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Assessment of Some Platelet Activating Markers and Secretory Status with Clinical Manifestations in Multiple Sclerosis Iraqi Patients

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ABSTRACT

Patients with Multiple sclerosis (MS) are presented with different degree of disability based on Expanded disability status scale (EDSS). Several studies reported that ABH non-secretors have higher prevalence in different auto-immune diseases, also platelets have a multi-form participation in development of MS. Therefore, this study aimed to assess the correlation of clinical manifestations in patients with platelet-activating factor (PAF), P-Selectin, matrix metalloproteinase-9 (MMP-9) and ABH secretor status. Fifty Iraqi patients with relapsing-remitting multiple sclerosis (RRMS) were enrolled and divided into two subgroups; 36 of them with no disability (D0), their EDSS score ranging between 0-1.5, and the rest 14 patients with different degrees of disability (D+), their EDSS score ≥ 2. Along with patients, 20 healthy subjects were involved to act as a control group. Results showed significant elevation of PAF, P-Selectin, and MMP-9 levels in patients compared with those in the control group. However, only PAF level in D+ patients is significantly higher than D0 and positively correlated with duration of disease and serum level of MMP-9. Moreover, the frequency of non-secretors among RRMS patients is significantly higher than those in the control group and have a higher level of MMP-9, so may act as a risk factor for getting MS disease via increasing the serum level of MMP-9.

KEYWORDS: Multiple sclerosis, Platelet activating markers, ABH secretory status.

الخلاصة

يتم تقديم المرضى المصابين بالتصلب المتعدد (MS) بدرجات مختلفة من الإعاقة بناءً على مقياس حالة الإعاقة الموسع (EDSS). ذكرت العديد من الدراسات أن الاشخاص غير الفارزين لـ ABH لهم فرصة أعلى للإصابة بأمراض المناعة الذاتية المختلفة، كما أن الصفائح الدموية لها مساهمة متعددة الأشكال في تطوير مرض التصلب العصبي المتعدد. لذلك، هدفت هذه الدراسة إلى تقييم ارتباط المظاهر السريرية في المرضى مع عامل تنشيط الصفائح الدموية (PAF) و P-Selectin و (MMP-9) وحالة إفراز ABH. تم تسجيل خمسين مريضا عراقيا يعانون من مرض التصلب المتعدد الانتكاس المتكرر (RRMS) وتم تقسيمهم إلى مجموعتين فرعيتين. 36 منهم ليس لديهم إعاقة (0D)، وتراوحت درجاتهم في EDSS بين 0-1.5 ، والباقي 14 مريضًا بدرجات مختلفة من الإعاقة (D +) ، ودرجة EDSS الخاصة بهم اكبر او يساوي 2. بالإضافة الى 20 شخصا اصحاء كمجموعة سيطرة. أظهرت النتائج ارتفاعًا معنويًا في مستويات PAF و P-Selectin و 9-MMP في المرضى مقارنةً بمجموعة السيطرة. ومع ذلك، فإن مستوى PAF فقط في مرضى D + أعلى بكثير من 0D ويرتبط بشكل طردي بمدة المرض ومستوى مصل 9-MMP. علاوة على ذلك، فإن تواتر عدم الأفراز بين مرضى RRMS أعلى بكثير من تلك الموجودة في المجموعة الضابطة ولديها مستوى أعلى من 9-MMP، لذلك قد يكون بمثابة عامل خطر للإصابة بمرض التصلب العصبي المتعدد عن طريق زيادة مستوى 9-MMP في المصل.

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune, demyelinating, and degenerative disease of the central nervous system (CNS) with a number of pathophysiological mechanisms, including axonal injury, neuronal damage, inflammation, demyelination, and astrogliosis [1]. Globally, the total number of people living with MS has increased to 2.8 million by 2020, with global prevalence of 35.9 per 100,000 people, which is 30% higher than in 2013 [2]. The degree of neurologic impairment in MS patients is calculated

by expanded disability Status Scale (EDSS) based on measurement of the impairment in eight functional systems. Patients with EDSS value equal to zero to 1.5 have no disability, and those with EDSS values ranging from 2 to 4.5 have mild to moderate disability, while those with values of 5 to 9.5 have severe disability in their walking, while death occurs at score 10 of EDSS [3].

ABH blood group antigens (A, B, and H) are well-known for their presence on red blood cells, lymphocytes, platelets, tissue cells, body fluids except cerebrospinal fluid (CSF), and secretions [4]. The secretion of ABH substances into body fluids are controlled by fucosyltransferase 2 (FUT2). "Se" and "se" are two alleles of the secretor gene. In terms of inheritance, the Se is dominant, while the Se is recessive. The dominant secretor phenotype is produced by homozygous (SeSe) and heterozygous (Sese) genotypes, while the recessive non-secretor phenotype is produced only by homozygous (sese)[5]. Non-secretors genes are more exposed to the cardiac and thrombotic diseases [6, 7]; also have higher prevalence of different varieties of autoimmune diseases including multiple sclerosis [8, 9].

On the other hand, it has been hypothesized that platelets may be play an important role in neurodegenerative disease because they are frequently the first cells to reach vascular injury sites,and possess different receptors and secretory products in addition to those who play a traditional role in hemostasis and thrombosis [10]. Several studies assumed that the direct cause of Blood–brain barrier (BBB) disruption is the interaction between blood platelets, leukocytes, and endothelium [11-13]. Once platelets are activated, they have a major influence on leukocyte and endothelial cell interactions via a broad range of mediators that regulate signalling pathways in selected cells [14, 15].

MATERIALS AND METHODS

Subjects

Case-control study was conducted on 50 Iraqi patients with relapsing remitting phenotype of multiple sclerosis (RRMS) and 20 normal subjects free from neurodegenerative diseases, matched in their age and gender to be act as control group. The MS patients were attending the consultation clinic for Multiple Sclerosis, Baghdad Teaching Hospital for 3 months from November 2020 to January 2021. The definitive diagnosis of disease and its phenotype carried out by a Neurologist based on MacDonald criteria in concerning with their clinical signs and symptoms and magnetic resonance imaging (MRI). The medical history of all patients was extracted from their profiles which include name, gender, age, disease duration, age at disease onset and disease course. The degree of disability in all patients was monitored and calculated according to expanded disability status scale (EDSS) with assistance of Neurologists [16].

Samples Collection & Preparation

About 5 ml of peripheral venous blood were aspirated from all subjects at 9:00-10:00 am by using a sterile syringe and transferred into gel activator tube (AFICO-DISP, Jordan) and left for 15 minutes at room temperature to be coagulated, then centrifuged at 3000 rpm for 10 minutes to separate serum to be used in determination of platelet-activating biomarkers including:- platelet-activating factor (PAF), P-Selectin, and matrix metalloproteinase-9 (MMP-9).

Simultaneously with blood sample collection, about 2-3 ml of whole saliva sample were collected from all subjects and transferred into sterile plain tubes and centrifuged at 3000 rpm for 10 minutes to eliminate any debris. Tubes containing saliva supernatant were placed in a water bath at 56 ^{0}C for 15-30 minute to inactivate salivary enzymes. The tubes were cooled and centrifuged at 3000rpm for 10 minutes, and then the collected supernatant was transferred into Eppendorf tubes (AFICO-DISP, Jordan) and stored at -20 ºC until to be used in determination of secretory status by hemagglutination inhibition test [17].

Determination of secretor status

Secretory status was determined based on hemagglutination inhibition technique [18] by using 5% RBC suspension and anti-sera against antigen-A and B and anti-H lectin (Lorne Laboratories / UK). Saliva samples resulted in positive agglutination of 5% RBC suspension indicate non-secretor status, while those with no agglutination indicate secretor status.

Determination of Platelet-activating markers

The serum levels of platelet-activating markers (MMP-9, P-selectin, and PAF) in all subjects (Patients and normal) were determined by using diagnostic kits from (CUSABIO / USA) based on

sandwich enzyme-linked immune sorbent assay technology (Sandwich ELISA) [19].

Statistical analysis

Vassar Stats Web Site for Statistical Computation is used for statistical analysis of results. The measurable data were expressed as mean ± standard deviation (M±SD), and the difference between two independent groups is statistically analyzed by t-test, while difference among three groups is analyzed by one-way analysis of variance test (ANOVA). However, categorical data were expressed as percentage values, and comparison of these data among different groups is carried out by using Chi square test and 95% confidence interval of proportion. The association between two variables is analyzed by Pearson's correlation calculator test. Any difference at P level less than 0.05 is considered significant.

RESULTS AND DISCUSSION

Table 1 showed that the average EDSS (M±SD) of all RRMS patients (N=50) is 1.35 ± 1.1, only 14 (28%) of them have different degrees of disability (abbreviated D+) with EDSS of 2.67 ± 1.15, while the rest 36 patients (72%) have no disability (abbreviated D0), and their EDSS is 0.83 ± 0.44.

Table 1. Grouping of RRMS patients according to their disability degree

Parameter		Patients group		Total
		D0	D+	
EDSS	Range	0 - 1.5	2 – 5.5	0 – 5.5
	M±SD	0.83 ± 0.44	2.67 ± 1.15	1.35 ± 1.1
Cases	Number (n)	36	14	50
	Percentage (%)	72%	28%	100%

* D0: group of patients without disability; D+: group of patients with disability; M±SD: mean ± standard deviation, EDSS: Expanded disability status scale

Concerning with clinical manifestations in patients' groups. Table 2 showed that there is no significant difference (P=0.461) in the age of patients at the onset of MS disease, between those in D0 (29 ± 7.6 year) and D+ (31 ± 11.1 year), but the duration of disease in patients of D+ (8.6 ± 5.5 year) is significantly (P=0.014) higher than those in D0 (5 ± 3.4 year). Also, this table showed that 28.6% of patients in group D+ at interview session in MS clinic are presented at relapse course of disease which is significantly (P=0.006) higher than 2.8% of patients in D0 group.

Table 2. Clinical manifestations in two groups of patients.

Character		D0 (n=36)	D+ (n=14)	P value
Age at disease onset (year) (M±SD)		29 ± 7.6	31 ± 11.1	0.461
Disease duration (year) (M±SD)		5 ± 3.4	8.6 ± 5.5	0.014
Disease course (n, %)	Relapse	1 (2.8%)	4 (28.6%)	0.006
	Remission	35 (97.2%)	10 (71.4%)	

The concentration of three biomarkers in table 3, platelet-activating factor (PAF), p-selectin, and matrix metalloproteinase-9 (MMP-9) in serum of D0 patients (33.7 ± 17.1, 17.6 ± 5.6, and 6.7 ± 2.8 ng/ml respectively) and D+ patients (45.8 ± 10.9, 18.4 ± 5.5, and 7.2 ± 1.6 ng/ml respectively) are significantly (P<0.0001) higher than those in the serum of control group (16.5 ± 14.2, 5.9 ± 2.8, and 3.1 ± 1.1 ng/ml respectively).

Moreover, there is no significant difference in the serum levels of p-selectin and MMP-9 between D0 and D+ patients' groups, but PAF concentration in the serum of D+ patients (45.8 ± 10.9 ng/ml) is significantly higher than its concentration in the serum of D0 patients (33.7 ± 17.1 ng/ml) as shown in Table 3.

Table 3. Concentration of platelets-activating markers in patients and control groups

Biomarker M±SD	Patients		Control (n=20)	P value
	D0 (n=36)	D+ (n=14)		
PAF (ng/ml)	33.7 ± 17.1^{b}	45.8 ± 10.9^{a}	16.5 ± 14.2^{c}	< 0.001
p-Selectin (ng/ml)	17.6 ± 5.6^{a}	18.4 ± 5.5^{a}	5.9 ± 2.8^{b}	< 0.001
MMP-9	6.7 ± 2.8^{a}	7.2 ± 1.6^{a}	3.1 ± 1.1^{b}	< 0.001

* Small letters indicate the significant difference among columns based on Tukey HSD test

The correlation of PAF concentration in serum of all patients is analyzed by Pearson's correlation coefficient test against disease duration, relapse course, p-Selectin, and MMP-9. Table 4 showed

no-significant correlation between PAF and each of relapse course, N/L ratio, P/L ratio, and p-selectin. However, its concentration showed a significant positive correlation with disease duration (R= 0.2793, P= 0.049) and MMP-9 concentration (R= 0.3332, P= 0.018).

Table 4. Correlation between serum level of PAF in patients and certain investigated parameters

Parameter	Correlation of serum level of PAF versus	
	R	P
Disease duration	0.2793	0.049
Relapse course	0.0372	0.797
p-Selectin	-0.2084	0.146
MMP-9	0.3332	0.018

In concerning with secretor status, Table 5 showed that 36% of total MS patients are non-secretors which is significantly (P= 0.029) higher than only 10% in control group. However, there is no significant difference in the frequency of non-secretors between D0 patients (36.1%) and D+ patients (35.7%).

Table 5. Secretor status in patients and control group.

Groups	Secretor status (n, %)	
	Secretor	Non-secretor
Patients (n=50)	32 (64%)	18 (36%)
Control (n=20)	18 (90%)	2 (10%)
P value	0.029	
D0 (n=36)	23 (63.9%)	13 (36.1%)
D+ (n=14)	9 (64.3%)	5 (35.7%)
P value	0.979	

The association between non-secretors of all individuals (patients and normal) and their serum levels of platelet-activating markers show that only MMP-9 level is significantly increased in the serum of non-secretors (R= 0.2802, P= 0.018) as shown in Table 6.

Table 6. Correlation of non-secretors with serum levels of biomarkers in patients and normal subjects.

Biomarkers	Correlation of non-secretors versus	
	R	P
PAF	0.0772	0.525
P-Selectin	0.1133	0.350
MMP-9	0.2802	0.018

The previous Iraqi studies obtained controversial results of EDSS value in comparison with the present findings for instances; the review of 1125 medical profile of Iraqi MS patients from 2000 to 2008 found that the average of EDSS was 4.15 ± 2.17 and it is more aggressive in adolescents than in children [20]. However, Mohammed *et al.*, [21] observed that the mean EDSS in 676 Iraqi MS patients depend on the phenotype of disease because its value in the RRMS group (3.5) was significantly lower than 4.6 in PPMS patients and 6.2 in SPMS, while Al-Hussainy and Hatem [22] noticed that the higher EDSS score is that in MS patients who are presented with older age and longer disease duration. Recently, Mohammed and Alallaf [23] in their study on MS patients in Mosul City found that the clinical phenotype of MS in the Mosul City is similar to what has been described in the other countries except for an earlier age at onset and a more aggressive clinical course leading to earlier disability. Similarly, the more recent Iraqi study found that EDSS in RRMS patients has significant positive correlation with their BMI [24]. According to these variations in the EDSS value, it seems that the degree of disability in MS patients might be attributed to several factors, our results in table 2 found a significant positive correlation between the degree of disability and the course and duration of disease. In agreement with this result, it has been reported that an increasing in duration of the disease, functional disabilities showed a progressive increase in frequency and more cases pass toward the moderate or the severe forms [21]. Another study suggested that the duration of disease, age, and phenotype of MS play an important role in the cognitive profile of patients [25].

According to Table 3, results found a significant elevation of PAF, P-selectin, and MMP-9 in patients compared with control, which is quietly indicate the hyperactivation of platelets in MS patients regardless their EDSS score. In agreement with these findings, Callea *et al.*, [26] identified an elevation in the serum level of platelet-activating factor (PAF) in RRMS patients. Since platelet-derived products such as fibrinogen has been demonstrated within or around MS lesions, it has been reported that platelets could be a new player in the development of MS due to their ability to cross BBB via the damaged vascular basal lamina [27, 28]. Therefore, the enhanced risk of cardiovascular events (ischemic stroke, myocardial infarction, and thrombosis) in MS patients is directly associated with aberrant platelet function and their increased pro-thrombotic activity [29, 30].

Furthermore, proteomic and genomic studies revealed about 3,000 distinct mRNA species that could be translated to diverse mediators via which platelets modulate inflammatory functions [31]. The most abundant transcripts represent proteins that produced in platelets include adhesive proteins, coagulation factors, proteoglycans, immunoglobulins, MMPs and protease inhibitors [10]. A further mechanism of platelet action is via the use of signaling molecules anchored on the plasma membrane such as P-selectin, its elevated level was demonstrated in RRMS patients and it is capable of binding to lymphocytes resulting in increased lymphocyte binding to the endothelium [32, 33]. Moreover, platelets under physiological and pathological conditioned released a heterogeneous population of small vesicles (100nm - 1μm in diameter) known as platelet microparticles (PMP), which are positively expressed PAF and P-selectin. These PMP are significantly increased in RRMS patients and could be used as biomarker to reflect the treatment effect with IFN-ß, or natalizumab drugs [33-35]. Therefore, activated platelets may provide a unique molecular epitope for early diagnosis of MS and for therapeutic drug-targeting, which holds great promise [36].

Additionally, our results in table 4 indicated that elevated PAF level in the serum of MS patients enhances the expression and production of MMP-9. Several researchers have been suggested that PAF enhances pulmonary metastasis of murine sarcoma cells by inducing MMP-9 expression, also PAF increased mRNA expression, protein synthesis, and activity of MMP-9 in transformed human vascular endothelial cell line (ECV 304 cells) [37, 38]. Furthermore, Seizer and May confirmed the presence of MMP-9 on platelets [39], but another study demonstrated the presence of MMP-9 in platelets and its secretion significantly increases during inflammation [40]. Moreover, other studies reported that activated platelets elevate MMPs concentrations at sites of vascular detriment, and then platelet-derived MMPs fundamentally contribute to a variability of pathologies by recruitment leukocytes on the endothelium cell surface and stimulation both of them to produce further MMPs that lead to tissue degradation, and finally inflammation [39, 41].

On the other hand, results of secretor status indicate two facts; frequency of non-secretors among MS patients is higher than control (table 5), and the serum level of MMP-9 is significantly elevated in non-secretors (Table 6). The importance of these findings highlights to investigate whether this trait is a genetic risk factor for getting the disease, exacerbate comorbidities and accelerate progression of MS disease, or serve as a protective genetic factor against other pathological conditions. Since some of MS patients have high risk for getting cardiovascular and gastrointestinal diseases, so ABH secretor status may influenced in these diseases. Ansari *et al.*, reported that Gastroduodenal complaints including duodenal ulcer, gastro-esophageal reflux, gastritis, and positive *H. pylori* were common among non-secretors [42]. Shaik and Sekhar found that group B non-secretors are more prone to ischemic heart diseases [43]. In contrast, Hu *et al.*, reported that the fucosyltransferase 2 (FUT2) was upregulated in Osteoarthritis (OA) leading to upregulation of MMPs which associated with extracellular matrix (ECM) degradation and chondrocyte apoptosis reaching to cartilage damage [44]. Therefore, inhibition of FUT2 expression resulted in beneficial effects against (OA) development [45].

According to the previous studies and articles that studied the association between MS and ABH secretor status are absent in Iraq country and very limited worldwide. However, Hosokawa *et al.*, found that serum MMP-9 concentrations were significantly higher in patients with neuromyelitis optica and MS than controls, and positively correlated with EDSS score, which indicate its crucial role in the pathogenesis of these diseases through the BBB disruption [46]. In addition, it is known that MMPs play a significant role in neuroinflammation and neurodegeneration such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis [47]. Other studies reported that the polymorphism of the gene encoded for MMP-9 is associated with drug therapy resistance, disease severity, MMP-9 plasma activity in MS patients [48-50]. A recent meta-analysis revealed significant association of MMP-9 Single-nucleotide polymorphism (SNP) with MS susceptibility that increased the disease risk [51].

CONCLUSIONS

According to these findings, it concluded that the degree of disability in RRMS patients is progressed in those who have longer duration of disease and more attacks (relapses). Also, the frequency of non-secretors among RRMS patients are significantly higher than those in control group and may act as risk factor for getting MS disease via increasing the serum level of MMP-9. Furthermore, the significant elevation of PAF, P-Selectin, and MMP-9 levels in patients compared with those in control group reflect the activation status of platelets that increases the risk of getting MS disease. However, only PAF may contributed to the progression of disability via increasing the serum level of MMP-9 particularly in patients who have long duration of disease. Therefore, it is very important to clarify the role of platelet mediators as well as secretor status in the volume, site, and number of MS lesions in CNS based on radiologic image.

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