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# CORRELATION BETWEEN INTEGRIN ALPHA-4 GENE POLYMORPHISMS AND FAILURE TO RESPOND TO NATALIZUMAB THERAPY IN IRAQI MULTIPLE SCLEROSIS PATIENTS

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**Abstract**

Genetic variation can impact the therapeutic response of natalizumab-treated multiple sclerosis patients; accordingly, screening for gene polymorphisms of the adhesion molecule α4β1-integrin that is specifically involved in lymphocyte transmigration into the CNS and the pathogenesis of human demyelinating disease will help to identify those with a predisposition to non-response. The aim is to assess the possible association between the rs200000911 (A > G,T) missense mutation at position 256 in the integrin α-4 subunit (ITGA-4) gene and the response to Natalizumab (NAT) in a sample of Iraqi patients with multiple sclerosis (MS). A sample of sixty-two patients with MS (45 females and 17 males; mean age of 31.6 years; age range of 15 to 52 years) receiving NAT for at least 12 consecutive months were involved in the study carried out from March to August 2022. The sample was categorized into two groups according to their response to NAT treatment (responders and non-responders). A polymerase chain reaction and Sanger's sequencing of the extracted deoxyribonucleic acid were performed to identify the polymorphism at the ITGA-4 gene promoter region. The rs200000911 (A > T) missense mutation was detected in both groups of patients (responders and non-responders to NAT treatment) with the absence of the rs200000911 (A > G) polymorphism. Additionally, the results revealed the existence of two intronic SNPs (rs936587744 (T > C) and rs2305588 (T > C)). All three polymorphisms appeared to have a non-significant impact on the responsiveness to NAT, serum concentration of Vascular Cell Adhesion Molecules-1 (VCAM-1), Expanded Disability Status Scale (EDSS), or rate of relapse change through the treatment period. The rs200000911 (A > T) missense mutation and the rs936587744 (T > C) and rs2305588 (T > C) intronic mutations are not correlated with the response to NAT in MS patients, with none of the genotypes appearing to increase the propensity to be a non-responder to NAT.

**Rezumat**

Variația genică poate influența răspunsul terapeutic al pacienților cu scleroză multiplă tratați cu natalizumab. Decelarea polimorfismului genetic al α4β1-integrina contribuie la identificarea genelor rezistente la terapie. Scopul acestui studiu a fost evaluarea asocierii între mutația rs200000911 (A > G, T) în poziția 256 a genei integrinei α-4 (ITGA-4) și răspunsul la natalizumab (NAT) într-un eșantion de pacienți irakieni cu scleroză multiplă (SM). În studiu au fost incluși 62 de pacienți cu SM care au primit NAT timp de cel puțin 12 luni consecutive în perioada martie-august 2022. Lotul a fost împărțit în două grupuri în funcție de răspunsul lor la tratamentul cu NAT (respondenți și non-respondenți). S-au efectuat polimerizarea și secvențierea *Sanger* a ADN-ului extras de la subiecți pentru a identifica polimorfismul genei ITGA-4. Mutația *missense* rs200000911 (A > T) a fost detectată în ambele grupuri de pacienți, cu absența polimorfismului rs200000911 (A > G). Toate cele trei forme polimorfe nu au avut un impact semnificativ asupra reactivității la NAT. Mutația *missense* rs200000911 (A > T) și mutațiile intronice rs936587744 (T > C) și rs2305588 (T > C) nu s-au corelat cu răspunsul la NAT în cazul pacienților cu SM.

**Keywords**: natalizumab, integrin α4 subunit gene polymorphism, multiple sclerosis

## Introduction

Multiple Sclerosis (MS) is a chronic neurodegenerative inflammatory disorder of the central nervous system (CNS) causing demyelination, gliosis and axonal destruction [1-3]. Patients with MS may experience considerable declines in quality of life due to a high level of disease-related prognostic variability and persistent degrees of disability [4]. It is brought on by lymphocyte migration across the blood-brain barrier (BBB), which relies on integrin 41 subunit (Very Late Antigen 4, VLA-4) interaction with Vascular Cell Adhesion Molecules-1 (VCAM-1) present on the endothelial cells of BBB [5]. Natalizumab (Tysabri®, Biogen-Idec Inc., NAT), a recombinant humanized monoclonal antibody (mAb) targeting the α4-integrin (ITGA4) component of the cell adhesion molecule, is one of the particularly efficient disease-modifying therapies (DMTs) used for the treatment of active relapsing remitting multiple sclerosis (RRMS) [5-7].
NAT connects to the integrin α4β1 subunit of the VLA4, preventing the entry of the lymphocytes into the brain *via* blocking VLA4 association with the corresponding ligand on the endothelium, VCAM-1 [8].

Its inhibitory mechanism is noncompetitive as it restricts the conformational space available to the VCAM-1 D2 subunit [9]. The approved dose of NAT is 300 mg injected intravenously (iv) every 28 days [10]. It seems to be highly efficacious and quite well tolerated by young MS patients [7]. Progressive multifocal leukoencephalopathy (PML) is a rare opportunistic infection of the brain, which represents the primary safety concern with NAT [11]. Furthermore, NAT is among the highly expensive DMTs, and these expenditures generate an enormous economic burden on the nation's health care system. The annual average cost of NAT treatment is around $36,000 *per* patient for the duration of treatment because of the high price and the necessity for repeated doses (12 doses *per* year) [12].

Numerous studies have been conducted on the safety and efficacy of natalizumab; however, data on NAT nonresponse are scarce. Emergent neurological impairment with NAT treatment is rarely a recurrence of multiple sclerosis and should necessitate a thorough evaluation of several potential causal factors, beginning with the exclusion of PML [13]. The second distinctive explanation of treatment failure is anti-natalizumab antibodies [14].

Recent studies suggest that genetic mutations can greatly impact the response to therapy in MS patients [15]. Any SNP located in the coding, non-coding, and intronic regions of the target gene may influence disease susceptibility and medication responsiveness [16]. Genome-wide association studies (GWAS) identify variants associated with disease predisposition. The next stage in MS genomics is to determine which genetic variants are associated with symptom severity, progress and treatment responsiveness [17].

Variants in the genes of VCAM-1, ITGA-4, and the integrin β1 subunit (ITGB-1) are interesting targets for studying the effect of genetic variation on NAT response [18]. The ITGA-4 gene is a promising candidate target for MS susceptibility and NAT responsiveness because it enhances the migration of leukocytes and other cells into the brain [5].

Clinical applications are still scarce despite advances in overall knowledge of the genetic processes underlying MS [19]. The primary objective of pharmacogenetics and pharmacogenomics in MS medication is to detect genetic mutations that can predict therapeutic response and toxicity. Therefore, genotype testing distinguishes between those with an effective pharmacological response and those with a poor or inadequate response [20].

In recent years, a rising number of studies have examined the link between target gene single nucleotide polymorphisms (SNPs) and MS susceptibility. Though the results of these studies varied by population due to pharmacogenetics-related ethnic and racial differences in allele types and frequencies [21]. Therefore, these results cannot be generalized. Additionally, genetics can influence environmental impacts. Accordingly, assessment of polymorphisms in different populations is required [19].

The Gln-152, Lys-201, and Lys-256 residues in the ITGA-4 gene have been identified as the main α4 residues to which NAT binds, and they can impact its action. The transversion of lysine (AAA) to arginine (AGA) at amino acid position 256 (rs200000911) has been sought as the cause of NAT non-responsiveness in rabbits (*Oryctolagus cuniculus*), as it represents the only different NAT binding site in nonresponsive rabbits from human ITGA-4 gene [22]. However, another study utilized a molecular dynamic model to study the mutation at position 256 and its impact on the molecular conformation of the whole integrin structure and the affinity of NAT binding. They found a non-significant correlation; instead, the mutated form revealed a higher binding affinity for NAT [9].

To date, no published study has investigated the effect of the rs200000911 (A > G,T) polymorphism at position 256 or the propensity for being a non-responder to NAT as ITGA-4 is its main target of action. We aimed to determine whether rs200000911 of the ITGA-4 gene may affect the response to NAT in a sample of Iraqi MS patients.

## Materials and Methods

*Patients*

A cross-sectional, single-centre study has been carried out at the Medical City Complex Baghdad Teaching Hospital, Department of Neurology, from March to August 2022. This study is part of a larger study that began using a convenient sample of seventy Iraqi MS patients. Since it is the exclusive site that administers NAT for MS patients, this clinic serves patients from all over Iraq, including city, rural and urban populations in the country. The research protocol has been approved by the College of Pharmacy Scientific and Ethical Committee, University of Baghdad, and also by the Neurology Department of the Baghdad Teaching Hospital.

In total, eighty-seven patients with relapsing-remitting MS who used NAT as monotherapy and fulfilled the inclusion criteria were investigated. Only 78 patients consented to be involved in the study. However, 70 of them met the research requirements.

*Inclusion criteria*

Confirmed MS diagnosis by specialized neurologists through clinical proof of CNS involvement, brain magnetic resonance imaging (MRI), and other diseases exclusion [23]. The patients must have taken NAT by intravenous infusion, regularly every 28 days, for at least 12 consecutive months.

*Exclusion criteria*

Patients who have been using NAT for less than 12 months. Patients using MS medications other than NAT. Patients with co-existing neurological diseases.

*Data Collection*

An information chart designed by the researchers was used to collect the demographic data, including age, gender, duration of disease, NAT dose number, and relapse number in the last 12 months, by direct interview. The baseline (before NAT treatment) Expanded Disability Status Scale (EDSS) has been collected from the patients' files, while the current EDSS was estimated by the neurologist.

*Groups of Patients and Clinical Assessment*

Sixty-two patients (45 females and 17 males, with a mean age 31.6 ± 8.37 years and their ages ranging from 15 to 52 years) were categorized into two groups; the first group (group A, n: 32) includes MS patients who clinically responded to NAT. While the second group (group B, n: 30) includes MS patients who did not respond to NAT as in Figure 1. The distribution into the groups was dependent on the value of the clinical response as measured by EDSS and the relapse rate for assessing the response to NAT therapy for at least 12 months.

The responsiveness of NAT-treated patients was determined by the presence of a steady or decreased EDSS and relapse absence. In contrast, NAT-unresponsive MS patients were diagnosed on the basis of an elevated EDSS despite treatment and the occurrence of relapses during the course of therapy [24, 25]. Furthermore, the development of anti-NAT neutralizing antibodies was investigated by a specific ELISA kit to exclude antibody-positive patients from the NAT-unresponsive group.

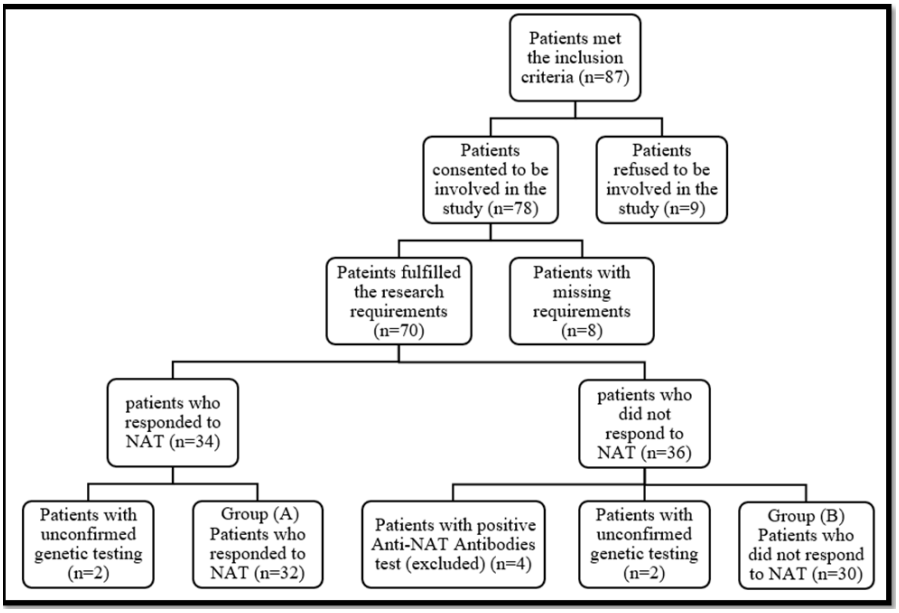


**Figure 1.**
Study Flow Chart

*Sample Collection and Deoxyribonucleic Acid (DNA) Extraction*

Venous blood (5 mL) was obtained from the forearm veins of each patient, taking 2 mL of blood to be utilized for the DNA extraction tube containing ethylenediaminetetraacetic acid (EDTA), and 3 mL of blood was transferred to the gel tube and centrifuged at 5000 rpm for 10 minutes to obtain serum.

The ReliaPrepTM Blood gDNA Miniprep System protocol (Promega, USA) was used to extract genomic DNA from the blood sample. The Master Taq polymerase enzyme and a hybrid thermal cycler were the components of the polymerase chain reaction (PCR) used to perform DNA amplification. The concentration of extracted DNA was then measured by the Quantus Fluorometer (Promega, USA).

*Primers and Primers Optimization*

The database of the National Centre for Biotechnology Information (NCBI) GenBank was used to get the ITGA-4 gene DNA sequence. The PCR primers were generated by Primer Premier 3 software (Table I). These primers were supplied by Macrogen Company (Macrogen, South Korea).

**Table I**

Primers' sequence with annealing temperature and base pair size

| Primer | Sequence 3`-5` | Annealing Temperature (°C) | Size (bp) |
|---|---|---|---|
| rs200000911-F | TGTAAAACGACGGCCAGTGTGTCACTCTCAACAGGATTT | 60 | 1008 |
| rs200000911-R | CAGGAAACAGCTATGACGTGGAATAAACCTTGCCAATTT | | |

rs200000911-F: Forward primer, rs200000911-R: Reverse primer

To establish the primers' best annealing temperature, the DNA template was amplified at 55, 58, 60, 63 and 65°C utilizing the corresponding primer sets (forward and reverse). After testing for the optimal primer annealing temperature, 60°C was selected.

*PCR Amplification and Sequencing of PCR Products*

Volumes of 20 µL containing 10 µL GoTaq Green Master Mix (2X) were used to perform PCR amplifications. Each primer (1 µL); nuclease-free water (6 µL) and template DNA (2 µL) were used. The PCR Express (Thermal Cycler, BioRad, USA) was used to complete PCR cycling with the specific temperature program as follows: 4 minutes for initial denaturation at 94°C then 30 cycles of denaturation at 94°C each for 30 seconds, followed by 30 seconds of annealing at 60°C; and finally, extension at 72°C for 30 seconds. The final extension incubation was for 7 minutes at a temperature of 72°C, followed by incubation at 4ºC for 10 minutes in order to stop the reactions. Macrogen Corporation's ABI3730XL, an automated DNA sequencing device, was employed to sequence PCR products by the Sanger method. The results were sent through email and evaluated using Geneious Prime software (Biomatters Ltd., New Zealand).

*Anti-NAT neutralizing antibodies ELISA kit*

Natalizumab antibody screening (qualitative) enzyme-linked immunosorbent assay (ELISA) kit (ELISA Genie Corporation, Ireland) was used to assess the occurrence of antibodies against NAT in serum samples of MS patients. The test was based on the sandwich principle, and the results were evaluated using a cut-off value [26].

*Vascular Cell Adhesion Molecule 1 (VCAM1) ELISA Kit*

A sandwich enzyme immunoassay kit for in vitro quantitative measurement of VCAM1 in human serum (Cloud-Clone Corporation, China) was used to measure the concentration of VCAM-1 in serum samples from MS patients [26].

*Statistical Analysis*

Version 24 of Statistical Package for the Social Sciences (SPSS) (IBM Corp., USA) was utilized to perform statistical analysis. Mean ± standard deviation (SD) was used to represent continuous variables. Numbers and frequencies were used to express alleles and genotypes. The normality of the results was tested by the Shapiro-Wilk test. Normally distributed demographic data and parameters were tested by an unpaired t-test to establish the differences between responders and non-responders. The Chi-square test was applied to investigate the data frequency difference between the groups. The correlation between genotypes and the non-response to NAT was evaluated using the phi correlation coefficient (phi). A p value of $< 0.05$ was considered statistically significant.

## Results and Discussion

*Demographic and Baseline Clinical Characteristics*

Demographic data and clinical variables for the patients are illustrated in Table II.

**Table II**

Demographic and clinical data of the study groups

| Grouping | Group A (Responders) n = 32 | | | Group B (Non-responders) n = 30 | | | p-value |
|---|---|---|---|---|---|---|---|
| | n | % | Mean ± SD | n | % | Mean ± SD | |
| **Age (year)** | | | 29.75 ± 7.565 | | | 33.57 ± 8.878 | 0.073[a] |
| **Gender** | | | | | | | |
| **Male** | 5 | 15.6 | | 12 | 40 | | 0.0315[b*] |
| **Female** | 27 | 84.4 | | 18 | 60 | | |
| **Disease Duration (year)** | | | 7.56 ± 4.196 | | | 8.67 ± 4.795 | 0.424[c] |
| **Number of NAT Doses** | | | 29.5 ± 14.476 | | | 18.57 ± 4.24 | 0.001[c*] |
| **VCAM-1 serum concentration (ng/mL)** | | | 385.67 ± 158.03 | | | 711.29 ± 254.31 | < 0.0001[c*] |
| **Baseline EDSS** | | | 2.438 ± 1.33 | | | 2.95 ± 1.89 | 0.554[c] |
| **EDSS after at least 12 months of NAT treatment** | | | 1.531 ± 1.397 | | | 4.873 ± 1.61 | < 0.0001[c*] |
| **EDSS change** | | | -0.906 ± 0.39 | | | 1.767 ± 1.265 | < 0.0001[c*] |
| **Relapse no. in the past 12 months** | 0 | 0 | | 14 | 46.67 | | < 0.0001[b*] |

SD: Standard deviation; NAT: Natalizumab; EDSS: Expanded Disability Status Scale; a: Independent 2 sample t-test; b: Chi-square test; c: Mann Whitney U test

*Prevalence of Alleles and Genotypes*

Genotypes and alleles frequencies in the two study groups are presented in Table III. The results revealed the presence of two intronic SNPs (rs936587744 (T > C) and rs2305588 (T > C)) in addition to the target SNP of this study (rs200000911 (A > G,T)). Notably, the results revealed the existence of AT variant of rs200000911 and the absence of the AG variant. In both rs200000911 and rs936587744, the wild form (AA and TT, respectively) genotypes have a higher prevalence than the heterozygote form (AT and TC, respectively), with zero incidence of the homozygote forms. While for rs2305588, the wild-type TT genotype has the highest incidence compared to the heterozygote and homozygote forms (TC and CC). All three SNPs have the reference allele at higher percentages as compared to their variant alleles.

**Table III**

Frequency of Genotypes and Alleles for the study population

| Genotypes | | | | |
|---|---|---|---|---|
| **rs200000911 (A > T)** | **Genetic Variants** | **AA** | **AT** | |
| | **No. (%)** | 45 (72.6) | 17 (27.4) | |
| | **Alleles** | **A** | **T** | |
| | **No. (%)** | 107 (86.3) | 17 (13.7) | |
| **rs936587744 (T > C)** | **Genetic Variants** | **TT** | **TC** | |
| | **No. (%)** | 35 (56.5) | 27 (43.5) | |
| | **Alleles** | **T** | **C** | |
| | **No. (%)** | 97 (78.2) | 27 (21.8) | |
| **rs2305588 (T > C)** | **Genetic Variants** | **TT** | **TC** | **CC** |
| | **No. (%)** | 45 (72.6) | 14 (22.6) | 3 (4.8) |
| | **Alleles** | **T** | **C** | |
| | **No. (%)** | 104 (83.9) | 20 (16.1) | |

Frequencies are represented by number and percentage

The results also present the genotype allocation between the two groups (Table IV). A non-significant difference in genotype prevalence between the two groups has been found for all three ITGA-4 polymorphic sites, with the wild forms being more frequent in both groups.

Concerning the difference in the prevalence of alleles between the two groups, the results revealed a non-significant difference in allele distribution between responders and non-responders, as seen in Table IV.

**Table IV**

Variation in Genotypes and Alleles prevalence between the groups

| Genotypes | | Group A (Responders) n = 32 | Group B (Non-responders) n = 30 | p-value |
|---|---|---|---|---|
| | | No. (%) | No. (%) | |
| **rs200000911 (A > T)** | **AA** | 23 (71.9) | 22 (73.3) | 0.896 |
| | **AT** | 9 (28.1) | 8 (26.7) | |
| | **A** | 55 (85.9) | 52 (86.7) | 0.905 |
| | **T** | 9 (14.1) | 8 (13.3) | |
| **rs936587744 (T > C)** | **TT** | 18 (56.3) | 17 (56.7) | 0.975 |
| | **TC** | 14 (43.8) | 13 (43.3) | |
| | **T** | 50 (78.1) | 47 (78.3) | 0.974 |
| | **C** | 14 (21.9) | 13 (21.7) | |
| **rs2305588 (T > C)** | **TT** | 22 (68.75) | 23 (76.7) | 0.063 |
| | **TC** | 10 (31.25) | 4 (12.5) | |
| | **CC** | 0 (0) | 3 (10) | |
| | **T** | 54 (84.4) | 50 (83.3) | 0.874 |
| | **C** | 10 (15.6) | 10 (16.7) | |

The statistical difference between the groups was determined using a Chi-square test

*Correlation between Genotypes and the incidence of NAT non-response*

By assessing the correlation between the incidence of non-response to NAT and each genotype through the use of the phi coefficient analysis. None of the studied genotypes seemed to increase the likelihood of NAT treatment failure (Table V). This indicates that these polymorphic sites, from the wild form to genotypes, cannot be used to predict the likelihood of NAT treatment failure.

**Table V**

Correlations between Genotypes and NAT treatment failure likelihood

| Genotypes | | Phi-coefficient | p-value |
|---|---|---|---|
| **rs200000911 (A > T)** | **AA (n = 45)** | 0.016 | 0.898 |
| | **AT (n = 17)** | -0.016 | 0.898 |
| **rs936587744 (T > C)** | **TT (n = 35)** | 0.004 | 0.974 |
| | **TC (n = 27)** | -0.004 | 0.974 |
| **rs2305588 (T > C)** | **TT (n = 45)** | 0.089 | 0.485 |
| | **TC (n = 14)** | -0.214 | 0.092 |
| | **CC (n = 3)** | 0.233 | 0.067 |

The association between each genotype and the propensity to become a non-responder was determined using the phi-correlation coefficient

*Correlation between Genotypes and VCAM-1 serum concentration*

Table VI revealed a non-significant difference in VCAM-1 serum levels among genotypes after at least 12 months of the NAT treatment, with non-significant post-HOC tests for all rs2305588 genotypes.

**Table VI**

Correlations between Genotypes and VCAM-1 serum concentration

| Genotypes | | VCAM-1 Serum Concentration (Mean ± SD) | p-value |
|---|---|---|---|
| **rs200000911 (A > T)** | **AA (n = 45)** | 524.01 ± 265.32 | 0.358[a] |
| | **AT (n = 17)** | 594.1 ± 266.17 | |
| **rs936587744 (T > C)** | **TT (n = 35)** | 501.95 ± 237.23 | 0.165[a] |
| | **TC (n = 27)** | 596.73 ± 293.59 | |
| **rs2305588 (T > C)** | **TT (n = 45)** | 546.52 ± 268.99 | 0.983[b] |
| | **TC (n = 14)** | 531.46 ± 275.76 | |
| | **CC (n = 3)** | 548.73 ± 241.59 | |

SD: Standard deviation; VCAM-1: Vascular Cell Adhesion Molecule-1; [a]:Mann Whitney U test was used to determine the difference between genotypes; [b]:ANOVA test was used to determine the difference between genotypes

*Correlation between Genotypes and EDSS change*

Table VII revealed a non-significant difference among the studied genotypes regarding the change in EDSS over the NAT treatment period. The only significant post-hoc analysis was between the TC and CC genotypes of rs2305588 (p-value 0.032).

**Table VII**

Correlations between Genotypes and EDSS change

| Genotypes | | EDSS change (Mean ± SD) | p-value |
|---|---|---|---|
| **rs200000911 (A > T)** | **AA (n = 45)** | 0.467 ± 1.66 | 0.536[a] |
| | **AT (n = 17)** | 0.176 ± 1.57 | |
| **rs936587744 (T > C)** | **TT (n = 35)** | 0.557 ± 1.68 | 0.353[a] |
| | **TC (n = 27)** | 0.167 ± 1.56 | |
| **rs2305588 (T > C)** | **TT (n = 45)** | 0.467 ± 1.63 | 0.082[b] |
| | **TC (n = 14)** | -0.214 ± 1.49 | |
| | **CC (n = 3)** | 2.0 ± 1.32 | |

SD: Standard deviation; EDSS: Expanded Disability Status Scale; [a]:Mann Whitney U test was used to determine the difference between genotypes; [b]:ANOVA test was used to determine the difference between genotypes

*Correlation between Genotypes and Relapse Rate in Non-responders*

The results showed a non-significant association between the genotypes and the occurrence of disease relapse in the non-responder group during the last 12 months of NAT therapy, as shown in Table VIII.

**Table VIII**

Correlations between Studied Genotypes and Relapse Rate in Non-responders

| Genotypes | | Relapse Rate in Non-responders | | p-value |
|---|---|---|---|---|
| | | n | % | |
| **rs200000911 (A > T)** | **AA (n = 22)** | 9 | 40.9 | 0.294 |
| | **AT (n = 8)** | 5 | 62.5 | |
| **rs936587744 (T > C)** | **TT (n = 17)** | 8 | 47.1 | 0.964 |
| | **TC (n = 13)** | 6 | 46.2 | |
| **rs2305588 (T > C)** | **TT (n = 23)** | 11 | 47.8 | 0.140 |
| | **TC (n = 4)** | 3 | 75 | |
| | **CC (n = 3)** | 0 | 0 | |

The difference between the genotypes was determined by Chi square test

For RRMS, natalizumab is recommended as monotherapy to reduce clinical exacerbations frequency, minimize brain lesions number and size as seen on magnetic resonance imaging (MRI) tests, and postpone physical disability progression [27]. Gene polymorphisms in genes that interact either directly or indirectly with drug actions and metabolism can impact a patient's response to therapy [28]. Patients may experience delays in receiving a treatment to which they can respond most fully, predisposing them to negative consequences with no tangible improvement and inflicting a tremendous strain on the healthcare system [29]. Pharmacogenomics researches the application of genetic information to improve therapeutic interventions and identifies individuals who have a higher likelihood of experiencing negative drug reactions or ineffectiveness [30]. The ITGA-4 molecule promotes immune cell recruitment into the CNS; NAT suppresses ITGA-4 and hinders lymphocyte translocation over the BBB [31]. In light of this, the ITGA-4 gene may be

considered a promising target gene for MS predisposition or NAT responsiveness [5]. The present study aimed to determine whether NAT ineffectiveness in a sample of patients with MS was related to the ITGA-4 gene rs200000911 (A > G,T) polymorphism at position 256.

Regarding this study's demographic characteristics, the results were similar to other studies on MS patients in Iraq. The baseline EDSS, mean age, and gender ratio were consistent with studies that involved MS patients and looked at therapy contentment, quality of life, and diagnostic markers [32-36].

The migration of lymphocytes across the BBB in MS is known to be significantly impacted by the binding of VLA-4 to VCAM-1 [37]. The current results revealed a significant decrease in VCAM-1 serum concentration in the responders as compared to the non-responders. Serum VCAM-1 levels were formerly observed to be higher in MS patients experiencing relapses as well as those with gadolinium-enhancing plaques on MRI [38]. Furthermore, a remarkably significant reduction in serum VCAM-1 concentration was detected in NAT-treated MS patients as compared to those without NAT treatment [39].

The mean EDSS after NAT treatment and the rate of relapse demonstrated significant differences between the groups, as they are being utilized as categorization criteria to classify the patients into responders and non-responders.

The development of neutralizing antibodies could render nearly all protein therapies ineffective [5]. Substantially reduced serum NAT concentrations and diminished therapeutic responses were caused by persistent anti-NAT antibodies [40]. According to data from the AFFIRM research, the recurrence rate among persistent antibody-positive patients was comparable to that of patients who received a placebo [41], mandating the exclusion of anti-NAT antibody-positive individuals from the non-responder group in the current study. The NAT-antibody immunologic complex's development is the putative explanation, as it ultimately leads to enhanced elimination and diminished effective serum levels of the medication [42]. The lack of routine anti-NAT antibody screening at the MS healthcare center in our study is the basis for not detecting antibody-positive patients earlier in the course of therapy.

In terms of genetic polymorphism outcomes, this study's findings, which included 62 MS patients receiving NAT, revealed the presence of the rs200000911 (A > T) missense mutation and the absence of the rs200000911 (A > G) mutation in the ITGA-4 gene at position 256. The prevalence of the AT variant represents about one quarter of genotypes in both responder and non-responder groups, with a notable absence of the homozygote TT variant. The A allele was the predominant one over the T allele. Unfortunately, no published studies have yet investigated this mutation in humans in terms of MS susceptibility or NAT responsiveness.

The present study unveiled a non-significant correlation of rs200000911 (A > T) with the inclination to be non-responders to NAT therapy in MS patients (a phi-coefficient of -0.016 for the AT variant and a p-value of 0.0898). A single SNP of the ITGA4 gene at amino acid position 256 resulting in the conversion of lysine (AAA) to arginine (AGA) (rs200000911, A > G) has been investigated as a possible target SNP to predict NAT response, as the Gln-152, Lys-201, and Lys-256 residues have been identified as the main α4 residues to which NAT binds. The Lys-256 residue was the only one that differed between NAT nonresponsive rabbits (*Oryctolagus cuniculus*) and humans and was thought to be responsible for NAT therapeutic failure [22]. In the context of pharmacogenetics, molecular dynamics modelling was utilized to examine the probable conformational alterations produced by the Lys-256 mutation on the entire integrin structure and the binding affinity of NAT in the non-mutant and mutant forms [9]. On the other hand, this research is the first, and no other published research has explored the rs200000911 (A > T) missense mutation with regard to NAT response in MS. However, these results should be authenticated by implementing larger-scale research encompassing various ethnic populations since the findings of such studies can be variable from population to population because of pharmacogenetic racial and ethnic inequalities brought on by the changeability in allele frequencies in various populations [21].

Every single SNP found in functional gene coding regions, non-coding regions and intronic regions may affect disease risk and medication sensitivity [16]. This investigation revealed the presence of two intronic SNPs (rs936587744 (T > C) and rs2305588 (T > C)). Previous studies revealed a significant association between various intronic SNPs in Glypican-5 (GPC5), interleukin-2 receptor alpha-chain gene (IL2RA), tumour necrosis factor receptor superfamily member 1A (TNFRSF1A) and tumour necrosis factor receptor superfamily member 14 (TNFSF14) and MS risk and susceptibility [43-46].

When considering the prevalence of rs936587744 (T > C) genotypes, the TC variant displayed a slightly lower incidence than the wild TT genotype, with the absence of a homozygote CC genotype and the C allele representing about one fifth of the whole allele frequency. The heterozygote TC genotype has a non-significant correlation with the likelihood of non-responsiveness to NAT (a phi-coefficient of -0.004 for the TC variant and a p-value of 0.974). Due to a lack of research encountering this polymorphism's prevalence or correlation with diseases or medication response, the current results remain inconclusive enough to be generalized.

On the other hand, the prevalence of rs2305588 (T > C) genotypes showed the abundance of the wild TT genotype over TC and CC variants. The C allele

denotes about one-sixth of the total allele incidence. All variants demonstrated a non-significant association with NAT unresponsiveness propensity (phi-coefficients of -0.214 and 0.233 with p-values of 0.092 and 0.067 for TC and CC variants, respectively). To date, only two published studies have involved this intronic mutation's relation to drug response in irritable bowel disease (IBD) and its correlation with melanocyte-to-lymphocyte ratio (MLR) [47, 48]. The findings of the first research correlated new genetic polymorphisms, including rs2305588 (T > C) in IBD patients with individual responsiveness variability to the anti-inflammatory drug BIRB796 (Doramapimod); this mutation was among the top twenty linked SNPs [47]. On the other hand, the second study revealed that rs2305588 was among a total of eleven ITGA-4 SNPs that proved to be correlated with the change in MLR [48]. Regretfully, no similar study has been undertaken and examined this intronic mutation with regard to disease vulnerability or NAT response, to which the results could be compared.

The present research couldn't verify a significant correlation between the three studied SNPs and the serum concentration of VCAM-1. Several previous studies proved that NAT reduced serum VCAM-1 concentrations in MS patients [39]. This explains the noticeably elevated serum levels of VCAM-1 in the NAT-non-responsive patients. Still, no studies have evaluated the impact of these mutations on serum VCAM-1 levels.

All three mutations in this study were observed to have a non-significant impact on the EDSS change. Nevertheless, the TC genotype of rs2305588 is the only one associated with a negative EDSS change compared to other genotypes. In the AFFIRM study, the primary outcomes for NAT treatment were the annual relapse rate at the first year and the cumulative disability progression, defined as an increase in the EDSS from baseline at 2 years. Natalizumab was confirmed to reduce the relapse rate and disability progression at 1 and 2 years, respectively [41, 49, 50]. Though our study failed to confirm a significant association between the three missense and intronic mutations and the relapse rate. The abundance of relapses in the non-responder group exclusively can be explained by the fact that NAT has been demonstrated to diminish the relapse rate and by utilizing the occurrence of relapse as a classification parameter in this research.

The primary limitation of the current study is the small sample size, which is attributable to the limited proportion of NAT-treated MS patients who fulfilled the inclusion criteria. Moreover, the correlation between the response and the NAT dose number was not examined. Furthermore, because this study only encompassed a single centre, the outcomes can't be extrapolated to all Iraqi MS patients. Nevertheless, this facility handles MS patients from all of Iraq's governorates. Additionally, due to inadequate facilities and a lack of funding, this study was unable to be expanded to encompass a reasonable follow-up duration for patients with particular genotypes.

## Conclusions

According to the results of this study, patients who did not respond to NAT showed greater VCAM-1 serum levels than those who did respond. Sanger sequencing of the ITGA-4 gene revealed three SNPs, including the primary target SNP of this study (rs200000911 (A > G,T)) in addition to the presence of two other intronic SNPs (rs936587744 (T > C) and rs2305588 (T > C)). The three polymorphisms had no significant effect on the propensity to be a NAT non-responder in Iraqi MS patients. The impact of these SNPs on NAT responsiveness has not been confirmed by this study. In addition, no previous research has explored their influence on NAT therapy response in other populations. Hence, larger-scale studies involving a wide variety of ethnic populations are recommended to prove or disprove the outcomes of this study.

## Conflict of interest

The authors declare no conflict of interest.

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