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Analysis of Mesencephalic Astrocyte-derived Neurotrophic Factor in Multiple Myeloma

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Abstract. *Background/Aim: Multiple myeloma (MM) is characterized by high production of immunoglobulins resulting in a constant source of endoplasmic reticulum (ER)-stress. Mesencephalic astrocyte-derived neurotrophic factor (MANF) was identified as a possible circulating biomarker that could help in monitoring ER-stress mediated diseases. Materials and Methods: To assess the relevance of MANF in MM, we performed in silico and in vitro analysis in malignant cell lines including the myeloma cell line RPMI 8226. Serum MANF concentration was compared between healthy subjects (n=60), patients with MM (n=68), or those with monoclonal gammopathy of undetermined significance (MGUS) (n=73). Results: MANF mRNA expression was upregulated in the RPMI 8226 cell line, and higher secretion of MANF was measured in RPMI 8226 supernatant. Serum MANF levels were not significantly different between MM or MGUS patients and those in age- and sex-matched healthy controls. Conclusion: MANF was not validated as a biomarker of interest in MM patients. Its potential implication in myeloma pathogenesis should be investigated.*

Multiple myeloma (MM) is a chronic hematological malignancy typically characterized by a clonal expansion of plasma cells in bone marrow with production of monoclonal immunoglobulin (Ig) or its components (free k or l light chains) and several organ dysfunctions (1). MM accounts for 13% of all hematological cancers in developed countries with an estimated 80,000 deaths per year worldwide (1), and is still considered as an incurable disease (2). Several studies have highlighted the role of the unfolded protein response (UPR) in MM biology and chemotherapy escape (3-6).

The protein-folding machinery in the endoplasmic reticulum (ER) is particularly challenged in plasma cells in response to the requirement of high protein synthesis, resulting in a constant source of ER stress (7, 8). UPR^{ER} is a signaling network that preserves ER homeostasis by down-regulation of mRNA translation, promoting new chaperone synthesis and degradation of misfolded proteins (8). UPR^{ER} represents a survival strategy for myeloma cells because of increased production of proteins (*i.e.* Ig and cytokines) and stromal changes (3, 4). UPR^{ER} is involved in the response to proteasome inhibitors (5), and clinical evaluation of UPR^{ER} could identify early-diagnosed patients with high risk of drug resistance or relapse (6).

Mesencephalic astrocyte-derived neurotrophic factor (MANF) has been identified as a possible circulating biomarker that could help in monitoring ER stress-mediated pathologies (9-13). MANF is an 18 kDa soluble protein, also called ARP (Arginine-rich protein) or ARMET (Arginine rich mutated in early-stage tumors). The MANF gene promoter carries the sequence endoplasmic reticulum stress response element (ERSE) recognized by UPR transcription factors (14). MANF can promote survival of a wide variety of cells and tissues by driving UPR^{ER} under conditions of physiologic and pathologic stimuli of ER stress (9, 14-22). Moreover, MANF could prevent terminal UPR-induced cell death by negatively regulating UPR^{ER} pathways upstream (9,

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17) and by directly inhibiting pro-apoptotic UPR-induced factors (16, 21).

Although MANF is predominantly located in the ER lumen (14, 19), it can also be partially secreted in the intercellular space (19, 23) providing access for biological measurement reflecting UPR activation (9-13, 24, 25). The aim of our study was to assess the relevance of MANF in the context of MM. First, we investigated the possible use of MANF as a biomarker in MM through *in silico* and *in vitro* analysis. Second, we addressed the clinical utility of MANF measurement in predicting clinical outcome, prognosis, and response to treatment in a cohort of patients with plasma cell dyscrasia.

Materials and Methods

In silico analysis. An *in silico* analysis was performed based on the National Cancer Institute-60 (NCI-60) database. NCI-60 is an open access resource introduced in 1990 for the identification and characterization of new candidate drugs with anticancer properties (26). It includes sixty human tumor cell lines from nine common types of cancer, namely myeloid and lymphoid blood malignancies and breast, lung, colon, prostate, ovary, kidney, and brain cancers.

Cell culture. Human MM cell lines (MM.1S [CRL-2974] and RPMI 8226 [CCL-155]) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human hepatocellular carcinoma (HCC) cell lines (PLC/PRF5, Hep3B, Huh7) were obtained from Dr Wychowski (Biology Institute, Lille, France). Human glioblastoma cell lines (H4, A172, U87) were purchased from Sigma-Aldrich (Lyon, France), and squamous cell carcinoma cell lines (SCC9, BICR18, PECA/PJ42, PECA/PJ34) from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). Cells were incubated at 37˚C in a humid atmosphere with 5% CO_2 and cultured in Dulbecco's Modified Eagle's Medium High glucose (Sigma-Aldrich, Saint Louis, MO, USA) with 10% fetal bovine serum (Jacques Boy, Reims, France), decomplemented for MM lines, and enriched with 1% glutamine and 1% penicillin/streptomycin. The difference in cell adherence between MM lines (non-adherent cells) and solid tumor lines (adherent cells) was taken into consideration for culture and experiments.

Measurement of MANF concentration in vitro. Basal intracellular and extracellular MANF protein concentration levels were measured in the culture supernatants and cellular lysates of the cell lines by ELISA (Human MANF ELISA kit, ab215417, Abcam, Cambridge, UK) according to the manufacturer's procedures. Absorbance was measured at 450 nm (Labsystems iEMS Reader MF, Vantaa, Finland) with Ascent Software version 2.6. To determine the ratio of secreted to intracellular MANF, we measured the MANF levels secreted in the cell supernatant after 18 h of cell culture.

Intracellular MANF concentration was measured after lysis of a cell pellet in 200 µl RIPA lysis buffer [20 mM Hepes KOH, 5 mM NaCl, 100 nM NaF, 50 nM βglycérophosphate, 1 mM Na3VO4, 1 mM NaPP, and a protease inhibitor cocktail (P8340, Sigma-Aldrich)]. A count of the total number of cells present in each sample was determined by Trypan blue and optical microscopy. Results are expressed as amount (ng) of MANF protein per 100,000 cells present in each sample.

Measurement of MANF concentration in human serum samples. MANF concentration was determined in the serum samples of patients and healthy subjects using the same ELISA technique according to the manufacturer's procedures. After verification, duration of serum storage did not have any influence on MANF levels.

Healthy subjects. MANF was measured in 60 healthy subjects aged 20-80 years (five men and five women per age group of ten years) with no medical history except for tobacco use, high blood pressure dyslipidemia, and/or chronic alcohol consumption without hepatic disease. Healthy subjects were recruited from the general population.

Patients with monoclonal gammopathy. We retrospectively included patients (≥18 years) followed in the Department of Internal Medicine (Amiens-Picardie University Medical Center, Amiens, France) for MGUS (IgA or IgG) or MM according to International Myeloma Working Group (IMWG) criteria (1). Human samples were obtained from the "BioResource Center (BRC) of the Amiens-Picardie University Hospital de Picardie (BRIF: BB-0033-00017). Blood samples were previously collected from September 1, 1995 to December 31, 2018. We did not include patients with IgM MGUS, Waldenström's disease, lymphoplasmocytic lymphoma, Ig heavy or light-chain deposition disease including primary amyloidosis, POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes), plasma cell leukemia, or solitary plasmacytoma. Demographic, clinical, biological, and cytogenetic data were collected from medical reports (DX-CARE software).

Ethics. Conforming to the Declaration of Helsinki and French legislation, all persons included in this study had previously given their informed and signed consent to store a serum sample for further clinical investigations. Data processing was in accordance with the reference methodology MR-004 of the French data protection authority (CNIL).

Statistical analysis. The Shapiro-Wilk test was used as a normality test. Continuous variables with a normal distribution are presented as mean±standard deviation and were analyzed by Student's *t*-test. Continuous variables without a normal distribution are presented as median [interquartile range] and were analyzed by Wilcoxon-Mann-Whitney and Kruskal-Wallis tests. Qualitative data are described as frequency (%, n/N) and were analyzed by Chi^2 test (Fischer's exact test if required). We investigated correlations between MANF levels and variables by either Pearson (variables with normal distribution) or Spearman (variables without normal distribution) correlations. A *p*-value <0.05 in two-tailed tests was considered statistically significant. All statistical analyses were performed with SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA).

Results

In silico analysis. An *in silico* analysis was performed to identify genes with expression highly correlated with UPR gene expression (*i.e.* a threshold $r^2>0.3$ according to Pearson correlation, $p<0.05$) (Table I). We found 8 genes with expression strongly correlated with UPR activation, namely *HSPA5, HSP90B1, HYOU1, HERPUD1, EDEM1, SEL1L, ATF4,* and *ATF6,* as they encode proteins playing a crucial

Table I. *Correlation between UPR genes and MANF gene expression in malignant human cell lines.*

Gene	r^2	*p*-Value
HSPA5	0.59	1×10^{-6}
HERPUD1	0.56	2×10^{-6}
HSP90B1	0.5	5×10^{-5}
HYOU1	0.42	0.0009
EDEM1	0.41	0.0012
ATF6	0.39	0.0022

Data were obtained from the National Cancer Institute-60. Statistical analyses were performed by Pearson correlation (threshold $r^2>0.3$ and $p<0.05$).

role in the *ER-stress* response. The expression of six of the genes was also highly and significantly correlated with MANF mRNA levels (Table I). These genes encode ER chaperones, namely heat shock protein 90 beta family member 1 (*HSP90B1*) or Grp94, heat shock protein family a member 5 (*HSPA5*) or Grp78, hypoxia up-regulated 1 (*HYOU1*) or Grp 170, ER degradation enhancing alpha-mannosidase like protein 1 (*EDEM1*) and homocysteine inducible ER protein with ubiquitin like domain 1 (*HERPUD1*), and factor ATF6. Moreover, mRNA expression of MANF varies with types of solid tumors and blood malignancies (Figure 1). The human myeloma cell line RPMI 8226 appears as one of human malignant cell lines (solid tumors and hematological malignancies) with the highest mRNA expression of MANF at the basal state (Z-score=+1.8). In addition, the MANF transcriptional levels in the MM cell line were higher than those of cell lines of other hematologic cancer types.

In vitro MANF assay. Intracellular expression of MANF was significantly higher in the RPMI 8226 cell line compared with that in MM1.S ($p=0.013$) (Figure 2). Furthermore, extracellular levels of MANF were also significantly higher than those of other malignant human cell lines studied ($p=0.018$). For all cell lines, intracellular MANF levels were higher than those found in cell supernatants. Intracellular and secreted MANF concentrations were significantly correlated (according to Pearson correlation with $r^2=0.17$, $p=0.0037$). Hence, the secreted form of MANF reflects its intracellular protein pool.

Cohort characteristics. The healthy population included 30 women and 30 men. Mean age was not statistically different between men and women in both groups. We also included 73 MGUS patients and 68 MM patients. Demographic and clinical characteristics of this population are presented in Table II. During follow-up, only one patient with MGUS (1.4%) progressed to symptomatic MM. Usual biological data were collected for both groups (Table III).

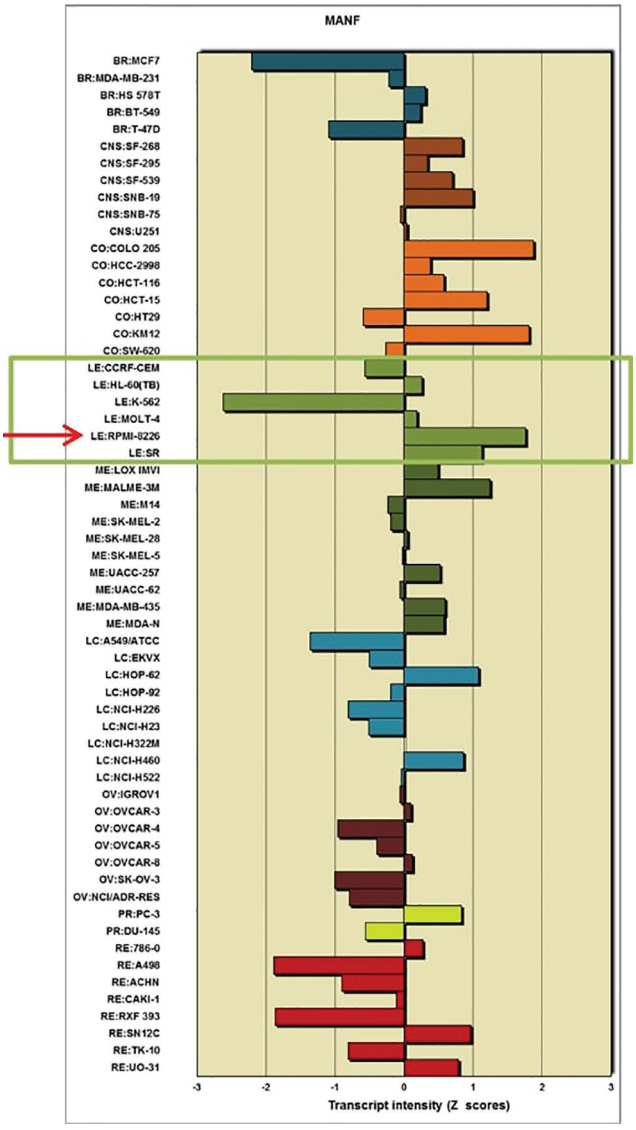

Figure 1. *MANF mRNA levels in different human solid and blood malignancy cell lines. Data were obtained from the National Cancer Institute-60. BR: Breast cancer; CNS: central nervous system cancer; CO: colon cancer; LE: leukemia; ME: melanoma; LC: lung cancer; OV: ovarian cancer; PR: prostate cancer; RE: renal cancer. The red arrow points to the RPMI 8226 MM cell line and the green box includes hematological cancer lines.*

Among MM patients, most (78%) had a symptomatic myeloma (Table II). Renal injury was reported in six of 67 patients (8.9%): myelomatous tubulopathy (n=1), Randall disease (n=1), AL amyloidosis (n=3), and glomerular nephropathy that was neither amyloid nor Randall (n=1). Prognostic factors of MM patients are presented in Table I. Cytogenetic data were available for 40 MM patients, and only 4 (10%) had a "high-risk" cytogenetic abnormality, represented by 17p deletion (n=3) and 4;14 translocation

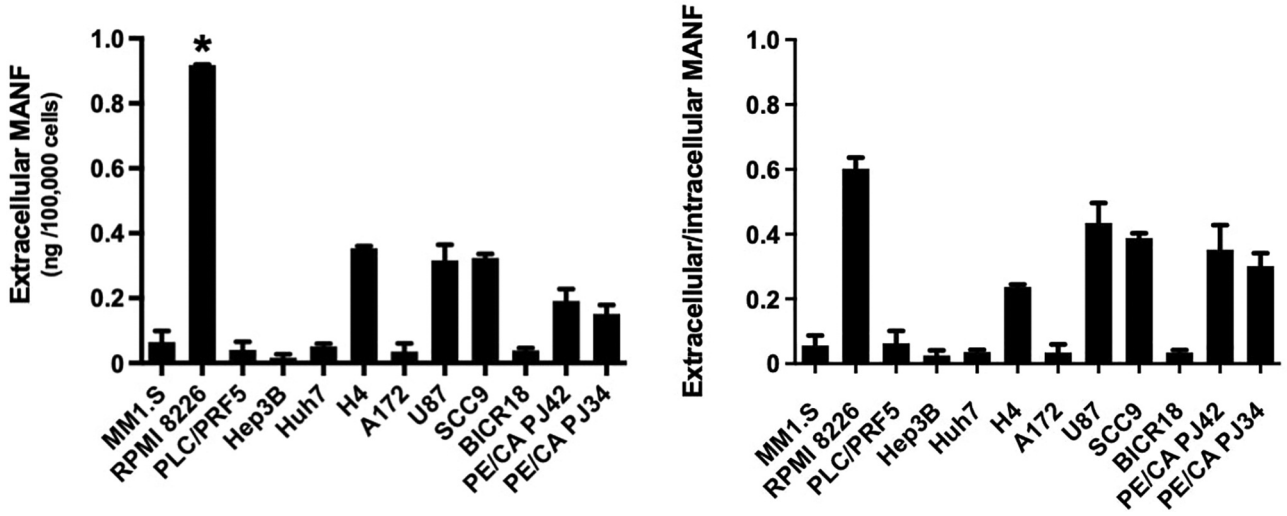

Figure 2. *Differences in intracellular expression and secretion of MANF protein in different malignant cell lines (MM and solid cancer cells). A) Extracellular MANF levels. Twelve tumor cell lines were studied: 2 MM lines (MM1.S and RPMI 8226), 3 hepatocellular carcinoma lines (PLC/PRF5, Hep3B, Huh7), 3 glioblastoma lines (H4, A172, U87), and 4 head and neck squamous cell carcinoma lines (SCC9, BICR18, PECA/PJ42, PECA/PJ34). Four duplicate seeded cell plates were made (5×10^5/ml for MM lines and 2×10^5/ml for other lines). ELISA was used for each supernatant after sample centrifugation to assay for MANF protein values. Results are expressed in ng per 100,000 sample cells (using Trypan blue). *significant difference between the RPMI 8226 cell line and the other cell lines ($p<0.05$). B) Ratio of extracellular/intracellular MANF levels. The cell pellet, obtained after sample centrifugation, was lysed in a lysis buffer in order to measure intracellular MANF concentration levels.*

(n=1). Two-thirds of the patients with indolent myeloma (68.8%) progressed to symptomatic disease requiring treatment. The median time between blood collection and disease progression was 15 months.

Among all 68 MM patients, 8 (11.7%) had already received at least one treatment line prior to the date of serum sample collection [no published data]. The response at the 6th month was evaluated for all 51 treated patients: 9 (17.6%) patients had progressive disease, 6 (11.8%) had stable disease, 9 (17.6%) had a partial response, 10 (19.6%) had a very good partial response, and 13 had (25.5%) a complete response. Median PFS and OS were 87.6 months and 98.2 months, respectively.

MANF concentrations in serum samples

Healthy subjects. The median MANF level was 4.84 ng/ml and 8.96 ng/ml in women and men, respectively (p=0.028). We estimated MANF concentration in three equal age groups (Table IV). Serum MANF concentration decreased significantly with age in the healthy population (p=0.0077), particularly in the healthy women group (p=0.0155). A decrease in MANF concentration with age was also observed in the sub-group of men, but it was not statistically significant. The MANF level did not correlate with body mass index (BMI) (r^2=–0.04 based on Spearman correlation, p=0.76).

Patients with monoclonal gammopathy. The median MANF level was 4.81 ng/ml in MGUS patients (n=73) and 5.615 ng/ml in MM patients (n=68, p=0.23) (Table III). In both groups, no statistically significant difference in MANF concentrations was found between sex and age, as seen in healthy subjects (Table IV). We compared serum MANF levels in MM patients with age- and sex-matched healthy controls and found no statistically significant differences (n=33, 6.50 ng/ml *vs*. 4.87 ng/ml respectively, p=0.19). Similar results were found with MGUS patients (n=36; 4.87 ng/ml *vs*. 4.88 ng/ml respectively, p=0.84). We identified a statistically significant correlation between MANF concentrations in MM patients and serum lactate dehydrogenase (LDH) *(*Table V*)* but not with International Staging System (ISS) or Revised International Staging System (R-ISS) prognostic scores. No correlation was found with PFS and OS.

Discussion

Despite evidence supporting interaction between both MANF and UPR and the involvement of UPR in pathogenesis of MM, no previous study, to our knowledge, has reported the possible relevance of MANF in patients with myeloma. Based on data from bioinformatic analysis, we found that mRNA expression of MANF was upregulated in the myeloma cell line RPMI 8226. In a second step using *in vitro* analysis, we have shown significantly higher secretion of MANF in the supernatants of the myeloma cell line RPMI 8226 compared to numerous solid tumor cell lines and another myeloma cell line, MM1.S. These preliminary

Table II. *Demographics and clinical characteristics of MM and MGUS patients*.

	MM (n=68)	MGUS (n=73)	*p*-Value
Follow-up duration, months			
Median [IQR]	78.1 [66.6]	51.6 [59]	**0.0218**
Age (years)			
Mean±SD	66.3±10.1	66.4±12.1	0.98
Female			
n/N (%)	36/68 (52.9)	45/73 (61.4)	0.38
BMI (kg/m)2			
n	38	39	
Median [IQR]	25.2 [6.6]	25.3 [6.2]	0.95
Cardiovascular risk factors			
n/N (%)			
Hypertension	32/65 (49.2)	31/72 (43)	0.58
Dyslipidemia	14/65 (21.5)	26/72 (36.1)	0.09
Tobacco	10/65 (15.3)	12/72 (16.7)	0.84
Obesity	5/65 (7.7)	6/72 (8.3)	0.89
Chronic alcoholism	3/65 (4.6)	3/72 (4.2)	1.00
Clinical history			
n/N (%)			
Cardiovascular disease	10/65 (15.4)	18/72 (25)	0.16
Neurologic disease	6/65 (9.2)	9/72 (12.5)	0.54
Autoimmune disease	4/65 (6.2)	8/72 (11.1)	0.37
Diabetes	3/65 (4.6)	12/72 (16.7)	**0.03**
Type 1	0/65 (0)	0/72 (0)	1.00
Type 2	8/65 (12.3)	3/72 (4.2)	0.12
Reason for serum sample			
n/N (%)			
Diagnosis	53/67 (79.1)	64/72 (88.9)	0.16
Relapse	6/67 (8.9)	-	
Follow-up	8/67 (11.9)	8/72 (11.1)	1.00
Heavy chain			
n/N (%)			
IgA	13/54 (24.1)	11/73 (15.1)	
IgG	41/54 (75.9)	62/73 (84.9)	0.25
Light chain			
n/N (%)			
Kappa	43/67 (64.2)	43/73 (59.9)	
Lambda	24/67 (35.8)	30/73 (41.1)	0.6
CRAB criteria			
n/N (%)			
Hypercalcemia	8/67 (11.9)	-	-
Anemia	24/67 (35.8)	-	-
Renal failure	8/67 (11.9)	-	-
Bone lesions	43/67 (64.2)	-	-
≤1	10/67 (14.9)	-	-
>1	33/67 (49.3)	-	-
Symptomatic			
n/N (%)	52/67 (77.6)	-	-
High-risk cytogenetic abnormalities			
n/N (%)	4/40 (10)	-	-
ISS			
n/N (%)			
1	34/66 (51.5)	-	-
2	17/66 (25.8)	-	-
3	16/66 (24.2)	-	-
R-ISS			
n/N (%)			
1	19/36 (52.8)	-	-
2	17/36 (47.2)	-	-
3	0/36 (0)	-	-

BMI: Body mass index; IQR: interquartile range; ISS: International Staging System; R-ISS: revised International Staging System. Significant *p*-Values are shown in bold.

Table III. *Laboratory data of patients with MM and MGUS*.

	MM (n=68)		MGUS (n=73)	
	n		n	
Median [IQR]				
Serum:				
MANF (ng/ml)	68	5.615 [7.595]	73	4.81 [3.237]
Hemoglobin (g/dl)	62	11.9 [2.4]	67	13.5 [1.6]
Protein (g/l)	62	81 [20]	73	72 [7]
Albuminemia (g/l)	62	40 [5.3]	72	40.9 [4.5]
Calcium (mmol/l)	61	2.32 [0.13]	72	2.3 [0.12]
CRP (mg/l)	60	3.1 [7.6]	61	3.1 [1.5]
Creatinine (µmol/l)	60	85 [28.3]	73	80 [21]
M protein§ (g/l)	48	25.3 [15.9]	72	11.6 [5.8]
Free light chain (mg/l)	49		56	
Kappa		21.1 [117.9]		14.4 [14.6]
Lambda		13.3 [50.7]		15.4 [16.3]
LDH, UI/l	67	362 [141.5]	66	398.5 [98.8]
β2microglobulin (µg/l)	68	3,340 [2,504]	67	2,190 [827.5]
Urine:				
Proteinuria (mg/24 h)	56	199 [1,290]	56	89.5 [57.8]
Bone marrow:				
Dystrophic plasma cells, %	40	15 [21]	62	2 [1]

CRP: C-reactive protein; LDH: lactate dehydrogenase; IQR: interquartile range; MM: multiple myeloma; MGUS: monoclonal gammopathy of undetermined significance. §Only MM with whole Ig.

results prompted us to validate the utility of MANF as an ER-stress biomarker in MM.

We did not find any significant difference in MANF serum levels between MM or MGUS patients and those in age- and sex-matched healthy controls. Furthermore, no difference was observed between MM and MGUS patients in terms of MANF concentration. Significantly different serum MANF levels have been reported in human diseases with involvement of ER stress compared with healthy controls. These results are consistent with data obtained *in vitro* (17, 23) and in animal models (22, 27).

Our study has some limitations such as small number of patients and the study's retrospective nature. MANF showed high biological variability, similar to that observed in different MM cell lines *in vitro*. Similarly, Steiner *et al*. showed a large variability in the amount of BiP in peripheral and bone marrow blood from patients with MM or MGUS without showing significant differences between the two groups (28). MM is a heterogenous disease with different genotypes and phenotypes. There is inter-tumoral and intra-tumoral heterogeneity that could explain, in part, the large variability of MANF in MM. We could suggest that measuring MANF in medullar plasma would probably be more appropriate, more specifically reflecting MANF secretion by tumor plasma cells.

Table IV. *Median [IQR] serum MANF concentration (ng/ml) in age-matched groups.*

	Healthy subjects							MGUS							MM						
Age (years)	20-40		41-60		61-80		*p*-Value	20-40		41-60		61-80		*p*- Value	41-60		61-80		>80		*p*-Value
	n		n		n			n		n		n			n		n		n		
Total	20	7.98 [8.56]	20	5.31 [4.20]	20	4.08 [3.26]	**0.0077**	1	9.45 [0]	20	4.98 [2.29]	42	4.48 [4.19]	10	5.73 [3.72]	0.33	17	9.87 [7.88]	44	4.84 [4.01]	7 [13.1]	7.31	**0.040**
Women	10	7.77 [5.11]	10	4.84 [1.52]	10	3.72 [1.46]	**0.0155**	1	9.45 [0]	12	5.89 [2.71]	24	4.74 [4.46]	8	5.23 [4.68]	0.36	8	7.03 [8.78]	23	5.61 [5.68]	5 [7.77]	3.58	0.68
Men	10	8.67 [12.00]	10	7.80 [7.94]	10	5.91 [6.60]	0.335	-	-	8	4.49 [1.59]	18	4.42 [2.76]	2	6.78 [1.89]	0.32	9	11.54 [6.32]	21	4.04 [3.42]	2 [8.72]	11.67	**0.011**

MM: Multiple myeloma; MGUS: Monoclonal gammopathy of undetermined significance. Significant *p*-Values are shown in bold.

Table V. *Correlations between serum MANF levels and clinical and biological parameters in patients with MM.*

Gene	n	r^2	*p*-Value
Age	68	–0.22	0.085
BMI	38	0.0234	0.45
Creatinine	60	–0.001	0.51
Hemoglobin	62	–0.0701	0.17
LDH	67	**0.088**	**0.015**
Albumin	62	–0.167	0.92
CRP	60	–0.061	0.70
β2microglobulin	68	0.0034	0.60
Monoclonal protein	48	0.0012	0.31
Plasma cell infiltration	40	–0.023	0.99
Protein	56	–0.045	0.83

r^2: Spearman correlation coefficient, $p<0.05$. BMI: Body mass index CRP: C-reactive protein; LDH: lactate dehydrogenase; IQR: interquartile range; MM: multiple myeloma; MGUS: monoclonal gammopathy of undetermined significance. Significant *p*-Values are shown in bold.

In our study, we found a decrease in circulating MANF concentrations in a gender mixed population of healthy subjects. Nevertheless, we did not observe this in our MM and MGUS patients who were also older than controls ($p<0.001$). Sousa-Victor *et al*. showed a decline in MANF protein levels in fly and mouse tissues with aging (25). This decrease was linked with deterioration of gut metabolic function in flies and with hepatic fibrosis in mice (25). Serum MANF concentration also decreased with age in an all-male healthy cohort (25). Recent studies have emphasized crucial roles of MANF in both immune response and in the regulation of energy metabolism, two major components of the aging process (16, 18, 29, 30).

However, in our healthy population we also found a statistically significant difference between men and women in terms of MANF concentrations. Surprisingly, in most previous , sex was not always considered as a possible confounding factor (11-13). Therefore, a MANF biomarker assay could not be considered without a prior and reliable determination of physiologic reference values in humans. Further investigations with large cohorts that will various physiologic and pathologic confounding influences, some of which are likely still unknown, are required.

In our study of patients with MM, we did not identify MANF as a prognostic factor or as predictive for treatment response. Furthermore, we failed to show that MANF could be a prognostic factor for malignant transformation of MGUS. On average, about 1% of MGUS patients progress to MM each year (1). Therefore, the small number of patients included in the MGUS group was not sufficient to consider MANF useful for identifying MGUS patients with a higher risk of developing MM. In sera from MM patients, we found a positive correlation between MANF and LDH.

Interestingly, increased LDH levels measured in many solid tumors and blood malignancies are not only reflective of tumor burden, but also of ER stress in malignant cells (31).

Some recent studies have considered the potential role of MANF in cancer development. It has been shown that MANF may be a potential diagnostic and prognostic indicator of hepatocellular carcinoma (32). Liu *et al*. recently suggested that MANF may act as a tumor suppressor by inhibiting NF-κB signaling (33). As a result, MANF would restrict malignant invasion by preventing epithelial-mesenchymal transition of hepatocarcinoma cells (33). It is known that the NF-κB pathway also plays a critical role in myeloma pathogenesis (34) and can be induced by UPR (8). Further large cohort studies of MM patients are required to explore the possible involvement of MANF in tumorigenesis.

Conclusion

In this study, MANF was not validated as a biomarker of interest in MM patients. Its potential implication in myeloma pathogenesis should be investigated. Further studies may clarify the physiological factors that influence the circulating levels of MANF in humans.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

AD, AG and VS designed the research study. AD, CL and CG performed the *in vitro* experiments. AD, AG and ZS performed the *in silico* analysis. AD, PD and VS managed patients and provided clinical datas. AD, CS and VS wrote the paper and analyzed the data. YEH managed the serum bank. All Authors read and approved the manuscript.

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