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Assessing the Impact of *HBB* Gene Polymorphisms on the Risk of Beta Thalassemia in the Population of Diyala Governorate, Iraq

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Abstract

Objective: The current research aims to investigate the genetic polymorphisms of the *HBB* gene and their relationship to the incidence of beta-thalassemia.

Methods: The study included 30 patients with beta-thalassemia major. Samples were collected during the period between August 2023 and February 2024. Their ages ranged from 2–31 years, in addition to 30 healthy people who did not suffer from hereditary blood diseases as a control group. Their ages ranged from 1–35 years. The exon region of the *HBB* gene was sequenced using nucleotide sequencing in order to identify the kind and location of mutations that alter the amino acid sequence that makes up the hemoglobin protein.

Results: The results of DNA sequencing of the exon region of the *HBB* gene, which consists of 382 base pairs, showed the presence of the rs713040 variant. This variant revealed that the GG genotype and the G allele, and the AA genotype and the A allele, were more frequent in the patient group compared to the healthy group and may be associated with risk. When comparing some physiological variables with the genotypes of the *HBB* gene between the group of patients and healthy controls at the rs713040 variant site, the results showed that there were significant differences ($P < 0.001$, $P < 0.05$) in the levels of WBC, HB, PLT, PCT, ALP, and Vitamin D in the three genotypes of the variant GG, GA, and AA.

Conclusion: The findings suggest that the rs713040 variant of the *HBB* gene may be associated with an increased risk of beta-thalassemia and that there are significant physiological differences between the genotypes of this variant in patients and healthy controls. Further research is needed to understand the underlying mechanisms and the potential clinical implications of these genetic differences.

Keywords: SNP, *HBB* gene, polymorphism rs713040, Beta-thalassemia major

Introduction

One of the most prevalent genetic disorders, beta-thalassemia, is brought on by mutations in the *HBB* gene, which codes for the postnatal form of the hemoglobin beta component. The *HBB* gene, which is expressed in neonates, codes for two beta globin subunits and two alpha subunits of hemoglobin tetramers after birth. Prior to then, one of the two HBG genes—which are active throughout the fetal stage and often silenced after birth—codes for beta globin. β-thal is a disease that affects children from an early age and is caused by a variety of mutations in the *HBB* gene that cause less or no *HBB* protein. Patients with β-thal have been shown to have over 200 distinct forms of *HBB* gene mutations, which can occur anywhere within a ~1600 base pair DNA tract that contains the three coding exons, splice sites, and elements. additional institution.[1] Individuals who have mutations in both *HBB* alleles, which drastically limit the synthesis of *HBB* protein (referred to as β-thal major or Cooley anemia), experience severe bone abnormalities and anemia, as well as a high death rate and shorter life expectancy. if treatment is not received.[2] The beta-globin *HBB* gene, found on chromosome 11, produces hemoglobin protein *HBB*, one of the hemoglobin components.[3] Adult hemoglobin (HbA), the most prevalent kind of hemoglobin, is formed when two beta-globin molecules attach to two alpha-globin molecules. In order to stop the formation of insoluble α-globin complexes, *HBB* is essential for maintaining the proper ratio of globin chains (α:β). An hereditary recessive blood condition induced by an anomaly in the *HBB* gene can be attributed to variations influencing the stability and synthesis of the β-globin chain at the transcription or translation level.[4]

Several mutations in the *HBB* gene, including translocation, deletion, and substitution mutations in the population tested positive for the beta-thalassemia trait, were found in Iraqi major thalassemia patients using molecular analysis. In southern Iraq, where thalassemia major is a major problem, this screening may be helpful for premarital genetic counseling as well as for preventative and therapy purposes.[5]

Mutations in the *HBB* gene result in a large number of *HBB* variations, and certain mutations change the production of the *HBB* chain entirely (β0) or partially (β+).[6] A characteristic of the extremely common blood condition beta thalassemia is decreased *HBB* chain production, which results in a decrease in the quantity of functional Hb.[7] Additionally linked to other hereditary blood diseases such as sickle cell disease and beta thalassemia are variations in the *HBB* protein. The most prevalent hemoglobin variations are hemoglobin E (HbE), sickle hemoglobin (HbS), and hemoglobin C (HbC), which are caused by point mutations in the *HBB* gene. More than 1,700 hemoglobin variants have been discovered to date, with more than 900 variants in hexa BDE.[8] A point mutation at position 26 in *HBB* caused glutamic acid to be replaced with lysine, resulting in the development of extremely unstable HbE α and β and a phenotype typical of a moderate form of beta thalassemia.[9] A point mutation at position VI in the *HBB* gene, which substitutes a valine codon (GTG) for the glutamic acid codon (GAG) during HbS synthesis, is the most frequent cause of sickle cell disease.[8] Furthermore, lysine can be substituted for glutamic acid to create HbC, a Hb variation linked to sickle cell disease.[10] However, there are several variables that could be advantageous. For instance, it is known that people with HbC have varying degrees of protection against the malaria parasite Plasmodium falciparum.[11]

The research aims to investigate the genetic polymorphisms of the *HBB* gene at the variant site rs713040 and their relationship to some physiological variations in the occurrence of beta thalassemia.

Materials and Methods

Samples Collection

Blood samples were obtained for beta thalassemia patients from baaquba Teaching Hospital/Diyala Specialized Center for Hematology who had previously been diagnosed with beta thalassemia. The total number of research samples was 60, with 30 samples for patients and 30 samples for healthy people. Two milliliters of venous blood from each patient and a healthy participant sample were taken for the study in the Molecular Genetics Laboratory at the College of Education for Pure Sciences, University of Diyala. The blood was then put in tubes containing the anticoagulant EDTA. The patients' ages varied from 2 to 31 years, whereas the control group's ages ranged from 1 to 35 years. Molecular genetic tests are then run on these samples after the K3 and this tube are gently shaken to combine the blood with the anticoagulant.

Genotyping

A Taiwanese company's Geneaid Blood Protocol micro genomic kit was used to extract the DNA, which was subsequently electrophoresed on a 1% agarose gel.

HBB Gene Amplification Utilizing the SNP-PCR Technique

The PCR technique and related responses were used to study the genetic polymorphisms of the *HBB* gene. The supervisor used the National Center for Biotechnology Information (NCBI) to design specific primers that duplicated the exon segment of the gene in order to study the kind and extent of mutations in the variant location of rs713040 of the *HBB* gene, as indicated in Tables 1 and 2.

The materials were moved to the polymerase chain reaction apparatus once the preceding components had been well mixed and the reaction conditions were established for the fragment to be amplified, as shown in Table 3.

Table 1. **The primers used to amplify the *HBB* gene's exon segment at the heterozygous location rs713040**

Primer	Sequence of primer	PCR product size
Forward primer	TGCACTGACCTCCCACATTC	382 bp
Reverse primer	TGAGTCCAAGCTAGGCCCTT	

Table 2. **Components of the PCR mixture for *HBB* gene amplification**

No	Ingredients	Size (microliter)
1	GoTaq premix	5
2	Forward primer	1.5
3	Reverse primer	1.5
4	Deionized water	14
5	DNA	3
	Total count	25

Table 3. ***HBB* gene amplification using the polymerase chain reaction program**

No	Step	Temperature	Time	Cycles
1	Initial denaturation	94	5 min	1
2	Denaturation	94	30 sec	35
3	Annealing	60	30 sec	35
4	Extension	72	30 sec	35
5	Final extension	72	5 min	1
	Hold		4 min	

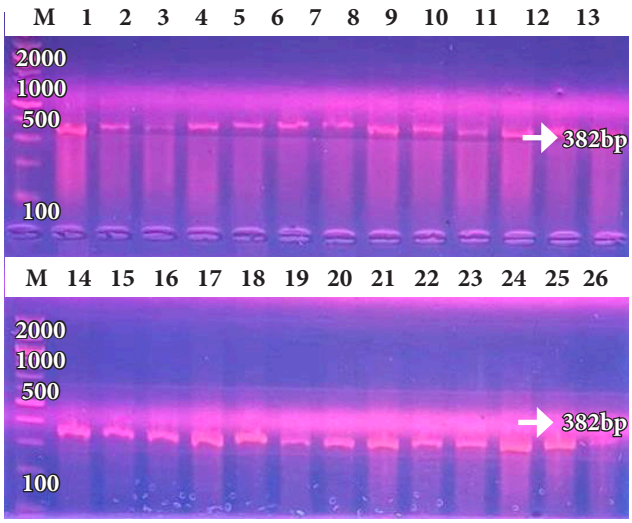

Fig. 1 **The result of amplification of the *HBB* gene for the coding region containing the heterozygous rs713040 A/C/G in individuals with thalassemia and healthy individuals in the Diyala Governorate community. Agarose gel was run at a concentration of 1.5% for 1.5 hours at a voltage differential of 90 volts. Numbers 1–13 show the healthy samples, whereas Numbers 14–26 show the patient samples.**

Statistical Analysis

The Hardy and Weinberg laws were used to compute the genotype and allele distribution. The Fisher's test was also used to determine the risk factor (OR) and confidence limits (C.L.) at the probability level of $P < 0.05$. The Hardy-Weinberg equilibrium law and the WINPEPI tool were used to extract the observed and predicted values in order to examine the genotype data. Next, a comparison was made between the significantly different percentages using the chi-square test for analysis.

Results and Discussion

The outcome of amplifying the *HBB* gene from beta thalassemia patients' DNA is depicted in Figure 1. The amplification findings indicated that the molecular weight of the resultant sample bands was 382 bp. The presence of rs713040 heterogeneity in the samples was further demonstrated by the molecular analysis results. These heterozygotes, which are found at the location of the exon's coding region, are point mutations of the transversion type.

This was investigated in a previous study on *HBB* polymorphism, which employed the HbA2 marker to investigate heterozygous beta thalassemia mutations of the *HBB* gene in the Chinese population with beta thalassemia.[12] The Okayama mutation arises from a change in homocysteine (His>Gln) at codon 2 (CD), which is a change in the nucleotide sequence at position 70603 from T to A or C (cat >CAA CAG) within the rs713040 heterogeneity site. This mutation is a characteristic of screening for silent beta-thalassemia mutations, making them challenging to identify. Another study on the related *HBB* gene in the Indian community discovered seven different types of mutations in the western state of Uttar Pradesh, India. In patients with frame shift beta thalassemia major, these new mutations included the first-ever deletion of four nucleotides from codon 66/67 (AAAG) in the exon 2 region and more frequent occurrences of IVS 1–5 G>C and codon 41–42 (CTTT).[13]

Comparing Beta Thalassemia Patients and Healthy Controls for the Genotypes and Allelic Frequency of the *HBB* Gene at the rs713040 Variant Location

Two groups were compared as the foundation for this investigation. In the *HBB* gene at the heterozygosity site rs713040, samples from beta thalassemia patients were included in the first group, while samples from the control group (healthy individuals) were included in the second group.

The current study's results are displayed in Table 4, where it is observed that there were 22 patients with thalassemia who carried the same genetic type, GG, and 48 patients with allele G. There was also a slight, noticeable decrease in the thalassemia patient group compared to the healthy second group, with percentages of 73.33% and 80%, respectively, and 60% and 76.67%, according to Fisher probability

Table 4. **The genotype distribution and allelic frequency of the *HBB* gene at the rs713040 A/C/T heterozygous locus in the research groups for patients with thalassemia major based on the Hardy-Weinberg law**

Hardy P-values	Allele frequencies		Genotype// rs713040 A/C/T					Groupe
	A	G	AA	GA	GG	Nu		
0.0014**	12	48	4	4	22	Nu	Observed	30 Patients
	20	80	13.33	13.33	73.33	%		
	Not diagnosed		1.2	9.6	19.2	Nu.	Expected	
			4	32	64	%		
0.7082 NS	14	46	2	10	18	Nu.	Observed	Control 30
	23.33	76.67	6.66	33.33	60	%		
	Not diagnosed		1.63	10.73	17.63	Nu.	Expected	
			5.44	35.78	58.78	%		

*($P \leq 0.05$), **($P \leq 0.01$), NS: Non-Significant.

Table 5. **Analysis of the association between alleles at the rs713040 A/C/T heterozygous region of the *HBB* gene and genotype in individuals with thalassemia major**

Genotype// rs713040 A/C/T	Patients No. (%)	Control No. (%)	Fisher's/P-value	O.R. (C.I.)
GG	22 (73.33%)	18 (60%)	0.291 NS	1.83 (0.61 – 5.63)
GA	4 (13.33%)	10 (33.33%)	0.078 NS	0.31 (0.08 – 1.13)
AA	4 (13.33%)	2 (6.66%)	0.433 NS	2.15 (0.35 – 17.80)
Total	30 (100%)	30 (100%)		
Allele		*Frequency*		
G	48 (80%)	46 (76.67%)	O.R. (C.I.) = 1.22 (0.50 – 2.96)	
A	12 (20%)	14 (23.33%)	O.R. (C.I.) = 0.82 (0.34 – 1.99)	

*($P \leq 0.05$), NS: Non-Significant.

($P = 0.291$ and $P = 0.078$) among thalassemia patients compared to the healthy group. Table 5 indicates that the homozygous genotype GG and allele G are thought to be protective factors against the illness, based on their respective findings of 1.83% and 1.22% with a probability of 0.61 and 0.5. Fisher's probability ($P = 0.433$ and $P = 0.82$), respectively, revealed that the thalassemia patients had a significantly higher AA genotype and A allele, with values of 13.33% and 20%, respectively, compared to the healthy group, which recorded 6.66% and 23.33%, respectively as indicated by Table No. 5. The odds ratio values for the homozygous genotype AA and the A allele reached 2.15 and 0.82, respectively, and are thought to be causal factors for the illness; in contrast, the heterozygous genotype GA and the G allele showed a 20% rise in thalassemia patients. In contrast, 33.33% and 23.33% were recorded by the healthy group, respectively. The GA genotype and the A allele are the disease's causal factors, according to Fisher's probability ($P = 0.078$ and $P = 0.82$), as indicated by the odds ratio values of 1.13% and 1.99%, respectively, with a probability of 0.31 and 0.82. Table 5.

Six genetic variations were identified with high genotype frequencies in recent research on the Senegalese population that evaluated genetic variants of the *HBB* gene: rs7946748, rs7480526, rs10768683, rs713040, rs35209591, and Hbs rs334. Thalassemia is also caused by severe malaria, and the HbS study demonstrated a substantial correlation with protection against it. However, the HbC polymorphism is not protective, as indicated by the big *P* value of 0.033, the likelihood coefficient of 0.38–95%, and the confidence coefficient of 0.16–0.91. The research population's variable.[14]

Chinese Zhuang beta thalassemia patients had a relationship between HbF and genetic variations of rs7482144, rs28384513, rs4895441, and rs46713934, according to a prior study.[15] Patients with beta-thalassemia who had elevated HbF levels had a comparatively greater frequency of rs4895441(G) on HMIP. The four genetic components had synergistic effects. This genetic variant has cumulative effects on high HbF levels in people with Chinese Zhuang beta thalassemia. We believed there would be a synergistic impact.

Comparison of Physiological Variables with Genotypes of the rs713040 A/C/G/T *HBB* Gene in Beta Thalassemia Patients and Healthy Controls

The current study's results showed that patients with all genotypes had high levels of WBC, PLT, PCT, and ALP. The WBC values were AA (3.27 ± 11.8), GA (6.46 ± 15.95), and GG (2.1 ± 12.65); the PLT values were AA (134.62 ± 460), GA (104.05 ± 472.5), and GG (52.88 ± 453.64); the PCT values were AA (0.18 ± 0.5), GA (0.08 ± 0.45) and GG (0.05 ± 0.46), and the ALP values were AA (12.37 ± 205.5), GA (48.34 ± 271.25), and GG (19.72 ± 177.55). There were notable variations when compared to healthy individuals ($P < 0.05$). However, compared to healthy subjects, there were significant differences ($P < 0.05$) in the levels of HB and vitamin D3 in patients of all genotypes. Specifically, HB values were lower in AA (0.31 ± 8.05), GA (0.18 ± 7.83), and GG (0.3 ± 7.68), and vitamin D3 in AA (3.26 ± 12.08), GA (4.8 ± 14.52), and GG (0.97 ± 8.17). The three genotypes (GG, AA, and AG) of the rs713040 A/C/G/T *HBB* gene did not vary significantly ($P > 0.05$) for patients or healthy individuals, or for any of the physiological measures WBC, HB, PCT, and PLT. When comparing the ALP and vitamin D3 variables to the other kinds, GA had the highest values (271.25 ± 48.34 and 14.52 ± 4.8), with a statistically significant difference ($P < 0.05$). Table 6.

Table 6. **Comparison of physiological variables with genotypes of the rs713040 A/C/G/T *HBB* gene in beta thalassemia patients and healthy controls**

rs713040 A/C/G/T *HBB*		Patients		Controls		*P*-value
		Mean	St. error	Mean	St. error	
	AA	11.8	3.27	6.43	0.23	$P < 0.05$*
WBC	GA	15.95	6.46	7.59	1.03	$P < 0.05$*
	GG	12.65	2.1	7.75	0.51	$P < 0.05$*
P value		$P > 0.05$		$P > 0.05$		
	AA	8.05	0.31	10.4	0.9	
HB	GA	7.83	0.18	12.9	0.42	$P < 0.05$*
	GG	7.68	0.3	13.46	0.42	$P < 0.05$*
P value		$P > 0.05$		$P > 0.05$		
	AA	460	134.62	275	13	$P < 0.05$*
PLT	GA	472.5	104.05	283	18.53	$P < 0.05$*
	GG	453.64	52.88	277.89	13.33	$P < 0.05$*
P value		$P > 0.05$		$P > 0.05$		
	AA	0.5	0.18	0.28	0.01	$P < 0.05$*
PCT	GA	0.45	0.08	0.25	0.02	$P < 0.05$*
	GG	0.46	0.05	0.26	0.01	$P < 0.05$*
P value		$P > 0.05$		$P > 0.05$		
	AA	205.5	12.37	73.5	1.5	$P < 0.001$**
ALP	GA	271.25	48.34	115.6	29.08	$P < 0.001$**
	GG	177.55	19.72	117.44	16.62	$P < 0.05$*
P value		$P < 0.05$*		$P < 0.05$*		
	AA	12.08	3.26	25.65	16.95	$P < 0.05$*
V.D3	GA	14.52	4.8	17.86	4.08	$P > 0.05$
	GG	8.17	0.97	26.16	4.27	$P < 0.001$**
P value		$P < 0.05$*		$P < 0.05$*		

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

A parallel study carried out on the Iranian population revealed that CD36/37, with a greater rate of 28.9%, was the most prevalent mutation, followed by VIVS1-110 and IVSll-1. Both the value of sLOX-1 and the iron index values varied considerably over the total number of mutations. According to Yaghooti et al. (2021), hemochromatosis and splenomegaly were shown to be more important in the IVSl-110 mutation group, where they were determined to be considerably greater.[16] The B-globin gene has five different mutations, two of which are mutations, and the other three are transformation mutations. These mutations have been linked to elevated levels of the liver enzymes ALP, AST, and ALT as well as hemolysis in erythrocytes in children with beta thalassemia, according to a recent study done in the Mosul Governorate.[17]

Conclusion

It is noteworthy that our study is the first in Iraq to identify the relationship between beta thalassemia major and the rs713040 heterozygosity site in the amplified region of the *HBB* gene (382 bp). When comparing a sample of beta thalassemia patients to healthy controls, the molecular study's findings revealed the existence of genetic variation at the heterozygous site A/C/G rs713040 of the *HBB* gene. In contrast, a percentage of the homozygous genotype GG and the G allele in the *HBB* gene was observed, which is a protective pattern against beta-thalassemia. The molecular study's results showed that the homozygous genotype AA and the A allele of the *HBB* gene at the heterozygous A/C/G site of the *HBB* gene recorded the highest percentage of the risk factor, and this genotype is beta-thalassemia.

Conflict of Interest

The authors declare no conflict of interest.

References

1. Weatherall, D. J. (2001). Phenotype—genotype relationships in monogenic disease: lessons from the thalassaemias. Nature Reviews Genetics, 2(4), 245–255.
2. Cai, L., Bai, H., Mahairaki, V., Gao, Y., He, C., Wen, Y., ... & Cheng, L. (2018). A universal approach to correct various *HBB* gene mutations in human stem cells for gene therapy of beta-thalassemia and sickle cell disease. Stem Cells Translational Medicine, 7(1), 87–97.
3. Wienert, B., Martyn, G. E., Funnell, A. P., Quinlan, K. G., & Crossley, M. (2018). Wake-up sleepy gene: reactivating fetal globin for β-hemoglobinopathies. Trends in Genetics, 34(12), 927–940.
4. Zakaria, N. A., Bahar, R., Abdullah, W. Z., Mohamed Yusoff, A. A., Shamsuddin, S., Abdul Wahab, R., & Johan, M. F. (2022). Genetic Manipulation Strategies for β-Thalassemia: A Review. Frontiers in Pediatrics, 10, 901605.
5. Al-Musawi, A. H. O., Aziz, H. M., Khudair, S., & Saleh, T. H. (2022). Molecular characterization of *HBB* gene mutations in beta-thalassemia patients of Southern Iraq. Biomedicine, 42(5), 1040–1043.
6. Zhao, Y. L., Lin, Q. F., He, X. W., Li, Y. Q., & Liang, L. (2021). Hb Hezhou [β64(E8)Gly⟶Ser; *HBB*: c.193G>A]: a novel variant on the β-globin gene. Hemoglobin, 45(2), 133–135.
7. Alaithan, M. A., AbdulAzeez, S., & Borgio, J. F. (2018). A comprehensive review of the prevalence of beta-globin gene variations and the co-inheritance of related gene variants in Saudi Arabians with beta-thalassemia. Saudi Medical Journal, 39(4), 329.
8. Aldakeel, S. A., Ghanem, N. Z., Al-Amodi, A. M., Osman, A. K., Al Asoom, L. I., Ahmed, N. R., ... & Borgio, J. F. (2020). Identification of seven novel variants in the β-globin gene in transfusion-dependent and normal patients. Archives of Medical Science, 16(2), 453–459.
9. Fucharoen, S., & Weatherall, D. J. (2012). The hemoglobin E thalassemias. Cold Spring Harbor Perspectives in Medicine, 2(8), a011734.
10. Fasola, F. A., Babalola, O. A., Brown, B. J., Odetunde, A., Falusi, A. G., & Olopade, O. (2022). The effect of alpha thalassemia, HbF, and HbC on hematological parameters of sickle cell disease patients in Ibadan, Nigeria. Mediterranean Journal of Hematology and Infectious Diseases, 14(1).
11. Lopez-Perez, M., Viwami, F., Doritchamou, J., Ndam, N. T., & Hviid, L. (2022). Natural acquired immunity to malaria antigens among pregnant women with hemoglobin c trait. The American Journal of Tropical Medicine and Hygiene, 106(3), 853.
12. Ray, R., Biswas, A., Bhattacharjee, S., & Bhattacharyya, M. (2018). Phenotypes of Hb Okayama Mutation. Blood, 132, 4898.
13. Chauhan, W., Afzal, M., Zaka-ur-Rab, Z., & Noorani, M. S. (2021). A novel frameshift mutation, deletion of *HBB*: C. 199_202delAAAG [Codon 66/67 (-AAAG)] in β-thalassemia major patients from the western region of Uttar Pradesh, India. The Application of Clinical Genetics, 77–85.
14. Thiam F, Diop, G., Coulanges, C., Derbois, C., Mbengue, B., Ndiaye, R., Thiam, A., ... & Dieye, A. (2020). *G6PD* and *HBB* polymorphisms in the Senegalese population: prevalence, correlation with clinical malaria. PeerJ, 10, e13487.
15. Lai, Y., Zhou, L., Yi, S., Chen, Y., Tang, Y., Yi, S., ... & He, S. (2017). The association between four SNPs (rs7482144, rs4671393, rs28384513 and rs4895441) and fetal hemoglobin levels in Chinese Zhuang β-thalassemia intermedia patients. Blood Cells, Molecules, and Diseases, 63, 52–57.
16. Yaghooti, H., Mirlohi, M. S., Shirali, S., & Aminasnafi, A. (2021). Characterization of Common β-Thalassemia Major Mutations in Southwest Iran with Respect to Biochemical Parameters, Oxidative Status and Complications. Journal of Advanced Biomedical Sciences, 11(1), 3715–3724.
17. Hamed, O. M., Al-Taii, R. A., & Jankeer, M. H. (2021). Biochemical and Genetic Study in Blood of β–Thalassaemia Children in Mosul City, Iraq. Iraqi Journal of Science, 62(8), 2501–2508.