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Exploring Phenotypic Diversity of Human Iris Features and Skin Color in Punjabi and Khyber Pakhtunkhwa Population of Pakistan

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SB and MS designed the study, collected data, participated in data interpretation and drafted the manuscript. MS assisted in data analysis. MSA assisted in data acquisition. AAS coordinated the study and helped to draft the manuscript. All authors gave final approval for the publication.

Key words
Pigmented traits, Eye color, Skin color, Iris features, Pakistani population

ABSTRACT

Phenotypic variations of eye color, skin color, and iris surface features have been well-explored in certain populations. However, there has been comparatively little research on variations in these features in Pakistani population. The aim of this study is to discover phenotypic diversity and correlations of pigmented traits and iris surface features in Punjab and Khyber-Pakhtunkhwa (KPK) province of Pakistan**.** Digital images of eyes and skin were examined by investigators to determine color using Fitzpatrick Phototype Scale. Similarly, iris patterns were characterized by Edward iris feature software and association studies were conducted through SPSS program. Intermediate eye color was frequent in KPK (44%) while brown was higher in Punjab (47%). Contrarily, light to medium brown skin color was recurring (55%) in Punjab whereas lighter skin color prevailed in KPK (69%). Furthermore, Fuchs' crypts were significantly correlated with contraction furrows in both populations. Likewise, crypts were significantly associated with Wolfflin nodules and furrows were significantly related to conjunctival melanosis and pigment spots in KPK sample set. Based on unique iris patterns, these phenotypic traits would be helpful for individuals' discrimination in the population**.** In future, there is need to explore genetic associations and functional differences of these traits.

INTRODUCTION

Eye and skin color are two of the most discernible and variable phenotypic traits of humans. Pigmentation in these polymorphic characters is highly complex and regulated by genetic and environmental factors (Sturm and Duffy, 2012). Human variability in pigmented traits depends on the type, amount and distribution of melanin which exhibits remarkable phenotypic diversity in different populations (Maranduca *et al*., 2019). Melanin is major pigment which controls the coloration of eye, skin and hair. It is synthesized in melanocytes and packaged in highly specialized vesicles, melanosomes, where excessive pigment is present (Schadendorf *et al*., 2015). There are two main types of melanin, eumelanin and pheomelanin, which produce blackish-brown and yellowish red pigments respectively (Bonaventure *et al*., 2013).

Brown eyes and dark skin color are ancestral phenotypes present worldwide. Increase in pigment diversity has been greatly influenced by evolutionary selective forces during prehistoric times (Jablonski and Chaplin, 2017). It is hypothesized that blue eyes have European origin taking advantage of rare color during sexual selection (Cavalli-Sforza *et al*., 1994; Eiberg *et al*., 2008). European population depicts full range of eye color variations while moderate diversity is observed in Middle East, North Africa and some parts of South Asia. However, different shades of brown ranging from light yellowish-red to dark blackish-brown are frequent in rest of the world (Walsh *et al*., 2012). On the other hand, observed variability in skin pigmentation displays strong association with latitude. High levels of ultraviolet radiations (UVR) cause darker skin in tropical and equatorial regions (Australia, sub-Saharan Africa, Melanesia and South Asia) than in areas far off the equator (Del Bino *et al*., 2018). Evolutionary hypothesis suggests that major driving forces responsible for obvious trends in skin color are latitude, natural selection and variable expression of melanin and vitamin D in response to UVR intensity (Jablonski and Chaplin, 2010).

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The human eye consists of five unique layers. The posterior-most layer, the iris pigment epithelium (IPE) is rich in melanin and does not differ significantly among individuals (Peles *et al*., 2009). Adjacent to the IPE, two muscle layers, the sphincter muscle and the dilator muscle are present (Eagle Jr, 1988). Most of the variations in eye color and surface features are attributed to the anterior border and stromal layer (Wilkerson *et al*., 1996). Broadly, eye is divided by collarette into two regions: the pupillary zone and the ciliary zone (Oyster, 1999). Furthermore, iris texture also reveals marked variations mainly due to unique set of surface features such as Fuchs' crypts, conjunctival melanosis, pigment spots, Wolfflin nodules and contraction furrows (Fig. 3). Fuchs' crypts are rhombus shaped cavities that vary in size and cover anterior layer of iris. They are common in ciliary region and mostly originate from collarette (Purtscher, 1965). Conjunctival melanosis is common in dark eyes with brown spotting on the sclera and pigmented ring around the iris (Damato and Coupland, 2008). Pigment spots are deeply pigmented discrete regions on the iris surface. They may deform the stromal layer (nevi) or may appear as freckles (Rennie, 2012). Wolfflin nodules are circular small whitish-orange collagen deposits distributed on the outer border of the iris (Williams, 1981). Lastly, contraction furrows result from expansion and contraction of pupil forming rings that cover the outer boundary of the iris (Eagle Jr, 1988) (Fig. 3).

Previously, considerable studies have been conducted to determine variations in eye and skin color in European, East Asian and South Asian populations (Edwards *et al*., 2016b; Liu *et al*., 2010; Walsh *et al*., 2017). However, there is need to have deep insight on variations in iris surface features and their relationship with eye and skin color in Pakistani population. These textural elements are of great interest in forensic, ophthalmologic and biomedical sciences. It is speculated that iris features influence overall perception of eye color and provide valuable information to predict individuals' health status (Horn *et al*., 1994; Mackey *et al*., 2011). Currently, iris patterns have been primarily characterized in European populations (Larsson *et al*., 2011; Sturm and Larsson, 2009). Only limited studies have concentrated on non-European ancestry and these studies have highlighted global differences in frequency of surface features (Qiu *et al*., 2006; Quillen *et al*., 2011). For example, when texture traits were analyzed in Brazil, Portugal and Cape Verdean populations, increasing European biogeographical ancestry was strongly correlated with contraction furrows, pigment spots, crypts and eye color (Quillen *et al*., 2011). Similarly, Malaysian population was examined for crypts and contraction furrows, both features were found to be related with iris thickness and angle closure (Sidhartha *et al*., 2014a, b). More recently, considerable variations in frequency were observed in South Asian, East Asian and European populations, depicting that all five surface features are highly population dependent (Edwards *et al*., 2016a).

In present study, two conspicuous traits i.e., eye and skin color along with iris patterns were investigated in Pakistani population. This manuscript has three major goals: (i) to look for phenotypic variations (based on eye and skin color) in Punjabi and KPK population; (ii) to evaluate frequency variations in iris patterns between two populations; (iii) to find out correlations between five iris textures and their associations with eye color, skin color, iris diameter, gender and age in each of the sample sets.

MATERIALS AND METHODS

Sample collection

After approval from Institutional Ethical Committee (letter no. D-1644-UZ), the study was conducted on 514 unrelated and healthy volunteers i.e., 334 males and 180 females from different regions of Punjab and Khyber Pakhtunkhwa (KPK) province of Pakistan. Among them 298 samples were from KPK and 216 from Punjabi population. All participants ranged in age between 10-85 years and they were asked to fill consent form and questionnaire detailing about gender, age, ethnic group and place of birth of ancestors.

Phenotyping

Digital captures of eyes and skin from the inner side of upper arm of each individual were recorded at a distance of 10 cm using 24.2-megapixel camera, Canon EOS 80D equipped with 18-135mm lens. All images were taken thricely at a shutter speed of 1/100, ISO 200, ensuring equal distance and constant light conditions.

Sample binning

Eye color was determined qualitatively according to Fitzpatrick Phototype Scale (Fig. 1) (Edwards *et al*., 2016a). For simplification purpose, images were grouped into three categories; 1: blue (equivalent to 1 and 2 in Fitzpatrick classification); 2: intermediate (equivalent to 3 in Fitzpatrick classification) where intermediate is combination of green, blue/green, brown/green pigments and 3: brown (equivalent to 4 and 5 in Fitzpatrick classification). Similarly, based on Fitzpatrick categorization system, three categories were used to classify skin color (Fig. 2); 1: white and beige skin color; 2: light brown to medium brown and 3: dark brown to black skin (Dario *et al*., 2015; Fitzpatrick, 1988; Gupta

and Sharma, 2019). Eye and skin digital images were independently inspected by two different investigators under uniform environmental conditions. In order to avoid discrepancies, a further detailed analysis of all photographs was performed until consensus assignment of phenotype took place.

Fig. 1. Eye color images collected from Pakistani population and characterized according to the Fitzpatrick Phototype Scale (A-E). This scale specifies five categories of eye color: A, blue; B, blue grey; C, green or light brown/green/hazel; D, light to dark brown and E, brownish black. For simplification, images were broadly assembled in three groups: 1, blue (equivalent to A and B in Fitzpatrick classification), 2, intermediate (equivalent to C in Fitzpatrick classification), 3, brown (equivalent to D and E in Fitzpatrick classification).

Fig. 2. Skin color images collected from Pakistani population (A-E) and characterized according to the Fitzpatrick Phototype Scale. This scale specifies six shades of skin color: A, very pale; B, pale; C, light brown; D, medium brown; E, dark brown; F, brownish black. Based on this scale, we used three categories to characterize skin color: 1, white and beige skin color (equivalent to A and B in Fitzpatrick classification), 2, light brown to medium brown (equivalent to C and D in Fitzpatrick classification), 3, dark brown to brownish black skin (equivalent to E and F in Fitzpatrick classification).

Characterization of iris patterns

Iris surface patterns were accurately characterized using a web-based application (http://iris.davidcha.ca/) designed by David Cha (Edwards *et al*., 2016a). Account was set up on request. Following instructions and guidelines of the program, right eye of each individual was analyzed for presence of Fuchs' crypts, pigment spots, melanosis, Wolfflin nodules and contraction furrows. After analysis of 514 irises, the program created an EXCEL spreadsheet providing complete information about the categories of all five surface features, the prevalence of these features in different quadrants and diameter of iris. Furthermore, accurate position and size of each of the pigment spots and crypts were also determined. Iris feature categorization system developed in a prior study was used to determine categories of iris textures in Pakistani population (Edwards *et al*., 2016a).

Statistical analysis

Statistical analysis was conducted through IBM STATISTICS SPSS v. 22.0. Gamma statistic was used to determine correlations among iris features, eye and skin color. Both *p*-value and G-value were reported for each of the correlation. Eye features were considered to be correlated with each other or with eye and skin color if $p < 0.05$. Moreover, ordinal regression was executed to explore associations between iris patterns and gender, eye color, skin color, iris diameter and age. Goodness of fit and proportional odds were also tested. Furthermore, chi-square test was carried out to highlight significant variations in iris features, eye color, skin color with respect to gender and age between provinces. Differences in iris diameter between two sample sets were evaluated with the help of one-way ANOVA. Before starting the statistical analysis, normality was checked by Q-Q plots.

RESULTS

A total of 514 samples (334 males and 180 females) were collected from Pakistani population. Overall, brown eyes were prevalent in both provinces but brown eyed individuals were more frequent in Punjab (47%) than KPK population (41%). In contrast, frequency of intermediate eye color was comparatively higher in KPK population (44%) than in Punjab (38%). However, blue eye color was reported with equal frequency in either population (15%). Females depicted brown eye color with higher frequency than males while intermediate and blue eyes were more common among males of both groups (Table I). Eye color phenotypes observed in Pakistani population are presented in Figure 1.

Individuals from Punjab portrayed full array of skin color phenotype. Light brown to medium brown skin color was most recurring (55%), followed by fair skin tone (33%) and dark brown color (12%). Contrarily, in KPK population lighter skin color (69%) prevailed than medium brown (31%) and none of the individuals were reported with dark brown skin color (0%) (Table I). In our study, number of females with fair skin color was much greater than males while medium brown to dark brown skin phenotype was persistent in males. Figure 2 displays different skin color variations perceived in Pakistani population.

Edward iris feature categorization software was used to analyze 298 KPK and 216 Punjab irises. Among these, 6 irises from KPK region were found to be significantly masked or blurry and were eliminated from the study. Two participants

Table I. Frequency distribution of eye and skin color in Punjab and KPK province of Pakistan.

Phenotype	Punjab						KPK					
	Males		Females		Overall		Males		Females		Overall	
	Count	Frequency	Count	Frequency	Count	Frequency/ (%)	Count	Frequency	Count	Frequency	Count	Frequency/ (%)
Eye color												
Brown	27	0.28	75	0.62	102	0.47 (47)	89	0.38	31	0.52	120	0.41 (41)
Intermediate	45	0.48	37	0.31	82	0.38 (38)	104	0.45	24	0.41	128	0.44 (44)
Blue	23	0.24	9	0.07	32	0.15 (15)	40	0.17	4	0.07	44	0.15 (15)
Total	95	1.00	121	1.00	216	1.00 (100)	233	1.00	59	1.00	292	1.00 (100)
Skin color												
White and beige	29	0.31	42	0.35	71	0.33 (33)	153	0.66	48	0.81	201	0.69 (69)
Light brown to medium brown	63	0.66	57	0.47	120	0.55 (55)	80	0.34	11	0.19	91	0.31 (31)
Dark Brown to black	3	0.03	22	0.18	25	0.12 (12)	0	0.00	0	0.00	0	0.00 (0)
Total	95	1.00	121	1.00	216	1.00 (100)	233	1.00	59	1.00	292	1.00 (100)

with ocular diseases were also not made part of study. Analysis was carried out on remaining 292 KPK and 216 Punjab healthy individuals. Iris diameter was found to be significantly different among two provinces (F= 8.989, p=0.003). Participants from KPK had comparatively smaller iris width mean=350.80 pixels than Punjabi population iris diameter mean= 363.97 pixels.

Extension of contraction furrows exhibited significant negative correlation with Fuchs' crypts grades in KPK (G= -0.167, p =0.031) and Punjab (G= -0.201, p= 0.035). Similarly, extension of contraction furrows revealed significant negative correlation with sclera pigmentation (G= -0.221, p= 0.030) and a significant positive association with number of pigment spots (G= 0.232, p= 0.027) in KPK province. Furthermore, a significant positive correlation was observed between grades of Fuchs' crypts and Wolfflin nodules in KPK (G= 0.400, p=0.002). Whereas, eye color was not found to be significantly related to any of the five surface features in both provinces. However, lighter skin color showed significantly negative correlation with sclera pigmentation in Punjab (G= -0.273, p =0.008).

Crypt grade was remarkably related with gender in KPK population (Nagelkerke R^2=0.015, p=0.040), particularly males had notable higher grades of crypts than females (male/female OR= 2.05). The other four eye patterns were not linked to gender in any population. Moreover, significant associations were depicted between iris diameter and sclera pigmentation (Nagelkerke R^2=0.033, p=0.024) in Punjab and KPK (Nagelkerke R^2=0.020, p=0.037). However, eye and skin color were not associated to iris diameter in either population. Although age was not significantly correlated with eye color and iris patterns but strong associations were reported between skin color and age in both groups (KPK: Nagelkerke R^2=0.041, p=0.001, Punjab: Nagelkerke R^2=0.036, p=0.009). Ageing had profound effect on skin pigmentation and texture (Maddodi *et al*., 2012; Ortonne, 1990; Yagi and Yonei, 2018).

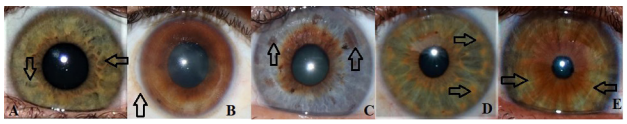

Fig. 3. Images of iris features found in Pakistani population (A-E). Arrows are directing towards the iris patterns in images. Fuchs 'crypts (A) are small or large diamond shaped cavities which appear in the anterior border layer of iris and arise during the constriction of pupillary membrane. Conjunctival melanosis (B) is brownish ring around the iris and/or isolated pigmented spots on sclera. Pigment spots (C) are distinct and well-defined brownish black regions of hyperpigmentation observed on iris surface. Nevi are pigmented spots that deform stromal layer while pigment dots that do not deform stromal layer are known as freckles. This feature is common in brown eyes. Wolfflin nodules (D) are small circular aggregates of collagen which are whitish orange in color and surround the outer boundary of iris. Contraction furrows (E) are discontinuous rings which look like wrinkles and formed during contraction and expansion of pupil. They expand along the outer border of iris.

Among five iris surface features, only pigment spots manifested significant frequency variations between two provinces (X^2 =7.983, p= 0.018). Individuals from KPK region had higher number of pigment spots (25.68%) than Punjab (15.28%). In contrast to eye color, skin color was found to be significantly different across two sample sets (X^2 =82.565, p= < 0.001) in which Punjabi population showed light brown to medium brown skin color while individuals of KPK region had comparably more fair skin tone.

Table II. Iris surface traits with their categories, count and percentage in Punjab and KPK province.

Category	Count n (%)		
	Punjab	KPK	Overall
Fuchs' crypts			
0	14(6.48)	39(13.36)	53(10.43)
1	93(43.05)	105(35.96)	198(38.98)
2	60(27.78)	75(25.68)	135(26.57)
3	49(22.69)	73(25.00)	122(24.02)
Total	216(100.00)	292(100.00)	508(100.00)
Contraction furrows			
0	83(38.42)	123(42.12)	206(40.55)
1	30(13.89)	40(13.70)	70(13.78)
2	103(47.69)	129(44.18)	232(45.67)
Total	216(100.00)	292(100.00)	508(100.00)
Pigment spots			
0	183(84.72)	217(74.32)	400(78.74)
1	20(9.26)	53(18.15)	73(14.37)
2	13(6.02)	22(7.53)	35(6.89)
Total	216(100.00)	292(100.00)	508(100.00)
Wolfflin nodules			
0	197(91.20)	253(86.65)	450(88.58)
1	11(5.1)	22(7.53)	33(6.50)
2	8(3.70)	17(5.82)	25(4.92)
Total	216(100.00)	292(100.00)	508(100.00)
Melanosis			
0	92(42.59)	114(39.04)	206(40.55)
1	124(57.41)	178(60.96)	302(59.45)
Total	216(100.00)	292(100.00)	508(100.00)

In both sample groups, highest percentage of pigment spots and nodules was observed in 0 category, crypts and melanosis fell in category 1 while furrows had category 2 (Table II). In both provinces iris patterns were notable in third quadrant except large crypts and nodules which were frequent in fourth quadrant of Punjabi irises and second quadrant of KPK irises respectively (Fig. 4).

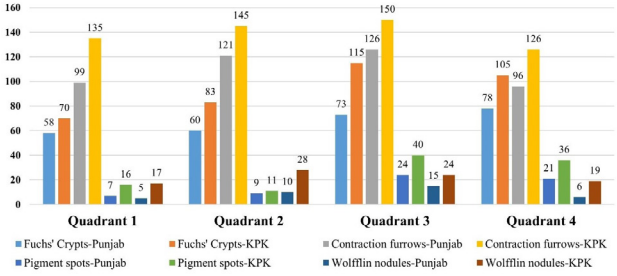

Fig. 4. Count of surface features in four quadrants of iris.

DISCUSSION

Previous studies mainly focused on variations in pigmented phenotypic traits in populations of European and non-European origin (Liu *et al*., 2010; Rawofi *et al*., 2017; Walsh *et al*., 2017). There are few studies which concentrated on associations of iris surface features with eye color in populations of European, South Asian and East Asian ancestry (Edwards *et al*., 2016a; Sidhartha *et al*., 2014a, b). However, it is the first study of this kind to address variations in iris features and their associations with phenotypic traits i.e., eye and skin color in Pakistan. The 508 out of 514 samples tested served as good representative of KPK and Punjabi population of Pakistan.

Eye and skin color phenotype was determined by visual qualitative methods reported by earlier studies (Dario *et al*., 2015; Edwards *et al*., 2016a). No doubt, quantitative methods used for classification of color provide more accurate data. However, this subjective manner of classification cannot be set aside as it is essence of eyewitness testimonies and routinely practiced in forensic investigations.

Brown eye color being ancestral phenotype prevailed in both regions followed by intermediate and blue color. A similar trend of eye color frequency was observed in a recent study conducted in the Swat district of Pakistan (Rahat *et al*., 2020). In contrast, fair skin tone was more apparent in KPK population than Punjabi population where light brown to medium brown skin color was prominent. It is observed that frequency of non-brown eyes and fair skin color increases from Southern Europe to Northern Europe while frequency of brown eyes and medium brown to dark brown sk4in color increases from Northern to Southern Europe (Walsh *et al*., 2017; 2012). Moreover, in both groups, frequency of males with non-brown eyes

and darker skin color was much greater than females while brown eye color and lighter skin was common in females than males. Our results show consistency with Spanish, Poland, Holland, Australia and France population data. (Branicki *et al*., 2009; Duffy *et al*., 2007; Katsara and Nothnagel, 2019; Martinez-Cadenas *et al*., 2013; Pośpiech *et al*., 2016; Rahat *et al*., 2020; Sulem *et al*., 2007).

In our study, we made use of Edward iris feature categorization system to have a deep insight on iris patterns. The use of software proved to be advantageous as compared to descriptive methods. It permits retrieval and storage of huge amount of data which includes iris features categories, iris diameter, precise position and size of crypts and pigment spots as well as distribution of patterns across different quadrants of eye.

In both sample sets, the substantial number of individuals had crypts in category 1. However, individuals of KPK had greater likelihood of having higher crypt grade than individuals of Punjab. In KPK, 25% of participants had crypts in category 3, compared with 22.69% of Punjabi population (Table II). There may be different justifications for this output. Participants from Punjab had comparatively greater frequency of dark brown eyes and larger iris width than KPK population. It is speculated that considerable quantity of melanin in dark eyes makes the iris less susceptible to expansion of crypts. However, it may be unlikely because development of crypts is not significantly related to iris width and eye color (Edwards *et al*., 2016a; Larsson and Pedersen, 2004). It is likely that several other developmental and genetic differences exist between populations. In contrast to Punjabi population, gender was notably associated with crypts in KPK population, specifically males had higher grades of crypts than females. This might be due to larger sample size and greater frequency of males in KPK population in comparison to Punjab. We were unable to reproduce any association of crypts with age and skin color (Larsson and Pedersen, 2004).

Pigmented spots on iris surface were significantly recurrent in KPK population than Punjab (Table II). Keeping in view that frequency of intermediate eye color was comparatively higher in KPK than in Punjab. About 25.68% individuals of KPK exhibited various degree of pigment spots compared with only 15.28% of Punjabi population (Table II). This result is not so astonishing. As pigment spots are distinct areas of black or brown color, we would anticipate fewer number of visible spots in dark-colored irises. Our results are inconsistent with European, East Asian and South Asian populations (Edwards *et al*., 2016a). In both populations, pigment spots were most probably located in third quadrant (lower temporal) of iris (Fig. 4). Various studies have highlighted the presence of pigment spots specially in lower section of iris (Lee *et al*., 2013). As this region of iris is merely covered by eyelids; maximum exposure from sun results in occurrence of hyper-pigmented regions (Edwards *et al*., 2016a). Gender, age, iris width and skin color were not found correlated to pigment spots.

It is postulated that sclera pigmentation appears to be more frequent in individuals with darker eyes (Edwards *et al*., 2016a). As brown eyes were prevalent in both provinces, we noticed conjunctival melanosis in more than half of KPK (61%) and Punjabi (57.4%) population (Table II). This outcome is obvious because primary factor responsible for expansion of melanosis on sclera is long-term exposure to intense UVRs. Our results are inconsistent to South Asian study (Edwards *et al*., 2016a). Iris width was found to be significantly associated with sclera pigmentation in both regions. These findings emphasize that pigmentation on sclera does not seem to be completely linked to iris width and eye color. In fact, global disbursement of this feature is highly complex and variable among different populations (Edwards *et al*., 2016a). Lastly, age or gender had no effect on sclera pigmentation. Instead, darker skin color was positively correlated with intensity of melanosis on sclera.

In both sample sets, very few irises had Wolfflin nodules that belonged to highest category (Table II). According to a previous study, Wolflinn nodules have a well-established relationship with iris color, being more widespread in light-colored eyes than in dark-colored eyes (Kim *et al*., 2002). As intermediate eyes were more common in KPK population than Punjab, about 5.8% individuals of KPK had nodules that stretched more than 180^{o} of iris compared to only 3.7% individuals of Punjab (Table II). Moreover, we could not find any signification relation of Wolffliin nodules with age, gender, skin color and iris width.

In our data, individuals of Punjab had comparatively higher furrow grade (48%) than KPK population (44%) (Table II). This is because contraction furrows had been found to be strongly linked to dark eyes and iris width in Singapore, Brazil and Portugal (Larsson and Pedersen, 2004; Quillen *et al*., 2011; Sidhartha *et al*., 2014a). Thus, it is unsurprisingly to notice higher contraction furrow grade in brown eyes of Punjabi individuals with greater iris width. Furthermore, no significant associations were revealed between contraction furrows and iris diameter, age, skin color and gender. These findings point out that there is need to explore other underlying factors accountable of frequency differences between populations.

Correlations were inspected between surface features

and eye color, skin color, age, iris width and gender in two groups. Within KPK population, a number of significant correlations were replicated which were pin pointed by earlier studies: (i) less extended furrows were correlated with highest crypts and sclera pigmentation category (Larsson and Pedersen, 2004); (ii) more extended furrows were correlated with greater number of pigment spots (Larsson and Pedersen, 2004); and (iii) higher grades of crypts and Wolfflin nodules were related to each other (Larsson and Pedersen, 2004; Sturm and Larsson, 2009). However, in Punjabi population, a similar correlation was observed between grades of crypts and extensions of contraction furrows as in KPK population. None of the remaining surface features were significantly correlated with each other. Important probable reasons for this trend are: (i) some of the associations might be masked by eye color; (ii) existence of potential dissimilarities between populations might be cause of non- significant associations; or (iii) our capacity to discover noteworthy associations in Punjab was reduced due to smaller data set.

Lastly, we also investigated consequences of ageing on eye and skin color. In our study, individuals did not report major changes in eye color during their lifetime (Wuerger, 2013). Instead, decreased vision, yellowing and thinning of oculus along with appearance of bluish hue around peripheral region were observed in people with advanced age (Salvi *et al*., 2006). Contrastingly, striking effects of age were visible on skin color of Pakistani population. It is presumed that growth and density of melanocytes in skin exposed to sunlight is two folds greater than non-exposed skin (Ortonne, 1990). Therefore, excessive exposure to harmful UVR causes the skin color to darken (Maddodi *et al*., 2012) as well as skin appears to be uneven, saggy, transparent and fragile with time (Yagi and Yonei, 2018).

CONCLUSIONS

Iris patterns have remarkable role in various fields related to health assessment and individual identification. In our study, we focused on phenotypic distribution of iris surface features with respect to eye and skin color in Punjab and KPK population. Only skin color and pigment spots showed significant frequency differences across two population groups. Moreover, majority of iris patterns correlations were replicated in KPK population than Punjab. In future, there is need to explore genetic associations of these traits in Pakistani population which would be helpful for forensic scientists and law enforcement agencies to resolve crime cases.

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Ethical statement

The study was conducted after approval from Institutional Ethical Committee (letter no. D-1644-UZ). Participants provided written informed consent.

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Data accessibility

The relevant data of manuscript will be accessible after publication for any interested researcher.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Bonaventure, J., Domingues, M.J., and Larue, L., 2013. Cellular and molecular mechanisms controlling the migration of melanocytes and melanoma cells. *Pigment Cell Melanoma Res*., **26**: 316-325. https://doi.org/10.1111/pcmr.12080

Branicki, W., Brudnik, U., and Wojas-Pelc, A., 2009. Interactions between HERC2, OCA2 and MC1R may influence human pigmentation phenotype. *Annls Hum. Genet*., **73**: 160-170. https://doi.org/10.1111/j.1469-1809.2009.00504.x

Cavalli-Sforza, L.L., Cavalli-Sforza, L., Menozzi, P., and Piazza, A., 1994. *The history and geography of human genes*. Princeton University Press.

Damato, B., and Coupland, S.E., 2008. Conjunctival melanoma and melanosis: A reappraisal of terminology, classification and staging. *Clin. Exp. Ophthalmol*., **36**: 786-795. https://doi.org/10.1111/j.1442-9071.2008.01888.x

Dario, P., Mourino, H., Oliveira, A.R., Lucas, I., Ribeiro, T., Porto, M.J., Costa Santos, J., Dias, D., and Corte Real, F., 2015. Assessment of IrisPlex-based multiplex for eye and skin color prediction with application to a Portuguese population. *Int. J. Legal Med*., **129**: 1191-1200. https://doi.org/10.1007/s00414-015-1248-5

Del Bino, S., Duval, C., and Bernerd, F., 2018. Clinical and biological characterization of skin pigmentation

diversity and its consequences on UV impact. *Int. J. mol. Sci*., **19**: 2668. https://doi.org/10.3390/ijms19092668

Duffy, D.L., Montgomery, G.W., Chen, W., Zhao, Z.Z., Le, L., James, M.R., Hayward, N.K., Martin, N.G., and Sturm, R.A., 2007. A three single-nucleotide polymorphism haplotype in intron 1 of OCA2 explains most human eye-color variation. *Am. J. Hum. Genet*., **80**: 241-252. https://doi.org/10.1086/510885

Eagle Jr, R., 1988. Iris pigmentation and pigmented lesions: An ultrastructural study. *Trans. Am. Ophthalmol. Soc*., **86**: 581.

Edwards, M., Cha, D., Krithika, S., Johnson, M., Parra, E.J., 2016. Analysis of iris surface features in populations of diverse ancestry. *R. Soc. Open Sci*., **3**: 150424. https://doi.org/10.1098/rsos.150424

Eiberg, H., Troelsen, J., Nielsen, M., Mikkelsen, A., Mengel-From, J., Kjaer, K.W., and Hansen, L., 2008. Blue eye color in humans may be caused by a perfectly associated founder mutation in a regulatory element located within the HERC2 gene inhibiting OCA2 expression. *Hum. Genet*., **123**: 177-187. https://doi.org/10.1007/s00439-007-0460-x

Fitzpatrick, T.B., 1988. The validity and practicality of sun-reactive skin types I through VI. *Arch. Dermatol*., **124**: 869-871. https://doi.org/10.1001/archderm.124.6.869

Gupta, V., and Sharma, V.K., 2019. Skin typing: Fitzpatrick grading and others. *Clin. Dermatol*., **37**: 430-436. https://doi.org/10.1016/j.clindermatol.2019.07.010

Horn, E.P., Hartge, P., Shields, J.A., and Tucker, M.A., 1994. Sunlight and risk of uveal melanoma. *J. Natl. Cancer Inst*., **86**: 1476-1478. https://doi.org/10.1093/jnci/86.19.1476

Jablonski, N.G., and Chaplin, G., 2010. Human skin pigmentation as an adaptation to UV radiation. *Proc. Natl. Acad. Sci*., **107**: 8962-8968. https://doi.org/10.1073/pnas.0914628107

Jablonski, N.G., and Chaplin, G., 2017. The colours of humanity: The evolution of pigmentation in the human lineage. *Philos. Trans. R. Soc. Lon. B Biol. Sci*., **372**: 20160349. https://doi.org/10.1098/rstb.2016.0349

Katsara, M.A., and Nothnagel, M., 2019. True colors: A literature review on the spatial distribution of eye and hair pigmentation. *Forensic Sci. Int. Genet*., **39**: 109-118. https://doi.org/10.1016/j.fsigen.2019.01.001

Kim, J.H., Hwang, J.M., Kim, H.J., and Yu, Y.S., 2002. Characteristic ocular findings in Asian children with Down syndrome. *Eye* (Lond), **16**: 710-714. https://doi.org/10.1038/sj.eye.6700208

Larsson, M., Duffy, D.L., Zhu, G., Liu, J.Z., Macgregor, S., McRae, A.F., Wright, M.J., Sturm, R.A., Mackey, D.A., and Montgomery, G.W., 2011. GWAS findings for human iris patterns: Associations with variants in genes that influence normal neuronal pattern development. *Am. J. Hum. Genet*., **89**: 334-343. https://doi.org/10.1016/j.ajhg.2011.07.011

Larsson, M., and Pedersen, N.L., 2004. Genetic correlations among texture characteristics in the human iris. *Mol. Vis*., **10**: 821-831.

Lee, R.Y., Huang, G., Porco, T.C., Chen, Y.-C., He, M., and Lin, S.C., 2013. Differences in iris thickness among African Americans, Caucasian Americans, Hispanic Americans, Chinese Americans, and Filipino-Americans. *J. Glaucoma*, **22**. https://doi.org/10.1097/IJG.0b013e318264ba68

Liu, F., Wollstein, A., Hysi, P.G., Ankra-Badu, G.A., Spector, T.D., Park, D., Zhu, G., Larsson, M., Duffy, D.L., Montgomery, G.W., Mackey, D.A., Walsh, S., Lao, O., Hofman, A., Rivadeneira, F., Vingerling, J.R., Uitterlinden, A.G., Martin, N.G., Hammond, C.J., and Kayser, M., 2010. Digital quantification of human eye color highlights genetic association of three new loci. *PLoS Genet*., **6**: e1000934. https://doi.org/10.1371/journal.pgen.1000934

Mackey, D.A., Wilkinson, C.H., Kearns, L.S., and Hewitt, A.W., 2011. Classification of iris colour: review and refinement of a classification schema. *Clin. exp. Ophthalmol*., **39**: 462-471. https://doi.org/10.1111/j.1442-9071.2010.02487.x

Maddodi, N., Jayanthy, A., and Setaluri, V., 2012. Shining light on skin pigmentation: the darker and the brighter side of effects of UV radiation. *Photochem. Photobiol*., **88**: 1075-1082. https://doi.org/10.1111/j.1751-1097.2012.01138.x

Maranduca, M.A., Branisteanu, D., Serban, D.N., Branisteanu, D.C., Stoleriu, G., Manolache, N., and Serban, I.L., 2019. Synthesis and physiological implications of melanic pigments. *Oncol. Lett*., **17**: 4183-4187. https://doi.org/10.3892/ol.2019.10071

Martinez-Cadenas, C., Peña-Chilet, M., Ibarrola-Villava, M., and Ribas, G., 2013. Gender is a major factor explaining discrepancies in eye colour prediction based on HERC2/OCA2 genotype and the IrisPlex model. *Forensic Sci. Int. Genet*., **7**: 453-460. https://doi.org/10.1016/j.fsigen.2013.03.007

Ortonne, J.P., 1990. Pigmentary changes of the

ageing skin. *Br. J. Dermatol*., **122** (**Suppl 35**): 21-28. https://doi.org/10.1111/j.1365-2133.1990.tb16121.x

Oyster, C.W., 1999. *The human eye*. Sinauer, Sunderland, MA

Peles, D.N., Hong, L., Hu, D.N., Ito, S., Nemanich, R.J., and Simon, J.D., 2009. Human iridal stroma melanosomes of varying pheomelanin contents possess a common eumelanic outer surface. *J. Phys. Chem. B*., **113**: 11346-11351. https://doi.org/10.1021/jp904138n

Pośpiech, E., Karłowska-Pik, J., Ziemkiewicz, B., Kukla, M., Skowron, M., Wojas-Pelc, A., and Branicki, W., 2016. Further evidence for population specific differences in the effect of DNA markers and gender on eye colour prediction in forensics. *Int. J. Legal med*., **130**: 923-934. https://doi.org/10.1007/s00414-016-1388-2

Purtscher, E., 1965. On the development and morphology of iris crypts. *Acta Ophthalmol*., **43**: 109-119. https://doi.org/10.1111/j.1755-3768.1965.tb06376.x

Qiu, X., Sun, Z., and Tan, T., 2006. *Global texture analysis of iris images for ethnic classification*. International Conference on Biometrics. Springer, pp. 411-418. https://doi.org/10.1007/11608288_55

Quillen, E.E., Guiltinan, J.S., Beleza, S., Rocha, J., Pereira, R.W., and Shriver, M.D., 2011. Iris texture traits show associations with iris color and genomic ancestry. *Am. J. Hum. Biol*., **23**: 567-569. https://doi.org/10.1002/ajhb.21183

Rahat, M.A., Khan, H., Hassan, I., Haris, M., and Israr, M., 2020. DNA-based eye color prediction of Pakhtun population living in District Swat KP Pakistan. Adv. Life Sci., **7**: 157-163. http://www.als-journal.com/739-20/

Rawofi, L., Edwards, M., Krithika, S., Le, P., Cha, D., Yang, Z., Ma, Y., Wang, J., Su, B., Jin, L., Norton, H.L., and Parra, E.J., 2017. Genome-wide association study of pigmentary traits (skin and iris color) in individuals of East Asian ancestry. *PeerJ*, **5**: e3951. https://doi.org/10.7717/peerj.3951

Rennie, I., 2012. Don't it make my blue eyes brown: heterochromia and other abnormalities of the iris. *Eye*, **26**: 29-50. https://doi.org/10.1038/eye.2011.228

Salvi, S.M., Akhtar, S., and Currie, Z., 2006. Ageing changes in the eye. *Postgrad. med. J*., **82**: 581-587. https://doi.org/10.1136/pgmj.2005.040857

Schadendorf, D., Fisher, D.E., Garbe, C., Gershenwald, J.E., Grob, J.J., Halpern, A., Herlyn, M., Marchetti, M.A., McArthur, G., and Ribas, A., 2015. Melanoma. *Nat. Rev. Dis. Primers*, **1**: 1-20. https://doi.org/10.1038/nrdp.2015.3

Sidhartha, E., Gupta, P., Liao, J., Tham, Y.-C., Cheung, C.Y., He, M., Wong, T.Y., Aung, T., and Cheng, C.-Y., 2014a. Assessment of iris surface features and their relationship with iris thickness in Asian eyes. *Ophthalmology*, **121**: 1007-1012. https://doi.org/10.1016/j.ophtha.2013.11.028

Sidhartha, E., Nongpiur, M.E., Cheung, C.Y., He, M., Wong, T.Y., Aung, T., and Cheng, C.Y., 2014b. Relationship between iris surface features and angle width in Asian eyes. *Invest. Ophthalmol. Vis. Sci*., **55**: 8144-8148. https://doi.org/10.1167/iovs.14-15402

Sturm, R.A., and Duffy, D.L., 2012. Human pigmentation genes under environmental selection. *Genome Biol*., **13**: 248. https://doi.org/10.1186/gb-2012-13-9-248

Sturm, R.A., Larsson, M., 2009. Genetics of human iris colour and patterns. *Pigment Cell Melanoma Res*., **22**: 544-562. https://doi.org/10.1111/j.1755-148X.2009.00606.x

Sulem, P., Gudbjartsson, D.F., Stacey, S.N., Helgason, A., Rafnar, T., Magnusson, K.P., Manolescu, A., Karason, A., Palsson, A., Thorleifsson, G., Jakobsdottir, M., Steinberg, S., Palsson, S., Jonasson, F., Sigurgeirsson, B., Thorisdottir, K., Ragnarsson, R., Benediktsdottir, K.R., Aben, K.K., Kiemeney, L.A., Olafsson, J.H., Gulcher, J., Kong, A., Thorsteinsdottir, U., and Stefansson, K., 2007. Genetic determinants of hair, eye and skin pigmentation in Europeans. *Nat. Genet*., **39**: 1443-1452. https://doi.org/10.1038/ng.2007.13

Walsh, S., Chaitanya, L., Breslin, K., Muralidharan, C., Bronikowska, A., Pospiech, E., Koller, J., Kovatsi, L., Wollstein, A., and Branicki, W., 2017. Global skin colour prediction from DNA. *Hum. Genet*., **136**: 847-863. https://doi.org/10.1007/s00439-017-1808-5

Walsh, S., Wollstein, A., Liu, F., Chakravarthy, U., Rahu, M., Seland, J.H., Soubrane, G., Tomazzoli, L., Topouzis, F., Vingerling, J.R., Vioque, J., Fletcher, A.E., Ballantyne, K.N., and Kayser, M., 2012. DNA-based eye colour prediction across Europe with the IrisPlex system. *Forensic Sci. Int. Genet*., **6**: 330-340. https://doi.org/10.1016/j.fsigen.2011.07.009

Wilkerson, C.L., Syed, N.A., Fisher, M.R., Robinson, N.L., Wallow, I.H., and Albert, D.M., 1996. Melanocytes and iris color. Light microscopic

findings. *Arch. Ophthalmol*., **114**: 437-442. https://doi.org/10.1001/archopht.1996.01100130433014

Williams, R.B., 1981. Brushfield spots and Wolfflin nodules in the iris: An appraisal in handicapped children. *Dev. Med. Child Neurol*., **23**: 646-650. https://doi.org/10.1111/j.1469-8749.1981.tb02047.x

Wuerger, S., 2013. Colour constancy across the life span: Evidence for compensatory mechanisms. *PLoS One*, **8**: e63921. https://doi.org/10.1371/journal.pone.0063921

Yagi, M., and Yonei, Y., 2018. Glycative stress and anti-aging: 7. Glycative stress and skin aging. *Glycative Stress Res*., **5**: 50-54.