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Genotype Association of rs3129878 and rs9268651 in HLA-DRA Gene with the risk of Non-Obstructive Azoospermia in a Sample of Iraqi Infertile Males

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Abstract

Non-Obstructive azoospermia is a common multifactorial disease. Many genome-wide association studies (GWAS) have discovered genetic variants that are associated with increased risk of NOA in HLA class II region. HLA class II region have been reported to be associated with reproduction efficiency in males, however the effect of HLA region variants on NOA remain largely unspecified. Moreover, the majority of GWAS studies were done using Asian populations of China and Japan with no replication in different ethnic groups. Therefore, in this study we will investigate the presence and association of two HLA-DRA variants (rs3129878 and rs9268651) with NOA risk and their potential effect on sperm production and on fertility hormonal level in a sample of Iraqi infertile men. Study based on case-control design with genotyping for both variants and semen analysis was done by using blood and semen samples for 91 Iraqi males. The results show no individual association of SNPs with NOA risk, yet deviation from HWE (p=0.031) in patients only for the variant rs3129878 suggested for association trend. The combined effect of specific haplotype C-G reached significance level (p= 0.021) with linkage Disequilibrium analysis showed a D' value=0.99 and R^2=0.046 between the two SNPs, additionally the variants rs3129878 show significant association (p=0.0178) with deceased sperm concentration in fertile men. Finally, the two variants appear to have different correlation association patterns with fertility hormones in NOA and controls. In conclusion, NOA is heterogeneous disorder influenced by several genetic, hormonal, environmental and idiopathic factors. Individual variants like (rs3129878 and rs9268651) with modest effect size might be diluted in case-control study yet the combined effect of the two SNPs in HLA region haplotype on NOA risk remain valuable.

Key words: Infertility, Non-obstructive azoospermia, Sperm, HLA Class II region, GWAS

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Introduction

Male infertility has prevalence rate with 10-15% among couples across different populations, with male factor almost contribute to half of the cases, and non-obstructive azoospermia (NOA) is a sever manifestation of spermatogenesis failure. Spermatogenesis failure is the disruption of sperm production which leads to significant reduction or complete absence of sperms in the semen [1]. NOA poses important genetic counseling and medical management implications because processes like testicular sperm extraction with intracytoplasmic injection (TESE-ICSI) can mediate pregnancy but with potential risk of transmitting genetic lesions to the infant [2]. The etiology of NOA shows heterogenous patterns as both non-genetic and genetic factors can be considered causality factors. Non-genetic factors such as hormonal imbalances including Luteinizing hormone (LH), follicle stimulating hormone (FSH) and testosterone, Prolactin (PRL) can all impact sperm production [3]. Candidate gene and genome studies characterize NOA as complex disorder with congenital, chromosomal abnormalities, Y-chromosomal microdeletion (AZF) region, and potentially autosomal or HLA region contribution to spermatogenetic failure [4]. Genome-Wide association studies have been ongoing for the last 2 decades in attempt to explore the role of SNP variations and their impact on NOA. A meta-analysis across 121 genes has identified 199 SNPs associated with idiopathic male infertility, with significant associations with rs3129878 and rs498422, displaying odd ratio 1.36 and 1.3 respectively [5]. Lower frequency variants were identified to be associated with NOA in Han chines males GWAS, variants that reached genome significance are re200847762 (OR=0.11) and rs2298090 (OR=0.30) [6]. HLA class II genes modulate adaptive immune response and have been constantly involved in reproductive biology; HLA genetic variations have investigated in spermatogenetic function and semen quality; several variants were mapped in this region with reported significant association [7]. Despite these findings, the replication of some NOA susceptibility loci is weak among association studies, which highlight the need for replicated studies in various populations to explore and confirm NOA-related genetic variants. In this study we aimed to investigate the statistical effect of variant rs3129878 with combination of closely variant rs9268651 on the risk of non-obstructive azoospermia in a sample of infertile Iraqi males.

Materials and Methods

Study Samples

The participants of this study were recruited from private clinic in Baghdad city during the year of 2025 informed consent was obtained from all participants. A total number of (91) male samples were collected, (61) samples of unrelated idiopathic NOA and (30) controls with normal sperm production level. Inclusion criteria included participants of men with reproductive age (18-60 years old) and have diagnosed with primary infertility with no successful conception attempts for the last 5 years. Also, complete absence of sperm in the ejaculate is confirmed by at least 2 earlier semen analysis tests and with normal karyotype. Exclusion criteria included patients with obstructive azoospermia, Cryptorchidism, patients with testicular surgery or trauma, patients with medical history of systematic disease such as diabetes. Furthermore, patients with confirmed Y-chromosome microdeletion. All NOA samples were previously check by the clinic including no tumor history, scrotal ultrasound, hormonal analysis and finally physical examination. NOA subjects have no sperm in ejaculation whereas controls have normal motility, morphology and count of sperm in ejaculate after centrifugation according to World Health Organization criteria[8].

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Sample Collection

10 ml of venous blood from each subject was carefully drawn into appropriate sample tubes. Samples were allowed to form blood clot for 5-10 minutes, serum is separated and centrifugation was done at 4000 rpm for 10 minutes. Three assays are included to obtain hormonal levels of testosterone (TESTO), Prolactin (PRL) and Follicle-stimulating hormone (FSH) using BioSource ELISA kit and according to manufacturer's instructions. For semen collection, each subject is allocated to private room where semen is collected after masturbation. Semen analyzed by andrologist according to WHO gaudiness[8] where azoospermia is declared if there are no semen in the ejaculate. Other parameters were also collected for each sample by the result of semen analysis including total semen concentration and total semen count.

DNA Extraction, Amplification and Genotyping

In this research, Genomic DNA was extracted from peripheral blood samples utilizing DNeasy QIAamp DNA Blood purification Kit (QiAGEN, Hilden, Germany). DNA purity was tested by Nanodrop spectrophotometer Cleaver /UK. The targeted DNA sites were amplified using new design-specific primer that covers both SNPs, Provided by Macrogen Company in South Korea, and designed using (Geneious primer) software (http ://WWW.macrogen.com), as shown in Table (1). The primer preparation was done by dissolving it in nuclease-free water to have a concentration of 100 pmol/µL with storing it in deep-freeze as a ready stock. To use the primers for PCR reaction we needed final concentration of 10 pmol/µL which was prepared by mixing 10-fold dilution of the stock primers with 90 µL nuclease-free water[9].

Table 1: Primer of the study

Sequence (5'…….3')	Product Size (bp)
Rs878F -AGTAAGGAAATGGGGAATCTG Rs878R -TTTGGTTTGGTTGTTTGAGAC	625 bp

All PCR reactions were carried out in a 25 µL final volume and according to the manufacturer's instructions. The PCR machine from applied Biosystems where used, program conditions were designed for this study as shown in table (2).

Table 2: The PCR program for *HLA-DRA* gene

Step	Temperature (°C)	Time	Cycle
Initial denaturation	94	4 min	1
Denaturation	94	30 sec	35
Annealing	57	30 sec	
Extension	72	30 sec	
Final extension	45	5 min	1

To confirm successful amplification, Final PCR product was electrophoresed using gel electrophoresis from cleaver science – UK at 80 V for 80 minutes. 1% agarose gel and 100 bp DNA ladder

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utilized to define the product size. The presence of a separate, distinct band of the presumed size was proven by gel documentation system with a high-resolution camera from Cleaver Scientific –UK which used to visualize and capture the DNA bands images.

Genotyping single nucleotide polymorphism under investigation rs3129878 and rs9268651 was done by using Sanger sequencing method. Sequencing was achieved by using ABI3730XL platform (Macrogen Corporation, Seoul, Korea) in one direction. The genotyping data were analyzed with Geneious Prime software (Geneious, Auckland, New Zealand).

Statistical Analysis

The allele and genotype frequency for the target SNPs was compared between NOA patients and controls by using chi-square test in SPSS 28.0 (SPSS, Inc.). The same software was used to test Hardy-Weinberg equilibrium using Pearsons's chi-square test. Moreover, expectation- maximization algorithm was used for haplotype analysis with chi-square for linkage disequilibrium measures. Median (25^{th} - 75^{th} percentile) was used as the case/control number are not equal with variables of non-normal distribution, it's used to report continues variables including semen parameters considering WHO guidelines for semen analysis is in percentile [8,10].

Results and Discussion

The present study investigates the association of two *HLA-DRA* gene SNPs (rs3129878, rs9268651) with Non-Obstructive Azoospermia susceptibility and their functional correlation on fertility hormones and sperm parameter in a sample of Iraqi males.

Characterization of Study Participants

A variety of demographic and clinical characteristics were assessed from NOA patients and control groups presented in table using median (25th75th percentile) for continuous age and body mass index (BMI) variables and n% for categorical smoking status variable. No statical differences were observed between the NOA and control groups in the tested parameters as in table (3). These findings imply that the groups are well-matched for baseline characteristics, which are unlikely to act as co-founding for subsequent analysis.

Table 3: Characteristics of study subjects

Parameters		AZO (n=61)	Control (n=30)	P-value
Age, median, (IQR), Years		30.0 (26.0-35.0)	29.0 (28.0-33.0)	0.567
BMI, median, (IQR), Kg/m^2		26.56 (25.31-29.39)	27.96 (25.25-30.59)	0.317
Smoking status freq. (%)	Yes	33 (54.1)	13 (43.3)	0.334
	No	28 (45.9)	17 (56.7)	

Mann-Whitney U test statistically significant at α=0.05

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PCR product verification

Image taken by gel documentation system show single, distinct DNA band of 625 bp in size for all samples, indicating accurate amplification process as shown in figure 1. The amplified product is part of *HLA-DRA* gene, The influence of *HLA-DRA* region on male fertility can be explored through virous biological mechanisms. For example, HLA class II regulates testicular immune privilege which is essential to maintain spermatogenesis hemostasis. Moreover, the expression of *HLA-DRA* molecules in testicular macrophages, Leydig and Sertoli cells, will modulate cell-cell interaction and cytokines production which are important for efficient sperm production [12,13]. Both SNPs are located with *HLA-DRA* gene, the gene located on chromosome 6p21.3 in the major histocompatibility complex (MHC) class II, encoding the alpha chain of the HLA-DRA heterodimer, which is curtail component of the adaptive immune response.

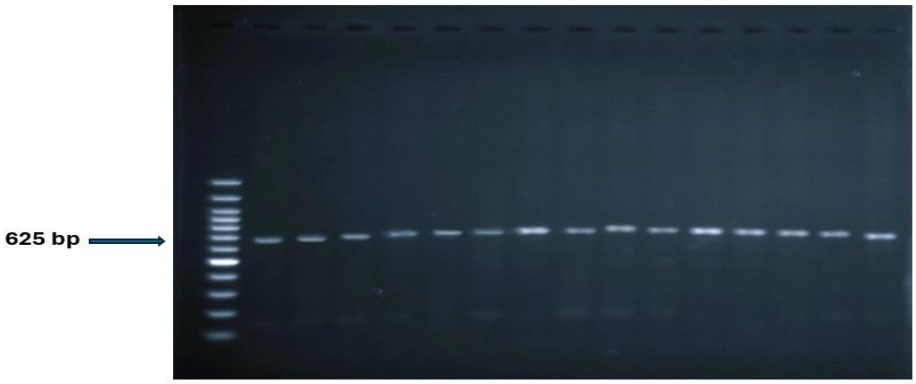

Figure 1. Profile of Gel Electrophoresis for PCR-amplified DNA of *HLA-DRA* gene (lane 1-15) Using 80 V for 80 minutes and 1% agarose gel with 100 bp DNA band size as ladder.

Single nucleotide polymorphism Sequencing

Two single nucleotide polymorphisms were discovered in the amplified sequence of 625 bp of *HLA-DRA* gene. The first one is rs3129878 located on chr6:32440958 (only genotype AA and AC) is found in our subjects, shown in figure 2. The second SNP is rs9268651 located on chr6:32441269 where all genotypes detected in our subjects as in figure 3.

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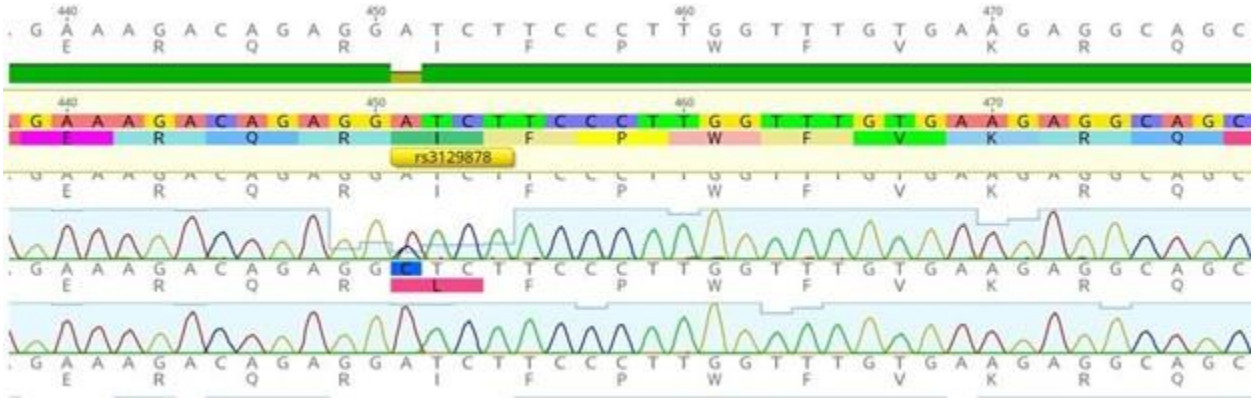

Figure 2: A chromatogram of DNA base pair sequencing on a region containing SNP (rs3129878) A>C

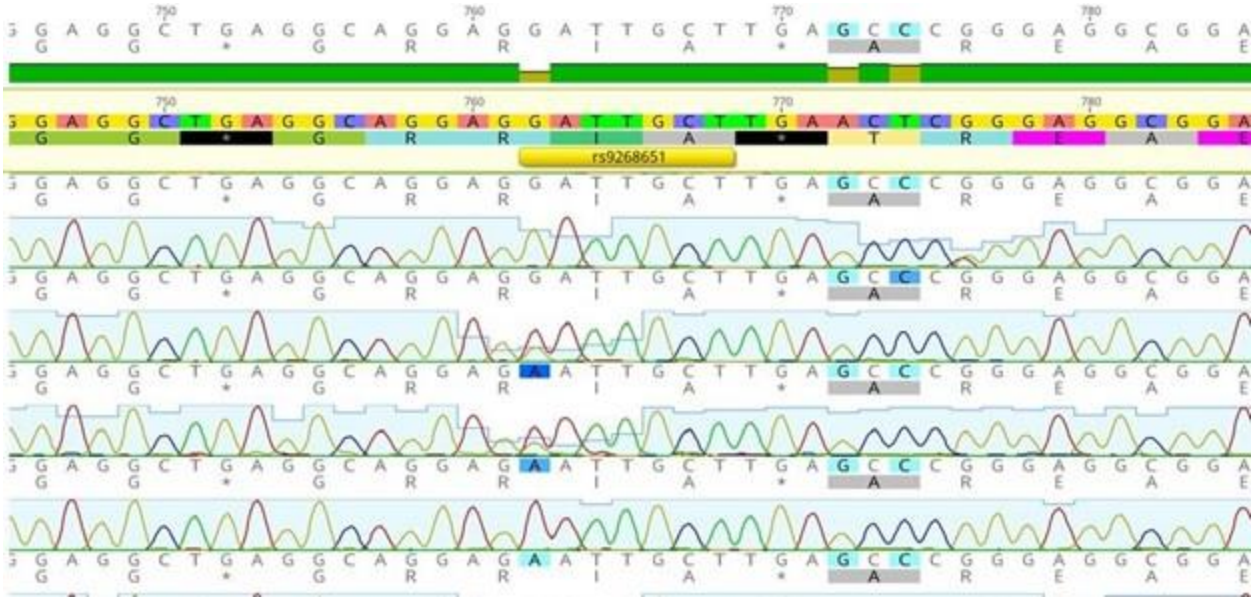

Figure 3: A chromatogram of DNA base pair sequencing on a region containing SNP (rs9268651) G>A

Hardy-Weinberg Equilibrium Analysis

Hardy-Weinberg equilibrium test analysis of SNP rs3129878 in both patients and controls exhibited contrasting patterns as in table (4). The control group (n=30) was in HWE (P=0.56) while the patient group (n=61) showed significant deviation from HWE (P=0.031). The deviation shown in patients is due to excessive heterozygosity of genotype AC (30 observed vs. 22.6 under expected HWE). This pattern of deviated HWE in cases and equilibrium in controls can be depicted as potential association of rs3129878 with azoospermia susceptibility. The analysis test for the SNP rs9268651 appeared to be in HWE which supports the assumption of random mating.

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Table 4: Hardy-Weinberg Equilibrium for genotype frequencies among non-azoospermia and controls.

	SNP	Non-Azoospermia (n=61)		Control (n=30)	
		Observed	Expected	Observed	Expected
rs3129878	AA	31 (51.0)	34.7	20 (67.0)	20.8
	AC	30 (49.0)	22.6	10 (33.0)	8.30
	CC	0 (0.0)		0 (0.0)	
	HWE	**P-value 0.031***		P-value 0.560	
rs9268651	GG	47 (77.0)	46.3	22 (73.3)	22.7
	GA	13 (21.3)	14.1	8 (26.7)	6.9
	AA	1 (1.6)	0.7	0 (0.0)	0.3
	HWE	P-value 1.0		P-value 1.0	

Chi-Square test statistically significant at α=0.05

This deviation remained after data quality control steps were applied, ruling up genotyping errors. Furthermore, the homogenous population of Baghdad city where samples are collected from has no ethnic admixture making population structure unlikely to appear. The excess of heterozygosity in patients might carry potential biological mechanism. Firstly, heterozygosity may occur where individuals carry AC genotype have improved survival or less severity of other reproductive disease in comparison with homozygous individuals [14]. Secondly, the HLA region is reported to be subjected to strong balancing selection because of pathogen-driven diversity. The observed deviation may reflect actual biological selection at the *HLA-DRA* locus driven by immune reproductive trade-offs, where heterozygosity offers advantages in immune functions that partially compensate for fertility disadvantages [15]. Van Dar Ven et. al. (2000) has shown that HLA class II allele frequencies (DQA1, DRB1, DQB1) are significantly different between males with andrological infertility and normal fertile males, supporting the role played by HLA class II variation in sperm productivity [16]. This in return might suggest that HLA heterozygosity may modulate immune-mediated process in the testis, possibly clarifying the observed deviation in HWE.

Association of rs3129878 and rs9268651with NOA

Association analysis was performed for the 2 SNPs (rs3129878 and rs9268651) for both NOA patients and control group as summarized in table (5). For rs3129878, it is observed that C allele is more frequent in patients (25%) than in controls (17%) with OR 1.63 (95% CI: 0.74-3.59, P=0.258). Genotype distribution shows higher frequency rate of the AC heterozygous genotype in patients (49.0%) against controls (33.0%, P=0.182) with no detection of genotype CC in either group. For rs926865, allele and genotype frequencies are similar in both groups, (P= 0.62 and P=0.68), respectively. Neither SNPs reached statistical significance, yet the pattern trend detected in rs3129878 suggested a possible contribution in NOA pathogenesis.

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Table 5. Association analysis of HLA-DRA (rs3129878 and rs9268651) and Non-Obstructive Azoospermia

SNP (gene)	Allele	Allele frequency And distribution				Genotype	Genotype frequency and distribution		
		Patients no. (%)	Control no. (%)	OR (95% CI)	*P* value		Patients no. (%)	Control no. (%)	*P* value
rs3129878	*A*	92 (75.0)	50 (83.0)	0.61 (0.28-1.35)	0.258	AA	31 (51.0)	20 (67.0)	0.182
	C	30 (25.0)	10 (17.0)	1.63 (0.74-3.59)		AC	30 (49.0)	10 (33.0)	
						CC	0 (0.0)	0 (0.0)	
rs9268651	*G*	13 (11.0)	8 (13.0)	0.78 (0.30-1.97)	0.626	GG	47 (77.0)	22 (73.3)	0.68
	A	109 (89.0)	52 (87.0)	1.29 (0.51-3.28)		GA	13 (21.3)	8 (26.7)	
						AA	1 (1.6)	0 (0.0)	

Chi-Square test statistically significant at α=0.05

While neither SNPs show significant association with NOA risk on individual allele or genotype level, yet the rs3129878 variant, formerly detected in NOA genome-wide association studies (GWAS) with genome wide significant, maps directly or close to *HLA-DRA* locus, suggesting its relation to spermatogenesis failure. The milestone GWAS by Zhoe et.al. (2012) has identified the variant rs3129878 as strongly statically associated (combined p-3.70x10-16, OR=1.37) with NOA risk in a sample of Han-Chinese population [17]. The same variant was confirmed by GWAS study in 2013 using Japanese subjects (p=3.98x10-4, OR=1.32) [18]. This finding was later replicated in different independent cohort studies, Tu et.al in 2015 validated the variant to be NOA risk factor in southwest chines cohort [19] and Zou et.al. (2017) reported the strongest association in their meta-analysis (p=6.75x10-21, OR=2.26) [20].

Our results of no significant individual SNP association with NOA (rs3129878: p=0.258, OR=1.63 and rs9268651: p=0.626, OR=1.29) contrast with mentioned GWAS, yet it is consistent with the known heterogenetic nature of NOA etiology along with population specific genetic architecture. The modest odd ratio and absence of statistical significance might be reflected by several factors including: the limited sample size (NAO=61, control=30), the complex polygenic nature of NOA may require larger sample size to detect individual variants with small effect size, and the differences in allele frequency caused by the difference between Iraq and east Asian populations [21,22]. It is important to mention that the extreme pattern of male infertility that involve several spermatogenesis and immune loci, might propose that NOA genetic susceptibility could be based on the combined effect of multiple variants rather than single dominant loci [23].

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Association of rs3129878 and rs9268651 Haplotype with NOA

Haplotype analysis for the two SNPs (rs3129878 and rs9268651) using the expectation-maximization algorithm[11] recognized 4 haplotypes (A-G, A-A, C-G, C-A). The analysis show that haplotype frequencies is significantly different between AZO patients and healthy control group ($\chi^2 = 17.41$, $p < 0.001$). While C-G haplotype has the strongest association with azoospermia risk (AZO 24.59% vs controls 8.99%, OR 3.08), the C-A haplotype was absent in all AZO patients but appears in 7.7% of controls which might offer a protective function as in table (6). For Linkage disequilibrium analysis, it can be seen there is strong LD in AZO patients (D' = -0.999) and weak LD for controls (D' = 0.491) as in table (7). These findings may suggest that haplotype combination is more informative than individual single nucleotide polymorphism analysis for non-obstructive azoospermia risk assessment.

Table 6. Association of HLA-DRA SNPs (rs3129878 and rs9268651) haplotypes with the risk of NOA.

Haplotype	AZO (n=61)	Controls (n=30)	Odds Ratio	95% CI	P-Value
A-G	0.63 (63.1%)	0.777 (77.7%)	0.50	(0.25, 1.01)	0.70
A-A	0.123 (12.3%)	0.057 (5.7%)	2.12	(0.67, 6.73)	0.256
C-G	0.246 (24.6%)	0.090 (9.0%)	3.08	(1.20, 7.91)	0.021*
C-A	0.000	0.077 (7.7%)	0.04	(0.00, 0.83)	<0.01

Overall haplotype association $\chi^2 = 17.41$, $p < 0.001$

Although SNPs acting individually did not reveal any significant association, yet haplotype analysis indicated the presence of significant association of C-G haplotype (rs3129878-C and rs9268651-G) with elevated NOA risk (OR=0.08, 95% CI (1.20, 7.91), p=0.021) and C-A haplotype (rs3129878-A and rs9268651-C) is appeared to have protective effect (p-<0.01, OR=0.04). This outcome implies that the combined effect of multiple polymorphisms might be more informative than individual ones, which is aligned with the principal concept of haplotype analysis that recognizes functional variations among connected regulatory elements. The importance of haplotypes in HLA region has been previously demonstrated in different studies. A study by Van der van et.al. (2000) identified significant differences by analyzing three-locus HLA class II in infertile males and controls [16]. The same pattern was observed in Matuszuka et.al. (2002) study where they mapped NOA risk to HLA-DR/DQ subregion and observed that haplotypes (DRB11302 and DQB10604) are in significant association with NOA increased risk, proven that multi-locus haplotypes in HLA region can be crucial where single SNP do not capture the signal [24].

Table 7. Linkage Disequilibrium measures

Group	D	**D'**	**r^2**
AZO patients	-0.030	-0.999	0.046
controls	+0.055	+0.491	0.186

The results of linkage disequilibrium between rs3129878 and rs9268651 has shown a significant difference between tested groups (r2=0.046 in cases vs. r2=0.186 in controls), supporting the suggestion

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that haplotype recombination event might occur or selection acting differently on those loci in NOA individuals. A study in Japanese population has reported the rs3129878 variant and several HLA alleles are in LD, making single marker to be more complicated as causality factor [18]. The result of strong negative D` in patient group (-0.999) suggests that specific allele combinations are depleted, which supports the non-random association pattern modeled by disease impact or by selection.

Association of SNP Genotypes and Sperm Parameters in Fertile Males

To examine the potential effect of the SNPs under study on spermatogenesis proficiency, we analyzed the correlation between the SNPs and sperm productivity through comparison of sperm concertation and total sperm count between normal fertile males with risk and non-risk genotype as in table (8). The analysis of the thirty normozoospermia males show the evidence for one statistical significance association between NOA associated SNPs and related sperm parameters. For the first parameter, the rs3129878 (A>C) risk genotype (AC) was significantly associated with decreased sperm concentration relatively to wild genotype AA (20.5 (17.25-25.75) vs. 31.0 (20.75-38.5) million/ml, p=0.0178), reporting 33.5% reduction with moderate negative correlation (r=-0.429). For rs9268651 (G>A) risk genotype (GA) did not show any sign of association with sperm production. For the second parameter, total sperm count, neither SNPs show significant association. The outcome of this analysis may suggest that the risk genotype of rs3129878 can be a potential risk factor for decreased sperm productivity even in fertile individuals.

Table 8. Comparisons of sperm productions between fertile males with the risk and non-risk genotype of (rs3129878, rs9268651)

SNP	Sperm Parameter	Risk genotype Median (25th–75th percentiles)	Wildtype Median (25th–75th percentiles)	correlation	P-value
rs3129878	Sperm concentration	20.5(17.25-25.75)	31.0(20.75-38.5)	-0.429	**0.0178**
	Total sperm count	66.5(53.5-71.5)	55.5(42.5-67.5)	0.171	0.3642
rs9268651	Sperm concentration	26.0(23.75-28.5)	23.5(20.0-37.5)	0.567	0.7660
	Total sperm count	65.0(58.0-1.005)	55.5(37.5-67.5)	0.292	0.1174

Mann-Whitney U test statistically significant at α=0.05
Sperman test for correlation coefficient.

Despite the absence of direct association with NOA, the SNP rs3129878 appeared to have significant functional impact on sperm parameters in fertile men. The AC risk genotype was associated with 34% decreased sperm concentration when compared to AA wild-genotype (median 20.5 vs. 31.0 million/ml, p=0.018, r= -0.429), indicating a subclinical effect on sperm production efficiency even in males with active productivity status. This outcome supports a model where rs3129878 effect fit in continuous contribution on spermatogenesis phenotype rather than binary infertile/fertile pattern. An interesting study by Tu et.al has described that the presence of specific risk genotypes such as rs3129878 and others had higher levels of total sperm count in fertile men compared with non-risk genotypes, demonstrating that the behavior of

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NOA risk alleles is different in responds to decreased sperm concentration in health males [19]. The lack of rs3129878 association with total sperm count in our study may suggest that the effect of rs3129878 mainly lies on sperm concentration per ejaculate volume rather than complete sperm production, possibly modifying epididymal function, seminal vesical contribution and other ejaculatory characteristics. The absence of positive association between rs9268651 and sperm parameters must be discussed with caution. In genetic association studies such null findings can occur due to several reasons, including a true absence of biological effect, population-specific architecture or due to the limitation of statical power in our study.

Genotype Association with Fertility Hormones

To explore the potential impact of SNPs (rs3129878, rs9268651) on fertility hormones, we analyzed the 91 samples (61 NOA, 30 Normal) which revealed significant correlations between genotypes and some fertility associated hormones as in table (9). The hormones are FSH, Testosterone and Prolactin which differ by fertility status. In NOA males, rs3129878, the AC risk genotype demonstrate a significant associated with higher level of prolactin production compared to wild type AA (median 15.10 (8.64-18.56) vs. 12.09 (7.60-13.13) ng/mL, p=0.048, r=-0.296). The rs9268651 genotypes show no significant association with selected hormones. For normal healthy males, no significant association is observed with selected hormones for rs3129878 genotypes, while the risk genotype of rs9268651 appears to be significantly associated with elevated level of FSH compared to non-risk genotype (median 25.87(24.08-26.48) vs. 5.83(3.14-7.28) mIU/mL, p<0.001, r=-0.966). Neither of the SNPs show significant association with testosterone in either group. These finding my indicate fertility condition-dependent hormonal effect of these azoospermia susceptibility alleles.

Table 9. Correlation association between risk-genotypes and hormonal level in NOA and controls

Group	SNP	Hormone	Risk genotype Median (25th–75th percentiles)	Wildtype Median (25th–75th percentiles)	Correlation	P-value
AZO	rs3129878	FSH	15.53(7.77-24.07)	8.21(4.20-19.13)	-0.212	0.157
		PRL	15.10(8.64-18.56)	12.09(7.60-13.13)	-0.296	**0.048**
		TESTO	3.61(2.61-4.84)	3.33(2.55-5.80)	0.015	0.925
	rs9268651	FSH	7.95(4.21-22.98)	9.02(4.45-21.72)	0.071	0.963
		PRL	11.27(6.71-16.29)	13.30(8.54-17.62)	0.146	0.359
		TESTO	4.00(3.16-4.90)	3.42(2.51-4.65)	0.248	0.165
Normal	rs3129878	FSH	14.92(5.81-25.88)	6.95(3.23-8.59)	0.180	0.441
		PRL	14.38(5.38-24.67)	10.75(8.55-12.03)	0.120	0.613
		TESTO	2.71(2.05-3.96)	3.76(3.04-5.31)	0.330	0.153
	rs9268651	FSH	25.87(24.08-26.48)	5.83(3.14-7.28)	-0.966	**<0.001**
		PRL	7.10(3.9 5-13.40)	11.41(9.61-13.45)	0.347	0.159
		TESTO	2.15(1.90-5.95)	3.76(2.85-4.15)	0.364	0.139

Mann-Whitney U test statistically significant at α=0.05
Sperman test for correlation coefficient.

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The hormonal analysis has uncovered genotype-phenotype pattern that is vary by fertility ability. NOA subjects with AC genotype of the variant rs3129878 appeared to be associated with significant increased level of prolactin hormone compared with wild type (p=0.048, median 15.10, vs. 12.9 ng/ml). The over production of prolactin (hyperprolactinemia) is widely known to be causation factor for male infertility through the suppression of gonadotropin-releasing hormone (GnRH) which subsequently leads to secretion reduction in luteinizing hormone (LH) and follicle stimulating hormone (FSH) [25,26]. The present study did not reveal association between rs3129878 and remaining tested hormones in either group. This result is biologically plausible knowing that the HLA region mainly encodes immune-related genes that play role in local tissue immunity response and antigen presentation[17,19]. The pathogenic mechanism of rs3129878 may act through local testicular immune interaction rather than systematic changes of the testis. Hence the genetic effect might be specific to spermatogenesis related aspects such as germ-cell survival, maturation and testicular immune tolerance without recognizing alterations in the concentration of circulating hormones[18].

Another hormonal association was found between the risk genotype of variant rs9268651 with increased FSH levels in fertile men ($p<0.00$) only. This paradoxical result may imply for intricate compensatory mechanism, where the elevated FSH levels may compensate for normal sperm production with the existence of minor testicular disfunction [27,28]. Malcher et.al (2024) has supported the concept that the genetic variation of HLA region could modulate hormonal response. They demonstrated that combined treatment of recombinant human chorionic gonadotropin (hCG) and recombinant FSH hormone (rFSH) stimulates sperm production in a group with clinical history of NOA, and they reported a difference between responders and non-responders of HLA-DQB1transcript suggesting the response of gonadotropin can be associated with HLA gene expression pattern [29]. Although, the constant replication studies across different Asian populations have discovered several statistically associated genetic risk variants with non-obstructive azoospermia yet moving from etiology to pathology remain needed to fully understand the biological and functional effect of these variants.

Conclusion

This study is designed to test the association of two *HLA-DRA* variants (rs3129878 and rs9268651) on the risk of non-obstructive azoospermia in sample of Iraqi infertile males. The study demonstrated that although there is no individual association of each SNP with NOA risk, it has yet exhibited significant functional impact on sperm productivity and reproduction hormones depending on fertility status. The critical role of *HLA-DRA* genetic alterations in the complicated genetic structure of male infertility is supported by the collective finding of the study including significant HWE in patients suggesting selection pressure, C-G haplotype association, and the subclinical influence in fertile males. These finding emphasis on the need to move beyond regular case-control associations to explore functional intermediate phenotypes in reproductive genomics. Future studies with larger sample size, functional validation of tested variants and multi-ethnic replication are needed to provide comprehensive role of *HLA-DRA* genetic variation in male infertility.

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Author's Declaration

- I hereby confirm the manuscript and all the included table are original and created by me.
- The study protocol was approved by the ethics committee of the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies/University of Baghdad.

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