Pakistan J. Zool., pp 1-10, 2021. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20210325000338

Polymorphism in *MCM6*-Gene Associated with Lactose Non-Persistence in Pakistani Patients

Maria Qibtia[1], Sehrish Faryal[1], Muhammad Wasim[1], Farzana Chowdhary[1], Muhammad Tayyab[1], Ahmed Mansouri[2], Zeeshan Ahmed[2], Muhammad Hamid[3] and Ali Raza Awan[1,*]

[1]*Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, 54000 Lahore, Pakistan*

[2]*Max-Planck Institute for Biophysical Chemistry Molecular Cell Differentiation Group, Am Fassberg 11, 37077 Göttingen, Germany*

[3]*University of Ulm, Energiewandlung und-speicherung, Albert-Einstein-Allee 47, 89081 Ulm, Germany*

ABSTRACT

Lactose persistence (LP) is a genetically determined phenotypic trait, generally related to pastoralism and milk consumption. However, Lactose non-persistence (LNP) results from the progressive decline of Lactase-phlorizin hydrolase (LPH) activity in enterocytes. The Single Nucleotide Polymorphism (SNPs) associated with occurrence of LP/LNP are different for European, Asian, African-American, Northern African and Arab populations. In this study, we studied, the association of these SNPs with LNP in Pakistani patients. Our analysis has evaluated the association between the development of LNP trait and two common variants in intron 13 and 9 (13910-C/T and 22018-G/A) along with the P-value significance level. Therefore in this study, we aimed to access genetic predisposition and clinical manifestations of LNP and LP in 80 subjects (30 subjects with LP and 50 LNP). The presence of T-13910, C-13913, G-13915, G-13907, C-3712, C-13779, A-13937, G-14009, C-14010, T-14011, T-14044, T-14091, A-14107, C-14176, A-14156 and A-22018 polymorphic variants in *MCM6*-gene were also accessed as reported in different populations. Genomic DNA was extracted from peripheral blood. Consequently, SNPs were analyzed with the PCR-sequencing method. The statistical analysis was performed in SPSS using the method of chi-square test. All the SNPs were evaluated for the association of polymorphism. In the current study 20 out of 30 LP subjects were presented with the following SNPs: C/T-13910 (intron 13) and G/A-22018 (intron 9), representing a frequency level of 0.67 for association. Similar to this frequency of 0.22 and 0.44 was also found in LNP patients. All the SNPs were heterozygous in LP subjects, and for the particular SNP: G/A-22018 (intron 9) 22% heterozygosity was also shown in LNP subjects. This is the first report of SNP in *MCM*-6 gene studied in Pakistani patients. It may help in better understanding of different diseases having underlying cause related to lactose intolerance.

Article Information
Received 25 March 2021
Revised 29 Apil 2021
Accepted 05 May 2021
Available online 13 December 2021 (early access)

Authors' Contribution
MQ conducted the research as part of her PhD work. ARA and ZA supervised the research. MW and FC served as member of supervisory committee. MT helped in checking plagiarism of the manuscript. SF helped in lab research. MA supervised the research at Max-planck Institute for Bio-physical chemistry. MH helped in every aspect of the research and manuscript-writing.

Key words
Lactose persistence (LP), Lactose non-persistence (LNP), Lactase-phlorizin hydrolase (LPH/LCT), Transcription Factor (TF), *MCM-6* gene, Screening, Sequencing.

INTRODUCTION

Most mammals including 65% of humans adult cannot digest lactose after weaning. During childhood, the production of lactase enzyme reduces to about 10-15% of its original level at birth. It is genetically programmed, due to which irreversible reduction of lactase enzyme occurs in about 75% of the world's population (Alizadeh and Sadr-Nabavi, 2012; White *et al*., 2012-2020a). Lactose persistence (LP) opposite of lactose non-persistence (LNP) is considered to be associated with polymorphism, due to cis-acting factors found in *MCM*-6 gene. LP is frequently variable among different human populations, across continents. It is most prevalent in Northwestern Europe, with the highest frequency in Scandinavian countries, and it shows a decline in frequency in Southern Europe (Swallow *et al*., 2003; White *et al*., 2012-2020; Bodlaj *et al*., 2006; Jarvela, 2005). Recent findings have shown, LNP is considered as a wild type pathway, in which after weaning, production of lactase is significantly reduced. In LP the C to T variant at –13910 and G to A variant at–22018, shows greater transcription factor (Oct-1) binding than other variants. LNP occurs when the presence of undigested lactose in the colonic lumen causes gastrointestinal symptoms such as abdominal pain, bloating, flatulence and diarrhea (Alizadeh and Sadr-Nabavi, 2012). There are a lot of abdominal diseases which could easily be confused with LNP. They include celiac disease, enteritis, Crohn's disease, bacterial/parasitic infections, and Bowl's syndrome that can temporarily or permanently cause loss of lactase production (Raz *et al*., 2013).

Initial studies involving molecular analysis of cis-

* Corresponding author: arawan77@uvas.edu.pk
0030-9923/2021/0001-0001 $ 9.00/0

acting factors identified two LP/LNP associated SNPs : 13910-C/T and 22018-G/A upstream of the *LCT*-gene located within intron 13 and 9 of *MCM*-6 gene (Enattah *et al*., 2002; Jarvela *et al*., 2003). Different SNPs in the binding site of lactase transcription factors are considered to be associated with the continuation of lactase production into adulthood (Ingram *et al*., 2007; Jones *et al*., 2013; Leseva *et al*., 2018; Lewinsky *et al*., 2005; Troelsen *et al*., 2005; White *et al*., 2012-2020b). The significant association of these two variants: 13910-C/T and 22018-G/A with lactase activity in the intestinal wall has facilitated large-scale population studies on the development of different diseases (Alizadeh and Sadr-Nabavi, 2012). However, the necessary information regarding the polymorphisms within Pakistani patients is lacking. Our research outlays the cis-acting polymorphisms through DNA sequencing in *MCM*-6 gene of Pakistani subjects.

Table I.- Socio-demographic data of subjects enrolled in this study.

Socio-demographic parameters	LP (%) n =30	LNP (%) n=50	N (%) n=80
Gender			
Male	14(46.67)	25(50)	39(48.75)
Female	16(53.33)	25(50)	41(51.25)
Age (years)			
<5	0(0)	6(12)	6(7.5)
5-14	0(0)	6(12)	6(7.5)
15-24	4(13.33)	2(4)	6(7.5)
25-34	14(46.667)	18(36)	32(43.75)
35-44	4(13.33)	11(22)	15(18.75)
45-54	2(6.667)	3(6)	5(6.25)
55-64	6(20)	4(8)	10(12.5)
Ethnic group			
Urdu speaking	5(16.66)	36(72)	41(51.25)
Punjabi speaking	18(60)	7(14)	25(31.25)
Kashmiri speaking	6(20)	6(12)	12(15)
Pashtun speaking	1(3.33)	0	1(1.25)
Turk speaking	0	1(2)	1(1.25)

MATERIALS AND METHODS

Study participants

This study enrolled 80 unrelated individuals; 30 with LP and 50 with Lactose non-persistence. The LP group included asymptomatic individuals, apparently healthy, recruited after careful investigation through filled out Performa. Diagnosis of LNP was performed through intolerance symptoms; blotting, diarrhea, constipation, weight loss after drinking milk 1 cup to 1 glass and symptoms must appear within half to 2 h to be considered as a case of LNP. It must be studied after the age of weaning till 65 years. Exclusion criteria in both groups were presence of any other illness that is celiac, crohn's disease, ulcer, allergies, temperature, blood in stool and pus in stool. This study was approved by Institutional Review Board (IRB) at the Institute of Biochemistry and Biotechnology (IBBT), the University of Veterinary and Animal Sciences (UVAS) in Lahore, Pakistan with registration No. DAS / 3067 (dated 11.12.2012).

To verify the reliability of our analysis in *MCM6*-gene (NG_008958.1), a written informed consent was taken from the LP and LNP subjects, explicitly belonging to Pakistan. All individuals answered a standard questionnaire to obtain information related with their clinical sign and symptom, family history, gender, age, area, cast / ethnic group, alcohol consumption, smoking habit. Individual with secondary LNP due to some other diseases were excluded from the analysis. Table I depict the data in relation to age, gender and ethnic belonging.

Genetic analysis

Genomic DNA was extracted from peripheral blood sample using GeneJET kit (thermo scientific: GeneJET whole blood genomic DNA purification mini kit catalogue No. #K0781, #K0782). Sixteen polymorphic sites were included in the study: T-13910, C-13913, G-13915, G-13907, C-3712, C-13779, A-13937, G-14009, C-14010, T-14011, T-14044, T-14091, A-14107, C-14176, A-14156 and A-22018. Nine primers were used as shown in Table II, a similar approach to this one was adopted by Ranciaro *et al*. (2014), which amplified and performed melting analysis at annealing temperature of 61.5°C. This PCR approach is implemented for the exon spliced intronic region (Ranciaro *et al*., 2014). PCR products showing a clear band of required lengths were purified in three necessary steps through GENECLEAN™ Spin Kit. The PCR products were eluted with 15µl elution buffer and diluted 1:10 with water. Purified PCR products were sequenced with both forward and reverse primers using an automated ABI PRISM 3100 Genetic Analyzer (SeqLab, Germany). Nucleotide sequence of all of the amplicons were used for multiple sequence alignments, which were performed with ClustalW freeware (http://www.ebi.ac.uk/Tools/clustalw2), and results were analyzed by using ApE, software. Association between the LP trait and variants found in intron 13 and 9 (C/T-13910 and G/A-22018) were evaluated using statistical methods.

Table II.- Primers used for *MCM-6* gene amplification self-designed through ApE software.

No		Sequence (5'-3')		
		Forward	Reverse	
21	I9.1-M6	ACCAGTGGTAAAGCGTCCAG	AACAGCAAACACACGTGCTC	618
22	I9.2M6	TGCATTGAGCCAAGATTGTG	TAGCCAGGTGTGGTGGTGTG	633
23	I9.3M6	TCCCTGTGGTAGCAGACTTTG	TCCCGCACGTCCATCTTATC	593
24	I13.1M6	ATCTCCGCCAGAGAGATGG	GCTTTGGTTGAAGCGAAGAT	616
25	I13.2M6	GTTCTTTGAGCCCTGCATTC	AGGTTCGGGGGTACACATGC	695
26	I13.3M6	AGATACCCTGGGACAAGGTC	TCATAGATGTTTTCAATTCTTCAAGT	694
27	I13.4M6	GGATCTCCTTTTGGACTTTCC	TTCAACAAGAAACACTGAAAAACA	716
28	I13.5M6	GTGAGCCATGTGCTTTCTCC	GCACGGTGGCTCATGTCTAT	644
29	I13.6M6	TCTTCTTTCTCAGCCTCCTG	TGGACCTAAACCAATAATGATGAA	613

Statistical analysis

The two polymorphic sites tested were in partial association with LP trait. We used Statistical Package for the Social Sciences (SPSS v20.0) for all analyses. Data were evaluated with chi-square test as required. To evaluate the associations between polymorphisms and LI, the genetic models were tested for bloating, abdominal pain, diarrhea, constipation and weight loss. A p-value of less than 0.05 was considered statistically significant (Table III). The age gender and ethnic group was also studied for considering association in both LI and LP individuals shown in Table I.

RESULTS

This study encompasses all socio-demographic parameters, such as gender, age and ethnicity of the subjects as presented in Table I. The presence of symptoms that define the LNP phenotype was accessed and compared between the LP and the LNP. Overall, the most prevalent symptoms observed are: abdominal pain (100%), bloating (100%) and diarrhea (100%). On the other hand, constipation (16.66%), and weight loss (3.33%) were found non-significant as presented in Table III.

The SNPs investigated in this study were T-13910, C-13913, G-13915, G-13907, C-3712, C-13779, A-13937, G-14009, C-14010, T-14011, T-14044, T-14091, A-14107, C-14176, A-14156 and A-22018 as reported in earlier studies (Ingram *et al*., 2007; Jones *et al*., 2013; Leseva *et al*., 2018; Breton *et al*., 2014; Enattah *et al*., 2008; Friedrich *et al*., 2012; Macholdt *et al*., 2014; Tishkoff *et al*., 2007). During this research, an encouraging correlation of Pakistani individuals was found between two SNPs: C/T-13910 and G/A-22018. These two variants have been reported in European populations (Enattah *et al*., 2002; Raz *et al*., 2013; Ranciaro *et al*., 2014; Bulhoes *et al*., 2007; Campbell *et al*., 2005; Enattah *et al*., 2007; Ponte *et al*., 2016; Tomar, 2014), and similar to study reported in Northern India as being descendants of Aryans, near Punjab (Pigott *et al*., 1977; Babu *et al*., 2010; Tomar, 2014). For the rest of SNPs analyzed regarding variant role of polymorphism it was not found (Fig. 1) (McIntosh and Scheinfeldt, 2012).

Table III.- Distribution of clinical symptoms among the LP and LI groups in Punjabi population.

Symptoms	LP (%) n_{total}=30	LNP (%) n_{total}=50	p-value
Bloating			
No	0(0)	35(70)	<0.001
Yes	30(100)	15(30)	
Total	30(100)	50(100)	
Abdominal pain			
No	0(0)	30(60)	<0.001
Yes	30(100)	20(40)	
Total	30(100)	50(100)	
Diarrhea			
No	0(0)	40(80)	<0.001
Yes	30(100)	10(20)	
Total	30(100)	50(100)	
Constipation			
No	25(83.3)	47(94)	0.124
Yes	5(16.66)	3(6)	
Total	30(100)	50(100)	
Weight loss			
No	29(96.66)	45(90)	0.273
Yes	1(3.33)	5(10)	
Total	30(100)	50(100)	

Results of analysis of sequencing of *MCM6*-gene in Pakistani subjects revealed encouraging pattern of polymorphic association. At nucleotide position -13910, in intron 13 frequency association of 1.5 was found in LP individuals. Twenty out of thirty (66.6%) LP individuals showed heterozygosity, and rest of 33.33% present were homozygous. On the other hand, in LNP 100%

homozygosity was found with a frequency association of 0.22. These results confirmed that homozygosity at this particular nucleotide position is indicative of LNP in Pakistanis (Lewinsky *et al*., 2005; Babu *et al*., 2010; Burger *et al*., 2007; Krawczyk *et al*., 2008; Lehtimaki *et al*., 2006; Lukito *et al*., 2015; Madry *et al*., 2010; Malmstrom *et al*., 2010; Miquel *et al*., 2011; Szilagyi *et al*., 2010; Tarabra *et al*., 2010) (Figs. 1, 2; Table IV). At nucleotide position -22018 in intron 9 again frequency of 1.5 was found in LP individuals. However, the level of heterozygosity was same as for the variant -13910. On the other hand in LNP subjects the same varaint was also present at a frequency of 0.44 level, as given in Table IV and Figure 3. Both varaiants C/T-13910 and G/A-22018 were presented at the same frequency levels shows 1.5 and same levels of heterozygosity in LP subjects. Only variant -22018 was also presented with 22 % heterozygosity in LNP subjects.

The current study shows the significance of genetic testing for LP/LNP diagnosis with the help of cis-acting factors: C/T-13910 and G/A-22018 present in *MCM6*-Gene. They are considered to be encouragingly associated with LNP phenotype. Such findings are important and suggestive for composing a future diagnostic test for LNP, as already shown in other populations (Swallow, 2003; Enattah *et al*., 2002; Jarvela *et al*., 2003; Raz *et al*., 2013; Breton *et al*., 2014; Campbell *et al*., 2005; Enattah *et al*., 2007, 2008; Friedrich *et al*., 2012; Macholdt *et al*., 2014; Ponte *et al*., 2016; Tishkoff *et al*., 2007; Tomar *et al*., 2014; Babu *et al*., 2010; Burger *et al*., 2007; Krawczyk *et al*., 2008; Miquel *et al*., 2011; Tarabra *et al*., 2010; Malmstrom *et al*., 2010; Johnson *et al*., 1993; Marton *et al*., 2012; Pohl *et al*., 2010; Rasinpera *et al*., 2004; Savaiano *et al*., 2013).

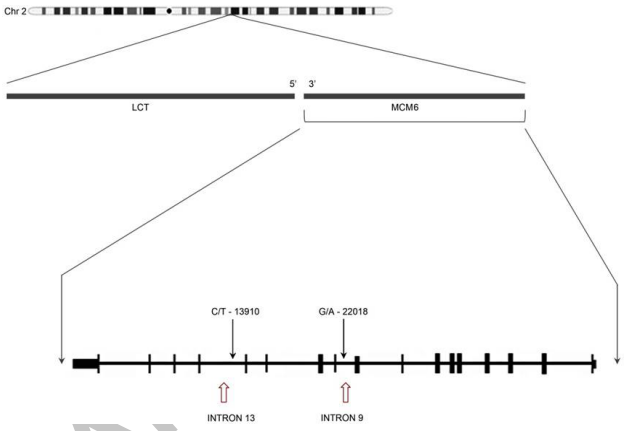

Fig. 1. Genetic variations in *MCM6*-gene. The chromosome 2 with LCT and *MCM6* gene (McIntosh and Scheinfeldt, 2012) is shown with two specific SNPs at intronic positions 13 and 9.

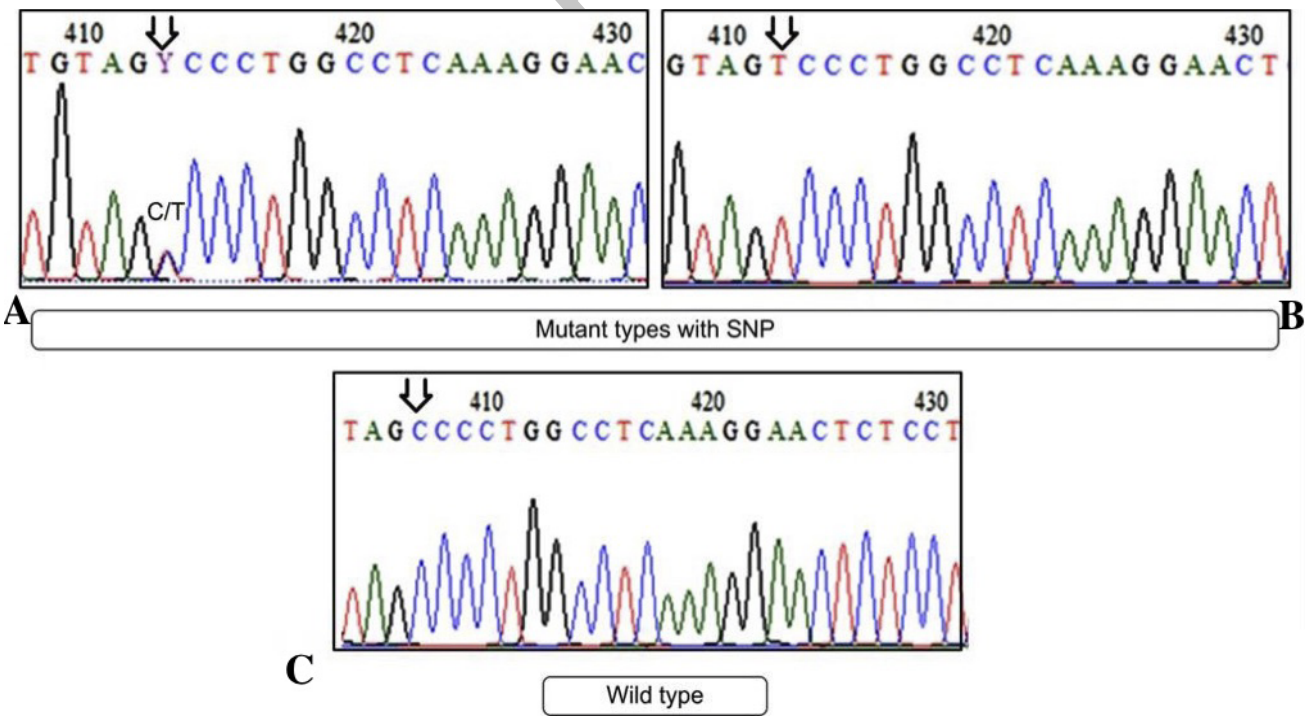

Fig. 2. Chromatograms of SNP at INTRON 13 in the *MCM6*-gene. In mutant types A shows the partial heterozygosity with both alleles C/T. In mutant type B shows allele T in place of allele C. In wild type the actual gene with C allele is shown.

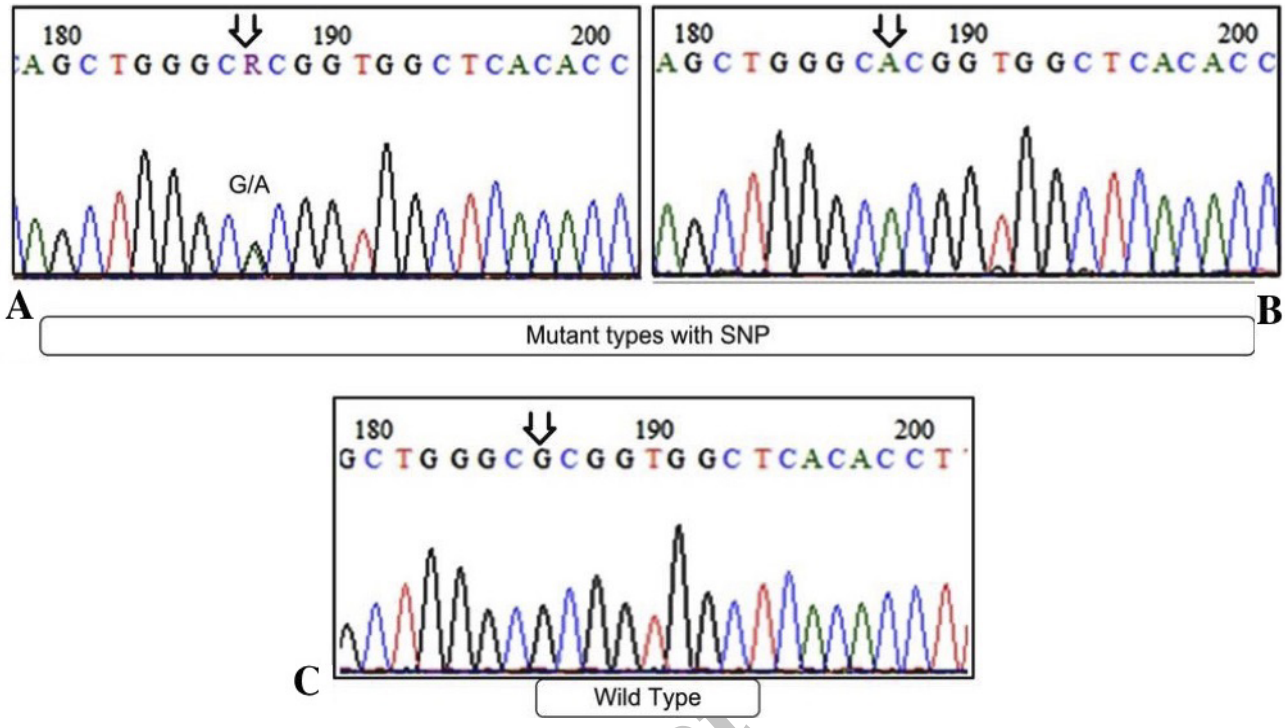

Fig. 3. Chromatograms of SNP at INTRON 9 in theMCM6-Gene. In mutant type A shows the partial heterozygosity with both alleles G/A is shown. In mutant type B allele A in place of allele G is shown. C, in wild type the actual gene with G allele is shown.

Table IV.- SNPs in *MCM6*-Gene associated with lactose persistence/ lactose non-persistence.

SNP	SNP position	Variant	Exonic / Intronic / Promoter	Frequency-LI/LNP	% in LI	% in LP	P-value
I	c.(1362+117)G>A g.-22018G/A	G>A	Intron 9	0.44/1.5	22	66(100% Heterozygosity)	<0.001
II	c.(1917+326)C>T g.-13910C/T	C>T	Intron 13	0.22/1.5	44(50% Heterozygosity)	66(100% Heterozygosity)	<0.001

DISCUSSION

LP is a common phenotype in humans. It varies widely depending of ethnicity, as humans do not typically consume milk in adulthood (Raz *et al*., 2013; Vesa *et al*., 2000). Thus the production of lactase is unnecessary or wasteful at a cellular level. Age-dependent lactase production of this sort occurs at the transcription level (Swallow *et al*., 2003; White *et al*., 2012-2020b). The regulation of lactase synthesis over developmental time is the factor that separates LP from LNP individuals. The latest research relating the development of LP in humans does not focus on mutation in the lactase *LCT*-gene; previous studies have focused on enhancers within the introns of *MCM-6* gene (Enattah *et al*., 2002, 2007; Jarvela *et al*., 2003; Raz *et al*., 2013; Lewinsky *et al*., 2005; Troelsen, 2005; Bulhoes *et al*., 2007; Campbell *et al*., 2005; Ponte *et al*., 2016; Tomar *et al*., 2014; Babu *et al*., 2010; Burger *et al*., 2007; Krawczyk *et al*., 2008; Lehtimaki *et al*., 2006; Lukito *et al*., 2015; Madry *et al*., 2010; Malmstrom *et al*., 2010; Miquel *et al*., 2011; Szilagyi *et al*., 2010; Tarabra *et al*., 2010). Different SNPs in the binding site of lactase transcription factors (TF) are associated with the continuation of lactase production up to adulthood (EFSA, 2010), after binding with enhancers. These enhancer sites are far away from the actual gene, but due to DNA loop formation, they produce their effect (Troelsen *et al*., 2005).

Possible theories that are considered for the development of LP : 1) selective pressure, which was proposed to be linked in the Middle-East during the

Neolithic era (Burger *et al*., 2007; Malmstrom *et al*., 2010). 2) Bio-cultural co-evolution; observed in ancient human population, the remains of juvenile cattle found in the Middle East around 10,500 years ago matched the one found in Greece and Balkans and it was confirmed that LP was spread across the Europe 5000-7000 years ago (White *et al*., 2012-2020a; Lewinsky *et al*., 2005; Sahi, 2001). 3). Convergent evolution was proposed for the development of LP in different populations by different variants. Among these, the most studied and reported variants are T-13910, A-22810 in Europe and G-13915, G-13907 in Africa (Kenya and Sudan) (White *et al*., 2012-2020a). These variants are capable of enhancing differential transcriptional activation of the lactase promoter for LP phenotype in humans (Ingram *et al*., 2007; Jones *et al*., 2013; Leseva *et al*., 2018; Lewinsky *et al*., 2005; Troelsen *et al*., 2005). Such correlation was evident in various studies conducted in Finns, Brazilian, Indians, Italians, Germans, and many other populations (Raz *et al*., 2013). The selective pressure and convergent theories are considered to be overlapping. Different studies are presented in different regions in lieu of the same. In Americans, C>T 13910 SNP was studied, and its frequency was correlated with less consumption of milk due to the cost, preferences and social habits (Miquel *et al*., 2011). In North Americans, this position was also studied, and it showed that Hispanics having European background exhibited a higher frequency of LP as compared to the Americans (Miquel *et al*., 2011). In Polanders, a study was reported in which 7.7 % of subjects confirmed LNP incidence due to genetic predisposition (Campbell *et al*., 2005). Israeli population presented a high correlation of both SNPs: C/T-13910 and G/A-22018, among jews of different ethnicity and also C/T-13915 in African and Arab Bedouins with LNP (Raz *et al*., 2013). In Germans, a complete association of CC-13910 with positive Breath Hydrogen Test (BHT +ve) was presented (Ponte *et al*., 2016; Krawczyk *et al*., 2008). Among Indians, a strong correlation of BHT and C/T-13910 polymorphism was found. The LNP was reported in 66.2% of population in North India, and 88.2% of population in Southern India, based on Gastro Intestinal symptoms with more or less same finding reported in two other studies (Tomar, 2014; Babu *et al*., 2010; Asmawi *et al*., 2006). All these studies lead to Aryan ancestry and pastoralism (Pigott *et al*., 1977). Among Pakistanis, Enattah (2007) studied the variation C/T-13910 in twelve ethnic groups and found an increased prevalence of LP in Balaei, Sindhi, Brahui and Pathans. Punjabis specifically were not considered in that group study (Enattah *et al*., 2007). Baseer and Rab (1976) studied Pakistani patients w/o involving genetic predisposition and reported high intestinal Lactase activity i.e. LP. Ahmad and Abbass (1983) reported 55% LP in Pakistanis. Afghani and Irani population showed a relatively low incidence of LP (Tomar *et al*., 2014; Enattah, 2007; Ahmad and Abhas, 1983; Baseer and Rab, 1975; Rahimi *et al*., 1976). In this study molecular analysis (C/T-13910 and G/A-22018) for the development of LNP/LP in association of cis-acting factors in Pakistani subjects of Punjab is carried out for the first time. These variants in intronic regions of MCM-6 Gene provided more reliable results for genetic screening of LNP/LP individuals. Colonic bacteria are also suggested to have an impact on LP-trait in Somalian population where people are consuming milk in adulthood and LNP is not developed. However, they possess a different colonic bacteria population that helps in the metabolism of lactose (Ingram *et al*., 2007; Szilagyi *et al*., 2010; White *et al*., 2012-2020a). In the current study, none of the LNP subjects was presented with heterozygosity: C/T at position -13910. Only 11 were found with T/T homozygosity at the same position. On the other hand, 20 out of 30 LP subjects confirmed heterozygosity presenting 66%, including ten subjects who showed symptoms without heterozygosity 44%. The same correlation was found for position -22018 of LP subjects. On the contrary, 11 LNP subjects were found with SNP G/A-22018 and 11 with A/A homozygosity at same position. The results are more inclined towards the role of heterozygosity in lactase production. As per our study, C/T-13910 and G/A-22018 SNPs are in strong correlation with the development of LP (Table IV). We conclude that the post-weaning decline could be either due to the insufficient binding of nuclear factors to the C-13910 variant or increased binding of repressors to the LPH-promoter. On the other hand T-13910 acts as enhancer as by ensuring accessibility of transcription factor (TF) (Ingram *et al*., 2007; Jones *et al*., 2013; Leseva *et al*., 2018; Lewinsky *et al*., 2005; Troelsen *et al*., 2005). The development of LP due to SNPs cis-acting elements, during adulthood, is considered as a "Mutant-type Pathway". LNP on the other hand, is considered as "Wild type Pathway" (White *et al*., 2012-2020b).

Different SNPs are reported in different studies in different parts of the world including: T-13910, C-13913, G-13915, G-13907, C-3712, C-13779, A-13937, G-14009, C-14010, T-14011, T-14044, T-14091, A-14107, C-14176, A-14156 and A-22018 in *MCM 6* gene (Enattah *et al*., 2002, 2007, 2008; Jarvela *et al*., 2003; Raz *et al*., 2013; Ingram *et al*., 2007; Jones *et al*., 2013; Lewinsky *et al*., 2005; Troelsen *et al*., 2005; Breton *et al*., 2014; Ranciaro *et al*., 2014; Tishkoff *et al*., 2007; Bulhoes *et al*., 2007; Campbell *et al*., 2005; Ponte *et al*., 2016; Tomar, 2014; Babu *et al*., 2010; Burger *et al*., 2007; Krawczyk *et al*., 2008; Lehtimaki *et al*., 2006; Lukito *et al*., 2015; Madry *et al*., 2010; Malmstrom *et al*., 2010; Miquel *et al*., 2011; Szilagyi *et al*., 2010; Tarabra *et al*., 2010). Our findings

suggest that two SNPs: C/T-13910 and G/A-22018 are encouragingly associated with transcriptional regulation of LPH-Gene (Tishkoff *et al*., 2007). -14010*C allele reported in Tanzanian, Kenya, and Southern African Khoisan population with a strong signal of positive selection was not found in the current study,and same found for LP allele -13907*G, -3712*C, -13913*C, -13915*G,-13779*C, -13937*A, G-14009*G,-14011*T, -14044*T, 14091*T, -14107*A, 14176*C and 14156*A which were found at low and varying frequencies in Africans (Macholdt *et al*., 2014).

CONCLUSION

The integration of these genome-wide studies with microbiome, anthropological, archaeological, and paleobiological data will also help to elucidate the history of pastoralism within Pakistan. Also, it will help in studying genetic and non-genetic factors contributing to the phenotypic variance of LNP in human populations. These findings can help us suggest the prognosis of complicated diseases by simple measures: of foods consumption of fermented and mature dairy products, use of Probiotics in order to assist digestion of lactose (Campbell *et al*., 2005; Savaiano *et al*., 2013; Tomar *et al*., 2014; Usai-Satta *et al*., 2012). This is the first report of genetic polymorphism in *MCM6*-Gene Intronic region in Pakistani LNP patients. The association of two significant SNPs has been found at encouraging levels in Pakistani patients, in contrast to near by other countries. These cis-acting factors of MCM-6 gene must be validated at larger scale for future reference.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the members of the Max-Planck Institute for Biophysical Chemistry, Gottingen, Germany for help in managing the sequencing PCR-results. The authors also thank Mr. Muhammad Bilal for assistance in compiling the results for MCM6 Gene polymorphic association through STAT. The research leading to these results received funding from HEC under Grant Agreement No. (a) 1-8/HEC/HRD/2015/5148 PIN. IRSIP 30 BMS 24, and (b) 17-5-6/HEC-Ind-Sch/2010.

Ethics approval

All parts of the research have been approved by the ethical committee and comply with the ethical standards.

Statement of conflict of interest

The authors have declared no conflict of interests.

REFERENCES

Ahmad, M. and Abbas, H., 1983. Persistence of high intestinal lactase activity in Pakistan. *Hum. Genet*., **64**: 277-278. https://doi.org/10.1007/BF00279410

Alizadeh, M. and Sadr-Nabavi, A., 2012. Evaluation of a genetic test for diagnose of primary hypolactasia in Northeast of Iran (Khorasan). *Iran. J. Basic med. Sci*., **15**: 1127-1130.

Asmawi, M.Z., Seppo, L., Vapaatalo, H. and Korpela, R., 2006. Hypolactasia and lactose intolerance among three ethnic groups in Malaysia. *Indian J. med. Res*., **124**: 697-704.

Babu, J., Kumar, S., Babu, P., Parasad, J.H. and Ghoshal, U.C., 2010. Frequency of lactose malabsorption among healthy southern and northern Indian populations by genetic analysis and lactose hydrogen breath and tolernace tests. *Am. J. clin. Nutr*., **91**: 140-146. https://doi.org/10.3945/ajcn.2009.27946

Baseer, A. and Rab, S.M., 1976. High intestinal lactase concentration in adult Pakistanis. *Br. med. J*., **21**: 436. https://doi.org/10.1136/bmj.1.6007.436

Bodlaj, G., Stocher, M., Hufnagl, P., Hubmann, R., Biesenbach, G., Stekel, H. and Berg, J., 2006. Genotyping of lactase-phlorizin hydrolase -13910 polymorphism by Light Cycler PCR and implications for the diagnosis of lactase intolerance. *Clin. Chem*., **52**: 148-151. https://doi.org/10.1373/clinchem.2005.057240

Breton, G., Schlebusch, C.M., Lombard, M., Sjodin, P., Soodyall, H. and Jakobsson, M., 2014. Lactase Persistence Alleles Reveal Partial East African Ancestry of Southern African Khoe Pastoralists. *Curr. Biol*., **24**: 852-858. https://doi.org/10.1016/j.cub.2014.02.041

Bulhoes, A., Goldani, h. A.S., Oliveria, F.S., Matte, U.S., Mazzuca, R.B. and Silveria, T.R., 2007. Correlation between lactose absorption and C/T-13910 and G/A-22018 mutations of the lactase phlorizin hydrolase (LCT) gene in adult-type hypolactasia. *Brazil. J. Med. biol. Res*., **40**: 1441-1446. https://doi.org/10.1590/S0100-879X2007001100004

Burger, J., Kircner, M., Bramanti, B., Haak, W. and Thomas, M.G., 2007. Absence of the lactase persistence-associated allele in early Neolithic Europeans. *Proc.natl. Acad. Sci. U.S.A*., **104**: 3736-3741. https://doi.org/10.1073/pnas.0607187104

Campbell, A.K., Mathews, S.B., Waud, J.P. and Roberts, A.G., 2005. Systemic lactose intolerance: A new perspective on an old problem. *Postgrad. med. J*., **81**: 167-173. https://doi.org/10.1136/

pgmj.2004.025551

EFSA, 2010. Scientific opinion on lactose thresholds in lactose intolearnce and galactosaemia. *Eur. Fd. Safe. Assoc. J.*, **8**: 1777. https://doi.org/10.2903/j.efsa.2010.1777

Enattah, N.S., Sahi, T., Savilahti, E., Terwilliger, J.D., Peltonen, L. and Jarvela, I., 2002. Identification of a variant associated with adult-type hypolactasia. *Nat. Genet.*, **30**: 233-237. https://doi.org/10.1038/ng826

Enattah, N.S., Jensen, T.G., Nielsen, M., Lewinski, R., Kuokkanen, M., Rasinpera, H., El-Shanti, H., Seo, J.K., Alifrangis, M. and Khalil, I.F., 2008. Independent introduction of two lactase-persistence alleles into human populations reflects different history of adaptation to milk culture. *Am. J. Hum. Genet.*, **82**: 57-72. https://doi.org/10.1016/j.ajhg.2007.09.012

Enattah, N.S., Trudeau, A., Pimenoff, V., Maiuri, L., Auricchio, S., Greco, L., Rossi, M., Lentze, M., Seo, J.K. and Rahgozar, S., 2007. Evidence of still-ongoing convergence evolution of the lactase persistence T-13910 alleles in humans. *Am. J. Hum. Genet.*, **81**: 615-625. https://doi.org/10.1086/520705

Friedrich, D.C., Santos, S.E.B, Ribeiro-dos-Santos, A.K. and Hutz, M.H., 2012. Several different lactase persistence associated alleles and high diversity of the lactase gene in the admixed Brazilian population. *PLoS One*, **7**: e46520. https://doi.org/10.1371/journal.pone.0046520

Ingram, C., Elamin, M.F., Mulcare, C.A., Weale, M.E., Tarekegn, A., Raga, T.O., Bekele, E., Elamin, F.M., Thomas, M.G., Bradman, N. and Swallow, D.M., 2007. A novel polymorphism associated with lactose tolerance in Africa: Multiple causes for lactase persistence? *Hum. Genet.*, **120**: 779-788. https://doi.org/10.1007/s00439-006-0291-1

Jarvela, I., Kuokkanen, M., Enattah, N.S., Oksanen, A., Salvilahti, E. and Orpana, A., 2003. Transcriptional regulation of the lactase-phlorizin hydrolase gene by polymorphisms associated with adult-type hypolactasia. *Int. J. Gastroentrol. Hepatol.*, **52**: 647-652. https://doi.org/10.1136/gut.52.5.647

Jarvela, I.E., 2005. Molecular genetics of adult-type hypolactasia. *Annls. Med.*, **37**: 179-185. https://doi.org/10.1080/07853890510007359

Johnson, A.O., Semenya, J.G., Buchowski, M.S., Enwonwu, C.O. and Scrimshaw, N.S., 1993. Correlation of lactose maldigestion, lactose intolerance, and milk intolerance. *Am. J. clin. Nutr.*, **57**: 399-401. https://doi.org/10.1093/ajcn/57.3.399

Jones, B., Raga, T.O., Liebert, A., Zmarz, P., Bekele, E., Danielsen, E.T., Olsen, A.K., Bradman, N., Troelsen, J.T. and Swallow, D.M., 2013. Diversity of lactase persistence alleles in Ethiopia: Signature of a soft selective sweep. *Am. J. Hum. Genet.*, **93**: 538-544. https://doi.org/10.1016/j.ajhg.2013.07.008

Krawczyk, M., Wolska, M., Schwartz, S., Gruenhge, F., Terjung, B., Portincasa, P., Sauerbruch, T. and Lammert, F., 2008. Concordance of genetic and breath tests for lactose intolerance in a Tertiary Referral Centre. *J. Gastrointestin. Liver Dis.*, **17**: 134-139.

Lehtimaki, T., Hemminki, J., Rontu, R., Mikkila, V., Rasanen, L., Laaksonen, M., Hutri-Kahonen, N., Kahonen, M., Viikari, J. and Raitakari, O., 2006. The effects of adult-type hypolactasia on body height growth and dietary calcium intake from childhood into young adulthood: A 21-year follow-up study- the cardiovascular risk in young finns study. *Pediatrics*, **118**: 1553-1559. https://doi.org/10.1542/peds.2006-0542

Leseva, M.N., Grand, R.J., Klett, H., Boerries, M., Busch, H., Binder, A.M. and Michels, K.B., 2018. Differences in DNA methylation and functional expression in lactase persistent and non-persistent individuals. *Scient. Rep.*, **8**: 5649. https://doi.org/10.1038/s41598-018-23957-4

Lewinsky, R., Jensen, T.G., Moller, J., Stensballe, A., Olsen, J. and Troelsen, J.T., 2005. T-13910 DNA variant associated with lactase persistence interacts with Oct-1 and stimulates lactase promoter activity *in vitro*. *Hum. Mol. Genet.*, **14**: 3945-3953. https://doi.org/10.1093/hmg/ddi418

Lukito, W., Malik, S.G., Surono, I.S. and Wahlqvist, M.L., 2015. From 'lactose intolerance' to 'lactose nutrition'. *Asia-Pac. J. clin. Nutr.*, **24**(Suppl 1): S1-8.

Macholdt, E., Stoneking, M., Lede, V., Barbieri, C., Mopoloka, S.W., Chen, H., Slatkikn, M. and Pakendorf, B., 2014. Tracing pastoralist migrations to sothern Africa with lactase persistence alleles. *Curr. Biol.*, **24**: 875–879. https://doi.org/10.1016/j.cub.2014.03.027

Madry, E., Lisowska, A., Kwiecien, J., Marciniak, R., Korzon-Burakowska, A., Drzymała-Czyż, S., Mojs, E. and Walkowiak, J., 2010. Adult-type hypolactasia and lactose malabsorption in Poland. *Acta Biochim. Pol.*, **57**: 585-588. https://doi.org/10.18388/abp.2010_2448

Malmstrom, H., Linderholm, A., Liden, K., Stora, J., Molnar, P., Holmlund, G., Jakobsson, M. and Goterstrom, A., 2010. High frequency of lactose

intolerance in a prehistoric hunter-gatherer population in northern Europe. *BMC Evol. Biol*., **10**: 89. https://doi.org/10.1186/1471-2148-10-89

Marton, A., Xue, X. and Szilagyi, A., 2012. Meta-analysis: The diagnostic accuracy of lactose breath hydrogen or lactose tolerance tests for predicting the North European lactase polymorphism C/T-13910. *Aliment. Pharmacol. Therapeut*., **35**: 429-440. https://doi.org/10.1111/j.1365-2036.2011.04962.x

McIntosh, S.K. and Scheinfeldt, L.B., 2012. It's getting better all the time: Comparative perspectives from Oceania and West Africa on genetic analysis and archaeology. *Afr. Archaeol. Rev*., **29**: 131-170. https://doi.org/10.1007/s10437-012-9122-z

Miquel, J., Morales, E., Azocar, L., Maul, X., Perez, C. and Chianale, J., 2011. The European lactase persistance genotype determines the lactase persistence state and correlates with gastrointestinal symptoms in the Hispanic and Amerindian Chilean population: A case-control and population-based study. *BMJ Open*, **1**: e000125. https://doi.org/10.1136/bmjopen-2011-000125

Pigott, S., Jalal, H., Merriam, A., Qayyum, A. and Stacey, T., 1977. *The Indus Valley civilization*. Stacey International, Pakistan, pp. 72.

Pohl, D., Savarino, E., Hersberger, M., Behlis, Z., Stutz, B., Goetze, O., Eckardstein, A.V., Fried, M. and Tutuian, R., 2010. Excellent agreement between genetic and hydrogen breath tests for lactase deficiency and the role of extended symptom assessment. *Br. J. Nutr*., **104**: 900-907. https://doi.org/10.1017/S0007114510001297

Ponte, P., de Medeiros, P.H.Q.S., Havt, A., Caetano, J.A., Cid, D.A.C., Prata, M.d.M.G., Soares, A.M., Guerrant, R.L., Mychaleckyj, J. and Lima, A.A., 2016. Clinical evaluation, biochemistry and genetic polymorphism analysis for the diagnosis of lactose intolerance in a population from northeastern Brazil. *Clinics*, **71**: 82-89. https://doi.org/10.6061/clinics/2016(02)06

Rahimi, A., Delbruck, H., Haeckel, R., Goedde, H.W. and Flatz, G., 1976. Persistence of high intestinal lactase activity (lactose tolerance) in Afghanistan. *Am. J. Hum. Genet*., **34**: 57-62. https://doi.org/10.1007/BF00284435

Ranciaro, A., Campbell, M.C., Hirbo, J.B., Ko, W.Y., Froment, A., Anagnostou, P., Kotze, M.J., Ibrahim, M., Nyambo, T. and Omar, S.A., 2014. Genetic origins of lactase persistence and the spread of pastoralism in Africa. *Am. J. Hum. Genet*., **94**: 496-510. https://doi.org/10.1016/j.ajhg.2014.02.009

Rasinpera, H., Savilahti, E., Enattah, N.S., Kuokkanen, M., Totterman, N., Lindahl, H., Jarvela, I. and Kolho, K.L., 2004. A genetic test which can be used to daignose adult-type hypolactasia in children. *Gut*, **53**: 1571-1576. https://doi.org/10.1136/gut.2004.040048

Raz, M., Sharon, Y., Yerushalmi, B. and Birk, R., 2013. Frequency of LCT-13910 C/T and LCT-22018G/A single nucleotide polymophisms associated with adult-type hypolactasia/lactase persistence among Israelis of different ethnic groups. *Gene*, **519**: 67-70. https://doi.org/10.1016/j.gene.2013.01.049

Sahi, T., 2001. Genetics and epidemiology of adult-type hypolactasia with emphasis on the situation in Europe. *Scand. J. Nutr.*, **45**: 161-162. https://doi.org/10.3402/fnr.v45i0.1799

Savaiano, D.A., Ritter, A.J., Klaenhammer, T.R., James, G.M., Longcore, A.T., Chandler, J.R., Walker, W.A. and Foyt, H.L., 2013. Improving lactose digestion and symptoms of lactose intolerance with a novel galacto-oligosaccharide (RP-G28): A randomized, double-blind clinical trial. *Nutr. J*., **12**: 160. https://doi.org/10.1186/1475-2891-12-160

Swallow, D.M., 2003. Genetics of lactase persistence and lactose intolerance. *Annu. Rev. Genet*., **37**: 197-219. https://doi.org/10.1146/annurev.genet.37.110801.143820

Szilagyi, A., Shrier, I., Heilpern, D., Je, J., Park, S., Chong, G., Lalonde, C., Cote, L.F. and Lee, B., 2010. Differential impact of lactose/lactase phenotype on colonic microflora. *Can. J. Gastroenterol*., **24**: 373-379. https://doi.org/10.1155/2010/649312

Tarabra, E., Pazienza, P., Borghesio, E., Actis, G.C., Tappero, G., Farmarin, L., Ayoubi, M., Castellino, F., Leone, N., Sansoe, G., Paolis, P.D., Comandone, A. and Rosina, F., 2010. LCT-13910C>T polymorphism-associated lactose malabsorption and risk for colorectal cancer in Italy. *Dig. Liver Dis*., **42**: 741-743. https://doi.org/10.1016/j.dld.2010.02.013

Tishkoff, S., Reed, F.A., Ranciaro, A., Voight, B.F., Babbitt, C.C., Silverman, J.S., Powell, K., Mortensen, H.M., Hirbo, J.B., Osman, M., Ibrahim, M., Omar, S.A., Lema, G., Nyambo, T.B., Ghori, J., Bumpstead, S., Pritchard, J.K., Wray, G.A. and Deloukas, P., 2007. Convergent adaptation of human lactase persistence in Africa and Europe. *Nat. Genet*., **39**: 31-40. https://doi.org/10.1038/ng1946

Tomar, B.S., 2014. Lactose intolerance and other disaccharidase deficiency. *Indian J Pediatr*., **81**: 876-886. https://doi.org/10.1007/s12098-014-1346-2

Troelsen, J.T., 2005. Adult-type hypolactasia and regulation of lactase expression. *Biochim. biophys. Acta*, **1723**: 19-32. https://doi.org/10.1016/j.bbagen.2005.02.003

Usai-Satta, P., Scarpa, M., Oppia, F. and Cabras, F., 2012. Lactose malabsorption and intolerance: What should be the best clinical management? *World J. Gastrointest. Pharmacol. Therap.*, **3**: 29-33. https://doi.org/10.4292/wjgpt.v3.i3.29

Vesa, T.H., Marteau, P. and Korpela, R., 2000. Lactose intolerance. *J. Am. Coll. Nutr.*, **19**: 165s-175s. https://doi.org/10.1080/07315724.2000.10718086

White, P.J.T., Smith, J. and Heidemann, M., 2012-2020. *Cases for evolution education. Cell biology*. Evo-Ed. Available at: http://www.evoed.org/pages/Lactase/anthro_biogeogr.html (accessed on 25 July, 2021).

White, P.J.T., Smith, J. and Heidemann, M., 2012-2020a. *Cases for evolution education. Anthropology and biogeography*. Evo-Ed. Available at: http://www.evo-ed.org/pages/Lactase/anthro_biogeogr.html (accessed on 25 July, 2021).

White, P.J.T., Smith, J. and Heidemann, M., 2012-2020b. *Cases for evolution education. Molecular genetics*. Evo-Ed. Available at: http://www.evo-ed.org/pages/Lactase/anthro_biogeogr.html (accessed on 25 July, 2021).