MMJ

ORIGINAL ARTICLE

Allele frequencies of Human Platelet Antigen 1 and Human Platelet Antigen 5 in the Maltese

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Background

The human platelet antigen systems consist of antigenic polymorphisms that arise from single base pair substitutions leading to amino acid changes in platelet glycoproteins. These polymorphisms cause a variety of clinically significant conditions where platelet typing is essential for accurate diagnosis and subsequent treatment. The aim of this study was to determine the allele frequencies of Human Platelet Antigen-1 (HPA-1) and Human Platelet Antigen-5 (HPA-5) in the Maltese population and to compare these frequencies to those in other populations.

Methods

This study was conducted on a total of 508 population DNA samples. Polymerase chain reaction was used to amplify segments of DNA spanning the single nucleotide polymorphism of interest for both the HPA-1 and HPA-5 systems. A restriction enzyme digest was then used to differentiate between the genotypes. The data was analysed by gender and nationality.

Results and Conclusion

From this study it was determined that, for these two polymorphisms, the Maltese population is in Hardy-Weinberg equilibrium and that the local allele frequencies are similar to frequencies of geographically close populations. The frequencies of these two HPA systems are: HPA-1a/1a; 71.6%, HPA-1a/1b; 25.5%, HPA-1b/1b; 2.9%, HPA-5a/5a; 77.4%, HPA-5a/5b; 22.0% and HPA-5b/5b; 0.6%.

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INTRODUCTION

Platelet glycoprotein genes are polymorphic and these give rise to the platelet surface antigens which make up the Human Platelet Antigen (HPA) system. Twenty-nine HPA systems on 6 different glycoprotein complexes have been identified until now.[1] The HPA system is an immunogenic system of alloantigens which, in the case of alloimmunization, may lead to a number of clinically significant conditions. Most HPA systems are biallelic including a high frequency antigen termed as 'a' and a lower frequency antigen termed as 'b'.[2] The HPA-5 polymorphism is situated in the GPIa part of the glycoprotein GPIaIIa,[3] a collagen receptor on the surface of platelet membranes. It is caused by a single nucleotide polymorphism (SNP) on the gene that codes for the GPIa; the *ITGA2* gene.[4] The polymorphism causes a single base change of Guanine into Adenine, which leads to an amino acid change of Glutamic acid to Lysine located between the first and second divalent cation binding domain of the GPIa.[5] On the other hand, the HPA-1 polymorphism is located on platelet GPIIa at amino acid residue 33.[6] The Leucine (HPA-1a) to Proline (HPA-1b) SNP is positioned in exon 3 of the *ITGB3* gene.[7]

Alloimmunization against such antigens can lead to 3 main clinical conditions which include Foetal and Neonatal Alloimmune Thrombocytopenia (FNAIT), Post Transfusion Purpura (PTP) and Refractoriness to Platelet Transfusion (RTP). Other conditions such as drug-induced and transplant-associated thrombocytopenia have also been reportedly associated.[1] Recent studies have shown the association between some HPA systems and Hepatitis C virus (HCV) infection. HCV not only invades hepatocytes but also numerous other types of cells including B-cells and T-cells,[8] macrophages and monocytes[9] and platelets.[10] Studies have also shown the relevance and possible association of HPA-5b and HCV carriers, where a higher allele frequency of HPA-5b was present in HCV carriers.[11]

FNAIT is a rare condition, with an incidence rate of 1 in 1000 to 1 in 2000 live births. [12-15] The condition is characterised by either severe thrombocytopenia in a newborn at birth or 7 days after birth, with a platelet count falling below 100,000 X 10^9/L, or foetal intracranial haemorrhage (ICH) with no other cause.[16] FNAIT occurs during pregnancy when the maternal immune system detects foetal platelet specific antigens that are different from those present in the mother and in turn produces immunoglobulin G antibodies against these platelet antigens. These antibodies target and destroy foetal platelets,[17] resulting in extravascular lysis of platelets[18] and reduced production[19]. The most common HPA to cause FNAIT is alloimmunisation against HPA-1a[12] causing 75 % of FNAIT cases followed by alloimmunisation to HPA-5b[20] causing 16 % of all FNAIT cases.

PTP is an immuno-haematological disorder that may be observed in patients a week or several weeks after being transfused with a blood product containing platelets or platelet membranes.[21] It has been reported in 1:50,000-100,000 transfusions[22] and results in widespread purpura associated with fever, chills and bronchospasms around 7 - 10 days after receiving the transfusion. In general, such patients would have already been exposed to allogeneic platelets either through pregnancy or previous blood transfusions. The alloimmunisation causes the destruction of both the foreign alloantigenic platelets as well as the patient's own platelets resulting in a

severe thrombocytopenia. RTP is a similar but milder condition where a low platelet count is noted after the patient has received an allogeneic random donor platelet transfusion.[21]

The population frequency of the HPA polymorphisms in the Maltese population are unknown and determining their frequencies would be useful for better detection, prevention and prompt treatment of all conditions described above, including identifying those who may be at risk to developing PTP and RTP.

MATERIALS AND METHODS

A total of 508 sequential cord blood samples were obtained from an anonymised Cord Blood Bank collection maintained by the Laboratory of Molecular Genetics (ethical approval 48/2002) and consisted of all samples from those born over a 2-month period in 2010. The only demographic data available included gender and whether the parents were Maltese or foreign. DNA was extracted from these samples using the Salting out technique.[23] The quantity of the DNA was measured using the Nanodrop 2000c spectrophotometer and the DNA integrity was checked by agarose gel electrophoresis using a 0.7 % agarose gel. Each DNA sample was diluted to a concentration of 50 ng/µL.

In order to amplify the HPA-1 and the HPA-5 gene fragments of 193 bp and 256 bp respectively (Figure 1), specific primers were selected and polymerase chain reaction (PCR) was optimised (Table 1). The restriction enzyme *Nci*I (NEB, UK) was used to digest HPA-1 PCR products (Figure 2A) using specific primers[24]. For HPA-5, the reverse primer had an altered nucleotide (A to T) 3 nucleotides from the 3' end, in order to create a restriction enzyme cutting site for restriction enzyme *Dde*I[25]. The restriction enzyme *Dde*I (NEB, UK) cuts only HPA-5a PCR products but not HPA-5b PCR products (Figure 2B). The restriction enzyme digest products were separated by agarose gel electrophoresis and genotypes called based on the fragment pattern.

Statistical analysis included the calculation of allele and genotype frequencies for the Maltese population, as well as the χ^2 test to determine if these were in Hardy-Weinberg equilibrium (HWE). Allele and genotype frequencies were calculated for the entire cord DNA collection as well as the subgroup with 2 Maltese parents. The 'difference between 2 population proportions' calculation was used to determine if there were any differences between frequencies of the subgroup with 2 Maltese parents (representing the traditional Maltese gene pool) and the collection as a whole (representing the current Maltese gene pool).

Figure 1 *PCR products of HPA-1 and HPA-5.* **(A)** HPA-1: Lane 1 represents the 100 bp DNA ladder. Lane 2 - 8 are 193 bp PCR products of HPA-1 and Lane 9 is the negative control. **(B)** HPA-5: Lane 1 represents the 100 bp DNA ladder. Lanes 2 - 5 are 256 bp PCR products of HPA-5. Lane 6 is empty and Lane 7 is the negative control.

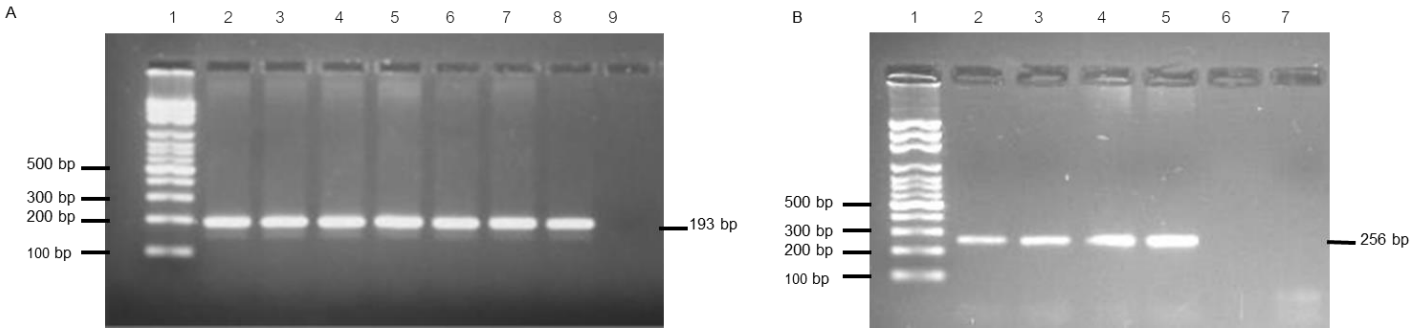

Table 1 **Primer sequences and PCR annealing temperature for HPA-1 and HPA-5.** The nucleotide highlighted in bold and underlined is the mismatched nucleotide used to create a DdeI restriction enzyme site.

	Primer Sequence		PCR Product (bp)	Annealing Temperature (°C)
HPA-1	**F**	**5'** TCTTTGGGCTCCTGACTTAC **3'**	193	52
	R	**5'** CTGGGGACTGACTTGAGTGA **3'**		
HPA-5	**F**	**5'** CTCTCATGGAAAATGGCAGTA **3'**	256	50
	R	**5'** AGGAAGAGTCTACCTGTTTACTATC**T**AA **3'**		

F=forward primer; R=reverse primer; bp=base pairs

RESULTS

A total of 508 samples were tested to represent the Maltese population and these consisted of 51 % males and 49 % females. The HPA-1 and HPA-5 gene fragments were successfully amplified by PCR for these samples (Figure 1) and genotyped using *Nci*I and *Dde*I respectively (Figure 2).

Genotype frequencies were calculated for each polymorphism (Table2) and found to be in HWE using the Chi-squared test. The allele frequencies were determined as follows: 0.844 for HPA-1a, 0.156 for HPA-1b, 0.884 for HPA-5a and 0.116 for HPA-5b. From the genotype frequencies, the allele frequencies were calculated and compared to frequencies of other worldwide populations[26-36] (Table 3). Statistical analysis using the χ^2 test confirmed that there is no statistically significant difference between allele frequencies in Malta and the other countries for which data is available.

Figure 2 *Restriction enzyme digest for HPA-1 and HPA-5.* **(A)** *Nci*I digests the HPA-1 PCR fragment of 193 bp when the C nucleotide is present in the HPA-1b polymorphism. This results in fragmentation into a 33 bp and 160 bp fragment. The top panel shows a typical gel and the bottom panel shows a restriction map. In the gel, Lane 1 shows the 100 bp DNA ladder, Lanes 2, 3, 5-9, 11-14, 16 show HPA-1a homozygous samples with an intact 193 bp PCR fragment, Lane 4 is a HPA-1b homozygous sample with a 160 bp fragment and a faint 33 bp fragment, and Lanes 10, 15 and 17 show HPA-1a/1b heterozygous samples. **(B)** *Dde*I digests the HPA-5a homozygous PCR fragment of 256bp into 2 fragments of 25bp and 231bp. The top panel shows a typical gel and the bottom panel shows a restriction map. In the gel, Lane 1 is the 100 bp DNA ladder, Lanes 2-4, 6, 8, 9 and 11 show HPA-5a homozygous samples which were cleaved into a 231 bp and a non-visible 25 bp fragment, Lane 7 is a HPA-5a/5b heterozygous sample and Lane 10 is a HPA-5b homozygous sample with an intact 256 bp PCR fragment.

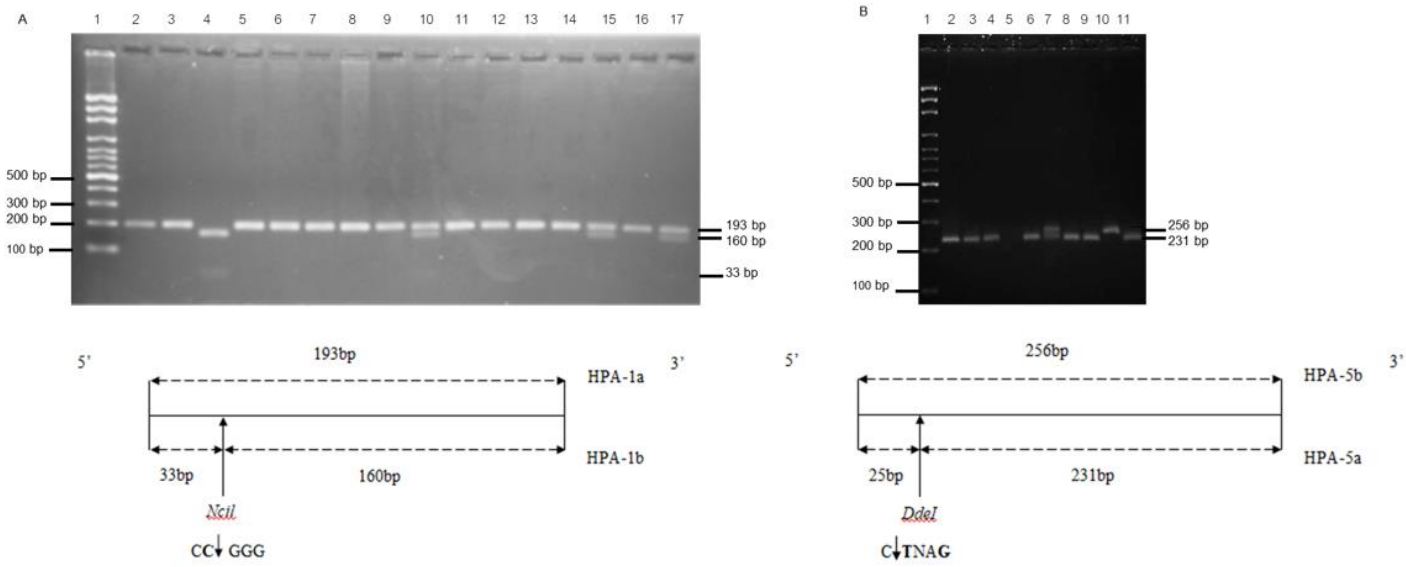

Table 2 **Genotype frequencies of HPA-1 and HPA-5 in the Maltese.** For χ^2 tests p-values > 0.05, the population is in Hardy Weinberg Equilibrium

HPA system	aa genotype (%)	ab genotype (%)	bb genotype (%)	χ^2 test P value
HPA-1	71.6	25.5	2.9	0.486
HPA-5	77.4	22.0	0.6	0.113

Table 3 Allele frequencies for different worldwide populations

Population	Number of samples tested		Allele frequencies			
	HPA-1	HPA-5	HPA-1a	HPA-1b	HPA-5a	HPA-5b
Greek [21]	58	58	0.67	0.33	0.85	0.15
Tunisian [22]	90	90	0.75	0.25	0.78	0.22
Moroccan Berber [23]	110	110	0.75	0.25	0.86	0.14
Slovenian [24]	152	152	0.81	0.19	0.89	0.11
Swiss [25]	500	500	0.81	0.19	0.93	0.07
Spanish [21, 26]	727	454	0.81	0.19	0.88	0.12
Danish [21, 27]	557	427	0.83	0.17	0.92	0.08
German [21, 28]	1583	1643	0.84	0.16	0.92	0.08
Maltese	**489**	**492**	**0.84**	**0.16**	**0.88**	**0.12**
French [21]	800	6192	0.85	0.15	0.87	0.13
Italian [21]	144	144	0.85	0.15	0.90	0.10
Irish [21]	250	250	0.88	0.12	0.91	0.09
African [29]	6382	922	0.89	0.11	0.84	0.16
Congolese [30]	125	125	0.90	0.10	0.73	0.27
Chinese [31]	1000	1000	0.99	0.01	0.99	0.01

DISCUSSION

Genotyping the frequencies of HPA systems is useful in collecting data on the genetics of the population, but may also be useful in clinical scenarios. The allele frequencies of HPA-1 and HPA-5 systems tested in 508 cord blood samples representing the Maltese population were found to be very similar to other Caucasian populations which are geographically close to Malta, such as the Italian, French and German populations (Table 3). When observing the allele frequencies calculated for individuals with one or both parents being foreign, the allele frequencies were also very similar to southern European and northern African populations since most immigrants in Malta originate from these countries.

The incidence rate of FNAIT in several Caucasian populations was found to be 1 in 1000 to 1 in 2000 live births. Last reported data from the National Statistical Office of Malta reported a total of 4444 live births in 2018.[37] Since the HPA-1 and HPA-5 allele frequencies in the Maltese population are similar to other European countries, one should expect to observe approximately 2-3 cases of FNAIT per year. The number of cases of FNAIT in Malta are much lower [Dr. Laspina personal communication]. This low local incidence rate of neonates with FNAIT may be due to several reasons including neonates with FNAIT being born healthy and asymptomatic, or because the HPA incompatibility leads to severe complications such as miscarriages. A similarly low frequency of FNAIT was observed in the Irish population, suggested to be due to under-recognition of the condition.[38]

With the data collected from this study, prospective implementations may be recommended such as donor and patient platelet antigen typing prior to transfusion. Anti-HPA screening is not recommended as this only indicates a possible risk factor for the development of FNAIT and therefore has several ethical issues, including the prediction and management of alloimmunised pregnancies. Post-natal management of FNAIT is therefore more favourable, including setting up genotype screening of newborns showing clinical symptoms requiring transfusion, for appropriate diagnosis and treatment with platelet products. Other possible applications of these findings include using the optimised methods for genotype screening of adults for PTP and RTP, as well as genotyping donor and patient prior to platelet transfusions.[38]

SUMMARY

- Platelets have different surface antigens which make up the Human Platelet Antigen (HPA) system.
- These different glycoproteins on platelet surfaces contribute to different clinical conditions which include FNAIT, PTP and RTP.
- Each country has different allele frequencies for these HPA systems.
- This study was carried out to determine the allele frequency of two of the most common HPA systems, HPA-1 & HPA-5, in the Maltese population.
- PCR and a restriction enzyme digest were used to genotype 508 random DNA samples.
- The local frequencies of these two HPA systems are: HPA-1a/1a; 71.6%, HPA-1a/1b; 25.5%, HPA-1b/1b; 2.9%, HPA-5a/5a; 77.4%, HPA-5a/5b; 22.0% and HPA-5b/5b; 0.6%.

ACKNOWLEDGEMENTS

Financial support was obtained from a University of Malta research grant held by R. Farrugia.

REFERENCES

1. Curtis BR. Genotyping for human platelet alloantigen polymorphisms: applications in the diagnosis of alloimmune platelet disorders. *Sem Thromb Hemost* 2008;34:539–548.
2. Santoso S, Kiefel V. Human platelet-specific alloantigens: update. *Vox Sang* 1998;74: 249-253.
3. Santoso, S., Kiefel, V., & Mueller-Eckhardt, C. (1989). Immunochemical characterization of the new platelet alloantigen system Bra/Brb. British Journal of Haematology, 72(2), 191-198.
4. Peterson, E. T., Sutherland, R., Robinson, D. L., Chasteen, L., Gersh, M., Overhauser, J., . . . Grady, D. L. (1999). An integrated physical map for the short arm of human chromosome 5. Genome Research, 9(12), 1250-1267.
5. Santoso, S., Kalb, R., Walka, M., Kiefel, V., Mueller-Eckhardt, C., & Newman, P. (1993). The human platelet alloantigens Br(a) and Brb are associated with a single amino acid polymorphism on glycoprotein Ia (integrin subunit alpha 2). The Journal of Clinical Investigation, 92, 2427-2432.
6. Goldberger, A., Kolodziej, M., Poncz, M., Bennett, J. S., & Newman, P. J. (1991). Effect of single amino acid substitutions on the formation of the PlA and Bak alloantigenic epitopes. Blood, 78, 681-687.
7. Ensembl [internet]. 2020 [cited November 2020]. Transcript: ITGB3-201. Available from: https://www.ensembl.org/Homo_sapiens/Transcript/Exons?db=core;g=ENSG00000259207;r=17:47253827-47313743;t=ENST00000559488.
8. Pavio N, Lai MMC. The hepatitis C virus persistence. How to evade the immune system? *J Biosci* 2003;28:287–304.
9. Muller HM, Pfaff E, Goeser T, Kallinwski B, Solbach C, Thielman L. Peripheral blood leukocytes serve as a possible extra hepatic site for hepatitis C virus replication. *J Gen Virol* 1995;74:669.
10. Pugliese A, Gennero L, Cutufia M, Enrietto M, Morra E, Pescarmona P, Ponzetto A. HCV infective virions can be carried by human platelets. *Cell Biochem Funct* 2004;22:353–358.
11. Verdichio-Moraes CF, Toralles-Pereira C, Grotto RM, Silva GF, Pardini MI. Allelic frequencies of HPA-1 to 5 human platelet antigens in patients infected with hepatitis C virus. *J. Med. Virol.* 2009;81:757–759.
12. Mueller-Eckhardt C, et al. 348 cases of suspected neonatal alloimmune thrombocytopenia. *Lancet* 1989;18:363-366.
13. Reznikoff-Etievant M, Kaplan C, Muller JY, Daffos F, Forestier F. Alloimmune thrombocytopenias, definition of a group at risk: a prospective study. *Current Studies in Haematology and Blood Transfusion* 1988;55:119-124.
14. Kaplan C, Schlegel N, Durand-Zaleski I, Tchernia G, Blum-Boisgard C. Anti-HPA-la materno-fetal platelet alloimmunisation: costs and benefits of a prospective routine program. *Thrombosis and Hamostasis* 1993;69:191.
15. Blanchette VS, Chen L, de Friedberg ZS, Hogan VA, Trudel E, Decary F. Alloimmunization to the PIA1 platelet antigen: results of a prospective study. *British journal of Haematology* 1990;72:209-215.
16. Petermann R, Bakchoul T, Curtis BR, Mullier F, Miyata S, Arnold DM. Investigations for fetal and neonatal alloimmune thrombocytopenia: communication from the SSC of the ISTH. *J Thromb and Haemost* 2018;16:2526–2529.
17. McCrae K R, Samuels P, Schreiber AD.Pregnancy-Associated Thrombocytopenia: Pathogenesis and Management. *Blood* 1992;80:2697-271.
18. Cohen DL, Baglin TP. Assessment and management of immune thrombocytopenia in pregnancy and in neonates. *Arch Dis Child* 1995;72:71-76.

19. Kaplan C. Neonatal alloimmune thrombocytopenia. *Haematologica* 2008;93:805- 807.

20. Kaplan C, et al. HPA-5b (Br(a)) neonatal alloimmune thrombocytopenia: clinical and immunological analysis of 39 cases. *Br J Haematol* 1991;78:425-429.

21. Murata M, Furihata K, Ishida F, Russell SR, Ware J, Ruggeri ZM. Genetic and structural characterization of an amino acid dimorphism in glycoprotein Ib alpha involved in platelet transfusion refractoriness. *Blood* 1992;79:3086–3090.

22. Padhi P, et al.Post-transfusion purpura: a rare and life-threatening aetiology of thrombocytopenia. BMJ Case Rep. 2013; 2013:brc2013008860.

23. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988;16:1215.

24. Newman PJ, Derbes RS, Aster RH. The Human Platelet Alloantigens, PlA1and PlA2 Are Associated with a Leucine33/Proline33 Amino Acid Polymorphism in MembraneGlycoprotein Ilia, and Are Distinguishable by DNA Typing. *J Clin Invest* 1989;83:1778-1781.

25. Liu TC, Shih MC, Lin CL, Lin SF, Chen CM, Chang JG. Gene frequencies of the HPA-1 to HPA-8w platelet antigen alleles in Taiwanese, Indonesian, and Thai. *Ann Hematol* 2002;81:244-248.

26. EMBL-EBI [internet]. 2020 [cited May 2020]. Immuno Polymorphism Database: HPA Allele Frequencies. Available from: http://www.ebi.ac.uk/ipd/hpa/freqs_1.html.

27. Mojaat N, et al. Gene frequencies of human platelet antigens in the Tunisian population. *Tissue Antigens* 1999;54:201-204.

28. Ferrer G, et al. Analysis of human platelet antigen systems in a Moroccan Berber population. *Transfus Med* 2002;12:49-54.

29. Rozman P, Drabbels J, Schipper RF, Doxiadis I, Stein S, Claas FH. Genotyping for human platelet-specific antigens HPA-1, -2, -3, -4 and -5 in the Slovenian population reveals a slightly increased frequency of HPA-1b and HPA-2b as compared to other European populations. *Eur. J. Immunogen.* 1999;26:265-269.

30. Boehlen F, Bulla O, Michel M, Reber G, de Moerloose P. HPA-genotyping and antiplatelet antibodies in female blood donors. *The Hematology Journal* 2003;4:441-444.

31. Muñiz-Díaz E, Arilla M, Ibáñez M, Bosch MA, Pastoret C, Madoz P. Frequency of platelet alloantigens in the Spanish population. *Sangre* 1993:38; 289-293.

32. Steffensen R, Kaczan E, Varming K, Jersild C. Frequency of platelet-specific alloantigens in a Danish population. *Tissue Antigens* 1996;48:93-96.

33. Carlsson LE, Greinacher A, Spitzer C, Walther R, Kessler C. Polymorphisms of the Human Platelet Antigens HPA-1, HPA-2, HPA-3, and HPA-5 on the Platelet Receptors for Fibrinogen (GPIIb/IIIa), von Willebrand Factor (GPIb/IX), and Collagen (GPIa/IIa) Are Not Correlated With an Increased Risk for Stroke. *Stroke* 1997;28:1392-1395.

34. National Library of Medicine [internet]. 2020[cited May 2020]. dbSNP. Available from: https://www.ncbi.nlm.nih.gov/snp/rs5918#frequency_tab.

35. Halle L, et al. HPA polymorphism in sub-Saharan African populations: Beninese, Cameroonians, Congolese, and Pygmies. *Tissue Antigens* 2005;65:295-298.

36. Feng ML, et al. Establishment of an HPA-1- to -16-typed platelet donor registry in China. *Transfus Med* 2006;16:369-374.

37. Population, Migration and Crime Statistics Unit, National Statistics Office [internet]. 2019 [cited July 2020]. News Release; World Population Day: 2019. Available from: https://nso.gov.mt/en/News_Releases/View_by_Unit/Unit_C5/Population_and_Migration_Statistics/Documents/2019/News2019_108.pdf.

38. Davoren A, McParland P, Barnes CA, Murphy WG. Neonatal alloimmune thrombocytopenia in the Irish population: a discrepancy between observed and expected cases. *J. Clin. Pathol.* 2002; 55:289-292.