

# The highest Duffy (FYX) allele frequency ever reported for Scottish population: A cross-sectional study

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## Abstract

**Background and Aim:** The Duffy (FY) blood group system has six known antigens among which the Fy<sup>a</sup> and Fy<sup>b</sup> are known as major antigens. Fy<sup>x</sup> phenotype forms as a result of two point mutations in the *FYB* allele leading to instability of Duffy protein and so reduction of Duffy antigen expression in the cells. This study aimed to investigate the FYX allele frequency in the Scottish population.

**Methods:** The Duffy blood group system was serologically and molecularly investigated in 222 samples collected from donors of Aberdeen Regional Blood Transfusion Center (BTC). The haemagglutination and BeadChip microarray chemistry methods were used for phenotyping and genotyping. Confirmatory tests were also used to check the discrepant results.

**Results:** In this study, the frequency of Duffy blood group phenotypes including Fy<sup>a+</sup>, Fy<sup>a+b+</sup>, and Fy<sup>b+</sup> were 17.57%, 42.79%, and 39.64%, respectively. Furthermore, the frequency of *FYA/FYA*, *FYA/FYB*, and *FYB/FYB* genotypes was estimated to be 14.41%, 45.95%, and 39.64%, respectively, using the Bioarray method. In the present study, based on Duffy DNA sequencing results, 12 samples (5.41%) had just one FYX allele.

**Conclusion:** The frequency of the FYX allele in this study was estimated to be 0.0270% which is more than the results reported so far.

## KEYWORDS

BeadChip microarray, Duffy blood group, FYX allele, Scottish population

## 1 | INTRODUCTION

The Duffy blood group system is a clinically remarkable blood group system.<sup>1</sup> It comprises six antigens (Fy<sup>a</sup>, Fy<sup>b</sup>, Fy<sup>3</sup>, Fy<sup>4</sup>, Fy<sup>5</sup>, Fy<sup>6</sup>), and four main phenotypes; Fy(a+b+), Fy(a-b+), Fy(a+b-), and Fy(a-b-).<sup>2,3</sup> The antigens are expressed on a glycosylated erythrocyte membrane protein known as the Duffy antigen receptor for chemokines (DARC).

The absence of DARC on the red blood cell surface is correlated with various conditions such as inflammation, human immunodeficiency virus infection, and malignancies. Duffy blood group negativity is prevalent among Africans causing erythrocytes resistance to invasion by *Plasmodium vivax* and *Plasmodium knowlesi*.<sup>4</sup> In 1950, Fy<sup>a</sup> was discovered after finding anti-Fy<sup>a</sup> in the serum of a hemophilic person who had received several transfusions.<sup>5</sup> One year later, after finding

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anti-Fy<sup>b</sup> in the serum of a woman who had been pregnant three times, Fy<sup>b</sup> was discovered.<sup>6</sup> The Fy<sup>x</sup> phenotype is a weak form of Fy<sup>b</sup> that was reported later by two other groups.<sup>7,8</sup> The FYX allele has a frequency of 2%–3.5% in Caucasian people while it is not seen in Blacks.<sup>9</sup> FYA and FYB alleles differ by a single G to A nucleotide substitution at position 125 in exon 2.<sup>10</sup> The molecular basis of the Fy (a- b<sup>-</sup>) phenotype is related to the mutation in the GATA promoter region of the FYB (-67, T to C). Disturbance at the binding site for the GATA-1 erythroid transcription factor can be considered a result of this nonsense mutation.<sup>9</sup> Moreover, a similar mutation is observed in the GATA-1 promoter region of the FYA.<sup>11</sup> The highest frequency for Fy<sup>b</sup> was observed when a group studied the frequency of Fy<sup>a</sup> and Fy<sup>b</sup> in Blacks using the polymerase chain reaction with restriction fragment length polymorphism (PCR-RFLP) method.<sup>12</sup> Owing to the high incidence of discrepancies between Fy<sup>b</sup> phenotyping results versus genotyping outcomes on the collected samples from Scottish blood donors through the PCR-RFLP method, Murphy suggested that the frequency of Fy<sup>x</sup> in Caucasians was more than the previously reported 0.015%.<sup>13,14</sup> To determine the pregnancies at risk for HDFN, Hessner et al. used a validated PCR-ASP assay for prenatal genotyping in 1999. The method utilized was able to determine the FYA, FYB, null FYA, and null FYB alleles.<sup>15</sup> One year later, the two missense mutations related to FYX were defined using the PCR with sequence-specific primers (PCR-SSP) method in 300 samples.<sup>16</sup> Schmid and co-workers reported a frequency of 1% for Fy<sup>x</sup> haplotypes in 54 samples of African American blood donors in 2011.<sup>17</sup> In 2015, Silvia Manfroi and co-workers used the BLOOD-chip<sup>®</sup> IDcor<sup>+</sup> technique to determine the FYX/FY null and FYA/FY null genotypes in a Caucasian thalassemia family from Sardinia and investigated the FYX and FY null alleles frequency in Caucasian and black people.<sup>9</sup> In the present study, the prevalence of the FYX allele was measured in a Scottish population using high-sensitivity molecular assays.

## 2 | MATERIALS AND METHODS

To study the Duffy blood group serologically and molecularly, blood samples were taken from 222 blood donors. Forty-seven of the donors (male = 19, female = 28) were healthy and partially known reference panel donors of Aberdeen Regional Blood Transfusion Center (BTC), and the other blood samples were taken from active, healthy platelet donors ( $n = 175$ , male = 126, female = 49) who consented to participate in the study. The collection of blood samples began after the project was approved by the Grampian Local and Regional Ethics Committee (LREC) and the Scottish National Blood Transfusion Services (SNBTS) Clinical Governance committee. The purified genomic DNA (gDNA) of buffy coat from whole blood EDTA samples was genotyped by BeadChip microarray chemistry, that is, BioArray HEA BeadChip<sup>™</sup> and their red blood cells were used for phenotyping. The commercial kits, READY GENE KKD PCR-SSP, in-house DNA-PCR SSP assay, and serological methods were used for re-genotyping and re-phenotyping due to

the existence of discrepant results. The mentioned methods are overviewed in the section below.

### 2.1 | BeadChip microarray

Microarray-based technologies are known as powerful techniques to study gene expression patterns on a genome-wide scale.<sup>18–20</sup> Recently, the human erythrocyte antigen (HEA) BeadChip array of bioarray solutions, which has been used in the present study to investigate the samples, has become more popular as microarray chemistry. In our study, the HEA24 and HEA28 BeadChip kits were used for genotyping of donor samples. The following five steps are necessary to use these kits; multiplex PCR amplification of target markers on the gDNA, cleaning up of the PCR products using ExoSAP-IT, generating single-strand DNA (ssDNA) products using Lambda Exonuclease, hybridization/and elongation of the ssDNA to the specific probes on the BeadChips and finally reading the images using a Microarray AIS400 system (Bioarray Solutions) and analyzing the data online.<sup>21</sup>

The results of allele frequency, allele detection rates (ADR), corrected allele detection rate (CADR), and concordance rates (CoR) were calculated based on the following formula, respectively:

$$F(\%) = \frac{n \times AA + [(n' \div 2) \times AB]}{N \times 2} \times 100, \quad (1)$$

where  $n$ ,  $AA$ ,  $n'$ ,  $AB$ , and  $N$  denote the number of detected homozygous, homozygous, the number of detected heterozygous, heterozygous, and total sample number, respectively.

$$ADR(\%) = \frac{N \times 2 - [(nIC + n'LS) \times 2]}{N \times 2} \times 100, \quad (2)$$

where  $N$  is the total number of the tested samples,  $nIC$  is the total number of samples that showed intermediate call (IC) and  $n'LS$  is the total number of samples with low signal.

$$CADR(\%) = \frac{N \times 2 - (nIC + n'LS)}{N \times 2} \times 100. \quad (3)$$

This formula was used when the warning message for a blood group was for only one allele and the  $s^{nd}$  allele gave a correct result.

$$CoR(\%) = \frac{N \times 2 - [(nIC + n'LS) \times 2] - n''DA}{N \times 2 - [(nIC + n'LS) \times 2]} \times 100, \quad (4)$$

where  $N$ ,  $nIC$ ,  $n'LS$ , and  $n''DA$  show the total number of tested samples, the total number of samples with intermediate calls, the total number of samples with low signal, and the total number of alleles with discrepancy versus predicted results based on serologic results, respectively.

### 2.2 | Serological test

All the blood samples collected for the BioArray study (BA study) including platelets and reference panels were phenotyped

serologically for Fy<sup>a</sup> and Fy<sup>b</sup>. The blood group phenotyping was performed based on the technical recommendations from suppliers for each monoclonal antibody/antiserum. All the samples with a discrepancy of serological results for Fy<sup>b</sup> were re-phenotyped using two different polyclonal anti-Fy<sup>b</sup> to check and confirm the donors' phenotype results.

### 2.3 | In-house PCR-SSP

To produce large amounts of a specific DNA fragment of defined length and sequence from a small amount of a template, the PCR can be used as an in-vitro method.<sup>21,22</sup> In this study, we used optimized and validated PCR-SSP. *Fy* and *human growth hormone (HGH)* primers were synthesized by MWG at a scale of 0.01 μM. All the stock and working primers were made up at the concentration of 100 and 2 μM, respectively. Additionally, each PCR experiment included an internal positive control (HGH) and a negative DNA control known as No Template Control (NTC) which the DNA sample was replaced by dH<sub>2</sub>O.<sup>23</sup>

### 2.4 | Commercial PCR-SSP kits

These kits are produced by different companies and are expected to perform more robustly than in-house protocols since they usually pass different stages of the validation process and quality controls before being introduced to the market. In this study, the commercial PCR-SSP kit (READY GENE KKD PCR-SSP Kit) was used as an additional method for confirmation and verification of some achieved results.

### 2.5 | Automated DNA sequencing

Sequencing of cloned/cleaned up DNA products for the BioArray study was performed by DNA Sequencing and Services (MRCPPU,

College of Life Sciences, University of Dundee, Scotland) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. The sequencing results of the FyX allele-positive donors were compared with the outcomes of FyX-associated mutations in white American donors.<sup>24</sup>

## 3 | RESULTS

The serological blood group phenotyping and the HEA *BeadChip*<sup>™</sup> genotyping results are as follows:

### 3.1 | Haemagglutination blood group phenotyping results

The haemagglutination blood group phenotyping results are presented in Table 1.

### 3.2 | Microarray blood group genotyping results

The results of the blood group genotyping, the determined allele frequencies, overall ADR for the Duffy blood group as well as CoR between predicted genotypes from haemagglutination and genotyping results of microarray assay are shown in Table 1.

In the present study, 0 of 222 samples showed IC or LS. ADR and CADR were 100%.

As it is shown in Table 1, genotype discrepancies versus serological phenotyping outcomes occurred in seven donors possessing Fy<sup>b</sup> ( $n = 7$ ; BA048, BA078, BA115, BA141, BA155, BA162, and RP35, which are based on the sample ID). As mentioned before, the discrepant samples were re-phenotyped and re-genotyped and this will be discussed in the following section.

**TABLE 1** Summary of Duffy blood group phenotypes and actual genotype results from hemagglutination and microarray typing of 222 platelet and reference panel donors from Aberdeen Regional SNBTS.

Blood group	Duffy								
Associated SNPs tested by BioArray BeadChips	FYA/B			FY-265			GATA		
Predicted genotypes based on serologic results	Fy <sup>a</sup> /Fy <sup>a</sup>	Fy <sup>a</sup> /Fy <sup>b</sup>	Fy <sup>b</sup> /Fy <sup>b</sup>	NA			NA		
Number phenotyped (Serology)	39	95	88	NA			NA		
BioArray genotypes	AA	AB	BB	AA	AB	BB	AA	AB	BB
Number genotyped (BioArray)	32	102	88	210	12	0	220	2	0
Number of samples with discrepancies	7/222			NA			NA		
Allele discrepancy rate versus serologic results	7/444			NA			NA		
CoR (%) between predicted genotypes from haemagglutination versus genotyping	98.42			NA			NA		

Abbreviations: NA, not applicable; SNP, single-nucleotide polymorphism.

**TABLE 2** Summary of the results from KKD PCR-SSP typing for seven discrepant and a control patient sample with SCD (MA).

Block specificity	Duffy			
	5	6	7	8
Reaction No.	5	6	7	8
Glycoprotein	Fy <sup>a</sup>	Fy <sup>b</sup> Fy <sup>b</sup> weak	Fy null	Fyb weak
Specificity of the products	FYA	FYB	FY* Null01	FYX
PCR-SSP Product size (bp)	720	720	720	187
Presence of 434 bp Internal control band**	+	+	+	+
BA 048	+	+	-	+
BA 087	+	+	-	+
BA 115	+	+	-	+
BA 141	+	+	-	+
BA 155	+	+	-	+
BA 162	+	+	-	+
RP 35	+	-	+	-
MA	-	-	+	-

Note: The positive (+) and negative (-) signs indicate the appearance and absence of a PCR product band.

\*This sometimes tends to show very weak bands, however, the genuine reactions are clearly distinguished by the strength and intensity of the bands.

\*\*This indicates that for all the samples this internal control band appeared, indicating the PCR protocol had not failed and there was enough gDNA for amplification.

### 3.3 | Re-phenotyping of discrepant samples

The re-phenotype results with different antisera were in good agreement with the original serological results that were obtained using antisera from Alba Bioscience with slight differences in their grades of hemagglutination ( $\pm 1$  grade variation of reaction at the most).

### 3.4 | Re-genotyping of discrepant samples

To validate the results obtained by HEA BeadChip assays for the FYB discrepant samples, the Inno-TRAIN KKD PCR-SSP kit was used as one of the confirmatory tests. A summary of the results is presented in Table 2.

Moreover, the in-house PCR-SSP has been also used as a confirmatory test and its results are in good agreement with the results of HEA BeadChip assays and commercial conventional PCR-SSP kits.

### 3.5 | DNA sequencing of discordant samples

The results of DNA sequencing for discordant donor samples (BA048, BA087, BA115, BA141, BA155, BA162, RP35) and a control patient sample with Sick Cell Disease (SCD) (MA), observed mutations, and also the amino acid substitutions have been presented as first 8 samples in Table 3.

To investigate the GATA mutation at the promoter region, the positions of nucleotide 125 G > A (Nt 797), which encoded the FYA/

**TABLE 3** Summary of final results for all Duffy DNA sequencing assays.

Region specificity		GATA			Fy <sup>a</sup> /Fy <sup>b</sup>		Fy <sup>x</sup>			Confirmed Genotype
		Allele	Nt -67	Nt 125	Aa 42	Nt 265	Aa 89	Nt 298	Aa 100	
6 (BA048, BA087, BA115, BA141, BA155, BA162)	1st	T	G	Glycine	C	Arginine	G	Alanine	FYA	
	2nd	T	A	Aspartic acid	T	Cysteine	A	Threonine	FYX	
1 (RP35)	1st	T	G	Glycine	C	Arginine	G	Alanine	FYA	
	2nd	C	A	Aspartic acid	C	Arginine	G	Alanine	FY	
1 (MA)	1st	C	A	Aspartic acid	C	Arginine	G	Alanine	FY	
	2nd	C	A	Aspartic acid	C	Arginine	G	Alanine	FY	
4 (BA017, BA083, BA128, BA154)	1st	NT	A	Aspartic acid	C	Arginine	G	Alanine	FYB	
	2nd	NT	A	Aspartic acid	T	Cysteine	A	Threonine	FYX	
2 (BA042, BA055)	1st	NT	A	Aspartic acid	C	Arginine	A	Threonine	FYB	
	2nd	NT	A	Aspartic acid	T	Cysteine	A	Threonine	FYX	

Denotes missense mutations                      Denotes an SNP                      Denotes missense mutations without a significant phenotypic change

Note: To avoid confusion, standard three-letter codes have been used for all correspondence Amino acids in this table.

Abbreviations: A, Alanine; Aa, Amino acid; C, Cysteine; D, Aspartic acid; G, Glycine; MA, a homozygous GATA positive control sample; NT, not tested; Nt, nucleotide; R, Arginine; T, Threonine.

FYB alleles, and two nucleotide substitutions at 265 C > T and 298 G > A (Nt 937 and Nt 970, respectively) that are known as mutations related to FYB/FYX, the entire amplified FY gene for all eight samples has been sequenced with different forward and reverse primers. As shown in Table 3, the sequence analysis of exon 2 in all of these samples shows the existence of a 'G' nucleotide in one of the alleles at position 125. The sequencing results for the rest of the coding region for both exons (exon 1 and exon 2) and the promoter regions were similar to the previously reported FY gene sequences deposited in GenBank from normal Duffy individuals. Furthermore, for six of these eight samples, the sequence analysis of exon 2 in the 2<sup>nd</sup> alleles indicated an 'A' nucleotide at position 125 with two extra nucleotide substitutions, T for C at position 265, and G for A at position 298 that these mutations are the specification of the FYX allele. Moreover, sample RP35 was identified as heterozygous for the GATA mutation (see Table 3).

Of 12 donors with AB genotype FY-265 by microarray (see Table 1), the six samples with Fy(a<sup>-</sup> b<sup>+</sup>) phenotypes with no discrepancy between the serological and microarray genotyping results, were examined by direct DNA sequencing as a complementary investigation. The summary of final direct DNA sequencing results for these samples is listed in Table 3 (the final six samples). All of these six samples are homozygous FYB, due to the presence of the polymorphic site (125 G > A) which is the characteristic of the Duffy phenotype. Moreover, the molecular presence of the polymorphic sites 265 C > T and 298 G > A that are indicative of the FYB/FYX indicates that samples BA128, BA083, BA154, and BA017 are heterozygous FYB/FYX genotypes for both polymorphic markers due to the presence of two simultaneous peaks at both positions. However, the BA042 and BA055 samples are heterozygous for FYB/FYX at position 265 but homozygous mutant for nucleotide 298. Consequently, in the present study, the existence of 265 C > T mutation reduced antigen expression on the surface of cells leading to negative serological results. It has to be noted that the 265 C > T missense mutation always occurs with 298 G > A mutation while the 298 G > A missense mutation can be found alone. However, the 298 G > A mutation without 265 C > T mutation can not decrease the antigen expression and lead to positive serological results. 50% of 12 FYX allele-positive donors were phenotyped as Fy(a<sup>+</sup> b<sup>-</sup>) and the remaining as Fy(a<sup>-</sup> b<sup>+</sup>) by the serological test. Whereas, by the HEA BeadChip method through recognition of mutation at nucleotide 265, heterozygous FYA/FYB and homozygous FYB/FYB were genotyped, respectively. The sequencing results of the 12 samples were compared with the outcomes of FYX-associated mutations in 47 white American donors (Table 4).<sup>24</sup>

## 4 | DISCUSSION

In the present study, in 12 of 222 Scottish donors (5.41%), one FYX allele was discovered (see Table 3). The frequency of the FYX allele was estimated to be 0.0270%. This is in agreement with a study conducted by Murphy et al.,<sup>14</sup> where they examined 109

**TABLE 4** FYX-associated polymorphisms in the Scottish and White American.

Genotype	FYX-associated mutations		Number of donors <sup>a</sup>	
	265 C > T	298 G > A	Scottish	American
FYA/FYB	wt/wt	wt/m	0	4
	wt/m	wt/m	6	2
	wt/m	m/m	0	0
FYB/FYB	wt/wt	wt/m	0	9
	wt/m	wt/m	4	0
	wt/m	m/m	2	0

Abbreviations: m, mutated allele; wt, wild type allele.

<sup>a</sup>The number of donors found with the FYX-associated mutations within 222 genotyped Scottish donors' samples in this study and between 47 investigated White American donors.

serologically Fy(a<sup>+</sup> b<sup>-</sup>) typed samples of Scottish donors using the PCR-RFLP method. Due to the high incidence of the discrepancy between Fy<sup>b</sup> phenotyping and genotyping results, they concluded that the frequency of Fy<sup>x</sup> was more than previously reported 0.015%.<sup>13</sup> Thirteen of 109 samples (11.92%) investigated by Murphy et al. were genotyped as FYA/FYB, while in the present study, 6 of 39 (15.38%) serologically typed Fy(a<sup>+</sup>b<sup>-</sup>) samples were genotyped as FYA/FYB with two FYX-associated mutations. The donors who participated in the present study, their parents, and their grandparents were Caucasians. Hence, this is the highest prevalence of FYX that has been reported in the Scottish population.

Several blood groups exist on red cell membrane which are grouped into different systems.<sup>25</sup> After the ABO system, Duffy is the second most significant blood grouping system.<sup>26</sup> Fy<sup>a</sup> and Fy<sup>b</sup> are the two major antigens of the Duffy blood group system.<sup>27</sup> The substitution c.265 C > T attenuates Fy<sup>b</sup> antigen leads to the Fy<sup>x</sup> phenotype.<sup>28</sup> The frequency of the FYX allele varies greatly in different populations. Hongfongfa et al.<sup>29</sup> in a study on malaria patients in Thailand, did not find the mutation of the FYX at nucleotide position 265 in 167 FYB negative samples. Van Alsten et al.<sup>30</sup> reported one patient with Fy<sup>x</sup> in 3537 non-Hispanic white participants. Sheppard and colleagues<sup>31</sup> in a study on a Hispanic population, reported a frequency of 1.2% for the Fy<sup>x</sup> phenotype. Chown et al. estimated a frequency of 2% for the FYX allele in a Caucasian population.<sup>8</sup> Frequencies of 0.025 in Swedish blood donors and 0.013 in Australian donors have also been estimated for the FYX allele.<sup>19,32,33</sup>

As mentioned earlier, the molecular basis of the FYX allele is due to the existence of two mutations, including the substitution of C to T at nucleotide 265 and G to A at nucleotide 298.<sup>9,34</sup> These mutations lead to the substitution of Arginine to Cysteine at position 89 and Alanine to Threonine at position 100, respectively. Instability and reduction of Fy<sup>b</sup> protein in the cell surface result from the mentioned amino acid changes.<sup>35,36</sup> According to Table 3, in two donors (BA042 and BA055) the missense mutation at position 298 G > A was found in just one allele, however, both



FYX-associated mutations were in the other allele. Since these two samples were Fy<sup>b+ve</sup> by the usual serologic typing, missense mutation at 298 position alone does not seem adequate to change the Fy<sup>b</sup> expression at the significant level and prevent the other FYB allele from being read. The existence of this mutation has been reported in 33% of Caucasians.<sup>32</sup> In the present study, in contrast to the 265 C > T mutation, the 298 G > A polymorphism did not lead to instability of Fy<sup>b</sup> protein antigen and reduction of antigen expression in erythrocytes.

## 5 | CONCLUSION

This study aimed to investigate the FYX allele frequency in the Scottish population. The frequency of the FYX allele was estimated to be 0.0270% which is, to the best of our knowledge, the highest frequency reported so far in the Scottish population. However, further studies with larger sample sizes are recommended to evaluate the FYX allele frequency more precisely.

## AUTHOR CONTRIBUTIONS

**Sylvia Armstrong-Fisher:** Conceptualization. **Stan Urbaniak:** Conceptualization. **Arian Karimi Rouzbahani:** Writing—original draft. **Mona Hemmati Chegeni:** Formal analysis. **Golnaz Mahmoudvand:** Writing—original draft. **Ali Mohammad Varzi:** Conceptualization; supervision; formal analysis; writing—review and editing.

## ACKNOWLEDGMENTS

We are thankful to all the staff of Aberdeen Blood Transfusion Service, particularly the Donor Apheresis Unit and Blood Bank staff for their kindness and cooperation in collecting blood samples for the BioArray study. Financial support by the Iranian Ministry of Health and Medical Education, Lorestan University of Medical Sciences is gratefully acknowledged. This study was supported by the College of Life Sciences and Medicine, University of Aberdeen.

## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## TRANSPARENCY STATEMENT

The lead author Ali Mohammad Varzi affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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**How to cite this article:** Armstrong-Fisher S, Urbaniak S, Karimi Rouzbahani A, Hemmati Chegeni M, Mahmoudvand G, Varzi AM. The highest Duffy (FYX) allele frequency ever reported for Scottish population: a cross-sectional study. *Health Sci Rep*. 2023;6:e1314. doi:10.1002/hsr2.1314