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Association of Methylenetetrahydrofolate Reductase Gene Polymorphisms with increased Risk of Multiple Sclerosis in a Sample of Jordanian Patients

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Abstract

Multiple sclerosis (MS) is an autoimmune disorder that is characterized by the destruction of myelin in the central nervous system and mainly affects young adults. Genetic and environmental factors play an important role in the MS etiology. This study is the first to investigate the genetic aspects of MS in Jordan. Therefore, the possible association of the individual and combined C677T and A1298C polymorphisms of the methylenetetrahydrofolate reductase (MTHFR) gene with MS susceptibility in Jordan was investigated in 100 (18 males and 82 females) patients and 100 age- and gender-matched healthy controls. The Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique revealed significant differences in the genotype and allele frequencies of A1298C polymorphisms between MS patients and controls (p =0.012218 and p =0.0275, respectively). The heterozygous AC [OR= 2.02 (95% CI: 1.119-3.6981, p = 0.0211)] and the homozygous recessive CC [OR= 3.52 (95% CI: 1.294-9.6028, p= 0.0137)] genotypes showed 2 and 3.5-fold increase in MS risk, respectively. No significant difference was observed in the C677T polymorphism between MS patients and controls (p =0.13466 and p=0.2321, respectively). Combined genotypes with 1-3 mutated alleles were detected in 89% of MS patients and 76% of controls. The prevalence of the combined heterozygous CT/AC genotypes was the highest (32%) in patients. None of the patients or controls exhibited the combined homozygous TT/CC genotypes. Polymorphisms were not associated with MS clinical and demographic characteristics.

In conclusion, the C677T polymorphism was not significantly associated with the risk of developing MS in our cohort of Jordanian patients. Conversely, a significant association was observed for the A1298C polymorphism, as the CC genotype correlated with elevated MS risk and may lead to a higher incidence of the disease. These findings provide additional support to the genetic basis of MS susceptibility and the potential role of the MTHFR gene in the physiopathology of MS. Thus, this gene could be a potential therapeutic target to halt neurodegeneration in MS patients.

Keywords: Multiple sclerosis; MTHFR gene; MTHFR C677T; MTHFR A1298C; MTHFR polymorphism; Jordan.

1. Introduction

Multiple sclerosis (MS) is a chronic neurodegenerative autoimmune disease in which the oligodendrocytes and the myelin sheets surrounding the axons in the central nervous system (CNS) are destroyed (Brownell and Hughes 1962; Mahad et al., 2015). Demyelination slows down and disrupts the message transmitted from and to the brain, causing motor and sensory impairment (Bitsch et al., 2000; Kutzelnigg et al., 2005). The disability in MS patients can be measured using Kurtzke's Expanded Disability Status Scale (EDSS), where 10 is the worst on a score of 0–10 (Kurtzke, 1983). MS is classified into four types: clinically isolated syndrome (CIS), relapsing remitting MS (RRMS), secondary progressive MS (SPMS), and primary progressive MS (PPMS) (Buck et al., 2013; Lublin et al., 2014). The exact etiology of this disease remains elusive and includes a complex interaction between environmental and genetic factors (Olsson et al., 2017; Ferrè et al., 2020; Goodin et al., 2021; Horjus et al., 2022; Jia et al., 2023). Most genetic studies have focused on identifying common and rare genetic variants in different genes contributing to MS susceptibility (Dashti et al., 2020; Zrzavy et al., 2020; Horjus et al., 2022; Esposito et al., 2022; Jia et al., 2023). Although several promising genes were nominated, some stood out due to their biological function, including the methylenetetrahydrofolate reductase (MTHFR) gene, which is significantly up-regulated in the brain of MS cases (Jia et al., 2023). This gene produces methylenetetrahydrofolate reductase (MTHFR), an enzyme essential for homocysteine metabolism, S-adenosylmethionine (SAM) synthesis, and nucleic acid synthesis. This enzyme converts 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-methyltetrahydrofolate (5-MTHF), essential for remethylating the neurotoxic intermediate homocysteine to methionine. In one-carbon metabolism, methionine is subsequently converted to SAM, essential for CNS myelination (Bagley et al., 1998). Moreover, the substrate

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(5,10-MTHF) of MTHFR is crucial for nucleic acid synthesis. Genetic variants that influence MTHFR activity and cause a disorder in the one-carbon metabolic pathway may be closely associated with the pathogenesis of MS (Jia et al., 2023). The association of two MTHFR C677T (rs1801133) and MTHFR A1298C (rs1801131) polymorphisms with MS has been validated in individuals of various descent (Klotz et al., 2010; Zhu et al., 201; Mrissa et al., 2013; Cevik et al., 2014; Naghibalhossaini et al., 2015; Erkan-Asci and Karahalil, 2017; Cakina et al., 2019). The MTHFR C677T point mutation includes a C to T transition at nucleotide 677 (677C>T), resulting in alanine to valine substitution in MTHFR and reduced enzymatic activity (Weisberg et al.,1998). Individuals with the homozygous genotype (TT) have higher serum homocysteine (hcy) levels than healthy controls (Van der Put et al., 1998; Hiraoka and Kagawa, 2017). The MTHFR A1298C polymorphism includes A to C transition at nucleotide 1298 (1298A>C), resulting in glutamate to alanine substitution in MTHFR and decreased enzymatic activity, causing mild hyperhomocysteinemia (Weisberg et al., 1998; Yamada et al., 2001). Also, the decreased activity affects the regeneration of oxidized tetrahydrobiopterin from 5-MTHF, which is involved in monoamine neurotransmitter synthesis (Miller 2008).

Previous research on MS in Jordan was limited and focused on characterizing the clinical, demographic, and epidemiological features of multiple sclerosis (Al-Din et al., 1995; El-Salem et al., 2006). Therefore, the current study represents the first molecular neuroepidemiological investigation conducted in Jordan that provides valuable insight into the genetics of MS susceptibility. It aimed to investigate a possible association of the individual and combined MTHFR C677T and A1298C polymorphisms with susceptibility to MS in a group of Jordanians. This study contributes to the global efforts in understanding the genetics of MS that may help identify potential molecular targets for future therapeutic strategies.

2. Materials and Methods

2.1. Subjects

A total of 100 unrelated MS patients (18 males and 82 females) who attended the neurology clinics at Al-Bashir Hospital in Amman, the capital of Jordan, were recruited to participate in this study. The patients were diagnosed by the neurologists according to McDonald's MS criteria for classification (Polman et al., 2005). Physical disability was assessed using the EDSS score. Also, a total of 100 age, gender, and ethnicity-matched unrelated healthy control subjects without signs or history of immunological, neurological, or genetic diseases were recruited from the Jordan University Hospital in Amman to participate in this study. Those subjects attended the hospital for regular checkups. The study was conducted according to the Declaration of Helsinki. The protocol and the consent forms were approved by the institutional Ethics Committee of Jordan University Hospital (10/ 2018/ 2916) and Al-Bashir Hospital (8590). All subjects were provided with information on this study and gave their informed consent for inclusion before they participated in the study.

2.2. MTHFR gene polymorphism

Human genomic DNA was extracted from whole blood samples using the Wizard DNA genomic purification kit (Promega, USA) and according to the manufacturer's instructions. The presence and integrity of the extracted DNA were detected in a 1% agarose gel. The quantity and purity of DNA were determined using the spectrophotometer at OD_{260} and OD_{280} (Sambrook and Russel., 2001). The MTHFR C677T (rs1801133) and A1298C (rs1801131) polymorphisms were analyzed by the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique (Skibola et al., 1999). The PCR products were detected in a 2% agarose gel and then digested by a restriction enzyme. The PCR products (198 bp) of the MTHFR C677T polymorphism were digested separately in a 25µl reaction mixture by the restriction enzyme *Hin*fI (New England Bio Lab, USA) because the C→T base pair substitution generates a *Hin*fI restriction site (Skibola et al., 1999). In the MTHFR A1298C polymorphism, the A→C substitution abolishes a *Mbo*II restriction site. Therefore, the PCR products (163 bp) of MTHFR A1298C were separately digested in a 22µl reaction mixture by *Mbo* II (Thermo Fisher, USA) according to the manufacturer's instructions. The genotype of individuals was determined based on the DNA profile in a 3% agarose gel as reported by Skibola et al (1999).

2.3. Statistical analysis

The distribution and the genotypic and allelic frequencies of the MTHFR gene polymorphisms in MS patients were compared with those of controls using Fisher's exact test (Rodriguez et al., 2009). Statistical difference was considered significant for *p*-values less than 0.05. The relationships between the two types of polymorphisms and the clinical and demographical characteristics of patients were analyzed using Pearson's Chi-square test (Rodriguez et al., 2009). The association between these genotypes and the risk of MS was estimated by calculating the odds ratios (ORs) and their 95% confidence intervals (CIs) using the binary logistic regression analysis. *p*-values less than 0.05 were considered statistically significant. Hard-Weinberg equilibrium was corroborated for the allelic frequencies in the control group (Smith and Baldwin, 2015).

3. Results

3.1. Demographic characteristics of participants.

The demographic and clinical characteristics of MS patients and healthy control subjects are presented in Table 1. Each group included 82 females and 18 males aged 16-66 years. There was no statistically significant difference (p= 0.7602) between the mean age of MS patients and that of the control subjects. The mean age of onset in MS patients is 29.63±9.205 years. Most patients (93%) have RRMS clinical MS type. The mean EDSS score for MS patients is 1.74±1.36, indicating that these patients can walk without any aid.

Table 1. Demographic and clinical characteristics of MS patients and healthy controls.

Demographic and Clinical Characteristics	MS Patients (n=100)	Healthy (n=100)
Gender, male/female, n (%)	18/ 82 (18/82)	18/ 82 (18/82)
Age (mean ± SD), y	37.04 ±11.95	36.55 ±11.84
Age of onset (mean ± SD), y	29.63 ±9.205	
CIS, n (%)	0 (0)	
RRMS, n (%)	93 (93)	
SPMS, n (%)	2 (2)	
PPMS, n (%)	5 (5)	
EDSS score (mean ± SD)	1.74 ±1.37	

MS: Multiple Sclerosis; n: Number; SD: Standard deviation; y: Year; CIS: Clinically isolated syndrome; RRMS: Relapsing-remitting MS, SPMS: Secondary progressive MS, PPMS: Primary progressive MS; EDSS: Expanded disability status scale.

3.2. MTHFR gene polymorphism

The C677T polymorphism-specific PCR products (198 bp) and A1298C polymorphism-specific PCR products (163 bp) were detected in a 2 % agarose gel (Figures 1 and 2).

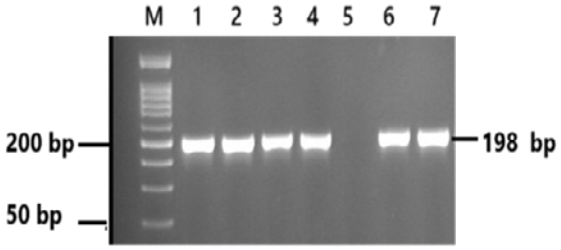

Figure 1. A representative ethidium bromide-stained 2% agarose gel for the target sequence of MTHFR C677T. M: 50 bp marker (GenedireX, Taiwan); Lanes 1-4, 6-10: the PCR product (198 bp); Lane 5: PCR blank.

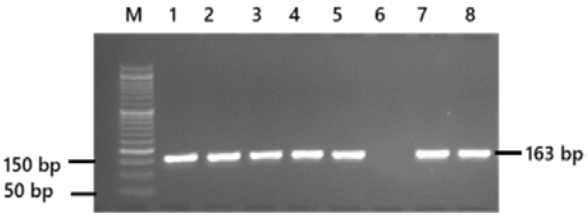

Figure 2. A representative ethidium bromide-stained 2% agarose gel for the target sequence of MTHFR A1298C (rs1801131). M: 50 bp marker (GenedireX, Taiwan); lanes 1-5, 7-8: the PCR product (163 bp); Lane 6: PCR blanks.

To determine the C677T polymorphism, the specific PCR products (198 bp) were separately digested by *Hin*f I, and the genotypes of the participants were identified based on the DNA profile in agarose gel (Figure 3). The normal wild genotype (CC) of individuals was identified by detection of the undigested 198 bp PCR products, while the heterozygote genotype (CT) was identified by detection of both the 175 bp and 198 bp *Hin*fI DNA fragments. The generated small 23 bp *Hin*fI fragment reported by Skibola et al (1999) was not detected in the gel. The homozygote genotype (TT) was identified by detection of the 175 bp DNA fragment without the small 23 bp *Hin*fI fragment reported by Skibola et al (1999).

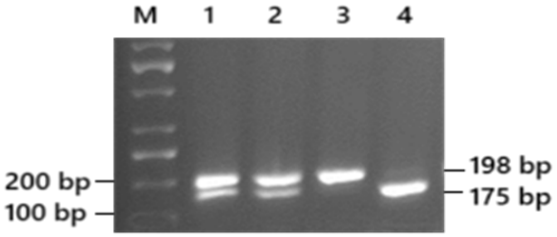

Figure 3. A representative ethidium bromide-stained 3% agarose gel for polymorphism analysis of MTHFR C677T PCR products digested by *Hin*fI. M: 100 bp marker (GenedireX, Taiwan); Lanes 1, 2: Heterozygous MTHFR 677CT genotypes generating 198 bp and 175 bp DNA fragments; Lane 3: normal wild MTHFR 677CC genotypes generating 198 bp DNA fragment; Lane 4: Homozygous MTHFR 677TT genotype generating 175 bp DNA fragment.

The wild type (1298AA) genotype was identified by detection of 18-, 31-, 28-, 56-, 30-bp DNA fragments, the homozygous variant (1298CC) genotype was identified by detection of 18-, 31-, 84-, 30-bp DNA fragments, and the heterozygous (1298AC) genotype by detection of 84-,56-, 31-, 30-, 28-,18-bp DNA fragments in the gel (Figure 4).

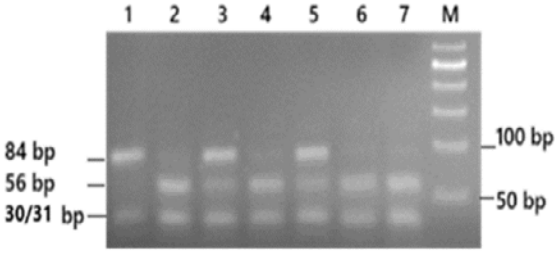

Figure 4. A representative ethidium bromide-stained 3% agarose gel for polymorphism analysis of MTHFR A1298C PCR products digested by *Mbo*II. M: 50 bp marker (GenedireX, Taiwan); Lane 1: the homozygous MTHFR 1298 CC generating 84 and 30/31 bp DNA fragments; Lane 2,4,6 and 7: the normal wild MTHFR 1298 AA genotype generating 56, and 30/31 bp DNA fragments; Lanes 3 and 5: the heterozygous MTHFR 1298 AC genotypes generating 84, 56, and 30/31 bp DNA fragments.

3.3. Genotypic and allelic frequencies of the MTHFR gene polymorphisms.

The genotypic and allelic frequencies of the MTHFR gene polymorphisms in both MS patients and healthy controls are shown in Table 2. The distribution of C677T genotypes was in accordance with Hardy-Weinberg equilibrium (HWE) in controls (p = 0.058). No significant difference was observed in the C677T genotypic (p = 0.1346) and allelic (p = 0.2321) frequencies between MS patients and controls, except for the CT genotype, which was significantly (p = 0.0169) higher in patients. The frequency of the heterozygous CT genotype (53%) and allele C (66.5%) was the highest in the MS patients (Table 2). The distribution of A1298C genotypes was in accordance with HWE in controls (p = 0.322). A significant difference was observed in the A1298C genotypic (p = 0.012218) and allelic (p = 0.0275) frequencies between MS patients and controls. The frequency of the AA, AC, and CC genotypes in MS patients was significantly (p = 0.0066, 0.0211, and 0.0137, respectively) higher than that of the controls. Moreover, the frequency of the mutant C allele in patients (40.5%) was significantly (p = 0.0275) higher than that in controls (29.5%) (Table 2).

Table 2. Genotypic and allelic frequencies of the MTHFR gene polymorphisms in the study population.

MTHFR gene polymorphisms	Genotype and alleles	Frequency (%)		
		Patients (N*=100)	Control (N=100)	Total (N=200)
C677T (rs1801133)	Genotypes			
	CC	40	53	93
	CT	53	39	92
	TT	7	8	15
	p-value[a] = 0.13466			
	Alleles			
	C	66.5	72.5	69.5
	T	33.5	27.5	30.5
	p-value[a] = 0.2321			
	HWE *p*-value[b] =0.058			
A1298C (rs1801131)	Genotypes			
	AA	31	50	82
	AC	54	43	96
	CC	15	7	22
	p-value[a] = 0.012218			
	Alleles			
	A	59.5	70.5	65
	C	40.5	29.5	35
	p-value[a] = 0.0275			
	HWE *P*-value[b] =0.322			

*N= number; [a] Fisher's exact test *p*-value; [b] Hardy-Weinberg equilibrium *p*-value assessed in control; Statistically significant results are shown in boldface.

3.4. Association analysis of C677T and A1298C polymorphisms with MS

In the C677T polymorphism, only the CT genotype was significantly (OR= 1.8, 95% CI:1.005-3.22, *p* =0.0169) associated with MS (Table 3). No statistically significant association (OR=1.7, 95% CI: 0.9656 -2.9631, *p*= 0.061) was observed when comparing patients to controls according to CC genotype versus the combined heterozygous and homozygous variant genotypes (CT+TT). Also, there was no significant association with MS in the recessive (TT) inheritance models of this polymorphism (OR= 1.15, 95% CI: 0.3881-3.4600, *p*= 0.791). For the A1298C polymorphism, both the heterozygous AC (OR= 2.02, 95% CI: 1.119-3.6981, *p* = 0.0211) and the homozygous CC (OR= 3.52, 95% CI: 1.294-9.6028, *p*= 0.0137) genotypes were significantly associated with MS (Table 3). Moreover, a significant association with MS risk (OR =2.2371, 95% CI: 1.2569-3.9829, *p*=0.0062) was observed when the dominant inheritance model for the heterozygous and homozygous genotypes (AC+CC) was evaluated (Table 3). Additionally, a significant association (OR=3.52, 95% CI: 1.2942-9.6028, *p*=0.0137) with MS was observed in the recessive (CC) inheritance models of this polymorphism. When assessing the association with MS by alleles, a statistically significant positive association **(**OR=1.8360, 95% CI: 1.2104-2.7849, *p*=0.004) was found in the C allele of the A1298C polymorphism (Table 3).

Table 3. Association analysis of C677T and A1298C polymorphisms with MS.

Polymorphisms	Control/ Case (n=100/100)	OR (95% CI)	*p*-value
C677T			
CC	53/40 (ref.)	1.00	
CT	39/53	1.8 (1.005-3.22)*	0.0169
TT	8/7	1.15 (0.3881-3.4600)	0.7911
Inheritance models			
CT+TT	47/60	1.7 (0.9656 - 2.9631)	0.061
TT[r]	8/7	1.15 (0.3881-3.4600)	0.7911
Alleles			
C	145/133	1.00	
T	55/67	1.3 (0.8611-2.0361)	0.1931
A1298C			
AA	51/31(ref.)	1.00	
AC	43/53	2.02 (1.1119-3.6981)	0.0211
CC	7/15	3.52 (1.2942-9.6028)	0.0137
Inheritance models			
AC+CC	145/115	2.2371 (1.2569-3.9829)	0.0062
CC[r]	57/83	3.52 (1.2942-9.6028)	0.0137
Alleles			
A	145/115	1.00	
C	57/83	1.8360 (1.2104-2.7849)	0.004

The statistically significant results are shown in boldface; * Significant *p*-value for trend ($p<0.05$). [r]Recessive inheritance model; ref: controls with the wild-type CC and AA genotypes were used as a reference category.

3.5. The prevalence of combined genotypes

The combined genotypes of both C677T and A1298C polymorphisms with 1-3 mutated alleles in the MTHFR gene were detected in 89% of MS patients and 76% of controls (Table 4). The CC/AA wild-genotype combination was the highest (24%) in controls, and its prevalence was significantly (*p*=0.0158) higher than that in MS patients. The prevalence of the combined heterozygous CT/AC genotypes was the highest (32%) in patients, but there was no significant difference (*p*=0.1121) in its prevalence between patients and controls. Importantly, none of the patients and controls exhibited the combined homozygous TT/CC genotypes. The prevalence of the other combined genotypes of the two polymorphisms did not exhibit a significant difference (*p*=0.0817- 0.7073) between patients and controls (Table 4).

Table 4. The frequency of the combined MTHFR C677T/ A1298C genotypes in MS patients and controls.

Polymorphisms		Number of mutated alleles in combined genotypes	Patients (N= 100)	Controls (N= 100)	*P* value
C677T	A1298C				
CC	AA	0	11 (11)	24 (24)	0.0158
	AC	1	18 (18)	23 (23)	0.3823
	CC	2	11 (11)	7 (7)	0.3242
CT	AA	1	18 (18)	16 (16)	0.7073
	AC	2	32 (32)	22 (22)	0.1121
	CC	3	3 (3)	1 (1)	0.3136
TT	AA	2	4 (4)	7 (7)	0.3533
	AC	3	3 (3)	0 (0)	0.0817
	CC	4	0	0	0
Total			100 (100)	100 (100)	

3.6. Clinical and demographical characteristics of patients stratified according to C677T and A1298C polymorphism.

The clinical and demographical characteristics of MS patients stratified according to C677T and A1298C gene polymorphisms are summarized in Tables 5 and 6. Parameters including gender, age, age of onset, disease duration, EDSS score, and family history of MS patients were analyzed. Notably, no statistically significant associations were observed between C677T and A1298C polymorphisms and any of the studied clinical and demographic characteristics of the MS patients.

Table 5. Clinical and demographical characteristics of patients stratified according to MTHFR gene C677T polymorphism.

Characteristic	Total (N=100)	CC (N=40)	CT+TT (N=60)	*p* - value
Gender, male/female, N (%)	18/82 (18/82)	9 /31 (22.5/77.5)	9/51 (15/85)	0.4274
Age, mean ± SD , y	37.04 ±11.95	38 ±11.53	36.04 ±12.05	0.4168
Age of onset, mean (SD), y	29.63 ±9.205	30.15 ±8.43	29.28 ±9.74	0.6457
Disease duration, mean (SD), y	8.06 ±9.660	7.72 ±6.5	8.28 ±11.33	0.7779
EDSS score (mean ± SD)	1.74 ±1.37	1.7 ±1.33	1.75 ±1.39	0.8581
MS types, N (%):				
RRMS + SPMS	95 (95)	37 (92.5)	58 (96.6)	0.3864
PPMS	5 (5)	3 (7.5)	2 (3.33)	
Family History, N (%)	18 (18)	4 (10)	14(23.3)	0.0914

N: Number; y: year; SD: standard deviation.

Table 6. Clinical and demographical characteristics of patients stratified according to MTHFR gene A1298C polymorphism.

Characteristic	Total (N=100)	AA (N=31)	AC+CC (N=68)	*p*-value
Gender, male/female, N (%)	18/82 (18/82)	5 /26 (15.15/84.84)	13/55(19.4/80.6)	0.7834
Age, mean (SD),y	37.04 ±11.95	36.93 ±12.93	37.35 ±11.35	0.7050
Age of onset, mean (SD), y	29.63 ±9.205	29.12 ±1.25	29.88 ±8.71	0.6200
Disease duration, mean (SD), y	8.06 ±9.660	7.09 ±5.36	8.53 ±11.19	0.4859
EDSS score, mean (SD)	1.74 ±1.37	2.07 ±1.61	1.57 ±1.22	0.0869
MS types, N (%)				
RRMS + SPMS	95 (95)	31 (93.93)	64 (95.5)	0.664
PPMS	5 (5)	2 (6.06)	3 (4.47)	
Family History, N (%)	18 (18)	4 (12.12)	14(20.89)	0.2855

N: Number; y: year; SD: standard deviation.

4. Discussion

Elucidating the pathogenesis of MS is a key goal of human genetics research. Genetic susceptibility to MS is thought to be an important factor in MS pathogenesis, which remains obscure. Studies on genetic polymorphisms provide insights into the role of individual susceptibility in MS development. Therefore, the possible association of the MTHFR gene polymorphism with MS susceptibility in a Jordanian population was investigated for the first time in the present study. A total of 100 MS patients from the middle of Jordan and 100 age, gender- and ethnicity-matched controls were analyzed for the presence of the MTHFR C677T and A1298C polymorphisms. The mean age of MS onset was found to be 29.6 ±9.2 years, which is consistent with those of previous findings in Jordan: 29.6 ±8.1 years (Al-Din et al., 1995), 29.3 ±9.6 years (El-Salem et al., 2006), 28.6 years (Al-Shimmery and Bzaini, 2008), and 29 years (Ahram et al., 2014). The mean age of MS onset varies globally and ranges from 25 to 35 years in European and North American MS patients (Khan and Hashim, 2015; Gbaguidi et al., 2022). Recently, MS onset shifted towards older age in some European countries, including Italy (Prosperini et al., 2022), Spain (Romero-Pinel et al., 2022), and Norway (Habbestad et al., 2024). Notably, 93% of the MS patients in our study were diagnosed with RRMS, indicating an early stage of the disease and not advanced disability. This prevalence is consistent with the findings of El-Salem et al. (2006), who reported that nearly 90% of MS patients have RRMS. The predominance of RRMS in our cohort aligns with the global pattern, as RRMS represents about 69-90% of MS cases (Bayas et al., 2022; Gracia et al., 2017). Also, the

mean EDSS score for the MS patients in the current study was 1.74, which is within that (1-8.5) reported for the European, Australian, Canadian, and American MS patients (Manouchehrinia et al., 2017; Braune et al., 2021; Fuh-Ngwa et al., 2023; Romeo et al., 2021).

The findings of this study suggest no association between the MTHFR C677T polymorphism and MS. There was no significant difference in the genotypic and allelic frequencies between MS patients and controls, except for the heterozygous CT genotype, where the *p*-value was 0.047 (Table 2). On the other hand, an association between the A1298C polymorphism and MS was observed in the studied population (Table 2). The frequency of the wild homozygous AA genotype in healthy controls was higher than in the MS patients, indicating a potential protective role of homozygosity for the A allele against MS development. This result was similar to that reported in Germany (Klotz et al., 2010). In our study, the heterozygous AC and the homozygous CC genotypes were 2 times and 3.5 times, respectively associated with increased risk of MS. These associations were observed when patients were compared with the controls according to the AA genotype versus the AC + CC genotypes. Additionally, the C allele was significantly 1.8 times more prevalent in the patient group (Table 3). The significant association of MS susceptibility in our patients with MTHFR A1298C polymorphism and the lack of association with MTHFR C677T are in agreement with other studies in German (Klotz et al., 2010) and Tunisian (Mrissa et al., 2013) MS patients. Also, there was no association between MTHFR C677T polymorphism and MS development in Swedish (Huang and Hillert, 1997), Australian (Tajouri et al., 2006), and Polish (Chorąży et al., 2019) patients. In contrast, the MTHFR C677T genotype was associated with MS susceptibility in Turkish MS patients, with statistically significant differences in genotype and allele frequencies between patients and controls (Cevik et al., 2014). In Iran, the association of MS and the MTHFR C677T polymorphism was observed in 194 MS patients, and the T allele was 1.7 times more prevalent in the patient group than in the healthy controls. Patients with the T allele developed MS almost 4 years earlier than those with other genotypes, indicating that carrying the T allele of the C677T polymorphism might predispose to an earlier onset of MS (Alatab et al., 2011). Other Iranian (Naghibalhossaini et al., 2015) and recent Turkish (Cakina et al., 2019) studies reported a significant association between genotypes of both C677T and A1298C and MS development. In Iran, the CT and TT genotypes increased the risk for MS development by 2.9 and 6.23-fold, respectively (Naghibalhossaini et al., 2015). In contrast, there was no significant association between A1298C polymorphisms and MS susceptibility in Australia (Szvetko et al., 2007) and Poland (Chorąży et al., 2019). These discrepancies highlight the ethnic and geographic variability in genetic susceptibility to MS, possibly due to lifestyle differences, environmental factors, or gene-environment interactions **(**Olsson et al., 2017). Despite all these diverse data from published reports, the positive association of the MTHFR A1298C polymorphism with MS in the present study and other studies highlights the crucial role of the MTHFR gene in MS physiopathology. It was reported that the A1298C polymorphism is associated with reduced MTHFR activity, particularly in the homozygous (CC) genotype compared to the heterozygous (AC) genotype (Van der Put et al., 1998). The reduced enzymatic activity affects the regeneration of oxidized tetrahydrobiopterin from 5-MTHF, which is involved in monoamine neurotransmitter synthesis (Miller 2008).

The combined effect of MTHFR 677 and 1298 polymorphisms on MS risk was investigated in the present study. It was found that the combined genotypes were detected in 89% of MS patients and 76% of controls (Table 4). In some combined genotypes, e.g. CT/AC, CC/CC, and TT/AC, a slight but not significant increase in the number of MS patients compared to the controls was observed, which could be due to the small number of the studied population. The prevalence of the combined CT/AC heterozygous genotypes was the highest (32%) in MS patients (Table 4). In Southern Iran, there was no significant difference in the prevalence of the combined genotypes between MS patients and controls except for the CT/AC, TT/AC, and TT/AA combinations that increase the risk of MS development by 5.3, 13.9, and 4.9-folds, respectively (Naghibalhossain et al., 2015). It was reported that this combined heterozygosity of both MTHFR mutations (CT/AC) is associated with reduced MTHFR-specific activity, higher Hcy, and decreased plasma folate levels, resulting in an outcome similar to that observed in TT homozygous individuals (van der Put et al., 1998).

Our study did not reveal a relationship between C677T and A1298C polymorphisms and any studied clinical and demographical characteristics of MS patients (Tables 5, 6). Similarly, no statistically significant association was observed between the clinical and demographical characteristics of MS patients from the North of Turkey and MTHFR gene C677T polymorphism (Cevik et al., 2014), and in MS patients from the West of Turkey and the two MTHFR polymorphisms (Cakina et al., 2019). Also, no statistically significant differences in frequencies of the MTHFR A1298C genotypes were found in Iranian MS patients stratified by age, sex, and disease type (Naghibalhossain et al., 2015). In Poland, there was no statistically significant association between MTHFR gene C677T and A1298C polymorphisms and the clinical characteristic features of MS patients (except for duration of disease and EDSS scale score) (Chorąży et al., 2019).

In conclusion, our study contributes to the growing knowledge of the complex interplay between genetic factors and MS. The significant association of A1298C polymorphism with the risk of MS development was observed in our cohort of Jordanian patients, providing additional support for the genetic basis of MS susceptibility and indicating a potentially significant role for the MTHFR gene in the physiopathology of MS. This gene could be a potential therapeutic target to halt neurodegeneration in MS. If this association is validated through further research on higher number of patients, this polymorphism could serve as a possible genetic marker for assessing MS risk in the Jordanian population. Despite the absence of statistically significant differences in genotypic and allelic frequencies between patients and controls, except for the heterozygous CT genotype, this finding does not entirely rule out the potential involvement of the MTHFR C677T gene polymorphism in predisposition to the risk of MS development in Jordan. The lack of statistical significance may be attributed to the modest

sample size, limiting our ability to detect a significant association if it exists. Thus, we recommend conducting a more extensive study on a larger sample set to validate these results and to highlight the possible contribution of the C677T polymorphism in MS susceptibility in Jordan. Expanding our investigation to include variants in other genes encoding key enzymes in the one-carbon metabolism pathway, such as the MTR gene coding for methionine synthase and the MTRR gene coding for methionine synthase reductase, could provide further insights. Future studies should also consider integrating these genetic data with environmental and lifestyle factors to better understand their combined effects on MS risk.

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