Original Research Article

Determining the genotype of the RH blood group system in Maltese blood donors

ABSTRACT

Aims: The Rh blood group system is a complex protein based system having the D, C, c, E and e as the most immunogenic antigens. This blood group system has a vital role in transfusion medicine thus by DNA typing, important medical issues in transfusion practice can be resolved when serological techniques fail. The aim of the study was to determine the frequency of the *RH* genotype in the Maltese blood donors using molecular techniques.
Methodology:797 blood donor samples were enrolled in this study. An allele-specific polymerase chain reaction (AS-PCR) method was used to determine the presence of *RHD, RHCE*E* and *RHCE*e*, while multiplex PCR was used to test for *RHCE*C/c*.
Results: Out of 797 samples studied, the most common allele was *RHCE*e* with a percentage of 98. The most common genotype in RhD positive samples was DCcee and in RhD negative samples, the most common was dccee. The most frequent haplotype was DCe.
Conclusion: Like in previous studies, this research also concludes that the distribution of the *RH* genotype varies in different geographical areas. Further studies working with larger sample size and better techniques would share better light on the molecular function of these loci.

Keywords: [Rh blood group system, RH frequency, blood group genotyping, allele specific PCR, multiplex PCR]

1. INTRODUCTION

The Rh blood group system is considered of great clinical significance, second to the ABO blood group, since it is involved in several life threatening diseases such as haemolytic disease of the foetus and the newborn (HDFN) and haemolytic transfusion reaction (HTR). The five most important antigens from this blood group system are the D, C, c E and e since they are highly immunogenic. The C, c, E and e antigens are less immunogenic than the D antigen but can still cause immunogenic reactions[28]. The RH locus is bigenic consisting of the *RHD* and *RHCE* genes located on the short arm p34-36 of chromosome 1 with an order of 5'-*RHD*-3'-3'-*RHCE*-5' **Error! Reference source not found.** They are very similar, closely linked loci with the *RHD* locus carrying the gene for the *RHD* polypeptide and express the D antigens, while the *RHCE* locus carrying the gene for the *RHCE* polypeptide expresses the C, c, E and e antigens[7][17].
Testing for the Rh blood group system in Malta is done using serological techniques. Serological typing is faster and is the first choice for routine testing but has its own limitations, such as testing is only done on red blood cells (RBC's), thus only the phenotype is known and reagents are sometimes costly[19]. In addition haemagglutination assays are not ideal for recently transfused patients, patients with autoantibodies and it cannot distinguish between different RHD variants [28],[35].In these cases serology techniques are difficult or impossible to carry out or else give inconclusive results, thus in Malta, the sample is referred abroad for genotyping. Sending samples overseas increases the turnaround time and related costs. As a consequence to overcome these limitations, genotyping procedures are being considered at the Hospital Blood Bank laboratory in Malta to complement the routine serological techniques.

There is no data on the prevalence of *RH* genotypes or phenotypes in the Maltese population to enable prediction of the risk of alloimmunization to Rh blood group antigens. The main aim of this research was to determine the frequency of the *RH* genotype in the Maltese population so that in the future *RHD* and *RHCE* genotyping can be used when serology is inconclusive.

2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY

2.1. SELECTION OF SAMPLES AND DETERMINATION OF THE Rh PHENOTYPE USING SEROLOGICAL TECHNIQUES

EDTA samples were collected randomly from 797 Maltese blood donors, upon donating blood at the National Blood Transfusion Service (NBTS), Gwardamanga, Malta. Initially, samples were submitted for serological typing first using column agglutination gel cards technology (CAT) for comparison with DNA genotyping. Gel typing cards (Grifols, Spain) were performed as per manufacturer's instructions to determine the presence or absence of the D, C,c,E and e antigens on red cells using monoclonal antibodies; anti-C, anti-c, anti-E and anti-e within each gel matrix. The results were read and recorded using the DG Reader (Grifols, Spain).

2.2. DNA EXTRACTION

Genomic DNA was extracted using the Qiacube work station (Qiagen®, Hilden, Germany) . Extracted DNA was quantified using Nanodrop 2000c UV-VIS spectrophotometer (Thermo Fischer Scientific™ Waltham, Massachusetts, USA) and diluted to the concentration of 50ng/μl and stored at -20°C until used for genotyping.

2.3. MOLECULAR TYPING

The primers chosen for *RH* genotyping may be viewed at Table 1. For *RHCE*E*, *RHCE*e* and *RHD* molecular analysis, allele specific PCR (AS-PCR) reactions were used while for *RHCE*C*, multiplex PCR was used. For *RHD*, *RHCE*E* and *RHCE*e*, the PCR amplification was performed in a total volume of 20μl containing 1μl genomic DNA (50 ng/μl), 4μl 5X FIREPol Master Mix (Solis Biodyne, Estonia), 0.1μl of both forward and reverse primers and 14.8μl sterile water. Multiplex PCR reaction was also carried out in a total volume of 20μl and consist of of 4μl of 5X FIREPol Master mix (Solis Biodyne, Estonia), 0.1μl of *RHCE*C* forward and reverse primers and 0.05μl of *RHCE**c of forward and reverse primers, together with 1μl genomic DNA (50 ng/μl). A negative amplification control was utilised using 1ul of sterile water instead of DNA with each run. All PCR runs were performed using the GeneAMP® PCR system 9700 (Applied Biosystem, USA).

Table 1: Primers used for *RHD* and *RHCE* molecular assay.

Allele Specificity	Primer	Primer sequence 5' to 3'	Amplicon size (bp)
D[38]	Forward	5'-ACGATACCCAGTTTGTCT-3'	2 amplicons were generated: • control fragment of 1200bp for the *RHCE* gene • *RHD* positive specific fragment of 600
	Reverse	5'-TGACCCTGAGATGGCTGT-3'	
C[24]	Forward	5'-CAGGGCCACCACCATTTGAA-3'	320
	Reverse	5'-GAACATGCCACTTCACTCCAG-3'	
C [24]	Forward	5-'TCGGCCAAGATCTGACCG-3'	177
	Reverse	5'-TGATGACCACCTTCCCAGG-3'	
E[24]	Forward	5'-CCAAGTGTCAACTCTC-3'	108

	Reverse	5'-TGACCCTGAGATGGCTGT-3'	
E [24]	Forward	5'-TGGCCACGTGTCAACTCTG-3'	143
	Reverse	5'-CATGCTGATCTTCCTTTGGG-3'	

References: [38](Simsek, et al., 1995); [24] (Hojjati, Einollahi, Nabatchian, Pourfathollah, & Mahdavi, 2011)

PCR was performed under the following conditions. To amplify the *RHD* allele, an initial hot start was conducted at 95°C for 5 minutes. The PCR program continues with 30 cycles of 1 minute at 95°C and 1.5 minute at 57°C and 2.5 minutes at 72°C. The last step was a final extension for 10 minutes at 72°C. To amplify the *RHCE*E* allele, an initial hot start was conducted at 95°C for 5 minutes. The PCR program continues with 28 cycles of 50 seconds at 95°C and 1.5 minute at 57°C and 1.5 minutes at 72°C. The last step was a final extension for 10 minutes at 72°C. To amplify the *RHCE*e* allele, an initial hot start was conducted at 94°C for 10 minutes, followed with 28 cycles of 30 seconds at 94°C and 45 seconds at 58°C and 45 seconds at 72C. The last step was a final extension for 5 minutes at 72°C.

For *RHCE**C/c touchdown PCR was used to improve specificityand the thermal cycler's profile for *RHCE*C/c* includes and initial hot start of 5 minutes at 95C. The program continues with 10 cycles of 10 seconds at 94°C and 20 cycles with 1 minute at 65°C, 30 seconds at 94°C, 1 minute at 61°C and 30 seconds at 72°C. Finally, the final extension was of 5 minutes at 72°C.

PCR products were analysed by electrophoresis and different concentrations of agarose gel were used depending on the DNA size and were visualized under a blue-light transilluminator. For *RHD*, the PCR products were separated on 1% agarose gel, 2.7% agarose gel was used for *RHCE**E and *RHCE*e* and a 2% agarose gel was used for *RHCE*C/c.*

To increase validity and reliability of our research, DNA samples from 100 randomly genotyped blood donors were retested by molecular typing with the same conditions as the first round testing to validate our in-house PCR techniques for *RH* molecular assay.

2.4. Statistical Analysis

Statistical Package for the Social Sciences (SPSS) software Version 22 (SPSS, Chicago, USA) was used to measure and produce graphical representation of the allele and genotype frequencies. The exact P value test for Hardy Weinberg equilibrium (HWE) was calculated for the C and E loci separately using an online web program (Cohen's Kappa (κ)[10] with 95% confidence interval was used to estimate the accordance between results based on serological and molecular- genetics methods. The 8 different haplotypes were estimated using Haploview [2].

3. RESULTS AND DISCUSSION

3.1. RESULTS

Following method 3 of the study of Simsek et al.[38] for *RHD,* 2 amplicons were amplified; one of 1200bp and another of 600bp. The 1200bp was obtained for the *RHCE* gene (control fragment) while the 600bp is obtained specifically for the *RHD* gene. As a result both product were obtained in an *RHD* positive individual, but the 600bp was missing when the individual was *RHD* negative.

For *RHCE*C* and *RHCE*c* genotyping, multiplex PCR was used which generated 2 amplicons while for *RHCE*E* and *RHCE*e* genotyping, two separate PCR reactions for each allele were performed[24]. In our samples 91.59% of blood donors were *RHD* positive while only 8.41% were *RHD* negative. Of the five alleles that were tested by PCR, the maximum percentage prevalence was found for the *RHCE*e* allele (98.24%) followed by *RHD* (91.59%). The lowest percentage was obtained for the *RHCE*E* allele (22.96%) as shown in table 2. The heterozygosity and homozygosity for *RHCE* is shown in table 2 with ee as the most frequent (77%). No significant deviation for Hardy Weinberg equilibrium was observed for the C and E loci (P<.01).

Table 2: Allele percentages in the Maltese population together with the heterozygosity/ homozygosity percentages for the *RHCE* allele

Allele	Cases	Percentages	Homo/hetero	Cases	Percentages
RHD	730	91.59	**Cc**	408	51.20
RHCE*C	614	77.04	**CC**	185	23.21
RHCE*c	592	74.27	**cc**	204	25.59
RHCE*E	183	22.96	**Ee**	169	21.20
RHCE*e	783	98.24	**EE**	14	01.75
			ee	614	77.05

The frequency of the 18 possible genotype combinations was also calculated (table 3). No individuals were detected with the following genotypes DCCEE, dCCEE, dCcEE, dccEE, dCCEe, dCCEe and dCCee. The most common genotypes in the Maltese population were DCcee (38.27%) followed by DCCee (24.46%). The most common genotype in *RHD* negative individuals was dccee (6.9%).

A rare blood donor phenotype occurs ≤1: 1000 random samples in a given population[27]. With a frequency of = 0.0, the *RH* genotypes DCCEE, dCCEE, dCcEE, dccEE, dCCEe, dCCEe dCCee can only be considered as rare in the Maltese population, being privy to the fact that larger sample size in needed to obtain more robust conclusions.

Using Cohen's Kappa statistical test (K=1) the result of the Rh blood group by PCR showed no discrepancy between red cell serology and DNA typing. The test statistics also gave a strong agreement with those obtained by serological haemagglutination testing. In addition, no serological weak D phenotypes were observed during serological testing. Given that the *RHD* and *RHCE* are close to each other, and strongly linked, a Haploview linkage disequilibrium graph of the

*RHD*and *RHCE* genes was produced as shown in figure 1. The three Rh genotypes of the *RHD* and *RHCE* genes were in high linkage disequilibrium and with these polymorphisms it was possible to construct seven haplotypes, with four having a higher frequency rate. The most common haplotype was DCe followed by dce, as shown in table 3. The dCE haplotype was not detected.

Table 3: *RHD and RHCE* genotype percentages and haplotype percentages in the Maltese population

Rh D Positive			Rh D Negative				
Genotype	**Cases**	**Percentage**	**Genotype**	**Cases**	**Percentage**	**Haplotype**	**Percentages**
DCCEE	0	00.00	**dCCEE**	0	00.00	**Dce**	3.90
DCcEE	2	00.25	**dCcEE**	0	00.00	**DcE**	11.0
DccEE	12	01.50	**dccEE**	0	00.00	**DCe**	49.70
DCCEe	11	01.38	**dCCEe**	0	00.00	**DCE**	0.70
DCCee	195	24.46	**dCCee**	0	00.00	**dce**	33.64
DCcEe	93	11.67	**dCcEe**	1	00.14	**dcE**	0.40
DccEe	53	06.64	**dccEe**	3	00.38	**dCe**	0.75
DCcee	305	38.27	**dCcee**	5	00.63	**dCE**	0.00
Dccee	62	07.78	**dccee**	55	06.90		

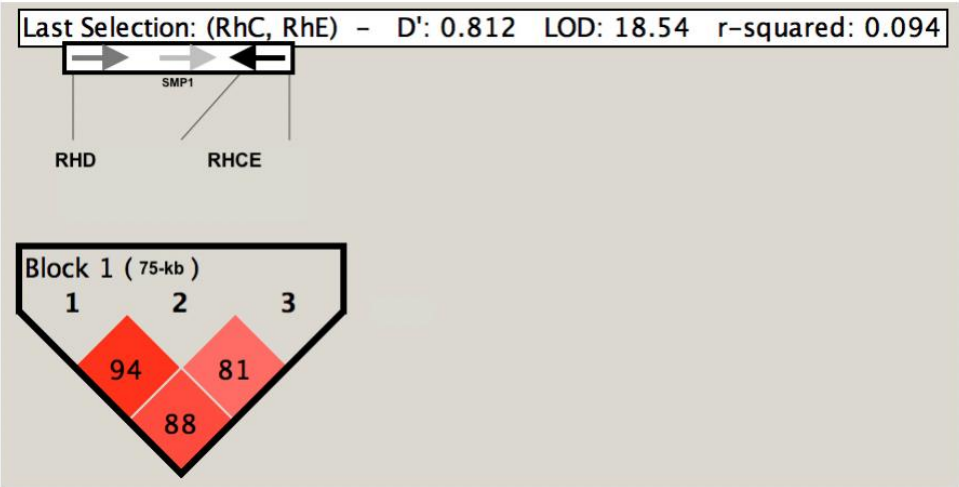

Figure 1:Pairwise linkage disequilibrium coefficients D' X 100 are shown in each cell (D' values of 1.0 are not shown.*Standard colour scheme of Haploview was applied for linkage disequilibrium and colour display (logarithm of odds [LOD] score ≥2 and D' = 1, shown in bright red).*

3.2. **DISCUSSION**

Data of ethnicity is important when determining the most probable genotype but, unfortunately there is no data on the *RHD* and *RHCE* genes in Malta at a molecular level. This study is the first that looks into the *RHD* and *RHCE* genes in Malta, coupled with serology and antigen typing. The total number of subjects was 797 of both sex and different age to determine the frequency of *RH* genotypes in the Maltese population. Geographically a higher frequency of RhD positive was reported in East Asia, such as China and Africa, with studies reporting a range between 94 to 99% rate of RhD positives in China[29][44] and also in Africa such as Tanzania (95%) [40] and Ghana (93%) [14]. Europeans has a lower frequency of RhD positives with a fraction of around 80% or lower[13][28][41][42]. Comparing our research with the literature , we report a higher RhD percentage rate (91.59%) with 8.41% of the population are RhD negative. In a comparison study between populations done by Weinstock [42], most of the European countries reported a prevalence of Rh D negatives between 11 to 29% .

Same as RhD, the *RHCE**C is found to be higher (76%) in our population study visive Caucasian population which is around 68%[29][34].Other populations studies done in China [29][44] ,India [20][26][30][37] and UAE [1][23][39] reported a C antigen percentage of around 93, 87 and 73 respectively. *RHCE*C*is more commonly found in D positive individuals than RhD negatives. One of the reasons may be that, since a higher percentage of RhD positive individuals are reported in our population and populations such as China [29][44] than, the more the *RHCE**C is detected. Alternatively, the C antigen seems to be less prevalent in Blacks [6][25][34]. Studies done on blood donors in West Africa [6] and Nigeria [25] reported a frequency of 21.97% and 17% respectively which contrast well with our study and other studies done on different populations worldwide . In the current research ,*RHCE** chad a percentage of 74% which is a quite comparable with Caucasians (80%*)* [34] while it is much higher in Africans [6][25][34] (around 96%) and less in Indians [20][24][26][27][30] (around 58%). It was also noted that *RHCE**c seems to be more common in D negative individuals. The presence of *RHCE*E* also varied according to the D status and is more commonly found when D is present. E allele is the least prevalent (22.96%) which is similar to other studies from around the world such as in India [20][24][26][27][30], Pakistan[26], Oman [1], Southern Eastern Europe [28]and others. Interestingly, studies done in China reported a higher prevalence of E antigen (39%) [23]. Further to that studies done on the population in Mainland China reported even a higher prevalence of 51%[44]. On the other hand, the distribution of the most common allele, i.e. *RHCE*e*, did not make much difference with the absence or presence of the *RHD* allele. Since 98.24% of the samples carry *RHCE*e*, thus when it comes to providing red cell units for transfusion it would be more difficult to find antigen negative units for patient with an allo anti-e antibody. Similar prevalence was observed in various studies raging from Brazil[11] Pakistan[16], India [20][24][26][27][30][3] and Europe[28][34][32] , that report the e is the most common antigen in their population. Daniels also mentioned the fact that the e-antigen being the most common is frequently noted worldwide, among various races [13].

Looking through a study done by Costa et al[11] we were able to compare the hetero/homozygosity for the *RHCE* allele with two other populations. Almost similar percentage prevalence was observed between the population of Santa Caterina (Brazil), Naples (Italy) and Malta. In all three populations the highest is *RHCE**ee with a percentage of 72, 79 and 77 followed by *RHCE** Cc with percentages of 43, 48 and 51 [11].

In the Maltese population DCcee was found to be the most common *RH* genotype (38.27%) with its probable genotype DCe/dce (41.40%). The probable genotype was calculated from the haplotype percentages. Given that patients and blood donors population is genetically homogenous in Malta, it can be assumed that the same type of *RH* genotypes that are seen in blood donors, would be common in patients too. *RH* genotype frequencies demonstrate a wide range of variations in different races. This study is similar to other studies worldwide ranging from Middle East, such as Omani[1]

and Riyadh [31] to Pakistan [16] and the other studies done in UK [32] and Southern Eastern Europe [28]. These previous studies all reported DCe/dce as the most common genotype. Alternatively these studies contrasts well with others done in India [20][24][26][27][30] and South Africa population [6][34] where DCe/DCe and Dce/dce are the most common.

Thanks to a study done by Ben, Raoudha, Pedro, Esther, & Hassen[5] we were able to compare the haplotype frequency in this study to other populations. All our Rh haplotype frequencies compare a lot to that of Europeans and it was noticed that Malta falls in intermediary position between Italy and Spain (table 4). The most common haplotype in the study was DCe with a percentage of 49.7% (Table 3), This is also expressed in the fact that DCcee and DCCee were the most common genotypes with a percentage of 38.27 and 24.46 respectively (table 3). All EU countries mentioned have a DCe frequency ranging from 0.172 to 0.643 and is the most common. As the study done by Ben, Raoudha, Pedro, Esther, & Hassen[5] also pointed out, it is interesting to note that DCe, Dce and dce are the most haplotypes that stick out most and highlights genetic differences between populations. As seen in table 4, Sub Saharan Africans show the highest Rh haplotype frequency in Dce and lowest in DCe. Thus, Dce is more specific to Sub Saharan African population ranging from 0.590 to 0.891 . This contrasts well with EU countries and Asian ones where Dce is low, and in our study it scored 0.025. We also looked at two other population studies, one done in UK [32] and another done in Western Germany [28]. As can be seen in table 4, difference and similarities in certain Rh haplotypes were observed between Malta and these 2 populations.

Table 4: Rh Haplotype frequencies in Europe, Asia, Sub Saharan Africa and North Africa

Rh haplotypes								
Populations	**Rh haplotypes frequencies in Europe**							
	DCE	**DCe**	**DcE**	**Dce**	**dCE**	**dCe**	**dcE**	**dce**
Malta	0.007	0.490	0.110	0.039	-	0.007	0.008	0.336
England [32]	0.024	0.475	0.140	0.025	-	0.009	0.019	0.386
W. Germany [41]	-	0.431	0.136	0.021	-	0.011	-	0.394
France [5]	0.004	0.425	0.130	0.028	-	0.013	0.005	0.390
Spain [5]	0.015	0.409	0.147	0.040	-	0.004	0.007	0.378
Bulgaria [5]	-	0.424	0.140	0.074	-	0.014	0.001	0.309
Greece [5]	-	0.561	0.101	0.015	-	0.014	0.001	0.309
Crete [5]	-	0.430	0.148	0.050	-	0.057	-	0.315
Italy: Lazio [5]	0.008	0.486	0.121	0.025	-	0.014	0.001	0.346
Italy: Sardinia [5]	0.020	0.643	0.089	0.036	-	0.025	-	0.187
Italy: Sicily [5]	0.010	0.539	0.109	0.015	-	0.016	-	0.311
Turkey [5]	-	0.482	0.171	0.013	-	0.014	-	0.320
	Rh haplotype frequencies in Asia							
India (North West) [5]	0.007	0.592	0.146	0.047	-	0.015	-	0.190
China [5]	0.004	0.729	0.187	0.033	0.003	0.018	-	0.023
Japan [5]	-	0.608	0.280	0.001	-	-	-	0.040
Cambodia [5]	0.014	0.734	0.173	0.059	-	0.014	0.003	0.001
	Rh haplotype frequencies in Sub-Saharan Africa							
Nigeria [5]	-	0.060	0.115	0.590	-	0.031	-	0.202
Senegal [5]	0.002	0.016	0.079	0.632	-	-	0.003	0.268
	Rh haplotype frequencies in North Africa							
Libya [5]	0.028	0.354	0.078	0.191	-	0.014	0.014	0.302
Tunisia (North) [5]	0.049	0.221	0.068	0.120	0.021	0.113	0.082	0.334
Algeria [5]	-	0.441	0.098	0.198	-	0.012	-	0.251
Morocco [5]	0.012	0.383	0.104	0.131	0.003	0.011	0.014	0.341

References: 32. (Race, Mourant, Lawler, & Sanger, 1948); 34. (Wagner, Kasulke, Kerowgan, & Flegel, 1995); 5 (Ben, Raoudha, Pedro, Esther, & Hassen, 2015)

It is worth noting that a number of limitations were encountered in this study. This is the first study done locally at a molecular level thus limited data on this subject was available. Although the genotyping results were in agreement with the phenotyping results, one must take into note that discrepancies might take place in rare cases due to transmission of silent alleles at the *RH* locus [13] . Additionally, the amorph "d" can be due to gene deletion, non-functional gene or RHD-RHCE-RHD hybrid gene as well as others. Various studies analysed the *RHD* and *RHCE* system in terms of gender but in our case this was not possible due to data protection. Further studies can also evaluate the *RHD* zygosity which could not be evaluated in this study due to time constraints. Future studies working with larger sample size and better techniques such as Sanger sequencing, high-throughput sequencing, microarray and multiplex technologies instead of gel electrophoresis would shed better light on the molecular function of these loci; how they are controlled and expressed and their protein product (or absence) in generating the Rh positive or Rh negative phenotypes.

Albeit these limitations, this study entailed various molecular tools such as multiplex based PCR and allele specific PCR. Taken together these molecular tools offer a very fast in-house testing system to obtain the *RHD* and *RHCE* genotype status and gives the Hospital Blood Bank a direction to prevent alloimmunisation.

Knowledge of ethnicity is important when determining the most probable genotype and thus we have presented the frequency of the most immunogenic *RH* alleles and *RH* genotypes in the Maltese population together with the haplotype frequencies. We have also presented a reliable and simple PCR-based method that can be used to determine the *RHD* and *RHCE* blood group system with the aim to minimise alloimmunisations to some extent not only in multi transfused patients (examples include oncology, sickle cells and thalassaemia patients)[4][9][21]but also in other groups such as obstetric patients[18] Phasing in *RH* genotyping has many advantages including more precise decision making in transfusion medicine and obstetric practice with the use of modern genomic techniques for more personalised and accurate medical care [35].

4. CONCLUSION

RH genotypes in the Maltese population together with the haplotype frequencies. We have also presented a reliable and simple PCR-based method that can be used to determine the *RHD* and *RHCE* blood group system with the aim to minimise alloimmunisation's to some extent not only in multi transfused patients (examples include oncology, sickle cells and thalassaemia patients) but also in other groups such as obstetric patients. Phasing in *RH* genotyping has many advantages including more precise decision making in transfusion medicine and obstetric practice with the use of modern genomic techniques for more personalised and accurate medical care [35].

CONSENT

Not applicable.

ETHICAL APPROVAL

The ethical approval for this research was sought from the University Research Ethics Committee (UREC), University of Malta, with ethics number 49/2015.

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