INTRODUCTION

Multiple sclerosis (MS) is a central nervous system disease characterized by demyelization, inflammation, and neuronal destruction. Genetic and environmental factors are coupled with the danger of developing MS, other than the precise reason still remains undisclosed. Among the well-recognized environmental hazard factors in MS were EBV, smoking, and vitamin D deficiency. The risk of rising MS is enlarged by infectious mononucleosis, which is caused by delayed primary infection with EBV. Potentially the EBV acts together with both genetic and additional environmental risk factors to amplify receptiveness and severity of MS disease [1]. EBV is a ubiquitous gamma-herpesvirus universally found in every geographic place and more frequently in developing countries [2]. Infection with EBV is very much common worldwide and around 90% of adults become antibody-positive before 30 years of the age [3]. Primary acute infection with EBV usually occurs during childhood sub-clinically, and subsequently a latent infection of B lymphocytes is established by the virus as the EBV continues for life [4]. In all actively dividing EBV-infected cells, EBNA-1 is expressed and is responsible for fusion of the viral episome to the mitotic cellular DNA, confirming duplication and transport of virus genome to all daughter cells [5]. EBNA-1, the vital EBV antigen for virus latency, makes up a principal antigen for both cell-mediated and humoral immune responses to the virus, and in MS the deregulation of immunity specific for EBV has been reported principally for this antigen [6]. Antibody responses specific for EBNA-1 may furthermore foretell alteration from clinically isolated syndrome (CIS) to MS [7]. Additionally, an enlarged occurrence of EBV DNA in serum for the duration of re-lapses, compared to periods of remission, has been reported [8]. Also, increased risk of MS has been associated with the presence of EBV DNA in the plasma [9].

THE AIM

This study aimed to compare the seroprevalence of Anti-EBNA among MS patients and controls and to study the frequency of active EBV in patients’ plasma in comparison to the controls and, finally, to find out whether there is a relation between the disease severity and anti-EBNA titer and/or the level of EBV viremia.

MATERIALS AND METHODS

A case-control study was conducted in 120 patients with multiple sclerosis, aged 12-42 years, from November 2020...
to June 2021. Blood samples were obtained from MS patients in the clinic of multiple sclerosis in Baghdad Teaching Hospital of Medical City, and from 120 controls, who were noticeably healthy age and sex-matched volunteers collected (their blood was obtained from blood donation centers). From all subjects we got informed consents before taking the samples. This study was approved by the ethical committee of the Al-Nahrain University, College of Medicine (No.20200980 on 17/11/2020).

The study was conducted in the labs of microbiology department in the College of Medicine-Al-Nahrain University. From all MS patients and controls, 3 ml of whole blood were collected and then divided into two parts: the first part was put in EDTA tubes and the second one (1.5ml) - in gel tubes. The blood samples in each tube were centrifuged at 5000 RPM for 5 minutes to get plasma from EDTA tubes and serum from the gel tubes. Plasma and serum were preserved in (-20 ºC); plasma was used for viral DNA extraction and serum for ELISA. Viral DNA was extracted from 100μl of plasma using WizPrep™ Viral DNA/RNA Mini Kit (V2) (South Korea). EBV Real-TM Quant Kit (Sacace, Italy) was used for detection of LMP-gene of EBV. EBV LMP DNA amplification was detected on JOE (Yellow) channel, whereas the IC glob gene DNA amplification was revealed on FAM (Green) channel and Master mix. 15 μl was added to all PCR tubes and 10μl of (DNA sample, negative control, positive control and standards) were added to master mix. The real time-PCR instrument used in the study was SaCycler-96 open (Sacace, Italy). The thermal protocol for Quantification Kit of Sacace consisted of an initial step of denaturation at 95 ºC for 15 min, for activation of the Hot Star Taq DNA Polymerase, followed by five cycles of thermal cycling at 95 ºC for 15 s, and 60 ºC for 20 s, and 72 ºC for 30 s, and then 20 cycles of 95 ºC for 10 sec, and 60 ºC for 40 sec, and 72 ºC for 15 sec. The ELISAs kits (Abnova /Taiwan) for anti-EBNA-1 IgG antibodies measurement, depended on the binding of antibodies in the sample with EBNA-1antigen that coat the wells of ELISA plate and the antibodies being in complexes with antigen are later recognized by animal anti-human IgG antibodies labeled with horseradish peroxidase. The labeled antibodies are revealed by an enzymatic reaction with a Chromogenic substrate. For quantitative evaluation the sample antibody titers in artificial units (AU/mL) were computed as follows: 1. A calibration curve was constructed by plotting the units of Standards (x-axis) to absorbance of Standard (y-axis). The place, where the absorbance of tested samples intersect calibration curve were found and the corresponding values (AU/mL) were marked on the X axis.

STATISTICAL ANALYSIS
SPSS version 21 was used for statistical analysis, categorical data were formulated as count and percentages, and Chi-square test was used to describe the association of these data. Numerical data were described as the mean with standard deviation. And independent sample t-test was used for comparison between two groups. The lower level of statistical significant difference was regarded as ≤ 0.05.

RESULTS
Among the 120 MS patients; 48(40%) were males and 72 (60%) were females. The median age of MS patients was 32 years (Percentile 25=26, Percentile 75=38). There was no statistically significant difference between the median age of the MS patients and controls (P=0.594), and no statistically significant difference between the age of the MS patients and controls for the different age groups indicating that they were of a comparable age (P=0.465). Most of MS patients (76.67%) 92 out of 120 were having a low number of relapses of equal or less than 3 relapses, and the lower percentage of patients (23.33%) 28 out of 120 were having a higher number of relapses of more than 3 relapses. The treatments used by MS patients included Avonex (5%) (β-interferon-1a), Betaferon (28%) (β-interferon-1b), Rebif (8%) (β-interferon-1a), Gilenya (7.5%) (Fingolimod), Natalizumab (41.5%) (Tysabri), and the last group without treatment with newly diagnosed cases (10%). Large number of MS patients (49%) treated with the second line therapy that included (Natalizumab or Gilenya). The results of quantitative real-time PCR showed that all the 240 samples were negative for EBV LMP-gene. However; all of them were positive for internal control, and the positive control gave positive results also. The results of ELISA showed that anti EBNA-1 IgG antibody was positive in 51.7% (62/120) of MS patients and 39.2% (47/120) of controls. The median of anti EBNA-1 IgG level of MS patients was 81.08U/ml (Percentile 25=57.88, Percentile 75=101.40), and of control was 67.73 U/ml (Percentile 25=54.62, Percentile 75=103.93), table I. Statistically, the sero-positivity and median level of anti EBNA-1 antibodies (IgG) were significantly higher in the MS patient than in the controls (P=0.035). The rate of sero-positivity and median of EBNA-1 IgG level of the MS patients were significantly higher than the controls in relation to the age groups: (<21 years) (P=0.050,0.006), and (21-30) (P=0.003, <0.001) respectively; whereas in the remaining age groups (31-40), (40), there was no significant differences in the median level and sero-positivity rate of EBNA-1 IgG between MS patients and controls, table I. Statistically, in MS patients the frequency of positive EBNA-1 IgG and median level were significantly higher in the MS patients than in the controls (p=0.004). Conversely, there was no significant differences in EBNA-1 IgG positivity and median level among the different sexes in controls (p=0.995), table II.

The rate of sero-positivity of EBNA-1 IgG is significantly higher in patients who have the disease for less than 2 years (83.33%), than in patients with a disease duration more than 2 years (41.11%) (p<0.001). In addition, the median of EBNA-1 IgG level in patients with a disease duration <=2 years is 82.81(Percentile 25=55.43, Percentile 75=126.14), which is significantly higher than in the patients with a
disease duration >2 years - 64.22 (Percentile 25=53.81, Percentile 75=97.37), (p=0.029), table III.

On the other hand, results of this study observed no statistically significant association of EBNA-1 IgG sero-positivity with number of relapses (p=0.812). Finally, table IV illustrated that there was no statistically significant association of the EBNA-1 IgG results with line of treatment (p=0.549).

**DISCUSSION**

Multiple sclerosis (MS) is the most common chronic inflammatory autoimmune illness of the central nervous system (CNS). Although the pathological characteristics of this persistent demyelinating disease are well recognized [10], little is presently known regarding the complex mechanisms that guide to the inflammatory process related with MS. But similar to other autoimmune diseases, MS...
might be elicited by an infectious agent [11]. A number of studies communicate Epstein-Barr virus (EBV) with MS [12], while others locate no association [13]. In the current study, all the representative samples showed negative EBV viremia, these findings suggest that the existence of EBV DNA is not a familiar incident in plasma and/or serum of MS patients and do not boost a direct role for systemic EBV infection in the MS pathogenesis, which agrees with other studies (Franciotta D et al., 2009, Villegas E et al., 2011) that showed absence of EBV viremia in all plasma samples, both in MS patients and control with EBV DNA negative [14,15]. While other researchers (Cocuzza CE et al., 2014, Hollsberg P et al., 2005) showed only small percentage of EBV DNA in plasma of MS patients; in addition to that, the same studies also reported the presence of EBV DNA in control groups [16,17]. While another study that was conducted on the serum sample using nested PCR failed to detect any viral DNA in examined sample [18]. On the other hand, a number of studies reported that EBV DNA and high viral load were detected in the plasma or serum of MS patients during relapses or at the time of exacerbation [19]. Wandinger et al. in 2000 have revealed that active replication of the virus (increased IgA and IgM responses to EBV EA), and positive EBV DNA in the serum (by quantitative polymerase chain reaction, qPCR) could be found in more than 70% of MS patients with exacerbations through the study time, but not in patients with stable disease [8].

Also Villegas E et al. in 2011 mentioned that the investigations of plasma and serum would only reveal viral genome in currently ill patients or in those with a towering systemic load of EBV [15]. This finding explains the negative results for EBV DNA by q-PCR in plasma of MS patients in the current study, since nearly all patients were taken during remission stage or the period of clinically stable disease. Alternatively, absence of viremia may be owing to the low specimen size subjected for PCR, and/or to the extremely low viral load in these individuals who are asymptomatically infected (i.e. viral load could be lower than the detection limit) [20]. In the present study, anti-EBNA-1 IgG antibody was positive in 51.7% (62/121) of MS patients and 39.2% (47/121) of controls. To the best of our knowledge, there are two previous studies regarding the seroprevalence of anti EBNA-1 IgG in Iraq, the more recent study carried out by Abd WS and Abd Al Kareem RM in 2020 among the Iraqi female patients with MS who were admitted to Clinic of Multiple Sclerosis in neuro-science hospital in Baghdad and they reported that mean serum level was significantly higher in female patients than in healthy controls [21]; another study, conducted in Rizgary teaching hospital in Erbil /Iraq, also showed significantly higher level in MS patients than in healthy controls (Bakir SH et al., 2017) [22]. The significantly higher seropositivity of anti EBNA-1 IgG in the MS patients than in the controls, in accordance with other studies [8, 23], however, other study, which was carried out in children, failed to manifest any relationship between the virus and MS [24].

The presence of an antigen such as the myelin basic protein (MBP), peptide derived from the myelin, sheaths surrounding an axon having a homology to EBV viral proteins. Kumar et al have illustrated molecular mimicry of viral EBNA-1 to MBP that could prompt T-cell autoimmune to myelin sheaths. For this reason, one of the most

| Table III. The relation between EBNA-1 IgG serology and disease duration |
|---------------------------|-----------------|-----------------|
| EBNA-1 IgG                | Disease duration | P value         |
|                          | <=2 years       | >2 years        |
| Negative                 | 5               | 53              |<0.001          |
| %                        | 16.67%          | 58.89%          |
| Positive                 | 25              | 37              |
| %                        | 83.33%          | 41.11%          |
| Median                   | 82.81           | 64.22           |
| Percentile 25            | 55.43           | 53.81           |
| Percentile 75            | 126.14          | 97.37           |

| Table IV. The relation between EBNA-1 IgG serology and line of treatment |
|---------------------------|-----------------|-----------------|
| EBNA-1 IgG                | Line of treatment | P value         |
|                          | No treatment    | 1st line        | 2nd line        |
| Negative                 | 5               | 22              | 31              | 0.549NS         |
| %                        | 41.67%          | 44.00%          | 53.45%          |
| Positive                 | 7               | 28              | 27              |
| %                        | 58.33%          | 56.00%          | 46.55%          |
| Median                   | 94.87           | 63.59           | 66.67           |
| Percentile 25            | 64.17           | 54.42           | 53.2            |
| Percentile 75            | 144.13          | 101.26          | 102.32          | 0.390NS         |
relevant non-self-antigens that is thought to induce MS is EBNA-1 [25]. The current study showed that the median of anti-EBNA-1 IgG titer was significantly higher in females compared to the males, MS is a disease of females, the females to males ratio is about 2:1 [26]. In addition, females have higher percentage of IgG seropositivity than males (59.5% in females versus 39.1 in males), as shown in the table IV. Foroutan-Pajoohian1 Pet al. in 2018, also have reported higher seropositivity of anti-EBNA-1 IgG in MS females than males [27]. The above data may possibly be correlated with the more competent immune response advanced by estrogens compared to the immunosuppressive function of androgens. Females have a better humoral immune response than males, as manifested by higher titers of serum immunoglobulin, and a larger antibody response to a variety of antigens after immunization [28]. The current study revealed significantly higher anti-EBNA-1 IgG titer at young age group as compared to the controls could be explained by Levin LI et al. showed in 2005 that anti-EBV antibody titers among cases compared with controls were already significantly elevated for 5 or more years before the onset of MS. He suggests that the increased antibody response to EBV is not a consequence of MS, but rather may be an early event in the pathological process that leads to demyelination and clinical disease; he was also noted that this increase in EBNA-1 IgG occurred between the late teens and the mid to late 20s, independently from the age of MS onset. On the other hand, the incidence of infectious mononucleosis peaks at this age (29) that clarified the relation between EBV infection and MS.

In support of these theories, this study found that the seroprevalence is significantly higher at early stages of the disease both qualitatively and quantitatively as shown in table III; in addition, table IV revealed that the median IgG titer is higher in patients at early diagnosis (i.e. before starting therapy). Drosu et al. in 2018 found that Zidovudine (a nucleoside analogues), a component of combivir, is known to inhibit EBV DNA replication and recommended in accordance with standard of care and well-established guidelines for Combivir treatment in newly diagnosed MS cases [30].

CONCLUSIONS
In conclusion, anti-EBNA-1 antibody could have an important triggering role of MS because of significantly higher levels both quantitatively and qualitatively in MS patients than in controls, especially at younger age groups, and at early stages of the disease (in those who haven’t start treatment yet), and also is higher in females who are well known to have risk factor for MS.

REFERENCES


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