RESEARCH ARTICLE

DOI: 10.4274/tjh.galenos.2024.2023.0469

Do Alarmins Have a Role in Multiple Myeloma?

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December 17, 2023
February 29, 2024

Abstract
Objective: Calprotectin (CLP), S100A6, and High Mobility Group Nucleosome-Binding Protein 1 (HMGN1), known as alarmins, are involved in the pathogenesis of many tumors. In this study, we aimed to inve stigate the relationship of serum CLP,
S100A6, and HMGN1 levels with clinical and laboratory findings in Multiple Myeloma (MM) patients and their role in the pathogenesis of MM.
Materials and Methods: We measured serum CLP, S100A6, and HMGN1 levels in
55 newly diagnosed patients and 32 healthy controls (HC).
Results: We determined significantly decreased serum CLP, S100A6 ve HMGN1 levels in
MM patients compared to HC (p=0.012, p=0.001, p=0.030, respectively).
ROC analysis was used to determine a diagnostic cut-off value for serum CLP,
S100A6 and HMGN1; the cut off value for CLP was <98 ng/ml (AUC = 0.663, 95% CI 0.554-0.761, p=0.009), S100A6 was <1174.5 pg/ml (AUC = 0.706, 95% CI 0.598-0.799, p=0.001), and HMGN1 was <440.18 pg/ml (AUC = 0.640, 95% CI 0.530-0.740, p:0.03).
CLP level was found to be statistically significantly in light chain MM patients (
91.58±22.57) higher than in heavy chain MM patients (79.42±15.83) (p=0.03).
A negative correlation was observed between CLP and M protein, IgG, globulin, and beta
2 microglobulin (correlation coefficient: -0,361; -0,370; -0,279; -0,300, p=0,024, p=006, p=0,04, p=0,0033).
Conclusion: In this study, we found that serum CLP,
S100A6, and HMGN1 levels were statistically lower in newly diagnosed MM patients compared to HC. These results suggest that CLP may binds to the paraprotein produced by heavy chain MM in the blood and therefore its blood levels are found to be low. Additionally, low levels of HMGN1, which is involved in
DNA repair, suggest that HMGN1 may contribute to the complex genetic abnormalities found in MM.
Key words: S100A8/9, calprotectin, S100A6, HMGN1, 1q21 gain/amplification

ÖZET
Amaç: Alarminler olarak bilinen Kalprotektin (CLP), S100A6 ve High Mobility Group Nucleosome-Binding Protein 1 (HMGN1), birçok tümörün patogenezinde rol almaktadır. Bu
çalışmada, Multiple Myelom (MM) hastalarında serum CLP, S100A6 ve HMGN1 düzeylerinin klinik ve laboratuvar bulgularıyla ilişkisini ve MM patogenezindeki rolünü araştırmayı amaçladık.
Yöntem: Yeni tanı almış 55 MM hastası ve 32 sağlıklı gönüllünün serum CLP, S100A6 ve HMGN1 düzeyleri ELISA yöntemiyle ölçüldü. Hastaların medikal kayıtları tarandı.
Bulgular: Hastaların tanıda bakılan CLP, S100A6 ve HMGN1 seviyeleri kontrol grubuna göre istatistiksel olarak anlamlı derecede düşük bulundu (sırasıyla p=0,012, p=0,001, p=0,030). ROC analizinde MM için CLP

<98 ng/ml (AUC = 0,663, %95 CI 0,554-0,761, p=0,009), S100A6 <1174,5 pg/ml (AUC = 0,706, %95 CI 0,598-0,799, p=0,001), HMGN1 için ise <440,18 pg/ml (AUC = 0,640, %95 CI 0,530-0,740, p:0,03) tanısal cut-off değeri olarak belirlendi. CLP seviyesi, hafif zincir MM hastalarında (Mean± standart sapma; 91,58±22,57), ağır zincir MM hastalarına (79,42±15,83) göre istatistiksel olarak anlamlı derecede yüksek bulundu (p=0,03). CLP ile M protein, IgG, globülin ve beta 2 mikroglobulin arasında negatif korelasyon gözlendi (sırasıyla korelasyon katsayısı: -0,361; -0,370; -0,279; -0,300, p=0,024, p=006, p=0,04, p=0,0033).
Sonuç: Çalışmamızda yeni tanı almış MM hastalarında CLP, S100A6 ve HMGN1 serum seviyeleri tanısal anlam taşıyacak düzeyde düşük bulunmuştur. Bu sonuçlar, CLP'nin kanda ağır zincir MM tarafından üretilen paraproteine bağlanabileceğini ve dolayısıyla kan seviyelerinin düşük bulunduğunu göstermektedir. Ayrıca DNA tamirinde rol alan HMGN1'nin düşük düzeyleri, HMGN1'in MM'de bulunan komplex genetik anormalliklere katkı sağlayabileceğini düşündürtmektedir.

INTRODUCTION

Multiple myeloma (MM) is a haematological malignancy characterised by clonal proliferation of plasma cells. The incidence of MM is approximately 160.000 cases/year worldwide. [1] In the last two decades, significant progress has been made in understanding the pathophysiology of MM. With the introduction of new generation agents, the survival of MM patients has improved significantly. However, MM is still recognised as an incurable disease. [2,3] Therefore, the identification of new pathways and biomarkers involved in the pathogenesis of MM is extremely important for the identification of new therapeutic targets.

Alarmins are present intracellularly in granules, the nucleus or cytosol, and are rapidly released as a result of degranulation, cell damage/death or immun induction. In the extracellular compartment they behave as cytokins and act as early warning signals for the immune system, to promote innate and adaptive immune responses. Alarmins can be classified into several categories including AMPs, heat shock proteins, nucleotides/metabolites, certain degradation products of the extracellular matrix, nuclear binding proteins (eg, HMGB1, HMGN1), and ion-binders (eg, S100A6, A8 and A9). [4]

The incidence of chromosome 1q21 gain and amplification in MM increases with the occurrence of relapses and is approximately 40%. [5] In many studies, acquisition and especially amplification of 1q21 has been found to be associated with poor prognosis independent of other poor risk factors. [6, 7] Most of the genes belonging to the S100 protein family (S100A1-16) are encoded in the 1q21 region. The S100 protein family, a subgroup of calcium-binding EF-hand type proteins, consists of members that tend to form homodimeric and/or heterodimeric complexes with each other. They are involved in many processes such as cell proliferation, differentiation, apoptosis, and inflammation and calcium haemostasis. They bind to various receptors such as Toll Like Receptor 4 (TLR4), receptor for Advanced Glycation End Products (RAGE) and activate JAK-STAT, Nuclear Factor Kappa B (NF-κB) and Mitogen-Activated Protein Kinase (MAPK) pathways. [8-10] Increased expression of S100 family members has been identified in various cancer types and has been correlated with tumour cell proliferation, invasion, metastasis and angiogenesis. [11-14] Calprotectin (CLP) has a heterodimeric structure consisting of S100A8 and S100A9 subunits. [15] S100A6 is another member of the S100 protein family. In the literature, there are few studies directly investigating the correlation between MM and CLP, S100A6.

High Mobility Group Nucleosome-Binding Protein 1 (HMGN1) is a protein located in the cell nucleus that plays a role in the regulation of DNA repair, transcription and replication. (16, 17) It has important functions in host defence and tissue repair. [18] Rat experiments predict that HMGN1 may be used as a therapeutic agent in the treatment of malignancy, as well as tumour suppressor effects, and may have a potential role in vaccine applications. [19] Our literature search did not reveal any study investigating the correlation between HMGN1 and MM.

In this study, we aimed to determine serum CLP, S100A6 and HMGN1 levels in newly diagnosed MM patients and to investigate their possible roles in MM pathogenesis by evaluating their relation with clinical findings.

MATERIAL AND METHOD

In this study, 55 patients with MM newly diagnosed in Department of Hematology, Medical Faculty, Kocaeli University and 32 healthy volunteers participated. MM diagnosis was determined according to the International Myeloma Study Group criteria. [19] Patients with rheumatological disease, active infection or concurrent malignancy were excluded. Staging was performed according to the International Staging System (ISS) and Revised ISS (R-ISS) criteria. Bone marrow biopsy was performed in all patients at the time of diagnosis. Positron Emission Tomography (PET) was preferred to evaluate bone involvement. Medical records of the patients were reviewed.

Written informed consent was obtained from all participants. Ethical approval was obtained from the faculty ethics committee on 21.10.2021 (Project no: 2021/285, Decision no: GOKAEK -2021/18.06).

Blood samples obtained from patients and control group were centrifuged at 3500 rpm for at least 15 minutes. The serum obtained was stored at -80 °C for the duration of the study. S100A6, CLP and HMGN1 levels of these samples were determined by sandwich enzyme-linked immune-sorbent assay (Sandwich ELISA) method (Cat. No: FINE TEST EH1923, Cat. No: ELABSCIENCE E-EL-H2357, Cat. No: FINE TEST EH2476) were analysed. The sensitivity of these tests are 37.5 pg/ml, 0.94 ng/mL, < 0.188 pg/ml, respectively. Both intra-assay and inter-assay coefficient of variation (%CV) values of all three tests were below 10%.

Statistical analysis was performed using IBM-Statistical Package for the Social Sciences (SPSS) program version 20 software package. Demographic variables were analyzed using descriptive analyses. Numeric variables were presented with mean ± standard deviation or median (minimum-maximum or 25th-75th percentile). Categorical variables were summarized as numbers (percentages). The normality of distribution of continuous variables was checked using Kolmogorov-Smirnov and the Shapiro-Wilk test. Continuous variables which distributed non-parametric were compared using the Mann-Whitney U-test and Kruskal Wallis test. Continuous variables which distributed parametric were compared using Independent Samples T-test and Annova test. Categorical parameters were analyzed using the Chi-square test and Fisher's Exact test. Paired comparison analysis was performed to determine which entity is preferred over others.). Associations between numeric variables were determined by Spearman correlation analysis. Receiver operator characteristic (ROC) analysis was used to determine AUC, sensitivity, specificity and cut-off values. All statistical analyses were carried out with 5% significance and a two-sided p-value <0.05 was considered statistically significant.

RESULTS

In this study, the results of 55 MM patients and 32 healthy volunteers as a control group were analysed. The two groups were similar in terms of age, gender and comorbidities (p=0.328, p=0.64, p=0.359, respectively). (Supplement 1) The median age of the patients was 63 years (range, 40-81 years) and 38.2% were female. The characteristics of the patients are shown in Table 1.

CLP, S100A6 and HMGN1 levels in MM patients were found as follows, respectively; (Median, 25th- 75th percentile); 80.7 ng/ml (69.4—97.1); 932 pg/ml (727.2-1120.5); 439.4 pg/ml (338.1-517.5). In the control group, CLP, S100A6 and HMGN1 levels were determined as follows, respectively; 93.9 ng/ml (78.9—130.9); 1201.2 pg/ml (861.8-1536.7); 488 pg/ml (443.5-558.3)). CLP, S100A6 and HMGN1 levels of the patients at the time of diagnosis were found to be statistically significantly lower than the control group (p=0.012, p=0.001, p=0.030, respectively). (Figure 1)

In the ROC analysis, CLP <98 ng/ml cut-off value for MM was diagnostic (p=0.009). With this cut-off value, the sensitivity and specificity of the test were found to be 78% and 50%, respectively. A cut-off value of <1174.5 pg/ml for S100A6 level was found to be diagnostic with a sensitivity of 89.09% and a specificity of 53.1%. (p=0.001). In the analysis performed for HMGN1, a cut-off value of <440.18 pg/ml was determined (p:0.03). With this cut-off value, the sensitivity and specificity of the test were 52.7% and 78.1%, respectively. ROC curves are shown in Figure 2.

CLP level was found to be statistically significantly higher in patients with light chain MM (mean ± standard deviation; 91.58±22.57) than in patients with heavy chain MM (79.42±15.83) (p=0.03). The correlation between calprotectin, S100A6, HMGN1 levels and clinical and laboratory findings are summarised in Table 2.

There were only 9 patients who had chromosome 1q21 gain and amplification analysis performed and 2 of them were positive. S100A6 and CLP levels were determined as 657.4 pg/ml (248.9-1065.8), 93.5 ng/ml (82.07-105) in positive cases and 966.3 pg/ml (756-1160.3), 76.2 ng/ml (67.2-89.3) in negative cases, respectively.

The correlation between CLP, S100A6, HMGN1 levels and clinical and laboratory values are summarised in Table-3.

DISCUSSION

The role of MAPK and NF-κB, which are activated by the RAGE pathway in which CLP is a ligand, in the pathogenesis of MM is known. [21-23] However, there are limited number of studies in the literature on the role of CLP in the pathogenesis of MM. S100A9 has previously been shown to increase the secretion of cytokines such as TNF-α, IL-6 and IL-10 from myeloid derived suppressor cells, which play an important role in the survival and proliferation of myeloma cells. [24] Lin et al., in their study on cell lines and rats, suggested that CLP contributes to MM progression by increasing megakaryopoiesis and indirectly angiogenesis. They also found that CLP levels were higher in the bone marrow of MM patients than in peripheral blood, but they did not compare it with healthy controls. [25] In another study, faecal CLP levels were found to be significantly higher in MM patients compared to the healthy control group. When newly diagnosed MM patients and treated MM patients were compared, faecal CLP levels were found to be higher in the newly diagnosed group. [26] In our study, CLP levels were examined in serum instead of faeces as in studies conducted in solid malignancies and rheumatological diseases. Serum CLP levels were found to be low enough to have diagnostic significance in the MM patient group compared to the healthy control group. A significant negative correlation was found between CLP levels and serum M protein, Ig G and globulin levels. In addition, serum CLP levels were found to be statistically significantly higher in light chain MM patients than in heavy chain patients. These results suggest that CLP may binds to the paraprotein produced by heavy chain MM in the blood and therefore its blood levels

are found to be low. The negative correlation between beta 2 microglobulin, which is a prognostic factor for MM, and CLP level suggests that low CLP level may be associated with poor prognosis. However, this hypothesis needs to be confirmed by survival analyses.

In a recent study conducted mostly on relapsed refractory MM patients, increased S100A6 gene expression and high protein levels in CD138 positive cell samples were found. The association of increased gene expression with advanced stage disease and decreased overall survival was shown in the same study. [27] In our study, serum S100A6 levels were significantly lower in patients with newly diagnosed MM compared to healthy controls. The low serum levels of S100A6 despite its increased intracellular level may be due to increased clearance, limited release into the extracellular space, or rapid reuptake by binding to its cellular receptors. As a matter of fact, it is known that the secretion of S100A6, which is located in the cytoskeleton, from neutrophils is very limited and conditional, unlike CLP. [28] In addition, Wnt/β-catenin pathway is activated in MM and S100A6 increases intracellular β-catenin by interacting with calcyclin-binding protein/Siah-1-interacting protein. [29,30] As a result, S100A6 may be primarily involved in intracellular pathways in the pathogenesis of MM.

Since analysis was not performed on a sufficient number of patients, the effect of chromosome 1q21 gain and amplification on CLP and S100A6 could not be evaluated.

Genetic analyses have revealed that both the progression of myeloma precursor conditions to MM and MM disease progression are associated with clonal evolution as a result of accumulating mutations. [31, 32] HMGN1 has global roles in the repair of DNA lesions and local roles in the transcriptional control of proto-oncogenes and tumour suppressor genes. It has been shown that proto-oncogenes and pro-metastastic genes such as c-fos, BCL3 and N-cadherin are upregulated in HMGN1 negative cell lines.*[33,34]* In our study, serum HMGN1 levels were found to be significantly lower in MM patients compared to the control group. If low serum HMGN1 levels are a reflection of low intracellular levels; this can be interpreted as HMGN1 may contribute to the genetic abnormalities seen in MM. However, the presence of autoantibodies against HMGN1 in the blood in some autoimmune diseases has been shown in the literature, and the low levels we detected in MM patients may be of immune origin. [35-37] The immunomodulatory effects observed in studies evaluating HMGN1 combinations in cancer treatment suggest that HMGN1 may be a promising molecule in the treatment of MM in which the microenvironment plays an important role in the pathogenesis. [19, 38-40]

The limitations of this study can be listed as having a relatively small cohort, lack of consecutive sampling, and not being supported by immunohistochemistry and in vitro cell line models. However, the easily accessible ELISA method provides an advantage in terms of reproducibility and verifiability.

In conclusion, we found that serum levels of CLP, S100A6 and HMGN1 were significantly lower in newly diagnosed MM patients. Further studies using molecular, genetic and immunohistochemical methods with larger and consecutive samples are needed to clarify the roles of CLP, S100A6 and HMGN1 in MM pathogenesis.

Conflict of Interest

No conflicts of interest

Funding

This study was supported by Kocaeli University Scientific Research Projects Coordination Unit. Project Number: TSA-2022-2836

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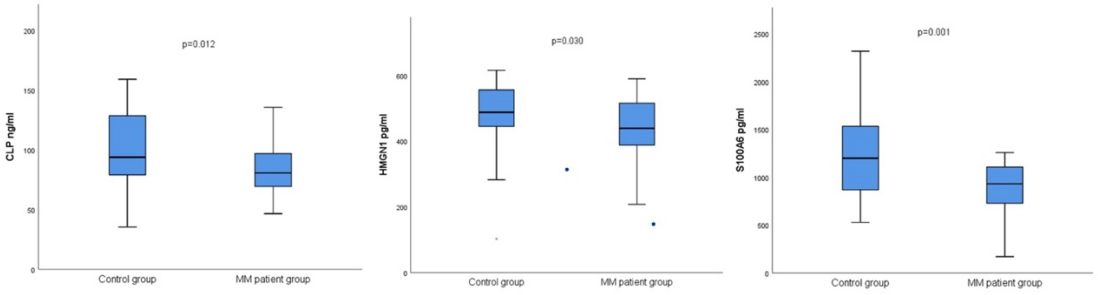

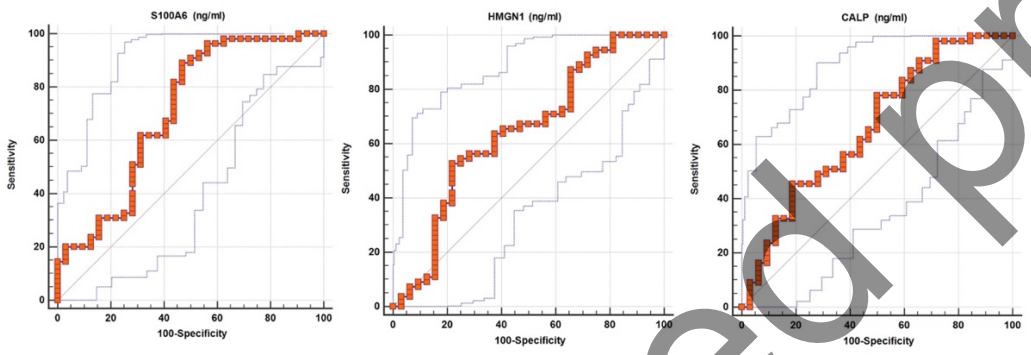

Feature	n (%)	Feature	n (%)
*Stage (ISS)**		*Lytic bone lesions****	
I	10 (19,6)	Not exist	11 (21,2)
II	17 (33.4)	<4	10 (19,2)
III	24 (47)	4-10	11 (21,2)
*R-ISS***		>10	20 (38,4)
I	6 (18,2)	*Pathological fracture****	
II	18 (54,5)	Exist	11 (21,2)
III	9 (27,3)	Not exist	41 (78,8)
Type of MM		*Plasmacytoma****	
IgA kappa	5 (9)	Exist Not exıst	10 (19,2) 42(80,8)
IgA lambda	3 (5,5)	*Comorbidity*	
IgG kappa	18 (32,7)	CAD	5 (9,1)
IgG lambda	14 (25,5)	HT	8 (14,5)
Lambda light chain disease	5 (9,1)	DM+HT	7 (12,7)
Kappa light chain disease	8 (14,5)	No exist	25 (45,5)
Non-secretory	2 (3,6)	Others	10 (18,2)

Abbreviations: ISS, International Staging System; R-ISS, revised ISS; CAD, coronary artery disease; HT, hypertension; DM, diabetes mellitus;

** The data of 4 patients is missing*
*** The data of 33 patients is exist*
**** The data of 3 patients is missing*

	Calprotectin (ng/ml) median (min-max)		**S100A6 (pg/ml) median (min-max)**		**HMGN1 (pg/ml) median (min-max)**	
Sex						
Female	76,93 (556,02-111,48)	p=0.390	893,4 (181,33-1980,7)	p=0.291	436,75 (363,72-582,06)	p=0.96
Male	82,7 (46,72-135,82)		981,39 (136,33-1260)		445,68 (207,90-590,12)	
Comorbidite						
Exist	77,9 (52,7-117,9)	p=0.417	950,9 (777,4-1056,2)	p=0.800	436,49 (271,3-588,39)	p=0.642
Not exist	80,7 (46,7-135,8)		932 (172,06-1260,06)		440,18 (207,9-590,12)	
Type of MM						
Light chain	90,01 (58,02-135,82)	*p=0.030*	1065,8 (172-1231)	p=0.192	449,1 (370,3-590,1)	p=0.88
Heavy chain	77,18 (46,72-111,48)		923,5 (136,3-1980,7)		436,2 (207,9-588,39)	
ISS						
I	78,36 (62-102,23)	p=0.825	979,7 (172-1980)	p=0.725	437,3 (207,9-582,06)	p=0.677
II+III	81,24 (46,72-135,82)		954,56 (136,33-1260)		438,11 (271,30-590,1)	
R-ISS						
I	75,17 (62-90,01)	P=0.228	979,76 (172,06-1980,7)	p=0.845	405,28 (207,90-524,51)	p=0.217
II+III	85,24 (52,7-135,82)		923,53 (243,46-1251)		436,24 (271,3-590,12)	
Lytic bone lesions						
<4	83,75 (60,93-111,48)	p=0.507	1003 (172,06-1260)	p=0.825	436,24 (360,72-588,39)	p=0.900
>4	77,18 (46,72-135,82)		972,46 (181,33-1980,73)		439,8 (207,90-590,1)	
Pathological fracture						
Exist	95,5 (60,9-135,8)	p=0.170	896,33 (538,05-1010,91)	p=0.190	494 (207,90-590,12)	p=0.710
Not exist	80,78 (46,72-117,98)		985,66 (842,64-1047,56)		436,7 (271,3-588,3)	
Plasmacytoma						
Exist	76,93	p=0.591	987,06	p=0.703	494	

	(52,7-105)	(181,33-1251)	(367,27-582,06)	
Not exist	81,71 (46,72-135,82)	972,46 (172,06-1980,7)	434,54 (207,9-590,1)	p=0.061

Abbreviations: ISS, International Staging System; R-ISS, revised ISS

		1	2	3	4	5	6	7	8	9	10	11
1-S100A6	r	1,000										
	p											
2-Calprotectin	r	**,256***	1,000									
	p	**,017**										
3-HMGN1	r	**,534****	**,357****	1,000								
	p	**,000**	**,001**									
4-Beta 2 microglobulin	r	-,063	**-,300***	-,088	1,000							
	p	,661	**,033**	,538								
5-CRP	r	,173	,114	,185	**,289***	1,000						
	p	,205	,407	,177	**,040**							
6-M protein	r	-,056	**-,361***	-,045	**,439****	,068	1,000					
	p	,733	**,024**	,784	**,008**	,683						
7-Age	r	-,087	-,060	-,167	,259	,264	**,353***	1,000				
	p	,422	,582	,123	,066	,051	**,027**					
8-IgG	r	-,018	**-,370****	-,176	**,289***	,119	**,701****	**,483****	1,000			
	p	,897	**,006**	,204	**,042**	,392	**,000**	**,000**				
9-IgA	r	-,075	,092	,129	-,209	,057	-,249	-,257	**-,528****	1,000		
	p	,586	,502	,349	,141	,680	,126	,059	**,000**			
10-Globulin	r	-,145	**-,279***	-,027	**,318***	,098	**,906****	**,342***	**,679****	-,157	1,000	
	p	,295	**,041**	,847	**,024**	,481	**,000**	**,011**	**,000**	,257		
11-Plasma cell ratio	r	,062	-,086	,032	,172	,079	**,405***	,090	,022	-,262	,111	1,000
	p	,669	,551	,826	,249	,585	**,014**	,534	,879	,066	,449	