Evaluation of Serum ficolin-2, Sialic Acid, and High sensitivity C - reactive protein in Patients with Multiple Sclerosis

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ABSTRACT— ***the aim of the present study was to evaluate inflammatory blood markers in multiple sclerosis (MS) and to evaluate them with its clinical progressive forms. This study included 60 MS patients (35 with relapsing-remitting MS (RRMS) and 25 with progressive MS clinical forms) and 30 healthy individuals. Peripheral blood samples were obtained to determine serum levels of ficolin-2, high sensitivity C-reactive protein (hs-CRP), and total sialic acid (SA). Average level of ficolin-2 in MS group was lower (29.35 + 19.88µmol/L) in comparison to (45.17 + 17.39 µmol/L) in the control group this difference appeared highly significant (p<0.0001). Mean levels of sialic acid and hs-CRP were significantly higher in the MS group (2.334+0.7046µmol/L) and (31.15 + 12.4326) respectively, as compared to the controls (1.468+0.7046 µmol/L) (20.1416+4.2496 µmol/L) (p <0.001). Furthermore, Ficolin-2 levels inversely correlated significantly with severity of the disease as measured by EDSS, this correlation has shown to be positive in regard to hs-CRP and Sialic acid.***

In the logistic regression, ficolin-2 and hs-CRP showed positive association with MS and were predictors of MS development. SA, were negatively associated with MS (p< 0.0001). Using this regression analysis, 83.33% of all subjects were correctly classified with a sensitivity of 86.67% and a specificity of 76.67%. In conclusion, SA was predictor of MS diagnosis, whereas ficolin-2 and hs-CRP was predictor that differentiated RRMS from the progressive clinical forms of MS.

Keywords— Ficolin-2, Sialic acid, High sensitivity C-reactive protein, Multiple sclerosis, progression

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1. INTRODUCTION

Multiple sclerosis (MS) is a chronic neuroinflammatory disorder affecting central nervous system which initiated and propagated by autoreactive T cells directed against myelin sheath. Both genetic and environmental elements are known risk factors for MS development (1) viral infection at certain ages are also blamed of increasing the risk (2) there has been increasing attention toward innate immunity, including humeral factors and myeloid dendritic cells driving Progressive Multiple Sclerosis (3). However the mechanisms responsible for disease progression are still unknown and studies on lectin components of the innate immunity are new candidates to gain more attention for exploring hidden immunological pathways and possible biochemical targets at the molecular levels that may promote or cause transformation of patients from early stages of the disease known as remitting relapsing disease (RRD) which characterized by attacks of neurological manifestations followed by a recovery but with minor deficit left behind, into a more advanced progressive none resolving stage of the disease.

Ficolin-2 is one of the humeral innate immunity molecules synthesized by the liver and present in the serum has been

associated with different diseases for its role in complement activation and other biological functions (4) recently ficolin-2 became focus of several disease association studies due to its wide biochemical specificity. Ficolin-2 can recognize and subsequently bind different molecular patterns including mannan, acetylated sugars, lipoteichoic acid (5) in addition to glycoproteins (E1 and E2) of the hepatitis C virus envelope (6) as well as sialic acid (7), its role has not been studied in Multiple sclerosis so far, Except for recent proteomic study done by Helen Tremlett (8) on MS who observed change in the protein level with the clinical form. Besides complement activation, other inflammatory processes are also known to contribute to the pathogenesis of the MS (9) (10). Among them, CRP-associated processes were studied. In 2005, Di Napoli et al (11) summarized evidence for CRP as an independent predictor of cerebrovascular events in at risk individuals and its usefulness in prediction of progression. It was also demonstrated that hs- C reactive protein predicts the prognosis of patients with MS (12) furthermore, The role of CRP had been studied in Multiple sclerosis in relation to the disease progression (13) Recently, Ormstad et al. (14) provided evidence that CRP plays an important role in the progression of cerebral tissue injury. In addition, complement activation and elevated CRP levels were independently associated with the clinical severity and different outcome measures of ischemic stroke (15) Although CRP is well known as a clinical marker for inflammation, its primary function in demyelination and neuroinflammation remains unclear. Beside its pivotal role in complement activation, (16) CRP has also shown to bind complement inhibitory molecules such as factor H (17) and C4BP (18), Thus, CRP can play regulatory and anti-inflammatory function in addition to its proinflammatory roles during acute infection.

Therefore, serum concentrations of hs-CRP, ficolin-2 levels together with SA were examined in 60 MS patients with two distinct clinical forms (RRMS and progressive forms) and healthy controls. In order to assess the clinical significance of the results, the correlation of serum concentrations of these inflammatory markers with EDSS which reflect the severity of the disease, were analyzed. The correlations of the markers were also examined with the progression state of the disease.

2. MATERIAL AND METHODS

2.1 Subjects.

The MS population In this prospective case- control study was obtained from patients attending neurology clinics at Baghdad Medical city and neurological sciences Hospital in Baghdad with definite diagnoses of multiple sclerosis made by a neurologist according according to the revised Mc Donald diagnosing criteria (19), were included as study cases which was comprised of two clinical subtypes: subtypes: 35 stable relapsing–remitting MS (RRD) and 25 progressive MS disease PD consisted of both 25 secondary progressive MS (SP-MS), and 5 primary progressive MS (PP-MS). The control group was also obtained from healthy volunteers from the general population, which had been matched for age, gender, and ethnicity. Each volunteer agreed to take part in this study upon, agreement on an informed consent form that had been approved by Institutional Review Board (I.R.B.) of College of Medicine/ Al-Nahrain University. Inclusion criteria for cases were patients eligible to give consent, and being above 18 years of age with definite diagnosis of MS who has complete medical record. Exclusion criteria were subjects having other chronic pathophysiology such as cancer hypertension and diabetes or current pregnancy. The disease severity score EDSS was measured by a qualified neurologist, based on the criteria outlined by the (20).

The current study included 60 patients diagnosed with Multiple sclerosis (MS) in comparison to age and sex matched 30 control subjects. The MS patients were 38 female and 22 male, with mean ± standard deviation (SD) age of (36.03 + 8.93) years, while the healthy controls were 18 female and 12 male, with mean (34.93 +10.27). The mean EDSS (±SD) at the time of recruitment in the study was (4.14 + 2.16), and disease duration was (5.08± 3.63) years. Table (3-1) shows baseline Characteristics data in controls and MS patients

2.2 Blood sampling.

Ten mL of blood was taken from all participants in the study, blood placed in a plane tube. Sera was separated by centrifugation at 1500xg for 10 min at 4C0, divided into small aliquot, and stored at -20 C0 for later analysis

2.3 High sensitivity C-reactive protein assay: Serum hs- C-reactive protein (hs-CRP) levels were determined, in accordance with the manufacturer's instructions by using a double-antibody sandwich enzyme-linked immunosorbent one-step process assay (ELISA) with Assay range: 6.25ng/ml- 400ng/ml

2.4Ficolin-2 assay*:* Ficolin-2 was measured in duplicate by a double-antibody sandwich enzyme-linked immunosorbent one-step process assay (ELISA). Assay range: 15.6pg/ml- 1000pg/ml

2.5 Total sialic acid assay: SA was determined in duplicate using a colorimetric method the Assay principle is by measuring the absorbance of colored complex that is formed by sialic acid (SA) reaction with Resorcinol Reagent with oxidizing reagent as previously described (21)

2.6 Statistical analysis.

Data are presented as mean ± SD. Analysis of variance was performed with Fisher'stest. Correlations were performed with standard regression analysis. Statistical analysis was performed with XLSTAT ad on Microsoft excel 2010 and SPSS Statistics Version 17 for Windows (SPSS Inc., Chicago, IL) and P values < 0.05 were considered significance.

3. Results

3.1 Characteristics of the MS Patients and Controls

Table (1) shows clinical data in MS patients divided into those with remitting relapsing clinical form (RRD) and progressive clinical forms (PD) that included primary progressive MS (PPMS) and secondary progressive MS (SPMS). Clinical data were obtained using a standard questionnaire and medical records. Age was significantly higher in subjects with a PD versus RRD clinical form subjects. EDSS scores were significantly higher in subjects with a PD versus RRD clinical form. Likewise, duration of illness was significantly higher in subjects with PD form than in those with a RRD form. There were no significant differences in gender ratio between the two clinical forms. All participants came from the same ethnic background so there were no ethnic differences among the study groups.

Table 1: Baseline characteristics of the MS patients and controls

Variable	MS pateints (n=60)	Controls (n=30)	p-value
Number of patients n. (%)	60	30	
Age (year)			
Mean $\pm$ SD	36.03 $\pm$ 8.93	34.93 +10.27	0.606
Gender n (%)			
Male	22 (36.66)	12(40)	
Female	38 (63.33)	18(60)	
MS clinical forms n (%)			
RRMS	35 (58.33%)		
progressive forms	25 (41.66%)		
Age at onset of MS – (years)	30.16 $\pm$ 8.98	-------	
EDSS	4.14 $\pm$ 2.16		
Disease duration – (years)	5.08 ± 3.63		

The continuous variables are expressed as mean ± standard deviation (SD) MS multiple sclerosis, RRMS relapsing-remitting multiple sclerosis, EDSS estimated disease severity score, Independent t-test, * Significant at 0.05 level

3.2 Blood Marker Differences between MS Patients and Controls

All MS patients' plasma concentration of the SA was significantly elevated compared to control (Table 2). In contrast, ficolin-2 was decreased in both groups of MS patients ($P < 0.01$) compared to controls (Figure 1). hs-CRP was higher in progressive clinical form as compared with the RRMS and control groups. When the relationship among the inflammatory markers ficolin-2, SA, and hs-CRP was examined, there was a significant positive association between the acute-phase

inflammatory markers hs-CRP and SA in MS groups investigated in this study (Table 2). In addition there was a significant positive association between the hs-CRP and SA in MS groups investigated. In contrast, there was a significant negative association between the soluble innate molecule, ficolin-2 and the acute phase inflammatory marker hs-CRP and SA in both MS groups (Table 2).

Table 2: Characteristics of the two MS Patients groups remitting relapsing (RRD) and Progressive disease (PD)

Characteristics	**groups**		t-test P value [a] = Chi test
	RRD No. 35	**PD No. 25**	
Sex	19/16	19/6	0.085[a]
Age	33.77+9.10	39.20+7.64	0.2
Age of onset	29.14+9.44	31.60+8.08	0.30
Duration of MS	3.71+2.96	7.00+3.62	<0.001
Number of relapse	1.57+1.29	4.88+1.70	<0.001
EDSS	2.84+1.54	5.96+1.49	<0.001

We further investigated the relationship among the markers in the study and other biological markers by subgrouping the patients into three classes according to their dissimilarities in the biological parameters. Fig 1 show that lower ficolin levels are associated with higher EDSS,hs-CRP, SA, number of relapses and duration of the disease

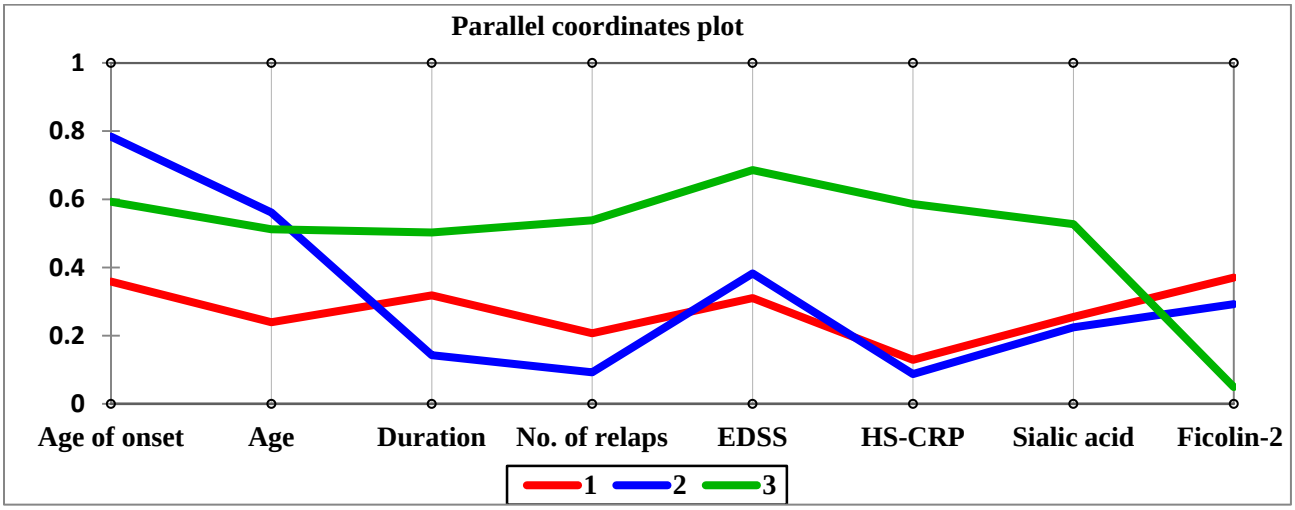

Figure 1: Parallel coordinates plot showing three classes of MS patient categorized according to their dissimilarities in the parameters indicated on X axis. The three classes are obtained by Agglomerative Hierarchical Clustering (AHC) analysis of data

Table 3 shows the outcome of a logistic regression analysis with MS group as dependent variable and the controls as the reference group and the blood markers as explanatory variables ($\chi 2$=46.761, df = 3, p< 0.0001; Nagelkerke = 0.562). We found that SA was significantly and negatively associated with MS, whereas hs-CRP and ficolin-2 was positively associated with MS. Using this regression analysis, 83.33% of all subjects were correctly classified with a sensitivity of 86.67% and a specificity of 76.67%

Table 3: shows the outcome of a logistic regression analysis with MS group

Source	Standard error	Wald	*P* value	Odds ratio	Lower bound (95%)	Upper bound (95%)
hs-CRP	0.0552	2.8951	0.0889	0.9104	0.8171	1.0144
Sailic acid	0.8794	11.3147	0.0008	0.0519	0.0093	0.2910
Ficolin-2	0.0165	0.7236	0.3950	1.0142	0.9818	1.0475

Average level of ficolin-2 in MS group was lower (29.35 + 19.88µmol/L) in comparison to (45.17 + 17.39 µmol/L) in the control group this difference appeared highly significant (p<0.0001) Fig 2 and 3. Mean levels of sialic acid and hs-CRP were significantly higher in the MS group (2.334+0.7046µmol/L) and (31.15 + 12.4326) respectively, as compared to the controls (1.468+0.7046 µmol/L) (20.1416+4.2496 µmol/L) (p <0.001). Fig. 4 and 5. Furthermore, Ficolin-2 levels inversely correlated significantly with severity of the disease as measured by EDSS, this correlation has shown to be positive in regard to hs-CRP and Sialic acid.

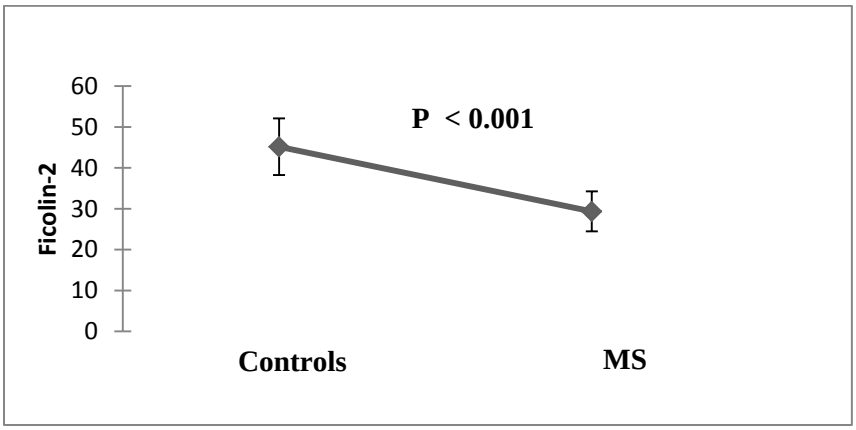

Figure 2: line curve showing Ficolin-2 means and confidence intervals for MS and control groups

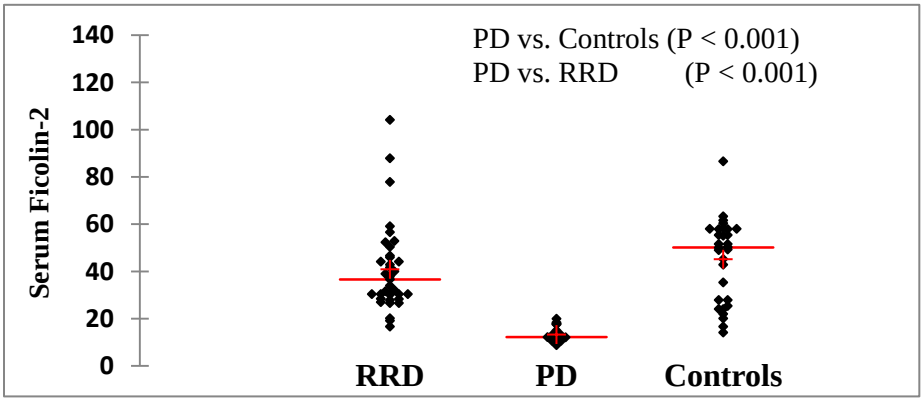

Figure 3: scatter gram showing serum ficolin-2 concentration among different groups in the study. RRD = reemitting relapsing disease, PD= progressive disease

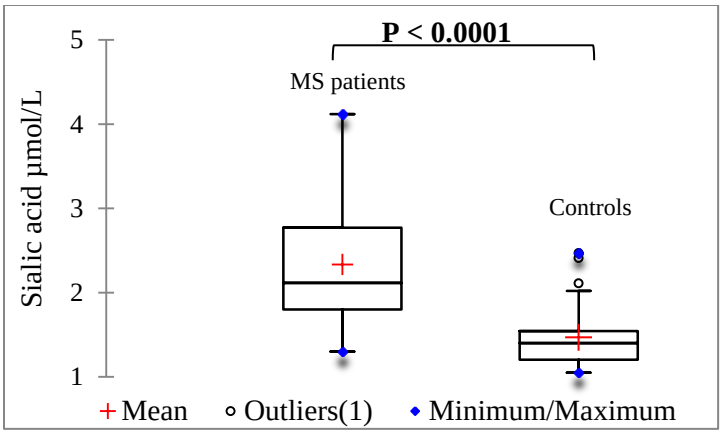

Figure 4: Box plot showing Means, outliers, maximum and minimum values of serum Sialic acid in MS pateints and control groups

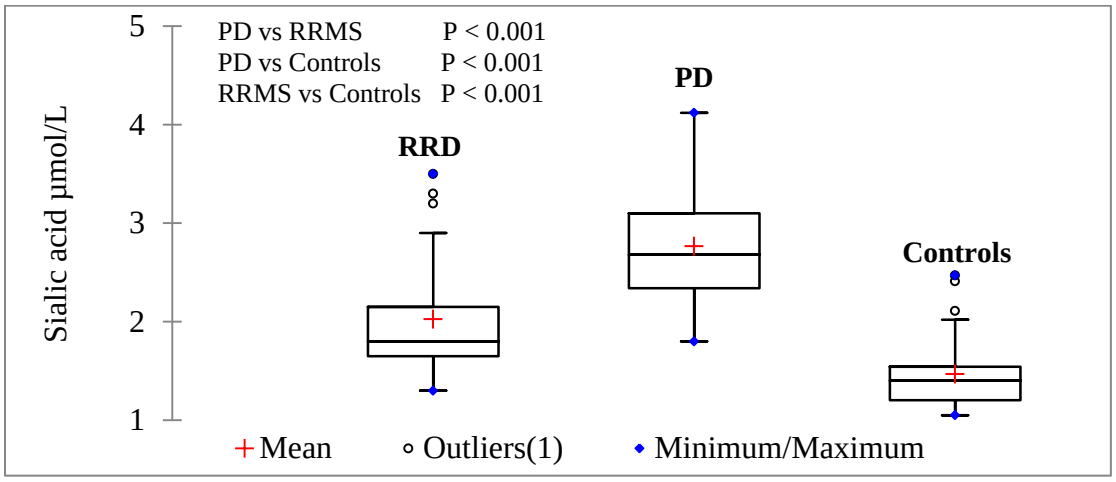

Figure 5: Box plot showing Means, outliers, maximum and minimum values of serum Sialic acid in (RRD) remmiting relapsing disease, (PD) progressive disease and Control groups.

Table 4 ANOVA (Tukey) analysis of hs-CRP for various groups in the study

Tukey (HSD) / Analysis of the differences between the categories with a confidence interval of 95%:					
Contrast	Means µmol/L	Difference	Critical value	P Value	Significant
PD vs Controls	43.31 vs 20.14	23.16	2.3847	< **0.0001**	Yes
PD vs RRD	43.31 vs 22.47	20.83	2.3847	< **0.0001**	Yes
RRD vs Controls	22.47 vs 20.14	2.33	2.3847	0.2864	No
Tukey's d critical value: 3.3724					

Table 5: Pearson correlation among different parameters in the study

Correlations	EDSS	hs-CRP	Sialic acid	Ficolin-2
EDSS	1	.587**	.347**	-.564**
		.000	.007	.000
hs-CRP	.587**	1	.407**	-.582**
	0.000		.001	.000
Sialic acid	.347**	.407**	1	-.408**
	.007	.001		.001
Ficolin-2	-.564**	-.582**	-.408**	1
	.000	.000	.001	
**. Correlation is significant at the 0.01 level (2-tailed).				

4. Discussion

Multiple sclerosis characterized heterogenic etiology with complex immunological pathogenesis. The damaged parts of CNS disrupt the nervous system communication ability, resulting in the wide range of signs and symptoms of the disease. The objectives of this study was to investigate differences in a three inflammatory markers in multiple sclerosis compared to normal healthy controls, and to further investigate differences in these markers in the disease progressive stage and whether they can predict the disease progressive form. The present study is the first to evaluate the pattern recognition receptors (PRRs) ficolin-2 together with inflammatory marker hs-CRP and Sialic acid as a candidate biomarkers of MS disease. Serum concentrations of these markers are correlated with severity score obtained from two sets of MS patients namely stable disease status including remitting period of remitting relapsing clinical form (RRMS) and active disease process of progressive clinical forms (PD) that included both primary MS (PPMS) and secondary progressive MS (SPMS)

the results from the current study were that the SA is significantly elevated in serum of MS patients compared to healthy controls and this elevation observed in PD group as compared with other groups ($P< 0.05$) While ficolin-2 was significantly decreased in MS compared to controls. Again Ficolin-2 was further decreased in progressive MS form. hs-CRP only increased in progressive disease . MS disease is a complex disease in which both the adaptive and innate immune systems play major role in the disease pathogenesis and contribute to the development of the progressive state. Alterations in innate immunity include both cellular and soluble components, reflected by elevated acute-phase proteins (22) MS has long been hypothesized to have an immunological basis, with excessive immune activation contributing to the demyelination evident in MS. In many studies, the association between elevated hs-CRP and MS have not clarified. However, Several lines of studies have reported increased CRP or hs-CRP levels in MS progression and relapses than in remission or during relieving stages of MS (23), (24) and (12) As our data show, there is a profound and significant increase in the circulating concentration of hs-CRP in patients with progressive phenotype compared to RRMS group or controls. Furthermore, SA is also an acute-phase inflammatory marker, and has long been studied for its role in atherosclerosis and coronary heart disease and stroke mortality

(25) and recently it has been found that SA is altered in different diseases like sarcopenia (26) and Oral Leukoplakia (27). Similar to CRP, SA is significantly elevated during progression, and that SA is significantly correlated with hs-CRP. Moreover, significant differences in SA concentrations were observed between two MS groups fig 4. Elevated serum levels of SA during the inflammatory response may be due to release from damaged tissues or increased synthesis, wheather or not this increased serum SA is associated with cellular hyposialylation remains to be further investigated. Recent data suggest decisive role of the production of reactive oxygen species in the course of MS (28). Notably The production of reactive oxygen species is linked with cellular hyposialylation, and Sialic acid deficiency is associated with oxidative stress (29) ficolin-2 is involved in complement activation and has been a limited investigation of ficolin-2 in MS. However, ficolin-2 was lower in MS patients when compared in controls and between the MS clinical forms. The decreased ficolin-2 in MS patients could be explained by either irreversible modifications of the protein induced by oxidative stress, such as protein oxidation, are generally associated with permanent loss of protein function and subsequent degradation

Interestingly, ficolin-2 was significantly correlated with hs-CRP in MS patients suggesting direct link between CRP and ficolin-2 or complement activation observed in MS during progressive stage of the disease. This finding highlights the possible role of complement in the pathogenesis of progressive disease course. Knowing that, both proteins are involved in complement activation by different pathways. The CRP activate classical pathway and ficolin-2 activate lectin pathway. More interesting here is the significant correlation between hs-CRP and ficolin-2 which can be explained as CRP increasing nonspecifically during acute inflammation while ficolin-2 which is not proven yet as an acute reactant may have different course of action especially it have been reported in many disease situations either to be increased or in other diseases is proved to decrease (30), as in our results in the present study.

One possible explanation for these findings may be that ficolin-2 is consumed by the virtue of neuroinflammation, apoptosis and degenerative process seen in the MS particularly during progressive disease stage also as a result of innate immune system activation, Limitations of this study include the measurement of inflammatory markers in MS was a single-point measurement and the relative small number of individuals with progressive MS clinical forms. In contrast, the strengths of the study include the quantification of new promising inflammatory markers, they are involved in the complement system each with different pathway

In conclusion, we have observed that the inflammatory markers SA is significantly elevated in MS patients. hs-CRP and SA are significantly increased during progressive stage of the disease these changes accompanies with significant decrease in ficolin-2 level. These data strengthen the hypothesis that MS is marked by a significant activation of the innate immune system, and that this activation is further exaggerated in the progressive course of the MS. furthermore, Our results indicate that neuroinflammation is a continuous process during all courses of MS and only exaggerated during progressive state , our finding suggest that the ficolin-2 -dependent activation of lectin complement pathway and SA which is likely resulting from elevated levels of richly sialylated acute-phase glycoproteins dependent regulation of the complement system together with CRP-dependent processes contribute to the progression and severity of MS. These findings help in the introduction of new therapeutic and diagnostic approaches in the management of a disease lacking definite therapeutic and diagnostic arsenals at present.

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