nodes or other organs accompanied by serum monoclonal immunoglobulin IgM production[1-3]. It is classified as a separate entity of the lymphoplasmacytic lymphoma type in the WHO classification[4]. Twenty-five to forty percent of patients are asymptomatic and do not need treatment, at least until disease progression, while the majority presents a wide clinical spectrum including fatigue, hyperviscosity manifestations, lymphadenopathy, organomegaly, recurrent infections, peripheral neuropathy and other, as well as varying laboratory findings such as anemia or other cytopenias, blood lymphocytosis,

hypoalbuminemia, autoimmune manifestations, increased β_2 -microglobulin (β_2 -M) and LDH, polyclonal hyper- or more frequently hypo-gammaglobulinemia, independently of IgM levels[5-8]. As a consequence of disease variability, survival ranges from 60 to 120 months. Symptomatic patients should be treated. Response to treatment is evaluated by the regression of signs and symptoms and the decrease of monoclonal IgM serum levels; serum IgM fluctuations is used for disease monitoring[9]. Thus IgM detection, including quantification by serum protein electrophoresis, densitometry and immunofixation, is mandatory for diagnosis and monitoring of WM patients. The accuracy of the tests depends upon the position of the migrating monoclonal protein, the quantification of which requires skilled personnel[10-12]. Furthermore, user operator error may account for unexpected results particularly at low concentration.

New methods for paraprotein free light and heavy chain quantification were recently developed and manufactured [13]. The serum free light chain assay (sFLC; Freelite™ test, The Binding Site, Birmingham, UK) is a decade old by now.

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Using antibodies that bind exclusively to the hidden FLC molecules, it allows sensitive nephelometric quantification of circulating unbounded serum kappa and lambda light chains. sFLC measurements as well as the resultant FLC ratio (sFLCR) proved to be of clinical and prognostic utility in plasma cell dyscrasias[13-17] and also in B-cell lymphoproliferative disorders including chronic lymphocytic leukemia[18-20].

The most recent Hevylite™ immunoassay uses antibodies that target unique junctional epitopes between the heavy and light chains of each Ig molecule and determines immunoglobulin heavy chain/light chain (HLC) pairs[10,21]. The serum IgM Hevylite assay has been very recently released on the market; it specifically quantifies IgMkappa and IgMlambda separately allowing the calculation of the deriving ratios (HLCR IgMkappa/IgMlambda and vice versa)[21].

Immunoglobulin (Ig) quantification by Freelite™ and Hevylite™ assays overcome some of the technical bias, while in addition they allow a much closer evaluation of the absolute value of the monoclonal intact and free component. Likewise when, for example, the monoclonal component is IgM-kappa, classical IgM measurements include the monoclonal IgM-kappa along with the remaining polyclonal IgM-kappa and the polyclonal IgM-lambda; Freelite™ measurement quantifies the monoclonal free kappa (monoclonal and polyclonal) light chain and separately the polyclonal free lambda light chain and Hevylite™ assay determines the monoclonal IgM-kappa along with the remaining polyclonal IgM-kappa while the polyclonal IgM-lambda is quantified separately.

In the present study, we evaluated the contribution of the new Ig biomarkers (Freelite™ and Hevylite™) in patients with WM.

2. Materials and Methods

2.1. Patients Selection

Seventy WM patients diagnosed and followed in 2 Hellenic centers were included in the study with their characteristics as shown in Table 1.

They were 33 women and 37 men with a median age 66 years. Forty-four, 28 and 28 were in WM-IPSS[22] stage 1, 2 and 3 respectively. Anemia (Hb < 10g/L) was present in 29% of the patients, thrombocytopenia (PLT < 140x10⁹/L) in 13%, and blood absolute lymphocytosis (Lymphocytes > 4x10⁹/L) in 13%. Hypoalbuminaemia (serum albumin < 3.5 g/dL) was present in 22% and increased LDH (above normal) in 16%. Twenty-two percent of patients had high levels of β_2 -M (≥ 5.5 mg/L) while in 43% an extensive BM lymphoplasmacytic infiltration ($\geq 50\%$) was found. Splenomegaly was present in 13% and lymphadenopathy in 17% of our patients. Paraprotein by serum immunofixation were IgM-kappa in 52 patients and IgM-lambda in 18 patients. Median IgM paraprotein level at presentation as routinely determined by

nephelometry was 24.55 g/L (range 1.5-110), median IgG 8.83 g/L (range 1.96- 47.50) and median IgA 0.93 g/L (range 0.22-10.9).

Fifty-one patients (73%) were or became symptomatic during follow up and received treatment according to the international criteria for treatment initiation. The 19 asymptomatic patients (27%) were only followed. Patients' median follow-up was 47 months (range 0.5-155 months)

Table 1. Patients' characteristics

Number of patients	70
Sex M/F	37/ 33
Median age	66 years (range 44-91)
Median follow-up	47 months
Disease Characteristics	%
Lymphadenopathy	17
Splenomegaly	13
Symptomatic	68
WM-IPSS stage	
1	44
2	28
3	28
Hb < 10 g/L	29
PLT < 140x10 ⁹ /L	13
Lymphocytes > 4x10 ⁹ /L	13
alb < 3.5 g/dL	22
LDH above normal	16
B2M ≥ 3.5 mg/L	40
B2M ≥ 5.5 mg/L	22
Immunofixation	
IgMkappa	74
IgMlambda	26
BM lymphoplasmacytic infiltration ($\geq 50\%$)	43

2.2. Sample Collection and Data Generation

Frozen sera collected from the 70 WM patients at the time of diagnosis and from 120 blood donor healthy individuals (HI) were retrospectively analyzed using polyclonal sheep antibodies (Freelite™ and Hevylite™, Binding Site, UK). HLCs measurements were performed by nephelometry on a Dade Behring BN™II nephelometer at the Binding Site laboratory in Birmingham. sFLCs measurements were also performed on a Dade Behring BN™II nephelometer either at the Binding Site laboratory or at the Laikon's Immunology laboratory, using Freelite™ assay, according to the manufacturers instructions.

The FLCRs were calculated with the involved sFLC as numerator. The HLCRs were calculated with the involved intact Ig as numerator and the polyclonal intact Ig of the same class as denominator, meaning that the ratio was calculated as IgM-kappa/IgM-lambda and IgM-lambda/IgM-kappa in IgM-kappa- and IgM-lambda- patients respectively.

Abnormal FLCR and HLCR were defined as any value above the 95th percentile range of HI. Abnormal polyclonal sFLC was also defined using as cut-off values the 95th percentile range of the HI. For kappa-restricted patients, lambda values above 32.1 mg/L were considered as polyclonal sFLC raise and values below 12.7 mg/L as polyclonal sFLC suppression. While, for lambda-restricted patients, kappa values above 14.9 mg/L were taken as polyclonal raise and that below 1.88 mg/L as suppression.

Systemic IgG and IgA hypogammaglobulinemia (IgG-SH and IgA-SH) were defined using our laboratory lower cut-off values as IgG < 7g/L and as IgA < 0.7g/L respectively. Immunoparesis of the same class (ISC) was defined as any polyclonal HLC value below the 95th percentile range of polyclonal HLCs in healthy individuals' (HI) sera. Thus IgM-kappa patients were considered to have ISC when their IgM-lambda was below 0.17 g/L and IgM-lambda when their IgM-kappa was below 0.29 g/L.

"High" sFLC, HLC and ratios were defined as any value above median. HLCs, HLCR, sFLC and FLCR values were compared with disease parameters such as beta2-microglobulin (β_2 M), haemoglobin (Hb), serum albumin (alb), platelets (PLT) lymphocytosis, LDH, albumin (alb), bone marrow (BM) infiltration presence of splenomegaly and lymphadenopathy and survival.

2.3. Statistical Analysis

Statistical analysis of the generated data was performed using SPSS v15.0 for windows. Hazard ratios and prognostic significance of "high" sFLC, HLC and ratios were determined by univariate Cox regression analysis. Kaplan Meier method was used for pictorial representation of survival and time to treatment. Categorical variables were compared with the chi square test (χ^2). $P < 0.05$ was considered statistically significant.

2.4. Ethical Consideration

All sera were obtained with informed consent and the use of frozen sera for the retrospective evaluation of sFLCs and HLCs was approved by the local ethical committee.

3. Results

3.1. sFLC, FLCR, HLC and HLCR in Healthy Individuals

Serum FLCs and FLCRs, IgM-kappa, IgM-lambda and HLCRs (IgM-kappa/IgM-lambda and IgM-lambda/IgM-kappa) values are shown in Table 2.

3.2. sFLC, FLCR, HLC and HLCR in Patients

Median involved kappa-sFLC was 45.65 mg/L (range 7.64 to 3090mg/L) and lambda-sFLC was 78.3 mg/L (range 4.49 to 1400 mg/L). Involved sFLC values above median correlated significantly with low serum albumin levels ($p=0.038$; $p<0.05$), BM infiltration $\geq 50\%$ ($p<0.001$; $p<0.05$)

and β_2 -M values above 3.5 mg/L or 5.5 mg/L ($p=0.019$; $p<0.05$ and $p=0.035$; $p<0.05$ respectively).

Median sFLC kappa+lambda was 67.4 mg/L (range 18.44-3093.36 mg/L) and levels above median correlated significantly with anemia ($p=0.016$; $p<0.05$), β_2 -M values above 3.5 mg/L or 5.5 mg/L ($p=0.023$; $p<0.05$ and $p=0.002$; $p<0.05$ respectively) BM infiltration $\geq 50\%$ ($p=0.006$; $p<0.05$) and with advanced IPSS stage ($p=0.01$; $p<0.05$).

FLCR levels were abnormal in 57/70 patients at diagnosis. Median FLCR in kappa restricted patients was 2.78 (range 0.55-9.19) and 6.95 in lambda ones (range 0.94-161.47). FLCR values above median correlated significantly with BM infiltration $\geq 50\%$ ($p=0.001$; $p<0.05$) and anemia ($p=0.016$; $p<0.05$). In 22/70 (31%) of patients polyclonal sFLC were low and it only correlated significantly with BM infiltration $\geq 50\%$ ($p=0.029$; $p<0.05$).

Median involved IgM-kappa was 25.45 g/L (range 0.11 to 173) and IgM-lambda was 34.80 g/L (range 0.08 to 138). Involved HLC-IgM above median correlated with BM infiltration $\geq 50\%$ ($p=0.007$; $p<0.05$) and with low serum albumin levels ($p=0.041$; $p<0.05$).

HLCR was abnormal in 66/70 patients at diagnosis. Median HLCR in IgM-kappa WM was 185.57 (range 0.90-15000) and in IgM-lambda WM 101.9 (range 2.86-1000). HLCR values above median correlated with BM infiltration $\geq 50\%$ ($p=0.013$) and the presence of splenomegaly ($p=0.028$).

In 24/70 (34%) IgG-SH was observed, in 28/70 (40%) IgA-SH and in 27/70 (39%) ISC were observed respectively. Altogether 49/70 (70%) patients had some type of polyclonal hypogammaglobulinemia (IgG-SH, IgA-SH, ISC or polyclonal sFCL) and it significantly correlated only to anemia ($p=0.023$; $p<0.05$).

Table 2. HIs' and Patients' results

	Median	95%-ile range
Kappa sFLC (mg/L)	8.85	1.88 - 14.9
Lambda sFLC (mg/L)	20.5	12.7 - 32.1
Kappa+Lambda sFLC (mg/L)	30.15	17.5 - 44.5
Kappa/Lambda FLCR	0.43	0.09 - 0.83
Lambda/Kappa FLCR	2.34	1.21 - 13.1
IgM kappa (g/L)	0.63	0.29 - 1.82
IgM lambda (g/L)	0.49	0.17 - 0.94
IgM kappa/IgM lambda HLCR	1.59	0.96 - 2.30
IgM lambda /IgM kappa HLCR	0.63	0.43 - 1.04

3.3. Relationship between sFLC, HLC and Corresponding Ratios with Time to First Treatment

Involved sFLC, sFLC (kappa+lambda) and FLCR values above median correlated significantly with time to first

treatment (TFT) ($p=0.002$; $p<0.05$, $p=0.002$; $p<0.05$ and $p=0.011$; $p<0.05$ respectively)

Involved HLC and HLCR above median correlated with TFT (both $p<0.001$). “Figures 1A and B” showed TFT according to FLCR and HLCR. Indeed, increased total IgM paraprotein measured by standard methods was also related to TFT ($p<0.001$). The presence of SH, ISC sFLC polyclonal suppression or raise was not associated with TFT

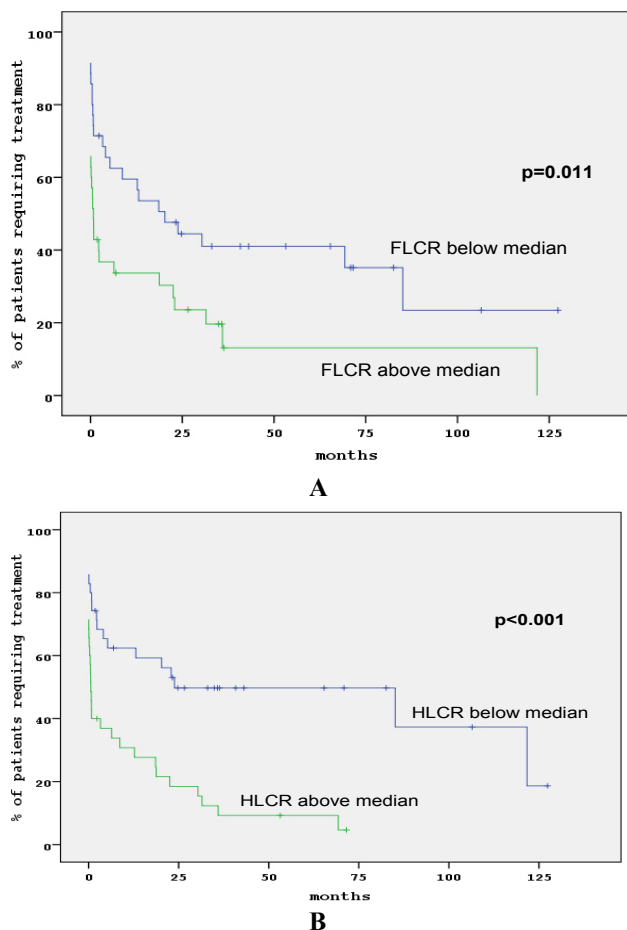


Figure 1. TFT in correlation to F LCR (A) and HLCR (B)

3.4. Relationship Between sFLC, HLC, Corresponding Ratios and Hypogammaglobulinemia with Overall Survival

Disease variables with an adverse prognostic impact on survival were WM-IPSS stage ($p<0.001$), high β_2M levels ($p<0.001$), abnormal LDH ($p=0.001$; $p<0.05$), the involved sFLC values above median ($p=0.003$; $p<0.05$) was shown in Figure 2A and sFLC(kappa+lambda) above median ($p=0.002$; $p<0.05$) was also shown in Figure. 2B.

In addition, patients with any type of hypogammaglobulinemia (IgG-SH, IgA-SH or ISC) had a tendency for longer overall survival (OVS) than the ones without hypogammaglobulinemia ($p=0.139$; $p<0.05$) as shown in Figure. 2C

In multivariate analysis, high β_2M levels (≥ 5.5 mg), abnormal LDH, involved sFLC values above median kept their significance.

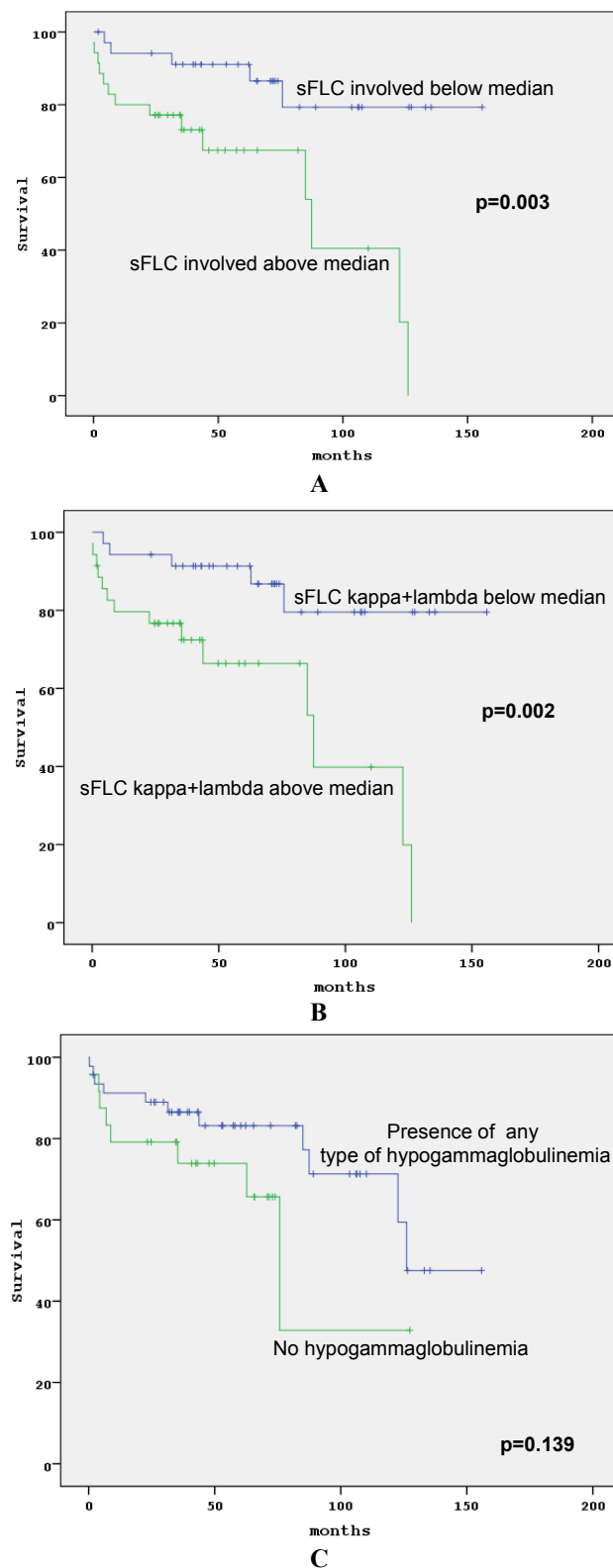


Figure 2. OVS in correlation to sFLC (A), sFLC kappa+lambda (B) and presence of hypogammaglobulinemia (C)

4. Discussion

Due to the WM wide range of manifestations and varying phenotype, resembling either a lymphoma or a frank plasma

cell dyscrasia or even an IgM gammopathy of undetermined origin, the exact paraprotein quantification and sensitive prognostication are needed for improved diagnosis, monitoring and management[3,5].

IgM paraprotein, like any other immunoglobulin (Ig), is composed of two Ig heavy chains and two light chains. It was shown, as already mentioned, that Ig light chains are normally secreted in slight excess [10] and that the amount of the excess may consistently increase and carry prognostic information in monoclonal diseases.

With regard to the contribution of sFLC levels in WM patients at diagnosis, a restricted number of studies reported that they can be increased and in such case, correlate with markers of disease activity, such as increased β 2M, anemia [23,24], low serum albumin levels as well as with a shorter time to treatment[25,26]. sFLC levels were also found to constitute a marker of response and of disease progression[23]. We confirmed previous results and showed, in addition, that increased sFLC levels at diagnosis correlated significantly with a shorter overall survival. Intriguingly, sFLC levels were more valuable for monitoring and prognostication than FLCR; this was not observed in multiple myeloma [16,17] and chronic lymphocytic leukemia [27].

In the present study, we also demonstrated that increased HLC-IgM correlated significantly with markers of disease activity such as extensive BM infiltration and low serum albumin levels, while high HLCR values with extensive BM infiltration also and the presence of splenomegaly. A recent study[28] suggested that, in WM, IgM secretion is related to the number of BM infiltrating plasma cells, as evaluated by CD138 staining, a finding in keep with results from our group, showing that bone marrow infiltration by CD138 expressing lymphoplasmacytes in WM strongly correlated with IgM levels[29]. In any case, although paraprotein levels are not considered reflecting tumor burden, an association with the percentage of malignant infiltrating cells has been found. Thus, it is suggested that HLC-IgM and HLCR better reflect tumor burden than IgM as classically determined.

In addition, due to the wide range of clinical manifestations and varying survival of WM, the establishment of prognostic factors is needed. Retrospective studies had highlighted a number of adverse factors for survival in WM, including advanced age, anemia, thrombocytopenia, hypoalbuminemia, elevated serum β 2-M, increased serum LDH, high serum IgM concentrations, a poor performance status, the pattern or the percentage of BM infiltration and the presence of lymphadenopathy and organomegaly[6-8, 30-38]. In addition, several investigators have applied and validated in WM[7, 39,40], the staging systems used in similar disorders such as IPI[41] for high grade lymphoma, ISS[42] for multiple myeloma and FLIPI[43] for follicular lymphoma. Five years ago, 7 cooperative research teams designed and successfully applied a scoring system for WM patients requiring therapy, the International Prognostic Scoring System for Waldenström's Macroglobulinemia (ISSWM)[22], that was

further validated by others[44,45].

In this study, the adverse prognostic impacts of increased serum β 2M, LDH levels and WM-IPSS staging system were observed again, while in addition, monoclonal sFLC and sFLC (kappa+lambda) above median correlated with a shorter overall survival. Recently, the prognostic superiority of the sum of monoclonal and polyclonal sFLC compared to the FLCR was shown in chronic lymphocytic leukemia [19,20], a disease with similarities to WM. Hence, in the present study was found that the sum also apply prognostically for TFT and OVS in WM.

Polyclonal hypogammaglobulinemia is a common finding in WM; however a consistent proportion of patients present normal or even increased uninvolved Igs[7].

In the present study, we found that the presence of hypogammaglobulinemia involving Igs of the same and/or of other classes was in favour of a longer survival, while on the contrary, increased uninvolved Igs was not. This finding is in keep with the observation that in CLL, uninvolved light chain increase confer a worse outcome compared to patients with a normal secretion[19,20]. The finding is intriguing and opens issues on the biologic role of the reactive lymphocytic component in indolent lymphoproliferative disorders. Others unanswered issues on possible suppressive inherent mechanisms, such as TGF β [46] and others that affect both normal and malignant lymphocytes and may contribute in disease control should also be explored. However, others reported on the contrary that low levels of serum IgG and IgA were associated with disease progression[47] and further investigations are needed.

5. Conclusions

Serum FLC measurements appear useful for prognostic purposes, HLCR powerfully predicted time to first treatment and therefore may be useful in diagnostic and prognostic settings, while HLC and HLCR also help for follow-up of WM patients. In addition the Hevylite assay provides information about the amounts of the uninvolved Ig subsets that may reflect biology important mechanisms. Further studies are awaited.

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