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Association between the concentration of CYP24A1, 25-OH vit D3 and calcium-phosphate metabolism with an increased risk of multiple sclerosis in Iraqi patients

S A Soud¹, S H N Al-Rubaei²*and A T Salman²

¹Biotechnology Division, Applied Science Department, University of Technology, Baghdad-Iraq.
²Mustansiriyah University, College of Science, Department of Chemistry, Baghdad-Iraq.
*Corresponding: salwahnaser@gmail.com

Abstract. Growing research has shown that multiple sclerosis (MS) patients have lower levels of 25-OHvitD3 than healthy controls. The purpose of this study was to evaluate the serum concentration of 25-OHvitD3, CYP24A1 enzyme in MS patients and calcium-phosphate metabolism indices depending on the different disease lines and gender. Furthermore, we elevated the relationship between study parameters and gender with degree of motor disability according to the Expanded Disability Status Scale (EDSS). Serum of 110 Iraqi MS patients (50 male and 60 female) and 63 healthy individuals (30 male and 33 female) as control groups were used in our study. Serum level of 25-OHvitD3, CYP24A1 and PTH were measured by ELISA. Serum level for calcium, phosphorus and magnesium were performed with a BioSystems A25 and A15 analyzers. Significantly lower level of CYP24A1 and 25-OHvitD3 was found in MS patients when compared to control groups. The Levels of CYP24A1 and 25-OHvitD3 were positively correlated in female and significantly decreased with EDSS in female groups. Also, in MS patients a highly significant decrease in calcium, a considerable increase in level of PTH, phosphorous and magnesium. In this study the negative correlation between 25-OHvitD3 and the degree of motor disability, according to EDSS in MS patients was confirmed. We identified a non-significant difference in the level of 25-OHvitD3 regarding on gender. Still, we confirmed the association between gender and MS disease development regarding the levels of 25-OHvitD3, PTH and phosphorus.

Keywords: Multiple sclerosis, 25-OHvitD3, CYP24A1, EDSS, Calcium, Phosphorus, Parathyroid hormone.

1. Introduction

Multiple sclerosis (MS) is a multifactorial, autoimmune disorder of the central nervous system (CNS) in young adults between the ages of 20 and 40 years, affecting more than 2.5 million people worldwide, which is characterized by focal inflammation, demyelination, and axonal injury [1]. Moreover, women have about three times increased compared with men for developing MS [2]. According to McDonald and his colleagues the first acute clinical episode in 85% of the patients, diagnosis with the clinically isolated
syndrome (CIS) together with paresthesia, optic neuritis, fatigue and paresis [3,4]. The CIS then with a time can be converted into the first line of MS known as a relapsing-remitting (RRMS), between 15 and 20 years, as well as after, into the second line identify as a secondary-progressive (SPMS) type, hence, long-term of disability. The lasting type of MS identifies as primary-progressive (PPMS) found in about 15% of patients [5,6]. Until now, the underlying cause of MS, which is still unknown. Many studies found to increase disease susceptibility correlated with genes modestly and relevant environmental factors including (vitamin D, susceptibility to ultraviolet light, Epstein-Barr virus infection, obesity and smoking) [7]. The relation between MS and low level of vitamin D (25-OHvitD3) has recommended with several analytical epidemiological studies [8-11]. Moreover, numbers of protective effects for 25-OHvitD3 have been related to MS risk [12]. Such as decrease in relapse rate and radiological inflammatory activities [13]. Thus, 25-OHvitD3 has vital roles in maturation of the immune cells, homeostasis of the epithelial barrier, and inhibiting proliferation and induces differentiation for a different type of cell [14]. Furthermore, 25-OHvitD3 is responsible for calcium-phosphate metabolism with parathyroid hormone (PTH), phosphorus, and calcium. The most significant extracalcemic function is to restrict autoimmune progression in MS, primarily due to increased anti-inflammatory response [15]. The most important inactivation mechanisms of vitamin D catalyzes by mitochondrial CYP24A1 [16]. The CYP24A1 is one of the seven human cytochromes (CYP) normally localized in mitochondria of kidney and some other places in the human body [17]. The objective of this study is to set the serum concentrations of 25-OHvitD3, CYP24A1 enzyme and the calcium–phosphate metabolism indices regarding on the gender and different disease lines with and without immuno-modulatory treatments. Furthermore, we elevated the relationship between study parameters and degree of motor disability according to the Expanded Disability Status Scale (EDSS).

2. Experimental section

2.1. Study Group
The present study was carried out in Baghdad Teaching Hospital Medical-City in Baghdad- Iraq, from November 2019 to February 2020. The study included 110 MS patients (50 male and 60 female) were diagnosed according to McDonald criteria with the age range 18-55 years. Group I were involved 31 relapsing-remitting multiple sclerosis patients without any immuno-modulatory treatment (N-RRMS), group II was involved 34 relapsing-remitting multiple sclerosis patients with Betaferon® treatment (RRMS), and the group III were included 45 secondary-progressive multiple sclerosis patients with Tysabri® treatment (SPMS). Furthermore, control group were included 63 healthy individuals (30 male and 33 female) with age range 18-55 years.

2.2. Blood collection
After being informed, 5 ml fasting blood samples were taken and placed into gel tubes for 15 minutes at 25 °C and were centrifuged at 2000×g for 15 min to collect sera. Then serum stored at -40 °C until used.

2.3. Anthropometric measurements
The Body Mass Index (BMI) was identified through dividing weight in (kg) by height² in (m²). Patients were deemed obese if their index of body mass was 29.9 (kg/m²) [18].

2.4. Laboratory analysis
Serum levels of 25-OHvitD3, CYP24A1 and PTH were measured by Enzyme-Linked Immunosorbent Assay (ELISA) kits (Mybiosource, USA). Serum calcium, phosphorous and magnesium were analyzed with a (BioSystems A25 and A15 analyzers, Spain)
2.5. Statistical analysis
All study data are shown as mean±standard division and analyses statistically with the Graph Pad prism 9. One-way variance analysis (ANOVA) was used to measure the difference among groups. The statistical analysis results were deemed statistically significant when the level of significance was \( p \leq 0.05 \).

3. Results
The demographic of MS patients and control groups as revealed in Table 1. There was non-significant differences were observed within study groups in terms of age, weight, length and BMI (\( P > 0.05 \)). The mean ± SD value of serum level of CYP24A1, 25-OHvitD3, PTH, calcium, phosphorus and magnesium in MS patients with different disease lines and control groups were revealed in Table 2. Post hoc analysis showed a significantly decreased in the serum level of CYP24A1, 25-OHvitD3 and calcium in all patients groups with control groups (\( P \leq 0.01 \)). There was a significantly decreased in 25-OHvitD3 level in RRMS patients as compared to N-RRMS (\( P \leq 0.05 \)). While only significantly increase in the serum level of PTH in SPMS group as compared to N-RRMS and controls groups was found (\( P \leq 0.05 \)). The significantly difference was found in serum level of magnesium in N-RRMS with all study groups (\( P \leq 0.01 \)). Furthermore, a significantly difference was indicated in the level of phosphorus among N-RRMS and control groups only (\( P \leq 0.01 \)) Figure 1.

Table 1. Demographic of the study groups.

| Parameters        | Patients N=110 mean± SD | Controls N=63 mean± SD | P-Value |
|-------------------|-------------------------|------------------------|---------|
| Age (years)       | 33.600±7.856            | 35.507±9.264           | NS      |
| Weight (kg)       | 72.436±11.654           | 71.500±10.089          | NS      |
| Length (cm)       | 170.736±8.412           | 171.22±8.041           | NS      |
| BMI (kg/m²)       | 24.844±3.743            | 24.356±2.788           | NS      |
| Disease duration (years) | 4.8±3.9             | -                      | -       |
| EDSS level mean (range) | 1.0 (0-6)            | -                      | -       |

N: Number of subjects, SD: Standard division, EDSS: Expanded Disability Status Scale (0-10), NS: Non-significant at \( P > 0.05 \).

Table 2. Serum level of CYP24A1, 25-OHvitD3, PTH and related parameters in patients with different disease lines and control groups.

| Parameters    | Control N=63 Mean± SD | N-RRMS N=31 Mean± SD | RRMS N=34 Mean± SD | SPMS N=45 Mean± SD |
|---------------|-----------------------|----------------------|--------------------|--------------------|
| CYP24A1 (ng/mL) | 4.050±1.586           | 1.443±0.671          | 1.284±0.591        | 1.497±0.640        |
| 25-OHvitD3 (ng/mL) | 70.394±16.75          | 55.702±13.703        | 43.082±19.177      | 47.686±19.368      |
| PTH (pg/mL)   | 46.986±13.80          | 51.152±8.794         | 53.423±11.251      | 52.622±12.185      |
| Calcium (mg/dL)| 8.789±0.687           | 8.251±0.727          | 8.203±0.673        | 8.196±0.701        |
Magnesium (mg/dL)  1.731 ±0.397  2.214± 0.320  1.914± 0.219  1.891 ± 0.334  
Phosphorus (mg/dL)  2.766±0.521  3.273 ± 0.860  2.970  ± 0.641  2.904  ± 0.710

N: Number of subjects, SD: Standard division.

Figure 1. Multiple comparisons analysis for CYP24A1, 25-OHvitD3, PTH and related parameters in patients with different disease lines and control groups. NS: Non-significant at P> 0.05,* Significant at P≤0.05 and ** Significant at P≤ 0.01.

Subsequently, the study parameters were assessed depending on the gender. As shown in Table 3 the mean ± SD values for the serum levels of study parameters were higher in male than female patients groups. An exception was found in higher levels of PTH and phosphorus with in N-RRMS and SPMS female than male patients groups respectively. Post hoc analysis depending on gender was indicated there was different in the levels of study parameters among female and male patients groups. Thus, there was a significantly decreased in the serum level of CYP24A1, 25-OHvitD3 and calcium among all female patients groups than female control groups (P≤0.01). Moreover, a significantly increase was found in level of PTH between SPMS and control female groups (P≤0.01). Similarly, multiple comparisons analysis for male groups were showed a significant decreased (P≤0.01) in the CYP24A1 and 25-OHvitD3 levels, but the level of 25-OHvitD3 was significantly decreased in RRMS and SPMS than male control groups only. Moreover, a non-significant differences were found (P>0.05) in all male groups in both PTH and calcium levels. In female groups a significantly increased was found in level of magnesium between N-RRMS than control and SPMS groups (P≤0.01). Also, a significantly decreased in in level of magnesium between RRMS and N-
RRMS (P≤0.05). While, in male a significantly increase were found in levels of magnesium between N-RRMS and SPMS than control groups (P≤0.01) and (P≤0.05) respectively. Whereas, there was a significantly decreased in in level of magnesium between N-RRMS than SPMS (P≤0.05). A non-significant difference (P>0.05) phosphorus level in female patients groups when compare to control groups was also found. A significantly increased (P≤0.01) was found in levels of phosphorus between N-RRMS than control groups, a significant increase among N-RRMS than RRMS and SPMS male groups (P≤0.05) and (P≤0.01) respectively Figure 2 and 3. Finally, regardless different disease lines groups comparison between male and female patients concerning serum concentration of all study parameters was done also, and significant difference (P≤0.05) was noticed in the calcium level only.

Table 3. Serum level of CYP24A1, 25-OHvitD3, PTH and related parameters of the study groups, depending on gender.

| Parameters        | Gender | N-RRMS N=31 | RRMS N=34 | SPMS N=45 | Control N=63 |
|-------------------|--------|-------------|-----------|-----------|--------------|
|                   |        | mean± SD    |           |           |              |
| CYP24A1 (ng/mL)   | F      | 1.421±0.627 | 1.274±0.620 | 1.446±0.618 | 4.052±1.575 |
|                   | M      | 1.482±0.772 | 1.290±0.598 | 1.540±0.657 | 4.049±1.625 |
| 25-OHvitD3 (ng/mL)| F      | 55.000±12.648 | 40.569±19.425 | 43.136±20.12 | 70.617±14.822 |
|                   | M      | 56.977±16.016 | 45.372±19.783 | 52.298±17.36 | 70.147±18.904 |
| PTH (pg/mL)       | F      | 47.148±6.397 | 48.149±9.175 | 55.217±18.58 | 43.099±15.341 |
|                   | M      | 46.613±6.661 | 54.490±8.256 | 57.902±16.81 | 51.261±10.560 |
| Calcium (mg/dL)   | F      | 8.178±0.724 | 8.022±0.619 | 8.094±0.755 | 8.848±0.728 |
|                   | M      | 8.247±0.596 | 8.360±0.775 | 8.420±0.658 | 8.722±0.644 |
| Magnesium (mg/dL) | F      | 2.180±0.303 | 1.881±0.247 | 1.847±0.363 | 1.773±0.374 |
|                   | M      | 2.273±0.354 | 1.946±0.193 | 1.938±0.294 | 1.684±0.422 |
| Phosphorus (mg/dL)| F      | 2.998±0.766 | 2.942±0.613 | 2.987±0.728 | 2.731±0.498 |
|                   | M      | 3.772±0.824 | 3.010±0.704 | 2.816±0.679 | 2.804±0.550 |

N: Number of subjects, M: male, F: female, SD: Standard deviation.
Figure 2. Multiple comparisons analysis for serum level of CYP24A1, 25-OHvitD3, PTH and related parameters in female patient and control groups. NS: Non-significant at P>0.05, * Significant at P≤0.05 and ** Significant at P≤0.01.

Figure 3. Multiple comparisons analysis for serum level of CYP24A1, 25-OHvitD3, PTH and related parameters in male patient and control groups. NS: Non-significant at P>0.05, * Significant at P≤0.05 and ** Significant at P≤0.01.
The results of correlation between CYP24A1 with other parameters regarding patients groups with different disease lines and gender revealed that CYP24A1 have a significant positive correlation (P≤0.01) with 25-OHvitD3, negative correlation (P≤0.01) with calcium in SPMS and a significant positive correlation (P≤0.05) with magnesium in RRMS female patients groups. While only a negative correlation (P≤0.01) with calcium in male SPMS patients group (Table 4,5A and 5B). In this study EDSS negatively correlated (P≤0.01) with both CYP24A1 in SPMS patients and 25-OHvitD3 in all groups with different disease linesTable 6. Also, in female patients EDSS negatively correlated (P≤0.01) with both CYP24A1 in SPMS and with 25-OHvitD3 in RRMS and SPMS groups. In male patients group EDSS negatively correlated (P≤0.01) with 25-OHvitD3 in all different disease lines groups (Table 6,7A and 7B).

Table 4. The correlation of CYP24A1 with other parameters regarding patients with different disease lines groups.

| Component | N-RRMS | RRMS | SPMS |
|-----------|--------|------|------|
| CYP24A1   | R²     | r    | P    | R²   | r    | P    | R²   | r    | P    |
| 25-OHvitD3| 0.003  | -    | NS   | 0.017| 0.130| NS   | 0.190| 0.436**| 0.003|
| PTH       | 0.042  | 0.205| NS   | 0.014| 0.120| NS   | 0.007| 0.083 | NS   |
| Calcium   | 0.007  | -    | NS   | 0.013| 0.112| NS   | 0.176| -0.419**| 0.004|
| Magnesium | 0.114  | 0.337| NS   | 0.171| 0.414**| 0.01  | 0.001| 0.037 | NS   |
| Phosphorus| 0.029  | 0.171| NS   | 0.000| 0.016| NS   | 0.006| -0.077| NS   |

N:-Number of subjects, R²: Coefficient of determination, r: Pearson’s correlation coefficients, NS: Non-significant at P> 0.05, * Significant at P≤0.05 and ** Significant at P≤ 0.01.

Table 5A. The correlation of CYP24A1 with other parameters regarding female patient groups.

| Component | N-RRMS | RRMS | SPMS |
|-----------|--------|------|------|
| CYP24A1   | R²     | r    | P    | R²   | r    | P    | R²   | r    | P    |
| 25-OHvitD3| 0.133  | -0.365| NS   | 0.017| -0.131| NS   | 0.327| 0.572**| 0.004|
| PTH       | 0.028  | 0.166| NS   | 0.015| 0.123 | NS   | 0.000| 0.020 | NS   |
| Calcium   | 0.000  | -0.020| NS   | 0.130| 0.360 | NS   | 0.224| -0.473* | 0.023|
| Magnesium | 0.099  | 0.315| NS   | 0.284| 0.533* | 0.028| 0.004| 0.062 | NS   |
| Phosphorus| 0.016  | 0.128| NS   | 0.004| 0.065 | NS   | 0.016| 0.128 | NS   |

Number of subjects, R²: Coefficient of determination, r: Pearson’s correlation coefficients, NS: Non-significant at P> 0.05, * Significant at P≤0.05 and ** Significant at P≤ 0.01.
Table 5B. The correlation between CYP24A1 with study parameters regarding male patients groups.

| Component | N-RRMS | RRMS | SPMS |
|-----------|-------|------|------|
|            | N=11  | N=17 | N=22 |
| CYP24A1    |       |      |      |
| 25-OHvitD3 | 0.063 | 0.169 | 0.080 |
| PTH        | 0.073 | 0.014 | 0.019 |
| Calcium    | 0.045 | 0.011 | 0.185 |
| Magnesium  | 0.130 | 0.066 | 0.000 |
| Phosphorous | 0.052 | 0.001 | 0.071 |

N: Number of subjects, R²: Coefficient of determination, r: Pearson’s correlation coefficients, NS: Non-significant at P>0.05, * Significant at P≤0.05 and ** Significant at P≤ 0.01.

Table 6. Correlation of EDSS and other parameters regarding patients with different disease lines groups.

| Component | N-RRMS | RRMS | SPMS |
|-----------|-------|------|------|
|            | N=31  | N=34 | N=45 |
| EDSS       |       |      |      |
| CYP24A1    | 0.007 | 0.012 | 0.002 |
| 25-OHvitD3 | 0.223 | 0.797 | 0.790 |
| PTH        | 0.031 | 0.002 | 0.000 |
| Calcium    | 0.009 | 0.004 | 0.000 |
| Magnesium  | 0.024 | 0.002 | 0.000 |
| Phosphorous | 0.015 | 0.056 | 0.007 |

N: Number of subjects, R²: Coefficient of determination, r: Pearson’s correlation coefficients, NS: Non-significant at P>0.05, * Significant at P≤0.05 and ** Significant at P≤ 0.01.

Table 7A. The correlation of EDSS and study parameters regarding female patients groups.

| Component | N-RRMS | RRMS | SPMS |
|-----------|-------|------|------|
|            | N=20  | N=17 | N=23 |
| EDSS       |       |      |      |
| CYP24A1    | 0.001 | 0.000 | 0.352 |
| 25-OHvitD3 | 0.041 | 0.865 | 0.755 |
| PTH        | 0.009 | 0.108 | 0.045 |
| Calcium    | 0.004 | 0.001 | 0.014 |
| Magnesium  | 0.029 | 0.003 | 0.004 |
| Phosphorous | 0.001 | 0.008 | 0.010 |

N: Number of subjects, R²: Coefficient of determination, r: Pearson’s correlation coefficients, NS: Non-significant at P>0.05, * Significant at P≤0.05 and ** Significant at P≤ 0.01.
Table 7B. The correlation of EDSS and study parameters regarding male patients groups.

| Component     | N-RRMS N=11 | RRMS N=17 | SPMS N=22 |
|---------------|-------------|-----------|-----------|
|               | $R^2$ | $r$ | P | $R^2$ | $r$ | P | $R^2$ | $r$ | P |
| CYP24A1       | 0.029 | -0.169 | NS | 0.064 | -0.252 | NS | 0.066 | -0.256 | NS |
| 25-OHvitD3    | 0.689 | -0.830** | 0.000 | 0.733 | -0.856** | 0.000 | 0.810 | -0.900** | 0.000 |
| PTH           | 0.080 | -0.282 | NS | 0.015 | -0.122 | NS | 0.067 | 0.258 | NS |
| Calcium       | 0.164 | -0.405 | NS | 0.000 | -0.022 | NS | 0.023 | -0.151 | NS |
| Magnesium     | 0.038 | -0.194 | NS | 0.010 | 0.098 | NS | 0.003 | 0.052 | NS |
| Phosphorous   | 0.010 | 0.100 | NS | 0.123 | -0.350 | NS | 0.047 | 0.217 | NS |

N: Number of subjects, $R^2$: Coefficient of determination, $r$: Pearson’s correlation coefficients, NS: Non-significant at $P> 0.05$, * Significant at $P \leq 0.05$ and **Significant at $P \leq 0.01$.

4. Discussion

There is compelling evidence to indicate that a significantly lower level of CYP24A1 and 25-OHvitD3 are associated with an increased risk of disease in MS patients, regarding different disease lines and gender when compared to controls. In this paper, CYP24A1 and 25-OHvitD3 serum levels were positively correlated and significantly decreased with EDSS in female patients at advance stages of disease. While, in male only the serum level of 25-OHvitD3 was negatively correlated with EDSS in both early and advance stages of disease. A number of studies identified the considerable deficiency of 25-OHvitD3 in female occurs between 15 and 45 years of age during the greatest fertility at the same time with the prevalence peak for MS occurs [19-21]. Which, specified the significant decrease in 25-OHvitD3 and calcium levels between female groups than control. Many previous studies were consistent with our findings regarding to the lower serum concentration of 25-OHvitD3 in patients with MS [22-24]. Similarly to our finding several investigators have indicated lower serum level of 25-OHvitD3 and was correlated with a higher degree of disability measured by the EDSS [25-26]. Thus, lower serum level of 25-OHvitD3 result in irregular bone mineralization and, subsequently, walking problems. Considering that MS patients with higher EDSS scores are less likely to remain outdoors (thus reduced 25-OHvitD3 dermal synthesis) [15]. Analysis of variance for different patients groups regarding disease lines was identified significant decrease ($P \leq 0.01$) in 25-OHvitD3 level of RRMS in compare with N-RRMS. This suggested that MS patients had lower concentrations of 25-OHvitD3 at the advanced stage of the disease than patients at the early stage of MS [24]. The higher level of PTH in MS patients than controls resulting from a highly significant decrease in the level of 25-OHvitD3, calcium and significant increase in the level of phosphorous [27,28], this finding was confirmed by [29], while dissenting opinion with results of previous study [28],that indicated the level of PTH did not differ between patients and controls. Due to, the level of calcium and phosphorous in serum is mainly dependent on their dietary intake[15]. Thus, the high dietary calcium intake was reduced the expression of CYP24A1. That explained the negative correlation between CYP24A1 and calcium level in both male and female patients SPMS groups [30]. Numbers of researchers have recommended elevation of PTH level above the upper limit in MS patients, which also assessed in 28.88% of SPMS group in our results [31,23]. In this analysis, a significant increase in mean PTH concentration with development of MS lines with a substantial simultaneous decrease in mean 25-OHvitD3 concentration was observed only in female groups with different MS lines than control groups. This will demonstrate the effect of the duration of the disease on the indices studied [23]. Moreover, significant increase in the level of magnesium in MS patients than healthy controls may be explained by the regular use of magnesium containing supplements by MS patients [32]. The positive correlation
between CYP24A1 and magnesium serum level is following the results of Qi Dai and colleagues that reported the activity of CYP24A1 is magnesium depended [33].

5. Conclusion
In contrast to healthy control, substantially lower in the levels of CYP24A1 and 25-OHvitD3 are considered a risk factors in female MS patients. Thus, MS patients had a lower level of 25-OHvitD3 at the advanced stage than patients at the early stage of the disease. We confirmed the negative correlation between the 25-OHvitD3 serum level and the degree of motor disability according to EDSS in MS patients. We identified a non-significant different in the level of 25-OHvitD3 regarding on gender. Still, we confirmed the association between development of MS lines with PTH, phosphorus and 25-OHvitD3 serum levels are gender depended.

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7. References
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