

HLA-DRB1 ALLELE DISTRIBUTION IN HEALTHY INDIVIDUALS CLASSIFIED BASED ON MIZAJ (TEMPERAMENT) OF UNANI SYSTEM OF MEDICINE

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ABSTRACT

Objective: Assessment of Mizaj of 500 healthy individuals between the age group of 25 and 40 years of the age group of either gender was carried out by validated Unani questionnaire and Sheldon's somatotype. The present study was designed to investigate if the frequency of HLA-DRB1 alleles in the healthy adult population has any association with four Mizaj.

Methods: Mizaj of the healthy individuals (n=500) was assessed by a validated Unani questionnaire based on 10 parameters related to phenotypic and psychological entities known as *Ajnase-e-Ashra*. Mizaj of the same subjects was further assessed by Sheldon's somatoplot. Polymerase chain reaction-sequence-specific typing method was used for human leukocyte antigen (HLA) genotyping of 180 healthy volunteers.

Conclusion: An expected correlation between HLA-DRB1 alleles and Mizaj types is observed. Five single-nucleotide polymorphisms ($p < 0.05$) were significantly associated with different Mizaj. The study suggests that the Mizaj classification of the Unani system of medicine has a genetic basis. This study would be one step toward a personalized approach as per the Mizaj of an individual to disease proneness, lifestyle modification, and medicine.

Keywords: Mizaj, Temperament, Sheldon's somatoplot, Polymerase chain reaction-sequence-specific typing, Human leukocyte antigen-DRB1, Disease management.

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INTRODUCTION

The present study on Mizaj (temperament) concept of human constitution of Unani system of medicine was inspired by earlier studies on *Prakriti* concept of *Ayurveda* as well as constitution classification of TCM [1,2]. Among the traditional systems of medicine practiced all over the world, Unani has a documented history dating back to 460–377 BC and remains highly popular, especially in South Asia [3]. As per the Unani system, the human body is composed of four basic elements: Earth (*Mitti*), water (*Paani*), air (*Hawa*), and fire (*Aag*). Each of these basic elements possesses a *Kaifiyat* (inbuilt property which can be called a specific heat capacity in terms of Physics) two dominant (*Faail*) *Kaifiyat* hot (*Haar*) and cold (*Barid*), and two *Mafool* (recessive), *Kaifiyaat* wet (*Ratab*) and dry (*Yaabis*) [4]. The body fluids are classified into four humors: Blood (*Dum*), phlegm (*Balghum*), yellow bile (*Safra*), and black bile (*Sauda*). These humors have their own *Kaifiyat*, blood has hot and wet *kaifiyat*, phlegm is cold and wet, yellow bile is hot and dry while black bile is cold and dry. The quality and quantity of four humors affect the state of health and their disproportion produces disease in the body. Although all four humors exist in every human being and in different proportions, one is dominant based on which an individual's Mizaj is determined. Like *Prakriti*; Mizaj could be considered as a discreet phenotype that is based on physical, physiological, psychological, and behavioral traits. They are independent of social, ethnic, and geographical variables [5]. In Unani system, human beings are classified into the four Mizaj as *Damvi* (Sanguine), *Balghami* (Phlegmatic), *Safrawi* (Choleric), and *Saudavi* (Melancholic) [6]. Mizaj is evaluated by a validated questionnaire having 10 parameters based on physical characteristics such as touch, distribution of muscles and fats in the body, distribution of hair, its color and texture on body and scalp, physique, physiological status such as sleep and wakefulness, receptivity of organs to external environment/climate, digestion, food liking, functional state of organs, bladder, and bowel movement, and psychological parameters such as depth of sorrow, irritability, and sensitivity. The

details of which are viewed in Table 1. These parameters are called as *Ajnase-Ashra* [7]. Various traditional systems of medicines such as *Ayurveda* and Chinese have classified the human population. Even psychologists have also classified humans into different personality types, for example, American psychologist William Sheldon has also classified human beings into three types of personalities and termed them somatotypes. Sheldon's somatotypes are based on physical characteristics or physique. These parameters are similar to the parameters (*Physique*) of *Ajnase-Ashra* of Unani questionnaire. He has expressed them numerically and named them endomorphy, mesomorphy, and ectomorphy, for they appeared to be derived from the three layers of the human embryo, the endoderm, the mesoderm, and the ectoderm [8]. Sheldon's body types are assessed by 10 anthropometric measurements such as stretch stature, body mass, four skin-folds (triceps, subscapular, suprascapular, and medial calf), two bone breadths (bi-epicondylar humerus and femur), and two limb girths (arm [flexed and tensed] and calf) [9]. William Sheldon also believes in the *Hippocrates'* theory of humors [10] and Mizaj. He has incorporated Mizaj and somatotypes and plotted them on a curvilinear triangle with their score (Fig. 1). The central portion of the curvilinear triangle represents a combination of sanguine Mizaj (dominant) with phlegmatic, choleric, and melancholic. The upper corner of the triangle represents a combination of choleric Mizaj (dominant) with phlegmatic, sanguine, and melancholic. The right lower corner of the curvilinear triangle represents phlegmatic Mizaj (dominant) with sanguine, choleric, and melancholic. The left lower corner represents melancholic Mizaj (dominant) with sanguine, choleric and phlegmatic Mizaj. A pilot study has been conducted to explore the Mizaj types by validated Unani questionnaire and by Sheldon's Somatotypes of the same subjects to find out any relationship between these two methodologies and to test if there is any statistical correlation. The study showed a great degree of similarity between Mizaj assessed by Unani questionnaire and by Sheldon's somatotypes with 80% concordance [11]. In Unani practice, Mizaj of healthy individuals and

Table 1: Questionnaire for the assessment of Mizaj (temperament)

Pro forma for the assessment of Mizaj (temperament)						
Name						
Father's Name						
Native Place						
Mother's Name						
Native Place						
Place of Birth						
Native Place						
Date of Birth				SunSign		
Age	Sin-e-Numu	Sin-e-Shabab	Sin-e-Kuhulat	Sin-e-Shekhukhat		
Years	(Upto 25 years)	(25-40 years)	(41-60 years)	(>60 years)		
Gender	Male	Female		LMP		
Phoneno						
Address						
Occupation				NA		
Socioeconomic Status	HIG	MIG	LIG	BPL	NA	
Dietary Habits	PureVeg		Mixed Diet			
Pulse						
Blood Group						
S. No.	Ajnās	Parameters	MIZĀJ (Temperament)			
			DAMWĪ	BALGHAMI	SAFRĀWĪ	SAWDĀVĪ
1	Adilla-i-Lams	Touch (Feel of the body)	Hot and Moist Smooth3	Cold and Moist Smooth2	Hot and Dry Rough4	Cold and Dry Rough1
2	Adilla-i-Lahm-o-shahm	Muscularity (WHOLE BODY SKELETAL MUSCLE)	F:>35.4%4 M:>44.1%4 Shahm: F: 21-33%2 M: 8-19%2	F: 30.4-35.3%3 M: 39.4-44.0%3 Shahm: F:>39%4 M:>25%4	F: 24.3-30.3%2 M: 33.3-39.3%2 Shahm: F: 33-39%3 M: 19-25%3	F:<24.3%1 M:<33.3%1 Shahm: F:<21%1 M:<8%1
3	Adillah-e-sha'r	Scalp and Body Hair				
		1.Distribution	Normal3	Scanty1	Moderate2	Excessive4
		2.Growth	Average3	Slow1	Rapid4	Slow1
		3.Texture	Fine2	Fine2	Coarse4	Coarse4
		4.Shape	Wavy3	Straight2	Straight2	Curly4
		5.Color	Brown2	Brown2	Brown/Black2/4	Black4
4	Adilla-i-Lawn-e-Jild	Skin Color	Towards reddish3	Towards whitish2	Towards Yellowish4	Towards blackish1
5	Adilla-i-Hay'at	Physique	Well built, Broad chest, Blood vessels prominent3	Average built, wide chest, blood vessels non-prominent2	Thin built, narrow chest, blood vessels prominent4	Thin built, narrow chest, blood vessels superficial1
6	Adilla-i-Harkat-o-Sukūn	Receptivity of Organs (receptivity to the Surrounding with Environment or With food)	Gradually warmed (gradual Response to heat) and Slowly cooled (gradual Response to cold) 3	Quickly cooled (quick response To cold) and Slowly warmed (slow response To heat) 2	Quickly warmed (quick response To heat) and Slowly cooled (slow response To cold) 4	Gradually cooled (Gradual Response to cold) and Slowly warmed (slow response To heat) 1
7	Adilla-i-Nawm-o-Yaqza	Sleep and Wakefulness				
		1.Quality and Duration	Moderate and Sound Sleep 6-8 h 3	Excessive and Sound Sleep More than 8Hrs2	Less and Restless Sleep Less than 6 h 4	Less and interrupted Sleep1
		2.Dreams (Dominant Color And Character)	Red Sentimental and romantic3	White Peaceful dream2	Yellow and fiery Passionate4	Black and Fearful Scary1
8	Adilla-i-Af'al-i-A'zā'	Functional state Of organs	Moderately active3	Lethargic and Sluggish2	Hyperactive4	Sluggish1
	Adilla-i-Nabz	1. Pulse	NabzAzim (Strong and voluminous) 3	NabzBatiwa Laiyyin (Slow and Soft) 2	NabzSariwa Mutawatir (Rapid and in succession) 4	NabzSulbWa Bati (Hard and Slow) 1
	Adilla-i-Zā'iqa	2. Taste	Sweet3	Tasteless1	Bitter4	Sour2
	Adillah	3. Food liking	Cold and dry	Hot and dry	Cold and wet	Hot and wet
	Adillah-i-Zawq	(Provide reference)	Salty1	Spicy4	Sour2	Sweet3

(Contd...)

Table 1: (Continued)

Pro forma for the assessment of Mizaj (temperament)					
Adilla-i-'Atash	4. Thirst	Moderate ²	Less ¹	Extreme ⁴	Excessive ³ PAGE-2
Adilla-i-Ishtihā'	5. Appetite	Good ³	Poor ²	Very good /Excessive ⁴	V.poor ¹
Adilla-i-Hazm	6. Digestion	Moderate ³	Slow ²	Very good ⁴	Poor ¹
Adilla-i-Nafsāniyya	7. Nervous System Activity (MOTOR)				
	a. Activeness	Active ³ i-when Climbing stairs Do you usually Take them Two at a time? ii-do you Usually, finish Your meals Hurriedly for No reason?	Sluggish ¹ i-are you inclined To be slow in Your actions?	HyperActive ⁴ i-are you always On the go?	Bidirectional ² i-do you Sometime feel Energetic and Self-motivated?
	adventurousness	Moderate ³ i-do you quite Enjoy taking risks?	Low ¹ i-do you always We are safety Belt while traveling?	High ⁴ i-would you Enjoy fast driving?	Bidirectional ²
	c. Aggressiveness	ii-would you Prefer a job involving Traveling and Variety even If its in secure? Moderate ³ i-do you ever Get so angry That you swear And yell?	ii-would you Prefer a job with Less challenges And secure?	ii-would you do Almost anything For a dare?	Bidirectional ² i-are you Considered a gentleperson? ii-do you like to watch something Which includes Fight or extreme behavior? V.Low ¹ i-do you consider Yourself a failure?
	d. Boldness	ii-do you often Grind your teeth Consciously or unconsciously Moderate ³ i-are you Pretty sure of yourself? ii-do you feel confident when someone appraises you?	ii-would you Rather agree on Something then arguea boutit? Low ² i-do you think You have little To be proud of? ii-are you shy and self-conscious mostofthe time?	ii-do you often Indulge in arguments? High ⁴ i-do you usually Feel you can Accomplish what You want? ii-are you pretty Confidentof your appearanceand attitude, in general?	ii-do you suffer fromfeelings ofinferiority?
	e. Gait	Fast ³	Slow ¹	VeryFast ⁴	Bidirectional ²
	f. Fatigability	Moderate ³	V.Early ¹	Late ⁴	Early ²
	g. Yawning	Infrequent ³	VeryFrequent ¹	Rarely ⁴	Frequent ²
	8. Nervous System Activity (SENSORY)				
	a. Feeling of Heaviness? (Afternightfall)	Moderate ³	High ⁴	Mild ²	Negligible ¹
	b. Receptivity Externalsenses	High ³	Low ²	VeryHigh ⁴	VeryLow ¹
	c. Alertness	High ³	Low ¹	VeryHigh ⁴	VeryLow ¹
9	Adilla-e-Istifrāgh				
	Excretion				
	Bawl (Urine) Colour	Reddish ⁴	Whitish ²	Yellowish ³	Greyish ¹
	Bawl (Urine) Odour	Strong ³	Mild ²	VeryStrong ⁴	Negligible ¹
	Araq (Sweat) Quantity	Moderate ³	Less ²	Excessive ⁴	VeryLess ¹
	Araq (Sweat) Colour	Reddish ⁴	Whitish ²	Yellowish ³	Greyish ¹
	Araq (Sweat) Odour	Moderate ³	Mild ²	Strong ⁴	Negligible ¹
	BarazColour	Reddish ³	Whitish ²	Yellowish ⁴	Greyish ¹
	BarazOdour	Strong ³	Mild ²	VeryStrong ⁴	V.Mild ¹
	BarazQuantity	Moderate ³	Less ²	Strong ⁴	V.less ¹

(Contd...)

Table 1: (Continued)

Pro forma for the assessment of Mizaj (temperament)						
10	Adilla-i-Quwā Nafsāniyya	Psychological Activity 1. Intensity of Anger 2. Depth of Sadness and Sorrow (let go or hold on) 3. Initiativity 4. Sensitivity 5. Irritability 6. Optimism (positive thinking) 7. Sympathy 8. Apprehensions (worry about future) ASSOCIATIONAL 1. Intelligence 2. Decision taking power 3. Firmness	Moderate 3 i-doyou stamp your feet and kick things when you are in rage? Moderate 2 i-doyou derive a good deal of happiness from your life? ii-doyou often suffer from loneliness? Moderate 3 i-Doyou like to be the first person to initiate group activity? Moderate 3 i-doyou get upset when you get to know about unfortunate people? High 3 i-doyou feel somewhat rattled if things don't go as planned? Moderate 3 i-would you try for a task even if it seems unachievable? High 4 i-would you help someone monetary even if you are in tight position? Low 2 i--doyou often worry about your future? Normal 3 Quick 3 Moderate 3 i-doyou argue a point if you are right? Winter 1	Low 1 i-would you hold back from engaging in fights? High 3 i-doyou seem to have more than of your share of bad luck? ii-doyou feel lonely even when with other people? Low 2 i-Doyou Usually, avoid to take first step in any group activity? High 4 i-would you refrain from expressing your opinion if others might be offended by it? Low 1 i-are you usually calm and not easily upset? Low 2 i-Doyou give up easily in unfavourable circumstances? Moderate 3 i-doyou tend to help people who are sick or unfortunate? Moderate 3 i-doyou always worry about your future? Low 1 Slow 2 Low 2 ii-are you easily convinced by others? Summer 4	High 4 i-have you ever felt some time genuinely to kill somebody? Low 1 i-In general are you satisfied with your life? ii-doyou generally feeling good spirits? High 4 i-Are you always the first person to initiate group activity? Low 2 i-Doyou express your opinion freely irrespective of other persons feeling? Very High 4 i-are you easily irritated by things being out of place/order? High 4 i-despite of any difficulties you think you can accomplish task? Low 2 ii-doyou feel content and find pleasure in helping others? V.Low 1 i--doyou never plan about your future? High 4 Very Quick 4 High 4 i-doyou never change your decision?	Bidirectional 2 i-doyou usually manage to be patient even with fools? Very High 4 i-when things go wrong, do you wish you were dead? ii-doyou feel miserable for no reason? Very Low 1 i-You never initiate group activity? Least 1 i-doyou like to play pranks on other people? Moderate 2 i-are you seldom irritated by things being out of place/order? Very Low 1 i-Are you always unsure to accomplish the task? Very Low 1 ii-are you detached in your dealings with people even if it required to be considerate? Very High 4 i-doyou always feel insecure about your future? Moderate 2 Very Slow 1 Very Low 1 ii-are you always uncertain about your opinions?
11	Adilla-i-Fusūl Depending upon the history and symptoms of the patients	Seasonal Comfortability	Winter 1	Summer 4	Winter 1	Rainy 2
ANTHROPOMETRY MEASUREMENTS						
	Stretch Stature (Height in cm)	Body Mass (Weight in Kg)	Skin Folds Triceps	Subscapular	Supraspinale	Medial Calf

(Contd...)

Table 1: (Continued)

Pro forma for the assessment of Mizaj (temperament)		LimbGirths	BoneBreadths	Bipicondylar
Body Composition	%General Fat Area	Flexed Arm Bodyage Subcutaneous Fatin%	Calf BMI Skeletal Muscle in%	Humerus RMK Cal Sign Femur %Visceral Fat
Somatoplot Score	Whole Body Trunk Legs Arms			Name of Physician Date
Temperament by questionnaire				
Temperament by somatoplot				

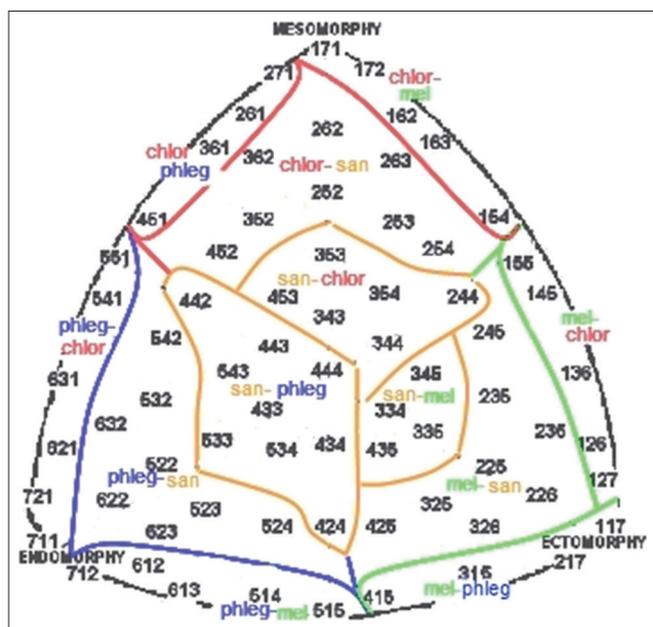


Fig. 1: The four temperaments mapped onto standard somatotype chart

patients is identified so that most suited individual therapy could be planned for patients and the most suited healthy lifestyle could be suggested to healthy individuals. The Eastern Medical Systems of Ayurvedic, Chinese, and Greek Unani medicine had existed for centuries and were based on holistic and vitalistic concepts. Many papers reporting the genetic basis of Prakriti classification, TCM, and Korean system are available however genetic basis of Mizaj concept of Unani system of medicine is unexplored [1,2,12]. In the present work, we assessed if different Mizaj show human leukocyte antigen (HLA)-DRB1 gene polymorphism.

METHODS

Mizaj was assessed by the validated Unani Questionnaire and by Sheldon's somatoplot method [11].

Assessment of Mizaj by validated Unani questionnaire based on "Ajnas-e-Ashra"

Informed consent was obtained from healthy participants before recruitment. The study design and protocol were approved by the Institutional Ethics Committee of Z.V.M. Unani Medical College and Hospital, Pune, India. The trial was registered on the Clinical Trial Registry of India: CTRI (CTRI/2017/01/007734). A total of 500 healthy participants between 25 and 40 years of the age group of either gender were assessed. Mizaj assessment has been carried out at Z.V.M. Unani Medical College and Hospital, Pune. For the assessment

of Mizaj, a questionnaire based on the Ajnas-e-Ashra was constructed (Table 1). As all the 10 parameters mentioned in the Ajnas-e-Ashra qualitative parameters, they have been quantified on 1-10 scales. Quantification was performed as described by Unani Hakeem Balinas [13]. The quantification is based on qualities, that is, Kafiyat. Validation and reliability test for the questionnaire were carried out using IBM SPSS software version 20 (Trial version), n=60 of either gender, and 52 parameters were tested. Data for validity test were uploaded in MS Excel sheet. The result was interpreted. Cronbach's alpha obtained was 0.871 which was statistically significant. It was important to measure Mizaj objectively, hence, the 10 qualitative parameters mentioned in Table 1 have been quantified on 1-10 scales. Assessment of Lahm-wo-Shahm (Muscles and fats): Quantification of Lahm-woshahm for the specific Mizaj types was done by the NIH/WHO guideline. Lahm-wo-Shahm were assessed by Omron's body composition analyzer. Skeletal muscles, subcutaneous fat, and visceral fats were expressed in percentage (Tables 2 and 3). The distribution of the percentage of fats and muscles is assigned to each Mizaj types (Tables 4 and 5). The questionnaire for the assessment of Mizaj has four columns representing each Mizaj, that is, Damwi, Balghami, Safrawi, and Saudawi. These parameters consist of the physical and psychological status of a person. Mizaj assessment questionnaire was explained to all the subjects. The subjects were asked to select the most suitable parameters as per their choice. Attempting of all the questions was compulsory. Scores are summed up and finalized in each Mizaj column. The Mizaj column scoring the highest was the dominant Mizaj and the second highest score was the recessive/second dominant Mizaj.

Assessment of Mizaj by Sheldon's somatotype

For further confirmation of Mizaj of the healthy 500 participants, Sheldon's somatotypes were carried out. Ten anthropometric dimensions were recorded to calculate the anthropometric somatotypes. These were stretch stature, body mass, four skin-folds (triceps, subscapular, supraspinal, and medial calf), two bone breadths (bi-epicondylar humerus and femur), and two limb girths (arm flexed and tensed, calf).

Plotting the Somatoplot chart

All the above 10 anthropometry measurements of 500 subjects were uploaded on Monte Goulding software (Sweat Technologies, Mitchell Park, South Australia) to get a score of Sheldon's somatoplot. The score obtained was then compared with Sheldon's curvilinear Somatoplot chart having score with Hippocrates' four basic humors and their combinations (Fig. 1). Thus, Mizaj was also assessed by Sheldon's somatotypes as earlier reported [11]. Out of 500 participants, 180 subjects having 80% concordance in Mizaj assessed by Unani questionnaire and by Sheldon's method of somatotypes were recruited in this study for HLA typing. Characteristics of recruited volunteers are summarized in Table 6.

DNA isolation and HLA genotyping

Venous blood was collected from healthy volunteers (n=180) 45 blood samples of each Mizaj types, in an EDTA-coated BD Vacutainer

(Becton, Dickinson Company Franklin Lakes, NJ, USA). Genomic DNA from peripheral blood mononuclear cells was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The purity and yield (ng) of isolated DNA was determined using NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, USA). The DNA samples used for genotyping had a purity ratio (260/280) between 1.7 and 2. Low-resolution HLA typing was performed by polymerase chain reaction-sequence-specific primer (PCR-SSP) techniques using Micro SSP DNA Typing Trays DRB (One Lambda Inc., Canoga Park, California, USA) according to the manufacturer's instruction. The PCR was performed using the Eppendorf Mastercycler® nexus (Hamburg, Germany). Amplified DNA fragments were detected using 2% agarose gel electrophoresis at 120 v for 15 min, stained with ethidium bromide in UV transillumination system as previously described [14]. One Lambda HLA Fusion software version 3.0 was used to detect specific HLA-DRB1 alleles.

Statistical analysis

The frequencies of HLA-DRB1 alleles within groups were determined by direct counting. Statistical analysis of the distribution of allele frequencies between four different Mizaj was performed by GraphPad PRISM software v5.0 (San Diego, CA, USA). Categorical data were analyzed using Fisher's exact test and the goodness of fit: χ^2 test. Odds ratio and 95% confidence intervals were also calculated. $p < 0.05$ was considered statistically significant.

Table 2: Skeletal muscle chart

Gender	Age	Low (-)	Normal (0)	High (+)	Very high (++)
Female	18-39	<24.3	24.3-30.3	30.4-35.3	≥35.4
	40-59	<24.1	24.1-30.1	30.2-35.1	≥35.2
	60-80	<23.9	23.9-29.9	30.0-34.9	≥35.0
Male	18-39	<33.3	33.3-39.3	39.4-44.0	≥44.1
	40-59	<33.1	33.1-39.1	39.2-43.8	≥43.9
	60-80	<32.9	32.9-38.9	39.0-43.6	≥43.7

Source: Omron Healthcare

Table 3: Body fat percentage chart

Gender	Age	Low (-)	Normal (0)	High (+)	Very high (++)
Female	20-39	<21.0	21.0-32.9	33.0-38.9	≥39.0
	40-59	<23.0	23.0-33.9	34.0-39.9	≥40.0
	60-79	<24.0	24.0-35.9	36.0-41.9	≥42.0
Male	20-39	<8.0	8.0-19.9	20.0-24.9	≥25.0
	40-59	<11.0	11.0-21.9	22.0-27.9	≥28.0
	60-79	<13.0	13.0-24.9	25.0-29.9	≥30.0

Source: NIH/WHO guidelines for BMI, Source: Gallagher et al., American Journal of Clinical Nutrition, vol. 72, Sept 2000

Table 4: Distribution of % of skeletal muscle assigned to each Mizaj

Muscularity	DAMWĪ	BALGHAMI	SAFRĀWĪ	SAWDĀVĪ
	Muscular	Flabby (Adipose)	Lean	Very Lean
(WHOLE-BODY SKELETAL MUSCLE)	F:>35.4% (4) M:>44.1% (4)	F: 30.4-35.3% (3) M: 39 (4) 44.0% (3)	F: 24.3-30.3% (2) M: 33.3-39.3% (2)	F:<24.3% (1) M:<33.3% (1)

Table 5: Distribution of % of subcutaneous fat assigned to each Mizaj

Adiposity	DAMWĪ	BALGHAMI	SAFRĀWĪ	SAWDĀVĪ
	Shahm:	Shahm	Shahm	Shahm
(WHOLE-BODY SUBCUTANEOUS FAT)	F: 21-33% (2) M: 8-19% (2)	F:>39% (4) M:>25% (4)	F: 33-39% (3) M: 19-25% (3)	F:<21% (1) M:<8% (1)

RESULTS

We explored the HLA-DRB1 alleles in four different Mizaj and their combinations categorized by Unani medical system in populations ($n=45/Mizaj$). The presence of HLA DRB1*01 allele in Sanguine Mizaj ($\chi^2=4.44$, $p=0.035$) and in Choleric Mizaj ($\chi^2=4.09$, $p=0.043$) was found to be significant. The frequency of the HLA DRB1*03 was significantly higher in Melancholic Mizaj, ($\chi^2=4.58$, $p=0.032$), Phlegmatic Mizaj ($\chi^2=4.14$, $p=0.042$), and in Choleric Mizaj ($\chi^2=3.89$, $p=0.048$). HLA-DRB1*11 allele in phlegmatic Mizaj ($\chi^2=4.19$, $p=0.040$) reached significance. The HLA-DRB1*14 was well distributed in phlegmatic Mizaj ($\chi^2=4.61$, $p=0.032$), while HLA-DRB1*15 alleles were well distributed in sanguine ($\chi^2=3.96$, $p=0.047$) and melancholic Mizaj ($\chi^2=4.49$, $p=0.034$) (Table 7).

DISCUSSION

In India, other than allopathic medicine, different forms of scientifically appropriate and acceptable systems of indigenous medicine, such as Ayurveda, Yoga, Unani, Naturopathy, Siddha, and Homeopathy (AYUSH) system, are practiced in different parts of the nation [15]. Scientific research on fundamental concepts of AYUSH remains important in mainstreaming and improving the scope of AYUSH. Ayurveda and Yoga have been explored scientifically to some extent to understand fundamental concepts along with the beneficiary role of the various therapies such as panchakarma and pranayama [16,17]. However, limited reports are available on scientific exploration of the basic concepts of Unani system of medicine. Mizaj is one of the major components of Unani system of medicine used for advising preventive measures and diets to healthy individuals. Diagnosis of the disease and line of treatment are also ascertained as per the Mizaj of the patient. Assessment of Mizaj is based on sets of physical and psychological features of an individual. There is a paucity of scientific research on fundamentals of Unani system. Ayugenomics study correlating Prakriti of Ayurveda with genetics inspired us to undertake similar study for constitution classification of Unani system of medicine with genetics. This prompted us to undertake a study on exploring HLA allele frequencies in different Mizaj and also look at Sheldon's somatotype classification ascertaining Mizaj. We reported 80% concordance between the Mizaj assessed by Unani questionnaire and by Sheldon's method of somatotypes. This opens an opportunity to develop an objective software-based method for the assessment of Mizaj like the one developed by C-DAC for Prakriti [18]. Our study for the 1st time has generated genetic-based scientific evidence about Mizaj and its association with HLA DRB1 alleles. Mizaj and Prakriti seem to be overlapping concerning the association of HLA-DRB1 alleles. As per Unani system of medicine diseases have also been classified as per the involvement of the humors, so the diseases can be sanguine, phlegmatic, choleric, and melancholic, for example, Damwiamraz (sanguine disorder), balghamiamraz (phlegmatic disorders), SafrawiAmraz (choleric disorder), and Saudawiamraz (Melancholic disorders). The detailed signs and symptoms of these diseases have been discussed in Unani manuscripts. Unani physicians predict disease proneness on the basis of Mizaj of an individual and, accordingly, can advise preventive measures

to their patients. In Unani classical text, it is mentioned that the sanguine and choleric *Mizaj* are prone to rheumatoid arthritis (hot type) [19]. HLA-DRB1 analysis showed a significant presence ($p < 0.05$) of HLA-DRB1*01 in sanguine and choleric population. HLA-DRB1*01 genotype variant is considered a predisposing factor for the development of rheumatoid arthritis. The most common genotype in rheumatoid arthritis patients was HLA DRB1*01/DRB1*13, which showed increased frequency and high relative risk [20]. Golmoghaddam *et al.* demonstrated that HLA-DRB1*01 may have a protective role against atherosclerosis in Iranian individuals [21]. HLA-DRB1*01 was found to be significant in choleric people. According to Unani philosophy, one of the important functions of *Safra* (yellow bile) is the emulsification of fat. Hence, it might play an important role in the prevention of atherosclerotic plaque in choleric persons [22]. A case reported suggested that the HLA-DRB1*03 allele may explain a common etiology underlying the comorbidity of type 2 diabetes, Graves' disease, and schizophrenia in the individual [23]. HLADRB1*03:01 is the strongest genetic modifier of disease severity in autoimmune hepatitis [24]. HLA-DRB1*03 is associated with early age of onset, severe inflammatory activity, less responsiveness to therapy, and greater frequency of liver transplantation seen in North American and North European patients than in patients with HLA-DRB1*04 [25]. Our results indicated a significant presence of HLA-DRB1*03 ($p < 0.05$) in phlegmatic, choleric, and melancholic *Mizaj*. Our results are in line with ancient Unani literature which stated that phlegmatic *Mizaj* persons are prone to diabetes [26] whereas choleric person is prone to liver disorders [27] and melancholic *Mizaj* people are prone to psychological disorders like schizophrenia [28]. Chaudhuri *et al.* suggested that HLA-DRB1*11 allele may have a protective role in human breast cancer [29]. In contrast, deficiency of the HLA-DRB1*11 allele potentially increased the risk factor for the aggregation of hepatocellular carcinoma. In addition, decreased frequency of HLADRB1*11 has been associated with protection from chronic hepatitis C virus infection [30]. A study in

Iranian population depicted individuals with positive history of multiple sclerosis showed that HLA-DRB1*11 allele has a significantly low rate [31]. Unani literature states that *Safravi* (choleric) are prone to liver disorders whereas *Saudavi Mizaj* people are prone to psychological disorders like schizophrenia [32]. HLA-DRB1*03 and DRB1*04 are associated with atrophic gastritis in an Italian population [33]. In this study, HLA-DRB1*03 is significant in choleric *Mizaj*, which supports the Unani theory of choleric person having tendency of gastritis [32]. In systemic juvenile idiopathic arthritis, the strongest risk factor was HLA-DRB1*11, which conferred at least a 2-fold increase in disease risk [34]. Our results indicated significant presence of ($p < 0.05$) HLA-DRB1*11 allele in phlegmatic *Mizaj*. As per Unani system of medicine, phlegmatic *Mizaj* persons are prone to arthritis (cold type) [35]. Rheumatic heart disease patients had a history of repeated attack of sore throat. As per Unani system, phlegmatic persons are prone to sore throat and respiratory tract infection [36]. A study in North Indian population showed that HLA-DRB1*14 gene polymorphism revealed two single-nucleotide polymorphisms with rheumatic heart disease patients [37]. Toro *et al.* results indicated that HLA-DRB1*14 allele exerts resistance to multiple sclerosis in a highly admixed Colombian population [38]. Our results indicated significant presence ($p < 0.05$) HLA-DRB1*14 allele in phlegmatic *Mizaj*. Asthma is a chronic inflammatory disease associated with both the genetic and environmental factors. A meta-analysis showed that HLA-DRB1*15:01 allele protects from asthma susceptibility [39]. Moreover, DNA methylation as a mediator of HLA-DRB1*15:01 haplotype acts as a protective variant in multiple sclerosis [40]. We found a significant presence of HLA-DRB1*15 allele in melancholic and sanguine *Mizaj* individuals. Sanguine persons are hot and wet, melancholic persons are having cold and dry *Mizaj*, hence are less likely to have asthma and sclerosis, respectively. Unani literature shows *Mizaj*-related disease association, HLA-DRB1 alleles have important role in immune pathogenesis of diseases. Our research shows indirect evidence for *Mizaj* and its association with HLA-DRB1 alleles and diseases. This study indicates close association of *Mizaj* and alleles of HLA DRB1 with proneness and prevalence of disease. Limitation of our study is a small number of study subjects; however, our study highlights the usefulness of HLA association in *Mizaj* as it is shown in the case of *Prakriti* and TCM. We would like to explore further *Mizaj* and disease proneness for early prognosis and prevention.

Table 6: Characteristics of recruited volunteers

Temperaments (<i>Mizaj</i>)				
Number of subjects with gender distribution	Sanguine	Phlegmatic	Choleric	Melancholic
Total (n) 45	Total (n) 45	Total (n) 45	Total (n) 45	Total (n) 45
Male (M)	22 (48.89%)	22 (48.89%)	23 (51.11%)	23 (51.11%)
Mean age (M)	33±3.68	38.6±4.70	32.3±5.05	38.0±3.87
Female (F)	23 (51.11%)	23 (51.11%)	22 (48.89%)	22 (48.89%)
Mean age (F)	36.5±4.15	34.3±4.37	27.0±5.09	32.33±4.46

Table 7: HLA DRB1 allele frequency distribution among different Mizaj

Sanguine		Phlegmatic				Choleric				Melancholic						
Phenotypic frequency		Phenotypic frequency				Phenotypic frequency				Phenotypic frequency						
HLA DRB1 Allele	S (n=45)	P+C+M (n=135)	χ^2	p	p	S+C+M (n=135)	χ^2	p	C (n=45)	S+P+M (n=135)	χ^2	p	M (n=45)	S+P+C (n=135)	χ^2	p
1	4	34	4.44	0.035	7	30	0.55	0.456	4	33	4.09	0.043	15	26	3.04	0.081
2	4	7	0.29	0.590	4	8	0.11	0.730	3	10	0.00	1.000	5	10	0.21	0.640
3	6	35	2.36	0.124	5	37	4.14	0.042	6	40	3.89	0.048	18	30	4.58	0.032
4	1	1	0.00	1.000	1	4	0.00	1.00	1	1	0.00	1.00	1	3	0.00	1.000
7	9	35	0.36	0.548	10	25	0.10	0.745	8	32	0.38	0.535	9	14	2.01	0.156
8	5	10	0.21	0.640	3	11	0.00	1.00	4	8	0.11	0.730	4	6	0.56	0.452
9	8	17	0.38	0.534	3	15	0.32	0.560	6	11	0.54	0.462	2	12	0.41	0.520
10	6	12	0.32	0.566	5	10	0.21	0.640	14	24	2.84	0.092	6	27	0.60	0.436
11	17	30	3.46	0.063	2	25	4.19	0.040	9	20	0.34	0.558	7	24	0.01	0.909
12	3	5	0.17	0.676	2	8	0.00	1.00	3	5	0.17	0.676	2	6	0.00	1.000
13	4	12	0.00	1.000	3	13	0.09	0.762	6	10	0.82	0.364	3	13	0.09	0.762
14	10	40	0.59	0.442	20	35	4.61	0.032	11	34	0.00	1.000	10	48	2.17	0.141
15	12	17	3.96	0.047	18	40	1.22	0.269	10	31	0.00	1.000	5	38	4.49	0.034

11 and HLADRB1* 14 while HLADRB1* 15 alleles were well distributed in Sanguine and Melancholic Mizaj. These findings suggest that the Mizaj of Unani System of Medicine has a genetic basis. However multi centric research studies with higher sample size should be carried out in future.

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AUTHORS' CONTRIBUTIONS

Dr. Ghazala Mulla: Principal investigator: The assessment of Mizaj is done under her guidance by the SRF Dr. Farhan Qureshi. Dr. Jalis Ahmed: Coinvestigator: He carried out a clinical assessment of Mizaj. Dr. Kalpana Joshi: Coinvestigator: She guided SRF Tejas Shah to carry out DNA isolation and HLA genotyping. Dr. Farhan Qureshi: First SRF carried out the assessment of Mizaj. Mr. Tejas Shah: Second SRF: He carried out DNA isolation and HLA genotyping.

CONFLICTS OF INTEREST

The authors report that there are no conflicts of interest regarding the publication of this paper.

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